Dissolved carbon fixation by sponge–microbe consortia of deep water coral mounds in the northeastern Atlantic Ocean

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ABSTRACT: We studied dissolved organic and inorganic carbon fixation in 2 sponge species from deep water coral mounds, viz. the demosponge Higginsia thielei and the hexactinellid sponge Rossella nodastrella. Sponges were collected between 500 and 700 m depth on coral mounds in the Rockall Trough (NE Atlantic). Prokaryote densities in sponge associations were on average 2.0 × 10^8 cm^-3 in H. thielei and 2.5 × 10^8 cm^-3 in R. nodastrella (ca. 7 to 30% Archaea, 36 to 65% Bacteria, counted after DAPI staining). Sponge samples were incubated in ultra-filtered seawater with 3H-leucine and 14C-bicarbonate. Mean leucine-based carbon production was 4 nmol C cm^-3 sponge d^-1 for H. thielei and 2 to 4 nmol C cm^-3 d^-1 for R. nodastrella. Average bicarbonate fixation by box-cored H. thielei was 0.7 nmol C cm^-3 sponge d^-1 and up to 4.5 nmol C cm^-3 d^-1 by R. nodastrella. Bicarbonate fixation by sponges was enhanced by (NH_4)_2SO_4 addition to the incubations. Net ammonia oxidation and nitrite production were established for both sponge species and indicated the presence of sponge-associated nitrifiers. Results suggest that sponge-associated chemoheterotrophic/mixotrophic nitrifying prokaryotes may contribute to the observed CO2 assimilation. On average, dissolved carbon was processed 100 to 150 times faster by sponge–microbe consortia of H. thielei and R. nodastrella than by planktonic microbes in ambient water. Preliminary estimates suggest that the assimilation of (in)organic carbon contributed up to 10% of total carbon assimilation by sponge–microbe consortia of H. thielei and R. nodastrella.

KEY WORDS: Deep water coral reef · Sponge-associated prokaryotes · Leucine incorporation · Bicarbonate fixation · Nitrification

INTRODUCTION

Sponges are well represented on cold water coral mounds (Reitner & Hoffmann 2003, Van Soest et al. 2007a), which are most common in the dark ocean between 400 and 1000 m depth (Roberts et al. 2006). Coral mound distribution is restricted by temperature (maximum 13°C) and hard substrata for settlement (Rogers 1999). Cold water coral reefs in the North East Atlantic are built primarily by the branching stony corals Lophelia pertusa and Madrepora oculata, which provide hard substrata with diverse spatial complexity (e.g. rubble pavement to more complex 3-dimensional structures) harboring a large variety of benthic organisms. The density, diversity and cover of benthic suspension and filter-feeding organisms are especially remarkable on cold water coral reefs (e.g. Jensen & Frederiksen 1992, Roberts et al 2006); this assemblage is often dominated by sponges (Van Soest et al. 2007).

The food sources that support deep water coral communities are not well understood. Hydrocarbon seeps are thought to provide food (Hovland & Thomsen 1997), but, at least for the NE Atlantic, there are no indications that hydrocarbon seeps contribute to the food requirements of cold water coral communities (Masson et al. 2003). It is more likely that the system is fuelled mainly by particulate and dissolved organic matter derived from the euphotic zone (Frederiksen et
al. 1992, Duineveld et al. 2004, Hopkinson & Vallino 2005). At times, relatively ‘fresh’ particles characterized by labile lipids such as polyunsaturated fatty acids reach the sea floor (Kiriakoulakis et al. 2004). These particles release labile dissolved organic matter (DOM) that is consumed mainly by prokaryotes. However, prokaryote abundance and productivity in the deep sea are usually low (e.g. Herndl et al. 2005); hence prokaryotes may be an inadequate year-round food energy source for sponges (which feed predominantly on prokaryotes, e.g. Pile et al. 1996, Yahel et al. 2007) in cold water coral reef habitats. Alternatively, DOM in ambient seawater may also be used directly as a nutrition source by benthic organisms, as reported for shallow water tropical coral reefs (De Goeij & Van Duyl 2007).

More than 40% of the biomass of marine sponges may consist of prokaryotes (e.g. Vacelet & Donadey 1977, Wilkinson 1978a,b). Sponge-associated prokaryotes are thought to mediate dissolved organic carbon (DOC) removal by the sponge, and they can be a source of nutrition for the sponge cells in oligotrophic environments (e.g. Reiswig 1981). Substantial DOM removal by the tropical demosponges has been reported only recently (Yahel et al. 2003, De Goeij et al. 2008). Moreover, rapid proline uptake by sponge-associated prokaryotes and subsequent transfer of proline to sponge cells suggests a trophic link between DOM, symbionts and host (Wilkinson & Garrone 1979). Whatever the pathway might be, DOM consumption by sponge–microbe consortia would allow the sponges to survive in areas with low levels of particulate organic carbon (e.g. dark ocean). Whether DOM is a source of carbon for sponges living on cold water coral reefs is as yet unknown and has been addressed in this study.

Inorganic carbon fixation has not yet been established for sponges living in the dark ocean, but it is considered likely in the light of recent findings of chemooautotrophy at these depths (Lam et al. 2004, Herndl et al. 2005, Ingalls et al. 2006). The dominance of Crenarchaeota below 100 m depth in the NE Atlantic (op. cit.) and their capacity to fix bicarbonate (Wucherer et al. 2003, Hallam et al. 2006) by ammonia oxidation (Könneke et al. 2005) opens up new pathways in the ocean carbon cycle. Many sponge species harbor a high diversity of Bacteria and Archaea (Taylor et al. 2007). Nitrification by sponges and the presence of ammonia and nitrite oxidizing prokaryotes (chemoautotrophs) in sponges (e.g. Diaz & Ward 1997, Hentschel et al. 2002, Jiménez & Ribes 2007) is likely coincident with bicarbonate fixation.

We hypothesized that DOM and dissolved inorganic carbon (DIC) are incorporated as a supplementary nutrient source in deep water coral reef sponges of the dark oligotrophic ocean. We determined the abundance of sponge-associated prokaryotes and tested the ability of sponge–microbe consortia of the demosponge Higginsia thielei and the hexactinellid sponge Rossella nodastrella to assimilate leucine at nanomolar concentrations for protein synthesis and to fix DIC in the dark. Additional experiments were conducted to detect nitration by chemolithoautotrophic/mixotrophic prokaryotes associated with these sponges. Carbon fixation rates of DOC and DIC in the sponges were compared with C-fixation rates in the ambient seawater and estimates of particulate organic matter incorporation by the sponges.

**MATERIALS AND METHODS**

**Study site and sampling.** Sponge and water samples were collected from deep water coral mounds on the southeastern part of Rockall Bank (Logachev mounds) from 24 June to 12 July 2005 (Fig. 1). Two complexes were visited, the Clan mounds (Artur mound 55.444° N, 16.0755° W) and the Haas mounds (center: 55.494° N, 15.7894° W), which lie approximately 20 km apart (Van Duyl & Duineveld 2005). Coral mounds on the Rockall Bank slope rise up from ~850–900 m to 550 m depth. Bottom water was collected on the mounds between 570 and 785 m depth using a 1000 l water box whose lids closed when a mechanical release trigger touched the bottom. It collected water from 50 cm above the bottom. In addition, water samples were taken from approximately 2.5 m above the bottom at the Haas mound complex (W-mound 55.4948° N, 15.8059° W) with a CTD array equipped with Noex water bottles. Seawater from the water box and the CTD array was collected in clean 1 l polycarbonate bottles for immediate processing. A box corer (stainless steel cylindrical barrel: 50 cm inner diameter, 55 cm high) was used to collect intact bottom cores with overlying seawater. The box-core water was siphoned into a 5 l glass bottle that had been rinsed with 0.1 M HCl and box-core water prior to sampling. The box-core water was usually very turbid and larger particles were allowed to settle for 1 h at the in situ temperature (9°C) before subsampling. Water samples from the water box, the CTD water bottles and the box corer were analysed for inorganic nutrients, DOC and DIC, prokaryote abundance and production.

Box cores were screened for the presence of sponges. We selected 2 abundant sponge species, Higginsia thielei Topsent, 1904 (small, round, rigid, whitegreenish in color: Class Demospongiae, Order Halichondrida, Family Heteroxyidae) and Rossella nodastrella Topsent, 1915 (white, large, thin-walled tubular-trumpet shape: Class Hexactinellida, Order Lyssacinosida, Family Rossellidae) (Van Soest et al.
2007b). *H. thielei* was collected in the Haas and Clan mound complexes between 570 and 785 m depth. *R. nodastrella* was collected by box core at 585 m depth and with a short trawl with a small triangular dredge at the Haas mound complex at around 529 m depth. Collected sponges were kept alive in tanks in a cool container at about 9°C until enough material was collected to start the incubation experiments (maximum 5 d holding time). Tank water was refreshed with bottom water every 2 d.

**Inorganic nutrients, dissolved organic and inorganic carbon.** Duplicate water samples for NH$_4^+$, NO$_2^-$, NO$_x$ (= NO$_2^-$ + NO$_3^-$), and PO$_4^{3-}$ were filtered over 0.2 µm Acrodisc filters, collected in 6 ml pony vials and stored at 4°C. Within 15 h of collection, samples were analysed using a Technicon TRAACS 800 autoanalyzer according to Grasshoff et al. (1999). Samples for DIC were fixed with 10 µl saturated HgCl$_2$ solution using a 5 ml glass vial filled to the rim to avoid inclusion of air. The DIC concentrations in the water were determined spectrophotometrically by a continuous flow set-up on a Technicon TRAACS 800 autoanalyzer (Stoll et al. 2001). DOC was gravity-filtered over 0.2 µm polycarbonate filters. Filters were pre-washed 3 times with sterile 0.1 M HCl and subsequently twice with sample water before the DOC sample was collected. Twenty ml samples were pipetted into combusted glass ampoules and fixed with 5 drops of a 1:1 (v/v) phosphoric acid and Milli-Q water solution. Ampoules were sealed using a gas burner. DOC concentrations were determined by high-temperature combustion on a Shimadzu TOC-5000.

**Bottom water incubations with $^3$H-leucine and $^{14}$C-DIC.** Heterotrophic prokaryote production was measured with $^3$H-leucine in the overlying seawater of the box core (n = 4), in seawater of the large water box (n = 8), and in seawater of the CTD water bottles (n = 4). Duplicate samples of 10 ml were incubated with a final concentration of 30 nM leucine, of which 50% was labelled with $^3$H-leucine (Amersham Biosciences, specific activity 167 Ci mmol$^{-1}$). Test experiments showed that a 30 nM final concentration was sufficient to just saturate leucine uptake (authors’ unpubl. data). Incubations with $^3$H-leucine were conducted at 9°C in the dark. Control samples were pre-incubated for 7 min with 0.5 ml of 0.2 µm filtered 37% formaldehyde before the radioactive label was added. Incubations lasted 2 h and were terminated by addition of 0.5 ml 0.2 µm filtered 37% formaldehyde (2% final concentration). The proteins synthesized during the incubation were precipitated with ice-cold trichloroacetic acid (TCA, 5% solution) on a polycarbonate filter (0.2 µm pore size, 3 washes) and subsequently washed with bacteria-free seawater. Filters were transferred to 20 ml plastic scintillation vials and stored at −20°C until analysis.

**Incorporation of $^{14}$C-DIC into prokaryotic cells** was determined in seawater samples of the water box (n = 5). Using 50 ml Greiner tubes, 50 µCi $^{14}$C-labeled sodium bicarbonate was added to 50 ml of seawater leaving negligible headspace to avoid degassing of CO$_2$. Samples were incubated in triplicate in the dark at an in situ temperature (9°C) and terminated at $t = 0$ (control) and $t = 72$ h with 0.2 µm filtered formaldehyde, 2% final concentration. Subsequently, the samples were filtered over a 0.2 µm pore size polycarbonate filter and the free $^{14}$C-labeled and unlabeled CO$_2$ was fumed from the sample in an excicator with concentrated HCl for 30 to 35 min. Subsequently, filters were transferred to plastic scintillation vials and stored at −20°C until analysis.

Radioactivity in filter samples was counted on a Wallac 1211 Rack Beta Scintillation Counter with an external standard. We added 10 ml of Ultima Gold XR Scintillation cocktail to each filter and corrected for quench. Heterotrophic carbon incorporation was determined according to Simon & Azam (1989) assuming an internal isotope dilution of 2. Total DIC fixation was based on the fraction of ambient DIC that was incorporated in biomass.

**Sponge incubations with $^3$H-leucine and $^{14}$C-DIC.** Intact live *Higginsia thielei* specimens with a maximum volume of 3.8 cm$^3$ and pieces of live *Rossella nodastrella* with maximum volumes of 11 cm$^3$ (carefully cut in filtered seawater) were used for the incuba-
tions. The conditions of whole sponges and sponge pieces were judged on basis of color (sponges showing color shifts from white to brownish were not used), firmness of skeleton and absence of debris/sediment on skin and dermalia (protruding spicules). The absence of debris indicates that sponges are able to clean themselves. Only sponges that met these criteria were used for experiments. Biochemical tests revealed 2-5A synthetase activity and ATP N-glycosidase activity in H. thielei and R. nodastrella, demonstrating the presence of live sponge cells in our samples (M. Kelve pers. comm.). We did not observe active pumping in either sponge species. Whether or not there was sponge pumping during the dark incubations, we considered turbulence in 50 ml Greiner incubation vials adequate to supply the sponge–microbe consortia with a passive flow of dissolved carbon.

Before the incubation experiments, sponges were carefully washed 3 times in ultra-filtered seawater. Sponge samples were placed in 50 ml Greiner tubes with 20 or 50 ml ultra-filtered seawater containing a natural DOC concentration of 70 to 80 µM and incubated with 20 or 50 ml ultra-filtered seawater containing a passive flow of dissolved carbon.

For the analysis of dry weight and C- and N-contents of sponges, we measured with calipers the individual sponges or sponge pieces submerged in filtered seawater. The length, width and height were taken, occasionally at several distances along the main axes of the sponges to estimate volumes. The morphology of intact H. thielei was variably characterized, depending on geometry, by a (truncated) cone, a sphere or a cylinder or a combination of 2 different or identical forms. For experiments with R. nodastrella, we cut the in situ vertical, peripheral curving wall into either circular or rectangular pieces of 5 to 12 cm² and the average thickness of the slices was estimated by measuring the thickness in different slice sections.

For the analysis of dry weight and C and N contents of sponges, we stored frozen specimens until processing. Sponges were thawed, flushed with distilled water and blotted dry. Dry weight was determined after 48 h...
drying to constant weight in an oven at 50°C. The carbon and nitrogen contents of sponge pieces were determined with a Carlo Erba Na-1500 HCN analyser (Verardo et al. 1990).

**Abundances of prokaryotes in sponge tissue and ambient seawater.** Sponge samples in 6 ml pony vials were fixed with a 1:1 (v/v) phosphate buffered saline (PBS) and paraformaldehyde solution for at least 30 min, but less than 12 h, at 4°C in a refrigerator. After fixation, samples were washed twice with 1 x PBS and stored at –20°C in 6 ml pony vials with PBS:ethanol (1:1 v/v). For dissociation of sponge cells and harvesting of associated prokaryotic cells, we adjusted the protocol of Müller et al. (1999). Sponge samples were cut in small pieces of ca. 50 mm³. Samples were blotted dry of Müller et al. (1999). Sponge samples were cut in

Seawater samples from the box cores (20 ml) were fixed with 1.2 ml of 0.2 µm filtered formaldehyde (37%) for at least 1 h, but no longer than 24 h. The samples were subsequently filtered over a white 0.2 µm polycarbonate filter (GTTP) on a support filter (0.45 µm cellulose nitrate). After filtration, the filters were rinsed with 5 to 10 ml MQ water and air-dried. The filters were stored in 2 ml Eppendorf cups at –20°C until processing.

Water samples from the water box and CTD bottles (10 ml) were fixed with 0.8 ml 0.2 µm filtered glutaraldehyde (25%) and stained in darkness with a 2.5 ml DAPI solution (10 ml MacIlvain buffer with 200 µl DAPI solution at a concentration of 5 mg ml⁻¹) for at least 30 min, but no longer than 24 h (4°C in a refrigerator) (Porter & Feig 1980). After staining, samples were filtered over a black 0.2 µm polycarbonate filter supported by a 0.45 µm pore size cellulose nitrate filter and air-dried. Filters were mounted in immersion oil and stored at –20°C until counting.

Prokaryotes in the dissociated sponge tissue and in the ambient seawater of the same box cores were double stained with DAPI and catalyzed reporter deposition fluorescent for in situ hybridization (CARD-FISH). Total counts of prokaryotes were made with the DAPI staining, while the molecular probes applied with the CARD-FISH method distinguished between Bacteria and Archaea. The following probes were used: EUBIII mix for Bacteria and Cren537 and Eury806 for Crenarchaeota and Eur yarchaeota, respectively. All filters for CARD-FISH were embedded in 0.1% agarose and dried at 37°C. For the full protocol of permeabilization, hybridization and amplification of the prokaryotes on the filters see Teira et al. (2004).

Filters were counted under a Zeiss Axioplan or Zeiss Axiovert epifluorescence microscope with a DAPI and FITC filter and a Plan-Neofluar objective. Up to 60 fields filter⁻¹ were counted, or 20 fields when fields contained 10 or more prokaryotes on average. Replicate counts were made on each sponge filtrate or water sample. For estimating the prokaryotic biomass, we assumed a C content of 10 fg C cell⁻¹ (Ducklow 2000).

**Statistical analyses.** Data were analysed using standard statistical tests (SYTAT® 10). We ran 1-sided, 2-sided and full ANOVAs (general linear models) on flux data to assess the effects of (1) chemical treatment, (2) method of collection of sponges and (3) sponge species on in-transformed leucine incorporation and CO₂ fixation rates by sponges. With the transformation, normal distribution of the flux data was obtained. For comparison of fluxes in live and dead sponges, we applied a 1-sided probability test and rejected the null hypothesis (no difference) when p ≤ 0.05. In the comparison of live treatments, we considered a 2-sided probability of <0.1, but >0.05 as significant.

**RESULTS**

**Bottom water characteristics**

The nutrient concentrations in bottom water of coral mounds with live corals and sponges between 560 and 781 m depth are presented in Table 1. The box-core water samples were particularly enhanced in PO₄⁻³, NH₄⁺ and DOC compared with the Noex bottle CTD and water box samples. The physical impact of the box core on the bottom induced resuspension of material in the overlying water. The CTD samples were taken 4 times at 581 m depth at the same location over 24 h. The clustered CTD sampling in space and time is reflected in the low SDs of the variables measured. In situ temperatures were measured only with the CTD and with temperature sensors connected to landers (Duineveld et al. 2004). During the tidal cycle, temperature varied from 8.4 to 9.7°C (CTD measurements 10 to 11 July 2005). The water samples taken with the water box were different from those of the other 2 devices. Based on the significantly lower NO₃⁻ concentrations, it is likely that the water box leaked and exchanged water with ambient water during transit to the ship.
The total microbial production in bottom water ranged from 0.04 to 0.11 nmol C cm$^{-3}$ d$^{-1}$ at a microbial abundance of 3.1 to 4.9 $\times$ 10$^5$ microbes cm$^{-3}$, with the higher values in water box samples (Table 2) in which the production and abundance were, on average, ca. 3-fold and up to 1.6-fold higher, respectively, than in the box core and Noex bottle CTD water samples. DIC fixation was measured only in the water box samples. For the specific growth rates of prokaryotes, we summed heterotrophic production based on leucine incorporation and DIC fixation (as measured in the water box) of prokaryotes. DIC fixation was 4% of the total prokaryotic biomass production in the water box (Table 2).

### 3H-leucine incorporation by sponge–microbe consortia

The incubations of intact live *Higginsia thielei* and pieces of live *Rossella nodastrella* with 3H-leucine in filtered seawater demonstrated a significant incorporation of leucine compared to the control incubations (with formaldehyde) (*H. thielei*, Fig. 2a: ANOVA, $F_{1,8} = 23.8, n = 10, p < 0.05$; *R. nodastrella*, Fig. 2b,c: ANOVA, $F_{3,10} = 8.473, n = 12, p < 0.05$). Control incubation levels were relatively high, suggesting that pre-incubations with formaldehyde for 5 to 10 min may have been too short, or the final concentration of 2% was too low to kill the sponges. Most of the leucine incorporated into proteins was associated with the hydrolysed tissue (mean ± SD; 79 ± 17% in *H. thielei* and 57 ± 24% in *R. nodastrella*). The remainder of the incorporated leucine was found in released particulate matter, which was collected on filters before hydrolysis. Leucine incorporation rates into protein (including a 2-fold isotope dilution) were on average 0.031 nmol leucine cm$^{-3}$ sponge d$^{-1}$ for *H. thielei* (= 4 nmol C cm$^{-3}$ sponge d$^{-1}$) and 0.014 and 0.028 nmol leucine cm$^{-3}$ sponge d$^{-1}$ for box-cored and dredged *R. nodastrella* (= 2 and 4 nmol C cm$^{-3}$ sponge d$^{-1}$), respectively. Rates measured in pieces of the dredged *R. nodastrella* were slightly higher than rates in the box-cored specimen. Rates per unit volume of sponge were 50 to 100 times higher than in the ambient water. DOC concentration in ultra-filtered incubation water ranged from 69 to 81 µM, and was comparable to reef ambient DOC concentrations (mean = 73 µM in box-core water).

### 14C-DIC fixation by sponge–microbe consortia

The different live treatments (with and without allythiourea and with and without ammonium sulfate) had no significant effect on DIC incorporation by *Higginsia thielei* (Fig. 3a) (1-way ANOVA, $n = 18, p > 0.10$) and *Rossella nodastrella* (Fig. 3b,c) (2-way ANOVA, treatment and method, $n = 17, p > 0.10$). The large variations between different incubations within one treat-
ment may be due to the fact that they were all performed with different individuals or sponge pieces. Moreover, 'killed' controls appeared to fix CO2, reducing the difference between presumed dead and live sponges. Since there were no significant differences between live treatments, we tested the difference in CO2 fixation between live and 'killed' sponges. Overall, the average DIC fixation in live intact *H. thielei* specimens significantly exceeded that of the 'killed' controls (1-way ANOVA, 1-sided probability, n = 24, p = 0.05). The average net CO2 fixation by *H. thielei* was 0.7 ± 0.8 nmol C cm\(^{-3}\) d\(^{-1}\), which was on average up to 170 times higher than the DIC fixation rate of prokaryotes in the ambient bottom water.

Across all live sponge incubations without allylthiourea shown in Fig. 3, the incubations with (NH4)\(_2\)SO\(_4\) were slightly higher than the live sponge incubations without (NH4)\(_2\)SO\(_4\). This difference was not significant within sponge species or within sampling methods (dredging or box coring). However, after combining the data from different sponge species and collection methods, the ammonium addition contributed significantly to the variation in CO2 fixation (GLM, 2-sided probability; species \(F_{1,16} = 139.090, p < 0.05\); method \(F_{1,16} = 346.956, p < 0.05\); treatment \(F_{1,16} = 3.313, 0.5 < p < 0.1; n = 19\). Interactions between species, method and treatment were not significant. This implies that (NH4)\(_2\)SO\(_4\) stimulated CO2 fixation by the sponge–microbe consortia.

Most DIC fixation in tissue was found in the hydrolysed tissue of the sponges (mean ± SD, 74 ± 14% in *H. thielei*, and 61 ± 4% for dredged and 53 ± 13% for box-cored *Rossella nodastrella*, respectively); the remainder was recovered in particulate matter filtered before tissue hydrolysis. Dissolved 14C-labeled metabolites released during incubation were not accounted for in the average DIC fixation rates presented in Fig. 3, but were found to contribute substantially to the total DIC incorporated. More than 80% of the DIC fixed was recovered in the DOC fraction of live *H. thielei* incubations (n = 5) and 77% of live dredged *R. nodastrella* incubations (n = 3) after correction for controls (authors’ unpubl. data). This implies that we have underestimated CO2 fixation rates by a factor of 5.

**Ammonia oxidation by sponge–microbe consortia**

The presence of sponge specimens in incubation tubes resulted in inorganic N concentration increases over the 24 h incubation period. NH\(_4^+\), NO\(_2^-\), NO\(_3^-\) did not significantly change in the absence of sponges. The net ammonium release rate (mean ± SD), by *Higginsia thielei* was 999 ± 330 nmol cm\(^{-3}\) d\(^{-1}\) (n = 4) and 36 ± 13 nmol cm\(^{-3}\) d\(^{-1}\) (n = 7) by the dredged *Rossella nodastrella*. No consistent effects of the treatments ((NH4)\(_2\)SO\(_4\) and/or allylthiourea) were found on the NH\(_4^+\) release by sponges. *H. thielei* excreted more N than it could have taken up under natural conditions,
and there may have been tissue degeneration during the incubation. Nitrite was released in all but one incubation (H. thielei, live with allylthiourea), indicating ammonia oxidation to nitrite (Fig. 4a,b). Net rates varied from –1 to 26 nmol NO$_2^-$ cm$^{-3}$ sponge d$^{-1}$ for H. thielei and 0.4 to 0.8 nmol NO$_2^-$ cm$^{-3}$ sponge d$^{-1}$ for the dredged R. nodastella. (NH$_4$)$_2$SO$_4$ addition did not enhance the release rates of NO$_2^-$ by sponges. Allylthiourea addition inhibited nitrite release rates in R. nodastella. In H. thielei the effect of allylthiourea was only evident in the incubation without (NH$_4$)$_2$SO$_4$. Preliminary estimates of sponge nitrification were made by subtracting the effluxes of NO$_2^-$ in the presence and absence of allylthiourea (Table 3). For comparison, we also added the net release of nitrite from the sponges as a measure of nitrification. No ammonia oxidation could be established in the ultra-filtered seawater used for the sponge incubations.

**Dry weight and carbon and nitrogen contents of sponges**

Dry weights cm$^{-3}$ sponge were derived from the relation between volume and dry weight (dry wt) for each sponge species. The average dry wt of Higginisia thielei was 143 mg cm$^{-3}$ sponge ($y = -19 + 162x$, $r = 0.85$, $n = 6$). The dry wt of Rossella nodastrella was lower, with an average of 126 mg cm$^{-3}$ sponge ($y = 109 + 17x$, $r = 0.78$, $n = 21$). Variations in dry wt cm$^{-3}$ sponge were large due to the difficulty of making co-
Prokaryote abundances in sponges and sponge contact water

Numbers of prokaryotes in different specimens of Higginsia thielei varied from 7.12 × 10^7 to 4.05 × 10^8 cells cm^{-3} (mean = 2.00 × 10^8 cells cm^{-3}, Table 4). Rossella nodastrella harbored, on average, more prokaryotes than H. thielei and contained 1.3 × 10^8 to 3.4 × 10^8 cells cm^{-3} (mean = 2.53 × 10^8 cells cm^{-3}). When R. nodastrella was present, prokaryotic counts were higher in box-core water (sponge contact water) than in the absence of this sponge. This was not the case for H. thielei (Tables 2 & 4). Box cores containing R. nodastrella were not used for the counts in Table 2. R. nodastrella and H. thielei were found in 13 and 38% of box cores collected over coral mounds, respectively.

Specific growth rates of sponge–microbe consortia

In Table 5, there is an overview of carbon production rates of the sponge–microbe consortia of Higginsia thielei and Rossella nodastrella in ultra-filtered seawater. The ^14^C-DIC incubations with allylthiourea are paired with the respective treatments without allylthiourea (i.e. without and with (NH_4)_2SO_4). The stimulating effect of (NH_4)_2SO_4 addition on the bicarbonate fixation remained (Table 5). Specific growth rates were based on ‘total dissolved carbon incorporation’, which is defined as the sum of the leucine based carbon production and the DIC fixation divided by the carbon content of the holobiont (sponge and sponge-associated prokaryotes) and the carbon content of associated prokaryotes, respectively. It is evident that

Table 3. Higginsia thielei and Rossella nodastrella. Nitrification rates based on the difference in nitrite release rate of sponges in the presence and absence of allylthiourea and on the net nitrite release rates. (NH_4)_2SO_4 was added to half of the incubations to prevent potential NH_4^+ limitation of nitrification.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Prokaryote n (nmol cm^{-3} sponge d^{-1})</th>
<th>Allylthiourea</th>
<th>Net nitrite release</th>
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</thead>
<tbody>
<tr>
<td>H. thielei (box-cored)</td>
<td>None</td>
<td>27.1</td>
<td>26.10</td>
<td></td>
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<tr>
<td></td>
<td>(NH_4)_2SO_4</td>
<td>1</td>
<td>3.53</td>
<td></td>
</tr>
<tr>
<td>R. nodastrella (dredged)</td>
<td>None</td>
<td>0.13</td>
<td>0.61</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(NH_4)_2SO_4</td>
<td>2</td>
<td>0.34</td>
<td>0.77</td>
</tr>
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</table>

Table 4. Higginsia thielei and Rossella nodastrella. Abundances of associated prokaryotes in sponges and planktonic prokaryote abundances in sponge contact water (overlying boxcore water).

<table>
<thead>
<tr>
<th>Sponge associated</th>
<th>Prokaryotes (n cm^{-3})</th>
<th>Bacteria (%)</th>
<th>Archaea (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean ± SD</td>
<td>n</td>
</tr>
<tr>
<td>H. thielei</td>
<td>5</td>
<td>2.00 × 10^8</td>
<td>1.36 × 10^8</td>
</tr>
<tr>
<td>R. nodastrella</td>
<td>3</td>
<td>2.53 × 10^8</td>
<td>1.07 × 10^8</td>
</tr>
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<td>Sponge-contact water</td>
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<td>3.14 × 10^5</td>
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<td>R. nodastrella</td>
<td>3</td>
<td>4.83 × 10^5</td>
</tr>
</tbody>
</table>

Both sponges harbored Archaea (Cren- and Euryarchaea) and Bacteria. Cells of Bacteria were more abundant than those of Archaea in both sponges (means of 48% of DAPI counts in H. thielei and 55% in R. nodastrella). The abundances of Crenarchaea and Euryarchaeae were 12 and 7% of the DAPI counts, respectively in H. thielei and 9 and 2%, respectively in R. nodastrella. Approximately 30 to 40% of the DAPI counts of sponge-associated prokaryotes could not be identified with the bacterial and archaeal CARD-FISH probes applied. In the sponge contact water, Archaea cells were present in only 2% of the total DAPI count; Bacteria made up ca. 30%. The proportion of cells in the total count stained with CARD-FISH was much lower in the ambient water than in the sponges. The abundance of prokaryotes associated with sponges was on average 500 to 640 times higher than the abundance of planktonic prokaryotes in sponge contact water (Table 4). Prokaryotes amounted to 2 µg C cm^{-3} in Higginsia and 2.5 µg C cm^{-3} in Rossella (based on the average C-content of prokaryotes in oceanic waters, viz. 10 fg C cell^{-1}; Ducklow 2000). These values represent 0.024 and 0.039% of the total organic carbon content of H. thielei and R. nodastrella, respectively.
sponges cannot grow quickly on ambient DOC and DIC. Up to 287 yr are required for biomass doubling at these uptake rates. If DOC and DIC incorporations are attributable to associated prokaryotes only, we arrive at specific growth rates of 0.02 to 0.04 d⁻¹, which are 3 to 15 times slower than the specific growth rate of prokaryotes in ambient water (Table 2). The contribution of DIC fixation to the total dissolved carbon incorporation differed between *H. thielei* and *R. nodastrella*; in *R. nodastrella* production on DOC and DIC was dominated by DIC-fixation (70 to 80%). Specific growth was highest for the box-cored *R. nodastrella* and lowest in the dredged *R. nodastrella* (due to the lower bicarbonate fixation in the dredged *R. nodastrella*). In *H. thielei*, the contribution of DIC fixation to total biomass production on dissolved carbon was 16 to 17%. In ambient water, the contribution of DIC fixation to total prokaryotic production was only 4% (Table 2). DIC incorporation represents a substantial fraction of the total dissolved C incorporation of sponge–microbe consortia in ultra-filtered seawater.

**DISCUSSION**

This study provides the first functional evidence of carbon incorporation from ambient dissolved organic and inorganic carbon by sponge–microbe consortia of deep water coral reefs. The association with prokaryotes apparently enables the sponges to exploit carbon sources that are generally believed to be unavailable to these organisms (Simpson 1984). Moreover, we found evidence for ammonia oxidation that may have fuelled the observed chemosynthetic metabolism of the sponge–microbe consortia. We are aware that our data set is not exhaustive. Replicates are limited and variations between replicates were occasionally substantial, an inherent difficulty of collecting sufficient live sponge material for experiments from these deep water coral reefs. However, our results are consistent overall and contribute to a better understanding of the productivity of sponges on cold water coral reefs.

**Dissolved organic carbon incorporation in sponges**

We established leucine incorporation into proteins in the deep water coral mound sponges *Higginsia thielei* and *Rossella nodastrella*. Protein synthesis in sponges in the absence of particulate organic matter (ultra-filtered seawater) is most likely due to DOM assimilation. This implies that these sponges actually take up and incorporate bulk DOM from ambient water in a manner comparable to DOM removal by the sponge *Theonella swinhoei* (Yahel et al. 2003). *H. thielei* and *R. nodastrella* harbor sponge-associated prokaryotes that likely mediate DOM assimilation (Wilkinson & Garrone 1979, Reiswig 1988). The choanocytes of the sponges may also consume DOM (taking into consideration their resemblance to DOM-consuming choanoflagellates, Sherr 1988, Tranvik et al. 1993). At nanomolar concentrations; however, leucine is primarily incorporated into proteins by prokaryotic rather than eukaryotic cells (Kirchman et al. 1985). Therefore, we...
assumed that during our 5 h incubations protein synthesis was performed predominantly by the sponge-associated prokaryotes. Most of the acquired DOM for synthesis of prokaryotic biomass probably came from the ambient water, but we cannot exclude input of exudates by sponge cells and by cell lysis during the incubations.

Leucine-based carbon production was 2 to 4 nmol C cm\(^{-3}\) sponge d\(^{-1}\), which is evidently higher than prokaryote production in ambient bottom water (0.03 to 0.04 nmol cm\(^{-3}\) d\(^{-1}\)). Whether DOM incorporation by associated prokaryotes is beneficial to the sponge cells is still unknown. Attributing production to synthesis of prokaryotic biomass probably came from associated prokaryotes. Most of the acquired DOM for incorporation; they were 0.7 and 4.5 nmol cm\(^{-3}\) sponge d\(^{-1}\) for \(H.\) \(thielei\) and \(R.\) \(nodastrella\), respectively. These rates of incorporation were orders of magnitude lower than nitrification estimates of shallow water tropical sponges (Corredor et al. 1988, Diaz & Ward 1997, Hentschel et al. 2002) and, more recently, for temperate sponges (Jiménez & Ribes 2007). Our estimated nitrite production rates by \(H.\) \(thielei\) and \(R.\) \(nodastrella\) were orders of magnitude lower than nitrification estimates of shallow water tropical sponges (Corredor et al. 1988, Diaz & Ward 1997), but higher than ammonia oxidation rates reported for a deep-sea hydrothermal plume (Lam et al. 2004). Our nitrification rates indicate that ammonia is an energy source for chemolithoautotrophy in \(H.\) \(thielei\) and \(R.\) \(nodastrella\).

Ammonium limitation of bicarbonate fixation by \(H.\) \(thielei\) and \(R.\) \(nodastrella\) could not be established, despite the observed increases of bicarbonate fixation after ammonium sulfate addition. Sponges showed net excretion of ammonium during 24 h incubations regardless of (NH\(_4\))\(_2\)SO\(_4\) addition. Moreover, ambient ammonium concentrations near the reef bottom of coral mounds were quite high (140 to 298 nM), comparable to NH\(_4\)+ concentrations in a deep sea hydrothermal plume, with ammonia oxidation rates of up to 91 nM d\(^{-1}\) (Lam et al. 2004). Therefore, it is unlikely that ammonia oxidation was limited by NH\(_4\)+ in \(H.\) \(thielei\) and \(R.\) \(nodastrella\). This is surprising, because NH\(_4\)+ may be a profitable energy source for chemotrophs in such oligotrophic habitats. There may be other pathways of bicarbonate fixation (e.g. inorganic sulfur oxidation); it is more likely that nitrifying \(Bacteria\) are not the sole agents of nitrification in sponges. In our sponges, 11 to 18% of FISH positive prokaryotes were \(Archaea\), with a dominance of \(Crenarchaeota\). There is recent evidence that certain \(Crenarchaeota\) are capable of oxidizing ammonia and fixing CO\(_2\) (Könecke et al. 2005). The sponge-associated \(Crenarchaeote\) \(Cenarchaeum\) \(symbiosum\), which was isolated from the sponge \(Axinella\) \(mexicana\) (Preston et
al. 1996), appears also to have the capacity to oxidize ammonia for energy and incorporate CO₂ (Hallam et al. 2006). The ammonia monoxygenase which catalyzes ammonia oxidation may be different between Bacteria and Archaea (Francis et al. 2005, Hallam et al. 2006). Therefore allylthiourea, which has, as far as we know, only been related to ammonia oxidizing and methane oxidizing Bacteria (Bédard & Knowles 1989), may not operate as an inhibitor of ammonia oxidation in Archaea. This implies that sponge-associated Archaea may have contributed to bicarbonate fixation.

We conclude that bicarbonate fixation occurs in Rossella nodastrella and Higginsia thielei and that we underestimated bicarbonate fixation by not accounting for the bicarbonate fixed in DOC. We argue that bicarbonate fixation may at least be partly due to sponge-associated nitrifying prokaryotes.

Significance of dissolved carbon incorporation by cold water coral reef sponges

Bicarbonate fixation rates in Higginsia thielei and Rossella nodastrella fell within the range of carbon production estimated with the leucine incorporation method, even without accounting for production of 14C-DOC by the sponge–microbe consortia. This suggests that bicarbonate fixation was quantitatively more important than leucine incorporation in the deep sea sponges. Strictly autotrophic prokaryotes may not be able to incorporate the leucine supplied into proteins. Therefore we added the DIC fixation rates to the leucine based carbon production rates to arrive at ‘total dissolved C assimilation rates’ of around 4.5 nmol C cm⁻³ sponge d⁻¹ in H. thielei (685 µmol C cm⁻³) and 4 to 9 nmol C cm⁻³ sponge d⁻¹ in R. nodastrella (542 µmol C cm⁻³). If sponge-associated prokaryotes are responsible for this fixation rate, prokaryote production in sponges was 2 orders of magnitude higher than the production in ambient water. The specific growth rate in sponges was, however, 5 to 6 times lower than the planktonic prokaryote rate in bottom water; the disparity may be explained by our observation of sponge prokaryote densities 3 orders of magnitude higher than in ambient water. The sponges may keep prokaryote growth under control and antimicrobial substances may inhibit growth of associated prokaryotes (e.g. Hentschel et al. 2001).

Assuming that sponge–microbe consortia rely mainly on dissolved carbon for their carbon production (key carbon source DOM, Reiswig 1990), we may estimate sponge biomass doubling times of up to 287 yr for Higginsia thielei and up to 150 yr for box-cored Rossella nodastrella. These times seem unrealistically long, even at the slow growth rates recorded for such cold water sponges (Dayton 1979, Leys & Lauzon 1998). Therefore, it is likely that these sponges filter particulate organic carbon in addition to consuming dissolved carbon.

Assuming a 2% assimilation efficiency of dissolved C by the sponge–microbe consortia (= growth yield of planktonic prokaryotes in the deep sea, Reinthaler et al. 2006), we arrive at a gross dissolved carbon consumption of 225 nmol C cm⁻³ d⁻¹ for the sponge–microbe consortium in Higginsia thielei and 200 to 350 nmol C cm⁻³ d⁻¹ in Rossella nodastrella. To obtain a comparable amount (225 and 200 to 350 nmol C cm⁻³ sponge d⁻¹, respectively) of particulate carbon in the form of planktonic prokaryotes, H. thielei would have to filter 0.008 cm³ cm⁻³ sponge s⁻¹ and R. nodastrella 0.008 to 0.014 cm³ cm⁻² sponge s⁻¹ (based on the ambient prokaryote biomass, viz. 3.58 x 10⁵ cm⁻³ and a C content of 10 fg C cell⁻¹) at 100% retention efficiency. Such pumping rates fall within the reported ranges of 0.002 to 0.84 cm³ cm⁻³ sponge s⁻¹ for pumping (Simpson 1984). The assumed retention efficiency is at the higher end of reported particle retention rates of 28 to 99% (e.g. Reiswig 1981, Pile 1997, Yahel et al. 2007). The efficiency with which POC is retained is orders of magnitude higher than for dissolved carbon; thus, despite its lower concentration (0.3 µM prokaryotic carbon), POC is as quantitatively abundant for sponges as dissolved carbon at the assumed pumping rate of 0.01 cm³ cm⁻³ sponge s⁻¹. With an assimilation efficiency of 2% for dissolved carbon and 40% for POC, POC feeding (100% retention, pumping rate of 0.01 cm³ cm⁻³ sponge s⁻¹) yields 15 to 26 times more carbon for synthesis (>90% of the complete diet) than dissolved carbon. We argue that H. thielei and R. nodastrella use dissolved as well as particulate carbon, with POC as the most important food source. It remains to be seen whether the capacity to use dissolved carbon is crucial for the survival and maintenance of these sponges during periods of low particulate food concentrations in the deep sea.

CONCLUSIONS

In cold water coral reef systems in the dark ocean, the sponges Higginsia thielei and Rossella nodastrella assimilated dissolved carbon at ambient concentrations. Chemoautotrophy was found to be as quantitatively important as heterotrophy on DOC for these sponges. Energy for CO₂ fixation by the sponges was partly derived from ammonia oxidation. The carbon incorporation of sponge–microbe consortia, along with their filter-feeding abilities, provide these sponges with a suite of nutritional capabilities in their oligotrophic, cold water coral reef habitat.
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LITERATURE CITED


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