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Functional convergence of microbes associated with temperate marine sponges

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Summary

Most marine sponges establish a persistent association with a wide array of phylogenetically and physiologically diverse microbes. To date, the role of these symbiotic microbial communities in the metabolism and nutrient cycles of the sponge-microbe consortium remains largely unknown. We identified and quantified the microbial communities associated with three common Mediterranean sponge species, *Dysidea avara*, *Agelas oroides* and *Chondrosia reniformis* (*Demospongiae*) that cohabitate coralligenous community. For each sponge we quantified the uptake and release of dissolved organic carbon (DOC) and nitrogen (DON), inorganic nitrogen and phosphate. Low microbial abundance and no evidence for DOC uptake or nitrification were found for *D. avara*. In contrast *A. oroides* and *C. reniformis* showed high microbial abundance (30% and 70% of their tissue occupied by microbes respectively) and both species exhibited high nitrification and high DOC and NH_4^+ uptake. Surprisingly, these unique metabolic pathways were mediated in each sponge species by a different, and host specific, microbial community. The functional convergence of microbial consortia found in these two sympatric sponge species, suggest that these metabolic processes may be of special relevance to the success of the holobiont.

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Introduction

The ways in which organisms obtain the carbon and energy needed for growth and cellular functioning is highly diverse across the different domains of the tree of life. Organisms in the domain *Eukarya* fall into two basic metabolic strategies in terms of carbon and energy source and electron donor (chemoorganoheterotrophs and photolithoautotrophs); in contrast, prokaryotes exhibit a diverse range of metabolic strategies, with numerous potential combinations (Madigan *et al*., 2003). Partnerships between microbes and metazoans can provide the consortium with alternative metabolic pathways not available for eukaryotes. A major challenge in environmental sciences is to understand how microbes become the biogeochemical engineers of life on Earth and identify the processes that control their activity at both the molecular and ecosystem levels (Falkowski *et al*., 2008).

Marine sponges are ancient metazoans that dominate many of the hard-bottom benthic substrates around the world (Hooper and van Soest, 2002, Díaz and Rützler, 2001). Sponges filter large volumes of water, up to 100 000 times their own volume per day (Weisz *et al*., 2008) and up to several cubic meters per individual per day. Sponges are also efficient filter feeders that can clean up the water of most microbes and organic particles in the 0.2–2 μm size range (Pile, 1997; Coma *et al*., 2001). With a lower efficiency, they can also remove larger particles up to a few hundred microns (Pile, 1997; Ribes *et al*., 1999) and smaller particles such as viruses (Hadas *et al*., 2006). As a result of the oxidation of the particulate organic matter ingested by sponges, release of inorganic dissolved compounds such as ammonia and phosphate is expected (Fig. 1A). Release of dissolved organic carbon, nitrogen and phosphorus (DOC, DON and DOP respectively) is expected as a result of incomplete oxidation of the ingestion matter and due to the excretion of metabolic waste (Brusca and Brusca, 1990). These characteristics make sponges potential key players in benthic–pelagic coupling in ecosystems where they are abundant (Gili and Coma, 1998).

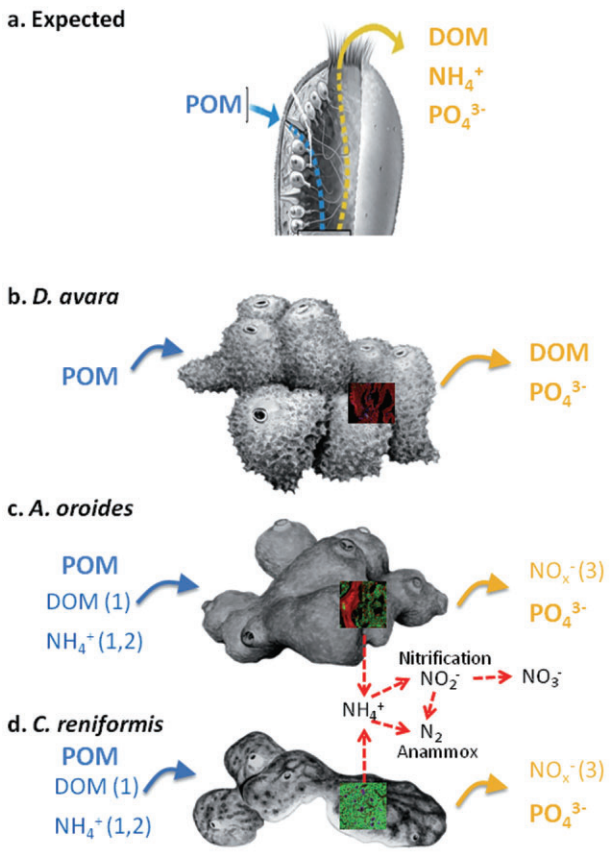

Fig. 1. Expected (A) and observed (B–D) nutrient fluxes in the three sponge species studied. Blue arrows indicate removal and yellow arrows indicate excretion. Bolded compounds names indicate that the flux is due to sponge metabolism. Un-bolded compound names indicate process related to microbial symbionts. Numbers indicate potential processes involved in the observed fluxes: (1) microbial heterotrophy, (2) microbial photoautotrophy, (3) microbial chemoautotrophy (nitrification and anammox indicated by red arrows). Pictures are from confocal microscopy of the sponge tissue using FISH with universal bacteria probes (bright green area) to visualize the relative abundance of symbiotic bacteria in each sponge. POM, Particulate organic matter; DOM, Dissolved organic matter.

Most marine sponges establish a persistent association with microbes including archaea, bacteria and protists. Microbial associates are hypothesized to contribute to the health and nutrition of sponges in different ways, such as by producing protective antibiotics, acquiring limiting nutrients and processing metabolic waste (Hoffmann *et al*., 2005; Taylor *et al*., 2007; Siegl *et al*., 2008). Based on the density of the microbial communities they host, sponges can be divided into 'high-microbial-abundance' (HMA; formerly 'bacteriosponges', e.g. Reiswig, 1981) which are sponges that contain dense and host-specific microbial populations that exceed the microbial density of the surrounding water by two to four orders of magnitude (e.g. Weisz *et al*., 2008). HMA species have dense tissues and low pumping rates (Siegl *et al*., 2008; Weisz *et al*., 2008 and references therein). In 'low-microbial-abundance' (LMA) sponges, the microbial community resembles the nearby seawater, both in concentration (Vacelet and Donadey, 1977; Reiswig, 1981; Wilkinson, 1983; Hentschel *et al*., 2006) and in phylogenetic composition (Schmitt *et al*., 2007). LMA species have well-irrigated tissues and high specific pumping rates.

Most of our current knowledge about the microbes associated with marine sponges is based on 16S rRNA gene library construction, functional gene surveys and metagenomics, which are used to infer alternative metabolic routes for sponge metabolism. These metabolic routes include a variety of processes with alternative energy (photo- or chemotrophic) and carbon (hetero- or autotrophic) sources under different oxygen conditions (see Hoffmann *et al*., 2005; 2009; Hentschel *et al*., 2006; Taylor *et al*., 2007). An outcome of each of these processes is the growth of microbial biomass in the consortium, but the adaptive value of these processes to the host sponge and/or the consortium is not well understood. Moreover, while a phylogenetically and physiologically diverse array of microbes associate with sponges, their roles in nutrient cycles remains largely unknown (Hentschel *et al*., 2006; Taylor *et al*., 2007; Moya *et al*., 2008; Hoffmann *et al*., 2009).

To elucidate the relationships between sponges, their microbial symbionts and nutrient cycles, we use molecular tools to quantify the microbial community composition and nutrient uptake and excretions, for three Mediterranean desmosponges *Dysidea avara*, *Agelas oroides* and *Chondrosia reniformis*. The selected species were formerly classified as either LMA (*D. avara*) or HMA (*A. oroides* and *C. reniformis*) on the basis of earlier qualitative observations (Turon *et al*., 1997; Vacelet and Donadey, 1977; Bayer *et al*., 2008). In a previous study using incubation chambers, we found contrasting dissolved nitrogen (DN) fluxes and a lack of balance between nitrogen released and removed from particulate food (Jiménez and Ribes, 2007). We suggested that alternative sources of organic nitrogen, such as dissolved organic nitrogen, might have been used by the consortium. In this study, we used a direct sampling method to compare the dissolved and particulate content of the water inhaled and exhaled by the sponge. This method allowed us to reliably examine the uptake and excretion of DON and DOC and address the hypothesis that the ability of sponges to handle dissolved compounds and the resulting nutrient flux is related to particular metabolic processes mediated by specific microbial associations. Thus, marked differences in the metabolic output should exist between species hosting contrasting microbial associates.

Results

Nutrient removal and excretion

The nutrient budget of the LMA sponge *D. avara* was markedly different from the budgets of the HMA sponges (*C. reniformis* and *A. oroides,* Fig. 1). While HMA sponges removed large amounts of dissolved organic carbon (8–10 μmol l^{-1} DOC) from the inhaled water, DOC was not significantly excreted or removed by *D. avara* (Table 1). DON was significantly excreted by *D. avara* but no differences were found between inhaled and exhaled concentrations in the HMA species. Similarly, NH_4^+ was removed by both *C. reniformis* (47%) and *A. oroides* (41%) but not by *D. avara*. The NO_x^- (NO_2^-+NO_3^-) budget also highlighted differences between *D. avara* and the other two species. *C. reniformis* and *A. oroides* had significant net excretion of NO_x^-, increasing ambient concentrations > 35%, whereas *D. avara* did not show significant differences between inhaled and exhaled concentrations (Table 1). Significant amounts of phosphate (PO_4^{3-}, 24–55%) were excreted by all three sponges (Table 1).

Microbial abundance

Each of the three sponge species support a specific microbial community containing both bacteria and archaea (Fig. 2) but in very different proportions. Bacteria dominated the microbial community in *A. oroides* and *C. reniformis* forming a compact layer, so dense, that single-cell enumeration was impossible in most of the sponge tissue (Figs 2A and 3). Among archaea, only *A. oroides* hosted both *Crenarchaea* and *Euryarchaea* (Figs 2B and 3). *Dysidea avara* had the lowest microbial abundance per tissue volume, with only 6% of the sponge volume occupied by microbes (Figs 2 and 3). In contrast, ~ 30% of the tissue volume of *A. oroides* was occupied by microbes (28% bacteria, 2% *Crenarchaea* and 0.1% *Euryarchaea*). The highest abundance of microbes was found in *C. reniformis*, with ~ 70% of its tissue occupied (60% bacteria and 6% *Crenarchaea*) (Figs 2 and 3). Using a non-EUB probe as negative control excluded the possibility of false non-specific or auto fluorescence in our measurements (Fig. S2).

Ammonium-oxidizing groups

Ammonium uptake and excretion of NO_x^- (nitrification) was detected in *C. reniformis* and *A. oroides* but not in *D. avara*. The presence and diversity of potential nitrifiers, including bacteria and archaea, was analysed by targeting *amoA* genes using PCR-based techniques. Surprisingly, a unique population of ammonia oxidizers microbes was present in each of the three sponges and these belong to different and remote taxonomic groups. *Dysidea avara* samples yielded positive PCR products only with β-proteobacteria primers (44 clones sequenced); *A. oroides* samples yielded positive PCR products only with archaeal primers (65 clones sequenced); *C. reniformis* samples yielded positive PCR product only with γ-proteobacteria primers (83 clones sequenced). Water column samples were positive only with archaeal primers (50 clones sequenced).

Four different sets of primers were used to amplify the 16S rRNA genes of putative anammox bacteria, including Planctomycetes and Scalindua (Table 2). All of the PCRs were compared with a positive control culture of Brocardia sp. (Sànchez-Melsió *et al.*, 2009). Neither the sponge samples nor water column samples showed

Table 1. Average concentration (± SD) and average net differences of dissolved nutrients at the inhaled and exhaled water for each species.

		Concentration (mean ± SD)		Net difference (In-Ex)		
Specie	Nutrient	Inhaled (μM)	Exhaled (μM)	N	Con (μM)	% Inhaled
D. avara	DOC	84 ± 16	85 ± 18	12	**–0.7** ± 2.8	
	NH_4^+	2.76 ± 0.89	2.47 ± 0.87	7	0.29 ± 1.20	
	NO_x^-	2.28 ± 0.21	2.12 ± 0.16	12	0.16 ± 0.27	
	DON	4.48 ± 0.50	4.96 ± 0.85	5	**–0.48** ± 0.51	
	PO_4^{3-}	0.10 ± 0.03	0.12 ± 0.03	12	**–0.02** ± 0.01*	24
C. reniformis	DOC	78 ± 7	70 ± 5	9	7.8 ± 3.4*	10
	NH_4^+	3.33 ± 0.63	1.64 ± 0.59	11	1.69 ± 1.15*	47
	NO_x^-	2.86 ± 0.32	3.86 ± 0.43	11	**–0.99** ± 0.27*	35
	DON	4.76 ± 0.39	4.90 ± 1.04	9	**–0.14** ± 1.13	
	PO_4^{3-}	0.10 ± 0.03	0.12 ± 0.02	10	**–0.02** ± 0.01*	29
A. oroides	DOC	80 ± 6	70 ± 4	9	9.9 ± 4.0*	12
	NH_4^+	2.40 ± 0.60	1.82 ± 0.67	15	0.57 ± 0.8*	41
	NO_x^-	2.61 ± 0.34	3.60 ± 0.58	15	**–0.99** ± 0.26*	37
	DON	4.90 ± 0.26	4.89 ± 0.47	15	0.01 ± 0.25	
	PO_4^{3-}	0.07 ± 0.02	0.10 ± 0.04	15	**–0.04** ± 0.02*	55

N: number of InEx pairs. The net nutrient difference is presented as the mean of paired differences and means of the percentage of the difference from the inhaled concentration (standard deviations were omitted for clarity of presentation). Negative values denote excretion (bolded). The asterisk (*) denotes significant differences between inhaled and exhaled concentrations ($P < 0.05$, two-tailed Wilcoxon test).

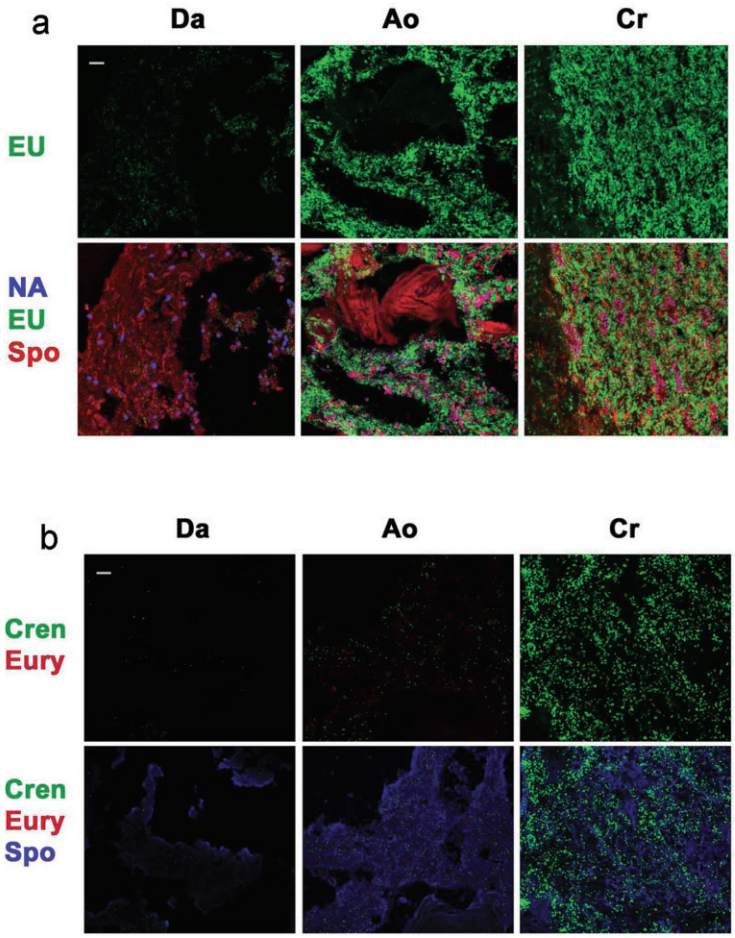

Fig. 2. Representative confocal microscopy (CLSM) images of the tissue of the three sponge species (Da, *Dysidea avara*; Ao, *Agelas oroides*; Cr, *Chondrosia reniformis*). CARD-FISH was used to visualize different microbial populations (upper panels in A and B) in combination with nucleic acid and sponge cells staining (lower panels in A and B). Different probes had different colours as follow: (A) *Eubacteria*: green for *Eubacteria* (EU), red for sponge cells (Spo) and blue for nucleic acids (NA); (B) *Archaea*: green for *Euryarchaea* (Eury), red for *Crenarchaea* (Cren) and blue for sponge cells (Spo).

positive PCRs products for 16S rRNA genes from anammox bacteria.

Analysis of photoautotrophic microbes

The presence and diversity of phototrophic microbes, potential consumers of NH_4^+, NO_x^- and PO_4^- and producers of DOC, was analysed by small subunit-rRNA and DGGE fingerprinting and sequence identification using both general bacterial primers and specific cyanobacterial primers. To insure that DGGE results were representative we use several replicates per sample for each of the molecular markers analysed (bacterial and cyanobacterial 16S rRNA genes). The different replicates were highly reproducible yielding nearly identical DGGE patterns. To confirm the identity of the DGGE bands, we sequenced DNA retrieved from the same positions in several replicates and sponge samples. All three sponges seem to

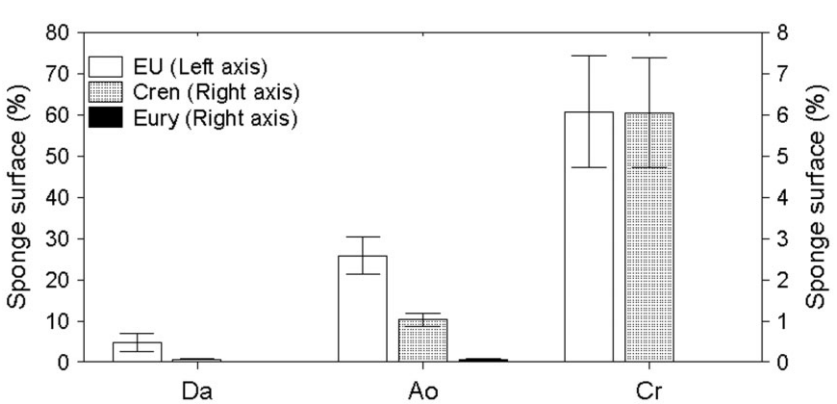

Fig. 3. Percentage of the sponge tissue occupied by different microbial populations. Abbreviations as in Fig. 2.

Table 2. Primers used for PCR amplification of *amoA* and 16S rRNA genes.

Primer sets	Target group	Reference
Nitrifiers	*amoA*	
amoA-1F/*amoA*-2R	β-*Proteobacteria*	Rotthauwe *et al*. (1997)
amoA-3F/*amoB*-4R	γ-*Proteobacteria*	Purkhold *et al*. (2000)
Arch-*amoAR*/Arch-*amoAF*	*Crenarchaeota*	Francis *et al*. (2005), de la Torre *et al*. (2008)
Anammox	16S rRNA	
Brod541F/Brod1260R	Candidatus *Scalindula*	Penton *et al*. (2006)
Pla46F/Amx368R	Anammox bacteria	Sànchez-Melsió *et al*. (2009)
Amx368F/BS820R	Anammox bacteria	Amano *et al*. (2007)
Amx368F/1392R	Anammox bacteria	Mohamed *et al*. (2010)
Bacteria		
357F/907RM	Universal	Muyzer *et al*. (1997), Sánchez *et al*. (2007)
CYA106F/CYA781 Ra/b	*Cyanobacteria*	Nübel *et al*. (1997)

PCR conditions are indicated in the cited reference.

harbour both photoautotrophic and photoheterotrophic microbial components. DGGE results showed significant differences between the three sponge species investigated (Table 3). As shown in Table 3 only DGGE bands SJ1, 3, 5 and 9 (most affiliated to chloroplasts of eukaryotes) were shared by more than one species of sponge. Sequences obtained from those bands and the ones at the same position in the gel were found identical (99–100% similarity). Sequencing specific bands from our 16S rRNA-DGGE profiles indicate the presence of several bands that shared some similarity to the photoheterotrophic members of *Chloroflexi*, a green non-sulfur group of bacteria (Table 3). These phylotypes were well represented in *C. reniformis* (DGGE bands B2 and Sj15) and *A. oroides* (DGGE bands B5, B13 and B15, and Sj12) but were absent in *D. avara*. In addition, bands related to other bacteria, such as Acidobacteria (DGGE bands B11) and Proteobacteria (DGGE bands B1 and Sj13–14), were also present in *A. oroides* and *C. reniformis* respectively (Table 3).

Using 16S rRNA primers specific to cyanobacteria, our analysis revealed a low level of cyanobacteria diversity with a distinct pattern for each of the three sponge species. Analysis of the most dominant DGGE bands revealed several photoautotrophic cyanobacteria closely related to unicellular *Chroococcales* of the genus *Synechococcus* (Table 3). These *Synechococcus*-like representatives may be an important part of the phototrophic microbial community associated with *D. avara* (DGGE band Sj6) and *A. oroides* (DGGE band Sj5). Indeed, *Synechococcus*-like chroococcales and phototrophic eukaryotes were the only phototrophic organisms associated with *D. avara*. In both *A. oroides* and *D. avara* sponges, the DGGE bands Sj1, 3, 9 and 10 were affiliated with chloroplasts of diatoms and pelagophytes (Table 3).

Phylogenetic analysis of ammonia oxidizers

Clone sequences of β-proteobacteria *amoA* in *D. avara* and archaea in *A. oroides* and water column samples were used for phylogenetic analyses. Rarefaction curves for the three clone libraries approached an asymptote when clustering sequences at a 97% similarity threshold (Fig. S1), indicating that we were close to sample the total diversity of each functional group. From the 83 clone sequences of γ-proteobacteria amoA in *C. reniformis*, seven showed a similarity of 100% with *Nitrosococcus oneani* amoA gene. The remaining 76 clone sequences presented a similarity of 99–100% between them but no significant similarity with amoA sequences from the Gene bank.

The β-proteobacteria *amoA* genes from *D. avara* fell into three different clusters (Fig. 4). Cluster 1 was dominated by *D. avara* sequences, which shared ~ 99% amino acid similarity with uncultured β-proteobacteria ammonia oxidizers bacteria from an aquarium sand biofilter in Tokyo [Cluster B2 (AB373584, AB373599) and cluster B3 (AB373428), respectively, in Urakawa *et al*., 2008]. Sediment sequences from three different sites also fell into cluster 1. Cluster 2 included sponge sequences from the Florida Keys and the deep Caribbean that were unrelated to *D. avara* sequences. Cluster 3 included seven *D. avara* clones that were 98–99% identical to uncultured *Nitrosospira* spp. clones from Mediterranean, Caribbean and Pacific Ocean marine sponges (sequences with Accession No. EF5296XX; Bayer *et al*., 2008). *Dysidea avara* clones in cluster 3 were also 98% identical to uncultured bacteria from sediments and sequences from the sponge *Mycale laxissima* (Florida Keys) reported by Mohamed and colleagues (2010).

Crenarchaeal ammonia oxidizers from *A. oroides* and water column samples primarily fell into three of the five different clusters (Fig. 5). Cluster 1 contained most of our water column samples, as well as seawater samples from the Pacific, Atlantic and Arctic oceans. Cluster 1 also included a few sequences of archaea associated with benthic invertebrates (three sequences from *A. oroides*, five sequences from sponges from Sydney (Australia) and

Table 3. 16S rRNA gene sequence identities of bacterial DGGE bands from sponge samples.

DGGE band accession No.	16S rRNA gene/primer set	Sponge	Nearest relative/GenBank accession No.	Source location	% identity	Phylogenetic affiliation	Reference
B1-JN314396	Universal *Bacteria*	*C. reniformis*	Uncultured alphaproteobacteria clone SHFH749; FJ203650	*Montastraea faveolata* San Cristobal, Panamá	86	*Alphaproteoacteria*	Sunagawa *et al.* (2009)
B2-JN314397	Universal *Bacteria*	*C. reniformis*	Uncultured sponge symbiont clone PAWS52F; AF186417	*Theonella* sp. Caroline Isl, Palau	94	*Chlorofexi*	Hentschel *et al.* (2002)
B5-JN314398	Universal *Bacteria*	*A. oroides*	Uncultured *Chloroflexi* clone W04IS5E12; EF629745	*Ircinia strobilina* Conch reef Key Largo, Fl	90	*Chloroflexi*	Mohamed *et al.* (2008)
B11-JN314399	Universal *Bacteria*	*A. oroides*	Uncultured bacterial clone CC13; DQ247947	*Corticium* embryo Palau	85	*Acidobacteria*	Sharp *et al.* (2007)
B13-JN314400	Universal *Bacteria*	*A. oroides*	Uncultured *Chloroflexi* clone E29; FJ529321	*Svenea zeai* San Salvador Is, Bahamas	79	*Chloroflexi*	Lee *et al.* (2009)
B15-JN314401	Universal *Bacteria*	*A. oroides*	Uncultured *Chloroflexi* clone AD020; EF076163	*Antho chartacea* Southeastern Australia	83	*Chloroflexi*	Taylor *et al.* (2007)
Sj1-JN314402	Specific *Cyanobacteria*	*D. avara* and *A. oroides*	Plastid *Skeletonema pseudocostatum*; X82155	*Skeletonema pseudocostatum*	99	Diatom chloroplast	Medlin *et al.* (1995)
Sj3-JN314403	Specific *Cyanobacteria*	*D. avara* and *A. oroides*	Plastid *Skeletonema pseudocostatum*; X82155	*Skeletonema pseudocostatum*	98	Diatom chloroplast	Medlin *et al.* (1995)
Sj5-JN314404	Specific *Cyanobacteria*	*D. avara* and *A. oroides*	Uncultured sponge symbiont clone Hg5a2D10; EU817117	*Haliclona* sp. northern Pacific, Monterey harbour	94	*Cyanobacteria*	Sipkema *et al.* (2009)
Sj6-JN314405	Specific *Cyanobacteria*	*D. avara*	Uncultured sponge symbiont clone Hg5a2D10; EU817117	*Haliclona* sp. Northern Pacific, Monterey harbour	99	*Cyanobacteria*	Sipkema *et al.* (2009)
Sj9-JN314406	Specific *Cyanobacteria*	*D. avara* and *A. oroides*	Plastid *Aureococcus anophagefferens* strain CCMP	*Aureococcus anophagefferens*	97	Pelagophyte chloroplast	Ong *et al.* (2010)
Sj10-JN314407	Specific *Cyanobacteria*	*A. oroides*	Plastid uncultured phototrophic eukaryote clone HF770_25L02; EU361155	North Pacific Subtropical Gyre	94	Eukaryotic chloroplast	Pham *et al.* (2008)
Sj12-JN314408	Specific *Cyanobacteria*	*A. oroides*	Uncultured *Chloroflexi* bacterium clone CC10; DQ247942	*Corticium embryo* Palau	90	*Chloroflexi*	Sharp *et al.* (2007)
Sj13-JN314409	Specific *Cyanobacteria*	*C. reniformis*	Uncultured bacterium clone i153; FM160905	*Aplysina fulva* Brazil	97	*Deltaproteobacteria*	Hardoim *et al.* (2009)
Sj14-JN314410	Specific *Cyanobacteria*	*C. reniformis*	Uncultured bacterium clone 27H6; EU 183804	*Rhopaloeides odorabile* Pelorus Isl, Australia	97	*Deltaproteobacteria*	Webster *et al.* (2008)
Sj15-JN314411	Specific *Cyanobacteria*	*C. reniformis*	Uncultured *Chloroflexi* clone E117; FJ529342	*Svenzea zeai* Salvador Isl, Bahamas	95	*Chloroflexi*	Lee *et al.* (2009)

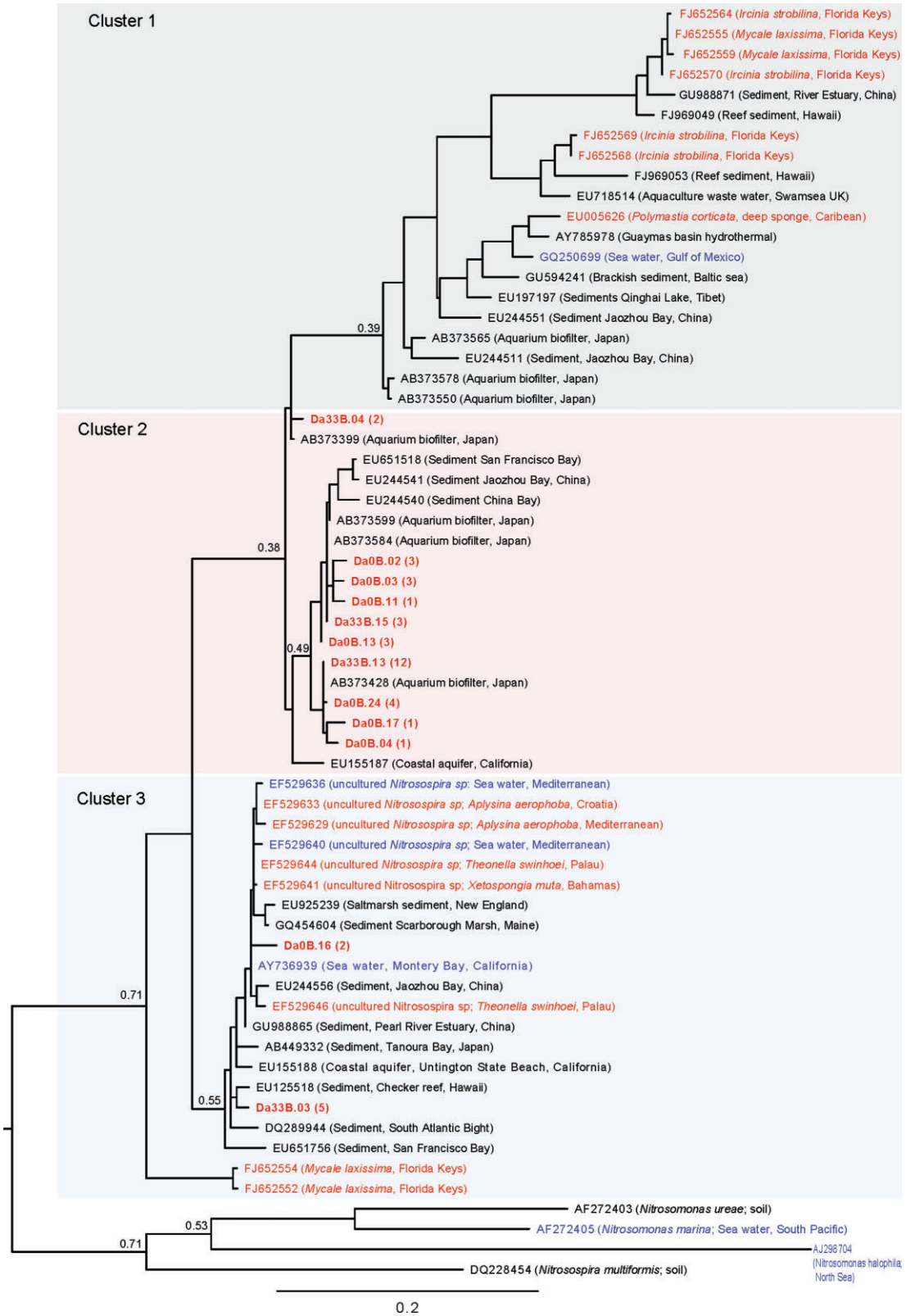

Fig. 4. Maximum likelihood phylogenetic tree based on β-proteobacteria *amoA* DNA sequences (420 informative positions). Sequences for *D. avara* are in red and boldface and database sequences for other marine sponges are in red. Database water column sequences are in blue. Sequences are identified by GenBank accession numbers. Bootstrap values for important nodes are displayed. Brackets refer to number of sequences.

two sequences from a Red Sea coral). Cluster 3 included most of the *A. oroides* sequences that were related to archaea from the sponge *Luffariella* and from a coral from Panama. None of our sequences fell in cluster 4 with *Cenarchaeum symbiosum*; only two of our water column samples fell into cluster 4 with *Nitrosopumilus maritimus*, which also included sediment, aquaculture and aquaria biofilter samples, as well as ground and freshwater.

Discussion

Observed nutrient fluxes and related microbial types

The nutrient fluxes we observed for the three studied sponges can be related to at least three different metabolic pathways: heterotrophy, photoautotrophy and chemoautotrophy (Fig. 1). Dissolved organic carbon taken up by the sponge is transferred to and metabolized by heterotrophic bacteria and archaea (Wilkinson and Garrone, 1980; De Goeij *et al*., 2008a,b), therefore a high abundance of heterotrophic microbes is expected to result in high DOC removal. Ammonium (NH_4^+) can support both photoautotrophy and chemoautotrophy. While phototrophs use ammonium as the preferred nitrogen source, chemoautotrophic ammonia oxidizers use ammonium as an energy source. In the later case, the decrease in ammonium excretion should be linked with enhanced NO_x^- excretion (Fig. 1). Other chemoautotrophic processes such anammox may also contribute to NH_4^+ removal (Fig. 1), nevertheless annamox bacteria were not detected in any of the three sponge species.

Removal of DOC and microbial abundance

Removal of dissolved organic carbon was associated with high abundance of bacteria and archaea. *Dysidea avara* has the lowest abundance of microbes (6% of the sponge volume occupied by microbes) and accordingly showed no significant DOC removal. In contrast, *A. oroides* and *C. reniformis* had much higher densities of associated microbes (~ 30 and 70% of sponge volume respectively) and consequently, high DOC uptake (average > 10%) was evident in 93% of the In-Ex pairs in *A. oroides* and 100% of the In-Ex pairs in *C. reniformis*.

The ability of sponges to remove large quantities of DOC from the water they pump has been reported for coral reef sponges (Yahel *et al*., 2003; De Goeij and van Duyl, 2007; De Goeij *et al*., 2008a,b), indicating that the removal of DOC by reef sponges may play a major role in the trophic dynamics of coral reefs. In other tropical systems, Southwell and colleagues (2008a) found contrasting levels of DOC uptake for LMA versus HMA, suggesting that total respiration in HMA sponges is largely fuelled by DOM uptake as previously suggested by Reiswig (1981).

While marine sponges are now routinely categorized into the LMA and HMA groups depending on their microbial abundance, these classifications are mostly based on visual assessments either with electron microscopy (Vacelet and Donadey, 1977; Friedrich *et al*., 1999) or by fluorescent *in situ* hybridization (FISH) and polarized/epifluorescent light microscopy (Friedrich *et al*., 1999; Pape *et al*., 2006; Bayer *et al*., 2008). In the HMA species we studied, the microbial populations were arranged in compact and dense structures resembling microbial biofilms. Quantification of the individual cell number in such dense communities is nearly impossible by standard microscopy. Therefore, previous workers, relying on epifluorescent light microscopy and related techniques (Schläppy *et al*., 2010), were forced to select sparse tissue regions which likely result in underestimate of the real microbial densities. We conclude that confocal microscopy is required for reliable quantification of similarly structured microbial communities in HMA sponges. This is the first attempt to quantify the percentage of sponge tissue being occupied by microbial cells in marine sponges using confocal laser scanning microscopy.

Presence of photoautotrophs

Photoautotrophs are potential consumers of DIN and producers of DOC. The unique DGGE profile of each sponge species provides a clear representation of the specific microbial communities that it harbours. Using universal bacterial primers and specific cyanobacterial primers, we identified bacterial phylotypes that are closely related to *Chloroflexi* in *C. reniformis* and *A. oroides* and *Chroococcales* unicellular cyanobacteria in *A. oroides* and *D. avara*. Both groups resemble previously reported photosynthetic symbionts from other sponges worldwide (Hentschel *et al*., 2002; Sharp *et al*., 2007; Taylor *et al*., 2007; Hardoim *et al*., 2009; Lee *et al*., 2009; Sunagawa *et al*., 2009). Only a few bands were related to cyanobacteria and only in *A. oroides* and *D. avara* and none was recovered with a bacterial universal primer, suggesting a low relative abundance for these organisms in our samples. The low abundance of photoautotrophs present in *D. avara*, was apparently insufficient to elicit net ammonia removal. In contrast, high diversity of *Chloroflexi*-like phylotypes recovered by our bacterial and cyanobacterial 16S rRNA gene analysis suggests that this group of potentially photosynthetic organisms is an important symbiont in the investigated species. However, not all *Chloroflexi* bacteria are phototrophs, and thus a phototrophic function of the *Chloroflexi*-like bacteria within the sponge we examined is yet to be confirmed. The overall contribution of phototrophic components to nutrient fluxes in the sponge consortium needs to be further studied.

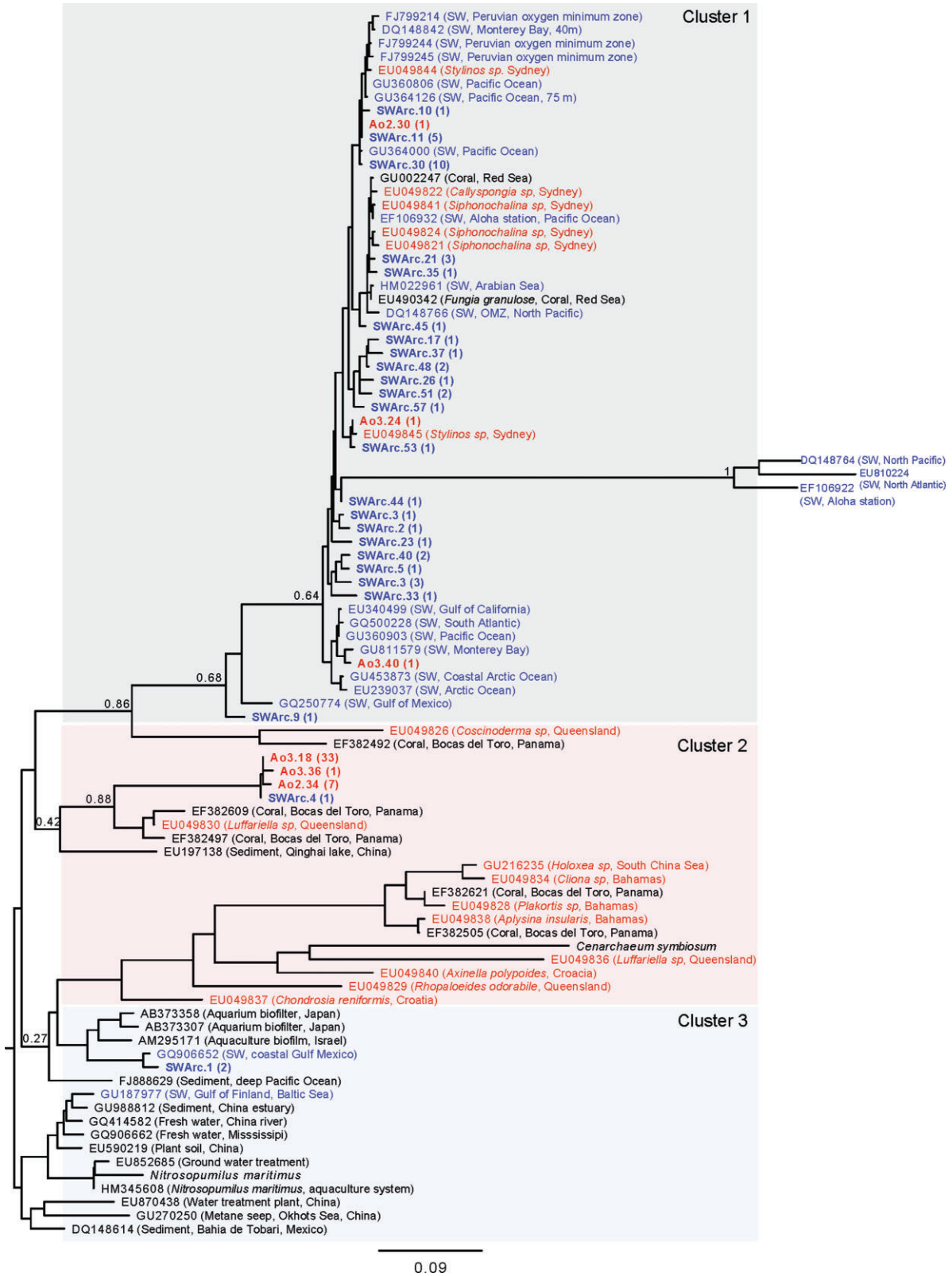

Fig. 5. Maximum likelihood phylogenetic tree based on archaeal *amoA* DNA sequences (570 informative positions). Sequences for *A. oroides* are in red and boldface, sequences for other sponges are in red. Database water column sequences are in blue and water column sequences from our sampling site are in blue and boldface. Database sequences are identified by GenBank accession numbers. Bootstrap values for important nodes are displayed. Brackets refer to number of sequences.

Release of NO_x^- and presence of ammonia oxidizers

Large amounts of excess NO_x^- (> 35% or 1 μmol l^{-1} per litre pumped) were excreted by the two HMA sponges *A. oroides* and *C. reniformis* but not by the LMA sponge *D. avara* (Fig. 1, Table 1). Ammonia oxidation, the first and limiting step in nitrification, can be performed by β- or γ-proteobacteria and by crenarchaeota from Marine Group 1 (Francis *et al*., 2005; 2007). Both groups of microorganisms have been previously reported in sponges (Taylor *et al*., 2007). Each of the three sponge species we studied appears to harbour its own nitrifier group: γ-proteobacteria (putative) in *C. reniformis*, β-proteobacteria in *D. avara* and archaea in *A. oroides*.

The phylogenetic tree based on β-proteobacteria ammonia oxidizers shows that only few of the *amoA* sequences derived from *D. avara* are related to *amoA* sequences of the *Nitrosospira* lineage. Clusters 1 and 2 were dominated by sequences that shared ~ 99% amino acid similarity with uncultured β-proteobacteria ammonia oxidizers from an aquarium biofilter in Tokyo (Clusters B2 and B3, respectively, in Urakawa *et al*., 2008).

The crenarchaeal ammonia oxidizers phylogenetic tree shows that *A. oroides*-derived sequences are clearly separated from their environmental water sample sequences, as well as from seawater samples from different oceans and sediments. Coral-derived *amoA* sequences from Panama and the sponge *Luffariella* sp. from Queensland, Australia, were the most closely related to the *amoA* sequences obtained from *A. oroides* whereas *amoA* sponge sequences reported in Steger and colleagues (2008) [cluster 4] were not related to *A. oroides amoA* sequences.

It was not possible to generate a phylogenetic tree with γ-proteobacteria ammonia oxidizers derived from *C. reniformis* as only 7 sequences were found to be related to a known GenBank sequences (*Nitrosococcus oceani*). Further studies will be performed with the remaining 76 sequences as they can be pointing out the presence of an unknown group of ammonia oxidizers.

A relationship between the amount of nitrification and specific pumping rates in marine sponges has previously been suggested (Weisz *et al*., 2008). Southwell (2007) and Southwell and colleagues (2008b) related nitrification rates to the amount of microbes associated with sponges and pumping rates. They found that 12 of 13 HMA sponges (with low specific pumping rates) showed nitrification, whereas nitrification was not detected in LMA sponges (with high specific pumping rates). Our results are consistent with this interpretation. The similar specific pumping rates of *C. reniformis* and *A. oroides* (both showing high nitrification rates) were three times lower than those of *D. avara* that showed no evidence for significant nitrification (M. Ribes unpubl. data). Active nitrification in HMA sponges with low specific pumping rate may be preventing the toxic effect of ammonia on sponge tissues.

Differences between ammonia oxidation in bacteria and archaea can depend on rates, carbon fixation mechanisms, environmental controls and competitive ability in the biogeochemical process (Francis *et al*., 2007). Crenarchaeal ammonia oxidizers appear to greatly outnumber their bacterial counterparts in soil systems (Leininger *et al*., 2006) and in the marine water column (Francis *et al*., 2005). However, it remains unclear whether archaea or bacteria are the main ammonia oxidizers in sponges (Taylor *et al*., 2007). Our results indicate that both bacteria and archaea may act as the main ammonia oxidizers group depending on the sponge species. The particular case of *A. oroides* shows that even though the bacterial population dominates in microbial abundance, the high nitrification rate of the species is likely attributed to its small archaeal population.

NO_x^- concentration did not change significantly in the water inhaled and exhaled by *D. avara* despite the presence of small populations of ammonia oxidizer microbes. In contrast, Schläppy and colleagues (2010) did measure net NO_x^- excretion by the *D. avara* specimens they studied. The differences between the two studies may be related to methodological biases or to seasonal differences in sponge metabolism. Schläppy and colleagues (2010) used closed vessels and long incubation times (24 h). A comparison of the direct In-Ex method we used with closed vessel incubations indicate that assessing dissolved compound fluxes in sponges is consistently biased by prolonged incubation times (M. Ribes, unpubl. data). Nevertheless, temporal variation in activity and bacterial symbiont composition are also possible because Schläppy and colleagues (2010) worked in early spring (March) whereas our experiments were performed in May–July.

Removal of ammonia

Ammonia uptake is an important source of nitrogen for autotrophic and heterotrophic microorganisms (Kirchman *et al*., 1990, Hoch and Kirchman, 1995), but also a potential energy source for ammonia-oxidizing microorganisms. Data about the ability of sponges to remove DIN (NH_4^+, NO_x^-) from the water column is still scarce and only reported for coral reef sponge (Schubauer, 1988; Díaz and Ward, 1997; Yahel *et al*., 2003; Southwell *et al*., 2008a). While all three sponges we studied contained ammonia oxidizer associate microorganisms, considerable ammonium removal was found only in HMA *A. oroides* and *C. reniformis* (41% and 52% respectively) but not in LMA *D. avara*. HMA sponges also showed high nitrification rates. While it is tempting

to associate nitrification with ammonia removal, since representative of all three potential ammonium consumers were present in each of the studied sponges, a more complex nitrogen pathways may exists. These findings call for attention to DIN consumption that appears as an important mechanism of nutrient acquisition in temperate sponges.

Release of PO_4^{3-}

The three sponges studied showed high and significant net excretion of PO_4^{3-} (~ 20%, 30% and 60% for *D. avara*, *C. reniformis* and *A. oroides* respectively) without any apparent relationship to microbial abundance or composition. To the best of our knowledge, phosphorus metabolism has not previously been studied in marine sponges (Taylor *et al.*, 2007). Our results suggest that sponges are important recyclers of phosphorus in the coralligenous communities. It is also evident that P is not a limiting nutrient for the microbial symbionts of the studied sponges, and that the already know particulate feeding by sponges seems to provide an excess amount of this nutrient for the consortium during the study period.

The composition of microbial components of the studied sponges and the surrounding water was focused on the most abundant groups, which we assumed were the most likely to account for gross metabolic processes. Nevertheless, more detailed analysis (e.g. pyrosequencing) may reveal rare microbial groups unaccounted for here and may bring new insights on specificity of microbial components in marine sponges.

Analysis of the microbial communities associated with the studied sponges indicated that each sponge species harboured a unique microbial community, different from those of the water column, and with a specific set of associated microbes. Surprisingly, these distinct communities converged to similar metabolic pathways in the HMA sponges, exhibiting high nitrification and high DOC and NH_4^+ uptake, and similar net nutrient fluxes. In each consortium, these similar metabolic activities and nutrient dynamic processes were accomplished by contrasting species or microbial communities. The results points to a functional convergence of microbial partners in these sympatric sponge species suggesting that these metabolic processes are of special relevance to the sponge consortium success.

Experimental procedures

Specimen collection

Six specimens from each of the three sponge species (*D. avara*, *A. oroides* and *C. reniformis*) were collected by scuba divers from the coralligenous community of the Montgri Coast (NW Mediterranean Sea, 42°3′N, 3°13′E) at a 15 m depth. Whole specimens were transported within a few hours to the Experimental Aquaria Zone (ZAE) of the Institute of Marine Sciences (ICM-CSIC) and maintained in 125 l tanks with a flow-through system of fresh seawater that renewed the total volume every 15 min.

Parallel to specimen collection, water samples (4 l) were collected from the water column near to the sponge community. Three replicates of 300–500 ml were filtered in the laboratory through 0.2 μm polycarbonate filters. Filters were frozen in liquid nitrogen and stored at –80°C until microbial DNA extraction was performed (see below).

Quantification of nutrient removal and excretion

Nutrient removal and excretion by each sponge specimen was measured using a direct method (Yahel *et al.*, 2005). In each experiment, two 0.55 mm ID Teflon tubes were used to sample simultaneously the water inhaled and exhaled (In-Ex) by the sponge at rates of < 1 ml min^{-1}. One tube was positioned a few millimetres inside the osculum to sample exhaled water, and the other was placed next to the sponge to sample inhaled water. Sampled water was collected in darkened glass bottles kept in ice during the sampling (6–10 h). For each sponge species we collected at least three In-Ex pairs from each of the five collected specimens. Experiments were conducted between May and June 2007. The temperature was recorded at the beginning and end of the experiments.

To avoid contamination from microbes in the water, after the experiments, small tissue samples (1 cm^3) of each individual were positioned in sterile 2 l containers filled with filtered seawater (0.22 μm) for 5 h. Each piece was then rinsed with filtered seawater before being frozen with liquid nitrogen and stored at –80°C for further microscopy observations and molecular analysis.

Analysis of dissolved compounds

Water samples were collected in acid-rinsed, 50 ml plastic bottles, frozen and analysed for inorganic nutrients with an Alliance autoanalyser following the method of Grasshoff and colleagues (1983). The following dissolved compounds were measured: NH_4^+ (ammonium), NO_x^- [NO_3^-+NO_2^-] and PO_4^{3-} (phosphate). The DN (including DIN and DON) concentration was analysed using a Bran-Luebbe AA3 autoanalyser after a double oxidation with UV light and persulfate (Grasshoff *et al.*, 1983). DON concentration was calculated by subtracting the DIN concentration (NH_4^++NO_x^-) from the DN. Samples for DOC determination were filtered through pre-combusted (450°C for at least 2 h) GFF filters with a baked glass filtration system. Water for DOC analysis was collected in 10 ml pre-combusted glass ampoules and acidified with concentrated, trace-metal grade, hydrochloric acid (0.1% final concentration). Ampoules were heat-sealed and stored at –20°C until analysis. DOC concentrations were determined using high-temperature catalytic oxidation method on a Shimadzu TOC-V analyser (Sharp *et al.*, 2002). The accuracy of the DOC determination was evaluated by daily comparisons with DOC reference materials (Yahel *et al.*, 2003).

FISH and confocal microscopy

We used catalysed reporter deposition (CARD)-FISH and confocal laser-scanning microscopy (CLSM) to quantify the microbial community associated with each sponge specimen. Tissue blocks of the sponges fixed in 1% paraformaldehyde were embedded in paraffin, and 10 μm slices were obtained. Three specimen per specie and three slices from each specimen were hybridized using CARD-FISH with probes for Bacteria (Eub 338 I+II+III; Amann *et al*., 1990; Daims *et al*., 1999) and Archaea (Cren 537 and Eury 806; Teira *et al*., 2004), following the protocol described in Pernthalerand collaborators (2002). Non-EUB probe was used as negative control following the same procedures (Non338 5′-ACT CCTACGGGAGGCAGC-3′, Wallner *et al*., 1993). The structure of the sponge was stained with CellMask™ Deep Red (Molecular Probes, Invitrogen, Carlsbad, CA, USA), and the nucleic acids were labelled with Hoechst 33342 (Molecular Probes, Eugene, OR, USA). Samples were observed with a Leica TCS-SP5 confocal spectral microscope (Leica Microsystems Heidelberg GmbH; Mannheim, Germany) using a Plan-Apochromatic 63 × 1.4 (oil HC × PL APO lambda blue objective). A series of images (xyz) was taken in four randomly selected areas of each sample to visualize the emission signal of Alexa 488 (for Eub 338 and Cren 537), Alexa 546 (for Eury 806), Hoechst and CellMask. The resulting images (11 images per area) were processed with Metamorph imaging software (Universal Imaging Corporation, West Chester, PA, USA) in order to calculate the percentage of sponge tissue volume occupied by microbes.

DNA extraction and PCR amplification

Tissue samples (~ 2 mm^3) from each specimen were dissected into small sections using a sterile scalpel. Total genomic DNA was extracted from sponge samples using a DNeasy Tissue Kit (Qiagen) following the manufacturer's protocol with the following modifications: removal of water excess before the DNA extraction; incubation with lysis buffer overnight instead of few hours; final elution in 100 μl of Buffer AE instead of 200 μl (A. Blanquer and S. López-Legentil, pers. comm.). Putative *amoA* gene fragments from the total microbial DNA of proteobacteria and archaea were amplified by PCR with the primers listed in Table 2 and using GoTaq Flexi Dna Polymerase (Promega). For PCR amplification we followed the conditions described in the reference papers with the following modifications: for γ-proteobacteria (Purkhold *et al*., 2000) we ran 35′ cycles and a final elongation step of 10′ was added. For archaea *amoA* (de la Torre *et al*., 2008) we used an annealing temperature of 56°C. Specific 16S rRNA genes for different groups of annamox organisms were amplified by PCR with the primers listed in Table 2 and following original references conditions. Reactions of 25 μl were carried out in an automated thermocycler (Bio-Rad) in parallel for the three sponge species and water column samples. A positive control was used to validate the PCR methodology in each run. An agarose gel was run in order to check PCR products with a molecular weight standard (Low DNA Mass Ladder, Invitrogen). The gel was visualized with UV in the Quantity One-Chemidoc software (Bio-Rad).

Cloning and sequencing

Amplified PCR products for β-proteobacteria (~ 420 bp) and for archaea (~ 570 bp) from two or three individuals were pooled and purified with a PCR Purification kit (Qiagen) and cloned using the TOPO-TA Cloning kit (Invitrogen) according to the manufacturer's instructions. Putative positive colonies were picked and transferred to a multiwell plate with LB-7% glycerol medium plus kanamycin. After an overnight incubation at 37°C the plates were stored at –80°. The presence of the insert in the colonies was checked by PCR reamplification with the original primer set. Positive clones were sent to Macrogen Sequencing Service (Korea) to be purified and sequenced. All chromatograms were visually inspected to minimize the sequencing errors. After this first examination, sequences were submitted to the BLAST search (Altschul *et al*., 1997) for a first phylogenetic affiliation and to remove putative chimeras. Forty-four clones were sequenced for β-proteobacteria ammonia oxidizers from *D. avara,* four of them were chimeric sequences. Sixty-five clones were sequenced for crenarchaeal ammonia oxidizers from *A. oroides* and 50 clones for crenarchaeal ammonia oxidizers from water samples. No chimeric sequences were detected in *A. oroides* or water samples.

Phylogenetic analysis

DNA sequences were aligned with MAFFTusing the slow and iterative refinement method FFT-NS-i (Katoh *et al*., 2002). The alignment was checked manually and edited using Seaview 3.2 (Galtier *et al*., 1996), to retain the longest region that was common to most sequences. Maximum likelihood (ML) phylogenetic trees were constructed with RAxML (Stamatakis, 2006) using the evolutionary model GTR+G + I″ that best fits our data in the freely available University of Oslo Bioportal (http://www.bioportal.uio.no). Repeated runs on distinct starting trees were carried out to select the tree with the best topology (the one having the best Likelihood of 1000 alternative trees). Bootstrap ML analysis was carried out using 1000 pseudo-replicates. Trees were edited with the FigTree v1.3.1. Rarefaction analysis estimates were performed in the program MOTHUR (Schloss *et al*., 2009) after grouping sequences at 97%, 98% and 99% similarity thresholds.

16S rRNA-DGGE fingerprinting

Denaturing gradient gel electrophoresis (DGGE) was used to compare bacterial and cyanobacterial communities associated with the different investigated sponge species. PCR amplifications were performed using the bacterial universal and cyanobacterial-specific 16S rDNA oligonucleotide primers (Table 2). DGGE was performed with a DGGE-2000 system (CBS Scientific) as described by Díez and colleagues (2001). Electrophoresis was performed using 0.75-mm-thick, 6% polyacrylamide gels (37.5:1 acrylamide: bisacrylamide) submerged in 1× TAE buffer (40 mM Tris, 40 mM acetic acid and 1 mM EDTA, pH 7.4) at 60°C with a linear gradient of denaturing agents from 45% to 75% for both cyanobacteria and bacteria. After electrophoresis, the gel was stained in 1×

TAE buffer containing SYBRGold Nucleic Acid Stain (1:10 000 dilution; Molecular Probes) to reveal the band patterns. The results were recorded using a molecular imager (Chemi Doc XRS system, Bio-Rad™). To obtain the gene sequences of the DGGE bands, polyacrylamide fragments were excised from the gel using sterilized razor blades, resuspended in 20 μl of MilliQ water and stored at 4°C overnight. An aliquot of the eluted DNA was re-amplified using PCR with the same primers and conditions as before. The re-amplified PCR products were sequenced (with the corresponding forward primer) at the Macrogen Sequencing Service (Korea).

Nucleotide sequence accession numbers

Sequences obtained in this study were submitted to GenBank under Accession No. JN409473–JN409512 (for *D. avara* bacterial *amoA* genes), JN409513–JN409554 (for *A. oroides* archaea *amoA* genes), JN409555–JN409596 (for seawater archaea *amoA* genes), JQ280408–JQ280414 (for *C. reniformis* bacterial *amoA* genes) (JN314396–JN314401 (for bacterial 16S rRNA genes) and JN314402–JN314411 (for cyanobacterial 16S rRNA genes).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Rarefaction curves for ammonia-oxidizers groups. (A) β-Ammonia-oxidizing bacteria from *D. avara*, (B) ammonia-oxidizing archaea *amoA* clone libraries for *A. oroi-*

des and (C) ammonia-oxidizing archaea *amoA* clone libraries for water samples. OTUs were defined at a 99, 98 and 97 sequence similarity thresholds.

Fig. S2. Representative confocal microscopy (CLSM) images of the tissue of the three sponges species (Da, *Dysidea avara*; Ao, *Agelas oroides*; Cr, *Chondrosia reniformis*).
A. Negative controls using a non-EUB probe.
B. Autofluorescence controls without probe (see Fig. 2).

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