



## Functional convergence of microbes associated with temperate marine sponges

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## Functional convergence of microbes associated with temperate marine sponges

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Running title: Functional role of microbial associations in marine sponges

## Summary

20 Most marine sponges establish a persistent association with wide arrays 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 identify and quantify  
the microbial communities associated with three common Mediterranean  
25 sponge species, *Dysidea avara*, *Agelas oroides* and *Chondrosia reniformis*  
(Desmospongiae) that cohabitate in the coralligenous community. For each  
sponge we also quantified its particulate diet and the uptake and release of  
dissolved organic carbon, phosphate, dissolved organic nitrogen (DON) and  
carbon (DOC), and inorganic nitrogen. Low microbial abundance (<6% of the  
30 tissue occupied by microbes) and no evidence for DOC uptake or nitrification  
was found for *D. avara*. In contrast *O. 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  
 $\text{NH}_4^+$  uptake. Surprisingly, these unique metabolic pathways were mediated in  
35 each sponge species by a different, and host specific, microbial community. The  
results point to a functional convergence of microbial consortia in some  
sympatric sponge species suggesting that these metabolic processes may be of  
special relevance to the success of the holobiont.

## Introduction

40           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); by contrast, prokaryotes  
45 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  
50 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  
55 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- to 2- $\mu\text{m}$  size range.  
With a lower efficiency, they can also remove larger particles up to a few  
60 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)  
65 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 any ecosystem where they abound (Gili and Coma, 1998).

70           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).

75 Based on the density of the microbial communities they host, sponges can be functionally divided into two groups. “High-Microbial-Abundance” (HMA; formerly “bacteriosponges”, e.g., Reiswig (1981)) sponges contain dense and host specific microbial populations that exceed the microbial density of the surrounding water by 2-4 orders of magnitude (e.g., Weisz et al., 2008). HMA  
80 species have dense tissues and low pumping rates (Weisz et al., 2008; Siegl 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; Wilkinson, 1983; Reiswig, 1981; Hentschel et al., 2006) and in phylogenetic composition (Schmitt et al., 2007).  
85 LMA species have well-irrigated tissues and high specific pumping rates.

Most of our current knowledge about the microbial diversity associated with marine sponges is based on 16S rRNA gene library construction, functional genes surveys and metagenomics, which are used to infer alternative metabolic  
90 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; Hentschel et al., 2006; Taylor et al., 2007; Hoffmann et al., 2009). An outcome of each of these processes is the growth of microbial biomass in the  
95 consortium, but the adaptive value of these processes to the hosting sponge and/or the consortium is not well understood. Moreover, while a wide array of phylogenetically and physiologically diverse microbes associated with sponges has been described, their role in nutrient cycles remains largely unknown (Hentschel et al., 2006; Taylor et al., 2007; Moya et al., 2008; Hoffmann et al.,  
100 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 *D. avara*, *A. oroides* and *C. reniformis*. The

105 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; Bayer et al., 2008; Vacelet and Donadey., 1997). 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  
110 particulate food (Jimenez 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 allows us to reliably examine the uptake  
115 and excretion of dissolved organic nitrogen (DON) and carbon (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  
120 contrasting microbial associates.

## Results

### *Nutrient removal and excretion*

125 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  
 $\mu\text{mol L}^{-1}$  DOC) from the inhaled water, DOC was not significantly excreted or  
removed by *D. avara* (Table 2). Similarly,  $\text{NH}_4^+$  was removed by both *C.*  
130 *reniformis* (47%) and *A. oroides* (41%) but not by *D. avara*. The  $\text{NO}_x^-$  ( $\text{NO}_2^-$   
 $+\text{NO}_3^-$ ) budget also highlighted differences between *D. avara* and the other two  
species. *C. reniformis* and *A. oroides* had significant net excretion of  $\text{NO}_x^-$ ,  
increasing ambient concentrations > 35%, whereas *D. avara* did not show  
significant differences between inhaled and exhaled concentrations (Table 2).  
135 Significant amounts of phosphate ( $\text{PO}_4^{3-}$ , 24-55%) were excreted by all three  
sponges (Table 2).

### **Microbial abundance**

The three sponge species support specific microbial communities containing both eubacteria and archaea (Fig. 2) but in very different proportions. Eubacteria dominated the microbial community in *A. oroides* and *C. reniformis* forming a compact biofilm, so dense, that single-cell enumeration was impossible in most of the sponge tissue (Fig. 2a, Fig. 3). Among archaea, only *A. oroides* hosted both Crenarchaea and Euryarchaea (Fig. 2b, Fig 3). *D. 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).

### *Ammonium oxidizing groups*

Ammonium uptake and excretion of  $\text{NO}_x^-$  (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 analyzed by targeting *amoA* genes using PCR-based techniques. Surprisingly, a unique population of ammonia oxidizing microbes was present in each of the three sponges and these belong to different and remote taxonomic groups. *D. avara* samples yielded positive PCR products only with  $\beta$ -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  $\gamma$ -proteobacteria primers (only in two out of 10 PCR trials). In the later case, sequences obtained did not have any similarity with any  $\gamma$ -proteobacteria sequences in GenBank, so phylogenetic analysis was not possible and these sequences are reported as “putative  $\gamma$ -proteobacteria *amoA*”. 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

170 1). All of the PCRs were compared to a positive control culture (Ca Brocadia Sanchez-Melsio et al. 2009). Neither the sponge samples nor water column samples showed positive PCRs products for 16S rRNA genes from anammox bacteria.

#### 175 *Analysis of photoautotrophic microbes*

The presence and diversity of phototrophic microbes, potential consumers of  $\text{NH}_4^+$ ,  $\text{NO}_x^-$ , and  $\text{PO}_4^-$  and producers of DOC, was analyzed by small subunit-rRNA-DGGE fingerprinting and sequence identification using both general bacterial primers (Fig. 4A) and specific cyanobacterial primers (Fig. 4B).  
180 The reproducibility of DGGE results were confirmed by the use of two replicates per sample for each of the molecular markers analyzed (16S rRNA and 18S rRNA). Figure 4A and B shows only a replicate per sample and genetic marker analyzed. The sequences retrieved from the DGGE bands gels were confirmed by sequencing different bands with the same position along samples and  
185 replicates. All three sponges seem to harbor both photoautotrophic and photoheterotrophic microbial components. DGGE results showed significant differences between the three sponge species investigated. Sequencing specific bands from our 16S rRNA-DGGE profiles indicate the presence of several bands that are phylogenetically affiliated with the photoheterotrophic  
190 members of Chloroflexi, a green non-sulfur group of bacteria (Table 3). These phylotypes were well represented in *C. reniformis* (DGGE bands B2 and B10 in Fig. 4A, and Sj15 in Fig. 4B) and *A. oroides* (DGGE bands B5, B13 and B15, in Fig. 4A, and Sj12 in Fig. 4B) but were absent in *D. avara*. In addition, bands related to other bacteria, such as Acidobacteria and Firmicutes (DGGE bands  
195 B11, Fig. 4A) and Proteobacteria (DGGE bands B1, Fig. 4A, and Sj13-14 in Fig. 4B), 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 but with a distinct pattern for each of the  
200 three sponge species (Fig. 4B). 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 microbial community associated with *D. avara* (DGGE band Sj6) and *A. oroides* (DGGE band Sj5).  
 205 Indeed, they seem to be the only phototrophic organisms associated with *D. avara* aside from phototrophic eukaryotes. DGGE bands Sj1, 3, 9 and 10 are affiliated with chloroplasts of diatoms and pelagophytes (Fig. 4B and Table 3) in both *A. oroides* and *D. avara* sponges.

#### 210 *Phylogenetic analysis of ammonia oxidizers*

Clone libraries of  $\beta$ -proteobacteria *amoA* sequences 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. 1, SI),  
 215 indicating that we had correctly sampled the total diversity of each functional group. The  $\beta$ -proteobacteria *amoA* genes from *D. avara* fell into three different clusters (Fig. 5). Cluster 1 was dominated by *D. avara* sequences, which shared ~99% amino acid similarity with uncultured ammonia-oxidizing bacteria from an aquarium sand biofilter in Tokyo [Cluster B2 (AB373584, AB373599) and cluster  
 220 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,  
 225 Caribbean and Pacific Ocean marine sponges (EF5296XX sequences; Bayer et al., 2008). *D. 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 et al., (2010).

230 Archaea ammonia oxidizers from *A. oroides* and water column samples primarily fell into three of the five different clusters (Fig. 6). 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.*  
 235 *oroides*, five sequences from sponges from Sydney (Australia) and 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 5 with  
 240 *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  
 245 related to at least three different metabolic pathways: heterotrophy, photoautotrophy and chemoautotrophy (Fig. 1). Dissolved organic carbon taken by the sponge is transferred to and metabolized by heterotrophic bacteria and archaea (Wilkinson and Garrone, 1980; de Goeij et al., 2008a), therefore high abundance of heterotrophic microbes is expected to result in high DOC  
 250 removal. Ammonium ( $\text{NH}_4^+$ ) 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  $\text{NO}_x^-$  excretion (Fig. 1). Other chemoautotrophic processes such  
 255 anammox may also contribute to  $\text{NH}_4^+$  removal (Fig. 1).

### *Removal of DOC and microbial abundance*

Removal of dissolved organic carbon was associated with high abundance of bacteria and archaea. *D. avara* has the lowest abundance of  
 260 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 correspondingly, 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.*  
 265 *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, 2008a, 2008b), 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 et al., (2008a) found contrasting levels of DOC uptake for LMA vs. HMA, suggesting that total respiration in HMA sponges is largely fueled by DOM uptake as previously suggested by Reisinger, (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 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.

290

#### *Removal of ammonia and presence of photoautotrophs*

Ammonium removal from pumped water (41 and 52%) was detected in *A. oroides* and *C. reniformis*, respectively. Using universal bacterial primers, we identified bacterial phylotypes that are closely related to *Chloroflexi* (*C. reniformis* and *A. oroides*) and *Chroococcales* unicellular cyanobacteria (*A. oroides* and *D. avara*). Both groups resemble previously reported photosynthetic symbionts from other sponges worldwide (Sunagawa et al., 2009; Lee et al., 2009; Hentschel et al., 2002; Taylor et al., 2007; Sharp et al.,

10

2007; Hardoim et al., 2009). The high diversity and abundance of *Chloroflexi*-  
300 like phylotypes recovered by our bacterial and cyanobacterial 16S rRNA gene  
analysis suggest that this group of photosynthetic symbionts is an important  
partner in the investigated species. The unique DGGE profile of each sponge  
species provides a clear representation of the specific microbial communities  
that it harbors. While photoautotrophs were also present in *D. avara*, it seems  
305 that their abundance was insufficient to elicit net ammonia removal.

Data about the ability of sponges to remove DIN from the water column is  
still scarce. Schubauer, (1988) was probably the first to report depletion of  $\text{NO}_3^-$   
and  $\text{NH}_4^+$  in tropical sponges. Diaz and Ward, (1997) also reported DIN uptake  
in two of four tropical sponges under light conditions. Similar findings were also  
310 reported by Yahel et al., (2003) for *Theonella swinhoei* and Southwell and  
collaborators (2008a) measured the uptake of  $\text{NH}_4^+$  by two sponge species in  
coral reefs ecosystems. Our results highlight the potential role of sponge  
phototrophic symbionts even under the moderate light conditions found in the  
sponge habitat. These findings also call for attention to DIN consumption that  
315 appears as an important mechanism of nutrient acquisition in temperate  
sponges.

#### *Release of $\text{NO}_x^-$ and presence of ammonia oxidizers*

Large amounts of excess  $\text{NO}_x^-$  (> 35% or  $1 \mu\text{mol L}^{-1}$  per liter pumped)  
320 were excreted by the two HMA sponges *A. oroides*, and *C. reniformis* but not by  
the LMA sponge *D. avara* (Fig. 1, Table 2). Ammonia oxidation, the first and  
limiting step in nitrification, can be performed by  $\beta$ - or  $\gamma$ -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).  
325 Each of the three sponge species we studied appears to harbor its own nitrifier  
group:  $\gamma$ -proteobacteria (putative) in *C. reniformis*,  $\beta$ -proteobacteria in *D. avara*  
and archaea in *A. oroides*.

The phylogenetic tree based on ammonia-oxidizing  $\beta$ -proteobacteria  
shows that only few of the *amoA* sequences derived from *D. avara* are related  
330 to *amoA* sequences of the *Nitrosospira* lineage. Clusters 1 and 2 were  
dominated by sequences that shared ~99% amino acid similarity with

uncultured ammonia-oxidizing bacteria from an aquarium biofilter in Tokyo (Clusters B2 and B3, respectively, in Urakawa et al., 2008).

335 The ammonia-oxidizing archaea 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  
340 reported in Steger et al., (2008) [cluster 4] were not related to *A. oroides* AmoA sequences.

A relationship between the amount of nitrification and specific pumping rates in marine sponges was suggested by Martens, Lindquist and co workers (Weisz et al., 2008). Southwell et al., (2008b; 2007) related nitrification rates to  
345 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  
350 high nitrification rates) were three times lower than those of *D. avara* that showed no evidence for significant nitrification (Ribes unpublished 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  
355 depend on rates, carbon fixation mechanisms, environmental controls and competitive ability in the biogeochemical process (Francis et al., 2007). Ammonia-oxidizing archaea 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  
360 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-oxidizing 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  
365 small archaeal population.

$\text{NO}_x^-$  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 co-workers, (2010) did measure  
net  $\text{NO}_x^-$  excretion by the *D. avara* specimens they studied. The differences  
370 between the two studies may be related to methodological biases or to  
seasonal differences in sponge metabolism. Schläppy and collaborators, (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  
375 prolonged incubation times (Ribes unpublished data). Nevertheless, temporal  
variation in activity and bacterial symbiont composition are also possible  
because Schläppy et al., (2010) worked in early spring (March) whereas our  
experiments were done in May-July.

#### 380 *Release of $\text{PO}_4^{3-}$*

The three sponges studied showed high and significant net excretion of  
 $\text{PO}_4^{3-}$  (~ 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  
385 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 particulate feeding by  
sponges provided an excess amount of this nutrient for the consortium during  
390 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  
395 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 harbored 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 high microbial abundance (HMA) sponges, exhibiting high nitrification and high DOC and  $\text{NH}_4^+$  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 pointed to a functional convergence of microbial partners in some sympatric sponge species suggesting that these metabolic processes may be of special relevance to the sponge consortium success.

## 410 **Experimental procedures**

### ***Specimen collection***

Sponge specimens 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  $\mu\text{m}$  polycarbonate filters. Filters were frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  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  $< 1 \text{ ml min}^{-1}$ . One tube was positioned

a few millimeters inside the osculum to sample exhaled water, and the other  
430 was placed next to the sponge to sample inhaled water. Sampled water was  
collected in darkened glass bottles that were immersed in ice during the  
sampling time (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  
435 beginning and end of the experiments.

To avoid contamination from microbes in the water, after the  
experiments, small tissue samples (1 cm<sup>3</sup>) 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  
440 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 analyzed for inorganic nutrients with an Alliance autoanalyzer  
445 following the method of Grasshoff et al., (1983). The following dissolved  
compounds were measured: NH<sub>4</sub><sup>+</sup> (ammonium), NO<sub>x</sub><sup>-</sup> [NO<sub>3</sub><sup>-</sup> + NO<sub>2</sub><sup>-</sup>], and PO<sub>4</sub><sup>3-</sup>  
(phosphate). The DN (including DIN and DON) concentration was analyzed  
using a Bran-Luebbe AA3 autoanalyzer after a double oxidation with UV light  
and persulfate (Grasshoff et al., 1983). DON concentration was calculated by  
450 subtracting the DIN concentration (NH<sub>4</sub><sup>+</sup> + NO<sub>x</sub><sup>-</sup>) from the DN. Samples for DOC  
determination were filtered through precombusted (450° C for at least 2 hours)  
GFF filters with a baked glass filtration system. Water for DOC analysis was  
collected in 10 mL precombusted glass ampoules and acidified with  
concentrated, trace-metal grade, hydrochloric acid (0.1% final concentration).  
455 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 analyzer (Sharp et al., 2002). The accuracy of  
the DOC determination was evaluated by daily comparisons with DOC  
reference materials (Yahel et al., 2003).

460

## ***Molecular techniques***

### *FISH and Confocal Microscopy*

We used catalyzed reporter deposition (CARD) fluorescent in situ  
465 hybridization (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  $\mu\text{m}$  slices were obtained. Three slices from each specimen  
were hybridized using CARD-FISH with probes for Eubacteria (Eub 338 I+II+III;  
470 Amann et al., 1990; Daims et al., 1999) and Archaea (Cren 537 and Eury 806;  
Teira et al., 2004), following the protocol described in Pernthaler et al., (2002).  
The structure of the sponge was stained with CellMask™ Deep Red (Molecular  
Probes, Invitrogen, Carlsbad, CA, USA), and the nucleic acids were labeled  
with Hoechst 33342 (Molecular Probes, Eugene, Oregon, United States).  
475 Samples were observed with a Leica TCS-SP5 confocal spectral microscope  
(Leica Microsystems Heidelberg GmbH; Mannheim, Germany) using a Plan-  
Apochromatic 63 $\times$ 1.4 (oil HC x 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  
480 546 (for Eury 806), Hoechst and CellMask. The resulting images 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.

### 485 *DNA extraction and PCR amplification*

Tissue samples ( $\sim 2 \text{ mm}^3$ ) 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  
490 DNA extraction; incubation with lysis buffer overnight instead of few hours; final  
elution in 100  $\mu\text{l}$  Buffer AE instead of 200  $\mu\text{l}$  (Blanquer and López-Legentil,  
personal communication). Putative AmoA gene fragments from the total

genomic DNA of proteobacteria and archaea were amplified by PCR with the primers listed in Table 1 and using GoTaq Flexi Dna Polymerase (Promega).  
495 For PCR amplification we followed the conditions described in the reference papers with the following modifications: for  $\gamma$ -proteobacteria (Purkhold et al., 2000) we run 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  
500 were amplified by PCR with the primers listed on Table 1 and following original references conditions. Reactions of 25  $\mu$ l 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  
505 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 beta proteobacteria (~420 bp) and for  
510 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  
515 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  
520 (Altschul et al., 1997) for a first phylogenetic affiliation and to remove putative chimeras. 44 clones were sequenced for  $\beta$ -ammonia-oxidizing bacteria from *D. avara*; 65 clones were sequenced for ammonia-oxidizing archaea from *A. oroides* and 50 clones for ammonia-oxidizing archaea from water samples.

525 *Phylogenetic analysis*

DNA sequences were aligned with MAFFT using the slow and iterative refinement method FFT-NS-i (Kato et al., 2002). The alignment was checked manually and edited using Seaview 3.2 (Galtier et al., 1996), to retain the longest region that is common in 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 (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 MOTHR (Schloss et al., 2009) after grouping sequences at several similarity thresholds.

540 *16S rRNA-DGGE fingerprinting*

Denaturing gradient gel electrophoresis (DGGE) was used to reveal the resemblance of the bacteria and cyanobacteria associated with the different investigated sponge species. PCR amplifications were performed using the bacterial universal and cyanobacterial-specific 16S rDNA oligonucleotide primers (Table 1). DGGE was performed with a DGGE-2000 system (CBS Scientific Co.) as described by Díez et al., (2001). Electrophoresis was performed using 0.75 mm thick, 6% polyacrylamide gels (37.5:1 acrylamide: bisacrylamide) submerged in 1x 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 1x 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, BioRad™). To obtain the gene sequences of the DGGE bands, polyacrylamide fragments were excised from the gel using sterilized razor blades, resuspended in 20 µl 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).

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*Nucleotide sequence accession numbers*

Sequences obtained in this study were submitted to GenBank under accession numbers XXXXX (for amoA genes), XXXXX (for bacterial 16S rDNA genes) and XXXXX (for cyanobacterial 16S rRNA genes).

565

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570

575 **References**

Altschul, S. F., Madden, T. L., Schäfer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**: 3389-3402.

580

Amann, R.I., Binder, F.J., Olson, R.J., Chisholm, S.W., Devereux, R., and Stahl D.A. (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Applied and Environmental Microbiology* **56**: 1919-1925.

585

Amano, T., Yoshinaga, I., Okada, K., Yamagishi, T., Ueda, S., Obuchi, A., *et al.* (2007) Detection of anammox activity and diversity of anammox related 16S rRNA genes in coastal marine sediments in Japan. *Microbes Environ* **22**: 232–242.

590

Bayer, K., Schmitt, S., and Hentschel, U. (2008) Physiology, phylogeny and in situ evidence for bacterial and archaeal nitrifiers in the marine sponge *Aplysina aerophoba*. *Environm Microbiol* **10**: 2942-2955.

595

Brusca, R., and Brusca, G. (1990) Phylum Porifera: the sponges. . In *The invertebrates*. Sinauer, A. (ed). Sunderland, MA, USA: Sinauer Press, pp. 181-210.

600

Daims, H., Brühl, A., Amann, R., Schleifer, K.H., and Wagner, M. (1999) The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. *Systematic and Applied Microbiology* **22**: 434-444

605

De Goeij, J.M., and van Duyl, F.C. (2007) Coral cavities are sinks of dissolved organic carbon (DOC). *Limnol Oceanogr* **52**: 2608-2617.

De Goeij, J.M., Moodley, L., Houtekamer, M., Carballeira, N.M., and van Duyl, F.C. (2008a) Tracing <sup>13</sup>C-enriched dissolved and particulate organic carbon in

- 610 the bacteria-containing coral reef sponge *Halisarca caerulea*: Evidence for  
DOM-feeding. *Limnol Oceanogr* **53**: 1376-1386.
- 615 De Goeij, J.M., van den Berg, H., van Oostveen, M.M., Epping, E.H.G., and Van  
Duyl, F.C. (2008b) Major bulk dissolved organic carbon (DOC) removal by  
encrusting coral reef cavity sponges. *Mar Ecol-Prog Ser* **357**: 139-151.
- 620 De La Torre, J.R., Walker, C.B., Ingalls, A.E., Könneke, M., and Stahl,  
D.A.(2008) Cultivation of a thermophilic ammonia oxidizing archaeon  
synthesizing crenarchaeol. *Environ Microbiol* **10**: 810–818.
- 625 Díaz, M.C., and Ward, B.B. (1997) Sponge-mediated nitrification in tropical  
benthic communities. *Mar Ecol Prog Ser* **156**: 97-109.
- Díaz, M.C., and Rützler, K. (2001) Sponges: an essential component of  
Caribbean coral reefs. *Bull Mar Sci* **69**: 535–546.
- 630 Falkowski, P.G., Fenchel, T., and Delong, E.F. (2008) The microbial engines  
that drive earth's biogeochemical cycles. *Science* **320**: 1034-1039.
- 635 Francis, C.A., Roberts, K.J., Berman, J.M., Santoro, A.E., and Oakley, B.B  
(2005) Ubiquity and diversity of ammonium-oxidizing archaea in water columns  
and sediments of the ocean. *Proceedings of the National Academy of Sciences*  
**102**: 14683-14688.
- 640 Francis, C.A., Berman, J.M., and Kuypres, M.M.M. (2007) New processes and  
players in the nitrogen cycle: microbial ecology of anaerobic and archaeal  
ammonia oxidation. *ISME J* **1**: 19-27.
- Friedrich, A.B., Merkert, H., Fendert, T., Hacker, J., Proksch, P., and Hentschel,  
U. (1999) Microbial diversity in the marine sponge *Aplysina cavernicola*  
(formerly *Verongia cavernicola*) analyzed by fluorescence in situ hybridization  
(FISH). *Mar Biol* **134**: 461-470.

- Galtier, N., Gouy, M., and Gautier, C. (1996) SEAVIEW and PHYLO\_WIN: two graphic tools for sequence alignment and molecular phylogeny. *Comput Appl Biosci* **12**: 543–548.
- 645
- Gili, J.M., and Coma, R. (1998) Benthic suspension feeders: their paramount role in littoral marine food webs. *Trends Ecol Evol* **13**: 316-321.
- 650
- Grasshoff, K., Ehrhardt, M., and Kremling, K. (1983) *Methods on sea-water analysis*. Weinheim. Verlag Chemie.
- Hadas, E., Marie, D., Shpigel, M., and Ilan, M. (2006) Virus predation by sponges is a new nutrient-flow pathway in coral reef food webs. *Limnology and Oceanography* **51**: 1548-1550.
- 655
- Hardoim, C.C., Costa, R., Araujo, F.V., Hajdu, E., Peixoto, R., Lins, U. et al. (2009) Diversity of bacteria in the marine sponge *Aplysina fulva* in Brazilian coastal Waters. *Appl. Environ. Microbiol.* **75**: 3331-3343.
- 660
- Hentschel, U., Hopke, J., Friedrich, A.B., Wagner, M., Hacker, J., and Moore, B.S. (2002) Molecular evidence for a uniform microbial community in sponges from diVerent oceans. *Appl Environ Microbiol* **68**: 4431- 4440.
- 665
- Hentschel, U., Usher, K.M., and Taylor, M.W. (2006) Marine sponges as microbial fermenters. *FEMS Microbiol Ecol* **55**: 167-177.
- Hoffmann, F., Larsen, O., Thiel, V., Rapp, H.T., and Reitner, J. (2005) An anaerobic world in sponges. *Geomicrobiol J* **22**: 1-10.
- 670
- Hoffmann, F., Radax, R., Woebken, D., Holtappels, M., Lavik, G., Rapp, H.T. et al. (2009) Complex nitrogen cycling in the sponge *Geodia barretti*. *Environ Microbiol* **11**: 2228–2243.

675 Hooper, J.M.A., and van Soest, R.W.M. (2002) *Systema Porifera: a guide to the classification of sponges*. New York, NY.: Kluwer Academic/Plenum Publishers.

Jiménez, E., and Ribes, M. (2007) Sponges as a source of dissolved inorganic nitrogen: nitrification mediated by temperate sponges. *Limnol Oceanogr* **52**:  
680 948-958.

Kato, K., Misawa, K., Kuma, K., and Miyata, T. (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res* **30**: 3059–3066.

685

Lee, O.O., Chui, P.Y., Wong, Y.H., Pawlik, J.R., and Qian, P.Y. (2009) Evidence for vertical transmission of bacterial symbionts from adult to embryo in the Caribbean sponge *Svenzea zeai*. *Appl Environ Microbiol* **75**: 6147-6156

690 Leininger, S., Urich, T., Schloter, M., Schwark, L., Qi, J., Nicol, G.W. et al. (2006) Archaea predominate among ammonia-oxidizing prokaryotes in soils. *Nature* **442**: 806-809.

Madigan, M.T., Martinko, J.M., and Parker, J. (2003) Metabolic diversity. In  
695 *Brock biology of microorganisms*. Madigan MT, Martinko JM, and Parker J (eds). Upper Saddle River, N.J.: Pearson Education Inc.

Medlin, L.K., Cooper, A., Hill, C., Wrieden, S., and Wellbrock, U. (1995) Phylogenetic position of the Chromista plastids based on small subunit rRNA  
700 coding regions. *Curr. Genet.* **28**: 560-565

Mohamed, N.M., Colman, A.S., Tal, Y., and Hill, R.T. (2008) Diversity and expression of nitrogen fixation genes in bacterial symbionts of marine sponges. *Environmental Microbiology* **10**: 2910-2921.

705

Mohamed, N.M., Saito, K., Tal, Y., and Hill, R.T. (2010) Diversity of aerobic and anaerobic ammonia-oxidizing bacteria in marine sponges *The ISME J* **4**: 38-48.

- 710 Moya, A., Peretó, J., Gil, R., and Latorre, A. (2008) Learning how to live together: genomic insights into prokaryote-animal symbioses. *Nature* **9**: 218-229.
- 715 Muyzer G, Brinkhoff T, Nübel U, Santegoeds C, Schaäfer H, and Wawer C (1997) Denaturing gradient gel electrophoresis (DGGE) in microbial ecology. . In *Molecular Microbial Ecology Manual*. Akkermans DL, van Elsas JD, and de Bruijn FJ (eds). Dordrecht, the Netherlands: Kluwer Academic pp. 1-27.
- 720 Nübel, U., Garcia-Pichel, F., and Muyzer, G. (1997) PCR primers to amplify 16S rRNA genes from cyanobacteria. *Appl. Environ. Microbiol.* **63**: 3327-3332.
- Ong, H.C., Wilhelm, S.W., Gobler, C.J., Bullerjahn, G., Jacobs, M.A., McKay, J et al. (2010) Analyses of the complete chloroplast genome sequences of two members of the Pelagophyceae: *Aureococcus anophagefferens* CCMP1984 and *Aureoumbra lagunensis* CCMP1507. *J. Phycol.* **46**: 602-615.
- 725 Pape ,T., Hoffmann, F., Queric, N.V., von Juterzenka, K., Reitner, J., and Michaelis, W. (2006) Dense populations of Archaea associated with the desmosponge *Tentorium semisuberites* Schmidt, 1870 from Arctic deep-waters. *Polar Biol* **29**: 662-667.
- 730 Penton, C.R., Devol, A.H., and Tiedje, J.M. (2006) Molecular evidence for the broad distribution of anaerobic ammonium-oxidizing bacteria in freshwater and marine sediments. *Appl Environ Microbiol* **72**: 6829–6832.
- 735 Pernthaler, A., Pernthaler, J., and Amann, R. (2002) Fluorescence in situ hybridization and catalyzed reporter deposition for the identification of marine bacteria. *Appl Environ Microbiol* **68**: 3094–3101.
- 740 Pham, V.D., Konstantinidis, K.T., Palden, T., and DeLong, E.F. (2008) Phylogenetic analyses of ribosomal DNA-containing bacterioplankton genome

fragments from a 4000 m vertical profile in the North Pacific Subtropical Gyre. *Environ. Microbiol.* **10**: 2313-2330.

745 Pile, A.J. (1997) Finding Reiswig's missing carbon: quantification of sponge feeding using dual-beam flow cytometry. *Proceedings of the 8th International Coral Reef Symposium 2*: 1403-1410.

750 Purkhold, U., Pommering-Röser, A., Juretschko, S., Schmid, M.C., Koops, H.P., and Wagner, M. (2000) Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and amoA sequence analysis: implications for molecular diversity surveys. *Appl. Environ. Microbiol.* **66**: 5368-5538.

755 Reiswig, H.M. (1981) Partial carbon and energy budgets of the bacteriosponge *Verongia fistularis* (Porifera: Demospongiae) in Barbados. *PSZNI: Mar Ecol 2*: 273-263.

760 Ribes, M., Coma, R., and Gili, J.M. (1999) Natural diet and grazing rate of the temperate sponge *Dysidea avara* (Demospongiae, Dendroceratida) throughout an annual cycle. *Mar Ecol Prog Ser 176*: 179-190.

765 Rotthauwe, J.H., Witzel, K.P., and Liesack, W. (1997) The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. *Appl Environ Microbiol* **63**: 4704-4712.

770 Sánchez, O., Gasol, J.M., Massana, R., Mas, J., and Pedrós-Alió, C. (2007) Comparison of different denaturing gradient gel electrophoresis primer sets for the study of marine bacterioplankton communities. *Appl Environ Microbiol* **73**: 5962-5967.

Sánchez-Melsió, A., Cáliz, J., Balaguer, M.D., Colprim, J., and Vila, X. (2009) Development of batch-culture enrichment coupled to molecular detection for

- 775 screening of natural and man-made environments in search of anammox  
bacteria for N-removal bioreactors systems. *Chemosphere* **75**: 169-179.
- Schläppy, M.L., Schöttner, S.I., Lavik, G., Kuypers, M.M., de Beer, D., and Hoffmann, F. (2010) Evidence for nitrification and denitrification in high and low microbial abundance sponges. *Mar Biol* **157**: 593-602.
- 780 Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B. et al. (2009) Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* **75**: 7537-7541.
- 785 Schmitt, S., Wehrl, M., Bayer, K., Siegl, A., and Hentschel, U. (2007) Marine sponges as models for commensal microbe-host interactions. *Symbiosis* **44**: 43-50.
- 790 Schubauer, J.P. (1988) Metabolism and nutrient cycling by marine sponges. In: University of Georgia.
- Sharp, J.H., Carlson, C.A., Peltzer, E.T., Castle-Ward, D.M., Savidge, K.B., and Rinker, K.R. (2002) Fianl dissolved organic carbon broad community  
795 intercalibration and preliminary use of DOC reference materials. *Mar Chem* **77**: 239-253.
- Sharp, K.H., Eam, B., Faulkner, D.J., and Haygood, M.G. (2007) Vertical transmission of diverse microbes in the tropical sponge *Corticium sp.* *Appl*  
800 *Environ Microbiol* **73**: 622-629.
- Siegl, A., Bayer, K., Kozytska, S., Hentschel, U., and Schmitt, S. (2008) Sponges and microbes- New frontiers in an ancient symbiosis. *Vie et Milieu- Live and environment* **58**: 165-174.
- 805

- Sipkema, D., Holmes, B., Nichols, S.A., and Blanch, H.W. (2009) Biological characterisation of *Haliclona* (?gellius) sp.: sponge and microorganisms. *Microb. Ecol.* **58**: 903-920.
- 810 Southwell, M.W. (2007) Sponges impacts on coral reef nitrogen cycling, Key Largo, Florida. In. North Carolina.: Chapel Hill.
- Southwell, M.W., Popp, B.N., and Martens, C.S. (2008a) Nitrification controls on fluxes and isotopic composition of nitrate from Florida Keys sponges. *Marine*  
815 *Chemistry* **108**: 96-108.
- Southwell, M.W., Weisz, J.B., Martens, C.S., and Lindquist, N. (2008b) In situ fluxes of dissolved inorganic nitrogen from the sponge community on Conch Reef, Key Largo, Florida. *Limnol. Oceanogr.* **53**: 986-996.
- 820 Stamatakis, A. (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**: 2688–2690.
- 825 Steger, D., Ettinger-Epstein, P., Whalan, S., Hentschel, U., de Nys, R., Wagner, M., and Taylor, M.W. (2008) Diversity and mode of transmission of ammonia-oxidizing archaea in marine sponges. *Environ Microbiol.* **10**: 1087-1094.
- Sunagawa, S., DeSantis, T.Z., Piceno, Y.M., Brodie, E.L., DeSalvo, M.K.,  
830 Voolstra, C.R. et al. (2009) Bacterial diversity and White Plague Disease-associated community changes in the Caribbean coral *Montastraea faveolata*. . *ISME J* **3**: 512-521.
- Taylor, M.W., Radax, R., Steger, D., and Wagner, M. (2007) Sponge associated  
835 microorganisms: evolution, ecology and biotechnological potential. *Microbiol Mol Biol Rev* **71**: 295-347.

- 840 Teira, E., Reinthaler, T., Pernthaler, A., Pernthaler, J., and Herndl, G.J. (2004) Combining Catalyzed Reporter Deposition-Fluorescence In Situ Hybridization and Microautoradiography To Detect Substrate Utilization by Bacteria and Archaea in the Deep Ocean. *Applied and Environmental Microbiology* **70**: 4411-4414.
- 845 Turon, X, Galera, J., and Uriz, M.J. (1997) Clearance rates and aquiferous systems in two sponges with contrasting life-history strategies. *J Exp Zool* **278**: 22-36.
- 850 Urakawa, H., Tajima, Y., Numata, Y., and Tsuneda, S. (2008) Low temperature decreases the phylogenetic diversity of ammonia-oxidizing archaea and bacteria in aquarium biofiltration systems *Appl Env Microb* **74**: 894-900.
- 855 Vacelet, J., and Donadey, C. (1977) Electron microscope study of the association between some sponges and bacteria. *J Exp Mar Biol Ecol* **30**: 301–314.
- Walsh, D.A., Zaikova, E., Howes, C.G., Song, Y.C., Wright, J.J., Tringe, S.G. et al. (2009) Metagenome of a Versatile Chemolithoautotroph from Expanding Oceanic Dead Zones. *Science* **326**: 578-582
- 860 Webster, N.S., Cobb, R.E., and Negri, A.P. (2008) Temperature thresholds for bacterial symbiosis with a sponge. *ISME J* **2**: 830-842.
- 865 Weisz, J.B., Lindquist, N., and Martens, C.S. (2008) Do associated microbial abundances impact marine desmosponge pumping rates and tissue densities? *Oecologia* **155**: 367-376.
- 870 Wilkinson, C.R., and Garrone, R. (1980) Nutrition in marine sponges. Involvement of symbiotic bacteria in the uptake of dissolved carbon. In *Nutrition in the lower Metazoa*. Smith DC, and Tiffon Y (eds). Oxford: Pergamon Press, pp. 157-161.

Wilkinson, C.R. (1983) Net primary productivity in coral reef sponges. *Science* **219**: 410-412.

875 Yahel, G., Sharp, J.H., Marie, D., Hase, C., and Genin, A. (2003) In situ feeding and element removal in the symbiont-bearing sponge *Theonella swinhoei*: Bulk DOC is the major source for carbon. . *Limnology and Oceanography* **48**: 141-149.

880 Yahel, G., Marie, D., and Genin, A. (2005) InEx - a direct in situ method to measure filtration rates, nutrition, and metabolism of active suspension feeders. *Limnol and Oceanogr-Methods* **3**: 46-58.

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## Figure legends

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Figure 1.- Expected (a) and observed (b,c,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.

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Figure 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 colors 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).

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

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Figure 4.- Denaturing gradient gel electrophoresis (DGGE) images using 16S rRNA- universal bacterial primers (A) and 16S rRNA- specific cyanobacterial primers (B) on the three sponges species collected in May-June 2007 (Cr: *C. reniformis*; Da: *D. avara*; Ao: *A. oroides*). Identical DGGE profiles were observed in three slices (from the exterior to the interior of the tissue) from each of the three different sponges (not shown). Targeted DGGE bands that have

been sequenced are denoted by black circles (white circles for chloroplast bands in b). Sequence information is given in Table 3.

920 Figure 5.- Maximum Likelihood phylogenetic tree based on  $\beta$ -proteobacteria *amoA* DNA sequences (420 informative positions). Sequences for *D. avara* are in boldface and database sequences for other marine sponges are in red. Database sequences are identified by GenBank accession numbers. Bootstrap values for important nodes are displayed.

925 Figure 6.- Maximum Likelihood phylogenetic tree based on archaeal *amoA* DNA sequences (570 informative positions). Sequences for *A. oroides* are in boldface and water column sequences from our sampling site are boldface and italics. Database sequences for other marine sponges are in red. Database sequences are identified by GenBank accession numbers. Bootstrap values for  
930 important nodes are displayed.

## a. Expected

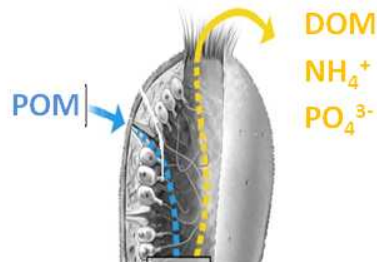
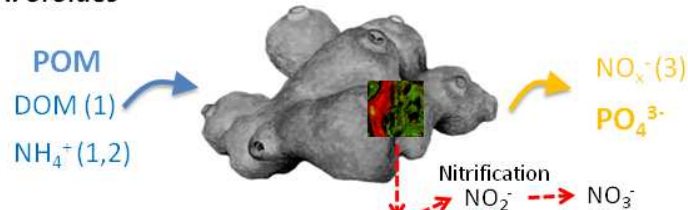
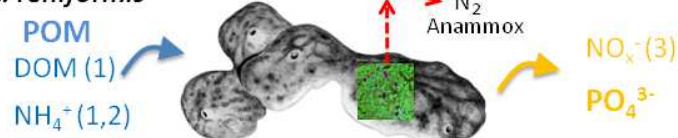
b. *D. avara*c. *A. oroides*d. *C. reniformis*

Figure 1.- Expected (a) and observed (b,c,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.

190x254mm (96 x 96 DPI)

Figure 2.-

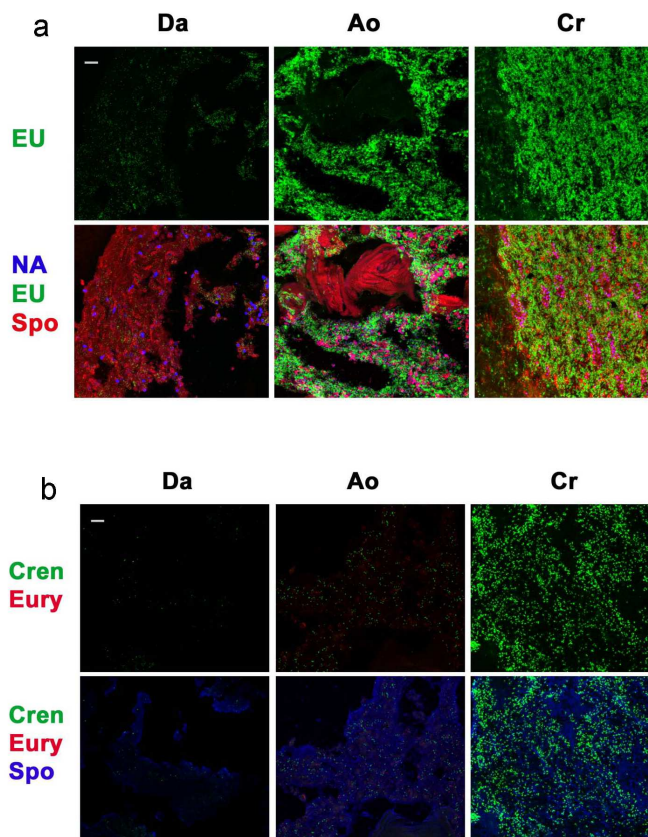


Figure 2.- Representative confocal microscopy (CLSM) images of the tissue of the three sponge species (Da: *Dysidea avara*, Ao: *Agelas oroides*, Cr: *Chondrosia reniformis*). CARD-FISH was used to visualize different microbial populations (upper panels in A and B) in combination with nucleic acid and sponge cells staining (lower panels in A and B). Different probes had different colors 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).  
210x297mm (220 x 220 DPI)

Figure 3.

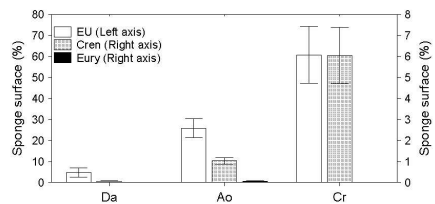


Figure 3.- Percentage of the sponge tissue occupied by different microbial populations.  
Abbreviations as in Figure 2.  
210x297mm (200 x 200 DPI)

Figure 4.

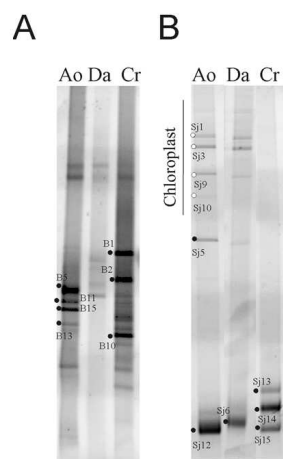


Figure 4.- Denaturing gradient gel electrophoresis (DGGE) images using 16S rRNA- universal bacterial primers (A) and 16S rRNA- specific cyanobacterial primers (B) on the three sponges species collected in May-June 2007 (Cr: *C. reniformis*; Da: *D. avara*; Ao: *A. oroides*). Identical DGGE profiles were observed in three slices (from the exterior to the interior of the tissue) from each of the three different sponges (not shown). Targeted DGGE bands that have been sequenced are denoted by black circles (white circles for chloroplast bands in b). Sequence information is given in Table 3.

210x297mm (150 x 150 DPI)

Figure 5.-

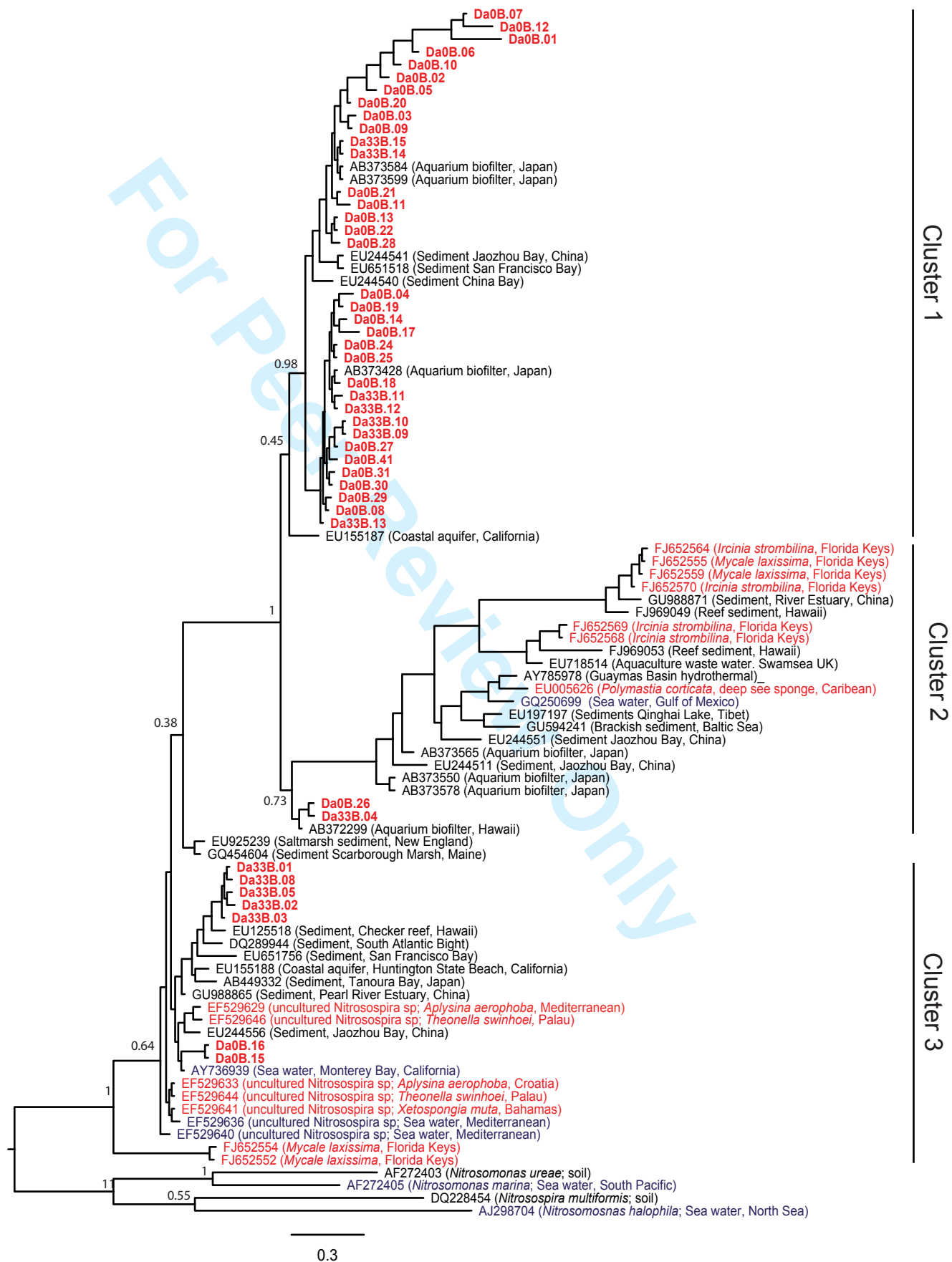


Figure 6.-

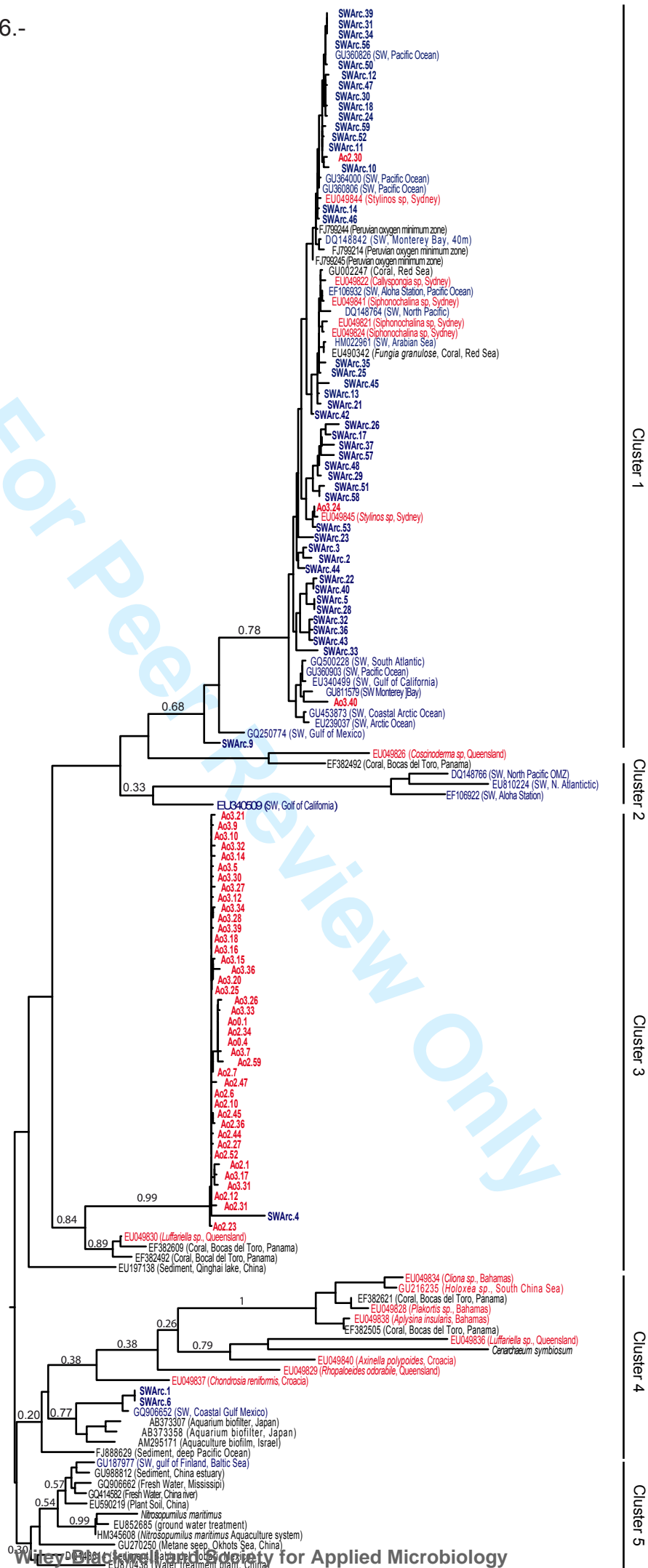


Table 1.- Primers used for PCR amplification of amoA, 16S rRNA and 18S rRNA genes. PCR conditions are indicated in the cited reference

Primer sets	Target group	Reference
Nitrifiers		
	amoA	
AmoA-1F/Amo A-2R	$\beta$ proteobacteria	Rotthauwe et al. 1997
AmoA-3F/AmoB-4R	$\gamma$ 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 <i>Scalindula</i>	Penton et al 2006
Pla46F/Amx368R	Anammox bacteria	Sanchez-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, Sanchez et al 2007
CYA106F/CYA781 Ra/b	Cyanobacteria	Nübel et al 1997

Table 2.- Average concentration ( $\pm$  SD) and average net differences of dissolved nutrients in the inhaled and exhaled water for each species. 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). \* denotes significant differences between inhaled and exhaled concentrations ( $p < 0.05$ , two tailed Wilcoxon test).

Specie	Nutrient	Concentration (Mean $\pm$ SD)			Net difference (In-Ex)	
		Inhaled ( $\mu$ M)	Exhaled ( $\mu$ M)	N	Con ( $\mu$ M)	% Inhaled
<i>D. avara</i>	DOC	84 $\pm$ 16	85 $\pm$ 18	12	<b>-0.7</b>	
	NH <sub>4</sub> <sup>+</sup>	2.76 $\pm$ 0.89	2.47 $\pm$ 0.87	7	0.29	
	NO <sub>x</sub> <sup>-</sup>	2.28 $\pm$ 0.21	2.12 $\pm$ 0.16	12	0.16	
	DON	4.48 $\pm$ 0.50	4.96 $\pm$ 0.85	5	<b>-0.48*</b>	11
	PO <sub>4</sub> <sup>3-</sup>	0.10 $\pm$ 0.03	0.12 $\pm$ 0.03	12	<b>-0.02*</b>	24
<i>C. reniformis</i>	DOC	78 $\pm$ 7	70 $\pm$ 5	9	7.8*	10
	NH <sub>4</sub> <sup>+</sup>	3.33 $\pm$ 0.63	1.64 $\pm$ 0.59	11	1.69*	47
	NO <sub>x</sub> <sup>-</sup>	2.86 $\pm$ 0.32	3.86 $\pm$ 0.43	11	<b>-0.99*</b>	35
	DON	4.76 $\pm$ 0.39	4.90 $\pm$ 1.04	9	<b>-0.14</b>	
	PO <sub>4</sub> <sup>3-</sup>	0.10 $\pm$ 0.03	0.12 $\pm$ 0.02	10	<b>-0.02*</b>	29
<i>A. oroides</i>	DOC	80 $\pm$ 6	70 $\pm$ 4	9	9.9*	12
	NH <sub>4</sub> <sup>+</sup>	2.40 $\pm$ 0.60	1.82 $\pm$ 0.67	15	0.57*	41
	NO <sub>x</sub> <sup>-</sup>	2.61 $\pm$ 0.34	3.60 $\pm$ 0.58	15	<b>-0.99*</b>	37
	DON	4.90 $\pm$ 0.26	4.89 $\pm$ 0.47	15	0.01	
	PO <sub>4</sub> <sup>3-</sup>	0.07 $\pm$ 0.02	0.10 $\pm$ 0.04	15	<b>-0.04*</b>	55

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

DGGE band accession #	Sponge	Nearest relative/ GenBank accession #	Source Location	% identity	Phylogenetic affiliation	Reference
B1-xxx	<i>C. reniformis</i>	Uncultured alphaproteobacteria clone SHFH749; FJ203650	<i>Monttastrea faveolata</i> San Cristobal, Panamá	86	Alphaproteobacteria	Sunagawa et al. 2009
B2-xxx	<i>C. reniformis</i>	Uncultured sponge symbiont clone PAWS52F; AF186417	<i>Theonella sp</i> Caroline Isl, Palau	94	Chloroflexi	Hentschel et al. 2002
B5-xxx	<i>A. oroides</i>	Uncultured Chloroflexi clone W04IS5E12; EF629745	<i>Ircinia strobilina</i> Conch reef Key Largo, FI	90	Chloroflexi	Mohamed et al. 2008
B10-xxx	<i>C. reniformis</i>	Uncultured Chloroflexi clone SGSC785; GQ347250	Symbiont of deep-sea clams, Izu Bonic Arc	78	Chloroflexi	Walsh et al. 2009
B11-xxx	<i>A. oroides</i>	Uncultured Bacterial clone CC13; DQ247947	<i>Corticium embryo</i> Palau	85	Acidobacteria	Sharp et al. 2007
B13-xxx	<i>A. oroides</i>	Uncultured Chloroflexi clone E29; FJ529321	<i>Svenea zeai</i> San Salvador Is, Bahamas	79	Chloroflexi	Lee et al. 2009
B15-xxx	<i>A. oroides</i>	Uncultured Chloroflexi clone AD020; EF076163	<i>Antho chartacea</i> Southeastern Australia	83	Chloroflexi	Taylor et al. 2007
Sj1-xxx	<i>D. avara</i> & <i>A. oroides</i>	Plastid <i>Skeletonema pseudocostatum</i> ; X82155	<i>Skeletonema pseudocostatum</i>	99	Diatom chloroplast	Medlin et al. 1995
Sj3-xxx	<i>D. avara</i> & <i>A. oroides</i>	Plastid <i>Skeletonema pseudocostatum</i> ; X82155	<i>Skeletonema pseudocostatum</i>	98	Diatom chloroplast	Medlin et al. 1995
Sj5-xxx	<i>D. avara</i> & <i>A. oroides</i>	Uncultured sponge symbiont clone Hg5a2D10; EU817117	<i>Haliclona sp.</i> northern Pacific, Monterey harbour	94	Cyanobacteria	Sipkema et al. 2009
Sj6-xxx	<i>A. oroides</i>	Uncultured sponge symbiont clone Hg5a2D10; EU817117	<i>Haliclona sp.</i> northern Pacific, Monterey harbour	99	Cyanobacteria	Sipkema et al. 2009
Sj9-xxx	<i>D. avara</i> & <i>A. oroides</i>	Plastid <i>Aureococcus anophagefferens</i> strain CCMP 1984; GQ231541	<i>Aureococcus anophagefferens</i>	97	Pelagophyte chloroplast	Ong et al. 2010
Sj10-xxx	<i>D. avara</i> & <i>A. oroides</i>	Plastid uncultured phototrophic eukaryote clone HF770_25L02; EU361155	North Pacific Subtropical Gyre	94	Eukaryotic chloroplast	Pham et al. 2008
Sj12-xxx	<i>A. oroides</i>	Uncultured Chloroflexi bacterium clone CC10; DQ247942	<i>Corticium embryo</i> Palau	90	Chloroflexi	Sharp et al. 2007
Sj13-xxx	<i>C. reniformis</i>	Uncultured bacterium clone i153; FM160905	<i>Aplysina fulva</i> Brazil	97	Deltaproteobacteria	Hardoim et al. 2009
Sj14-xx	<i>C. reniformis</i>	Uncultured bacterium clone 27H6; EU 183804	<i>Rhopaloeides odorabile</i> Pelorus Isl, Australia	97	Deltaproteobacteria	Webster et al. 2008
Sj15-xxx	<i>C. reniformis</i>	Uncultured Chloroflexi clone E117; FJ529342	<i>Svenea zeai</i> Salvador Isl, Bahamas	95	Chloroflexi	Lee et al. 2009

## Supporting information

SI Figure 1.- Rarefaction curves for ammonia-oxidizers groups. A)  $\beta$ -ammonia-oxidizing bacteria from *D. avara*, B) Ammonia-oxidizing archaea *amoA* clone libraries for *A. oroides* and C) Ammonia-oxidizing archaea *amoA* clone libraries for water samples. Operational taxonomic units (OTUs) were defined at a 5% sequence cut-off.

