

# First insights into the microbiome of a carnivorous sponge

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## Keywords

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## Abstract

Using 454 pyrosequencing, we characterized for the first time the associated microbial community of the deep-sea carnivorous Demosponge *Asbestopluma hypogea* (*Cladorhizidae*). Targeting the 16S rRNA gene V3 and V6 hypervariable regions, we compared the diversity and composition of associated microbes of two individual sponges of *A. hypogea* freshly collected in the cave with surrounding seawater and with one sponge sample maintained 1 year in an aquarium after collection. With more than 22 961 high quality sequences from sponge samples, representing *c.* 800 operational taxonomic units per sponge sample at 97% sequence similarities, the phylogenetic affiliation of *A. hypogea*-associated microbes was assigned to 20 bacterial and two archaeal phyla, distributed into 45 classes and 95 orders. Several differences between the sponge and seawater microbes were observed, highlighting a specific and stable *A. hypogea* microbial community dominated by *Proteobacteria* and *Bacteroidetes* and *Thaumarchaeota* phyla. A high relative abundance of ammonia-oxidizing archaea and a dominance of sulfate oxidizing/reducing bacteria were observed. Our findings shed lights on the potential roles of associated microbial community in the lifestyle of *A. hypogea*.

## Introduction

Sponges (Phylum *Porifera*) are the oldest of the extant metazoans, of great interest due to their ecological and biotechnological potential. With more than 8000 described species, mostly filter feeders, marine sponges appear to be a rich source of secondary metabolites, whose bacterial origin has been reported in numerous studies (Piel, 2009). Indeed, marine sponges live in association with a complex microbial community of exceptional diversity, which may reach up to 37% of the sponge volume (Vacelet, 1975). A combination of vertical and horizontal transmission has been demonstrated in the maintenance of these complex sponge–microbe symbioses (Ereskovsky *et al.*, 2005; Enticknap *et al.*, 2006; Taylor *et al.*, 2007; Lee *et al.*, 2009; Schmitt *et al.*, 2012a).

In the past decades, numerous studies have focused particularly on the cultivable bacterial symbionts with the aim of producing the metabolites of interest by biotechnology (Taylor *et al.*, 2007; Kennedy *et al.*, 2009; Sipkema *et al.*, 2011; Abdelmohsen *et al.*, 2012; Webster & Taylor, 2012). Given the low amount of microorganisms cultivable

in laboratory conditions (< 1% of the total microbial community; Amann *et al.*, 1995), several strategies have been developed to improve the cultivable microbial diversity, which include use of varying growth conditions and innovative culture methods such as floating filter culture systems (Alain & Querellou, 2009; Sipkema *et al.*, 2011).

Two ecological sponge types were described based on their microbial abundance, diversity and pumping rate (Vacelet & Donadey, 1977). Sponges qualified as high microbial abundance sponges have a microbial density between 10<sup>8</sup> and 10<sup>10</sup> cells per gram of sponge tissue, a dense mesohyl and long, narrow canals which can be correlated with a slow rate of water flow through the sponge. In contrast, low microbial abundance sponges have a microbial density between 10<sup>5</sup> and 10<sup>6</sup> cells per gram of sponge tissue, a lower density and a higher pumping rate, receiving a greater amount of nutrition from transient microbes (Hentschel *et al.*, 2006; Weisz *et al.*, 2008; Giles *et al.*, 2013). However, this classification could be re-evaluated using fluorescent *in situ* hybridization (FISH) experiments as suggested by some authors (Thacker & Freeman, 2012).

Recent advances in culture-independent methods, such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), 16S rRNA gene clone library construction and the exciting development of high throughput sequencing techniques including 454 pyrosequencing, have also demonstrated their significant impact on access to the uncultivable microbial diversity (Zhu *et al.*, 2008; Lee *et al.*, 2011; Hentschel *et al.*, 2012; Jackson *et al.*, 2012; Schmitt *et al.*, 2012a, b; White *et al.*, 2012). Molecular data based on 16S rRNA gene sequences revealed sponge-specific prokaryote communities, different from those of the surrounding seawater (Hentschel *et al.*, 2002; Taylor *et al.*, 2007) and their stability was demonstrated through spatio-temporal explorations (Lee *et al.*, 2011). So far, 29 bacterial phyla as well as two major lineages of archaea and a wide variety of microeukaryota have been identified in sponges (Taylor *et al.*, 2007; Webster *et al.*, 2010; Webster & Taylor, 2012). Six phyla were frequently recovered from sponges: *Proteobacteria* (with a majority of *Alpha*-, *Gamma* and *Deltaproteobacteria*), *Chloroflexi*, *Actinobacteria*, *Acidobacteria*, *Nitrospirae* and *Poribacteria*. The latter was postulated as a new, sponge-specific, candidate phylum (Fieseler *et al.*, 2004; Hentschel *et al.*, 2012).

The current knowledge obtained by metagenomics about the sponge–microbe associations is restricted to filtering demosponges.

Carnivorous sponges, in contrast to filtering demosponges, have lost their aquiferous systems and their choanocyte cells (Vacelet & Boury-Esnault, 1995). Around 119 species of carnivorous sponges were described, all belonging to the family of *Cladorhizidae* and mostly deep-sea sponges (Van Soest *et al.*, 2012). Their morphologic adaptation is correlated with their nutrient-poor deep-sea environment. Our model of study, *Asbestopluma hypogea*, was surprisingly discovered in a Northwestern Mediterranean littoral cave at 17 m depth near Marseille (La Ciotat, France; Vacelet & Boury-Esnault, 1996). Although usually found in deeper sites, *A. hypogea* was also discovered in two shallow water caves in Southern France (Jarre Island) and Croatia (Garmenjask Island; Bakran-Petricioli *et al.*, 2007; Aguilar *et al.*, 2011). This carnivorous species passively captures prey such as small crustaceans on its filaments. Capture is followed by extracellular and intracellular digestion and phagocytosis within 10 days (Vacelet & Dupont, 2004).

We investigated the microbial community associated with *A. hypogea* combining microscopy studies and 454 high throughput pyrosequencing. Targeting the V3 16S rRNA gene region, we compared the diversity of bacteria associated to two sponge samples of *A. hypogea* collected in the cave with surrounding seawater. As we observed the long-term survival of a sponge specimen in an aquarium,

we also used the V6 primer set on one of the two sponges investigated with the V3 primer and on one specimen maintained 1 year in an aquarium, aiming to compare the bacterial and archaeal communities of two sponge samples with different lifestyles and to assess the stability of the microbial symbionts of *A. hypogea*.

## Materials and methods

### Sponge and seawater sampling

*Asbestopluma hypogea* (class: *Demospongiae*, order *Poecilosclerida*, family: *Cladorhizidae*) is a carnivorous sponge isolated from a Mediterranean shallow-water submarine cave (3PP; La Ciotat, France). The sponge colonizes the rocky surface of the cave at around 20 m depth from the inlet. This tiny sponge is formed of an ovoid body with numerous filaments on its surface, supported by a thin peduncle. Samples were collected in July 2009 and May 2011 by SCUBA diving in the 3PP cave (La Ciotat, France) at a depth of 17 m.

Sponges were collected with a fragment of the rocky cave wall to preserve the sponge integrity and transferred to the laboratory. A sponge sample (CQ, July 2009) was held at 13 °C in a dark chamber with a monthly change of water and was fed with living *Artemia* sp. *nauplii* for 1 year. The sponge was starved 1 month before the analysis. Two samples (CA and CB, May 2011) were flushed with sterile artificial seawater (ASW) directly after collection and were kept at –80 °C for further analysis. Two additional samples (CC and CD, May 2011) were used for electron microscopy and placed in fixative for 24 h immediately after collection. No evidence of prey capture was observed in sponge samples (CC and CD) through microscopic observation. Three liters of seawater (SW) were also collected in the 3PP cave close to the sponges (May 2011) and were filtered through a 0.22-µm polycarbonate membrane (47 mm; Millipore) and stored at –80 °C.

### Transmission electron microscopy (TEM)

Immediately after collection, the two samples (CC and CD) were flushed with ASW and fixed for 24 h in a fixative containing 2.5% glutaraldehyde, 0.4 M sodium cacodylate and seawater with 1% of saturated picric acid (1 : 4 : 5, 1120 mOsm, pH 7.4), then rinsed in the same buffer. Post-fixation was performed in 2% osmium tetroxide in seawater for 2 h. After dehydration through a graded ethanol series, samples were embedded in Spurr low viscosity resin for ultrathin sections. Blocks were then sectioned and counterstained with 2% uranyl acetate in ethanol solution. *Asbestopluma hypogea* tissue sections

were observed (seven sections per sample) under a Hitachi H-7100 electron microscope equipped with an image analyzer system (CCD camera Hamamatsu coupled with AMT software).

### Metagenomic DNA extraction from sponge

Extraction of the metagenomic DNA from *A. hypogea* followed published procedures (Piel *et al.*, 2004). Sponges were thawed on ice and flushed with ASW to remove loosely attached microbes and debris. Sponges were ground to a fine powder under liquid nitrogen in a sterile mortar and were frozen twice in 200  $\mu$ L extraction buffer [100 mM Tris-HCl, 100 mM EDTA, 100 mM NaPPi, 1.5 M NaCl, 1% cetyl trimethyl ammonium bromide (CTAB), pH 8.0]. A treatment with proteinase K was performed at a final concentration of 1 mg mL<sup>-1</sup> and then incubated at 37 °C for 30 min with regular inversion every 5 min. A second treatment was carried out with 360  $\mu$ L of 20% sodium dodecyl sulfate (SDS) and incubated at 65 °C for 2 h with gentle inversion every 20 min. The supernatant was collected after 10 min of centrifugation at 14 000 g at room temperature. Cell pellets were extracted again with 100  $\mu$ L of extraction buffer and 10  $\mu$ L of 20% SDS, then vortexed for 10 s and incubated at 65 °C for 10 min. After incubation, a second centrifugation at 14 000 g for 10 min was performed and the supernatants were combined. Purification of genomic DNA (gDNA) was carried out with 330  $\mu$ L of phenol : chloroform : isoamyl alcohol (25 : 24 : 1) and vortexed for 20 s. After a centrifugation at 14 000 g for 10 min, 0.6% of isopropanol was added to the aqueous phase and incubated at room temperature for 1 h. The supernatant was separated from the pellet by a centrifugation at 15 000 g for 20 min at room temperature and the pellets were washed with 100  $\mu$ L of a cold solution of 70% ethanol and dried at room temperature for 1 h. Total gDNA was recovered in 50  $\mu$ L of deionized water and stored at -20 °C.

### Metagenomic DNA extraction from seawater

Filter membranes (0.22  $\mu$ M) were cut aseptically and gDNA was extracted following the procedure described in the paragraph above.

### PCR amplicon library preparation for V3–V6 pyrosequencing runs

The V3 and V6 hypervariable regions of the 16S rRNA gene were amplified by PCR labeled with specific MID for each amplicon (Supporting Information, Table S1). These two regions are commonly used to obtain a sufficient coverage of bacterial phyla present in the samples (Armougom &

**Table 1.** Sequences and position of primers targeting V3 and V6 regions on the 16S rRNA gene

| Primers                     | Sequence 5'-3'            | Targeted region | Amplification length (bp) |
|-----------------------------|---------------------------|-----------------|---------------------------|
| <i>Bacteria and Archaea</i> |                           |                 |                           |
| U789F*                      | TAGATACCCSSGTAGTCC        | V6              | 279                       |
| U1068R*                     | CTGACGRCRGCCATGC          | V6              |                           |
| <i>Bacteria</i>             |                           |                 |                           |
| E338F†                      | ACTCCTACGGGAGGCA<br>GCAGT | V3              | 167                       |
| E505R†                      | CTGCTGGCACGDAGTTAGCC      | V3              |                           |

\*Lee *et al.* (2009).

†Wang & Qian (2009).

Raoult, 2009). PCR reactions were carried out in 50- $\mu$ L final volume reaction mixture containing 1.5 mM MgCl<sub>2</sub> buffer (VWR), 0.2 mM of each dNTP (Promega), 2.5 units of Taq DNA polymerase (VWR), 0.6  $\mu$ M of each primer (Table 1) and 2  $\mu$ L DNA at 30 ng  $\mu$ L<sup>-1</sup>. PCR reactions were conducted in a Veriti™ Thermal Cycler (Applied Biosystem) with an initial denaturing step (95 °C for 4 min) followed by 30 cycles of denaturation at 95 °C for 1 min, primer annealing at 55 °C for 30 s and extension at 72 °C for 30 s, and a final extension step at 72 °C for 7 min. Each PCR reaction was duplicate and combined to avoid potential PCR biases.

Amplicons were visualized on agarose (1.5%) electrophoresis gel in a TAE buffer. Quantification of DNA samples was measured by Genoscreen company ([www.genoscreen.fr](http://www.genoscreen.fr)) on a Agilent 2100 Bioanalyzer (Table S2). For multiplexing library preparation, an equimolar mix was realized at a final concentration of 10<sup>9</sup> molecules  $\mu$ L<sup>-1</sup>. Two sequencing runs were performed on the same library using the Roche 454 FLX titanium platform (454, Life Science).

### Pyrosequencing data analysis

Raw pyrosequencing data containing a set of barcoded multiplex pyrosequences were first analyzed for demultiplexing and size selection using GENEIOUS® PRO 5.6.6 (Biomatters Ltd). All these data were submitted to NCBI Short Reads Archive Database (SRR767734 and SRR767737). Sequences shorter than 100 bp and which may contain homopolymer sequences or ambiguous nucleotides were removed. Chimeric sequences detection and exclusion were conducted using the results from the DECIPHER FIND CHIMERAS web tool from the University of Wisconsin (<http://decipher.cee.wisc.edu/FindChimeras.html>; Wright *et al.*, 2012). Denoising, alignment of sequences and clustering (cutoff 0.01, 0.01 and 0.03) were done using the SSH.FLOWS protocol MOTHUR platform, which is an implementation of the PyroNoise for MOTHUR,

and SHHH.FLOWS uses a expectation-maximization algorithm to correct flowgrams (version 1.25.1; Schloss *et al.*, 2009). Sequence identification and taxonomical assignment were performed using the MOTHUR protocols against the release 111 of the SILVA 16S rRNA gene database and taxonomy (Pruesse *et al.*, 2007; Quast *et al.*, 2013), the RDP CLASSIFIER (Wang *et al.*, 2007) and BLASTN (Altschul *et al.*, 1990) search against prokaryotic division of GenBank (release 192 – Oct 2012) and NCBI Taxonomy database. Clustering was done using the average neighbor algorithm. Operational taxonomic units (OTUs) were defined at the same cutoff value and were used to construct rarefaction curves, to estimate richness (Chao1 and Ace), and to calculate diversity indices (Shannon and Simpson). In our study, OTUs were defined as sequence groups in which sequences differed by 3%. Sample comparisons were performed based on the abundance-based Sorenson dissimilarity index (Sorabund) and Jaccard index (Jclass) computed using the MOTHUR platform.

## Results

### TEM observations

Ultrathin sections were performed on the ovoid body of *A. hypogea* and revealed numerous symbiotic bacteria within intracellular bacteriocytes (2–10 cells per vacuole; Fig. 1a and b). Extracellular bacteria were also detected near the sponge cells (Fig. 1c) located in the extracellular matrix and did not form accumulations. Two preponderant morphotypes, rod (Fig. 1c) and coccoid cells (Fig. 1d), of numerous shape and size, were detected within *A. hypogea* tissues.

Symbiotic bacteria up to 5 µm in length and 2 µm in diameter were detected in bacteriocytes (Fig. 1a and b). The nucleoid region is narrow and filamentous. No flagella or pili at the surface of the bacterial wall were observed. Based on the thickness of the outer membrane, Gram-positive and Gram-negative morphotypes were distinguished within bacteriocytes (Fig. 1b). Gram-negative cells harbored an inner and external membrane (Fig. 1d) and Gram-positive cells possessed a single membrane with a large amount of peptidoglycans (Fig. 1e and f).

### General analyses of the pyrosequencing-derived dataset

After pyrosequencing, more than 60 000 sequences were recovered from three samples of sponge and one sample of the seawater column, amplified on 16S rRNA gene spanning the V3 and V6 hypervariable regions. Sequences were screened first for their quality and length fragments (Table 2).

Taxonomic assignments of 22 961 high quality tag sequences for sponge and 3582 for the surrounding seawater were obtained using the average read length of *c.* 150 and 250 bp for the V3 and V6 regions, respectively. More than 17 765 high quality sequences were obtained for the V3 region (7621 for CA; 6562 for CB and 3582 for SW) and 8778 sequences for the V6 region (4055 for CA and 4723 for CQ; Table 3). Rarefaction curves were established on these 26 543 sequences at 97% sequence similarity level for OTUs identification (Fig. 2). The rarefaction analysis showed, first, that the sampling curves of sponge were more saturated than those from seawater and, secondly, that the sponge samples (CA and CB) exhibited similarly saturated curves.

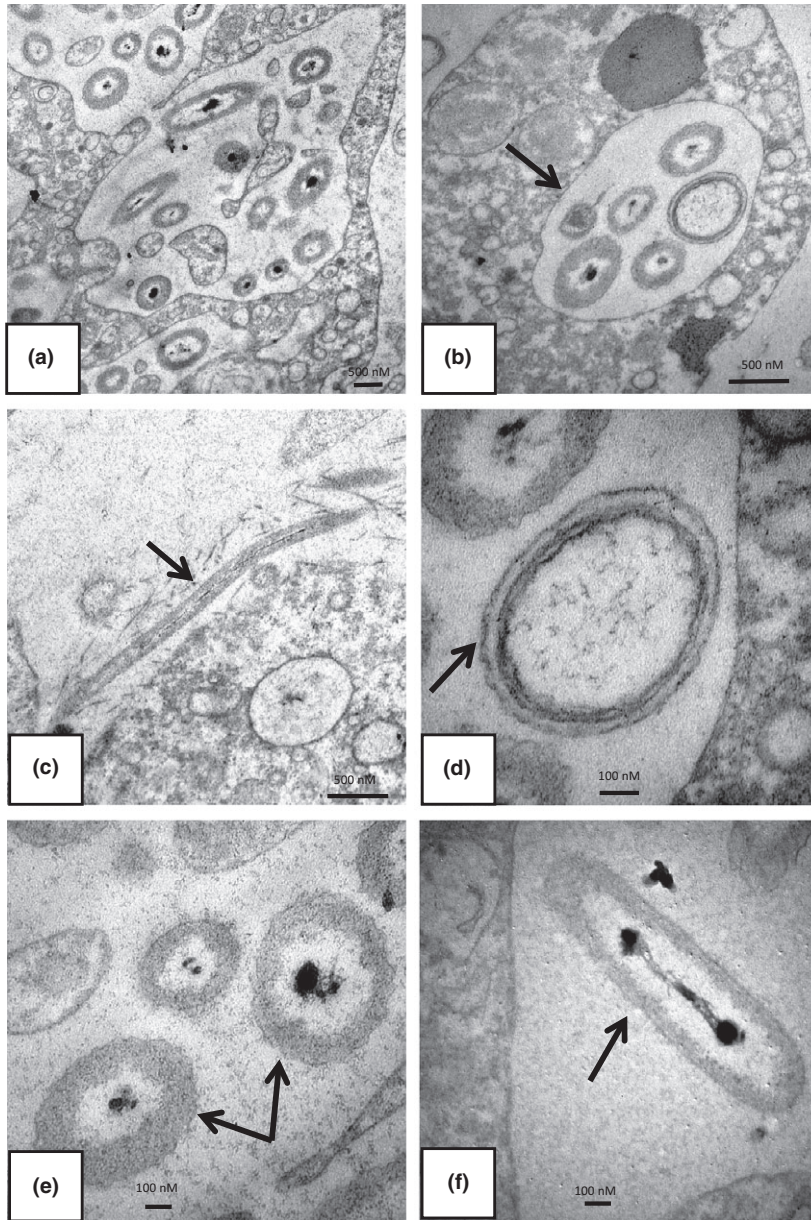
The 26 543 sequences derived from sponge and seawater samples were classified to domain level on the SILVA database using a 97% pairwise-identity threshold.

### Bacterial diversity of the 16S rRNA gene V3 hypervariable region

The comparison of the species richness indices Chao1 of the two *A. hypogea* samples with that of the seawater surrounding the sponge in natural conditions showed similar values at 97% sequence similarity (3035 for CA; 3732 for CB and 3385 for SW). However, the Simpson indice allows to the sponge and seawater samples to be distinguished (7024 for CA, 7030 for CB and 5989 for SW). More than 800 OTUs were obtained from each sponge sample, indicating that the sponge microbiome was highly complex. Among them, 283 OTUs were found both in CA and CB, whereas fewer than 40 OTUs were common to both sponge (CA and CB) and surrounding seawater (SW) samples. The Shannon and Simpson indices of diversity were determined for the three samples, and indicated the highest homogeneity of microbial diversity in seawater at 97% sequence similarity, and a specific microbial community within the sponge samples (CA:  $H' = 4.10$  and Simpson = 0.06; CB:  $H' = 4.08$  and Simpson = 0.06; SW:  $H' = 5.47$  and Simpson = 0.02; Table 3).

Taxonomic V3-tag assignments via the SILVA 16S rRNA gene database revealed that replicates of the same sample type (CA and CB) contained similar microbial communities, whatever the examined phylogenetic level (Figs 3a and 4a), demonstrating that different individuals of the same environment harbor similar microbiomes. All OTUs were assigned to phylum level. The vast majority of OTUs contained in sponge were affiliated, at 97% sequence similarity, with 13 bacterial phyla and two candidate phyla for both sponge samples, and 17 bacterial phyla and one candidate phyla for the seawater sample (Fig. 3a).

In addition, more than 98% of V3 reads from sponge samples (CA and CB) were affiliated with the two dominant



**Fig. 1.** Transmission electron micrographs of the mesohyl. (a: CC, b: CD) Overview of intracellular bacteriocyte. (c: CC) Rod-shape extracellular symbiotic bacteria. (d: CD) Gram-negative ovoid cell type. (e: CC, f: CC) Gram-positive ovoid and rod type cell. The arrows indicate the bacteriocyst and bacteria into the mesohyl of *Asbestopluma hypogea*.

phyla *Proteobacteria* (82%) and *Bacteroidetes* (16%). The phyla of *Acidobacteria*, *Actinobacteria*, Candidate division TM7, Candidate division OP3, *Cyanobacteria*, *Chloroflexi*, *Fibrobacteres*, *Firmicutes*, *Gemmatimonadetes*, *Lentisphaerae*, *Planctomycetes*, *Spirochaetes*, and *Synergistetes* were found to be minor (< 1.5%). Similar results were observed with the seawater sample but with a higher proportion of *Cyanobacteria* (1.7%) and *Actinobacteria* (0.8%) and the presence of additional minor phyla including *Chlamydiae*, *Defferibacteres*, *Deinococcus-Thermus*, *Fusobacteria*, *Nitrospirae*, *Thermodesulfobacteria*, *Verruimicrobia* and the candidate division TM6. The phyla of *Fibrobacteres*, *Spirochaetes*, *Synergistetes* and the two

candidate divisions TM7 and OP3 were absent in the surrounding seawater sample (Fig. 3a, Table S3a). At the class level, 45 classes were detected in sponge samples and 35 classes in the surrounding seawater sample. These SILVA assignments showed a dominance of *Gammaproteobacteria*, *Alphaproteobacteria* and *Flavobacteria* in sponge and seawater samples. In addition, three additional classes, *Deltaproteobacteria* (> 11%), *Sphingobacteria* (> 5%) and Milano-WF1B-44 (> 2.8%) were only present in sponge samples (Fig. 4a, Table S4a).

In addition, comparisons of the relative abundance of OTUs in CA, CB and SW using a heatmap representation, revealed the dominance of 18 OTUs including

**Table 2.** Overview of the results for sponge and seawater samples.

|   | Sponge | Seawater |
|---|--------|----------|
| Total no. of samples                    | 4      | 1        |
| Total raw sequences                     | 45 786 | 13 275   |
| Sequences below length requirement      | 22 825 | 9693     |
| Sequences passing quality filtering (%) | 99     | 99       |
| Average of putative chimeric reads (%)  | 1      | 1        |
| Final high-quality sequence count       | 22 961 | 3582     |
| Average reads per sample                | 5740   | –        |
| Average of OTUs per sample              | 2348   | 2582     |
| Unique phyla detected (BLAST)           | 17     | 16       |

Otu0268 (*c.* 10%), Otu2300 (*c.* 10%) and Otu0432 (*c.* 20%) (corresponding to *Ulvibacter* genus of *Flavobacteria*, *Nitrospina* genus of *Deltaproteobacteria* and BD2-7 genus group of *Gammaproteobacteria*, respectively) in the sponge samples, whereas they were in low abundance in seawater sample. In addition, both OTUs, Otu0269 (*c.* 5%), Otu0436 (*c.* 8%) (corresponding to *Ulvibacter* genus of *Flavobacteria* and *Glaciecola* genus of *Gammaproteobacteria*, respectively) were detected in seawater samples and were absent in the two sponge samples (Fig. 5). Replicates from the sponge samples exhibited a highly conserved bacterial community at all taxonomic level assignments.

To confirm these identifications, two additional assignments were performed by comparison with the RDP and GenBank databases.

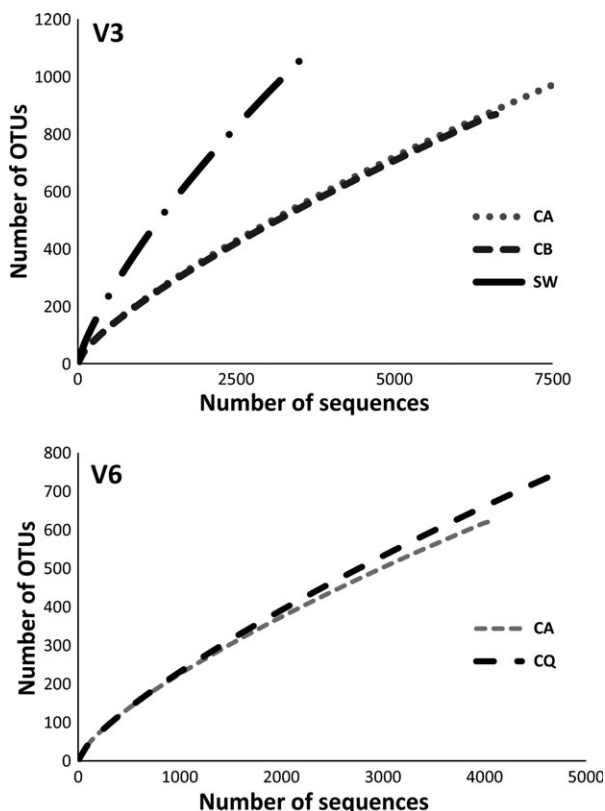
The vast majority of OTUs contained in sponge and seawater samples were affiliated with only 16 bacterial phyla belonging to *Proteobacteria* and *Bacteroidetes*, confirming the results obtained by *SILVA* analysis, although fewer classes ( $n = 26$ ) and orders ( $n = 49$ ) were observed (Figs S1a, S2a, S3a and S4a).

These analyses also revealed a high average number of deep-sea sequences derived from sediments or from symbiotic associations such as sequences affiliated with the sulfur-oxidizing-symbiont group (Fig. S4a). Comparisons at the order level between sponge and seawater samples revealed some specific orders present exclusively in seawater (*Vibrionales*, *Oceanospirillales*, *Rhodobacterales*) and

**Table 3.** OTU analyses. Abundance estimators (Chao1 and Ace) and diversity estimators (Shannon and Simpson) in sponge and seawater samples of the V3 and V6 16S rRNA gene hypervariable regions

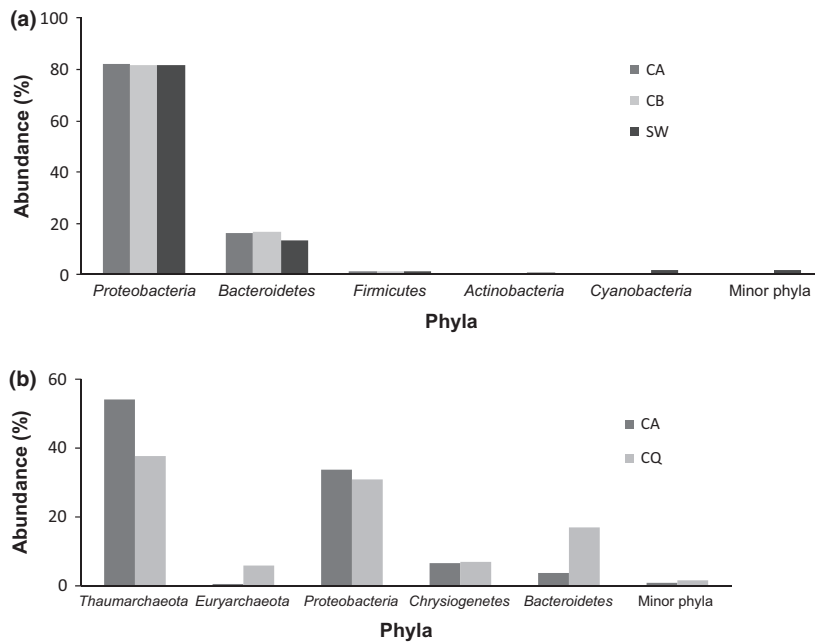
| Sample name | No. of high quality reads | No. of OTUs | Chao1*  | Ace*    | Shannon ( $H'$ )* | Simpson* |
|-------------|---------------------------|-------------|---------|---------|-------------------|----------|
| <b>V3</b>   |                           |             |         |         |                   |          |
| CA          | 7621                      | 982         | 3035.48 | 7024.54 | 4.10              | 0.06     |
| CB          | 6562                      | 868         | 3732.53 | 7030.15 | 4.08              | 0.06     |
| SW          | 3582                      | 1072        | 3385.00 | 5989.94 | 5.47              | 0.02     |
| <b>V6</b>   |                           |             |         |         |                   |          |
| CA          | 4055                      | 624         | 1977.08 | 4872.82 | 4.04              | 0.05     |
| CQ          | 4723                      | 748         | 2515.06 | 5640.94 | 4.15              | 0.06     |

\*Estimates at a 3% dissimilarity.

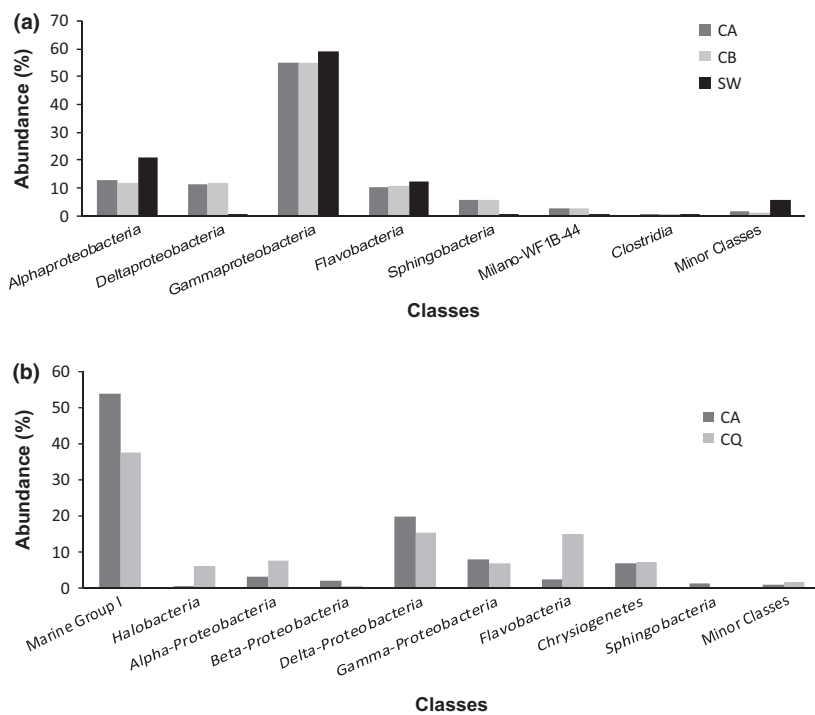
**Fig. 2.** Diversity of sponge-associated prokaryotic communities and prokaryotes in seawater. Rarefaction curves of each sample indicating the observed number of OTUs at 97% sequence similarity on the two hypervariable 16S rRNA gene V3 and V6.

led to the conclusion that major differences exist between sponge and seawater samples (Fig. S4).

Statistical analyses were performed to compare the sponge microbiome and the microorganisms contained in the surrounding seawater column at different levels of dissimilarity (96%, 97% and 99%). Sorënsen (Sorabund) and Jacard (Jclass) showed a high dissimilarity between the two sponge samples and the surrounding sea water column (at 97% sequence similarity, Sorabund = *c.* 0.80; Jclass =  $\pm$  0.70; Table 4a and b).



**Fig. 3.** Taxonomic distributions of assigned V3 (a) and V6 (b) tag sequences at the phylum level using the SILVA database. Bars represent for each sample the proportion (expressed as percentage) of tags that belong to a given prokaryotic phylum. All phyla with a lower abundance (< 0.1%) were combined in the 'minor group' cluster. See Table S2 for details of the abundance of each phylum.

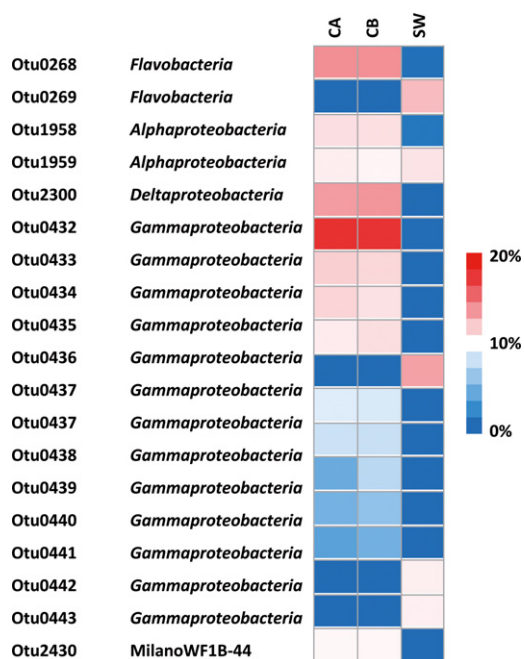


**Fig. 4.** Taxonomic distribution of assigned V3 (a) and V6 (b) tag sequences at the class level using the SILVA database. Bars represent for each sample the proportion (expressed as percentage) of tags that belong to a given prokaryotic phylum. All classes with a lower abundance (< 0.1%) were combined in the 'minor group' cluster. See Table S3 for details of the abundance of each class.

### Microbial diversity of the 16S rDNA gene V6 hypervariable region

Approximately 8778 high quality sequences were obtained from both samples of *A. hypogea* analyzed using the V6 region (Table 3). To estimate the richness of these two samples, the abundance estimators (Chao1 and Ace), the

diversity estimators (Shannon and Simpson) and OTU analyses were calculated at 97% sequence similarity and demonstrated a higher number of OTUs in *A. hypogea* from the aquarium (CQ, 748 OTUs) than in freshly collected *A. hypogea* (CA, 624 OTUs; Table 3). However, the abundance estimates indicate that the totality of expected diversity was not obtained. Similar values for estimated



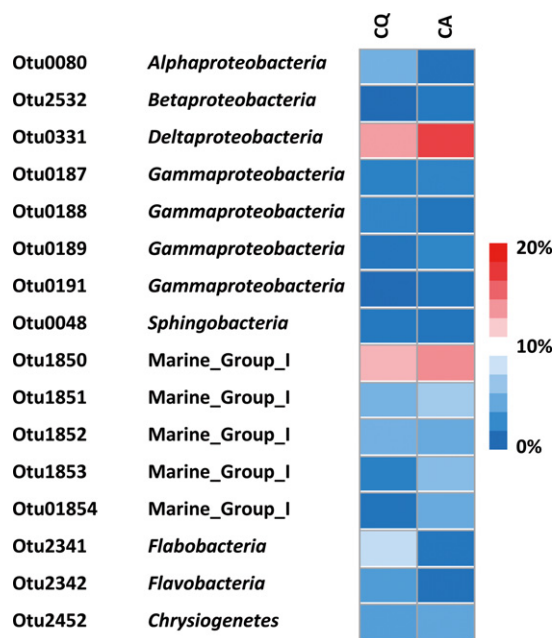
**Fig. 5.** Occurrence of 'major OTUs' clusters derived from V3 reads in sponge and seawater sample. Heatmap showing the relative abundance (expressed as percentage) of major OTUs, color code indicates relative abundance, ranging from blue (low abundance) via blank to red (high abundance).

**Table 4.** Matrix of dissimilarity between the sponge and the surrounding seawater samples based on Sorabund (a) and Jclass (b) indices at 97% sequence similarity

|              | CA   | CB   | SW |
|--------------|------|------|----|
| (a) Sorabund |      |      |    |
| CA           | 0    | –    | –  |
| CB           | 0.13 | 0    | –  |
| SW           | 0.80 | 0.86 | 0  |
| (b) Jclass   |      |      |    |
| CA           | 0    | –    | –  |
| CB           | 0.07 | 0    | –  |
| SW           | 0.67 | 0.76 | 0  |

microbial diversity were obtained for the two *A. hypogea* samples (CA,  $H' = 4.04$  and Simpson = 0.05; CQ,  $H' = 4.15$  and Simpson = 0.06; Table 3).

Taxonomic V6 assignment was firstly carried out by comparison of sequences with those present in the SILVA database (Figs 3b and 4b). A large proportion of archaea was observed in both sponge samples (> 50% for CA and < 40% for CQ). Phylum level composition of prokaryotic communities was highly conserved in both samples (Fig. 3, Table S3b). All OTUs were assigned to 13 bacterial and two archaeal phyla. The five dominant phyla were *Bacteroidetes* (3.7% in CA and 17% in CQ), *Chrysiogenetes*



**Fig. 6.** Occurrence of 'major OTUs' clusters derived from V6 reads in sponge and seawater sample. Heatmap showing the relative abundance (expressed as percentage) of major OTUs, color code indicates relative abundance, ranging from blue (low abundance) via blank to red (high abundance).

(7% in CA and CQ), *Crenarchaeota* (54% in CA and 38% in CQ), *Euryarchaeota* (0.7% in CA and 6% in CQ) and *Proteobacteria* (33% in CA and 30% in CQ), present in high s in the two sponge samples (Fig. 3b). *Deinococcus-Thermus*, *Firmicutes*, *Acidobacteria*, *Actinobacteria*, *Planctomycetes*, *Verrumicrobia*, *Chlorobi*, *Deferribacteres*, *Nitrospirae* and *Thermodesulfobacteria* were found only in small numbers (< 1%). At the class level, 29 classes and two candidate classes were detected, with the majority of OTUs belonging to Marine Group I (37% in CQ and 50% in CA), *Delta-* (16% in CQ and 20% in CA), *Gamma-* (7% in CQ and 8% in CA) and *Alphaproteobacteria* (8% in CQ and 3% in CA), *Halobacteria* (6% in CQ and < 1% in CA) and the *Flavobacteria* (15% in CQ and 2.5% in CA) classes (Fig. 4b, Table S4b). The bacterial community composition of individual samples was evaluated by the comparison of relative abundance of all OTUs. A heatmap constructed from the major OTUs showed that the two samples clustered tightly together (Fig. 6).

Further analyses were realized using RDP CLASSIFIER and BLASTN softwares. Examining reads at the phylum level, the most dominant phyla among those assigned on the RDP and GeneBank database were *Proteobacteria*, *Bacteroidetes*, *Thaumarchaeota* and *Euryarchaeota*, divided into 21 classes (Figs S1b, S2b and S3b). Archaeal reads represented more than 50% of the total sequences, with a large



part of archaea affiliated to *Nitrosopumilales* order (Fig. S5). Results obtained with the GenBank assignments confirmed the presence of *Deltaproteobacteria* (*Desulfobacterales*, *Desulfovibrionales*) previously detected on the assignment of V3 reads. Both latter analyses (RDP and BLASTN) confirmed the taxonomic identification obtained from the SILVA database.

The dissimilarity coefficient of Sorensen (Sorabund) and Jacard (Jclass) revealed a low dissimilarity of microbiome between the two *A. hypogea* (Sorabund = 0.20; Jclass = 0.1). The microbial composition of both sponge samples was similar, which suggests the establishment of a close relationship between microorganisms and their host over time, and when sponges were maintained under artificial conditions.

## Discussion

The sponge–microbe ecosystems are complex associations with significant ecological and biotechnological impacts (Taylor *et al.*, 2007). Over the past decade, the identification of their microbiomes has emerged as essential for understanding the symbiotic relationships between the sponge hosts and their associated microbial communities. However, recent studies conducted using the pyrosequencing technology were restricted to filtering sponges (Hentschel *et al.*, 2012). Here we report for the first time the microbial diversity associated with the carnivorous sponge *A. hypogea* using pyrosequencing technology.

The presence of numerous and diverse symbiotic microorganisms has been observed in *A. hypogea* within bacteriocytes with 5–10 microbes per vacuole. From these ultrastructural observations, *A. hypogea* could be qualified as a bacteriosponge, as previously described by Reiswig (1981). These results prompted us to explore the *A. hypogea* microbial community.

Two sponge samples of *A. hypogea* freshly collected in the cave were analyzed using 16S rRNA gene tag pyrosequencing and compared with a surrounding seawater sample and with one sponge sample maintained 1 year in an aquarium after collection.

Read analyses of *A. hypogea* samples allowed the identification of 20 bacterial phyla and two archaeal phyla using the SILVA database, whereas 10 bacterial phyla and two archaeal phyla were assigned in the RDP database with 10–50% of unclassified reads, and 16 bacterial phyla and two archaeal phyla were assigned using BLASTN software with only 5–10% of unclassified reads. These results revealed a better efficiency and a higher quality of taxonomic identification using the SILVA database and highlighted the importance of using two sets of primers targeting the hypervariable V3 and V6 regions of 16S rRNA genes. A lower diversity at the phylum level and no

new candidate phylum were observed in comparison with other reported sponge pyrosequencing investigations.

Our results revealed diverse bacterial and archaeal phyla associated with *A. hypogea* that are distinct from those present in the surrounding seawater. They represent a typical bacterial profile of bacteriosponges (Hentschel *et al.*, 2002; Taylor *et al.*, 2007) with *Proteobacteria*, *Bacteroidetes* and *Firmicutes* as major bacterial phyla. The presence of *Thaumarchaeota* as the major prokaryotic phylum in *A. hypogea* is uncommon. This phylum, recently split from the *Crenarchaeota* phylum (Brochier-Armanet *et al.*, 2008), was frequently reported to be associated with sponges (Hoffmann *et al.*, 2009; Turque *et al.*, 2010; Radax *et al.*, 2012a, b), sometimes in large amounts (65% of the whole microbiome), as exemplified by the Californian sponge *Axinella mexicana* (Margot *et al.*, 2002). Previous studies have identified 29 bacterial phyla including 10 candidate phyla and two major archaeal lineages from demosponges from Mediterranean Sea (*Aplysina aerophoba*, *Aplysina cavernicola*, *Ircinia variabilis*, *Petrosia ficiformis*, and *Pseudocorticium jarrei*), Red Sea (*Hyrtios erectus*, *Stylissa carteri* and *Xestospongia testudinaria*), Caribbean Sea (*Axinella corrugata*) and Pacific Ocean (*Ircinia ramosa*, *Ianthella basta* and *Rhopaloeides odorabile*; Webster *et al.*, 2010; Lee *et al.*, 2011; Schmitt *et al.*, 2012a, b; White *et al.*, 2012). The sponge microbial communities, remarkably similar in these studies, revealed the phyla *Proteobacteria*, *Chloroflexi*, *Actinobacteria*, *Acidobacteria*, *Nitrospirae* and the candidate phylum *Poribacteria* as dominant. The lack of significant discrepancy between the *A. hypogea* and the microbial communities of the filtering sponge may be attributed to the presence of underrepresented sponge-specific species. Worthy of note, the *Poribacteria* candidate phylum, found exclusively in marine sponges, is missing in *A. hypogea* pyrosequencing data and in TEM observations. The *Poribacteria* can be easily recognized in microscopic observations by their morphology, which is characterized by the presence of a membrane-bound nucleoid (Fieseler *et al.*, 2004). The lack of *Poribacteria* in the pyrosequencing dataset could reflect a bias of the specificity of primers used in this study, which may have failed to detect this specific phylum. Indeed, using the same set of primers, Arellano *et al.* (2012) have recently reported the lack of *Poribacteria* in the *Myxilla methanophila* deep-sea sponge. In addition, although methanotrophic symbionts have already been detected in TEM observations of the carnivorous deep-sea sponge *Cladorhiza* sp. (Vacelet *et al.*, 1996; Vacelet & Boury-Esnault, 2002), no thioautotrophic bacteria were detected in *A. hypogea*, either by ultrastructural cell observations or by taxonomic affiliations.

To evaluate the stability of the microbial association in different living conditions, we have compared the

microbial diversity of one sponge sample freshly collected in the cave with one sample maintained for 1 year in the aquarium. In these two *A. hypogea* samples, reads spanning the V6 region were dominantly affiliated to the *Proteobacteria* and *Thaumarchaeota* phyla and in less amount to the *Bacteroidetes*, *Chrysiogenetes* and *Euryarchaeota* phyla. At the class level, we observed a higher proportion of the *Halobacteria*, *Flavobacteria* and *Alphaproteobacteria* classes in the sponge maintained under artificial life conditions. In contrast, reads affiliated to *Thaumarchaeota* of Marine Group I class and to the *Deltaproteobacteria* class appeared to be higher in the freshly collected sponge sample. Furthermore, the same proportion of major OTUs were found in these samples. Overall, these results suggest that *A. hypogea* harbors a specific and stable microbial community over time and under different life conditions. Remarkably, *A. hypogea* does not seem to be stressed by the life conditions in the aquarium, and therefore it could be used as a useful experimental model to study carnivorous sponges. Other studies have been carried out to evaluate the stability of sponge microbiomes on a temporal scale (Taylor *et al.*, 2007; Erwin *et al.*, 2012) and under artificial life conditions for several filter-feeding demosponges (Friedrich *et al.*, 2001; Mohamed *et al.*, 2008; Webster *et al.*, 2011). Under artificial conditions, the associated microbiome was stable in *A. aerophoba* and *R. odorabile*, but an increase in microbial diversity was noticed in *Ircinia strobilina*.

Furthermore, our findings shed light on the functional roles of associated microbes in the lifestyle of *A. hypogea*. The high relative abundance of ammonium-oxidizing archaea and a dominance of sulfate-reducing bacteria in the sponge suggest their involvement in the detoxification process and nitrogen/carbon cycles. To verify this hypothesis, further studies by metatranscriptomics as previously conducted with the sponge *Phakellia fusca* and *Geodia barretti* will be required (Han *et al.*, 2012; Radax *et al.*, 2012b). Another potential role of the microbial community could be related to the digestion process. It has been observed by TEM that the density of symbiotic bacteria increased during the early stage of crustacean prey digestion (Vacelet & Duport, 2004). A culture-dependent approach to analyze the microbes with key genes in digestion, for example chitinase genes, would help to clarify this hypothesis. Studies on the cultivable microbial diversity are underway to complement these first insights into the carnivorous *A. hypogea* microbiome.

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## Supporting Information

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

**Fig. S1.** Taxonomic identification of assigned V3 (a) and V6 (b) tag sequences at the phylum level using RDP classifier. The number of reads per phylum is illustrated. Graph was divided in two parts with two scales: left part represent major phyla and the right part shows minor phyla.

**Fig. S2.** Taxonomic identification of assigned V3 (a) and V6 (b) tag sequences at the phylum level using BLASTn Software. The number of reads per phylum is illustrated. Graphs were divided in two parts with two scales: left part represent major phyla and right part shows the minor phyla.

**Fig. S3.** Taxonomic identification of assigned V3 (a) and V6 (b) tag sequences at the class level using BLASTn Software. The number of reads per class is illustrated. Graphs were divided in two parts with two scales: left part represent major phyla and the right part shows minor phyla.

**Fig. S4.** Taxonomic identification of reads derived from sponge (a: CA & b: CB) and surrounding seawater (c: SW) 16S rDNA amplifications on the hypervariable 16S rDNA region V3 at the order level using BLAST procedure.

**Fig. S5.** Taxonomic identification of reads derived from sponge (a: CQ & b:CA) 16S rDNA amplifications on the hypervariable 16S rDNA region V6 at the order level using BLAST procedure.

**Table S1.** List of adaptator sequences and their corresponding sample.

**Table S2.** Sequences and position of primers targeting V3 and V6 regions on the 16S rDNA gene.

**Table S3.** Abundance of each phylum from V3 (a) and V6 (b) hypervariable sequences using SILVA database.

**Table S4.** Abundance of each class from V3 (a) and V6 (b) hypervariable sequences using SILVA database.