1 Removing environmental sources of variation to gain insight on
2 symbionts vs. transient microbes in High and Low Microbial
3 Abundance sponges

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15 Running tittle: Symbionts vs transient bacteria in HMA and LMA sponges

Abstract

In this study, we pursue unraveling the bacterial communities of 26 sponges, belonging to several taxonomical orders, and comprising Low Microbial Abundance (LMA) and High Microbial Abundance (HMA) representatives. Particularly, we searched for species-specific bacteria, which could be considered as symbionts. To reduce temporal and spatial environmentally-caused differences between host species we sampled all the sponge species present in an isolated small rocky area in a single dive. The bacterial communities identified by pyrosequencing the 16S rRNA gene showed that all HMA species clustered separated from LMA sponges and seawater. HMA sponges often had highest diversity but some LMA sponges had also very diverse bacterial communities. Network analyses indicated that no core bacterial community seemed to exist for the studied sponges, not even for such a space- and time-restricted sampling. Most sequences, particularly the most abundant ones in each species, were species-specific for both HMA and LMA sponges. The bacterial sequences retrieved from LMA sponges, despite being phylogenetically more similar to seawater did not represent transient seawater bacteria. We conclude that sponge bacterial communities depend more on the host affiliation to the HMA or LMA groups than on host phylogeny.

50 **Introduction**

51 Diverse bacterial assemblages that are virtually absent from seawater have been
52 found in high abundances in geographically and/or phylogenetically distant
53 sponge hosts (e.g., Hentschel *et al.*, 2002; Taylor *et al.*, 2007; Webster *et al.*, 2010;
54 Schmitt *et al.*, 2012). The specificity of these sponge-associated microbial
55 assemblages and their vertical transmission from the parental sponge tissue to
56 their propagules (e.g., Usher *et al.*, 2001; Oren *et al.*, 2005; De Caralt *et al.*, 2007;
57 Schmitt *et al.*, 2008; Lee *et al.*, 2009; Uriz *et al.* 2012) strongly suggests a symbiotic
58 relationship, and one could conclude that microbial symbionts have a key role in
59 the evolutionary history and ecological success of sponges (Taylor *et al.*, 2007;
60 Hentschel *et al.*, 2012, Thacker and Freeman, 2012). However, an evolutionary
61 scenario allowing co-evolution of hundreds of different bacteria species and the
62 associated host seems unlikely, which suggests that the symbionts may be
63 concealed among the many transient bacteria found in sponge tissues.
64 Determining which bacteria detected in sponge tissue really are sponge symbionts
65 remains an important question for understanding the origin and maintenance of
66 these associations. We considered consistent species-specific bacteria-sponge
67 associations as an indicator of possible symbiotic relationships.

68 Sponges have been traditionally classed in two ecologically distinct groups
69 according to the characteristics and abundance of their associated microbiota. In
70 some sponge species, microbial communities can contribute up to 38% of the
71 sponge volume (Vacelet and Donadey, 1977). Such bacteria-rich sponges were
72 called bacteriosponges (Reiswig, 1981), and, more recently, High Microbial
73 Abundance (HMA) sponges (Hentschel *et al.*, 2003). Many other species were
74 considered to be devoid of associated microorganisms (based on electron

75 microscope observations) and were called non-bacteriosponges (Reiswig, 1981) or
76 Low Microbial Abundance (LMA) sponges (Henstchel *et al.*, 2003). They were later
77 shown to harbor bacteria although at lower concentrations, similar to those of the
78 water column (Henstchel *et al.*, 2003).

79 The difference in microbial biomass between LMA and HMA sponge groups
80 may represent two contrasting life strategies. HMA species have a denser mesohyl
81 that can host large and tightly packed communities of microorganisms. They also
82 have more complex aquiferous systems that favor longer water residence times
83 within the sponge tissues and may enhance the capture of bacteria from seawater.
84 Conversely, the LMA sponges have a less dense mesohyl and their bodyplan moves
85 large quantities of water through their tissues (Weisz *et al.*, 2008); their associated
86 bacteria have been reported to be more similar to seawater bacteria (Weisz *et al.*,
87 2007; Kamke *et al.*, 2010). LMA sponges would then contain mostly transient
88 bacteria acquired horizontally from the surrounding water and HMA species
89 would contain preferably sponge-specific microbes acquired vertically (Schmitt *et*
90 *al.*, 2008). These suppositions arose from studies focusing on other ecological
91 questions (Weisz *et al.*, 2007; Kamke *et al.*, 2010) and have only rarely been tested
92 explicitly. For example, differences between HMA and LMA sponges have been
93 investigated in a study on the bacterial phylum *Chloroflexi*. The results indicated
94 that HMA sponges harbor more diverse and abundant *Chloroflexi* representatives
95 than LMA sponge species (Schmitt *et al.*, 2011), and that HMA species had more
96 sponge specific *Chloroflexi* clusters than LMA sponges, which harbored more
97 bacteria similar to those from the surrounding seawater. A recent study on
98 bacterial symbionts of LMA and HMA sponges by DGGE showed that the two
99 groups harbored distinct communities (Gerçe *et al.*, 2011), and that again, LMA

sponges did harbor a more transient bacterial community similar to that in the environment. However, the DGGE fingerprinting method used and the lack of replicates in this study did not allow a complete description of the bacterial community structure. In contrast, a more recent study targeting specifically LMA sponges (using 16S rDNA clone libraries without replicates) reported that these sponges also harbored different bacterial communities (Giles *et al.*, 2013). Questions remain about the extent of the species-specific bacterial symbionts in LMA sponges compared to that in HMA sponges, and whether the bacterial clades in LMA are consistently specific to these sponges or acquired randomly and/or enriched from the surrounding seawater.

Temporal and spatial variations of bacterial assemblages in the water column could affect the composition of the bacterial communities found in sponges. For instance, a large-scale study showed a strong environmental influence on the sponge bacterial assemblages (Schmitt *et al.*, 2012). The data revealed that bacterial communities from tropical sponges belonging to distant taxonomic orders were more similar than bacteria from taxonomically-related sponges found in tropical and subtropical areas (Schmitt *et al.*, 2012). At smaller spatial scales, habitat specific conditions such as irradiance can also structure sponge microbial communities (Erwin *et al.*, 2012b). On the other hand, although temporal variability may be low for the most abundant species-specific bacteria, strong seasonal changes have been reported for low abundance bacteria (Anderson *et al.*, 2010).

In this study, our objective was to improve identification of sponge specific bacterial communities by reducing environmental variations and that may be responsible for between-species differences. By sampling water and all the

sponges at the same time (three replicates *per* species and seawater), we minimize temporal variations, while spatial variability was reduced by targeting a set of sponges living on a same isolated rocky substrate. The sponge bed included representatives of the main taxonomic orders of Demospongiae (Hooper and van Soest, 2002) and Homoscleromorpha (Gazave *et al.*, 2012), and of both HMA and LMA species. We applied parallel tag sequencing combined with robust statistics to produce a precise picture of the bacterial community structure. LMA and HMA sponges were used to test whether all sponges harbor species-specific bacteria comparable to symbionts or if some have transient communities more similar to the surrounding seawater.

Results

Alpha diversity

Approximately 165 000 raw reads of the 16S rRNA gene were obtained from the 26 sponge samples corresponding to the nine species (pyrosequencing failed for one replicate of *Pleraplysilla spinifera*), and 26 000 raw reads from the surrounding seawater. After removing poor-quality reads, a total of 136 700 reads remained. The total number of operational taxonomic units (OTUs) identified as Bacteria at a sequence similarity of 97% was 3 435. Only 22 OTUs could not be identified at the domain level (representing 140 sequences).

The Shannon diversity index (H') varied between sponge species and ranged from 1.1 (*Oscarella lobularis*) to 3.8 (*Pleraplysilla spinifera* and *Hexadella topsenti*), while an H' value of 2.8 was obtained for seawater samples (Table 1). LMA sponges had often a lower microbial diversity than both the seawater samples and HMA species. However, there was no significant relationship between

150 H' and the sponge ecological type (HMA or LMA; $p=0.410$; t-test), and both
151 *Hexadella topsenti* and *Pleraplysilla spinifera*, which are considered LMA species,
152 had the highest diversity indices. The dominance index (Simpson D), which
153 measures the relative abundance of species or evenness in a community, can
154 theoretically range from 0 (all taxa are equally present) to 1 (one taxon dominates
155 the community completely). In the studied sponges dominance ranged from 0.1 to
156 0.2 for HMA sponges and from 0.1 to 0.6 (*Oscarella lobularis*) for LMA sponges.
157 However, a significant cut off between HMA and LMA sponges could not be
158 established based on the dominance index ($p= 0.194$; t-test; Table 1).

159

160 *Beta diversity*

161 Comparison of the bacterial community at the OTU level between sponge species
162 by cluster analysis using the Bray-Curtis similarity index showed a marked
163 species-specificity: all individuals from the same species clustered together (Fig 1).
164 The level of variation among replicates, however, depended on the sponge species.
165 For example, the bacterial community of *Cliona viridis* was nearly the same among
166 replicates (>90 % Bray-Curtis similarity), while that of *Hexadella topsenti* showed
167 the highest inter-individual variation (<35% similarity). The similarities among
168 replicates of the remaining species ranged between these two extreme values (Fig
169 1). The bacterial assemblages of all sponge species analyzed were different from
170 those of the water.

171 The three HMA species (*Petrosia ficiformis*, *Chondrosia reniformis* and
172 *Agelas oroides*) clustered together, but showed distinct microbial assemblages.
173 LMA sponges and the water samples grouped together; the bacterial assemblages
174 of *Cliona viridis* and *Hemimycale columella* were more similar than those of the

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other LMA sponges and the water samples. The *Oscarella lobularis* communities emerged as a sister group of those of *Hexadella topsenti*, *Axinella polypoides*, *Pleraplysilla spinifera* and seawater. The bacterial communities of the latter sponge were closest to those of the water samples.

No phylogenetic signal was observed in the sponge clustering at the Order level (Fig 2). For instance, according to the similarity of the bacterial community composition, Haplosclerida, Chondrosida and Agelasida (three orders of the class Demospongiae) clustered together, while representatives of the order Hadromerida clustered with the orders Poecilosclerida, Halichondrida, Dictyoceratida and Verongida (Fig 1). Furthermore, the recently created Class Homoscleromorpha (Gazave *et al.*, 2012) clustered within the Demospongiae orders.

Association network

Based on the MINE statistics of all OTUs (Fig 3), two distinct and unconnected networks distinguished HMA from LMA hosts. OTUs in the HMA network showed strong species-specific associations, but the relationships between species was not strong (Fig 3). Only two OTUs connected all three HMA sponges. These were neither detected in LMA sponges nor in the surrounding seawater and were identified as *Gammaproteobacteria*. They were 99% similar to sequences reported in other HMA sponge species elsewhere (i.e., *Geodia barretti* (Radax *et al.*, 2012), *Ircinia strobilina* (unpublished), *Ancorina alata* (Kamke *et al.*, 2010), *Desmacidon* sp. (Jensen *et al.*, 2008)). Seven OTUs were connected to both *Agelas oroides* and *Chondrosia reniformis* among which the most abundant (OTU17) was identified as a *Chloroflexi*. This OTU has also been found in *Chondrosia reniformis* in a previous

200 study (clone CrT-1, Gerçe *et al.*, 2011). Six OTUs connected *Petrosia ficiformis* to
201 *Chondrosia reniformis* with a *Nitrospirales* being the most abundant bacteria
202 (OTU72).

203 In general, LMA sponges were associated to only a few OTUs that were
204 species-specific (Fig 3). There were more differences between LMA species than
205 between HMA sponges. For instance, *Axinella polypoides* had many species-specific
206 OTU connections compared to *Hemimyscale columella*, which had only a few OTUs
207 that were present in low abundances. *Cliona viridis* appeared as the most distant
208 species in the LMA network, indicating that its species specific OTUs had few
209 relationships with other LMA bacteria. With the exception of *Cliona viridis*, seven
210 OTUs were present in the other LMA sponges. The most abundant of these non-
211 species specific OTU belonged to a *Synechococcus* (*Cyanobacteria*). Interestingly,
212 the LMA sponge bacteria network included the seawater OTUs. Some sponges'
213 (*Oscarella lobularis*, *Axinella polypoides* and *Hemimyscale columella*) bacteria were
214 more closely associated to those detected in seawater than others (*Cliona viridis*
215 and *Hexadella topsenti*).

216

217 *Bacterial community composition*

218 Both seawater samples and LMA sponges harbored *Proteobacteria*, and more
219 precisely, *Alphaproteobacteria*, as the dominant bacterial class (79 % and 67 % on
220 average, respectively (Fig 4). In contrast, *Alphaproteobacteria* only accounted for
221 10% of the total number of sequences on average in HMA sponges (Fig 4).
222 However, the LMA *Alphaproteobacteria* assemblage, differed from that in
223 seawater: the SAR116 clade accounted for 46% of the sequences of LMA sponges,
224 while the SAR11 clade represented 67% of the bacterial sequences in seawater

225 samples (Supplementary Figure S1A-B). On the other hand, *Gammaproteobacteria*
 226 were more abundant in both LMA and HMA sponges (12% and 11% on average,
 227 respectively) than in seawater samples (5%). Another conspicuous difference
 228 among the three groups of samples was the high abundance of *Chloroflexi*
 229 sequences in HMA sponges (44%, Fig 4) versus the near absence of these bacteria
 230 in LMA sponges and seawater samples. Other relatively frequent sequences were:
 231 *Cyanobacteria* (10%), and chloroplasts (7%) in LMA sponges; *Acidobacteria* (7%)
 232 and *Nitrospiraceae* (5%) in HMA sponges; and chloroplasts (7%) and
 233 Bacteroidetes (5 %) in the seawater samples. The candidate Poribacteria phylum
 234 was not detected due to a primer mismatch (Fieseler *et al.*, 2004).

235 Rare taxonomic bacterial groups (with a relative contribution of less than
 236 1%) were also characteristic of each sample type (HMA and LMA sponges, and
 237 seawater. In LMA sponges, rare groups were *Bacteroidetes* (0.6%), *Actinobacteria*
 238 (0.6%), and *Nitrospiraceae* (0.3%), Leptonemaceae (0.3%), the clade SAR406
 239 (0.1%), and the order Lactobacillales (0.06%). For HMA sponges, the rare clades
 240 also consisted of the *Bacteroidetes* (0.9%), as well as *Gemmatimonadetes* (0.8%),
 241 the genus *Entotheonella* (0.6%), chloroplasts (0.4%), family *Spirochaetaceae*
 242 (0.2%), Verrucomicrobia (0.08%), and the order Clostridiales (0.05%). In seawater,
 243 the bacteria taxa with lower abundances than 1% of the total sequences were the
 244 phylum *Firmicutes* (0.2%), the class *Anaerolineae* (0.02%), the *Alphaproteobacteria*
 245 NAC1-6 (0.8%), the subclass Acidimicrobidae, within the *Actinobacteria* (0.6%)
 246 and the genus *Polaribacter* within the class *Flavobacteriales*, phyla *Bacteroidetes*
 247 (0.6%).

248

249 *Species-specific bacteria*

Species-specific bacteria were defined as 16S rRNA sequences found in all three replicates of the sponge species and not more than once in any of the other analyzed sponge species or in the surrounding seawater. All HMA sponges contained more than 50% species-specific bacterial sequences while the percentage was lower in some LMA (2.1% and 0.5% for *Pleraplysilla spinifera* and *Hemimyscale columella*, respectively) (Table 1). *Hemimyscale columella* harbored the lowest number of species-specific bacteria (0.5%) because its most abundant OTU was shared with *Cliona viridis*. The percentage of species-specific sequences for *H. columella* could reach 24% if that shared OTU was included. The LMA *Oscarella lobularis* sponge had the highest proportion of species-specific sequences found in this study (80.9 %), illustrating the wide range of values observed for species-specific sequences in LMA sponges. Species-specific bacteria were represented by a low number of abundant OTUs that contained a high number of sequences. However, we also detected rare OTUs (<0.1% in abundance) that were species-specific.

As for the taxonomic position of species-specific bacterial OTUs (Fig 5), some OTUs were identical to those that had been found before in the same species. This is the case for two HMA sponges *Agelas oroides* (OTU Aoro_183) and *Petrosia ficiformis* (OTU Pfici_9) and the two LMA sponges *Oscarella lobularis* (OTU Olob_57) and *Axinella polypoides* (OTU Apoly_3, Apoly_34, Apoly_194) (Fig 5). Some purported species-specific sequences appeared not to be species-specific when they were compared to databases (such as, OTU Hcol_73 of *Hemimyscale columella* or OTU Pfici_42 of *Petrosia ficiformis*).

Discussion

275 The study was designed to capture sponge microbial diversity with the lowest
276 environmental variability possible. The aim was to clearly distinguish constant
277 bacterial assemblages, which are likely to represent symbionts, from transient
278 bacteria without relevant biological roles for the sponges. Our results reveal that
279 both HMA and LMA sponges harbored real symbiotic associations *sensu* Moya et al.
280 (2008) as species from both groups had large proportions of species-specific
281 bacteria different from the ones present in the surrounding water. HMA sponges
282 were known to harbor species-specific symbionts (Taylor *et al.*, 2007), while LMA
283 sponges were first thought to contain mostly seawater bacteria (Weisz *et al.*, 2007).
284 Some studies have suggested that LMA sponges may also have their own bacterial
285 communities although less diverse than those of HMA sponges, and containing
286 bacteria that do not belong to a so-called 'sponge specific' group (i.e., those
287 previously described from sponges; Hentschel *et al.*, 2006; Kamke *et al.*, 2010;
288 Schmitt *et al.*, 2012). Our results showed that the associations of LMA sponges with
289 species-specific bacteria are maintained in replicate samples, which points to a
290 symbiotic role for these microorganisms.

291 Bacterial communities from LMA species clearly differed from those in the
292 surrounding seawater. Even though *Alphaproteobacteria* dominated both LMA
293 sponges and water samples, the OTUs were different between these two sample
294 types. The *Alphaproteobacteria* SAR11 cluster represented two thirds of all
295 sequences from seawater samples, but only 3% of the LMA bacterial assemblages.
296 In contrast, the *Alphaproteobacteria* SAR116 cluster represented almost half of all
297 sequences from LMA sponge bacteria. Thus, the *Alphaproteobacteria* present in
298 our LMA sponges were not the result of a temporary enrichment of seawater
299 bacteria, as suggested previously (Weisz *et al.*, 2007; Gerçe *et al.*, 2011).

300 Our HMA species mostly contained more diverse communities than LMA
301 species confirming general earlier findings (Taylor *et al.*, 2007; Gerçe *et al.*, 2011;
302 Giles *et al.*, 2013). However, two clear exceptions to this generalization were found.
303 Two LMA species, *Hexadella topsenti* and *Pleraplysilla spinifera*, showed
304 exceptionally high bacterial diversity indices. Indeed *Pleraplysilla spinifera* had the
305 highest diversity index among the studied sponge species. Thus, our data suggest
306 that LMA sponges do not always have a lower bacterial diversity, as previously
307 thought. In addition, the dominance index showed an uneven distribution of LMA
308 bacterial communities. The high values of D for *Oscarella lobularis* (0.6) and *Cliona*
309 *viridis* (0.4) indicate that these two sponges had the most skewed OTU distribution
310 with one or a very few OTUs dominating the communities. These dominating OTUs
311 were *Proteobacteria* (*Alphaproteobacteria*). Such uneven community distribution
312 could be typical for some LMA sponges as suggested by Giles *et al.*, (2013). Among
313 HMA sponges, *Petrosia ficiformis* had the highest dominance index (0.2) and thus
314 the most uneven bacterial community, but its dominant OTU was not a
315 *Proteobacteria* like in LMA, and was a *Synechooccus* (*Cyanobacteria*).

316 The species-specificity of many bacteria in both HMA and LMA sponges
317 indicates that bacteria are not randomly associated with sponges (Webster *et al.*,
318 2010; Giles *et al.*, 2013). Indeed, the presence of the same bacterial sequences in
319 individuals of the same species independent of the habitat or locality is additional
320 evidence of a non-random bacteria-host association. Since our target sponges lived
321 very close to each other, we can assume that they grow under similar
322 environmental conditions. Thus, differences in physico-chemical conditions cannot
323 be argued to explain the differences in the sponge bacterial communities. Overall,
324 our data support host-related, evolutionary features (e.g., whether HMA or LMA)

325 rather than environmental conditions, as the main cause shaping the structure of
326 the sponge microbial communities.

327 To harbor or not to harbor a huge quantity of bacteria in the sponge mesohyl
328 (i.e., to be a HMA or a LMA sponge), which is independent of the sponge phylogeny,
329 are two strategies that have prospered evolutionarily. A certain relationship
330 between the complexion of the sponge mesohyl, the characteristics of the
331 aquiferous system, and the type of strategy (i.e., to be a HMA or a LMA sponge) has
332 been reported (Vacelet and Donadey, 1977; Weisz *et al.*, 2008). HMA species have
333 a denser mesohyl, smaller choanocyte chambers, and a more complex, tortuous,
334 aquiferous system than LMA species. It may be that HMA sponges, which have a
335 less developed pumping system (Vacelet and Donadey, 1977; Weisz *et al.*, 2008),
336 could cover their nutritional needs preferentially from dissolved organic carbon
337 though their high bacterial biomass (Weisz *et al.*, 2007). Conversely, the higher
338 pumping rates of LMA sponges would allow them to acquire sufficient organic
339 matter from the water column to supply their energetic demands while bacteria
340 there would play a minor role (Belarbi *et al.*, 2003; Blanquer *et al.*, 2008; De Caralt
341 *et al.*, 2010).

342 The high abundance of *Chloroflexi* sequences in HMA sponges, which has also
343 been reported in another study (Schmitt *et al.*, 2011), and their absence from LMA
344 sponges may be related to the particular N and P needs of these bacteria.
345 *Chloroflexi* might have found an appropriate niche in the dense sponge mesohyl of
346 HMA sponges where they could benefit from N and P-rich excretions from the
347 sponge and other bacterial metabolisms (Ribes *et al.*, 2012). Finally it is also
348 possible that sponges could use carbon from photosynthetic bacteria such as
349 cyanobacteria (Thacker and Freeman, 2012).

350 There was no relationship between the bacterial community composition
351 and the host phylogeny at high taxonomic levels (i.e. Order level), but some
352 similarities have earlier been found in some genera. For instance, two species from
353 the genus *Xestospongia* from two distant oceans have been reported to share
354 bacteria (Montalvo and Hill, 2011). Erwin and collaborators (2012a) also detected
355 a phylogenetic signal within three close Mediterranean species of the genus *Ircinia*.
356 Similarly, some of the species-specific sequences retrieved from the Mediterranean
357 *Agelas oroides* (current study) had also been found in *Agelas dilatata* from the
358 Caribbean Sea (Taylor *et al.*, 2007).

359 No core bacterial community was common to the sponges from our study
360 location. Interestingly, *Cliona viridis* and *Hemimycale columella* shared an
361 *Alphaproteobacteria* OTU that accounted for 52% and 63% of their sequences
362 respectively. This sequence was absent from any other sponge species and the
363 seawater samples. On the other hand, the three HMA species shared two
364 *Gammaproteobacteria* OTUs similar to sequences detected earlier in other HMA
365 sponges. Some core HMA bacteria, distinct from LMA bacteria, may thus be
366 identified.

367 In summary, this study, which was designed to reduce to a minimum the
368 possible variations due to environmental conditions, has revealed that not only
369 HMA sponges but also LMA sponges harbor a high number of species-specific
370 symbiotic bacteria. The species-specificity of bacteria in LMA sponges makes them
371 unlikely to be the result of neutral processes of accumulation from the
372 surrounding seawater as suggested before (Weisz *et al.*, 2007; Kamke *et al.*, 2010).
373 The consistency of these data from a typical Mediterranean sponge assemblage
374 indicates that sponge microbial communities are primarily defined by their

375 affiliation to the HMA or LMA groups of sponges rather than to the sponge
376 phylogeny or the environmental conditions.

377

378 **Experimental Procedures**

379 *Sampling*

380 Sampling was done by SCUBA diving on 13 January 2011 at Arenys de Mar (41°
381 34'N, 2°33'E) on the central Catalan coast (NW Mediterranean). The sampling area
382 was a rocky bar (ca. 1m high, 10 m wide, 25 m long and 29 m water depth), which
383 runs parallel to the coastline (from NE to SW), and was completely surrounded by
384 sandy bottom sediments. We collected three replicates of all the conspicuous
385 sponge species living in the area, covering representatives of most orders of the
386 classes Demospongiae and Homoscleromorpha (Gazave *et al.*, 2012). Species
387 studied were: Demospongiae HMA species, *Petrosia ficiformis* (O. Haplosclerida),
388 *Chondrosia reniformis* (O. Chondrosida) and *Agelas oroides* (O. Agelasida);
389 Demospongiae LMA species, *Cliona viridis* (O. Hadromerida), *Hemimyscale columella*
390 (O. Poecilosclerida), *Hexadella topsenti* (O. Verongida), *Axinella polypoides* (O.
391 Halichondrida), and *Pleraplysilla spinifera* (O. Dictyoceratida); and
392 Homoscleromorpha LMA species, *Oscarella lobularis* (O. Homosclerophorida). The
393 assignment of species to either LMA or HMA group was based on previous studies:
394 *Petrosia ficiformis*, *Agelas oroides*, *Axinella polypoides*, *Pleraplysilla spinifera* and
395 *Oscarella lobularis*, by Vacelet and Donadey (1977), and *Chondrosia reniformis*,
396 *Cliona viridis*, *Hemimyscale columella* and *Hexadella topsenti* by Uriz *et al.* (1992).
397 The species sampled are representatives of the most abundant sponges in a typical
398 Mediterranean pre-coralligenous assemblage (Ballesteros, 2006). Fig 2 shows the

399 phylogenetic relationships of the Orders of the species analyzed in the present
400 study.

401 Sponge samples were put in individual receptacles underwater, until they
402 could be fixed in ethanol after the dive. Three replicate volumes of seawater (1.5
403 liters each) were collected from the vicinity of the sponges in parallel. Each
404 replicate volume was filtered through an individual 0.2 µm polycarbonate sterile
405 filter (GE Osmonics, Minnetonka, MN, USA). The filters were stored frozen at -80 °C
406 until analysis.

407

408 *DNA extraction, PCR and sequencing*

409 DNA from all sponges, except *Hemimyscale columella*, and the filters were extracted
410 with a DNeasy blood and tissue kit (Qiagen). DNA from *H. columella* samples was
411 extracted with the Qiaamp DNA stool kit (Qiagen), which worked better with this
412 particular encrusting species. DNA concentration and quality were assessed using
413 Qubit fluorometer (Invitrogen) and a Biomate spectrophotometer (Thermo
414 Scientific), respectively. Bacterial 16S rRNA genes were amplified using primers
415 28F TTTGATCNTGGCTCAG and 519R GTNTTACNGCGGCKGCTG with a single-step
416 28 cycles PCR using the Hot Star Taq Plus Master Mix Kit (Qiagen, Valencia, CA).
417 The PCR conditions were: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30
418 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation
419 step at 72°C for 5 minutes was performed. Following PCR, all amplicon products
420 from the different samples were mixed in equal concentrations and purified using
421 Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA).
422 Pyrosequencing was done with a Roche 454 FLX using commercially prepared

423 Titanium reagents by a commercial laboratory (Research and Testing Laboratory,
424 Lubbock, TX).

425

426 *Sequence data analyses*

427 First, sequences were quality controlled by removing all reads that had a mismatch
428 with the 16S rRNA primers, contained ambiguous nucleotides (N) and that were
429 < 270 nucleotides long after the forward primer. A stringent quality trimming
430 criteria was applied to remove reads having $\geq 3\%$ of bases with Phred values <27
431 (0.2% per-base error probability). This is recommended to ensure that when
432 clustering at 97% the influence of erroneous reads is minimized (Huse *et al.*, 2010;
433 Kunin *et al.*, 2010). Sequences were then de-replicated and clustered at a 97%
434 threshold using Uclust (Edgar, 2010). Read quality filtering and length trimming,
435 dereplication, clustering at 97% sequence identity, taxonomic classification and
436 dataset partitioning based on barcodes were conducted with Pyrotagger (Kunin
437 and Hugenholtz, 2010).

438 Sequences from each OTU were classified by comparison to the Greengenes
439 database (DeSantis *et al.*, 2006). The taxonomic affiliations of the most abundant
440 OTUs (>1 % of the sequences) were further verified against sequences from the
441 NCBI databases using BLAST (Altschul *et al.*, 1990).

442 To compare bacterial communities for diversity analysis, all samples were
443 randomly resampled to the size of the sample containing fewest sequences
444 (n=1396) using Daisy Chopper (Gilbert *et al.*, 2009). The Shannon diversity index
445 (H'), the dominance index (D) and cluster analysis were conducted using the
446 software PAST (Hammer *et al.*, 2001).

447

448 *Phylogenetic tree and network analysis*

449 OTUs were defined as species-specific when their representative sequences were
450 found in all three replicates of a species and not more than once in any other
451 sample. A phylogenetic tree was constructed from the species-specific OTUs only.
452 For the phylogeny, raw data were reanalyzed to obtain only sequences that were
453 ca. 400bp long. This reduced the size of the dataset by 41% as fewer longer
454 sequences meet the clean-up criteria. However, this did not impact the
455 phylogenetic analysis, because the abundant OTUs that we targeted remained
456 unchanged. Sequences included in the tree were deposited in GenBank under
457 accession numbers HF678679- HF678716. Representative sequences for each OTU
458 together with their best match from Genbank were aligned using MUSCLE (Edgar,
459 2004) and phylogenetic analyses were completed with the program PHYLIP
460 (Felsenstein, 1989). DNADIST was used to calculate genetic distances using
461 Kimura-2 model. The distance tree was estimated with the phylogenetic algorithm
462 FITCH in the PHYLIP program.

463 For the network analysis, the relationship between bacterial OTUs was
464 characterized through maximal information-based nonparametric (MINE)
465 statistics by computing the maximal information coefficient (MIC) between each
466 pair of OTUs (Reshef *et al.*, 2011). MIC captures associations between data and
467 provides a score that represents the strength of a relationship between data pairs.
468 The matrix of MIC values >0.3 was used with Cytoscape 2.8.3 to visualize the
469 network of associations (Smoot *et al.*, 2011). In these visualizations, bacterial OTUs
470 are represented as nodes and are connected by lines that are proportional in
471 length to the MIC value. The force-directed layout was edge-weighted by the MIC
472 value.

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Table and Figure legends

Table 1. Shannon (H') and dominance (D) indices, and proportion of species-specific bacterial sequences and OTUs for High Microbial Abundance sponges (HMA), Low Microbial Abundance Sponges (LMA) and the surrounding seawater. Indices and OTU numbers were calculated after summing sequences from all triplicates for each sponge species.

Sponge type	Sponge Species	Species-specific OTUs (%)	Species-specific sequences (%)	OTU total	H'	D
HMA	<i>Agelas oroides</i>	30 (15.2)	2984 (71.3)	197	3.4	0.1
	<i>Chondrosia reniformis</i>	26 (18.8)	2101 (50.2)	138	3.2	0.1
	<i>Petrosia ficiformis</i>	45 (17.1)	2736 (65.3)	263	3.6	0.2
LMA	<i>Axinella polypoides</i>	11 (4.7)	2514 (60)	235	2.9	0.2
	<i>Hexadella topsenti</i>	7 (1.5)	1494 (35.7)	458	3.8	0.1
	<i>Cliona viridis</i>	7 (17.9)	1970 (47)	39	1.3	0.4
	<i>Oscarella lobularis</i>	3 (2.6)	3387 (80.9)	114	1.1	0.6
	<i>Pleraplysilla spinifera</i>	16 (6.3)	58 (2.1)	255	3.8	0.1
	<i>Hemimycale columella</i>	2 (0.8)	23 (0.5)	253	2.2	0.4
Seawater		10 (3.6)	57 (1.4)	280	2.8	0.3

661 Fig 1. Dendrogram based on the Bray-Curtis index showing the similarity between
662 16S rRNA bacterial community composition for the three high microbial
663 abundance sponges (HMA), the six low microbial abundance sponges (LMA) and
664 the surrounding seawater.

665

666 Fig 2. Phylogenetic relationships of the Porifera Orders containing the species in
667 the present study. A. Reconstruction of the Demospongiae and
668 Homoscleromorpha Orders based on 18S rRNA data after Borchellini *et al.* (2004).
669 B. Reconstruction of the G4 clade, within the Demospongiae, based on 28S rRNA
670 data after Morrow *et al.* (2011). The studied species are placed in brackets. HMA
671 sponges are in bold.

672

673 Fig 3. Association network of the sponge associated bacterial communities in
674 which nodes correspond to OTUs and edges correspond to relationships calculated
675 with the MINE statistics. Node size is proportional to the number of sequences
676 contained in an OTU.

677

678 Fig 4. Bacterial community composition for High Microbial Abundance Sponges
679 (HMA), Low Microbial Abundance Sponges (LMA) and the surrounding water. The
680 composition is based on average values over all triplicates of all sponges.

681

682 Fig 5. Phylogenetic tree representing the position of the species specific sequences
683 found in High Microbial Abundance sponges (HMA), Low Microbial Abundance
684 sponges (LMA). HMA sequences are in blue and LMA in red. The best matching
685 sequences from the database are in black and their origin is specified.

Figure 2. Phylogenetic relationships of the Porifera Orders to which belong the species in the present study. A. Reconstruction of the Demospongiae and Homoscleromorpha Orders based on 18S data after Borchellini et al. (2004). B. Reconstruction of the G4 clade within the Demospongiae based on 28S data after Morrow et al. (2011). The studied species are placed in brackets. HLM sponges are in bold.

Agelas oroides

Chondrosia reniformis

HMA

Cliona viridis

Petrosia ficiformis

Oscarella lobularis

Hexadella topsenti

water

**Hemimycale
columella**

Axinella polypoides

LMA