Symbiosis (2010) 51:239–243
DOI 10.1007/s13199-010-0082-2

Quantitative comparison of bacterial communities in two Mediterranean sponges

Charlotte Noyer · Alastair Hamilton · Oriol Sacristan-Soriano · Mikel Aingeru Becerro

Received: 2 March 2010 / Accepted: 20 July 2010 / Published online: 1 September 2010

Abstract Marine sponges can host in their tissues abundant and diverse bacterial communities. Lack of truly quantitative data on bacterial abundance and dynamics limits our understanding of the organization and functioning of these endobiotic communities. In this technical note, we describe a quantitative polymerase chain reaction approach to quantify the relative abundance of multiple clades of three major sponge-associated bacterial phyla: *Chloroflexi*, *Acidobacteria*, and *Actinobacteria*. To test our approach we used the Mediterranean sponges *Spongia lamella* and *Aplysina aerophoba*. We designed five out of the six primer sets used in our study. We tested the new primer sets for specificity and optimized their conditions. Our preliminary data showed that *Spongia lamella* had larger bacterial abundance than *Aplysina aerophoba*, except for one clade of *Chloroflexi*. The two *Chloroflexi* clades investigated in our study amplified a fraction of the *Chloroflexi* present in *Spongia lamella* and most of what is present in *Aplysina aerophoba*, suggesting a more diverse *Chloroflexi* population in *Spongia lamella* than in *Aplysina aerophoba*. This quantitative technique has a great potential to provide a rapid and robust assessment of sponge microbial target and could contribute to deciphering the complexity of these largely unknown host-symbiont interactions.

Keywords Porifera · Real time PCR · Sponge-associated bacteria · *Spongia agaricina-lamella* · *Aplysina aerophoba* · Symbiosis

C. Noyer · A. Hamilton
Integrin Advanced Biosystems, Marine Resource Centre,
Barcaldine, PA37 1SE,
Oban, Argyll, UK

C. Noyer · O. Sacristan-Soriano · M. A. Becerro (✉)
Center for Advanced Studies of Blanes (CEAB, CSIC),
Acc Cala S Francesc 14,
17300 Blanes, Girona, Spain
e-mail: mikel@ceab.csic.es

A. Hamilton
Veromara, European Centre for Marine Biotechnology,
Dunstaffnage Marine Lab,
Dunbeg, Oban PA37 1QA Argyll, UK

1 Introduction

Sponges are one of the most abundant and widespread groups of marine benthic ecosystems (Diaz and Rutzler 2001). Sponges are sessile, filter-feeder organisms that can host abundant microbial consortia in their tissues up to 40% of the total sponge biomass (Wilkinson 1987; Hentschel et al. 2006). These large biomasses consist of a rich and complex diversity of microorganisms including archaea, unicellular algae, viruses, fungi, and bacteria (Taylor et al. 2007). These microbial communities can be a food source for the sponge and are involved in important sponge functions such as nutrient acquisition, stabilization of the sponge skeleton, processing of metabolic waste, and secondary metabolite production (Thakur and Müller 2005; Hentschel et al. 2006; Taylor et al. 2007).

Molecular techniques have represented a leap forward in our understanding of these microbial communities and there is a great demand for information on sponge microbial ecology (Taylor et al. 2007; Becerro 2008). Our current perception mainly stems from qualitative data on bacterial composition and it is therefore limited by the lack of quantitative data. For example, some studies reveal consistent bacterial communities in various sponge species from different oceans (Hentschel et al. 2002) while others have

challenged the stability or uniformity among bacterial communities in sponges (Wichels et al. 2006; Mohamed et al. 2008; Hardoim et al. 2009). Until recently, quantitative analyses were performed by fluorescence in situ hybridization (Webster et al. 2001; Hoffmann et al. 2006). The recent development of quantitative real time polymerase chain reaction (qPCR) has emerged as a practical method to quantify microorganisms in multiple environments (Skovhus et al. 2004; Fierer et al. 2005; Galand et al. 2009). Despite the increasing interest in sponge symbiosis, the use of qPCR to quantify microbial symbionts in sponges has been reported only once (Cassler et al. 2007).

In this study, we describe the development of a quantitative real-time polymerase chain reaction (SYBR® Green qPCR) method to quantify different bacterial clades at broad taxonomic levels in two marine sponges: *Aplysina aerophoba* and *Spongia lamella* (the Mediterranean Elephant ear previously and generally named *S. agaricina*; Castritsi-Catharios et al. 2007; Pronzato and Manconi 2008). We tested six individual qPCR assays to quantify bacteria from 3 phyla: *Chloroflexi*, *Acidobacteria* and *Actinobacteria*, commonly found in high abundance in sponges.

2 Materials and methods

We used the universal bacterial primers BAC358F (5′- CCT ACG GGA GGC AGC AG -3′) and BAC907RM (5′- CCG TCA ATT CMT TTG AGT TT -3′) to amplify fragments approximately 560 bp long in the sponges *Aplysina aerophoba* and *Spongia lamella* to perform bacterial fingerprints using DGGE, and obtain a first insight into the bacterial communities inhabiting the sponges. The main representatives of these bacterial communities were affiliated with *Chloroflexi*, *Actinobacteria*, *Acidobacteria*, *Bacteroidetes*, and *Cyanobacteria* (the latter in *A. aerophoba* only) (Noyer 2006; Sacristan-Soriáno 2007). We focused our work on *Chloroflexi*, *Acidobacteria*, and *Actinobacteria*, which are the most frequently recovered in sponge surveys (Hentschel et al. 2006). We selected primer sets from the literature (Gich et al. 2001; Bachar et al. 2007) and designed five new primer pairs in the BAC358F-BAC907RM fragment to amplify clade-specific bacteria (Table 1). Primer sets were checked for specificity (Blast search and Probe Match, Cole et al. 2005) and compatibility (FastPCR, Kalendar and Schulman 2009). We used metagenomic DNA from *S. lamella* and strains of target and non target phylogenetic groups *Acidobacterium capsulatum* (DSMZ 11244), *Bacteroides fragilis* (ATCC700781), *Chloroflexus aurantiacus* (DSMZ 11244), and *Escherichia coli* (NCTC12241) to further assess for specificity over a temperature gradient, amplification efficiency, and dissociation curve analysis.

Real-time-qPCR assays were conducted using a Stratagene Mx3005P QPCR system (Stratagene 2007). Each 25 µl reaction contained 12.5 µl of 2× Brilliant SYBR® Green QPCR Master Mix (Stratagene), 0.4 µM of each primer, 125 mM BSA (Promega), 30 nM ROX reference dye, and 2 µl of template DNA at the previously determined optimal dilution. PCR conditions were 10 min at 95°C, followed by 40 cycles of 95°C for 1 min, 30 s at the annealing temperatures (Table 1) and 72°C for 1 min. All PCR products were then subjected to a melting curve analysis to check for primer-dimers and non-specific amplification products. Threshold cycle (Ct) values were then determined and averaged over triplicates for each sample.

For each primer set, we used DNA from *S. lamella* to generate a plasmid standard containing the target region. Amplified products were resolved on a 1.5% agarose gel to confirm amplification specificity, purified using a Montage™ PCR Centrifugal Filter Devices (Millipore), and cloned into pGEM-T Easy Vector (Promega). Plasmids were transformed

Table 1 Characterization of the clade-specific primers used for the qPCR assays

Target group	Primers	GenBank accession number	Primer sequence (5′–3′)	Approximative amplicon length (bp)	Annealing temp (°C)
Chloroflexi	GNSB-941F GNSB-1340R		AGCGGAGCGTGTGGTTT CGCGGTTACTAGCAAC	400	58
Chloroflexi	Cl1-372F Cl1-666R	GU732290	CAGTAAGGGATATTGCACAATGG ACTTCCCTCTATTTCTCTCTAGTT	290	58
Chloroflexi	Cl2-372F Cl2-665R	GU732291	CAGCAGGGAATCTTGGGCAATGG CTTACCTCTGCTGTCCTCAAGTCG	290	58
Acidobacteria	Ac1-372F Ac1-746R	GU732292	GCAGTAGGGAATTGTTCGCAATGG ACCGGTCCAGGATGCCGCCT	375	64
Acidobacteria	Ac2-371F Ac2-746R	GU732293	GCAGTGAGGAATTTTTGTCAATGG ACCGGTCCAGGGAGACGCCT	375	64
Actinobacteria	Actino-440F Actino-818R	GU732294	GGGTCGTAAACCCCTTTCAGCA CCCAACGTTTACGGCGTGGACTA	380	58

into *Escherichia coli* cells (DH5alpha cells, Invitrogen) following the manufacturer's instructions. We amplified ten clones per transformation using the universal primers M13f and M13r and each primer pair to confirm presence of the correct length inserts. PCR products of four of those clones from each set were then purified and sequenced to confirm the correct identity of the inserts. Plasmid extraction was performed using the Wizard Plus® SV Minipreps DNA purification System (Promega) and sequenced to re-verify that the correct fragment of 16S rDNA was present. We measured DNA concentrations by agarose gel determination using ImageJ analysis software calibrated using a serial dilution of known lambda DNA concentration (Promega). We generated standard curves using duplicate 10-fold dilutions of plasmid DNA. We used at least seven nonzero standard concentrations per assay. Plasmid concentration ranged from 10^{-2} to 10^{-10} of initial plasmid DNA. Target copy numbers for each following reaction were calculated from the standard curves (approximate molecular weight of double − strand DNA : nucleotide number × 607.4 + 157.9; http://www.ambion.com/techlib/append/na_mw_tables.html).

For the six assays, the log of the plasmid DNA copy number was strongly correlated with Ct (R^2 varied from 0.990 to 0.999, Fig. 1). Amplification efficiencies were automatically calculated by Stratagene MxPro QPCR software as the slope of the curve and varied from 89.9% to 109.2% (Fig. 1).

We tested and optimized the qPCR using DNA extracted from *Aplysina aerophoba* and *Spongia lamella*. We collected underwater eight specimens from each species in Es Caials, Spain (42°17′N, 3°18′E). Sponge tissues were manually cleaned and rinsed with ethanol before storage in absolute ethanol. We extracted sponge metagenomic DNA from the mesohyl using DNAeasy Blood and Tissue kit (Qiagen) according to the manufacturer's instructions, except for a 5 min incubation time before elution in a total volume of 75 µl manufacturer's elution buffer. DNA concentrations were then determined by Qubit® Quantitation (Invitrogen). We performed the six assays on all specimens as described above. From the obtained Ct values, and for each primer set and specimen, we calculated the copy numbers μg^{-1} of DNA given the standard curve equation, the DNA concentrations, and the dilution applied. As

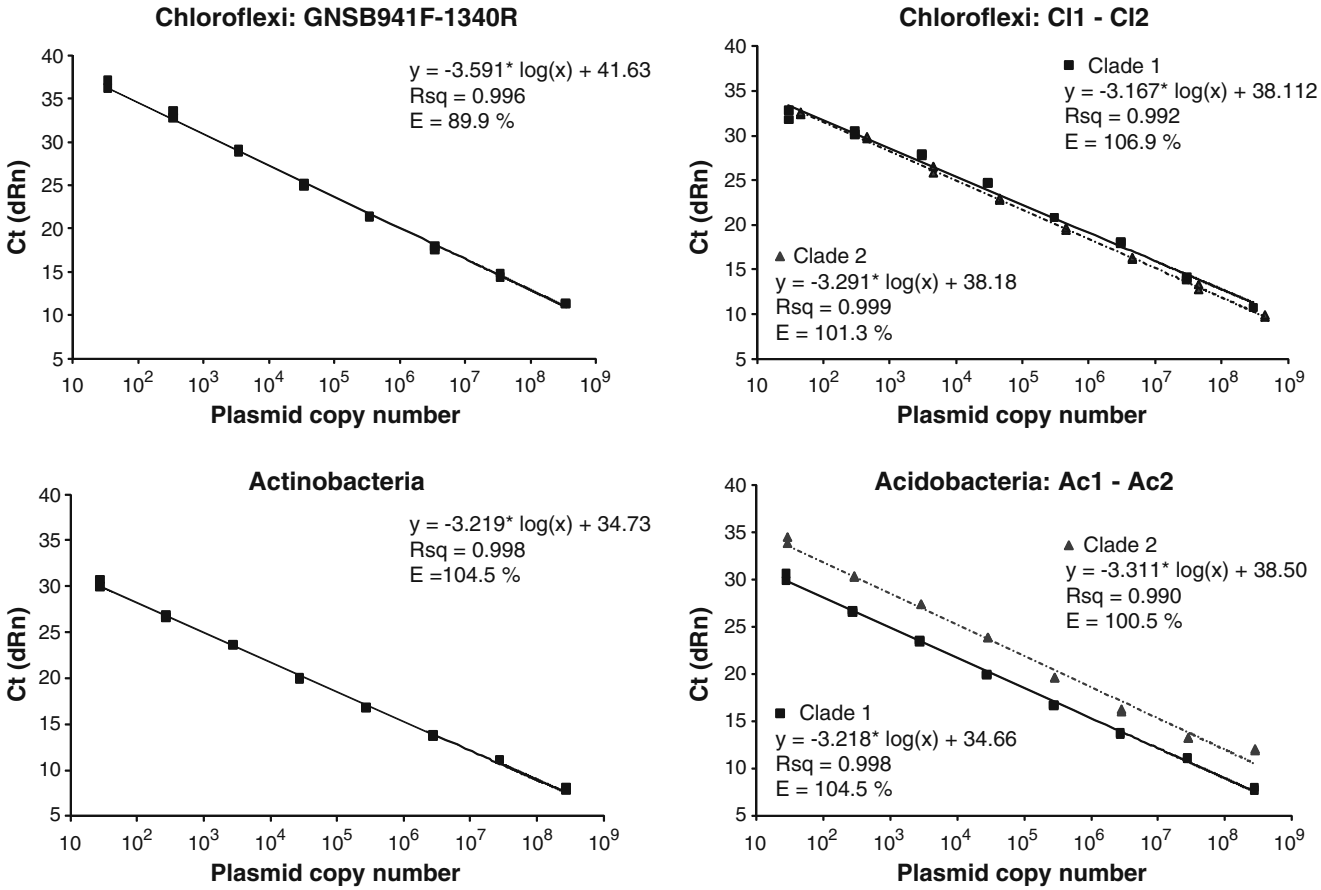

Fig. 1 Standard curves. Correlations between cycle threshold and copy numbers of purified plasmid DNA from one random clone per primer set from: *Acidobacteria* (Ac1 and Ac2), *Actinobacteria* (Actino), and *Chloroflexi* (Cl1, Cl2, and GNSB-941F). Rsq: R-squared, coefficient of determination; Eff: efficiency or PCR performance

heterogeneity in ribosomal operon number may affect relative estimated abundances of certain bacterial groups (Tourova 2003), we used each primer pair as an independent variable. We tested for differences in bacterial clades between the two sponge species using multivariate analysis of variance (MANOVA) on rank-transformed abundance.

3 Results

The six primer pairs showed successful clade-amplification in both sponges. We found significant differences in bacterial abundance between sponge species (MANOVA, Wilks' Lambda F=8.623, p=0.003). *Spongia lamella* presented larger bacterial abundance than *Aplysina aerophoba* (Fig. 2) except for the *Chloroflexi* clade 2 (Univariate Test, F=1.987, p=0.181; Fig. 2). *Chloroflexi* were the most abundant bacterial phylum investigated in this study.

4 Discussion

Although studies on sponge-associated microbial communities originated a few decades ago, recent advances in molecular genetics have fostered new interest in this field (Taylor et al. 2007; Becerro 2008). Most studies focus on the diversity and specificity of microbial symbionts in sponges, including *A. aerophoba* (Friedrich et al. 2001; Hentschel et al. 2002; Taylor et al. 2007). Studies on the dynamics or interactions of symbiotic bacterial communities have received considerably less attention. Constraints such as the acquisition of fresh material, the difficulties inherent in objective analyses of FISH images (Levsky and Singer 2003), or the necessity of sequencing a large number of clones in libraries (Hentschel et al. 2002; Hardoim et al. 2009) may have limited the use of quantitative analyses. This could hinder progress in sponge microbial ecology. In our study, we have provided evidence for the suitability and ease of use of a qPCR technique to quantify and compare major bacterial clades among sympatric sponges.

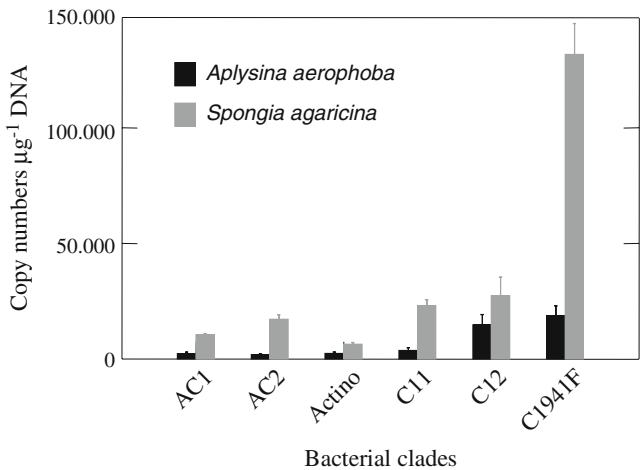

Fig. 2 Relative abundance, as estimated by qPCR analysis, of six bacterial clades including *Acidobacteria* (Ac1 and Ac2), *Actinobacteria* (Actino), and *Chloroflexi* (Cl1, Cl2, and GNSB-941F) in the sponge species *Aplysina aerophoba* and *Spongia lamella*. Vertical bars are standard errors (n=8 samples for each sponge species). *Spongia lamella* had greater bacterial abundance than *Aplysina aerophoba* (Wilks' Lambda F=8.623, p=0.003) except for Cl2 (Univariate tests: Ac1, Ac2, Cl1 and GNSB-941F, p<0.001; Actino, p=0.001; and Cl2, p=0.181)

We designed five primer sets and obtained an additional one from the literature suitable for our samples to quantify three major bacterial groups in *Aplysina aerophoba* and *Spongia lamella*. We found evidence for significant variation in the abundance of bacterial clades that are shared by the two Mediterranean sponges. We also found that *Chloroflexi* is more abundant than *Acidobacteria* and *Actinobacteria*. *Acidobacteria* and *Chloroflexi* are the phylotypes mainly recovered from 16S rRNA gene libraries (Taylor et al. 2007). Our data also suggest that the diversity of *Chloroflexi* is larger in *S. lamella* than in *A. aerophoba*. In *A. aerophoba*, *Chloroflexi* primer pairs of clades 1 and 2 amplified most of *Chloroflexi* present, as compared to the universal primer pair GNSB-941F- GNSB-1340R. In *S. lamella*, the two *Chloroflexi*-specific primers failed to amplify the large abundance obtained with the universal primers, which emphasizes a high abundance and diversity of *Chloroflexi* in *S. lamella*.

The qPCR technique described here offers a reliable procedure to quantify the abundances of clade-specific bacteria in diverse microbial consortia. Some potential limitations of our method need to be clarified. First, variation in DNA extraction and PCR efficiencies might alter the estimated abundances (Fierer et al. 2005). Second, we designed sponge bacteria primers at broad taxonomic level and for bacterial clades well represented in our sponge species. Third, cross-amplification may also occur between the two clades within *Chloroflexi* and *Acidobacteria* phylum. However, our results showed differences in these clade-specific amplifications within the same phyla. Caution must be exercised when comparing different sponge species. Bias in favor of a bacterial type in one sponge, does not necessarily imply that the same bias occur in other sponges species. This could lead to overestimation of the abundance of that bacterial type in the first sponge. *S. lamella* showed higher abundance of almost all bacterial clades; however *A. aerophoba* could harbor a higher abundance of other uninvestigated bacteria. For instance, *Cyanobacteria* form abundant communities in the outer layer of *A. aerophoba* (Becerro et al. 2003; Taylor et al. 2007) and they remain to be quantified. Furthermore, quantifying the abundance of bacterial clades not only per quantity of metagenomic DNA, but also proportionally to

the total bacterial community would add further information of relative proportion and importance of bacterial clades in the host species. This could potentially unravel interactions between different members of bacterial communities. The evidence for the interspecific quantitative variations presented here could help clarify adaptations of bacterial clades to sponge species or environmental conditions, as they probably represent specific ecological niches. It could also shed light on the proportion of bacteria vertically inherited versus environmentally acquired or help understand spatial and temporal patterns in bacterial communities. Quantitative analyses of host-associated bacteria will reveal a natural variation that remains elusive to date. Quantifying this unexplored level of diversity and understanding the factors behind its variation are critical steps to progress not only in sponge microbial ecology but in microbial-host symbiotic interactions in general.

Acknowledgements We thank Allison Hardie, Stuart Dent, Anja Nusser and Claire Moss for their help. We also thank Dr Richardson and an anonymous reviewer, whose comments helped increased the quality of this manuscript. Financial support was provided by the BIOCAPITAL project (MRTN-CT-2004-512301) of the European Union and the Spanish Ministry of Education and Science (CTM2007-66635 and CTM2010-17755).

References

Bachar A, Omoregie E, de Wit R, Jonkers HM (2007) Diversity and function of Chloroflexus-like bacteria in a hypersaline microbial mat: phylogenetic characterization and impact on aerobic respiration. Appl Environ Microbiol 73:3975–3983

Becerro MA (2008) Quantitative trends in sponge ecology research. Mar Ecol 29:167–177

Becerro MA, Turon X, Uriz MJ, Templado J (2003) Can a sponge feeder be a herbivore? *Tylodina perversa* (Gastropoda) feeding on *Aplysina aerophoba* (Demospongiae). Biol J Linn Soc 78:429–438

Cassler M, Peterson CL, Ledger A, Pomponi SA, Wright AE, Winegar R et al (2007) Use of real-time qPCR to quantify members of uncuturable heterotrophic bacterial community in a deep sea marine sponge, *Vetulina* sp. Microb Ecol 55:384–394

Castritsi-Catharios J, Magli M, Vacelet J (2007) Evaluation of the quality of two commercial sponges by tensile strength measurement. J Mar Biol Assoc UK 87:1765–1771

Cole JR, Chai B, Farris RJ, Wang Q, Kulam SA, McGarrell DM et al (2005) The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. Nucleic Acids Res 33: D294–D296

Diaz MC, Rutzler K (2001) Sponges: an essential component of Caribbean coral reefs. Bull Mar Sci 69:535–546

Fierer N, Jackson JA, Vilgalys R, Jackson RB (2005) Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. Appl Environ Microbiol 71:4117–4120

Friedrich AB, Fischer I, Proksch P, Hacker J, Hentschel U (2001) Temporal variation of the microbial community associated with the Mediterranean sponge *Aplysina aerophoba*. FEMS Microbiol Ecol 38:105–113

Galand PE, Lovejoy C, Hamilton AK, Ingram RG, Pedneault E, Carmack EC (2009) Archaeal diversity and a gene for ammonia oxidation are coupled to oceanic circulation. Environ Microbiol 11:971–980

Gich F, Garcia-Gil J, Overmann J (2001) Previously unknown and phylogenetically diverse members of the green nonsulfur bacteria are indigenous to freshwater lakes. Arch Microbiol 177:1–10

Hardoim CCP, Costa R, Araujo FV, 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

Hentschel U, Hopke J, Horn M, Friedrich AB, Wagner M, Hacker J, Moore BS (2002) Molecular evidence for a uniform microbial community in sponges from different oceans. Appl Environ Microbiol 68:4431–4440

Hentschel U, Usher KM, Taylor MW (2006) Marine sponges as microbial fermenters. FEMS Microbiol Ecol 55:167–177

Hoffmann F, Rapp HT, Reitner J (2006) Monitoring microbial community composition by fluorescence in situ hybridization during cultivation of the marine cold-water sponge Geodia barretti. Mar Biotechnol 8:373–379

Kalendar RLD, Schulman AH (2009) FastPCR software for PCR primer and probe design and repeat search. Genes, Genomes, Genomics 3:1–14

Levsky JM, Singer RH (2003) Fluorescence in situ hybridization: past, present and future. J Cell Sci 116:2833–2838

Mohamed NM, Enticknap JJ, Lohr JE, McIntosh SM, Hill RT (2008) Changes in bacterial communities of the marine sponge *Mycale laxissima* on transfer into aquaculture. Appl Environ Microbiol 74:1209–1222

Noyer C (2006) Genetic and bacterial diversity of the endangered sponge *Spongia agaricina*. Master "Environments Méditerranées et Développement Durable", Université de Perpignan, p 38

Pronzato R, Manconi R (2008) Mediterranean commercial sponges: over 5000 years of natural history and cultural heritage. Mar Ecol 29:146–166

Sacristán-Soriano O (2007) Chemical and microbial profiles of the sponge *Aplysina aerophoba*. Master en Biología Experimental, University of Barcelona, p 42

Skovhus TL, Ramsing NB, Holmstrom C, Kjelleberg S, Dahllof I (2004) Real-time quantitative PCR for assessment of abundance of *Pseudoalteromonas* species in marine samples. Appl Environ Microbiol 70:2373–2382

Stratagene (2007) Introduction to quantitative PCR: methods and applications guide. Stratagene an Agilent Technologies Company. p 58

Taylor MW, Radax R, Steger D, Wagner M (2007) Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. Microbiol Mol Biol Rev 71:295–347

Thakur NL, Müller WEG (2005) Sponge-bacteria association: a useful model to explore symbiosis in marine invertebrates. Symbiosis 39:109–116

Tourova TP (2003) Copy number of ribosomal operons in prokaryotes and its effect on phylogenetic analyses. Microbiology 72:389–402

Webster NS, Webb RI, Ridd MJ, Hill RT, Negri AP (2001) The effects of copper on the microbial community of a coral reef sponge. Environ Microbiol 3:19–31

Wichels A, Wurtz S, Dopke H, Schutt C, Gerdts G (2006) Bacterial diversity in the breadcrumb sponge *Halichondria panicea* (Pallas). FEMS Microbiol Ecol 56:102–118

Wilkinson CR (1987) Significance of microbial symbionts in sponge evolution and ecology. Symbiosis 4:135–145