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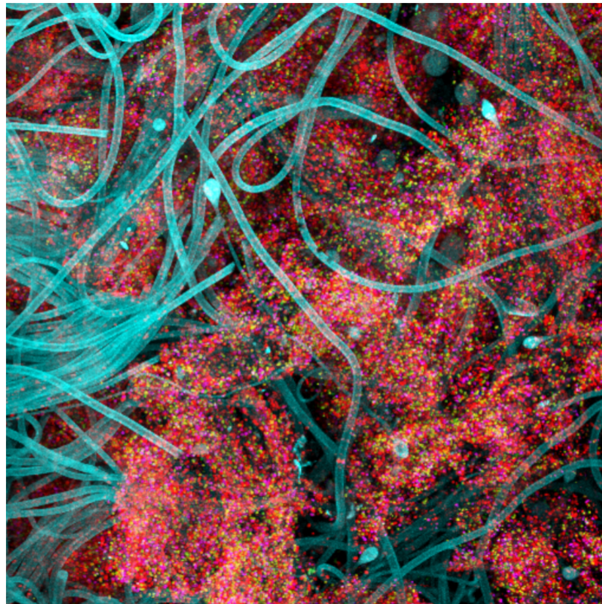
Microbiome diversity and composition in the phylogenetically related marine sponges *S. spinosulus* and *I. variabilis*

Cristiane Cassiolato Pires Hardoim



Ph.D in Biological Sciences
Speciality in Microbiology

Thesis supervised by
Dr. Rodrigo Costa
Dr. Cymon J. Cox

Faro
2013

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Declaração de autoria de trabalho

Cristiane Cassiolato Pires Hardoim

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluído

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To my husband,

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“Eu poderia suporta, embora não sem dor, que tivessem morrido todos os meus amores..., mas enlouqueceria se morressem todos os meus amigos!”

Vinícius de Moraes

ABSTRACT

Microbiome diversity and composition in the phylogenetically related marine sponges *Sarcotragus spinosulus* and *Ircinia variabilis*

In this Thesis the theory of a uniform prokaryotic community associated with marine sponges was challenged. To this end, an in-depth inspection of the abundance, diversity, and composition of prokaryotic communities in the phylogenetically related marine sponges *S. spinosulus* and *I. variabilis* was undertaken. The within-habitat, between-habitat and temporal dynamics of these communities were disclosed. Further, an innovative approach to measure bacterial cultivation bias in the characterization of these communities was employed. Using state-of-the-art imaging technologies, both sponge species were classified as high microbe abundance sponges and bacterial cells were shown to be mainly associated with sponge cells and to neglect the sponge skeleton. PCR-DGGE fingerprinting was initially used and revealed that, within the same habitat, distinct bacterial communities were associated with *S. spinosulus* and *I. variabilis*. This was latter confirmed by 454 pyrosequencing of the same communities. Further, when two different cultivation-independent methods were applied to profile the bacterial communities associated with these hosts, a similar structure was obtained for *S. spinosulus* specimens, whereas the same was not true for *I. variabilis*. Using a common cultivation-dependent method, an alike bacterial community was detected in both sponge species, as opposed to the species-specific profiles obtained via cultivation-independent methods. Unexpectedly, around half of the OTUs recovered with the cultivation-dependent method was exclusive to this procedure. When between-habitat comparisons were made, replicates from the same sponge species were more similar to one another than replicates of different species independently of the sampling sites. Furthermore, the bacterial community associated with *S. spinosulus* displayed a state of dynamic stability over three consecutive years, whereby about half of the observed *S. spinosulus* could be detected in all sampling years. Remarkably, the archaeal community associated with *S. spinosulus* was dominated by one single OTU affiliated with *Nitrosopumilus* sp., a known ammonia-oxidizer. Overall, the prokaryotic community associated with *S. spinosulus* and *I. variabilis* was species-specific, and these communities were also maintained across biogeographical and temporal gradients, however the environmental also played a role.
Keywords: microbial communities, symbiosis, 454-pyrosequencing, microbial biogeography, temporal stability, ammonia-oxidizers.

RESUMO

Diversidade e composição microbiana nas esponjas marinhas filogeneticamente próximas *Sarcotragus spinosulus* e *Ircinia variabilis*

Esponjas marinhas são conhecidas por abrigar uma comunidade microbiana diversa e complexa. Inúmeros compostos bioativos têm sido isolados destes animais e acredita-se que os simbiontes sejam os produtores de pelo mesmo parte destes metabolitos, revelando assim um grande potencial biotecnológico. Uma das teorias desenvolvidas na área de microbiologia de esponjas marinhas estabelece que a composição da comunidade procariótica associada a estes animais é uniforme. Com o objectivo de testar esta teoria com acurácia, a abundância, diversidade e composição da comunidade procariótica em duas espécies de esponja pertencentes à família Irciniidae (classe Demospongiae, ordem Dictyoceratida), nomeadamente *Sarcotragus spinosulus* e *Ircinia variabilis*, foram investigadas em profundidade na presente Tese. Estas comunidades simbióticas foram caracterizadas em espécimes de ambas as espécies recolhidas em um mesmo habitat (costa do Algarve), em habitats diferentes (na costa do Algarve assim como nos arquipélagos da Madeira e dos Açores) e ao longo de três anos consecutivos (costa do Algarve). No último caso, foram coletadas apenas espécimes de *S. spinosulus*. Para além das espécimes de esponja, foram também recolhidas, em réplicas, amostras da água do mar circundante às esponjas e de sedimentos. Uma abordagem inovadora foi implementada para averiguar a eficiência de um método tradicional de cultivo na caracterização das comunidades procarióticas. Inúmeras técnicas foram empregadas à avaliação destas comunidades, especialmente de biologia molecular, tais como PCR-DGGE (reação em cadeia da polimerase – eletroforese em gel de gradiente denaturante) e pirosequenciação em massa com o uso da tecnologia 454. A abundância procariótica analisada com microscopia de epifluorescência em espécimes coletadas em um mesmo habitat revelou que: i) *S. spinosulus* abriga uma abundância procariótica significativamente maior quando comparada com *I. variabilis* e ii) a abundância detectada nas duas espécies de esponjas é significativamente superior, em 4 a 5 ordens de magnitude, à abundância procariótica encontrada na água do mar. Com base nesses resultados, *S. spinosulus* e *I. variabilis* foram classificadas como esponjas de alta abundância microbiana (“high microbial abundance sponges”). A comunidade bacteriana foi inicialmente investigada via PCR-DGGE, revelando em *S. spinosulus* uma menor variabilidade entre as réplicas e foi diferente da observada em *I. variabilis*, que mostrou maior variabilidade entre os

replicados. Além disso, as comunidades bacterianas associadas às duas espécies de esponja foram distintas da observada na água do mar coletada nas proximidades das esponjas. Consequentemente, a comunidade bacteriana associada com *S. spinosulus* e *I. variabilis* é específica de cada espécie de esponja e distinta do ambiente. Os efeitos dos métodos independentes de cultivo e dependente de cultivo para a obtenção do DNA (ácido desoxirribonucleico) microbiano associado às esponjas foram determinados via PCR-DGGE e pirosequenciação em massa. A estrutura das comunidades bacterianas associadas à *S. spinosulus* acedidas pelos métodos independentes de cultivo foi semelhante, enquanto o oposto foi observado para *I. variabilis*. Portanto, os resultados obtidos com a pirosequenciação em massa confirmaram que a comunidade bacteriana é especificamente associada a cada espécie de esponja. Porém quando o método dependente de cultivo foi usado, a comunidade bacteriana nas duas espécies de esponja foi similar, em oposição aos perfis específicos das espécies obtidas por métodos independentes de cultivo. Surpreendentemente, por volta da metade das OTUs (Unidades Taxonómicas Operacionais definidas a uma similaridade de 97% entre sequências do gene 16S do RNA ribossômico) obtidas com este método foi exclusiva deste procedimento. Este resultado demonstra o potencial deste procedimento para a seleção e detecção de filotipos bacterianos menos abundantes que são enriquecidos pelo meio e condições de cultivo, como temperatura e tempo de incubação. Para além disso, a localização e distribuição de células bacterianas associadas às esponjas *S. spinosulus* e *I. variabilis* foram determinadas via hibridação *in situ* por fluorescência juntamente com microscopia eletrônica confocal de varredura (FISH-CLSM). Esta análise revelou que a grande maioria das células procarióticas foram encontradas associadas às células do mesoílo das esponjas e raramente associadas às fibras e filamentos (estruturas de suporte das esponjas), indicando existir uma troca de metabolitos entre a esponja e os simbiontes. De maneira geral a maioria das células bacterianas tinham a forma cocoíde e estavam entre as células das esponjas, de onde colônias bacterianas com alta abundância foram observadas. Quando as comparações entre habitat foram feitas, incluindo as espécies de esponjas, água do mar circundante às esponjas e de sedimentos, observou-se a formação de cinco grupos distintos. Independentemente dos locais onde as amostras foram recolhidas, todas as réplicas de *S. spinosulus*, *Ircinia* spp. e *Spongia* sp. agruparam entre si. O mesmo foi observado com as réplicas de sedimentos. Entretanto, as amostras de água do mar formaram três grupos distintos de acordo com o local de recolha. Para finalizar, a comunidade

bacteriana associada à *S. spinosulus* exibiu um estado de estabilidade dinâmica ao longo de três anos consecutivos, sendo que cerca de metade dos simbiontes observados em *S. spinosulus* pode ser detectado em todos os anos de amostragem. Notavelmente, a comunidade de *Archaea* associada à *S. spinosulus* foi dominada por uma única OTU afiliada com *Nitrosopumilus* sp., conhecida por sua capacidade em oxidar amônia em condições aeróbicas. Todos os resultados apresentados nessa Tese sugerem uma importância fundamental dos simbiontes para a funcionalidade das esponjas marinhas e, devido à proximidade das células bacterianas com as células ativas da esponja é bastante provável que as mesmas executem funções vitais para manutenção da saúde e desenvolvimento das esponjas. Esta Tese suporta a teoria que a comunidade procariótica é na verdade específica de cada espécie de esponja, então refutando a visão de uniformidade. Está comunidade também foi mantida em diferentes gradientes biogeográficos e temporal. Finalmente, fatores ambientais também desempenham uma importante função como reservatório de bactérias simbiontes para as esponjas marinhas, com destaque para a comunidade encontrada nos sedimentos.

Termos chave: comunidades microbianas, simbiose, pirosequenciação em massa, biogeografia microbiana, dinâmica temporal, oxidadores de amônia.

LIST OF CONTENTS

Acknowledgements 1
Abstract 3
Resumo 4
Table of Contents 7
List of Figures 11
List of Tables 21
List of Abbreviations and Acronyms 22

CHAPTER I - General Introduction 25
1 Phylum Porifera 25
1.1 Evolutionary History, diversity, and classification 25
1.2 Sponge body structure and gross morphology 28
1.3 Sponge nutrition 29
1.4 Sponge macroecology 30
2. Sponge microbiology 31
2.1 Defining symbiosis 31
2.2 The bacterial diversity of sponges 32
2.2.1 Early surveys 32
2.2.2 State-of-the-art assessments of sponge-associated bacterial diversity 33
3. Archaea associated with marine sponges 41
4. Nitrification 42
5 Outline of the Thesis 45
5.1 Objectives 45
5.2 The hosts 46
5.2.1 Irciniidae family 46
5.2.2 Sampling sites 47
5.3 Outline of this thesis 48

CHAPTER II - Phylogenetically and spatially close marine sponges harbour divergent bacterial communities 55

Abstract 56
Introduction 57
Results 59
Discussion 71
Material and Methods 77
Acknowledgments 83
Author contributions 83
Supplementary material 84

CHAPTER III - Degrees of host specificity, effects of sample handling and extent of cultivation bias elucidated for bacterial communities in closely related marine sponge.89
Abstract 90
Introduction 91
Material and Methods 93
Results 99
Discussion 114
Conclusions 116
Acknowledgments 116
Supplementary material 117

CHAPTER IV - Tackling the specificity of the marine sponge microbiome: a biogeographical approach 129
Abstract 130
Introduction 131
Material and Methods 133
Results 136
Discussion 148
Supplementary material 155

CHAPTER V Temporal maintenance of symbiont communities and ammonia oxidation potential in the marine sponge *Sarcotragus spinosulus* 157
Abstract 158

Introduction 159
Material and Methods 160
Results 164
Discussion 177
Conclusions 181
Acknowledgments 181
Supplementary material 182

CHAPTER VI - Microbial communities in marine sponges of the family Irciniidae and their bioactive compounds – a review 187
Abstract 188
1. Introduction 189
2. The Irciniidae family as a model taxon in sponge microbiology research 190
3. Microbial diversity and bioactivities in the family Irciniidae 192
3.1 Early microbiology studies 193
3.2 Bacteria 194
3.2.1 Diversity 194
3.2.1.1 Culture-dependent approaches 194
3.2.1.2 Cultivation-independent approaches 195
3.2.2 Bioactivities 201
3.2.3 Bioactive compounds 202
3.3 Archaea 203
3.3.1 Diversity 203
3.3.2 Bioactivities 204
3.3.3 Bioactive compounds 204
3.4 Fungi 204
3.4.1 Diversity 204
3.4.2 Bioactivities 205
3.4.3 Bioactive compounds 206
3.5.1 Other microeukaryotes 206
4. Vertical transmission of sponge symbionts 207
5. Bacterial communication and signalling molecules 209

6. Diseases affecting irciniids ... 211
7. Experimental microbial ecology ... 212
8. Metagenomics-based discovery of secondary metabolites biosynthetic gene clusters ... 214
9. Cultivation of Irciniidae species ... 215
9.1 Sponge mariculture ... 215
9.2 Sponge cell culture ... 217
9.3 Sponge larviculture ... 218
10. Concluding remarks and outlook ... 219
Supplementary material ... 221

CHAPTER VII General Discussion ... 231

Bibliography ... 241

LIST OF FIGURES

Figure 1.1. Underwater photographs of marine sponges: (A) *Spongia azorica*; (B) *Ciocalypta penicillus*; (C) *Axinyssa aurantiaca*; (D) *Haliclona* sp.; (E) *Hexadella racovitzai*; (F) *Haliclona fistulosa*; (G) *Aplysina aerophoba*; (H) *Clathrina coriacea*; (I) *Petrosia ficiformis* and (J) *Oscarella lobularis.* Photographs courtesy of Ms. Francisco R. Pires.........................27

Figure 1.2. Schematic overview of a demonsponge from the Irciniidae family. Mesohyl (M); ostium (Ot); pinacoderm (Pi); porocyte (Po); choanocyte (C); archeocyte (A), osculum (Oc) and horny (H)..29

Figure 1.3. Geographical distribution of *Sarcotragus spinosulus* and *Ircinia variabilis.* Sampling sites: Az, M, and A: Azores archipelago, Madeira Island, and Algarve, respectively. 48

Figure 2.1. Phylogenetic inference of the Irciniidae family based on the cytochrome oxidase gene, subunit 1. The Maximum Likelihood tree (-ln likelihood: 1383.921591) is shown, with sequences retrieved in this study highlighted in bold. Numbers at tree nodes are bootstrap values and posterior probabilities calculated in Maximum Likelihood and MCMC Bayesian analyses, respectively, and values above 70/0.95 are shown..60

Figure 2.2. Epifluorescence counts. Microscopy pictures taken from *S. spinosulus* (A), *I. variabilis* (B), and Seawater (C) are shown. Arrows exemplify counted bacterial cells. Values in panel (D) are expressed as means ± standard errors of log-transformed total cell numbers (TCN))...61

Figure 2.3. PCR-DGGE fingerprints and canonical analyses. PCR-DGGE 16S rRNA gene fingerprints of *S. spinosulus*, *I. variabilis*, and seawater DNA samples generated with "total-community" bacterial primers (A) and specific primer systems for *Actinobacteria* (C) and *Alphaproteobacteria* (E). The arrows indicate bands that were excised from DG-gels and sequenced. Corresponding ordination biplots of PCR-DGGE fingerprints and qualitative environmental variables are shown in panels B, D, and F. Symbols: ▲ *S. spinosulus*, ■ *I. variabilis*, and ● Seawater. Labels displayed on the diagram axes refer to the percentage variations of PCR-DGGE ribotypes - environment correlation accounted for the respective

axis. The "star" symbols represent the centroid positions of the environmental variables in the diagram. Variables that significantly ($p<0.05$) influence the bacterial community composition are indicated by an asterisk..64

Figure 2.4. Phylogenetic inference of *Actinobacteria* 16S rRNA genes. The modified ARB database generated by Simister *et al.,* (2012c) used long sequences (≥1200bp) to infer the phylogeny and shorter sequences were added using the ARB parsimony interactive tool. Sequences from the sponge-specific cluster 22 (SC22) (Simister *et al.,* 2012c) along with sequences closely related to band 1 and outgroup sequences were selected for further phylogenetic analysis. The Maximum Likelihood tree (-ln likelihood: 4501,317092) is shown, with sequences retrieved in this study highlighted in bold. Numbers at tree nodes are bootstrap values and posterior probabilities calculated in Maximum Likelihood and MCMC Bayesian analyses, respectively, and values above 70/0.95 are shown..................................67

Figure 2.5. Phylogenetic inference of *Gammaproteobacteria* 16S rRNA genes. Tree construction procedure was as described for Figure 2.4, except that sequences closely related to band 2 were selected as well as sequences from SC155 (Simister *et al.,* 2012c), which were used as outgroup. The Maximum Likelihood tree is shown (-ln likelihood: 2696,593494)....68

Figure 2.6. Phylogenetic inference of *Chloroflexi* 16S rRNA genes. Tree construction procedure was as described for Figure 2.4, except that sequences from SC46 were selected along with sequences from SC47, which were used as outgroup (Simister *et al.,* 2012). The Maximum Likelihood tree is shown (-ln likelihood: 3791,095)...69

Figure 2.7. Phylogenetic inference of *Alphaproteobacteria* 16S rRNA genes. Tree construction procedure was as described for Figure 2.4, except that sequences close related to band 8 were selected along with sequences from SC84 (Simister *et al.,* 2012), which were used as outgroup. The sequences shown in a box were isolated from different environments. The Maximum Likelihood tree is shown (-ln likelihood: 3253,686594)................................70

Figure S2.1. Sponge species. *In situ* pictures of *S. spinosulus* (A) and *I. variabilis* (B).........84

Figure S2.2. Cluster analysis. Cluster analysis of PCR-DGGE fingerprints obtained for *Bacteria* (A), *Actinobacteria* (B) and *Alphaproteobacteria* (C). *S. spinosulus*: Alg10/08, Alg10/09, Alg10/10 and Alg10/11; *I. variabilis*: Alg10/12, Alg10/13, Alg10/14 and Alg10/15 and Seawater: SW07, SW22, SW23 and SW24...85

Figure 3.1. PCR-DGGE 16S rRNA gene fingerprinting of bacterial communities associated with the marine sponges *Sarcotragus spinosulus* (Ss) and *Ircinia variabilis* (Iv). Profiles obtained under the three methods of sample processing ("direct", "indirect", and "plate washing") are shown (a) along with their corresponding ordination by redundancy analysis (b). M: 16S rRNA gene marker used to control the DGGE run. Samples on the ordination diagram: ▲ - *S. spinosulus* direct processing, ■ - *S. spinosulus* indirect processing, ● - *S. spinosulus* plate washing processing, △ - *I. variabilis* direct processing, □ - *I. variabilis* indirect processing, ○ - *I. variabilis* plate washing processing. Labels displayed on the diagram axes refer to the percentage variations of PCR-DGGE ribotypes - environment correlation accounted for the respective axis. The ''star'' symbol represents the centroid positions of the canonical variables (i.e., sponge species and sample processing methods) in the diagram. Variables that significantly ($p<0.05$) influenced bacterial community composition are highlighted with an asterisk...100

Figure 3.2. Quantitative analysis of bacterial richness and diversity in marine sponges and seawater. Observed and estimated (Chao1) richness measures [panels (a) and (b)] and Shannon diversity indices [panels (c) and (d)] of bacterial OTUs determined at 97% 16S rRNA gene similarity are displayed using normalized depths of 1.236 [includes seawater libraries, panels (a) and (c)] and 3.866 [sponge libraries only, panels (b) and (d)] sequences per library. Values on bars are means ± standard deviations of 4 replicates within each sample category, except for seawater where 3 replicates were used. Bars labelled with different letters represent statistically distinct sample categories in terms of richness and/or diversity values. In panels (a) and (b), uppercase letters define differences in estimated richness across sample categories, whereas lowercase letters define differences in observed richness. Ss_D: *Sarcotragus spinosulus* under the direct processing method, Ss_I: *S. spinosulus* under the indirect processing method, Ss_PW: *S. spinosulus* under the plate washing processing method, Iv_D: *Ircinia variabilis* under the direct processing method; Iv_I: *I. variabilis* under

the indirect processing method, Iv_PW: *I. variabilis* under the plate washing processing method, SW: seawater..103

Figure 3.3. Phylum- (a) and class-level (b) bacterial community composition in marine sponges and seawater. Compositional data for *S. spinosulus* and *I. variabilis* handled with the direct, indirect and plate washing processing methods are shown. Results obtained using pooled replicate samples (n=4) within each sample category are displayed. Bacterial community composition in each replicate sample is shown as supplemental material to this article (Fig. S3.2). In panel (b) the top 15 bacterial classes are listed and all remaining taxa are labelled as “others”. Labelling of sample categories is as described in legend to Fig. 3.2....106

Figure 3.4. Bacterial OTU networking (a) and Venn diagrams (b-g). All OTUs detected across all analysed samples are included in the analysis. In the network diagram, gradients of green correspond to *S. spinosulus* samples (light green: plate washing processing, green: indirect processing, dark green: direct processing), and gradients of pink to *I. variabilis* samples (light pink: plate washing processing, pink: indirect processing, dark pink: direct processing) along with their corresponding OTUs. Seawater samples and their corresponding OTUs are shown in blue. Venn diagrams (b) and (c) enumerate OTUs within *S. spinosulus* and *I. variabilis*, respectively, shared by and exclusive to the different methods of sample processing. Diagram (d) shows all OTUs detected in, shared by and exclusive to *S. spinosulus* and *I. variabilis* by merging the data obtained with the three processing methods. Diagrams (e) and (f) depict the degree of exclusiveness and sharedness between each species (under each of the three processing methods) and seawater, whereas diagram (g) compares marine sponge and seawater microbiomes as determined by cultivation-independent methods only (i.e. plate washing sponge processing excluded). Labelling of sample categories is as described in legend to Fig. 3.2. An equivalent analysis including only OTUs containing ≥ 50 sequences is shown as supplemental material to this article (Fig. S3.3)...............................109

Figure 3.5. Principal coordinates analysis (PCoA) of bacterial community profiles at the phylotype (OTUs) level. Analyses embracing sponge and seawater samples (a) and sponge samples only (b) are shown, and were performed with normalized sequencing depths [1.236 seqs/sample in panel (a) and 3.668 seqs./sample in panel (b)] using the Unifrac metric. The

ten most dominant bacterial taxa (at the phylum or class level) assigned to the OTUs detected in this study are plotted on both diagrams. Symbol sizes of bacterial taxa correspond to their respective, mean relative abundances across the whole dataset. The position of bacterial taxa in the ordination space is determined by the correlation between their relative abundances and the sample categories defined in this study. Coloured circles represent replicates within each sample category. Labelling of sample categories is as described in legend to Fig. 3.4, except for both sponge species under plate washing method, which is coloured in red. Alternatively, constrained canonical ordination was performed using normalization of abundance data (and not of sequence size) to determine whether sample categories significantly influence variation in 16S rRNA gene profiling by 454 pyrosequencing. These analyses are shown as supplemental material to this article (Fig. S3.4)..111

Figure 3.6. Confocal laser scanning microscopy images of fluorescent *in situ* Hybridization (FISH)-stained bacteria in *S. spinosulus* and *I. variabilis*. Volume rendering images (left in each panel) and their corresponding 3D reconstructions (right in each panel) are shown for hybridizations coupling the Cy3-labeled universal bacterial probe (red cells) to ALEXA488- or Cy5-labeled group-specific probes targeting the *Alpha*- and *Gammaproteobacteria* classes and the phylum *Acidobacteria*. When solely used in combination with the universal probe [panels (a)-(d), (g) and (h)], cells of these taxonomic groups appear as yellowish cells in the volume rendering images and as green objects in the 3D reconstructions. For co-hybridizations including bacterial, alpha and gammaproteobacterial probes [panels (e) and (f)], the latter two groups are represented by yellowish and pink cells, respectively, except in the 3D reconstruction image of *I. variabilis* (f), in which gammaproteobacterial cells appear in purple. Sponge background structure, vastly dominated by profuse spongin filaments, is shown overall in cyan or blue, except in panel (f) where it is displayed in semi-transparent brown..113

Figure S3.1. Observed and estimated richness measure and Shannon diversity indices of bacterial OTUs (97% cut-off) detected in *I. variabilis*, *S. spinosulus* and seawater, disregarding the de-noising methodology employed in this study. Figure details are the same as provided in legend to Figure 2, except for sequence-depth: 3.540 [includes seawater

libraries, panels (a) and (c)] and 4.366 [sponge libraries only, panels (b) and (d)] sequences per library..121

Figure S3.2. Phylum- (a) and class-level (b) bacterial community composition in each replicate sample of *S. spinosulus*, *I. variabilis* and seawater. Figure details are the same as provided in legend to Figure 3.3..122

Figure S3.3. Network (a) and Venn diagrams (b-g) constructed for OTUs containing ≥ 50 sequences. Figure details are the same as described in legend to Figure 3.4.........................123

Figure S3.4. Constrained ordination analysis of bacterial communities profiled by 454 pyrosequencing. Ordination diagrams represent canonical correspondence analysis (CCA) embracing all sample categories (n = 7: seawater, *I. variabilis* with direct, indirect and plate washing methods, and *S. spinosulus* with direct, indirect and plate washing methods) (a); redundancy analysis (RDA) with both sponge species under the culture-independent methods (b); RDA with *I. variabilis* under culture-independent methods (c), and RDA with *S. spinosulus* under culture-independent methods (d). Labelling of sample categories is as described in legend to Fig. 4, except for both sponge species under the plate washing method, which are coloured in red. Arrows indicate OTUs that were specific or enriched in *S. spinosulus* or *I. variabilis* under direct or indirect methods. Labels displayed on the diagram axes refer to the percentage variations of OTU abundance - environment correlation accounted for the respective axis. The ‘‘star’’ symbol represents the centroid positions of the canonical variables (i.e., sponge species and sample processing methods) in the diagram. Variables that significantly (p<0.05) influenced bacterial community composition are highlighted with an asterisk...124

Figure 4.1. Relative abundance of bacterial phyla in sediment samples from all locations. The stacked bar plot (A) shows the relative abundance (%) of each phylum per sample on the y-axis. The identity of each triplicate sample per site is given on the x-axis. SD=sediment. Pie charts (B) show the average values of triplicates for the main phyla present in the samples. Graphs only represent those phyla present at percentages equal or higher than 1%..............138

Figure 4.2. Relative abundance of bacterial phyla in seawater samples from all locations. The stacked bar plot (A) shows the relative abundance (%) of each phylum per sample on the y-axis. The identity of each triplicate sample per site is given on the x-axis**.** SW=seawater. Pie charts (B) show the average values of triplicates for the main phyla present in the samples. Graphs only represent those phyla present at percentages equal or higher than 1%.............139

Figure 4.3. Relative abundance of bacterial phyla in sponge samples from all locations. The stacked bar plot (A) shows the relative abundance (%) of each phylum per sample on the y-axis. The identity of each triplicate sample per site is given on the x-axis. IRC=*Ircinia* spp.; SAR=*Sarcotragus spinosulus*; SPO=*Spongia* sp.. Pie charts (B) show the average values of triplicates for the main phyla present in the samples. Graphs only represent those phyla present at percentages equal or higher than 1%..141

Figure 4.4. Comparison on bacterial richness and diversity among sample types. The estimated (Chao 1) and observed richness averages for each sample group. (A). The Shannon diversity index from the same groups (B). Alg=Algarve; Azo=Azores; Mad=Madeira; SD,=Sediment; SW=Seawater; Irc=*Ircinia* spp.; Sar=*Sarcotragus spinosulus*; Spo=*Spongia* sp..142

Figure 4.5. Principal Coordinates Analysis (PCoA) based on the unweighted UniFrac distance metric. The graph shows the grouping of seawater samples by locality, whereas sediment and sponge samples do not present a geographical pattern. They group instead by sample type..143

Figure 4.6. Number of phylotypes exclusive and common to all sample categories at each sampling site. (A), Algarve; (B), Azores; and (C), Madeira. The Venn diagrams were constructed using the complete OTU table (i.e., both dominant and rare OTUs included) as input data. 144

Figure 4.7. Common and habitat-specific bacterial OTUs in sponge samples. (A), *Ircinia* spp.; (B) *Sarcotragus spinosulus*; (C), *Sarcotragus*-*Spongia* spp. group. The Venn diagrams

were constructed using the complete OTU table (i.e., both dominant and rare OTUs included) as input data. 144

Figure 4.8. Common and habitat-specific bacterial OTUs in sponge samples, with removal of "rare" symbionts from the analysis. (A), Using OTUs with at least 50 reads in the whole data set; (B), using OTUs with at least 50 reads in the sponge-derived libraries………………..145

Figure 4.9. Bacterial OTUs common to all sponge species and specific to *Ircinia* and *Sarcotragus*-*Spongia* spp. groups. (A), Using the whole OTUs dataset; (B), Using OTUs with at least 50 reads in the whole dataset; (C), Using OTUs with at least 50 reads in the sponge libraries dataset. 146

Figure S4.1. Phylogenetic inference of the Irciniidae/Spongiidae family based on the cytochrome oxidase gene, subunit 1. The Maximum Likelihood tree (-ln likelihood: 1343.504072) is shown, with sequences retrieved in this study highlighted in bold. Numbers at tree nodes are bootstrap values and posterior probabilities calculated in Maximum Likelihood and MCMC Bayesian analyses, respectively, and values above 70/0.95 are shown. 154

Figure 5.1. Phylogenetic inference of the Irciniidae family based on cytochrome oxidase gene subunit 1 sequences. The maximum likelihood tree (-ln likelihood: -1204.8294) is presented, with sequences recovered in this study highlighted in bold. Number at tree nodes are bootstrap values and posterior probabilities calculated in Maximum Likelihood and MCMC Bayesian analyses, respectively, and values above 75/0.95 are shown.................................165

Figure 5.2. Redundancy analysis of bacterial 16S rRNA gene (a), archaeal 16S rRNA gene (b) and bacterial *amoA* gene (c) PCR-DGGE profiles. Symbols: *S. spinosulus* specimens collected ○ in 2010, □ in 2011, and △ in 2012. Labels displayed on the diagram axes refer to the percentage variations of PCR-DGGE ribotypes - environment correlation accounted for the respective axis. The ''star'' symbol represents the centroid positions of the environmental variables in the diagram. Variables that significantly ($p<0.05$) influence the bacterial community composition are indicated by an asterisk...167

Figure 5.3. Phylum- (a, b) and class-level (c, d) bacterial community composition in *S. spinosulus*. The compositions of each replicate sample (a, c) and of pooled replicate samples (b, d) are shown..169

Figure 5.4. Bacterial OTU networking and maintenance in the marine sponge *Sarcotragus spinosulus* across time. Networks with all OTUs per sponge replicate (a) and with all OTUs in composite samples where sponge replicates were pooled according to the sampling year (b) and Venn diagrams (c) with all OTUs and (d) with OTUs containing ≥ 50 sequences are shown. In the diagrams, red, green, and purple correspond to the sampling years of 2010, 2011, and 2012, respectively..171

Figure 5.5. Ordination analysis of bacterial OTUs with ≥ 97% sequence similarity. (a): Principal Coordinate Analysis of OTUs using the UniFrac metric. Red, green, and purple correspond to the sampling years of 2010, 2011, and 2012, respectively. The ten most dominant bacterial taxa (at phylum or class level) are shown in the diagram. Symbol sizes of bacterial taxa correspond to their respective, mean relative abundances across the dataset. The position of bacterial taxa in the ordination space is determined by the correspondence between their relative abundances and the year of sampling. (b): Constrained ordination of bacterial OTUs via Redundancy Analysis. Symbols: ○ *S. spinosulus* collected in 2010, □ in 2011, and △ in 2012. The ''star'' symbol represents the centroid positions of the environmental variables in the diagram. Variables that significantly (p<0.05) influence the bacterial community composition are indicated by an asterisk...174

Figure 5.6. Structure of archaeal communities in *Sarcotragus spinosulus* across time. (a): Heatmap showing the distribution of the archaeal 16S rRNA gene taxonomic affiliation in each *S. spinosulus* specimens; (b): OTU network with all OTUs at 97% sequence similarity, where red, green and purple correspond to the sampling years of 2010, 2011, and 2012, respectively; (c): PCoA of the archaeal OTUs; (d): UPGMA clustering, and (e): RDA of the archaeal OTUs. Symbols: *S. spinosulus* collected ○ in 2010, □ in 2011, and △ in 2012. The ''star'' symbol represents the centroid positions of the environmental variables in the

diagram. Variables that significantly ($p<0.05$) influence the bacterial community composition are indicated by an asterisk..176

Figure S5.1. PCR-DGGE fingerprinting of bacterial 16S rRNA gene (a), archaeal 16S rRNA gene (b) and bacterial *amoA* gene (c) fragments associated with the marine sponges *S. spinosulus*...182

Figure S5.2. Observed and estimated richness for bacterial (a) and archaeal (b) OTUs (97% cut-off) and diversity indices for bacterial (c) and archaeal (d) OTUs (97% cut-off) in the marine sponge *S. spinosulus* along three consecutive years..182

LIST OF TABLES

Table 2.1. Closest 16S rRNA gene relatives of seawater-derived and “cosmopolitan” PCR-DGGE bands. 71

Table S2.1. PCR-DGGE band richness, diversity and evenness..85

Table 3.1. FISH probes used in this study...99

Table 3.2. Sequence data summary...102

Table S3.1. Statistical analysis of PCR-DGGE band richness (a) factorial ANOVA (b) TukeyHSD and diversity (c) factorial ANOVA (d) TukeyHSD measures............................125

Table 4.1. Number of filtered sequences and OTUs per sample type and locality…………136

Table 4.2. Ten more abundant OTUs common to all sponge specimens………………….. 147

Table 5.1: The Pan microbiome associated with *S. spinosulus*..172

Table 5.2: The essential core associated with *S. spinosulus*..173

Table S5.1. Taxonomic classification and absolute abundance of bacterial OTUs found exclusively in each sampling year (a) 2010, (b) 2011 and (c) 2012......................................183

LIST OF ABBREVIATIONS AND ACRONYMS

ASW = Artificial Seawater

ANOVA = One Way Analysis of Variance

BLAST = Basic Local Alignment Search Tool

BSA = Bovine Serum Albumin

CCA = Canonical Correspondence Analysis

CFU = Colony Forming Unit

CLSM = Confocal Laser Scanning Microscope

CMFASW = Calcium Magnesium Free Artificial Seawater

CO1 = mitochondrial cytochrome oxidase subunit 1

DBUA. Por = Department of Biology zoological collection, University of Azores

RDA = Redundancy Analysis

DNA = Deoxyribonucleic acid

DGGE = Denaturing Gradient Gel Electrophoresis

dNTPs = Deoxynucleoside triphosphates

DMSO = dimethyl sulfoxide

EMBL = European Molecular Biology Laboratory

FISH = Fluorescence *in situ* Hybridization

GTR = General Time Reversible

HMA = High Microbial Abundance

OTU = Operational Taxonomic Unit

PCR = Polymerase Chain Reaction

QIIME = Quantitative Insights Into Microbial Ecology

RDP = Ribosomal Database Project

rRNA = ribosomal ribonucleic acid

SC = sponge-specific cluster

SINA = SILVA Incremental Aligner

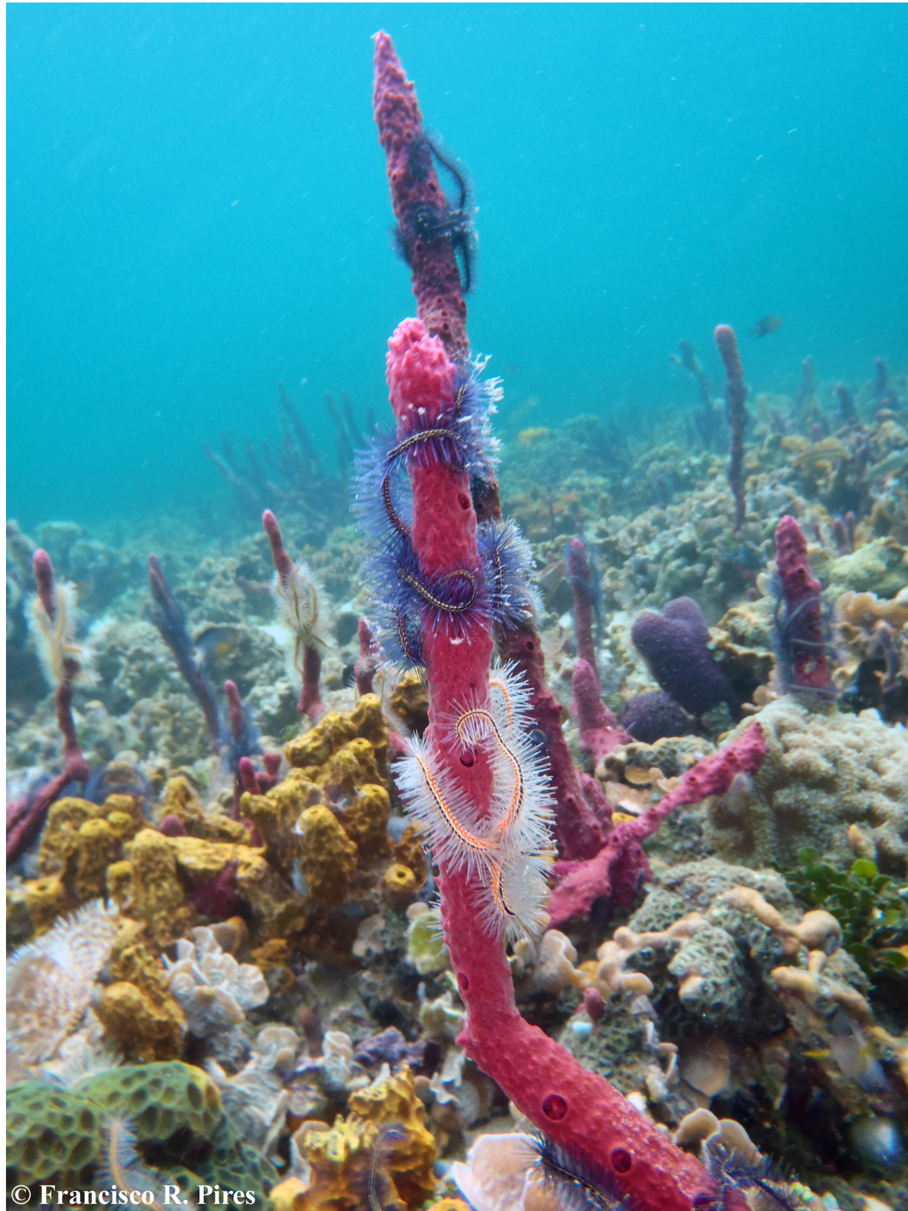
MEGA = Molecular Evolutionary Genetics Analysis

NCBI = National Center For Biotechnology Information

UPGMA = Unweighted Pair Group Method with mathematical Averages

UV = ultraviolet

CHAPTER I



General Introduction

1. Phylum Porifera

1.1 Evolutionary history, diversity, and classification

The phylum Porifera (latin porus, “pore”; ferre, “to bear”) contains the animals known as sponges. They are one of the oldest metazoans, with a chemical fossil record dating back approximately 635 million years (MYA) to the Ediacaran epoch within the Neoproterozoic era (Love *et al*., 2009). Since the late Cambrian (509 MYA), sponges have maintained their essential body plan. Throughout the Phanerozoic eon and until the late Mesozoic era (543 to 65 MYA), sponge species were very important reef builders. Today, the phylum Porifera is one of the most diverse and successful of the 28 aquatic invertebrate phyla with respect to the number of species as well as the range of morphological characteristics (Brusca and Brusca, 2002; Hooper and van Soest, 2002). According to the World Porifera Database (http://www.marinespecies.org/porifera/), so far more than 8.500 sponge species have been described. Most likely, this is only a portion of their real richness, which is estimated in 17.000 species (van Soest *et al*., 2012). Sponges show a wide range of shapes (e.g. tube, barrel, ball, vase, encrusting, rope), colours (e.g. green, yellow, white, blue, purple, brown, black), sizes (e.g. a few millimetres to nearly two meters or even more) and reside in diverse aquatic habitats (e.g. marine, brackish, freshwater) (Brusca and Brusca, 2002) (Fig. 1.1). They also occur at all depths, however especially rich faunas are most probably found in non-polluted littoral and tropical reef habitats where sponges can contribute or even dominate the fauna in terms of biomass (Brusca and Brusca 2002; van Soest *et al*., 2012). Sponges are classified into four classes designated Calcarea (discrete cells, calcareous spicules, ubiquitous fibrillar collagen), Hexactinellida (syncytial choanoderm, discrete cells and pinacoderm, siliceous triaxone spicules, ubiquitous fibrillar collagen, mostly deeper water), Demospongiae (discrete cells, siliceous monaxome or tetraxone spicules, ubiquitous fibrillar collagen), and Homoscleromorpha (flagellated opinacocytes and a basement membrane lining choanoderm and pinacoderm, large choanocytes with oval to spherical chambers, and viviparous cinctoblastula larva). They contain 25 orders, 128 families and 680 valid genera (van Soest *et al*., 2012). Demospongiae is by far the largest sponge class, encompassing around 83% of all described sponge species. Within this class, the majority are marine species but some can be found in freshwater habitats. Demosponges possess a wide range of occurrence from intertidal zones to the deepest seas, covering the full latitudinal and longitudinal spectra of the planet.

Currently, demosponges encompass 11 orders, 97 families and more than 550 valid genera (van Soest *et al*., 2012). The sponge species described in this chapter belong to demosponges, unless otherwise stated.

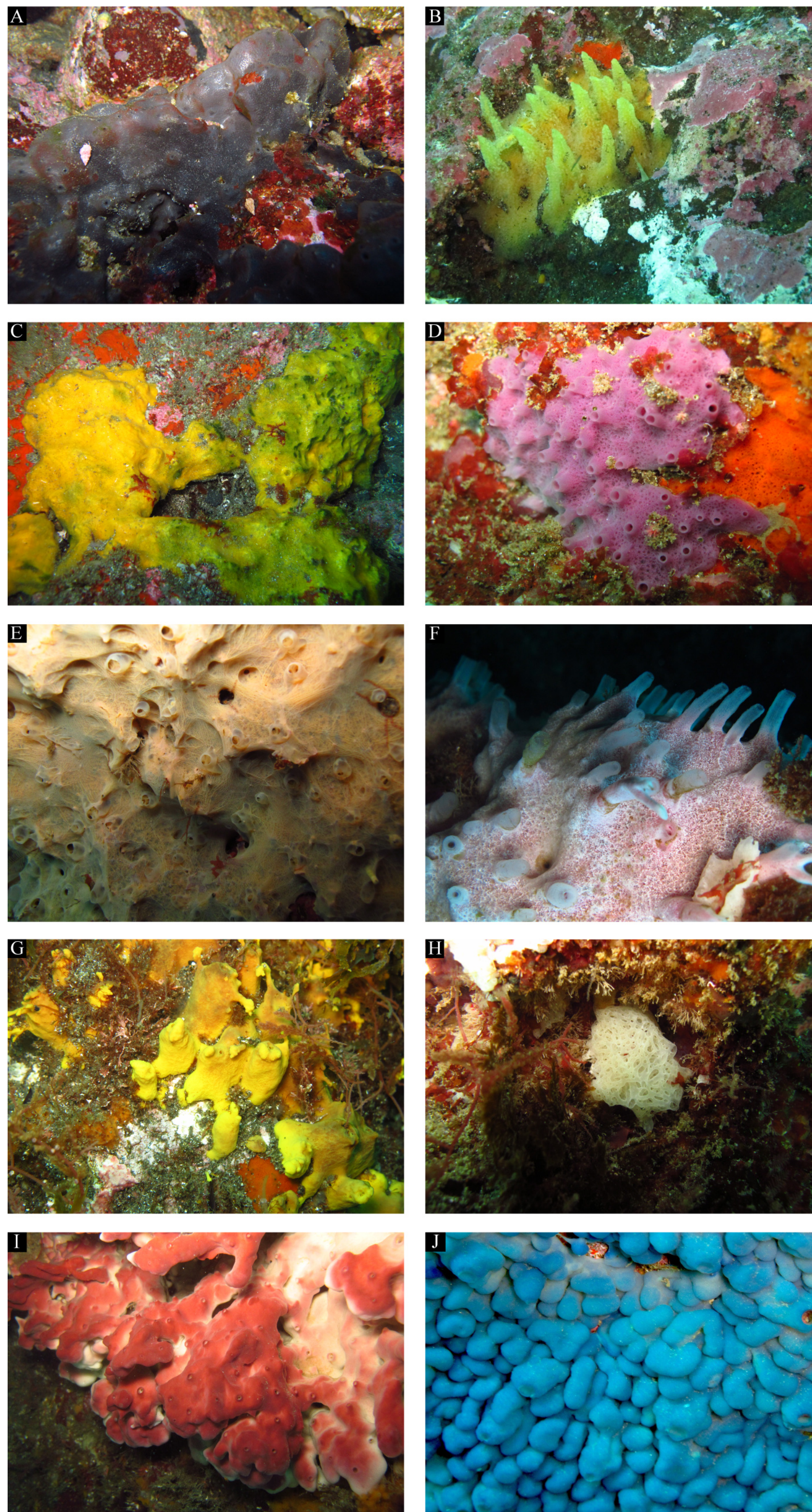


Figure 1.1 - Underwater photographs of marine sponges. (A) *Spongia azorica*, (B) *Ciocalypta penicillus*, (C) *Axinyssa aurantiaca*, (D) *Haliclona* sp., (E) *Hexadella racovitzai*, (F) *Haliclona fistulosa*, (G) *Aplysina aerophoba*, (H) *Clathrina coriacea*, (I) *Petrosia ficiformis,* and (J) *Oscarella lobularis.* Photographs courtesy of MSc. Francisco R. Pires.

1.2 Sponge body structure and gross morphology

The Porifera are sessile metazoans and the only phylum with parazoan body construction, which means that they lack the true embryological germ layer and do not possess true tissues and organs. Nevertheless distinct types of cells are found in the sponge body. Two exclusively organizational features define sponges and play essential roles in their success: the aquiferous system (system of canals -incurrent and excurrent- and choanocytes chambers where the water circulates in the sponge interior, Fig. 1.2) and the highly totipotent (cells capable of forming any cell type) nature of their cells. Pinacocyte cells form the pinacoderm, which covers the outer surface of the sponge as well as all incurrent and excurrent canals or channels, whereas the inner surface is lined by the choanocytes (flagellated cells) making the choanoderm; both sheets are composed of a single cell layer (Fig. 1.2). The space between the outer and inner surface is an extracellular matrix made of fibrils of collagen that forms the mesohyl (Fig. 1.2). The pinacoderm is punctured by small holes named dermal pores or ostia. The first is surrounded by several cells, while the latter by only one cell (Fig. 1.2). The porocyte cells of the pinacoderm form the ostia, they are contractile and able to open and close the pores as well as regulate their diameter (Fig. 1.2). Water is inhaled by these openings, crosses the incurrent canals in the direction of the choanocytes chamber, and is expelled through excurrent canals and the larger exhalant osculum (large pore in the body wall of sponge through which water flows out from the body cavity, Fig. 1.2). The unidirectional water currents within the sponge body are maintained by the flagellated choanocyte cells. Several types of cells are found in the mesohyl and play fundamental roles in sponge metabolism. For instance, part of the function of choanocytes is to remove oxygen and food particles from the water, whereas archaeocytes, which are ameboid motile cells with extraordinary totipotent capacity, are involved in digestion of food and transport of the products retrieved from water throughout the sponge body (Fig. 1.2). These cells are as well responsible for excretory activities. Collencytes, lophocytes, and spongocytes are very mobile ameboid cells also located in the mesohyl and capable to move freely within it. The first two secrete and disperse fibrillar collagen found intercellularly in the mesohyl, whereas the latter generates the fibrous supportive collagen known as spongin. However, only members of the Demospongiae contain true spongin. The calcareous and siliceous spicules are produced by sclerocytes, situated in the mesohyl. Myocytes are the contractile cells of the sponge observed

in the mesohyl. Spherulous cells are present in the mesohyl and often contain secondary metabolites (Brusca and Brusca, 2002; Hooper and van Soest, 2002).

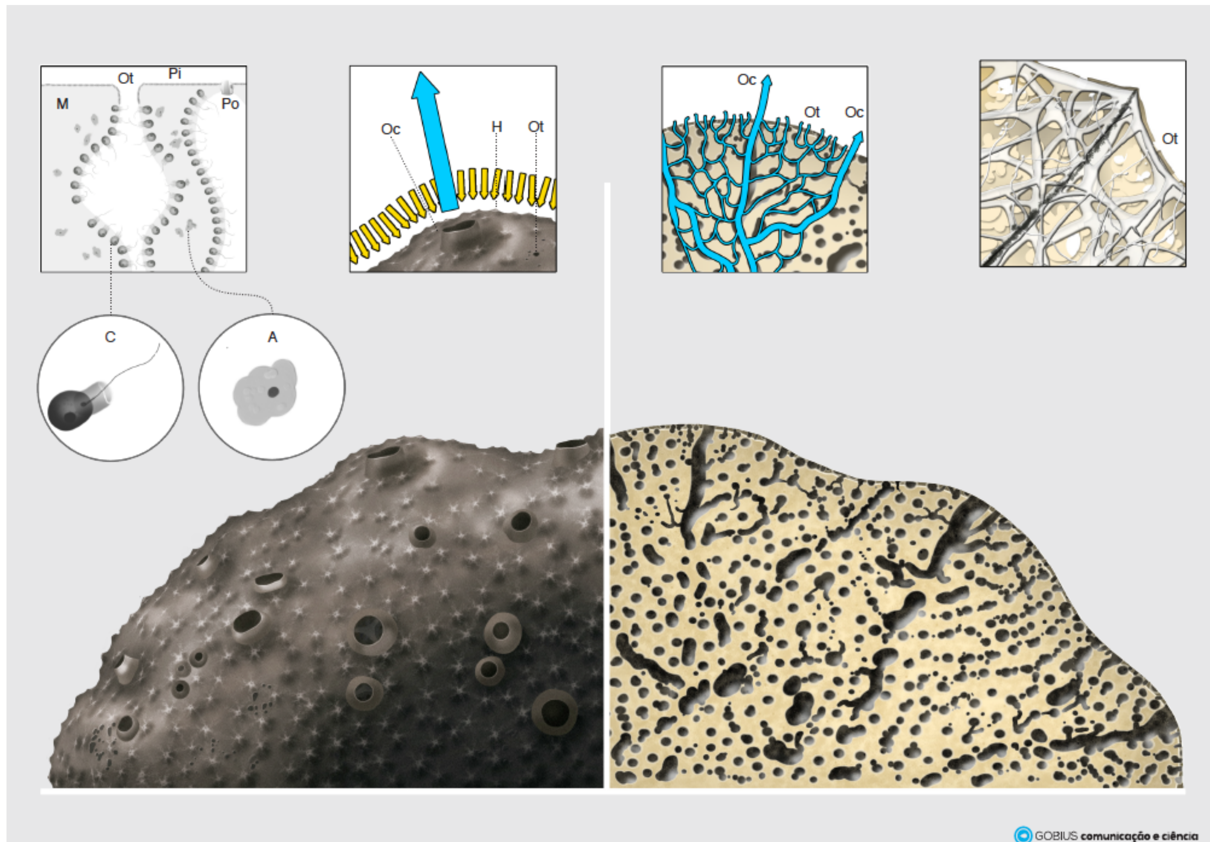


Figure 1.2 - A schematic overview of a demonsponge from the family Irciniidae. Mesohyl (M), ostium (Ot), pinacoderm (Pi), porocyte (Po), choanocyte (C), archeocyte (A), osculum (Oc), and horny (H).

1.3 Sponge nutrition

The majority of sponges depend on intercellular digestion, and consequently on phagocytosis and pinocytosis, as means of food capture. The aquiferous system plays an essential role in this process by maintaining an almost continuous movement of water through the sponge body and carrying microscopic food particles. The size of the food particles is limited by the size of the components of the aquiferous system, especially the size of the incurrent canal, which in most sponges ranges from 5 to 50µm in diameter. Food particles ranging in size from 2 to 5µm (e.g. bacteria, small protists, unicellular algae, and organic detritus) are internally captured by phagocytic motile archaeocytes that move to the lining of the incurrent canals. As the water current continuously passes over the choanoderm, whirlpools are created

around the choanocytes, which trap in the mucous reticulum small particles between 0.1 and 2.0µm that will further be moved to the choanocytes cell body where ingestion takes place by phagocytosis or pinocytosis. When phagocytosis is performed by archaeocytes, the digestion occurs in a food vacuole formed in the moment of the capture, whereas food particles captured by the choanocytes are partially digested by these cells and then rapidly moved to the archaeocytes for final digestion. In both cases, the transport of nutrients throughout the sponge body happens because of the mobility of the mesohyl cells (Brusca and Brusca, 2002). A survey performed with three Jamaican demosponges revealed that 80.5% of the organic matter ingested by these species was below the detection limit of the light microscope, whereas microscopically resolvable particulate material (MPOC) accounted for 19.5% of the particulate organic carbon. These three sponge species retained available MPOC within the size range of 0.3 to 50µm with high efficiencies. Moreover, it was shown that the main components of the MPOC diet of these sponge species encompassed primarily bacteria and dinoflagellates (Reiswig, 1971).

1.4 Sponge macroecology

Sponges are very abundant organisms, dominating many benthic and shallow marine habitats with a solid substrate, such as coral reefs and submarine caves. The majority of the tropical and subtropical rock littoral regions harbour a large number of sponge species. Recent evidence indicates that they also occur in large numbers and sizes in the Antarctic continent (Brusca and Bruca, 2002; Muricy and Hadju, 2006; Muricy *et al*., 2008). Several functional roles have been described for sponges in ecosystem functioning, for instance as bioeroders which process solid carbonate into smaller fragments and fine sediments. However some other fundamental and presumed roles of sponges such as reef creation, stabilization, consolidation, and regeneration remain poorly studied. Sponges have also been suggested to take part in carbon, nitrogen, and silicon cycling (Bell, 2008 and references therein).

Many animals prey on sponges, however the damage caused by predators is usually slight. Some tropical fishes, turtles, molluscs (mainly gastropods), sea urchins, and starfishes are able to harvest certain types of sponge. As sponges are sessile animals, they developed alternative mechanisms of defence. The presence of linear mineral spicules help to keep a portion of the predators away by providing a physical deterrent. However, the primary

defence system is based on the production of a variety of toxic compounds. These may assist the sponge in deterring predators, avoiding microbial infections and/or fouling and competing for space with other invertebrates such as bryozoans, ascidians, corals, and even other sponges (Brusca and Bruca, 2002; Muricy and Hadju, 2006; Muricy *et al*., 2008). It was demonstrated that a Mediterranean nudibranch (a type of gastropod), when disturbed, secreted a white slime that contained secondary metabolites known to be present in the sponge species that it fed on, suggesting that this animal was capable of using sponge-derived metabolites for its own defence (Marin *et al*., 1997).

Comensalistic and positive (i.e. protocooperation and mutualism) interactions involving sponges and varied partners are found quite often in the seas. The aquiferous system and the antipredator defence mechanisms of sponges offer protection to a variety of small invertebrates (e.g. crustaceans, brittle stairs, and polichaetes) and some fishes. Several novel invertebrate species, particularly of polichaetes and crustaceans, have been described in association with marine sponges (Brusca and Brusca, 2002; Musco and Giangrande, 2005; Muricy and Hadju, 2006; Wulff, 2006a; Iseto *et al*., 2008; Winfield *et al*., 2009; Fiore and Jutte, 2010; Lattig and Martin, 2011; Ortiz *et al*., 2011). Many of these species are thought to take advantage of the shelter provided by sponges to keep their population stable in the juvenile phase. Other organisms use sponges as cover or camouflage: many crab species excise small pieces from distinct sponges and cultivate them on their shells. Another very common relationship occurs between sponges and their symbiotic bacteria (Brusca and Brusca, 2002; Muricy and Hadju, 2006). This particular interaction, which has attracted considerable scientific interest in the last decade or so for several reasons, is the central focus of this Thesis and as such is presented in detail below.

2. Sponge microbiology

2.1 Defining symbiosis

The term symbiosis when used in the present work will maintain the original definition given by Anton de Bary in 1869 that refers to two organisms belonging to distinct species that live together in close physical association over a long period of time: "Symbiosis is the living together of parasite and host" Anton de Bary (1869) *apud* Pound (1893).

2.2 The bacterial diversity of sponges

2.2.1 Early surveys

Even though bacterial cells are consumed by marine sponges as food particles as demonstrated by Reiswig (1971), pioneering work done by Vacelet and Donadey (1977) and Wilkinson (1978c, b, a) revealed that bacterial cells were observed in consistent association with the mesohyl of these animals. In the study by Vacelet and Donadey (1977), thirteen sponge species (two calcareous and eleven demosponges) collected from distinct sampling sites were analysed by electron microscopy and showed that intact bacterial cells were present in the mesohyl of all specimens. In addition, these authors demonstrated that massive sponge species with dense mesohyl contained distinct bacterial morphotypes, whereas well-irrigated species with low mesohyl density hosted only one scarcely distributed bacterial morphotype and that in some specimens bacterial cells accounted for up to 38% of the sponge's wet weight. In the study of Wilkinson (1978c) four sponge species were collected from the Great Barrier Reef, Australia, which revealed small and large numbers of heterotrophic bacteria in *Pericharax heteroraphis* (Calcarea) and *Jaspis stellifera*, respectively, whereas moderate counts were registered for *Ircinia wistarii* and *Neofibularia irata* when compared to surrounding seawater. *J. stellifera* and *I. wistarii* were characterized by narrow ostia and a complex aquiferous system with small choanocytes canals and high filtering efficiencies, while *P. heteroraphis* and *N. irata* possessed wider ostia and a simple aquiferous system with few large canals and lower filtering activity (Wilkinson, 1978c). It was also verified that *J. stellifera* and *I. wistarii* contained a dense mesohyl with numerous bacteria, while *P. heteroraphis* and *N. irata* possessed thin mesohyl and few bacteria (Wilkinson, 1978c, a). Thus, the former two sponge species would be classified as "high microbe abundance" (HMA) sponges, whereas the latter two species would be classified as "low microbe abundance" (LMA) sponges following the definitions provided by Hentschel *et al.* (2006). In a following study, Wilkinson (1978b) isolated more than 500 heterotrophic bacterial strains from the same four sponge species and surrounding seawater. These isolates possessed unique characteristics and could metabolize a wide range of compounds, some of which were thought to be important for the removal of waste products when water was not being expelled by the sponge. The isolates were grouped into six clusters based on their morphology, physiology, and metabolic features. One cluster was found almost exclusively within sponge species and was further characterized as strictly symbiont, whereas the other five groups contained a

mixture of sponge symbionts and bacteria isolated from surrounding seawater (Wilkinson, 1978b). The author considered it unlikely that substantial nutritional energy was gained by the sponge through digestion of the obligate, and persistent bacterial symbionts, and that therefore these bacteria most likely performed other functions in the association with their hosts (Wilkinson, 1978a).

2.2.2 State-of-the-art assessments of sponge-associated bacterial diversity

The last decade or so has seen an enormous increase of scientific interest in sponges, and in particular in the marine species belonging to the class Demospongiae. This is firstly due to the conspicuousness of their microbiota, as sponge species show a unique capacity to host a wide variety of phylogenetically distinct microorganisms (Taylor *et al*., 2007; Hentschel *et al*., 2012; Webster and Taylor, 2012) and, secondly, due to the several bioactive secondary metabolites retrieved from these animals (e.g. Faulkner, 1997, 1999, 2002; Blunt *et al*., 2004, 2006; Blunt *et al*., 2008, 2009; Blunt *et al*., 2011; Blunt *et al*., 2013). It is assumed that these two factors – complex sponge microbiomes and biochemistry - are frequently interconnected (Bewley *et al*., 1996; Piel *et al*., 2004b).

Culture-dependent methods for the assessment of sponge-associated bacterial diversity

To investigate their beneficial properties and putative function, considerable effort has been made to cultivate bacterial symbionts from marine sponges. For instance, using transmission electron microscopy, distinct heterotrophic bacteria were observed in the mesohyl of the sponge *Ceratoporella nicholsoni*, and further cultivation onto modified marine agar medium revealed that between 3.4 to 11% of the total number of bacteria inhabiting this species were recovered (Santavy *et al*., 1990). In another study, it was demonstrated that only a small fraction (0.15%) of the bacterial population obtained from the sponge *Aplysina aerophoba* could be cultivated on marine agar medium (Friedrich *et al*., 2001). Using the same medium, approximately 0.1% of the total bacterial community observed in *Rhopaloeides odorabile* was cultivated (Webster and Hill, 2001). All surveys defined cultivability as the number of colony forming units (CFU) observed on the culture medium divided by the total number of bacteria detected in the sponge samples by microscopy. However, the main difference observed between the first and the two latter studies could be partially explained by the way in which

the microscopy data were generated and assessed. Specifically, Santavy *et al*. (1990) estimated the bacterial cell concentration in the sponge by conversion of the bacteria counted in the transmission electron microscopy images to a three-dimension model, whereas Friedrich *et al*. (2001) and Webster and Hill (2001) used epifluorescence microscopy to estimate the concentration of the bacterial cells in the sponge body.

In the present Thesis, bacterial abundances in marine sponges are also determined via cultivation onto marine agar and epifluorescence microscopy preparations (Chapter 2). However, rather than comparing numbers of CFU on culture plates with numbers of bacterial cells detected under the microscope, which disregards the identity of colonies or cells under evaluation, cultivability is here defined as the diversity of bacterial phylotypes that can be retrieved from cultivation-dependent methods in comparison to that obtained by cultivation-independent, DNA-based, analyses (Chapter 3). Several cultivation surveys have verified that the majority of bacteria isolated from marine sponges belong to *Proteobacteria*, especially within the classes *Alpha*- and *Gammaproteobacteria* (Hentschel *et al*., 2006), whereas molecular-based, cultivation-independent studies usually unveil many more of sponge-associated bacterial phyla (Taylor *et al*., 2007; see below). Thus, measuring the discrepancy between culturable and unculturable bacterial diversities in marine sponges was one of the major goals of the present study. To this end, an approach that circumvents the need to isolate and purify bacterial cultures in diversity assessments was employed in Chapter 3.

In the last few years, the development of new or innovative cultivation strategies has increased the culturable bacterial assemblage retrieved from marine sponges. For instance, Sipkema *et al*. (2011) used three different methods to isolate bacteria from *Haliclona* (*gellius*) sp.. More than 3.900 isolates were obtained from agar plates, liquid, and floating filter cultures using 17, 7, and 12 different medium formulations, respectively. The isolates were assigned to 205 operational taxonomic units (OTUs). The majority of cultures (2.278) and OTUs (151) originated from agar plates, whereas 11 and 23 OTUs were specific to liquid and floating filter cultures, respectively. By applying these approaches, less commonly isolated phyla such as *Plactomycetes*, *Verrucomicrobia,* and *Deltaproteobacteria* were recovered from *Haliclona* (*gellius*) sp., along with *Actinobacteria*, *Bacteroidetes*, *Firmicutes,* and *Alpha*-, *Beta*-, and *Gammaproteobacteria.* By defining cultivability as the percentage of OTUs acquired with 16S rRNA gene libraries from sponge internal body when compared to the total numbers of 16S rRNA gene sequences from the isolates (i.e. using the cultivability criterion

employed in this Thesis) between only 10 and 14% of the bacterial diversity associated with *Haliclona* (*gellius*) sp. was recovered (Sipkema *et al*., 2011). In a separate study (Schupp, personal communication), diffusion growth chambers were designed to allow the incubation of sponge-associated bacteria inside the host body. Diffusion chambers were inserted into *Rhabdastrella globostellata* specimens in the field and kept inside the sponges for four weeks. After this period the chambers were retrieved, the contents were collected, diluted, and plated onto three distinct culture media. Partial sequence data revealed that many bacterial isolates were either previously uncultured or showed <97% 16S rRNA sequence similarity with known cultured strains.

Culture-independent techniques

In the early stages of the scientific development of sponge microbiology, Vacelet and Donadey (1977) made a correlation between the thickness of the aquiferous system and the density of bacteria in the sponge mesohyl, and this correlation was used to distinguish among LMA and HMA sponges (Hentschel *et al*., 2006). It has been demonstrated that the bacterial abundance in HMA sponges might reach 10^8 to 10^{10} bacteria per gram of sponge wet weight, which normally exceeds those recovered from seawater by 2 to 4 orders of magnitude. Conversely, bacterial abundance in LMA sponges is usually very similar to that detected in surrounding seawater, 10^5 to 10^6 bacteria per gram of sponge wet weight (Friedrich *et al*., 2001; Hentschel *et al*., 2006; Weisz *et al*., 2007). In this Thesis the microbial abundance of the sponge species *Sarcotragus spinosulus* and *Ircinia variabilis*, is evaluated (Chapter 2), allowing their further classification into HMA or LMA sponges.

The advent of molecular technologies to the study of microbial communities, most of which are based on the analysis of 16S/18S rRNA gene fragments, has revolutionized the field of microbial ecology. These tools are being very important for revealing the community structure and diversity of the bacteria associated with marine sponges by overcoming the limitations inherent to culture-dependent methodologies. One of the first studies using culture-independent approaches was performed with two taxonomically different sponge species, *Aplysina aerophoba* and *Theonella swinhoei*, collected at several sampling sites (Hentschel *et al*., 2002). In total 160 clones of the 16S rRNA gene were obtained and they were affiliated with the phyla *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Nitrospira*, *Spirochaetes,* and *Proteobacteria* (*Alpha*, *Gamma,* and *Delta*

classes). In addition, the bacterial community found in both marine sponges was distinct from marine plankton and sediment. Coverage analysis with OTUs defined at 95% 16S rRNA sequence similarity showed that 60 and 58% of the microbial diversity of *A. aerophoba* and *T. swinhoei,* respectively, were recovered. This survey was the first evidence for the existence of monophyletic, sponge-specific bacterial clusters and that a uniform bacterial community composition in marine sponges could be observed at a global scale (Hentschel *et al*., 2002). Thereafter, several studies have addressed bacterial community structure in distinct marine sponges by constructing 16S rRNA gene libraries by PCR and/or by excising and sequencing bands from Denaturing Gradient Gel Electrophoresis (DGGE) assays. In broad terms, sponge-associated microbial 16S rRNA sequences have been broadly identified as belonging to the phyla *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chlamydiae*, *Chloroflexi*, *Cyanobacteria*, *Deferribacteres*, *Deinococcus-Thermus*, *Firmicutes*, *Fusobacteria*, *Gemmatimonadetes*, *Lentisphaerae*, *Nitrospira*, OP10, OP11, *Planctomycetes*, *Proteobacteria* (*Alpha*, *Beta*, *Delta*, *Epsilon,* and *Gamma* classes), *Spirochaetes*, *Tenericutes*, TM6, TM7, *Verrucomicrobia,* and WS3 (Taylor *et al*., 2007; Webster and Taylor, 2012). Moreover, the discovery of the candidate phylum "*Poribacteria"*, found almost exclusively associated with marine sponges, was highly significant (Fieseler *et al*., 2004), although they have since then been found in several marine sponge species collected worldwide (Schmitt *et al*., 2008; Lafi *et al*., 2009; Mohamed *et al*., 2010; Yang *et al*., 2011; Hardoim *et al*., 2013). Additional, phylogenetic studies revealed the existence of three or four poribacterial sponge-specific clades (Lafi *et al*., 2009; Hardoim *et al*., 2013). It is now known that these organisms, instead of existing exclusively in marine sponges, occur in very low numbers in seawater (Pham *et al*., 2008; Mohamed *et al*., 2010) - thus belonging to the so-called rare marine biosphere - and are likely enriched in the sponge body through as-yet unknown mechanisms (Webster *et al*., 2010; Taylor *et al*., 2013). This mode of environmental symbiont acquisition might likewise apply to several other distinct bacterial lineages commonly observed in sponges and, in combination with the mechanism of vertical symbiont transmission (see below), could shape the distinct composition of the marine sponge microbiome. Indeed, multiple surveys have demonstrated that bacterial community structures in marine sponges largely differ from those of their surrounding seawater (Hentschel *et al*., 2006; Taylor *et al*., 2007; Hardoim *et al*., 2009; Hentschel *et al*., 2012; Webster and Taylor, 2012). Comprehensive phylogenetic inferences performed with more than 7.000 sponge-derived 16S rRNA gene sequences revealed 173

monophyletic, "sponge-specific" bacterial clusters identified based on the criteria defined by Hentschel *et al*. (2002) as groups containing at least three 16S rRNA gene sequences that (i) have been retrieved from distinct sponge species and/or from the same species but sampled at different geographic sites, (ii) are more related to each other than to any sequence from other source (e.g. sediment, seawater, and so on), and (iii) are supported by independent methods of tree construction (Hentschel *et al*., 2002; Simister *et al*., 2012c). The maintenance of this complex and diverse bacterial community in marine sponges might be explained by the mechanism of vertical transmission in which specific sponge symbionts are transferred from parental to the next generation via reproductive cells. In the sponge *Halisarca dujardini* a single bacterial morphotype was observed in the mesohyl of adults as well as in all phases of embryonic development (Ereskovsky *et al*., 2005). Clones affiliated to *Actinobacteria*, *Chloroflexi*, *Proteobacteria,* and *Nitrospira* were detected in the embryos of the sponge *Corticium* sp. (Homoscleromorpha) and thus it was demonstrated that complex and diverse bacteria were vertically transmitted from adults to embryos (Sharp *et al*., 2007). In another study, at least ten bacterial phyla were retrieved from adults of the sponges *Agelas conifera*, *A. wiedenmayeri*, *Ectyoplasia ferox*, *Smenospongia aurea*, and *Xestospongia muta* and from their offspring, strengthening the concept that complex communities of several bacterial lineages might be vertically transmitted in marine sponges (Schmitt *et al*., 2008).

Recently, a new era in the field of molecular biology has started with the use of high-throughput sequencing technologies such as 454-pyrosequencing, whereby 25 million nucleotides can be generated in one four-hour run with 99% or better accuracy (Margulies *et al*., 2005). Using this approach, 200.000 reads were obtained from three sponge species collected at four sampling sites along the Red Sea coast (Saudi Arabia) and surrounding seawater, and after quality assessments 140.000 reads with average length of 290-300bp were further analysed (Lee *et al*., 2011). The highest number of 97% OTUs was detected in *Hyrtios erectus* (1.020 OTUs), whereas only 182 97% OTUs were found in the seawater from the same location. Estimated ACE and Chao1 richness indices for *H. erectus* were 2.386 and 2.054, respectively, whereas for surrounding seawater these values dropped to 331 (ACE) and 292 (Chao1). Overall, *Proteobacteria* and *Cyanobacteria* dominated seawater communities, although a further 13 bacterial phyla were also detected but in much lower numbers. Strikingly, in total more than 20 bacterial phyla were recovered from the sponges *H. erectus*, *Xestospongia testudinaria,* and *Stylissa carteri* (Lee *et al*., 2011). *Proteobacteria*, *Firmicutes,*

and *Chloroflexi* constituted between 52 and 73% of the bacterial community associated with *H. erectus* and *X. testudinaria*, whereas *Proteobacteria* and *Bacteroidetes* were the main phyla representing 60 and 33% of the sequences, respectively, associated with the sponge *S. carteri.* These results suggested that the composition of bacterial communities in the analysed sponges was species-specific (Lee *et al*., 2011). In another study, next generation sequencing was also used to assess the bacterial community associated with 32 distinct marine sponges collected worldwide (Schmitt *et al*., 2012). Overall 2.567 distinct 97% OTUs were detected and up to 364 different 97% OTUs from a single sponge species were observed. Sixteen bacterial phyla, namely *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chlamydia*, *Chloroflexi*, *Cyanobacteria*, *Deinococcus-Thermus*, *Firmicutes*, *Fusobacteria*, *Gemmatimonadetes*, *Lentisphaerae*, *Planctomycetes*, *Nitrospira*, *Proteobacteria* (*Alpha*, *Beta*, *Delta*, *Gamma*, and *Epsilon* classes), *Spirochaetes*, and *Verrucomicrobia*, nine candidate phyla (CAB-I, OD1, OP3, OP10, OS-K, SBR1093, TM6, TM7, and *Poribacteria*) and one unclassified lineage (SAUL - sponge-associated unclassified lineage) were identified (Schmitt *et al*., 2012). However, the core bacterial community– that is, the number of 97% OTUs commonly recovered from almost all 32 sponge species - was very reduced (~ 0.001%) (Schmitt *et al*., 2012). Taken together, the studies by Lee *et al*. (2011) and Schmitt *et al*. (2012) using deep sequencing technologies not only provided novel insights into the extent of bacterial diversity in marine sponges but their interpretation also favoured the hypothesis of species-specific microbial community composition in these animals, contrasting earlier suggestions of microbiome uniformity across different sponge hosts and geographical locations (Hentschel *et al*., 2002). Nevertheless, it is not possible to rule out that the observations made by Lee *et al.* (2011) and Schmitt *et al.* (2012) have been biased by 1) experimental design, whereby distantly-related sponge hosts sampled in varied geographic locations were surveyed, and 2) the incorporation of typical next-generation sequencing artefacts into the final analysis dataset as a result of the use of less-stringent noise filtering methods in comparison with those available today. Both of these factors could have simultaneously contributed to the measurement of large discrepancies between the communities. Thus, the debate concerning species specific versus uniform microbial communities in marine sponges still persists and has not just been restricted the measurement of diversity, but also extended to their functional attributes as well (Thomas *et al*., 2010a; Fan *et al*., 2012). In this Thesis, a dedicated experimental design is used to circumvent the

limitations of previous analyses and specifically answer the question of microbiome specificity versus uniformity in ecologically and biotechnologically relevant marine sponges. The results reveal microbiome diversity and composition in phylogenetically close hosts belonging to the family Irciniidae across spatial (Chapters 2-4) and temporal (Chapter 5) scales, using PCR-DGGE fingerprinting, high-throughput sequencing technology and state-of-the-art data processing pipelines and analyses that prevent the interference of methodological artefacts in ecological observations.

Biotechnological potential of sponge bacteria

The importance of bioactive secondary metabolites recovered from marine environments is most clearly demonstrated by the large number of articles and reviews appearing on the subject during the late 90s and early 2000s (e.g. Faulkner, 1997, 1999, 2002; Blunt *et al*., 2004, 2006; Blunt *et al*., 2008, 2009; Blunt *et al*., 2011; Blunt *et al*., 2013). Although several marine microorganisms and invertebrates have been shown to produce or possess numerous bioactive compounds, to date sponges are recorded as being the most prolific producers of such substances. The secondary metabolites recovered from marine sponges encompass distinct classes, such as terpenoids, polyketides, alkaloids, and nucleosides, and these compounds exhibit a wide range of activity from antimicrobial to anticancer (e.g. Faulkner, 1997, 1999, 2002; Blunt *et al*., 2003, 2004; Blunt *et al*., 2008, 2009; Blunt *et al*., 2011; Blunt *et al*., 2013). The beneficial properties towards human health have been demonstrated in several studies. For instance, from marine sponges collected in Florida (USA) unusual arabino and ribo-pentosyl nucleosides were obtained. The identification of these compounds led to the development of the chemical derivatives ara-A (vibarabine) and ara-C (cytarabine) that have significant anticancer properties that have been in clinical application for decades (Molinski *et al*., 2009). Furthermore, several antitumor compounds recovered from marine sponges have been in preclinical or phase I and II clinical trials (Newman and Cragg, 2004; Molinski *et al*., 2009). Norhalichondrin A, B, and C, homohalichondrin A, B, and C, and halichondrin B and C were isolated from extracts of *Halichondria okadai* collected in the Miura Peninsula, south of Tokyo, from which halichondrin B showed surprising *in vivo* antitumor activity (Uemura *et al*., 1985; Hirata and Uemura, 1986). Based on the natural product halichondrin B a synthetic analogic eribulin mesylate (E7389) was developed and is

in phase III clinical trials for the treatment of cancer (Alday and Correia, 2009; Smith *et al.*, 2010; McBride and Butler, 2012; http://www.clinicaltrials.gov/ct2/results?term=Eribulin+ mesylate).

The most important question with respect to sponge secondary metabolites is whether are produced by the sponge itself or by their bacterial symbionts. Recently, several studies have proposed that at least some bioactive compounds detected in marine sponges, for instance complex polyketides and nonribosomal peptides, are most likely produced by the symbiont bacteria. This conclusion relies mainly on the high similarity of sponge metabolite and metabolites known to be produced by bacteria, or on the metabolites being of distinct classes that are usually encountered in bacteria (Faulkner *et al.*, 2004; Piel, 2004; Piel *et al.*, 2004b; Kim and Fuerst, 2006; Thomas *et al.*, 2010b; Leal *et al.*, 2012). In a novel study performed by Bewley *et al.* (1996), the localization of natural products within the microorganisms associated with *Theonella swinhoei* was investigated. Differential centrifugation was applied to separate the cell populations of the sponge samples and the fractions were chemically analyzed. With this approach, it was demonstrated that the cytotoxic macrolide compound swinholide A and the peptide P951 were obtained from the extracts of heterotrophic unicellular bacteria and filamentous heterotrophic bacteria, respectively, and therefore implying a prokaryotic origin to their biosynthesis (Bewley *et al.*, 1996). Moreover, it was shown that these metabolites were neither found in the associated *Cyanobacteria* (*Aphanocapsa feldmanni*) as previously suggested nor in the sponge cells (Bewley *et al.*, 1996). A complex and diverse bacterial community is known to be associated with *T. swinhoei* (Hentschel *et al.*, 2002), and the polyketides onnamide A and theopederin A were obtained from this species (Piel *et al.*, 2004b). The onnamide A and theopederin A biosynthesis genes were shown to have a bacterial architecture based on the following features: gene clustering, lack of promoters, polyadenylation sites, and introns, the presence of putative Shine-Dalgarno sequences, and a bacterial transposase A (Piel *et al.*, 2004b). Altogether, 26 demosponge families belonging to 11 orders have been reported to synthesize bioactive secondary metabolites in association with their symbionts (Thomas *et al.*, 2010b). *Actinobacteria*, *Cyanobacteria*, *Firmicutes,* and *Proteobacteria* (*Alpha* and *Gamma* classes) from different marine sponge species emerge as the main bacterial phyla responsible for the biosynthesis of secondary metabolites displaying several properties from antibacterial to antifungal, antitumor, and, anti-HIV, among others (Thomas *et al.*, 2010b).

3. Archaea associated with marine sponges

The number of marine archaeal cells has been estimated to be as high as 1.3 x 10^{28} (Karner *et al*., 2001) and have, unsurprisingly, been found in marine sponges. The first insights into the diversity of *Archaea* associated with marine sponges were obtained by a molecular approach (PCR amplification with an *Archaea*-specific primer pair) whereby an archaeon was found in *Axinella mexicana* (formally accepted taxon: *Dragmacidon mexicanum*), but absent from aquarium surrounding seawater (Preston *et al*., 1996). The authors proposed the name *Cenarchaeum symbiosum* for this sponge-associated archaeon. Analysis performed with captive *A. mexicana* revealed that this symbiosis was stable over time. In addition, it was shown that between 47 and 63% of the prokaryotic rRNA gene fragment extracted from aquaria-maintained *A. mexicana* was from the archaeal community (Preston *et al*., 1996). Metagenomic library construction subsequently showed the coexistence of at least two closely related lineages of *C. symbiosum* in *A. mexicana* (Schleper *et al*., 1998). A filamentous archaeon was also observed within the collagen that surrounds the siliceous spicules of the Mediterranean sponges *Axinella* sp., *A. verrucosa* and *A. domicornis*. This archaeon was classified as marine group I *Crenarchaeota*, being closely related to *C. symbiosum*. This symbiosis was shown to be species-specific and stable over time and space (Margot *et al*., 2002). Additionally, the archaeal community associated with the sponge *Rhopaloeides odorabile* was composed of one dominant OTU assigned to marine group I *Crenarchaeota* and another less abundant OTU affiliated to group II *Euryarchaeota* (Webster *et al*., 2001a). The authors also demonstrated that the archaeal cells were found in all regions of the sponge body with significantly higher number in the pinacoderm compared to the mesohyl area (Webster *et al*., 2001a). Four distinct archaeal 16S rRNA gene sequences were obtained from three Antarctic marine sponges, which grouped in the marine group I *Crenarchaeota*, whereas no archaeal sequence was found in the surrounding seawater (Webster *et al*., 2004). To date, marine group I *Crenarchaeota* and marine group II *Euryarchaeota* have been recovered from several sponge species collected worldwide (Holmes and Blanch, 2007; Taylor *et al*., 2007; Bayer *et al*., 2008; Cheng *et al*., 2008; Meyer and Kuever, 2008; Turque *et al*., 2010; Han *et al*., 2012; Ribes *et al*., 2012; Yang and Li, 2012). A novel archaeal phylum named *Thaumarchaeota* was proposed, which encompassed sequences previously classified as marine group I *Crenarchaeota* or mesophilic *Crenarchaeota*, including *C. symbiosum* and its relatives and *Candidatus* Nitrosopumilus maritimus (Brochier-Armanet *et al*., 2008; Spang *et*

al., 2010; Pester *et al*., 2011). So far, 103 *Thaumarchaeota* and 10 *Euryarchaeota* distinct 16S rRNA gene sequences have been retrieved from marine sponges, from which 41% of the *Thaumarchaeota* sequences were distributed in five sponge-specific clusters (Taylor *et al*., 2007; Simister *et al*., 2012c; Webster and Taylor, 2012). More recently, with the use of high-throughput sequencing technology (i.e. 454-pyrosequencing) eight archaeal orders belonging to four classes (*Thermoplasmata*, *Methanomicrobia*, *Halobacteria,* and *Archaeoglobi*) of *Euryarchaeota* and four orders in the class *Thermoprotei* of *Crenarchaeota* were recovered with distinct relative abundances from the Red Sea marine sponges *Hyrtios erectus*, *Stylissa carteri,* and *Xestospongia testudinaria* (Lee *et al*., 2011).

In general, archaeal communities in marine sponges have so far been found to be less diverse than bacterial communities. However, archaeal symbionts of marine sponges remain poorly studied in comparison with their bacterial counterparts, and further research is still needed for a more comprehensive perspective of the range of occurrence, degree of conservation and functioning of archaea in these hosts. Regarding the latter aspect, several members of the *Thaumarchaeota* were shown to oxidize ammonia to nitrite, the first step in the nitrification process within nitrogen cycling (Spang *et al*., 2010; Pester *et al*., 2011). Moreover, recent evidence from the genomic analysis of *C. symbiosum* demonstrated the potential role of this archaeon in the nitrification process. In this Thesis, the diversity and temporal stability of archaeal communities in the highly microbe selective marine sponge *Sarcotragus spinosulus* is determined using an in-depth approach which combines archaeal-specific PCR-DGGE and 454-pyrosequencing profiling methodologies (Chapter 5). The potential participation of *S. spinosulus* archaeal symbionts in the nitrification process (see below) within the host is also addressed.

4. Nitrification

Nitrification is an essential process in the marine nitrogen cycle and encompasses the oxidation of ammonia (NH_3) into nitrite (NO_2^-) and further into nitrate (NO_3^-). Ammonia oxidation is the first step in the nitrification and is performed by ammonia oxidizing *Bacteria* (AOB) belonging to the *Beta-* and *Gammaproteobacteria* classes and ammonia oxidizing *Archaea* (AOA) belonging to the group I *Crenarchaeota* within the *Thaumarchaeota* phylum (Rotthauwe *et al*., 1997; Venter *et al*., 2004; Schleper *et al*., 2005; Brochier-Armanet *et al*.,

2008; Spang *et al*., 2010; Pester *et al*., 2011). Ammonia oxidation initiates with the oxidation of ammonia to hydroxylamine, which is catalysed by the enzyme ammonia monooxygenase (AMO). The *amoA* gene encodes for the catalytic α-subunit of the AMO enzyme and has been extensively applied as a genetic marker to detect AOB and AOA (Rotthauwe *et al*., 1997; Francis *et al*., 2005). The genome of the sponge symbiont *C. symbiosum* was shown to contain gene homologues possibly associated with chemolithotrophic ammonia oxidation, including ammonia monooxygenase encoding genes (Hallam *et al*., 2006).

AOA *amoA* gene sequences obtained from *Aplysina aerophoba* and surrounding seawater collected in two distinct sampling sites in the Mediterranean Sea revealed that the sponge-derived sequences were closely affiliated with *C. symbiosum* and *Candidatus* Nitrosopumilus maritimus, whereas no seawater sequence showed resemblance with archaeal *amoA* genes (Bayer *et al*., 2008). *Candidatus* N. maritimus was isolated from a tropical marine tank and shown to grow chemolithoautotrophically by aerobically oxidizing ammonia to nitrite (Könneke *et al*., 2005), which might indicate that the archaeon associated with *A. aerophoba* could also oxidize ammonia in the sponge host (Bayer *et al*., 2008). Bacterial *amoA* gene sequences were as well retrieved from *A. aerophoba* but also from seawater. All sponge- and seawater-derived sequences fell into a distinct and robust *Nitrosospira* cluster along with sequences obtained from *Theonella swinhoei* (Palau) and *Xestospongia muta* (Bahamas). Probes designed for the detection of *Nitrosospira* cluster I and marine crenarchaeal group I confirmed the presence of these microorganisms in *A. aerophoba*, but at rather low abundance. These results suggest that AOA formed a consistent association with *A. aerophoba*, whereas AOB were most likely filtered from seawater and did not represent a true symbiont (Bayer *et al*., 2008). Moreover, phylogenetic analysis performed with AOA *amoA* gene sequences recovered from the sponge *Geodia barretti* collected between 100-200 m, formed a monophyletic and specific cluster affiliated with *Thaumarchaeota (*Hoffmann *et al.,* 2009). The ammonia oxidation observed in *G. barretti* might be attributed to the activity of archaea, since a lower amount of bacterial ammonia oxidizers was detected (Hoffmann *et al*., 2009). It was demonstrated that all three AOA OTUs (cut off at 97% sequence similarity) retrieved from the sponge *Phakellia fusca* clustered with each other and were closely related to sponge-derived sequences collected worldwide (Han *et al*., 2012). Quantitative Real-Time PCR revealed the presence of 1.2 x 10^5 copies of archaeal 16S rRNA and 1.6 x 10^3 copies of *amoA* genes ng^{-1} of sponge metagenomic DNA, indicating that AOA accounted for 1.3% of

the total archaeal community in this sponge. The presence of AOA and the absence of AOB suggested that *Archaea* and not *Bacteria* were the main players in the ammonia oxidizing process in *P. fusca*, even though AOA corresponded only to a small part of the archaeal community associated with this sponge (Han *et al*., 2012). Similar results were further verified in the tropical *Halisarca caerulea* and in the cold-water *Higginsia thielei* and *Nodastrella nodastrella* (Hexactinellida) sponges: more archaeal *amoA* copies were observed than bacterial *amoA* copies, suggesting a greater role of archaea in the process of ammonia oxidation in marine sponges, in spite of much higher bacterial than archaeal abundances in all investigated sponge species (Cardoso *et al*., 2013).

Interestingly, phylogenetic analysis generated from *amoA* gene sequences of *Thaumarchaeota* ammonia oxidizers obtained from the HMA sponge *Agelas oroides* and surrounding seawater revealed that the majority of the sequences retrieved from the sponge formed a cluster almost exclusively dominated by sponge and coral sequences, whereas surrounding seawater sequences formed two distinct clusters (Ribes *et al*., 2012). The authors also speculated that although the bacterial community dominated *A. oroides* in terms of microbial abundance, the high nitrification rates observed in this species could be attributed to its small archaeal community (Ribes *et al*., 2012). Ammonia oxidizing *Archaea* affiliated with the phylum *Thaumarchaeota* were further recovered from distinct sponge species collected in the Mediterranean Sea (*Axinella polypoides* and *Chondrosia reniformis*), Caribbean Sea (*Aplysina insularis*, *Cliona* sp., and *Plakortis* sp. (Homoscleromorpha)), the Great Barrier Reef (*Coscinoderma* sp., *Luffariella variabilis*, and *Rhopaloeides odorabile*), and South-eastern Australia (*Callyspongia* sp., *Siphonochalina* sp., and *Stylinos* sp.- accepted name: *Hymeniacidon*) (Steger *et al*., 2008). Phylogenetic analysis revealed a large cluster formed by many sponge-derived AOA *amoA* gene sequences collected at the Great Barrier Reef, Mediterranean, and Caribbean Seas together with *C. symbiosum*, whereas no sequence from seawater and sediment was found in this cluster. It was also demonstrated that a single, identical *amoA* gene sequence was obtained from adult and larvae of *R. odorabile,* suggesting that vertical transmission of the corresponding symbiont occurs (Steger *et al*., 2008). In this Thesis the temporal dynamics of bacterial and archaeal *amoA* genes in the marine sponge *Sarcotragus spinosulus* is investigated.

5. Outline of the Thesis

Marine sponges can harbour a diverse and complex prokaryotic community, from both the bacterial and archaeal domains. Indeed, to date 28 bacterial phyla and two main lineages of *Archaea* (*Euryarchaeota* and *Thaumarchaeota*) have been associated with the from marine sponge microbiota (Taylor *et al*., 2007; Hentschel *et al*., 2012; Simister *et al*., 2012c; Webster and Taylor, 2012). Nevertheless, the question of whether microbial community composition in marine sponges is conserved, as suggested in early studies (Hentschel *et al*., 2002), or display a species-specific pattern, as indicated in recent surveys (Lee *et al*., 2011; Schmitt *et al*., 2012), remains controversial. This Thesis makes use of a specific experimental design to evaluate the hypothesis that a uniform community composition is found associated with marine sponges independent of geographical settings and sponge species. To this end, the abundance, composition, diversity, and host-specificity of microbial communities in the phylogenetically related sponge species *Sarcotragus spinosulus* and *Ircinia variabilis* (Demospongiae, Dictyoceratida, Irciniidae) was accurately determined across spatial and temporal scales. A suite of tools consisting mainly of culture-independent methods - i.e. epifluorescence analysis, polymerase chain reaction-denaturing gradient electrophoresis (PCR-DGGE), fluorescent *in situ* hybridization coupled with confocal laser scanning microscopy (FISH-CLSM), and 454-pyrosequencing - was applied to investigate the targeted symbiont communities. This Thesis also reveals the extent cultivability of the bacterial community present in the surveyed sponge hosts by comparing the diversity of bacteria readily grown on a commonly used growth medium with that determined by cultivation-independent, DNA-based procedures.

5.1 Objectives

General:

- To determine the spatiotemporal stability, degree of host-specificity, and extent of cultivability of microbial communities associated with marine sponges

Specific:

- To unveil the bacterial abundance and molecular diversity associated with two phylogenetically close marine sponges (*Ircinia variabilis* and *Sarcotragus spinosulus*) that co-exist in spatial proximity

- ❖ To disclose the effects of sample handling methods prior to DNA extraction and the extent of cultivation bias on the bacterial diversity and community composition observed in both target sponge species
- ❖ To determine the localization and distribution of bacterial cells in the sponge body
- ❖ To reveal the extent to which the shape of sponge-associated bacterial communities is driven by the host sponge and its biogeographical background
- ❖ To evaluate the temporal stability of bacterial and archaeal symbiont communities and their ammonia oxidation potential in *Sarcotragus spinosulus*
- ❖ To establish how selective the sponge-associate microbiome is by contrasting it with the microbiota of its immediate environmental surroundings (i.e. seawater and sediments)

5.2 The hosts

5.2.1 Irciniidae family

The family Irciniidae (Demospongiae, Dictyoceratida) was selected based on the following criteria:

- ❖ Several species have been registered in the Atlanto-Mediterranean region. For instance, they are known to occur along the coast of the Algarve, the southernmost Portuguese continental shore, as well as in the oceanic archipelagos of Madeira and Azores;
- ❖ The majority of the surveys have been performed with tropical irciniid sponges, whereas very few studies have been carried out with North Atlantic irciniids. The use of novel sponge targets would thus bring novelty to the field of sponge microbiology besides allowing comparisons between tropical and temperate marine sponge microbiomes;
- ❖ Some tropical *Ircinia* specimens had already been classified as high microbial abundance sponges, whereas for *Sarcotragus* species there was no data available. Looking after HMA sponges was a pre-requisite in this project due to the putative higher metabolic and genetic diversity of their symbionts, which bear potential benefits to host fitness and survival, and irciniids would fulfil this requirement on the basis of available data;

- ❖ A wide range of bioactive secondary metabolites has been reported for Irciniidae species (*Ircinia*, *Sarcotragus,* and *Psammocinia*). Chemical complexity is indicative of a likewise complex and diverse microbiome in marine sponges. In this context, irciniids were particularly attractive as besides serving as excellent models for microbial diversity and host-symbiont interaction studies, they bear relevance as likely reservoirs of microorganisms of potential use in biotechnology.

5.2.2 Sampling sites

Three sampling campaigns took place between 2010 and 2012:

- The first one occurred in June of 2010 at Galé Alta, Armação de Pêra, at the coast of the Algarve (Fig. 1.3), where several specimens of *S. spinosulus* and *I. variabilis* were collected along with surrounding seawater. Chapters 2 and 3 cover the results of this sampling campaign and tackle bacterial community specificity in marine sponges sharing the same habitat as well as effects of sample handling methods on sponge microbiome diversity and composition.
- The next campaign took place between August and September of 2011 at three sampling sites to know (i) the island of São Miguel in the Azores archipelago, situated around 950 miles from the Portuguese mainland, (ii) the Madeira Island located approximately 360 miles off the southwestern coast of Portugal, and (iii) at Galé Alta, Armação de Pêra, Algarve (Fig. 1.3). This time, several *S. spinosulus* and *I. variabilis* specimens were sampled along with surrounding seawater and sediment. Results related with this sampling campaign are reported in Chapter 4 and shed light on the maintenance of microbial symbionts across large spatial scales and the influence of the environmental microbiota on symbiont community composition.
- The last sampling campaign took place in October 2012 and was carried out as described for the first one, where few specimens of *S. spinosulus* were collected. This sampling enabled the confection of Chapter 5, where the stability of microbial communities in *S. spinosulus* along three consecutive years is addressed.

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Figure 1.3. Geographical distribution of *Sarcotragus spinosulus* and *Ircinia variabilis.* Sampling sites: Az, M, and A: Azores archipelago, Madeira Island, and Algarve, respectively.

5.3 Outline of this thesis

As already mentioned, complex prokaryotic communities are known to be associated with marine sponges and some of them might be responsible for secondary metabolites frequently detected in the hosts. An increasing number of publications related with the abovementioned topics has been seen in the last couple of years, however several questions remain unanswered: i) Is there any resemblance between the bacterial diversity and community structure associated with phylogenetically and spatially close sponge species? ii) Do distinct sample processing methods prior to DNA extraction affect observations on sponge microbiome diversity and composition? iii) Is the bacterial community structure alike over distinct biogeographic settings? iv) How the bacterial community observed in surrounding seawater and sediment influences the one recovered from marine sponges? v) Is there any shift in the bacterial and archaeal community structure along successive years? To address these questions, a stringent experimental design was created and a range of molecular tools

was used - with special attention to the application of high-throughput sequencing technology - to the characterization of the marine sponge microbiome. Thus, the approach presented in this Thesis "as-a-whole" allowed a more in-depth and extensive analysis of the prokaryotic diversity encrypted in the marine sponge holobiont, and contributes to the emergence of the Irciniidae family as a suitable and desired model taxon in sponge microbiology studies. The temperate marine sponges *Sarcotragus spinosulus* and *Ircinia variabilis* (Demonspongiae, Irciniidae) were selected as model hosts as they are sympatric across several habitats spanning wide spatial distribution in the North Atlantic, and are a promising source of biologically active compounds (see topic 5.2.1 above for a more comprehensive list of criteria justifying their choice).

Chapter 1 provides basic information about sponges (Porifera) as well as an overview of the bacterial and archaeal communities associated with distinct sponge species collected worldwide and the main players involved in the marine nitrification process. In addition, it exemplifies the techniques that have been applied to investigate the marine sponge microbiome, many of which used in the Thesis.

Chapter 2 establishes the first assessment of bacterial abundance, diversity and community composition associated with the phylogenetically and spatially close related marine sponges *S. spinosulus* and *I. variabilis*. To this end, four specimens of each sponge species were collected. An internal piece of each sponge body was excised and ground generating a homogenate that was further used for plate counting and epifluorescence microscopy analyses. PCR-DGGE analysis was carried out, from which the dominant bands were selected and sequenced. No significant difference was found in the counts of heterotrophic bacteria associated with both sponge species. Epifluorescence microscopy analysis enabled us to classify *S. spinosulus* and *I. variabilis* as HMA sponges. PCR-DGGE profiles of *S. spinosulus* and *I. variabilis* were very different. The PCR-DGGE bands revealed divergent bacterial communities associated with both sponge species. Overall, the results obtained in this chapter confirmed the hypothesized host-specific composition of bacterial communities between *S. spinosulus* and *I. variabilis.*

Chapter 3 determines the effects of three pre-processing methods of sample handling prior to DNA extraction in the study of bacterial community composition associated with *S. spinosulus* and *I. variabilis*. The study compared bacterial diversity and composition in marine sponges as determined by two cultivation-independent methods in comparison with one cultivation-dependent method. This was achieved via PCR-DGGE and 454-pyrosequencing profiling of bacterial 16S rRNA genes amplified from total community DNA samples. In addition, the localization, abundance, and distribution of bacterial cells in the sponge body were investigated by fluorescence *in situ* hybridization coupled with confocal scanning laser microscopy (FISH-CLSM). The cultivation-independent methods demonstrated a species-specific community structure in *S. spinosulus* and *I. variabilis*, whereas the cultivation-dependent method showed highly similar bacterial assemblages in both sponge species. Cultivation-independent methods led to the detection of 15-18 bacterial phyla in both sponge species, with *Actinobacteria*, *Acidobacteria*, and *Proteobacteria* as the most prevalent, and *c*. 200 and 220 OTUs in *S. spinosulus* and *I. variabilis*, respectively. Conversely, the cultured bacterial fraction was dominated by the phylum *Proteobacteria* and only 33 and 39 OTUs were registered in *S. spinosulus* and *I. variabilis*, respectively. Unexpectedly, about half of the OTUs retrieved by cultivation was exclusive to this procedure. FISH-CLSM revealed that bacterial cells were almost exclusively observed in-between sponge cells. This chapter strengthened the view of species-specific sponge bacterial communities suggested in chapter 2. It further demonstrated that interpretation of sponge microbiome composition and diversity data might be influenced by the choice of methodology in a case-by-case manner (i.e. depending on the sponge species under study), and revealed a sharp bias induced by cultivation in the analysis of bacterial community composition and diversity in both sponge hosts.

Chapter 4 inspects the extent to which the shape of bacterial communities associated with *Sarcotragus* spp., *Ircinia* spp. (Irciniidae) and *Spongia* spp. (Spongiidae) is driven by the host organism and/or by its biogeographical background, addressing concomitantly the contribution of the surrounding environment (i.e. seawater and sediments) as “seedbanks” of eventual bacterial symbionts which could then experience an enrichment in abundance when associated with the sponge host. To this end, three specimens of each sponge species were collected at the Algarve coast, the Madeira Island and Azores archipelago. PCR-DGGE and

454-pyrosequencing 16S rRNA genes analyses were applied to unveil bacterial community composition in the targeted species and in the environment. *Ircinia* spp. showed a more variable bacterial community compared to *Sarcotragus* spp. and *Spongia* spp.. Surprisingly, bacterial communities from *Sarcotragus* spp. and *Spongia* spp. were more similar to one another than *Ircinia* spp. and *Sarcotragus* spp. communities. Thus, although species-specific communities were again registered within each of the studied sites, these results contradict the hypothesis of higher levels of microbiome resemblance between phylogenetically closer sponge hosts. The predominant taxonomic groups in *Sarcotragus* and *Spongia* were *Acidobacteria* and *Actinobacteria* (*c.* 20% relative abundance), followed by *Proteobacteria*, *Poribacteria*, PAUC34f, *Bacteroidetes,* and *Chloroflexi* (*c.* 10% relative abundance each group). The aforementioned abundances remained similar in these sponges regardless of the sampling locality. Conversely, phylum-level abundances shifted across localities in *Ircinia* spp.. In these hosts, the *Chloroflexi* displayed dominance at the Madeira site (31%) but was found to be a minority taxon at the Algarve (7%) and Azores sites (3%), where *Proteobacteria*, *Acidobacteria,* and *Actinobacteria* prevailed. *Ircinia* spp., the most variable host regarding bacterial community composition, maintained a common pool of symbionts, with 93 out of 403 detected bacterial OTUs (*c.* 23%) shared between specimens from all examined sample sites. The number of shared symbionts in these hosts rose to 35 – 38% when only specimens from two sample localities were compared. Overall, our results suggest that an intricate and complex interaction of host and environmental forces cooperatively shape symbiont community composition in marine sponges at the approximate species (i.e. OTU) level. Further, compelling evidence for the recognition of marine sediments as the pivotal environmental source of highly specialized and abundant sponge-associated *Acidobacteria* was found.

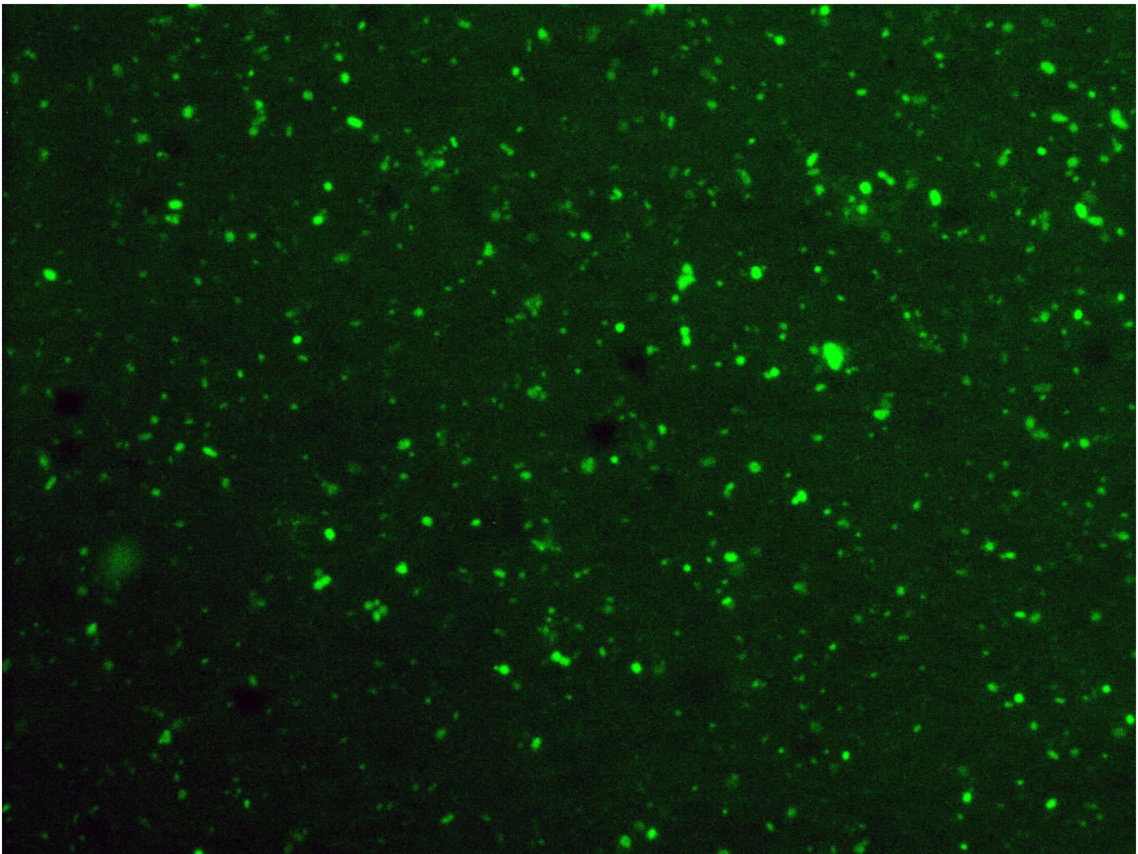
Chapter 5 tackles the stability of the bacterial and archaeal communities associated with *S. spinosulus* as well as the prokaryotic ammonia-oxidizers (*amoA* gene) in a temporal manner. Specimens of *S. spinosulus* were collected in three successive years and subjected to PCR-DGGE and 454-pyrosequencing of 16S rRNA genes. *S. spinosulus* was found to be dominated by nine bacterial phyla, from which only two showed significant differences in relative abundances over the years. The "pan microbiome" of *S. spinosulus*, that is, the sum of all bacterial phylotypes found in this host species, was composed by 205 OTUs, whereas only

27 OTUs were detected in all 12 sponge replicates. Except for one host specimen, the archaeal community was dominated by a single symbiont. PCR-DGGE profiles of bacterial ammonia oxidizers revealed that few phylotypes were observed over the years. The results from this chapter reveal that the prokaryotic communities associated with *S. spinosulus* display a dynamic stability.

Chapter 6 presents a review covering the literature on microbial communities associated with Irciniidae species and addressing the biotechnological potential of the irciniid sponge holobiont.

Chapter 7 summarizes the main results obtained in the distinct surveys of this Thesis.

CHAPTER II



Phylogenetically and spatially close marine sponges harbour divergent bacterial communities

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Abstract

Recent studies have unravelled the diversity of sponge-associated bacteria that may play essential roles in sponge health and metabolism. Nevertheless, our understanding of this microbiota remains limited to a few host species found in restricted geographical localities, and the extent to which the sponge host determines the composition of its own microbiome remains a matter of debate. We address bacterial abundance and diversity of two temperate marine sponges belonging to the Irciniidae family - *Sarcotragus spinosulus* and *Ircinia variabilis* – in the Northeast Atlantic. Epifluorescence microscopy revealed that *S. spinosulus* hosted significantly more prokaryotic cells than *I. variabilis* and that prokaryotic abundance in both species was about four orders of magnitude higher than in seawater. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) profiles of *S. spinosulus* and *I. variabilis* differed markedly from each other – with higher number of ribotypes observed in *S. spinosulus* – and from those of seawater. Four PCR-DGGE bands, two specific to *S. spinosulus*, one specific to *I. variabilis*, and one present in both sponge species, affiliated with an uncultured sponge-specific phylogenetic cluster in the order *Acidimicrobiales* (*Actinobacteria*). Two PCR-DGGE bands present exclusively in *S. spinosulus* fingerprints affiliated with one sponge-specific phylogenetic cluster in the phylum *Chloroflexi* and with sponge-derived sequences in the order *Chromatiales* (*Gammaproteobacteria*), respectively. One *Alphaproteobacteria* band specific to *S. spinosulus* was placed in an uncultured sponge-specific phylogenetic cluster with a close relationship to the genus *Rhodovulum*. Our results confirm the hypothesized host-specific composition of bacterial communities between phylogenetically and spatially close sponge species in the Irciniidae family, with *S. spinosulus* displaying higher bacterial community diversity and distinctiveness than *I. variabilis*. These findings suggest a pivotal host-driven effect on the shape of the marine sponge microbiome, bearing implications to our current understanding of the distribution of microbial genetic resources in the marine realm.

Introduction

Marine sponges have been the focus of increasing microbiology research interest mainly because of their symbiotic association with abundant and diverse bacteria and production of biologically active secondary metabolites (Taylor *et al*., 2007; Hentschel *et al*., 2012). For so-called High Microbial Abundance (HMA) sponges, it has been shown that up to 38% of sponge wet weight is composed of bacterial cells (Vacelet and Donadey, 1977), and that such bacterial abundance surpasses that of seawater by two to four orders of magnitude (Friedrich *et al*., 1999; Hentschel *et al*., 2002; Taylor *et al*., 2007; Hardoim *et al*., 2009). It has been suggested that HMA sponges harbour several bacteria involved in the production of secondary metabolites, which might, for example, improve protection against predation of the sponge host (Taylor *et al*., 2007). The synthesis of bioactive compounds derived from sponge-microbe associations has already been reported for 26 of the 92 families in the Demospongiae (Thomas *et al*., 2010b), the most diversified class of the phylum Porifera. Currently, the use of high-throughput sequencing technology is extending our knowledge of microbial diversity in marine sponges, with more than 25 bacterial phyla detected in sponges by this means (Webster *et al*., 2010; Lee *et al*., 2011). Taken together, these features foreshadow marine sponge holobiomes as valuable reservoirs of microbial genetic and metabolic diversity of potential use in biotechnology.

Despite such remarkable advances, current understanding of symbiont community structure in marine sponges remains restricted to certain regions and host species (Taylor *et al*., 2007). This holds true for species within the family Irciniidae (Demospongiae, Dictyoceratida), from which the majority of surveys undertaken so far have been limited to tropical latitudes and to the species *Ircinia felix*, *I. strobilina*, and *I. ramosa* (Usher *et al*., 2006; Schmitt *et al*., 2007; Weisz *et al*., 2007; Mohamed *et al*., 2008a; Mohamed *et al*., 2008b; Mohamed *et al*., 2008c; Mohamed *et al*., 2010; Webster *et al*., 2010; Yang *et al*., 2011). Electron microscopy analyses unveiled abundant and diverse bacterial morphotypes in *I. felix* (Usher *et al*., 2006; Schmitt *et al*., 2007; Weisz *et al*., 2007), whereas five (Mohamed *et al*., 2008b) and seven (Yang *et al*., 2011) bacterial phyla were revealed in association with *I. strobilina* by cloning-and-sequencing of 16S rRNA gene fragments. By means of high-throughput sequencing, 16 phyla and 1.199 bacterial operational taxonomic units (OTUs) at 95% sequence similarity were found in association with *I. ramosa* (Webster *et al*., 2010), highlighting the complexity of the *Ircinia*-associated "bacteriome". The detection of

Acidobacteria, *Alpha*-, and *Gammaproteobacteria* in adult, larva, and juvenile samples of *I. felix* (Schmitt *et al*., 2007) supports the hypothesis that a portion of this microbiota might be vertically transmitted throughout successive host generations.

Conversely, the microbial ecology of temperate irciniids remains underexplored. Only recently a study first approached the diversity of bacterial communities in Mediterranean *Ircinia* spp. - namely *I. variabilis*, *I. fasciculata*, and *I. oros* - revealing eight bacterial phyla across these hosts and species-specific OTUs (Erwin *et al*., 2012c). Because of their global distribution, encompassing both tropical and temperate species, Irciniidae sponges constitute a valuable taxon for the study of the ecology and evolution of symbiotic relationships. In addition, a great variety of cytotoxic compounds has been retrieved from Irciniidae species, which indicates they are potentially of high biotechnological importance (Cichewicz *et al*., 2004a; Emura *et al*., 2006; Liu *et al*., 2006b; Xu *et al*., 2008; Wätjen *et al*., 2009; Orhan *et al*., 2010). Furthermore, two studies performed with the temperate *I. muscarum* and *I. variabilis* described the production of cyclic peptides by cultivated bacteria (De Rosa *et al*., 2003b; Mitova *et al*., 2003), whereas psymberin – which resembled the pederin family of polyketides – was recovered from *Psammocinia* sp. and shown to have a bacterial symbiont origin (Cichewicz *et al*., 2004a; Fisch *et al*., 2009). In this context, addressing microbial diversity and distribution in widespread and chemically complex marine sponges is not only relevant to the study of symbiosis and co-evolutionary relationships, but also bears importance to our understanding of the extent of marine genetic and metabolic resources.

In light of the recent evidence for divergent bacterial communities across different sponge species or even specimens (Lee *et al*., 2011; Schmitt *et al*., 2012), a feature that has also been observed for other eukaryotes that support complex bacterial consortia (Fierer *et al*., 2010; Kvennefors *et al*., 2010; Arumugam *et al*., 2011), this study uses a stringent experimental design to test the hypothesis of host-specific assemblages of dominant symbionts in marine sponges. To this end, we address bacterial abundance and diversity in the temperate marine sponges *Sarcotragus spinosulus* Schmidt, 1862 and *Ircinia variabilis* Schmidt, 1862 (Demospongiae, Dictyoceratida, Irciniidae), two closely related species that co-exist in spatial proximity at the coast of the Algarve (southern Portugal), a region with a Mediterranean climate located in the Northeast Atlantic. We use the 16S rRNA gene as a phylogenetic marker in polymerase chain reaction – denaturing gradient gel electrophoresis (PCR-DGGE) analyses of the domain *Bacteria*, the phylum *Actinobacteria* and the class

Alphaproteobacteria in these hosts, thus allowing the inspection of bacterial community structures across different taxonomic ranks with concomitant focus on abundant and biotechnologically relevant sponge-associated microorganisms (Bull and Stach, 2007; Thomas *et al*., 2010b; Webster and Taylor, 2012). Phylogenetic analysis of dominant bacterial populations (i.e. PCR-DGGE bands) consistently and specifically found in association with these species is performed, and their status as "sponge-specific bacterial clusters" (Simister *et al*., 2012c) is verified. We also determine the degree of dissimilarity between sponge-associated bacterial communities and that of their neighbouring bacterioplankton. To assure accurate identification of the target sponges, we infer host phylogenies based on cytochrome oxidase gene sequence relationships. This is the first study of bacterial community structure and diversity in North Atlantic Irciniidae.

Results

Sponge identification.

Sponge specimens (Fig. S2.1) were identified as *Sarcotragus spinosulus* and *Ircinia variabilis* based on macro- and microscopic analyses using morphological criteria. Analysis of 636 bp-long sequences of the subunit I of the mitochondrial cytochrome C oxidase (CO1) gene obtained for all specimens (accession numbers HE797930 to HE797937) showed no intraspecific variation among our sequences of *I. variabilis* or *S. spinosulus*, whereas a 4.7% genetic distance (p-distance) was found between our sequences of these two species. Genetic distances between our *S. spinosulus* sequences and those available on NCBI GenBank ranged from 0 to 0.6%, whereas for *I. variabilis* a distance of 0.5% was observed between our sequences and those of *I. variabilis/fasciculata* collected in the Northwestern Mediterranean. Phylogenetic reconstructions based both on Maximum Likelihood and Bayesian inference confirmed the identification of our sponge specimens. Indeed, *I. variabilis* and *S. spinosulus* CO1 sequences sampled in this study formed well-supported clades with CO1 sequences from *Ircinia* spp. and *Sarcotragus* spp. retrieved elsewhere (Fig. 2.1).

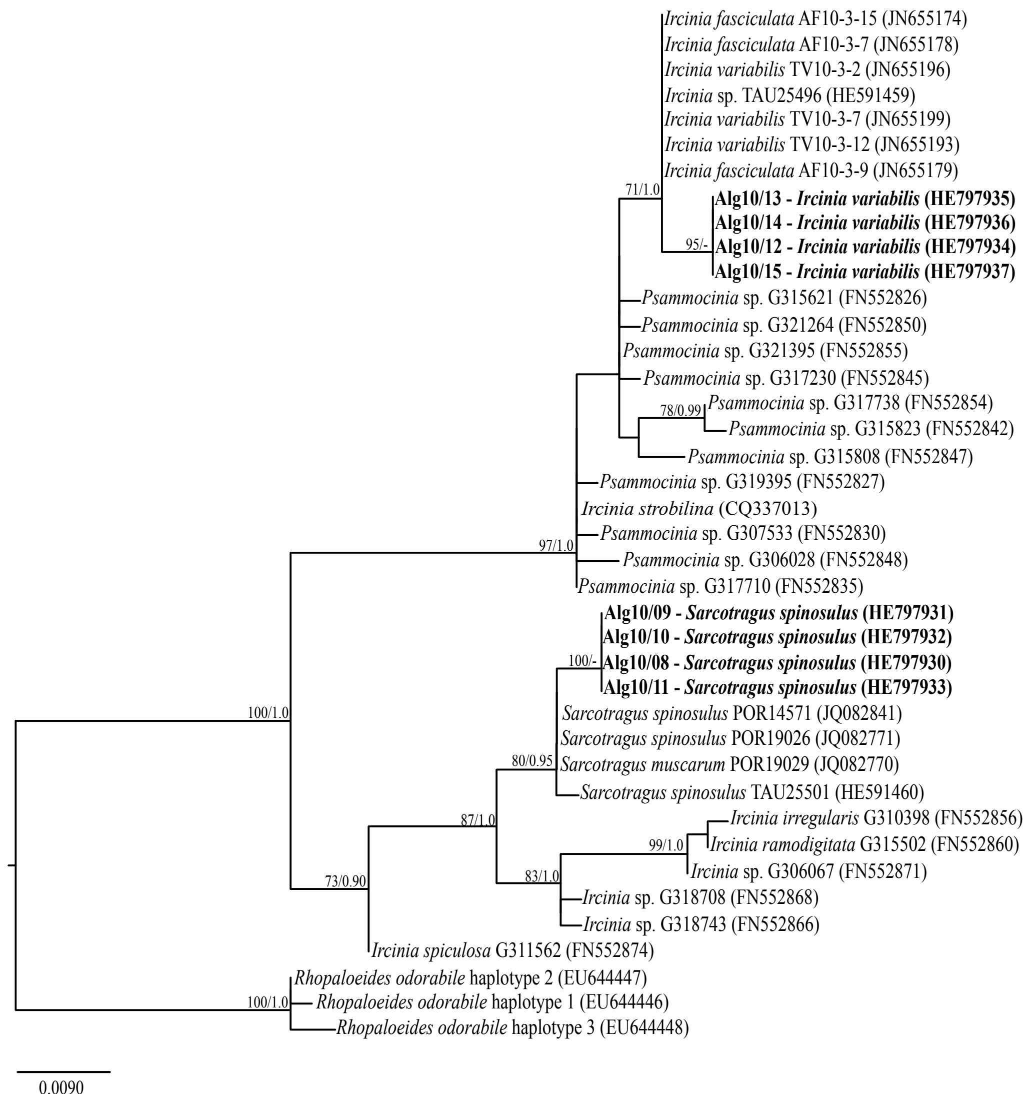


Figure 2.1 - Phylogenetic inference of the Irciniidae family based on the cytochrome oxidase gene, subunit 1. The Maximum Likelihood tree (-ln likelihood: 1383.921591) is shown, with sequences retrieved in this study highlighted in bold. Numbers at tree nodes are bootstrap values and posterior probabilities calculated in Maximum Likelihood and MCMC Bayesian analyses, respectively, and values above 70/0.95 are shown.

Counting of heterotrophic culturable bacteria.

The colony forming units (CFU) counts of heterotrophic bacteria on marine agar revealed no significant difference (p>0.05) between sponges species, with $3.21 \pm 2.03 \times 10^6$ CFU and 1.63 $\pm 0.61 \times 10^6$ CFU g^{-1} of fresh sponge for *S. spinosulus* and *I. variabilis,* respectively.

Epifluorescence microscopy.

Analyses showed that *S. spinosulus* harboured significantly (p<0.05) higher number of prokaryotic cells (average of 1.37 x 10^{10} cells g^{-1} of fresh sponge), as surveyed in this study, when compared to *I. variabilis* (average of 3.81 x 10^{9} cells g^{-1} of fresh sponge). The abundance of prokaryotic cells in surrounding seawater (average of 4.63 x 10^{5} cells mL^{-1}) was significantly (p<0.05) lower than in both sponge species (Fig. 2.2).

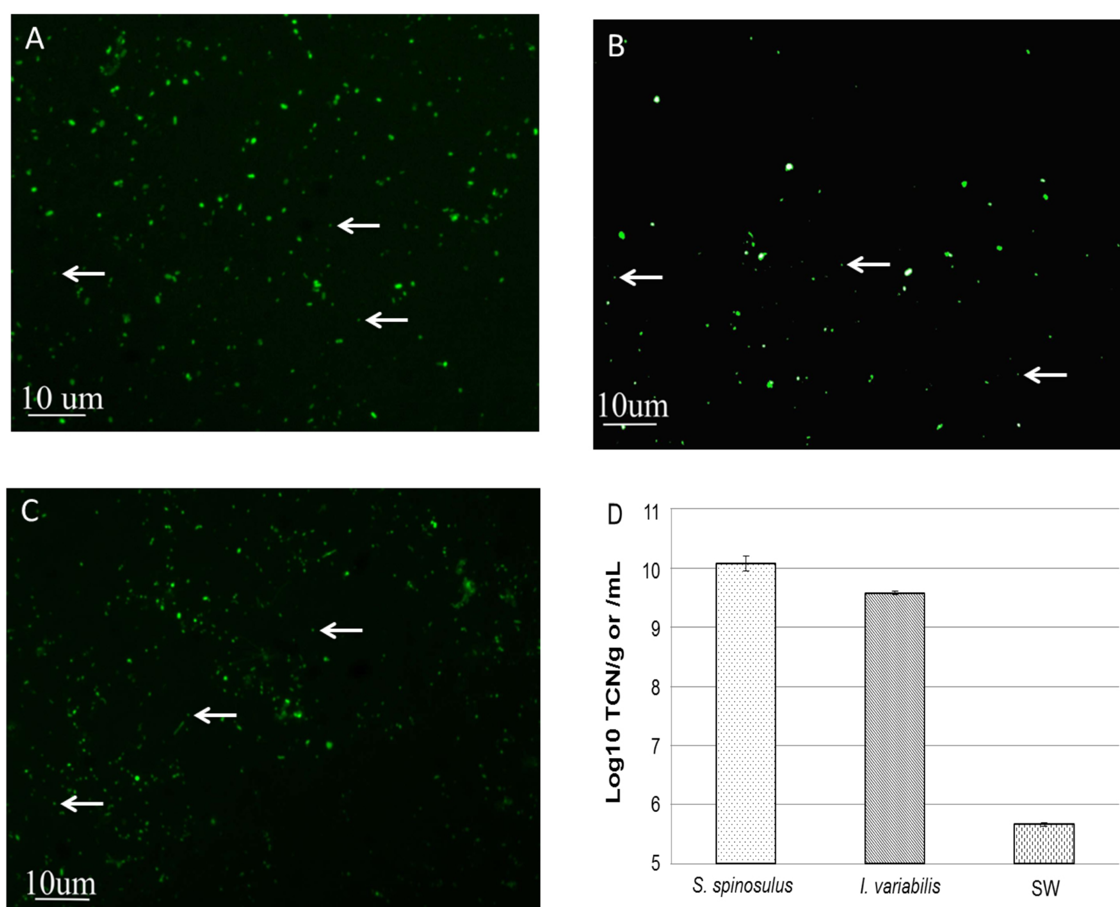


Figure 2.2 - Epifluorescence counts. Microscopy pictures taken from *S. spinosulus* (A), *I. variabilis* (B) and Seawater (C) are shown. Arrows exemplify counted bacterial cells. Values in panel (D) are expressed as means ± standard errors of log-transformed total cell numbers (TCN).

PCR-DGGE analysis of bacterial communities.

(i) *Bacteria PCR-DGGE profiles*. The bacterial PCR-DGGE profiles of *S. spinosulus* were characterized by five dominant bands and a large number of fainter bands (16 to 30) whereas those of *I. variabilis* comprised one dominant band in addition to five to 26 fainter bands (Fig.

2.3a, Table S2.1). Seawater DGGE profiles showed seven dominant bands and a large number of fainter bands (28 to 30). While the similarity within seawater and *S. spinosulus* replicates was high, profiles of *I. variabilis* specimens displayed large within-replicate heterogeneity (Fig. 2.3a). Clearly contrasting profiles were observed between seawater and sponge samples, and between both sponge species. Indeed, the UPGMA cluster analysis (Fig. 2.S2a) revealed two main groups, one formed exclusively by all sponge specimens and other containing only seawater samples. These two groups shared less than 10% similarity whereas *S. spinosulus* and *I. variabilis* PCR-DGGE profiles shared around 20% similarity. Ordination via canonical correspondence analysis (CCA) of the DGGE band data and environment variables revealed that sponge species and seawater significantly influenced band variation in the DGGE profiles ($p<0.05$, Fig. 2.3b). The horizontal axis of the diagram, which accounted for 54.8% of the dependent (i.e. DGGE bands) – independent (i.e. sample classes) variables correlations, mainly distinguished the sponges *S. spinosulus* and *I. variabilis* from seawater (Fig. 2.3b). The vertical axis grouped replicates from sponge *S. spinosulus* clearly apart from those observed in *I. variabilis*.

(ii) *Actinobacteria PCR-DGGE profiles*. The actinobacterial PCR-DGGE profiles of *S. spinosulus* consisted of few (two to four) strong bands along with more than four detectable bands, while those of *I. variabilis* comprised one to three dominant bands along with one to five fainter bands (Fig. 2.3c). Significant reduction in actinobacterial diversity and richness were determined for the latter species in comparison with the former (Table S2.1). A large heterogeneity was observed within *I. variabilis* profiles. In comparison with the sponge fingerprints, the seawater PCR-DGGE profiles displayed higher diversity of bands, especially against *I. variabilis* profiles (Table S2.1), and contained 3 strong bands along with more than 6 fainter bands, and much lower within-replicate variability (Fig 2.3c). Two main groups were revealed by cluster analysis, one formed exclusively by all sponge specimens and other containing only seawater samples (Fig. S2.2b). These two groups shared about 10% similarity. However, there was also a clear difference between the profiles of both sponge species, which shared *c.* 20% similarity according to cluster analysis. Ordination via CCA discriminated both sponge species and seawater across the horizontal axis of the diagram, which represented around 60% of the overall PCR-DGGE – sample correlations (Fig. 2.3d).

All independent variables (i.e. the sample classes seawater, *S. spinosulus* and *I. variabilis*) significantly (p<0.05) affected the PCR-DGGE banding patterns (Fig. 2.3d).

(iii) *Alphaproteobacteria PCR-DGGE profiles*. The *Alphaproteobacteria* profiles of *S. spinosulus* contained one to three dominant bands, in addition to more than seven detectable fainter bands, whereas the profiles of *I. variabilis* revealed one to three strong and fainter bands (Fig. 2.3e). Significantly greater richness, diversity, and evenness were found for *S. spinosulus* alphaproteobacterial PCR-DGGE profiles in comparison with those of *I. variabilis* (Table S2.1). The seawater profiles showed two strong bands along with more than seven fainter bands. The variability within sponge specimens and among sponge species was relatively high. Conversely, seawater samples displayed highly homogeneous profiles (Fig. 3e). Cluster analysis revealed a clear separation between seawater and sponge samples and high similarity scores for the former (Fig. S2.2c). The latter grouped into two further clusters in which the visible, higher degrees of within-sample variability could be numerically depicted (Fig. S2.2c). Sample outliers were detected, as one replicate from *I. variabilis* grouped with a cluster dominated by three *S. spinosulus* samples, and the same effect was observed for one replicate from *S. spinosulus,* which clustered with *I. variabilis* specimens (Fig. 2.3f, S2.1c). Nevertheless, CCA showed that all factors significantly (p<0.05) influenced the patterns of band distribution in alphaproteobacterial PCR-DGGE profiles. Ordination via CCA revealed that 63% of total PCR-DGGE band – independent variables correlations was explained by the horizontal axis of the diagram, which mainly differentiated *S. spinosulus* from seawater (Fig. 2.3f), whereas the residual variability in the vertical axis of the diagram (37%) discriminated most *I. variabilis* from seawater and *S. spinosulus* samples (Fig. 2.3f).

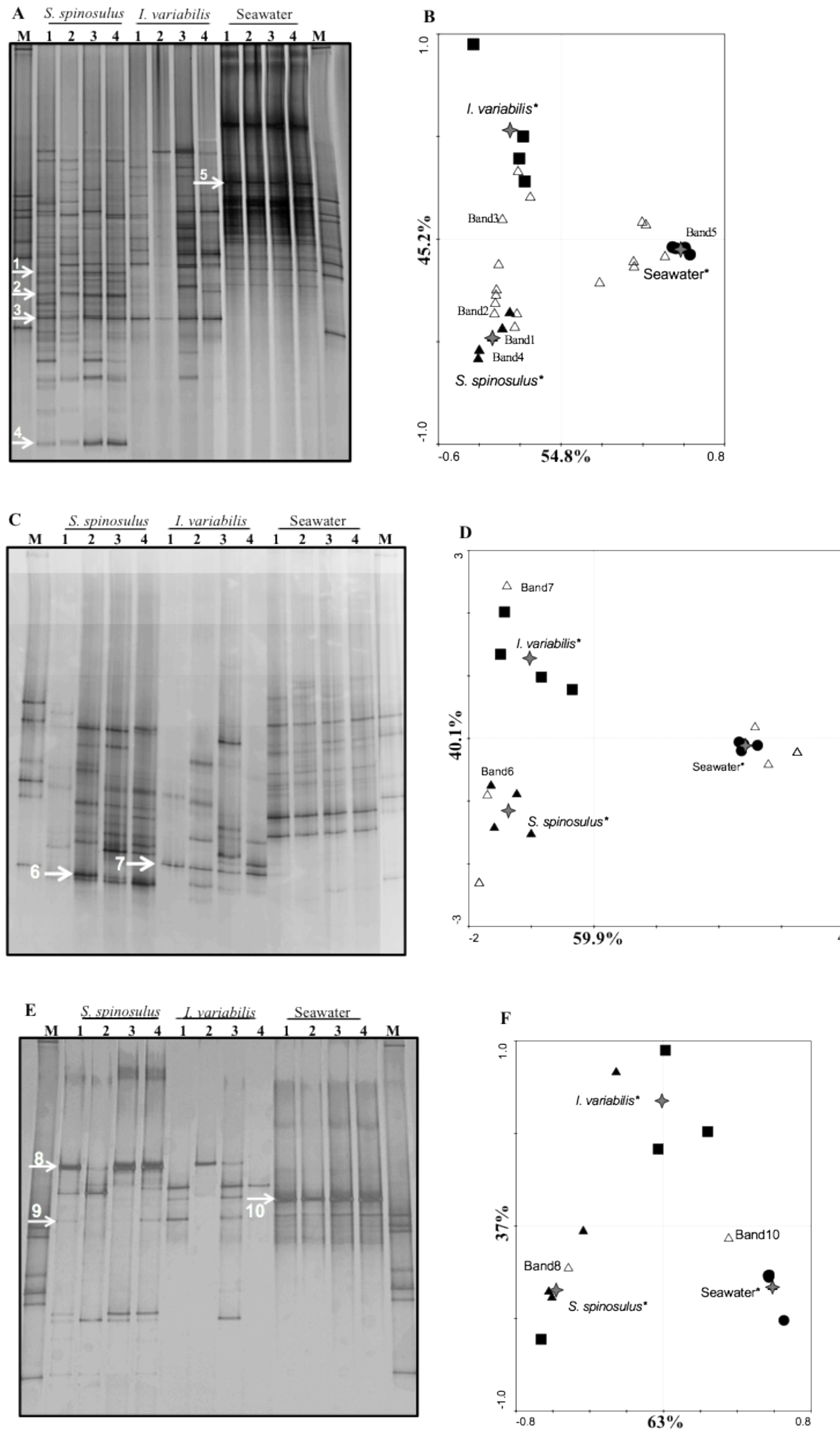


Figure 2.3 - PCR-DGGE fingerprints and canonical analyses. PCR-DGGE 16S rRNA gene fingerprints of *S. spinosulus*, *I. variabilis*, and seawater DNA samples generated with "total-community" bacterial primers (A) and specific primer systems for *Actinobacteria* (C) and *Alphaproteobacteria* (E). The arrows indicate bands that were excised from DG-gels and sequenced. Corresponding ordination biplots of PCR-DGGE fingerprints and qualitative environmental variables are shown in panels B, D, F. Symbols: ▲ *S. spinosulus*, ■ *I.*

variabilis, and ● Seawater. Labels displayed on the diagram axes refer to the percentage variations of PCR-DGGE ribotypes - environment correlation accounted for the respective axis. The "star" symbols represent the centroid positions of the environmental variables in the diagram. Variables that significantly ($p<0.05$) influence the bacterial community composition are indicated by an asterisk.

Analysis of sequences of dominant and discriminating PCR-DGGE bands

(i) *Bacteria PCR-DGGE bands*. Three dominant bands labelled 1, 2, and 4 (see arrows in Fig. 2.3a) were exclusively found in all replicates of *S. spinosulus*. Bands 1 and 4 were directly sequenced whereas band 2 was subjected to cloning and sequencing. From band 1, one sequence was retrieved and affiliated with the *Actinobacteria* order *Acidimicrobiales*. The phylogenetic analysis showed that this band affiliated with an uncultured and apparently diverse lineage containing sponge-derived bacterial sequences of worldwide origin (Fig. 2.4). Further, two clones were sequenced from band 2 and found to be highly alike, with five different nucleotides between them. They were assigned to the *Gammaproteobacteria* order *Chromatiales.* These sequences belonged to a well supported *Chromatiales* phylogenetic clade containing uncultured bacteria retrieved exclusively from marine sponges sampled in several geographical localities (Fig. 2.5). From band 4, one sequence was obtained and classified in the *Chloroflexi* phylum. Phylogenetic analysis revealed that this sequence belonged to a sponge-specific bacterial phylogenetic cluster as determined by (Simister *et al.*, 2012c) (Fig. 2.6). A dominant band labelled 3 (Fig. 2.3a) was found in all sponge specimens. Two identical sequences were recovered and assigned to the *Actinobacteria* order *Acidimicrobiales*. They also affiliated with an uncultured sponge-specific lineage previously suggested by (Simister *et al.*, 2012c) (Fig. 2.4). A dominant band labelled 5 (see arrow in Fig 2.3a) was exclusively found in all seawater samples. This band possessed high discriminating power, as obviated by its centroid position in the CCA diagram. Two identical sequences were obtained for this band and assigned to the *Alphaproteobacteria* family *Rhodobacteraceae* (Table 2.1). They belonged to a supported, uncultured bacterial phylogenetic clade containing sequences retrieved solely from seawater (data not shown).

(ii) *Actinobacteria PCR-DGGE bands*. The dominant bands labelled 6 and 7 (Fig. 2.3c) were recovered from three specimens of *S. spinosulus* and from all *I. variabilis* specimens, respectively. These bands were subjected to cloning and sequencing. Three clones were

obtained from each band, which contained two and three dissimilar nucleotides for bands 6 and 7, respectively. All sequences were assigned to the order *Acidimicrobiales*. Phylogenetic analysis revealed that these sequences fell into a sponge-specific bacterial phylogenetic clade (Simister *et al*., 2012c) from which no cultured representative has so far been registered (Fig. 2.4).

(iii) *Alphaproteobacteria PCR-DGGE bands*. The dominant band labelled 8 (Fig. 2.3e) appeared in all specimens of *S. spinosulus*. Three identical sequences were recovered and assigned to the order *Rhodobacterales*. Phylogenetic reconstruction revealed that these sequences affiliated with bacterial phylotypes retrieved almost solely from marine sponges distributed worldwide. No cultured representative isolated from marine sponges has been observed in this cluster (Fig. 2.7). One band found almost in all samples in addition to a dominant band found exclusively in seawater samples labelled, respectively, 9 and 10 (Fig. 2.3e) were subjected to sequencing. One and three sequences were obtained from bands 9 and 10, respectively. They all shared high similarity at the primary sequence level (up to three nucleotide differences detected), belonged to a phylogenetic cluster containing several uncultured bacterial phylotypes retrieved only from seawater, and affiliated with the family *Rhodobacteraceae* (Table 2.1).

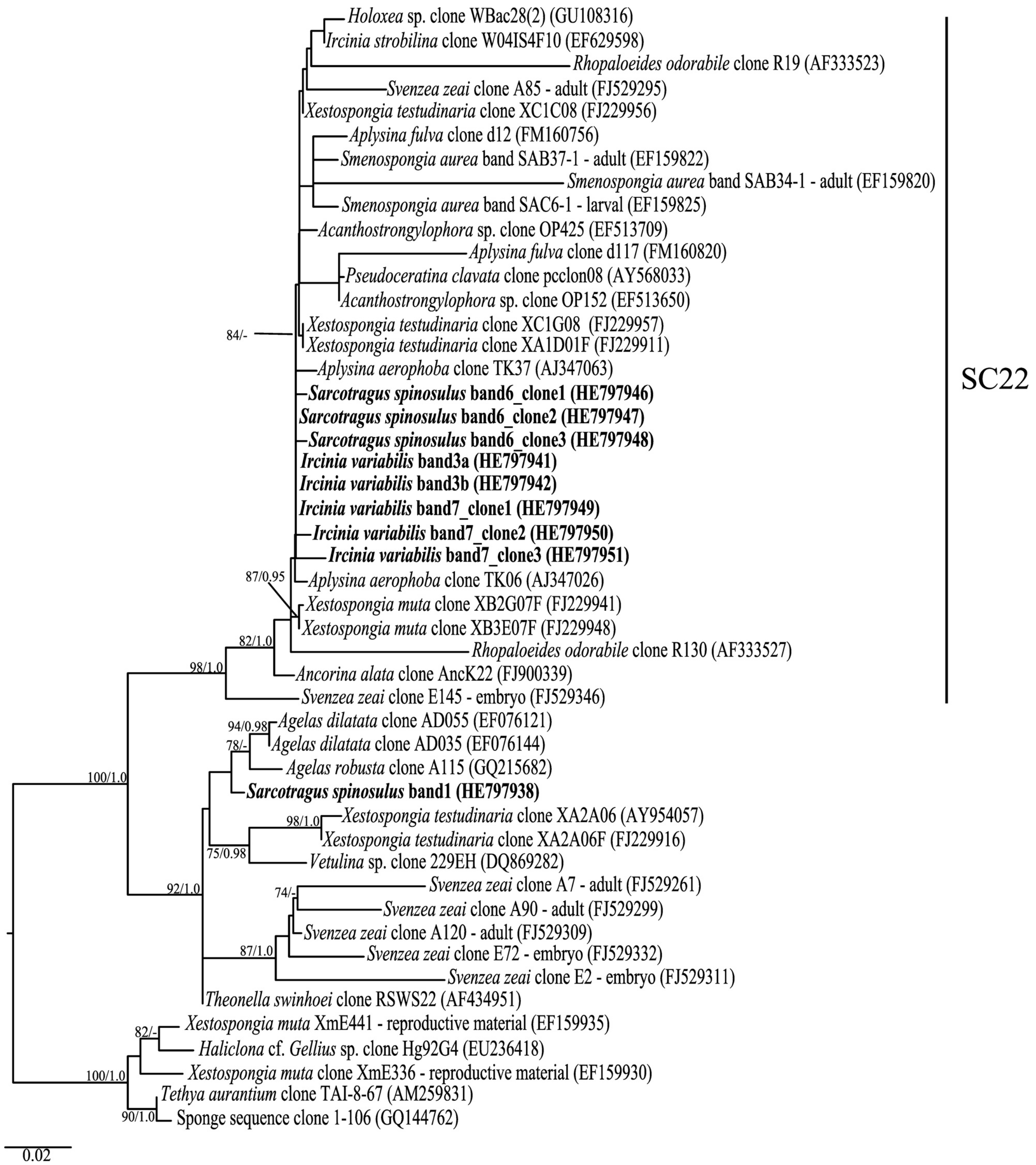


Figure 2.4 - Phylogenetic inference of *Actinobacteria* 16S rRNA genes. The modified ARB database generated by Simister *et al.* (2012c) used long sequences (≥1200bp) to infer the phylogeny and shorter sequences were added using the ARB parsimony interactive tool. Sequences from the sponge-specific cluster 22 (SC22) (Simister *et al.*, 2012c) along with sequences closely related to band 1 and outgroup sequences were selected for further phylogenetic analysis. The Maximum Likelihood tree (-ln likelihood: 4501,317092) is shown, with sequences retrieved in this study highlighted in bold. Numbers at tree nodes are bootstrap values and posterior probabilities calculated in Maximum Likelihood and MCMC Bayesian analyses, respectively, and values above 70/0.95 are shown.

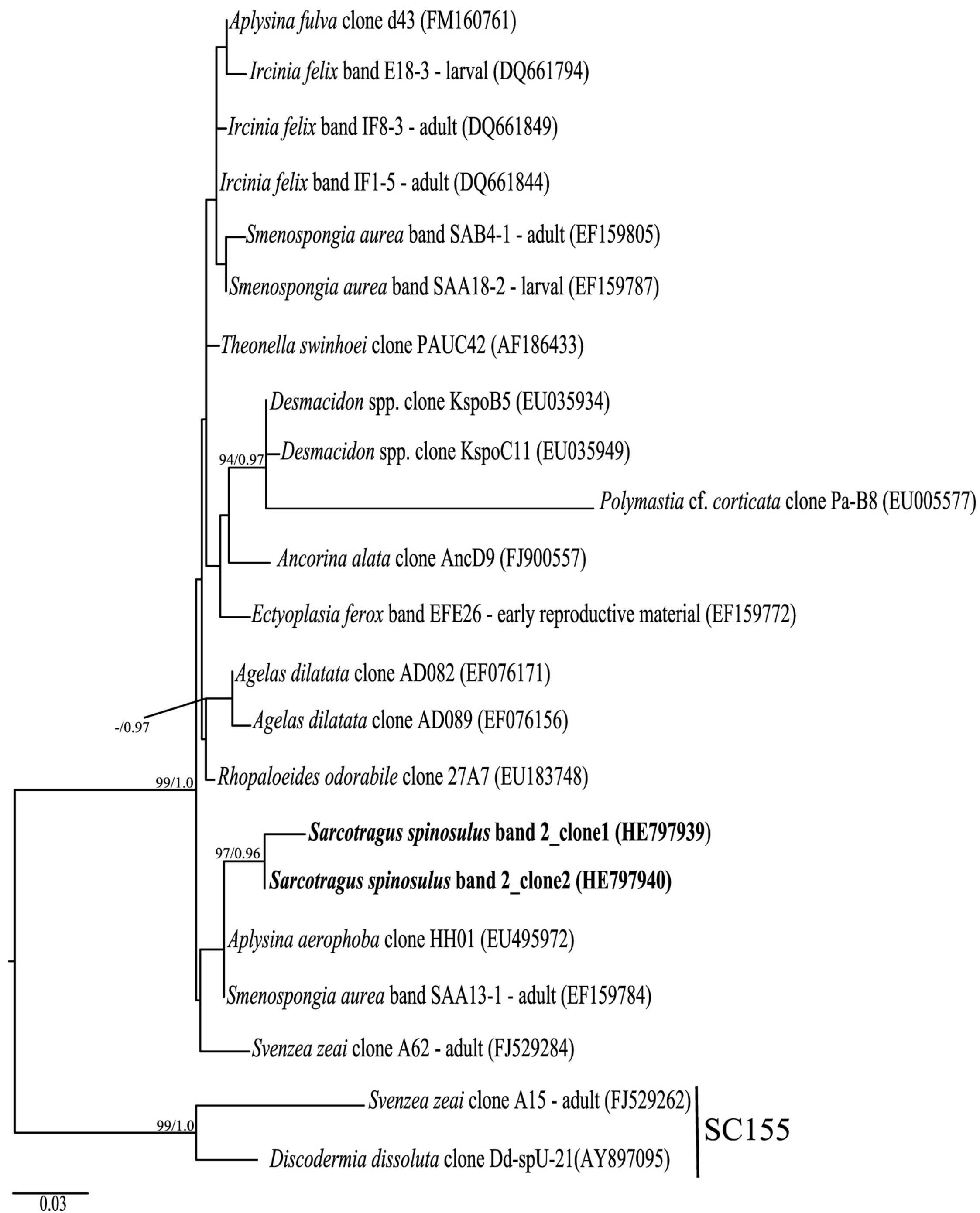


Figure 2.5 - Phylogenetic inference of *Gammaproteobacteria* 16S rRNA genes. Tree construction procedure was as described for Figure 2.4, except that sequences closely related to band 2 were selected as well as sequences from SC155 (Simister *et al*., 2012c), which were used as outgroup. The Maximum Likelihood tree is shown (-ln likelihood: 2696,593494).

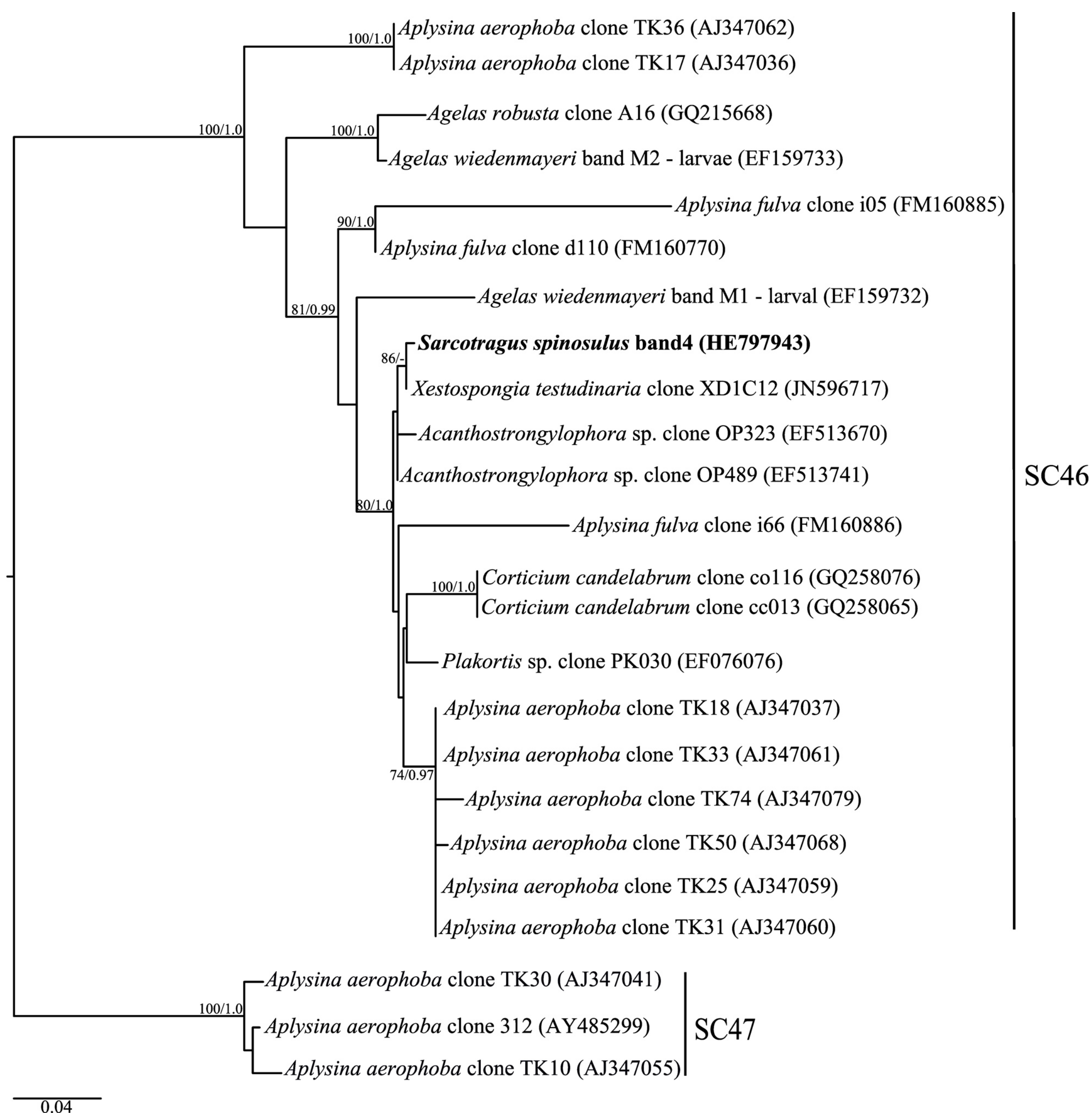


Figure 2.6 - Phylogenetic inference of *Chloroflexi* 16S rRNA genes. Tree construction procedure was as described for Figure 2.4, except that sequences from SC46 were selected along with sequences from SC47, which were used as outgroup (Simister *et al*., 2012c). The Maximum Likelihood tree is shown (-ln likelihood: 3791,095).

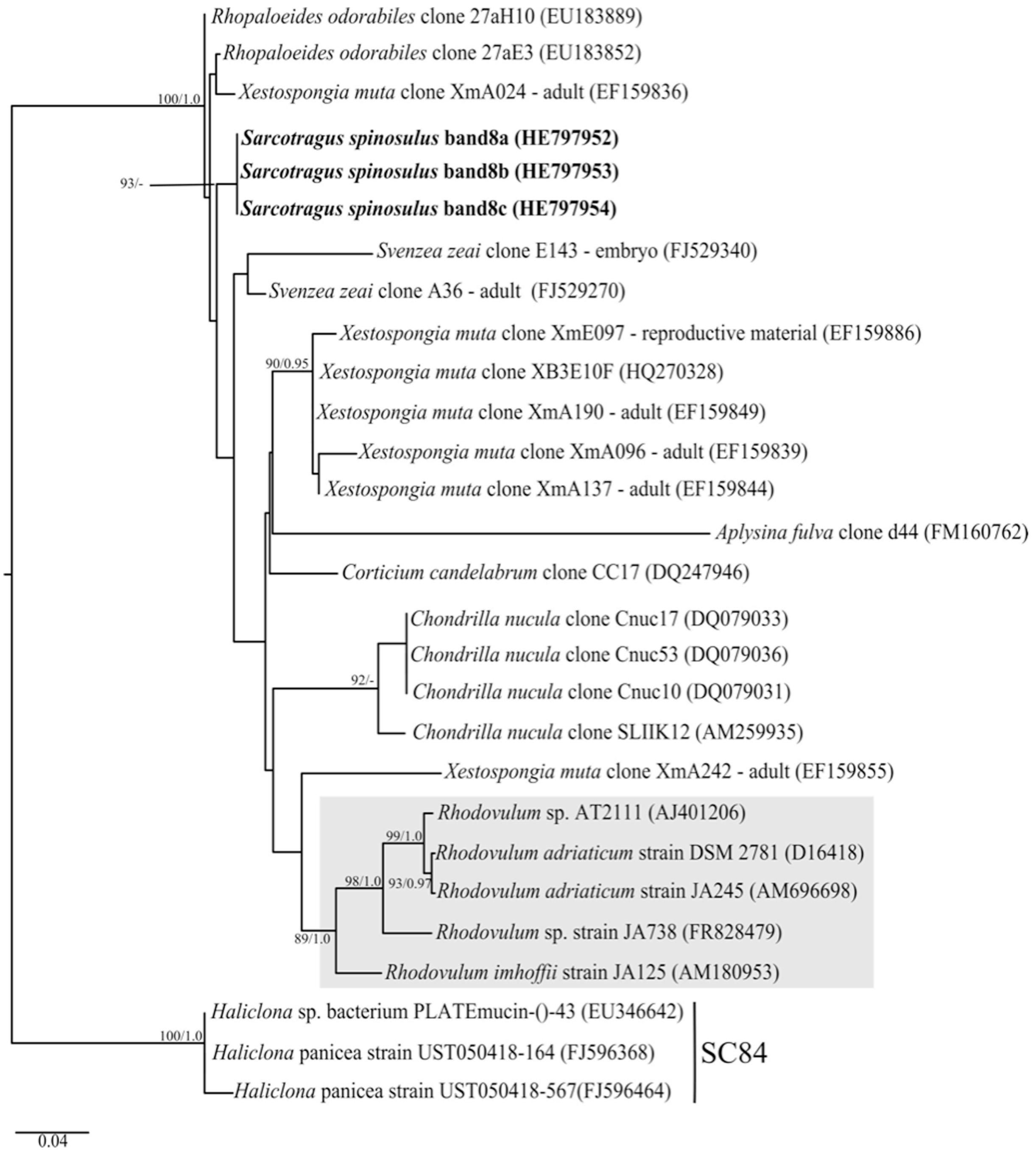


Figure 2.7 - Phylogenetic inference of *Alphaproteobacteria* 16S rRNA genes. Tree construction procedure was as described for Figure 2.4, except that sequences close related to band 8 were selected along with sequences from SC84 (Simister *et al.*, 2012c), which were used as outgroup. The sequences shown in a box were isolated from different environments. The Maximum Likelihood tree is shown (-ln likelihood: 3253,686594).

Table 2.1 - Closest 16S rRNA gene relatives of seawater-derived and "cosmopolitan" PCR-DGGE bands

Band id (Accession number)	RDP closest match[1] (Accession number)	NCBI closest match[2] (Percent similarity, accession number)
5a (HE797944)	Uncultured *Roseobacter* sp. (AY627365)	Uncultured *Rhodobacteraceae* bacterium clone GG101008Clone5 (99%, JN591908)
5b (HE797945)	Uncultured *Roseobacter* sp. (AY627365)	Uncultured *Rhodobacteraceae* bacterium clone GG101008Clone5 (99%, JN591908)
9a (HE797955)	Uncultured *Roseobacter* sp. (AY627365)	Uncultured *Rhodobacteraceae* bacterium clone GG101008Clone5 (99%, JN591908)
10a (HE797957)	Uncultured *Roseobacter* sp. (AY627365)	Uncultured *Rhodobacteraceae* bacterium clone GG101008Clone5 (100%, JN591908)
10b (HE797958)	Uncultured *Roseobacter* sp. (AY627365)	Uncultured *Rhodobacteraceae* bacterium clone GG101008Clone5 (100%, JN591908)
10c (HE797956)	Uncultured *Roseobacter* sp. (AY627365)	Uncultured *Rhodobacteraceae* bacterium clone GG101008Clone5 (100%, JN591908)

[1]Closest 16S rRNA gene relative using the "sequence match" tool of the Ribosomal Database Project (RDP).
[2]Closest 16S rRNA gene relatives as determined by the blast-n search in the National Center for Biotechnology Information (NCBI) database.

Discussion

This survey addresses bacterial abundance, diversity and specificity in the Atlanto-Mediterranean sponges *Sarcotragus spinosulus* and *Ircinia variabilis* (Demospongiae, Dictyoceratida, Irciniidae). These species are widely distributed along the southern Portuguese coast (http://www.marinespecies.org/porifera/). Both species were initially identified by traditional taxonomic methods. However, species within the Order Dictyoceratida to which the family Irciniidae belongs are, along with the Order Dendroceratida, known as 'keratose' sponges, which usually lack a suite of morphological features making their classification problematic (Cook and Bergquist, 2002; Erpenbeck *et al*., 2002). In recent years, molecular characterization of sponges by sequencing of standard genetic markers – known as DNA barcoding – is being used increasingly as a means to facilitate identification and to complement the description of new species (Wörheide and Erpenbeck, 2007). Almost invariably, analyses involve the use of the subunit I of the

cytochrome C oxidase gene (CO1) (Erpenbeck *et al*., 2007; Wörheide and Erpenbeck, 2007; Cardenas *et al*., 2009). The genetic variation (p-distance) found in our host species' CO1 gene, i.e. no intraspecific variation and a 4.7% genetic distance between *I. variabilis* and *S. spinosulus*, are within the range of values observed for other irciniids using the same marker. In a barcoding study of Indo-Pacific irciniids, (Pöppe *et al*., 2010) observed no intraspecific variation within any of the analysed species, low interspecific variation between congeners (0.2-2.7% in *Ircinia* spp. and 0.2-1.7% in *Psammocinia* spp.), and higher differentiation levels between members of the two genera (p-distances of 3.1-5.8%) (Pöppe *et al*., 2010). In a second study comparing the bacterial symbionts in three species of *Ircinia* in the Mediterranean Sea, (Erwin *et al*., 2012c) found no intraspecific variation within any of the studied species (nor between *I. variabilis*/*fasciculata*) and a p-distance of 0.6-1.8% between the different species. Not unexpectedly we found some genetic distance (p-distance 0-0.6%) between the sequences of our Atlantic specimens and those available on GenBank from Mediterranean specimens. This may indicate some level of genetic isolation and differentiation between conspecific populations occurring in these areas as previously observed in other sponge taxa (e.g. Xavier *et al*., 2010a). Overall, host phylogenetic inference can be a suitable and complementary tool in sponge microbiology studies - as shown in early (Erpenbeck *et al*., 2002; Thacker and Starnes, 2003) and recent (Montalvo and Hill, 2011; Erwin *et al*., 2012c) reports on host-symbiont co-evolutionary relationships. Its use seems especially well suited to the study of sponge hosts displaying smooth gradients of phylogenetic relatedness or unresolved taxonomies such as the members of the Irciniidae family and its relevance in such studies is likely to rise with the analysis of multiple phylogenetic markers in concatenation.

In the present survey, the abundance of culturable bacteria associated with *S. spinosulus* and *I. variabilis* was similar. It is well-known that many aspects affect bacterial cultivation and the use of standard culture media has so far allowed the assessment of only a minor fraction (e.g. from 0.1 to 1%) of bacteria associated with marine sponges (Santavy *et al*., 1990; Friedrich *et al*., 2001; Webster and Hill, 2001). This might sharply compromise the comparative assessment of bacterial abundance in sponges when solely using this technique. To circumvent the limitations inherent from cultivation, epifluorescence microscopy was applied to estimate abundance by enabling the count of all detectable nucleic-acid containing cells present in the samples. Based on the cell counts retrieved with this method, about three

orders of magnitude higher than the registered CFU counts, *S. spinosulus* and *I. variabilis* can be regarded as HMA sponges, supporting previous observations obtained for tropical Irciniidae species such as *I. felix* and *I. strobilina* (Vicente, 1990; Weisz *et al*., 2007; Weisz *et al*., 2008).

Bacteria, *Actinobacteria*, and *Alphaproteobacteria* PCR-DGGE fingerprinting revealed a clear difference in bacterial diversity and community composition between sponge and seawater samples. This expected trend has been reported in several previous sponge microbiology surveys (Taylor *et al*., 2004; Taylor *et al*., 2005; Taylor *et al*., 2007). In agreement with our results, the bacterial PCR-DGGE profiles from *I. felix* collected at two sites in Key Largo, Florida, revealed distinct band patterns in comparison with seawater samples (Weisz *et al*., 2007), whereas bacterial PCR-DGGE profiles of wild and captivated *I. strobilina* specimens were likewise distinct when compared with surrounding seawater and water used in sponge aquaculture, respectively (Mohamed *et al*., 2008b). In contrast with species of the genus *Ircinia*, knowledge of bacterial abundance and diversity in *Sarcotragus* specimens is virtually nonexistent. Here we showed *S. spinosulus*-specific profiles that strongly differed from those found in *I. variabilis*, with several species-specific PCR-DGGE bands detected and further identified (see below). *S. spinosulus* exhibited greater bacterial community diversity and richness, and homogeneity across individual specimens than the latter. Further, evidence for greater prokaryotic abundance in *S. spinosulus* was found. In a recent survey, Erwin *et al*. (2012c) could detect bacterial OTUs exclusive to the species *I. oros*, *I. fasciculata* or *I. variabilis* in the Mediterranean Sea. Taken together, these studies hint at a fundamental role of the host in shaping the structure and promoting diversity of symbiont communities within closely related sponge hosts. Interestingly, functional equivalence and evolutionary convergence of symbiont communities have been suggested as an evolutionary model applicable to the complex sponge microbiota, based on the share of core microbial functions between six phylogenetically distant sponge species with different symbiont community structures (Fan *et al*., 2012). In this context, it is tempting to speculate that the less studied *Sarcotragus* also establishes close interactions with selected bacterial communities, which regardless their degree of distinctiveness might have intrinsic functions like those observed for *Ircinia* spp. (Schmitt *et al*., 2007; Mohamed *et al*., 2008b; Mohamed *et al*., 2010). Future studies addressing microbial functioning in sympatric and

phylogenetically close hosts will certainly shed further light on our current understanding of symbiont evolution within sponges.

We successfully identified several sponge-specific bacterial populations by PCR-DGGE. Four dominant symbionts - two from *S. spinosulus*, one from *I. variabilis*, and one found in both sponge species - were affiliated with an uncultured actinobacterial lineage within the order *Acidimicrobiales* (Stackebrandt *et al*., 1997). Three of these ("bands" 3, 6, and 7 in Figs. 2.3 and 2.4) belonged to a cluster of sponge-specific sequences collected worldwide and called SC22 by Simister *et al*. (2012c), whereas the fourth grouped into a different and diverse cluster dominated by sponge-derived bacterial sequences (Fig. 2.4). Within cluster SC22, sequences were obtained from adult of *Svenzea zeai* and *Smenospongia aurea* along with their reproductive material, which suggests that vertical transmission of this particular phylotype is likely to occur (Schmitt *et al*., 2008; Lee *et al*., 2009). The same observation was made for the cluster formed by the fourth symbiont in the *Acidimicrobiales* group ("band 1" in Fig. 2.4) and related sequences, from which two sequences from adult *S. zeai* and one from its embryo were found (Lee *et al*., 2009). These results indicate an intimate pattern of relationship between sponge-associated *Acidimicrobiales* and their hosts. The order *Acidimicrobiales* contains mesophilic and moderate thermophilic species and all members are obligatory acidophilic found in iron-, sulphur- or mineral-sulphide rich environments. Species within this order are capable of ferrous iron and sulphur oxidation and ferric iron reduction (Clark and Norris, 1996; Clum *et al*., 2009; Davis-Belmar and Norris, 2009; Johnson *et al*., 2009). However, the physiological properties exhibited by cultivated *Acidimicrobiales* might not necessarily match those of marine sponge symbionts, as these usually share lower relatedness to cultured species at the 16S rRNA gene level, and therefore further research is needed to unveil the ecology and functioning of these symbionts in marine sponges.

A prevailing symbiont found exclusively in *S. spinosulus* was affiliated with uncultured *Gammaproteobacteria* within the order *Chromatiales* (Imhoff, 2005b). These sequences belonged to a cluster of sponge-specific sequences acquired worldwide (Fig. 2.5). Among them, adult sequences from *Ircinia felix*, *Smenospongia aurea*, and *Svenzea zeai* were observed along with sequences from reproductive material of *I. felix*, *S. aurea*, and *Ectyoplasia ferox* (Schmitt *et al*., 2007; Schmitt *et al*., 2008; Lee *et al*., 2009). The order *Chromatiales* encompasses members of the purple sulphur bacteria that are capable of performing anoxygenic photosynthesis using hydrogen sulphide as electron donor (Imhoff,

2005b). Furthermore, many *Chromatiales* species have been shown to perform fixation of molecular nitrogen (Proctor, 1997; Imhoff, 2005b). These functions might be highly valuable for sponge survival, and the consistency with which members of this group are found in marine sponges at a global scale indeed suggests that *Chromatiales* species play an important role in their association with such hosts.

Another phylotype solely recovered from *S. spinosulus* was affiliated with an uncultured, sponge-specific lineage in the *Chloroflexi* phylum, named SC46 by Simister *et al*. (2012c) (Fig. 2.6). The *Chloroflexi* is regarded as one of the most abundant and diverse bacteria phyla associated with a wide variety of marine sponges, with many sponge-specific clusters identified (Hardoim *et al*., 2009; Schmitt *et al*., 2011; Simister *et al*., 2012c). So far, only one *Chloroflexi* species was isolated from the sponge *Geodia* spp., which also clustered with sequences exclusively obtained from marine sponges (Bruck *et al*., 2010). In shallow waters, members of *Chloroflexi* are able to fix atmospheric carbon through photosynthesis, and thus these bacteria could provide carbonaceous compounds to the sponge host (Bruck *et al*., 2010). Recently, a *Chloroflexi* bacterium was pointed as the likely producer of a novel non-ribosomal peptide synthase (Siegl and Hentschel, 2010). Thus, *Chloroflexi* strains might play important roles in sponge nutrition and defence.

Using a taxon-specific fingerprinting approach to the *Alphaproteobacteria,* a dominant symbiont exclusive to *S. spinosuls* was uncovered ("band 8" in Fig. 2.3) and found to be closely related to an uncultured alphaproteobacterium within the family *Rhodobacterales* (Garrity, 2006). Sequences representing this symbiont formed a concise cluster with sequences retrieved from marine sponges in several geographical backgrounds in addition to cultured representatives obtained from different environments such as microbial mats, seawater, soil from saltpan, water, and marine aquaculture pond (Hiraishi and Ueda, 1995; Srinivas *et al*., 2007) (Fig. 2.7). This clade contained sequences obtained from adults of *Xestospongia muta* and *Svenzea zeai* along with their reproductive material (Schmitt *et al*., 2008; Lee *et al*., 2009). This symbiont is closely related to *Rhodovulum* species, in which many type strains have been mostly retrieved from marine habitats. This genus contains species that undertake diverse metabolic pathways such as photoautotrophic, photoheterotrophic, and chemotrophic and occur mainly in marine and hypersaline environments under oxic, microoxic, and anoxic conditions (Imhoff, 2005a). The metabolic versatility of *Rhodovulum* species indicate that they are able to use the waste generated by

sponges. For instance, ammonia, which is a toxic metabolic waste product that could accumulate within the sponge body, especially during low pumping activity, might be used as nitrogen source for *Rhodovulum* species (Brusca and Brusca, 2002; Imhoff, 2005a; Taylor *et al*., 2007). In addition, some strains of *Rhodovulum* could be involved in nitrogen and sulphur cycling, once they are capable to use dinitrogen, sulphur, sulphite, sulphate, and thiosulphate (Imhoff, 2005a). Vertical transmission has also been documented for members of this genus in marine sponges, (Schmitt *et al*., 2008) suggesting *Rhodovulum* as a likely, relevant constituent of the sponge-associated microbiome.

The present study provides first insights into the bacterial abundance and diversity in Atlantic *S. spinosulus* and *I. variabilis*. In spite of their sympatric occurrence, the inspected species hosted bacterial communities that differ from each other and from those found in seawater. Interestingly, all bands excised from PCR-DGGE profiles that were exclusive to sponge samples affiliated with previously identified sponge-specific sequence clusters (Simister *et al*., 2012c) or with potentially novel sponge-specific clusters found in the present survey. Thus, the approach used here enabled not only straightforward assessment of overall trends in bacterial community structures, but also direct identification of symbionts of putative relevance in association with their hosts, given their dominance and consistent patterns of occurrence in the analysed specimens, and their presumed sponge-specific life histories as inferred by 16S rRNA gene phylogenies. Notably, bacterial phylotypes regarded as "*S. spinosulus*-specific" or "*I. variabilis*-specific" in this study shared high degrees of resemblance with sponge-derived sequences from other biogeographical settings and/or more distantly related sponge hosts. This picture, in which bacterial signatures not shared by co-occurring and taxonomically close sponge species are found in disparate sponge hosts and localities, most likely derives from factors of the host and of the environment – including vertical transmission *vs.* environmental acquisition of symbionts, specific habitat preferences and life stages of the host - that cooperatively shape the structure of the sponge-associated microbiome (Taylor *et al*., 2007; Erwin *et al*., 2012c). As a result, complex communities of specific composition at the host species or even specimen level (Lee *et al*., 2009; Schmitt *et al*., 2012) with concomitant sharing, across sponge species, of generalist symbionts displaying broad host range and/or widespread occurrence (Hentschel *et al*., 2002; Montalvo and Hill, 2011) have often been reported for marine sponges. Here, the distinct communities observed in *S. spinosulus* and *I. variabilis* within the same habitat, along with the detection of

symbionts showing broad host and geographical ranges as inferred by 16S rRNA gene phylogenies, hints at a pivotal role for the host in shaping the structure of its own microbiota while revealing versatile and widespread bacterial phylotypes with apparently intimate sponge-associated life histories. The high abundance and species-specific character of these assemblages suggest in-faunal microbial communities as overriding drivers of functioning and of genetic and metabolic diversities in coastal ecosystems.

Material and methods

Sponge and seawater sampling. Four specimens of *Sarcotragus spinosulus* and *Ircinia variabilis* (Demospongiae, Dictyoceratida, Irciniidae) were collected by scuba diving at depths around 15m at Galé Alta, Armação de Pêra (37º 04' 09.6"N and 8º 19' 52.1"W) in the coast of the Algarve, Portugal, in June 2010. Measurements of temperature, oxygen, and salinity during the sampling procedure were 14.6ºC, 5.95 mg L^{-1}, and 35.11 part per million (ppm), respectively. *In situ* pictures of the specimens were taken to aid laboratory identification (Fig. S2.1). The individual samples were placed, *in situ*, separately in plastic bags (type Ziploc®) containing natural seawater, transferred into cooling boxes, brought to the laboratory within few hours, and processed upon arrival. Four samples of seawater (1L each) from the vicinity of the sponges (i.e. 1 m above the specimens) were also collected as above. Prior to sample processing, the sponge specimens were rinsed with sterile Artificial Seawater (ASW) (McLachlan, 1964) to remove loosely associated organisms. Voucher samples were preserved in 90% ethanol for taxonomic identification and deposited in the Biology Department´s zoological collection of the University of the Azores (DBUA.Por). Because sampling did not involve endangered or protected species and did not occur within privately owned or protected areas, no specific permits were required for the described field studies.

Sponge identification. Specimens were identified from the analysis of general external morphological characters and internal skeletal features, i.e. thickness, degree of fasciculation, and presence of foreign debris within the spongin fibres and structure of the collagenous filaments. Genera within the family Irciniidae are distinguished by the presence of a cortical armour of sand (exclusive to *Psammocinia*), and presence (in *Ircinia*) versus absence (in *Sarcotragus*) of foreign debris within the primary fibres (Cook and Bergquist, 2002).

Phylogenetic inference of sponge specimens (commonly referred to as "sponge DNA barcoding") was used to aid species identification by molecular means. PCR amplifications were carried out on sponge total community DNA (see below) targeting the subunit I of the cytochrome oxidase gene with the primers dgLCO1490 and dgHCO2189 (Meyer *et al*., 2005). This fragment (*c.* 640 bp) encompasses the standard "barcoding" partition (Folmer *et al*., 1994). The reaction mixture (25µL) contained 1.5 µL of template DNA (~ 20 ng), 1X reaction buffer (Bioline, London, UK), 0.16 mM deoxynucleoside triphosphates (dNTPs), 4.0 mM $MgCl_2$, 0.64 mg mL^{-1} of bovine serum albumin (BSA), 0.24 µM of primers, and 0.625U of Bio*Taq*™ DNA polymerase (Bioline, London, UK). After initial denaturation at 95ºC for 3 min., 36 cycles of 45 sec at 94ºC, 60 sec at 51ºC, and 90 sec at 72ºC were carried out. A final extension of 10 min at 72ºC was used to finish the reaction. All PCR amplifications were carried out in a MyCycle thermal cycler (Bio-Rad, Hercules, CA, USA). Amplicons were checked after electrophoresis on 1% agarose gel under UV light. PCR products with right size were cleaned with Sephadex G50 (GE Healthcare Bio-Science AB, Uppsala, Sweden) columns, quantified with Image Lab™ Software (Bio-Rad, Hercules, CA, USA), and subjected to sequencing with the chain termination method in an Applied Biosystems 3130 genetic analyser using the forward primer. Closest relatives were searched using the megablast and blastn algorithms of the National Center for Biotechnology Information (NCBI) (Altschul *et al*., 1997). Closely related sequences from the NCBI and the Sponge Barcoding Project (www.spongebarcoding.org) databases were used to retrieve representative CO1 sequences for phylogenetic inference (see below).

Plate counting of heterotrophic bacteria. Per sponge specimen, 2.5 g of fresh internal body was cut and transferred to a 50 mL screw cap polypropylene tube containing 25 mL of Calcium/Magnesium Free Artificial Seawater (CMFASW) (Garson *et al*., 1998). The sponge samples were ground with sterile mortar and pestle. The resulting suspensions were collected and allowed to decant for 5 min. Serial 10-fold dilutions were then prepared with sterile ASW and plated in triplicate onto Marine broth (Carl Roth GmbH + Co, Germany) plus 1.5% agar. The plates were incubated at room temperature (~25ºC) and Colony Forming Unit (CFU) counting was performed after three, five, and seven days of incubation. Log-transformed CFU values fitted the normal distribution and were compared by One Way Analysis of Variance (ANOVA) using PASW Statistics 18 (SPSS Inc., Chicago, USA).

Epifluorescence microscopy. A cultivation-independent analysis of prokaryotic abundance based on epifluorescence microscopy was performed in this study. For the sponge samples, the suspensions prepared in the abovementioned procedure were first centrifuged at 500 *g* for 2 min to remove sponge cells and debris. Aliquots (100 µL) of the resulting supernatants were individually fixed in 2.5% glutaraldehyde and the volume was completed to 10 mL with sterile ASW. Seawater samples (9.2 mL) were also fixed in 2.5% glutaraldehyde. From the fixed material, 100 µL and 10 mL from sponge and seawater samples, respectively, were filtered through 0.2-µm-pore-size isopore^{TM} black membrane filters (Millipore, Bellerica, MA, USA). The filter was stained with the DNA-binding fluorochrome acridine orange, mounted on glass slides and analysed with an inverted research system microscope IX81 (Olympus Europa GmbH, Hamburg, Germany) where 25 photos per specimen were taken at random. Cells with a well-defined edge, usually ranging from 0.2 to 1 µm in diameter when coccoid, or reaching up to 5 µm in length when rod-shaped, were counted and served as proxy for prokaryotic cell abundance in the samples. Larger objects (> 5 µm) that could eventually account for eukaryotic organisms were not considered. Total prokaryotic numbers were log-transformed and analysed by One Way ANOVA using PASW Statistics 18 (SPSS Inc., Chicago, USA).

Total community DNA extraction. Genomic DNA of about 0.25 g of internal sponge body was extracted using UltraClean® Soil DNA isolation kit (Mo Bio, Carlsbad, CA, USA) according to the manufacturer's protocol. Based on preliminary PCR-DGGE assessments, this method led to a more reproducible depiction of bacterial community structures in the sponges when compared with a method that employs a cell-separation treatment prior to DNA extraction (Hardoim *et al.,* unpublished results), and was therefore chosen for the purpose of this study. Seawater samples (500 mL) were filtered through 0.2-µm-pore-size nitrocellulose filters (Millipore, Billerica, MA, USA) using a vacuum pump. The filters were cut into small pieces and directly used for DNA extraction as explained above.

Bacterial PCR for DGGE analysis. A nested PCR-denaturing gradient gel electrophoresis (PCR-DGGE) approach was chosen - based on its higher detection sensitivity and reproducibility when compared with a one-step amplification protocol in preliminary assays (data not shown) - to assess the total bacterial communities in all samples. Nearly full-length

16S rRNA gene fragments were amplified with the primer pair F27 and R1492 (Weisburg *et al*., 1991). The reaction mixture (25 µL) was prepared with 1 µL of template DNA (~20 ng), 1X Stoffel buffer (Applied Biosystems, Foster, CA), 0.2 mM dNTPs, 3.75 mM $MgCl_2$, 0.1 mg mL^{-1} of BSA, 2% (vol/vol) dimethyl sulfoxide (DMSO), 0.2 µM of each primer, and 1.25U of *Taq* DNA polymerase (Applied Biosystems, Foster, CA). After initial denaturation at 94ºC for 5 min, 30 cycles of 1 min at 94ºC, 1 min at 56ºC, and 2 min at 72ºC were performed, followed by a final extension for 10 min at 72ºC. The amplicons (1.5 µL) were used as template in a subsequent PCR for DGGE analysis (20 cycles) using the primer pair F984-GC and R1378 (Heuer *et al*., 1997). The PCR mixture and thermal cycling followed the protocol by Costa *et al*. (2006b), with half the quantity of *Taq* DNA polymerase (1.25 U) per reaction.

PCR of specific bacterial groups for DGGE analyses

(i) *Actinobacteria 16S rRNA gene fragments.* The first amplification of the nested PCR was carried out with the primers F243 (Heuer *et al*., 1997) and R1494 (Weisburg *et al.,* 1991) to generate *Actinobacteria*-specific amplicons. The reaction mixture and PCR conditions were carried out as described by Hardoim *et al*. (2009), except for the concentration of *Taq* DNA polymerase (1.25U), number of cycles (25 cycles), and extension period (1 min) in the present study. The amplicons (2 µL) were used in a second PCR for DGGE analysis using the primers F984-GC and R1378 (Heuer *et al*., 1997) as described previously for total bacteria, except for the number of cycles (30 cycles).

(ii) *Alphaproteobacteria 16S rRNA gene fragments*. The first reaction mixture of the nested PCR was prepared as described by Gomes *et al*. (2001), except that in the present study the primer concentration and *Taq* DNA polymerase were 0.2 µM and 1.25U, respectively, and that BSA was not used in the group-specific PCR. After initial denaturation at 94ºC for 7 min, 30 cycles of 1 min at 94ºC, 1 min at 56ºC, and 1 min at 72ºC were carried out. The reaction was finished with an extension of 10 min at 72ºC. Amplicons from the first reaction (2 µL) were used in a subsequent PCR for DGGE analysis as described previously, except for the number of cycles (25 cycles).

PCR-DGGE profiling. DGGE assays were carried out in a PhorU-2 gradient system (Ingeny International, Goes, The Netherlands). The 16S rRNA gene amplicons generated as explained above were applied in even concentrations onto polyacrylamide gels containing a 46.5 to 65%

gradient of denaturants (100% denaturants defined as 7M urea and 40% formamide) and a 6.2 to 9% gradient of acrylamide. Mixtures of PCR products of ten bacterial strains isolated from *Sarcotragus* sp. and *Ircinia* sp. (*Staphylococcus* sp.; *Ruegeria* sp.; *Pseudomonas* sp.; *Leisingera* sp.; *Corynebacterium* sp.; *Micrococcus* sp.; *Streptomyces* sp. and *Pontibacter* sp.) were loaded at the edge of the gels as markers. Electrophoresis was performed in a 1X Tris-acetate-EDTA buffer (pH 7.8) at 58ºC and 140V for 16 h. The gels were silver stained (Heuer *et al.,* 1997) and air dried, after which digital images were obtained by scanning.

Analysis of PCR-DGGE profiles. The software GelCompar II 5.1 (Applied Maths, Kortrijk, Belgium) was used to analyse the PCR-DGGE profiles as recommended by Rademaker and de Bruijn (2004). Briefly, pairwise Pearson correlation coefficients (r) were calculated as a measurement of the similarity between the community profiles. Cluster analysis was carried out with the unweighted pair group method with mathematical averages (UPGMA) using the similarity matrix generated with the calculated Pearson coefficients. In addition to cluster analysis, constrained (i.e. canonical) ordination was performed with the Canoco for Windows 4.5 software package (Microcomputer Power. Ithaca, NY) using a "sample x species" datasheet as input, in which the "species" data represent the presence and relative abundance of PCR-DGGE bands in each fingerprint, as described in detail by Costa *et al*. (2006a). This was used to infer whether sponge species and seawater significantly contributed to the observed variability in the PCR-DGGE profiles (see Costa *et al*., 2006a; Hardoim *et al*., 2009). The Shannon measure of diversity (H'), determined as $H' = -\sum pi.logpi$; where pi represents the relative abundance of the ith category (i.e. PCR-DGGE band) within the sample (i.e. PCR-DGGE fingerprint) was applied to estimate the diversity of each PCR-DGGE fingerprint generated in this study. The evenness ($J' = \frac{H'}{Hmax}$) of PCR-DGGE fingerprints was calculated based on the diversity indices obtained. Measures of richness (i.e. number of PCR-DGGE bands), diversity, and evenness of PCR-DGGE fingerprints were compared by One Way ANOVA using PASW Statistics 18 (SPSS Inc., Chicago, USA).

Identification of dominant bands in PCR-DGGE profiles. Sponge-associated and seawater exclusive bands were visually determined based on their occurrence across replicates (i.e. only bands detected in at least three of four replicates were sequenced). Further, their discriminating power was assessed, and only bands displaying high variance in relative

abundance as a response to the sample classes "*I. variabilis*", "*S. spinosulus*", and "Seawater" were selected. Discriminating bands were revealed by the species fit range function in the Canoco for windows 4.5 software, where only those bands displaying 50% fit range or more were considered for sequencing purposes. Discriminating bands were excised from DG-gels and re-amplified for PCR-DGGE analysis using the method of Costa *et al*. (2006a). The resulting amplicons were loaded onto DGGE with the original community DNA samples to verify their electrophoretic mobility. Excised bands that displayed the right melting behaviour when compared with the original band in the community profiles were used as templates in another PCR amplification, in which the forward primer F984 used had no GC clamp. PCR-DGGE reaction mixtures and thermal cycles were carried out as described above. The amplicons were then purified in Sephadex G50 columns, quantified with Image Lab™ Software, and subjected to sequencing as above mentioned. For some excised bands, no pure amplicon was recovered and thus a cloning procedure was undertaken using the pGEM-T Vector System II Kit (Promega, Madison, WI) as described elsewhere (Costa *et al*., 2006b; Costa *et al*., 2006a; Hardoim *et al*., 2009). Clones that showed the same electrophoretic mobility when compared to their original band were selected for sequencing as explained above. All sequences retrieved in this study were submitted to the EMBL Nucleotide Sequence Database under accession numbers HE797930 to HE797937 for sponge CO1 sequences and HE797938 to HE797958 for PCR-DGGE bands representing bacterial 16S rRNA genes.

Phylogenetic analyses. Sequences generated from sponge CO1 amplification and excised bacterial bands were quality-inspected and edited with the Sequence Scanner software V.1 (Applied Biosystems). For bacterial DGGE bands, taxonomic assignment of sequences was performed with the seqmatch and classifier tools of the Ribosomal Database Project II (http://rdp.cme.msu.edu) at 80% confidence threshold. Closest phylogenetic relatives were searched with the blast-n algorithm of NCBI. PCR-DGGE band sequences and their closest phylogenetic relatives were aligned using the SINA web aligner (Pruesse *et al*., 2007). Aligned sequences were then imported into a modified SILVA 16S rRNA gene database version 102, which included all sponge-derived 16S rRNA gene sequences available in early 2010 (Simister *et al*., 2012c), using the parsimony tool as implemented in the ARB software (Ludwig *et al*., 2004). The sponge database generated by Simister *et al*. (2012c) contained

phylogenetic inferences performed with long sequences (≥1200bp) using the program RAxML for all sponge-associated bacterial phyla, from which sponge-specific clusters were assigned (Simister *et al*., 2012c) according to the criteria described by Hentschel *et al*. (2002). Alignments were manually checked and corrected when necessary using the ARB alignment window. The sequences generated in this study were added to maximum likelihood trees inferred by Simister *et al*. (2012c) through the parsimony interactive tool available in ARB using 50% conservation filters for each of the corresponding bacterial phyla, and their affiliation with sponge-specific phylogenetic clusters was then ascertained. From the resulting trees, relevant sequences were selected for further phylogenetic analyses (see below). The CO1 gene sequences from each investigated specimen were aligned against selected sponge barcoding sequences using Clustal X in the MEGA5 software (Tamura *et al*., 2011). Phylogenetic inferences of bacteria and sponge sequences were performed as described by Hardoim *et al*. (2013). Briefly, an appropriate evolutionary model for all phylogenetic trees was determined using ModelGenerator version 85 (Keane *et al*., 2006) and found to be the general-time reversible model (GTR, Rodriguez *et al*., 1990) with a discrete gamma-distribution of among-site rate variation (Γ_4) and a proportion of invariant sites (I), except for CO1 inference, in which invariant sites did not fit. Maximum likelihood and Bayesian MCMC analyses were conducted using RAxML (vers. 7.0.4-MPI) and MrBayes (vers. 3.2.1), respectively (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003; Stamatakis, 2006).

Acknowledgments

This work was financed by the Portuguese Foundation for Science and Technology (FCT) through the research project PTDC/MAR/101431/2008. CCPH has a PhD fellowship granted by FCT (Grant No. SFRH/BD/60873/2009). JR Xavier research is funded by a FCT postdoctoral fellowship (grant no. SFRH/BPD/62946/2009). We acknowledge Msc. Sandra Mesquita, Dr Paulo J. Gavaia, and João C. Silva from the Centre of Marine Sciences (CCMar-UAlg) for their help in epifluorescence microscopy sample preparation and analysis. We thank Dr. Christin Zachow from the Institute of Environmental Biotechnology, Graz University of Technology, Austria, for her help with Gelcompar analysis. We are grateful to Dr. Michael Taylor for kindly providing the ARB database containing sponge-derived 16S rRNA gene sequences (Simister *et al*., 2012c) used in this study to diagnose sponge-specific clusters.

Author Contributions

Conceived and designed the experiments: JMSG, JRX, RC. Performed the experiments: CCPH, AISE, FRP, JRX. Analyzed the data: CCPH, CC, JRX, RC. Contributed reagents/materials/analysis tools: JMSG, CC, JRX, RC. Wrote the paper: CCPH, JRX, RC. Final approval of the version to be published: CCPH, AISE, FRP, JMSG, CC, JBTX, RC.

Supplementary Material

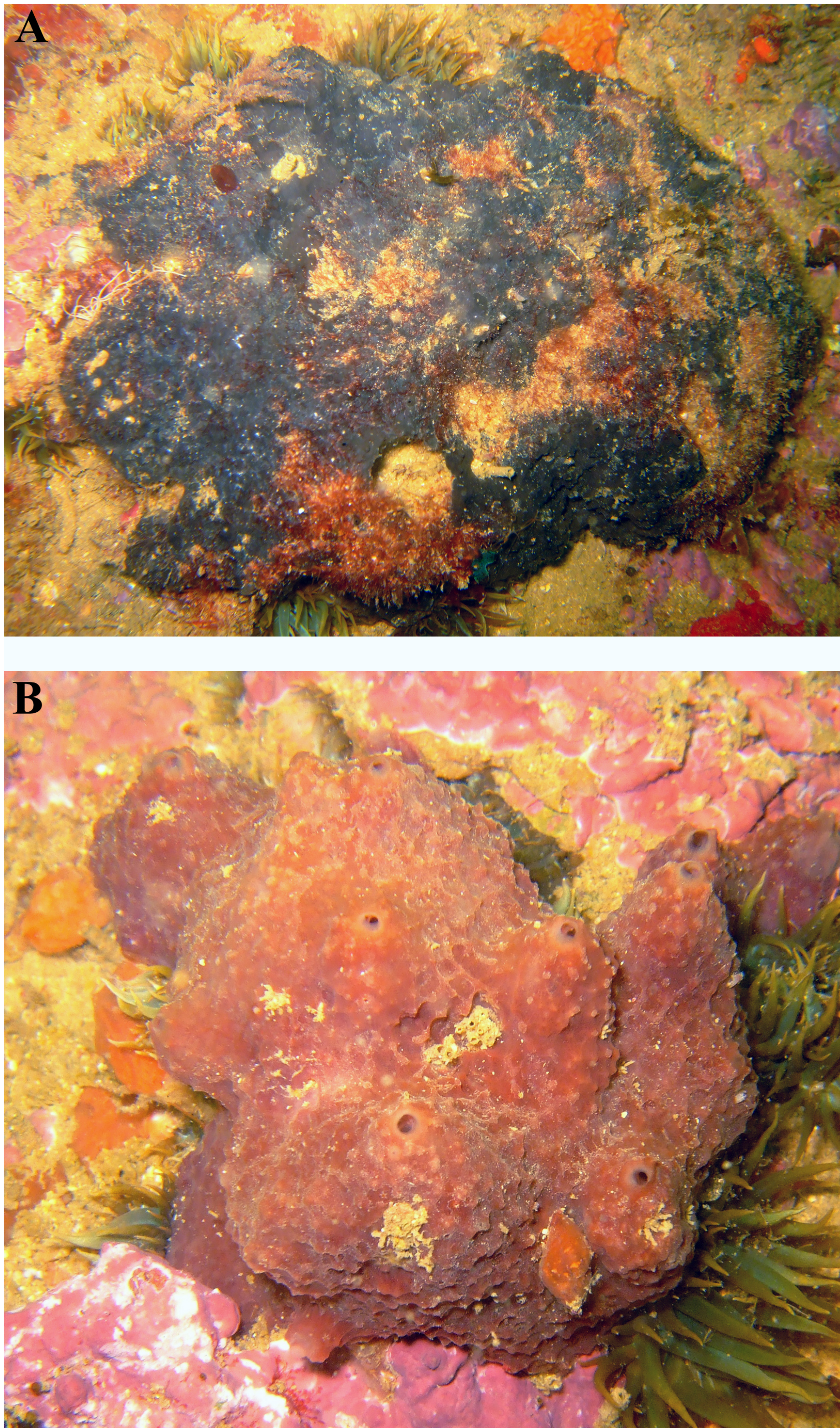


Figure S2.1 - Sponge species. *In situ* pictures of *S. spinosulus* (A) and *I. variabilis* (B).

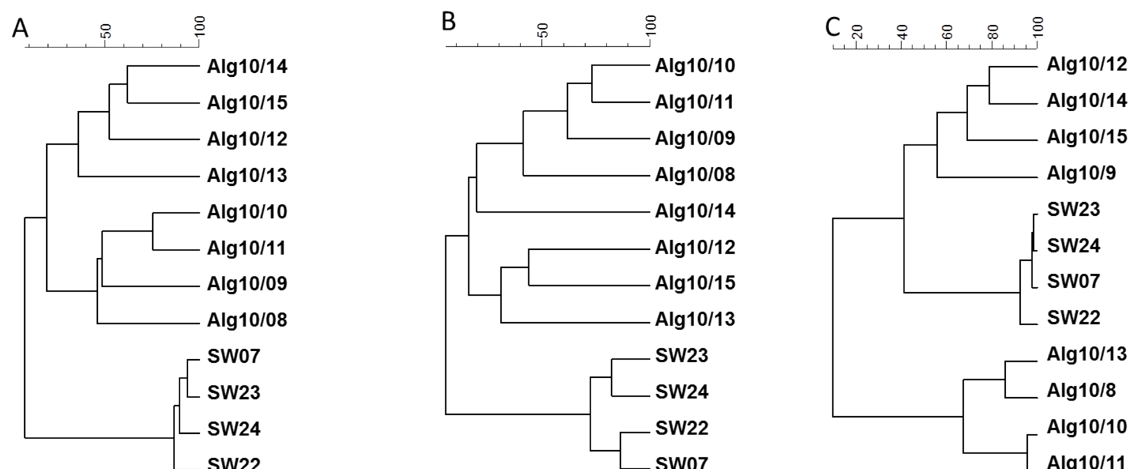


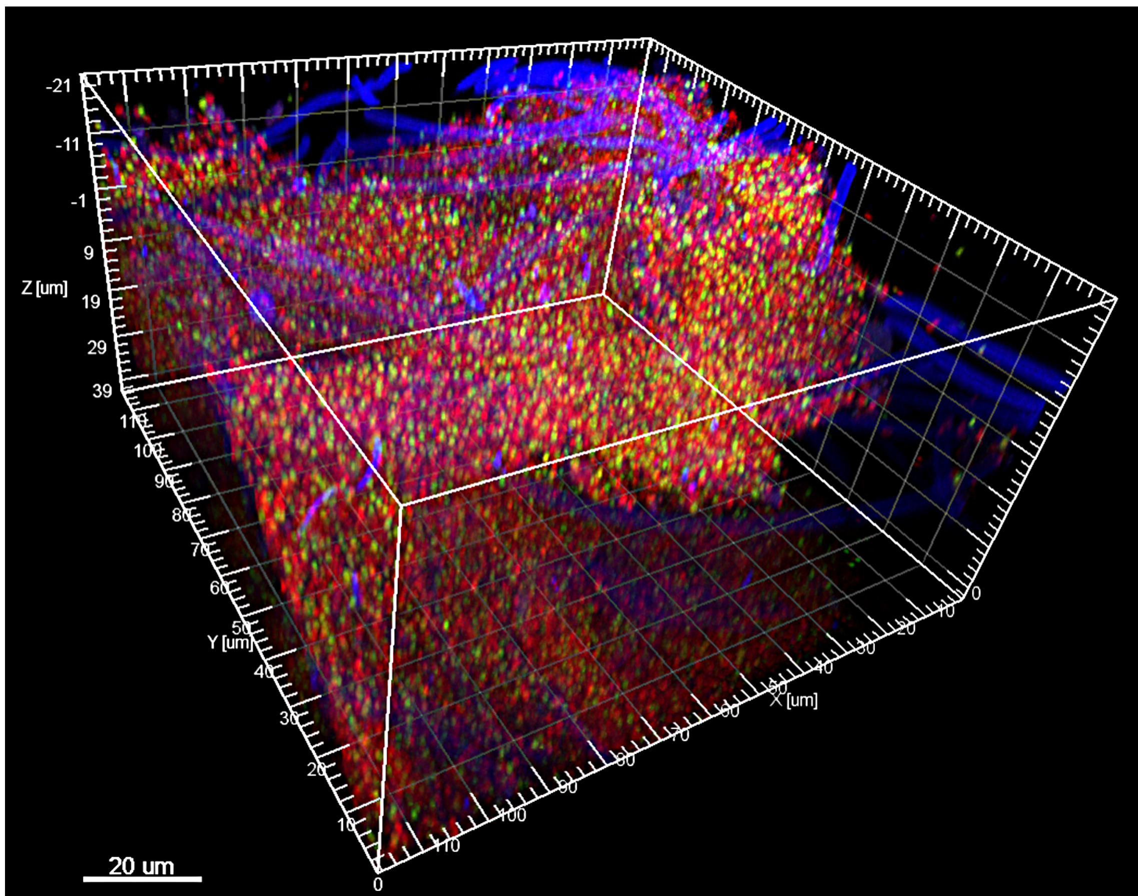
Figure S2.2 - Cluster analysis. Cluster analysis of PCR-DGGE fingerprints obtained for *Bacteria* (A), *Actinobacteria* (B) and *Alphaproteobacteria* (C). *S. spinosulus*: Alg10/08, Alg10/09, Alg10/10, and Alg10/11; *I. variabilis*: Alg10/12, Alg10/13, Alg10/14, and Alg10/15 and Seawater: SW07, SW22, SW23, and SW24.

Table S2.1 - PCR-DGGE band richness, diversity and evenness.[1]

Bacteria PCR-DGGE	**Richness**	**Diversity**	**Evenness**
S. spinosulus	28.25 ± 2.93 [a]	3.03 ± 0.15 [a]	0.91 ± 0.03 [a]
I. variabilis	15.25 ± 4.52 [b]	2.15 ± 0.52 [a]	0.79 ± 0.12 [a]
Seawater	36 ± 0.58 [a]	3.18 ± 0.09 [a]	0.88 ± 0.02 [a]
Actinobacteria PCR-DGGE	**Richness**	**Diversity**	**Evenness**
S. spinosulus	10.75 ± 0.94 [a]	2.10 ± 0.05 [a]	0.89 ± 0.03 [a]
I. variabilis	5.00 ± 1.29 [b]	1.31 ± 0.27 [b,c]	0.88 ± 0.03 [a]
Seawater	12.25 ± 1.03 [a]	1.51 ± 0.44 [a,c]	0.79 ± 0.02 [a]
Alphaproteobacteria PCR-DGGE	**Richness**	**Diversity**	**Evenness**
S. spinosulus	11.50 ± 1.44 [a]	1.66 ± 0.07 [a]	0.69 ± 0.03 [a]
I. variabilis	4.00 ± 0.71 [b]	1.12 ± 0.18 [b]	0.82 ± 0.04 [b]
Seawater	10.00 ± 0.71 [a]	2.19 ± 0.08 [c]	0.95 ± 0.01 [c]

[1] Values are expressed as means ± standard error of the mean. Within each group of values, those represented by different letters are significantly different as determined by One Way ANOVA ($p<0.05$).

CHAPTER III



Degrees of host specificity, effects of sample handling and extent of cultivation bias elucidated for bacterial communities in closely related marine sponges

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Chapter III

Abstract

Complex and distinct bacterial communities inhabit marine sponges and are believed to be essential to host survival, but our present-day inability to domesticate sponge symbionts in the laboratory hinders our access to the full metabolic breadth of these microbial consortia. Here, we address bacterial cultivation bias in marine sponges using a procedure that circumvents the need to isolate single colonies during richness surveys, and instead enables direct comparison of symbiont compositional and diversity data with cultivation-independent methods. Bacterial community profiling of the sympatric and phylogenetically close species *Sarcotragus spinosulus* and *Ircinia variabilis* (Irciniidae) was performed by PCR-DGGE and 454-pyrosequecing of 16S rRNA genes amplified from sponge-derived metagenomic DNA. Whereas cultivation-independent methods clearly unravelled species-specific community structures in these hosts, cultivation led to the detection of highly similar bacterial assemblages from both sponge species. Between 15 and 18 bacterial phyla were found in *S. spinosulus* and *I. variabilis* by cultivation-independent means. Conversely, *Proteobacteria* in the *Alpha*- and *Gammaproteobacteria* classes massively dominated the cultured bacterial community. Whereas cultivation-independent methods unveiled about 200 and 220 operational taxonomic units (OTUs, 97% gene similarity) in *S. spinosulus* and *I. variabilis*, respectively, only 33 and 39 OTUs were registered in these species via culturing. Surprisingly, around 50% of all OTUs recovered by cultivation was exclusive to this methodology, suggesting culturing is a means of enriching rare sponge-associated bacteria that escape detection by cultivation-independent, deep sequencing efforts. This study demonstrates that interpretation of ecological data on sponge microbiome diversity is dependent on the choice of methodology. It further encourages the development of alternative culturing technologies to capture the dominant sponge symbiont fraction that remains recalcitrant to laboratory manipulation.

Introduction

"The great plate count anomaly", as introduced by Staley and Konopka (1985), describes the difference observed, for any given sample, between the number of colony forming units (CFUs) grown on a culture medium and that of bacterial cells observed by microscopy. This way, it was estimated that only 0.1 to 1.0% of the total bacteria cells could be assessed via standard cultivation approaches (Staley and Konopka, 1985). This perspective was supported by several studies on free-living (Kogure *et al*., 1979; Gregory, 1979 *apud* Staley and Konopka, 1985; Amann *et al*., 1995) and host-associated microbial communities, such as those performed with the marine sponges *Aplysina aerophoba* (Friedrich *et al*., 2001) and *Rhopaloeides odorabile* (Webster and Hill, 2001). However, the plate count anomaly as originally described disregards the phylogenetic diversity of those CFUs grown on plates and uncultivated cells observed under the microscope (Donachie *et al*., 2007): the ratio CFU/microscopy cell counts, when directly used to describe "cultivation bias", assumes even relative abundances between all microbial species or phylotypes that constitute the community under study. In contrast with this assumption, natural communities (be it microbial or not) more than often display uneven species abundance ranks in which few members dominate the assemblage followed by a tail of diverse, but more rare, species or phylotypes (Sogin *et al*., 2006; Gomes *et al*., 2010; Webster *et al*., 2010; Hardoim *et al*., 2013). Nevertheless, microbial ecologists have arguably overlooked this consistent pattern in the last three decades or so when referring to our capacity to cultivate microbial species in the environment (Donachie *et al*., 2007). What is the diversity of phylotypes that we can cultivate and how it compares with that unlocked with the use of cultivation-independent methods? Are we domesticating the few dominant members of the community or rather accessing preferentially the rare ones? Do cultured *vs*. uncultured communities from a given habitat display a high share of microbial phylotypes or are these communities rather discrepant? Comparatively few studies have solidly addressed these questions (see Donachie *et al*., 2007 and references therein; Zhu *et al*., 2008; Li *et al*., 2011; Sipkema *et al*., 2011 for notable examples), severely restricting our knowledge of the actual extent of the culturable microbiome diversity from a multitude of habitats worldwide.

Marine sponges are true reservoirs of microbial genetic and metabolic novelties. Indeed, the richness of bacteria associated with these animals has been shown to encompass 28 bacterial phyla, including formerly described and candidate phyla, based on phylogenetic

analyses of 16S rRNA gene sequences from clone libraries and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) bands (Simister *et al*., 2012c). This conspicuous taxonomic diversification suggests functional variety, and several possible roles have been proposed for sponge bacterial symbionts upon interaction with their hosts (Taylor *et al*., 2007; Webster and Taylor, 2012). Among them, the putative chemical defence enabled by biologically active compounds produced by sponge-associated bacteria is receiving considerable attention (Piel *et al*., 2004b; Hochmuth and Piel, 2009; Siegl and Hentschel, 2010; Thomas *et al*., 2010b; Hentschel *et al*., 2012). It is thus clear that cultivating bacterial symbionts from marine sponges might be highly rewarding from both the phylogenetic and biotechnology viewpoints. Although several attempts have been made to cultivate sponge-associated bacteria (Muscholl-Silberhorn *et al*., 2008; Sipkema *et al*., 2011; Esteves *et al*., 2013), we still lack a clear perspective of how abundant the cultured phylotypes might actually be *in situ* and of the bias induced by cultivation procedures in diversity surveys of the sponge-associated microbiome.

The present study addresses the extent to which culturing and distinct sample handling procedures affect the observed bacterial community diversity and structure in marine sponges. To this end, the hereafter called "direct", "indirect" (cultivation-independent) and "plate washing" (cultivation-dependent) sample processing methods were employed prior to metagenomic DNA extraction from replicate specimens of the marine sponges *Sarcotragus spinosulus* and *Ircinia variabilis*. The 16S rRNA gene was used as a phylogenetic marker in PCR-DGGE and massively parallel 454 tag-pyrosequencing profiling of bacterial communities in these hosts. We hypothesized that a cultivation procedure commonly used in marine microbiology research rather approximates otherwise species-specific sponge symbiont communities (Hardoim *et al*., 2012) by sharply reducing the observable diversity breadth of the marine sponge microbiome as enabled by cultivation-independent analyses. In addition, we applied in-tube fluorescent *in situ* hybridization (FISH) of dominant sponge-associated bacteria to obtain insights into their localization and distribution in the sponge body, and to test whether relative abundances depicted by 16S rRNA gene analyses correspond to cell abundances observed under the microscope.

Material and Methods

Sponge and seawater sampling. Sampling took place at Galé Alta, Armação de Pêra (37º 04' 09.6"N and 8º 19' 52.1"W) off the coast of the Algarve, South Portugal, in June 2010. Four specimens of *Sarcotragus spinosulus* and *Ircinia variabilis* Schmidt 1862 (Demospongiae, Dictyoceratida, Irciniidae) and four samples of surrounding seawater (1L each, about 1 m above sponge specimens) were collected in sterile Ziploc® bags by scuba diving at about 15 m depth. Samples were placed in cooling boxes, transported to the laboratory (c. 2 h), and immediately processed for downstream analyses as described below. Sponge species were identified based on macro- and microscopic morphological criteria coupled to molecular phylogenetic inference (Hardoim *et al*., 2012). For more details on sampling procedures, see Hardoim *et al*. (2012).

Cultivation-independent and –dependent total community DNA extraction. For each collected sponge specimen, three different procedures of sample processing were undertaken prior to total DNA extraction for bacterial community analysis. These procedures are hereafter called "direct", "indirect", and "plate washing" methods of sponge sample processing. In the former method, 0.25 g (fresh weight) of internal sponge body was directly used for DNA extraction. For the indirect method, 2.5 g (fresh weight) of internal sponge body was ground with sterile mortar and pestle in 25 mL of Calcium/Magnesium Free Artificial Seawater (CMFASW) (Garson *et al*., 1998). The suspension was well mixed to produce a sponge-derived homogenate, which was further vortexed in a 50 mL sterile polypropylene tube and centrifuged at 500 *g* for 2 min to decant sponge cells and debris. The supernatant was transferred to a new 50 mL polypropylene tube and centrifuged at 9000 *g* for 30 min. The resulting microbial pellet was then used for DNA extraction. For the latter method, aliquots of homogenates prepared as above were serially diluted and plated onto Marine Agar (MA, Carl Roth GmbH+Co, Germany). After incubation for 5 days at 25ºC, all colonies grown on a given plate were re-suspended in 3 mL of sterile artificial seawater (ASW) with the aid of a Drigalsky spatula and transferred into a 50 mL polypropylene tube. This procedure was performed for 6 MA plates per sample (triplicates of 10^{-4} and 10^{-5} dilutions), resulting in a 15 mL cultured cell suspension prepared for each sample. The suspension was thoroughly mixed and centrifuged at 9000 *g* for 30 min, after which the supernatant was discarded and DNA extraction was performed using the resulting cell pellet as starting material. As opposed to the

direct and indirect methods, which enable cultivation-independent analyses of the sponge-associated microbiome, the "plate washing" methodology constituted a means of addressing, without purifying and singularizing colonies, the pool of sponge-associated bacteria readily cultured on MA. For each of the collected sponge specimens, all sample material prepared through the three procedures described above was individually subjected to total community DNA extraction using the UltraClean® Soil DNA isolation kit (MO BIO, Carlsbad, CA, USA) according to the manufacturer's protocol. The same kit was used for DNA extraction from bulk seawater samples as explained by Hardoim *et al*. (2012).

PCR-DGGE fingerprinting of bacterial communities. A nested PCR-denaturing gradient gel electrophoresis (PCR-DGGE) approach was used to fingerprint the bacterial communities associated with both sponge species under the three methods of sample processing. The first reaction was performed as explained by Hardoim *et al*. (2012), using 0.6 µM of both primers (F27: 5'-AGAGTTTGATCMTGGCTCAG-3' and R1492: 5'-TACGGYTACCTTGTTACG ACTT-3') and 25 thermal cycles. The resulting amplicons (2.0 µL) were used as templates in a subsequent PCR for DGGE analysis using the primer pair F984-GC (5'-5'CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGAACGCGAAGAAC CTTAC-3') and R1378 (5'-CGGTGTGTACAAGGCCCGGGAACG-3') (Heuer *et al*., 1997). Reaction mixture and thermal cycling were as described by Hardoim *et al*. (2012) except for the concentration of primers (0.6 µM).

PCR-DGGE profiling was then performed using a PhorU-2 gradient system (Ingeny International, Goes, The Netherlands). Gel gradient, marker constituents, electrophoresis conditions, and staining procedures were described previously (Hardoim *et al*., 2012). The software GelCompar II 5.1 (Applied Maths, Kortrijk, Belgium) was used to process the PCR-DGGE fingerprints as explained by Hardoim *et al*. (2009), delivering a contingency table species *vs.* samples containing the relative abundances of all bands in each profile. This was used as input data for unconstrained (Principal Coordinate Analysis - PCA) and constrained (Redundancy Analysis - RDA) ordination analyses of PCR-DGGE fingerprints using Canoco for Windows 4.5 (Microcomputer Power, Ithaca, NY) as described in detail by Costa *et al*. (2006a). PCR-DGGE band richness and diversity (Shannon-Wiener index) values were obtained for each sample using the Canoco software. These values were tested for significant differences between sample groups using the software package R (Team, 2010). Both datasets

fitted the normal distribution and were subjected to factorial Analysis of Variance (ANOVA) coupled to Tukey´s Honestly Significant Differences (TukeyHSD) Test for pairwise comparisons.

Preparation of samples for pyrosequencing. A barcoded pyrosequencing approach was employed for in-depth analysis of bacterial community composition and diversity. To this end, total community DNA samples were cleaned with Sephadex G50 columns (GE Healthcare Bio-Science AB, Uppsala, Sweden) and quantified with Nanodrop1000 (ThermoScientific, Delaware, USA). The V4 hypervariable region of the 16S rRNA gene was PCR-amplified using the primer set described in the pyrosequencing pipeline of the Ribosomal Database Project (V4_titF: 5'- AYTGGGYDTAAAGNG-3' and V4_tit_R: 5'-TACNVRRGTHTCTAATYC-3', http://pyro.cme.msu.edu/pyro/help.jsp#intro), which generates amplicons of around 248bp in length. Two PCR reactions of 25 μL were prepared per sample, each containing 1 μL of template DNA (~20 ng), 1X Bioline buffer (Bioline, London, UK), 0.2 mM deoxynucleoside triphosphates (dNTPs), 3.75 mM $MgCl_2$, 0.1 mg mL^{-1} of Bovine Serum Albumin (BSA), 5% (vol/vol) dimethyl sulfoxide (DMSO), 0.2 μM of forward and reverse primers, and 2.5U of *BioTaq*™ DNA polymerase (Bioline, London, UK). Each sample was tagged by different 8-mer barcodes attached to the reverse primer. After initial denaturation at 94ºC for 4 min, 31 cycles of 30 sec at 94ºC, 45 sec at 44ºC, and 60 sec at 68ºC were performed, followed by a final extension for 10 min at 68ºC. The amplicons were delivered for pyrosequencing on a 454 Genome Sequencer GS FLX Titanium platform (Roche Diagnostics Ltd, West Sussex, UK) at BIOCANT (Biotechnology Innovation Center, Cantanhede, Portugal). Pyrosequencing data were deposited in the National Center for Biotechnology Information Sequence Read Archive (SRA) under the accession number SRP021445.

Pyrosequencing data processing. 454 pyrosequencing raw data were processed using AmpliconNoise (Quince *et al.*, 2011) for noise filtering (for instance, homopolymers), chimera removal, sequence sorting, and trimming of maximum sequence length (≥ 260 bp). Trimming of minimum sequence length (≤ 150 bp, thus maintaining sequences between 150 and 260 bp) was achieved using the Galaxy software (https://main.g2.bx.psu.edu/). The Quantitative Insights Into Microbial Ecology (QIIME) software package (Caporaso *et al.*,

2010) was then applied to the filtered dataset for operational taxonomic units (OTUs) determination and taxonomic assignment, followed by the generation of a samples *vs*. OTUs table using customized scripts (supplementary material). Briefly, OTUs were determined within QIIME at 97% sequence similarity using the UCLUST method (Edgar, 2010). Representative sequences of each OTU were picked using QIIME default parameters, and aligned employing Infernal (Nawrocki *et al*., 2009) against a STOCKHOLM file of pre-aligned sequences and secondary structures. Taxonomy assignment of representative sequences was performed using the BLAST taxonomy assigner method with the Greengenes 12_10 reference sequence database (http://greengenes.secondgenome.com/downloads/database/12_10). Based on the assigned taxonomy, a file was created with undesirable OTUs, that is, OTUs classified as chloroplasts, *Archaea* (1 OTU containing 2 sequences from seawater), and with no assigned taxonomy at the domain level ("no-blast hit"). These, along with OTUs represented by one single sequence (singletons), were removed from the samples *vs.* OTU table using a specific QIIME script (supplementary material). A final OTU biom-format table was then created and used as input data for downstream analyses.

Pyrosequencing data analysis

Bar charts were created within QIIME depicting phylum and class-level taxonomic assignments for each replicate sample as well as for sample categories in which replicates were pooled. To avoid biases related with sequencing depth, libraries normalized for size were created by randomly picking sequences that were further used for alpha- and beta-diversity metrics. Two sequence-depth thresholds were defined, 1.236 and 3.688 sequence reads, which allowed the comparison of (1) all four replicate samples of both sponge species under the three methods of sample processing plus triplicate seawater samples and (2) all the sponge-derived libraries, respectively. Shannon-Wiener diversity measures (Shannon, 1948b, a) and Chao1 richness estimates (Chao, 1984; Chao and Lee, 1992) were calculated for each sample within the QIIME environment (Caporaso *et al*., 2010). Multivariate analysis of community composition at the OTU level (97% sequence cut-off) was performed using the beta-diversity weighted Unique Fraction metric (UniFrac), which was applied to determine measures of (dis)similarity in bacterial community composition between samples (Lozupone and Knight, 2005). The distance matrix generated by weighted UniFrac was used for

multivariate analysis by means of Unweighted Pair Group Method with Arithmetic means (UPGMA) clustering (Felsenstein, 2004) and Principal Coordinate Analysis (PCoA) (Krzanowski and Krzanowski, 2000). PCoA results were seen with the KiNG software package v. 2.21 (Chen *et al*., 2009). Jackknife beta-diversity analysis was used to estimate the uncertainty in hierarchical clustering and PCoA plots of bacterial communities in all sample categories. 3D PCoA biplots were generated showing the 10 most abundant bacterial phyla for both sequence-depths and, when possible, abundant bacterial classes were also shown on ordination diagrams. Alternatively, to enable constrained multivariate analysis of the data with libraries not normalized for size, the OTU biom-format table was transformed into a tab-delimited table (see supplementary material for script), where OTU absolute abundances were converted into relative abundances. These were employed in constrained (RDA and Canonical Correspondence Analysis - CCA) ordination analyses with the software Canoco 4.5 (see Costa *et al*., 2006a for details**)** using Hellinger-transformed OTU abundance data. Similar statistical analyses were performed as well for an unfiltered dataset, disregarding chimera and noise removal procedures, following the tutorial available at the QIIME webpage with the minimum and maximum sequence length added to the "split_library.py" script. In this case, two normalized sequence-depths were also used, with 3.640 and 4.366 sequence reads for comparisons with and without seawater samples, respectively (supplementary material).

In order to determine the extent of share and exclusiveness of bacterial phylotypes across sample categories, an approach coupling OTU networking and Venn diagrams construction was employed. To this end, the 4 replicates within each sample category (n=7; seawater, *I. variabilis* with direct, indirect and plate washing methods, and *S. spinosulus* with direct, indirect and plate washing methods) were pooled. OTU networks and Venn diagrams were created (1) using all OTUs observed across the whole dataset and (2) taking only OTUs containing 50 or more sequences into account (i.e. "rare" phylotypes discarded). The inputs for OTU networks were generated using QIIME as described above (Caporaso *et al*., 2010) and analysed with Cytoscape version 2.8.3 (Smoot *et al*., 2011). The constructed networks approximate sample categories in the diagram space in accordance with their OTU community composition, functioning thus as an exploratory proxy to cluster analysis. To determine the number of shared and exclusive OTUs to each sample category in several category combinations, diagrams were generated with the program "Venndiagram" as implemented in the software package R using default parameters (Chen and Boutros, 2011).

To achieve this, the final OTU biom-format table obtained within QIIME was converted into a tab-delimited table (see supplementary material for script) where the list of OTU IDs found in each sample category was organized and used as input data.

In-tube Fluorescent in situ Hybridization coupled with Confocal Laser Scanning Microscopy (FISH-CLSM).

Small pieces of around 125 mm^3 were cut from the inner part of the sponge specimens. Fixation of the sponge pieces was performed by incubation in 4% paraformaldehyde (PFA) at 4ºC for 6 h. Samples were then washed three times with ice-cold 1X phosphate buffered saline (PBS) to remove residual PFA, covered with an ice-cold mix (1:1) of 1X PBS and 96% ethanol, and stored at -20°C until further used. Prior to hybridization, small sections of the animals were prepared with forceps and scissors under magnifying glass, which showed to be an efficient method to maintain sponge internal structure. For the detection of all bacteria, an equimolar mixture of Cy3-labelled EUB338, EUB338II and EUB338III probes was used (Amann *et al*., 1990, Table 3.1). Samples were further hybridized with ALEXA488- or Cy5-labeled FISH probes specific for *Acidobacteria* (SS_HOl1400, Meisinger *et al*., 2007), *Alphaproteobacteria* (ALF968, Neef, 1997), and *Gammaproteobacteria* (Gam42a, Manz *et al*., 1992, Table 3.1). These taxa were selected based on their predominance revealed by 454-pyrosequencing. An unlabelled betaproteobacteria-specific probe (Gam42a-competitor, Table 3.1) was applied as a competitor together with probe Gam42a to avoid unspecific hybridization signals (Manz *et al*., 1992). A negative control was carried out in parallel with a non-sense FISH probe (NONEUB, Wallner *et al*., 1993, Table 3.1) labelled with the same fluorochromes used in the positive FISH. All FISH probes were purchased from Sigma-Aldrich, Vienna, Austria. In-tube FISH was performed as described by (Cardinale *et al*., 2008)**,** with some modifications as follows. The concentration of lysozyme was 0.5 mg mL^{-1}, the ethanolic series was carried out with 50-80-96% ethanol solutions, hybridization was performed at 43ºC, washing buffer was pre-warmed at 44ºC, no incubation with the unspecific nucleic acid stain Sytox Blue took place, and the sections were mounted with SlowFade®Gold antifade reagent (Invitrogen, Molecular Probes). CLSM was carried out with a Leica TCS SPE confocal microscope (Leica Microsystems, Mannheim, Germany). Fluorescent dyes Cy3, Cy5 and ALEXA488 were excited with 532, 635 and 488 nm laser beams, respectively; the emitted light was detected in the range of 545-634, 648-731 and 500-

539 nm, respectively. To acquire the autofluorescence of the sponge internal structure (i.e. spongin fibres and filaments) an additional channel (excitation at 405 nm; emission range 425-462 nm) was applied. Photomultiplier gain and offset were optimised for every channel and field of view to improve the signal/noise ratio. Confocal stacks were acquired with a Leica ACS APO x 63 OIL CS objective (NA: 1.30) by applying a Z-step of around 0.6 µm. The software Imaris 7.0 (Bitplane, Zurich, Switzerland) was used to generate volume renderings and three-dimensional reconstructions.

Table 3.1. FISH probes used in this study

Name	Sequence (5´-3´)	Target	Formamide Concentration (%)[a]	Reference
EUB338[b]	gctgcctcccgtaggagt	Most bacteria	20	(Amann *et al.*, 1990)
EUB338II[b]	gcagccacccgtaggtgt	*Planctomycetales*	20	(Daims *et al.*, 1999)
EUB338III[b]	gctgccacccgtaggtgt	*Verrucomicrobiales*	20	(Daims *et al.*, 1999)
ALF968	ggtaaggttctgcgcgtt	*Alphaproteobacteria*	40	(Neef, 1997)
Gam42a	gccttcccacatcgttt	*Gammaproteobacteria*	40	(Manz *et al.*, 1992)
Gam42a competitor	gccttcccacatcgttt	*Betaproteobacteria*	40	(Manz *et al.*, 1992)
SS_HO1140	ttcgtgatgtgacgggc	*Acidobacteria*	20	(Meisinger *et al.*, 2007)
NONEUB	actcctacgggaggcagc	-	[c]	(Wallner *et al.*, 1993)

[a]The percentage of formamide for hybridizations at 43ºC.
[b]Used as a mixture of equimolar concentration.
[c]Used as negative control with the same formamide concentration as used for positive FISH

Results

PCR-DGGE fingerprinting of bacterial communities

The PCR-DGGE profiles of *S. spinosulus* obtained with direct and indirect processing methods were visually very similar, encompassing *c.* 8 dominant and several fainter bands consistently found in all samples. Conversely, much larger band variation was observed between *I. variabilis* fingerprints generated with both cultivation-independent methods. Profiles obtained for both sponge species via "plate washing" stood in sharp contrast with those generated by the former methods, revealing 3 dominant bands across all samples and a diverse range of fainter bands (Fig. 3.1a). Ordination analysis of PCR-DGGE band composition data and independent variables clearly discriminated between particular sample groups (Fig. 3.1b). The amount of variation explained by the canonical variables (processing

methods and sponge species) was 30.6%. The horizontal axis of the diagram, accounting for 58.8% of the explained variation, differentiated the fingerprints obtained via cultivation-independent methods from those obtained via plate washing. The vertical axis of the diagram grouped all *S. spinosulus* fingerprints generated by both cultivation-independent procedures and *I. variabilis* fingerprints obtained with the indirect method clearly apart from *I. variabilis* fingerprints retrieved with the direct method. In terms of community structure, Montecarlo permutation tests revealed that the independent variables "*S. spinosulus*" and "direct" and "plate washing" processing methods significantly altered band composition in PCR-DGGE profiles. Conversely, likely due to the large variation in band numbers across profiles, no significant differences in PCR-DGGE richness and diversity measures could be solely attributed to a given sponge species (including all methods) or method (including both species) (p>0.05). Pairwise comparisons of replicates within and between sample groups further revealed statistically similar PCR-DGGE band richness and diversity measures in most cases (Table S3.1).

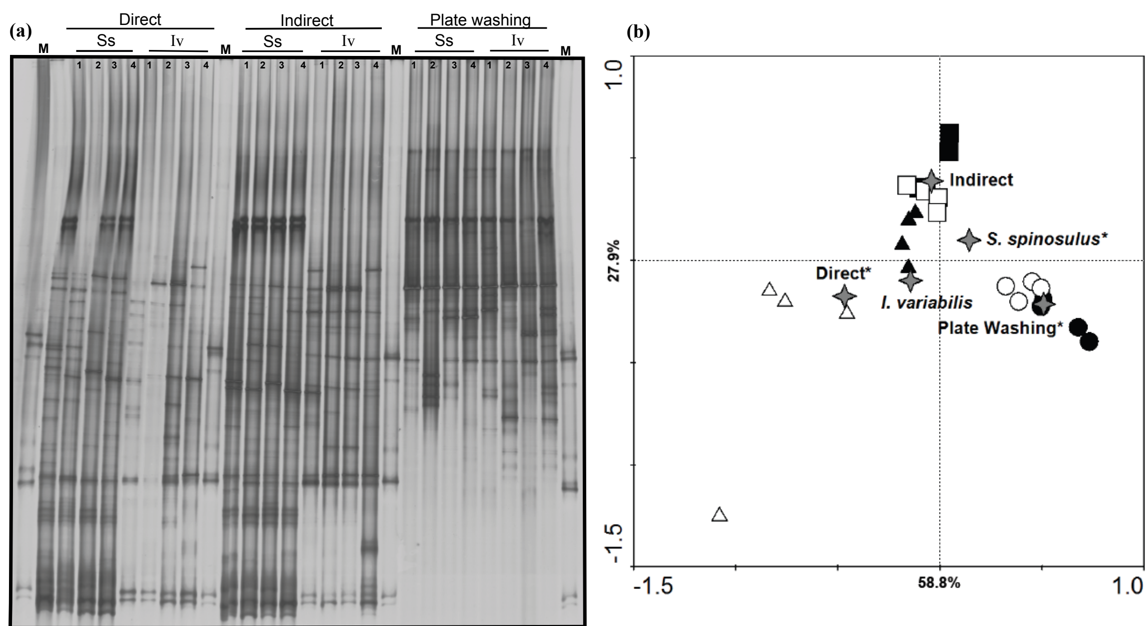

Figure 3.1. PCR-DGGE 16S rRNA gene fingerprinting of bacterial communities associated with the marine sponges *Sarcotragus spinosulus* (Ss) and *Ircinia variabilis* (Iv). Profiles obtained under the three methods of sample processing ("direct", "indirect", and "plate washing") are shown (a) along with their corresponding ordination by redundancy analysis (b). M: 16S rRNA gene marker used to control the DGGE run. Samples on the ordination diagram: ▲ - *S. spinosulus* direct processing, ■ - *S. spinosulus* indirect processing, ● - *S. spinosulus* plate washing processing, △ - *I. variabilis* direct processing, □ - *I. variabilis* indirect processing, ○ - *I. variabilis* plate washing processing. Labels displayed on the

diagram axes refer to the percentage variations of PCR-DGGE ribotypes - environment correlation accounted for the respective axis. The ‘‘star’’ symbol represents the centroid positions of the canonical variables (i.e., sponge species and sample processing methods) in the diagram. Variables that significantly ($p<0.05$) influenced bacterial community composition are highlighted with an asterisk.

454 pyrosequencing

Bacterial richness and diversity

Altogether, 237.773 16S rRNA V4-tag sequences that passed preliminary filtering on the 454 apparatus were obtained. After sequence trimming and further quality filtering through AmpliconNoise, 166.442 bacterial 16S rRNA gene V4-tag sequences were analysed (see supplementary material for analysis of all sequences). Filtered sequences were assigned 639 operational taxonomic units (OTUs) at a 97% similarity cut-off (Table 3.2). Considering all sequence libraries (depth=1.236 sequences/sample), higher bacterial richness was observed in seawater (129.5 ± 23.79 OTUs) than in sponge samples (Fig. 3.2a). Here, the latter were found to host from 43.2 ± 8.54 (*I. variabilis*, indirect) to 73.8 ± 4.93 (*S. spinosulus*, direct) bacterial OTUs under cultivation-independent methods (Fig. 3.2a). Bacterial richness in both sponge species increased significantly - and across all methods - when analyses were made with larger libraries (depth=3.668 sequences/sample). In this case, *S. spinosulus* and *I. variabilis* hosted averages of 95.8 ± 5.75 and 91.8 ± 7.99 bacterial OTUs/specimen under the direct method, and 89.9 ± 9.55 and 77.2 ± 15.89 OTUs/specimen under the indirect method, respectively (Fig. 3.2b). Regardless of the sequence depth, two major trends were found across the data. First, a drastic reduction in bacterial richness and diversity was observed for both sponge species because of culturing (Fig. 3.2a-d), with averages of 11.4 ± 2.52 and 14.0 ± 5.62 OTUs/specimen detected in *S. spinosulus* and *I. variabilis* via plate washing (Fig. 3.2a-d), with averages of 11.4 ± 2.52 and 14.0 ± 5.62 OTUs/specimen detected in *S. spinosulus* and *I. variabilis* via plate washing (Fig. 3.2b). This represented 11.9 and 15.25% of the bacterial OTU richness recorded for *S. spinosulus* and *I. variabilis*, respectively, under the direct method. Second, when only cultivation-independent methods were compared, contrasting results were obtained for each of the sponge species. Whereas no difference in richness and diversity values was found for *S. spinosulus* treated with the direct and indirect processing methods, handling of *I. variabilis* with the indirect method resulted in significant reduction of the observed bacterial richness and diversity in this host (Fig. 3.2a-d). Overall, Shannon

diversity indices were not affected by the size of libraries used in the comparisons (Fig. 3.2c,d), and seawater and *S. spinosulus* bacterial diversities obtained with culture-independent methods were of comparable magnitude (Fig. 3.2c) in spite of the significantly higher bacterial richness detected in seawater (Fig. 3.2a). Disregarding library size normalizations used in comparative analyses of significance, and thus using the full sequencing dataset, averages of *c.* 105 OTUs per sample were detected in both sponge species under the direct method, whereas *c.* 145 OTUs were recovered per seawater sample. Our sequencing effort was found to cover about 65, 81 and 84% of the estimated bacterial diversity in seawater, *S. spinosulus* and *I. variabilis* samples (estimates for sponge samples under the direct method), respectively, while the diversity found in the cultivatable bacterial fraction was fully covered. Richness and diversity measurements were also obtained from the non-filtered dataset, which revealed higher values for all sample categories when compared with filtered libraries (Fig. S3.1).

Table 3.2. Sequence data summary

Sample type	**Pre-processing method**	**n**	**454 filtering**		**454 + AmpliconNoise filtering**	
			Sequences	OTUs 97	Sequences	OTUs 97
S. spinosulus	Direct	4	35.198	739	29.174	199
	Indirect	4	34.988	705	29.227	184
	Plate washing	4	27.497	247	24.547	33
I. variabilis	Direct	4	29.503	671	24.222	215
	Indirect	4	25.899	541	22.686	225
	Plate washing	4	31.983	257	27.930	39
Seawater	n.a.	4	15.567	598	8.656	329
Total	n.a.	27	200.635	1.974	166.442	639

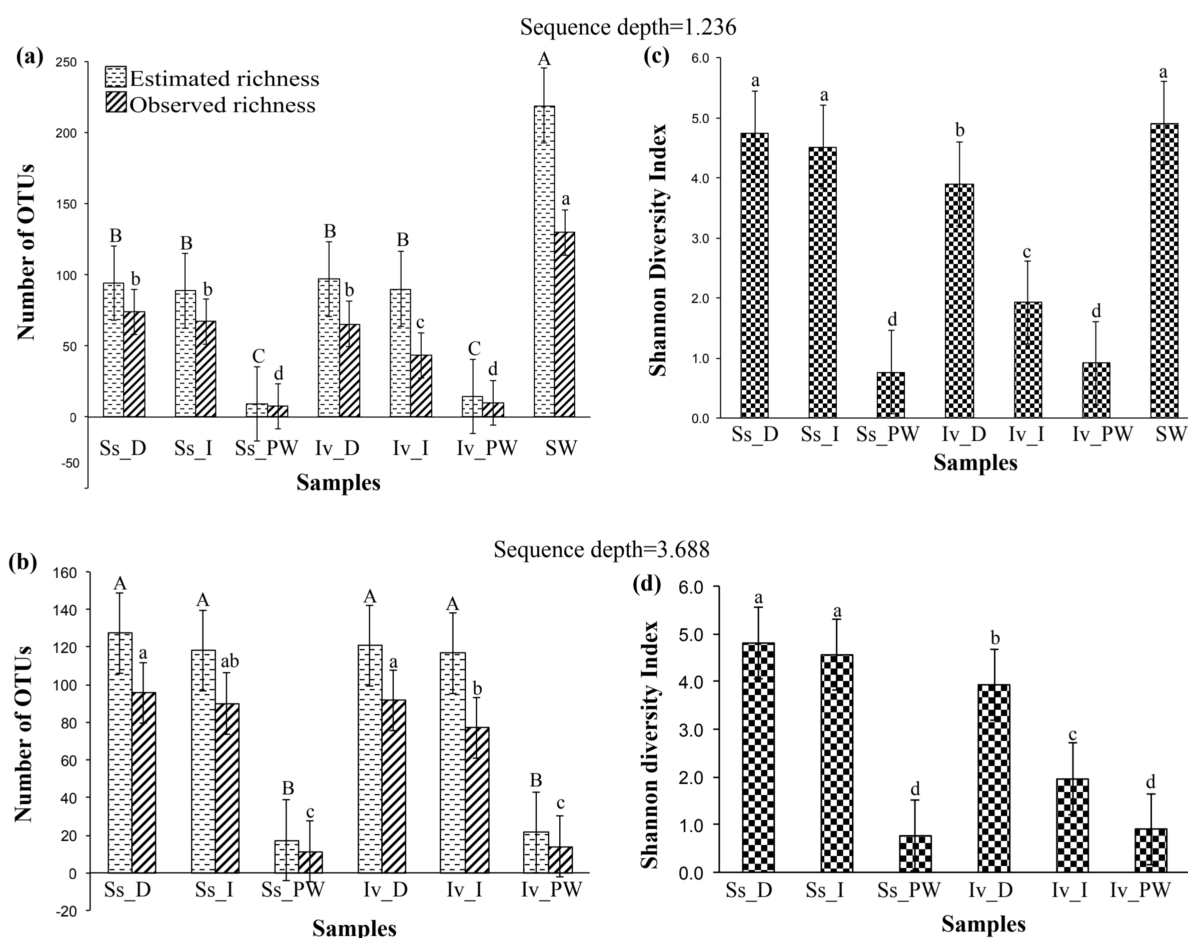

Figure 3.2. Quantitative analysis of bacterial richness and diversity in marine sponges and seawater. Observed and estimated (Chao1) richness measures [panels (a) and (b)] and Shannon diversity indices [panels (c) and (d)] of bacterial OTUs determined at 97% 16S rRNA gene similarity are displayed using normalized depths of 1.236 [includes seawater libraries, panels (a) and (c)] and 3.866 [sponge libraries only, panels (b) and (d)] sequences per library. Values on bars are means ± standard deviations of 4 replicates within each sample category, except for seawater where 3 replicates were used. Bars labelled with different letters represent statistically distinct sample categories in terms of richness and/or diversity values. In panels (a) and (b), uppercase letters define differences in estimated richness across sample categories, whereas lowercase letters define differences in observed richness. Ss_D: *Sarcotragus spinosulus* under the direct processing method, Ss_I: *S. spinosulus* under the indirect processing method, Ss_PW: *S. spinosulus* under the plate washing processing method, Iv_D: *Ircinia variabilis* under the direct processing method; Iv_I: *I. variabilis* under the indirect processing method, Iv_PW: *I. variabilis* under the plate washing processing method, SW: seawater.

Community composition at the phylum level

Contrasting results at the phylotype (OTU) level (Fig. 3.2), seawater presented much lower bacterial richness at the phylum level than sponges (Fig. 3.3a). Indeed, the bacterioplankton

community was massively dominated by *Bacteroidetes* (80 OTUs and 4.918 sequences) and *Proteobacteria* (156 OTUs and 3.401 sequences), which accounted for 57 and 40% of all sequences retrieved from seawater, respectively (329 OTUs and 8.656 sequences). Conversely, up to 21 bacterial phyla (438 OTUs in 157.786 sequences across all methods), among recognized and candidate taxa, could be detected in the sponge samples (Fig. 3.3a), with each individual specimen usually hosting 14 to 16 phyla (Fig. S3.2a). Bacterial communities cultured from both sponges consisted mainly of *Proteobacteria* (> 97% of all sequence hits), with dominance of the *Alpha*- and *Gammaproteobacteria* classes, and thus presented sharply reduced phylum diversification in comparison with uncultured sponge bacterial communities (Fig. 3.3a). The most dominant sponge-associated phyla were *Proteobacteria*, *Actinobacteria*, and *Acidobacteria* (Fig. 3.3a). They were retrieved from all replicates of both sponge species handled with cultivation-independent methods (Fig. S3.2a). Taking only these methods into account, *Proteobacteria* was the most diverse phylum in sponges with 188 OTUs detected in 16.377 sequences, whereas *Acidobacteria* and *Actinobacteria* harboured only 27 and 29 OTUs in totals of 30.346 and 26.931 sequences, respectively. Conspicuous differences in phylum relative abundances, without consequences for phylum OTU richness, could be observed between *I. variabilis* communities described by both cultivation-independent methods. To note in this regard was the higher abundance of *Acidobacteria* (direct: 15 OTUs and 5.041 sequences; indirect: 21 OTUs and 6.140 sequences) at the expense of much lower proportions of *Proteobacteria* (direct: 101 OTUs and 4.102 sequences; indirect: 103 OTUs and 1.239 sequences) and *Bacteroidetes* (direct: 21 OTUs and 3.424 sequences; indirect: 20 OTUs and 249 sequences) retrieved with the indirect *vs.* the direct method (Fig. 3.3a). Sample processing bias in *I. variabilis* could be further characterized among other, moderately dominant phyla such as PAUC34f (direct: 4 OTUs and 1.198 sequences; indirect: 2 OTUs and 41 sequences), *Chloroflexi* (direct: 18 OTUs and 411 sequences; indirect: 19 OTUs and 1.140 sequences), and AncK6 (direct: 2 OTUs and 1.051 sequences; indirect: 1 OTU and 61 sequences). Conversely, oscillations in phylum relative abundances because of sample handling were either much less pronounced or negligible in *S. spinosulus*. In this host, the most obvious differences involved enriched numbers of *Poribacteria* detected via the direct (6 OTUs and 3.726 sequences) against the indirect processing method (5 OTUs and 2.137 sequences), as also observed for *Acidobacteria* (direct: 15 OTUs and 4.947 sequences; indirect: 15 OTUs and 2.046

sequences), and the candidate phylum PAUC34f (direct: 4 OTUs and 3.759 sequences; indirect: 4 OTUs and 2.634 sequences).

Classes within the most abundant phyla were assigned when possible. Considering only the sponge samples and disregarding the sample processing methods and sponge species, the dominant bacterial classes were *Gammaproteobacteria* (113 OTUs and 14.270 sequences), *Alphaproteobacteria* (72 OTUs and 50.256 sequences), *Sphingobacteriia* (*Bacteroidetes*, 23 OTUs and 6.478 sequences), *Acidimicrobiia* (*Actinobacteria*, 21 OTUs and 26.820 sequences), *Deltaproteobacteria* (20 OTUs and 2.788 sequences), *Anaerolinea* (*Chloroflexi*, 14 OTUs and 2.994 sequences), and Sva075 (*Acidobacteria*, 8 OTUs and 26.599 sequences) (Figs. 3b and S2b). In seawater, *Flavobacteriia* (*Bacteroidetes*, 67 OTUs and 4.576 sequences), *Alpha*- (64 OTUs and 1.803 sequences), and *Gammaproteobacteria* (62 OTUs and 1.511 sequences) were the dominant classes (Figs. 3.3b and S3.2b).

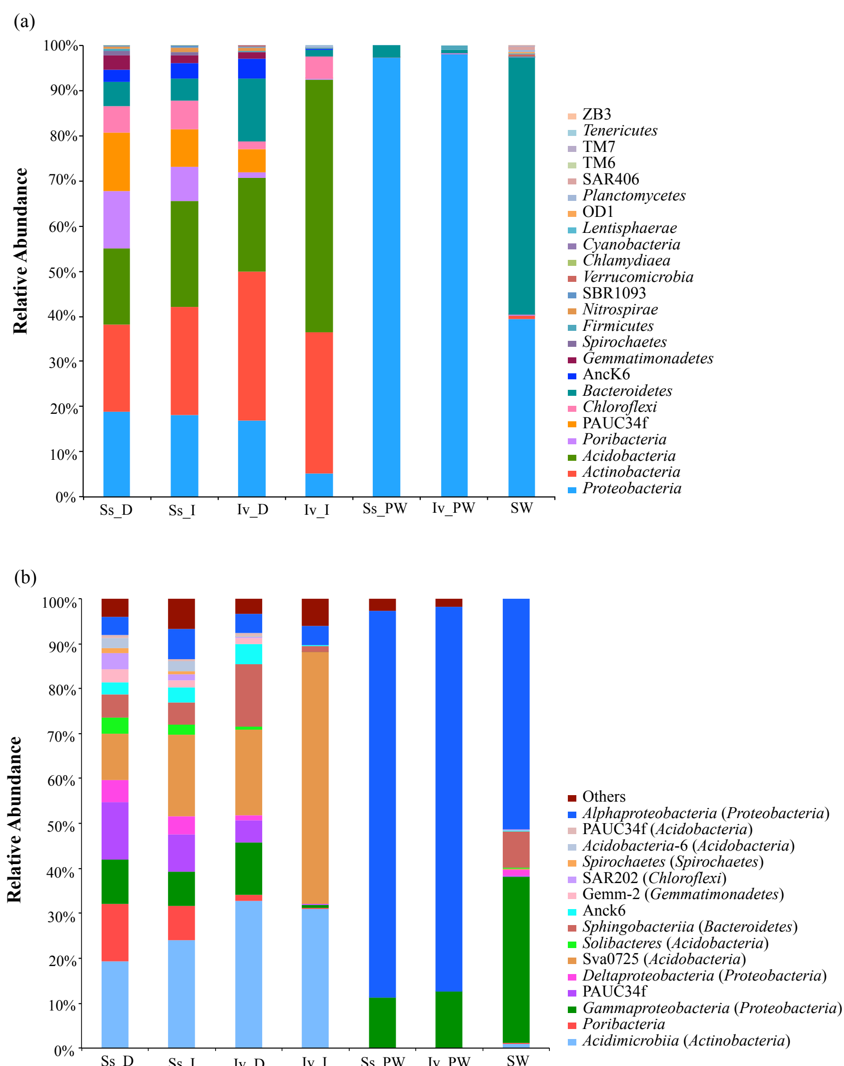

Figure 3.3. Phylum- (a) and class-level (b) bacterial community composition in marine sponges and seawater. Compositional data for *S. spinosulus* and *I. variabilis* handled with the direct, indirect and plate washing processing methods are shown. Results obtained using pooled replicate samples (n=4) within each sample category are displayed. Bacterial community composition in each replicate sample is shown as supplementary material to this article (Fig. S3.2). In panel (b) the top 15 bacterial classes are listed and all remaining taxa are labelled as "others". Labelling of sample categories is as described in legend to Fig. 3.2.

Specificities vs. commonalities: shared and exclusive OTUs

The distribution and extent of "shared" and "specific" bacterial phylotypes across each of the 7 sample categories inspected in this study was assessed using the cumulative number of OTUs detected in all replicates/sample category (Table 3.2) by means of OTU networking (Fig. 3.4a) and Venn diagram construction (Fig. 3.4b-g). As the positioning of samples in the network space reflects the distances between the surveyed communities based on their OTU

composition, this approach also functioned as a proxy to cluster analysis. Networking revealed that the majority of bacterial OTUs found in seawater were specific to this environment, placing bacterioplankton far apart from symbiotic communities (Fig. 3.4a). It also evidenced a closer similarity between *S. spinosulus* communities profiled with the direct and indirect processing methods in comparison with *I. variabilis* communities retrieved under the same procedures. Several bacterial OTUs exclusive to *I. variabilis* under either the direct or the indirect method could be clearly depicted, positioning those communities farther apart from one another than the corresponding *S. spinosulus* communities (Fig. 3.4a). Culturing seemed to approximate otherwise sponge species-specific bacterial communities as *I. variabilis* and *S. spinosulus* assemblages profiled via plate washing were very similar to one another yet displaying some specific OTUs (Fig. 3.4a). The abovementioned trends were further confirmed and accurately quantified using Venn diagrams (Fig. 3.4b-g). Strikingly, only 4 and 13 bacterial OTUs were common to all three methods of sample processing in *S. spinosulus* [*Alphaproteobacteria* (*Rhizobiales* and *Rhodobacterales*), *Gammaproteobacteria* (*Vibrionales*), and *Chloroflexi* (SAR202)] and *I. variabilis* [e.g. *Alphaproteobacteria* (*Rhizobiales*) and *Acidobacteria* (Sva0725)], respectively (Fig. 3.4b,c). The diagrams also demonstrated that both sponge species contained exclusive OTUs under each processing method, and that the proportion of OTUs specific to either the direct or the indirect method was higher in *I. variabilis* than in *S. spinosulus* (Fig. 3.4b,c). In *S. spinosulus*, 59 and 44 specific bacterial OTUs were assigned exclusively to the direct [OTUs classified as *Firmicutes* (*Clostridiales*) and *Betaproteobacteria* (*Burkholderiales*) as the most abundant] and indirect [OTUs classified as *Poribacteria* and *Alphaproteobacteria* (*Rhodobacterales*) as the most abundant] methods, respectively. For *I. variabilis* 93 OTUs specific to the direct method were found, with OTUs in the *Poribacteria*, *Gammaproteobacteria*, and *Deltaproteobacteria* (*Syntrophobacterales*) groups as the most representative, whereas 96 OTUs exclusive to the indirect method were observed, of which OTUs belonging to *Acidobacteria* (Sva0725), *Chloroflexi* (*Anaerolineae*), and *Planctomycetes* (*Planctomycetia*) were the most abundant. In spite of the reduced bacterial diversity retrieved from the plate washing method, this approach led to the unexpected detection of several “specific” OTUs not readily observable by cultivation-independent means. *S. spinosulus* contained 25 exclusive bacterial OTUs of which *Gammaproteobacteria* in the orders *Alteromonadales* and *Vibrionales* were the most abundant (Fig. 3.4b). *I. variabilis* encompassed 15 specific

bacterial OTUs among which *Gammaproteobacteria* (*Alteromonadales*) and *Alphaproteobacteria* (*Kiloniellales*) were the most abundant OTUs (Fig. 3.4c).

A further approach was used to detect OTUs assigned to each sponge species independently of processing methods. A total of 167 bacterial OTUs were common to *S. spinosulus* and *I. variabilis*, from which OTUs assigned with the *Alphaproteobacteria* (*Rhizobiales*), *Acidobacteria* (Sva0725), *Actinobacteria* (*Acidimicrobiia*), and *Poribacteria* clades revealed to be the most abundant (Fig. 3.4d), while 103 and 168 bacterial OTUs were found to be exclusively associated with *S. spinosulus* and *I. variabilis*, respectively. Interestingly, for each sponge species the most abundant "species-specific" OTU was affiliated with the *Bacteroidetes* phylum (class *Sphingobacteriia*).

A comparison between assigned OTUs from each sponge species obtained with the three processing methods and seawater revealed that as few as 4 bacterial OTUs were commonly found between *S. spinosulus* and seawater. These were classified as *Alphaproteobacteria* (*Rhizobiales* and *Rhodobacterales*), *Gammaproteobacteria* (*Vibrionales*), and *Chloroflexi* (SAR202) (Fig. 3.4e). Likewise, 5 bacterial OTUs were shared between *I. variabilis* and seawater, being classified as *Alphaproteobacteria* (*Rhizobiales*), *Acidobacteria* (Sva0275), *Gammaproteobacteria* (*Vibrionales*), *Actinobacteria* (*Rubrobacteria*), and *Firmicutes* (*Clostridia*) (Fig. 3.4f). These numbers rose to 32 bacterial OTUs shared by both sponge species processed via both cultivation-independent methods and seawater, from which the most abundant OTUs belonged to *Acidobacteria* (Sva0725), *Actinobacteria* (*Acidimicrobiia*), *Poribacteria*, and PAUC34f, even though they were very rare in seawater (< 10 sequences in each OTU) (Fig. 3.4g). OTU networking and Venn diagrams were also performed for OTUs with at least 50 sequences (i.e. "rare" phylotypes discarded). These analyses revealed that the vast majority of the thus inspected OTUs were commonly shared across samples; and that the number of specific OTUs in each sample category dramatically decreased (Fig. S3.3).

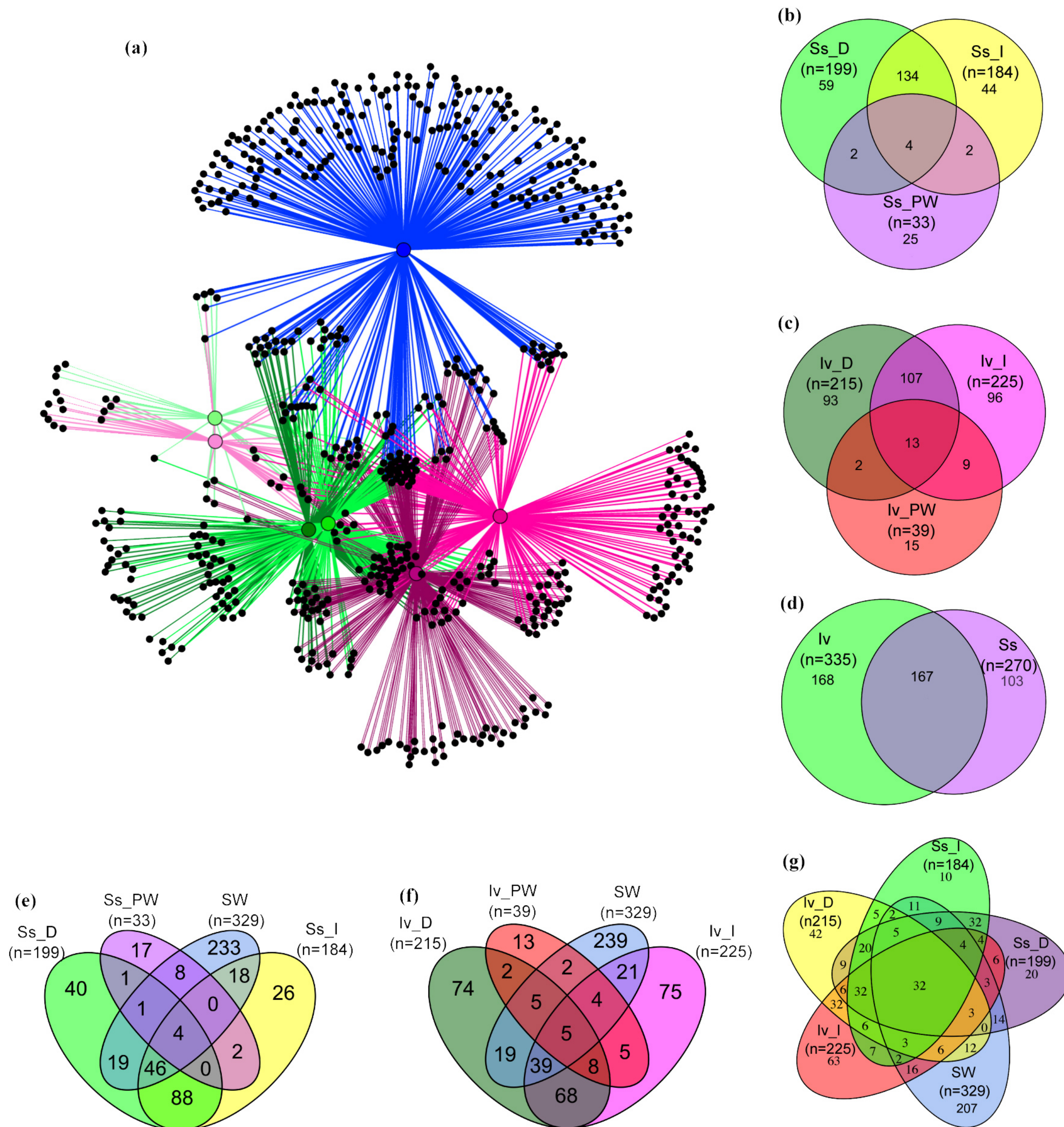

Figure 3.4. Bacterial OTU networking (a) and Venn diagrams (b-g). All OTUs detected across all analysed samples are included in the analysis. In the network diagram, gradients of green correspond to *S. spinosulus* samples (light green: plate washing processing, green: indirect processing, dark green: direct processing), and gradients of pink to *I. variabilis* samples (light pink: plate washing processing, pink: indirect processing, dark pink: direct processing) along with their corresponding OTUs. Seawater samples and their corresponding OTUs are shown in blue. Venn diagrams (b) and (c) enumerate OTUs within *S. spinosulus* and *I. variabilis*, respectively, shared by and exclusive to the different methods of sample processing. Diagram (d) shows all OTUs detected in, shared by and exclusive to *S. spinosulus* and *I. variabilis* by merging the data obtained with the three processing methods. Diagrams (e) and (f) depict the degree of exclusiveness and sharedness between each species (under each of the three processing methods) and seawater, whereas diagram (g) compares marine sponge and seawater microbiomes as determined by cultivation-independent methods only

(i.e. plate washing sponge processing excluded). Labelling of sample categories is as described in legend to Fig. 3.2. An equivalent analysis including only OTUs containing ≥ 50 sequences is shown as supplementary material to this article (Fig. S3.3).

Ordination of bacterial OTUs

For the first sequence threshold comparison, which included seawater samples in the analysis, two concise sample clusters could be clearly visualized by principal coordinate analysis (PCoA): (i) all seawater replicates, and (ii) all sponge specimens processed with the plate washing method (Fig. 3.5a). The remaining samples comprised all sponge replicates processed via cultivation-independent methods. Here, the higher similarity within *S. spinosulus* replicates treated with both direct and indirect methods could be depicted in contrast to the lower correspondence between methods and the more pronounced individual-to-individual variability detected for *I. variabilis* samples (Fig. 3.5a). After increasing the sequence depth by removing the seawater samples from the ordination analysis (Fig. 3.5b), the sharp dichotomy between sponge samples handled with cultivation-dependent and –independent methods persisted, whereas the divergence between *I. variabilis* specimens treated with direct *vs*. indirect cultivation-independent procedures became more apparent. For both sequence thresholds, the 3D plots were helpful in demonstrating the relationships between the samples and the most abundant bacterial phyla and/or classes, such as the prevalence of the *Alphaproteobacteria* in the culturable sponge fraction and the dominance of the *Bacteroidetes* classes *Flavobacteriia* and *Sphingobacteriia* in seawater and sponge samples, respectively (Fig. 3.5a,b).

Making use of constrained ordination analyses and measuring the relative contribution of each of the accounted independent variables (i.e. seawater, processing methods and sponge species) in determining compositional data variation, further trends could be clearly identified in our dataset (Fig. S3.4). First, CCA of the whole OTU data revealed that our independent variables could explain 46.8% of the total dataset variation. The discrepancies between cultivation-dependent *vs.* –independent methods and sponge (all methods included) *vs.* seawater samples accounted for 41.8 and 41.4% of the explained variability, respectively, surpassing the sole effects of the sponge species (*I. variabilis vs. S. spinosulus*, 12%) and cultivation-independent procedures (direct *vs.* indirect, 4.8%) on the total data variation. The resulting CCA ordination diagram sharply distinguished seawater, sponge samples handled with both cultivation-independent methods and sponge samples handled with the cultivation-

dependent method into three concise sample clusters (Fig. S3.4a). Patterns of species-specificity in sponge symbiont communities became more evident when only *I. variabilis* and *S. spinosulus* specimens characterized by cultivation-independent methods were contrasted (Fig. S3.4b). Here, constrained ordination analysis via RDA revealed that each sponge species held its own bacterial community. Further, whereas no distinct clustering of *S. spinosuslus* specimens handled with both cultivation-independent methods was observed, for *I. variabilis* the shape of the bacterial community was clearly influenced by the used methodology (Fig. S3.4b). Indeed, the use of a different cultivation-independent processing method was found to significantly determine compositional data of *I. variabilis* communities (Fig. S3.4c), whereas this was not the case for *S. spinosulus* specimens (Fig. S3.4d).

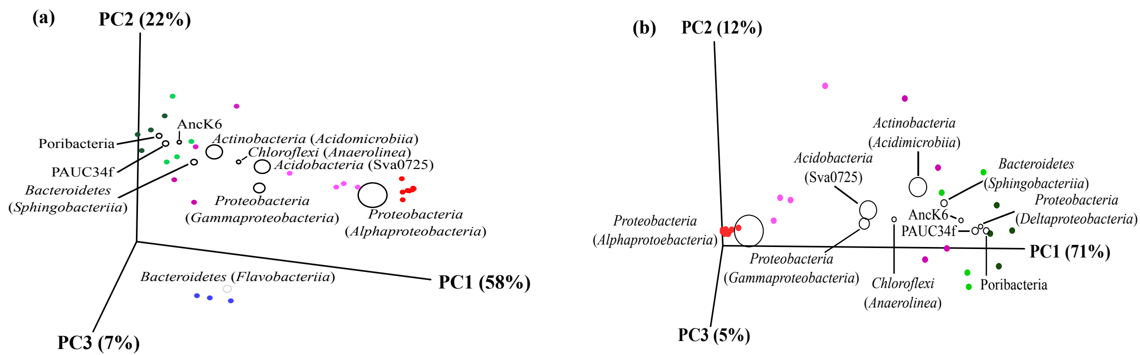

Figure 3.5. Principal coordinates analysis (PCoA) of bacterial community profiles at the phylotype (OTUs) level. Analyses embracing sponge and seawater samples (a) and sponge samples only (b) are shown, and were performed with normalized sequencing depths [1.236 seqs/sample in panel (a) and 3.668 seqs./sample in panel (b)] using the Unifrac metric. The ten most dominant bacterial taxa (at the phylum or class level) assigned to the OTUs detected in this study are plotted on both diagrams. Symbol sizes of bacterial taxa correspond to their respective, mean relative abundances across the whole dataset. The position of bacterial taxa in the ordination space is determined by the correlation between their relative abundances and the sample categories defined in this study. Coloured circles represent replicates within each sample category. Labelling of sample categories is as described in legend to Fig. 3.4, except for both sponge species under plate washing method, which is coloured in red. Alternatively, constrained canonical ordination was performed using normalization of abundance data (and not of sequence size) to determine whether sample categories significantly influence variation in 16S rRNA gene profiling by 454 pyrosequencing. These analyses are shown as supplementary material to this article (Fig. S3.4).

In tube FISH-CLSM

FISH-CLSM was used to localize and assess the abundance and distribution of *Alphaproteobacteria*, *Gammaproteobacteria,* and *Acidobacteria* cells at the microscale in *S. spinosulus* and *I. variabilis*. These groups were chosen because of their shared dominance in both species as revealed by 454 pyrosequencing. They also represent distinct scenarios regarding their prevalence across the sequencing dataset. Whereas *Proteobacteria* could be regarded as a generalist phylum presenting dominance in seawater and sponge communities (both cultured and uncultured), *Acidobacteria* constituted a specialized phylum with enhanced proportions within the uncultured sponge microbiome. The detected bacterial cells were mainly cocci found in between sponge cells, where colonies with high evenness could be observed, with no evidence for a taxon-dependent aggregation of bacteria within the sponge body. In all analysed samples, bacterial cells were seldom found on spongin filaments. The high abundance of detected cells precluded discrete counting of cell numbers, and taxon abundance data relative to total bacterial coverage was retrieved instead. The relative abundance of *Alphaproteobacteria* in both sponge species was similar: 27.14% in *S. spinosulus* (Fig. 3.6a,e) and 22.85% in *I. variabilis* (Fig. 3.6b,f), whereas the *Gammaproteobacteria* were more abundant in *S. spinosulus* (21.41%, Fig. 3.6c,e) than in *I. variabilis* (9.55%, Fig. 3.6d,f). Both *Alpha*- and *Gammaproteobacteria* were more abundant than *Acidobacteria* in *S. spinosulus* (9.09%, Fig. 3.6g). In *I. variabilis*, the relative abundance of *Acidobacteria* (6.20%, Fig. 3.6h) was similar to that of *Gammaproteobacteria* and lower than that of *Alphaproteobacteria*. These results contrasted relative abundance data obtained by 454-pyrosequencing. With this method, *Acidobacteria* sequence tags accounted for 16.91% and 20.68% of the total communities in *S. spinosulus* and *I. variabilis*, respectively, surpassing numbers obtained for *Alpha*- (3.90% for *S. spinosulus* and 4.27% for *I. variabilis*) and *Gammaproteobacteria* (*S. spinosulus* 9.78% and *I. variabilis* 11.57%) in both sponge species.

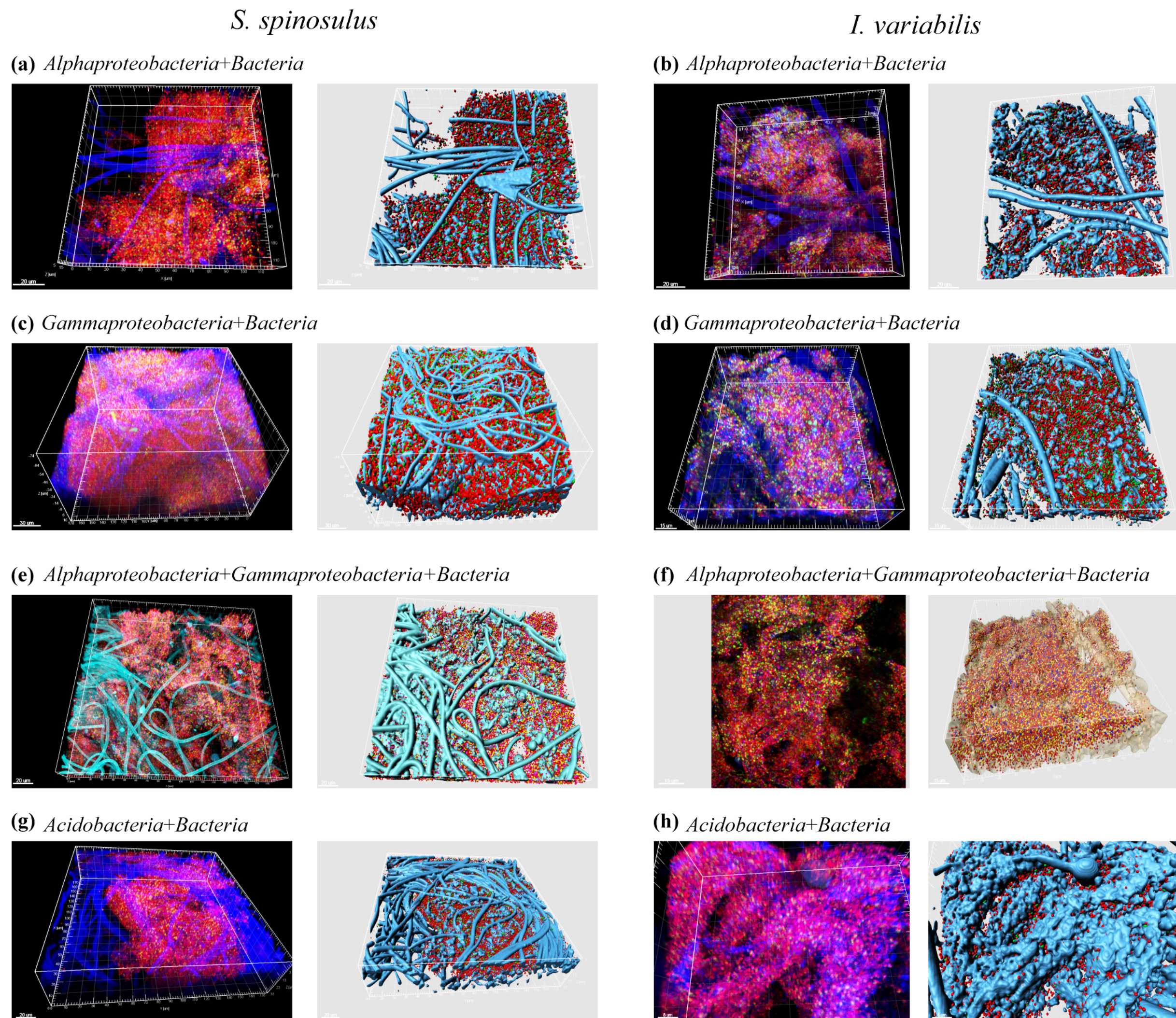

Figure 3.6. Confocal laser scanning microscopy images of fluorescent *in situ* Hybridization (FISH)-stained bacteria in *S. spinosulus* and *I. variabilis*. Volume rendering images (left in each panel) and their corresponding 3D reconstructions (right in each panel) are shown for hybridizations coupling the Cy3-labeled universal bacterial probe (red cells) to ALEXA488- or Cy5-labeled group-specific probes targeting the *Alpha*- and *Gammaproteobacteria* classes and the phylum *Acidobacteria*. When solely used in combination with the universal probe [panels (a)-(d), (g) and (h)], cells of these taxonomic groups appear as yellowish cells in the volume rendering images and as green objects in the 3D reconstructions. For co-hybridizations including bacterial, alpha and gammaproteobacterial probes [panels (e) and (f)], the latter two groups are represented by yellowish and pink cells, respectively, except in the 3D reconstruction image of *I. variabilis* (f), in which gammaproteobacterial cells appear in purple. Sponge background structure, vastly dominated by profuse spongin filaments, is shown overall in cyan or blue, except in panel (f) where it is displayed in semi-transparent brown.

Discussion

In this study, the effects of cultivation-independent (direct and indirect) and cultivation-dependent (plate washing) processing methods on the bacterial diversity and community composition associated with *S. spinosulus* and *I. variabilis* were assessed via PCR-DGGE and 454 pyrosequencing profiling. The results obtained from cultivation-independent methods with both approaches congruently indicated that fewer DGGE bands and bacterial OTUs dominated the microbiome associated with *S. spinosulus* and *I. variabilis* in comparison with a much larger number of more rare phylotypes, that is, fainter DGGE bands and less abundant bacterial OTUs. Further, both techniques revealed that the bacterial community associated with *S. spinosulus* was clearly distinct from the one observed in *I. variabilis*, and thus the bacterial microbiome was characterized as species-specific. Recently, few studies have suggested that the bacterial community associated with marine sponge was indeed species-specific (Webster *et al*., 2010; Lee *et al*., 2011; Erwin *et al*., 2012c; Hardoim *et al*., 2012; Schmitt *et al*., 2012), which contradicted earlier indications of microbiome conservation across distinct sponge hosts and geographical locations (Hentschel *et al*., 2002). Here, we demonstrated that the bacterial microbiome specificity was greatly determined by the rare symbionts (fainter DGGE bands and, especially, OTUs with <50 sequences), suggesting that previous evidences for conserved microbial community composition might have been influenced by the depth coverage of the molecular techniques employed.

The direct processing method has been widely used to disclose the microbial communities associated with marine sponges (Hentschel *et al*., 2002; Taylor *et al*., 2005; Hardoim *et al*., 2009; Hardoim *et al*., 2012) and in metatranscriptomic study (Radax *et al*., 2012), whereas the indirect processing method, where most of the sponge DNA cells can be eliminated, has been applied in metagenomic and metaproteomic surveys (Fieseler *et al*., 2006; Thomas *et al*., 2010a; Fan *et al*., 2012; Liu *et al*., 2012). In the present study, both approaches led to similar diversity and composition results for *S. spinosulus*. For instance, the bacterial diversity and richness did not significantly differ between methods for both sequence thresholds, the relative abundances of the dominant bacterial phyla and classes were alike in both approaches, similar community composition was observed with both procedures, and the majority of bacterial OTUs obtained for *S. spinosulus* under culture-independent methods were shared. Conversely, for *I. variabilis* the observed richness and diversity were significantly different between the direct and indirect processing methods. Likewise, each

culture-independent method favoured certain bacterial phyla and classes, which consequently affected their relative abundances under each method. Further, the numbers of OTUs shared by and exclusive to each method were equivalent in *I. variabilis*. These contrasting results might be related to the density of the collagenous filaments present in the mesohyl of these sponges, which is higher in *I. variabilis* making this species exceptionally tough and more difficult to tear or cut in comparison with other species in the Irciniidae family (Cook and Bergquist, 2002). This aspect might have led to differential efficiencies between both methods in regards with bacterial cell detachment and disruption from the sponge matrix leading to methodology-dependent, non-corresponding community structures retrieved from the same host. It appears thus that the congruence between direct and indirect methods might vary on a case-by-case manner and fast-paced fingerprinting of sponge communities by more traditional methods such as PCR-DGGE or T-RFLP may still function as a suitable tool to determine whether microbial cell enrichments usually prepared for metagenomics are likely to be representative of the actual community directly determined from the sponge body. Overall, these results indicate that the accuracy of distinct cultivation-independent methods in resembling the actual *in situ* sponge bacterial community might differ from species to species.

The bacterial microbiome disclosed for both sponge species under the plate washing processing method revealed reduced diversity and similar bacterial community structures when compared with culture-independent methods, which confirmed our hypothesis. However, unexpectedly, the cultivation method resulted in exclusive OTUs that could not be recovered with culture-independent methods. It has been previously demonstrated that some bacteria isolated with culture-dependent methods could not be detected with molecular tools (Donachie *et al*., 2007). For instance, from North Sea bacterioplankton only two bacterial species within *Proteobacteria* (*Alpha* and *Epsilon* classes) were commonly retrieved with culture-dependent and -independent approaches (Eilers *et al*., 2000). Similarly, both methods were used with the sponges *Suberites zeteki* (note by authors: unaccepted taxon) and *Gelliodes carnosa*, which revealed that *Gammaproteobacteria* was the only phylum recovered with both procedures (Zhu *et al*., 2008; Li *et al*., 2011). To overcome this bias, alternative strategies have enhanced the cultivability of the sponge associated microbiota. For instance, the simultaneous application of diverse cultivation procedures revealed that between 10 and 14% of the total bacterial diversity associated with the marine sponge *Haliclona* (gellius) sp. could be recovered (Sipkema *et al*., 2011), bearing testimony to the suitability of

using varying conditions to extend the sponge bacterial diversity captured with culture procedures. Another strategy was the development of growth chambers, which were incubated inside the sponge *Rhopaloeides odorabile* and showed that several of the obtained bacterial isolates were previously uncultivated or shared <97% 16S rRNA gene similarity with other cultured strains (Schupp, personal communication). Hence, investing in innovative cultivation methods can be very rewarding considering the high dominance of the as-yet-uncultured sponge associated bacteria. This might lead to the isolation of the essential bacterial symbionts, further extending our knowledge of the bacterial functioning in the host.

Conclusions

Culture-independent methods revealed that the bacterial communities associated with *S. spinosulus* and *I. variabilis* diverged from one another primarily due to the collective pool of less abundant phylotypes characteristic of each species, unveiling this way species-specific patterns of symbiont communities between phylogenetically close and sympatric marine sponges. Further, for *S. spinosulus* congruent results were obtained under culture-independent methods, whereas the opposite was observed for *I. variabilis*. This suggests that the ability to retrieve similar bacterial communities with distinct cultivation-independent methods might depend exclusively on the sponge species investigated and therefore differ from species to species. Conversely, under the plate washing method the bacterial community structure associated with both sponge species was remarkably similar, evidencing a large bias when sponge microbial communities are characterized via cultivation. The discrepancy between the bacterial community recovered with culture-independent and -dependent methods indicates that the most likely essential bacterial symbionts still remain recalcitrant to cultivation. This exemplifies the need of alternative culturing techniques to capture specific sponge symbionts and to unravell their function in the host microbiome.

Acknowledgments

This work was financed by the Portuguese Foundation for Science and Technology (FCT) through the research project PTDC/MAR/101431/2008. CCPH has a PhD fellowship granted by FCT (Grant No. SFRH/BD/60873/2009). JR Xavier research is funded by a FCT postdoctoral fellowship (grant no. SFRH/BPD/62946/2009).

Supplementary Material

Material and Methods

Pyrosequencing scripts for filtered dataset.

- Pyrosequencing analysis was carried out with the Quantitative Insights Into Microbial Ecology (QIIME, Caporaso *et al*., 2010)
- To assign the pyrosequencing sequences to samples a mapping file was created with six columns: SampleID, BarcodeSequence, LinkerPrimerSequence, ReversePrimer, Treatment and Description. The reverse primer column was added because the barcode was attached to it. This file was saved as pyro.txt and checked with the script: *check_id_map.py -m pyro.txt -o mapping_output -v*
- The pyro.sff file was fragmented by running the script: *process_sff.py -i* pyro.*sff -f -o,* which generated the file needed for ampliconnoise (pyro.sff.txt)
- AmpliconNoise (Quince *et al*., 2011) was used to remove chimera and noise, to trim the sequences maximum length ≥ 260 bp and to assign sequences to sample ID: *ampliconnoise.py -i pyro.sff.txt -m pyro.txt \ -o anoise_seqs --platform titanium -n 8 \--truncate_len 260*.
- Sequences ≤ 150 bp were removed from the fasta file created in the previously script. This was done with Galaxy software (https://main.g2.bx.psu.edu/). First the fasta file was uploaded (*Get data>Upload file from your computer>Browse>Execute*) and then sequences were trimmed (*FASTA manipulation>Filter sequences by length>minimum length>150*)
- The reverse primer was also removed from the fasta file produced in the previously step with *truncate_reverse_primer.py -f anoise_seqs_gal.fasta -m pyro.txt -o reverse_primer_removed/*; and the resulting file was renamed to *anoise_seqs_final.fna* and used for downstream analyses within QIIME pipeline
- Then the sequences were grouped to operational taxonomic units (OTUs) with 97% sequence similarity using the default method (UCLUST, Edgar, 2010): *pick_otus.py -i reverse_primer_removed/anoise_seqs_final.fna -o picked_otus/*
- Representative 97% OTUs were selected with *pick_rep_set.py -i picked_otus/anoise_seqs_final_otus.txt -f reverse_primer_removed/anoise_seqs_ final.fna -o rep_set.fna*

- Sequences were aligned with Infernal (Nawrocki *et al*., 2009) with *align_seqs.py -m infernal -i rep_set.fna -t seed.16s.reference_model.sto -o infernal_aligned/*
- Taxonomic assignment was carried out with the Greengenes database (http://greengenes.secondgenome.com/downloads/database/12_10) and the BLAST classifier: *assign_taxonomy.py -i rep_set.fna -r 97_otus.fasta -t 97_otu_taxonomy.txt -m blast*
- Alignment was filtered with some optimizations suggested by QIIME team, to know: -s suppressed the lanemask; -g allowed up to the value of gaps; -e entropy threshold in the script *filter_alignment.py -i infernal_aligned/rep_set_aligned.fasta -s -g 0.80 -e 0.10 -o filtered_alignment/*
- Phylogeny was generated with the alignment made previously: *make_phylogeny.py -i filtered_alignment/rep_set_aligned_pfiltered.fasta -o rep_phylo.tre*
- The OTU biom-format table was created with: *make_otu_table.py -i picked_otus/anoise_seqs_final_otus.txt -t blast_assigned_taxonomy/rep_set_tax_ assignments.txt -o otu_table.biom*
- Based on the taxonomic assignment of the sequences, a file (remove.txt) was made with all undesirable OTUs: chloroplast and sequences without assignment at domain level ("no blast-hit"); in addition singletons were also selected to be removed through the flag -n in *filter_otus_from_otu_table.py -i otu_table.biom -o otu_final.biom -n 2 -e remove.txt*
- The script *per_library_stats.py -i otu_final.biom* was used to check how many sequence reads were assigned to each library
- The biom-format of the OTU table was converted into a tab delimited table with *convert_biom.py -i otu_final.biom -o otu_final.from_biom.txt -b*, which was opened in an excel workbook and further used for the construction of Venn diagrams (Chen and Boutros, 2011) and Canoco 4.5 analyses
- An OTU network was created with *make_otu_network.py -m pyro.txt -i otu_final.biom -o otu_network* and analyzed in Cytoscape (Smoot *et al*., 2011)
- The summary of several taxa level was generated with *summarize_taxa_through_plots.py -i otu_final.biom -o taxa_summary -m pyro.txt*
- Before run the alpha diversity metric, the Shannon index of diversity was included with *echo "alpha_diversity:metrics shannon,chao1,observed_species, PD_whole_tree" >*

alpha_params.txt and then the alpha diversity script *alpha_rarefaction.py -i otu_final.biom -m pyro.txt -o wf_arare/-p alpha_params.txt -t rep_phylo.tre* was applied

- For beta diversity metrics, two sequence thresholds were selected allowing the comparison of (1) all libraries (n=1.236) and (2) only sponge-derived libraries (n=3.688) with the following script: *beta_diversity_through_plots.py -i otu_final.biom -o bdiv_even1236/ -t rep_phylo.tre -m pyro.txt -e 1236 --color_by_all_fields* and *beta_diversity_through_plots.py -i otu_final.biom -o bdiv_even3688/ -t rep_phylo.tre -m pyro.txt -e 3688 --color_by_all_fields*
- Jackknifed beta diversity was used to estimate the uncertainty in Principal Coordinate Analysis (PCoA) and hierarchical clustering for both sequence thresholds with the script: *jackknifed_beta_diversity.py -i otu_final.biom -t rep_phylo.tre -m pyro.txt -o wf_jack_blast -e 1.236* or *-e 3.688*
- In addition a bootstrapped tree was created with: *make_bootstrapped_tree.py -m wf_jack /weighted_unifrac/upgma_cmp/master_tree.tre -s wf_jack /weighted_unifrac /upgma_cmp/jackknife_support.txt -o wf_jack /weighted_unifrac/upgma_cmp /jackknife_named_nodes.pdf*
- To finalize a 3D plot was generated; for both sequence thresholds (n=1.236 and n=3.688); with the 10 most abundant phyla level with: *make_3d_plots.py -i bdiv_even1236/weighted_unifrac_pc.txt -m pyro.txt -t taxa_summary/otu_final_L3.txt --n_taxa_keep 10 -o 3d_biplot10* and *make_3d_plots.py -i bdiv_even3688/weighted_unifrac_pc.txt -m pyro.txt -t taxa_summary/otu_final_L3.txt --n_taxa_keep 10 -o 3d_biplot10*

Pyrosequencing scripts for unfiltered dataset.

- Pyrosequencing analysis was carried out with QIIME (Caporaso *et al*., 2010)
- A mapping file was generated with six columns: SampleID, BarcodeSequence, LinkerPrimerSequence, ReversePrimer, Treatment and Description and checked with *check_id_map.py -m pyro.txt -o mapping_output -v*
- *process_sff.py -i* pyro.*sff -f -o,* which generated the files needed for the next script
- *split_libraries.py -m pyro.txt -f pyro.fna -q pyro.qual -o split_library_output/ -b hamming_8 -l 150 -L 260 -z 'truncate_only'*

Chapter III

- *pick_otus.py -i split_library_output/seqs.fna -o picked_otus/*
- *pick_rep_set.py -i picked_otus/seqs_otus.txt -f split_library_output/seqs.fna -o rep_set.fna*
- *align_seqs.py -m infernal -i rep_set.fna -t seed.16s.reference_model.sto -o infernal_aligned/*
- *assign_taxonomy.py -i rep_set.fna -r 97_otus.fasta -t 97_otu_taxonomy.txt -m blast*
- *filter_alignment.py -i infernal_aligned/rep_set_aligned.fasta -s -g 0.80 -e 0.10 -o filtered_alignment*
- *make_phylogeny.py -i filtered_alignment/rep_set_aligned_pfiltered.fasta -o rep_phylo.tre*
- *make_otu_table.py -i picked_otus/seqs_otus.txt -t blast_assigned_taxonomy/rep_set_tax_assignments.txt -o otu_table.biom*
- *filter_otus_from_otu_table.py -i otu_table_blast.biom -o otu_final.biom -n 2 -e remove.txt*
- *per_library_stats.py -i otu_final.biom*
- *convert_biom.py -i otu_final.biom -o otu_final.from_biom.txt -b*
- *make_otu_network.py -m pyro.txt -i otu_final.biom -o otu_network*
- *summarize_taxa_through_plots.py -i otu_final.biom -o taxa_summary -m pyro.txt*
- *echo "alpha_diversity:metrics shannon,chao1,observed_species, PD_whole_tree" > alpha_params.txt* and then the alpha diversity script *alpha_rarefaction.py -i otu_final.biom -m pyro.txt -o wf_arare/-p alpha_params.txt -t rep_phylo.tre*
- *beta_diversity_through_plots.py -i otu_final.biom -o bdiv_even3640/* or *-o bdiv_even4366/ -t rep_phylo.tre -m pyro.txt -e 3640 or -e 4366 --color_by_all_fields*
- *jackknifed_beta_diversity.py -i otu_final.biom -t rep_phylo.tre -m pyro.txt -o wf_jack_blast -e 3640* or *-e 4366*
- *make_bootstrapped_tree.py -m wf_jack/weighted_unifrac/upgma_cmp/master_tree.tre -s wf_jack/weighted_unifrac/upgma_cmp/jackknife_support.txt -o wf_jack/weighted_unifrac/upgma_cmp/jackknife_named_nodes.pdf*
- *make_3d_plots.py -i bdiv_even3640/weighted_unifrac_pc.txt* or *-i bdiv_even4366/weighted_unifrac_pc.txt -m pyro.txt -t taxa_summary/otu_final_L3.txt --n_taxa_keep 10 -o 3d_biplot10* and

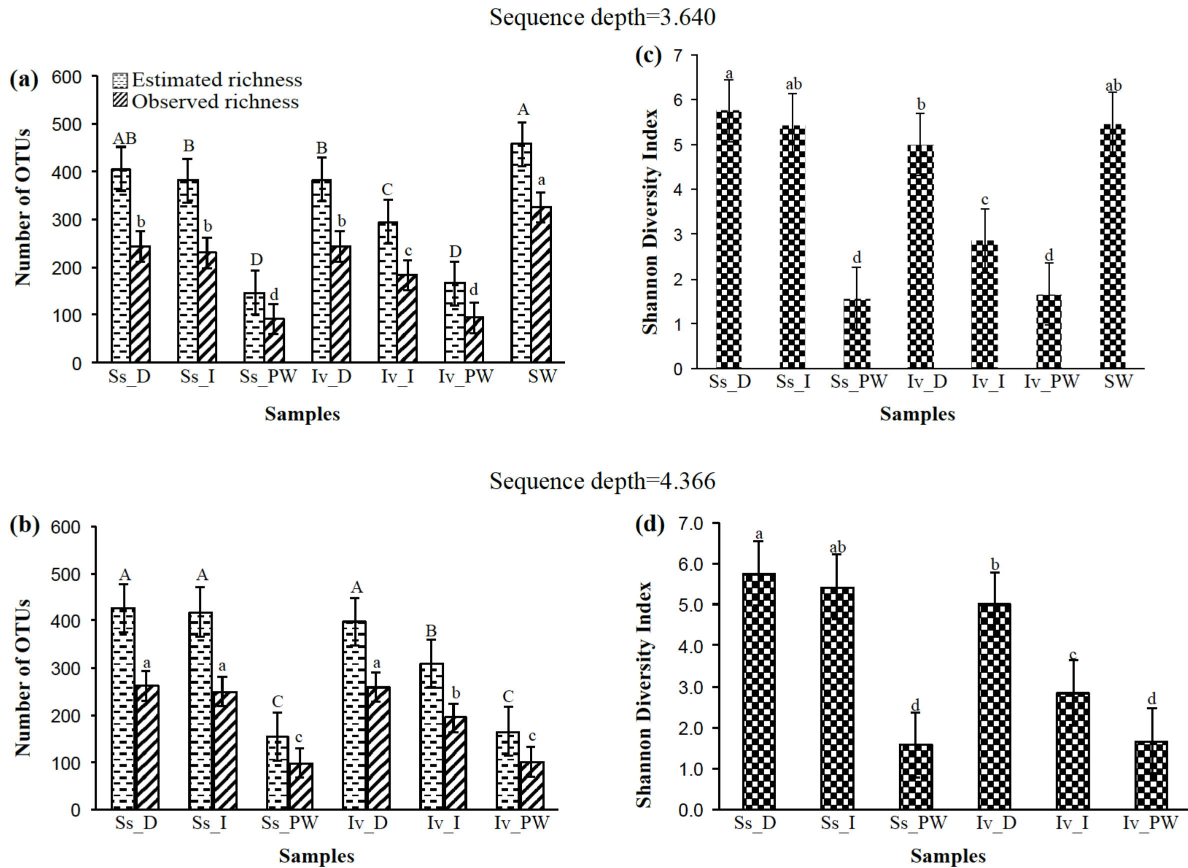

Figure S3.1. Observed and estimated richness measures and Shannon diversity indices of bacterial OTUs (97% cut-off) detected in *I. variabilis*, *S. spinosulus* and seawater, disregarding the de-noising methodology employed in this study. Figure details are the same as provided in legend to Figure 2, except for the sequence-depth: 3.640 [includes seawater libraries, panels (a) and (c)] and 4.366 [sponge libraries only, panels (b) and (d)] sequences per library.

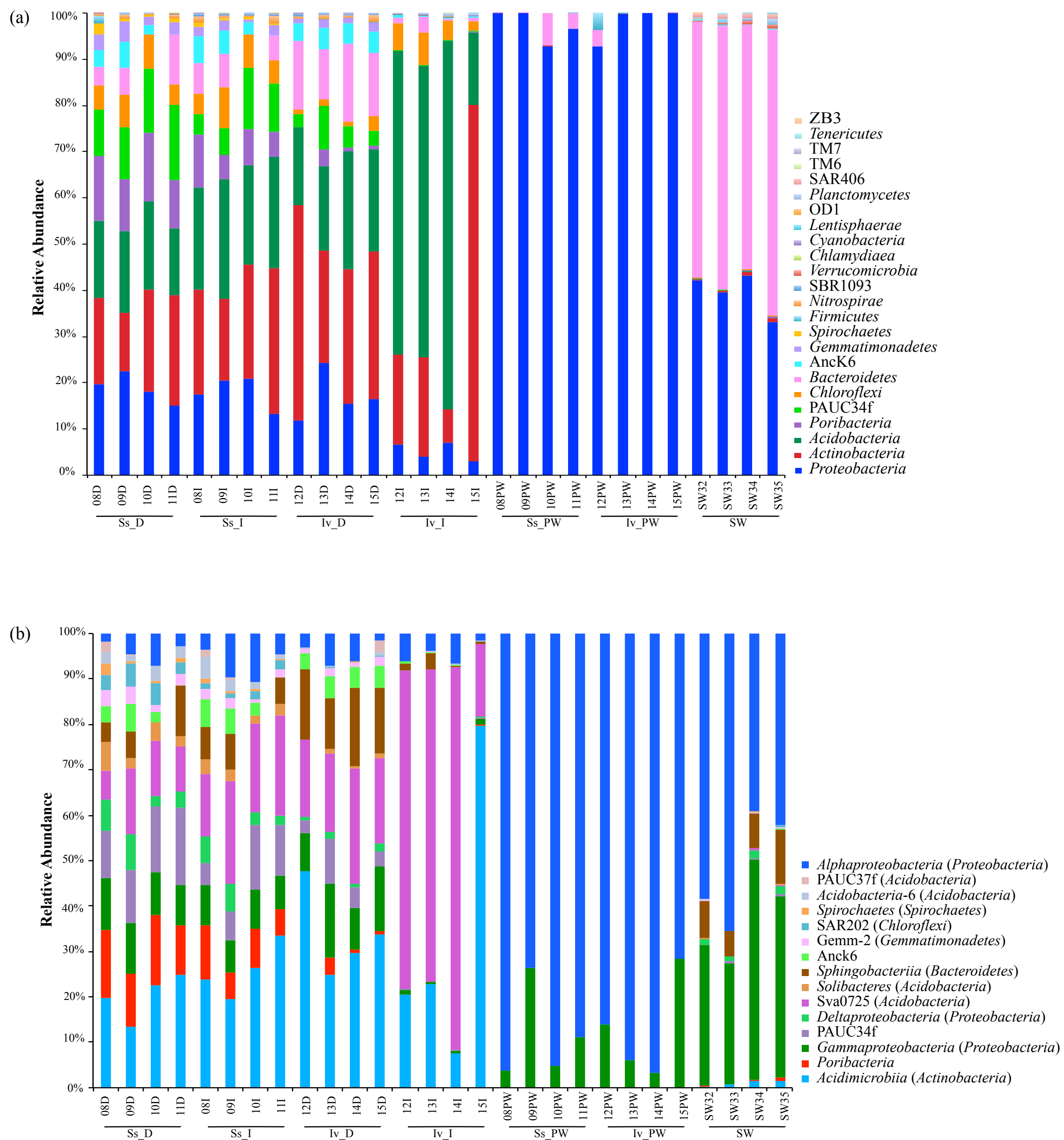

Figure S3.2. Phylum- (a) and class-level (b) bacterial community composition in each replicate sample of *S. spinosulus*, *I. variabilis* and seawater. Figure details are the same as provided in legend to Figure 3.3.

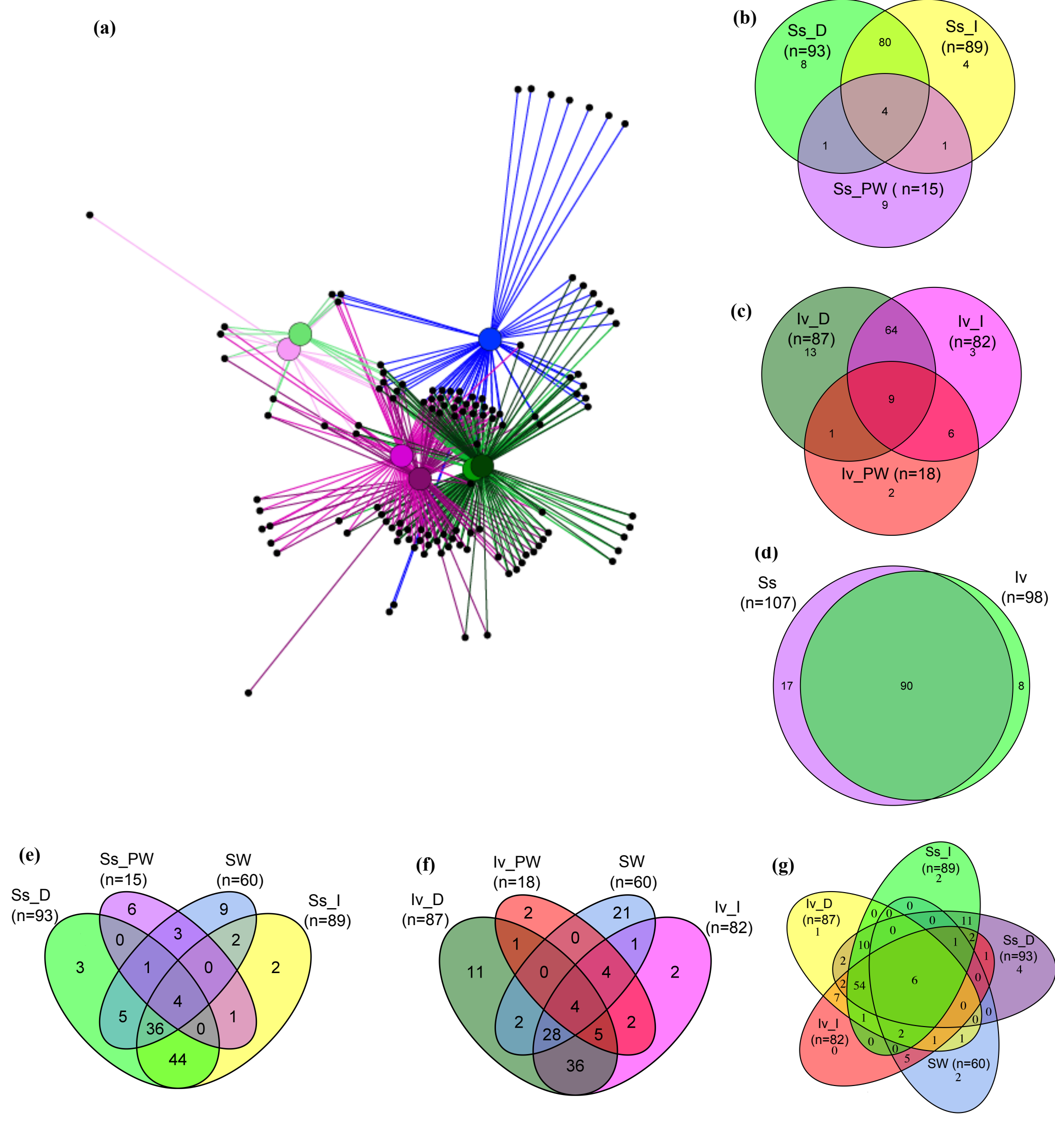

Figure S3.3. Network (a) and Venn diagrams (b-g) constructed for OTUs containing ≥ 50 sequences. Figure details are the same as described in legend to Figure 3.4.

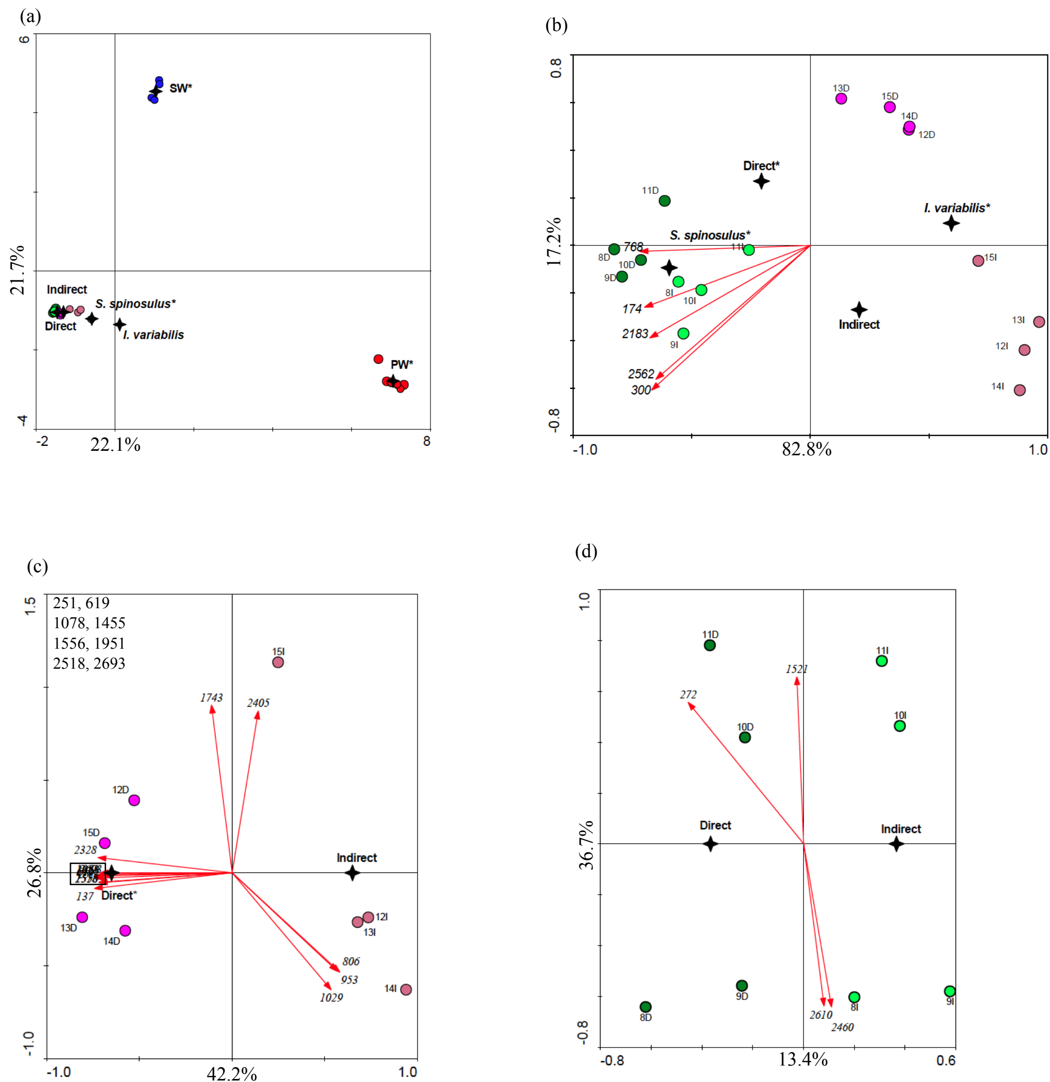

Figure S3.4. Constrained ordination analysis of bacterial communities profiled by 454 pyrosequencing. Ordination diagrams represent canonical correspondence analysis (CCA) embracing all sample categories (n = 7: seawater, *I. variabilis* with direct, indirect and plate washing methods, and *S. spinosulus* with direct, indirect and plate washing methods) (a); redundancy analysis (RDA) with both sponge species under the culture-independent methods (b); RDA with *I. variabilis* under culture-independent methods (c), and RDA with *S. spinosulus* under culture-independent methods (d). Labelling of sample categories is as described in legend to Fig. 4, except for both sponge species under the plate washing method, which are coloured in red. Arrows indicate OTUs that were specific or enriched in *S. spinosulus* or *I. variabilis* under direct or indirect methods. Labels displayed on the diagram axes refer to the percentage variations of OTU abundance - environment correlation accounted for the respective axis. The ''star'' symbol represents the centroid positions of the canonical variables (i.e., sponge species and sample processing methods) in the diagram. Variables that significantly (p<0.05) influenced bacterial community composition are highlighted with an asterisk.

Table S3.1. Statistical analysis of PCR-DGGE band richness (a) factorial ANOVA (b) TukeyHSD and diversity (c) factorial ANOVA (d) TukeyHSD measures.

(a)

	DF	SumSq	MeanSq	F value	Pr(>F)
Specie	1	105.5	105.5	1.041	0.322
Method	2	291.1	145.5	1.436	0.265
Specie:Method	2	1060.4	530.2	5.232	**0.017**
Residuals	17	1722.9	101.3		

(b)

Specie	diff	lwr	upr	p adj
Ss_Iv	4.287879	-4.578149	13.15391	0.3218551

Method	diff	lwr	upr	p adj
I-D	1.341991	-12.024182	14.70817	0.9641663
PW-D	8.091991	-5.274182	21.45817	0.2922774
PW-I	6.75	-6.162951	19.66295	0.3928006

Species:Method	diff	lwr	upr	p adj
Ss_D-Iv_D	19.916667	-4.677965	44.511298	0.15321
Iv_I-Iv_D	7.916667	-16.677965	32.511298	0.9014505
Ss_I-Iv_D	16.916667	-7.677965	41.511298	0.2873625
Iv_PW-Iv_D	25.666667	1.072035	50.261298	**0.0380796**
Ss_PW-Iv_D	12.666667	-11.927965	37.261298	0.5810021
Iv_I-Ss_D	-12	-34.770204	10.770204	0.5581326
Ss_I-Ss_D	-3	-25.770204	19.770204	0.9979853
Iv_PW-Ss_D	5.75	-17.020204	28.520204	0.962147
Ss_PW-Ss_D	-7.25	-30.020204	15.520204	0.905361
Ss_I-Iv_I	9	-13.770204	31.770204	0.799518
Iv_PW-Iv_I	17.75	-5.020204	40.520204	0.1805367
Ss_PW-Iv_I	4.75	-18.020204	27.520204	0.9833062
Iv_PW-Ss_I	8.75	-14.020204	31.520204	0.8169555
Ss_PW-Ss_I	-4.25	-27.020204	18.520204	0.9898365
Ss_PW-Iv_PW	-13	-35.770204	9.770204	0.4760904

(c)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Specie	1	0.1064	0.1064	2.282	0.1493
Method	2	0.0358	0.0179	0.384	0.6869
Specie:Method	2	0.7167	0.3584	7.683	**0.0042**
Residuals	17	0.793	0.0466		

(d)

Specie	diff	lwr	upr	p adj
Ss-Iv	0.1361803	-0.0540256	0.3263863	0.1492673

Method	diff	lwr	upr	p adj
I-D	0.05516365	-0.2315855	0.3419128	0.8753295
PW-D	0.09771765	-0.1890315	0.3844668	0.6632777
PW-I	0.042554	-0.234472	0.31958	0.9183697

Specie:Method	diff	lwr	upr	p adj
Ss_D-Iv_D	0.5325888	0.004951765	1.0602259	**0.0471701**
Iv_I-Iv_D	0.2173451	-0.310291985	0.7449822	0.7718312
Ss_I-Iv_D	0.4822008	-0.045436235	1.0098379	0.0843655
Iv_PW-Iv_D	0.5518168	0.024179765	1.0794539	**0.0375465**
Ss_PW-Iv_D	0.2328371	-0.294799985	0.7604742	0.7200901
Iv_I-Ss_D	-0.3152437	-0.803740753	0.1732533	0.3496245
Ss_I-Ss_D	-0.050388	-0.538885003	0.438109	0.9993781
Iv_PW-Ss_D	0.019228	-0.469269003	0.507725	0.9999946
Ss_PW-Ss_D	-0.2997517	-0.788248753	0.1887453	0.4010778
Ss_I-Iv_I	0.2648558	-0.223641253	0.7533528	0.5293855
Iv_PW-Iv_I	0.3344718	-0.154025253	0.8229688	0.2916672
Ss_PW-Iv_I	0.015492	-0.473005003	0.503989	0.9999982
Iv_PW-Ss_I	0.069616	-0.418881003	0.558113	0.997079
Ss_PW-Ss_I	-0.2493638	-0.737860753	0.2391333	0.5896977
Ss_PW-Iv_PW	-0.3189798	-0.807476753	0.1695173	0.337834

CHAPTER IV

Tackling the specificity of the marine sponge microbiome: a biogeographical approach

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Abstract

The ability of a given sponge host to maintain a core microbiota across biogeographical gradients has been poorly approached, in spite of being integral to our understanding of the marine sponge holobiont, its resilience and degree of specificity. Here, we used next generation sequencing technologies to determine the extent to which the shape of the sponge-associated microbiome is driven by the host organism and its biogeographical background, and to define the complementary role of seawater and marine sediments as "seed banks" of microorganisms, which make up its composition. To this end, specimens of the genera *Ircinia*, *Sarcotragus* (Irciniidae) and *Spongia* (Spongiidae) were sampled at the Algarve coast, the Madeira Island, and the Azores archipelago and subjected to 454 pyrosequencing profiling of bacterial 16S rRNA genes. After quality filtering, 232.251 sequences were obtained, representing 5.601 different OTUs. Sponge microbiomes presented sharply contrasting composition of bacterial phyla in comparison with those present in seawater and sediments. The predominant taxonomic groups in *Sarcotragus* and *Spongia* were *Acidobacteria* and *Actinobacteria*, followed by *Proteobacteria*, *Poribacteria*, PAUC34f, *Bacteroidetes* and *Chloroflexi*. In *Ircinia* spp., the *Chloroflexi* displayed dominance at the Madeira site but was found to be a minority taxon at the Algarve and Azores sites, where *Proteobacteria*, *Acidobacteria,* and *Actinobacteria* were dominant groups at varying degrees. Regarding the biogeographical patterns exhibited by the studied communities at the approximate species (i.e., OTU) level, only the seawater samples were clearly dependent of the sampling location. Sediments and sponges clustered otherwise together independently of the sampling site they were collected. Our results suggest that the composition of the sponge associated microbiome is shaped by an intricate and cooperative interaction between the host organism and its environmental background. Further, compelling evidence for the recognition of marine sediments as the pivotal environmental source of highly specialized and abundant sponge-associated *Acidobacteria* was found.

Introduction

Biogeography is a scientific field that addresses the ultimate causes driving the range of occurrence and patterns of abundance of organisms in nature. Research interest in microbial biogeography is not new, as demonstrated by the influential postulate of Becking "Everything is everywhere, but the environment selects" (Becking, 1934) of almost 80 years ago. Nevertheless, a much renewed interest and research effort surfaced recently thanks to new advances in molecular biology techniques (Martiny *et al*., 2006; Fierer, 2008; Hanson *et al*., 2012). These advances allow us to deeply interrogate microbial life in nearly all environments on Earth even if we cannot cultivate the microbes. Becking´s postulate implies that different contemporary environments maintain distinctive microbial assemblages, but also suggests that microorganisms have such enormous dispersal capabilities that they could rapidly erase the effects of past evolutionary and ecological events (Martiny *et al*., 2006). Both dispersal limitation and environmental selection are the main processes shaping free-living microbial communities (Martiny *et al*., 2006; Fierer, 2008), but host-related factors may also play a major role in structuring symbiotic communities (Pita *et al*., 2013a; Pita *et al*., 2013b). At present, evidence suggests geographical isolation plays a crucial role in driving microbial evolution and community structure in nature. For instance, high rates of bacterial endemism at the strain level are observed in soils geographically separated from each other (Cho and Tiedje, 2000). Likewise, microbial distributions among hot springs that share the same chemistry are better explained by geographical distance rather than by local selection of cosmopolitan and genetically similar microorganisms (Papke *et al*., 2003; Whitaker *et al*., 2003). In this context, the study of microbial symbionts in animal hosts of restricted mobility is of particular interest given their proposed contribution to host fitness and survival. Recent research on microbial symbionts of benthic marine invertebrates such as sponges (Porifera) and corals (Cnidaria) has unveiled high abundance and diversity of distinct prokaryotes, mainly within the domain *Bacteria*, which are proposed to benefit the fitness of their hosts (Rosenberg *et al*., 2007; Taylor *et al*., 2007; Webster and Taylor, 2012). Microorganisms often act as beneficial symbionts, providing their hosts with readily-available nutrients, protection from disease, chemical defence and removal of metabolic by-products. Thus, the notion of a collective holobiont has emerged, representing the host and all its associated symbionts (Rosenberg *et al*., 2007). A flexible hologenome – that is, the pool of genomes that

make up the holobiont – has been suggested as the genomic unit under natural selection which would drive the plasticity and resilience of the holobiont in nature.

The dawn of interactions between prokaryotes and higher organisms most likely lies in the origin of sponges and their contemporary metazoans, dating back to 600 million years ago (Love *et al*., 2009). Hence, the study of prokaryotic consortia in marine sponges has the potential to unveil unknown, ancient histories and modes of host-symbiont relationships. As typical filter-feeders, marine sponges are capable of pumping many thousands of litres of water a day (Hentschel *et al*., 2006). This ability makes sponges highly efficient "samplers" of aquatic microbial communities. Indeed, microbes may comprise up to 40% of the sponge biomass (Hentschel *et al*., 2006). Although a high proportion of such microorganisms makes part of the sponge diet, diverse, abundant, and "resident" bacterial communities are now known to populate the mesohyl layer - an intercellular matrix of the sponge body - of marine sponges (Taylor *et al*., 2007; Webster and Taylor, 2012). Thus, a selective process favouring particular bacteria, involving microbe-sponge interactions, is likely to occur. Increasing research interest in the sponge-associated microbiota has surfaced in the past few years due to the possible roles it plays in host defence, health, and disease (Taylor *et al*., 2007; Luter *et al*., 2010; Webster and Taylor, 2012), with substantial emphasis on the production, by microbial symbionts, of biologically-active secondary metabolites of pharmaceutical interest and their putative ecological functions (Hochmuth and Piel, 2009).

Only recently have surveys addressed microbial diversity in marine sponges using the sheer force of next generation sequencing (NGS) technologies, thereby circumventing the pitfalls of more traditional molecular assessments (Lee *et al*., 2011; Webster *et al*., 2010a). These studies revealed tremendous bacterial diversity in varied sponge hosts. Importantly, NGS could be used to demonstrate that two modes of transmission of bacterial symbionts in sponges - environmental acquisition from seawater and vertical transmission through sponge larvae – play a substantial role in shaping the sponge-associated microbiome (Webster *et al*., 2010), and indications for sponge-specific symbiont composition have been reported (Lee *et al*., 2011). However, such a perception of specificity derives from comparisons between phylogenetically distinct host sponges, sampled either in relatively confined areas (Lee *et al*., 2011) or sporadically spread across several locations in different oceans (Schmitt *et al*., 2012). The ability of a given sponge host, here understood as one single genetic unit, to maintain a core microbiota across biogeographical gradients has not yet been consistently

addressed, in spite of being integral to our understanding of the marine sponge holobiont, its resilience and degree of specificity.

Here, we address the hypothesized sponge-specific character of the marine sponge microbiome using a biogeography-driven experimental design. We determine the extent to which the shape of the sponge-associated microbiome is driven by the host sponge and its biogeographical background, and define the complementary roles of seawater and marine sediments as "seed banks" of microorganisms, which make up this microbiome. Specificity is defined as the maintenance, by a given host species, of an identifiable and exclusively sponge-derived core microbiota, regardless of the microbial composition of the environmental surroundings. Our target hosts were sponge species proven to bear specific bacterial communities when compared to one another in a given sampling location (Hardoim *et al*., 2012), in addition to other phylogenetically-related sponge taxa. Diversity and composition of bacterial symbionts in these model organisms were deciphered in three geographical settings – Algarve, Madeira Island and Azores Archipelago – by means of NGS. This work is the first biogeographical study of bacterial communities in coastal seawater, sediments and biotechnology-relevant marine sponges across a northeast-mid Atlantic transect encompassing the shores of continental Europe and of the underexplored oceanic archipelagos of Madeira and Azores.

Material and Methods

Sampling. Sponge specimens, surrounding seawater, and sediment were sampled between August and September of 2011 at three locations: the Algarve coast (Galé Alta, Armação de Pêra); the Madeira Island, located approx. 590 miles off the SW coast of Portugal; and São Miguel island in the Azores archipelago, located at approximately 970 miles from the Portuguese mainland. These sampling sites represent divergent geological and biogeographical backgrounds. The sponge specimens collected were *Sarcotragus* spp. and *Ircinia* spp. (Demospongiae, Irciniidae) from the Algarve coast and the Madeira Island, and *Spongia* sp. (Dictyoceratida, Spongiidae) and *Ircinia* spp. from the Azores archipelago. All samples were collected in triplicate by scuba diving at depths ranging from 12 to 16 m. Samples were transported to the laboratory in cooling boxes and immediately processed. Species identification was initially performed based on morphological criteria and then confirmed *a posteriori* using molecular phylogenetic inference (see below).

Sponge Identification. Sponge DNA barcoding analysis was used to identify all specimens. This was performed as explained previously by Hardoim *et al*. (2012). Briefly, total community DNA was extracted from sponge samples (see below) and used for PCR amplification of the subunit I of the cytochrome oxidase gene (COI) using the primer pair dgLCO1490 and dgHCO2189 (Meyer *et al*., 2005). PCR conditions, cleaning and sequencing of amplicons, as well as phylogenetic inference were done as described by Hardoim *et al*. (2012). The obtained sequences will be submitted to the EMBL Nucleotide Sequence Database.

DNA extraction. Genomic DNA was extracted from 0.25 g of internal sponge body using UltraClean® Soil DNA isolation kit (Mo Bio, Carlsbad, CA, USA) according to the manufacturer's protocol. Seawater samples (1 L), taken at about 2 m apart from the sampled sponge specimens, were filtered through 0.2-µm-pore-size nitrocellulose filters (Millipore, Billerica, MA, USA) using a vacuum pump. The filters were cut into small pieces and used for DNA extraction as explained above. Each sediment sample consisted of about 150 g of the top 10 cm sediment layer collected in sterile bags and mixed by sieving in the laboratory. DNA was extracted from 0.5 g of the sieve-homogenized sediment using the kit described above.

Preparation of samples for pyrosequencing. A barcoded pyrosequencing approach was employed for in-depth analysis of bacterial community composition and diversity. To this end, the V4 hypervariable region of the 16S rRNA gene was PCR-amplified (V4_titF: 5'-AYTGGGYDTAAAGNG-3' and V4_tit_R: 5'-TACNVRRGTHTCTAATYC-3') as described previously (Hardoim *et al*., Chapter 3). Each sample was tagged by different 8-mer barcodes attached to the reverse primer. The amplicons were sent for pyrosequencing on a 454 Genome Sequencer GS FLX Titanium platform (Roche Diagnostics Ltd, West Sussex, UK) at BIOCANT (Biotechnology Innovation Center, Cantanhede, Portugal).

Pyrosequencing data processing. 454 pyrosequencing raw data was processed using AmpliconNoise (Quince *et al*., 2011) for noise filtering (for instance, homopolymers), chimera removal and sequence sorting. Sequences were trimmed at maximum length of 260 bp. After trimming, they were filtered for the removal of any sequence with lengths shorter than 150 and longer than 260 bp using the FASTA manipulation tool at the Galaxy web-based

platform (https://main.g2.bx.psu.edu/) (Taylor *et al*., 2007a; Blankenberg *et al*., 2010). The Quantitative Insights Into Microbial Ecology (QIIME) software package (Caporaso *et al*., 2010) was then applied to generate a samples *vs*. OTUs table, OTU taxonomic assignments and some of the downstream statistical analyses using customized scripts. After the removal of unclassified OTUs, chloroplasts and singletons, a final OTU biom-format table was created and used for downstream analysis (see Hardoim *et al*., Chapter 3 for more details).

Pyrosequencing data analysis. Bar charts showing the taxonomic assignments were created in Excel using data obtained from the QIIME pipeline. Bacterial diversity and richness were calculated for each sample using the Shannon-Wiener diversity index (Shannon, 1948a, b) and the Chao1 richness estimator (Chao, 1984; Chao and Lee, 1992), respectively, within the QIIME environment (Caporaso *et al*., 2010). The results ("alpha diversity" graphics) were customized using Excel software. Multivariate analysis of community composition at the OTU level (97% sequence cut-off) was performed using the beta-diversity unweighted Unique Fraction metric (UniFrac), which was applied to determine measures of (dis)similarity in bacterial community composition between samples (Lozupone and Knight, 2005). The Jackknife protocol was used to assess the robustness of UniFrac hierarchical cluster nodes through statistical re-sampling (Hamady *et al*., 2010). A phylogenetic distance matrix was generated using the unweighted UniFrac method against the tree generated from Infernal. This matrix was used for multivariate analysis by means of Unweighted Pair Group Method with Arithmetic means (UPGMA) clustering (Felsenstein, 2004) and Principal Coordinate Analysis (PCoA) (Krzanowski and Krzanowski, 2000). PCoA results were displayed with the KiNG software package v. 2.21 (Chen *et al*., 2009).

In order to assess the extent of shared and exclusiveness of bacterial phylotypes across sample categories and observe similarities and differences among the datasets, Venn diagrams were created. 4-way Venn diagrams were constructed using the *Venny* software (http://bioinfogp.cnb.csic.es/tools/venny/index.htm, Oliveros, 2007), while 3-way Venn diagrams were generated with the *GeneVenn* freeware tool (http://simbioinf.com/mcbc/applications/genevenn/genevenn.htm, (Pirooznia *et al*., 2007). To this end, the final OTU biom-format table obtained within QIIME was converted into a tab-delimited table where the list of OTU IDs found in each sample category was organized and used as input data.

Results

Sponge identification

Sponge specimens were initially collected based on morphological criteria. They were then subjected to traditional and molecular identification using macro- and microscopic features along with COI phylogenetic inference, respectively. This analysis as a whole revealed that the specimens sampled at the Azores site, initially categorized underwater as *Sarcotragus* sp., indeed belong to one as-yet unidentified species in the genus *Spongia* (Dictyoceratida, Spongiidae, Fig. S4.1). The resulting COI phylogenetic tree also revealed a minimal degree of genotypic variation among all sponge samples classified as *Ircinia* sp. (Fig. S4.1), in spite of the fact that, according with traditional classification, specimens collected at the Madeira site were tentatively affiliated with *I. dendroides*, whereas those sampled at the Algarve and Azores sites were identified as *I. variabilis*. High COI conservation was also observed within all specimens identified as *Sarcotragus spinosulus* from the Algarve and Madeira sites (Fig. S4.1), corroborating the traditional classification results. We hereafter refer to all sampled *Ircinia specimens* as Ircinia spp., to all sampled *Sarcotragus* specimens as *Sarcotragus spinosulus* and to all sampled *Spongia* specimens as *Spongia* sp..

Bacterial community composition

In total, 317.333 sequences were processed using the AmpliconNoise software. After filtering, 232.251 high quality chimera- and noise-free sequences were obtained from the whole dataset (36 triplicate samples grouped in 12 populations: 4 sample types and 3 locations). These sequences were assigned to 5.601 unique OTUs (Operational Taxonomic Units) defined at a 97% sequence identity threshold. Table 4.1 shows the total number of sequences and OTUs per sample type and location.

Table 4.1. Number of filtered sequences and OTUs per sample type and locality.

	Algarve		Azores		Madeira	
Samples	**Seqs**	**OTUs**	**Seqs**	**OTUs**	**Seqs**	**OTUs**
Sediment	14.674	3.049	15.199	3.449	10.904	2.765
Seawater	24.579	231	13.386	395	17.102	296
***Ircinia* spp.**	23.456	211	20.397	255	23.142	192
***Sarcotragus* spp.**	20.746	166	-	-	24.532	184
***Spongia* spp.**	-	-	24.134	167	-	-

Taxonomic classification showed different bacterial composition depending on the sample type (Figs. 4.1-3). Sediment samples (Fig. 4.1) contained the larger number of bacterial phyla (59 among recognized and candidate phyla), two of which clearly dominated the sediment communities at the three locations. Only 15 phyla were present in a percentage higher than 1%. The two most abundant phyla were *Proteobacteria*, ranging from 37 to 40% in relative abundance across sites, and *Bacteroidetes*, with minimum and maximum abundance values of 25 and 40% at the Azores and Algarve sites, respectively. They were followed by *Planctomycetes* (7% in average), *Firmicutes*, *Actinobacteria*, *Acidobacteria*, and *Chloroflexi* (*c.* 2%). All sediment triplicates showed a very homogenous pattern of bacterial composition at the phylum level regardless of the sample localities. Figure 4.1 summarizes the bacterial taxonomic composition of the sediment samples for those phyla present in percentages equal or higher than 1%. Looking at lower taxonomic levels, 161 classes and 281 orders were recovered from sediments, of which only 25 and 27, respectively, were more abundant than 1%. The dominant classes in *Proteobacteria* were *Deltaproteobacteria* (*c.* 17% of relative abundance) and *Gammaproteobacteria* (*c.* 14%). For *Bacteroidetes*, the class *Bacteroidia* was the most abundant, especially at the Algarve site (20%). However, at the Azores site this class represented only 7% of the total community.

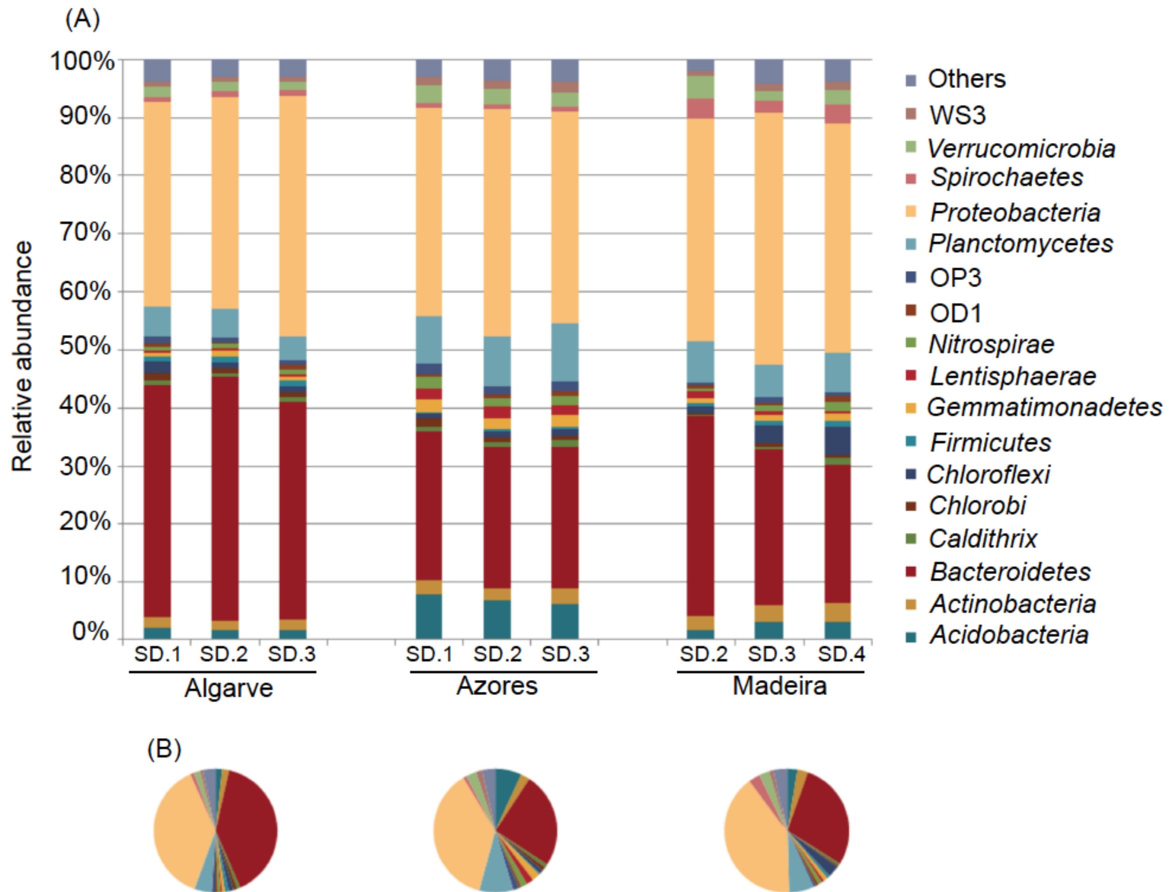

Figure 4.1. Relative abundance of bacterial phyla in sediment samples from all locations. The stacked bar plot (A) shows the relative abundance (%) of each phylum per sample on the y-axis. The identity of each triplicate sample per site is given on the x-axis. SD=sediment. Pie charts (B) show the average values of triplicates for the main phyla present in the samples. Graphs only represent those phyla present at percentages equal or higher than 1%.

Seawater samples encompassed, in total, 31 bacterial phyla from the three localities. However, this number decreased to 11 when only phyla displaying relative abundances higher than 1% were considered (Fig. 4.2). These samples showed more variability in bacterial community composition across localities than sediment (Fig. 4.1) and sponge (Fig. 4.3) samples. Replicates at the Madeira site displayed the most diverse and distinct bacterioplankton communities. Overall, the prevailing phyla in seawater were *Proteobacteria* (*c.* 50% of the relative abundance across the dataset) and *Bacteroidetes* (19% in average). However, at the Madeira site *Bacteroidetes* represented only 6%, in average, of the total bacterial communities. In these samples, some other bacterial phyla were also detected with a relatively high percentage, for instance, PAUC34f (12%), *Poribacteria* (9%), *Actinobacteria* (9%), and *Chloroflexi* (6%). Azores samples were heavily loaded with *Proteobacteria* - which

made up to 60% of the reads - and *Firmicutes* (7%). The latter phylum was detected at much lower levels in the other two locations. At the Algarve site, the candidate phylum SAR406 was one of the main phyla with relative abundance close to 20% (Fig. 4.2).

At lower taxonomic levels, 66 classes and 115 orders were detected in seawater, but only 17 and 28, respectively, represented more than 1% of the total community. *Alphaproteobacteria* (14% - 38%) and *Gammaproteobacteria* (14% - 20%) were among the more abundant classes. At the Algarve and Azores sites, *Flavobacteriia* (*Bacteroidetes*) was also quite abundant, with 28% and 19% respectively. In contrast, at the Madeira site this group showed a decreased abundance of about only 1%. Class-level bacterioplankton assemblages at the Maderia site also included *Deltaproteobacteria* (6%), PAUC34f (11%), *Poribacteria* (9%), and *Actinobacteria* (9%) in relatively high proportions

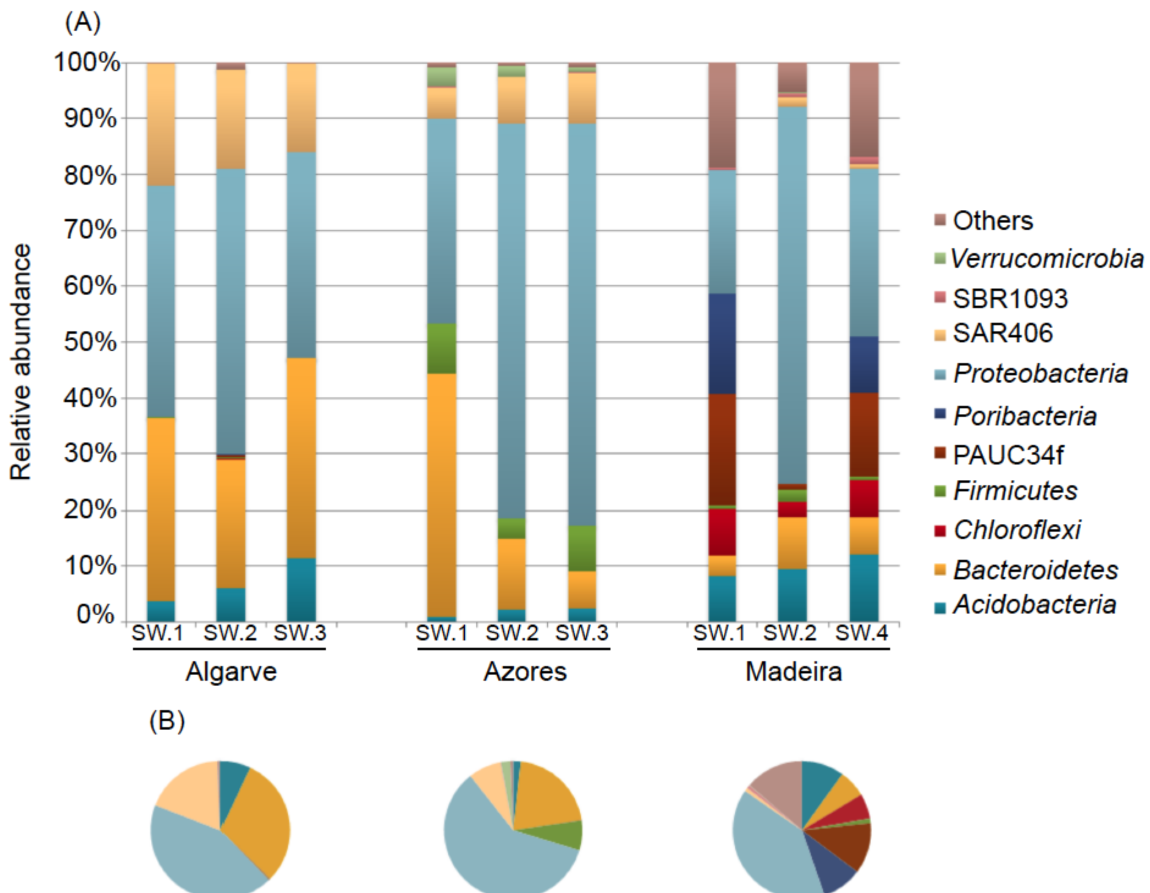

Figure 4.2. Relative abundance of bacterial phyla in seawater samples from all locations. The stacked bar plot (A) shows the relative abundance (%) of each phylum per sample on the y-axis. The identity of each triplicate sample per site is given on the x-axis**.** SW=seawater. Pie charts (B) show the average values of triplicates for the main phyla present in the samples. Graphs only represent those phyla present at percentages equal or higher than 1%.

Sponge microbiomes encompassed several phyla with quite abundant representatives. In *Ircinia* spp. 20 phyla were detected but only 9 were present in percentages higher than 1%. Microbiomes of *Sarcotragus spinosulus* and *Spongia* sp. were quite similar across the sampling sites. For both these sponge hosts, 25 bacterial phyla were detected, of which 11 were significantly abundant (i.e., ≥ 1%, Fig. 4.3). The predominant taxonomic groups in *S. spinosulus* and *Spongia* sp. were *Acidobacteria* and *Actinobacteria* (*c.* 20% relative abundance), followed by *Poribacteria* (*c.* 11%), PAUC34f (*c.* 10%), and *Proteobacteria* (*c.* 8%). The aforementioned abundances remained similar in these sponge species regardless of the sampling locality. Conversely, phylum-level abundances shifted across localities in *Ircinia* spp. In these hosts, *Chloroflexi* displayed dominance at the Madeira site (31%) but was a minor taxon at the Algarve (7%) and Azores sites (3%), where *Proteobacteria, Acidobacteria*, and *Actinobacteria* were dominant groups at varying degrees. At the Algarve site, *Acidobacteria* and *Actinobacteria* represented 62% of the total community associated with *Ircinia* specimens, whereas *Acidobacteria, Bacteroidetes*, and *Proteobacteria* were the prevailing taxa at the Azores site, summing up 74% of the total abundance in these communities.

When lower taxonomic levels were considered, 62 classes (52 from *Ircinia* spp. and 50 from *Sarcotragus*-*Spongia* spp.) and 100 different orders across all samples and localities were registered. The most representative classes were similar among the studied species, displaying eventual changes in relative abundances across different hosts. The class Sva0725 (*Acidobacteria*) was higher in *Ircinia* spp.. The same occurred with *Acidomicrobiia,* with the exception of samples from Azores where this order represented only 6% of the total community. Apparently, this class was replaced by the *Rhodothermi* class at the Azores site (20% abundance in *Ircinia* spp.). Higher levels were found within *Ircinia* specimens for the class *Anaerolineae* when compared with the *Sarcotragus*-*Spongia* group. On the other hand, PAUC34f and *Poribacteria* classes were quite higher in *S. spinosulus* and *Spongia* sp. compared with *Ircinia* spp.

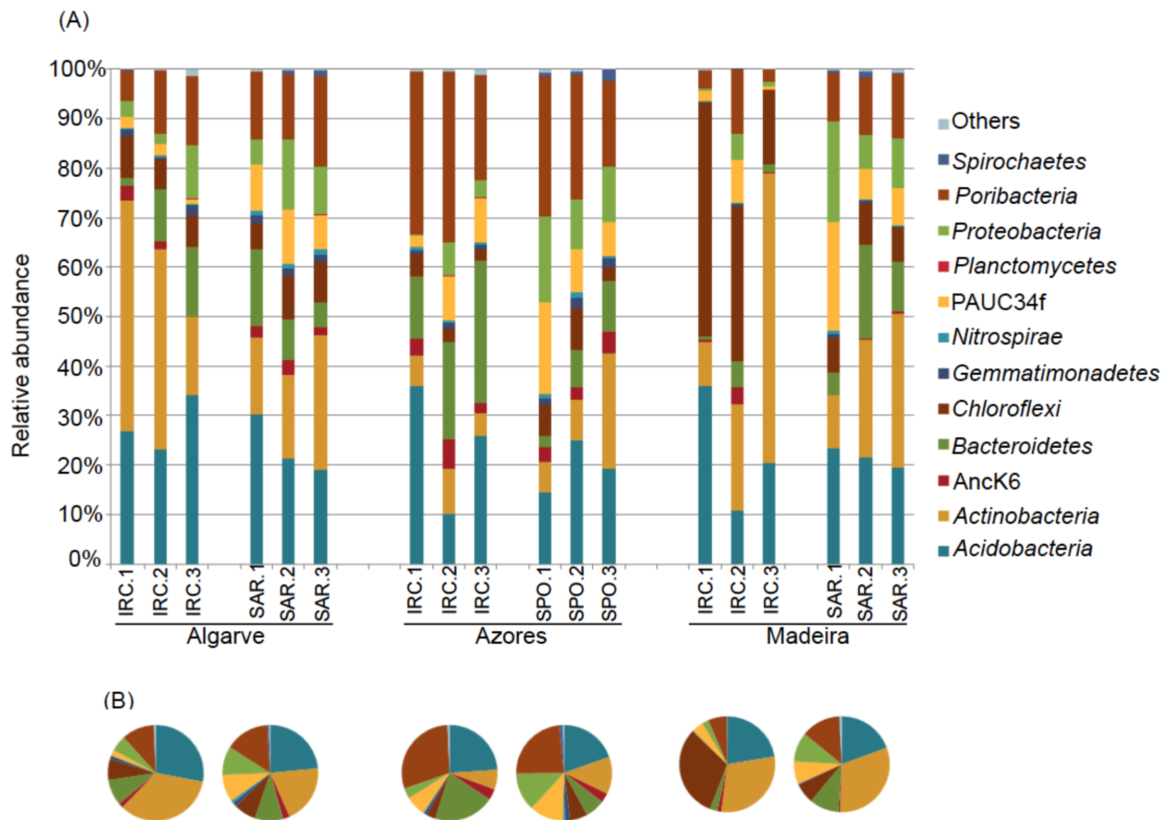

Figure 4.3. Relative abundance of bacterial phyla in sponge samples from all locations. The stacked bar plot (A) shows the relative abundance (%) of each phylum per sample on the y-axis. The identity of each triplicate sample per site is given on the x-axis**.** IRC=*Ircinia* spp.; SAR=*Sarcotragus spinosulus*; SPO=*Spongia* sp.. Pie charts (B) shows the average values of triplicates for the main phyla present in the samples. Graphs only represent those phyla present at percentages equal or higher than 1%.

Bacterial community richness and diversity

Diversity and richness of bacterial communities at the approximate species level (that is, OTUs defined at 97% gene similarity) were calculated using alpha diversity metrics such as the Shannon-Wiener diversity index and the Chao1 species richness estimator. Both “species” richness (Chao1 estimated and observed OTUs) and diversity (Shannon) results confirmed sediment samples as the group with the highest taxonomic richness and diversity in the dataset. The estimated (Chao1) OTU richness of sediment samples was 10- to 20-fold times higher than that of seawater and sponge samples, whereas the observed richness in sediments was between 8- and 15-fold higher than that detected in seawater and sponges. (Fig. 4.4a) The Shannon diversity index for sediment samples was twice as high in comparison with seawater

and sponge samples (Fig. 4.4b). Overall diversity indices of sponge and seawater samples were similar, with slightly lower values registered for *Ircinia* spp. (Fig. 4.4b).

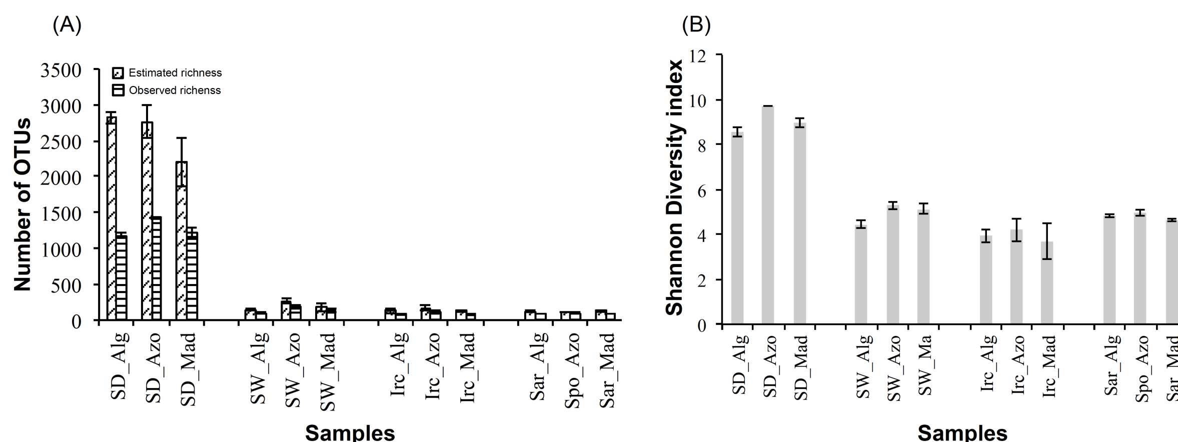

Figure 4.4. Comparison on bacterial richness and diversity among sample types. The estimated (Chao 1) and observed richness averages for each sample group. (A). The Shannon diversity index from the same groups (B). Alg=Algarve; Azo=Azores; Mad=Madeira; SD=Sediment; SW=Seawater; Irc=*Ircinia* spp.; Sar=*Sarcotragus spinosulus*; Spo=*Spongia* sp..

Bacterial community ordination

Principal Coordinates Analysis based on the Unweighted UniFrac metric was used to ordinate the bacterial communities across the three distinct geographical areas. Five groups were clearly different according with their clustering pattern in the ordination diagram: all sediment samples, all sponge species, and seawater samples from Algarve, Azores and Madeira (Fig. 4.5). Thus, regardless of the sampling sites, the bacterial community found in sediment and sponge samples was overall similar. Especially concerning the former samples, it was not possible to depict any distinct clustering of samples according to the different sponge species or families used in this analysis. In contrast, seawater samples fell into three separate groups, each corresponding to the three studied locations, revealing higher bacterial community similarities between replicates sampled within the same habitat.

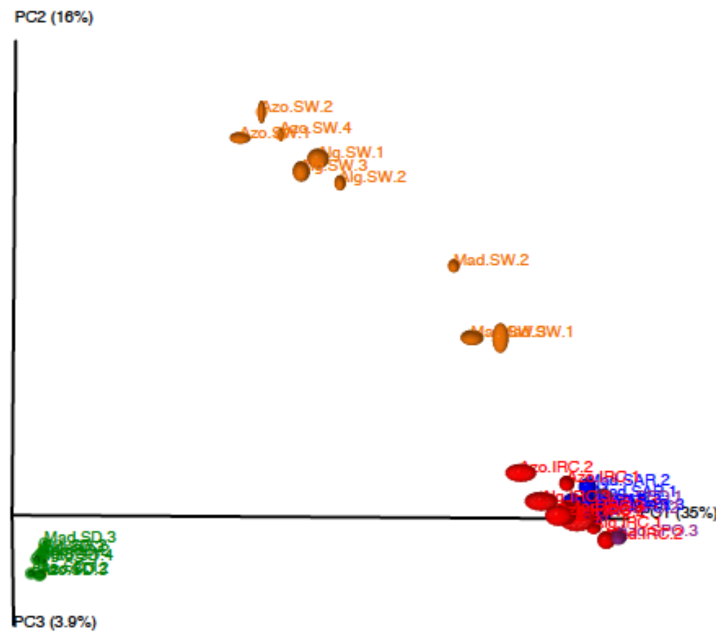

Figure 4.5. Principal Coordinates Analysis (PCoA) based on the unweighted UniFrac distance metric. The graph shows the grouping of seawater samples by locality, whereas sediment and sponge samples do not present a geographical pattern. They group instead by sample type.

Within-habitat share of bacterial phylotypes among sample types

At each of the surveyed sampling sites, a much reduced number of bacterial phylotypes (i.e. OTUs) was simultaneously present in all sample categories "sediment", "seawater", and "sponges" (Fig. 4.6). The total amount of the thus so-called "core" OTUs was indeed very low at all sites: only 2 at the Algarve, 6 at the Azores and 12 at Madeira within datasets comprising several thousands of OTUs. As expected, for all studied sites, a higher share of bacterial OTUs was found between the two sponge species under scrutiny than any other sample type combination. Interestingly, *Ircinia* specimens from the Algarve and Azores sites did share more bacterial OTUs with sediment than with seawater samples, whereas the opposite was observed at the Madeira site. Taken together, the Venn diagrams revealed a high degree of specificity in "bacteriome" composition for each sample category, with the vast majority of the OTUs detected across the dataset being rather exclusive to sediment, seawater, or sponges.

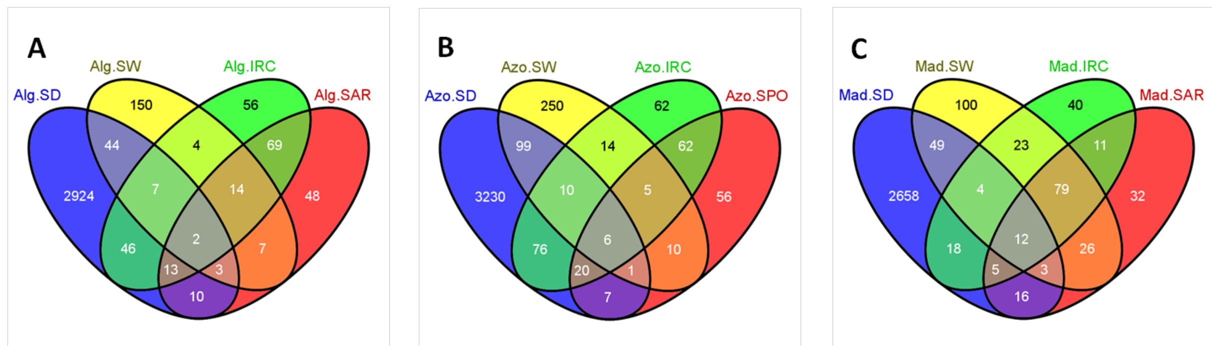

Figure 4.6. Number of phylotypes exclusive and common to all sample categories at each sampling site. (A), Algarve; (B), Azores; and (C), Madeira. The Venn diagrams were constructed using the complete OTU table (i.e., both dominant and rare OTUs included) as input data.

Between-habitat share of bacterial phylotypes among sponge samples

One of the main questions in this study dealt with the existence of a sponge-specific core of bacterial symbionts and/or a sponge species-specific one. To answer this question, Venn diagrams were also very useful to visualize the number bacterial phylotypes common to several sponge species combinations across habitats. This way, a common bacterial core comprising 22% of all OTUs found in *Ircinia* specimens at the three sites was revealed (Fig. 4.7a), whereas 44% of the total bacterial symbionts detected in *S. spinosulus* were common to specimens from Algarve and Madeira (Fig. 4.7b), and 29% were common to the *Sarcotragus-Spongia* sp. group (Fig. 4.7c). A high degree of "habitat-specific" bacterial symbionts has also been revealed at each sampling site (Fig. 4.7).

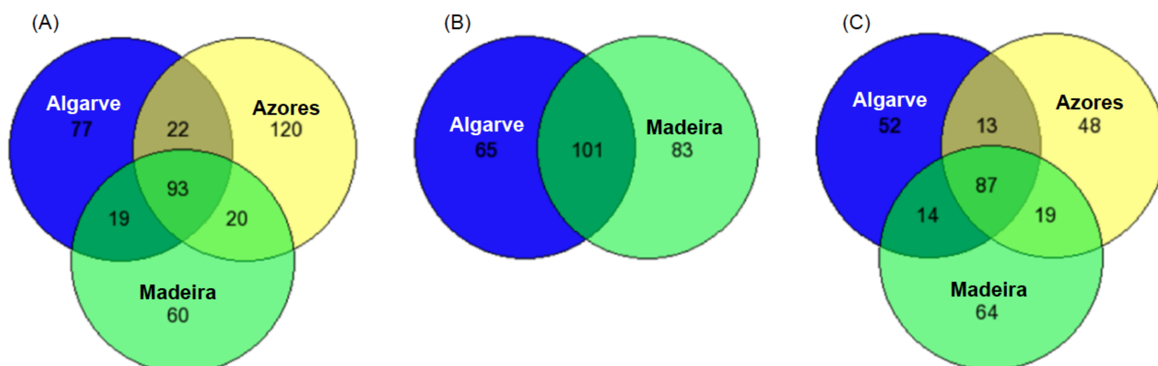

Figure 4.7. Common and habitat-specific bacterial OTUs in sponge samples. (A), *Ircinia* spp., (B) *Sarcotragus spinosulus*, and (C) *Sarcotragus-Spongia* spp. group. The Venn diagrams were constructed using the complete OTU table (i.e., both dominant and rare OTUs included) as input data.

To assess the extent to which outcomes shown in Figures 4.6 and 4.7 were influenced by the presence of only rarely or moderately abundant bacterial symbionts in our analyses, further Venn diagrams were constructed after removing those OTUs represented by less than 50 sequences from the dataset (Fig. 4.8). This was done for (i) OTUs containing less than 50 sequences in the whole dataset, including all sample categories (Fig. 4.8a) and (ii) OTUs containing less than 50 sequences taking only the sponge-derived libraries into account (Fig. 4.8b). In both cases, the number of the otherwise considered “habitat-specific” OTUs was dramatically reduced. Conversely, the sponge species cores sharply increased in proportions to 77% for *Ircinia* spp., 68% for *S. spinosulus* and 61% for the *Sarcotragus-Spongia* group when the whole dataset was considered, and to 77%, 81% and 76% respectively, when only the sponge-derived sequence libraries were approached.

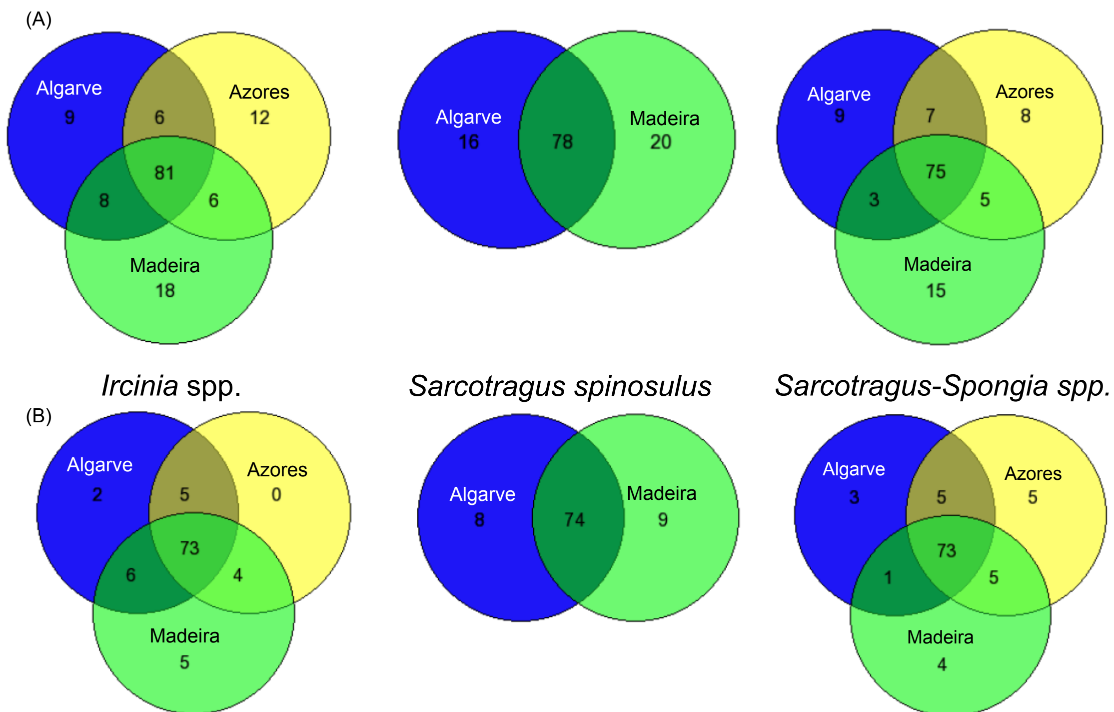

Figure 4.8. Common and habitat-specific bacterial OTUs in sponge samples, with removal of “rare” symbionts from the analysis. (A), Using OTUs with at least 50 reads in the whole data set; (B), using OTUs with at least 50 reads in the sponge-derived libraries.

Finally, the same process was applied to find the common core of the analysed marine sponges as a whole (regardless of the sponge species and sampling sites). This was done by creating Venn diagrams with cores obtained previously for *Ircinia* spp. and *Sarcotragus-Spongia* spp. as input data (Fig. 4.9). This way, a considerable common bacteriome was found

across all sponge species and sampling sites, amounting 55, 65 and 72% of all detected sponge symbionts depending on the inclusion/exclusion of rare phylotypes (Fig. 4.9a-c). Disregarding the rare symbionts present in the sponge libraries (Fig. 4.8b), 61 OTUs were common to all sponge species in comparison with 5-fold lower proportions of species-specific symbionts, delineating this way the magnitude of the sponge core. The two most abundant shared OTUs contained as much as 22.827 and 14.621 reads within the sponge libraries dataset and affiliated with the bacterial classes Sva0725 (*Acidobacteria*) and *Acidimicrobiia* (*Actinobacteria*), respectively (Table 4.2). Several phylotypes belonging to the phyla PAUC34f, Anck6, *Actinobacteria* (*Acidomicrobiia* and *Solibacteres* classes) and *Proteobacteria* in the *Alpha Delta* and *Gamma* classes were also quite abundant (Table 4.2).

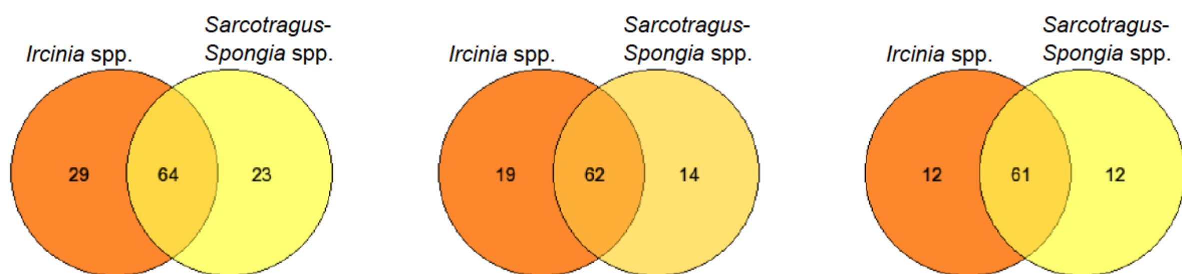

Figure 4.9. Bacterial OTUs common to all sponge species and specific to *Ircinia* and *Sarcotragus*-*Spongia* spp. groups. (A), Using the whole OTUs dataset; (B), Using OTUs with at least 50 reads in the whole dataset; (C), Using OTUs with at least 50 reads in the sponge libraries dataset.

Table 4.2. Ten more abundant OTUs common to all sponge specimens.

		Number of reads per OTU					
OTU id	**Taxon id**	**Alg.IRC**	**Azo.IRC**	**Mad.IRC**	**Alg.SAR**	**Azo.SPO**	**Mad.SAR**
10.705	p: *Acidobacteria*; c: Sva0725; o: Sva0725	6.141	4.354	3.845	2.537	2.825	3.125
664	p: *Actinobacteria*; c: *Acidimicrobiia*;o: *Acidimicrobiales*; f:TK06	2.545	439	5.372	1.847	1.672	2.746
8.912	p: *Bacteroidetes*; c: *Rhodothermi*; o: *Rhodothermales*; f: *Rhodothermaceae*	302	4.491	75	1.142	613	2.518
13.984	p: PAUC34f	206	1.160	491	1.268	1.454	1.699
2.517	p: *Chloroflexi;* c: *Anaerolineae*; o: *Caldilineales*; f: *Caldilineaceae*	258	230	4.288	294	198	375
5.143	p: *Actinobacteria*; c: *Acidimicrobiia*;o: *Acidimicrobiales*	1.879	274	1.063	762	530	349
7.162	p: *Poribacteria*	383	276	296	663	1.821	968
24.160	: PAUC34f	218	354	271	592	1.383	1.072
16.992	p: *Proteobacteria*; c*: Deltaproteobacteria*; o: *Entotheonellales*; f: *Entotheonellaceae*	269	81	75	597	1.220	616
23.023	p: *Actinobacteria*; c: *Acidimicrobiia*;o: *Acidimicrobiales*	1.709	27	529	141	103	200

p=phylum; c=class; o=order; f=family

Discussion

Next-generation sequencing (NGS) technologies offer the possibility to deeply describe the microbial richness hidden in open ecosystems, expanding our knowledge of these complex communities in the environment (DeLong, 2005; DeLong, 2006; Fierer *et al*., 2012; Hentschel *et al*., 2012). These techniques are also very useful for better understanding the relationship between host organisms and their guest microbes, being of fundamental relevance for recent advances in the study of the marine sponge microbiome (Webster *et al*., 2010; Lee *et al*., 2011; Jackson *et al*., 2012; Schmitt *et al*., 2012). Here, we apply NGS to inspect microbial biogeography and specificity in marine sponges across a “double” geography-host phylogeny gradient. Our experimental design included accurate molecular phylogenetic inference of chemically complex marine sponges of potential relevance in biotechnology sampled along a northeast – mid Atlantic geographical transect. It enabled us to address (i) the distinctiveness of these communities when contrasted with those from seawater and sediments, (ii) the contribution of the latter microenvironments in shaping symbiont communities in marine sponges, (iii) the maintenance, by a given sponge host, of symbiont communities across divergent biogeographical backgrounds, and (iv) the hypothesis of species-specific symbiont community composition within several habitats simultaneously. Importantly, beyond our sponge symbiont-centered approach, this is the first study to address the biogeography of coastal seawater and sediment bacterial communities from continental Europe and the oceanic Islands of Madeira and Azores. This effort considerably adds to previous knowledge acquired by our research group on the specificity, cultivability and temporal and spatial dynamics of microbial communities associated with marine sponges of the family Irciniidae (Hardoim *et al*., 2012; Esteves *et al*., 2013; Hardoim *et al*., Chapter 3). In these studies, we unveiled the species-specific nature of bacterial communities associated with the phylogenetically close and sympatric species *Sarcotragus spinosulus* and *Ircinia variabilis* at the Algarve site (Hardoim *et al*., 2012) and revealed that the shape of these communities stood in sharp contrast with that of the surrounding bacterioplankton (Hardoim *et al*., 2012; Hardoim *et al*., Chapter 3). We further demonstrated that commonly used bacterial cultivation procedures sampled a reduced, but distinct, diversity of bacterial phylotypes when compared to more comprehensive cultivation-independent methods (Hardoim *et al*., Chapter 3; Esteves *et al*., 2013). Here, we challenge the previously observed species-specific character of these communities by chasing bacterial symbionts in the

abovementioned species across divergent biogeographical settings and by qualitatively inspecting the cooperative contribution of sediments and seawater as environmental symbiont sources to these hosts. By extending our analyses to *Spongia* sp. specimens, we were able to address the hypothesis of increasingly similar symbiont communities in phylogenetically closer sponge species making use of a smooth gradient of host phylogenetic relationships.

Our data revealed that the bacterial composition of each type of sample was clearly different from each other. Indeed, several changes were observed in bacterial diversity and relative abundances between seawater, sediments, and sponges from the phylum to the "species" (i.e. OTU) hierarchic levels. This difference is consistent with data from previous reports, where sponge-associated microbial communities strongly differed from those of seawater (Taylor *et al*., 2007b; Webster *et al*., 2010; Hardoim *et al*., 2012; Schmitt *et al*., 2012; Taylor *et al*., 2013). In fact, seawater bacterial communities have been usually characterized along with symbiont communities in sponge microbiology studies (Hardoim *et al*., 2009; Webster *et al*., 2010). This reflects not only the obvious relevance of the bacterioplankton to sponge metabolism given the filter-feeding habit of these animals, but also the notion that seawater is the main environmental source through which marine sponges acquire their symbionts via horizontal transmission (Taylor *et al*., 2007). Conversely, in spite of our much improved knowledge of marine sediment microbial communities (Lyra *et al*., 2013; Wang *et al*., 2013), much less attention has been paid to the participation of these truly astounding reservoirs of bacterial diversity in structuring symbiotic consortia in marine ecosystems. Only recently have *in silico* analyses highlighted the relevance of marine sediments as environmental source of bacteria for marine sponges (Taylor *et al*., 2013). In our study, in spite of the fact that all of the surveyed microenvironments were distinguished from one another based on their microbiome composition, share of bacterial phylotypes among these systems was also observed at varying extents over multiple sample combinations. Notably, the high abundance of *Acidobacteria* in the sponges contrasted with the virtual absence of this phylum in seawater. However, *Acidobacteria* sequences represented 3% of the total bacterial hits obtained in sediment samples. The *Acidobacteria* is a quite recent group (Kuske *et al*., 1998) and one of the most abundantly distributed bacterial phyla in the environment (Janssen, 2006). This phylum has been detected in numerous 16S rRNA gene surveys from a wide range of environments that vary greatly in physical and biogeochemical characteristics, including soil, sediment, freshwater, marine, extreme, and polluted

environments (Lopez-Garcia *et al*., 2003; Janssen, 2006; Barns *et al*., 2007; Lee *et al*., 2008; Ward *et al*., 2009). Their sequences are particularly abundant in soils and sediments, comprising 10 to 50% of the total bacterial 16S rRNA gene sequences in clone libraries (Janssen, 2006; Lee *et al*., 2008; Ward *et al*., 2009). Interestingly, members of the most abundant *Acidobacteria* lineage in our sponge specimens, Sva075, have originally been detected in marine sediments (Ravenschlag *et al*., 1999), and only latter have molecular microbiology surveys uncovered their worldwide occurrence and high abundance in marine sponges (Taylor *et al*., 2007b; Hardoim *et al*., 2009; Simister *et al*., 2012c). Our findings suggest that sediments, rather than the water column, might be the primary environmental source of these bacteria, functioning thus as an important ecological "seed bank" of symbiont acquisition by marine sponges. At present, no single evidence exists for the possible functional roles these bacteria play in association with marine sponges. Functional metagenomic approaches are thus an imperative need that promises new insights into the ecology and evolution of the elusive and widespread *Acidobacteria* symbionts of marine sponges. A next intriguing evidence for the likely environmental acquisition of highly specialized symbionts by marine sponges concerns the candidate phylum *Poribacteria*. This group is known to be abundant in and widespread among sponges (Fieseler *et al*., 2004; Lafi *et al*., 2009), being also detected in our sponge samples at different percentages depending on the host, since *Ircinia* spp. sequence libraries contained 2-3 times less *Poribacteria* 16S rRNA gene hits than *Sarcotragus* and *Spongia* spp. libraries. Unexpectedly, in our survey *Poribacteria* sequences were quite abundant in two of the three replicates of Madeira seawater samples (9-18%). Although this phylum was initially described as strictly sponge-specific, several studies have already detected low abundances of *Poribacteria* 16S rRNA genes outside sponges (Pham *et al*., 2008; Mohamed *et al*., 2010; Schmitt *et al*., 2012; Taylor *et al*., 2013). This includes not only seawater but also sediments (Taylor *et al*., 2013), suggesting that these symbionts also possess adaptive strategies for free-living and/or particle-associated life styles. It cannot be excluded that the unusually high proportions of *Poribacteria* in the abovementioned seawater samples might have been influenced to some extent by sponge metabolism, considering that these samples have been collected at close proximity to our sponge specimens. Altogether, recent molecular evidence points to the existence of a most likely flexible *Poribacteria* metabolism enabling these microorganisms to

thrive in several environmental settings within the marine realm, while being notably enriched in marine sponges.

This study also disclosed the distribution of the bacterial communities associated with marine sponges and found in surrounding seawater and sediment across distinct biogeographical settings. Sediment samples displayed extreme bacterial diversity at both the phylum and approximate species (i.e. OTUs) levels. The composition of sediment bacterial communities was highly conserved across localities, thus rejecting the hypothesis that coastal sediments of distinct geological origins and mineral constitution would harbour divergent bacterial communities. Instead, the shape of these communities might be ruled by the quality of the organic matter reaching the sediment surface layer in coastal biomes, driven by the high primary productivity of these systems (Bienhold *et al*., 2012). In contrast, seawater samples contained much higher variability in microbial community composition both across localities and among replicates, which could be explained by the role of water currents in determining the dispersion of planktonic bacteria across the oceans (Friedline *et al*., 2012) or by intrinsic features of the microhabitat. Relatively conserved sponge-associated communities were observed across the data, especially when these were contrasted with the seawater assemblages. Indeed, independently of the sponge species (*S. spinosulus* and *Ircinia* spp. - Irciniidae, *Spongia* sp. -Spongiidae) and of the sampling site, all sponge samples grouped together in our whole-dataset ordination analysis (Fig. 4.5), suggesting the existence or prevalence of an “overall” sponge-associated bacterial core rather than a sponge species-specific core. *Ircinia* spp. presented a more variable microbiome when compared with the group formed by *Sarcotragus*-*Spongia* spp., which kept a more homogenous community across localities. Surprisingly, bacterial communities from *S. spinosulus* and the phylogenetically closer *Ircinia* spp. were less similar to one another than *Spongia* sp. and *S. spinosulus* communities. Lee *et al*. (2011) found similar results while studying the microbial communities associated with three different sponge species, whereby the phylogenetically more distant species hosted the more similar communities. Collectively, these results reject the hypothesis of higher degrees of microbial community distinctiveness between distantly related sponge taxa and are indicative of a more general, non-stringent recruitment process (i.e. host species-independent) of symbiont bacteria by marine sponges.

Nevertheless, dedicated analyses performed in our study with the use of Venn diagrams unveiled a considerable number of both host-specific bacterial symbionts within

each habitat (Fig. 4.6) and habitat-specific symbionts within each sponge host (Fig. 4.7). These numbers were in such proportions that the sum of species- or habitat-specific phylotypes nearly equalized and surpassed, respectively, the number of the common bacterial phylotypes determined in each analysis. Given this scenario, it seems contradictory that the sponge-associated bacterial communities inspected in this study presented neither a host-dependent nor a biogeographical clustering pattern in ordination diagrams. This is because specificity, both in terms of the habitat and of the sponge host, was solidly defined by the rare symbionts, as much more conserved sponge-associated communities were depicted across habitats and sponge species when Venn diagrams were constructed considering only the prevailing symbiont bacteria (Figs. 4.8 and 4.9). As ordination analysis considers both the diversity and the abundance of taxa to infer (dis)similarities between the communities, the common clustering of all sponge specimens in the ordination space was most likely ruled by the conservative pool of dominant symbionts found across localities and sponge hosts. We therefore posit that the views of conserved (Hentschel *et al*., 2002) *vs.* species-specific (Lee *et al*., 2011) bacterial communities in marine sponges, often regarded as opposite and mutually-exclusive perspectives, in fact make part of one single phenomenological process governing symbiont community make-up in marine sponges. In every single host, a substantial pool of dominant and rare phylotypes form the flexible structure of the marine sponge microbiome. Most likely, dominant and rare symbionts simultaneously determine the extents of commonality and specificity found across different host species and biogeographical settings, as determined in this study, and across time as determined elsewhere (Hardoim and Costa, Chapter 5). Thus, previous studies reporting on conserved, stable and/or uniform bacterial community composition in marine sponges - regardless of the hosts, habitats or seasons evaluated – (Hentschel *et al*., 2002; Taylor *et al*., 2005; Pita *et al*., 2013a; Pita *et al*., 2013b) do find support in our data when only the patterns of distribution of the dominant sponge symbionts are to be considered. Not surprisingly, most or the totality of the abovementioned studies have been conducted with the use of less-resolving molecular technologies, such as PCR-DGGE, t-RFLP and cloning-and-sequencing of bacterial 16S rRNA genes, which typically enable the assessment of the most dominant community members only. When inspected in sufficient detail by means of NGS, substantial evidence for specificities arise, even between sponge individuals (Lee *et al*., 2011; Schmitt *et al*., 2012)and are likely to be driven by the rare sponge microbiota. It remains to be determined what the collective role of

this rare microbiota might be in association with marine sponges. Based on existing data gathered by our team, rare and dominant sponge symbionts collectively display similar taxonomic composition at high hierarchical ranks (orders, classes, phyla). This suggests that the rare sponge-associated biosphere comprises genetic variants of functionally redundant symbionts that provide the holobiont with a flexible gene pool of presumed relevance for the resilience of these organisms in nature. Taken together, this study demonstrates that an intricate combination of habitat- and host-dependent factors cooperatively shape symbiont community structure in marine sponges, whereby both conserved and specific bacterial genetic signatures form the full genetic make-up of these complex bacterial consortia.

Supplementary Material

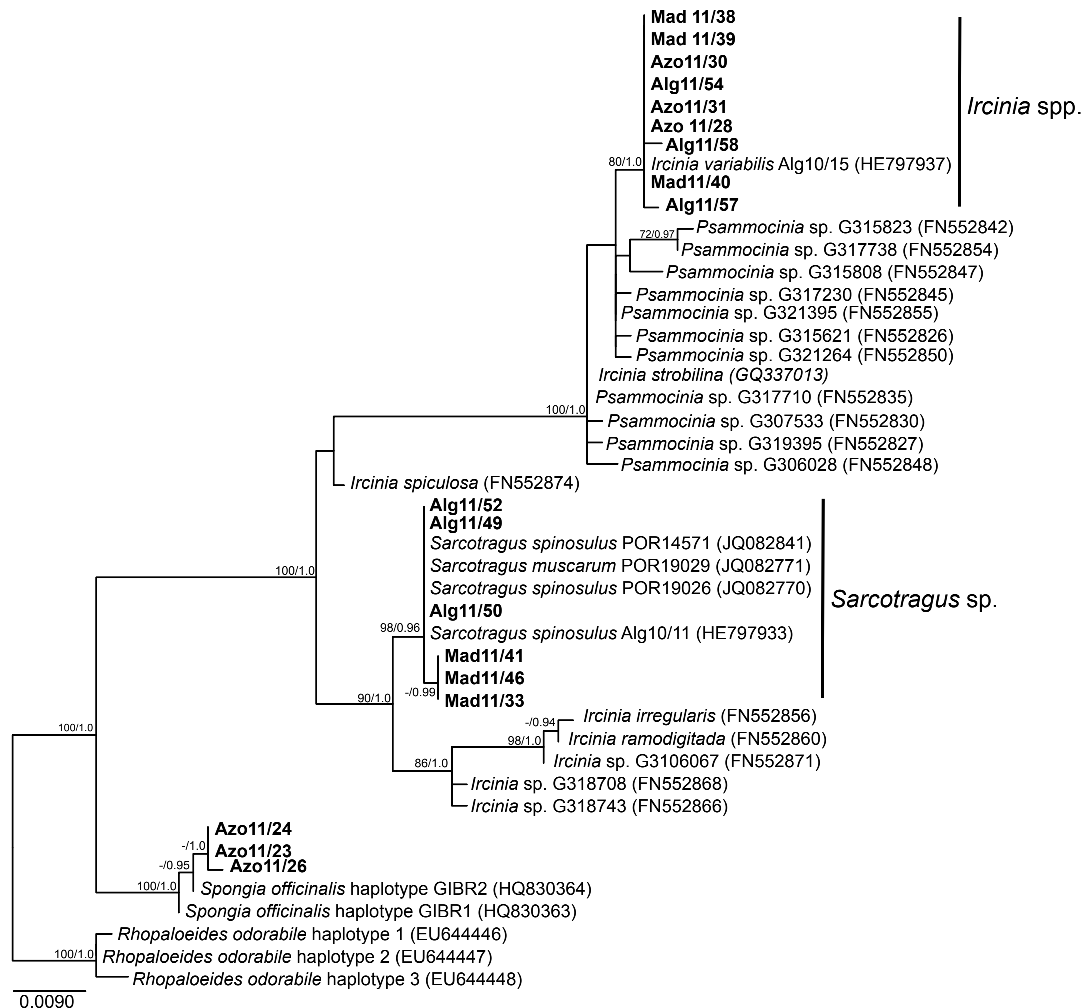

Figure S4.1 - Phylogenetic inference of the Irciniidae/Spongiidae family based on the cytochrome oxidase gene, subunit 1. The Maximum Likelihood tree (-ln likelihood: 1343.504072) is shown, with sequences retrieved in this study highlighted in bold. Numbers at tree nodes are bootstrap values and posterior probabilities calculated in Maximum Likelihood and MCMC Bayesian analyses, respectively, and values above 70/0.95 are show.

CHAPTER V

Temporal dynamics of prokaryotic symbiont communities and ammonia oxidation potential in the marine sponge *Sarcotragus spinosulus*

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Manuscript in preparation

Chapter V

Abstract

Prokaryotic communities compose a fundamental portion of the marine sponge holobiont. In spite of their putative relevance in host functioning, in-depth knowledge of the stability of sponge symbiont communities over time is extremely scarce. This study tackles the extent of temporal variation in archaeal and bacterial assemblages in the marine sponge *Sarcotragus spinosulus*. Specimens were collected in three successive years off the coast of the Algarve, South Portugal. Prokaryotic community profiling was performed by PCR-DGGE and 454-pyrosequencing of 16S rRNA genes amplified from sponge metagenomic DNA. Prevailing bacterial phyla in *S. spinosulus* were *Acidobacteria*, *Actinobacteria*, AncK6, *Bacteroidetes, Gemmatimonadetes*, *Chloroflexi,* PAUC34f, *Poribacteria,* and *Proteobacteria*, of which only *Bacteroidetes* and *Chloroflexi* showed significantly different relative abundances over the years. At the approximate species level (OTUs, operational taxonomic units defined at 97% gene similarity) no differences in bacterial richness were found in *S. spinosulus* through time. Low within-year and larger between-year variations in bacterial OTU composition were observed. Nearly 50% of all detected bacterial symbionts (96 in 205 OTUs) were found in the three sampling years, whereas 136 OTUs were detected in at least two sampling years. Except for one host specimen, archaeal communities were highly dominated by one single symbiont affiliated with the genus *Nitrosopumilus* (*Thaumarchaeota*), well known for its ability to aerobically oxidize ammonia to nitrite. Its remarkable prevalence and temporal persistence in *S. spinosulus* suggest an effective participation of this archaeon in host nutrient cycling and housekeeping functions. PCR-DGGE fingerprinting of bacterial ammonia oxidizers showed likewise that few phylotypes in these cohorts prevailed in *S. Spinosulus* across the years. The results reveal that the prokaryotic symbionts within *S. spinosulus* display a state of dynamic stability shaped by the interplay between the maintenance and turnover of community members, in time and across host individuals, with no apparent consequences to holobiont functioning.

Introduction

Marine sponges are well known to harbour complex and diverse prokaryotic assemblages. Based on 16S rRNA gene analyses, 28 bacterial phyla have been identified to establish close associations with these animals (Taylor *et al*., 2007; Hentschel *et al*., 2012; Webster and Taylor, 2012). *Proteobacteria* (especially *Alpha* and *Gamma* classes), *Actinobacteria*, *Firmicutes*, *Cyanobacteria*, *Bacteroidetes, Acidobacteria*, and *Chloroflexi* are among the most dominant bacterial phyla inhabiting these hosts. Sequences from the two main archaeal phyla - *Thaumarchaeota* and *Euryarchaeota* - have been as well recovered from marine sponges (Taylor *et al*., 2007; Simister *et al*., 2012c; Webster and Taylor, 2012). A comprehensive phylogenetic analysis carried out with more than 7.500 sponge-derived 16S rRNA gene sequences revealed 173 monophyletic "sponge-specific" bacterial clusters as well as five monophyletic "sponge-specific" archaeal clusters (Simister *et al*., 2012c) according to the definition of Hentschel *et al*. (2002). Recently, the application of high-throughput sequencing technology has shown that many of the so-called "sponge-specific" microbial lineages occur in rare numbers in the open environment, being thus pronouncedly enriched within the sponge body (Taylor *et al*., 2013).

Sponge prokaryotic symbionts are known to be involved in several biogeochemical cycles (Taylor et al., 2007). In particular, the nitrification step within the nitrogen cycle, that is, the conversion of ammonia into nitrite mediated by ammonia-oxidizing archaea and bacteria (AOA and AOB, respectively), is of supposed relevance for marine sponges. This is because ammonia, a highly toxic metabolic waste produced by these animals, can accumulate within the sponge body in periods of low pumping activity (Taylor et al., 2007, Webster and Taylor, 2012). The subunit A of the ammonia-monooxygenase gene (*amoA* gene) has been extensively applied as a genetic marker to detect prokaryotic ammonia-oxidizers in several ecosystems (Rotthauwe *et al*., 1997; Francis *et al*., 2005), including the marine sponge holobiome where AOA seem to prevail over AOB (Bayer *et al*., 2008; Hoffmann *et al*., 2009; Han *et al*., 2012; Cardoso *et al*., 2013). However, little is known about the spatial and temporal dynamics of these functional cohorts in the sponge host.

In the last ten years or so, we experienced a sharp increment in the knowledge of the diversity and function of sponge symbiont communities as highlighted in several reviews (Taylor et al., 2007; Hentschel et al., 2012; Webster and Taylor, 2012). However, our perspective on their temporal dynamics remain limited as the vast majority of sponge

microbiology surveys so far undertaken relied on single sampling events. Regardless of their diversity and extent of specificity, unveiling the spatiotemporal stability of the marine sponge microbiome is not only central to our understanding of the degree of intimacy between hosts and symbionts and their evolutionary history, but also to the management of marine genetic resources of potential biotechnological use. Nevertheless, few studies have disclosed the dynamics of the marine sponge microbiome along a time-series. Overall, these surveys have sustained that the bacterial community in these hosts was stable across time, from 6-month to 3-year periods (Taylor *et al*., 2004; Thiel *et al*., 2007; Erwin *et al*., 2012b; White *et al*., 2012). With one exception (White *et al*., 2012) surveys on the temporal stability of sponge symbionts used more traditional molecular tools to characterize these communities, such as rRNA gene clone libraries or polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE)/terminal restriction fragment length polymorphism (T-RFLP) fingerprinting. With ever-evolving resolving power, next generation sequencing technologies are much needed to overcome depth biases of previous protocols and will soon certainly enable novel insights into sponge microbiome dynamics across time.

Here, the temporal dynamics of prokaryotic communities associated with the temperate sponge *Sarcotragus spinosulus* Schmidt, 1862 (Demospongiae, Irciniidae) over a 3-year period is unravelled. This species was selected based on our previous observations of highly distinct and specific bacterial community composition at small and large geographical scales (Hardoim *et al*., 2012; Hardoim *et al*., Chapter 3; Lago-Léston *et al*., Chapter 4). The 16S rRNA gene was used as a phylogenetic marker in PCR-DGGE and 454-pyrosequencing profiling of the *Bacteria* and *Archaea* domains. Further, the *amoA* gene was used as a target to fingerprint prokaryotic ammonia-oxidizers associated with *S. spinosulus* via PCR-DGGE. This is the first in-depth, simultaneous analysis of the temporal dynamics of archaeal and bacterial communities in marine sponges.

Material and Methods

Sponge sampling. Four specimens of *Sarcotragus spinosulus* (Schmidt, 1862; Demospongiae, Irciniidae) were collected by scuba diving at depths around 15 m at Galé Alta, Armação de Pêra (37º 04' 09.6"N and 8º 19' 52.1"W) off the coast of the Algarve, South Portugal, in June 2010, September 2011, and October 2012. Samples were transported to the laboratory

separately in plastic bags (type Ziploc®) inside cooling boxes and processed upon arrival (for more details see Hardoim *et al*., 2012).

Sponge identification. Traditional classification was performed as explained elsewhere (Hardoim *et al*., 2012). Sponge phylogenetic inference was applied to aid in the identification of the specimens. To this end, total community DNA was extracted from the sponge samples (see below) and used for PCR amplification, which targeted the subunit I of the cytochrome oxidase gene (CO1) with the primer pair dgLCO1490 and dgHCO2189 (Meyer *et al*., 2005). PCR conditions, cleaning and sequencing of amplicons, and phylogenetic inference followed the procedures of Hardoim *et al*. (2012).

Total community DNA extraction. Genomic DNA of about 0.25 g of internal sponge body was extracted using UltraClean® Soil DNA isolation kit (Mo Bio, Carlsbad, CA, USA) according to the manufacturer's protocol.

PCR-DGGE analyses.

Bacterial 16S rRNA gene. A nested PCR-DGGE approach was selected to generate community profiles of the dominant bacterial symbionts in *S. spinosulus* using the primer pairs F27 (5'-AGAGTTTGATCMTGGCTCAG-3') and R1492 (5'-TACGGYTACCTTGTT ACGACTT-3') (first PCR) and F984-GC (5'- CGCCCGGGGCGCGCCCCGGGCGGGGCG GGGGCACGGGGGGAACGCGAAGAACCTTAC-3') and 1378R (5'-CGGTGTGTACAA GGCCCGGGAACG-3') (second PCR) as described previously (Hardoim *et al*., 2012).

Archaeal 16S rRNA gene. A nested PCR-DGGE approach was as well applied to profile the prevailing archaeal symbionts inhabiting *S. spinosulus*. The first PCR reaction mixture was prepared with the *Archaea*-specific primer pair ARC344f-mod (5'-ACGGGGYGCASSAGKCGVGA-3') and Arch958R-mod (5'-YCCGGCGTTGAVTCCAAT T-3') as described by Pires *et al.* (2012), using 3.75 mM $MgCl_2$, 0.1 mg mL^{-1} of bovine serum albumin, 2% (vol/vol) dimethyl sulfoxide, 0.625 U of Bio*Taq*™ DNA polymerase (Bioline, London, UK). The PCR-DGGE reaction mixture was prepared as explained by Pires *et al.* (2012) with the primer pair 524F-10 (5'-GCCGCGGTAA-3') and Arch958R-mod (GC) (5'-GC Clamp-CCGGCGTTGAVTCCAATT-3') using 2 µL of previous amplicons as DNA

template, 3.75 mM $MgCl_2$, and 0.625U of *BioTaq*™ DNA polymerase. Thermal cycling in both PCRs were as described by Pires *et al.* (2012).

Bacterial amoA gene. A semi-nested PCR-DGGE was chosen to address the ammonia-oxidizing bacteria associated with *S. spinosulus*. For the first PCR, the reaction mixture (25 µL) contained 1.0 µL of template DNA (~ 10 ng), 1X reaction buffer, 0.2 mM dNTPs, 3.75 mM $MgCl_2$, 4% (vol/vol) acetamide, 0.2 µM of primer pair amoA1F (5'-GGGGTTTCTACTGGTGGT-3') and amoA2R-GG (5'-CCCCTCGGGAAAGCCTTCTTC-3') (Nicolaisen and Ramsing, 2002), and 0.625 U of Bio*Taq*™ DNA polymerase. After initial denaturation at 92ºC for 1 min, 35 cycles of 30 sec at 92ºC, 30 sec at 57ºC, and 60 sec at 72ºC were performed. A final extension of 5 min at 72ºC was used to finish the reaction. The obtained amplicons [3.0-4.5 µL] were used as templates in a second PCR-DGGE using the primer pair amoA1F-GC and amoA2R-GG (Nicolaisen and Ramsing, 2002) in 15 thermal cycles using the same reaction mixture and cycling conditions described for the first PCR.

Archaeal amoA gene. One single amplification step was used to amplify archaeal *amoA* gene fragments for PCR-DGGE profiling. The reaction mixture was prepared as for the first PCR used in the amplification of the bacterial *amoA* gene*,* except for the primer pair (Crenamo23f: 5′-ATGGTCTGGCTWAGACG-3′ and CrenamoA616r: 5′-GCCATCCATCTGTATGTCCA-3′, Tourna *et al*., 2008) and template DNA quantity (~30 ng). After initial denaturation at 95ºC for 5 min, 35 cycles of 30 sec at 92ºC, 30 sec at 55ºC, and 60 sec at 72ºC were performed, followed by a final extension of 10 min at 72ºC.

PCR-DGGE profiling and analysis. PCR-DGGE was carried out in a PhorU-2 gradient system (Ingeny International, Goes, The Netherlands). The 16S rRNA and *amoA* gene amplicons were applied in even concentrations onto polyacrylamide gels containing a 40 to 75% gradient of denaturants (100% denaturants defined as 7M urea and 40% formamide) and a 6% of acrylamide, except for the bacterial *amoA* gene, where 8% acrylamide was used. Marker constituents, electrophoresis conditions, and staining procedures were as previously described (Hardoim *et al*., 2012). The PCR-DGGE profiles were analysed in the software GelCompar II 5.1 (Applied Maths, Kortrijk, Belgium) as explained by Hardoim *et al*. (2009). This analysis delivered a table species *vs*. samples encompassing the relative abundance of all

bands in each profile and it was further used as input for ordination analyses of PCR-DGGE fingerprints with Canoco for Windows 4.5 (Microcomputer Power, Ithaca, NY) as described in detail by Costa *et al* (2006a), using Hellinger-transformed abundance data.

Preparation of samples for pyrosequencing. A barcoded pyrosequencing method was applied for in-depth analysis of bacterial and archaeal community composition and diversity. The V4 hypervariable region of the 16S rRNA gene of *Bacteria* was PCR-amplified (V4_titF: 5'-AYTGGGYDTAAAGNG-3' and V4_tit_R: 5'-TACNVRRGTHTCTAATYC-3', http://pyro.cme.msu.edu/pyro/help.jsp#intro) as explained by Hardoim *et al*., (Chapter 3). For *Archaea*, the V4-V5 hypervariable region of the 16S rRNA gene was targeted and two independent PCR amplifications (25 µL) for pyrosequencing analysis were performed for each sample. To this end, amplicons from the first archaeal nested PCR (see above) were used as templates (2 µL) in the pyrosequencing reaction, which was carried out with 25 cycles using the primers 524F-10-ext (5'-TGYCAGCCGCCGCGGTAA-3') and Arch958R-mod (5'-YCCGGCGTTGAVTCCAATT-3') and conditions as described by Pires *et al*. (2012). Bacterial and archaeal amplicons were delivered for pyrosequencing on a 454 Genome Sequencer GS FLX Titanium platform (Roche Diagnostics Ltd, West Sussex, UK) at Biocant (Biotechnology Innovation Center, Portugal).

Pyrosequencing data processing. The bacterial 454-pyrosequencing data were processed as explained in details by Hardoim *et al*. (Chapter 3), except that the flag "truncate_len" in the AmpliconNoise script was removed and sequence trimming was then performed with Galaxy software (https://main.g2.bx.psu.edu) using the text manipulation tool (260 bp), while the minimum sequence length (150 bp) was filtered under the FASTA manipulation tool also with Galaxy. The same procedure was applied for archaeal 454-pyrosequencing analysis, where sequences were trimmed at 450 bp and those with < 200 bp were filtered out with Galaxy. Analyses of filtered sequences were carried out as previously explained. Analyses of filtered sequences were carried out as previously explained (Hardoim *et al*., Chapter 3), using the Quantitative Insights Into Microbial Ecology (QIIME, Caporaso *et al*., 2010) software package. Taxonomic assignments of bacterial and archaeal sequences were performed with the new greengenes 13_05 database release (http://greengenes.secondgenome.com/downloads/database/13_5) within the QIIME

environment. A final OTU table was generated after the removal of unclassified OTUs, chloroplasts and singletons, and further used for downstream analyses. The OTU tables generated from bacterial and archaeal 16S rRNA gene tag-pyrosequencing were converted from .biom to .txt format and employed as in-custom ordination analyses with the software package Canoco 4.5 using Hellinger-transformed OTU abundance data.

Statistical analysis

Homogeneity of variance tests were used to check the normal distribution of the richness and diversity measurements from DGGE fingerprints and 454-pyrosequencing. Analysis of Variance (ANOVA) was performed to test whether or not the mean values obtained for all sample groups were equal. A pairwise t-test - which analyses the significance between groups, in our case distinct sampling years - was then carried out. Homogeneity of variance and ANOVA were also used to compare the relative abundance data from tag-pyrosequencing of the most dominant bacterial phyla and classes found in *S. spinosulus* across the years. All statistical analyses were performed with the stat package in R programming (Team, 2010).

Results

Sponge identification

Sponge specimens collected in 2010 and 2011 were identified by traditional classification following Hardoim *et al*. (2012) and Lago-Léston *et al*. (Chapter 4) as *Sarcotragus spinosulus*, while all sampled specimens (2010, 2011, and 2012) were further investigated by molecular phylogenetic inference. Trees based on Maximum likelihood and Bayesian inferences using the CO1 gene revealed a well-supported cluster composed only by sequences from *Sarcotragus* specimens (Fig. 5.1). This group was clearly placed apart from phylogenetic clusters containing sequences from the other two genera of the family Irciniidae, namely *Ircinia* and *Psammocinia*, thus firmly confirming the status of our species as belonging to the genus *Sarcotragus* (Fig. 5.1). Analysis of 579 bp-long CO1 gene sequences obtained for all 12 sponge specimens analysed in this study and the other 3 remaining *Sarcotragus* spp. sequences available at NCBI showed a high level of gene conservation within the genus, with genetic distances (p-distance) between pairs of sequences ranging from 0 to 0.3%.

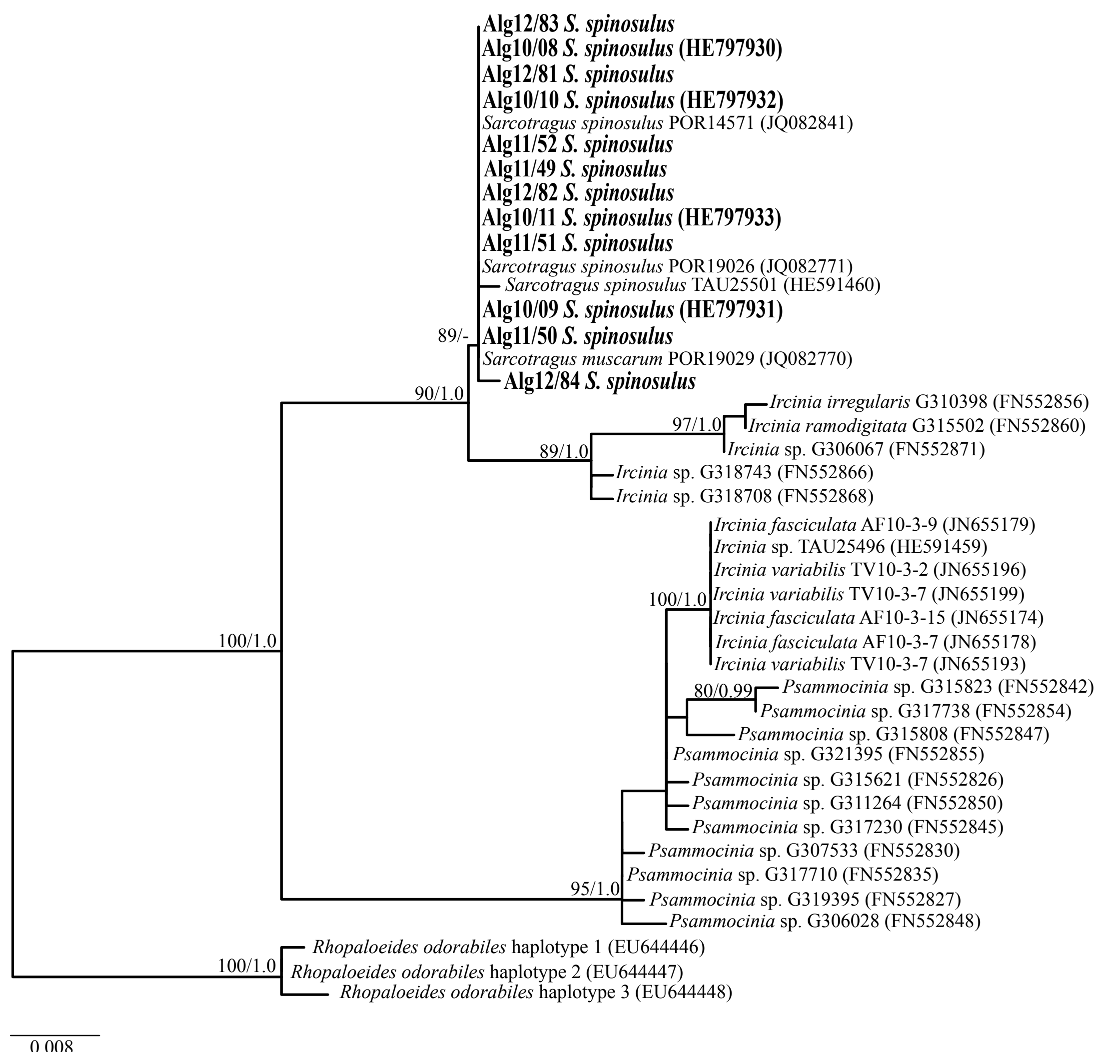


Figure 5.1. Phylogenetic inference of the Irciniidae family based on cytochrome oxidase gene subunit 1 sequences. The maximum likelihood tree (-ln likelihood: -1204.8294) is presented, with sequences recovered in this study highlighted in bold. Number at tree nodes are bootstrap values and posterior probabilities calculated in Maximum Likelihood and MCMC Bayesian analyses, respectively, and values above 75/0.95 are shown.

PCR-DGGE analysis

Bacterial 16S rRNA gene fragments.

The bacterial PCR-DGGE profiles of *S. spinosulus* collected in three consecutive years were characterized by about nine dominant along with 11 to 36 fainter bands (Fig. S5.1a). Most of the prevailing bands were present in all sponge specimens, whereas the fainter ones showed varied patterns of abundance or presence/absence across the profiles, subtly differentiating *S.*

spinosulus specimens according to the sampling year (Fig. S5.1a). Ordination of the PCR-DGGE band data via redundancy analysis (RDA) revealed that specimens collected in 2010 and 2012 significantly influenced band variation in the DGGE profiles ($p<0.05$, Fig. 5.2a). Overall, 23.4% of the total PCR-DGGE band data variation could be attributed to the factor "year of sampling". The horizontal axis of the diagram accounted for 64.7% of this explained variability and mainly distinguished all replicates acquired in 2012 from those obtained in 2010 (Fig. 5.2a). The remaining 35.3% of the explained variability was constrained to the vertical axis, which grouped replicates from 2010 and 2012 apart from 2011 (Fig. 5.2a). The PCR-DGGE richness and diversity measures of 2010 (35.33 ± 6.35 and 3.55 ± 0.17, respectively) were significantly different ($p<0.05$) from the values observed for 2011 (26.21 ± 4.87 and 3.25 ± 0.19, respectively) and 2012 (20.92 ± 1.49 and 3.03 ± 0.07 respectively), revealing a slight decrease in these two parameters along time.

Archaeal 16S rRNA gene fragments

The archaeal PCR-DGGE profiles of *S. spinosulus* collected in three successive years consisted of two conspicuous bands along with few (1 to 12) other detectable bands (Fig. S5.1b). Two dominant bands were observed in all *S. spinosulus* specimens, whereas the majority of the fainter ones were found in almost all sponge specimens and few were common to a specific sampling year (Fig. S5.1b). Here, 45.8% of the whole PCR-DGGE variation was attributed to the factor "year of sampling". In spite of the high degree of conservation of the two major bands in the gel, ordination via RDA revealed that 2010 and 2012 replicates possessed different archaeal community structures ($p<0.05$). 73.2% of the explained dataset variation was constrained to the horizontal axis of the ordination diagram, which placed 2010 and 2011 replicates apart from 2012 replicates (Fig. 5.2b). No significant difference ($p>0.05$) was found in the PCR-DGGE richness and diversity measurements across sampling years 2010 (3.39 ± 0.96 and 1.18 ± 0.33, respectively), 2011 (5.47 ± 1.52 and 1.67 ± 0.27, respectively), and 2012 (5.24 ± 1.16 and 1.64 ± 0.22, respectively).

Bacterial and Archaeal amoA gene fragments

The bacterial ammonia-oxidizing PCR-DGGE profiles of *S. spinosulus* sampled along three consecutive years revealed two dominant bands found across all sponge specimens and few fainter bands observed in almost all *S. spinosulus* patterns (Fig. S5.1c). RDA showed that 23.8% of the whole variation in PCR-DGGE profiles could be attributed to our independent variable (years of sampling), with replicates from 2012 significantly affecting band abundance data across samples (p<0.05, Fig. 5.2e). The horizontal axis of the diagram corresponded to 76.1% of the explained dataset variability and grouped replicates from 2010 and 2011 apart from 2012 (Fig. 5.2c). The vertical axis accounted for 23.9% of the explained dataset variability and distinguished 2010 from 2011 replicates. The PCR-DGGE richness and diversity measures were not significantly different across the sampling years 2010 (8.05 ± 3.94 and 1.98 ± 0.53, respectively), 2011 (10.71 ± 2.16 and 2.35 ± 0.22, respectively), and 2012 (10.68 ± 4.17 and 2.30 ± 0.43, respectively). For the archaeal *amoA* gene, no amplification was obtained from any of the *S. spinosulus* replicates.

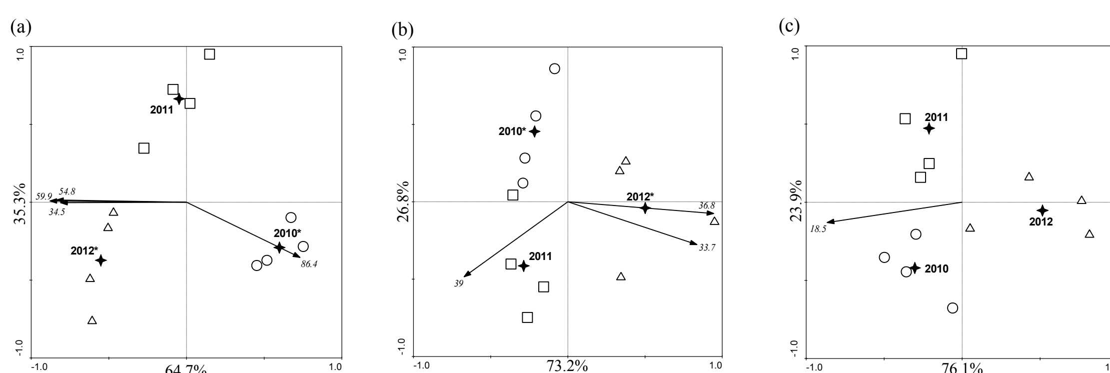


Figure 5.2. Redundancy analysis of bacterial 16S rRNA gene (a), archaeal 16S rRNA gene (b) and bacterial *amoA* gene (c) PCR-DGGE profiles. Symbols: *S. spinosulus* specimens collected ○ in 2010, □ in 2011, and △ in 2012. Labels displayed on the diagram axes refer to the percentage variations of PCR-DGGE ribotypes - environment correlation accounted for the respective axis. The ''star'' symbol represents the centroid positions of the environmental variables in the diagram. Variables that significantly (p<0.05) influence the bacterial community composition are indicated by an asterisk.

454-pyrosequencing analyses

Bacterial 16S rRNA gene dataset

In total, 109.741 bacterial 16S rRNA V4-tag sequences were obtained, which were reduced to 108.892 sequences after preliminary filtering on the 454 equipment. These were subjected to quality filtering and removal of noise and chimera with AmpliconNoise, and then to trimming and filtering by length with Galaxy, resulting in 77.125 16S rRNA V4-tag sequences, which were further analysed with QIIME. After passing the script that excluded unclassifiable OTUs and chloroplasts as well as singletons from the final OTU table, 71.404 sequences were assigned to 205 operational taxonomic units (OTUs) at 97% sequence similarity.

Bacterial community composition at high taxonomic ranks

Actinobacteria (average relative abundance of 20.95% across all sampled specimens), *Acidobacteria* (20.30%), *Proteobacteria* (13.58%), *Poribacteria* (11.45%), PAUC34f (9.97%), *Chloroflexi* (9.75%), *Bacteroidetes* (5.74%), AncK6 (3.86%), and *Gemmatimonadetes* (2.66%), together corresponding to 98.29% of all analysed sequences, dominated the bacterial community associated with *S. spinosulus* (Fig. 5.3a,b). Among these phyla, only *Chloroflexi* and *Bacteroidetes* showed significant differences ($p<0.05$) in relative abundances over the years (Fig. 3b). The largest individual-to-individual variability in phylum percentage abundances was observed for PAUC34f and *Acidobacteria* within 2012 specimens, with values ranging from 6.7 to 32.6% and from 9.4 to 33.1%, respectively (Fig. 5.3a). Conversely, the lowest levels of individual-to-individual variability were detected for the phyla *Gemmatimonadetes* in 2011 (from 0.7 to 1.6%) and *Acidobacteria* in 2010 (from 16.5 to 19.1%, Fig. 5.3a). Regarding the abundance of bacterial classes within phyla, *Acidimicrobiia* was found to massively dominate the pool of *Actinobacteria* hits in the sequence libraries. Likewise, Sva0725, *Sphingobacteriia*, and *Gammaproteobacteria* were the most abundant classes within the *Acidobacteria, Bacteroidetes,* and *Proteobacteria* phyla, respectively (Fig. 5.3c,d). Among the 8 dominant classes, only *Sphingobacteriia* significantly ($p<0.05$) varied over the years in terms of relative abundance (Fig. 5.3d). Highest and lowest individual-to-individual variations in relative abundance were registered for the class Sva0725 of the *Acidobacteria* (from 6 to 28.7% in 2012) and the *Deltaproteobacteria* within the *Proteobacteria* (from 0.5 to 3.5% in 2012), respectively (Fig. 5.3c).

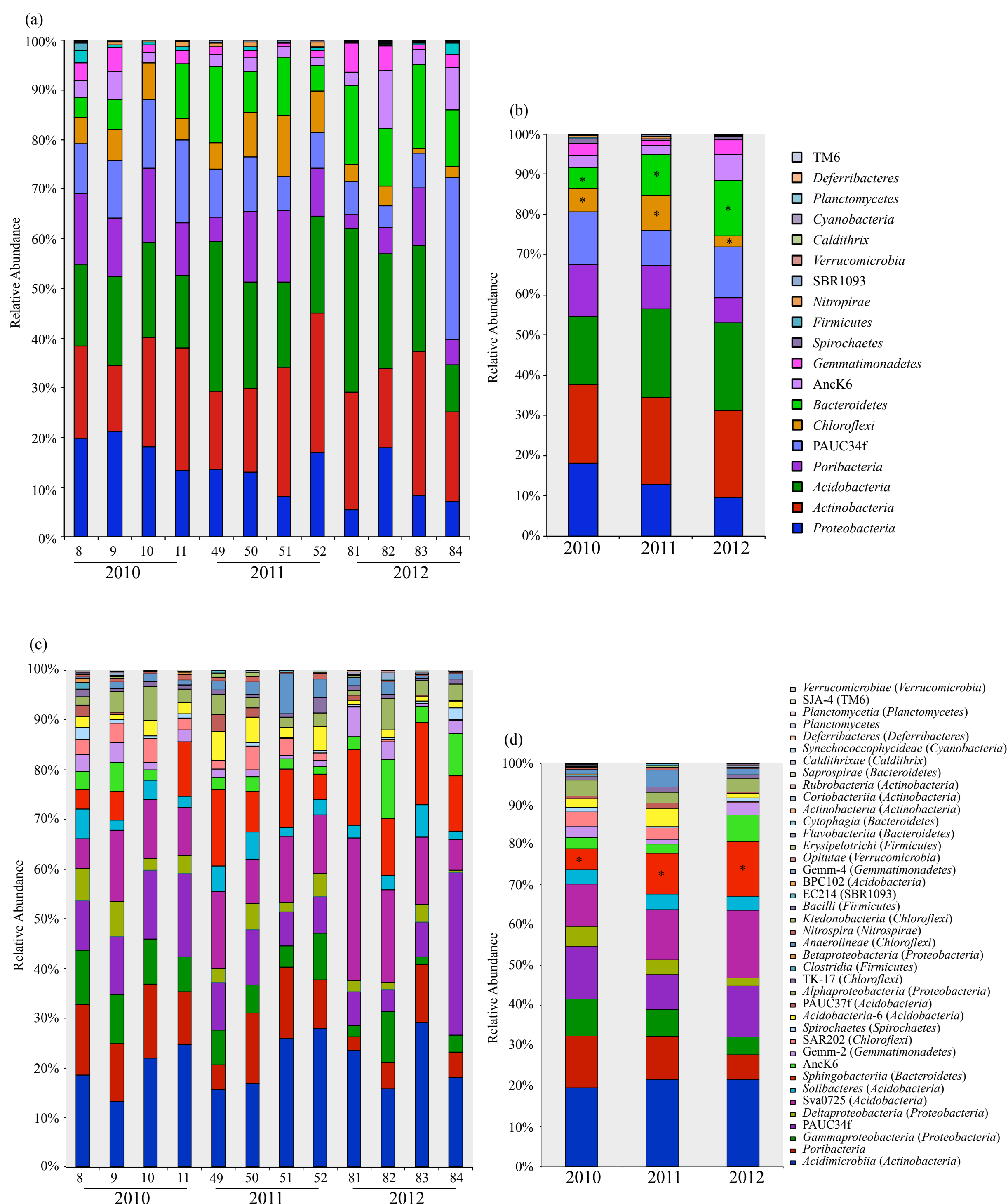


Figure 5.3. Phylum- (a, b) and class-level (c, d) bacterial community composition in *S. spinosulus*. The compositions of each replicate sample (a, c) and of pooled replicate samples (b, d) are shown.

Bacterial richness and diversity

One replicate from 2012 was removed from the richness and diversity assessments due to its low number of recovered sequences. In order to enable adequate quantitative comparisons, these analyses were performed with normalized library sizes set at 3.155 sequencing reads per sample. Under this sequencing depth, the observed bacterial richness found in *S. spinosulus* in years 2010 (105 ± 3.44), 2011 (94 ± 12.5), and 2012 (95 ± 8.33) did not significantly differ from one another (p>0.05, Fig. S5.2a). The Shannon diversity indices of 2010 (4.83 ± 0.23) and 2011 (4.71 ± 0.22) significantly differed (p<0.05) from the measurements observed in 2012 (4.18 ± 0.37) (Fig. S5.2b).

Specificity and sharedness of bacterial OTUs

The assignment of all 205 bacterial OTUs detected in this study to their source samples – *i.e.*, the 12 replicates of *S. spinosulus* - was depicted in an OTU network (Fig. 5.4a). The majority of the bacterial OTUs were common to two or more *S. spinosulus* specimens and thus only few were found to be exclusively associated with each sponge individual. When the biological replicates from the same year were pooled, the bacterial OTU network revealed that, although the majority of OTUs detected in *S. spinosulus* in any given year could be re-sampled in another year, still a considerable amount of "year-specific" OTUs were unveiled (Fig. 5.4b). Year-to-year maintenance and variation of bacterial OTUs in *S. spinosulus* were further investigated and precisely quantified with Venn diagrams (Fig. 5.4c,d). These analyses revealed that 34, 19, and 16 OTUs were found exclusively in each sampling year (2010, 2011, and 2012, respectively), whereas a comparatively greater "temporal core" of 96 in 205 OTUs was common to all sampling years (Fig 5.4c), but not necessarily to all sponge individuals (see below). Interestingly, the bacterial OTUs exclusively associated to each sampling year were classified into 7 to 10 bacterial phyla, of which several are typical constituents of the sponge microbiome such as *Acidobacteria*, *Actinobacteria*, PAUC34f, and *Poribacteria* (Taylor *et al*., 2007; Simister *et al*., 2012c; Webster and Taylor, 2012). These OTUs usually contained few sequences (from 2 to 49, Table S5.1) representing rare to moderately abundant *S. spinosulus* symbionts. representing rare to moderately abundant *S. spinosulus* symbionts. Thus, when only OTUs containing at least 50 sequences were considered, the total number of analysed OTUs dropped from 205 to 78, from which 70 OTUs comprised the temporal

bacterial core in *S. spinosulus*, and negligible OTU numbers were detected in one year or two (Fig. 5.4d). Interestingly, the "pan-microbiome" associated with *S. spinosulus*, hereby defined as the total number of bacterial phylotypes detected across all analysed specimens (205 OTUs, Fig. 5.3), was composed by 19 formally recognized and candidate bacterial phyla (Table 5.1), whereas the "essential core" of the sponge, that is, the pool of symbionts common to all 12 *S. spinosulus* specimens, consisted of only 27 OTUs belonging, nevertheless, to eight archetypical sponge-associated bacterial phyla and one candidate phylum (Table 5.2).

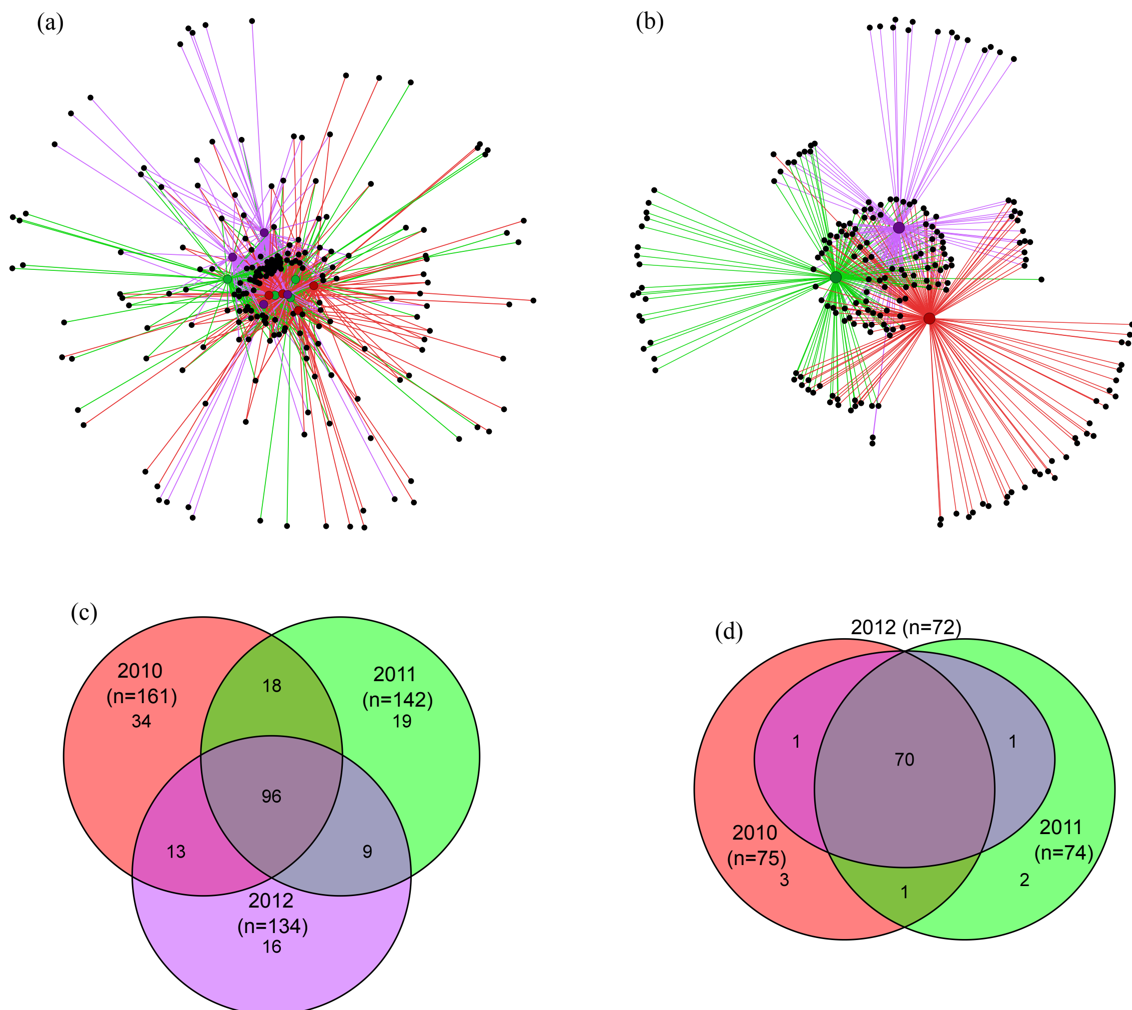


Figure 5.4. Bacterial OTU networking and maintenance in the marine sponge *Sarcotragus spinosulus* across time. Networks with all OTUs per sponge replicate (a) and with all OTUs in composite samples where sponge replicates were pooled according to the sampling year (b) and Venn diagrams (c) with all OTUs and (d) with OTUs containing ≥ 50 sequences are shown. In the diagrams, red, green, and purple correspond to the sampling years of 2010, 2011, and 2012, respectively.

Table 5.1. The Pan microbiome associated with *S. spinosulus*

Phylum level	Number of OTUs	Number of sequences	Class level	Number of OTUs	Number of sequences
Acidobacteria	17	14501	*Acidobacteria*-6	6	2013
			BPC102	1	44
			PAUC37f	1	661
			Solibacteres	5	2606
			Sva0725	4	9177
Actinobacteria	11	14381	*Acidimicrobiia*	8	14374
			Actinobacteria	1	2
			Coriobacteriia	1	3
			Rubrobacteria	1	2
AncK6	2	2706	na	2	2706
Bacteroidetes	17	6419	*Sphingobacteriia*	3	6354
			Saprospirae	1	2
			Cytophagia	2	4
			Flavobacteriia	11	59
Caldithrix	1	2	*Caldithrixae*	1	2
Chloroflexi	33	4203	*Anaerolineae*	10	1459
			Ktedonobacteria	2	210
			SAR202	16	1735
			TK17	5	799
Cyanobacteria	1	3	*Synechococcophycideae*	1	3
Deferribacteres	1	2	*Deferribacteres*	1	2
Firmicutes	5	119	*Bacilli*	1	8
			Clostridia	2	107
			Erysipelotrichi	1	4
Gemmatimonadetes	7	1947	Gemm-2	7	1947
Nitrospirae	2	388	*Nitrospira*	2	388
PAUC34f	6	8411	na	6	8411
Planctomycetes	6	29	unknown	1	2
			Planctomycetia	5	27
Poribacteria	6	7167	na	6	7167
Proteobacteria	84	10374	*Alphaproteobacteria*	29	2479
			Betaproteobacteria	2	57
			Deltaproteobacteria	10	2592
			Gammaproteobacteria	43	5246
SBR1093	1	130	EC124	1	130
Spirochaetes	2	584	*Spirochaetes*	2	584
TM6	1	3	SJA-4	1	3
Verrucomicrobia	2	35	*Opitutae*	1	33
			Verrucomicrobiae	1	2

na - not applicable

Table 5.2. The essential core associated with *S. spinosulus*

Phylum level	Number of OTUs	Number of sequences	Class level	Number of OTUs	Number of sequences
Acidobacteria	5	13000	*Acidobacteria*-6	3	1740
			Solibacteres	1	2093
			Sva0725	1	9167
Actinobacteria	6	14220	*Acidimicrobiia*	6	14220
AncK6	1	2703	na	1	2703
Chloroflexi	1	581	TK17	1	581
Gemmatimonadetes	1	474	Gemm-2	1	474
Nitrospirae	1	386	*Nitrospira*	1	386
PAUC34f	1	3809	na	1	3809
Poribacteria	1	4950	na	1	4950
Proteobacteria	10	6086	*Alphaproteobacteria*	3	1327
			Deltaproteobacteria	1	1566
			Gammaproteobacteria	6	3193

na - not applicable

Bacterial community ordination

The principal coordinate analysis (PCoA) performed on pyrosequencing data at the phylotype (OTU) level with a sequence-depth of 3.155 sequences (one replicate from 2012 was removed from the analysis) revealed no clear pattern of community composition variation along time (Fig. 5.5a). Indeed, several replicates from different years are proximate from each other in the ordination diagrams, with only a few observed sample outliers. Figure 5a also shows the distribution of the 10 most abundant phyla across the ordination space, whereby their central and overlapping positions reveal the absence of any taxon-sampling year correlations. Confirming the results retrieved via exploratory PCoA, constrained ordination of the OTU data and environmental variable via RDA showed that 23.8% of the total dataset variation could be attributed to the factor “year of sampling”. RDA also revealed that this factor did not influence the patterns of OTU distribution across the bacterial 16S rRNA V4-tag sequences ($p>0.05$, Fig. 5.5b). In spite of this, the horizontal axis of the diagram, representing 66% of the dataset variability explained by the sampling year, hints at a temporal transition in community structures from 2010 to 2012 bridged by the 2011 samples (Fig. 5.5b), resembling the patterns obtained via constrained ordination of PCR-DGGE profiles.

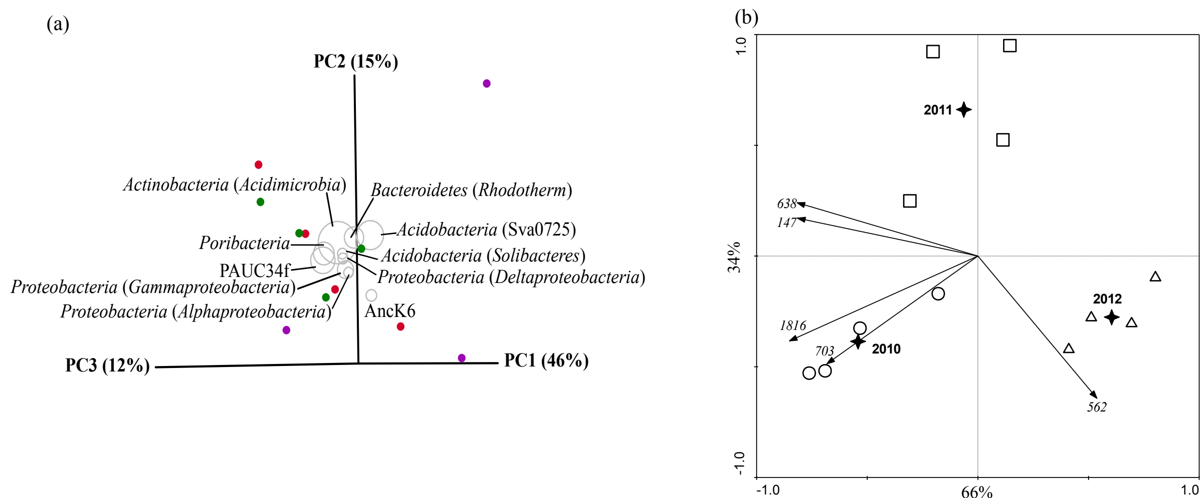


Figure 5.5. Ordination analysis of bacterial OTUs with ≥ 97% sequence similarity. (a): Principal Coordinate Analysis of OTUs using the UniFrac metric. Red, green, and purple correspond to the sampling years of 2010, 2011, and 2012, respectively. The ten most dominant bacterial taxa (at phylum or class level) are shown in the diagram. Symbol sizes of bacterial taxa correspond to their respective, mean relative abundances across the dataset. The position of bacterial taxa in the ordination space is determined by the correspondence between their relative abundances and the year of sampling. (b): Constrained ordination of bacterial OTUs via Redundancy Analysis. Symbols: ○ *S. spinosulus* collected in 2010, □ in 2011, and △ in 2012. The ''star'' symbol represents the centroid positions of the environmental variables in the diagram. Variables that significantly (p<0.05) influence the bacterial community composition are indicated by an asterisk.

Archaeal 16S rRNA gene dataset

Overall, 67.106 16S rRNA V4-V5-tag sequences passed the elementary filter on the 454 apparatus, which had originally generated 67.109 sequences. After quality filtering and removal of noise and chimera with AmpliconNoise, sequences were trimmed and filtered by length using Galaxy, resulting in 60.735 16S rRNA V4-V5-tag sequences that were further analysed with QIIME. The filtering of OTU table resulted in 60.729 sequences that were assigned to 14 OTUs at 97% sequence similarity.

Archaeal community composition

In 11 of 12 replicates, the archaeal community was dominated by the genus *Nitrosopumilus* (*Thaumarchaeota; Nitrosopumilales*), whereas the remaining replicate (Alg11/51) had *Cenarchaeum* (*Thaumarchaeota*, *Cenarchaeales*) as the dominant archaeon (Fig. 5.6a).

Archaeal richness and diversity

Rarefied libraries with 4.050 sequences were used for the assessment of the archaeal diversity and richness. The observed richness found in *S. spinosulus* in 2010 (2.30 ± 1.35), 2011 (4.60 ± 4.04), and 2012 (1.72 ± 0.48) were not significantly different ($p>0.05$, Fig. S5.2c). Likewise, no significant difference ($p>0.05$) was detected in the Shannon diversity index for 2010 (0.1 ± 0.16), 2011 (0.04 ± 0.03), and 2012 (0.23 ± 0.45) (Fig. S5.2d).

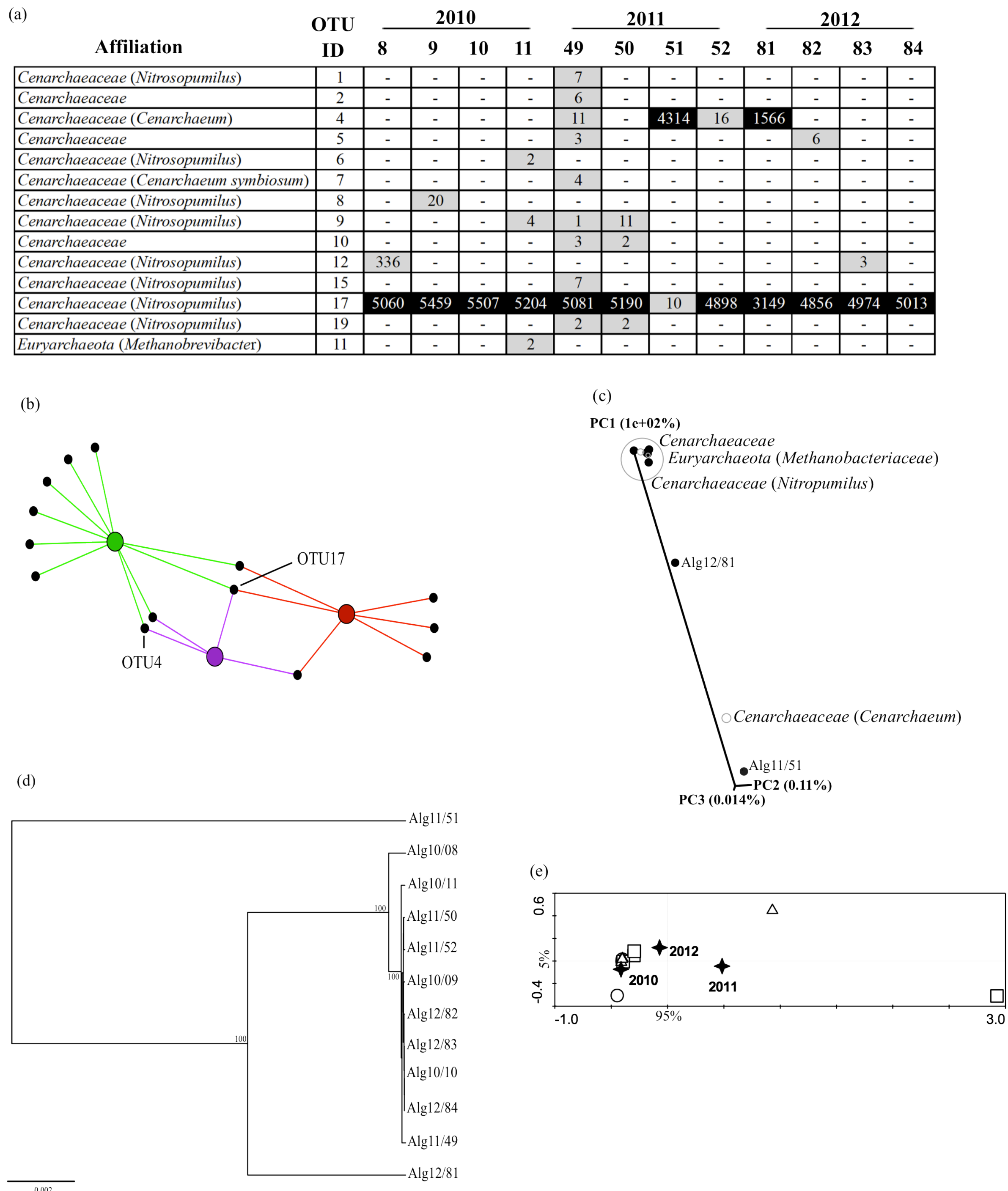
Specificity and sharedness of archaeal OTUs

The OTU network demonstrated that replicates within 2011 contained more specific OTUs than 2010 and 2012 (Fig. 5.6b). It also showed that the dominant OTU (OTU 17, *Nitrosopumilus*) was shared among the three sampling years and detected in all sponge individuals, whereas the second most dominant OTU (OTU 4, *Cenarchaeum*) was only common to 2011 and 2012 replicates (Fig. 5.6b).

Archaeal community ordination

The PCoA carried out with rarefied libraries containing 4.050 sequences demonstrated that 10 replicates grouped at the edge of the first ordination axis (PC1), whereas one replicate from 2012 (Alg12/81) was placed between this group and one further replicate from 2011 (Alg11/51) located near the PC2-PC3 extreme (Fig. 5.6c). The distribution of the main archaeal taxon groups across the samples is represented along the ordination diagram, highlighting their correlation with the source specimens in terms of relative abundance (Fig. 5.6c). The UPGMA dendrogram constructed by cluster analysis of archaeal community profiles confirmed the trend revealed by PCoA, as one replicate from 2011 (Alg11/51) and another from 2012 (Alg12/81) grouped apart from the rest of the replicates (Fig. 5.6d). RDA showed that 14.4% of the whole variation could be attributed to the independent variable “years of sampling”. RDA showed that 14.4% of the whole dataset variation could be attributed to the independent variable “year of sampling”, which was found not to significantly affect archaeal OTU composition across the sampled specimens ($p>0.05$, Fig. 5.6e). The horizontal axis of the diagram accounted for 95% of the dataset variability

explained by the independent variable and discriminated Alg11/51 and Alg12/81 from the rest of the replicates (Fig. 5.6e).



(a)

Affiliation	OTU ID	2010				2011				2012			
		8	9	10	11	49	50	51	52	81	82	83	84
Cenarchaeaceae (*Nitrosopumilus*)	1	-	-	-	-	7	-	-	-	-	-	-	-
Cenarchaeaceae	2	-	-	-	-	6	-	-	-	-	-	-	-
Cenarchaeaceae (*Cenarchaeum*)	4	-	-	-	-	11	-	4314	16	1566	-	-	-
Cenarchaeaceae	5	-	-	-	-	3	-	-	-	-	6	-	-
Cenarchaeaceae (*Nitrosopumilus*)	6	-	-	-	2	-	-	-	-	-	-	-	-
Cenarchaeaceae (*Cenarchaeum symbiosum*)	7	-	-	-	-	4	-	-	-	-	-	-	-
Cenarchaeaceae (*Nitrosopumilus*)	8	-	20	-	-	-	-	-	-	-	-	-	-
Cenarchaeaceae (*Nitrosopumilus*)	9	-	-	-	4	1	11	-	-	-	-	-	-
Cenarchaeaceae	10	-	-	-	-	3	2	-	-	-	-	-	-
Cenarchaeaceae (*Nitrosopumilus*)	12	336	-	-	-	-	-	-	-	-	-	3	-
Cenarchaeaceae (*Nitrosopumilus*)	15	-	-	-	-	7	-	-	-	-	-	-	-
Cenarchaeaceae (*Nitrosopumilus*)	17	5060	5459	5507	5204	5081	5190	10	4898	3149	4856	4974	5013
Cenarchaeaceae (*Nitrosopumilus*)	19	-	-	-	-	2	2	-	-	-	-	-	-
Euryarchaeota (*Methanobrevibacter*)	11	-	-	-	2	-	-	-	-	-	-	-	-

Figure 5.6. Structure of archaeal communities in *Sarcotragus spinosulus* across time. (a): Heatmap showing the distribution of the archaeal 16S rRNA gene taxonomic affiliation in each *S. spinosulus* specimens; (b): OTU network with all OTUs at 97% sequence similarity, where red, green and purple correspond to the sampling years of 2010, 2011, and 2012, respectively; (c): PCoA of the archaeal OTUs; (d): UPGMA clustering, and (e): RDA of the

archaeal OTUs. Symbols: *S. spinosulus* collected ○ in 2010, □ in 2011, and △ in 2012. The ‘‘star’’ symbol represents the centroid positions of the environmental variables in the diagram. Variables that significantly ($p < 0.05$) influence the bacterial community composition are indicated by an asterisk.

Discussion

This study addressed the temporal dynamics of bacterial and archaeal assemblages associated with the marine sponge *Sarcotragus spinosulus* along three consecutive years via PCR-DGGE fingerprinting and 454-pyrosequencing. *S. spinosulus* was used as a model organism because it is a high microbial abundance (Hardoim *et al*., 2012) and sharply selective sponge species hosting distinct and specific bacterial communities in comparison with those in the environmental surroundings or associated with other sponge hosts (Hardoim et al., 2012; Hardoim et al., Chapter 3; Lago-Léston et al., Chapter 4). Bacteria isolated from *S. spinosulus* have been further shown to display anti-bacterial activities *in vitro*, highlighting their potential use in biotechnological applications (Esteves *et al*., 2013). All *S. spinosulus* specimens sampled in this study displayed a high degree of intra-specific CO1 gene conservation, being thus almost identical genetically and permitting adequate temporal analysis of symbiont temporal dynamics within one single host genotype.

In this survey, the bacterial community associated with *S. spinosulus* was shown to be dynamically stable over three successive sampling years, where nine conspicuous PCR-DGGE bands were observed across most of the 12 replicates, while many distinctly fainter DGGE bands were particularly related to each sampling year. Hence, the significant differences observed in the DGGE profiles might be attributed to the fainter bands that characterized each sampling year. Bacterial community stability was demonstrated for *Chondrilla nucula* collected in three consecutive years at the Adriatic Sea, where several common PCR-DGGE bands were observed in all duplicate individuals sampled per year (Thiel *et al*., 2007). Several past studies, employing shorter temporal scales or sampling intervals, corroborate the hypothesis of host-associated community maintenance also observed here, however along with some extent of variability among less abundant phylotypes. For instance, a PCR-DGGE profiling study showed that the bacterial communities associated with three Australian sponges (*Cymbastela concentrica*, *Callyspongia* sp., and *Stylinos* sp.), collected once per season, were stable along five successive seasons (Taylor *et al*., 2004). Moreover, a study using T-RFLP revealed that the

bacterial community associated with each of three *Ircinia* spp. sampled every month was considered highly stable along a period of a year and half, regardless of changes in temperature and light intensity over the distinct seasons (Erwin *et al*., 2012b).

To circumvent the inherent limitations of fingerprinting techniques (*i.e.* less-resolving method and impossibility to identify all members of the prokaryotic community), we used 454-pyrosequencing to unravel the bacterial community structure associated with *S. spinosulus* over time. Using this approach, White *et al*. (2012) registered an overall stability of the majority of the bacterial members associated with *Axinella corrugata* collected in spring and fall within one single year. Here, PCR-DGGE and 454-pyrosequencing analyses congruently indicated that the bacterial community associated with *S. spinosulus* presents a pattern of dynamic, rather than stagnant, stability in which a core of dominant OTUs prevailed over the years along with temporally-transient OTUs which often represented rare or moderately-abundant sponge symbionts. Importantly, among the OTUs that were "specific" to each sampling year, many of them belong to typical sponge-associated phyla, for instance *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, PAUC34f, and *Poribacteria*, and thus are less likely to represent food or free-living bacteria eventually captured from the environment. These populations might therefore represent genetic variants of functionally-equivalent symbionts that could replace/succeed the more dominant phylotypes in face of changing host physiological or microenvironment conditions.

Among the nine bacterial phyla detected in *S. spinosulus* along the three successive years, only *Bacteroidetes* and *Chloroflexi* were shown to present significant shifts in their relative abundance over the years. Although some secondary metabolites have been reported for marine *Sphingobacteriia* (Spyere *et al*., 2003; Mincer *et al*., 2004) , which was the most abundant *Bacteroidetes* class in our sponge samples, its putative functioning in the sponge host is still to be unravelled. Conversely, members of the *Chloroflexi* comprise diverse metabolic and physiological properties and have been often retrieved from marine sponges worldwide, with 17 sponge-specific clusters detected so far (Bryant and Frigaard, 2006; Taylor *et al*., 2007; Bruck *et al*., 2010; Gupta, 2010; Schmitt *et al*., 2012; Simister *et al*., 2012c; Webster and Taylor, 2012). Although it is not possible, on the basis of available data, to propose a cause-effect relationship to explain the observed shifts in *Bacteroidetes* and *Chloroflexi* abundance over the three successive years, we noted for both phyla that there was no correlation between abundance and diversity changes. This suggests that the range of

potential metabolic capacities comprised within both phyla was rather conserved throughout the study period.

The "essential core" of *S. spinosulus* comprised 27 OTUs distributed in nine bacterial phyla, of which the vast majority (24 OTUs) have been often found in association with marine sponges (Taylor *et al*., 2007; Webster and Taylor, 2012). Several putative functions have been attributed to these bacterial phyla. For instance, marine *Actinobacteria* are well known for the production of a variety of secondary metabolites (Fenical and Jensen, 2006; Bull and Stach, 2007; Blunt *et al*., 2010), including several phylotypes isolated from marine sponges (Thomas *et al*., 2010b). Members of the phylum *Chloroflexi* might also synthesize antimicrobial compounds, as revealed by the non-ribosomal peptide synthase genes retrieved from a *Chloroflexi* cell sorted from *A. aerophoba* (Siegl and Hentschel, 2010). The phylum *Nitrospirae,* in its turn, encompasses species capable of oxidizing nitrate, nitrite, and iron, and reducing sulphur (Garrity and Holt, 2001). Representatives of the candidate phylum *Poribacteria* were shown to contain two intermediate enzymes of the anaerobic respiratory chain (nitrite and the nitric oxide reductases) and at least two polyketide synthases (Siegl and Hentschel, 2010; Siegl *et al*., 2011). The *Proteobacteria* is by far the most diverse bacterial phylum and exhibits a wide range of metabolic capabilities such as anoxygenic photosynthesis, sulphur-, iron-, hydrogen-, and nitrogen-dependent chemolithotrophy, methane oxidation, and chemoorganotrophic metabolism (Brenner *et al*., 2005). No information regarding the putative functions of marine *Acidobacteria*, AncK6, *Gemmatimonadetes*, and PAUC34f exists thus far. Because its composition cannot be explained by chance alone, it is tempting to speculate that the relatively poor bacterial core (at the phylotype level) shared by all *S. spinosulus* individuals might represent the minimal repertoire of symbionts (characterized by a nevertheless high phylum-level richness) needed to maintain the sponge holobiont health and functional.

The *Archaea* associated with *S. spinosulus* was stable over three successive years, encompassing few DGGE bands and 14 OTUs, with no significant difference in richness and diversity. To the best of our knowledge, this is the first temporal study of archaeal communities in marine sponges. Previous studies have also documented low diversity (usually ≤ four DGGE bands or OTUs) of archaeal symbionts in these animals (Webster *et al*., 2001a; Webster *et al*., 2004; Meyer and Kuever, 2008), highlighting the presence of a comparatively simple archaeal microbiota in sponges in comparison with their bacterial

counterparts. The assemblage of archaeal symbionts in *S. spinosulus* was largely dominated by *Nitrosopumilus* in the three consecutive years, except for one single sponge specimen that was dominated by *Cenarchaeum* sp.. It was shown that *Candidatus* Nitrosopumilus maritimus was capable to grow chemolithoautotrophically by aerobically oxidizing ammonia to nitrite (Könneke *et al*., 2005). *Cenarchaeum symbiosum* has been in its turn registered in different sponge species from various locations and even found to persist in one species kept in aquarium during a 6-month period (Preston *et al*., 1996; Schleper *et al*., 1998; Margot *et al*., 2002). Genomic analysis of *C. symbiosum* revealed homologues of genes related with chemolithotrophic ammonia oxidation, including ammonia monooxygenase encoding genes (Hallam *et al*., 2006). This indicates functional redundancy between both *Cenarchaeum* sp. and *Nitrosopumilus* sp. found in association with *S. spinosulus*. Thus, alterations in their shared prevalence in a given host individual, regardless of whether these changes were caused by a deterministc factor or stocastic event(s), does not seem to compromise the presumed roles archaeal sponge symbionts play in ammonia-oxidation.

The prokaryotic ammonia-oxidizers were also investigated in *S. spinosulus* using the functional *amoA* gene. The bacterial ammonia-oxidizers (AOB) associated with *S. spinosulus* comprised up to 14 DGGE bands. Previous studies are in congruence with our survey, for instance, PCR-DGGE profiling of the Caribbean *Halisarca caerulea* and the deep cold-water species *Higginsia thielei* and *Nodastrella nodastrella* (North Atlantic), generated from a single sampling, showed few bands associated with these hosts (Cardoso *et al*., 2013). Moreover, only two AOB OTUs were retrieved from the endosome of *Astrosclera willeyana* (Yang and Li, 2012) and the AOB associated with *Geodia barretti* was usually on the detection limit for PCR amplification (Hoffmann *et al*., 2009). In *S. spinosulus* the AOB community was shown to be stable along the three successive years with small variation among replicates and similar measurements of richness and diversity. In the present study no archaeal *amoA* gene amplicon was obtained from *S. spinosulus*, although archaeal ammonia-oxidizers (AOA) bands were observed in the PCR-DGGE profiles of *H. caerulea*, *H. thielei*, and *N. nodastrella* (Cardoso *et al*., 2013). A plausible explanation for our contrasting results might be on the primer selected for PCR-DGGE profiling, because 454-pyrosequencing analyses performed with universal archaeal primers showed that this community was dominated by *Nitropumilus* sp., known to be an ammonia-oxidizer. Altogether, these data

represent the first evidence of AOA and AOB stability in marine sponges over consecutive years.

Conclusions

To date this is the largest and most comprehensive temporal study of prokaryotic communities associated with a marine sponge host in the natural environment. Here, we show evidences for i) a slight community gradient being formed across time in *S. spinosulus* characterized by the maintenance and turnover of symbionts in equivalent numbers, ii) temporally-transient symbionts which do correspond to typical sponge-associated lineages but are more rare than the core temporal symbionts, and iii) subtle replacement of bacterial communities, within the study period, with likely functional maintenance. Thus, the microbiome of *S. spinosulus* appears to be in dynamic stability as supported by the community turnover concept in which the maintenance of the most dominant species is under constant assault by the emergency of transient species, which might replace the dominant ones when conditions favour (Gonzalez *et al*., 2011). This temporal dynamics has been observed in other environments, for instance, the human body (Costello *et al*., 2009; Caporaso *et al*., 2011), oceans (Gilbert *et al*., 2009), lakes (Shade *et al*., 2007), and soils (Costello and Schmidt, 2006; Griffiths *et al*., 2011). It is likely that deterministic factor(s) encountered in the environment (e.g. nutrient resources and pH) and variations thereof exert a strong influence in the process of microbial community structuring in marine sponges along with host genotype/metabolism. A more comprehensive understanding of the mechanisms that regulate the diversity, structure, and composition of symbiont communities is still necessary to better understand the functioning of marine sponges, aiding in the conservation of the natural genetic resources and biotechnological potential encrypted in their diversified microbiomes.

Acknowledgments

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Supplementary Material

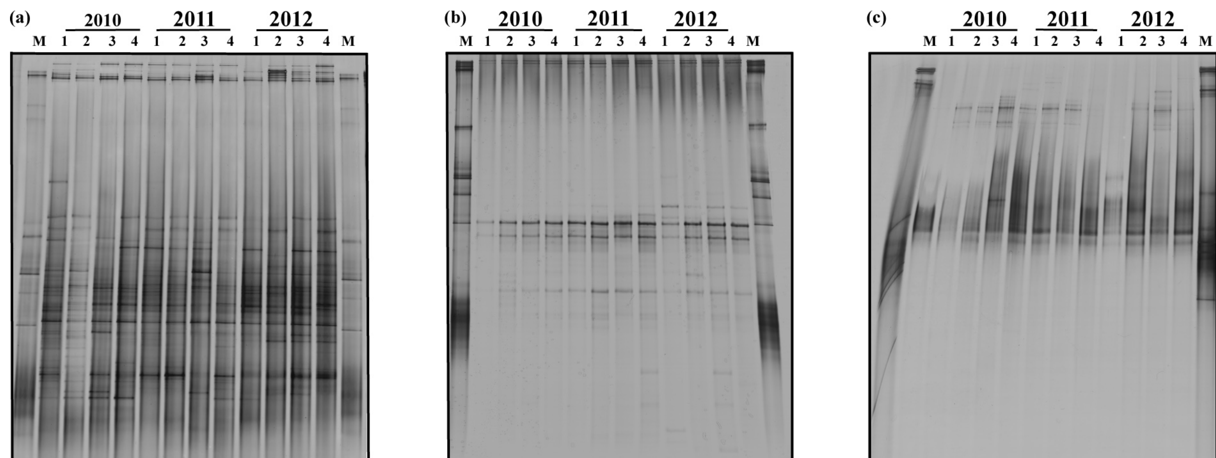


Figure S5.1. PCR-DGGE fingerprinting of bacterial 16S rRNA gene (a), archaeal 16S rRNA gene (b) and bacterial *amoA* gene (c) fragments associated with the marine sponges *S. spinosulus*.

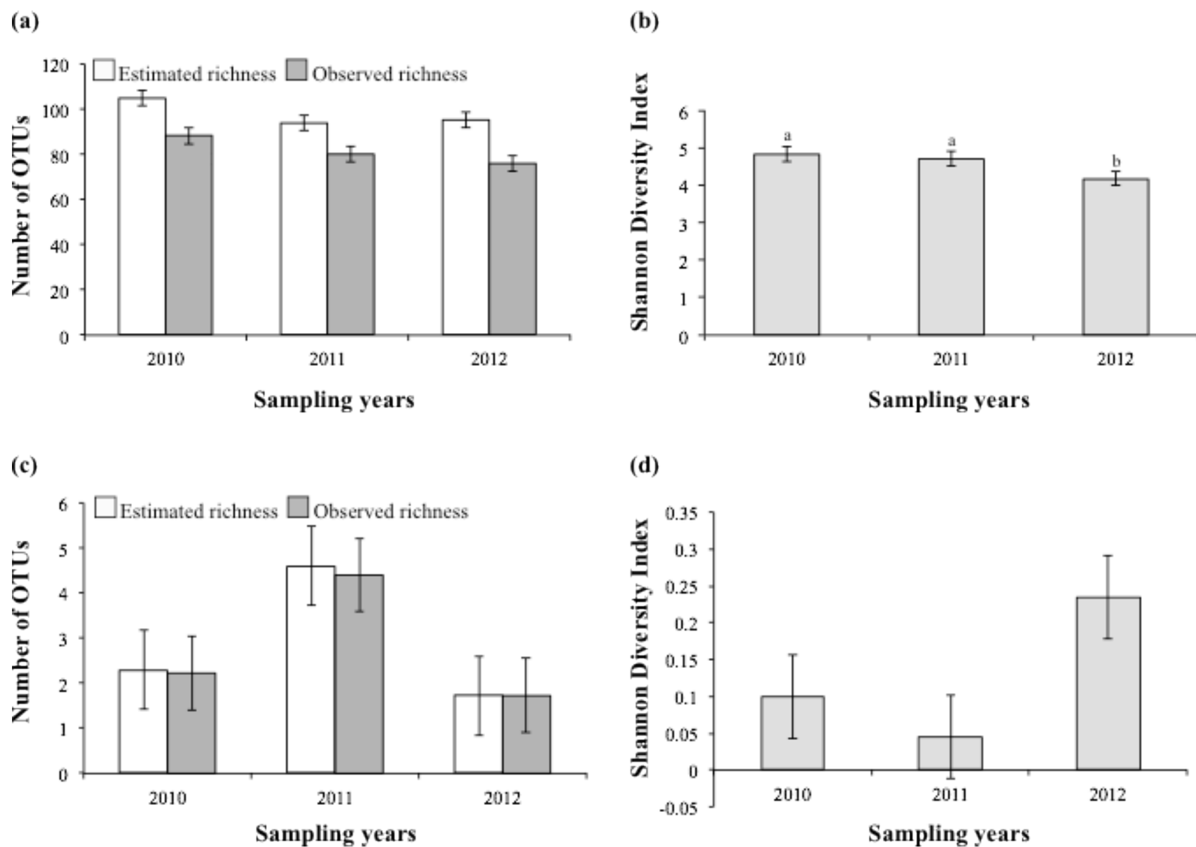


Figure S5.2. Observed and estimated richness for bacterial (a) and archaeal (b) OTUs (97% cut-off) and diversity indices for bacterial (c) and archaeal (d) OTUs (97% cut-off) in the marine sponge *S. spinosulus* along three consecutive years.

Table S5.1. Taxonomic classification and absolute abundance of bacterial OTUs found exclusively in each sampling year (a) 2010, (b) 2011 and (c) 2012.

A) 2010

Phylum level	Number of OTUs	Number of sequences	Class level	Number of OTUs	Number of sequences
Acidobacteria	1	2	*Rubrobacteria*	1	2
Bacteroidetes	7	16	*Cytophagia*	1	2
			Flavobacteriia	6	14
Chloroflexi	5	25	*Anaerolinea*	2	8
			SAR202	3	17
Deferribacteres	1	2	*Deferribacteres*	1	2
Firmicutes	4	116	*Bacilli*	1	8
			Clostridia	2	104
			Erysipelotrichi	1	4
Gemmatimonadetes	1	18	Gemm-2	1	18
Nitrospirae	1	2	*Nitrospira*	1	2
Planctomyecetes	2	4	*Planctomycetia*	2	4
Poribacteria	1	2	-	1	2
Proteobacteria	11	138	*Alphaproteobacteria*	2	67
			Betaproteobacteria	2	57
			Gammaproteobacteria	7	14

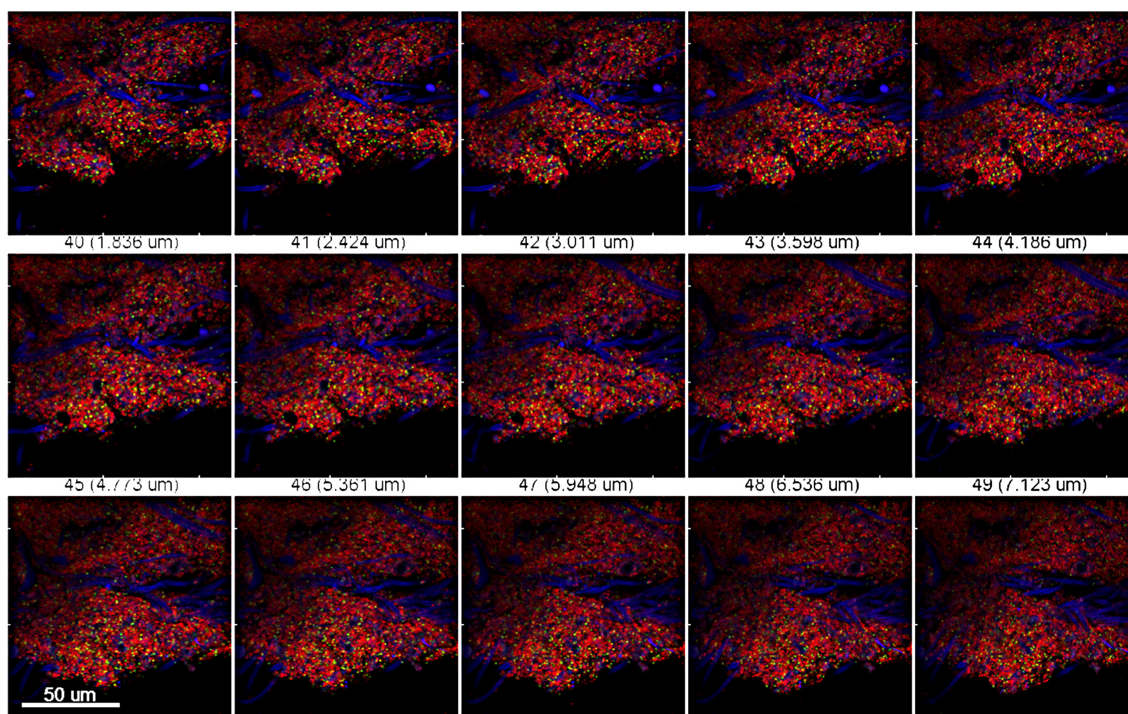
B) 2011

Phylum level	Number of OTUs	Number of sequences	Class level	Number of OTUs	Number of sequences
Acidobacteria	2	8	*Solibacteres*	1	5
			Sva0725	1	3
Actinobacteria	2	5	*Acidimicrobiia*	1	2
			Coriobacteriia	1	3
Anck6	1	3	-	1	3
Bacteroidetes	1	2	*Flavobacteriia*	1	2
Caldithrix	1	2	*Caldithrixae*	1	2
Cyanobacteria	1	3	*Synechococcophycideae*	1	3
Firmicutes	1	3	*Clostridia*	1	3
PAUC34f	1	3	-	1	3
Poribacteria	2	105	-	2	105
Proteobateria	7	77	*Alphaproteobacteria*	2	4
			Detaproteobacteria	1	2
			Gammaproteobacteria	4	71

C) 2012

Phylum level	Number of OTUs	Number of sequences	Class level	Number of OTUs	Number of sequences
Acidobacteria	1	2	Sva0725	1	2
Actinobacteria	1	2	*Actinobacteria*	1	2
Bacteroidetes	2	38	*Cytophagia*	1	2
			Flavobacteriia	1	36
Chloroflexi	2	9	*Anaerolineae*	2	9
PAUC34f	1	2	-	1	2
Planctomycetes	1	2	-	1	2
Proteobacteria	8	20	*Alphaproteobacteria*	3	6
			Deltaproteobacteria	2	6
			Gammaproteobacteria	3	8

CHAPTER VI



Microbial communities in marine sponges of the family Irciniidae and their bioactive compounds – a review

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Manuscript in preparation

Chapter VI

Abstract

Marine sponges are known to harbour dense and diverse microbial communities with substantial ecological and biotechnological relevance. Here, we provide numerous reasons that support the sponge family Irciniidae (Demospongiae, Dictyoceratida) as an appropriate model for microbiology and biotechnology investigations. Complex and intricate bacterial communities have been registered in several irciniid species and some studies have demonstrated that they are specific to each sponge host. Further, evidence for vertical transmission of several bacterial symbionts in an irciniid host has been documented, suggesting an intimate host-microbe pattern of relationship. Conversely, few surveys approached the archaeal and microeukaryotic diversities in these hosts, and improved knowledge in this area is much needed. Distinct bioactivities and bioactive compounds with putative biotechnological applications were recovered from bacterial and fungal symbionts isolated from irciniid species. So far, two out of five quorum-sensing molecules (QSM) were detected in irciniid sponges, indicating that chemical signalling between bacteria and eventually between host and symbionts is an important process governing the dynamics of this complex interactive system. Notably, Mediterranean irciniid species seem quite vulnerable to disease outbreaks, as several mass mortality events have been registered in the literature. However, the etiological agents underpinning such events still need to be ascertained. Although a great amount of secondary metabolites have been recovered from Irciniidae species, to date the true producer of such compounds was rarely unravelled. To address this and many other fundamental questions, the mariculture approach, as opposed to sponge tissue and/or aquaculture, seems to be the most feasible strategy to cultivate irciniids. Although knowledge regarding Irciniidae sponges is advancing, several fundamental questions concerning holobiont functioning remain unanswered, which certainly will require further efforts to achieve a comprehensive understanding of the relationship between the sponge host and its microbiome.

1. Introduction

The phylum Porifera (sponges) represents the oldest metazoan lineages on Earth, with fossil records dating back to around 580 million years ago (Li *et al*., 1998; van Soest *et al*., 2012). The ancestry of marine sponges might even be of greater magnitude than previously thought, as chemical records found for demosponges place the origin of these animals back to more than 645 million years ago at the transition between the Ediacaran (Neoproterozoic) and the Cambrian (Paleozoic) periods (Love *et al*., 2009). Sponges are sessile filter feeders with a great capability to filter thousands of litres of water per day (Vacelet and Donadey, 1977; Taylor *et al*., 2007; Hentschel *et al*., 2012). They are found in almost all of the 232 marine ecoregions of the World. Eleven such ecoregions encompass between 201 and 461 sponge species and are thus considered hot spots of marine sponge diversity (Spalding *et al*., 2007; van Soest *et al*., 2012). These animals are restricted to aquatic habitats and are divided into four classes: Demospongiae, Hexactinellida, Calcarea, and Homoscleromorpha, which are further distributed into 25 orders, 128 families, and 680 genera. The class Demospongiae is by far the richest and most widespread, containing about 83% of the 8.553 valid species described so far (van Soest *et al*., 2012).

Although prokaryotic microorganisms are the most important components of the sponge diet, these animals are capable to form symbiotic associations with abundant and diverse bacteria, and in some cases up to 38% of the sponge wet weight is composed by bacterial cells (Vacelet and Donadey, 1977; Taylor *et al*., 2007; Hentschel *et al*., 2012). So far, 28 bacterial phyla have been detected in marine sponges (Taylor *et al*., 2007; Hentschel *et al*., 2012; Webster and Taylor, 2012), and several lineages within these phyla have been shown to be involved in the production of secondary metabolites (Thomas *et al*., 2010b), which might enhance the host defence mechanisms against predators and invading pathogens. Besides bacteria, archaeal and fungal cells are consistently found as constituent members of the marine sponge microbiome, and specific host-related functions as well as bioactive potential have also been proposed or reported for these groups (Taylor *et al*., 2007; Thomas *et al*., 2010b). In the light of the enormous richness of marine sponges across the globe, their contribution as the most prolific sources of marine bioactive compounds and the actual participation of their microbial symbionts in secondary metabolites biosynthesis, it is reasonable to posit that marine sponge microbiomes constitute as-yet uncharted and extremely fertile reservoirs of genetic and metabolic novelties. In this context, the present review covers

microbial community structure, diversity, and bioactivities reported for marine sponges, making use of the family Irciniidae as a model taxon. This family displays wide geographical distribution and is well known for its chemical complexity and diverse microbiota. Here, we highlight bioactive compounds reported for irciniids possessing a microbial origin. Moreover, we revisit our current knowledge of host and symbiont cell culturing, metagenomics-based gene discovery, experimental microbial ecology applied to and diseases affecting marine sponges, and cell-cell signalling and its relevance to bioactive metabolite biosynthesis. It is not the scope of this review to describe all bioactive compounds retrieved from Irciniidae species. For this, we direct the reader to several previous and comprehensive reviews on marine secondary metabolites (Faulkner, 1997, 1998, 1999, 2000, 2001, 2002; Blunt *et al*., 2003, 2004, 2005, 2006; Blunt *et al*., 2007, 2008, 2009; Blunt *et al*., 2010, 2011; Blunt *et al*., 2012, 2013).

2. The Irciniidae family as a model taxon in sponge microbiology research

With more than 8.500 described and 17.000 estimated species, few relevant issues such as comprehensively describing microbial diversity, metabolism and functioning in marine sponges and how these features relate with host species, habitat, depth and environmental and geographical gradients, constitute a true challenge. Given this picture, the need of spotting and making use of model hosts in sponge microbiology research has been raised and discussed by specialists at recent meetings such as the "1st International Symposium on Sponge Microbiology" (Taylor *et al*., 2011), and the "Lower Invertebrates Symbiosis with Microorganisms Workshop" held in Eilat, Israel in 2012. In the past 15 years or so, intense molecular microbiology research performed on species such as *Aplysina aerophoba* from the Mediterranean Sea (Hentschel *et al*., 2002; Fieseler *et al*., 2004; Siegl and Hentschel, 2010; Schmitt *et al*., 2012; Bayer *et al*., 2013), *Rhopaloeides odorabile* from the Great Reef Barrier (Webster *et al*., 2008; Webster *et al*., 2010; Pantile and Webster, 2011; Simister *et al*., 2012a; Fan *et al*., 2013), and *Ircinia* spp. from the tropics (Mohamed *et al*., 2008c; Mohamed *et al*., 2008a; Mohamed *et al*., 2008b; Mohamed *et al*., 2010; Zan *et al*., 2011) have naturally turned these organisms into model sponge hosts. Strategic and long-term research on key host organisms will most likely improve our understanding of sponge-microbe interactions in a more mechanistic fashion, allowing the scientific community to extract pertinent information

from a core of relevant species that would well represent the whole. In this regard, a likely rewarding approach to the choice of model organisms is looking after taxonomic ranks above the species level. Such strategy would not only widen the biodiversity spectrum of the target animals, but also most likely span the breadth of geographical occurrence, phenotypic and genotypic plasticity, habitats, and depths under scope, thus facilitating the assessment of microbiome diversity and function as a response to environmental and host-related factors within a well contextualized phylogenetic framework. The family Irciniidae (Demospongiae, Dictyoceratida) is composed only by marine species divided into three genera namely *Ircinia* (Schmidt, 1862), *Sarcotragus* (Schmidt, 1862), and *Psammocinia* (Lendenfeld, 1889) with 75, 11, and 25 species, respectively, so far accepted according to the World Porifera Database (van Soest *et al*., 2013). Most species have been mainly recovered from tropical to temperate regions (Cook and Bergquist, 2002) and are encountered inhabiting the epipelagic layer (i.e. photic zone where there is enough light for the photosynthesis process), at depths ranging from 0 to 60 m. Nevertherless but few specimens have been even collected within the mesopelagic layer at about 365 m depth (Cook and Bergquist, 1999). Irciniidae species exhibit a wide variety of shapes (for a technical description, see Cook and Bergquist, 2002). Fine collagenous and terminally enlarged spongin filaments that increment the fibre skeleton, hence making the sponges very difficult to tear, are typical features of this family. Irciniids lack authentic spicules, the archetypical mineral skeleton-forming structures in the majority of demosponges (Wörheide *et al*., 2012). The spherical to oval diplodal choanocyte chambers and the mesohyl (an extracellular matrix that fills the space between outer and inner surfaces) penetrated with collagen are also characteristic of this family (Cook and Bergquist, 2002). Several reasons underpin the notion of the Irciniidae as a suitable model taxon for microbiology and biotechnology research, as follows:

1. The existence of several Irciniidae species allows analysis of evolutionary relationships between the hosts and testing the species-specific hypothesis of microbiome composition.
2. The wide geographical distribution of these species permits testing hypotheses of symbiont maintenance across biogeographical gradients.
3. Several bioactive secondary metabolites have been isolated from Irciniidae species (see supplementary material). This chemical complexity indicates intricate and diverse microbiome associated and emphasizes their biotechnological potential.

4. Tropical Irciniidae species have been reported as high microbial abundance sponges. This suggests high metabolic activity of their symbionts, which most likely play essential roles for host fitness and survival.
5. Bacterial species from at least 15 phyla have been found enriched in *Ircinia* spp. and *S. spinosulus* when compared with surrounding seawater. This diverse microbiome suggests an intrinsic relationship between host and their symbionts.
6. Several bacterial lineages associated with Irciniidae species are known for their putative functions that enhance the host phenotypic plasticity.
7. The order Dictyoceratida comprises four families - including Irciniidae - collectively known as 'keratosa' sponges because they lack mineral elements (i.e. true spicules), making these families particularly interesting in what concerns their microbiome composition.

In the following sections, we provide a historical perspective on the several findings and particular features pertaining to the marine sponge microbiome, with focus on irciniid hosts. The review presented here results from a comprehensive literature search made using *Ircinia*, *Sarcotragus,* and *Psammocinia* as keywords, covering all scientific papers publicly available from 1970 until April 2013.

3. Microbial diversity and bioactivities in the family Irciniidae

The majority of the microbial diversity surveys undertaken so far on Irciniidae specimens have focused on tropical species belonging to the genus *Ircinia* (Usher *et al*., 2006; Schmitt *et al*., 2007; Weisz *et al*., 2007; Mohamed *et al*., 2008c; Mohamed *et al*., 2008a; Mohamed *et al*., 2008b; Webster *et al*., 2010; Yang *et al*., 2011). Only recently the microbiome of *Ircinia* species in temperate latitudes have been more thoroughly investigated (Erwin *et al*., 2012c; Erwin *et al*., 2012a; Erwin *et al*., 2012b; Hardoim *et al*., 2012), but thus far no direct comparison between prokaryotic community structures from tropical and temperate irciniids has been performed. Previous studies classified members of the Irciniidae family as high microbial abundance (HMA) sponges (Weisz *et al*., 2007; Hardoim *et al*., 2012), highlighting their capability of hosting highly dense microbiomes, ranging from 10^8 to 10^{10} prokaryotic cells g^{-1} of sponge (fresh weight). Although several studies focused on the isolation of

secondary metabolites from these sponges (see supplementary material), little attention was paid to investigate the actual producer (i.e. host or symbiont) of such compounds.

3.1 Early microbiology studies

First insights into the microbial abundance and diversity associated with sponges were obtained with the utilization of electron microscopy - especially transmission electron microscopy (TEM) (Vacelet and Donadey, 1977; Wilkinson, 1978a), which is still being an important tool in a variety of studies (Webster and Hill, 2001; Fieseler *et al*., 2004; Hentschel *et al*., 2006; Hardoim *et al*., 2009; Kamke *et al*., 2010).

In the early 1970s, Sara (1971) showed by electron microscopy that the temperate *I. variabilis* was populated by the cyanobacteria *Aphanocapsa feldmanni* and *A. raspaigellae*. The former was detected in the cortical mesohyl and inside the sponge cells, while the latter was recorded only in the mesohyl and was involved by a lacunar space. Extracellularly, both species reproduced. The symbiotic relationship was confirmed, whereby *Aphanocapsa* spp. supplied the sponge with organic material originated from photosynthesis, protected the host from excessive illumination, and could use the nitrogenous compounds eliminated by the sponge, whereas *I. variabilis* offered shelter and protection for the *Cyanobacteria* species (SARA, 1971). Using scanning and transmission electron microscopies, Wilkinson (1978a,c) revealed higher abundance of heterotrophic bacteria in the dense mesohyl of *I. wistarii*, collected at Great Barrier Reef (Australia), when compared with surrounding seawater. These symbionts were concentrated around the inhalant canals of the sponge (Wilkinson, 1978c, a). Although analyses with TEM enabled first insights into the abundance and morphology of bacterial symbionts in sponges, their diversity and composition (including here the identity of most cells observed under the microscope) remained enigmatic until the first culture-dependent and –independent inventories of sponge-associated bacteria were created. These were ultimately responsible for an enormous increase of knowledge regarding sponge microbiome diversity in the last 35 years.

3.2 Bacteria

3.2.1 Diversity

3.2.1.1 Culture-dependent approaches

Wilkinson (1978b) isolated 87 bacterial colonies from *I. wistarii*, and performed a total of 76 tests measuring morphology, physiology, and metabolic capabilities. Fifty-nine isolates were grouped into 6 well-defined clusters, whereas 28 isolates were too ambiguous to be assigned to any clade. Surprisingly, these isolates were able to metabolize a wide range of compounds and, for the first time, comprehensive information about sponge-associated bacteria along with isolates from surrounding seawater (SSW) was provided (Wilkinson, 1978b). During the subsequent 25 years, no attempts were made to cultivate symbionts from Irciniidae species, while several other sponge hosts have been approached in this manner, usually reporting on *Alpha*- and *Gammaproteobacteria* as the prevailing members of the culturable sponge-associated microbiota (Hentschel *et al*., 2001; Webster and Hill, 2001; Hentschel *et al*., 2006). Likewise, using several media the culturable assemblage of *Ircinia* sp. (St. Giovanni, Croatia) was determined and found to be dominated by *Alpha* and *Gammaproteobacteria* (Muscholl-Silberhorn *et al*., 2008). In another study using marine agar, Esteves *et al*., (2013) isolated more than 270 bacterial strains from *S. spinosulus* and *I. variabilis*. These were classified into 17 genera and up to 10 putative new species were detected (Esteves *et al*., 2013). In accordance with several cultivation-dependent studies performed with other sponge hosts (Webster and Hill, 2001; Thiel and Imhoff, 2003; Enticknap *et al*., 2006; O' Halloran *et al*., 2011), the bacterial genera *Pseudovibrio*, *Ruegeria,* and *Vibrio* (*Proteobacteria*) prevailed among the retrieved symbionts (Esteves *et al*., 2013). Further, using six *Actinobacteria*-specific media (Tabares *et al*., 2011) isolated only *Arthrobacter* sp. BA51 from *I. felix*. The main advantage of cultivation technique is the possibility to determine the putative functions of the bacterial symbionts. For instance, Muscholl-Silberhorn *et al*. (2008) and Esteves *et al*. (2013) have tested the bacteria cultures for *in vitro* antimicrobial activity (see bioactivities section), which is a common approach widely used in sponge microbiology. However, the biases inherent to culture-dependent approaches, such as < 1% of total bacterial counts being recovered (Friedrich *et al*., 2001; Webster and Hill, 2001), with most of the isolates belonging to the *Proteobacteria* (Hugenholtz, 2002; Hentschel *et al*., 2006; Taylor *et al*., 2007), have favoured the use of molecular techniques in the characterization of bacterial communities associated with marine sponges.

3.2.1.2 Cultivation-independent approaches

The application of DNA-based, molecular approaches to the marine sponge microbiome, relying mainly on the sequencing of 16S rRNA genes, has illuminated our view of the bacterial community structure and diversity associated with marine sponges by circumventing the well-known limitations of culturing techniques. The first study using a molecular, cultivation-independent method to target microorganisms inhabiting an irciniid sponge was performed by Usher *et al*. (2004). Using a *Cyanobacteria*-specific primer pair, the authors detected two distinct denaturing gradient gel electrophoresis (DGGE) bands on the profile of *I. variabilis*. These were affiliated with *Candidatus Synechococcus spongiarum* with 99.7% sequence similarity and with *S. feldmannii* (Usher *et al*., 2004). However, a more comprehensive assessment of the Irciniidae microbiome was still needed. PCR-DGGE fingerprinting and clone-and-sequencing of the 16S rRNA gene fragments approaches using universal bacterial primers targeting the whole bacterial community were applied to determine the bacterial community associated with *I. felix* and *I. strobilina* from Key Largo, Florida, USA (Schmitt *et al*., 2007; Weisz *et al*., 2007; Mohamed *et al*., 2008b), *I. strobilina* from Sweetings Cay (Bahamas) (Yang *et al*., 2011), and the Mediterranean *I. variabilis*, *I. fasciculata* and *I. oros* (Erwin *et al*., 2012c). Overall, these studies revealed *Acidobacteria, Actinobacteria*, *Chloroflexi*, *Cyanobacteria*, *Gemmatimonadetes*, *Nitrospira*, and *Proteobacteria* (*Alpha*, *Delta,* and *Gamma* classes) as the prevailing bacterial phyla associated with *Ircinia* spp. (Schmitt *et al*., 2007; Weisz *et al*., 2007; Mohamed *et al*., 2008b; Yang *et al*., 2011; Erwin *et al*., 2012c). It was also demonstrated that irciniids harboured a distinct microbiome compared with SSW (Weisz *et al*., 2007; Mohamed *et al*., 2008b; Yang *et al*., 2011; Erwin *et al*., 2012c). Erwin *et al*. (2012c) showed that 38 out of 56 sequences fell within sponge-specific or sponge- and coral-specific clusters. The number of 99% OTUs (Operational Taxonomic Units, defined at 99% sequence similarity) for *I. fasciculata*, *I. oros*, and *I. variabilis* was 29, 33, and 34, respectively. Rarefaction curves showed that the sponge-associated bacteria approached the plateau, whereas the opposite occurred with seawater. Surprisingly, only 4 OTUs were registered in all three *Ircinia* species, indicating that each sponge species harboured its unique bacterial community and that intrinsic aspects of the host species - for instance habitat preference, specific geographic distribution, and evolutionary history - play important roles in shaping the structure of bacterial symbiont communities in these sponges (Erwin *et al*., 2012c).

To address temporal variation within symbiont communities, Erwin *et al*. (2012b) monitored, for 1.5 years, the bacterial community structure associated with three Mediterranean *Ircinia* spp. and SSW collected once every season. Terminal restriction fragment length polymorphism analysis revealed that the bacterial communities within each host were highly stable along time. Cloning-and-sequencing approach with universal bacterial primers was performed for all *Ircinia* spp*.,* where the same sponge individuals were collected in winter and summer (Erwin *et al*., 2012b). This showed that > 50% of the sponge symbionts was stable across seasons with no significant difference in genetic differentiation and community structure. Similar bacterial composition was detected within each host in both winter and summer libraries and differences between libraries were usually caused by changes in the rare bacterial OTUs. Further, seven of eight OTUs were closely related to sponge-associated bacteria, of which *Candidatus S. spongiarium* was the most dominant followed by OTUs affiliated with *Deltaproteobacteria*, *Acidobacteria*, *Gammaproteobacteria*, *Nitrospira,* and *Cyanobacteria*. The seasonal stability of the sponge microbiome observed in this study supports the host-specific hypothesis. Erwin *et al*. (2012a) also unravelled the *Cyanobacteria* diversity in *I. fasciculata* and *I. variabilis* using several approaches. *I. fasciculata* contained almost twice the level of chlorophyll *a* and high abundance of glycogen granules than *I. variabilis*. Cyanobacterial cells were found in the ectosome of both sponge species in dense populations. *Candidatus S. spongiarum* was dominant and detected intercellularly in the mesohyl, actively reproducing, and seemed to interact with host cells. *Synechocystis* sp. was seldom and sporadically found in *I. fasciculata*, and it was neither in reproductive process nor in close association with host cells. Rarefaction analysis revealed that the diversity of cyanobacterial present in both sponge species reached the plateau by the employed sequencing effort, showing 2 and 1 OTUs in *I. fasciculata* and *I. variabilis*, respectively. 16S rRNA gene analysis showed that ~85% of *I. fasciculata* and 100% of *I. variabilis* clones were closely related to *Candidatus S. spongiarum* (99% sequence identity), whereas the remaining clones from *I. fasciculata* matched the genus *Synechocystis*. Phylogenetic inferences of 16S–23S rRNA ITS gene sequences recovered from both sponge species showed a novel clade of *S. spongiarum*, whereas the analysis of partial 16S rRNA gene sequences retrieved only from *I. fasciculata* disclosed a new *Synechocystis* cluster and four robust and distinct clades were resolved, among them one was *I. fasciculata*-specific (Erwin *et al*., 2012a). These results corroborate the findings of Usher *et al*. (2004), whereby *Candidatus S. spongiarum* has been

consistently observed in *Ircinia* spp. and suggesting that cyanobacterium symbionts play an important role in photosynthesis activity within the sponge body (Erwin *et al*., 2012a).

Hardoim *et al*. (2012) determined the bacterial abundance, diversity, and community composition associated with the phylogenetically and spatially close related marine sponges *S. spinosulus* and *I. variabilis*. Both sponge species were classified as HMA sponges based on epifluorescence microscopy analysis, providing thus inaugural information on the prokaryotic abundance of temperate Irciniidae. PCR-DGGE profiles of *S. spinosulus* and *I. variabilis* were dissimilar, showing that each sponge species harboured its own bacterial community. This prompted these authors to claim that host-specific and divergent bacterial community structures might even characterize phylogenetically related sponge species living in sympatry, further strengthening the simultaneous findings of Erwin *et al*. (2012c) on host-specific symbiont composition of Ircinidae species in the Mediterranean Sea. These surveys revealed that iciniids held a complex and intrincate bacterial microbiome, however due to limitations inherent to PCR-DGGE fingerprints (i.e. impossibility to identify the entire prokaryotic community) and clone library (i.e. restrictions regarding library sizes), several questions regarding the diversity associated with Irciniidae species remained unanswered. To overcome these limitations, the application of next-generation sequencing (NSG, e.g. 454-pyrosequencing), which extends our knowledge of microbial diversity associated with marine sponges, was introduced in recent years (Webster *et al*., 2010; Lee *et al*., 2011).

Using 454-pyrosequencing, a total of 16 phyla and 1.199 bacterial OTUs (assigned at 95% sequence similarity) were recovered from *I. ramosa* collected at Great Barrier Reef (Australia), whereas 30 bacterial phyla and 6.800 OTUs were recovered from SSW (Webster *et al*., 2010). With this technology, Schmitt *et al*. (2012) uncovered 159, 179, 86, and 111 bacterial OTUs (97% cut-off) from *Ircinia* sp., *I. felix*, *I. variabilis*, and *I. gigantea* (note by authors: non-valid taxon) specimens sampled at the Indian Pacific, Caribbean and Mediterranean Seas, and the Great Barrier Reef, respectively. Overall, the dominant bacterial phyla associated with *Ircinia* spp. were *Acidobacteria*, *Chloroflexi*, *Poribacteria,* and *Proteobacteria* (*Alpha* and *Gamma* classes). Both surveys suggested that indeed the bacterial community associated with marine sponges was species-specific (Webster *et al*., 2010; Schmitt *et al*., 2012).

454-pyrosequencing was likewise employed as the overriding methodology in a series of recent studies covering cultivation bias, biogeographic patterns, and temporal stability of

microbial communities associated with Irciniidae species in the north Atlantic. Hardoim *et al*. (Chapter 3) disclosed the effects of three methods of sample handling on the bacterial community composition associated with *S. spinosulus* and *I. variabilis*. The authors, once more, observed with cultivation-independent methods that the bacterial community structure in *S. spinosulus* and *I. variabilis* was species-specific, hence further deepening the observations made by means of PCR-DGGE profiling, whereas a highly similar bacterial assemblage was detected in both sponge species via culturing. Around 200 and 220 OTUs (97% sequence similarity) in *S. spinosulus* and *I. variabilis*, respectively, and 15-18 bacterial phyla were registered in both sponge species with cultivation-independent methods. Instead, only 33 and 39 OTUs were detected in *S. spinosulus* and *I. variabilis*, respectively, via culturing. Most of these OTUs were classified into only one bacterial phylum, clearly revealing that the cultured bacterial fraction in these sponges displayed much reduced diversity than that unveiled by cultivation-independent analyses. Surprisingly, about half of the OTUs obtained with the cultivation-dependent approach was exclusive to this procedure, suggesting that the bacteria cultivated from the inspected specimens represented extremely rare symbionts not readily detected in sponge metagenomic DNA by NGS, which were probably sharply selected and enriched in number on cultivation plates. The authors also determined, for the first time, the bacterial localization in *I. variabilis* and *S spinosulus* by fluorescence *in situ* hybridization, which revealed that bacterial cells were almost exclusively observed between sponge cells and "neglected" on the spongin fibers that constitute the skeleton of Irciniidae sponges. Overall, culture-independent approaches favour the species-specific view of bacterial community composition in Irciniidae sponges, which could not be made solely based on cultivation-dependent techniques. Furthermore, the clearly different bacterial communities obtained with culture-independent and -dependent methods demonstrated that the dominant bacterial phyla recovered with the former approach were exactly the ones that could not be cultured. Thus, the dominant and most likely essential bacterial symbionts of marine sponges remain primarily recalcitrant to cultivation, and much more effort is still needed to improve cultivation techniques in order to capture them in the laboratory.

Lago-Lestón *et al*. (Chapter 4) unravelled the extent to which the structure of bacterial communities associated with *Sarcotragus* spp., *Ircinia* spp. (Irciniidae), and *Spongia* spp. (Spongiidae) is determined by the host organism and/or by its biogeographical background.

To test this hypothesis, the sponge species were collected at the Algarve coast, the Madeira Island, and the Azores archipelago (Portugal). *Sarcotragus* spp. and *Spongia* spp. possessed a less variable bacterial communities compared with *Ircinia* spp. Unexpectedly, bacterial communities associated with *Ircinia* spp. and *Sarcotragus* spp. were more dissimilar to one another than *Sarcotragus* spp. and *Spongia* spp. communities, which are phylogenetically more distantly related than *Ircinia* spp. and *Sarcotragus* spp.. Thus, although species-specific communities were again detected within each of the investigated places, these results contradict the hypothesis of higher levels of microbiome similarity between phylogenetically closer sponge species. In *Sarcotragus* spp. and *Spongia* spp. the dominant bacterial phyla were *Acidobacteria* and *Actinobacteria* (*c.* 20% relative abundance), followed by *Proteobacteria*, *Poribacteria*, PAUC34f, *Bacteroidetes,* and *Chloroflexi* (*c.* 10% relative abundance each group). These abundances were maintained in *Sarcotragus* spp. and *Spongia* spp. independently of the sampling sites. Conversely, in *Ircinia* spp. there was a change in the abundances at the phylum level: while *Chloroflexi* was the most dominant phylum (31%) in specimens collected at the Madeira site, it was registered as a minor taxon at the Algarve (7%) and the Azores (3%) sites, where *Proteobacteria*, *Acidobacteria,* and *Actinobacteria* prevailed. The most variable bacterial community composition was detected in *Ircinia* spp., which kept a common core (i.e. OTUs shared between specimens from all sampling sites) of 93 out of 403 registered bacterial OTUs. Overall these results indicate a complex and intricate collaboration between sponge host and surrounding environment that together shape the bacterial microbiome associated with marine sponges at the estimate species (i.e. OTU) level.

Hardoim *et al*. (Chapter 5) further unravelled the temporal dunamics of the bacterial community associated with *S. spinosulus* over three sucessive years. PCR-DGGE and tag-pyrosequencing showed that the bacterial assemblage was stable over time, with the majority of dominant bands and OTUs detected in all years. *S. spinosulus* was dominated by eight bacterial phyla, of which only two presented significantly different ($p<0.05$) relative abundances over the years. Twenty-seven OTUs distributed in eight bacterial phyla and one candidate phylum were detected in all 12 sponge specimens. Interestingly, in spite of its low diversity at the phylotype level, this minimal core of symbionts shared by all specimens displays high bacterial richness at the phylum level, quite comparable to that observed in a single sponge specimen hosting about 200 OTUs. This suggests that phylum diversification is fundamental to sponge functioning, and that high redundancy of phylotypes within each

phylum likely aids the sponge host in maintaining its diversified repertoire of bacterial phyla in face of local environmental changes or across different developmental stages. The low number of shared bacterial phylotypes in 12 replicate specimens of the same species also reveals some degree of individuality in symbiont community structure in *S. spinosulus*, in spite of the repeated detection of common symbionts over the years when composite samples (that is 4 individuals per year) are collectively analysed.

In summary, the application of NGS enabled a better understanding regarding the bacterial community structure associated with marine sponges compared to the cloning-and-sequencing approach. For instance, between 29 to 33 OTUs (99% sequence similarity) were associated with *Ircinia* spp. using the cloning-and-sequencing method (Erwin *et al*., 2012c), whereas a much more complex microbiome was registered with NGS where hundreds to thousands of OTUs (95 and 97% sequencing similarity) were recovered from irciniids (Webster *et al*., 2010; Schmitt *et al*., 2012; Hardoim *et al*., Chapter 3; Lago-Lestón *et al*., Chapter 4; Hardoim and Costa, Chapter 5). Therefore, the use of NGS will be the most suitable tool to unveil complex associations such as those established by sponges and their symbionts enabling a more comprehensive understanding of this interaction.

Besides the use of the 16S rRNA gene as phylogenetic marker, selected genes have also been applied to determine putative functions from sponge symbionts, especially those involved in biogeochemical cycling (Taylor *et al*., 2007). Among the elements essential to life, nitrogen is one of the most limiting factors affecting ecosystem functioning. Nitrogen cycling is a complex system that plays a critical role in ecosystem functioning (Fiore *et al*., 2010). The *nifH* gene, which encodes an iron-containing dinitrogenase reductase, has been widely used in diversity assessments of N-fixing bacteria in aquatic and terrestrial environments (Zehr *et al*., 1998; Zehr *et al*., 2003; Man-Aharonovich *et al*., 2007). Mohamed *et al*. (2008a) determined the expression of *nifH* genes in *I. strobilina.* The sponge-associated bacterial clones from *nifH*-deduced amino acids were assigned to one cluster consisting of *nifH* from conventional eubacterial Mo-Fe operons and some to *vnfH* (which encodes the dinitrogenase reductase of the V nitrogenase) and another group mainly related to anaerobic bacteria. Within the former, five groups were assigned based on >90% identity and were closely related to cultured *Azotobacter chroococum* (*Gammaproteobacteria*), *Methylocystis* sp. (*Alphaproteobacteria*), *Tolypothrix, Myxosarcina,* and *Leptolyngbya* sp. (*Cyanobacteria*). Whereas in the latter, sequences resembled two main groups, which had *Desulfovibrio*

salexigens (*Deltaproteobacteria*) as closely related cultured organism. From the reverse transcription-PCR cDNA clone library of *I. strobilina*, 44 *nifH* gene clones were retrieved and clustered within a group formed exclusively by cyanobacterial phylotypes. These results indicate that *Cyanobacteria* symbionts were likely the N-fixers in *I. strobilina*. Mohamed *et al*. (2010) also disclosed the diversity of aerobic ammonia-oxidizing bacteria (AAOB) associated with *I. strobilina* using clone-and-sequencing of the *amoA* gene (which encodes the catalytic α-subunit of the ammonia-monooxygenase enzyme). The *amoA* sequences fell into two clusters affiliated with *Nitrosospira amoA* (90-92%). No sequence was related to *amoA* from nitrite oxidizing bacteria (NOB). The absence of NOB and the affiliation of sponge-associated *amoA* genes with *Nitrosospira* suggested that AAOB were the main nitrifiers in *I. strobilina*. Moreover, no expression of *amoA* was detected in this sponge species (Mohamed *et al*., 2010). As sponges comprise a major portion of the biomass in many coral reefs, microbial mediated nitrogen metabolism in sponges is expected to have a major impact on the nitrogen budget of these environments (Mohamed *et al*., 2008c; Mohamed *et al*., 2010b). Likewise, Hardoim and Costa (Chapter 5) used the *amoA* gene in PCR-DGGE fingerprinting to unravel the temporal stability of ammonia oxidizers bacterial (AOB) in *S. spinosulus*. Few AOB phylotypes were detected in all PCR-DGGE profiles of *S. spinosulus* over the three successive years, suggesting that the AOB community associated with *S. spinosulus* was stable over time.

Overall, Irciniidae species revealed to harbour a complex and intricate bacterial microbiome. It was demonstrated that the bacterial community associated with irciniids was maintained over seasonal environmental conditions, successive years as well as across geographical regions. Additionally, it was shown that each irciniid species held its own bacterial community, which differ from each other and from the environment (i.e. seawater and sediment). Several bacterial taxa associated with Irciniidae species are known for their physiological and metabolic abilities, which indicate that they play essential roles for the establishment and maintenance of the sponge host.

3.2.2 Bioactivities

Few surveys have described biological activities obtained from bacterial strains - or from the respective metabolite extracts of these strains - isolated from Irciniidae species (Thakur and Anil, 2000; Thakur *et al*., 2004; Muscholl-Silberhorn *et al*., 2008; Esteves *et al*.,

2013). Although these studies usually do not unveil the identity of the compounds possibly underlying the observed activities, they collectively highlight a broad spectrum of functions of applied interest found in the culturable fraction of the sponge-associated microbiota. For instance, Thakur *et al*. (2004) determined the importance of sponge-associated bacteria in the epibacterial defense of the host. Twenty-five isolates were recovered from five collection periods. In two periods, the sponge crude extracts were active against isolates obtained for the respective sampling event, while the opposite was observed for isolates retrieved from the other periods. *Micrococcus* sp. and *Bacillus* sp. were responsible for the antibacterial metabolites exhibited in one and all other periods, respectively. The isolated bacteria were also effective against seven common fouling bacteria without perceptible differences in their activities. This highlights the potential of bacteria from both genera to inhibit the growth of epibiotic bacteria on the sponge surface. These results suggest that the bacteria associated with sponges can modulate the growth of their own cells as well as their neighbours by producing bioactive metabolites (Thakur et al., 2004). Esteves et al. (2013) described *in vitro* antagonistic activity of 155 distinct genotypes isolated from *S. spinosulus* and *I. variabilis* towards two relevant clinical strains: *Escherichia coli* NCTC 9001 (Gram-negative) and *Staphylococcus aureus* NCTC 6571 (Gram-positive). Few isolates (n=18, 12%) were active against both strains, whereas 27 (17%) and 44 (28%) isolates showed antimicrobial activity towards *S. aureus* and *E. coli*, respectively. The most active genus isolated from Ircinidae species was *Vibrio* with 27 (84%) and 12 (38%) of the isolates active against *E. coli* and *S. aureus*, respectively (Esteves et al., 2013). These studies emphasized the biotechnological potential of the culturable bacterial fraction associated with Irciniidae sponges.

3.2.3 Bioactive compounds

Few surveys identified the identification of bioactive compounds extracted from sponge-associated bacteria. Several cyclic peptides have been likewise retrieved from one *Bacillus* and one *Staphylococcus* strains isolated from *I. variabilis* at the Bay of Naples, Italy (De Rosa *et al*., 2003). Of those, the compound cyclo-(L-prolyl-L-tyrosine) could modulate the activity of the *LuxR*-based quorum sensing system of bacteria (Holden *et al*., 1999), whereas all other investigated compounds presented structural similarities with known compounds that could interact with *LuxR*-based biosensors (Holden *et al*., 1999; Degrassi *et al*., 2002). Thus, such cyclic peptides might play a role in the sponge microbiome as quorum sensing signal

molecules (De Rosa *et al*., 2003). Further, one strain of *Bacillus pumilus* was isolated from *Ircinia* sp. and found to produce five surfactin-like substances (cyclic acyldepsipeptides) called bacircines (Kalinovskaya *et al*., 1995; Prokof'eva *et al*., 1999). These compounds showed cytotoxic effects on the development of sea urchin eggs and one of them exhibited antitumour activity against Ehrlich ascites carcinoma and anti-HIV activity (Kalinovskaya *et al*., 1995; Prokof'eva *et al*., 1999). Altogether these studies demonstrate that screening for bioactivity within identified compounds extracted from sponge-associated bacteria is a promising, direct route to manifold interesting bioactivities, which nevertheless remains underexplored.

3.3 Archaea

In comparison with the multi-layered molecular research undertaken in the past decade on sponge-associated bacteria, much fewer surveys have thus far addressed archaeal community composition and diversity in marine sponges. Overall, these studies uncovered much less diversified archaeal assemblages from sponges than the corresponding bacterial communities (Taylor *et al*., 2007; Simister *et al*., 2012c; Webster and Taylor, 2012).

3.3.1 Diversity

Phylogenetic inference performed with three distinct archaeal 16S rRNA gene sequences obtained from *Sarcotragus* sp. (Jeju Island, Korea) revealed that they were affiliated to marine group I *Crenarchaeota* (nowadays known as *Thaumarcheota*, see Brochier-Armanet *et al*., 2008; Spang *et al*., 2010; Pester *et al*., 2011), and among them two grouped in clusters containing only sponge-derived sequences, but without bootstrap support (Lee *et al*., 2003). Further analysis showed that one of these sequences indeed belonged to a *Thaumarchaeota* sponge-specific cluster (SC175) according to Simister *et al*. (2012c).

Using PCR-DGGE and 454-pyrosequencing approaches, we demonstrated that the archaeal community associated with *S. spinosulus* was stable over three successive year. In 11 of 12 *S. spinosulus* specimens one single OTU affiliated to *Nitrosopumilus* dominated the archaeal community, whereas the other replicate was dominated by *Cenarchaeum* sp.. *Candidatus* Nitrosopumilus maritimus and *Cenarchaeum* sp. are known to be able to oxidize ammonia. This indicates that the shift in the archaeal symbionts observed in the *S. spinosulus* does not interfere with their functioning in ammonia-oxidation (Hardoim and Costa, Chapter

5). Overall, these findings are in agreement with previous studies, whereby the diversity of archaea associated with other sponge species was indeed encompassed by only few phylotypes (Webster *et al*., 2001a; Webster *et al*., 2004; Taylor *et al*., 2007; Simister *et al*., 2012c). Despite their low diversity, it appears that *Archaea*, and not *Bacteria*, are the active players in the process of ammonia-oxidation in other marine sponges (Hoffmann *et al*., 2009; Han *et al*., 2012; Cardoso *et al*., 2013). It still needs to be determined which other possible roles archaeal symbionts play in association with Irciniidae and marine sponge as a whole.

3.3.2 Bioactivities and 3.3.3 Bioactive compounds

Neither *in-vitro* bioactivities of archaeal cultures and their metabolite extracts nor identified bioactive compounds from sponge-associated *Archaea* were found in our literature survey.

3.4 Fungi

As pointed out by Webster and Taylor (2012), the study of the diversity and functioning of sponge-derived fungi is still an area requiring further research effort. They have been approached primarily in a cultivation-dependent manner, probably because of their higher amenability to laboratory domestication than that displayed by bacteria and archaea. Whereas this is an exceptional feature that facilitates functional screenings for manifold activities and full genome exploration of the obtained pure cultures, it is felt that comprehensive analyses of their full diversity and maintenance across temporal and spatial scales could benefit more from dedicated cultivation-independent studies.

3.4.1 Diversity

The diversity of fungi associated with *I. oros* and *I. variabilis* collected at Malta was assessed via a cultivation-dependent approach using several media whereby a small cube of the inner sponge body was placed onto each medium and allowed to incubate for long periods (> 30 days) at room temperature (~ 20°C) (Höller *et al*., 2000). *I. oros* and *I. variabilis* were dominated by *Aspergillus* spp. corresponding to 39.4 and 50%, respectively, of the total isolates followed by *Penicillium* spp. (13.6% in *I. oros* and 27% in *I. variabilis* of the total isolates). Notably, 13 and 10 fungal genera were found in *I. oros* and *I. variabilis*, respectively, while only five of them were shared by both sponge species (Höller *et al*., 2000). The diversity of culturable fungi was as well assessed in *Psammocinia* sp. (Sedot-Yam,

Israel), using potato dextrose medium with 250 mg l^{-1} chloramphenicol (Paz *et al*., 2010). Culture media amended with fungicides were used in two approaches were used: direct plating of inner sponge fragments and direct plating of sponge extracts ("sponge-compressed"). The Petri dishes were incubated at 25°C in the dark for 3 to 30 days. Sequencing of the internal transcribed spacer (ITS) of the 18S rRNA gene was carried out to identify the fungal strains. Overall, 220 pure cultures were isolated and assigned to 85 fungal taxa of which the majority was obtained with the sponge-compressed method (n=76). The vast majority of the isolated strains (94%) was identified as Ascomycota, whereas Basidiomycota and Glomeromycota were minor components representing 4 and 2% of the cultured fungal community, respectively. From Ascomycota, a total of 80 taxa were recovered and among them 25 were identified as *Eurotiales* (*Aspergillus* and *Penicillium* spp.), 5 as *Capnodiales* (*Cladosporium* spp.), 15 as *Pleosporales* (*Bionectria*, *Fusarium*, *Phoma*, and *Preusia* spp.), and 35 taxa as *Hypocreales* (*Acremonium* and *Trichoderma* spp.). To unravel whether inhibiting the growth of some taxa during the isolation method would increase the diversity of fungi obtained, sponge-compressed samples were plated onto medium amended with different fungicides. This resulted in the isolation of further 28 distinct taxa that were not obtained with previous approach. Therefore, the sponge-compressed method combined with fungicides enabled an increase in the diversity of fungi cultured from the sponge. Using a coverage-based algorithm, the authors estimated that only 15% of the fungi associated with *Psammocinia* sp. could be recovered (Paz *et al.*, 2010). Notably, even though both studies used distinct approaches, the fungal diversity obtained from *I. oros*, *I. variabilis* (Höller *et al*., 2000) and *Psammocinia* sp. (Paz *et al*., 2010) was alike.

3.4.2 Bioactivities

Accompanying their assessment of fungal diversity, Paz *et al*. (2010) did also investigate the inhibitory activities of fungi isolated from *Psammocinia* sp. by diffusion bioassay. Thirty-six fungal cultures showed *in vitro* antagonism against at least one of the test fungi (*Alternaria alternata*, *Rhizoctonia solani*, and *Neurospora crassafour*) or against the oomycete *Pythium aphanidermatum*. Few isolates (*Trichoderma*, *Acremonium*, *Bionectria*, *Verticillium*, *Penicillium*, and *Aspergillus* spp.) were able to secret inhibitory compounds into the growth medium, although those were not identified. It was demonstrated that distinct *Trichoderma*

spp. isolated from *Psammocinia* sp. were capable to mycoparasite *Fusarium equiseti* isolated from the same sponge species (Paz *et al*., 2010).

3.4.3 Bioactive compounds

Penicillium chrysogenum was isolated from the Mediterranean *I. fasciculata* collected in Bight of Fetovaia (Italy) and showed to produce the sorbicillinoid alkaloids sorbicillactones A and B, and sorbivinetone (Bringmann *et al*., 2005). The first compound exhibited remarkable cytotoxic activity towards murine leukaemia lymphoblast (L5178y) cells, and anti-HIV activity. In addition, sorbicillactone A might also be a promising neuroprotective metabolite. Moreover, three known sorbicillinoid fungal compounds oxosorbicillinol, sorbicillin, and bisvertinolone were identified, along with the alkaloids meleagrine and roquefortine C (Bringmann *et al*., 2005). The isolate *Microascus* sp. K14 from *I. variabilis* also produced the anti-fungal compound fungerin (Höller *et al*., 2000).

Overall, these surveys indicate that irciniids harboured a quite complex, readily culturable fungal diversity. One might speculate that the great biotechnological potential for novel fungal-derived bioactive compounds might emerge with the improvement of cultivation techniques and/or implementation of molecular surveys, such as whole genome sequencing. This will also improve our understanding of the likely roles played by sponge-associated fungi within the host.

3.5.1 Other microeukaryotes

Although diatoms and dinoflagellates have been detected inhabiting marine sponges (Taylor *et al*., 2007) and references therein), no study was recovered with irciniids specimens. This might highlight the need to unravel the microeukaryotes associated with Irciniidae specimens as well as to pursuit their biotechnological potential.

In summary, much of the microbial diversity research so far undertaken, considering both marine sponges as a whole and the family Irciniidae, has focused primarily on bacterial communities. Such an increased interest might reflect with fidelity the presumably higher relevance of this particular group of symbionts in host fitness. Nevertheless, it is felt that more efforts made to unveil the spatial-temporal diversity, composition and abundance of archaeal, and microeukaryote assemblages - and their corresponding biological activities – in marine sponges are needed for a balanced picture of the marine sponge holobiont. Within

members of the family Irciniidae, a clear bias towards higher research attention to the genus *Ircinia* was found. First attempts to approaching the microbial ecology of *Sarcotragus spinosulus* have been published only recently by our team (Hardoim *et al*., 2012; Esteves *et al*., 2013), and the highly selective and conserved character of its microbiome makes it a valuable species for comparative studies with other irciniids in the Atlanto-Mediterranean zone and beyond. Further, besides the recent study by Paz *et al*. (2010) on fungal diversity and inhibitory activities, very limited information exists on the microbial ecology of *Psammocinia* spp., in spite of their distinct geographical range and manifold bioactivities (see supplementary material). Collectively, approaching the microbiology of these three genera might constitute a rewarding strategy to understanding the microbiology of marine sponges at a global scale with a solid, underlying comparative framework.

4. Vertical transmission of sponge symbionts

Vertical transmission is an important mechanism by which sponge-specific symbionts are passed from the parental to the next generation via larvae or gametes. This mechanism has been investigated in several sponge species and, when present, has been usually interpreted as evidence for an intimate pattern of relationship between host and the transmitted symbionts (Ereskovsky *et al*., 2005; Enticknap *et al*., 2006; Schmitt *et al*., 2007; Sharp *et al*., 2007; Schmitt *et al*., 2008; Lee *et al*., 2009).

Vertical transmission was proposed as a mode of symbiont inheritance in *I. felix* through the detection of bacteria in adult, larval, and juvenile (1 to 3 days after settlement of larvae) stages of the sponge (Schmitt *et al*., 2007). These authors showed via TEM that adults contained large and complex bacterial communities, and that high bacterial abundance was also observed extracellularly in the inner region of the larvae, whereas the outer region was almost free of microorganisms. When the host entered the juvenile stage, bacterial cells were found primarily in the mesohyl. The vertical transmission view was further asserted by using a molecular approach. Bacterial 16S rRNA gene sequences obtained from the larvae and juveniles were affiliated with six and three bacterial phyla, respectively, that were usually recovered from adult sponges as well. Overall, 12 monophyletic clusters were registered containing 16S rRNA gene sequences from *I. felix* adults and offspring (larvae and/or juvenile), which belonged to four distinct bacterial phyla: *Acidobacteria*, *Chloroflexi*,

Gemmatimonadetes, *Proteobacteria* (*Alpha*, *Delta*, and *Gamma* classes), and one lineage of uncertain affiliation. In a subsequent study, Schmitt *et al*. (2008) detected several vertically transmitted sequence clusters (VT-clusters, which were composed by sequences obtained from adults and offspring). VT-clusters of *I. felix* encompassed: VT-*Acidobacteria*-I and -IV, VT-*Chloroflexi*-II, VT-*Gemmatimonadetes*-I, VT-*Nitrospira*, VT-*Alphaproteobacteria*-I, and -II, VT-*Gammaproteobacteria*-I, -III and IV, VT-*Deltaproteobacteria,* and VT-uncertain (Schmitt *et al*., 2008). These results demonstrated the importance of vertical transmission as a mechanism through which a complex bacterial consortium was formed and maintained within *I. felix*. We still do not know what might be their functioning in the larvae as well as in settlement, which may occur from few minutes to several hours after the release from the adult (Lindquist *et al*., 1997). Once settled, sponge larvae undergo a rapid metamorphosis to an early juvenile stage. Thus, the bacterial symbionts might protect the juvenile from predators by producing antibiotics and deterrent compounds. Arguably, the best example of such ecological function was obtained by Lopanik and co-workers (Lopanik *et al*., 2004b; Lopanik *et al*., 2004a; Lopanik *et al*., 2006; Lopanik *et al*., 2008) and Sudek *et al*. (2007), in which the bacterial symbiont *Candidatus* Endobugula sertula associated with the bryozoan *Bugula neritina* was found to produce the bioactive polyketide bryostatins, which protected the larvae against predators and was observed in all life stages of *B. neritina*. Bacterial symbionts in sponge larvae might as well perform important housekeeping functions or collectively constitute a source of food, once the larvae are unable to take up food particles from the seawater (Jaeckle, 1995).

Even though Schmitt *et al*. (2008) also described two archaeal VT-clusters, represented by 16S rRNA gene sequences retrieved from the marine sponges *Agelas conifera* and *Luffariella variabilis*, there is to this date no specific documentation of archaeal vertical transmission in Irciniidae sponges, although their existence can be presumed based on available data. Our understanding of fungal acquisition by marine sponges remains otherwise very limited, with neither dedicated studies of this group in sponge larvae or juveniles nor an existing conceptual model on how sponges recruit and maintain these symbionts.

5. Bacterial communication and signalling molecules

Quorum sensing (QS) is a mechanism by which microorganisms monitor and regulate their population size through chemical signalling (Bandara *et al*., 2012). When quorum-sensing molecules (QSM) released by microorganisms into the extracellular environment reach a threshold concentration, they initiate a signaling transduction cascade that regulates the expression of several target genes in an orchestrate response to the prevailing conditions. Thus, QS is a complex mechanism known to be involved in bacterial virulence, swarming motility, conjugal plasmid transfer, biofilm maturation, and antibiotic production and resistance (Bandara *et al*., 2012). As such, it affects several activities or areas relevant to human health (e.g. the emergence of multidrug resistance bacterial strains, aquaculture, water purification), the environment (e.g. ship industry), all of which of enormous economic importance, which might be severely impacted by negative feedbacks ensued from QS and its mediation of bacterial metabolism (Bandara *et al*., 2012; Kalia, 2013). Solutions to QS-derived drawbacks might nevertheless arise from the activities of the symbiont communities themselves. For instance, from the marine sponge *Luffariella variabilis,* the compounds manoalide, secomanolide, and manoalide monoacetate were obtained and showed to be important QS inhibitors (Skindersoe *et al*., 2008). Further, the alkaloids ageliferin and mauritamide B obtained from *Agelas conifera* and *A. nakamurai*, respectively, inhibited the QS of the bacterial reporter *Chromobaterium violaceum* CV017, whereas seven compounds isolated from distinct marine sponges inhibited the QS of *C. violaceum* CV017 and showed antibiotic properties (Dobretsov *et al*., 2011).

One of the most studied QSM, *N-acyl* homoserine lactones (AHLs), were recently investigated in *I. strobilina* (Key Largo, USA) inhabiting shallow waters in the Key Largo, Florida (Mohamed *et al*., 2008c). This was the first survey to report the detection of AHLs in the Irciniidae species, in which two *Alphaproteobacteria* and three *Gammaproteobacteria* were capable to produce AHL. AHL biosynthesis by sponge-associated *Proteobacteria* might suggest that QS systems play an important role in sponge microbiome dynamics as observed for other systems. Sponge niches offer a nutrient rich environment when compared to oligotrophic seawater, thus colonizing bacteria might be capable to grow and reach high densities allowing them to perform quorum-sensing.

Autoinducer-2 (AI-2) is the only well characterized molecule involved in interspecies communication (Bandara *et al*., 2012; Kalia, 2013). Briefly, during AI-2 biosynthesis, S-

adenosylmethionine is transformed into 4,5-dihydroxy-2,3-pentanedione (DPD) by a sequence of three enzymatic reactions, where the last one is catalyzed by the enzyme LuxS. Since DPD is unstable, it naturally cyclizes to generate a range of furanones in the presence of water. *Vibrio harveyi* detects the borate diester form of AI-2 using the LuxP/LuxQ signaling cascade, which may be exclusive to *Vibrio* organisms. AI-2 is associated with a broad range of functions, for instance virulence in *V. vulnificus*, virulence factor expression in *V. cholera*, type III secretion, protease production, luminescence, colony morphology, and siderophore production in *V. harveyi* (Bandara *et al*., 2012). Several surveys detected *Vibrio* spp. associated with distinct marine sponges (Hentschel *et al*., 2001; Thiel and Imhoff, 2003; Dieckmann *et al*., 2005; Anand *et al*., 2006; Muscholl-Silberhorn *et al*., 2008; Hoffmann *et al*., 2010; Flemer *et al*., 2012; Esteves *et al*., 2013), which indicate that *Vibrio* spp. might be capable to perform the array of functions described above in the host niche. From *I. strobilina* (Key Largo, USA), 10 out of 40 isolates were identified as *Vibrio* spp. based on 16S rRNA gene sequencing analysis (Zan *et al*., 2011), and assigned to be closely related to *V. harveyi* (n=5) and *V. campbelli* (n=4). Notably, all 10 *Vibrio* isolates were capable to synthesize AI-2 molecules. Further, a total of 30 distinct *Vibrio luxS* gene sequences were recovered, of which 28 were closely related to the *luxS* gene of *V. harveyi* 1DA3 (96-99% sequence identity), and the other two shared 94% identity with *luxS* gene of *V. parahaemolyticus* RIMD 2210633. Interestingly, two *luxS* gene sequences were distantly related to any known *Vibrio luxS* sequence and might indicate that this group encompassed a sponge-specific *luxS* cluster (Zan *et al*., 2011).

Two of the five known classes of QSM have so far been detected in the bacterial community associated with *I. strobilina*, and more might be found as QS research in sponges advances. Considering the complexity of the bacterial communities encountered in Irciniidae species, future investigations involving e.g. full genome sequencing of symbionts and deep sponge metagenomic mining for QS regulatory genes might enable the discovery of other classes of QSM. It is yet early to determine the potential function of QS in the gene regulation of both symbionts and even host metabolism. The biotechnological potential of QS can be nevertheless exemplified from other host-microbe associations in the marine realm. For instance, *Shewanella* sp. strain MIB010 isolated from the intestine of Ayu fish is an effective agent against the QS-regulated biofilm formed by the fish pathogen *V. anguillarum* (Kalia, 2013).

6. Diseases affecting irciniids

Several studies have shown that microorganisms might also cause disease in marine sponges as reviewed by Webster (2007 and references therein). For Irciniidae species, in 1884, a disease affected the Pacific Ocean *Ircinia* spp., where fungal filaments were shown to destroy the sponge body, leaving only the hard spongin fibres (Webster, 2007).

The abundance of *I. variabilis* decreased from 6.9% in 1986 to 3.0% in 1989 at the Marsala Lagoon (Italy), probably due to a disease characterized by white patches on the surface of affected individuals (Gaino *et al*., 1992). Wulff (2006b) observed that several *Ircinia* specimens (Guigalatupo, Panamá) were lost over the five censuses performed from 1984 to 1998, and spreading lesions were seen in individuals of *Ircinia* spp., *I. felix*, *I. campana*, and *I. strobilina* (Wulff, 2006b). A mass mortality event was reported in 1994 with the Mediterranean *S. spinosulus* and *Ircinia* sp., whereby the spongin filaments of these organisms decayed (Corriero *et al*. 1996 *apud* Webster, 2007). Because the putative agent was unknown, it was not possible to establish a cause-effect relationship.

Two disease outbreaks affected the Mediterranean *I. fasciculata–variabilis* complex and *I. variabilis* and *Sarcotragus spinosulus* (Maldonado *et al*., 2010; Cebrian *et al*., 2011). In the former the disease started with small pustules and the progression resulted either in death or recovery. Maldonado *et al*. (2010) suggested that a twisted rod bacterium was the etiological agent because it penetrated the sponge body, proliferated and outcompeted with the high diversity of bacterial symbionts, becoming the dominant bacterium in the mesohyl. The sponge primitive immune system was then shown to form a collagen barrier isolating the necrotic area and the phagocytic-totipotent cells engulfed and digested the rod bacterium. Eventually the rod bacterium was eliminated, but 30% of the specimens analysed died (Maldonado *et al*., 2010). Cebrian *et al*. (2011) observed yellowish spots in *I. fasciculata* specimens, which evolved into large necrotic areas within a few days, culminating with the death of the specimen. Very distinct features characterized the healthy and necrotic areas, such as the presence of healthy *Cyanobacteria* and heterotrophic symbiont bacteria, and abundant collagen bundles in the former region, and the absence of *Cyanobacteria*, the presence of an unidentified bacterium inhabiting the collagen areas and of several vesicles, broken membranes and remains of collagen in the latter. Cebrian *et al*. (2011) also reported two events of mass mortality in the summers of 2008 and 2009, mainly affecting *I. fasciculata* and reaching 80-100% of the surveyed individuals, whereas for *S. spinosulus* 10-30% of

injured specimens was observed from 2007 to 2010. A significant reduction in photosynthesis efficiency of *Cyanobacteria* at high temperature and degeneration or absence of this group was detected in injured specimens. The change in the *Cyanobacteria* community, probably triggered by the increase in water temperature, might have helped opportunistic bacteria normally present in low numbers to express their virulence (Cebrian *et al*., 2011).

In September 2009, a mortality event affecting *I. variabilis* occurred in the Adriatic and Ionian Seas (Stabili *et al*., 2012). In both sites, the disease affected up to 67.2% of the population. Nearly all-diseased specimens showed large areas of necrotic mesohyl and a survey performed some months after revealed that many of them were dead. The disease was characterized by whitish necrotic areas, which were regularly covered by a thin mucous coat constituted by bacteria. The most disturbed specimens showed partially or completed deterioration of their organization and a final exposure of the internal skeletal network. In the complete damage phase, the sponge cellular constituents were vanished and the sponge body was attacked by distinct bacterial morphotypes. However, no bacteria were found inside the skeletal elements. The abundance of surface bacteria was significantly higher in injured than in healthy specimens. Sucrose-fermenting vibrios were also more abundant in damaged specimens. Vibrios only grew from wounded areas, and all isolates were identical to the type strain *V. rotiferanus* LMG21460$_T$ with 99.65% identity (Stabili *et al*., 2012).

To this date, few surveys firmly identified bacterial species as the etiological agents of disease in sponges (Webster, 2007; Stabili *et al*., 2012). More often, a clear cause-effect relationship between a putative agent and the observed disease is difficult to achieve (Webster, 2007; Luter *et al*., 2010). The identification of the etiological agent(s) and how the biotic and abiotic features influence disease incidence will lead to a better understanding of disease development in sponges and might provide clues to prevent and/or manage outbreaks in order to preserve the integrity of sponge species and benthic ecosystems.

7. Experimental microbial ecology

One of the facets in the study performed with marine sponges is the possibility to keep these animals for long term in aquarium and thus test whether the structure and function of their associated bacterial communities shift during long maintenance periods. These surveys are done in order to validate the experimental set-up of the sponge holobiont under controlled

conditions. In this regard, several approaches were attempted with marine sponges (Mohamed *et al*., 2008d; Isaacs *et al*., 2009). They overall revealed that the bacterial community composition of specimens kept during 6 months in a recirculation system (Isaacs *et al*., 2009), or during 6 months and 2 years in a flow-through system (Mohamed *et al*., 2008d) changed when compared to the wild type.

Mohamed *et al*. (2008b) applied molecular techniques to characterize and monitor the bacterial communities associated with *I. strobilina* maintained in aquaculture for 3 and 9 months compared with wild specimens. For the wild specimens, 3-month, and 9-month cultivated, 35, 48, and 47 OTUs, respectively, were obtained and distributed in five or six bacterial phyla. Although the highest diversity was observed for the 3-month sponge, the 9-month showed an intermediate diversity between wild and 3-months, which suggested an adaptation to the aquaculture system. From the sponge maintained in aquaculture for 3 and 12 months, chemical fingerprints of small molecules including primary and secondary metabolites extracted with ethanol were analysed by liquid chromatography-mass spectrometry and compared to the wild type. These analyses revealed no major change in the natural product profile of *I. strobilina* upon transfer to aquaculture (Mohamed *et al*., 2008b). These results suggest that although the bacterial community from aquarium-maintained *I. strobilina* differs from wild type along the assay, the major putative functions are kept. This can be explained by the functional redundancy of the bacterial symbionts observed in other sponge species (Thomas *et al*., 2010a; Fan *et al*., 2012).

So far, no studies approached sponge-associated archaeal or fungal community during maintenance in aquarium.

It is still to be determined whether the microbial communities can be successfully stabilized in aquarium or not, perhaps longer surveys might shed light on this issue. To date, technical challenges to maintain sponges under control conditions for long periods have impaired the performance of important experimental approaches to elucidate how marine sponge microbiome might be affected by e.g. temperature increase (Simister *et al*., 2012b; Webster *et al*., 2013), water acidification (Duckworth and Peterson, 2013) or invasive pathogens (see section 6 above).

8. Metagenomics-based discovery of secondary metabolites biosynthetic gene clusters

Polyketide synthase (PKS) genes have attracted considerable attention due to their highly potent cytotoxic activities (Staunton and Weissman, 2001). However, the complexity of the bacterial assemblages associated with marine sponges makes the assessment of the actual PKS producers very difficult. Few techniques have been applied to overcome these limitations, for instance, cloning-and-sequencing of the biosynthetic gene clusters, metagenome, genome mining, and single-cell genome sequencing. Targeting the biosynthetic gene clusters, two pederin-type PKS systems putatively involved in the biosynthesis of antitumour PKSs were located in the metagenomic DNA of *Theonella swinhoei* (Piel *et al*., 2005). Likewise, metagenomic libraries generated from fractions enriched for filamentous bacteria associated with *Discodermia dissoluta* revealed that they comprised several non-ribosomal synthase (NRPS) as well as a mixed PKS-NRPS gene clusters (Schirmer *et al*., 2005). Moreover, genomic mining of two bacterial cells sorted from *Aplysina aerophoba* and belonging to the phyla *Poribacteria* and *Chloroflexi* revealed Sup-PKS ("sponge symbionts ubiquitous PKS gene") and NRPS gene clusters in the screened genomes, respectively (Siegl and Hentschel, 2010). Single-cell genomics further uncovered at least two PKSs, one of which affiliated with the sponge-specific "Sup-type PKS" from a *Poribacteria* cell sourced from *A. aerophoba* (Siegl *et al*., 2011).

Psymberin was also isolated from *Psammocinia* aff. *bulbosa* collected at Milne Bay (Papua New Guinea) (Fisch *et al*., 2009). The authors developed a new methodology consisting of the substrate specificity of ketosynthase (KS) domains, PKS components that catalyze polyketide chain elongations, that allowed rapid access to biosynthetic gene clusters and used a targeted approach PKS gene mining in their study. The complete sequencing of three fosmids revealed common features of bacterial architecture: no introns were found, the genes were preceded by Shine-Dalgano sequences, the space nearby genes suggested that the transcribed mRNA was polycistronic, and the close relationship to genes exclusively from bacteria. Taken together, these features clearly suggested a symbiont origin of the PKS gene clusters. Nevertheless, the low similarity to genes from *Pseudomonas* sp. or other prokaryotes hampered the correct identification of the bacterium (Fisch *et al*., 2009).

Finally, a strong correlation among bacterial abundance, the presence of *Poribacteria* and Sup-PKS was observed in the Caribbean *I. felix*, and it was proposed that *Poribacteria*

was most likely the producer of mid-chain-branched fatty acids (MBFAs) from this sponge (Hochmuth *et al*., 2010).

9. Cultivation of Irciniidae species

Even though marine sponges are the most prolific producers of secondary metabolites in marine ecosystems, very few compounds have reached commercial production. This is mainly because of the normally minute amounts readily found in the sponge body and of the impossibility to collect sufficiently large amounts of sponge biomass from the natural habitat, which is clearly intolerable given the foreseen impacts on the marine environment and natural sponge populations. To circumvent these obstacles, many techniques have been developed and applied to cultivate sponge species (Osinga *et al*., 1999; Rinkevich, 2005; Sipkema *et al*., 2005; Taylor *et al*., 2007). Below we highlight attempts to sponge cultivation using Irciniidae specimens. We divide these attempts into three technical modalities: sponge mariculture, sponge cell culture, and sponge larviculture.

9.1 Sponge mariculture

In an inaugural study, Wilkinson and Vacelet (1979) failed to transplant *I. variabilis* collected from Endoume (near Marseille) at 3-4 m from well-shaded cave walls and rock cliffs to a 7 m deep rock shelf around 30 m from the sampling place. All collected specimens died after several days after sampling, most likely due to severe damage caused during the sampling, cutting, and sewing of *I. variabilis* into plastic plaques.

Duckworth *et al*. (1997) determined the feasibility to cultivate *Psammocinia hawere in situ* using two culture systems along a depth gradient. Fifty specimens of *P. hawere* were collected from 10-20 m depths at 'Ti Point Reef', New Zealand. They were cut into cubes with four different sizes and transplanted to two locations along with small whole sponges representing a control with minimal damage. Three depths and two culture systems were used, and the experiment was conducted in summer (82 days) and winter (88 days). The survivorship was considered high (276 out of 360 explants), even though 2/3 of the explants lost weight (226 explants). *P. hawere* explants transported to deeper (10 and 17 m) water in winter showed the highest growth and survivorship. This was related with the lower UV radiation as well as the cooler water temperatures, which helped to accelerate the pinacoderm

healing. *P. hawere* is known to incorporate detritus from the sediment into its body or fibres, what might have enhanced the surface consolidation during the healing process. Growth and survivorship improved with the size of the explant and the proportion of the intact pinacoderm. Independently of the culture systems used, *P. hawere* displayed better growth and survivorship at 10 and 17 m (Duckworth *et al*., 1997).

In another study, van Treeck *et al*. (2003) sampled 52 and 100 specimens of *I. variabilis* at the north-western coast of Corsica in summer and spring, respectively. They were transplanted, to naturally-formed sand patches in-between seagrass meadows at 15 m depth. Prior to transplanting, the sponge specimens were cut into different sizes underwater to avoid exposure to air. The explants were placed between two frames, closed, mounted and transported to the experimental sites. The survival rates of *I. variabilis* were high within the first 6 months, then there was a reduction of 25% in population size, mostly likely due a strong storm, after which the sponge species did not stop to grow. The specimens stocked in spring presented higher survival rates. In the first 12 months, *I. variabilis* biomass increased by 90.17%, however to notice here was the great variation between maximum and minimum rates (220 and -43%, respectively) (van Treeck *et al*., 2003). Previously, *I. variabilis* was shown to be inadequate for mariculture, because this species seemed dead before transplants were created, and consequently, they were not transplanted. However, this might be related to *I. variabilis* recovery strategy, which started from the vital inner layer, and thus, it needed longer periods until recovery and growth could be observed.

I. ramosa was collected at Bone Lone reef (Indonesia), cut *in situ* into standard explant sizes of around 30 mm in length; where at least one side contained exopinacoderm, and 25 explants were used. A polyethylene rope was passed through the sponge body, each rope carrying around 9 explants was attached to a PVC frame and these frames were placed horizontally ~20 cm above the reef bottom at a depth between 12-15 m, and secured with iron pegs at the exposed side of the submerged Bone Lola reef. A high survival rate of 92% was observed for *I. ramosa* during the four months of mariculture experiment. A slight but significant increase in length was detected, and a change in shape was observed which could not be precisely measured. However, only few explants grew during trials (de Voogd, 2007).

9.2 Sponge cell culture

In a series of studies, De Rosa and co-workers (De Rosa *et al*., 2001; De Rosa *et al*., 2002; De Rosa *et al*., 2003b) meticulously monitored the chemical composition of *I. muscarum* cells grown on a simple culture medium under controlled conditions. Cell suspensions from *I. muscarum* (Gulf of Naples, Italy) were obtained by a combination of mechanical and chemical treatments, inoculated in a glucose-containing medium and incubated at 18°C in the darkness and at 22°C in the light for 5 days. The medium was changed every third day and a mixture of antibiotics was applied only in the first two weeks of cultivation. One week after inoculation, cells were observed attached to the bottom of culture chambers along with some aggregates. Cells were actively dividing in both culture conditions and the stationary phase of growth was reached after 4 days. Around 90% of the total free sterols in cultured *I. muscarum* were represented by Δ^{5-7} sterols; from which 7-dehydrocholesterol, ergosterol and 7-dehydrositosterol prevailed. Overall, 17 sterols were recovered. The medium was then supplemented with water-soluble cholesterol, which increased the number of observed cells by 70%. Oleic acid was one of the main acids in the lipids of the intact sponge and was found in low concentration in its cultured cells. When the media were supplemented with oleic and linoneic water-soluble fatty acids, no growth promotion was observed. However, they were completely metabolized by the cells. Overall, the concentration of total lipids was higher in the intact animal, and cells developed in the dark had higher total lipids than those grown under light. Several distinct structural groups of volatile compounds were observed, and their significant concentrations indicated that bacteria (from diet or symbionts) could be involved in their formation. A significant number of free amino acids were found in the intact sponge, whereas practically none was found in the cell cultures. The major difference in the carbohydrates composition was the presence in the cell cultures of γ-lactones of 3-desoxy-arabino-hexonic acid and 3-desoxy-ribohexonic acids in significant concentrations, which might be correlated to certain shifts in their carbohydrate metabolism. The culture cells did not produce any secondary metabolites as the major cell energy supplies had most likely been allocated to the biosynthesis of primary compounds (De Rosa *et al*., 2001; De Rosa *et al*., 2002; De Rosa *et al*., 2003b).

9.3 Sponge larviculture

One alternative way of generating sponge biomass for applied research concerns the cultivation of sponge larvae under controlled conditions. With the aim of culturing sponges from larvae, de Caralt *et al*. (2007) transported mature individuals of *I. oros* sampled at I'Escala (Mediterranean Sea) to an open aquaculture system where larvae were released after about 45 days of incubation. Swimming larvae were then collected and transferred to six-multi-well dishes. These were placed in aquaria with filtered seawater (0.7 µm pore diameter) at the same temperature of the field (20°C). Settlement of the larvae on the six-multi-well dish bottom initiated after 24 h and metamorphosed into juveniles after 5-7 days. One week was the time needed for the settled larvae to complete metamorphosis, and an exhalant tube should be formed after this period. However, no visible inhalant/exhalant orifices were observed in the settlers of *I. oros* and, at this point, most juveniles died before the skeletal fibres could be formed. The difference between settlement success and survival was considered high (94.5%). The authors suggested that the success in settlement could be related to favourable conditions such as still water and no substrate competition found by the larvae in laboratory conditions. During this experiment, the first 2 weeks of culture experienced the maximum increase in area, which is related to the rearrangement of the biomass as a result of the processes involved in metamorphosis and not true growth (de Caralt *et al*., 2007). It is felt therefore that yet unknown and specific stimuli and requirements from the environment are needed to enable full development of sponge larvae into mature individuals in the laboratory.

For Irciniidae species, mariculture is apparently the most feasible culturing methodology. However, it needs yet to be tested whether irciniids will produce secondary metabolites under mariculture. Osinga *et al*. (1999) described that five sponge species grown in mariculture were able to keep the biosynthesis of target metabolites demonstrating that this approach can be rewarding. Moreover, optimization of explants' size, depth of transplantation, temperature ranges, and exposure to light and water currents might as well improve the sponge productivity and bioactive compounds' availability. Besides mariculture, *ex-situ* culture and semi-synthesis might be alternative approaches. In the former, the sponge grows under controlled conditions, and the costs and yields of bioactive compounds would be comparable to mariculture, whereas in the latter a biosynthetic precursor produced by a genetically modified bacterium followed a limited number of chemical reactions to synthesize the final product. For instance, using the antibiotic cyanosafracin B, which was obtained from

Pseudomonas fluorescens via bacterial fermentation as starting point for the chemical synthesis, an analogue of the antitumor compound ecteinascidin 743 isolated from the tunicate *Ecteinascidia turbinata* was effectively synthesized (Sipkema *et al*., 2005). Overall, a combination of approaches (biological and chemical) might be the best option to cultivate sponges and recover the secondary metabolites.

10. Concluding remarks and outlook

Irciniidae-microbe interactions are intrinsic to host health and have been evolving since the host origins. In recent years, few fundamental questions regarding their interactions have been successfully addressed. For instance, we do know that selected symbionts are vertically transmitted, that the bacterial symbiont community is sponge-specific and stable along time and across geographical regions. Nevertheless, there are still many other essential questions to be answered: what are the deterministic factors allowing symbionts to form sponge-specific mutualistic cooperation? What are the functions played by the symbionts in host health and functioning? How does the host discriminate, if it does, between food source and symbiont microorganisms? How do the vertically transmitted microorganisms affect larvae and settlement and juvenile establishment? What are the benefits of quorum sensing for the host? Are the main secondary metabolites produced by the symbionts? How disease outbreaks can be efficiently avoided? For how long Irciniidae species can be kept in aquarium and how this would influence microbial community composition? Once well established, aquarium experiments can be very useful for the testing of a plethora of hypotheses related with e.g. changes in water temperature, pH and nutrients; shifts in light incidence and UV radiation; production of secondary metabolites and vertical transmission of symbionts. Furthermore, the majority of microbiology studies of Irciniidae species have so far only determined the bacterial diversity and community structure associated with these hosts. These inventories usually did not unravel the biotechnological potential of the bacterial symbionts. Other symbionts, such as archaea, fungi and microeukaryotes are left almost unaddressed as well as their putative biotechnological relevance. Only by integrating the whole holobiome in these surveys, a more comprehensive picture will emerge allowing detailed network of the host functioning. Nowadays, several advanced molecular approaches could help to answer these questions, for instance, metatranscriptomics, metaproteomics, metabolomics, single-cell

genomics, and whole genome sequencing. Thus, a clearly joint effort among interdisciplinary fields from microbiology and chemistry, to genetic and zoology is needed to advance the knowledge of the oldest extant metazoan-microbe interaction system on Earth. Here, we also reinforce the need to select sponge models and provide a multitude of reasons in favour of the Irciniidae family as one such model.

Supplementary Material

Secondary metabolites retrieved from Irciniidae specimens and the possible role of associated microorganisms in the production of such compounds

Increasing research interest in the sponge-associated microbiota can be to a large extent attributed to the production of an enormous diversity of biologically active secondary metabolites especially in the class Demospongiae (Taylor *et al*., 2007). Some studies suggested that certain bioactive compounds retrieved from marine sponges; for instance, complex polypeptides and nonribosomal peptides, are likely to be synthesized by the symbiont bacteria. This is due to their high resemblance with metabolites known to be produced by bacteria, or to the fact that they belong to a class that is commonly found in bacteria (Piel, 2004; Piel *et al*., 2004b; Kim and Fuerst, 2006; Thomas *et al*., 2010b; Leal *et al*., 2012). Species within the family Irciniidae host several classes of metabolites. Many of these metabolites have been directly extracted from the host animal and shown interesting biological activities. They are described below along with comments on their potential microbial origin whenever possible.

1- Terpenoids

Terpenoids or terpenes belong to a natural product class and related compounds formally derived from isoprene units and classified based on the number of C [monoterpenoids (C_{10}), sesquiterpenoids (C_{15}), etc] (**International Union of Pure and Applied Chemistry, IUPAC,** http://www.chem.qmul.ac.uk/iupac/class/terp.html#15; Wang *et al*., 2005). There are over 24.000 known terpenoids and many have been applied in food and pharmaceutical industries (Wang *et al*., 2005; Motohashi *et al*., 2007). The antimalarial Artemisinin derived from the plant *Artemisia annua* and the anticancer paclitaxel (Taxol®) synthesized by the endophytic fungus *Penicillium raistrickii* in the bark of *Taxus brevifolia*, which is being used to treat ovarian, lung, breast cancers, head, and neck carcinoma and melanoma (Wang *et al*., 2005), are examples of compounds used in human health research. Moreover, paclitaxel is in clinical trials for other forms of cancer (http://www.clinicaltrials.gov/ct2/results?term=paclitaxel). In 2002, the worldwide sales of terpenoids-based pharmaceuticals were around 12 billion dollars (Wang *et al*., 2005). Besides, terpenoids are also part of other compounds like meroterpenoids, which encompass terpenoids and non-terpenoid derived fragments, such as chromanols, polyprenylated quinones and hydroquinones (Sunassee and Davies-Coleman,

2012; http://dnp.chemnetbase.com/dictionary-search.do?method=view&id=9461310&si). These compounds are present in nature and possess a wide range of biological activities (Sunassee and Davies-Coleman, 2012). Terpenoids dominate the chemistry of marine cnidarians, while terpenoids, quinones, and hydroquinones have been detected in marine sponges and in marine bacteria (Faulkner, 2001; Blunt *et al*., 2008, 2009; Blunt *et al*., 2010; Gordaliza, 2010; Blunt *et al*., 2011; Solecka *et al*., 2012; Sunassee and Davies-Coleman, 2012). In this section, we describe the terpenoids and its derivatives isolated from Irciniidae species as well as their cytotoxic activities.

1.1 Cytotoxic activities

1.1.1 Anti-virus

The compounds penta-, hexa-, and heptaprenylhydroquinone 4-sulphates were obtained from an *Ircinia* sp. (Norfolk Ridge region) and showed to inhibit HIV-integrase enzymes (Bifulco *et al*., 1995). Although showing promising results as anti-HIV drugs, unfortunately later heptaprenylhydroquinone and its methylated derivative were found to be toxic to mammalian cells in culture (Loya *et al*., 1997). A MeOH/toluene extract from *Sarcotragus* sp., showed significant in vitro antiviral activity towards *Herpes simplex* I and *Polio* type I (Barrow *et al*., 1988b).

1.1.2 Antimicrobial activity

The mixture of variabilin and strobilinin isolated from *I. strobilina* showed high activity against *Staphylococcus aureus* and *Bacillus subtilis* (Rothberg and Shubiak, 1975; Martinez *et al*., 1997). Palominin isolated from *Ircinia* sp. (Palomino Island, Puerto Rico) inhibited the growth of *Proteus vulgaris* and *Shigella flexineri* (Garcia And Rodriguez, 1990). The 2-octaprenyl-1,4-hydroquinone isolated from *S. spinosulus* (Saronicos Gulf, Greece) was the most active metabolite against the development of marine bacteria and fungi compared to compounds obtained from *I. oros* and *I. variabilis* (Tsoukatou *et al*., 2002). Sulfircin obtained from a deep-sea *Ircinia* sp. (Andros, Bahamas) was active against *Candida albicans* (Wright *et al*., 1989). The ethanol fraction of *I. variabilis* inhibited the growth of marine fungi (Tsoukatou *et al*., 2002).

1.1.3 Anti-crustacean tests

Some compounds were highly toxic in the *Artemia salina* shrimp test. These encompassed palominin recovered from *Ircinia* sp. (Palomino Island, Puerto Rico) (Garcia And Rodriguez, 1990), ircinin-1 and -2 (furanosesterterpenes) isolated from *I. oros* (North Adriatic Sea) (Degiulio *et al*., 1990) and palinurin and fasciculation sulphates recovered from *I. variabilis* and *I. fasciculata* (Bay of Policastro), respectively (De Rosa *et al*., 1997). While sarcotin P (norsesterterpenpods) recovered from *Sarcotragus* sp. was also effective in the *A. salina* shrimp test (He *et al*., 2012). Additionally, three sulphated 2-prenylhydroquinone derivatives retrieved from *S. spinosulus* (Sutomixica, Zadar, Croatia) exhibited greater activity in the *A. salina* shrimp assay than the corresponding hydroquinones. This indicates that these sulphate compounds play a defensive role against macro-symbionts, which were indeed absent in *S. spinosulus* (De Rosa *et al*., 1995).

Ircinin-1 and -2 (furanosesterterpenes) were obtained from the Mediterranean *I. oros* and were very active in a settlement inhibition assay of the cyprids *Balanus amphitrite,* whereas the acetates of the compounds 2-octaprenyl-1,4-hydroquinone and 2-[24-hydroxy]-octaprenyl-1,4-hydroquinone isolated from *S. spinosulus* (Saronicos Gulf, Greece) reduced the settlement of *B. amphitrite* larvae (Tsoukatou *et al*., 2002; Tziveleka *et al*., 2002; Hellio *et al*., 2005).

***1.1.4 Activities against vertebrates* (fish and sea urchin)**

Tsoukatou *et al*. (2002) demonstrated that the mixture of ircinin-1 and -2 obtained from *I. oros* were responsible for the anti-feeding behaviour of the generalist predator fish *Thalassoma pavo*. Palinurin has been recovered from *I. variabilis* at different sampling sites (Alfano *et al*., 1979; De Rosa *et al*., 1997; Marti *et al*., 2003) and from *Psammocinia* sp. (Korea) (Choi *et al*., 2004) and moderate cytotoxicity was observed in the fish lethality test (De Rosa *et al*., 1997).

Variabilin have been recovered from other *I. variabilis*, *I. felix*, *I. strobilina*, *I. campana*, *Ircinia* sp. and *Sarcotragus* sp. sampled worldwide (Faulkner, 1973; Rothberg and Shubiak, 1975; Barrow *et al*., 1988a, b; Garcia and Rodriguez, 1990; Epifanio *et al*., 1999; Pawlik *et al*., 2002). Reef fishes avoided the crude extract of *I. strobilina* incorporated into carrageenan or calcium alginate strips at its natural concentration. Further analysis revealed variabilin as the deterrent agent, whereas pure variabilin added into calcium alginate was

responsible to deter the feeding of natural assemblage of reef fishes (Epifanio *et al*., 1999; Pawlik *et al*., 2002). Variabilin also inhibited the feeding of the generalist predator fish *T. pavo* (Tsoukatou *et al*., 2002). Fasciculatin isolated from *I. fasciculata*, *I. oros,* and *I. variabilis* sampled at distinct places (Cafieri *et al*., 1972; De Rosa *et al*., 1997; Hellio *et al*., 2005; Rifai *et al*., 2005), showed high toxicity in the fish lethality assay (De Rosa *et al*., 1997). Moreover, suvanine (FTA) recovered from *Ircinia* sp. (Fiji Island) displayed toxicity to goldfish and inhibited cell division of the sea urchin (Manes *et al*., 1985).

FTAs were found in the inner body of *I. felix* whereas in *I. campana* there was no significant difference between the concentration of FTAs in the inner and outer layers. This observation contradicts the previous idea that the antipredator compounds should be in the outermost 2 mm of the sponge body. The authors suggested that this high concentration of FTAs in the interior of the sponge might act to protect this important region where physiological processes take place (Freeman and Gleason, 2010). In another survey, Freeman and Gleason (2012) demonstrated that extracts from inner and outer compartments of *I. campana*, which contain similar amounts of defensive chemicals, did not differ in the capacity to deter generalist temperate reef fishes and the widespread sea urchin, whereas extracts from the inner region of *I. felix* did not show enhanced deterrence to generalist reef fish and one species of sea urchin. These results indicate that an increase in the concentrations of the defensive chemical did not improve the protection of the sponges from predators, and that other factors might play a role in the defensive compound allocation (Freeman and Gleason, 2012).

1.1.5 Anti-cancer

Several compounds recovered from *Sarcotragus* sp. showed marginal to significant cytotoxicity towards a panel of five human cancer cell lines [A549 (colon); SK-OV-3 (ovarian); SK-MEL-2 (skin); XF498 (Central Nervous System - CNS) and HCT-15 (colon)]. These comprehended sarcotins A to J, M, N, and O; sarcotins K and L (bisfuranoditerpenes) obtained as an inseparable mixture; ent-kurospongin; sarcotragins A and B (trinosesterterpene lactans); epi-sarcotins A, B, and, F (pyrrolosesterterpenoids); epi-sarcotrine A and B; ircinin-1 and -2; (7*E*,12*E*,18*R*,20*Z*)-variabilin; (8*E*,13*E*,18*R*,20*Z*)-strobilin; sarcotrine A to F; *epi*-sarcotrines A to C (pyrrolosesterterpenoids); and iso-sarcotrines E and F (pyrrolosesterterpenoids) (Liu *et al*., 2001; Shin *et al*., 2001; Liu *et al*., 2002b; Liu *et al*.,

2002a; Liu *et al*., 2003; Liu *et al*., 2006a; He *et al*., 2012). Ircinin-1 isolated from *Sarcotragus* sp., inhibited the proliferation of the skin cancer cell line (SK-MEL-2) (Choi *et al*., 2005). Furthermore, the crude extract containing sarcotragins A and B displayed moderate cytotoxicity towards the leukaemia cancer cell line K652 (Shin *et al*., 2001). Moreover, palinurin and isopalinurin were recovered from a Korean *Psammocinia* sp., the former exhibited moderate selective cytotoxicity against the SK-MEL-2, A549, SK-OV-3, XF498, and HCT15 cancer cell lines (Choi *et al*., 2004). Fasciculatin obtained from *I. fasciculata* (Bay of Naples) inhibited the growth of the human cancer cell lines MCF-7 (breast), NCI-H640 (lung) and SF-268 (CNS) on a dose-dependent manner (Rifai *et al*., 2005).

The kohamaic acids A and B (bicyclic sesterterpenoids) isolated from *Ircinia* sp. exhibited cytotoxicity against the P388 leukaemia cell line (Kokubo *et al*., 2001). From a Taiwan *Ircinia* spp., Su *et al*. (2011) isolated 15-acetylirciformonin B and 10-acetylirciformonin F, which exhibited strong and moderately cytotoxic activity, respectively, against the K562 (chronic myelogenous leukaemia), DLD-1 (colon adenocarcinoma), HepG2, and Hep3B (liver carcinoma) cancer cell lines. The authors suggested that the furan moiety was essential for the cytotoxic activity of C_{22} furanoterpenoids. Additionally, irciformonin B and F were retrieved and the former showed significant cytotoxicity against the K562, DLD-1, and HepG2 cancer cell lines, whereas the latter was active against the HepG2 cancer cell line (Su *et al*., 2011). Furthermore, heptaprenyl- and octaprenylhydroquinones, isolated from *S. spinosulus* (Callejones, Ceuta) inhibited cell metabolism and the number of cancer cells (K562) by most likely inducing apoptosis (Abed *et al*., 2011). Irciformonin C and D (trinorsesterterpenoids) isolated from *I. formosana* (Taiwan), exhibited mild cytotoxicity against WiDr (colon adenocarcinoma) cancer cell line (Shen *et al*., 2006).

Wätjen *et al*. (2009) retrieved hexa- and nona-prenylhydroquinone from *S. muscarum* and heptaprenylhydroquinone from *I. fasciculata* collected in Mersin and Fethiye (Turkey), respectively. These compounds inhibited NF-*k*B-signalling in H4IIE hepatoma cells, and heptaprehylhydroquinone was the most active. Hexa- and hepta-prenylhydroquinone disrupted the extracellular-signal regulated kinase signalling pathway by inhibition of the epidermal growth factor receptor (EGF-R), and heptaprenylhydroquinone also inhibited the activity of other kinases. Thus, especially the heptaprenylhydroquinone might be useful for the development of anti-cancer drugs (Wätjen *et al*., 2009).

1.1.6 Anti-inflammatory

The search for selective inhibitors of phospholipase A_2 and their capacity to control inflammatory processes appeared as an alternative to non-steroidal anti-inflammatory drugs. From *S. spinosulus* (Bay of Naples, Italy), the compounds 2-octaprenyl-1,4-hydroquinone and 2-[24-hydroxy]-octaprenyl-1,4-hydroquinone were recovered, and the greatest effect occurred on human recombinant synovial enzyme in a concentration-dependent manner compared to snake and bee venom (Gil *et al*., 1995). The same sponge species from Saronicos Gulf (Greece) also contained 2'-[28-hydroxy]heptaprenyl-1',4'-hydroquinone; 2-hexaprenyl- and 2-heptaprenyl-hydroquinones, which displayed the highest anti-inflammatory activities among the four metabolites isolated (Tziveleka *et al*., 2005). Moreover, octa- and nonaprenylhydroquinone sulphates retrieved from *Sarcotragus* sp. (Cleveland Bay, Australia) showed to inhibit α1,3-fucosyltransferase VII and thus might be a strong drug to treat inflammatory diseases (Wakimoto *et al*., 1999). Instead, the compound Sch599473 isolated from *Ircinia* sp. exhibited weak inhibitory activity in the CCR7 receptor binding assay which is involved in a biological cascade including inflammatory, allergic and metastasis processes (Yang *et al*., 2003).

1.1.7 Inhibitors of protein kinases

From a deep-sea *Ircinia* sp. (Norfolk Ridge region, New Caledonia) the compounds penta-, hexa-, and heptaprenylhydroquinone 4-sulphates were recovered and inhibited the tyrosine protein kinase (Bifulco *et al*., 1995). The cheilanthanes sesterterpenoids 25-hydroxy-13(24),15,17-cheilanthatrien-19,25-olide; 13,16-epoxy-25-hydroxy-17-cheilanthen-19,25-olide; 16,25-dihydroxy-13(24),17-cheilanthadien-19,25-olide, and 25-hydroxy-13(24),17-cheilanthadien-16,19-olide retrieved from an Australian *Ircinia* sp. inhibited the serine protein kinases MSK1 (mitogen and stress activated kinase) and MAPKAPK-2 (mitogen activated protein kinase), which are involved in signal transduction (Buchanan *et al*., 2001).

1.1.8 Others

Sarcochromenol sulphate A and sarcohydroquinone sulphates A to C isolated from *S. spinosulus* (Tasmanian Sea) repressed the activity of Na^+, K^+ -ATPase from the rat brain (Stonik *et al*., 1992).

The bicyclic sesterterpenoid kohamaic acids A was a potent DNA inhibitor (Kokubo *et al*., 2001). Additionally, 11 analogues of kohamaic acid A have been synthesized and it was proposed that the carboxylic acid was important for the inhibition of DNA polymerase (Takikawa *et al*., 2008).

From *S. spinosulus* (Saronicos Gulf, Greece) the compounds 2-octaprenyl-1,4-hydroquinone and 2-[24-hydroxy]-octaprenyl-1,4-hydroquinone were isolated (Tsoukatou *et al*., 2002; Hellio *et al*., 2005). The former showed strong interaction with 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) and had a moderate effect on lipid peroxidation, whereas the latter interacted extensively with DPPH and exhibited a significant effect against lipid peroxidation (Tsoukatou *et al*., 2002).

Irciformonin I (trinorsesterterpenoids) isolated from a Taiwan *I. formosana* showed significant inhibition on peripheral blood mononuclear cell proliferation induced by phytohemagglutinin (Shen *et al*., 2009).

The compounds chromarols A to D isolated from a Papua New Guinea *Psammocinia* sp. showed to be potent and effective inhibitors of human 15-lipoxygenase which is responsible for the signal pathway of atherosclerosis (Cichewicz *et al*., 2004b).

From an Australian *Ircinia* sp. and *Psammocinia* sp., three compounds (glycinyl lactam sesterterpene/sesterterpene tetronic acids) were retrieved, from which (12*E*,20*Z*,18*S*)-8-hydroxyvariabilin might lead the development of new drugs to treat temporal lobe epilepsy, while 8-hydroxyircinialactam A and B could be helpful for the treatment of movement disorders (Balansa *et al*., 2010).

In this section, the enormous amount of distinct terpenoids and its derivatives retrieved from Irciniidae species as well as their broad spectrum of activities were revealed. However, none of these studies discussed the possibility of these compounds to be produced by sponge-symbionts. It was demonstrated that marine *Bacteroidetes* and *Actinobacteria* were capable to produce terpenoids (Spyere *et al*., 2003; Mincer *et al*., 2004; Fenical and Jensen, 2006; Blunt *et al*., 2010), or that they contained the gene clusters needed to synthesize terpenoids (Williams, 2009). Angucyclinone (benz[α]anthraquinones) was isolated from the actinobacterium *Saccharopolyspora taberi* associated with a Tanzanian marine sponge, and was found to be very cytotoxic against three cancer cell lines (Sunassee and Davies-Coleman, 2012). Further, 2-methyl-1,4-naphthoquinone was produced by an unidentified bacterium closely related to *Alphaproteobacteria* that was isolated from *Dysidea avara* (Thomas *et al*.,

2010b). Considering the high diversity of bacterial phyla associated with *Ircinia* spp. and *S. spinosulus* (Webster *et al*., 2010; Schmitt *et al*., 2012; Hardoim *et al*., Chapter 3; Lago-Leston *et al*., Chapter 5), it is much likely that some of these compounds might be indeed produced by the bacterial symbionts. Further research is needed to determine the real producers of the metabolites detected in Irciniidae species and more insights might be obtained by using metagenomics and single-cell genomes approaches.

2. Polyketides

From an Australian *I. ramosa* 73-deoxychondropsin A was retrieved, whereas chondropsin C was isolated from *Ircinia* sp. collected in the Philippines. These compounds belong to the condropsin family of polyketide-derived macrolide lactams and showed cytotoxicity against the LOX (melanoma) and the MOLT-4 (leukaemia) cancer cell lines (Rashid *et al*., 2001).

Haterumalide NA to NE are cytotoxic chlorinated macrolides isolated from an Okinawan *Ircinia* sp. (Takada *et al*., 1999; Kita *et al*., 2006). Mycalolide A and C (trixazole macrolide) were isolated from *Sarcotragus* sp. (Liu *et al*., 2008). The former was originally obtained from the sponge *Mycale* sp., where it showed antifungal activities against many pathogenic fungi, and was also cytotoxic against the B-16 melanoma cancer cell line (Fusetani *et al*., 1989). Tedanolide C was retrieved from *Ircinia* sp. collected at Papua New Guinea and showed to be cytotoxic to the HCT-116 (colorectal) cancer cell line and might act as an inhibitor of protein synthesis (Chevallier *et al*., 2006).

Moreover, the compounds (+)-psymberin (resembles the pederin family of polyketides), (+)-pederin, (-)-variabilin, (-)-psymbamide A, (-)-preswinholide A, and (+)-swinholide A were obtained from *P.* aff. *bulbosa* (Papua New Guinea) (Robinson *et al*., 2007; Rubio *et al*., 2008). Psymberin showed strong cytotoxic activity against the HCT-116 (colorectal) cancer cell line (Robinson *et al*., 2007). In a pioneer study, Bewley *et al*. (1996) demonstrated that the occurrence of swinholide A isolated from *Theonella swinhoei* was limited to the mixed population of unicellular heterotrophic bacteria (Bewley *et al*., 1996). Furthermore, psymberin was also recovered from *Psammocinia* sp. and exhibited outstanding cytotoxicity against diverse human cancer cells lines, for instance MDA-MB-435 and T-47 (breast), HCT-116 (colon), and SK-MEL-5 and -28 (melanoma), and might be one of the most prominent candidates for drug development (Cichewicz *et al*., 2004a). In the beetle

Paederus fuscipes, an uncultured bacterium closely related to *Pseudomonas aeruginosa* was suggested to be the producer of pederin (Piel *et al*., 2004a)

Although it is not possible to determine whether or not all polyketides recovered from Irciniidae species have a bacterial origin, evidences from order sponge species and hosts (Piel, 2004; Piel *et al*., 2004b; Piel *et al*., 2005) strongly suggest that members of the complex irciniid microbiome might indeed produce many of these compounds.

CHAPTER VII

A pertinent debate in the field of sponge microbiology regards the views of conservative *vs.* sponge-specific prokaryotic community structures in marine sponges. This is the principal drive of the present Thesis, where rigorous experimental designs were implemented and several approaches were applied in order to disentangle the uniform *vs.* species-specific discussion. For the first time, the microbial communities associated with temperate species belonging to the Irciniidae family were assessed taking into consideration the deterministic factors: sponge species, geographical range and temporal dynamics. The results obtained in this Thesis favour the view of species-specific prokaryotic communities inhabiting marine sponges, and I further discuss our findings on the perspective of individual (specimen-specific) microbial community composition as an important factor for the hosts health and functioning. This Thesis also highlights the advantages and limitations of the PCR-DGGE approach to assess the marine sponge microbiome, as well as the advances enabled by 454-pyrosequencing that circumvented the limitations concerning microbial identification which are inherent to less resolving community profiling techniques. I also used epifluorescence microscopy analysis that allowed the counting of all detectable nucleic-acid containing particles in a given sample. Moreover, fluorescent *in situ* hybridization was applied to allow the exclusive detection of cells that hybridize with a taxon-specific, fluorescently-labelled oligonucleotide probe, thus being an alternative to the non-specific nature of epifluorescence microscopy. An argumentation is provided on the difficulties that need to be overcome to expand our ability to cultivate marine sponge symbionts in the laboratory. Finally, an outlook is provided summarizing the current status of our knowledge in marine sponge microbiology, the key challenges we currently face in this field and the future research initiatives that need to be set forth to overcome them.

In the majority of studies comparing the bacterial community associated with distinct marine sponges, researches normally collected the sponge specimens that were local to their facilities or associated laboratories. Therefore, the phylogenetic relationships among/between sponge species were usually only inferred after sampling, which might favour the view of a conservative microbial community associated with these hosts. **In Chapter II**, I and colleagues disclosed the bacterial abundance and diversity associated with two phylogenetically and spatially related marine sponges, *Sarcotragus spinosulus* and *Ircinia variabilis* (Demospongiae, Irciniidae). Both sponge species were first identified by traditional classification, which is a challenge because of the lack of morphologic features within the

family Irciniidae. This problem was partially circumvented by applying a molecular approach based on the sequencing of the subunit I of the cytochrome oxidase gene (COI), which further confirmed the identity of both sponge species as *Sarcotragus* sp. and *Ircinia* sp. as well as indicated that they were indeed distinct sponge species. Unfortunately, the resolution of the COI gene did not allow the identification at species level for members of the investigated family, and thus the use of alternative phylogenetic marker genes should be implemented in future studies to enhance our diagnostic accuracy, enabling us to identify our sponge specimens with more confidence. Examples of alternative genes with potential to increase our resolution power in molecular identification of sponges are the ALG11 (Belinky *et al*., 2012) and certain domains of the 28S rRNA (Chombard *et al*., 1997; McCormack *et al*., 2002; Borchiellini *et al*., 2004) genes or an alternative extension of the COI gene that overlaps about 60 bp with Folmer's 3' partition (Folmer *et al*., 1994) proposed by (Xavier *et al*., 2010b). The results from bacterial abundance revealed that both sponge species, *S. spinosulus* and *I. variabilis*, could be classified as HMA sponges, hence further advancing our knowledge regarding the bacterial abundance associated with these marine sponge species, especially considering *S. spinosulus*, from which no bacterial assay was previously performed. Although PCR-DGGE fingerprinting is considered a less-resolution technique, it was effective in providing first insights into the bacterial community structure associated with both sponge species. PCR-DGGE fingerprint analysis revealed that *S. spinosulus* and *I. variabilis* harbour their own bacterial community, which differed from each other as well as from surrounding seawater. This was an early indication that the phylogenetically related sponge species hosted distinct bacterial communities. Additionally, the bacterial community associated with *S. spinosulus* showed less individual-to-individual variability when compared to *I. variabilis.* Furthermore, phylogenetic analysis performed with the excised DGGE bands revealed that some symbionts fell within sponge-specific clusters. Therefore, the main outcome of this study confirmed that the bacterial community associated with *S. spinosulus* and *I. variabilis* was species-specific.

Despite the extensive knowledge gained in bacterial community structure associated with marine sponges, we do not know how it would be influenced by distinct sample handling procedure prior to DNA extraction. **In Chapter 3**, I and colleagues unveiled the effects of culture-independent methods ("direct" and "indirect") and culture-dependent ("plate washing") processing methods in the bacterial community structure associated with *S.*

spinosulus and *I. variabilis* using PCR-DGGE and 454-pyrosequencing approaches. The morphology, abundance and localization of active bacterial cells within the host sponges were determined by fluorescent *in situ* hybridization coupled with confocal laser scanning microscopy (FISH-CLSM). Congruent results were obtained for *S. spinosulus* and *I. variabilis* under the three pre-processing methods using PCR-DGGE and 454-pyrosequencing profiling. Both profiling approaches congruently indicated that the bacterial community associated with *S. spinosulus* was alike between direct and indirect methods. However, the opposite was observed for *I. variabilis*, where the direct method revealed a more complex bacterial community compared to the indirect method. One plausible explanation is the fact that the inner matrix of *I. variabilis* is remarkably tougher and more difficult to tear compared to *S. spinosulus* (Cook and Bergquist, 2002). Hence, event though the physical force (i.e. grinding with mortar and pestle) was well applied in the indirect method, the toughness of *I. variabilis* did not enable all bacterial cells to disrupt from the sponge matrix, whereas a more efficient release of the bacterial cells that were attached to the sponge matrix was obtained with the direct processing method. Based on this result, it was not possible to indicate which methodology should be implemented, once it varied between sponge species. Instead, the simplest and cheapest way to decide between direct and indirect approaches for other sponge species would be to carry out a PCR-DGGE fingerprint analysis to determine the bacterial community. Regarding the plate washing method, the bacterial community unveiled with this procedure was alike in both sponge species, suggesting that otherwise species-specific communities are rather approximated with the use of culturing. This highlights the bias inherent to cultivation techniques. However, culturing also enabled the assessment of a number of symbionts not readily detected by the cultivation-independent procedures. Remarkably, about half of the OTUs obtained with plate washing method was exclusive of this procedure, highlighting the potential of this methodology for the selection and detection of less abundant community members. The use of 454-pyrosequencing further confirmed the observation made in Chapter 2, where bacterial microbiome was species-specific. FISH-CLSM revealed that the living bacterial cells were mainly coccus observed intrinsically associated with sponge cells and very rarely found on the sponge fibres and filamentous, suggesting intercellular colonization among functional host cells. Although it is a mere speculation at this point, the close vicinity of bacterial cells to active host cells, but not to structural compartments of the sponges (e.g. fibres and filamentous) might indicate exchange

of metabolites between both patterns, which is often observed in host-symbiont relationships leading to mutualistic cooperation. Thus, the vast majority of sponge-associated microbes might have a primordial function for the host health and functioning.

The bacterial community structure associated with *S. spinosulus* and *I. variabilis* has so far been confirmed as species-specific. Based on this our research team challenged the species-specificity observed in *S. spinosulus* and *I. variabilis* over a biogeographical gradient. Thus, **in Chapter IV**, Lago-Lestón, I and colleagues unravelled the bacterial community structure associated with *Sarcotragus* spp. and *Ircinia* spp. collected at the Algarve coast, the Madeira Island and Azores archipelago along with surrounding seawater and sediment. Even though the sampling events were directed towards Irciniidae specimens, at the Azores archipelago the specimens initially collected as *S. spinosulus* was latter reassigned to *Spongia* spp. after sponge DNA barcoding analysis and traditional taxonomy assessments. The collected specimens and similar specimens from other surveys were, actually, used to describe a novel sponge species *Spongia* spp. registered in the Azores archipelago (Pires *et al*., manuscript in preparation). The bacterial community composition associated with *Ircinia* spp. presented high individual-to-individual variability within and among sampling sites, which might indicate that intrinsic characteristics inherent to each sponge specimen as well as environmental factors influenced the composition of the bacterial community. It would be important to investigate which factors play a role in the establishment of bacterial communities in *Ircinia* species, especially *Ircinia variabilis* which have been consistently shown to be less selective than other phylogenetically related sponge hosts (Chapters 2 and 3; Erwin *et al*., 2012c). A current discussion persists among marine sponge taxonomists concerning the status of *Ircinia variabilis* as a supra-species complex, encompassing several distinct genotypic and phenotypic variants (Xavier, personal communication). It cannot be excluded that the assignment of such different variants to one single species, *Ircinia variabilis*, can be an eventual confounding factor in the study of the genetics, physiology and associated microbiota of this species. Instead, similar bacterial community composition was observed in *Sarcotragus spinosulus* sampled at the Algarve coast and Madeira Island, with less individual-to-individual variation within and among replicates. It would be interesting to disclose through which mechanism this bacterial community was maintained in two contrasting biogeographical settings: vertical transmission of diverse symbionts through the sponge larvae and their consequent co-dispersal, or environmental acquisition of generalist

sponge symbionts dispersing freely in the water column? Moreover, the bacterial community associated with *Spongia* sp. was comparable to the observed in *S. spinosulus*, this could be related to their phylogenetic proximity, consequently more studies should be performed with several *Sarcotragus* spp. and *Spongia* spp. to unravel how consistent this observation is and whether or not it would influence their phylogenetic relationship. Regarding the environmental variables, sediment replicates grouped together even though they have distinct origins, whereas surrounding seawater samples were overall separated from one another according to the sampling site. This difference between the bacterioplankton communities might evidence water currents as a major determinant of bacterial structures in seawater. However, dedicated studies coupling microbial community and physical-chemical profiling of the studied sites would be needed to test this hypothesis decisively.

In the majority of the sponge microbiology studies, the sponge species were collected in just one sampling event. Consequently, few investigations using less-resolving techniques determined the maintenance of prokaryotic communities associated with sponge species kept in their natural habitat over the years. Therefore, **in Chapter V**, I and Costa unveiled the temporal dynamics of the prokaryotic communities associated with *S. spinosulus* over a 3-year period via PCR-DGGE and 454-pyrosequencing profiling based on 16S rRNA gene. Moreover, the *amoA* gene was used as a target to fingerprint prokaryotic ammonia-oxidizers associated with *S. spinosulus* via PCR-DGGE. The bacterial community associated with *S. spinosulus* was shown to be stable over the years and a persistent core of 27 OTUs (defined at 97% sequence similarity) was detected in all specimens investigated. These OTUs were distributed among eight bacterial phyla and one candidate phylum, among which some lineages are known for their putative functions. Hence, they might represent the minimum bacterial assemblage responsible to maintain the host health and functioning. In the future, it would be important to unravel the functions performed by these bacterial symbionts. Definitely a metatranscriptomic approach would further advance our knowledge by allowing the assessment of gene expression and putative functions performed by the microbial communities associated with marine sponges. The archaeal community revealed to be stable over the period analysed and it was dominated by one single OTU affiliated with *Nitrosopumilus* sp., except for one replicate that was dominated by *Cenarchaeum* sp.. Both archaeal symbionts are known to be ammonia-oxidizers and it would be interesting to disclose whether or not these symbionts were indeed oxidizing ammonia in *S. spinosulus*. Further,

bacterial ammonia-oxidizers were found to be maintained over the period inspected. However, no amplification was obtained for archaeal ammonia-oxidizers. As the host sponge was sampled always within the same annual season, it would be important to test the temporal dynamic and activity of the prokaryotic community over the four annual seasons, as well as for extended periods of time and distinct biogeographic settings. This would enable a broader perspective of the relevance of ammonia oxidation as a key microbial-mediated process aiding host´s metabolism. This is because ammonia, which can be toxic to eukaryotes, is the metabolic by-product of the sponges and could accumulate in their body during low pumping activity.

In this Thesis, several reasons favoured the investigation of sponge species within the Irciniidae family, and thus this family was selected as our model sponge taxon. Even though Irciniidae specimens are distributed worldwide, few studies unravelled the microbial community structure associated with species belonging to the three genera in this family (*Sarcotragus*, *Ircinia*, and *Psammocinia*). Hence, in **Chapter VI** I and Costa summarized the current knowledge regarding the Irciniidae species. The majority of the studies addressing the bacterial community were performed with *Ircinia* spp., whereas our research group was the first to investigate the temperate *Sarcotragus spinosulus* and no survey was performed so far with *Psammocinia* spp.. Several secondary metabolites were retrieved from bacterial strains isolated from *Ircinia* spp., and this fact highlights the biotechnological potential of this and most likely other species within the Irciniidae family. Moreover, very few studies disclosed archaeal, fungal, and other microeukaryotic communities associated with Irciniidae species. Indeed, when compared to bacterial assemblages, the sponge-associated archaeal communities are known to be less diverse. Nevertheless, they are consistently present and their functions are yet to be determined. In this Thesis I disentangled the archaeal community structure associated with *S. spinosulus* using high-throughput sequencing technology. Additionally, its maintenance was monitored over three consecutive years, suggesting a beneficial role played by these symbionts to the host. Fungal diversity has been so far only determined with culture-dependent approaches, enabling the isolation of several strains among which few produced bioactive secondary metabolites. Clearly, much more effort should be employed regarding the archaeal and fungal communities associated with Irciniidae species and marine sponges as a whole, mainly because of their putative functions. For instance, archaea are known to be involved in biogeochemical cycling, whereas fungi usually

synthesize interesting bioactive compounds. Even though this Thesis fulfilled several gaps of knowledge regarding the prokaryotic community associated with marine sponges, there are numerous related topics that should be pursued. For instance, several bacterial lineages were vertically transmitted in *I. felix*, but so far we do not know what are the functions played by these bacterial symbionts. Additionally, more Irciniidae species should be investigated to disentangle whether or not these symbionts are consistent over distinct sponge species and geographical locations. Moreover, it is remarkable the amount of secondary metabolites retrieved from Irciniidae species and their distinct bioactivities. However, no survey disclosed whether or not these compounds were produced by the microbial symbionts. Taken together, it might not come as a surprise that more intense research on Irciniidae specimens and their microbiomes is needed to deepen our understanding of the potential use of biologically active compounds recovered from sponge-microbe associations in the pharmaceutical industry.

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