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Antimicrobial activities and diversity of sponge derived microbes

A thesis presented to the National University of Ireland for the degree of Doctor of Philosophy

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This thesis is dedicated to my family, my inspiration

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I herewith declare that the thesis is my own work and has not been submitted for another degree, either at University College Cork or elsewhere.

Burkhardt Flemer _____

ABSTRACT

Marine sponges are the single best marine source for novel secondary metabolites with bioactivity and play host to a microbiota with a remarkable diversity. In this study, marine sponges collected in Irish waters were analysed for their associated microbiota. Bacteria and fungi were isolated from several sponge species and analysed for their diversity and antimicrobial activity and deep water sponges were analysed for their associated bacteria and *Archaea* employing 454 pyrosequencing.

In order to evaluate the diversity and antimicrobial activity of bacteria from the marine sponges *Suberites carnosus* (class *Demospongiae*) and *Leucosolenia* sp. (class *Calcaraea*) collected at Lough Hyne, Ireland, two hundred and thirty seven bacteria were isolated. Isolates from the phyla *Actinobacteria, Bacteroidetes, Firmicutes* and *Proteobacteria* were obtained. Isolates of the genus *Pseudovibrio* were dominant among the bacteria from *S. carnosus* whereas *Pseudoalteromonas* and *Vibrio* were the dominant genera isolated from *Leucosolenia sp.* Approximately 50% of the isolates from *S. carnosus* displayed antibacterial activity and ~15% of the isolates from *Leucosolenia sp.* demonstrated activity against the test fungal strains. The antibacterial activity observed was mostly from *Pseudovibrio* and *Spongiobacter* isolates, while the majority of the antifungal activity was observed from the *Pseudoalteromonas, Bacillus* and *Vibrio* isolates. Overall, both sponges possess a diverse range of bioactive and potentially novel bacteria. Differences observed from the sponge-derived groups of isolates in terms of bioactivity suggests that *S. carnosus* isolates may be a better source of antibacterial compounds, while *Leucosolenia sp.* isolates appear to be a better source of antifungal compounds. Both sponge species have been evaluated for the first time for their associated microbiota.

12 marine sponge samples from the same site, among them the *S. carnosus* and *Leucosolenia* sp. sample, were analysed for their associated culturable fungi and 71 fungal isolates were obtained. Based on their 18S-rRNA gene sequence the isolates were found to be very diverse with 22 OTUs determined at a sequence identity of 98.5% and different phylotypes present in some of the OTUs. Together with genera

or orders typically isolated from marine sponges such as *Hypocreales, Pleosporales* and *Eurotiales* some isolates were representatives of taxa seldomly found in marine sponges. All isolates were assessed for their antimicrobial activity against 2 Gram postitive and 1 Gram negative bacterium in the deferred antagonism assay and more than 60% of the isolates tested positive in at least one assay against at least one of the test strains. Isolates with clear and/ or broad range activity were also tested in the well diffusion assay and the disc diffusion assay against the same bacterial as well as 5 fungal test strains (4 yeasts and 1 mycelium forming fungi). Isolates grouping into three OTUs of the orders *Hypocreales, Pleosporales* and *Eurotiales* showed especially strong and broad range activity. One of the isolates, which was closely related to *Fusarium oxysporum* and showed activity against bacteria and fungi, was investigated for its secondary metabolite genes (NRPS and PKS). At least 5 different NRPS genes were identified in its genome based on partial gene sequences obtained from a clone library. The sequence identity to published NRPS sequences of one gene was as low as 50 % highlighting the likelihood that this isolate may be capable of producing potentially novel secondary metabolites.

A *Micromonospora* sp. was isolated from a *Haliclona simulans* sample collected in Irish waters. The isolate inhibited the growth of Gram positive bacterial test strains in three different antimicrobial assays. Several production media were subsequently employed to optimize the production of the antimicrobial compound(s) and a scale up culture with the best medium was extracted for its antimicrobial compounds. The purification of a yellow-orange compound with strong bioactivity against *B. subtilis, S. aureus* and *P. aeruginosa* was facilitated by bio-assay guided chromatography and the compound was tried to be characterized utilising LC-MS and NMR. By LC-MS two compounds of bioactivity were detected in the extracts, one with an m/z=617.21 (positive mode) and one with an m/z=619.21. Another compound with little or no bioactivity was also detected and had an m/z=445.17. Compounds with similar m/z-values have previously been reported from *Streptomyces* and *Micromonospora* isolates; quinocycline and isoquinocycline A with an m/z=619.21, the compounds quinocycline/kosinostatin and isoquinocline B with an m/z=617.21 and aglycons of all derivatives with an m/z=445.17. However, NMR studies of the isolated compounds did not confirm the presence of known (iso)quinocylines in the extracts

derived from the here presented *Micromonospora* isolate and thus the bioactive compounds could indeed be novel.

Two deep water sponges, *Lissodendoryx diversichela* and *Stelletta normani* were shown to host a remarkably different bacterial and archaeal diversity by application of 454 Pyrosequencing targeting a region of the 16S-rRNA gene common to *Bacteria* and *Archaea*. The *L. diversichela* community was dominated by a single γ-proteobacterial sponge symbiont, closely related to the non-phototrophic, chemoheterotrophic and aerobic genus *Granulosicoccus* and uncultured bacteria previously found in shallow water sponges. The *S. normani* sample hosted a largely sponge specific microbial community, even more diverse than has been previously reported for shallow water sponges. Organisms potentially involved in nitrification (*Crenarchaeota),* denitrification (*Nitrospira, Acidobacteria* and *Chromatiales),* sulphate reduction (*Desulfovibrio)* and secondary metabolite production (*Poribacteria, Chloroflexi* and *Actinobacteria)* were found to be spatially distributed in the sponge. Whereas reads classified as *Crenarchaeota*, *Nitrospira* and *Chromatiales* were more often encountered in the cortex, *Desulfovibrio* and *Chloroflexi* featured more heavily in the choanosome. The presence of a large proportion of unclassified reads in this sample highlights the potentially novel microbial community associated with this sponge. Even though *Archaea* were less abundant in both sponges than *Bacteria* they made up an important fraction of the prokaryotic community. While, on the higher taxonomic levels, many similarities between shallow- and deep water sponge-associated microbial communities were detected, by comparing shared OTUs from the presented study with shallow-water derived pyrosequencing reads, a deep sea specific population was implied.

Chapter 1

Introduction

INTRODUCTION

Sponges (phylum *Porifera*) can be found all over the world in marine and freshwater environments. They are often important members of the benthic community and can take up to 80% of the available surfaces (Hentschel et al., 2012). The phylum *Porifera* comprises 4 classes: *Demospongiae, Hexactinellida, Homoscleromorpha* and *Calcarea.* To date 8,553 sponges have been discovered and accepted as sponge species (Van Soest et al., 2012) with 83% of these being *Demospongiae*. Currently 200-250 of the discovered species are freshwater species, united in the suborder *Spongilina* (class *Demospongiae*). Thus approximately 8300 are marine species. A large number of sponges are still being described each year and it is estimated that the number of accepted species will rise to at least 12,000 by the end of this century (Van Soest et al., 2012). Sponges are fixed to the sea floor and live by drawing in water and filtering microscopic-size food particles from it and more recently it has been shown that they can also take up dissolved organic matter (Van Soest et al., 2012).

Body plan of marine sponges

Sponges are found in various shapes, such as cushion-shaped, cup-shaped and branching, while sizes can range from millimetres (crusts) to meters. The colour of the sponges varies greatly between species as does the consistency. Sponges can be soft, compressible, fragile or rock hard. Inter-species variations in colour and shape are known to occur due to environmental differences.

Demosponges

The class *Demospongiae* comprises 83% of all known sponge species and has also been the main target for researchers. Thus they are described in more detail in the following paragraphs. Despite the wide variety of sponge-morphologies all demosponges are build upon the same simple body plan (Figure 1). Water is taken up from a sponge through small pores called ostia and moves into the choanocyte chambers via canals in the sponge. The choanocytes are located in the choanocyte

chambers. They are flagellated cells which collectively form the choanoderm. The movement of the flagella is responsible for a current which provides the means for the uptake of sea water. The choanocytes are also responsible for the uptake of microorganisms and food particles and transfer them to the mesohyle, an extracellular matrix which makes up most of the sponge tissue. In the mesohyle, archaocytes are responsible for the digestion of food particles including microorganisms. But the mesohyle is also the place where symbiotic bacteria reside in the sponge. They have found a way to evade digestion by the archaeocytes. Siliceous spicules, which provide mechanic stability, are also found in the mesohyle (Hentschel et al., 2012).

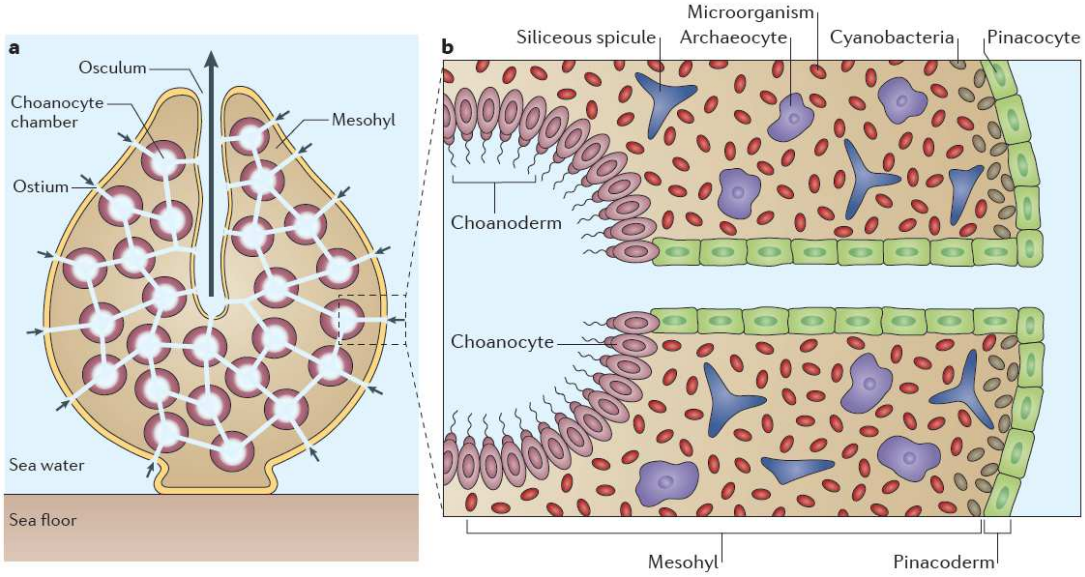


Figure 1.1: Body plan of a typical demosponge from Hentschel et al., 2012.

Microbial sponge associates

Marine sponges developed ~600 million years ago in a sea full of potentially pathogenic microorganisms and despite their simple body plan and lack of an adaptive immune system they have developed an ability to defend themselves from potential pathogens. In addition sponges have also learned to live with microorganisms and nowadays play host to a remarkable diversity of microbial organisms, including bacteria, eukaryotes and even viruses. In certain demosponges up to 35% of the biomass can be constituted of microorganisms and they can reach

densities of up to 10^{10} microbial cells per cubic centimetre of sponge tissue (Taylor et al., 2007).

In 1977, through electron-microscopy studies, sponges were firstly shown to harbour a variety of microorganisms in their tissue. Differences were noted between sponges with high tissue density, which hosted between 4-7 different bacterial phylotypes and well irrigated sponges with low tissue density, which hosted only one morphological type (Vacelet and Donadey, 1977). These sponge types were later called high microbial abundance (HMA) and low microbial abundance (LMA) sponges (Hentschel et al., 2006). HMA demosponges may host 10^{8}-10^{10} bacterial cells per gram sponge, 2-4 orders of magnitude higher than in the surrounding seawater. Contrastingly, LMA sponges in the same habitat will have bacterial densities in the range of seawater (10^{5}-10^{6} bacterial cells per gram sponge tissue). Despite the fact that sponges perform phagocytosis on bacterial cells in the mesohyle, a large number of bacterial cells are present in the mesohyle and are known to be metabolically active (Hentschel et al., 2006). Indeed bacterial cells have not only been found in the mesohyle, but in some sponges they may reside within host bacteriocytes (Vacelet and Donadey, 1977) and nuclei (Friedrich et al., 1999). Living in the sponge could in fact provide several benefits for the associated microorganisms. Nutritionally they could benefit from the ammonium being excreted by the sponge, thereby supplying nitrogen and also from carbohydrates and amino acids following digestion of food-particles (including microorganisms) (Hentschel et al., 2006). The mesohyl of the sponge can also be considered a stable habitat, with the consequence that the sponge associated microbiome is much more consistent over time than the microbiome of the surrounding seawater (Erwin et al., 2012).

Early work on marine sponges and their association with microbes employed microscopy to study the uptake of microorganisms by marine sponges (Reiswig, 1971). Three Caribbean marine sponges were found to retain ~96% of bacteria present in seawater. They also exhibited high levels of retention of other microorganisms. Microorganisms (including bacteria) together with particles not-resolved by microscopy were proposed as food sources (Reiswig, 1975, 1971). In 1976 anaerobic Cyanobacteria were isolated from marine sponges collected in the Mediterranean (Imhoff and Trüper, 1976). Wilkinson in 1978 described the microscopic studies of bacteria in 4 tropical sponges. He showed that the number of bacteria is proportional to the density of the sponge mesohyle, already implying

HMA and LMA sponges, and also identified Cyanobacteria as important members of the sponge-associated microbiota which are predominantly found in exposed tissue sections of sponges growing in shallow habitats. He also recorded differences in the composition of the sponge-associated microbiota according to the sponge species. The sponge *Ircinia wistarii* contained no cyanobacterial symbionts and was found in deeper habitats (Wilkinson, 1978a). Additionally, the sponges *Pericharax heteroraphis*, *Jaspis stellifera* and *Neofibularia irrata* hosted similar morphological types which were thought to be facultative anaerobes with good metabolic versatility, possibly involved in the removal of sponge waste products (Wilkinson, 1978b). Cyanobacterial sponge symbionts were also proposed to play a role in nitrogen fixation (Wilkinson and Fay, 1979). Contrastingly, the sponge *Ircinia wistarii* contained a mixed aerobic microbiota which was similar to that of the ambient seawater (Wilkinson, 1978b). Also, similar populations of Cyanobacteria in Mediterranean and Great barrier Reef sponges were identified (Wilkinson, 1978c; Wilkinson et al., 1981). Thus, by 1980 many important features of sponge-microbe associations have already been described. The role of Cyanobacteria as sponge symbionts was further evaluated in the following years. Marine sponges with cyanobacterial symbionts were shown to have faster growth rates when grown in light, whereas sponges without such symbionts grew preferably in the dark (Wilkinson and Vacelet, 1979). Sponges were also demonstrated to adapt their morphology to increase the surface area for phototrophic bacteria (Wilkinson and Vacelet, 1979; Wilkinson, 1983) and that many sponges in the Great Barrier Reef are net primary producers (Wilkinson, 1983). While it was accepted that many microbes reside in sponges (Wilkinson et al., 1981), it was in no way clear to what orders of magnitudes the diversity of sponge associated microorganisms would extent. With the exception of what has been described above, very little else was discovered during the 1980s in terms of sponge-microbe associations. This changed in 1990 when Santavy and colleagues (Santavy et al., 1990) analysed a Caribbean sclerosponge for its associated microbes. Apart from finding a variety of microbes in association with the sponge samples they also estimated that only 3-11% of the microbes present in the sponge were actually culturable.

Following this, again very little progress was made for several years until close to the end of the millennium. In the meantime it became clear that marine sponges are one of the richest producers of secondary metabolites with biological activity (Sarma

et al., 1993) and that at least some of the bioactive compounds isolated from sponges were actually of microbial origin (Althoff et al., 1998). This made sponges a more and more interesting target for researchers and spiked a boom in marine sponge related research in the following years. Additionally, in the 90s of the last century microbial ecology was revolutionized by the introduction of the use of molecular tools to study biodiversity (16S-rRNA genes) and the metabolic potential (e.g. secondary metabolite genes) of microbial ecosystems. In particular the discovery of PCR and molecular gene cloning together with improvements in gene sequencing (Sanger sequencing) made it possible to study the biodiversity and microbial community composition as well as the metabolic potential of marine sponges. Given that it became accepted that only ~1% of microbes actually present in any particular habitat were culturable (Amann et al., 1995; Streit and Schmitz, 2004), it was important that new culture-independent approaches were developed and employed to gain access to the vast majority of biodiversity present in any habitat. Thus the introduction of culture-independent approaches such as 16S-rRNA clone libraries, together with Fluorescent in situ hybridization (FISH) and denaturing gradient gel electrophoresis (DGGE) had a marked impact on microbial ecology in general and in marine sponge microbial molecular ecology in particular by increasing our understanding of sponge microbiology. The results obtained subsequently, especially during the first decade of this millennium, served to further strengthen many of the main concepts of sponge microbiology initially reported upon by Vacelet, Wilkinson and co-workers as previously described.

In 1998 Althoff and co-workers (Althoff et al., 1998) were one of the first to utilize these novel molecular techniques to study the microbial ecology of marine sponges by analysing the microbial community of sponges from the Baltic Sea, North Sea and the Mediterranean Seas. By employing a 16S-rRNA clone library and electron-microscopic analysis they identified a bacterium from the genus *Rhodobacter* which was present in all sponge samples but which was not present in the surrounding seawater. The sponge specificity which they observed correlated with earlier findings by Wilkinson (Wilkinson, 1978c; Wilkinson et al., 1981). Apart from the sponge specificity which was also discovered in sponges from the Great Barrier Reef (Burja and Hill, 2001), the earlier described spatial distribution of sponge-microbes, e.g. of cyanobacterial symbionts (Wilkinson, 1978a) was also observed for

Desulfovibrionaceae by FISH (Manz et al., 2000) and for *Rhopaloeides odorabile* (Webster and Hill, 2001).

In 2002 Hentschel and co-workers analyzed the then available sponge derived bacterial sequence data (from both culture-dependent and culture-independent studies), involving 190 sponge derived sequences. From this they were able to identify 14 sequence clusters which were specific to sponges, and subsequently coined the term "sponge-specific cluster". They defined a sponge-specific 16S-rDNA sequence cluster as "a group of at least three sequences that (i) have been recovered from different sponge species and/or different geographic locations, (ii) are more closely related to each other than to any other sequence from non sponge sources, and (iii) cluster together independent of the treeing method used." In their study, 70% of all sponge-derived sequences belonged to such a cluster and those sequences were spread through various bacterial phyla. By that time, sponge associated microbes have been affiliated with the following phyla: *Proteobacteria*, *Nitrospira*, *Bacteroidetes*, *Cyanobacteria*, *Spirochaetes*, *Actinobacteria*, *Acidobacteria* and *Chloroflexi*. In all of those phyla except in the *Spirochaetes* phylum, sponge specific clusters were found (Hentschel et al., 2002). In the following years many more sponge species were analysed for their associated microbes using culture dependent and independent methods, resulting in ever stronger evidence supporting the presence of sponge specific lineages (Hentschel et al., 2006). Most interestingly, Fieseler and co-workers discovered the sponge specific candidate phylum *Poribacteria* (Fieseler et al., 2004), while some other studies described sponges with a relatively low diversity of associated microorganisms (Gernert et al., 2005; Webster et al., 2004) and a lack of sponge-specific microorganisms (Gernert et al., 2005; Taylor et al., 2004; Webster et al., 2004).

In 2007 Taylor and colleagues (Taylor et al., 2007) followed on from the earlier Hentschel group study by analysing ~1,500 sponge derived 16S- or 18S-rRNA sequences which were then available in GenBank (on 28 February 2006). With the ~200 sequences contributed by the authors a total of 1694 sequences were studied (20 eukaryotic, 44 archaeal and 1630 bacterial sequences), in comparison to the 190 sequences analysed by Hentschel and co-workers 5 years earlier (Hentschel et al., 2002). According to that review, sponge-associated microbes were recorded belonging to 14 bacterial phyla (*Acidobacteria*, *Actinobacteria*, *Bacteroidetes*,

Chloroflexi, *Cyanobacteria*, *Deinococcus-Thermus*, *Firmicutes*, *Gemmatimonadetes*, *Lentisphaerae*, *Nitrospira*, *Planctomycetes*, *"Poribacteria"*, *Proteobacteria*, *Spirochaetes*, *TM6* and *Verrucomicrobia*), 2 archaeal phyla (*Crenarchaeota* and *Euryarchaeota*) and some eukaryotic lineages. In a similar study, Simister and colleagues analysed the publicly available 7546 sponge derived 16S- and 18S-rRNA sequences (as of February 2010) (Simister et al., 2012). By then, more than 25 bacterial phyla had been found in sponges and 18S-sequences of fungal sponge isolates were also available. While fungi had been isolated from sponges before the Taylor study in 2007, the 20 18S-sequences available at that time were not analysed in detail. But, despite the much larger dataset which was approximately four times greater than before, the bacterial phyla previously reported to be involved in microbial sponge associations remained the same: *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Firmicutes* and *Proteobacteria*; and to a lesser extent *Planctomyces*, *Poribacteria* and *Thaumarchaeota*. For the most part the affiliation of sequences to sponge specific clusters was confirmed in this study and some of the phyla which were found relatively seldomly were apparently very closely related to sponges, because many of the sequences belonged to sponge-specific clusters. This was especially true for members of the phyla *Gemmatimonadetes* (36% of all sequences belonged to sponge-specific clusters), *Nitrospirae* (39%), *SAUL* (71%), *Poribacteria* (79%), *Spirochaetes* (91%), *TM6* (43%), *TM7* (67%) and the *Thaumarchaeota* (41%). Of the abundant microbial phyla in sponges only the *Bacteroidetes* and *Firmicutes* were seldomly grouped into sponge-specific clusters. Some of the phyla, namely *Acidobacteria*, *Chloroflexi*, *Gemmatimonadetes*, *Nitrospirae* and *δ-Proteobacteria* were also often found in sponge-coral-specific clusters. Since the coral-associated microbiota is thought to be important for host health, e.g. by conferring resistance against certain diseases to the host (Reshef et al., 2006) a similar role for the sponge holobiont is conceivable. Even though the deposition of more and more sequences in public databases makes it likely that some of the sponge-specific clusters might be redefined in the future, nonetheless it is clear that many microbial organisms form an intimate relationship with their sponge host.

Highthroughput sequencing as a tool to estimate sponge-associated microbial diversity

Another revolutionary methodological development which has had a marked effect on the area of microbial ecology has been the introduction of next-generation sequencing, in particular the pyrosequencing approach. The application of this method to the analysis of DNA sequences from marine ecosystems has changed our perception of the diversity of the ocean's microbial life dramatically. Instead of an estimated maximum of a few thousand distinct microbial organisms per litre of seawater, marine environments are now known to harbour one to two orders of magnitudes larger microbial diversity (Sogin et al., 2006). The application of this approach to the analysis of DNA sequences from sponge metagenomic samples during the last 3-4 years has seen a similar increase in the estimated diversity of sponge-associated microbes. Typical observed diversities with classical clone libraries were e.g. up to 29 bacterial OTUs (97% sequence similarity) in seven phyla in *Haliclona simulans* (Kennedy et al., 2008). For the sponge *Ancorina alata* 43 OTUs (99% sequence similarity) or 31 OTUs (95%) in 8 phyla were reported (Kamke et al., 2010). A total of 263 different OTUs (99%) in 9 phyla and 1 candidate division was obtained from different tissue sections of the sponge *Tethya califonia* (Sipkema and Blanch, 2010). Culture dependent methods followed by a classification based on 16S-rRNA reported similar diversities (Flemer et al., 2012; Kennedy et al., 2009a). The numbers of sequences obtained in such studies is below 1,000 and up to 2010, 7546 such sponge derived sequences have been deposited in GenBank (Simister et al., 2012). Even though many bacterial, archaeal and eukaryotic sponge-associated phyla were reported through such efforts (i.e. more than 25 bacterial phyla in the review of Simister and colleagues) and the number of sponge-derived SSU-sequences increased rapidly over the last 10 years (190 in 2002, ~1500 in 2006, ~7500 in 2010;(Hentschel et al., 2002; Simister et al., 2012; Taylor et al., 2007), the analysis of sponge-associated microbiota was more comprehensive with the larger number of sequences obtained through pyrosequencing.

Studies employing pyrosequencing for the analysis of sponge-associated microbiota

In 2010 the first study using massive parallel tag-sequencing was published. It dealt with the microbiota of 3 sponges, including a larval sample, from the Great Barrier Reef and compared the bacterial diversity based on 16S-rRNA derived sequences with the water column (Webster et al., 2010; Figure 1.2). The authors stated that previous to their study a total of 20 bacterial phyla have been reported. Also, by that time, sponge-derived sequences in public databases numbered in the thousands (see e.g. (Simister et al., 2012). The first pyrosequencing study alone contributed another ~250,000 sponge derived sequences and detected 23 bacterial phyla in the analysed sponges. Six of the phyla were reported for the first time from sponges, thus raising the number of sponge associated bacterial phyla to 26. Moreover, the OTU-level diversity of the sponge *Rhopaloeides odorabile* was extraordinarily high, in both adult and larvae, with up to 3,500 OTUs being detected at a sequence-similarity cut off of 95%. Thus both in numbers of sequences obtained, but also in numbers of OTUs estimated to be present in one sponge species, this study increased the previous overall numbers of sponge derived sequences by up to 2 orders of magnitude. The analysis also revealed the likely vertical transmission of a large proportion of sponge associated microbes, because they were only found in larvae and adult samples of *R. odorabile* but not in the seawater. Interestingly, members of the sponge-specific candidate phylum *Poribacteria* were found in very low abundances in the seawater samples, which led to the assumption that members of the rare seawater biosphere may function as seed organisms for marine sponges.

This supports the assumption that sponge-microbe associations are shaped by both vertical and horizontal transmission as will be discussed later. Interestingly, while information on the microbiota might become more detailed with the application of next generation sequencing, as was the case with the introduction of culture-independent studies, our perception of the main groups or phlya of bacteria in marine sponges remains the same. Phyla previously often reported from sponges, namely *Acidobacteria, Actinobacteria, Chloroflexi, Nitrospira* and *Proteobacteria*, were also most often affiliated to the sequences obtained in the study from Webster and colleagues.

The next high-throughput sequencing study of sponge-microbiota was published in 2011 (Lee et al., 2011). By analysing three sponges collected in the Red Sea, the

study increased the bacterial phyla detected in marine sponges to 30. But more interestingly, the employed primer pair targeted both the 16S-rRNA of bacteria and *Archaea*. Sponge derived *Archaea* have been reported repeatedly and growing evidence was available of the importance of archaeal ammonia oxidizers in nitrogen metabolism in the sponge holobiont. Consequently the application of next generation sequencing identified a hitherto unknown wide diversity of *Archaea* in these sponges.

More evidence was also obtained to further support the sponge-specificity of many microbes. A *Raspailia ramose* sponge sample collected in Ireland was also shown to host a very diverse microbiota whereas the microbiota associated with a *Stelligera stuposa* sponge sample collected from the same location was considerably less diverse (Jackson et al., 2012). Even though the worldwide analysis of 32 marine sponge species by Schmitt and colleagues (Schmitt et al., 2012a, 2012b) did not assess the associated microbes of each of the samples to the same extent as the three previously mentioned studies, it was to date the most comprehensive study of sponge associated microbes. It defined the terms core, variable and species-specific community by analysing the shared OTUs of all samples. Surprisingly, only a very small fraction of the analysed OTUs were shared between a majority of sponge samples, whereas most of the associated bacteria were found to be sponge-species specific. This contrasted somewhat to the previous assumption that much of sponge-associated diversity is similar over wide geographical and host-taxonomical distances. However, the presence of a core-microbiota was further evidence for a horizontal symbiont transfer, whereas the sponge-species specific bacterial communities added further to the relevance of vertical symbiont transmission as a means to maintain sponge-microbe associations. Another interesting implication of this study was the presence of a tropical specific sponge-microbe association, because sponges sampled at geographically extremely distant tropical locations showed the closest similarity in their associated microbiota.

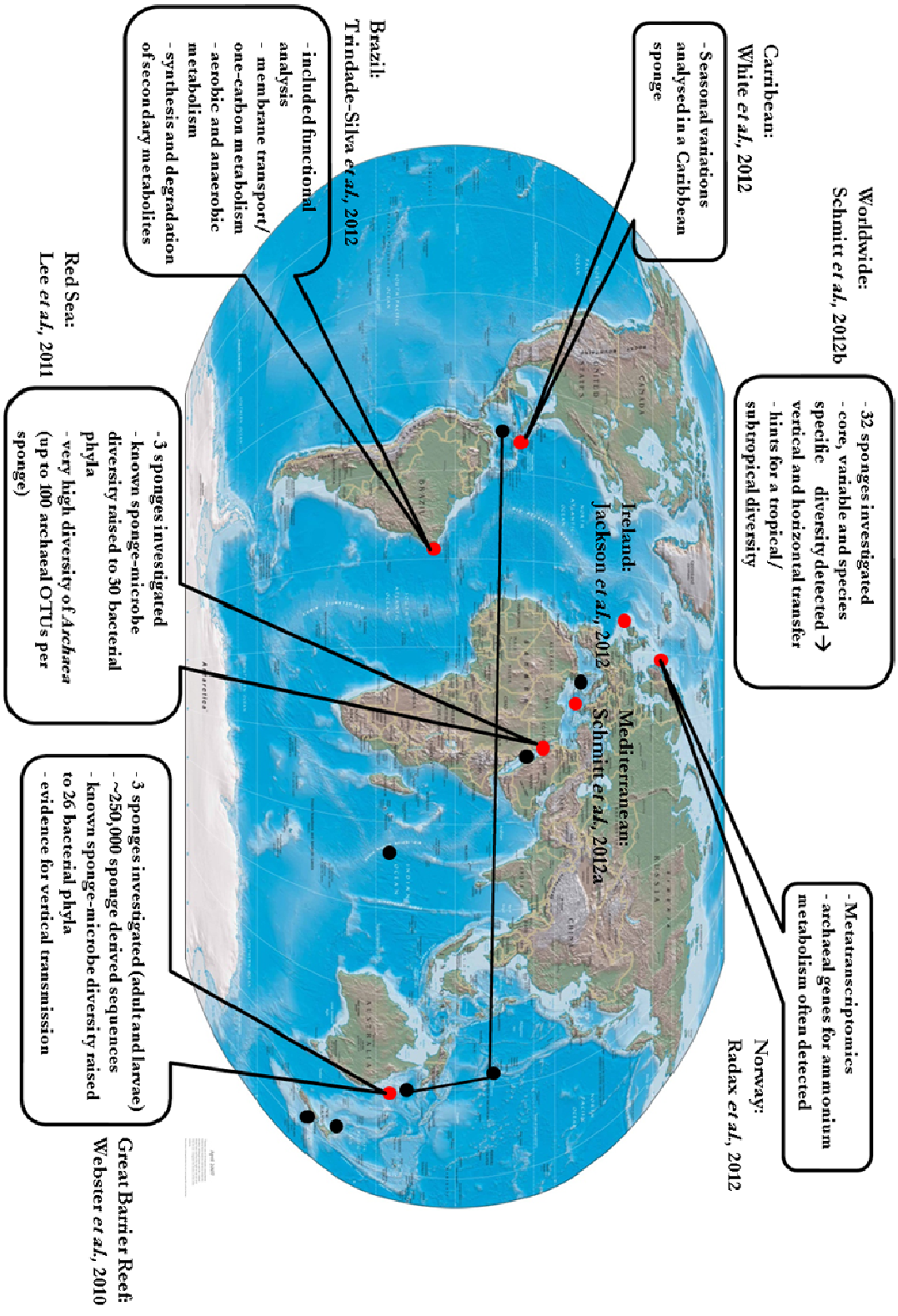


Figure 1.2: 454 pyrosequencing studies on sponge-associated microbiota

Figure 1.2 shows a summary of all published studies which applied the 454 pyrosequencing approach to assess diversity and/or function of the microbiota associated with marine sponges; ● approximate sampling locations for all studies except (Schmitt et al., 2012b); ●approximate sampling locations for a study dealing with 32 sponge species all over the world (Schmitt et al., 2012b). Samples from three tropical sampling locations were related and are connected by black lines.

Instead of analysing the microbial diversity of different sponges, White and colleagues analysed a Caribbean sponge for potential seasonal variations in its associated microbiota; but only observed slight changes highlighting the stable association of microbial organisms with marine sponges (White et al., 2012). This year has also seen metagenomic approaches designed to assess sponge microbes and their possible functional roles within the sponge ecosystem. Trindade-Silva and colleagues for example analysed a Brazilian sponge employing shotgun pyrosequencing to not only analyse the associated microbe-diversity but also the genetic diversity. They identified several genes for which the metagenome was enriched: genes for membrane-transport and one-carbon metabolism, aerobic and anaerobic metabolism and genes potentially involved in the synthesis and degradation of secondary metabolites (Trindade-Silva et al., 2012). Radax and colleagues for their part analysed the metagenomic RNA (metatranscriptomics) rather than the DNA in order to determine the microbes and genes which were metabolically active at the time of sampling (Radax et al., 2012a).

In summary, the application of high throughput sequencing has contributed significantly to our understanding of sponge-microbe associations. Even though the main groups of microbes present in marine sponges have not changed, the approach which have to date been employed have helped to describe the rare microbial biosphere in marine sponges. It also demonstrated the presence of sponges with an extraordinarily high diversity of associated microorganisms and sponges which do not appear to have such highly diverse symbiont communities. Especially interesting, and novel, is the observation of a large amount of sponge-species specific microbial organisms which is in stark contrast to a previously widely held theory of global, sponge specific community. Thus, the main contribution the application of highthroughput sequencing studies has had to date is to increase our understanding of sponge microbiology through the provision of a more comprehensive view of the community structures within various sponges and to provide initial insights into the subsets of these populations which may be playing metabolically roles within the sponge ecosystem. In addition it has enabled us to compare many sponge samples in a more exhaustive way than before and thereby making it possible to infer more robust conclusions with respect to sponge-microbe associations. In the future the use of metatranscriptomics holds much promise in providing further insights into sponge symbiont function.

Establishment and maintenance of sponge microbe interactions

It is now widely accepted that sponges host an extraordinarily diverse microbiota but how was this association established and how is it maintained? As previously mentioned, sponges are sessile filter feeders and even 600 million years ago aquatic environments microorganisms are likely to have been present at high levels (Hentschel et al., 2012); thus sponges were constantly exposed to a wide range of potential pathogens and parasites. But those organisms not only presented a severe threat to the sponge but they also enabled the sponge to build long lasting relationships with these microbial communities thereby potentially securing the sponges' evolutionary success. It is likely that ancient sponge filter feeders retained some food microorganisms as symbionts. But whether the acquisition of the symbiotic microbiota was an immediate process or if it occurred gradually over millions of years remains mere speculation (Hentschel et al., 2012). The main modes employed to maintain symbiotic relationships between invertebrate hosts and microorganisms are either horizontal or vertical transfer or a mixture of both. Horizontal transfer is the uptake of symbionts through the host environment as for example shown for the bobtail squid *Euprymna scolopes* (Nyholm and McFall-Ngai, 2004). Vertical transfer has for example been shown for the pea aphid *Acyrthosiphon pisum* and its bacterial symbiont *Buchnera aphidicola*. In marine sponges both vertical and horizontal modes of transmission are thought to be present. The first evidence of the presence of microbiota in larvae was gathered by electron microscopy, for example in the eggs of the sponges *Tethya citrina* (Gaino et al., 1987), *Hippospongia lachne, Spongia barbara, Spongia cheiris* and *Spongia graminea* (Kaye, 1991), *Chondrilla australiensis* (Usher et al., 2001), the arctic-sponge *Halisarca dujarin* (Ereskovsky et al., 2005) or *Corticium candelabrum* (María J. Uriz, 2007). The same alphaproteobacterial strain was isolated from both adult and embryos of the Caribbean sponge *Mycale laxissima*, implying vertical transmission (Enticknap et al., 2006). Molecular based studies have only relatively recently identified vertical transmission in marine sponges, which has been shown for cyanobacteria in the sponge *Diacarnus erythraenus* from the Red Sea (Oren et al., 2005). Molecular studies on a broader range of target organisms has identified at least four bacterial phyla which have been vertically transmitted in 13 sequence clusters obtained by DGGE (Schmitt et al., 2007). Sharp and colleagues

demonstrated vertical transmission of three selected taxa (*Actinobacteria*, *Proteobacteria* and a deep branching sponge specific clade) in a tropical *Corticium* sp. (Sharp et al., 2007). The bacterial communities of both adult and larvae in the Caribbean sponge *Svenzea zeai* was shown to comprise both *Acidobacteria* and *Chloroflexi* thus vertical transmission for members of those phyla was evident. In total 21 sequence clusters were identified in this study as being vertically transmitted (Lee et al., 2009). Schmitt and colleagues analysed five Caribbean sponges (adult and larvae) and all the available 16S-rRNA sequences (n=116) at that time, from sponge offspring for vertical transmission clusters and identified 28 clusters in 10 bacterial phyla and 1 archaeal phylum (Schmitt et al., 2008). They concluded that microbial symbionts of these Caribbean sponges were in large part transferred vertically. The lack of co-speciation of sponge-host and symbiont (Taylor et al., 2007) as well as repeating records of sponge-associated microbes in seawater (Webster et al., 2010) implies that horizontal transfer also plays a role in the shaping of sponge associated microbiota. The existence of sponge-coral specific clusters also implies a horizontal transfer of symbionts. The presence of horizontal symbiont transfer also implies, that the current sponge microbiota has developed only relatively recently (Webster et al., 2010).

Symbiont function

One of the research questions in the area of marine sponge microbiology which remains to be addressed is the question as to why these sponges play host to such a wide variety of microbial symbionts?

Many functions have to date been associated with microbial sponge symbionts, these include bacteria which photosynthetically fix carbon which are believed to provide ~50% of the sponges carbon requirements (Wilkinson, 1983). Some microbes are also believed to contribute to the host defence mechanism through the production of bioactive secondary metabolites as has been demonstrated by Unson and colleagues (Unson et al., 1994) or Schmidt and colleagues (Schmidt et al., 2000). Other roles include the elimination of toxic metabolic by-products by the microbial symbiont (Taylor et al., 2007) and the protection of the sponge host from UV-light (Steindler et al., 2002). In turn, the sponge could provide the symbiont with access to sunlight,

shelter from predators, a substrate for colonization and a supply of nutrients (Kennedy et al., 2009a).

Carbon metabolism

Due to their exposure to light tropical shallow water sponges are particularly well known for their association with photosynthetic microorganisms (Arillo et al., 1993; Cheshire and Wilkinson, 1991; Steindler et al., 2002; Wilkinson and Vacelet, 1979; Wilkinson, 1983). Glycerol (as a photosynthate) can be transferred by cyanobacteria to the host (Taylor et al., 2007) and glucose can be transferred by a chlorella-like green algae (Wilkinson, 1980). As previously mentioned phototrophic sponges have been shown to obtain more than 50% of their carbon requirements from cyanobacteria (Wilkinson, 1983) which enables them to thrive in environments such as those which exist in tropical reefs, characterized by their low nutrient availability and their high light intensity. Further evidence for the dependence of phototrophic sponges on their cyanobacterial symbionts can be found based on the distribution of sponges. They have been reported to be present in much higher abundances on outer reefs (up to 50% of total sponge biomass) but were less often found in the much more turbid inshore waters (Wilkinson and Trott, 1985). Also, sponges from the Great Barrier Reef, which live in extraordinarily clean waters, are much more likely to play host to cyanobacterial symbionts than Caribbean sponges which live in more turbid waters (Wilkinson, 1987). Some sponges may in fact be obligate phototrophs, a phenomenon which has been observed for sponges in the Great Barrier Reef which had a lower depth limit determined by the availability of light for photosynthesis (Cheshire and Wilkinson, 1991). Other sponges may not contain any phototrophic symbionts at all and may satisfy their carbon requirements solely by filter feeding; while others may combine both filter feeding and phototrophy (Taylor et al., 2007). Another form of microbial carbon uptake by sponges is believed to specifically occur in the case of the deep sea cladorhizid sponges which are believed to consume methanotrophs to satisfy a significant portion of their nutrition (Taylor et al., 2007).

Nitrogen metabolism

Nitrogen fixation has to date been somewhat underestimated in the marine environments but recent research shows that nitrogen fixation rates in the ocean are similar or may even exceed these of terrestrial habitats (Ward et al., 2007). A

possible role of cyanobacterial sponge symbionts in nitrogen fixation has already been proposed in 1979 when marine sponges were assessed for their nitrogen fixation ability using the acetylene reduction technique, a test which detects nitrogenase activity, the main enzyme involved in nitrogen fixation (Wilkinson and Fay, 1979). Nitrogen fixation could possibly benefit the sponge host in a low nutrient environment such as a coral reef. Further evidence on nitrogen fixation is scarce but includes incorporation of $^{15}N_2$-derived nitrogen into sponge-amino-acids (Taylor et al., 2007), isolation of nitrogen fixing bacteria from a *Halichondria* sp. (Shieh and Lin, 1994), microbial input of nitrogen in the sponges *Ircinia felix* and *Aplysina cauliformis* as shown by stable isotope probing studies (Weisz et al., 2007) and the expression of proteobacterial and cyanobacterial *nifH* genes in two Caribbean sponges (Mohamed et al., 2008).

Nitrification is the conversion of ammonia to nitrite and then to nitrate by ammonia-oxidizing and nitrite oxidizing bacteria, respectively. Since sponges excrete ammonium as a metabolic waste product it was perhaps not surprising therefore that a link between microbial ammonia oxidizers and sponges was established. Extensive nitrate release has been measured in the sponges *Chondrilla nucula, Pseudaxinella zeai* and *Plakortis halochondroides,* and has been reported to be potentially linked to their cyanobacterial symbionts (Diaz and Ward, 1997). Nitrite release was also recorded for the sponge *Oligoceras violacea* in the same study. Other studies also reported the release of nitrite and nitrate from tropical sponges and implied the occurrence of ammonia-oxidation (Jiménez and Ribes, 2007; Southwell et al., 2008) in marine sponges. Recently, molecular evidence of ammonia- and nitrite-oxidizers associated with sponges was found (Hentschel et al., 2002; Meyer and Kuever, 2008; Mohamed et al., 2010). So far aerobic ammonium and nitrite oxidation in sponges has been linked to the ammonia-oxidizing genera *Nitrosospira* and *Nitrosomonas,* the nitrite oxidizing *Nitrospira,* the sponge specific *Archaeum Cenarchaeum symbiosum* and marine group I *Crenarchaeota* (changed to *Thaumarchaeota*) (Bayer et al., 2008; Hallam et al., 2006a, 2006b; Mohamed et al., 2010; Off et al., 2010; Steger et al., 2008). Interestingly, the archaeal *amoA* genes found in marine sponges formed sponge specific clusters. Also, archaeal ammonia oxidizers were identified in sponge larvae by the presence of archaeal *amoA* and 16S-rRNA genes as well as by fluorescent *in situ* hybridisation which indicated vertical transmission and an intimate relationship with the host (Steger et al., 2008). In some recent sponge

studies *Archaea* have been reported to be the main drivers of ammonium metabolism (Radax et al., 2012a) and anaerobic ammonium oxidizers (anammox) from the phylum *Planctomycetales* have been found (Mohamed et al., 2010). Also, anaerobic denitrification was shown in the marine sponges *G. barretti* (Hoffmann et al., 2009), *Dysidea avara* and *Chondrosia reniformis* (Schläppy et al., 2010). Betaproteobacterial (Hoffmann et al., 2009) or poribacterial (Siegl et al., 2011) symbionts are believed to play a role in anaerobic denitrification. The presence of anaerobic metabolism in sponges is only a recently discovered phenomenon in sponges, which for a long time were thought to be well saturated with oxygen due to their pumping activity but recent publications have reported oxygen depletion in sponges with no or less pumping activity, in regions between 0.5-1 mm below the sponge surface (Gatti et al., 2002; Hoffmann et al., 2008, 2005a, 2005b; Schläppy, 2007). Consequently, marine sponges can harbour both anaerobic as well as aerobic microbial symbionts.

Sulphur metabolism

Sulphur metabolism by microorganisms seems to be widespread in marine sponges. In the early days of sponge microbiology Imhoff and Trueper isolated strictly anaerobic sulphur oxidizers from the alphaprotoebacterial family *Rhodospirillaceae* and the gammaproteobacterial family *Chromatiaceae* (Imhoff and Trüper, 1976). Green sulphur bacteria of the phylum *Chlorobi* have also been identified in marine sponges (Webster et al., 2001). Sulphur oxidizers metabolize reduced sulphur such as hydrogen sulphide (Taylor et al., 2007) which is presumably derived from sulphate reducers. Sulphate reducers have regularly been identified in marine sponges (Hoffmann et al., 2006, 2005a; Imhoff and Trüper, 1976; Manz et al., 2000) thus a sulphur cycle comprising of sulphate reduction and sulphide oxidation is likely to be present in sponges. Sulfate reducing genera such as *Desulfoarculus*, *Desulfomonile* and *Syntrophus* have been found in the sponge *Geodia barretti* and possible biomass transfer of sulphate reducers (or other sponge associated bacteria) has been reported (Hoffmann et al., 2005a). Indeed a sponge specific, endosymbiotic sulphur cycle as described for *Oligochaetes* (Dubilier et al., 2001) based on the discovery of sponge-specific, sulphur-oxidizing *Gammaproteobacteria* and *Alphaproteobacteria* and a sulphate-reducing archaeon has been proposed (Meyer and Kuever, 2008).

Sponge associated fungi

The “golden age of antibiotics” was initiated by the discovery of penicillin in cultures of *Penicillium rubens* (Houbraken et al., 2011) 1929 by Fleming. As early as the 1940s researchers also began to analyse marine fungi for their secondary metabolites and the compound cephalosporin C was discovered, the parent compound of the cephalosporin antibiotics (Proksch et al., 2008). Motivated by these early discoveries, researchers continued to explore marine fungi for their secondary metabolites. By 1998 more than 100 metabolites from marine derived fungi were described (Biabani and Laatsch, 1998) and by 2000, 40 metabolites from 15 sponge-derived fungi had been isolated (Höller et al., 2000). Most of the research focus at that time was from a Biodiscovery perspective rather than focusing on ecological aspects of the marine derived fungi; resulting in the discovery of novel metabolites with biological activity, e.g. the gymnastatins, novel cytotoxic metabolites (Numata et al., 1997). Interestingly, sponge derived marine fungi are thought to be a more likely source of antimicrobial compounds than other marine fungi (Bhadury et al., 2006; Bugni and Ireland, 2004). However the potential novel chemistry of many marine or sponge derived fungi was often under reported given that they were often related to their terrestrial counterparts and were thus often believed to result from terrestrial “run-off” (Höller et al., 2000). In 2000 Hoeller and colleagues isolated 681 fungal strains from six marine sponges and tested 92 of them for bioactivity against various targets including fungi, algae and bacteria. More than 80% of fungal isolates inhibited the growth of at least one test strain with isolates from the *Coniothyrium* and *Phoma* genera being especially likely to show inhibitory activity. Chemical analysis of 9 isolates resulted in the identification of 27 natural products, 5 of which possessed novel structures. Even though the rate of discovery of novel structures was not considered to be particularly high this was probably due to the isolation of mainly terrestrial species. But notwithstanding this many groups continued to target sponge derived fungi in the following years. The presence of a high proportion of terrestrial species even in offshore environments was however recorded again (Morrison-Gardiner, 2002). Pivkin et al. described a relationship between sponge morphology and rigidity and the diversity of sponge associated fungi (Pivkin et al., 2006), while fungi isolated from *Suberites domuncula* were shown to be diverse with 20 genera being obtained and 3 novel metabolites being identified (Proksch et al.,

2008). Molecular phylogeny using the 18S-rRNA as a tool to identifying sponge derived fungi was employed in a study on two sponges isolated from the China Sea (Wang et al., 2008). Despite the discovery that marine fungi are often derived from terrestrial environments, a coherent case for fungi involved in a symbiotic relationship with marine sponges was also advanced. A yeast was shown to be maternally transmitted in a *Chondrilla* sponge (Maldonado et al., 2005) and putative fungal genes were detected in sponge mitochondria (Rot et al., 2006). Yeasts were also shown to be a potential source of vitamins for marine sponges (Duckworth and Pomponi, 2005). The first fungal isolates obtained from sponges in Irish waters were recorded by Baker and colleagues who also analysed both sponge-derived DNA and RNA for the diversity of fungal 18S-rRNA. The diversity of the fungi isolated from *Haliclona simulans* was found to be similar to other sponges but interestingly the diversity recovered from the clone libraries was very low (4 and 8 phylotypes for DNA- and RNA, respectively). Further studies have reported on the isolation of fungi from three Hawaiian sponges (Li and Wang, 2009), from the Mediterranean sponges *Psammocinia* sp. (Paz et al., 2010) and *Tethya aurantium* (Wiese et al., 2011) and from China Sea sponges (Ding et al., 2011; Liu et al., 2010; Zhou et al., 2011). Zhou et al. also reported on the secondary metabolite genes in the obtained isolates and found 15 PKS genes and 4 NRPS genes (Zhou et al., 2011).

Overall, fungal associations with marine sponges are less understood than are microbial associations. This is partially due to the fact that metagenomic 18S-rRNA based studies typically encounter problems with respect to contamination with sponge rRNA coupled with difficulties in extracting fungal DNA. Consequently next generation sequencing has yet to be applied to analyse the diversity of sponge-associated fungi. Even though sponge specific clusters have recently been detected in the genus *Penicillium* (Simister et al., 2012) and 530 possibly obligate marine fungi have been described (Jones et al., 2009) many, if not most of sponge-derived fungal isolates or 18S-rRNA clones have been classified as genera typically found in terrestrial environments and many compounds derived from those isolates have also been shown to be present in their terrestrial counterparts. Thus a longer lasting relationship to the sponge appears to be unlikely. Nonetheless, the tremendous biochemical potential of fungi and the lack of understanding of sponge-fungi ecology should ensure an ongoing research interest in this area.

Deep sea sponges

The deep sea is as yet a largely underexplored environment in which sponges also live but much less is known about deep water marine sponges and their associated microbiota than about their shallow water counterparts. In 1996 Vacelet and colleagues discovered a large amount of carnivorous sponges near deep water mud volcanoes off the coast of Florida. Sponges were sampled from depths around 4700-4950 m and possibly vertically transmitted, methanotrophic bacteria were identified using stable isotopes and microscopy (Vacelet et al., 1996). Others also reported the presence of methanotrophic sponges in deep sea environments (Vacelet et al., 1995), including hydrothermal seeps (Harrison et al., 1994). In a study from 2005 Olson and colleagues analysed the microbial associates of two lithistid deep water sponges from depths of 242 m and 255 m. They used various cultivation media for the isolation of bacteria and analysed sponge-derived DNA with DGGE. They also used an elegant approach to compare metagenomic bacterial DNA and biodiversity of isolates by first extracting the DNA from several isolates, pooling it and subsequently running a DGGE. The two DGGEs (from sponge-metagenomic and isolate derived DNA) could then be compared. They found that the diversity when assessed by employing the two different approaches was in large part different and that sequences derived from DGGE bands were most closely related to GenBank entries derived from other sponge studies (Olson and McCarthy, 2005). A novel sponge-derived *Tsukamurella* species was later described (Olson et al., 2007). Romanenko and co-workers isolated two novel bacterial species from a *Pachastrella* sponge sampled at a depth of 750 m in the Phillippine Sea (Romanenko et al., 2008, 2005). The microbiota of Arctic deep water sponges, which make up most of benthic biomass in their habitat (Barthel and Tendal, 1993), has been shown to be dominated by *Archaea*, which constitute up to 90% of microbial cells, using lipid biomarker analysis and FISH probes (Pape et al., 2006). In an effort to obtain quantitative data on sponge microbes resident in deep water sponges, qPCR using TaqMan probes identified members of the phylum *Actinobacteria* and *Nitrospira* as being abundant in a *Vetulina* sp. collected in Curacao at a depth of 212 m. Evidence for the presence of an *Entotheonella* microbe in the sponge *Discoderma dissolute*, sampled at depth of ~150 m was reported by employing TEM, 16S-rRNA sequencing and fluorescent probes (Brück et al., 2008). This is particularly interesting as *Entotheonella*

palauensis which is found in the marine sponge *Theonella swinhoei* is thought to be the actual producer of the natural product theopalauamide (Schmidt et al., 2000). A highly sponge specific microbial diversity has been reported in the deep water sponge *Polymastia* cf. *corticata* collected at a depth of 1127 m. 79% of the 38 archaeal and bacterial 16S-rRNA phylotypes obtained by DGGE were either relatives to previously described sponge specific clusters of shallow water sponges (53%) or specific to the sponge under investigation (26%). A functional analysis revealed the presence of both environmentally derived and sponge specific sulphur- and ammonia-oxidizers, including a sulphate-reducing archaeon (Meyer and Kuever, 2008). Contrastingly, the microbiota of *Geodia* spp. collected at depths of ~200-700m was comparable to the microbiota of nearby sediment samples. The isolation of anaerobic microbes from the same samples confirmed the similarity. However, members of interesting taxa such as *Desulfovibrio*, *Clostridium* and for the first time *Chloroflexi* were also obtained (Brück et al., 2010). Sponges collected at hydrothermal vents at depths between 550 and 650 m off the Japan coast were shown to be associated with thioautotrophic bacteria (Nishijima et al., 2010). Very recently, ammonia oxidizing archaea were identified as the main driver of nitrification in four cold water sponges from 200-300m, among them *Geodia barretti* (Radax et al., 2012a). The sponge *Geodia barretti* was also analysed in the first published metatranscriptomic survey of sponge associated microorganisms. The dominance of archaeal ammonia oxidizers as found in the previous study was confirmed by analysis of the mRNA transcripts. The high relative abundance of rRNA sequences affiliated to *Chloroflexi, Poribacteria* and *Acidobacteria* implied their likely active contribution to the ecology of the sponge holobiont (Radax et al., 2012b).

Secondary metabolites from marine sponges and sponge derived microbes

One of the main reasons for the extensive research being carried out on sponges in laboratories all over the world is their associated microbe's capability to produce a vast array of secondary metabolites, with potential biopharmaceutical applications. During the past decades marine sponges have been the single most prolific invertebrate source for novel secondary metabolites from marine environments (Leal et al., 2012). It has been estimated that 48.2 % of all 9,812 marine derived novel

natural products discovered from 1990-2009 were isolated from members of the phylum *Porifera* and even though the phylum *Cnidaria* became more and more important as a source over the years, marine sponges still contribute more novel natural marine products per year than any other invertebrate phylum (287 in 2009, 283 in 2010 in comparison to 188 for *Cnidaria* in 2010 for example (Blunt et al., 2012)). Another recent review attributes more than 3,500 (out of ~12,000 analysed products) novel marine derived natural products as being from marine sponges (covering the years 1985-2008) further confirming their standing as the most prolific source (Hu et al., 2011).

Natural products themselves have begun to receive increased attention recently due to their potential utility as antibiotics and the growing threat of antibiotic resistant pathogens. Examples include multidrug resistant *E. coli* in cattle (Vidovic and Korber, 2006), *Salmonella typhimurium* strains which are resistant against ampicillin, chloramphenicol, streptomycin, sulphonamide and tetracycline (Gorman and Adley, 2004), ~25% multidrug resistant strains in a study on *Clostridium perfringens* (Tansuphasiri et al., 2005), a MRSA strain which can be spread from animals to humans (Vanderhaeghen et al., 2010) and the recent outbreak of the Shiga toxin producing enterohemorrhagic *E. coli* (EHEC) strain in Germany whose genome encoded for extended-spectrum *β*-lactamases and potentially for phenicol, tetracycline and streptomycin resistance (Brzuszkiewicz et al., 2011). Such strains are a significant threat to both human health and to the food processing industry. The discovery of novel antibiotics to counter this trend is therefore important and even though combinatorial chemistry and other alternative approaches have been employed, only one de novo combinatorial chemical entity has been approved worldwidec (the kinase inhibitor sorafenib) (Newman, 2008). Contrastingly, natural products continue to be an important source for novel drugs (Newman, 2008).

Potential of sponge-associated microorganisms for the production of bioactive metabolites

Because as previously mentioned sponges are sessile organisms they rely heavily on natural products or secondary metabolites to help them cope with infection, predators and competitors. It is well established that marine sponges play host to a considerable amount and diversity of microorganisms. Many of these microorganisms have been reported to produce (Baker et al., 2009; Flemer et al.,

2012; Kennedy et al., 2009a; Kim et al., 2006, 2005; Muscholl-Silberhorn et al., 2008; O'Halloran et al., 2011; Xi et al., 2012) or have the potential to produce (Kennedy et al., 2008; Kim et al., 2005; Siegl et al., 2011; Xi et al., 2012) bioactive compounds. A relationship between the likelihood to produce a bioactive compound and the presence of secondary metabolite genes in microbial genomes has by now been well established (Schneemann et al., 2010). A particularly interesting group of microorganisms are the *Actinobacteria* because of the fact that they are known to possess a wide diversity of secondary metabolite genes, especially NRPS and PKS (Goodfellow and Fiedler, 2010; Schneemann et al., 2010). The overall metabolic potential of many marine derived bacteria has been assessed in several genome sequencing projects. A *Rhodococcus* sp. has been shown to harbour 24 NRPS and 7 PKS genes (McLeod et al., 2006), while the genome of the industrial-scale erythromycin producer *Saccharopolyspora erythraea* contains at least 25 secondary metabolite genes for the production of terpenes, polyketides and non-ribosomal peptides (Oliynyk et al., 2007). The genome of *Salinispora tropica*, the source of the proteasome inhibitor salinosporamide A (Feling et al., 2003), harbours 17 PKS-, NRPS- or hybrid gene-clusters (Udwary et al., 2007). Among the *Actinobacteria*, especially *Streptomyces* spp. have been shown to host a wide range of secondary metabolite genes. Thirty such gene clusters have been identified in the genome of the avermectin producer *Streptomyces avermitilis* (Ikeda et al., 2003), and one of the most medically important bacterium, *Streptomyces coelicolor*, is known to harbour at least 20 such genes (Bentley et al., 2002). The importance of *Strepotmyces* as a potential source of novel bioactive molecule is highlighted by the fact that approximately two thirds of all antibiotics and other medicines currently in use such as anticancer drugs are produced by *Streptomyces* (Bentley et al., 2002). Even though secondary metabolite genes are also present in other bacteria, e.g. 4 in *Pseudomonas aeruginosa* (Stover et al., 2000), 3 in *Bacillus subtilis* (Kunst et al., 1997) and 2 in *Ralstonia solanacearum* (Salanoubat et al., 2002) these bacteria lack the metabolic diversity of *Actinobacteria*. Additionally, *Actinomycetes* are thought to produce ~45% of all microbial bioactive secondary metabolites with 7600 (80%) of them being produced by *Streptomyces* (Goodfellow and Fiedler, 2010). Consequently, Actinobacteria, and particularly *Streptomyces*, have often been the prime target in culture based approaches to obtain bioactive microorganisms from marine sponge samples (Abdelmohsen et al., 2012; Jiang et al., 2007; Kim et al.,

2006, 2005; Schneemann et al., 2010; Xi et al., 2012; Zhang et al., 2008). But many other taxa have been shown to produce bioactive compounds as well. Examples include *α-Proteobacteria* (Hentschel et al., 2001), *Pseudoalteromas* (Hentschel et al., 2001; Thakur et al., 2005), *Bacillus* (Anand et al., 2006; Hentschel et al., 2001; Kennedy et al., 2009a; Pabel et al., 2003; Phelan et al., 2012), *Pseudomonas* (Thakur et al., 2005), *Pseudovibrio* (Flemer et al., 2012; Kennedy et al., 2009a; O'Halloran et al., 2011) and *Vibrio* (Anand et al., 2006; Flemer et al., 2012). It has also been shown that compounds previously thought to be of sponge origin are likely to be produced by associated microorganisms instead. Examples include the polychlorinated peptides dysidin and dysidenin which were isolated from *Dysidea herbacea.* Cell separation experiments implied a cyanobacterial sponge-associate to be the actual producer of the peptide (Flowers et al., 1998) and closely related compounds, e.g. pseudosysidenin (Jiménez and Ribes, 2007), were also found in free-living Cyanobacteria. By comparison of the PKS genes of a beetle's *Pseudomonas*-symbiont (Piel, 2002), which produces pederin, a compound related to the *Theonella swinhoei* derived onnamide A, and the sponge's metagenome as a likely bacterial-source has been proposed (Piel et al., 2004).

Bioactive natural products from marine sponges

The efforts of researchers in the marine novel natural products discovery area are beginning to become more apparent with time, with an estimated 118 marine derived natural products now in preclinical trials, 22 in various stages of clinical trials and 3 products on the market (Nastrucci et al., 2012). The first FDA approved marine derived natural products were cytarabine (arabinosyl cytosine; Ara-C; anticancer) and vidarabine (arabinofuranosyladenine; Ara-A; antiviral), which are both synthetic products based on pyrimidine and purine nucleosides obtained from the marine sponge *Tethya crypta*. Spongothymidine and spongouridine were the original products (Newman et al., 2009). Eribulin mesylate (E7389; Halichondrin B; brand name Halaven) has only recently been approved for the treatment of metastatic breast cancer by the FDA in November 2010 and is the third, and so far last, marine sponge derived natural product to be approved as a drug (http://marinepharmacology.midwestern.edu/clinPipeline.htm). Only one more marine sponge derived product is currently in preclinical trials (phase I) but not more than the compound name (PM060184), the proposed activity (anti-cancer) and the

target (minor groove DNA) is publicly available. Other important marine natural products but which are not of sponge origin are Trabectedin (ET743; Yondelis^{R}), an anticancer alkaloid from the tunicate *Ecteinascidia turbinata*, and Ziconotide (Prialt), a peptide for pain relief isolated from the cone snail (Mayer et al., 2010). Interestingly with respect to Yondelis a recent report from the Sherman group which involved sequencing DNA from the microbial symbiont community associated with the tunicate *Ecteinascidia turbinata* resulted in the identification of 25 genes which constitute the core Non Ribosomal Peptide Synthase (NRPS) biosynthetic pathway cluster involved in the synthesis of Yondelis^{R}. It was subsequently shown that the Proteobacterium *Candidatus Endoecteinascidia frumentensis*, a symbiont of the tunicate, in fact produces the tetrahydroisoquinoline core of the ET-743 molecule (Rath et al., 2011). Further promising bioactive compounds have been isolated from marine sponges but have not as yet entered clinical trials. Among these are compounds with antibacterial (isoaaptamine), antifungal (neopeltolide), antimalarial (*(E)-oroidin*), antiprotozoal (plakortide B), antituberculosis (parguesterols A and B) and antiviral (mirabamides A, C and D) activities (Mayer et al., 2011).

Polyketide synthases (PKS) and Non ribosomal peptide synthases (NRPS)

PKS and NRPS are involved in the production of many natural products (Brakhage, 2013). NRPS are involved in the production of some of the most important currently available antibacterial, antifungal, antiviral, immunosuppressant, and anticancer drugs (Felnagle 2008) and thousands of natural products, including the cholesterol-lowering compound lovastatin, are PKS derived (Brakhage, 2013).

NRPSs

Early work on peptide synthesis revealed that some peptides are still produced even if ribosome-targeting inhibitors prevented the assembly of other proteins and that the addition of RNases did not abolish the production of such compounds as tyrocidine (Mach et al., 1963), implying a non-ribosomal production.

The minimal NRPS module consists of three domains in the order C-A-T as described in Figure 1.3, with different domains added for the modification of the growing amino acid chain. Three different NRPSs are described: linear NRPSs (Type A), in which each of the domains is used to proceed one amino acid and then

pass it onto the next module; iterative NRPSs (type B) in which each module is used more than once and non linear NRPSs (type C) which differ from the usual C-A-T module structure and in which certain domains may work more than once during the production of a single non ribosomal peptide (Felnagle et al., 2008). Despite their relatively simple structure, the diversity of products of NRPSs is enormous. This is due to the combination of different amino acids, their modification (e.g. between L- and D-form), their length and many other factors. Important NRPS products include: β-lactams (e.g. penicillin and cephalosporin), glycopeptides (e.g. vancomycin) and capreomycin, which is used in the treatment of multi drug resistant tuberculosis. NRPS genes can be amplified using NRPS specific oligonucleotide primers and this has been employed as a valuable tool in estimating the potential of e.g. isolated microbes for the production of bioactive secondary metabolites (Kennedy et al., 2009b).

PKSs

PKSs use simple molecules, malonyls, as building blocks. As with NRPSs, PKSs are organized in modules which consist of at least three essential domains: an acyl-transferase (AT) domain, the acyl-carrier protein (ACP) and a ketoacyl synthase domain (KS). The three domains form a β-ketothioester which is often further processed by β-ketoacyl reductase (KR) domains, dehydratase (DH) domains, enoyl reductase (ER) domains and methyltransferase domains. In order to confer additional structural functionalities, tailoring enzymes, such as oxygenases, glycosyltransferases and other transferases may further alter the polyketide (Brakhage, 2013). Fungi mainly harbour iterative type I PKSs, which are further subdivided into reducing, partially-reducing and non-reducing according to the presence of KR, DH and ER domains (Kroken et al., 2003). Generally, PKSs are subdivided into 3 types: Type I, II and III PKSs, type I PKSs can be iterative or modular (Watanabe and Ebizuka, 2004). Modular Type I PKSs consist of a single protein with multiple modules, are linear and found in bacteria, iterative type I PKSs consist of a single protein with one module and are found in fungi. Type II PKSs consist of multiple proteins, each with a single mono-

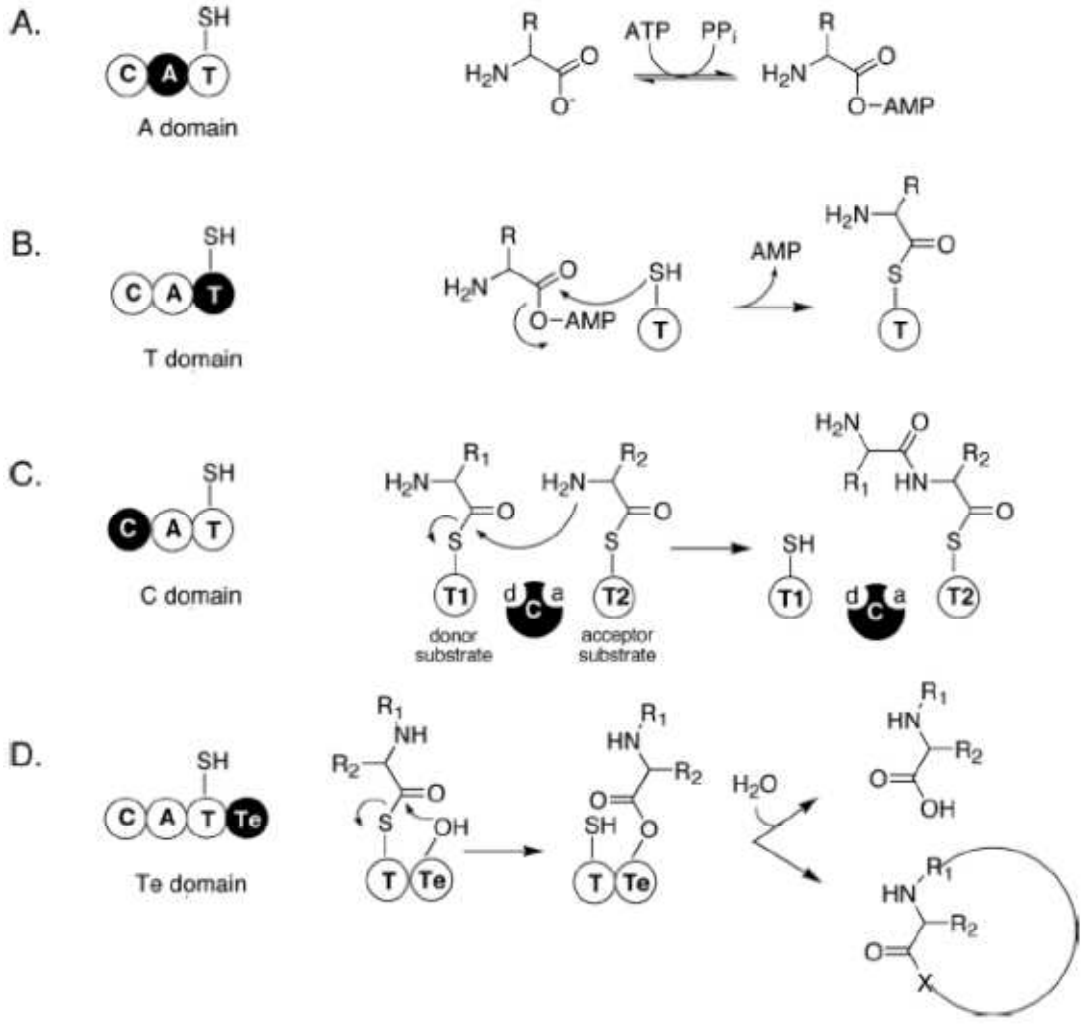


Figure 1.3: Core domains of NRPS and their catalyzed reactions (Felnagle et al., 2008)

A typical NRPS module consists of 3 domains: The adenylation domain (**A**), which selects and activates an amino acid by its conversion to aminaoacyl-AMP; The thiolation domain (**T**; or peptidyl carrier protein PCP) which forms an aminoacylthioester between aminoacyl-AMP and a cofactor; the T domain also works together with the condensation domain (**C**) in a "swinging arm" motion to transfer an aminoacid to a growing chain. The C-domain is responsible for the formation of the peptide bond between donor and acceptor substrate. The thioesterase domain (**Te**) is typically located at the C-terminus of the terminal CAT-domain and facilitates the release of the peptide and sometimes its intramolecular cyclization.

functional active site, are iterative and found in bacteria, Type III PKSs consist of a single protein with multiple modules, are also iterative and but found in bacteria and plants (Watanabe and Ebizuka, 2004). Important molecules at least partially produced by PKSs include erythromycin (Staunton and Wilkinson, 1997), produced by the *Actinomycete Saccharopolyspora erythraea*, leinamycin (Tang et al., 2004), daunorubicin and tetracycline (Zhang and Tang, 2009) or vancomycin (Austin and

Noel, 2003). Often, both NRPSs and PKSs are involved in the production of a single natural product, examples include leinamycin and vancomycin.

Aims and overview of the presented study

The here presented study set out, using culture dependent and culture independent techniques, to assess the microbial diversity associated with marine sponges collected in Ireland as well as the potential of isolated microbes for the production of bioactive secondary metabolites.

Chapters 1 and 2 describe the isolation of microorganisms from several marine sponges collected, at the same time, in Lough Hyne, Ireland. The isolation of bacteria from two marine sponges, *Suberites carnosus* and *Leucosolenia* sp. is reported in Chapter 1 along with their potential for the production of bioactive compounds. The culturable fungal diversity associated with 12 marine sponges is described in Chapter 2. All isolates are also tested for their bioactivity and one isolate with promising activity is analysed in more detail for the presence of PKS and NRPS genes. Chapter 3 reports the isolation of a *Micromonospora* sp. from the sponge *Haliclona simulans* and the purification of a culture extract for its bioactive compound(s). Chapter 4 finally deals with the unculturable biodiversity of two deep water marine sponges employing 454 pyrosequencing of the 16S-rRNA gene amplicon derived from sponge-metagenomic DNA.

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Chapter 2

Diversity and antimicrobial activities of microbes from two Irish marine sponges, *Suberites carnosus* and *Leucosolenia* sp.

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ABSTRACT

Aims: To evaluate the diversity and antimicrobial activity of bacteria from the marine sponges *Suberites carnosus* and *Leucosolenia sp.*

Methods and Results: Two hundred and thirty seven bacteria were isolated from the sponges *S. carnosus* (*Demospongiae*) and *Leucosolenia sp.* (*Calcarea*). Isolates from the phyla *Actinobacteria, Bacteroidetes, Firmicutes* and *Proteobacteria* were obtained. Isolates of the genus *Pseudovibrio* were dominant among the bacteria from *S. carnosus* whereas *Pseudoalteromonas* and *Vibrio* were the dominant genera isolated from *Leucosolenia sp.* Approximately 50% of the isolates from *S. carnosus* displayed antibacterial activity and ~15% of the isolates from *Leucosolenia sp.* demonstrated activity against the test fungal strains. The antibacterial activity observed was mostly from *Pseudovibrio* and *Spongiobacter* isolates, while the majority of the antifungal activity was observed from the *Pseudoalteromonas, Bacillus* and *Vibrio* isolates.

Conclusions: Both sponges possess a diverse range of bioactive and potentially novel bacteria. Differences observed from the sponge-derived groups of isolates in terms of bioactivity suggests that *S. carnosus* isolates may be a better source of antibacterial compounds, while *Leucosolenia sp.* isolates appear to be a better source of antifungal compounds.

Significance and Impact of the study: This is the first study in which cultured bacterial isolates from the marine sponges *S. carnosus* and a *Leucosolenia sp.* have been evaluated for their antibacterial activity. The high percentage of antibacterial isolates from *S. carnosus* and of antifungal isolates from *Leucosolenia sp.* suggests that these two sponges may be good sources for potentially novel marine natural products.

INTRODUCTION

Marine sponges are known to harbour a wide variety of bacteria with 26 bacterial phyla being detected in a recent study (Lee et al., 2011). One of the roles which has been proposed for these sponge associated microbes is protection of the sponge from predation, potential pathogens, competitors and fouling organisms; by producing biologically active secondary metabolites (Taylor et al., 2007). Marine sponges themselves have proven to be a very rich source of biologically active and pharmaceutically important natural products; many with biotechnological relevant properties, including anticancer, antiviral and anti-inflammatory activities (Brady et al., 2009; Cragg et al., 2009; Piel, 2009). Indeed sponges are the most prolific marine producers of novel compounds, with more than 3500 new metabolites being reported from sponges between 1985-2008 (Hu et al., 2011). These sponge-derived compounds include a wide variety of different chemical classes such as alkaloids, polyketides and terpenoids amongst others. The occurrence of structural similarities between some of these compounds from sponges and those from the sponge microbiota has led to the hypothesis that at least some of these bioactive compounds may in fact be of microbial origin (Wang, 2006). In addition a number of studies have shown that sponge associated bacterial isolates produced the same compounds which had been isolated from the sponges themselves. Thus it is clear that the sponge-microbe association makes sponges an ideal source for biologically active microorganisms producing potentially novel chemicals.

In recent years sponge-associated marine microorganisms have received renewed attention with respect to their production of secondary metabolites, with numerous studies focusing on culturing these microorganisms and screening them for the production of bioactive compounds (Hentschel et al., 2001; Muscholl-Silberhorn et al., 2008; Kennedy et al., 2009). This has involved culturing bacteria predominantly belonging to the actinobacteria, α-*Proteobacteria* and γ-*Proteobacteria* phyla, with limited reports from other bacterial phyla such as *δ-Proteobacteria*, *Planctomycetes* and *Verrucomicrobia* (Radjasa et al., 2011). Among bacterial isolates from marine sources actinobacteria are the most significant producers of secondary metabolites, with ~50% of the novel compounds derived from bacteria in 1985-2008 being from these genera (Hu et al., 2011). However α- and γ-*Proteobacteria* together with

Bacillus species have also been shown to produce compounds with antimicrobial activity (Hentschel et al., 2001; O'Halloran et al., 2011; Pabel et al., 2003; Thakur et al., 2005). The marine genus *Pseudovibrio* has only recently been described (Shieh et al., 2004) and to date comprises of three described species (*P. denitrificans*, *P. ascidiaceicola* and *P. japonicus*). Recent studies describe biologically active *Pseudovibrio* species isolated from different organisms including marine sponges (Hentschel et al., 2001; Thiel and Imhoff, 2003; Kennedy et al., 2009). Since little is currently known about the nature of these biologically active compounds derived from *Pseudovibrio spp.* they may prove to be a particularly promising source of potentially novel metabolites.

With the growing importance of drug-resistant infections over the last number of years, increases in infections by multidrug-resistant *Escherichia coli*, methicillin-resistant *Staphylococcus aureus* and other drug resistant bacteria such as *Salmonella spp.* have become a more and more prevalent problem in the food industry and in clinical settings. Thus there is an ever increasing need to discover new antibiotics, with the most recent fatal outbreak of the Shiga toxin producing *Escherichia coli* O104:H4 in Germany, which displayed resistance to multiple antibiotics providing a stark reminder of this fact (Bielaszewska et al., 2011).

Thus this study focused on the isolation and identification of bacteria from two marine sponges namely *Suberites carnosus* and *Leucosolenia sp.* and the subsequent screening of these bacteria for antibacterial activities against the three clinically relevant bacterial; *E. coli* (NCIMB 12210), *Bacillus subtilis* (IA40) and *S. aureus* (NCIMB 9518), and against five fungal test strains. While some work has been performed on *Suberites domuncula* with respect to biosilica production (Wang et al., 2011), and *Leucosolenia sp.* regarding the production of novel bioactive aminoimidazole alkaloids (Ralifo et al., 2007); these sponges have not previously been targeted for their culturable biodiversity nor for their potential as sources for novel antibiotics. In addition they represent two different classes of sponges, namely the *Demospongiae* (*S. carnosus*) and the *Calcarea* (*Leucosolenia sp.*), and in general the calcareous sponges to date remain a largely overlooked class with respect to biodiscovery.

MATERIALS AND METHODS

Sponge sampling

Samples of *S. carnosus* and *Leucosolenia sp.* were collected at a depth of 15m by SCUBA in Lough Hyne, Co. Cork, Ireland (N 51°50.556', W 09°30.389') in November 2008. Whole sponge samples were either transported to the on-site laboratory in seawater, rinsed with sterile artificial sea water and directly processed for the cultivation of bacteria; or frozen immediately following collection.

Isolation of bacteria

After collection 1 g of each sponge sample was finely chopped with a sterile razor blade, mixed with sterile glass beads and 1 ml seawater and vortexed for 30 seconds. The homogenate was then diluted and 100 µl of the dilutions 10^{-1} to 10^{-5} was spread on agar plates using sterile glass beads. For the cultivation three different media were employed: Starch-Yeast-Extract-Peptone-Seawater (SYP-SW) agar (1 L distilled water, 10g starch, 4 g yeast extract, 2 g peptone, 33.3 g artificial sea salts, 15 g agar), MMA-agar (1 L natural, filtered autoclaved seawater, 50 µg yeast extract, 0.5 mg Tryptone (enzymatic digest from casein), 0.1 mg Na-glycerol-PO_4, 15 g agar) and Chitin-Medium (1 L distilled water, 4 g colloidal chitin, 33.3 g artificial seawater, 15 g agar). All agar plates were then incubated at 18°C for ~2 months and checked regularly for the formation of distinguishable colonies. Colonies were picked from these isolation plates and re-streaked on SYP-SW agar plates. The colonies were not kept on the isolation medium in the case of MMA- and Chitin-agar because they grew faster on SYP-SW-agar. Colonies were selected based on their morphology and colour with the aim of picking as many different isolates as possible. Single colonies were repeatedly re-streaked to obtain pure colonies. For long term storage, isolates were grown in liquid SYP-SW for 3-5 days at 18ºC, sterile glycerol was added to 15% and stocks were stored at -80°C.

Deferred antagonism assays

These assays were performed as previously described (Kennedy et al., 2009). 5 µl of the stock culture were spotted onto an SYP-SW agar plate and incubated at 18ºC until the culture was ~0.5-1 cm in diameter (for most isolates 3-5 days of incubation

were sufficient). The culture was then overlayed with 10 ml of soft agar seeded with a fresh culture of bacterial or fungal test strains. For bacterial test strains (*E. coli NCIMB 12210, B. subtilis IA 40, S. aureus NCIMB 9518*) LB soft agar (1 l distilled water, 20 g LB broth, 5 g agar) was used. For fungal test strains (*Candida albicans Sc5314, Candida glabrata* CBS138, *Saccharomyces cerevisiae* BY4741, *Kluyveromyces marxianus* CBS86556 and *Aspergillus fumigatus* Af293) YPD soft agar (1 L distilled water, 10 g yeast extract, 20 g peptone, 20 g D-glucose, 7 g agar) was used. The overlayed plates were incubated for ~12-24 h at 28ºC for the fungal test strains and 37ºC for the bacterial test strains. A zone of clearance in the overlay agar indicated the production of an antimicrobial compound by an isolate.

Well diffusion assays

Isolates which showed clear activity in the deferred antagonism assay were also assayed in the well diffusion assay. The isolates were grown up in 50 ml liquid SYP-SW in 250 ml Erlenmeyer flasks at 28ºC and 180 rpm for 14 days. Twice a week 1 ml of the culture was taken and centrifuged at 20,238 x g for 10 min in order to obtain a cell-free supernatant. Parallel, LB and YPD agar plates were prepared for testing of bacterial and fungal test strains, respectively. Plates were first seeded with 100 µl of a 1:50 dilution of an overnight culture of each test-strain that were spread out on the plates using sterile glass beads. Holes were then punched into the agar with a heat-sterilised cork borer (diameter 5 mm). Then 100 µl of the cell-free supernatant or SYP-SW broth as a negative control were applied to the wells. The plates were incubated for 12-24 h at 28ºC for fungal test strains and 37ºC for bacterial test strains. Zones of inhibition around the punched wells indicating antimicrobial activity of the according supernatant were recorded.

PCR amplification of 16S-rRNA genes

A single colony of each isolate was added to 100 µl of autoclaved TE-buffer, pH 8.0 and incubated for 15 min at 98ºC. The mixture was centrifuged at 4,500 x *g* for 10 min and 3 µl of the crude extracts were used in the PCR reaction. To amplify the 16S rRNA gene, the universal primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3') were used (Lane, 1991), generating a PCR product of approximately 1500 bp. The following PCR conditions were used: initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for

30 s, 50°C for 30 s, and 72°C for 2 min, and a final elongation step of 72°C for 10 min. The reaction mixture (30 µl) contained: 3 µl 10x buffer (Fermentas), 3 µl dNTPs (2 mmol/l each; Fermentas), 1.5 µl 27f forward primer (10 µmol/l; Sigma-Aldrich), 1.5 µl 1492 r reverse primer (10 µmol/l; Sigma-Aldrich), 0.15 µl TAQ-polymerase (DreamTaq™ DNA polymerase, Fermentas, 5 U/µl), 17.85 µl molecular biology grade water and 3 µl DNA. The PCR products were analysed by agarose gel electrophoresis**.**

Sequencing and phylogenetic analysis of 16S-rRNA gene products

The 16S rRNA gene PCR products were purified and partially sequenced using primer 27f (carried out by Macrogen, Korea). The partial 16S rRNA gene sequences were manually checked for quality using Finch TV (http://www.geospiza.com/Products/finchtv.shtml) and then grouped into Operational Taxonomic Units (OTUs) based on 98.5 % sequence similarity with FastGroupII (Yu et al., 2006). Sequences representing isolates with antibacterial activity, antifungal activity and both antibacterial and antifungal activity (all from both sponges) and isolates from *S. carnosus* and *Leucosolenia sp.* without activity were uploaded and grouped separately. Representatives of each OTU obtained were aligned with neighbouring sequences obtained from RDP using the seqmatch tool (Cole et al., 2009). Only type strains with a sequence length of ≥1200 bp and of good quality were initially analysed. For isolates which formed deep branches in the phylogenetic tree the closest BLAST hit sequence was included in the analysis. The sequences were aligned and phylogenetic trees were calculated using MEGA5, using the neighbour joining algorithm (Tamura et al., 2011). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal trees are shown and the sum of branch lengths are given in the caption for each tree. The percentage of replicate trees (values below 50 deleted) in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic trees. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated.

RESULTS

Isolation of Bacteria

103 and 134 bacteria were isolated from the *S. carnosus* (W13) and *Leucosolenia sp.* (W15) samples, respectively. The bacteria were picked from the original plates based on colony appearance to obtain a diverse collection of isolates. For all 237 bacteria a 16S rRNA sequence was obtained. The phyla distribution of isolated bacteria from *S. carnosus* and *Leucosolenia sp.* is shown in Figure 2.1 and in supplementary Figures 2.1-2.5. In both sponges the most dominant isolates were *γ-Proteobacteria* (36% and 55% for *S. carnosus* and *Leucosolenia sp.* respectively), *α-Proteobacteria* (34% and 7%), *Actinobacteria* (11% and 5%), *Firmicutes* (10% and 11%), *Bacteroidetes* (7% and 22%) and *β-Proteobacteria* (3% and 0%). Thus on the phylum level the isolates from both sponges are dominated by bacteria belonging to the *Proteobacteria* with 72% and 63% of isolates from *S. carnosus* and *Leucosolenia sp.* grouping into this phylum, respectively. The remaining ~30% are shared more or less equally for both sponges between the phyla *Actinobacteria, Bacteroidetes* and *Firmicutes,* with the exception that isolates from the phylum *Bacteroidetes* were slightly more dominant and diverse among the bacteria from *Leucosolenia sp.*. Among the *Bacteriodetes* a total of 19 different OTUs were isolated from *Leucosolenia sp.* and only 5 OTUs from *S. carnosus* (Supplementary Figure 2.2).

Pseudovibrio were the most common genus isolated from *S. carnosus* (30 isolates), followed by *Spongiobacter* (10), *Microbulbifer* (8), *Pseudoalteromonas* (8), *Vibrio* (6), *Bacillus* (5), and *Staphylococcus* (4). For *Leucosolenia sp.* the most common isolates were of the genera *Pseudoalteromonas* (33) and *Vibrio* (27). Other frequently found genera were *Bacillus* (9), *Shewanella* (8), *Staphylococcus* (8), *Maribacter* (7), *Polaribacter* (5) and *Formosa* (5).

The four bacterial phyla obtained from the two sponges have previously been detected with culture dependent and independent methods (Taylor et al., 2007). This is also true on the genus level with representatives of e.g. *Pseudovibrio* (Kennedy et al., 2009; O'Halloran et al., 2011), *Pseuodoalteromonas* (Dieckmann et al., 2004; Kennedy et al., 2009), *Vibrio* (Dieckmann et al., 2004), *Spongiobacter* (Thiel et al., 2007; Kennedy et al., 2008; Mohamed et al., 2008), *Pseudomonas* (Thakur et al., 2005; Kennedy et al., 2008) and *Bacillus* (Webster and Hill, 2001; Kennedy et al., 2009) being commonly isolated from marine sponges or detected with culture

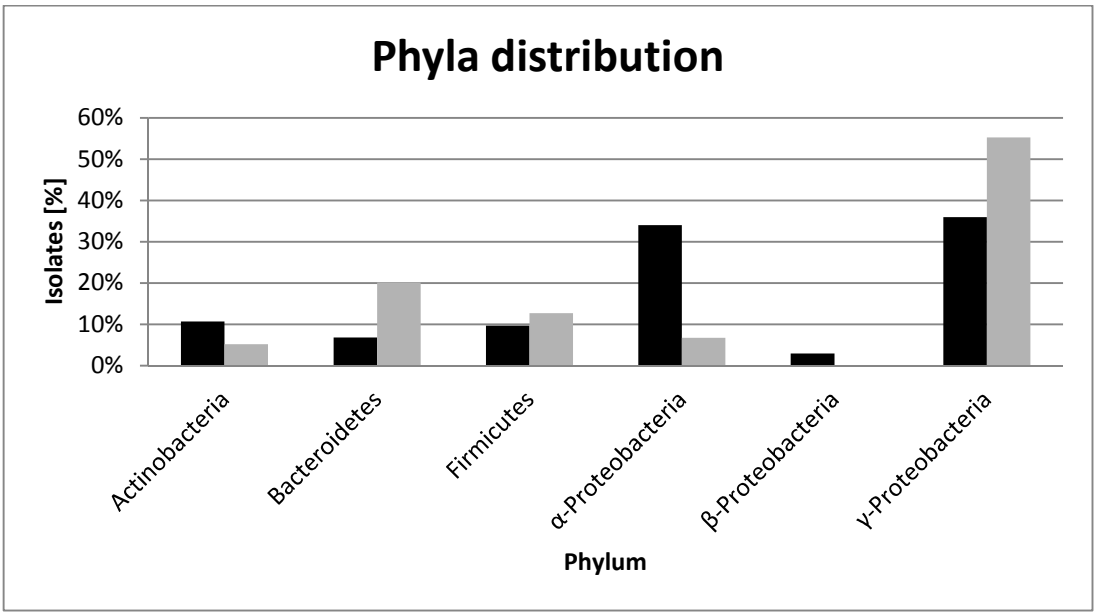


Figure 2.1: Phyla-distribution in percentage of isolates from *S. carnosus* (black bar) and *Leucosolenia sp.* (grey bar)

independent methods. But some isolates, especially from *Leucosolenia sp.* have a sequence similarity <98% to the closest hit in a BLAST search and therefore probably represent novel species or genera (data not shown). The majority of these potentially novel species are *Bacteroidetes*, with all but one isolated from the *Leucosolenia sp.* sponge sample. The bacteria were isolated on different growth media (Chitin-agar, Minimal-agar and SYP-SW-agar), with the distribution of bacterial groups shown in Table 2.1.

Table 2.1: Phyla distribution of 16S rRNA gene sequences obtained from isolates from *Leucosolenia sp.* (L) and *S. carnosus* (Sc) related to the isolation medium.

	Proteobacteria						Actinobacteria		Bacteriodetes		Firmicutes	
	α		β		γ							
	L	***Sc***	***L***	***Sc***	***L***	***Sc***	***L***	***Sc***	***L***	***Sc***	***L***	***Sc***
Chitin	33	57	-	33	16	16	0	18	19	29	29	20
MMA	44	11	-	33	12	27	43	73	52	71	24	10
SYP	22	31	-	33	72	57	57	9	30	0	47	70

Values are given as % of the total isolates of any given phylum, i.e. 33% of all α-*Proteobacteria* isolates from *Leucosolenia sp.* were isolated on Chitin-agar plates.

Antimicrobial activity - deferred antagonism assay

All isolates obtained from the two sponge samples were tested in an overlay assay against 3 clinically relevant bacterial and 5 fungal test strains: *E. coli* (NCIMB 12210), *B. subtilis* (IA40), *S. aureus* (NCIMB 9518), *C. albicans* (Sc5314), *C. glabrata* (CBS138), *S. cerevisiae* (BY4741), *K. marxianus* (CB86556) and *A. fumigatus* (Af293). In total 69 isolates showed clear activity against at least one of the test strains (Table 2.2) with 45 isolates from *S. carnosus* being active against bacterial test strains, 2 isolates active against fungal test strains and 2 isolates active against both fungal and bacterial test strains. Thus in total 49 isolates (i.e. 48%) of the 103 isolates from *S. carnosus* inhibited microbial growth in the initial activity assay. The main contributors to antimicrobial activity belonged to the phylum *α-Proteobacteria* (55% of the 49 active isolates from *S. carnosus* were *α-Proteobacteria*) followed by *γ-Proteobacteria* isolates (31%). Isolates from *Leucosolenia sp.* did not show antibacterial activity but 20 isolates (15%) showed activity against the fungal test strains. The main contributors to antifungal activity

Table 2.2: Active Isolates.

Isolate	Closest BLAST hit	Similarity [%]	EC	BS	SA	CA	CG	SC	KM	AF
W13M60	*Aquimarina muelleri strain KMM 6021*	98	-	-	+	-	-	-	-	-
W13M61A	*Aquimarina muelleri strain KMM 6021*	98	-	-	+	-	-	-	-	-
W13M62B	*Aquimarina muelleri strain KMM 6021*	98	-	-	+	-	-	-	-	-
W13C11	*Arthrobacter sp. 7A9S3*	100	+	+	+	-	-	-	-	-
W13S8	*Bacillus pumilus strain Ba9*	100	-	+	-	-	-	-	+	-
W13S32	*Bacillus sp. RS114(2010)*	99	(+)	+	+	-	-	-	-	-
W13M2	*Pseudoalteromonas sp. STAB201*	100	-	-	-	-	-	+	+	+
W13S34	*Pseudoalteromonas sp. STAB201*	100	+	+	-	-	-	-	-	-
W13S28	*Pseudoalteromonas tetraodonis*	100	-	-	-	-	-	+	-	-
W13M4	*Pseudomonas sp. C127*	99	-	+	+	-	-	-	-	-
W13C16	*Pseudovibrio sp. Ad2*	99	-	(+)	+	-	-	-	-	-
W13C12	*Pseudovibrio sp. Ad35*	99	(+)	+	+	-	-	-	-	-
W13C15	*Pseudovibrio sp. Ad42*	100	(+)	+	+	-	-	-	-	-
W13C24	*Pseudovibrio sp. Ad42*	100	(+)	+	(+)	-	-	-	-	-
W13C25	*Pseudovibrio sp. Ad42*	100	(+)	+	+	-	-	-	-	-
W13C28	*Pseudovibrio sp. Ad42*	100	-	+	+	-	-	-	-	-
W13C32	*Pseudovibrio sp. Ad42*	100	(+)	+	(+)	-	-	-	-	-
W13S49	*Pseudovibrio sp. Ad53*	99	+	+	+	-	-	-	-	-
W13C22	*Pseudovibrio sp. Ad54*	100	(+)	+	-	-	-	-	-	-
W13C30	*Pseudovibrio sp. Ad54*	99	-	+	+	-	-	-	-	-
W13S4	*Pseudovibrio sp. Ad57*	100	+	+	+	-	-	-	-	-

Isolate	Closest BLAST hit	Similarity [%]	EC	BS	SA	CA	CG	SC	KM	AF
W13S21	*Pseudovibrio sp. Ad58*	99	+	+	+	-	-	-	-	-
W13C10	*Pseudovibrio sp. Hs3*	100	(+)	+	-	-	-	-	-	-
W13C14	*Pseudovibrio sp. Hs3*	100	(+)	+	-	-	-	-	-	-
W13C17	*Pseudovibrio sp. Hs3*	100	(+)	+	+	-	-	-	-	-
W13C18	*Pseudovibrio sp. Hs3*	100	(+)	+	-	-	-	-	-	-
W13C21	*Pseudovibrio sp. Hs3*	100	(+)	+	-	-	-	-	-	-
W13C23	*Pseudovibrio sp. Hs3*	100	(+)	-	+	-	-	-	-	-
W13C27	*Pseudovibrio sp. Hs3*	100	-	(+)	+	-	-	-	-	-
W13C34	*Pseudovibrio sp. Hs3*	100	-	+	+	-	-	-	-	-
W13S13	*Pseudovibrio sp. Hs3*	100	+	-	+	-	-	-	-	-
W13S16	*Pseudovibrio sp. Hs3*	100	+	-	(+)	-	-	-	-	-
W13S22	*Pseudovibrio sp. Hs3*	100	+	+	nt	-	-	-	-	-
W13S23	*Pseudovibrio sp. Hs3*	99	+	+	+	-	-	-	-	-
W13S26	*Pseudovibrio sp. Hs3*	100	+	+	+	-	-	-	-	-
W13S31	*Pseudovibrio sp. Hs3*	99	+	+	+	-	-	-	-	-
W13C33	*Pseudovibrio sp. Pb2*	100	-	+	+	-	-	-	-	-
W13S54	*Psychrobacter faecalis strain UCL-NF 1590*	99	-	+	-	-	-	-	-	-
W13M7	*Ralstonia sp. 1F2 16S*	100	-	+	-	-	-	-	-	-
W13S29	*Shewanella sp. E505-7*	99	-	+	-	-	-	-	-	-
W13C2	*Spongiobacter sp. S2293*	98	(+)	+	-	-	-	-	-	-
W13C5	*Spongiobacter sp. S2293*	98	(+)	+	-	-	-	-	-	-
W13C7	*Spongiobacter sp. S2293*	99	(+)	+	-	-	-	-	-	-
W13S12	*Spongiobacter sp. S2293*	98	-	+	-	-	+	-	-	-
W13S18	*Spongiobacter sp. S2293*	98	-	+	-	-	-	-	-	-
W13S2	*Spongiobacter sp. S2293*	98	-	+	-	-	-	-	-	-
W13S30	*Spongiobacter sp. S2293*	98	-	+	-	-	-	-	-	-
W13S46	*Spongiobacter sp. S2293*	98	-	+	-	-	-	-	-	-
W13S51	*Spongiobacter sp. S2293*	98	-	+	-	-	-	-	-	-
W15C18a	*Bacillus amyloliquefaciens strain Z*	100	-	-	-	-	-	-	-	+
W15C2	*Bacillus amyloliquefaciens strain Z*	99	-	-	-	+	-	-	-	-
W15M1A	*Bacillus amyloliquefaciens strain Z*	99	-	-	-	-	+	+	+	+
W15S58a	*Bacillus simplex*	99	-	-	-	-	+	-	-	-
W15S84a	*Bacillus sp. A-05*	100	-	-	-	-	-	-	+	-
W15S84b	*Bacillus thuringiensis strain W8B-80*	99	-	-	-	-	-	-	+	-
W15S32	*Formosa algae strain F89*	99	-	-	-	-	-	-	-	+
W15S83	*Pseudoalteromonas issachenkonii*	99	-	-	-	-	+	-	-	-
W15S11	*Pseudoalteromonas sp. A2B10*	99	-	-	-	-	+	-	-	-
W15M16	*Pseudoalteromonas sp. K2B-2*	99	-	-	-	-	+	-	-	-
W15S10	*Pseudoalteromonas sp. LJ1*	100	-	-	-	-	+	-	-	-
W15S18	*Pseudoalteromonas sp. LJ1*	100	-	-	-	-	+	-	-	-
W15S14	*Pseudoalteromonas sp. S3178*	99	-	-	-	-	+	-	-	-
W15M34	*Staphylococcus saprophyticus*	100	-	-	-	-	-	-	-	+
W15S87a	*Staphylococcus sp. HJB003*	99	-	-	-	-	-	-	-	+
W15S67	*Vibrio litoralis strain MANO22P*	100	-	-	-	-	-	-	-	+
W15S24b	*Vibrio sp. SC-C1-5*	99	-	-	-	+	+	+	-	-

Isolate	Closest BLAST hit	Similarity [%]	EC	BS	SA	CA	CG	SC	KM	AF
W15C15	*Vibrio sp. BSw21697*	100	-	-	-	+	+	+	-	+
W15C16	*Vibrio splendidus LGP32*	99	-	-	-	+	+	+	-	+
W15C28	*Vibrio splendidus LGP32*	99	-	-	-	+	-	+	-	+

All 69 isolates which showed clear activity in the overlay assay against at least one test strain. Also given are the closest BLAST hits to the 16S rRNA sequence including the sequence similarity. W13 = isolate from *S. carnosus*; W15 = isolate from *Leucosolenia sp.*; C, M and S = isolate obtained from Chitin-, MMA- or SYP-agar, respectively; the test strains used were: *EC = E. coli, BS = B. subtilis, SA = S. aureus,* CA = C. *albicans* (Sc5314), CG = *C. glabrata* (CBS138), SC = *S. cerevisiae* (BY4741), KM = *K. marxianus* (CB86556) and AF = *A. fumigatus* (Af293); + = clear zone of inhibition visible in overlay agar; (+) = faint zone of inhibition; - = no zone of inhibition; nt = not tested

belonged to the phylum *γ-Proteobacteria* (55% of the 20 active isolates from *Leucosolenia sp.* were *γ-Proteobacteria*) followed by *Firmicutes* isolates (40%). Only one isolate (5%) from the phylum *Bacteroidetes* showed activity. Thus bacterial isolates from the phyla *Proteobacteria* and *Firmicutes* proved to be most active in this study.

All of the growth media used recovered bioactive isolates with Chitin agar and SYP agar resulting in the isolation of greater numbers and proportions of active isolates (Figure 2.2) than MMA. In terms of obtaining bioactive isolates Chitin-agar proved to be most successful for both sponges.

Antibacterial isolates (Table 2.3, Figures 2.3 and 2.4) were most commonly found in the genera *Pseudovibrio* (27), *Spongiobacter* (9), *Aquimarina* (3), and *Bacillus* (2). Antifungal isolates were most common from *Pseudoalteromonas* (8), *Bacillus* (7), *Vibrio* (5), and *Staphylococcus* (2). Representatives of many of those genera have been frequently shown to produce antimicrobial compounds (Gram et al., 2009; Hentschel et al., 2001; Kennedy et al., 2009; Muscholl-Silberhorn et al., 2008; O'Halloran et al., 2011) but only one report could be found for antimicrobial activity from a *Spongiobacter* isolate (Gram et al., 2009).

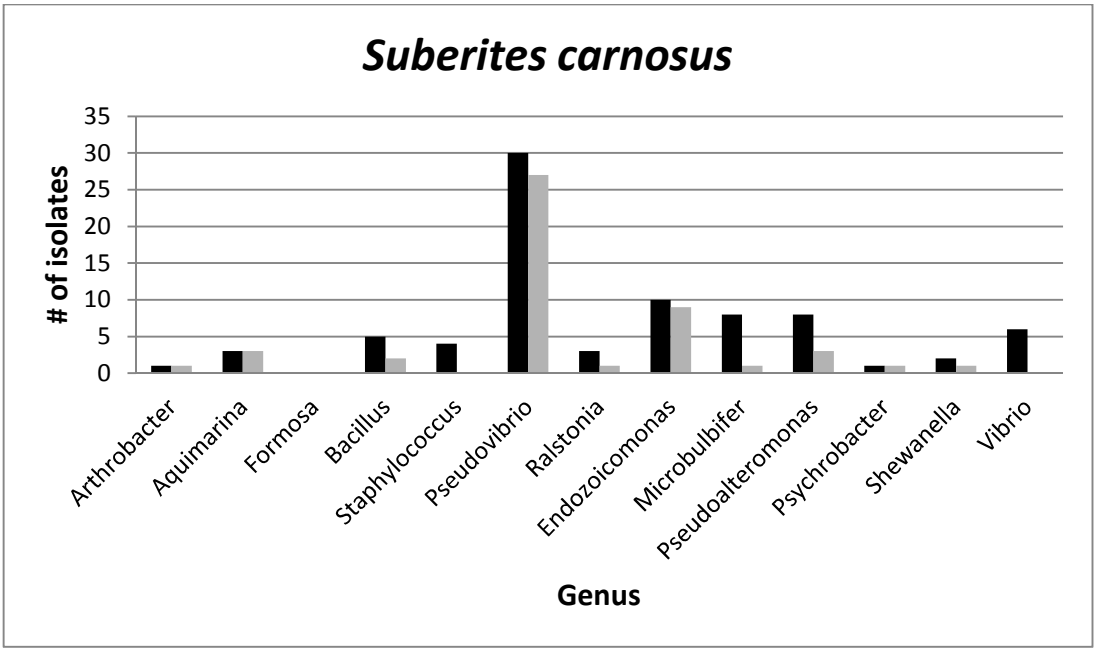


Figure 2.1: Total number of isolates from *S. carnosus* (black bar) and number of bioactive isolates (grey bar) for each genus

Table 2.3: Summary of antibacterial activities detected in isolates from *S. carnosus* and *Leucosolenia sp.*.

		S. carnosus					*Leucosolenia sp.*				
Phylum	**Genus**	**NA**	**B**	**B&F**	**F**	**%**	**NA**	**B**	**B&F**	**F**	**%**
Actinobacteria	*Arthrobacter*	-	1	-	-	100	3	-	-	-	0
Bacteroidetes	*Aquimarina*	-	3	-	-	100	1	-	-	-	0
Bacteroidetes	*Formosa*	-	-	-	-	-	4	-	-	1	20
Firmicutes	*Bacillus*	3	1	1	-	40	3	-	-	6	67
Firmicutes	*Staphylococcus*	4	-	-	-	0	6	-	-	2	25
α-Proteobacteria	*Pseudovibrio*	3	27	-	-	90	-	-	-	-	-
β-Proteobacteria	*Ralstonia*	2	1	-	-	33	-	-	-	-	-
γ-Proteobacteria	*Spongiobacter*	1	8	1	-	90	-	-	-	-	-
γ-Proteobacteria	*Microbulbifer*	7	1	-	-	13	1	-	-	-	0
γ-Proteobacteria	*Pseudoalteromonas*	5	1	-	2	38	27	-	-	6	18
γ-Proteobacteria	*Psychrobacter*	-	1	-	-	100	1	-	-	-	0
γ-Proteobacteria	*Shewanella*	1	1	-	-	50	8	-	-	-	0
γ-Proteobacteria	*Vibrio*	6	-	-	-	0	22	-	-	5	19

The different isolates are grouped into genera and are distinguished by their activity profile in the deferred antagonism assay: NA = not active, B = antibacterial activity, B&F = antibacterial and antifungal activity, F = antifungal activity, % = percentage of bioactive isolates relative to the total number of isolates in this genus.

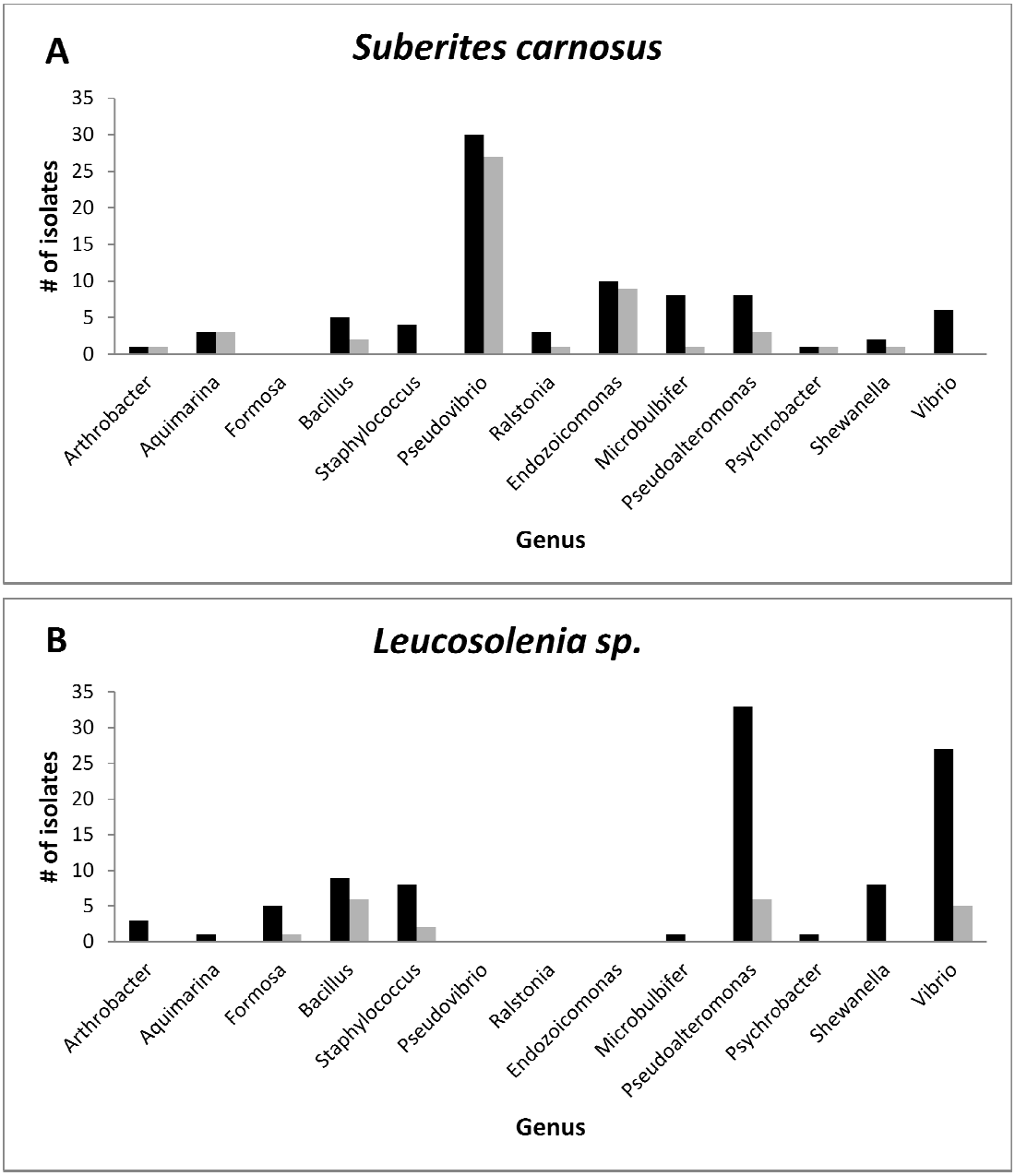


Figure 2.3: Total number of isolates (black bar) and number of bioactive isolates (grey bar) from *S. carnosus* (Figure 2.3A) and *Leucosolenia sp.* (Figure 2.3B).

Shown are genera for which at least one bioactive isolate in either of the two sponges was detected in the deferred antagonism assay.

Antimicrobial activity - well Diffusion Assay

All 69 bioactive isolates were tested in a well diffusion assay, with 9 isolates showing activity in this assay (3 from *S. carnosus* and 6 from *Leucosolenia sp.*). None of the supernatants tested showed activity against more than one indicator strain and no activity was found from any *S. carnosus* isolate against bacterial test strains. The 3 isolates from *S. carnosus* were active against *A. fumigatus*, the isolates from *Leucosolenia sp.* were active against *C. glabrata* (2 isolates) and *C. albicans* (4 isolates) (Table 2.4).

Table 2.4: Well diffusion assays.

Isolate	Closest BLAST hit	CA	CG	SC	KM	AF
W13M2	*Pseudoalteromonas sp. STAB201*	-	-	-	-	+
W13S28	*Pseudoalteromonas tetraodonis*	-	-	-	-	+
W13S8	*Bacillus pumilus strain Ba9*	-	-	-	-	+
W15C15	*Vibrio sp. BSw21697*	+	-	-	-	-
W15C18A	*Bacillus amyloliquefaciens strain Z*	+	-	-	-	-
W15C28	*Vibrio splendidus LGP32*	+	-	-	-	-
W15M16	*Pseudoalteromonas sp. K2B-2*	-	+	-	-	-
W15S14	*Pseudoalteromonas sp. S3178*	+	-	-	-	-
W15S87A	*Staphylococcus sp. HJB003*	-	+	-	-	-

In total 69 isolates were tested and 9 showed activity against one of the fungal test strains for which the results are shown. None of the isolates tested showed activity against any of the bacterial test strains. CA-C. *albicans* (Sc5314), CG-*C. glabrata* (CBS138), SC-*S. cerevisiae* (BY4741), KM-*K. marxianus* (CB86556) and AF- *A. fumigates* (Af293); + = clear zone of inhibition; - = no zone of inhibition

DISCUSSION

The bacteria isolated from both sponges were diverse, with microbes from 4 phyla and a combined total of 85 OTUs being isolated from both sponges. Whereas for *S. carnosus* this is perhaps not surprising since demosponges are known to host a diverse range of bacteria, calcareous sponges for their part have not to date been thoroughly analysed for their microbial biodiversity. Detailed phylogenetic analysis of these isolates showed that only 11 out of the 85 OTUs were obtained from both sponges. Both *Pseudovibrio* and *Spongiobacter*, the dominant isolates from the demosponge *S. carnosus,* have been commonly isolated from demosponges and

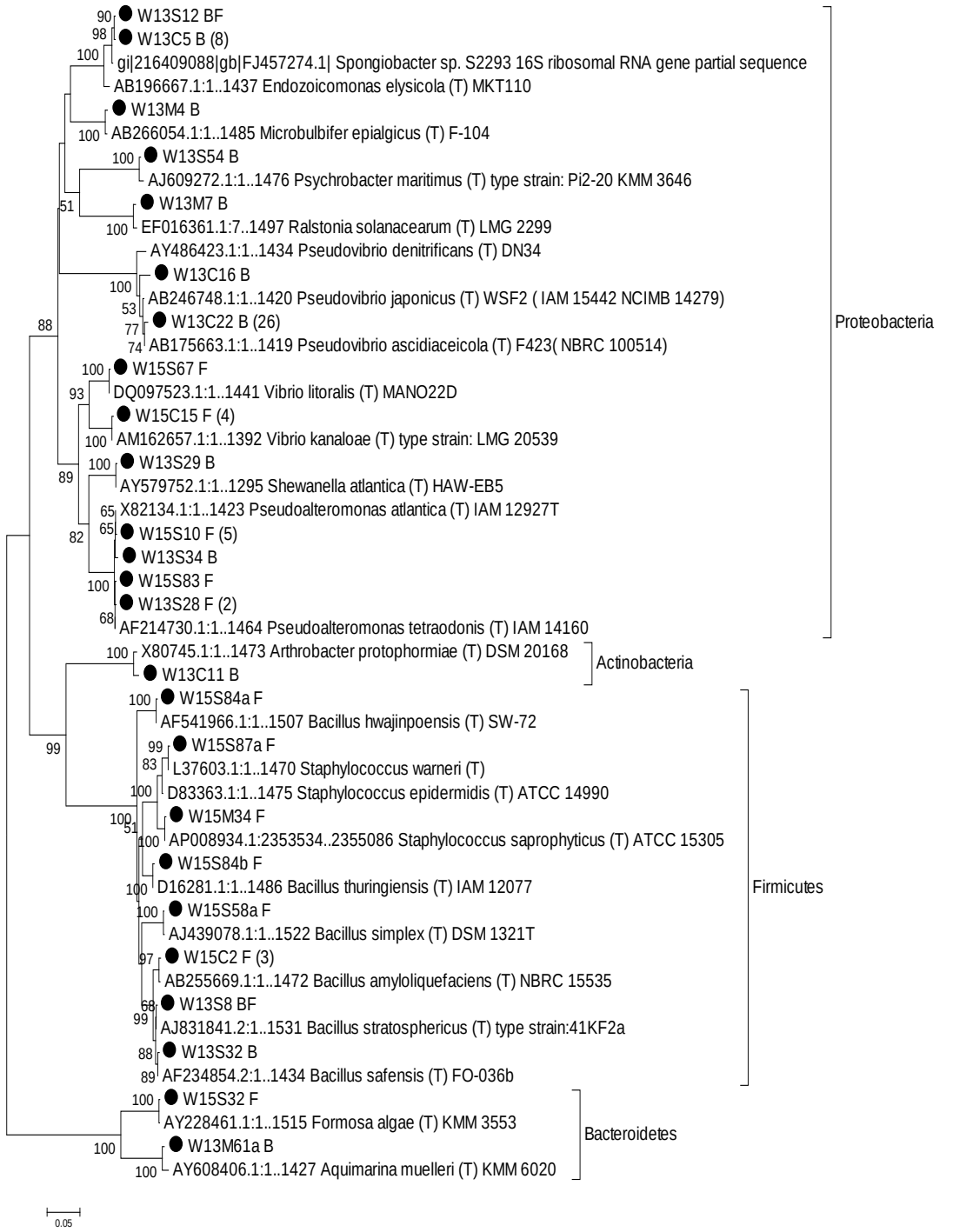


Figure 2.4: Phylogenetic analysis of 16S rRNA gene sequences from bioactive sponge isolates.

Sequences obtained in this study are marked (●). Where a strain represents more than one isolate the corresponding number is given in brackets. Bioactivities of isolates are marked with B (antibacterial), F (antifungal) or BF (both antibacterial and antifungal). W13 = isolate from *S. carnosus*, W15 = isolate from *Leucosolenia sp.*; C, M and S = isolate derived from Chitin-, MMA- or SYP-medium respectively. The optimal tree with the sum of branch length = 1.59214718 is shown. The analysis involved 50 nucleotide sequences. There were a total of 492 positions in the final dataset.

other marine invertebrates and are likely to have a symbiotic relationship with the sponge. The common isolates from *Leucosolenia sp*., *Pseudoalteromonas* and *Vibrio*, while also commonly isolated from sponges are also highly abundant in seawater, perhaps implying a less close, more opportunistic, relationship. In a parallel study metagenomic analysis of seawater collected at the same site and time revealed the most dominant phyla to be *Vibrio* and *Pseudoalteromonas* (Jackson et al., 2012).

Most of the isolates from both sponges have >98% sequence identity to 16S rRNA genes from cultured bacteria in the GenBank database. However several isolates, especially those derived from *Leucosolenia sp.* have a 16S rRNA gene sequence similarity equal to or less than 97% identity indicating potentially novel species or genera (data not shown). These isolates did not inhibit the growth of any of the test strains in the initial assays but none the less the potential of the investigated sponge as a source for novel microorganisms is evident. Since sponges of the class *Calcarea* have so far been almost largely overlooked it is perhaps not surprising that the calcareous sponge investigated in this study reveals more novelty in its culturable microbiota. This finding also highlights the potential of this genus of calcareous sponges as a potential source for novel metabolites, enzymes or other activities produced by their associated microbes. Some of the most promising isolates are currently being analysed for description as new species or potentially novel genera.

A wide range of bacteria with antimicrobial activity were isolated from both sponges. Representatives of 13 bacterial genera (21 OTUs) and 4 phyla exhibited activity against at least one of the test strains used (Table 2.3). The isolates from *S. carnosus*, especially of the genus *Pseudovibrio*, were more likely to exhibit antimicrobial activity (48% of all isolates showed activity) than isolates from *Leucosolenia sp.* (15%). Especially interesting in this respect is the observation that 46% of all *Suberites* isolates were biologically active against bacterial test strains and only 4% of isolates were antifungal (2% of *Suberites* isolates were both antibacterial and antifungal). Conversely, none of the isolates from *Leucosolenia sp.* inhibited bacterial growth in the deferred antagonism assay, while 15% displayed antifungal activity. Differences in the activity profiles of the sponge isolates are also reflected in the genera isolated from the sponges. For *Leucosolenia sp.* the clearly dominant genera were *Pseudoalteromonas* (27 isolates) and *Vibrio* (23), while the dominant genera isolated from *S. carnosus* were *Pseudovibrio* (30 isolates)

and *Spongiobacter* (10). The *Pseudovibrio* and *Spongiobacter* isolates comprised a large fraction of the antibacterial isolates from *S. carnosus* and neither of these genera were obtained from *Leucosolenia sp.*, which could explain the lesser extent of antibacterial activity. It is also apparent that both sponges share isolates of the same genera which demonstrated antibacterial activity if isolated from *S. carnosus* and no, or antifungal, activity if isolated from *Leucosolenia sp.*. This is true for isolates of the genus *Arthrobacter*, *Aquimarina*, *Bacillus*, *Microbulbifer*, *Psychrobacter*, *Shewanella, Staphylococcus* and *Vibrio* (Table 2.3). The only exception to this are isolates of the genus *Pseudoalteromonas* which showed antifungal activity regardless of their origin. While this study was designed to access culturable microbial diversity (rather than measure relative abundances) and is subject to the biases of all culture based approaches, the apparent sponge specific activity profile is striking, and could be due to subtle genetic differences between members of the same bacterial species residing in the different sponges (e.g. plasmid-encoded activities). In summary, the different activity profiles of isolates from the two sponges can partly be explained by the different genera and species isolated but intraspecies variation also appears to play a role.

It is especially interesting that isolates belonging to genera not often or not at all reported to exhibit antimicrobial activity were found to inhibit the growth of the test microbes. For good reason the focus of biodiscovery researchers has been on bacteria belonging to actinobacterial genera, particularly *Streptomyces* and *Micromonospora.* These bacteria have been routinely shown to be prolific producers of secondary metabolites and to be abundant members of the sponge-associated microbiota but it has also been reported that their abundance varies greatly from sponge to sponge. From the sponges targeted in the present study no isolate grouped with the above mentioned genera. However, representatives from different genera such as *Spongiobacter* and *Aquimarina* were shown to exhibit antimicrobial activity. To our knowledge this is one of the first reports for representatives of these genera to inhibit the growth of microbes. The other main genera found (*Bacillus, Pseudovibrio, Pseudoalteromonas* and *Vibrio*) have been previously reported to exhibit antimicrobial activity, while the genus *Pseudovibrio* has gathered the interest of researchers recently due to the broad range of activities detected in isolates of this genus (Hentschel et al., 2001; Kennedy et al., 2009; O'Halloran et al., 2011; Thiel and Imhoff, 2003). The different bioactivity profiles of *Pseudovibrio* isolates which

are phylogenetically similar indicate the production of different compounds from closely related bacteria. But very little is known yet about the bioactive compounds produced from *Pseudovibrio* strains. Only two antimicrobial compounds from a *Pseudovibrio* isolate (Sertan-de Guzman et al., 2007; Penesyan et al., 2011) have to date been characterised. The *P. denitrificans* strain Z143-1 was found to produce a red pigment, heptylprodigiosin, with anti-Staphylococcus aureus activity (Sertan-de Guzman et al., 2007) and the strain *Pseudovibrio* sp. D323 was reported to produce tropodithietic acid which exhibits activity against a variety of marine bacteria. The high number of pathways related to the biosynthesis of secondary metabolites found in the genome of *Psudovibrio* sp. JE062 (http://patricbrc.vbi.vt.edu/portal/portal/patric/Genome?cType=genome&cId=14949) obtained from the marine sponge *Mycale laxissima* further highlights the considerable potential of members of this genus to produce novel bioactive metabolites .

In summary, a wide variety of bacteria have been isolated with a high percentage exhibiting antimicrobial activity. The isolates of *Leucosolenia sp.*, a calcareous sponge, only showed activity against different fungal test strains whereas almost 50% of the isolates from *S. carnosus* showed activity against bacterial test strains. Also, biological activity was detected for genera which have not previously been shown to inhibit microbial growth. This not only highlights the potential of the genus *Suberites* but it also shows that the so far almost overlooked calcareous sponges could also play an important role in the biodiscovery of novel compounds. This is especially true because they appear to harbour hitherto unknown bacterial species. The differences in the antibacterial and antifungal profiles in the isolates from the two sponges are intriguing. While from this study it is clear that the isolates from the *Calcareous* sponge *Leucosolenia sp.* appear to be a better source for antifungal agents and those from *S. carnosus* a better source of antibacterial activity, the biological reason for this is unknown; and whether this is due to the different competing microbes or pathogens in the two niches, or biases in culturing may be worthy of further studies with additional samples. Finally while in this study many antimicrobial isolates were found, antimicrobial activity was subsequently difficult to establish in shake flask cultures. Other approaches such as extraction from agar plates, changing the culture medium and concentrating the broth before testing it in the bioassay have also been tried with selected isolates but have proven

unsuccessful. Little is known regarding antimicrobials produced by the genera *Spongiobacter*, *Aquimarina* and *Pseudovibrio*, however, new approaches to induce the production of bioactive compounds such as co-cultivation techniques (Nützmann et al., 2011; Pérez et al., 2011) and biofilm formation (Yan et al., 2003; Wilson et al., 2011) may help in the further characterisation of the bioactive component of these strains.

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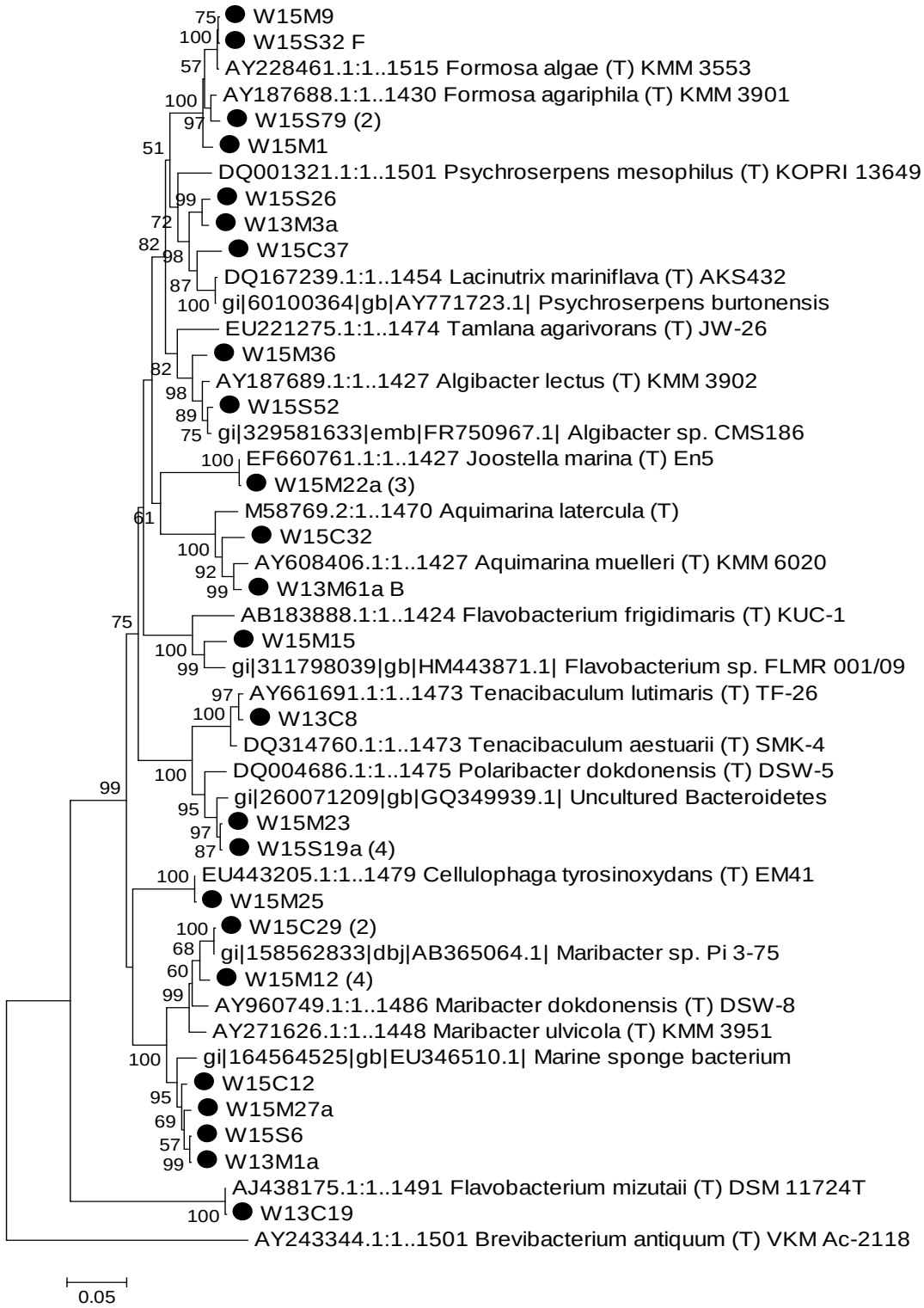
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SUPPLEMENTARY FIGURES



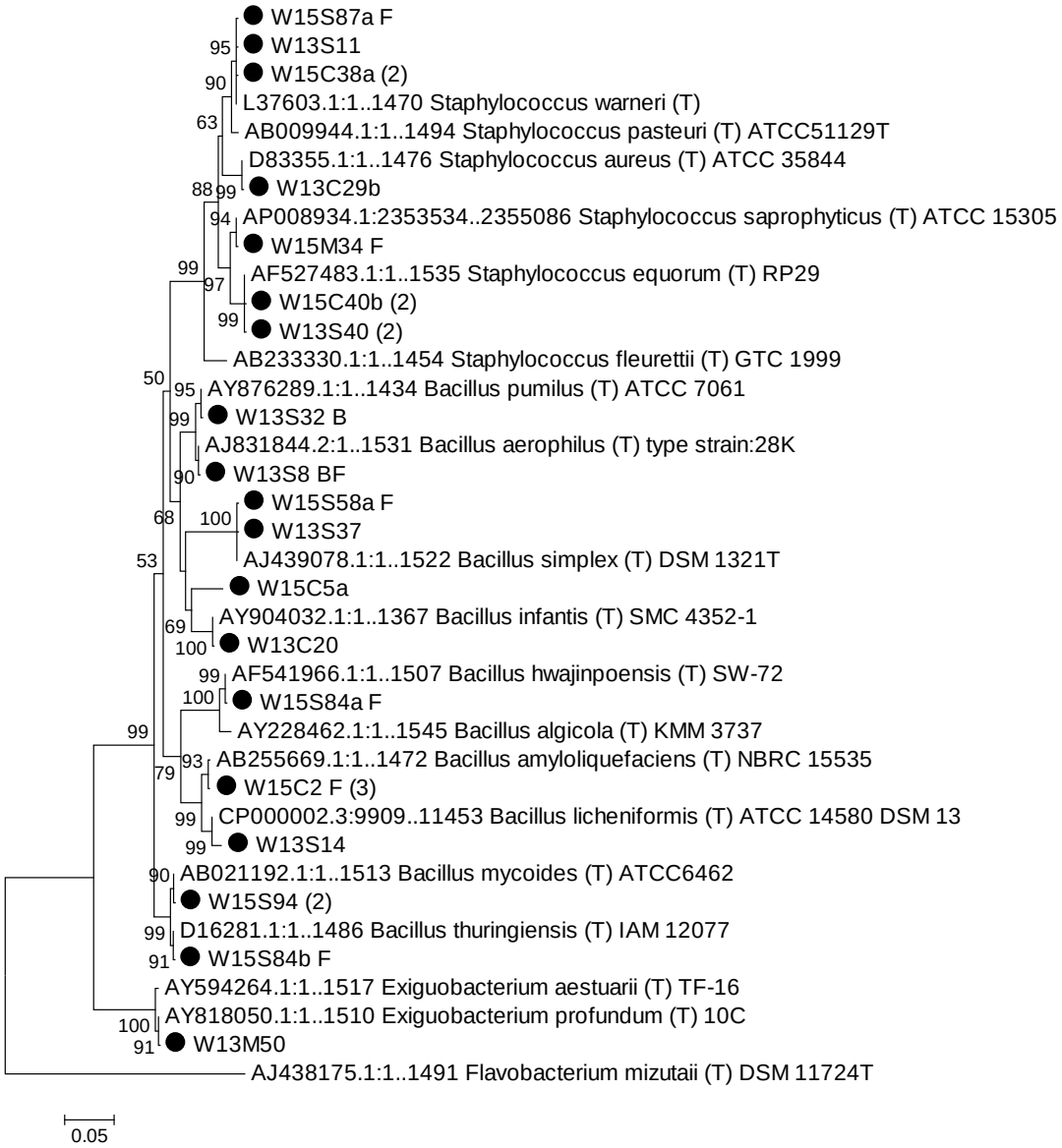
Supplementary Figure 2.1: Phylogenetic analysis of all actinobacterial sponge isolates.

Sequences obtained in this study are marked with a dot. Where a strain represents more than one isolate the corresponding number is given in brackets. Bioactivities of isolates are marked with B (antibacterial), F (antifungal) or BF (both antibacterial and antifungal). W13 = isolate from *S. carnosus*, W15 = isolate from *Leucosolenia sp.*; C, M and S = isolate derived from Chitin-, MMA- or SYP-medium respectively. The optimal tree with the sum of branch length = 0.64931053 is shown. The analysis involved 35 nucleotide sequences. There were a total of 456 positions in the final dataset.



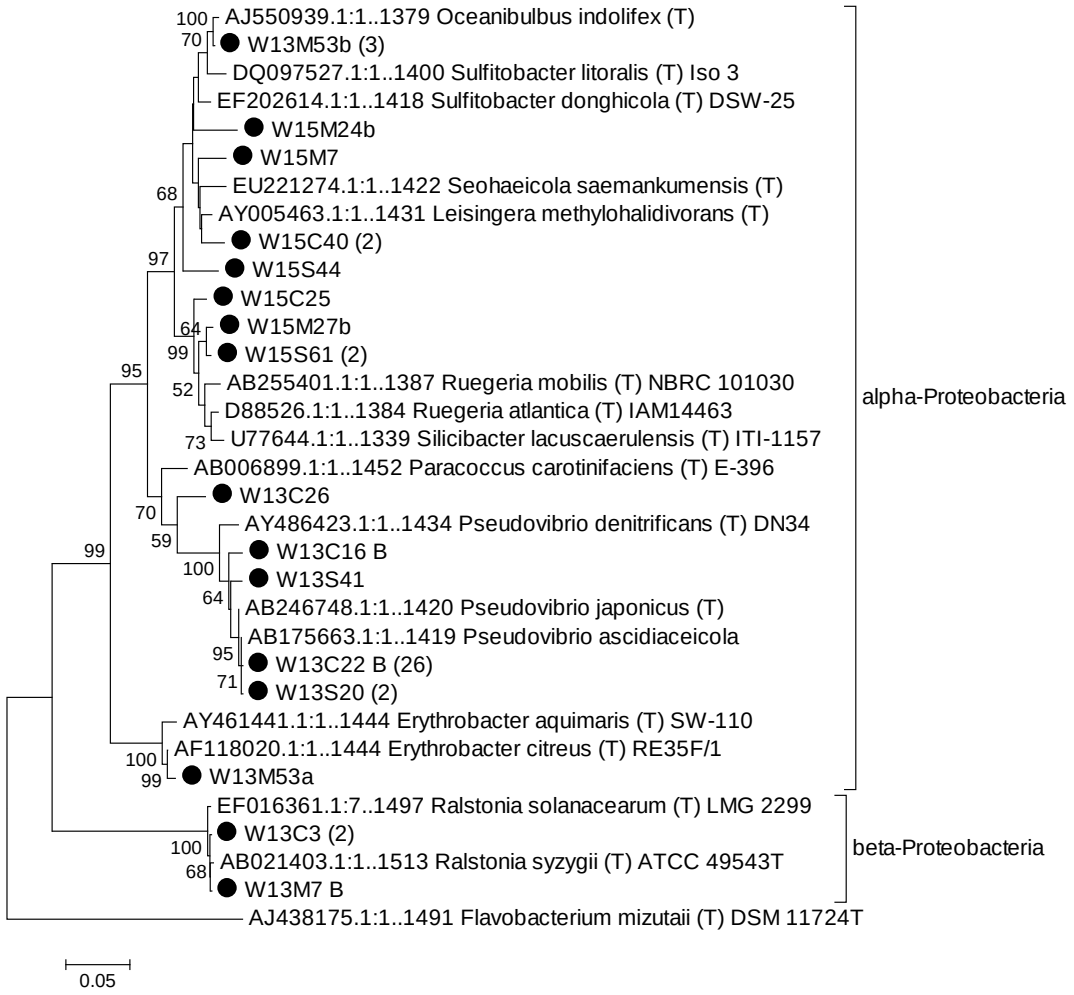
Supplementary Figure 2.2: Phylogenetic analysis of all *Bacteroidetes* sponge isolates.

Sequences obtained in this study are marked with a dot. Where a strain represents more than one isolate the corresponding number is given in brackets. Bioactivities of isolates are marked with B (antibacterial), F (antifungal) or BF (both antibacterial and antifungal). W13 = isolate from *S. carnosus*, W15 = isolate from *Leucosolenia sp.*; C, M and S = isolate derived from Chitin-, MMA- or SYP-medium respectively. The optimal tree with the sum of branch length = 1.39908229 is shown. The analysis involved 48 nucleotide sequences. There were a total of 608 positions in the final dataset.



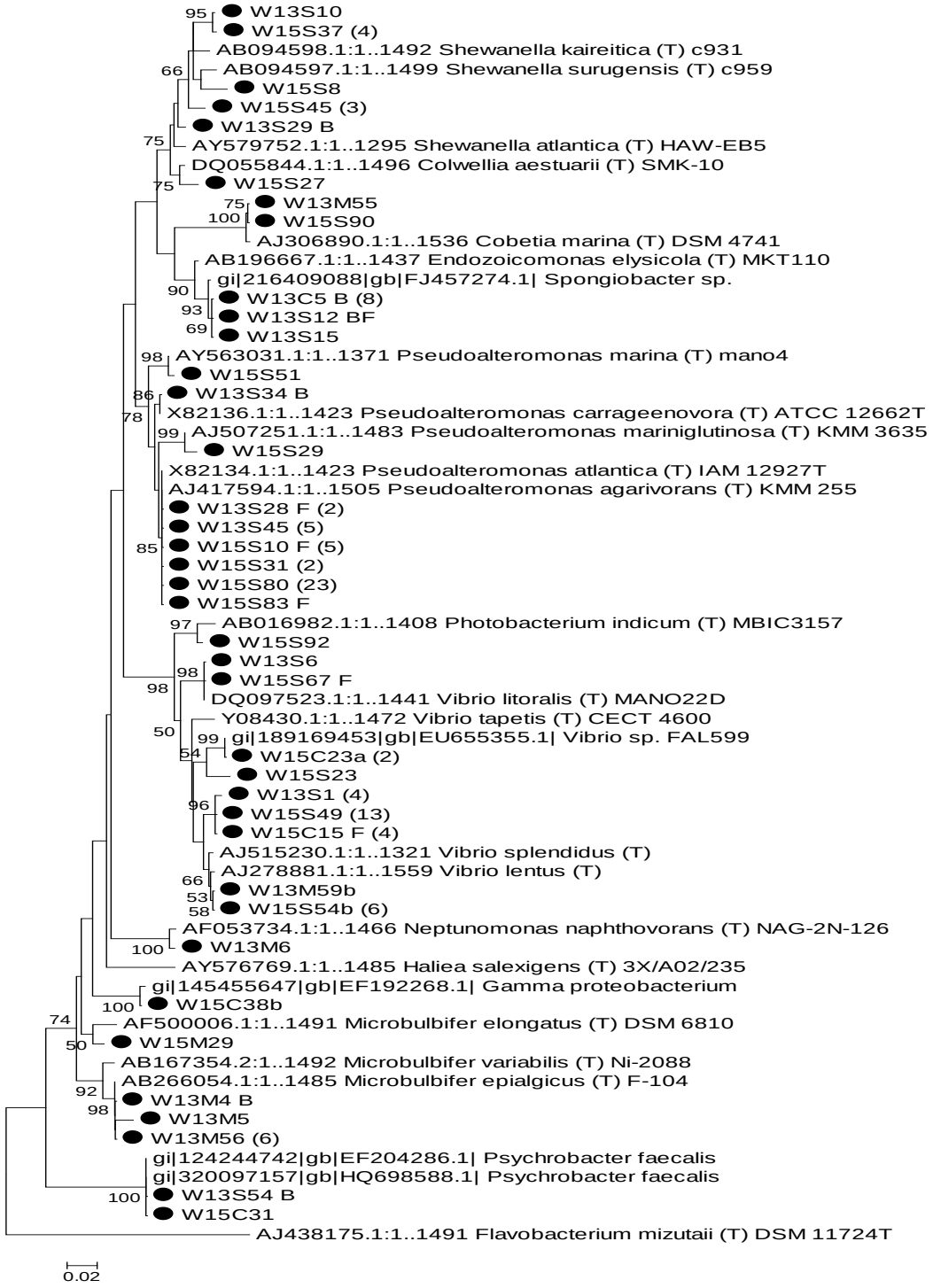
Supplementary Figure 2.3: Phylogenetic analysis of all *Firmicutes* sponge isolates.

Sequences obtained in this study are marked with a dot. Where a strain represents more than one isolate the corresponding number is given in brackets. Bioactivities of isolates are marked with B (antibacterial), F (antifungal) or BF (both antibacterial and antifungal). W13 = isolate from *S. carnosus*, W15 = isolate from *Leucosolenia sp.*; C, M and S = isolate derived from Chitin-, MMA- or SYP-medium respectively. The optimal tree with the sum of branch length = 0.92374162 is shown. The analysis involved 38 nucleotide sequences. There were a total of 352 positions in the final dataset.



Supplementary Figure 2.4: Phylogenetic analysis of all α- and β-proteobacterial sponge isolates.

Sequences obtained in this study are marked with a dot. Where a strain represents more than one isolate the corresponding number is given in brackets. Bioactivities of isolates are marked with B (antibacterial), F (antifungal) or BF (both antibacterial and antifungal). W13 = isolate from *S. carnosus*, W15 = isolate from *Leucosolenia sp.*; C, M and S = isolate derived from Chitin-, MMA- or SYP-medium respectively. The optimal tree with the sum of branch length = 0.92860151 is shown. The analysis involved 33 nucleotide sequences. There were a total of 475 positions in the final dataset.



Supplementary Figure 2.5: Phylogenetic analysis of all γ-proteobacterial sponge isolates.

Sequences obtained in this study are marked with a dot. Where a strain represents more than one isolate the corresponding number is given in brackets. Bioactivities of isolates are marked with B (antibacterial), F (antifungal) or BF (both antibacterial and antifungal). W13 = isolate from *S. carnosus*, W15 = isolate from *Leucosolenia sp.*; C, M and S = isolate derived from Chitin-, MMA- or SYP-medium respectively. The optimal tree with the sum of branch length = 0.98632771 is shown. The analysis involved 65 nucleotide sequences. There were a total of 263 positions in the final dataset.

Chapter 3

Diversity and antimicrobial activities of fungal isolates obtained from 12 marine sponges sampled in Lough Hyne, Ireland

ABSTRACT

12 marine sponge samples were collected in Lough Hyne, Ireland and 71 fungal isolates were obtained. Based on their 18S-rRNA gene sequence the isolates were found to be very diverse with 22 OTUs determined at a sequence identity of 98.5% and different phylotypes present in some of the OTUs. Together with genera or orders typically isolated from marine sponges such as *Hypocreales, Pleosporales* and *Eurotiales* some isolates were representatives of taxa seldomly found in marine sponges. All isolates were assessed for their antimicrobial activity against 2 Gram positive and 1 Gram negative bacterium in the deferred antagonism assay and more than 60% of the isolates tested positive in at least one assay against at least one of the test strains. Isolates with clear and/ or broad range activity were also tested in the well diffusion assay and the disc diffusion assay against the same bacterial as well as 5 fungal test strains (4 yeasts and 1 mycelium forming fungus). Isolates grouping into three OTUs of the orders *Hypocreales, Pleosporales* and *Eurotiales* showed especially strong and broad range activity. One of the isolates, which was closely related to *Fusarium oxysporum* and showed activity against bacteria and fungi, was investigated for its secondary metabolite genes (NRPS and PKS). At least 5 different NRPS genes were identified in its genome based on partial gene sequences obtained from a clone library. The sequence identity to published NRPS sequences of one gene was as low as 50 % highlighting the likelihood that this isolate may be capable of producing novel secondary metabolites.

INTRODUCTION

Drug-resistant microbes such as vancomycin-resistant *Enterococcus*, methicillin-resistant *Staphylococcus aureus* or *Salmonella spp.* are becoming an increasing threat in hospital settings and in the food industry. For this reason, there is a growing need for novel antibiotics to counter this trend.

Both the cultured and uncultured diversity of marine-sponge-associated bacteria has been well described (Simister et al., 2012). In contrast, even though sponge specific clusters for sponge derived fungi have been described (Simister et al., 2012) and terrestrial fungi have played an important role in drug discovery over the years; very little is known about the fungal diversity associated with marine sponges. Nonetheless, the potential of sponge-associated fungi has recently been demonstrated through the isolation of 81 fungi from the sponge *Tethya aurantium*. These fungi were subsequently characterised and various secondary metabolites identified in fungal-derived extracts, with compounds displaying antibacterial, antifungal, antiviral and many other activities being reported (Wiese et al., 2011). Large numbers of sponge-derived fungal isolates had also previously been reported with bioactivity against bacteria, algae and other fungi (Höller et al., 2000). Other studies have analysed the fungal biodiversity associated with Hawaiian sponges using either culture independent (Gao et al., 2008) and/or dependent approaches (Li and Wang, 2009; Wang et al., 2008). Fungi have also been isolated from sponges from the South China Sea, with polyketide synthase PKS and NRPS genes being identified in these fungi in an attempt to assess their bioactive potential (Ding et al., 2011; Zhou et al., 2011). The sponge *Haliclona simulans* was the first marine sponge collected from Irish waters analysed for its culturable fungal associates (Baker et al., 2009). Of the sponge-derived fungal isolates which have been studied to date many bioactive compounds have been characterized, with examples including antiviral (Peng et al., 2012), antibacterial (Li et al., 2012) and cytotoxic (Almeida et al., 2012) compounds. It has been estimated that up to 2008 only 300 chemicals have been described from marine fungi (Hu et al., 2011), in a total of approximately 22000 marine derived natural products which were identified up until then (Blunt et al., 2011). Also, considering the contribution of natural products derived from terrestrial fungi, e.g.

the discovery of penicillin, and the finding, that marine fungi are more often bioactive than their terrestrial counterparts and more often produce novel compounds (Cuomo et al., 1995), it is clear that marine fungi are an as yet largely underutilized or exploited source for potentially novel secondary metabolites. This highlights the vast chemical potential still hidden in marine fungi.

In this study the culturable diversity and antimicrobial potential of fungi associated with 12 marine sponges collected at Lough Hyne in Ireland, was evaluated.

MATERIALS AND METHODS

Sponge sampling

Samples of *Polymastia boletiformis* (J1), *Stelligera stuposa* (J2), *Eurypon major* (J9), *Suberites ficus* (J14), *Tethya citrina* (W1), *Cliona celata* (W2), *Amphilectus fucorum* (W4), *Raspailia ramosa* (W9), *Axinella dissimilis* (W11), *Suberites carnosus* (W13 and W14) and *Leucosolenia sp.* (W15) were collected at a depth of 15m by SCUBA in Lough Hyne, Co. Cork, Ireland (N 51°50.556', W 09°30.389') in November 2008. Whole sponge samples were either transported to the on-site laboratory in seawater, rinsed with sterile artificial sea water and directly processed for the cultivation of fungi; or frozen immediately following collection.

Isolation of fungi

After collection, 1 g of each sponge sample was finely chopped with a sterile razor blade, mixed with sterile glass beads and 1 ml seawater and vortexed for 30 seconds. The homogenate was then diluted and 100 µl of the dilutions 10^{-1} to 10^{-3} was spread on agar plates using sterile glass beads. For cultivation the following isolation medium was employed (MEG-medium). Solution I: 0.5 l distilled water, 30 g malt extract, 3 g peptone, 23.6 g Instant Ocean adjusted to pH 5.5 prior to autoclaving; Solution II: 0.5 l distilled water, 8 g gellan gum (gellan gum was dissolved prior to autoclaving by heating up to 80°C and stirring for 30 min). The solutions were autoclaved separately, kept at 70°C, mixed and 200 mg/L chloramphenicol added. All agar plates were incubated at 18°C for ~2 months and checked regularly for the formation of distinguishable colonies. Colonies were picked from these isolation

plates and re-streaked on plates containing agar instead of gellan gum (MEA-medium: 1 L distilled water, 30 g malt extract, 3 g peptone, 23.6 g Instant Ocean, 15 g agar). Colonies were selected based on their morphology and colour with the aim of picking as many different isolates as possible. Single colonies were repeatedly re-streaked to obtain pure colonies. For long term storage, pieces of mycelium were added to 15% glycerol and kept at -80°C.

Deferred antagonism assay

Mycelium was transferred to the centre of a MEA plate and incubated for 3-5 days until the diameter of the colony was 0.5-1cm. The culture was then overlayed with 10 ml of LB soft agar (1 l distilled water, 20 g LB broth, 5 g agar) seeded with a fresh culture of either *E. coli NCIMB 12210, B. subtilis IA 40* or *S. aureus NCIMB 9518*. The overlayed plates were incubated for ~12-24 h at 37ºC. A zone of clearance in the overlay agar indicated the production of an antimicrobial compound by an isolate.

Well diffusion assay

Isolates which showed clear activity in the deferred antagonism assay were also assayed in the well diffusion assay. The isolates were grown up in 50 ml liquid malt-extract (ME) medium in 250 ml Erlenmeyer flasks at 28ºC and 180 rpm for up to 28 days. Twice a week 1 ml of the culture was taken and centrifuged at 20,238 x g for 10 min in order to obtain a cell-free supernatant (CFS). LB (1 l distilled water, 20 g LB broth, 15 g agar) and YPD (1 L distilled water, 10 g yeast extract, 20 g peptone, 20 g D-glucose, 20 g agar) agar plates were prepared for testing the bacterial and fungal test strains, respectively. Plates were first seeded with 100 µl of a 1:50 dilution of an overnight culture of each test-strain that were spread out on the plates using sterile glass beads. Holes were then punched into the agar with a heat-sterilised cork borer (diameter 5 mm). Then 100 µl of the cell-free supernatant or ME-broth as a negative control were applied to the wells. The plates were incubated for 12-24 h at 28ºC for fungal test strains and 37ºC for bacterial test strains. Zones of inhibition around the punched wells indicating antimicrobial activity of the according supernatant were recorded. Bacterial test strains used were as in "Deferred antagonism assay", fungal test strains were as follows: (*C. albicans Sc5314, C.*

glabrata CBS138, *S. cerevisiae* BY4741, *K. marxianus* CBS86556 and *Aspergillus fumigatus* Af293).

Preparation and testing of antibiotic discs

Fungal isolates showing activity in the well diffusion assay were grown again in 50 ml ME broth. The culture broth was tested for antimicrobial activity in the well diffusion assay as described above and upon showing antimicrobial activity was centrifuged at 4,000 x g. If no firm pellet was obtained, the broth was instead sterile filtered (Filtropur V50 0.45, Sarstedt). 2 g of XAD-16 (Amberlite, SIGMA) was added to the cell free supernatant and after agitation for 2 h and the resin was filtered off using Miracloth (Calbiochem). The resin was then washed twice with 10 ml distilled water and the compounds were re-extracted with 2x10 ml methanol. The methanol extract and the aqueous wash were both dried down and redissolved in 1 ml of 50:50 (v/v) methanol or 1 ml distilled water, respectively. The aqueous wash and the culture broth after extraction were tested for antimicrobial activity in the well diffusion assay as previously described, the methanol extract was tested as follows; 25 µl of the redissolved extract were applied to Antibiotic Assay Discs (6mm, Whatman) and the discs were left to dry in the fume hood. When fully dry, discs were applied to LB (bacterial test strains) or YPD (fungal test strains) agar plates prepared in the same way as for the well diffusion assay (except that no wells were punched into the plates). Zones of inhibitions were recorded after growth for 12-24 h at 28°C (fungal test strains) or 37°C (bacterial test strains).

A scale up was performed for selected isolates. First, a seed culture was grown and tested every second day for antimicrobial activity in the well diffusion assay. Once the result was positive, 800 ml of the malt extract medium in a 2 l Erlenmeyer flask was inoculated with 16 ml of the seed culture. The scale up culture was tested regularly for antimicrobial activity in the well diffusion assay. Once the result was positive a cell free supernatant was obtained either by centrifugation or by sterile filtration (see above) and 40 g/l XAD-16 resin were added to the clarified culture broth. After shaking for 12 h at 28°C and 180 rpm, the resin was filtered off, washed twice with distilled water and extracted twice with methanol. The extract, wash and supernatant after extraction were then tested as describe above.

Isolation of genomic DNA from fungal isolates and PCR amplification of 18S-rRNA genes

The isolation of genomic DNA from fungal isolates was performed using the Nucleo Spin Plant II kit (Machery Nagel) and the support protocol for the extraction of fungal DNA. The DNA obtained was quantified using a NanoDrop2000 (Thermo Scientific). The quality of the DNA was checked on a 1% agarose gel. For amplification of the 18S-rRNA gene the universal eukaryotic 18S-rRNA primer pair EukA/ EukB (Díez et al., 2001) was used generating a PCR product of approximately 1.5 kb. The following PCR conditions were used: initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 2 min, and a final elongation step of 72°C for 10 min. The reaction mixture (30 µl) contained: 3 µl 10x buffer (Fermentas), 3 µl dNTPs (2 mmol/l each; Fermentas), 1.5 µl EukA forward primer (10 µmol/l; Sigma-Aldrich), 1.5 µl EukB reverse primer (10 µmol/l; Sigma-Aldrich), 0.15 µl TAQ-polymerase (DreamTaq™ DNA polymerase, Fermentas, 5 U/µl), 17.85-19.85 µl molecular biology grade water and 1-3 µl DNA (10-100 ng per reaction). The PCR products were analysed by agarose gel electrophoresis (1% agarose gel)**.**

Sequencing and phylogenetic analysis of 18S-rRNA gene products

The 18S-rRNA gene PCR products were purified and partially sequenced using primer EF4 (Smit et al., 1999) or EF60F (Weber et al., 2009). The sequencing was carried out by Macrogen, Korea. The partial 18S-rRNA gene sequences were manually checked for quality using Finch TV (http://www.geospiza.com/Products/finchtv.shtml) and then grouped into Operational Taxonomic Units (OTUs) based on 98.5 % sequence similarity with PreGap (Staden, 1996). Representatives of each OTU were aligned with neighbouring sequences obtained from Arb-Silva (http://www.arb-silva.de/aligner/). For isolates which formed deep branches in the phylogenetic tree, closest BLAST hit sequences (Altschul et al., 1990) were included in the analysis. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood was presented, while the percentage of trees in which the associated taxa clustered together is shown next to the branches (values below 50 deleted). Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of

common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 58 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 413 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

Analysis of secondary metabolite genes

DNA was extracted as described above and PCRs were carried out employing several primers targeting NRPS- and PKS-genes (Table 3.1). The PCR reactions were carried out as previously described (Bingle et al., 1999; Johnson et al., 2007; Neilan et al., 1999; Nicholson et al., 2001).

PCRs were analysed on 1% agarose gels and products were purified using QIAquick PCR purification kit (Qiagen) as described in their manual. Purified PCR-products were analysed on a NanoDrop ND-1000 (Thermo Scientific) and by agarose gel electrophoresis. Purified PCR products were cloned using TOPO® TA Cloning® Kit (Invitrogen) adhering to the suppliers manual. TOP10 chemically competent cells were used. The picked clones were transferred into storage medium (Table 3.2; supplemented with 50 µg/ml kanamycin, grown at 37˚C, 200 rpm over night and stored at -80˚C). Clones were analysed by PCR using primers M13 forward (-20) (5´-GTAAAACGACGGCCAG-3´) and M13 reverse (5´-CAGGAAACAGCTAT GAC-3´). The following PCR conditions were used: initial denaturation at 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 75 s and a final elongation step of 72°C for 10 min. The reaction mixture (30 µl) contained: 3 µl 10x buffer (Fermentas), 3 µl dNTPs (2 mmol/l each; Fermentas), 1.5 µl M13 forward primer (-20) (10 µmol/l; Sigma-Aldrich), 1.5 µl M13 reverse primer (10 µmol/l; Sigma-Aldrich), 0.15 µl TAQ-polymerase (DreamTaq™ DNA polymerase, Fermentas, 5 U/µl), 17.85 µl molecular biology grade water and 3 µl broth of the stored culture. The PCR products were analysed by agarose gel electrophoresis (1% agarose gel)**.** Clones with the correct insert size (~1 kb) were sent to BeckmanCoulter Genomics for sequencing with their M13-forward primer. The PCR-reactions were purified by BeckmanCoulter Genomics.

Table 3.1: Primer-pairs used for the amplification of PKS- and NRPS-genes in the genome of isolate W9F6

Primer	pair	sequence (5'-3')	Product size	Target	Reference
LC1	A	GATCCIAGITTTTTTAATATG	720 bp	wA-type PKS	(Bingle et al., 1999)
LC2c		GTICCIGTICCGTGCATTTC			
LC3	B	GCIGAACAAATGGATCCICA	680 bp	6-MSAS-type PKS	
LC5c		GTIGAIGTIGCGTGIGCTTC			
KS3	C	TTTGATGCIGCITTTTTTAA	~745 bp	PKS	(Nicholson et al., 2001)
KS4c		ATGATTIGGCATIGTIATICC			
MTF2	D	GCNGG(C/T)GG(C/T)GCNTA(C/T)GTNCC	~1,000 bp	NRPS	(Neilan et al., 1999)
MTR		CCNCG(AGT)AT(TC)TTNAC			
RJ017-F	E	TAYGGNCCNACNGA	250-350 bp	NRPS	(Johnson et al., 2007)
RJ017-R		ARRTCNCCNGTYTTRTA			

The primer pairs were chosen in order to target different regions of the NRPS and PKS genes in an attempt to increase the chances of obtaining a PCR product and to maximise the diversity of obtained gene sequences.

Table 3.2: Recipe for storage medium

Storage Medium	
Bacto-tryptone	**10 g**
Yeast Extract	**5 g**
NaCl	**5 g**
K_2HPO_4	**6.3 g**
KH_2PO_4	**1.8 g**
Na_3-citrate	**0.45 g**
$(NH_4)_2SO_4$	**0.9 g**
$MgSO_4.7H_2O$	**0.09 g**
Glycerol	**44 g**
Made up to 1 litre with water	
Autoclave at 121℃ x 15 minutes	

RESULTS

Isolation of Fungi

In total, 71 fungal isolates were obtained from the 12 different sponges (Table 3.3). Most isolates were obtained from *Axinella dissimilis* (W11; 13 isolates) and *Leucosolenia sp.* (W15; 12 isolates). These two sponges also yielded the most diverse isolates. The 13 isolates of *A. dissimilis* were grouped into 8 OTUs based on 98.5% sequence identity; while the 12 isolates from *Leucosolenia sp.* were grouped into 7 different OTUs. The least isolates were obtained from both *S. carnosus* samples (W13: 1 isolate and W14: 2 isolates) and from the sponge *Eurypon major* (1 isolate). Even though 5 isolates were obtained from *Suberites ficus* (J14) they all belonged to one OTU (order *Eurotiales*) (Table 3.3).

Antimicrobial activity – deferred antagonism assay

All isolates were tested twice in the deferred antagonism assay against three bacterial test strains (*E. coli*, *B. subtilis* and *S. aureus*). The results are shown in Table 3.5. A total of 46 isolates (65 % of all isolates) showed activity in at least one of the assays against at least one of the test strains employed. The results of the overlay assays were however quite inconsistent, i.e. the same zones of inhibitions was noted in both assays for only 11 isolates. Nonetheless, a high number of isolates inhibited growth of the test strains. The producers of antimicrobials according to the overlay assay belonged to most of the isolated orders of fungi. The most prolific producers were isolates from the orders *Pleosporales* (63% of isolates showed activity against at least one test strain in at least one assay), *Hypocreales* (69%) and *Eurotiales* (78%). They also often inhibited the growth of two or all of the employed test strains. Even though 50% of the ten yeasts antagonized bacterial growth, the expression of the antibiotic activity was often not reliable and/or only directed against one test strain.

Table 3.3: Sponge samples, the number of isolates obtained from each and the number of OTUs at 98.5% sequence identity

Sponge	# of isolates	# of OTUs (98.5%)
***Polymastia boletiformis* (J1)**	6	2
***Stelligera stuposa* (J2)**	5	4
***Eurypon major* (J9)**	1	1
***Suberites ficus* (J14)**	5	1
***Tethya citrina* (W1)**	2	2
***Cliona Celata* (W2)**	5	4
***Amphilectus fucorum* (W4)**	10	6
***Raspailia ramosa* (W9)**	9	6
***Axinella dissimilis* (W11)**	13	8
***Suberites carnosus* (W13)**	1	1
***Suberites carnosus* (W14)**	2	2
***Leucosolenia sp.* (W15)**	12	7

Most isolates were classified as *Ascomycota* (68 isolates) according to BLAST and molecular phylogeny. Only 3 isolates belonged to the phylum *Basidiomycota*. Most of the *Ascomycota* were of the orders *Eurotiales* (23 isolates), *Pleosporales* (16) and *Hypocreales* (13). Based on 98.5% sequence similarity, a total of 22 OTUs were isolated. Most diverse were the isolates of the order *Hypocreales* with 7 OTUs isolated followed by isolates from the order *Pleosporales* (5 OTUs). All 23 isolates of the order *Eurotiales* were grouped into one OTU based on 98.5 % sequence similarity. 10 isolates were identified as yeasts and clustered into 6 OTUs. A summary of this analysis can be found in Table 3.4. BLAST results for all isolates are given in Table 3.5 and a phylogenetic tree is shown in Figure 3.1

Table 3.4: Summary of the classification of all fungal isolates.

Phylum	Order/ class	# of isolates	# of OTUs
	Hypocreales	13	7
	Leotiomycetes - incertae sedis	1	1
	Sordariomyces - incertae sedis	1	1
Ascomycota	Pleosporales	16	5
	Capnodiales	8	2
	Eurotiales	23	1
	Saccharomycetales	6	2
	Cystofilobasidiales	1	1
Basidiomycota	Erythrobasidiales	1	1
	Sporidobolales	1	1

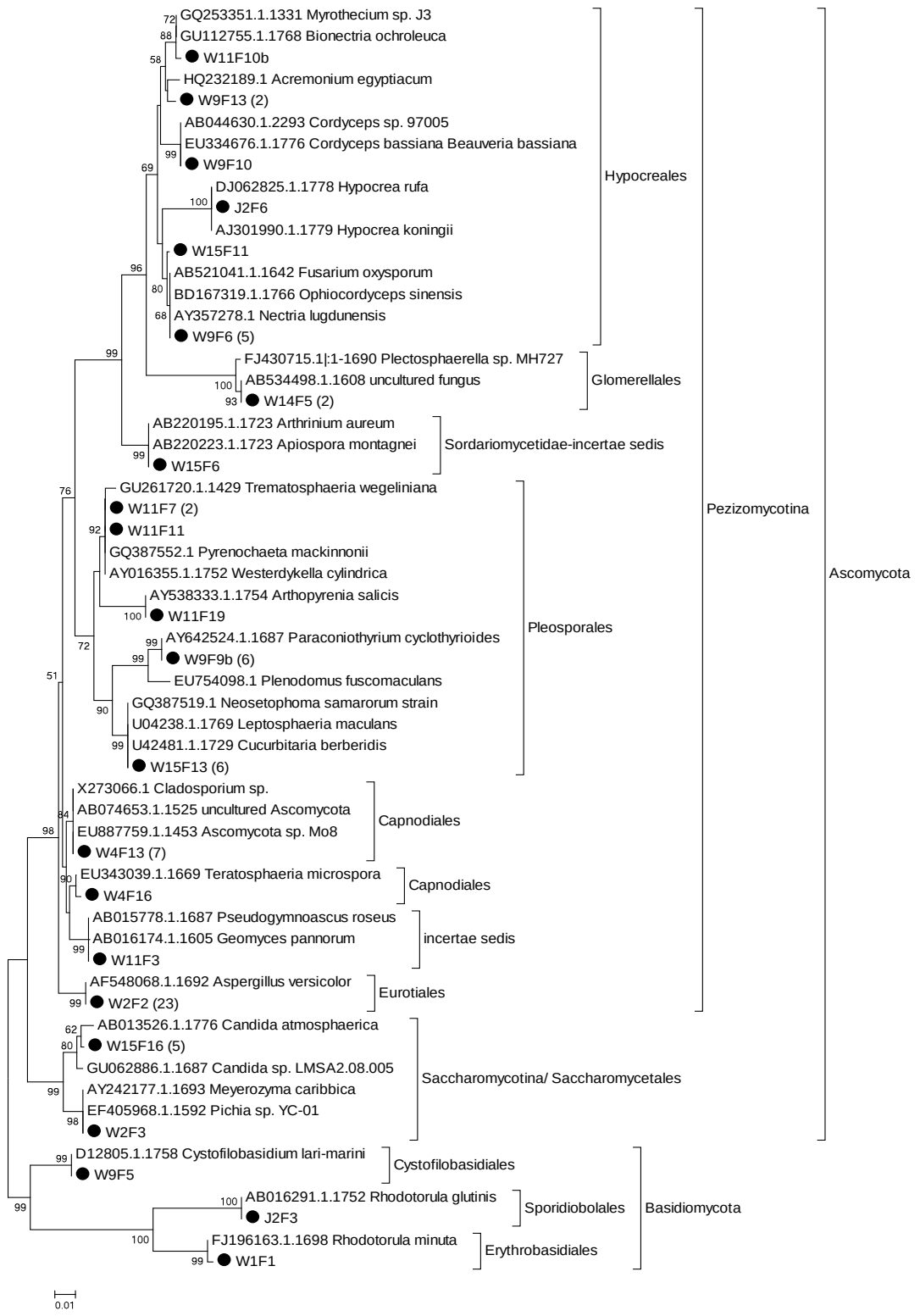


Figure 3.1: Phylogenetic tree of all fungal isolates from all sponges

Reference sequences of each OTU at 98.5% sequence identity were used together with SILVA sequences obtained as described in Materials and Methods to construct the phylogenetic tree. The treeing method was maximum likelihood. A neighbour joining tree was also constructed to confirm the placing of each OTU. Bootstrap values of 50 or higher are given next to each branch. ● isolates from this study; numbers in brackets = amount of isolates represented according to 98.5 % sequence similarity.

Table 3.5: Phylogenetic affiliation and antibiotic activities for all isolates.

0TU	Sample	Accession no.	Closest BLAST hit	%	EC	BS	SA
1	J1F1	FJ215704.1	*Cucurbitaria* sp.	100	v	+	+
1	J2F1	GU934572.1	*Didymella cucurbitacearum*	100	-	-	-
1	W15F13	DQ898287.1	*Pyrenochaeta nobilis*	99	v	+	+
1	W15F17	GU205238.1	*Fenestella fenestrata*	100	v	v	+
1	W15F4	FJ215704.1	*Cucurbitaria* sp.	100	-	-	-
1	W2F4	HQ696111.1	*Phoma* sp.	99	-	v	v
2	W11F17	AB665311.1	*Paraphaeosphaeria* sp.	99	-	v	v
2	W11F4	JN397390.1	*Pleosporales* sp.	99	-	-	-
2	W1F2	EU754098.1	*Plenodomus fuscomaculans*	99	v	+	-
2	W9F11	JN397390.1	*Pleosporales* sp.	99	v	v	-
2	W9F8	AB665311.1	*Paraphaeosphaeria* sp.	100	-	-	-
2	W9F9B	AB665311.1	*Paraphaeosphaeria* sp.	100	+	-	v
3	W13F1(Y)	AB013527.1	*Candida atlantica*	99	+	-	-
3	W15F16(Y)	AB013527.1	*Candida atlantica*	99	-	-	-
3	W15F9(Y)	AB013527.1	*Candida atlantica*	99	-	-	-
3	W2F1(Y)	AB013527.1	*Candida atlantica*	99	-	-	-
3	W4F3(Y)	AB013527.1	*Candida atlantica*	99	-	-	-
4	J14F1	HQ882177.1	*Penicillium chrysogenum*	100	+	-	+
4	J14F3	HQ263114.1	*Eladia saccula*	100	+	-	-
4	J14F5	GU733359.1	*Penicillium chrysogenum*	99	+	+	+
4	J14F8	HQ263114.1	*Eladia saccula*	99	v	-	-
4	J14F9	HM161749.1	*Penicillium* sp.	100	+	-	+
4	J1F2	HQ263114.1	*Eladia saccula*	99	-	-	-
4	J1F3	GU733359.1	*Penicillium chrysogenum*	99	v	-	-
4	J1F5	FJ717699.1	*Penicillium brevicompactum*	99	v	+	+
4	J1F6	HQ263114.1	*Eladia saccula*	99	v	v	-
4	J1F7	HQ882177.1	*Penicillium chrysogenum*	99	v	v	+
4	J2F8	HQ263114.1	*Eladia saccula*	99	-	-	-
4	J2F9	FJ717699.1	*Penicillium brevicompactum*	100	-	v	-
4	J9F1	HQ263114.1	*Eladia saccula*	99	v	-	-
4	W11F13A	HQ263114.1	*Eladia saccula*	99	v	-	+
4	W11F15	FJ716250.1	*Chromocleista* sp.	100	-	-	-
4	W14F4	HM161749.1	*Penicillium* sp.	100	-	-	-
4	W15F2A	AB293968.1	*Penicillium janthinellum*	99	-	-	-
4	W15F8	FJ717699.1	*Penicillium brevicompactum*	99	v	v	+
4	W2F2	AF548069.1	*Aspergillus versicolor*	99	-	v	-
4	W2F8	FJ717699.1	*Penicillium brevicompactum*	99	+	+	v
4	W4F18	FJ717699.1	*Penicillium brevicompactum*	100	+	+	-
4	W4F8	HQ263114.1	*Eladia saccula*	100	+	-	v
4	W9F14	GU733359.1	*Penicillium chrysogenum*	99	+	+	+
5	W11F13B	EU263604.1	*Cladosporium cladosporioides*	99	-	v	+
5	W15F1	AB521051.1	*Cladosporium cladosporioides*	100	-	-	-
5	W4F13	HQ696094.1	*Cladosporium* sp.	100	-	-	-

0TU	Sample	Accession no.	Closest BLAST hit	%	EC	BS	SA
5	W4F14	EU263604.1	*Cladosporium cladosporioides*	99	-	-	-
5	W4F1B	EU263604.1	*Cladosporium cladosporioides*	99	v	-	-
5	W4F2	FJ716247.1	*Cladosporium* sp.	99	-	v	-
5	W9F7	AB521051.1	*Cladosporium cladosporioides*	99	-	-	v
6	W11F12	AF141950.1	*Fusarium merismoides*	99	-	v	-
6	W15F15	HQ680636.1	*Cordyceps bifusispora*	100	-	v	-
6	W4F10	EF468961.1	*Metacordyceps liangshanensis*	99	-	-	-
6	W4F6	HQ680636.1	*Cordyceps bifusispora*	99	+	+	-
6	W9F6	HQ696108.1	*Fusarium* sp.	100	-	-	-
7	W11F14	JN939687.1	*Nectria pseudotrichia*	99	-	-	-
7	W9F13	HQ232189.1	*Acremonium egyptiacum*	97	v	v	-
8	W11F6	U42488.1	*Westerdykella dispersa*	99	-	-	+
8	W11F7	U42488.1	*Westerdykella dispersa*	100	v	-	-
9	W14F5	FJ430715.1	*Plectosphaerella* sp.	99	v	+	+
9	W15F10	FJ430715.1	*Plectosphaerella* sp.	99	-	v	-
10	J2F3(Y)	DQ854820.1	*Rhodosporidium diobovatum*	99	v	-	-
11	J2F6	HM152770.1	*Hypocrea koningii*	100	v	-	-
12	W1F1(Y)	FJ517758.1	*Rhodotorula* sp.	99	-	-	-
13	W2F3	JN941099.1	*Meyerozyma guilliermondii*	99	+	-	-
14	W4F16(Y)	GU323998.1	*Dothideomycetes* sp.	99	v	-	-
15	W9F5(Y)	DQ645524.1	*Cystofilobas. infirmominiatum*	99	v	v	-
16	W9F10	JF429899.1	*Isaria farinosa*	99	+	+	-
17	W11F3	JN657522.1	*Geomyces* sp.	99	-	-	-
18	W11F10B	GU112755.1	*Bionectria ochroleuca*	99	+	v	+
19	W11F11	GQ387552.1	*Pyrenochaeta mackinnonii*	95	-	-	-
20	W11F19	AB524482.1	*Roussoella pustulans*	99	-	-	-
21	W15F6	HQ696088.1	*Arthrinium* sp.	99	-	-	-
22	W15F11	AY357278.1	*Nectria lugdunensis*	99	-	-	-

The isolates are sorted according to their clustering into one of the 22 OTUs at 98.5 % sequence identity. Closest BLAST hits are shown. Antimicrobial activity was determined by deferred antagonism assay: + = zone of inhibition detected, - = no zone of inhibition detected, v = zone of inhibition only detected in one of the two carried out deferred antagonism assays; EC = *E. coli*, BS = *B. subtilis*, SA = *S. aureus*. Isolates identified as yeasts are indicated (Y).

Antimicrobial activity – well diffusion assay and antibiotic discs

Mainly based on inhibition in the deferred antagonism assay and partially based on phylogeny, isolates were subsequently chosen for further antimicrobial tests. Firstly, 32 isolates were grown in liquid medium and the cell free supernatant was tested for antimicrobial activity in a well diffusion assay against all bacterial and fungal test strains as previously described in Material & Methods. Isolates showing activity in this assay were subsequently grown up again and the culture broths were extracted with the non-ionic, hydrophobic, cross-linked polymer resin XAD-16. The results for both assays are shown in Table 3.6. Antibacterial activities were strongest and had the broadest spectrum for isolates from 3 OTUs belonging to the order *Pleosporales*, *Hypocreales* and *Eurotiales*. The extracts from isolates W15F14, W15F17, W15F4 which belonged to one OTU at 100 % sequence similarity, all strongly inhibited the growth of most of the fungal test strains. Contrastingly the extracts of J2F1, which belonged to the same OTU at 98.5 % sequence similarity, but not at 99 %, did not show any antibiotic activity. Isolates from another OTU, closely related to *Penicillium* and *Eladia* species (Order *Eurotiales*) also often showed antibiotic activity in the well diffusion assay and the bioactive compounds were often readily extractable with the applied method. The last group of isolates which showed strong antimicrobial activity in both supernatant and extract belonged to an OTU of the order *Hypocreales*. This OTU can be split into three groups due to colony appearance and sequence similarity. Isolates W9F6 and W11F12 not only have a different colony appearance but they also form two different OTUs at 99% sequence similarity. Isolates W4F6, W4F10 and W15F15 are morphologically similar. Isolates W9F6 (closest BLAST hit: *Fusarium sp.*) and W15F15 (closest BLAST hit: *Cordyceps bifusispora*) both show strong activity against a range of yeast/ fungal test strains and W9F6 also inhibited the growth of the Gram positive bacteria *B. subtilis* and *S. aureus*. Other isolates either did not grow in shake cultures, did not show antimicrobial activity in the well diffusion assay or the activity was not extractable with the method employed.

Secondary metabolite genes in the genome of W9F6

Due to its broad range of activities against Gram positive bacteria and fungi, the isolate W9F6 was analysed for secondary metabolite genes using a PCR based

approach. Two separate DNA extractions were performed employing 117 mg and 50 mg of fungal mycelium (Table 3.7). DNA quality was in each case confirmed by agarose gel electrophoresis.

Table 3.6: Antimicrobial activities of raw and extracted culture broths.

OTU	Sample	Closest BLAST hit	EC	BS	SA	CA	CG	SC	KM	AF
1	J1F1	*Cucurbitaria* sp.	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
1	J2F1	*Didymella cucurbitacearum*	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
1	W15F13	*Pyrenochaeta nobilis*	-/-	-/-	-/-	+/+	+/+	+/+	+/+	+/+
1	W15F17	*Fenestella fenestrata*	-/-	-/-	-/-	+/+	+/+	-/+	+/+	+/**nt**
1	W15F4	*Cucurbitaria* sp.	-/-	-/-	-/-	+/+	+/+	-	+/+	**(+)**
1	W2F4	*Phoma* sp.	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
2	W11F17	*Paraphaeosphaeria* sp.	not grown in shaking culture							
2	W1F2	*Plenodomus fuscomaculans*	-/-	+/-	(+)/-	+/-	+/-	-/-	(+)/-	-/-
2	W9F11	*Pleosporales* sp.	not grown in shaking culture							
2	W9F9B	*Paraphaeosphaeria* sp.	not grown in shaking culture							
4	J14F1	*Penicillium chrysogenum*	-/-	+/+	+/+	-/-	-/-	-/-	-/-	(+)/-
4	J14F5	*Penicillium chrysogenum*	-/na	+/na	+/na	-/na	-/na	-/na	-/na	-/na
4	J14F9	*Penicillium* sp.	-/-	-/-	-/-	-/-	-/-	(+)/-	-/-	-/-
4	J1F5	*Penicillium brevicompactum*	-/-	+/v	+/-	+/v	-/-	-/-	-/-	-/-
4	J1F7	*Penicillium chrysogenum*	-	-	-	-	-	-	-	-
4	J2F9	*Penicillium brevicompactum*	-/-	+/-	+/-	+/-	(+)/-	-/-	(+)/-	(+)/-
4	W11F13A	*Eladia saccula*	not grown in shaking culture							
4	W15F8	*Penicillium brevicompactum*	-	-	-	-	-	-	-	-
4	W2F8	*Penicillium brevicompactum*	-/-	+/+	-/-	+/+	-/-	+/(+)	+/+	+/+
4	W4F18	*Penicillium brevicompactum*	not grown in shaking culture							
4	W4F8	*Eladia saccula*	not grown in shaking culture							
4	W9F14	*Penicillium chrysogenum*	-/-	+/+	+/+	-/-	-/-	-/-	-/-	-/-
5	W11F13B	*Cladosporium cladosporioides*	not grown in shaking culture							
6	W11F12	*Fusarium merismoides*	-/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-
6	W15F15	*Cordyceps bifusispora*	-/-	-/-	-/-	+/+	(+)/+	(+)/-	+/+	nt/(+)
6	W4F10	*Metacordyceps liangshanensis*	-	-	-	-	-	-	-	-
6	W4F6	*Cordyceps bifusispora*	-/-	-/-	-/-	-/-	(+)/-	-/-	(+)/-	-/-
6	W9F6	*Fusarium* sp.	-/-	+/+	-/+	+/+	+/+	(+)/+	+/+	nt/nt
7	W9F13	*Acremonium egyptiacum*	-/-	+/-	+/-	-/-	-/-	-/-	-/-	+/-
9	W14F5	*Plectosphaerella* sp.	-	-	-	-	-	-	-	-
15	W9F5(Y)	*Cystofilobas. infirmominiatum*	not grown in shaking culture							
18	W11F10B	*Bionectria ochroleuca*	not grown in shaking culture							

Result given before slash stands for the well diffusion assay of untreated culture broth, result after slash for disc diffusion assay using extracts. The isolates are sorted for simple distinction between OTUs at 98.5% sequence identity. + = zone of inhibition detected, - = no zone of inhibition detected, v = in repeated experiment the result was not identical, nt = not tested; EC = *E. coli*, BS = *B. subtilis*, SA = *S. aureus*, CA = *C. albicans*, CG = *C. glabrata*, SC = *S. cerevisiae*, KM = *K. marxianus*, AF = *A. fumigatus.*

Table 3.7: DNA concentrations of two genomic DNA extractions from W9F6.

Sample ID	ng/ul	A260	A280	260/280	260/230	Constant
W9F6 1	1318.99	26.38	12.255	2.15	2.29	50
W9F6 2	135.87	2.717	1.322	2.06	2.05	50

W9F6/1: 117mg mycelium extracted, W9F6/2: 50 mg mycelium extracted

Both DNA extractions were used in PCR reactions targeting NRPS and PKS secondary metabolite genes. Different primer pairs were used as shown in Table 3.1. The primer pairs targeted wA-type (Lc1/2c; non-reducing KS domain), MSAS-type PKS genes (LC3/5c; partially-reducing KS domain) and highly-reducing KS-domains (KS3/4c). The NRPS-directed primer pairs targeted different regions of the adenylation domain, present in all NRPS-genes (RJ017-F/ RJo17-R and MTF2/MTR).

Products were obtained for primer pair D (Figure 3.2). Although nonspecific products of other than the expected size were also amplified (compare with Table 3.1) the PCR amplicons obtained with primer pair D (reaction 1; see Figure 3.2) were purified and used to prepare a clone library as described in Materials and Methods. In total, 96 clones were picked from the library and an M13-PCR was performed to check the insert sizes of the clones. In total, 16 reactions had a size which would be consistent with the correct size for a NRPS-gene-insert (~1 kb) and these clones were subsequently sequenced by Beckman-Coulter as described in Materials and Methods.

The sequences obtained were checked for quality using Finch-TV and edited sequences were analysed using blastx (Table 3.8). In total, 13 sequences were most closely related to NRPS-genes, 2 sequences were related to other genes and 1 sequence did not pass the quality check.

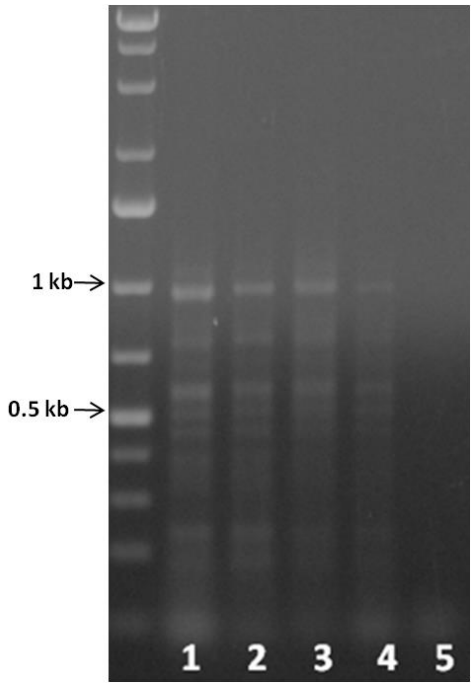


Figure 3.2: Results of NRPS PCR with primer set D

Agarose gel (1%) of PCR-reactions employing primer pair D. Many of the bands are probably due to unspecific products because the expected product size was ~1 kb. However, the band at ~1kb was the most intense one and therefore the PCR product 1 was cloned into a sequencing vector and analysed for the presence of NRPS related sequences (Table 3.8) **1**: PCR with DNA extract W9F6 1 (see Table 3.7); **2:** PCR with extract W9F6 1 diluted 1:10; **3:** PCR with DNA extract W9F6 2; **4:** PCR with DNA extract W9F6 1 diluted 1:10; **5:** Negative control; The marker size is indicated left of the gel picture.

Table 3.8: Blastx result of DNA-sequences from W9F6

Clone	Accession	Closest blastx hit	Identity
W9F6_27	EFY90995.1	peptide synthetase [Metarhizium acridum CQMa 102]	54%
W9F6_68	EFY90995.1	peptide synthetase [Metarhizium acridum CQMa 102]	54%
W9F6_72	EFY90995.1	peptide synthetase [Metarhizium acridum CQMa 102]	54%
W9F6_88	EFY90995.1	peptide synthetase [Metarhizium acridum CQMa 102]	53%
W9F6_90	EFY90995.1	peptide synthetase [Metarhizium acridum CQMa 102]	54%
W9F6_25	EFZ02057.1	peptide synthetase [Metarhizium anisopliae ARSEF 23]	52%
W9F6_96	EFZ02057.1	peptide synthetase [Metarhizium anisopliae ARSEF 23]	52%
W9F6_22	XP_391470.1	hypothetical protein FG11294.1 [Gibberella zeae PH-1]	84%
W9F6_34	XP_391470.1	hypothetical protein FG11294.1 [Gibberella zeae PH-1]	83%
W9F6_77	XP_391470.1	hypothetical protein FG11294.1 [Gibberella zeae PH-1]	83%
W9F6_79	EKJ71405.1	NPS12 [Fusarium pseudograminearum CS3096]	83%
W9F6_55	XP_002843358.1	peptide synthetase [Arthroderma otae CBS 113480]	53%
W9F6_59	BAL27709.1	NRPS related to anti-diabetes peptide [Aspergillus oryzae]	52%

Sequences from 13 clones which were most closely related to NRPS genes. The sequences are sorted according to their closest BLAST-neighbour and grouped according to their clustering in a phylogenetic tree (Figure 3.3).

A phylogenetic tree was also constructed which included the sequences from W9F6 and their closest BLAST-neighbours. Two sequences (W9F6-55 and W9F6-59) did not have any overlap in the alignment either with each other or with the other W9F6-derived sequences thus they were not included in the phylogenetic tree (Figure 3.3). The BLAST search and the phylogenetic tree imply the presence of at least 5 NRPS genes in the genome of W9F6. One of the clusters is most closely related to a NRPS gene previously detected in *Fusarium pseudograminearum*, an important wheat and barley pathogen in Australia (Gardiner et al., 2012). Two clusters are most closely related to peptide synthetase genes detected in the genome sequences of two *Metarhizium* species (Gao et al., 2011), while one sequence was most closely related to a peptide synthetase in an *Athroderma* species and W9F6-59 was most closely related to a NRPS-gene which was related to an anti-diabetes peptide in *Aspergillus oryzae* (not published).

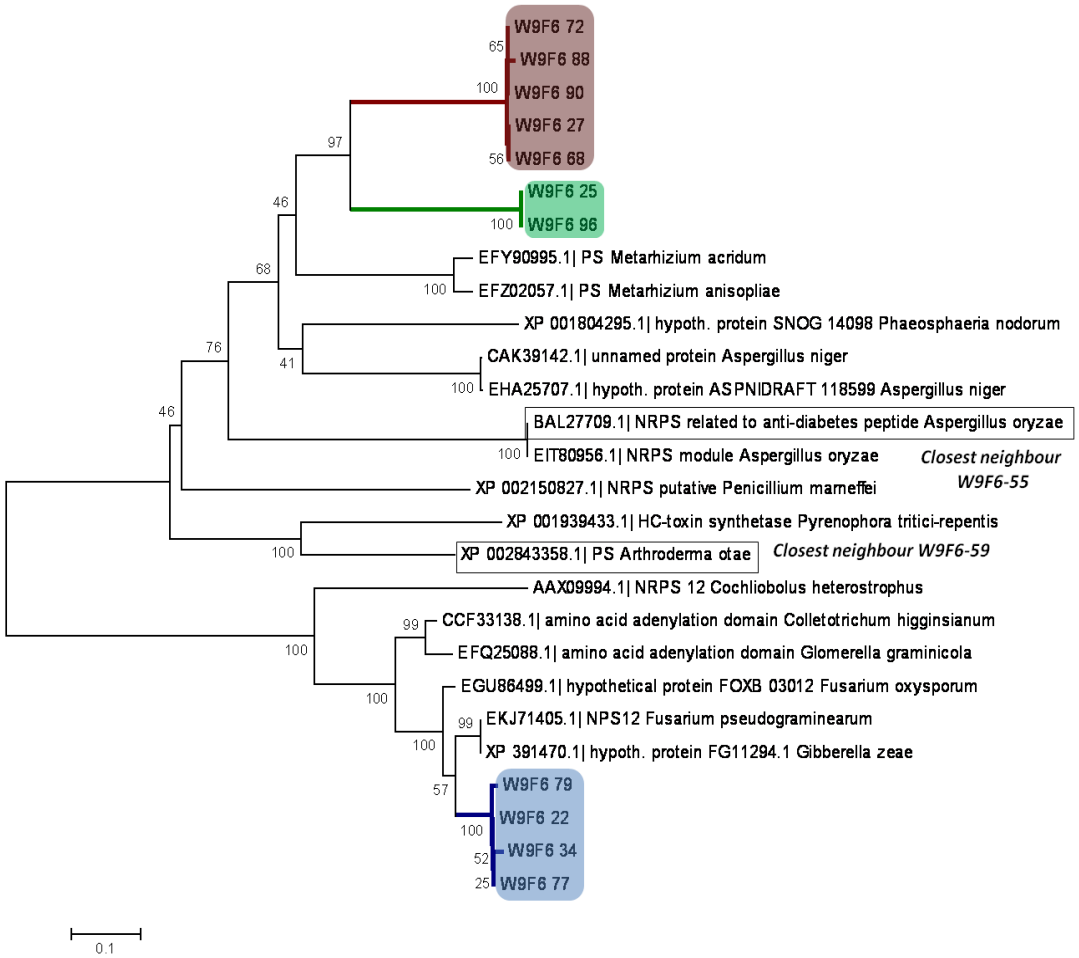


Figure 3.3: Neighbour-Joining tree of NRPS sequences obtained from W9F6

Included in the tree were predicted amino acid sequences derived from the DNA-sequences of each clone and their closest BLAST neighbours. Three groups of sequences are visible (highlighted in red, green and blue) indicating the presence of at least 3 NRPS-genes or gene-clusters in the genome of W9F6. Clones W9F6-55 and W9F6-59 did not overlap with the other W9F6-derived sequences when aligned and were not included in the tree. Their closest BLAST-neighbours are highlight with rectangles. The placing implies another 2 NRPS genes in the genome of W9F6.

DISCUSSION

Diversity

The fungal isolates recovered from the different sponges were very diverse with 22 OTUs being found at a sequence identity of 98.5 %. It is very likely that even more diverse isolates would have been obtained from the different sponge samples if different isolation media had been used, such as those employed by Ding and co-workers when culturing fungi associated with marine sponges *Clathrina luteoculcitella* and *Holoxea* sp. in the South China Sea (Ding et al., 2011). Due to the small dataset (a maximum of 13 isolates obtained for one sponge, only 1 isolate obtained for two of the sponges) a relevant comparison of the culturable diversity of each sponge is not possible. In order to compare the unculturable fungal diversity of each of the sponge samples, 3 sponges were chosen for the preparation of an 18S-rRNA-amplicon clone library but the experiment was not successful due to high levels of contamination of the clone libraries with sponge derived 18S-rRNA sequences. Similar problems have previously been observed in a study on Hawaiian sponges (Gao et al., 2008). The use of different primer sets and even the design of primers differentiating between sponge- and fungal 18S-rRNA did not improve the results, thus further insights into the fungal associates of the here studied sponges were not obtained.

Isolates of the orders *Pleosporales*, *Hypocreales*, *Eurotiales*, *Capnodiales* and *Saccharomycetales* have been routinely and abundantly isolated from sponges from different countries (Baker et al., 2009; Ding et al., 2011; Gao et al., 2008; Höller et al., 2000; Liu et al., 2010; Wiese et al., 2011). In contrast, isolates of the orders *Cystofilobasidiales*, *Sporidiobolales*, *Erythrobasidiales* and *Glomerellales* have

rarely been reported from marine sponges. Two isolates, W15F6 and W11F3, were most closely related to *Pseudogymnoascus* and *Geomyces* (W11F3) and *Apiospora* and *Arthrinium* (W15F6), respectively. These genera are *incertae sedis* and related isolates have only rarely been isolated from sponges. The available evidence in the literature does not support the presence of a large numbers of sponge associated fungi as has been shown for bacteria. Fungi are thought to be recognized by sponges via d-glucan carbohydrates on fungal surfaces (Perović-Ottstadt et al., 2004). Other evidence of sponge-fungi symbioses include; a possibly fungal intron observed in sponge mitochondria (Rot et al., 2006); a yeast shown to be vertically transmitted in a sponge (Maldonado et al., 2005) and; identification of some sponge specific clusters of fungus-derived 18S-rRNA sequences (Simister et al., 2012). On the other hand fungi isolated from marine sponges are often from genera which are also frequently isolated from terrestrial environments and molecular studies on sponge derived fungal genomic DNA appears to imply a low diversity and abundance of fungal 18S-r-RNA (Baker et al., 2009; Gao et al., 2008). Thus, the currently available evidence suggests that there may be a symbiotic relationship between sponges and a limited set of fungi but that the majority of fungi in sponges are more than likely to be only transient organisms, potentially derived from terrestrial "wash off". The fungi which were isolated in this study were from genera previously isolated from terrestrial environments and are therefore likely to be mainly derived from "wash off". The sampling site Lough Hyne is a relatively secluded environment with only a small canal connecting it to the open sea. Additionally, it is surrounded by hills which are subject to frequent rain showers, hence terrestrial "wash off" is probably a very common phenomenon in Lough Hyne, and the water circulation is reduced due to its secluded character. Thus fungi isolated from Lough Hyne derived sponges might be even more enriched for terrestrial species. This could mean that Lough Hyne fungi are not such a good target for the isolation of marine fungi. On the other hand, an analysis and comparison of the fungal diversity in the surrounding terrestrial environment, the seawater and the sponge could give insights into the relationship between sponges and fungi. Not only could possibly marine or sponge specific species be identified by the presence or absence in the corresponding environment, but also a mode of selection of fungi by sponges could be investigated.

Bioactivity

A very high percentage of fungal isolates (65%) were shown to inhibit the growth of a variety of test strains in the initial overlay assay. Even though the assay did not give repeatable results nonetheless it did demonstrate the potential of the isolated fungi to produce bioactive secondary metabolites. Most of the bioactive isolates were from the orders *Pleosporales*, *Eurotiales* and *Hypocreales*. Some isolates from OTUs 1, 4 and 6 showed activity in all three applied bioactivity assays. Despite their clustering into an OTU at 98.5% sequence identity the isolates in OTU 1 (*Pleosporales*) showed different activities in the assays. A similar situation was observed for isolates of OTUs 4 (*Eurotiales*) and 6 (*Hypocreales*). The isolates of OTUs 1 and 6 were thus analysed without grouping into OTUs (Figure 3.4). OTU 1 could be divided into 2 different groups according to bioactivity, morphology and phylogeny/ classification based on the 18S-rRNA sequence. OTU 6 could be divided into 4 groups (Figure 3.4).

Secondary metabolite genes

Secondary metabolite genes in the genome of isolate W9F6 (closest BLAST-hit to a *Fusarium sp.*) were analysed using primer sets targeting various regions of PKS and NRPS genes. Since PKS and NRPS gene products are well known to be responsible for the production of bioactive secondary metabolites they are a good target for estimating the bioactivity potential of an isolate. Following the PCR one NRPS primer set gave a product, indicating the likely presence of NRPS genes in the genome of W9F6. The NRPS PCR product was analysed more closely by cloning it into a sequencing vector. 13 of the analysed clones comprised of sequences most closely related to NRPS genes (Table 3.8).

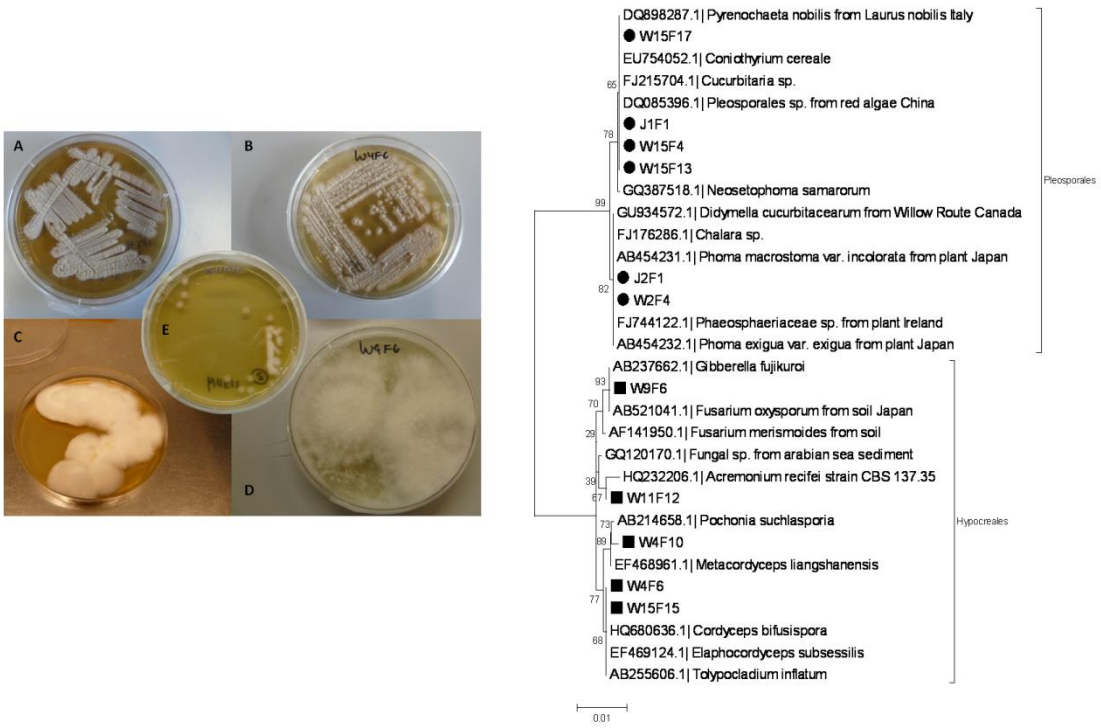


Figure 3.4: Phylogenetic and morphological characterisation of OTUs 1 and 6.

OTUs 1 (of the order *Pleosporales*) and 6 (*Hypocreales*) were analysed in more detail. Instead of only analysing a representative isolate for each OTU, all isolates were considered. For OTU 6 pictures of agar plate cultures on MEA for each isolate are shown (A-E). Isolates W15F15 (A) and W4F6 (B) had a similar morphology and appeared on the same branch in the phylogenetic tree, whereas isolates W4F10 (C), W9F6 (D) and W11F12 (E) all had differing morphologies and a different phylogeny. Isolates of OTU 1 appeared on two different *Pleosporales* branches in the phylogenetic tree. ●: isolates from OTU 1 (*Pleosporales*); ■ : Isolates from OTU 6 (*Hypocreales*)

Phylogenetic analyses revealed the presence of 5 different genes, most closely related to NRPS sequences from various fungi, including a *Fusarium sp.*, but also to *Metahrizium*, *Aspergillus* and *Arthroderma*. The sequence identity values (~50%) and the phylogenetic analysis indicated the likely presence of novel NRPS genes in the genome of W9F6, highlighting the potential of this fungus to produce novel bioactive secondary metabolites.

CONCLUSION

Fungal isolates from 12 Irish marine sponges have been identified based on their 18S-rRNA sequence. This is the first report of fungi being isolated and subsequently characterized from any of these sponge species. Additionally, a very high percentage of isolates were identified as being potential producers of bioactive substances. Many of the isolates belonged to orders which have commonly been isolated from marine sponges (*Pleosporales*, *Eurotiales* and *Hypocreales*) but some were identified as members of orders previously rarely encountered from marine sponges. The presence of secondary metabolite genes in the genome of isolate W9F6 highlighted the potential of this isolate for the production of novel secondary metabolites.

Overall, the fungi isolated from Irish marine sponges showed a very good potential for the production of secondary metabolites and should be analysed further in the future. Future research on the sponges could include the isolation of more fungi using different cultivation media as for example described in Baker et al., 2009 who isolated 80 fungi (19 phylotypes) from *Haliclona simulans* collected in Ireland. The fungi already obtained could also be analysed further using different screens for example for anticancer or antiviral activity. Isolates which did not show activity in either of the screens could be stimulated to produce bioactivity with epigenetic regulators (Brakhage, 2013; Cichewicz, 2010), by co cultivation (Brakhage, 2013) or by employing different production media (Bills et al., 2008; Wang et al., 2011). The isolates could also be analysed for their salt-preference in order to select isolates for further screens which are likely to be more adapted to the marine environment, and as such are more likely to be true marine fungi. Due to their bioactivity, particularly interesting targets among the obtained isolates were members of the OTUs 1, 4 and 6. It would also be interesting to analyse the isolates, both from OTUs 1, 4 and 6 and from other OTUs, which did not inhibit the growth of the test strains for secondary metabolite genes. Are such genes not present, or are they present and not expressed? If present, can they be expressed by the addition of e.g. epigenetic regulators or the change of growth conditions? Since fungi have previously been shown to harbour up to 50 secondary metabolite genes which are only expressed under certain conditions

(Brakhage, 2013), many possibilities for further Biodiscovery research exist even for the relatively small collection of fungi obtained in this study. For future studies it has to be noted that a de-replication of fungal isolates according to their sequence similarity alone is likely to underestimate the sampled diversity as has been shown e.g. for OTUs 1, 4 and 6. Thus the morphology of isolates in each OTU should also be considered. It is also noteworthy, that the initial bioactivity assay (the deferred antagonism assay) employed in this study did not produce reproducible results and that for example the isolate W9F6, which proved to be one of the most interesting isolates upon more detailed investigation, could have been overlooked based on the activity profile from this assay alone. Future work in this area should thus use both phylogenetic and morphological characterization in order to choose available isolates for further studies (as for example carried out in Wiese et al., 2011). The screening of isolates for novel secondary metabolite genes and their ability to preferentially grow under saline conditions could also improve the chances of obtaining novel and marine-specific fungi which may potentially produce novel bioactive compounds. Additionally, the deferred antagonism assay is (also based on the results obtained in Chapter 1) not recommended as a good tool to choose bacterial and fungal isolates for further tests. Due to the fungi's vast potential as producers of secondary metabolites but the demonstrated lack of actual production of many potentially bioactive compounds under "normal" conditions it is perhaps more prudent to in future study de-replicated samples in more depth employing e.g. different production media, the addition of epigenetic regulators and/or co-cultivation strategies.

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Chapter 4

Antimicrobial activity and chemical characterization of a secondary metabolite from a sponge derived *Micromonospora* sp.

ABSTRACT

A *Micromonospora* sp. was isolated from a *Haliclona simulans* sample collected in Irish waters. The isolate inhibited the growth of Gram positive bacterial test strains in three different antimicrobial assays. Several production media were subsequently employed to optimize the production of the antimicrobial compound(s) and a scale up culture with the best medium was extracted for its antimicrobial compounds. The purification of a yellow-orange compound with strong bioactivity against *B. subtilis*, *S. aureus* and *P. aeruginosa* was facilitated by bio-assay guided chromatography and the compound was tried to be characterized utilising LC-MS and NMR. By LC-MS two compounds of bioactivity were detected in the extracts, one with an m/z = 617.21 (positive mode) and one with an m/z = 619.21. Another compound with little or no bioactivity was also detected and had an m/z = 445.17. Compounds with similar m/z-values have previously been reported from *Streptomyces* and *Micromonospora* isolates. The compounds quinocycline and isoquinocycline A with an m/z = 619.21, the compounds quinocycline/kosinostatin and isoquinocline B with an m/z = 617.21 and aglycons of all derivatives with an m/z = 445.17. However, NMR studies of the isolated compounds did not confirm the presence of known (iso)quinocylines in the extracts derived from the here present *Micromonospora* isolate and thus the bioactive compounds could indeed be novel.

INTRODUCTION

The genus *Micromonospora* is a ubiquitous bacterial genus which has previously been isolated from soil environments such as from root nodule tissues of legumes and from marine environments including marine sponges (Alonso-Vega et al., 2012). Representatives have been found to possess a tremendous metabolic diversity. *Micromonospora* are known to produce a wide variety of bioactive substances, as well as possessing xylanolytic or cellulolytic activities; with members of the genus even being able to degrade natural rubber (Alonso-Vega et al., 2012). *Micromonospora* are also thought to play a role in nitrogen fixation (Hirsch and Valdés, 2010). In recent years their importance in soil ecology and their impact on plant growth and development has been recognized (Hirsch and Valdés, 2010). In particular it has been shown that *Micromonospora* can participate in biocontrol, in the promotion of plant growth, in root ecology and in the breakdown of plant cell wall material. By 2005 740 bioactive microbial metabolites produced by *Micromonospora spp.* were known (Bérdy, 2005). Examples of *Micromonospora*-derived bioactive substances include the aminoglycoside gentamicin and the antitumor anthraquinones lupinacidins A and B (Igarashi et al., 2007). More recently marine *Micromonospora* strains have also been evaluated for their potential as producers of bioactive substances. Because of the growing threat of antibiotic resistance in aquaculture, the EU and USA implemented bans or restricted the use of antibiotics in aqua-culture (Das et al., 2008). *Actinobacteria*, including *Micromonospora* are now being actively evaluated as potential probiotics in aquaculture (Das et al., 2008).

Sponges have been shown to be a rich source for bioactive *Micromonospora* strains, e.g. *Halichondria panacea* (Schneemann et al., 2010), furthermore sponge derived *Micromonospora* have been demonstrated to produce novel metabolites and have NRPS gene fragments in their genome. Bioactive metabolites isolated from marine *Micromonospora* include novel fluostatins (Zhang et al., 2012), the anti-oxidative metabolite diazepinomicin (Abdelmohsen et al., 2012) and kosinostatin (quinocyline B) (Furumai et al., 2002).

In this section the fermentation and isolation of antimicrobial compounds from the *Micromonospora* isolate FMC8 obtained from the marine sponge *Haliclona simulans* is reported.

MATERIALS AND METHODS

Sponge sampling

The *Haliclona simulans* sponge was collected by SCUBA as previously described (Kennedy et al., 2009) and frozen immediately after collection at -80˚C.

Isolation of microbes

The frozen sponge (~1g) was macerated by finely chopping with a razor blade, then re-suspended and vortexed in 10 mL sterile artificial seawater, followed by grinding with a Potter-Elvehjem Tissue Grinder. Serial dilutions of the sponge homogenate were then plated out onto SYP-SW-agar plates containing 50 µg/ml ciprofloxacin. The agar plates were incubated at 18°C for several weeks. *Micromonospora*-like colonies were picked and re-streaked several times on fresh SYP-SW-agar plates, with pure cultures of *Micromonospora* strains being subsequently obtained.

Deferred antagonism assays

These assays were performed as previously described (Kennedy et al., 2009). 5 µl of *Micromonospora* stock cultures were spotted onto SYP-SW agar plates and incubated at 18ºC until the culture was ~0.5-1 cm in diameter (for most isolates 3-5 days of incubation was sufficient). The cultures were then overlayed with 10 ml of soft agar seeded with a fresh culture of bacterial or fungal test strains. For bacterial test strains (*Escherichia coli* NCIMB 12210*, Bacillus subtilis* IA 40*, Staphylococcus aureus* NCIMB 9518) LB soft agar (1 l distilled water, 20 g LB broth, 5 g agar) was used.

The overlayed plates were incubated for ~12-24 h at 28ºC for the fungal test strains and 37ºC for the bacterial test strains. A zone of clearance in the overlay agar indicated the production of an antimicrobial compound by an isolate.

Well diffusion assays

Isolates which showed clear activity in the deferred antagonism assay were then subsequently assessed in the well diffusion assay. The isolates were grown up in 50 ml liquid SYP-SW in 250 ml Erlenmeyer flasks at 28ºC and 180 rpm for 14 days. Twice a week 1 ml of the culture was taken and centrifuged at 20,238 x g for 10 min in order to obtain a cell-free supernatant. Parallel, LB and YPD agar plates were prepared for testing of bacterial and fungal test strains, respectively. Plates were first seeded with 100 µl of a 1:50 dilution of an overnight culture of each test-strain that was spread out on the plates using sterile glass beads. For fungal test strains (*Candida albicans SC5314, Candida glabrata* CBS138, *Saccharomyces cerevisiae* BY4741, *Kluyveromyces marxianus* CBS86556 and *Aspergillus fumigatus* Af293) YPD soft agar (1 L distilled water, 10 g yeast extract, 20 g peptone, 20 g D-glucose, 7 g agar) was used. Holes were punched into the agar with a heat-sterilised cork borer (diameter 5 mm). Then 100 µl of the cell-free supernatant or SYP-SW broth as a negative control were applied to the wells. The plates were incubated for 12-24 h at 28ºC for fungal test strains and 37ºC for bacterial test strains. Zones of inhibition around the punched wells indicating antimicrobial activity of the according supernatant were recorded.

Fermentation and preparation of cell free supernatant (CFS) for extraction of antimicrobial compounds

For preparation of a seed culture, 50 ml of SYP-SW medium were inoculated with one colony of a freshly grown culture of the isolate and incubated for 7-10 days at 28°C and 200 rpm shaking. For the production of antibiotic activity the medium of choice was inoculated with 1/50 volume of the seed culture (e.g. 1 ml seed culture was added to 50 ml medium). Media used for the fermentation of FMC8 are given in Table 4.1.

Following between 8-10 days of growth, the culture was centrifuged at 6,000 g for 10 min and filtered through Miracloth (Calbiochem) to obtain a CFS. The pellet was either discarded (small scale fermentations of 50 ml culture) or extracted for antimicrobial compounds (large scale fermentations of 800 ml culture).

Table 4.1: Growth media

Substance	SGG-SW [g/l]	333-SW [g/l]	MMM-SW [g/l]	SYP-SW [g/l]
Glucose	10	5	10	-
Glycerol	10	-	-	-
Cornsteep Solids	2.5	-	-	-
Peptone	5	3	-	2
Soluble starch	10	10	20	10
Yeast extract	2	3	5	4
$CaCO_3$	3	2	1	-
NaCl	1	-	-	-
Instant Ocean	33.3	33.3	33.3	33.3
NH_4NO_3	-	3	-	-
Tryptone	-	-	5	-

Extraction of antimicrobial compounds

Metabolites were extracted from the CFS using the hydrophobic resin Amberlite XAD-16 (SIGMA). Prior to extraction 100g of the resin was autoclaved in 800 ml of distilled water. When added to the CFS the resin-water mixture was shaken well and 5 ml was added to each 50 ml of CFS. The CFS-resin mixture was then shaken at 200 rpm and 28°C for 2 hours. The resin was separated from the CFS by pouring over Miracloth and washed three times with 5 ml distilled water for extractions of 50 ml CFS or 80 ml for extractions of 800 ml CFS. The resin was then extracted with 2x200 ml MeOH per 1,000 ml CFS over a chromatography column. The MeOH fractions were pooled and dried down under vacuum. For a scale up extraction, the resin was first extracted with 2x200 ml n-Hexane per 1,000 ml CFS followed by the same amounts of dichloromethane, ethylacetate and methanol. The fractions were kept separately for each of the solvents. When collecting the first n-Hexane fraction, two phases formed. The phases were separated in a separating funnel, dried down and labelled as fraction “water in n-Hexane” (lower layer) and “n-Hexane” (upper layer).

Liquid-liquid extraction

In order to desalt extracts obtained by methanol extraction of the resin and acetone-methanol extracts of the pellet, the extracts were resolved in 2:1 EtOAc:dH_2O, shaken vigorously and the two layers were separated in a separating funnel and dried down under vacuum. The aqueous layer was then extracted twice more. The pooled EtOAc fraction was extracted once more in the same way.

Extraction of microbial pellet

In the scale up culture the remaining pellet following preparation of a CFS was also extracted, firstly with3x 1 L of acetone followed by 3x 1L of MeOH. All fractions from the pellet extraction were pooled and dried down. The dried down fraction was then dissolved in 50 ml dH_2O and shaken with 100 ml EtOAc. The phases were separated in a separating funnel and the remaining aqueous fraction was extracted again with 100 ml EtOAc. The two EtOAc fractions were pooled and the remaining aqueous fraction was extracted once more. The EtOAc fractions were pooled again, dried down and solved in 50 ml dH2O and 100 ml EtOAc. After separation of the phases, the aqueous phase was pooled with the aqueous phase of the previous partitioning and the remaining EtOAc fraction was again extracted twice with 50 ml dH2O. The final EtOAc and aqueous fractions were dried down and weighed.

Resazurin assay

Initially, an overnight culture of a bacterial test strain was prepared in 50 ml LB-broth (SIGMA) at 37˚C and 200 rpm. After 12-18 hr 5 ml of the bacterial culture were transferred to 45 ml iso-sensitest™ broth (Oxoid ltd.) and grown at the same conditions until OD 600 nm reached 1.0. Yeast test strains were freshly grown over night on YPD-agar plates at 28˚C. A colony of the culture was then dissolved in sterile dH_2O and the OD was adjusted to 0.12 at 540 nm. 100 µl of isosensitest broth (bacteria) or RPMI (yeast test strains) [2.6 g RPMI (SIGMA), 8.63 g Mops (SIGMA), pH7] were then pipetted into each well of a microtitre plate (multiple well plate 96-well flat bottom, Sarstedt), except for wells in column 11 which were filled with 90 µl isosensitest broth or RPMI. Next, 80 µl of isosensitest broth or RPMI were applied to the first column of the plate. 20 µl of a known concentration of the extract to be tested was then added to the first column, mixed well and 100 µl were transferred to the second column. Again, the solution was mixed well by pipetting.

This procedure was repeated until column 10 of the plate. 10 µl MeOH (or any other solvent used for the solution of the tested extracts) was added to the wells of column 11. A 100 µl of the test strain solution [50 ml isosensitest broth or RPMI, 100 µl test strain (OD 1.0 at 540 nm or OD 0.12 at 600 nm), 250 µl resazurin] was then added to each well of the columns 1-11 whereas 100 µl of SC [10 ml isosensitest broth or RPMI, 50 µl resazurin] was added to the wells of column 12. The plate was wrapped in parafilm and incubated at 37˚C for 48 h. The colour of each well was noted after 20 h and 48 h.

TLC

Thin layer chromatography was carried out using aluminium TLC silica gel 60 F_{254} plates (Merck), using the following solvent systems: **1a:** 30:6:5 EtOAc:MeOH:dH_2O + 0.1% TFA, **1b:** 30:6:5 EtOAc:MeOH:dH_2O, **2a:** 30:3:2 EtOAc:MeOH:dH_2O + 0.1% TFA, **2b:** 30:3:2 EtOAc:MeOH:dH_2O, **3a:** 30:2:1 EtOAc:MeOH:dH_2O + 0.1% TFA, **3b:** 30:2:1 EtOAc:MeOH:dH_2O, **4:** Chloroform:MeOH + 0.1% TFA. Preparative scale layer chromatography (PLC) was carried out using PLC siliga gel 60 F_{254} plates with concentrating zones (Merck).

Analytical procedures

Liquid chromatography–mass spectrometry (LC-MS) was used to analyze samples, which were run on A Dionex Ultimate 3000 Exactive system (Thermo Scientific, Germany) using the column: ACE 5 C18 (75x3.0 mm) (Highchrom Ltd, UK). The flow rate used was 0.3 µl/min, the injection volume 10 µl and the concentration of extracts added 1 mg/ml. The following linear solvent gradient was used: 10% acetonitrile/ 90% water (0.1% formic acid) to 100% acetonitrile (0.1% formic acid) in 30 minutes; 100% acetonitrile (0.1% formic acid) for 5 minutes.

Samples were also analyzed using Nuclear magnetic resonance (NMR) on a Jeol LA--400 FT-NMR spectrometer system with an AS400 magnet at 400MHz.

RESULTS AND DISCUSSION

A total of 55 isolates were obtained from the sponge *Haliclona simulans*. The analysis of the biodiversity is not part of this chapter but it will deal with an orange, spore- and branching mycelium forming isolate obtained from the sponge. The isolate was given the internal code FMC8. The characteristic colony and spore morphology suggested that the isolate was a *Micromonospora* sp.. This was confirmed by sequencing of the 16S-rRNA gene using the primer pair 27f and 1492r. Figure 4.1 shows a sporulating plate culture of FMC8, which is orange in the phylogenetic tree of *Micromonospora* isolates obtained from *Haliclona simulans* and micrographs of a relatively fresh, still orange culture (left-down) and a sporulating culture which already turned dark (down right) is shown. The typical morphology of *Micromonospora* spores is visible.

Isolate FMC8 was initially tested in a deferred antagonism assay against three different bacterial test strains (*E. coli*, *B. subtilis* and *S. aureus*) and FMC8 showed activity against *B. subtilis*. The isolate was then grown in liquid culture (SYP-SW) and the CFS and an Amberlite XAD-16 extract of the CFS was subsequently shown to be active against *B. subtilis* and *S. aureus*. Different growth media were then employed to determine optimal conditions for production of the anti-bacterial metabolite. These included MMM-SW, 333-SW, SGG-SW and SYP-SW (Table 4.1). The different MeOH extracts were diluted to 50 mg/ml in 50:50 MeOH:H_2O and then tested for bioactivity against the following test strains (*E. coli*, *B. subtilis*, *S. aureus*, *P. aeruginosa*, *C. albicans*, *C. glabrata*, *S. cerevisiae* and *K. marxianus*) in the resazurin based bioassay as described in Materials and Methods. The results are shown in Table 4.2 and Figure 4.2.

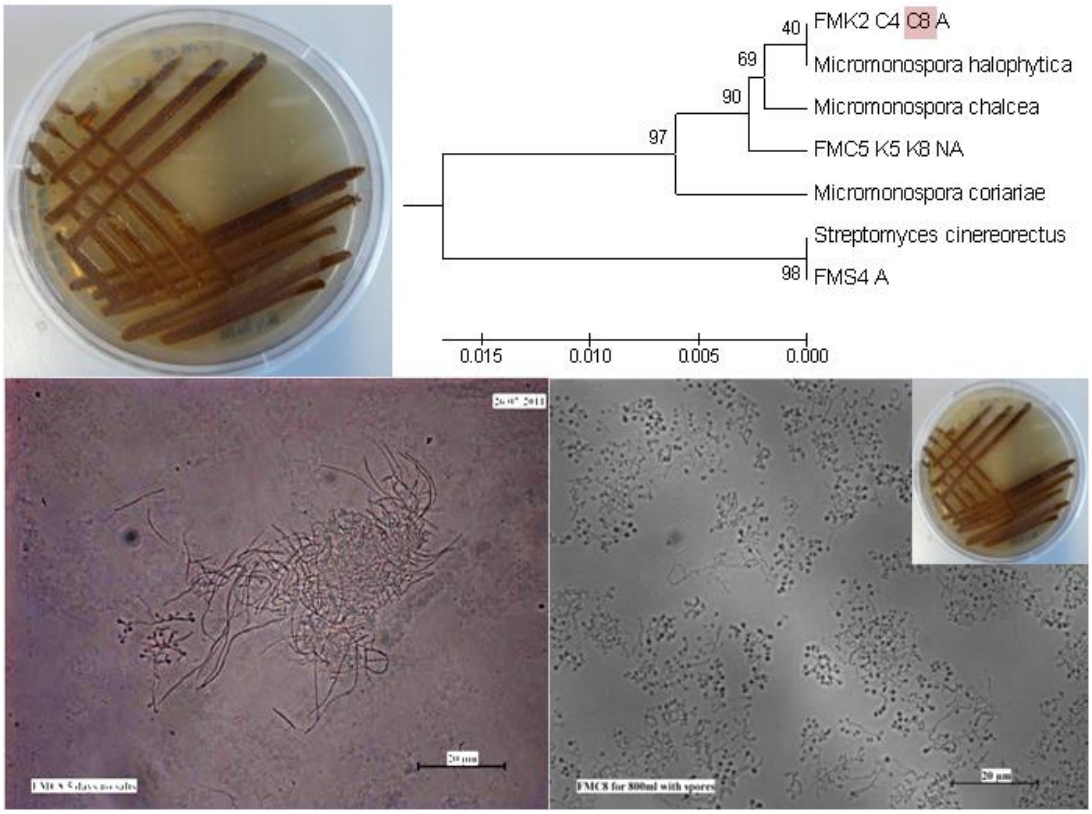


Figure 4.1: Microscopy and molecular identification of *Micromonospora* isolate FMC8

Top-left shows a plate culture which has already turned dark due to spore-formation but the characteristic orange colour of the isolate is also visible. Top-right shows a phylogenetic tree with all 55 isolates obtained from the sponge (tree inferred using the maximum likelihood method as described in Chapter 1, boot strapping values are given next to each branch). The 16S-rRNA sequences of the 6 *Micromonospora* isolates obtained clearly grouped with *Mircomonospora* derived sequences in GenBank, which were also included in the tree. Down-left shows a micrograph of a relatively fresh culture of FMC8 with the characteristic branching mycelium. Some spores are also already visible slightly left of the centre. Down-right shows a micrograph of the plate culture from top-left. Branching-mycelium is still present but dominated by the characteristic *Micromonospora* spores.

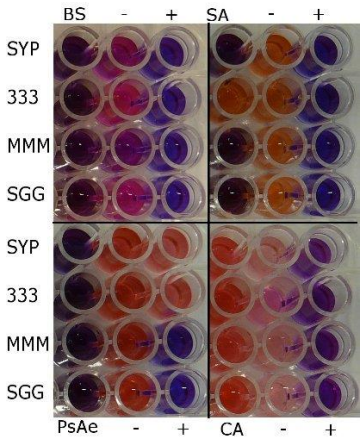


Figure 4.2: Antimicrobial activity detected in the resazurin assay for extracts of FMC8 (for full results please refer to Table 4.2).

Figure 4.2 shows the results obtained for all growth media tested, against the bacterial test strains *B. subtilis* (BS), *S. aureus* (SA) and *Ps. aeruginosa* (PsAe) as well as against the yeast *C. albicans* (CA). The column on the left hand side of each rectangle shows the activity of the extract added at 50 mg/ml against the corresponding test strain (blue: active; reddish-pink: not active), the middle columns returns the result for the solvent control (50:50 dH_2O:MeOH; expected: reddish-pink), the right hand column represents the sterility control (expected: blue).

Table 4.2: Results of the resazurin assay using different extracts from FMC8 at a concentration of 50 mg/ml.

Medium	EC	BS	SA	PsAe	CA	CG	SC	KM
SYP	-	+	+	+	-	-	-	-
333	-	+	-	+	-	-	-	-
MMM	-	+	+	+	-	-	(+)	-
SGG	-	+	+	+	-	-	-	-

EC=*E. coli*, BS = *B. subtilis*, SA = *S. aureus*, PsAe = *P. aeruginosa*, CA = *C. albicans*, SA = *S. cerevisiae* and KM = *K. marxianus.*

To further analyse difference in metabolite profiles between various growth media, extracts were subjected to TLC analysis using solvent system 1b (Figure 4.3), with the TLC plate being analysed under both UV light (254 nm and 366 nm) and day light. Plates were also sprayed with *p*-anisaldehyde and analysed under UV-light (366 nm) and day light. While many bands were observed in each of the extracts analysed, marked differences were observed in the compound production profiles of FMC8 grown on different growth media, with several compounds only being detected in one, two or three of the growth media employed or being present in different concentrations dependent on the growth medium.

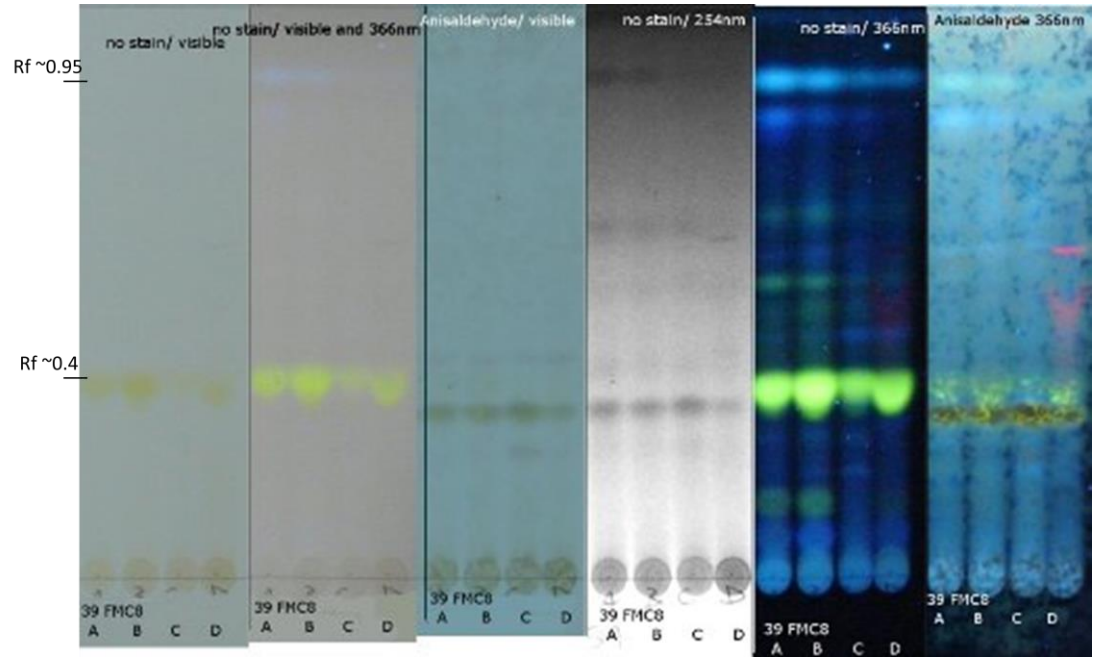


Figure 4.3: Analysis of a TLC plate using the solvent system 1b visualized under different light conditions and sprayed with *p*-anisaldehyde.

Spotted were equal amounts of crude extracts obtained from cultures grown in SYP-SW-broth (A), 333-SW-broth (B), MMM-SW-broth and SGG-SW-broth (D). The same TLC plate was visualized (from left to right) before staining under white light; under white light and 366 nm; after staining under white light; before staining under 254 nm; before staining under 366 nm and after staining under 366 nm. The Rf values for two prominent bands are given on the left hand side. Visible are both differences and similarities between the extracts derived from different growth media.

Extracts were then pre-purified using EtOAc:H_2O liquid-liquid extraction and run again on TLC plate using the solvent system 1b in order to compare the composition of the organic and aqueous fractions. The compounds were separated between the aqueous and EtOAc phase according to their Rf-value (Figure 4.4). That is, compounds with an Rf-value of ~0-0.3 in a TLC with solvent system 1b were predominantly present in the aqueous fractions and compounds with an Rf-value of ~0.3-1 could be found in the EtOAc fractions. Because EtOAc is the main component of the solvent system employed it was expected that compounds in the EtOAc fractions would run faster on the TLC-plate and generally less polar compounds will separate into the EtOAc layer and subsequently run faster on the relatively polar normal-phase silica. The TLC plate was then overlayed with *B. subtilis* in order to locate the bioactive compounds on the plate (Figure 4.4). Another TLC employing solvent system 1b was analysed using *p*-anisaldehyde. Pictures of the TLC plates are shown in Figure 4.4. The fractions were also tested for

antimicrobial activity in the resazurin assay and MIC values against *B. subtilis* were determined (Table 4.3).

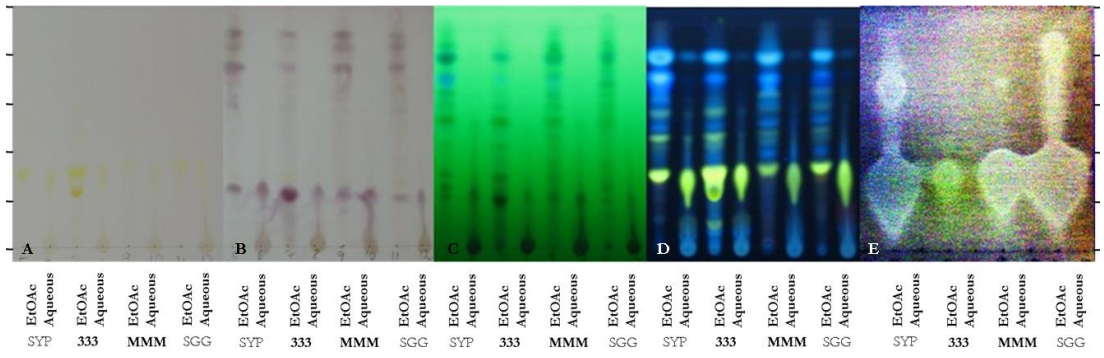


Figure 4.4: TLC plates developed with solvent system 1b

2 µl of each extract was applied at a concentration of 50 mg/ml. The preference of compounds with a low Rf-value for the aqueous phase and compounds with high Rf-values for the EtOAc phase was obvious for each extract. Also, the presence of different zones of inhibition in the different extracts (as shown in E) was noted. The largest zone of inhibition, detected in all EtOAc fractions, is thought to be due to an orange band just below the dominant yellow band (just visible for the EtOAc fraction in SGG). This can be deduced by comparing the Rf-values of the band and the centre of the zone of inhibition (both ~0.3).

A number of zones of inhibition (ZOI) were detected in the overlay TLC plate assay system (Figure 4.4E). This implies that either there is more than one compound which is displaying anti-*Bacillus* activity in some of the fractions or that the bioactive compound is present in two different states (e.g. protonated and neutral). The activity is almost exclusively detected in the EtOAc fractions regardless of the growth-medium employed. The largest zone of inhibition at Rf=0.2-04 is common to all EtOAc fractions in extracts from all four growth media, even though larger quantities appear to be present in extracts from the SGG growth medium and lower levels of activity appear to be present in extracts from the growth medium 333. Given that equal concentrations of the extracts were present in all fractions analysed this implies a higher relative activity for the EtOAc fraction of the SGG-SW- extract. For extracts from SYP-SW and MMM-SW zones of inhibition were also detected at Rf=0.6-0.7, while a zone of inhibition was also visible for the SGG-SW-extract at Rf=0.8-0.9.

The MIC results (Table 4.3) confirmed the results of the overlay TLC assay with the EtOAc fraction of the SGG-SW extract being the most active and the EtOAc fraction of the 333-SW-extract being the least active fraction. Even though no activity was

detected for the aqueous fractions in the overlay assay, activity was present in those fractions according to the resazurin based assay. This is most likely due to differences in sensitivity of the two assays. Nonetheless, Table 4.3 shows an 8 fold increase in the MIC for extracts from the growth media 333-SW, MMM-SW and SGG-SW following extraction with liquid-liquid partitioning using EtOAc:dH_2O. Data for SYP-SW is not available however because the MIC-test for the crude extract failed and insufficient material was available to repeat the assay.

Table 4.3: Results for the Resazurin assay with *B. subtilis*.

Medium	Fraction	MIC [mg/ml]	µg for 1 Unit	Weight [mg]	Units
SYP-SW	EtOAc	0.010	2	0.4	200
SYP-SW	Aq	1.250	250	22.9	92
333-SW	EtOAc	0.156	31	3.5	112
333-SW	Aq	-	-	20.8	-
MMM-SW	EtOAc	0.020	4	1.5	375
MMM-SW	Aq	1.250	250	38.2	153
SGG-SW	EtOAc	0.005	1	2.4	2400
SGG-SW	Aq	0.625	125	41.6	333
333-SW	Crude	1.25	250	33	132
MMM-SW	Crude	0.156	31	53	1699
SGG-SW	Crude	0.039	8	55	7051

Column 1: The growth culture medium from which the extract was derived; Column 2: either EtOAc, aqueous (Aq) or crude; Column 3: minimal inhibitory concentration (MIC); Column 4: µg necessary to inhibit the growth of the test strain in this assay (defined as 1 unit); Column 5: total dry weight of each fraction; Column 6: total units available from the fraction.

Despite overlaying of the TLC plate and aligning pictures of the different visualizations (white light, 254nm, 366nm) next to each other it was not possible to determine the TLC-band responsible for observed activity. It is clear from the TLC plate shown in (Figure 4.4) that the large zones of inhibition are not due to the bright yellow spot because there are clear differences when comparing the Rf-values of the centre of the zone of inhibition and this yellow spot. Another indication is that in the EtOAc-fraction of 333-SW the yellow compound appears to be most concentrated but the activity of this fraction is much lower than in the other EtOAc fractions. A small red band beneath the yellow compound correlates better with the Rf-values of

the zones of inhibition and is thus more likely to be the compound associated with the observed activity.

Scale up

In an attempt to obtain more material for the purification and potential characterisation of the bioactive compound(s), a scale up strategy was undertaken. Given that the growth medium SGG-SW was previously determined as the best of the growth media tested, for the production of at least two of the potential bioactive compounds; this growth medium was chosen for the scale up. 800ml liquid medium in a 2 l Erlenmeyer flask were inoculated with 16 ml of a 7 day seed culture (10x800ml cultures were prepared). Since the *Micromonospora* isolate FMC8 appeared to grow faster than had previously been observed on a smaller scale, the bioactivity of the supernatant was tested after 5 days in the well diffusion assay. All of the supernatants displayed anti-*Bacillus* and anti-*Staphylococcus* activity and therefore the cultures were extracted on day 6. In order to achieve a pre-purification of the complex crude extract which had previously been observed in the smaller scale experiment, the resin was eluted with a sequence of organic solvents according to their polarity index (Table 4.4) as described in Materials and Methods. The pellet was also extracted thus in total 7 different fractions were obtained: 2 fractions from the bacterial pellet and 5 fractions from the resin. Weights for the fractions are given in Table 4.5.

Table 4.4: Solvents used for the extraction of metabolites from XAD-16 resin

Solvent	Polarity index
n-Hexane	0
Dichloromethane	3.1
Ethyl Acetate	4.4
Methanol	5.1

Table 4.5: Pellet and XAD fractions from the scale up culture

Fraction	Weight [mg]
1 Pellet-aqueous	Nd
2 Pellet-EtOAc	Nd
3 XAD-n-Hexane	32 (oily)
4 XAD-aqueous in n-hexane	2,708.9
5 XAD-Dichloromethane	568.1 (oily)
6 XAD-Ethyl Acetate	111.1
7 XAD-Methanol	2,210.2

(nd) = not dry, some extracts would not dry down completely even after 24 h.

These fractions were subsequently analysed using the TLC assay plate system for both composition and activity (Figure 4.5). The zones of inhibition which were observed were however too large to allow differentiation between them but from aligning the visualizations with the overlay the bioactivity could be roughly correlated to an under UV-light (366 nm) orange band as postulated from the small-scale experiment. It could also be seen, that this compound was mainly present in the fractions pellet-EtOAc, XAD-dichloromethane and XAD-EtOAc (Figure 4.5). Interestingly, in these preparations the centre of the ZOI had an Rf~0.7 when compared to an Rf~0.3 in extracts from the small-scale experiments, possibly due to the different solvent systems used (1a and 1b).

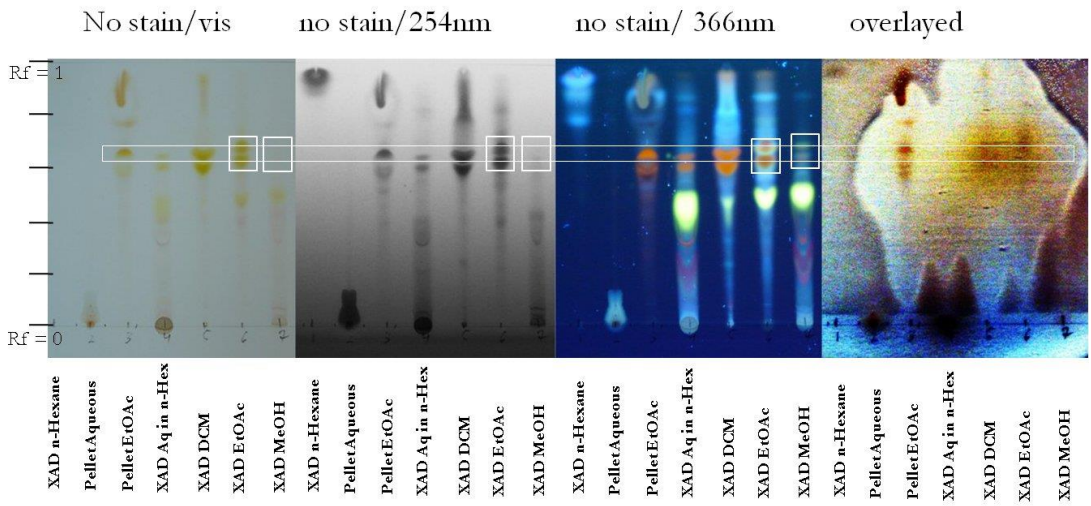


Figure 4.5: Fractions obtained from a scale up culture of FMC8 run with solvent system 1a.

Equal amounts of each fraction were run on the TLC plate and the same plate was visualised under different light conditions (from left to right): White light; 254 nm, 366 nm; overlay (*B. subtilis*) under white light. The plate was not stained. The centre of the largest zone of inhibitions visible in the overlay could be aligned with the upper yellow (white light) or orange (366 nm) band with an Rf value of ~0.6. This indicates that this compound is involved in the inhibition of the test strain.

The different Rf-values obtained for the upper orange bands when running the fractions were separated using the (2a) solvent system also implied that the compound(s) extracted from the pellet may be different than the compound(s) extracted from the supernatant (Figure 4.6).

Dilutions of the extracts were prepared in order to obtain a better correlation between zone of inhibition and bioactive compound(s). Again, the presence of the zones of inhibition correlated with the presence of the two orange bands, with the centre of the zones of inhibition correlating most closely with the upper of the two bands. The observed activity was strongest in fractions 1 (Pellet-EtOAc), 5 (XAD-DCM) and 6 (XAD-EtOAc) but was also present in fractions 4 and 7.

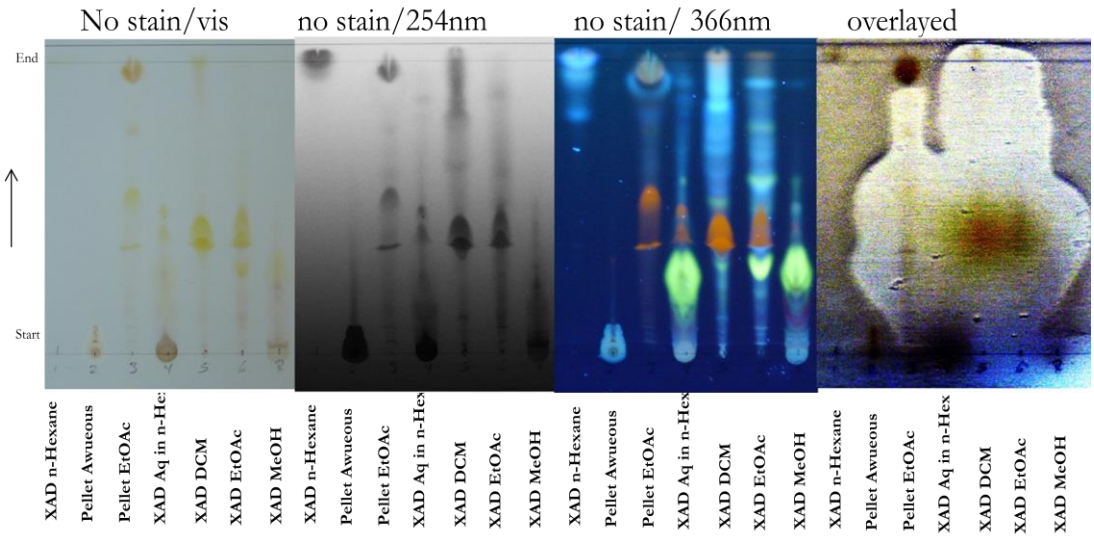


Figure 4.6: Fractions obtained from the scale up culture separated using the 2a solvent system.

Different orange bands (under 366 nm light) imply different, but related compounds being present in fractions Pellet-EtOAc and XAD-DCM/EtOAc. For a more detailed legend see Figure 4.5.

Purification

In order to purify the fractions for the bioactive compound(s), 100 µl of fractions 1, 5 and 6 were spotted horizontally on a 10x10 cm TLC plate. The plate was developed with solvent mix 2a and orange together with some other sharp bands were scraped off. The resolved fractions were then analysed on a TLC gel with solvent system 1a and MIC values against *B. subtilis* were obtained. Overall 19 fractions of relative purity were obtained from Pellet-EtOAc, XAD-EtOAc and XAD-DCM fractions. The purification for the pellet-EtOAc fraction is represented in Figure 4.7. Pictures of TLC plates with all fractions and their MIC-values against *B. subtilis* are shown in Figure 4.8. A selection of the fractions, based on activity, purity and location of the compound on the gel was subsequently analysed via LC-MS and NMR.

LC-MS and NMR analysis

A summary of fractions analysed via LC-MS and NMR is given in Table 4.6. Fraction 34 was analysed as a negative and contaminant control.

The analysis of the LC chromatograms and MS-spectra implied the presence of three compounds in the fractions (Figure 4.9). The most active fractions mainly comprised

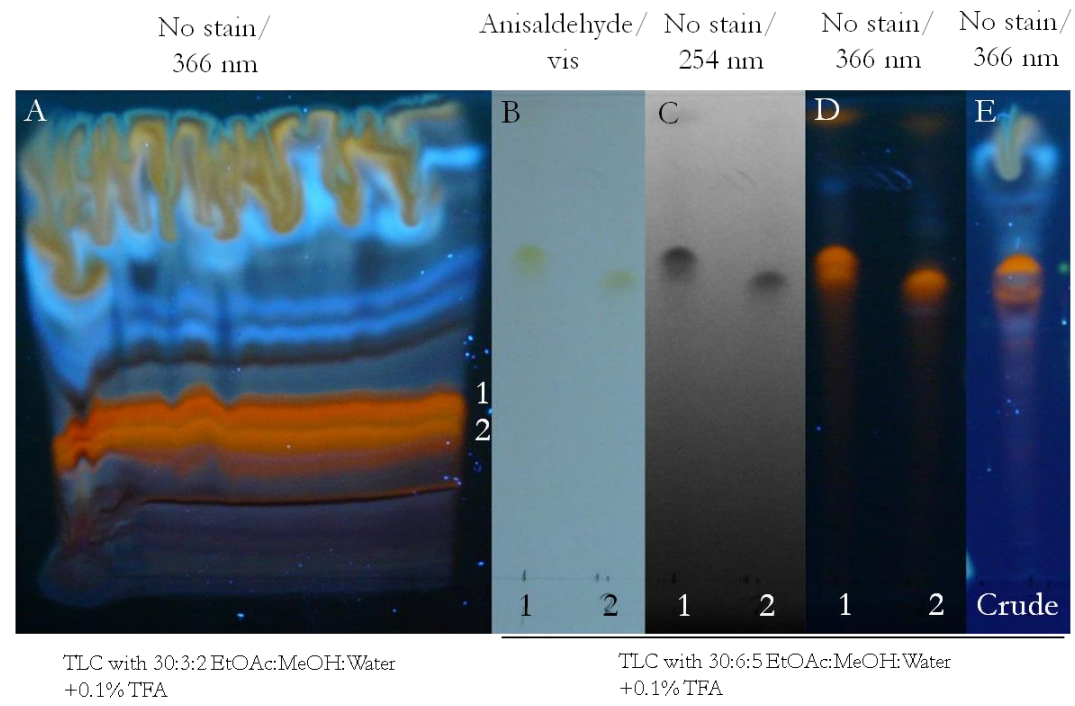


Figure 4.7: TLC plates developed with solvent system 2a (A) and 1a (B-E).

100 µl of the fraction pellet-EtOAc was spotted horizontally onto a PLC-plate. The plate was developed with solvent system 2a and the orange bands labelled 1 and 2 in (A) were scraped off and resolved from the silica gel using solvent system 2a. Fractions 1 and 2 were obtained and analysed on a TLC plate with solvent system 1a (B-D). A TLC of the crude extract (fraction Pellet-EtOAc) is aligned for comparison (E).

of a compound with an m/z = 617.21 whereas the fractions with less activity comprised mainly of a compound with an m/z = 445.14. For fraction 2 no MIC data was available but the LC-MS data implied the presence of a compound with an m/z = 619.22 (all m/z-values given as found in positive mode). The m/z values correspond well with the m/z values of (iso)quinocycline A and B. Quinocycline was originally detected in cultures of a *Streptomyces sp.* (Celmer et al., 1958). Isoquinocyline B and quinocycline B were later reported from the culture broth of a *Micromonospora* sp. isolated from Toyama Bay, Japan at 321 m depth (Furumai et al., 2002; Igarashi et al., 2002). Quinocycline derivatives can be found in the aglyconated forms with an m/z = 445.1393 (Igarashi et al., 2002). Thus the available MS-data for the compound fitted with the published data on Isoquinocycline B. However the NMR data obtained (in DMSO-d6) for the compound with m/z = 617.21 did not fit with published spectra for (iso)quinocycline B (Igarashi et al., 2002) (Figure 4.10).

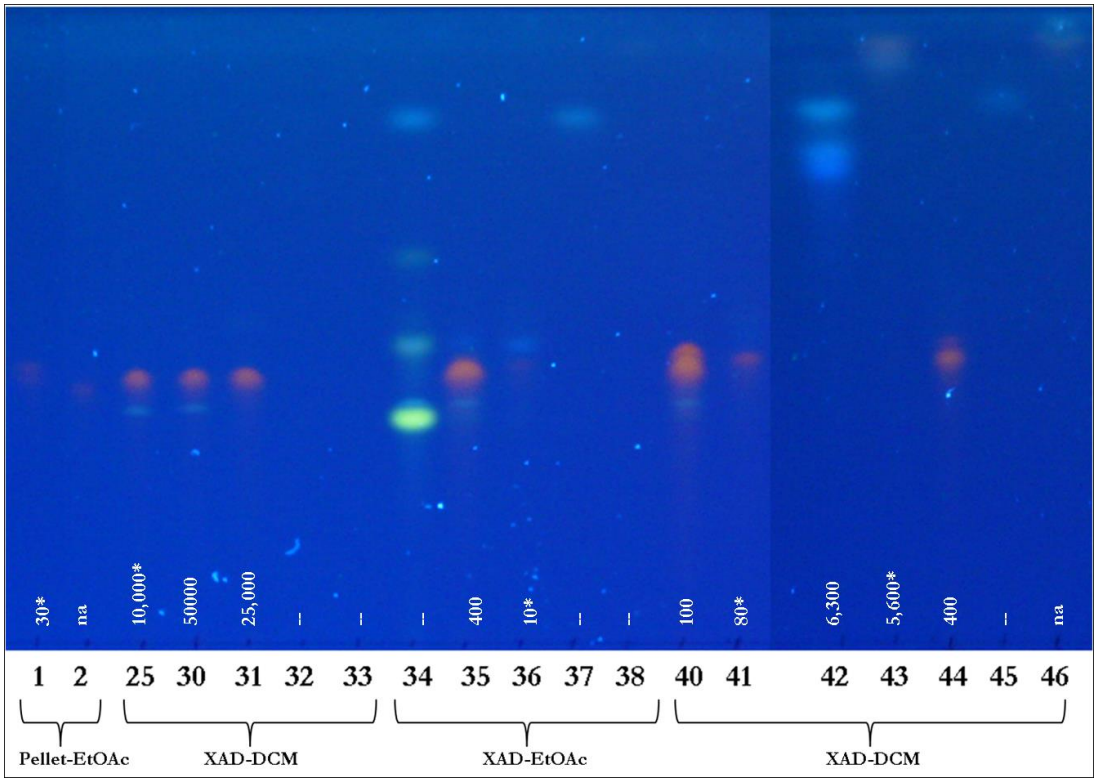


Figure 4.8: TLC analysis of the 19 fractions (fraction numbers below TLC-plate) obtained by scraping off sharp bands from TLC plates (Figure 4.8) employing solvent system 1a.

MIC values against against *B. subtilis* are depicted as white numbers above each fraction number; -- = no activity detected; *weight of fraction was below 1 mg and therefore the MIC value would need to be confirmed with more compound). The source of each fraction is given below the fraction number (Pellet-EtOAc, XAD-DCM and XAD-EtOAc).

Table 4.6: Fractions analysed via LC-MS.

Fraction	weight	MIC [ng/ml]
1	0.3	30 (or lower)
2	0	nd
31	3.2	25000
34	3.1	not active
35	2.3	400
41	0.7	80 (or lower)
44	1.7	400

The weight and the MIC values are given in column 2 and 3, respectively. For fraction 1 and 41 the lowest tested concentration tested was still active so the MIC value could be lower. However, because of the low weight (below 1 mg), MIC values for the two fractions must be treated with caution. Nd = not determined (because of low amount).

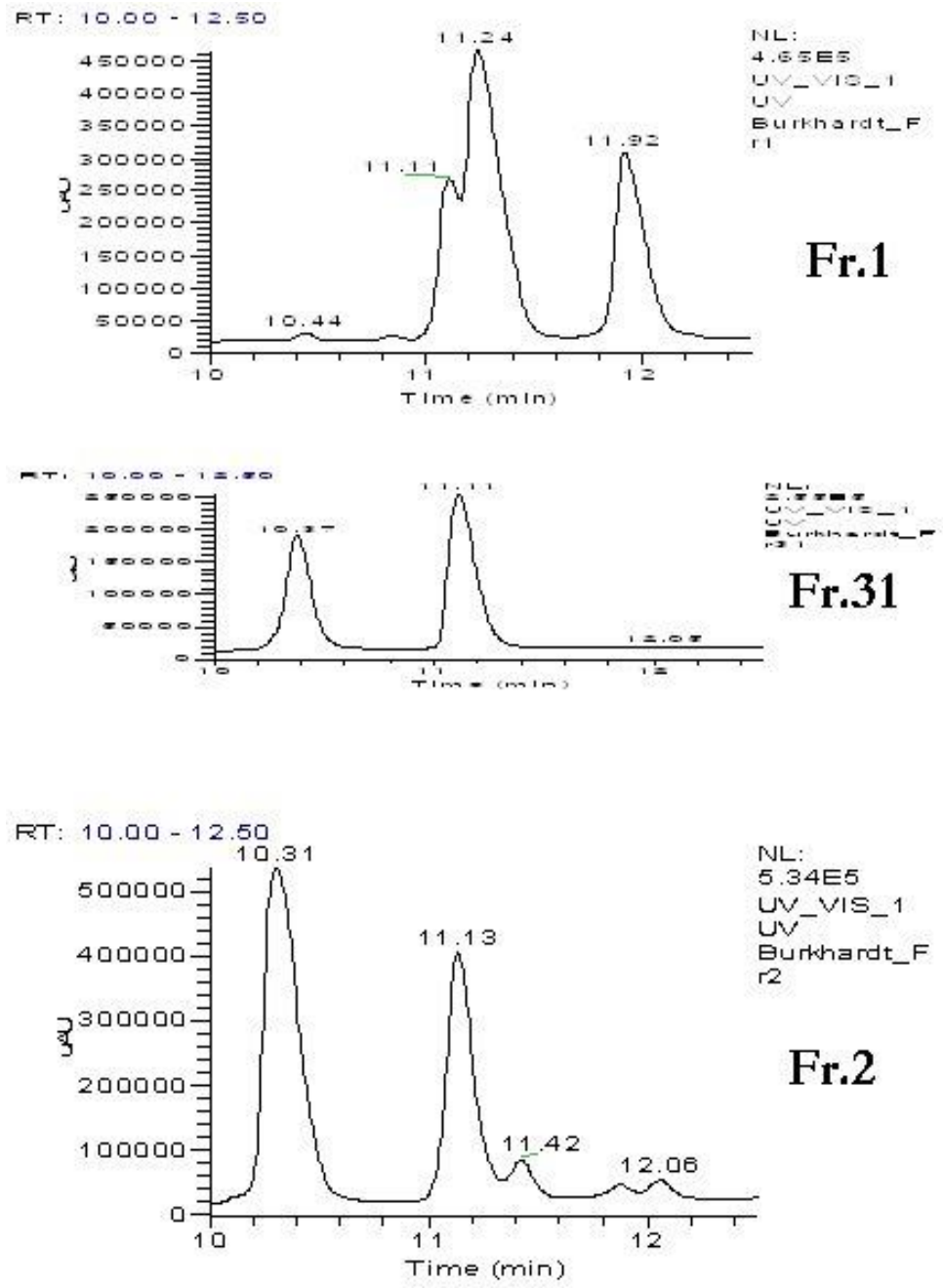


Figure 4.9: LC-MS chromatograms (UV-vis detection) obtained for fractions with varying MIC values.

A clear relation between activity and peak height was observed for peaks at Rt~11.30 and Rt~12.0 (compare fraction 31 with low activity with fraction 1 with strong activity). The peaks at Rt~10.3 and Rt~11.1 were highest in fractions with less activity. The spectra at the according times reveal a compound with m/z = 617.21 (positive mode) being present at both peaks in the active fraction. Whereas the compound corresponding to the peaks at Rt~10.30 and Rt~11.1 had an m/z = 445.14. Even though strong activity was assumed for fraction 2 (not reliably quantifiable due to too little fraction obtained) the two largest peaks in the chromatogram were at Rt~10.3 and Rt~11.1, implying low activity. But when considering the mass-spectrum at the two times a compound with m/z = 619.22 was detected. Thus apparently two compounds with relatively similar m/z values are strongly active against *B. subtilis* whereas the compound with m/z = 445.14 is (maximally) mildly active against *B. subtilis*.

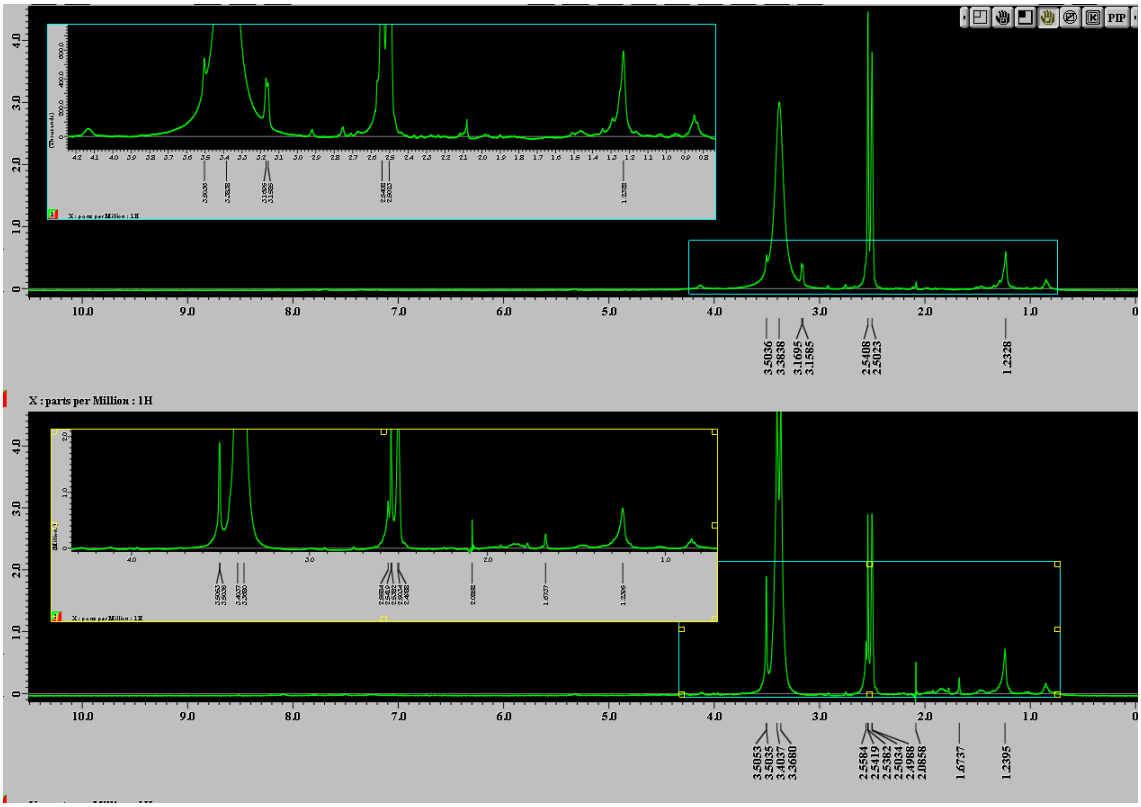


Figure 4.10: NMR spectra of fraction 1 (above) and fraction 31 (below).

Comparison with the published spectra for quinocycline and isoquinocycline B (Igarashi et al., 2002) implied that the isolated compounds are not (iso)quinocyline B or aglycons thereof.

In order to get better spectra for the three compounds, a larger quantity was purified from extracts 5 (XAD-DCM) and 6 (XAD-EtOAc). Scale up purification was performed using preparative LC (PLC). An overview of the fractions obtained is shown in Table 4.7, including MIC values against different test strains. Fraction 8 was subsequently split up into two fractions (a and b) because the scraped off silica-gel was first eluted with MeOH (fraction 8b) and then with solvent system 1b (fraction 8a).

The fractions were also analysed for purity and for the location of the bioactive compounds using TLC experiments. The amount spotted was equal for all fractions and the results of the quantitative resazurin assay were confirmed with fractions 6 and 9 being the most active fractions (Figure 4.11).

Table 4.7: PLC fractions obtained following preparative LC purification.

Source-fraction	Fraction	Weight [mg]	MIC [µg/ml]		
			BS	SA	PsAe
XAD-DCM	5	1.65	0.78	12.5	1.56
	6	2.3	0.024*	1.56	0.078
	7	14	0.20	25	1.25
XAD-EtOAc	8a	1.25	0.78	-	6.25
	8b	2.2	6.25	12.5	1.56
	9	3.3	0.024*	6.25	0.16
	10	26.3	0.39	25	6.25

Fractions 5-7 were obtained from XAD-DCM and fractions 8-10 from XAD-EtOAc. The MIC values against three different test strains are given. BS = *B. subtilis*, SA = *S. aureus*, PsAe = *Ps. aeruginosa.* *the lowest concentration tested was still active thus a lower MIC-value is possible.

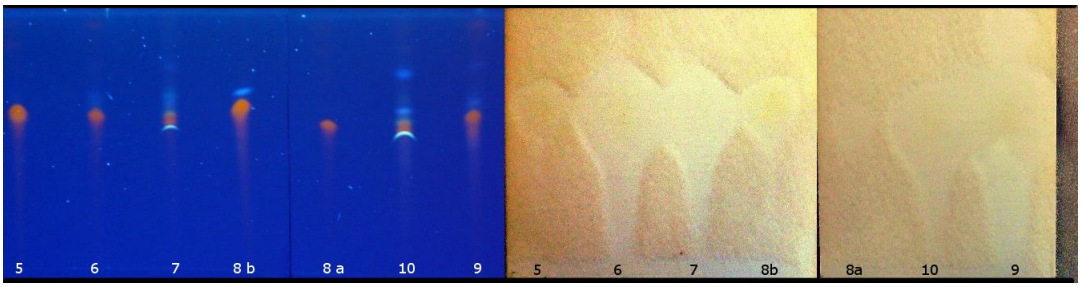


Figure 4.11: TLC of PLC-fractions developed with solvent system 1a.

The overlay confirmed strong activity particularly from fractions 6 and 9 and corresponded well with the MIC values against *B. subtilis*. For a list of fractions spotted see Table 4.7.

LC-MS of fractions 5, 6, 8a, 8b and 9

The fractions 5, 6, 8a, 8b and 9 were analysed on LC-MS and the m/z values present were similar to those obtained from previous experiments. In order to have a larger amount of compound for the NMR-experiment, fractions 5, 8a and 8b as well as fractions 6 and 9 were pooled based on their bioactivity, TLC-characteristics (see Figure 4.12 as well), HPLC-chromatograms (Figure 4.13) and m/z-values (Figure 4.14). Fractions 6 and 9 mainly comprised of a compound with m/z = 617.21 (positive mode) and fractions 5, 8a and 8b mainly comprised of a compound with m/z = 445.14 (positive mode).

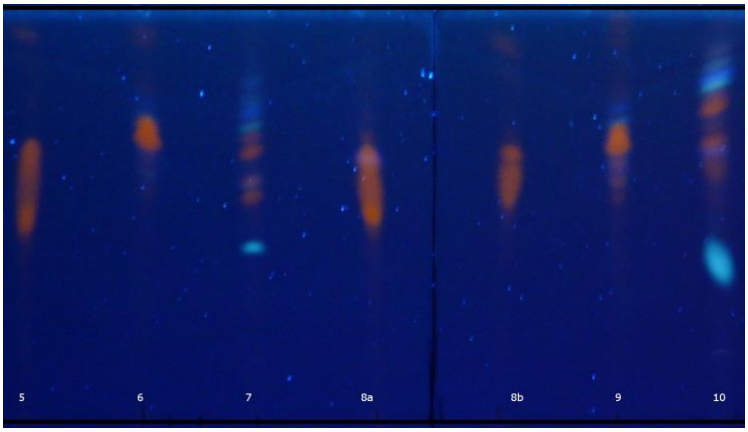


Figure 4.12: TLC of PLC-fractions developed with solvent system 4.

This different solvent system confirmed the close similarity of fractions 5, 8a and 8b.

The presaturation NMR in DMSO-d_6 is shown in Figure 4.15. As a comparison, the spectrum of quinocycline B (El-Naggar, 2007) is shown. The major peaks are not shared between the spectra implying different compounds in the fractions. C-spectra and 2 dimensional NMR spectra have not been obtained for either of the fractions to date, thus a structural determination has not as yet been possible.

CONCLUSION

The *Micromonospora* isolate FMC8 from the sponge *Haliclona simulans* has been shown to produce at least one compound with strong inhibitory activity against Gram positive bacteria. The m/z-value of the active compound was similar to that of previously reported antibiotics, the quinocyclines, but since the NMR spectra of the compounds isolated in this study differed markedly from the published NMR spectra of quinocycline B and isoquinocycline B it is tempting to speculate that the compounds may in fact be different and potentially novel. In order to obtain a full structure of the isolated compounds it will be necessary to further purify the fractions, e.g. using size exclusion chromatography for the separation of the compounds with m/z = 445 and m/z = 617. It will also be necessary to obtain more of a reasonably pure compound for better NMR measurements. It would also be

interesting to investigate the extracts obtained for other bioactive compounds since TLC-experiments implied the presence of at least two more such compounds.

Overall, the isolate FMC8 was shown to be a promising producer of antimicrobials which may also be an interesting target for genetic analysis; either the preparation of clone libraries with secondary-metabolite-gene-amplicons or for whole genome sequencing.

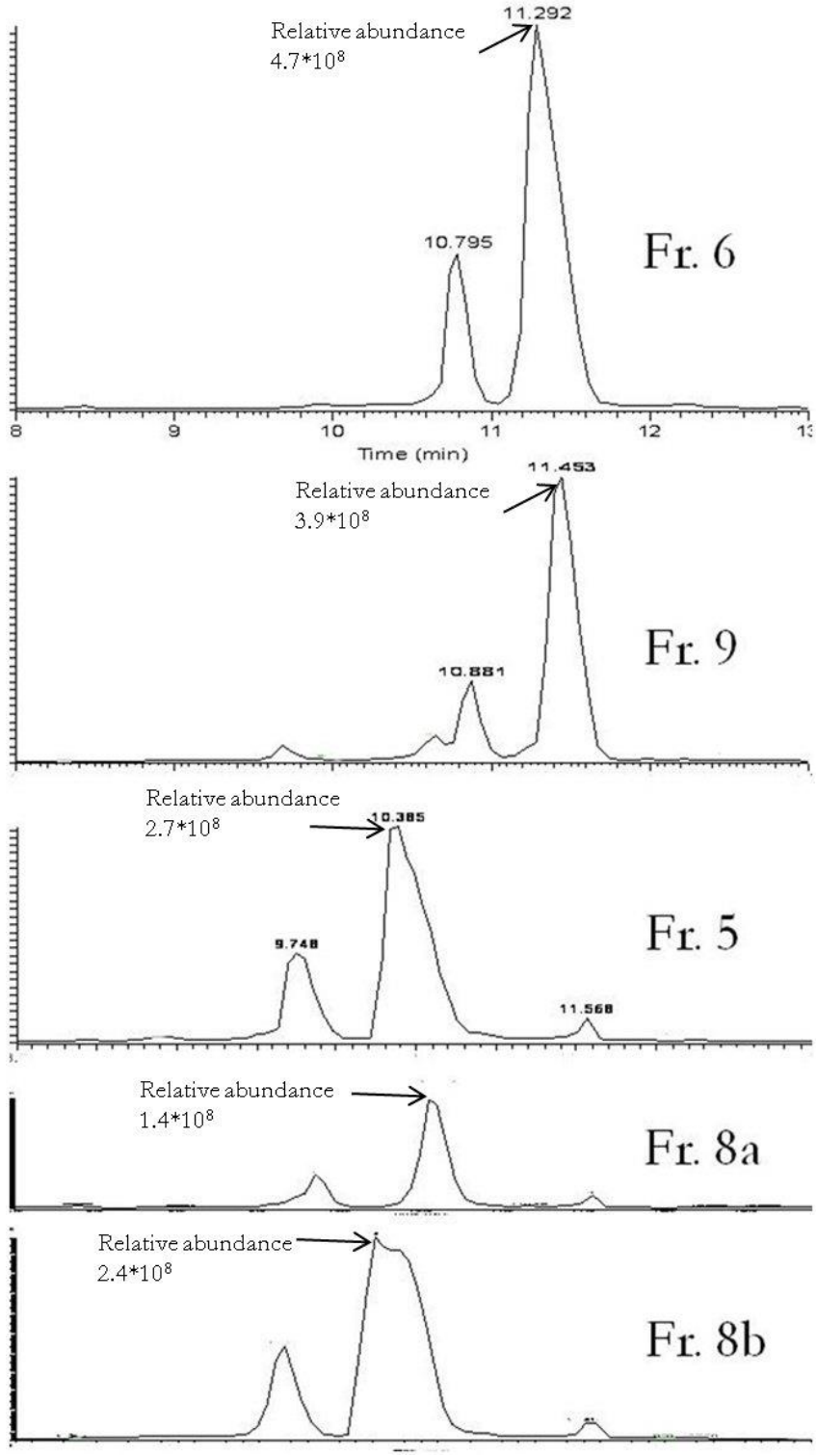


Figure 4.13: LC-MS chromatograms (showing minutes 8-13) for fractions 5, 6, 8a, 8b and 9.

The fractions are sorted according to their bioactivity (fractions with stronger activity on top) and the different chromatograms are shown in the same scale. The two peaks at Rt ~ 10.8-10.9 and Rt ~ 11.3-11.5 were largest for fractions 6 and 9, which also had the strongest activity vs. *B. subtilis*. The two peaks at Rt ~ 9.7 and 10.4 were present in fractions 5, 8a and 8b which had much lower activity against *B. subtilis.* For mass spectra of the most intense peak of each fraction please refer to Figure 4.15.

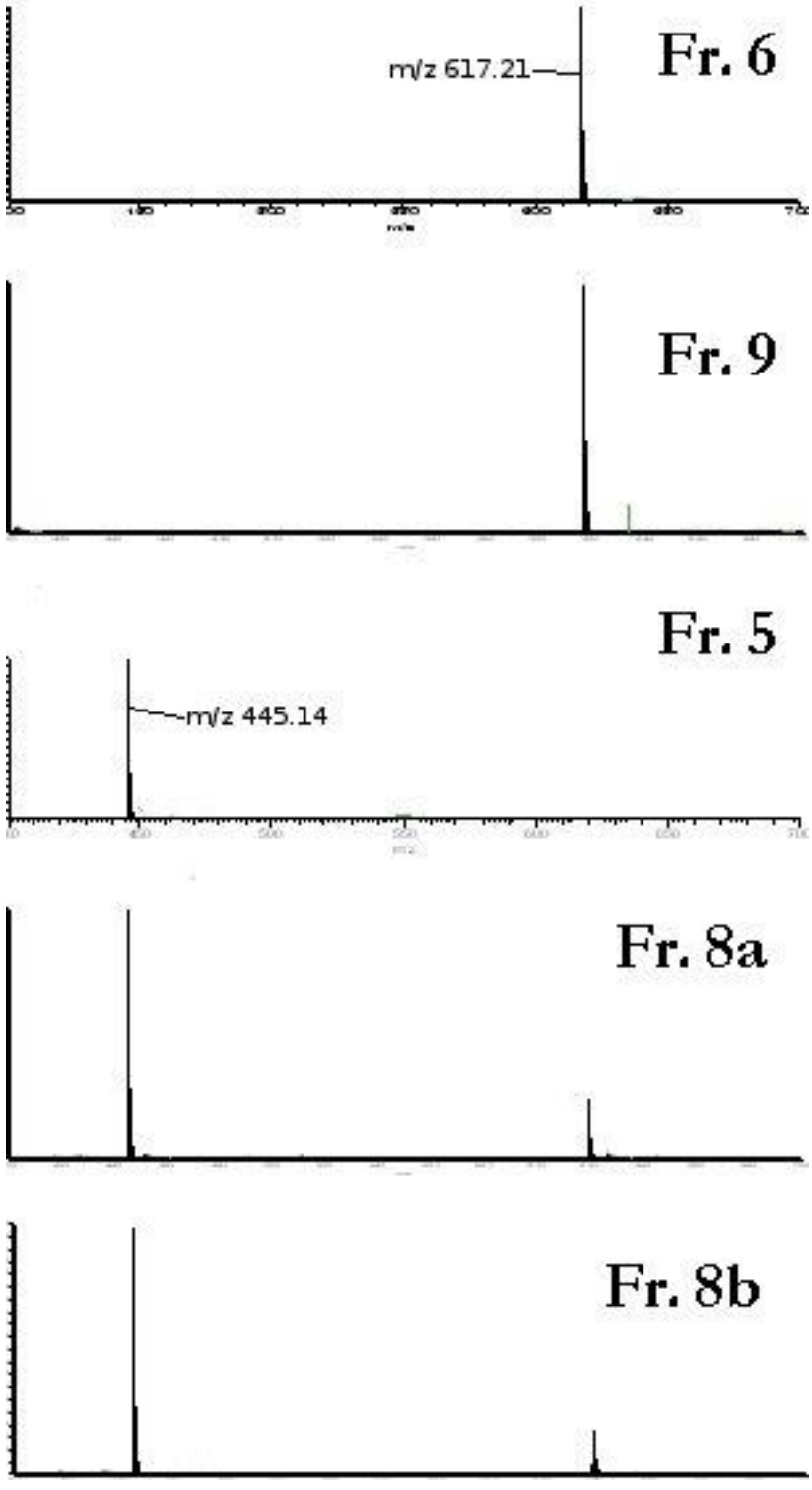


Figure 4.14: Mass-spectra of major peaks (see Figure 4.14) in the LC-chromatogram of each fraction.

Shown are m/z-values from 400-700. Fractions 6 and 9 (most active) comprise a compound with the m/z value of 617.21 (peak on right hand side); while this compound was also present in relatively small amounts in fractions 5, 8a and 8b (see Figure 4.13), the main peak of those fractions comprises a compound with the m/z value of 445.14 (peak on left hand side in mass spectrum).

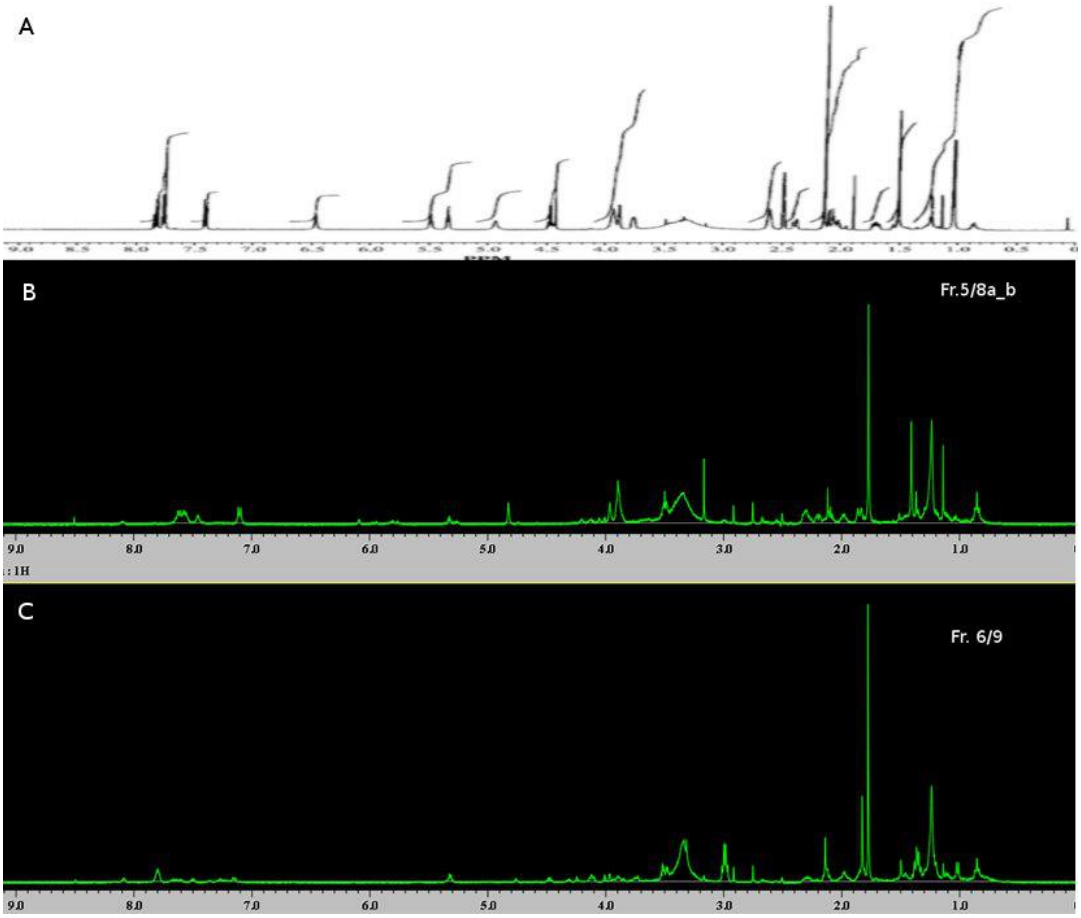


Figure 4.15: NMR spectra

Shown are spectra for B: fractions 5/8a/8b and C: fractions 6/9 compared to a published spectrum of A: Kosinostatin. Note the marked differences between the published spectrum and the spectra obtained for the compounds isolated from FMC8.

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Chapter 5

Distinct prokaryotic diversity and spatial distribution of microbes associated with deep sea sponges revealed by pyrosequencing

ABSTRACT

Two deep water sponges, *Lissodendoryx diversichela* and *Stelletta normani* were shown to host a remarkably different bacterial and archaeal diversity by application of 454 Pyrosequencing targeting a region of the 16S-rRNA gene common to *Bacteria* and *Archaea*. The *L. diversichela* community was dominated by a single γ-proteobacterial sponge symbiont, closely related to the non-phototrophic, chemoheterotrophic and aerobic genus *Granulosicoccus* and uncultured bacteria previously found in shallow water sponges. The *S. normani* sample hosted a largely sponge specific microbial community, even more diverse than has been previously reported for shallow water sponges. Organisms potentially involved in nitrification (*Crenarchaeota),* denitrification (*Nitrospira, Acidobacteria* and *Chromatiales),* sulphate reduction (*Desulfovibrio)* and secondary metabolite production (*Poribacteria, Chloroflexi* and *Actinobacteria)* were found to be spatially distributed in the sponge. Whereas reads classified as *Crenarchaeota, Nitrospira* and *Chromatiales* were more often encountered in the cortex, *Desulfovibrio* and *Chloroflexi* featured more heavily in the choanosome. The presence of a large proportion of unclassified reads in this sample highlights the potentially novel microbial community associated with this sponge. Even though *Archaea* were less abundant in both sponges than *Bacteria* they made up an important fraction of the prokaryotic community. While, on the higher taxonomic levels, many similarities between shallow- and deep water sponge-associated microbial communities were detected, by comparing shared OTUs from the presented study with shallow-water derived pyrosequencing reads, a deep sea specific population was implied.

INTRODUCTION

Marine sponges have gained much attention during recent years mainly because of their remarkably diverse community of microorganisms and their involvement in bioactive secondary metabolite production. Sponge associated microbes contribute to sponge biology in many ways, such as providing a chemical defence mechanism, carbon and nitrogen nutrition and as a food source (Taylor et al., 2007; Webster et al., 2010). Great efforts have been made to characterise the diversity of the microbial assemblages in shallow water sponges, using culture dependent (Flemer et al., 2012; Hentschel et al., 2001; Kennedy et al., 2009; Webster and Hill, 2001) and culture independent approaches (Kennedy et al., 2008; Thiel et al., 2007a; Webster et al., 2001). Up to 2007, 15 bacterial phyla (including the candidate phylum *Poribacteria*), 2 major archaeal lineages and many microbial eukaryotes have been reported from marine sponges (Taylor et al., 2007). The application of "next generation" sequencing in recent years has allowed access to the so called "rare biosphere" (Sogin et al., 2006) and increased the number of bacterial phyla detected in sponges to more than 30. Marine sponges from the Great Barrier Reef (Webster et al., 2010), the Red Sea (Lee et al., 2011), the Mediterranean (Schmitt et al., 2012a), the northern Atlantic (Jackson et al., 2012; Radax et al., 2012), the Carribean (White et al., 2012), Brazil (Trindade-Silva et al., 2012) and worldwide (Schmitt et al., 2012b) have been studied for their microbial diversity. More interesting than the sheer diversity of microbial communities in sponges is the sponge-specificity, i.e. OTUs almost exclusively found in sponges (Simister et al., 2012; Taylor et al., 2011). In a comprehensive study of sponge-microbe associations, Schmitt *et al.* have distinguished between core, variable and species-specific assemblages in sponges. Interestingly, only a very small proportion of 90 %, 95 % and 97 % OTUs was shared between different sponge species (Schmitt et al., 2012b). This "core" community of microbial OTUs found in most studied sponges implies a horizontal transfer of sponge-associated microbial diversity, i.e. through the surrounding seawater. In previous studies, by comparison of larvae and adult sponges, evidence for vertical symbiont transfer has also been shown (Webster et al., 2010). Thus potentially both vertical and horizontal transfer is likely to be involved in shaping

sponge-associated microbial communities. The data presented in the study from Schmitt *et al.* also suggests a tropical/ sub-tropical gradient of sponge microbial associates thus temperature and salinity may play a more important role in shaping sponge-microbe associations than the ocean currents. Other studies focused on describing the community structure in diverse sponges (Jackson et al., 2012; Webster et al., 2010), included archaeal diversity (Lee et al., 2011), determined seasonal variations in the community structure (White et al., 2012) or included a functional analysis of the sponge metagenome (Radax et al., 2012; Trindade-Silva et al., 2012).

In contrast, very little is known about the microbial diversity of deep water sponges. In 2005 and 2008 Romanenko and co-workers reported the isolation of two new bacterial species from a deep sea sponge (Romanenko et al., 2008, 2005). Brueck and colleagues identified an *Entotheonella* species in *Discodermia dissoluta* from a depth of 150 m (Brück et al., 2008) and later characterized the culturable anaerobes of *Geodia sp.* samples from depths of ~200-350 m (Brück et al., 2010). A culture independent study on *Polymastia cf. corticata* sampled at a depth of ~1100 m revealed that bacteria previously found in shallow water sponges are also present in deep water sponges and that the bacterial community has a spatial distribution in the sponge (Meyer and Kuever, 2008) which has also been described for a shallow water sponge (Thiel et al., 2007b). More evidence that deep water sponges share a microbial community with their shallow water counterparts was found by Olson (Olson and McCarthy, 2005). The potential role of microorganisms involved in sulphur- and ammonium metabolism in marine sponges has also been shown (Brück et al., 2010; Meyer and Kuever, 2008; Nishijima et al., 2010). Together with the finding of novel chemical entities from deep water sponges, including a *Lissodendoryx sp.,* (Gunasekera et al., 2003; Hickford et al., 2009) it is clear that deep sea sponges are interesting, yet an as yet largely understudied research area.
Thus in order to increase our understanding of the microbial diversity associated with deep water marine sponges, this study has applied the 454 sequencing approach to sponge samples from the bathypelagic zone. Two northern deep water species, *S. normani* and *L. diversichela,* which have not previously been studied for their associated microbiota, were collected at a depth of 1348 m and a water sample was included as a comparison. Additionally, the sponge sample *S. normani* was analysed for the spatial distribution of its microbial cohort in the choanosome and the cortex.

The primer pair targeting the V5-V6 region of 16S rRNA genes which is common between both *Archaea* and *Bacteria* has been chosen because *Archaea* have been reported to be particularly abundant in deep water marine sponges (Pape et al., 2006). Also, this primer pair yields sequence lengths of about 280 bp which enables the classification of sequence reads into lower taxonomic levels.

MATERIALS AND METHODS

Sponge sampling

The sponge samples used in this study were collected with the remotely operated vehicle (R.O.V.) *Holland I* during the Biodiscovery cruise 2010 aboard R.V. *Celtic Explorer*. Upon retrieval the sponge samples were washed with sterile, artificial seawater (33.3g/L Instant Ocean, Aquarium Systems – Blacksburg, VA, USA) and stored at -80˚C until molecular work was carried out in our laboratories in Cork. A part of each sample was also used for taxonomic identification by Christine Morrow, Queens University, Belfast. Additionally, a water sample was retrieved during a CTD (Conductivity-Temperature-Depth) measurement. 30 L water were collected as close as possible to the sponge sampling site, filtered through a 0.45 µm membrane filter (Whatman – Austin, TX, USA) and the filter was immediately frozen at -80˚C. The *L. diversichela* and the water sample were collected at the same sampling site whereas the *S. normani* sample was collected at the same depth ~400 m away. Duplicate samples were not collected due to sampling difficulties at such depths.

Metagenomic DNA extraction from seawater and sponge samples

DNA was extracted from filters using WaterMaster DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA) according to the manufacturer's instructions. Extracted DNA was stored at -20°C.

The sponge tissue (3-5g) was cut into fine pieces with a sterile razorblade and then ground to a fine powder under liquid nitrogen using a mortar and pestle. For the *S. normani* sample, the cortex was first separated from the choanosome, cleaned carefully with sterile artificial seawater and any remaining sediment from the surface was removed with a sterile razorblade. For the choanosome a cross section of the

ball shaped tissue was taken in order to include inner- and outer areas of the choanosome in the analysis. Choanosome and cortex were treated as a separate sample in all following steps including the sequence analysis. The powder was added to a Lysis buffer adapted from Brady (100 mM Tris, 100 mM EDTA, 1.5 M NaCl (w/v), 1% CTAB (w/v), 2% SDS (w/v); 5 ml buffer per 1 g sponge tissue; Brady, 2007) and incubated for 2 h at 70 ˚C. Metagenomic DNA was then extracted as described by (Kennedy et al., 2008). DNA solutions were analysed by gel electrophoresis, quantified by spectrophotometry (NanoDrop ND-1000-Wilmington, DE, USA) and then stored at -20°C.

PCR amplicon library preparation for pyrosequencing

PCR amplicon libraries of the V5-V6 region of 16S rRNA genes were prepared from all metagenomic DNAs. Universal primers U789f (5'-TAGATACCCSSGTAGTCC-3') and U1068r (5'-CTGACGRCRGCCATGC-3'), targeting both bacteria and archaea, were adapted for pyrosequencing by the addition of sequencing adapters and multiplex identifier (MID) sequences as per Supplementary Table 5.1. Each 50µl PCR reaction comprised 1X buffer, 0.2 mM dNTPs (both Fermentas, St. Leon-Rot, Germany), 0.1 µM of each primer (Sigma Aldrich, Arklow, Ireland), 2 U Taq polymerase (Fermentas), ~10 ng template DNA and dH_2O. PCR cycle conditions were as reported previously (Lee et al., 2011). To minimise PCR bias three individual reactions were performed per template and equimolar amounts of PCR products from each of the three reactions were pooled for pyrosequencing. PCR products were purified using Qiagen PCR Purification Kit (Qiagen Ltd., UK) as per the manufacturer's instructions. Barcoded samples were pooled and sequenced on a GS FLX Titanium platform (454 Life Sciences) at the University of Liverpool, Centre for Genomic Research, Liverpool, UK.

Pyrosequencing data analysis

Primer adapter and MID sequences were removed from all reads and remaining reads were filtered for quality using the Ribosomal Database Project (RDP) -Release 10.29, Pyrosequencing Pipeline (http://rdp.cme.msu.edu/). Reads with ambiguous bases 'N', average quality score <20 or shorter than 100 bp were discarded from further analysis. Individual sample libraries were aligned using the INFERNAL

aligner (Nawrocki et al., 2009). OTUs were determined using the RDP clustering tool (complete linkage clustering). .

Taxonomic classifications were determined using the 'Classifier' tool (naïve Bayesian rRNA classifier; (Wang et al., 2007)) at 50% confidence threshold by comparing to the database of 2 320 464 rRNA sequences. Rarefaction curves were generated from data obtained from the 'Rarefaction' tool; diversity indices (Shannon index & Chao1 species estimator) were obtained using the relevant tools at sequence similarities of 95% and 97%. Rank-abundance curves were derived from cluster analysis results. For the analysis of shared OTUs the sequence reads of interest were pooled and then aligned and clustered as a single file. Tagging of sequence read names with sample specific codes prior to pooling enabled the assignment of shared and non-shared OTUs later on. For example, an OTU containing reads from the cortex and the choanosome of *S. normani* was considered a shared OTU in regards to those two samples. The number of reads in an OTU derived from each sample could also be analysed. Obtained raw sequences were submitted to MG-Rast and have the MG-Rast Ids 4494748.3 (*L. diversichela*), 4494749.3 (*S. normani* choanosome), 4494750.3 (*S. normani* cortex) and 4494751.3 (Water sample) and are publicly available.

Preparation of Phylogenetic trees

Sequences were aligned using the aligner of the SILVA rRNA database project at http://www.arb-silva.de/aligner/ (Pruesse et al., 2012) and the two closest neighbours from the SILVA database were retrieved. Phylogenetic trees were then calculated in MEGA5 (Tamura et al., 2011) and closest BLAST neighbours were included into the alignment if necessary. Phylogenetic trees were calculated using Neighbour-joining (Tamura et al., 2004) and Maximum likelihood (Tamura and Nei, 1993) algorithms.

RESULTS

Sponge sampling

Two sponge samples and a water sample were obtained from a depth of 1348 m (Table 5.1). One of the sponge samples was identified as *L. diversichela*, the other sponge sample was identified as *S. normani* (Figure 5.1). The cortex and the choanosome of *S. normani* were processed as two separate samples in order to characterize the spatial distribution of bacteria in this sponge. The outer layer of the sponge was cleaned carefully with a sterile scalpel in order to remove any sediment attached to the sponge. This involved scraping off some of the tissue which was particularly heavily contaminated with sediment.

Table 5.1: Sampling and basic sequencing information of samples obtained during Biodiscovery cruise 2010 aboard *RV Celtic Explorer*.

Species	latitude	longitude	sampling depth [m]	quality filtered sequences	Average sequence length
Water	54.0580	12.5482	1348	8748	279
L.diversichela	54.0580	12.5482	1348	6108	281
***S. normani* choanosome**	54.0613	12.5517	1348	18171	282
***S. normani* cortex**	54.0613	12.5517	1348	8051	276

Initial analysis

A total number of 44,539 individual 16S rRNA sequence reads were obtained. After quality filtering in RDP, 8,748 (Water sample), 6,108 (*L. Diversichela*), 18,171 (*S. normani* choanosome) and 8,051 (*S. normani* cortex) sequences remained for the different samples (Table 5.1). The average sequence length of all quality filtered sequences was ~280 bp. The two samples derived from the sponge *S. normani* contained the highest diversity of associated microbes, indicated by their richness estimators (Chao and H') whereas the *L. diversichela* sample harboured a lower prokaryotic diversity than all other samples (Table 5.2). The rarefaction curve of the microbial associates in the choanosome of *S. diversichela* does not reach a plateau indicating an undersampling of the biodiversity. The rarefaction curves of the other samples approach an asymptote thus indicating that the biodiversity is well

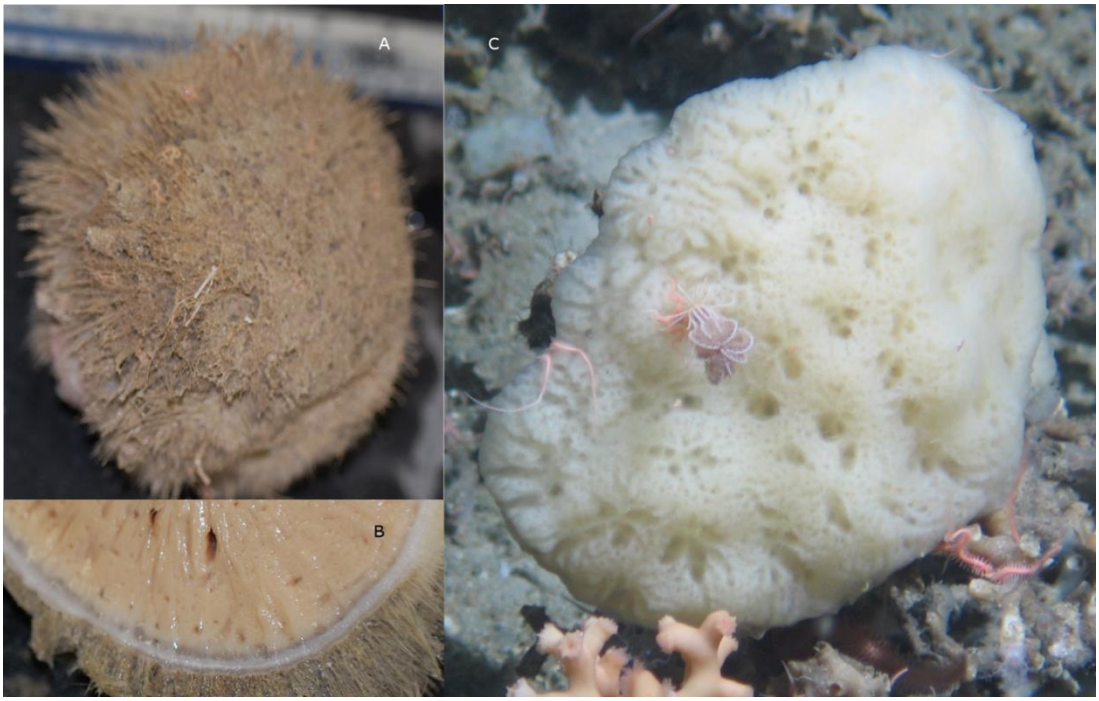


Figure 5.1: Pictures of sampled sponges.

A: *S. normani*; B: Cross section of *S. normani* showing the choanosome and the cortex. The sediment contaminated papillae were scraped off prior to DNA-extration; C: *L. diversichela* prior to collection.

represented by the obtained sequences (Figure 5.2). The rank abundance curves at 95% sequence similarity (Figure 5.3) also show that the community of the sponge *L. diversichela* is dominated by a relatively small number of OTUs, demonstrated through the steepest slope. The communities of the other samples appear to be more evenly distributed with the choanosome of *S. normani* having the most even distribution.

Table 5.2: Diversity estimators of the samples under investigation.

	3%			5%		
Sample	**OTUs**	**Chao**	**H'**	**OTUs**	**Chao**	**H'**
Water	599	1003	4.06	408	599	3.10
L. diversichela	302	574	2.61	219	369	1.20
***S. normani* choanosome**	5643	13623	7.24	3743	5816	6.56
***S. normani* cortex**	991	2515	5.13	718	1682	4.18

Obtained OTUs, Chao1 estimates and Shannon indices (H'), calculated for 95 % and 97 % sequence similarity, show the differences of the microbial diversity in the different samples.

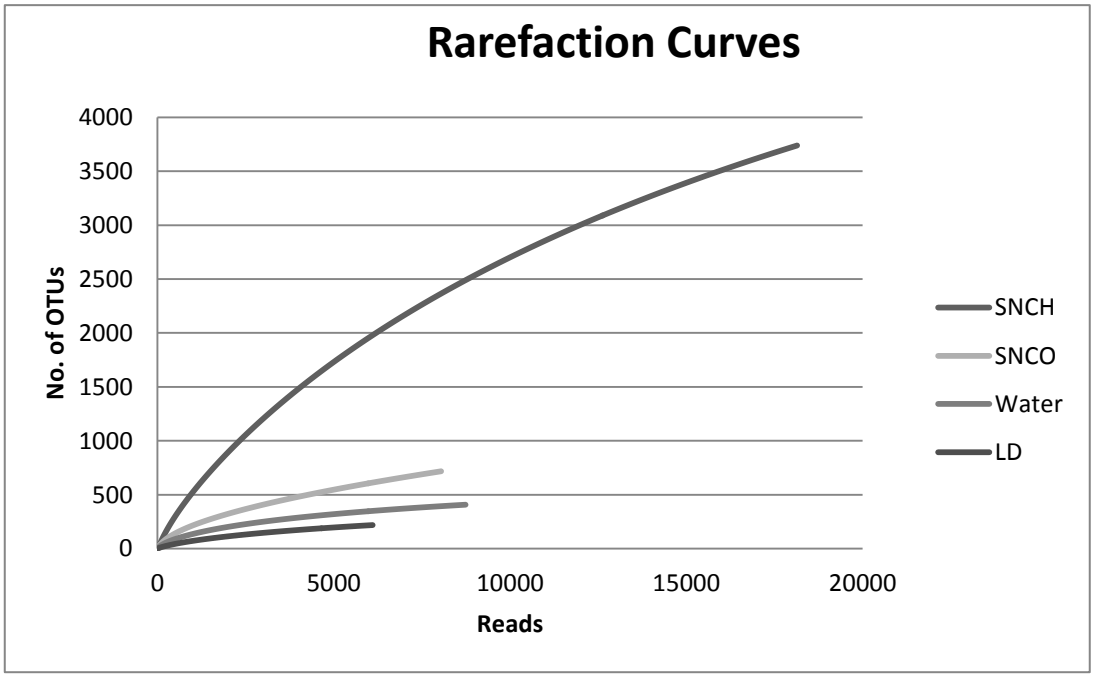


Figure 5.2: Rarefaction curves

Shown are rarefaction curves for all samples at 95 % sequence similarity. SNCH = *S. normani* choanosome, SNCO = *S. normani* cortex, LD = *L. diversichela*

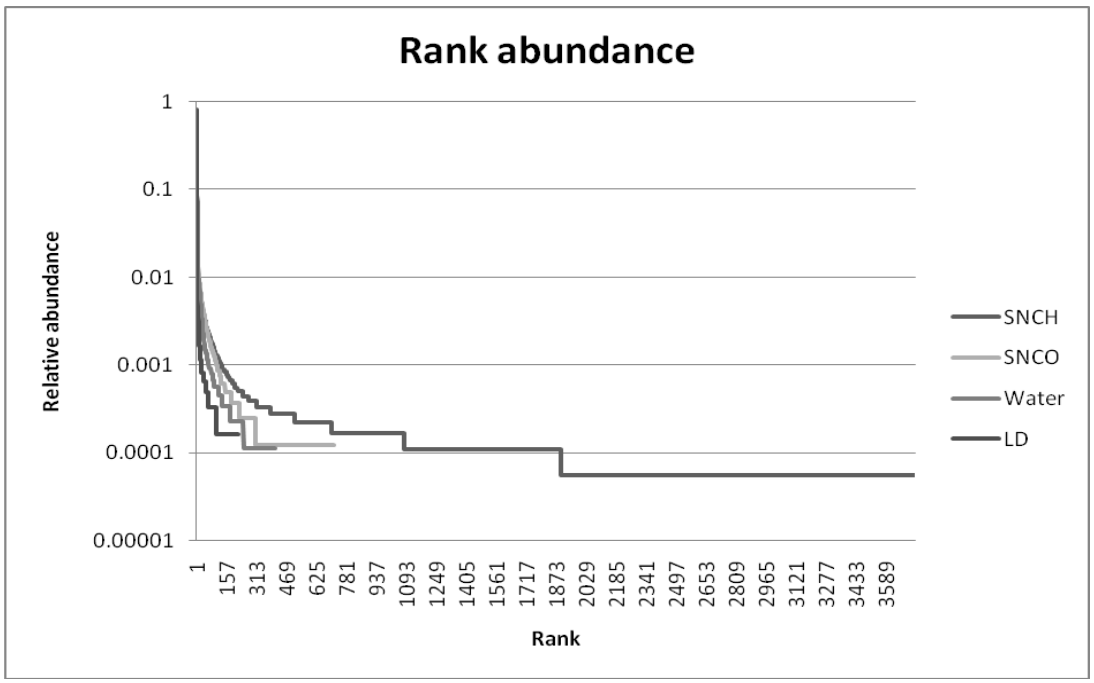


Figure 5.3: Rank abundance curves

Shown are rank abundance curves for all samples at 95 % sequence similarity. SNCH = *S. normani* choanosome, SNCO = *S. normani* cortex, LD = *L. diversichela*

Bacterial vs. archaeal diversity

The relative abundance of archaea in the samples ranged from 4.0% (*L. diversichela*) up to 38.8% (water sample). Archaea were more often found in the cortex of *S. normani* (25.1% relative abundance) than in the choanosome (9.8%) (Figure 5.4). In all samples, bacterial reads made up most of the remaining proportion and thus were the dominant group of microbial organisms. A negligible amount of sequencing reads could not be classified into either of the two prokaryotic domains of life.

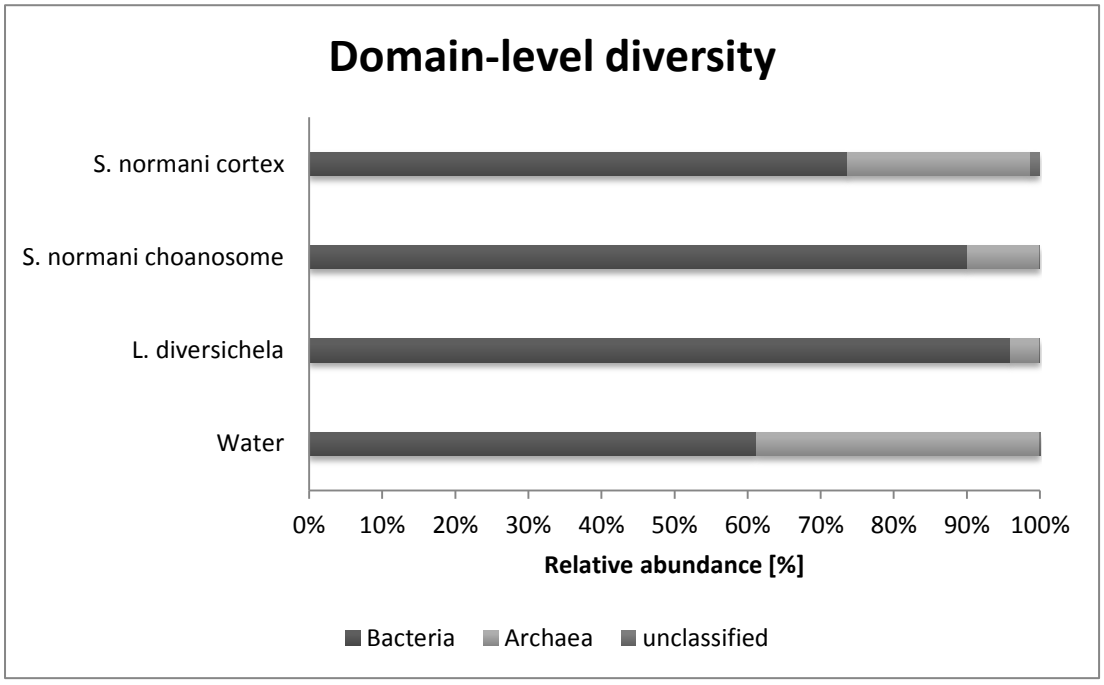


Figure 5.4: Prokaryotic domain level diversity

The diversity was determined through classification of sequences with the RDP classifier tool at 50 % confidence threshold.

Archaeal diversity

In each sample the two archaeal phyla *Euryarchaeota* and *Crenarchaeota* were detected. The three sponge samples were dominated by the *Crenarchaeota* (85-93% of all archaeal reads belonged to this phylum), whereas the *Euryarchaeota* only contributed 1-2.5 %. All crenarchaeal reads were classified as *Thermoprotei*, but the vast majority of the sequences (99-100% depending on the sample) could not be classified to lower taxonomic levels (Figure 5.5). Some sponge-derived sequences could be identified as *Desulfurococcales* or *Desulfurococcacaea* (in both samples of

S. normani but not in *L. diversichela*) and some sequences were classified as *Thermofilum* (only in the cortex of *S. normani*). The sponge-derived euryarchaeal reads could not even be classified on class-level with only a few exceptions, those being classified as *Methanomicrobia* (in both samples of *S. normani* but not in *L. diversichela*) or *Methermicoccus* (genus-level). The *Crenarchaeota* also contribute the highest proportion of reads in the water sample (47.7% of all archaeal reads) but the *Euryarchaeota* take up a much more prominent role (32.5%) than in the sponge.

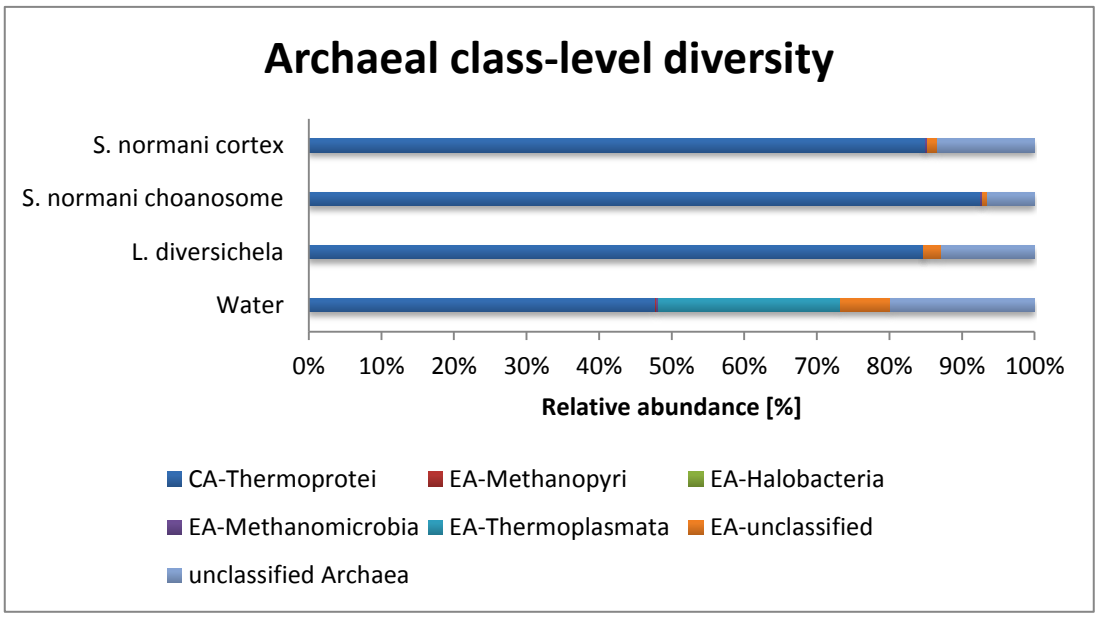


Figure 5.5: Archaeal diversity of all samples on the class level

The diversity was determined with the RDP classifier tool at 50 % confidence threshold. Relative abundances are given as proportions of all archaeal sequence reads of the respective sample. CA = *Crenarchaeota*, EA = *Euryarchaeota*

Again the *Thermoprotei* make up 100% of the crenarchaeal reads with only a few sequences classified down to order (*Desulfurococcales*) and genus level (*Caldisphaera*). Whereas 74.8% of the euryarchaeal reads were classified as *Thermoplasmata*, a class absent from sponge derived reads, 21.4% could not be classified to lower taxonomic levels than phylum, and the remaining 3.7% were classified into the classes *Methanopyri*, *Halobacteria*, and *Methanomicrobia* (Figure 5.5). Again few reads could be classified into lower taxonomic levels. Of the *Thermoplasmata* reads some were classified as *Ferroplasmaceae*, *Thermogymnomonas* and *Thermoplasma*. The *Methanopyri* reads were classified as

Methanopyrus, the *Halobacteria* as *Halobacteriaceae* and some *Methanomicrobia* reads were classified as *Methermicoccus*.

Bacterial diversity

As stated earlier, the dominant prokaryotic domain in all samples were the bacteria with 61.2% (water sample) to 96.0% (*L. diversichela*) of all sequences belonging to this domain (Figure 5.4). A very high proportion of unclassified bacterial sequences was obtained from the two *S. normani* samples (48.0 % of all bacterial reads for the cortex and 63.0 % for the choanosome); lower levels of unclassified sequences were found for water (7.4%) and *L. diversichela* (0.5%) derived reads. The choanosome of *S. normani* gave rise to sequences classified into 14 bacterial phyla followed by the cortex of the same sponge (11 phyla), the water (11) and the *L. diversichela* (9) sample (Figure 5.6). The communities of both the water and the *L. diversichela* sample were strongly dominated by the *Proteobacteria* (87.2% and 96.3% of all bacterial reads, respectively). Of the other phyla, only the *Acidobacteria* and the *Planctomyces* contributed more than 1% of all bacterial sequences in the water sample (1.7% and 1.3%, respectively). In *L. diversichela* only the *Planctomyces* made up more than 1% of all bacterial sequences (1.4%). Whereas *Proteobacteria* again featured heavily in both samples from *S. normani* (13.8% of all bacterial sequences for the choanosome, 26.9% for the cortex), other phyla also played an important role. These phyla were the *Acidobacteria* (13.7% and 13.6%), *Actinobacteria* (2.1% and 4.2%), *Chloroflexi* (4.0% and 2.9%) and *Firmicutes* (2.5% and 1.8%). The more rare bacterial community, comprising a combined proportion of less than 1% of all bacterial reads, consisted of the phyla *Deinococcus-Thermus, Bacteroidetes, Verrucomicrobia, Spirochaetes, Planctomyces, Aquificae, Thermotogae* and *TM7*. However, the single most dominant group comprised of the unclassified sequences (63.0% for the choanosome and 48.0% for the cortex).

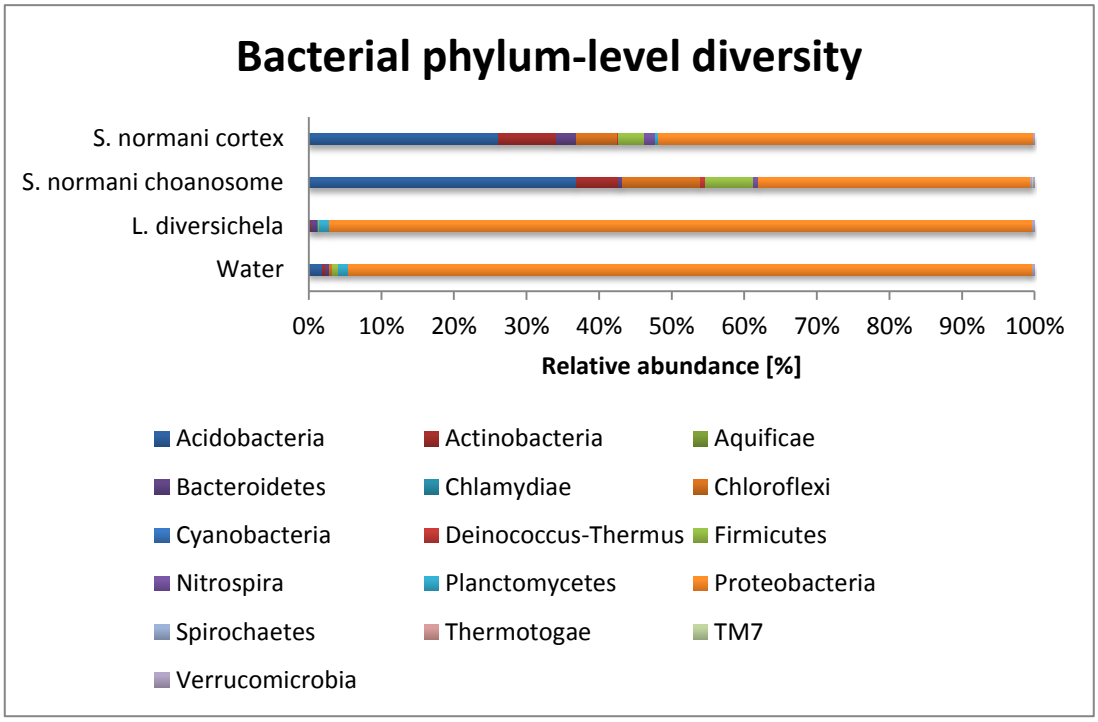


Figure 5.6: Diversity of classified bacteria on the phylum level.

Relative abundances are given as proportions of all classified bacterial sequence reads of the respective sample. Sequences not classified at bacterial phylum level make up 63.0% (*S. normani* choanosome), 48.0% (*S. normani* cortex), 0.5% (*L.diversichela*) and 7.4% (water sample) but are not included in the chart.

Classification into lower taxonomic levels

The sequences classified at bacterial phylum level were then further classified where possible, to genus level. In the sponge *L. diversichela* the dominant phylum of the *Proteobacteria* is itself dominated by the γ-*Proteobacteria*. They make up more than 93% of all bacterial reads. Most of the bacterial reads from this sponge were classified as unclassified γ-Proteobacteria (17.2%), *Granulosicoccus* (36.1%) and unclassified *Chromatiales* (38.7%). The analysis of the dominant OTUs at 95% sequence similarity revealed that the most dominant OTU comprised of 85.9% of all reads from this sponge and a representative sequence of this OTU was classified as *Granulosicoccus* by RDP classifier. A BLAST search of this sequence found it to be most closely related to an uncultured *γ-Proteobacterium* (96% sequence similarity) derived from a clone library of two *Xestospongia* sponge species (Montalvo and Hill, 2011). The closest cultured relatives are unclassified sulphur-oxidizing *γ-Proteobacteria* derived from gutless marine worms which are nutritionally dependent on their microbial endosymbionts (Thornhill et al., 2008) or marine clams

(Fujiwara et al., 2009). Another OTU, which comprised 1.1% of all reads, was classified into the same group. The relationship is highlighted in the phylogenetic tree shown in Figure 5.10 where the two OTUs are represented by the sequences HLJF0EA02EAR7L and HLJF0EA02EIE0K. Thus the vast majority of the microbial population of this sponge is dominated by organisms belonging to one bacterial genus of the proteobacterial order *Chromatiales*, potentially being involved in sulphur-oxidation. Some other relatively often encountered taxa were *Blastospirellula* and *Phycisphaera*, two genera of the phylum *Planctomycetes* (both 0.4% of all bacterial reads) and *α-Proteobacteria* (0.8%; ~ 1/3 of them were classified as *Rhodospirillales*).

In the water sample, γ-*Proteobacteria* also make up the vast majority of the proteobacterial reads and therefore are the single most dominant class in the bacterial community of this sample. The classification down to the genus level shows, that the community is dominated by the γ-proteobacterial genera *Pseudoalteromonas* (70.2% of all bacterial reads), *Psychrobacter* (1.6%), *Cobetia* (1.5%) and *Vibrio* (0.8%) none of which were found in the sponge samples (except for a tiny fraction of *Psychrobacter* reads in the *L. diversichela* sample). Only few other taxa appear to play a major role in this sample, namely the α-proteobacterial order *Rhodospirillales* (1.6%), some unclassified *Proteobacteria* (5.6%), γ-*Proteobacteria* (2.2%), α-*Proteobacteria* (0.8%) and *Acidobacteria* (0.7%) and the acidobacterial genus *Gp6* (0.7%).

The dominant taxa in the choanosome and cortex of *S. normani* were unclassified *Proteobacteria* (5.7% of all bacterial reads in the choanosome; 14.2% in the cortex), *Acidobacteria* (5.4%; 4.8%), *Chloroflexi* (3.8%; 2.8%) and γ-*Proteobacteria* (3.3%; 6.6%). The acidobacterial genera *Gp6* (2.9%; 2.3%), *Gp21* (2.1%; 1.8%), *Bryobacter* (1.1%; 1.0%) and *Gp11* (0.9%; 0.8%) all featured heavily in this sponge. Members of the *Firmicutes* order *Clostridiales* (1.4%; 0.6%) and the α-proteobacterial family *Rhodospirillaceae* (0.9%; 1.2%) were also found relatively often. Whereas most of the dominant taxa were found in approximately equal abundances in both the cortex and the choanosome, two genera made a notable exception; the actinobacterial genus *Lamia* (0.2% of all bacterial reads in the choanosome; 2.6% in the cortex) and the acidobacterial genus *Gp10* (0.3 %; 1.9%) were found much more often in the cortex than in the choanoderm.

The classification of all bacterial reads down to the order level is shown in Figure 5.7. A full classification down to genus level (wherever possible) of all bacterial and archaeal reads can be found in Supplementary Table 5.2.

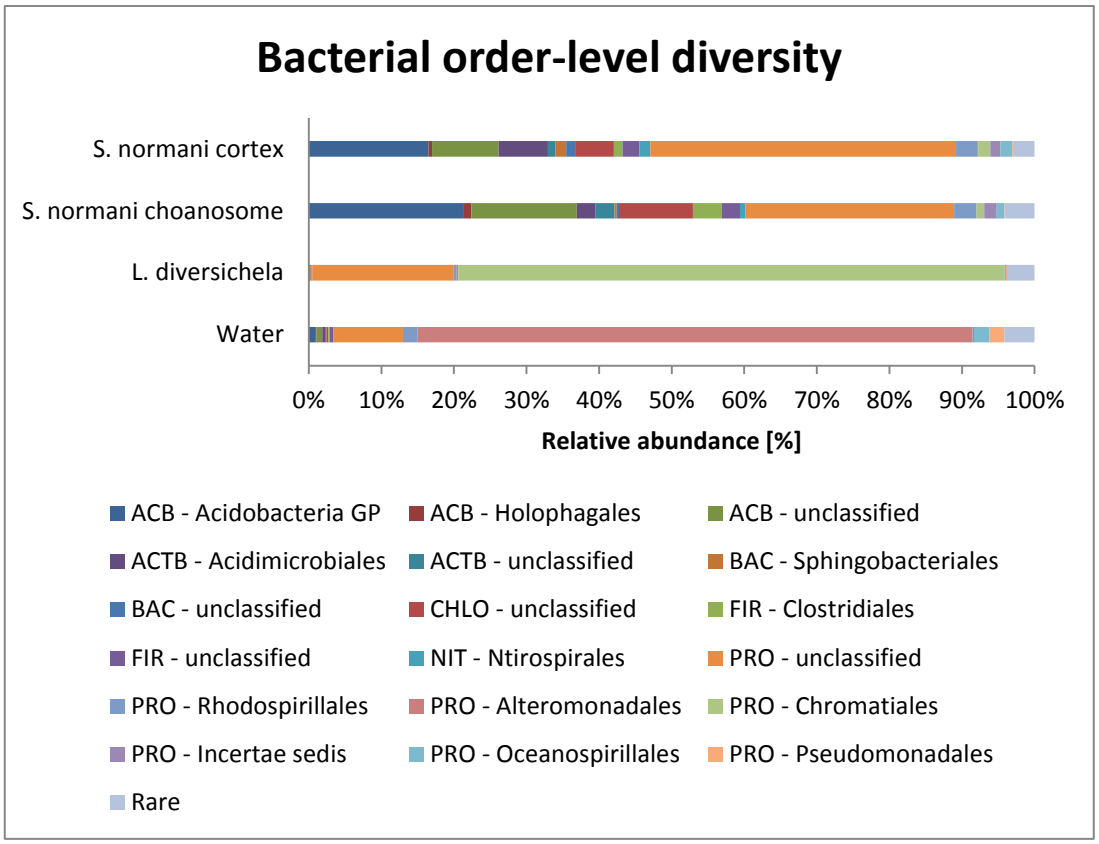


Figure 5.7: Classification of bacterial sequencing reads to order level.

Relative abundances are given as proportions of all classified bacterial sequence reads of the respective sample. Sequences not classified at bacterial phylum level make up 63.0% (*S. normani* choanosome), 48.0% (*S. normani* cortex), 0.5% (*L.diversichela*) and 7.4% (water sample) but are not included in the chart. ACB = *Acidobacteria*, ACTB = *Actinobacteria*, BAC = *Bacteroidetes*,CHLO = *Chloroflexi*, FIR = *Firmicutes*, NIT = *Nitrospira*, PRO = *Proteobacteria*

The extraordinarily large amount of unclassified sequence reads for the two samples from *S. normani* significantly hampered the analysis of the community structure of this sponge and the comparability with other sponge samples. In order to gain more insights into the bacteria associated with the studied sponges OTUs shared between samples were analysed as described in Material and Methods. This analysis was carried out for 95% and 97% sequence similarity OTUs, but since the trends observed were similar; only values for 95% sequence similarity are presented. The statistical analysis revealed striking differences in the community structure of the different samples (Table 5.3). Additionally, representative sequences of the most

dominant OTUs of each sample were submitted to a BLAST search. A total of 59 OTUs was analysed in this way, 21 OTUs from the *S. normani* choanosome (representing 29.5% of all sequence reads from this sample), 21 from the *S. normani* cortex (62.4%), 10 from *L. diversichela* (91.7%) and 22 from the water sample (82.5%); (numbers do not add up because of overlap between samples). Supplementary Table 5.3 gives a summary of this analysis including the closest neighbours obtained by the BLAST search for each OTU. Phylogenetic trees showing the evolutionary relationship between these abundant OTUs can be found in Figures 5.8-5.11. The phyla *Acidobacteria* and *Chloroflexi* are shown in Figures 5.8 and 5.9, respectively. Mainly OTUs comprising sequence reads from *S. normani* (choanosome and cortex) are present in those phyla because they were not abundant in the other two samples. The phylogenetic tree in Figure 5.10 represents abundant proteobacterial reads and Figure 5.11 deals with all other detected bacterial phyla and the *Archaea*. The OTUs from this study are highlighted. Closed and open circles represent OTUs which have the highest relative abundance in the choanosome and cortex of *S. normani*, respectively; closed triangles represent water derived OTUs and closed squares represent *L. diversichela* derived OTUs. The closest neighbours obtained by a BLAST search and from the "nearest neighbour" option in SILVA (www.arb-silva.de/aligner/) were included in the trees. The source of each sequence was added to the name. In this way it becomes clear that most dominant water and *L. diversichela* OTUs are closely related to deep water derived sequences whereas most dominant *S. normani* OTUs (both choanosome and cortex) are closely related to sponge-derived sequences. However, as mentioned before, the single most dominant OTU from *L. diversichela* is most closely related to an uncultured *γ-Proteobacterium* derived from a *Xestospongia testudinaria* sponge sample, implying a close relationship of this group of organisms with sponges.

Table 5.3: Comparison of shared OTUs between deep water samples.

Compared samples		Shared OTUs	Shared reads	% of reads in	
Sample 1	Sample 2	[%]	[%]	sample 1	sample 2
SNCH	**SNCO**	12.8	73.8	64.3	95.4
SNCH	**LD**	0.4	1.6	2.1	0.4
SNCH	**W**	0.8	3.9	5.3	1.2
SNCO	**LD**	2.2	2.9	2.7	3.1
SNCO	**W**	3.4	15.9	18.0	14.0
LD	**W**	17.1	24.5	8.4	35.8

Shown are the shared OTUs and the shared reads between two samples. Each of the possible 6 combination of samples is shown. In column 1 and 2 the two samples which are compared are shown, column 3 denotes the relative abundance of shared OTUs, columns 4 the relative abundance of shared reads and columns 5 and 6 return the distribution of the reads among the two compared samples. SNCH: *S. normani* choanosome, SNCO: *S. normani* cortex, LD: *L. diversichela*, W: Water.

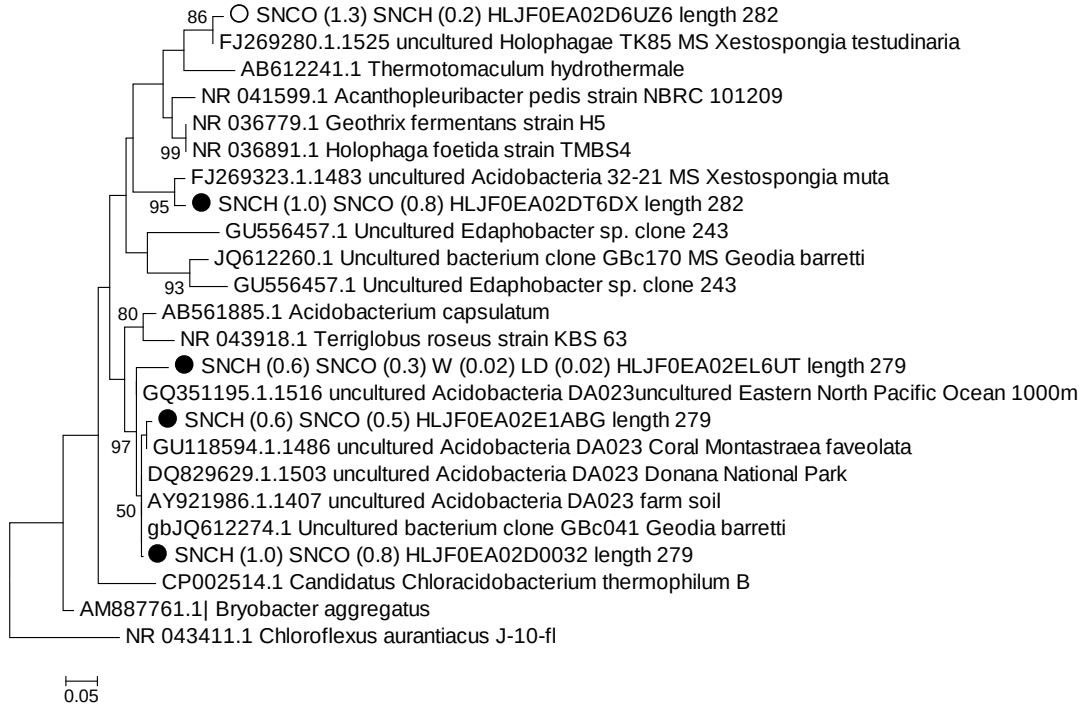


Figure 5.8: Phylogenetic tree (maximum likelihood) with dominant acidobacterial OTUs

OTUS are derived from the analysis of shared OTUs of the deep sea samples. Relative abundances in percentage of all reads for each OTU in each sample are shown in brackets behind the sample acronym. Additionally, the source organism/ environment was added to each reference sequence. MS: marine sponge, SNCH: *S. normani* choanosome, SNCO: *S. normani* cortex; LD: *L. diversichela*; W: Water; closed circle: reads from this OTU were found predominantly in SNCH; open circle: OTU predominantly in SNCO.

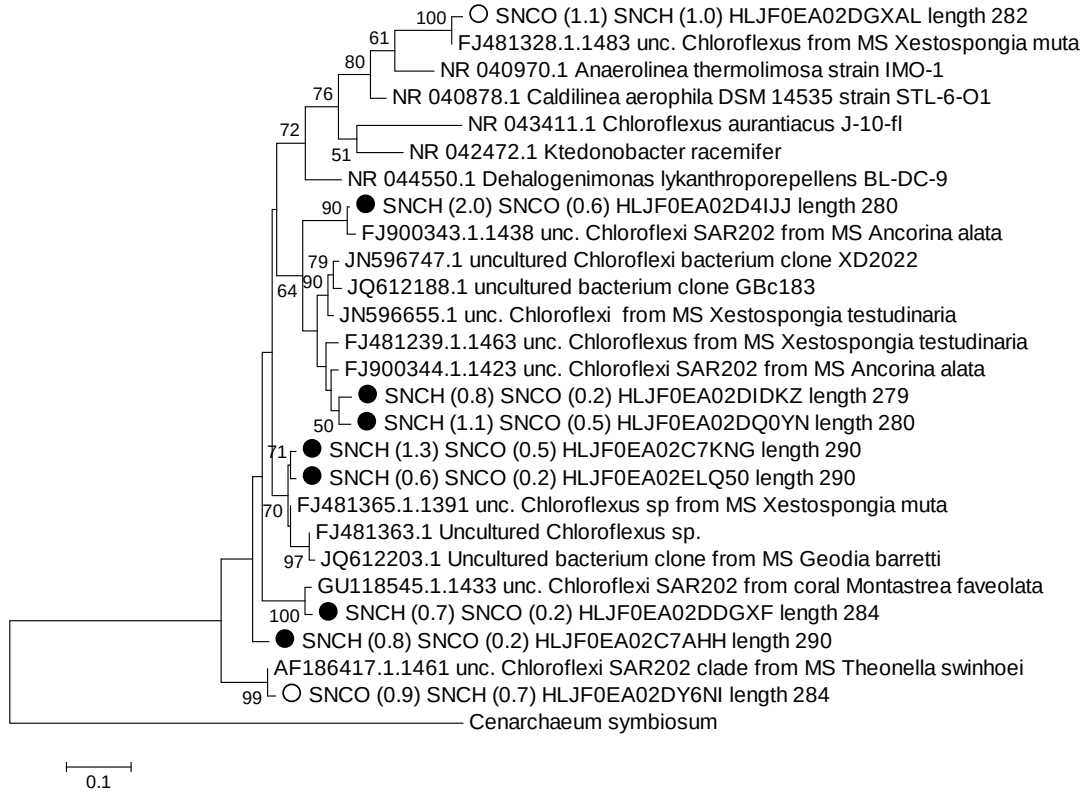


Figure 5.9: Phylogenetic tree (maximum likelihood) with dominant *Chloroflexi*-OTUs

OTUs are derived from the analysis of shared OTUs of the deep sea samples. Relative abundances in percentage of all reads for each OTU in each sample are shown in brackets behind the sample acronym. Additionally, the source organism/ environment was added to each reference sequence. MS: marine sponge, SNCH: *S. normani* choanosome, SNCO: *S. normani* cortex; LD: *L. diversichela*; W: Water; closed circle: reads from this OTU were found predominantly in SNCH; open circle: OTU predominantly in SNCO.

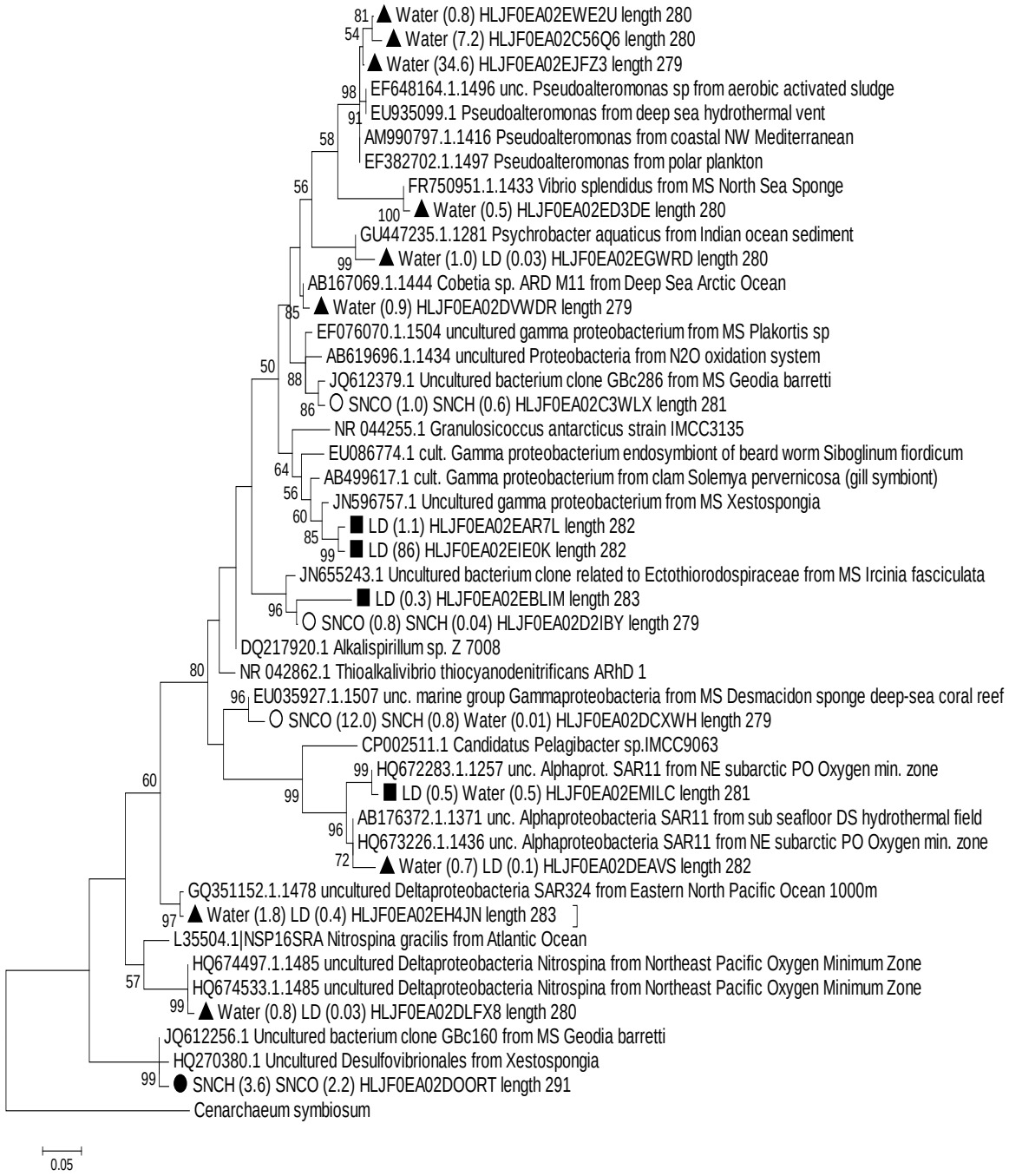


Figure 5.10: Phylogenetic tree (maximum likelihood) with dominant proteobacterial OTUs

OTUs are derived from the analysis of shared OTUs of the deep sea samples. Relative abundances in percentage of all reads for each OTU in each sample are shown in brackets behind the sample acronym. Additionally, the source organism/ environment was added to each reference sequence. MS: marine sponge, SNCH: *S. normani* choanosome, SNCO: *S. normani* cortex; LD: *L. diversichela*; W: Water; closed circle: reads from this OTU were found predominantly in SNCH; open circle: OTU predominantly in SNCO; closed triangle: OTU predominantly in W; closed quadrangle: OTU predominantly in LD.

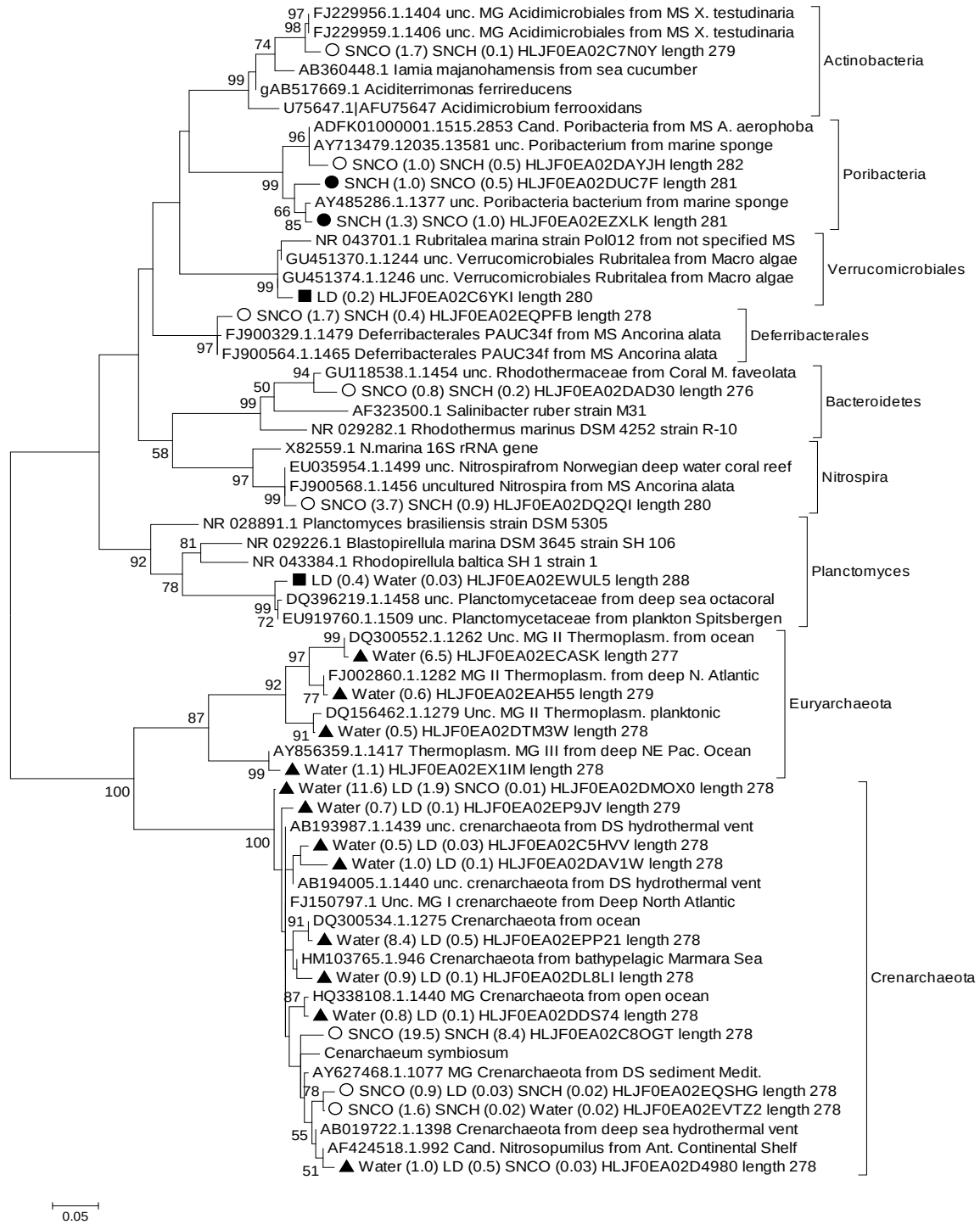


Figure 5.11: Phylogenetic tree (maximum likelihood) with all other dominant OTUs

OTUs are derived from the analysis of shared OTUs of the deep sea samples. Relative abundances in percentage of all reads for each OTU in each sample are shown in brackets behind the sample acronym. Additionally, the source organism/ environment was added to each reference sequence. MS: marine sponge, SNCH: *S. normani* choanosome, SNCO: *S. normani* cortex; LD: *L. diversichela*; W: Water; closed circle: reads from this OTU were found predominantly in SNCH; open circle: OTU predominantly in SNCO; closed triangle: OTU predominantly in W; closed quadrangle: OTU predominantly in LD.

DISCUSSION

Marine sponges have long been known to harbour a wide range of microbes. Whereas the microbial community of shallow water sponges has been studied extensively, very little is known about the microbes associated with deep water sponges, especially from the bathypelagic and deeper zones. By applying the 454 Pyrosequencing approach to assess the prokaryotic community of two deep-water sponge samples this study sheds light on this aspect of sponge-microbe associations.

Bacterial community

The sponges under investigation harbour very distinct bacterial communities. The sponge *L. diversichela* harbouring 2 archaeal and 9 bacterial phyla, was dominated by few organisms and had the lowest species richness estimates of all samples in this study. Most interestingly, the prokaryotic community of this sponge was dominated by one OTU at 95 % sequence similarity which comprised 85.9 % of all sequence reads and was classified as *Granulosicoccus* (order *Chromatiales*) by the RDP classifier at a 50 % confidence threshold. It was most closely related to an uncultured γ-*Proteobacterium* derived from a shallow water sponge, with 96 % sequence similarity. The presence of *Chromatiales* representatives has been shown before by other high-throughput studies of sponge associated microbes (Jackson et al., 2012; White et al., 2012), but the organisms found belonged to the family *Ectothiorodospiraceae.* Since most members of the *Ectothiorodospiraceae* belong to the phototrophic purple-sulfur bacteria it is not surprising that instead non-phototrophic representatives of this order were found in a habitat of complete darkness. However, the family *Granulosicoccaceae* has only been described recently (Lee et al., 2007) and so far comprises 2 species (Kurilenko et al., 2010). Interestingly, the two known species are both strictly aerobic (in contrast to the anaerobic or microaerophilic purple-sulfur bacteria of this order), implying that they are more likely to reside in compartments of the sponge which are more exposed to oxygen. Additionally, no *Granulosicoccus* reads were found in the water sample indicating a very close relationship of this group, which comprises more than 85% of all sequencing reads, with the sponge sample.

In comparison to *L. diversichela,* the community of the sponge *S. normani* has a very different profile. It is much more diverse with 14 bacterial phyla being detected in the combined dataset of cortex and choanosome, a much higher number of OTUs were found, together with much higher species richness estimators and a more even distribution of the community as shown by the rank abundance curves (Table 5.2, Figures 5.2 and 5.3, Figure 5.6). In fact, of the published high-throughput studies of sponge associated microbes to date, the *S. normani* choanosome sample has one of the highest recorded diversities as seen by the Chao1 estimator and the Shannon index. The next striking feature in comparison to the other samples in this study and other 454 based investigations of sponge associated microbes is the large amount of unclassified bacterial sequence reads with 56.8 % (choanosome) and 35.4 % (cortex) of all reads being classified as bacteria but no further (3791 and 470 OTUs at 97 % sequence similarity, respectively). Very high numbers of unclassified reads have also been encountered during a survey of Caribbean sponges (White et al., 2012), when ~36% of all reads, but comprising less than 200 OTUs, were not classified to the phylum level. Other studies reported a lower but still significant abundance of unclassified bacteria (Jackson et al., 2012; Lee et al., 2011; White et al., 2012), comprising less than 10% of the obtained reads. The huge number of unclassified reads (and OTUs) indicates a bacterial community that is to large extent novel and highlights this sponge species as particularly interesting for further studies. In this regard it is also worth noting that the sequence reads from the *L. diversichela* sample were classified to almost 100% thus the method applied to qualify and classify sequences does not seem to be the reason for the low percentage of classified reads. The RDP classifier has also been shown by others to be a valid method for classifying large amounts of sequence reads (Jackson et al., 2012; Lee et al., 2011). The combined relative abundances of the bacteria also differed from the other sponge sample (*L. diversichela)* and the water sample on the phylum level as the phyla *Acidobacteria, Chloroflexi, Firmicutes, Actinobacteria, Nitrospira* and *Bacteroidetes* featured more heavily in this sponge sample but the phylum *Proteobacteria* was less abundant. All named phyla have been consistently found as important members of sponge microbial communities in the aforementioned 454 sequencing studies of shallow water sponges and they have also been recorded in a comprehensive review of sponge derived sequences (Simister et al., 2012). Furthermore, representatives of all these phyla were linked to sponge specific

clusters, some more often (*Acidobacteria*, *Actinobacteria*, *Chloroflexi*, *Nitrospira*, proteobacterial classes) than others (*Bacteroidetes*, *Firmicutes*). The finding of 9 sequencing reads classified as *Thermotogae* in the choanosome was in need of more careful investigation. This phylum has previously been reported to be potentially present in sponges (Webster et al., 2010) but upon closer investigation the relevant sequencing reads turned out to belong to the phylum *Chloroflexi*. In the most recent 454 sequencing effort of sponge associated microbes (Trindade-Silva et al., 2012), *Thermotogae* were also claimed to be present at very low abundances. The nine reads from our study were submitted to a BLAST search and showed similarities of 88-93 % to uncultured *Chloroflexi* sequences, thus the presence of *Thermotogae* can not be claimed unequivocally. Bacterial phyla found relatively often in previous studies but not in this study include *Gemmatimonadetes* and *Cyanobacteria*. The lack of *Cyanobacteria* is, similar to the purple-sulfur bacteria, to be expected since no light is present below ~1.000 m. The candidatus phylum *Poribacteria* was not

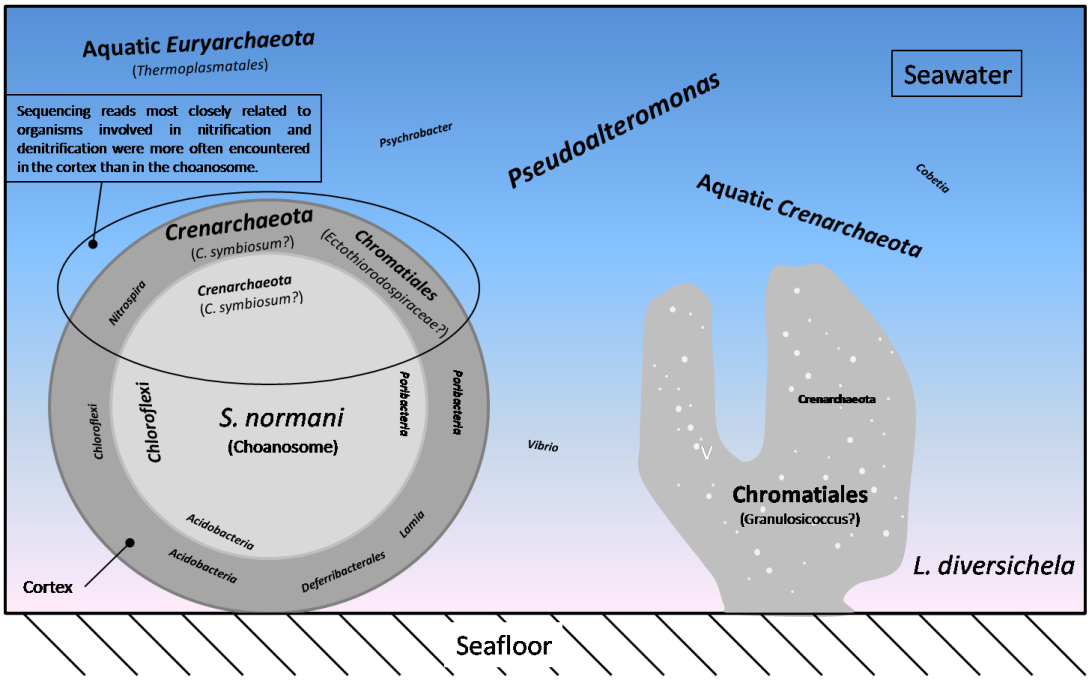


Figure 5.12: Schematic illustration of the microbial diversity detected in this study.

Shown is the distribution of microorganisms in the different habitats investigated. The most dominant organisms in the water sample were *Pseudoalteromonadaceae* and *Archaea*, *L. diversichela* hosted mainly *Chromatiales* and *S. normani* had a very diverse, spatially distributed set of microbial associates. The relative abundances of the detected organisms are roughly reflected by the font size.

found using the RDP classifier, but when representative sequences of dominant OTUs were submitted to a BLAST search, 3 OTUs were most closely related to poribacterial sequences (Figure 5.11). A schematic of the microbial diversity detected in the different samples of this study can be found in Figure 5.12.

Since most of the dominant bacterial orders present in the water sample were only seldomly detected in the sponge samples and vice versa, the bacterial associates of the sponges appear to have a close relationship with the sponges rather than being just transient.

Archaeal community

Members of the domain *Archaea* have previously been shown to be associated with marine sponges (Han et al., 2012; Margot et al., 2002; Pape et al., 2006; Preston et al., 1996). Similar to bacteria associated with sponges, sponge specific clusters as well as vertical transmission has been demonstrated (Sharp et al., 2007; Simister et al., 2012). Even evidence for the spatial distribution of *Archaea* has been found, with *Archaea* specific to the pinacoderm (Webster et al., 2001), collagen fibres (Margot et al., 2002), the sponge mesohyl (Pape et al., 2006) and inner/ outer areas of the sponge (Meyer and Kuever, 2008) being reported. Han *et al.* also reported that ammonia oxidizing genes found in a *Phakiella* sponge from China belonged exclusively to archaeal populations (Han et al., 2012), highlighting their potential role in nitrification, which is possibly beneficial for the sponge (Meyer and Kuever, 2008). The first pyrosequencing study targeting archaeal (and bacterial) diversity in marine sponges (Lee et al., 2011) revealed a higher archaeal diversity than was previously thought, with richness estimators of ~300 archaeal OTUs for one of the studied sponges. Additionally, *Crenarchaeota* were reported to be dominant in sponges whereas *Euryarchaeota* were more often found in the studied water samples.

The findings of this study concur with most of the previously reported characteristics of sponge-archaea associations. Of all sequence reads from the samples, approximately 4% (*L.diversichela*) to 40% (water sample) were classified as *Archaea*. Thus the *Archaea* are abundant in the samples but bacteria are the dominant prokaryotic domain. This is similar to the data from Lee and co-workers (Lee et al., 2011) but the levels of archaeal abundances are lower than reported by

Pape (Pape et al., 2006). A spatial distribution of archaeal reads was also observed, with two dominant OTUs being mainly present in the cortex of *S. normani* (Supplementary Table 5.3; OTUs 361 and 362). It was also noted that almost all archaeal reads retrieved from sponges belong to the phylum *Crenarchaeota* (mainly of the class *Thermoprotei*), whereas water-column derived reads were more often classified as *Euryarchaeota* (mainly of the order *Thermoplasmatales*). Contrastingly, Lee et al. reported *Euryarchaeota* being almost exclusively present in water samples and found higher levels of archaeal diversity in the sponge samples than in the water samples.

Analysis of shared OTUs

Only 5 OTUs were shared between all samples (representing 0.9 % of all sequencing reads in this sampling group) and 14 OTUs were shared between all sponge samples (1.8 %) highlighting the large differences of the communities. This corresponds well with the findings of Schmitt and colleagues (Schmitt et al., 2012b) that only a very small amount of OTUs, the "core" community, are shared between different sponge samples. The communities of the two *S. normani* samples (choanosome and cortex) are clearly more closely related to each other than to the water and *L. diversichela* samples. It is also noteworthy that the cortex has more common OTUs with the water sample than has the choanosome and that the sponge *L. diversichela* is much more closely related to the water sample, than are both other sponge samples and that it is also much more closely related to the water than to the other two sponge samples. Interestingly, the majority of the sequences from the *S. normani* sample are most closely related to GenBank entries derived from other sponge samples indicating their possible placing into sponge specific clusters (Simister et al., 2012). Contrastingly, many of the sequences derived from *L. diversichela* are closely related to GenBank entries derived from water samples.

It is conceivable that the dominant microorganism associated with this particularly porous and thus water-permeable sponge is apparently related to an aerobic microbe (*Granulosicoccus*), because oxygen depleted regions will be less prominent than in more solid sponges such as *S. normani* (see Figure 5.1). The dominance of this OTU and the fact that the closest relatives in a BLAST search were from sponge derived microbes is evidence of a symbiotic relationship between sponge and microorganism. When, in a community, which shares many OTUs with the

surrounding seawater, the dominant taxon is not found in the water sample it is very likely that this organism has a symbiotic relationship to the sponge. The more solid sponge *S. normani* and the discrete tissue sections present in the sponge makes it more likely to present a distinct ecological habitat to its associated microbes, especially in terms of oxygenation (Hoffmann et al., 2005). The abundance of anaerobic microorganisms such as *Clostridia* and sulphate reducing bacteria (*Desulfovibrio*) as well as sequences most closely related to sponge-derived GenBank entries fit well with this observation.

Whereas no reads were classified as *Poribacteria* by the RDP classifier, 3 of the dominant OTUs from *S. normani* (both cortex and choanosome) were found to belong to this candidate phylum by BLAST search (Supplementary Table 5.3) and phylogeny (Figure 5.11). The OTUs comprised 2.8% of all sequences from the choanosome of *S. normani* and 2.5% of sequences from the cortex, but the proportion could be much higher considering that only OTUs representing 31.8% of all choanosomal and 66.4% of all cortex derived sequence reads were analysed in this way. Thus the number of phyla associated with this sponge must be raised to at least 15 but it is likely that more phyla were missed and remained hidden in the unclassified reads.

Spatial distribution of sponge-associated microbes in *S. normani*

Even though the choanosome and cortex sample of *S. normani* showed a broadly similar microbial community structure, with 64% (choanosome) and 95% (cortex) of reads belonging to shared OTUs (Table 5.3), notable differences could also be observed. The difference in their relation to the water sample has been shown by comparing the shared OTUs of the samples. More strikingly, considering the classifications by RDP, the pattern of relative abundance was often A) Choanosome>Cortex>Water or B) Choanosome<Cortex>Water. The observance of such patterns shows a spatial distribution of some taxa associated with the sponge. When comparing commonly encountered reads it is striking that representatives of the acidobacterial genera Gp3 (*Bryobacter*), Gp6, Gp11 and Gp21 and the acidobacterial family *Halophagaceae* were present at 1.4-1.6 fold more relative abundance in the choanosome than in the cortex and almost completely absent from the water sample. An even clearer preference for the choanosome was observed for reads belonging to the clostridial order *Clostridiales* (2.7 fold higher relative

abundance than in the cortex; 10.8 fold more than in the water sample). Conversely, the actinobacterial genus *Lamia* was observed 10.5 fold more often in the cortex than in the choanosome and was not present in the water column. Other taxa showing pattern B as described above were the acidobacterial genus *Gp10* (5.9 fold more in cortex than choanosome; not present in the water sample), the γ-*Proteobacterial* genus *Ectothiorodospiraceae* (2.4 fold; n/a) and some unclassified *Nitrospiraceae* (2.3 fold; n/a). The presence of such patterns becomes even clearer considering the dominant OTUs presented in Supplementary Table 5.3 (for a more detailed characterization of the evolutionary relationship please refer to the phylogenetic trees in Figures 5.8-5.11). Especially OTUs 361 (most closely related to *Crenarchaeota*), 323 (*Ectothiorodospiraceae*) and 424 (*Nitrospira*) were significantly more abundant in the cortex than in the choanosome.

The reported oxygen free zones inside sponges (Hoffmann et al., 2005) may explain the high abundance of *Clostridiales* bacteria in the choanosome. The same study linked sulphite reducing strains to the choanosome of sponges and proposed them to have a synergistic relationship. The preference of several acidobacterial genera for the choanosome and, contrastingly, one genus (*Gp10*) for the cortex is curious. Since not much is known about the lifestyle of *Acidobacteria* in general, it is difficult to assign a role to this group of organisms, even though they have been linked to nitrite and nitrate reduction and the production of antimicrobial compounds (Ward et al., 2009), two features often associated with sponge derived microbes. It is clear that *Acidobacteria* are not only found in various sponges throughout the world but that different members can be spatially distributed, indicating a distinct function in the sponge holobiont.

Deep water sponges' microbiota vs. microbiota of shallow water sponges

Since deep sea sponges of the bathypelagic zones live in an environment very different in many aspects to that of shallow water sponges (no light, high pressure, low temperature) it is interesting to see if these environmental parameters are reflected in the sponge associated community of deep water sponges. A huge amount of sequence data derived from shallow water sponges is now available, but relatively little is known about deep sea sponges. Nonetheless, Meyer and Kuever proposed a sponge specific microbial community for deep water sponges, reported similar levels

of microbial diversity and similar taxa as found in shallow water sponges, and assumed a spatial distribution of the prokaryotic sponge community.

When comparing the diversity observed in this study with the relevant shallow water data it becomes apparent that on the phylum level, no distinct differences could be found. On the contrary, the similarities on the phylum level are clear since members of the phyla *Acidobacteria, Actinobacteria, Chloroflexi, Nitrospira* and *Proteobacteria* have been consistently reported to be dominant in shallow water sponges as it is the case for at least one of the deep water sponges investigated here. Other studies also reported sponges dominated by one OTU (Webster et al., 2010) or sponges with a relatively low biodiversity (Jackson et al., 2012), as found for the sponge sample *L. diversichela* in this study. In order to compare the diversity on lower taxonomic levels one has to bear in mind, that different studies used different regions of the 16S-rRNA (V1-V3 vs. V5/V6 regions) for amplification and different approaches to classify the obtained reads. Thus, for a more robust comparison, publicly available reads from shallow water sponges from the Red Sea (Lee et al., 2011) and the Great Barrier Reef (Webster et al., 2010), which targeted the same or parts of the same region as in our study (V5/V6), were processed with the RDP pyrosequencing pipline. The sponge derived reads were put into a joint alignment with all sponge-derived sequences obtained in this study and then clustered using the RDP classifier allowing the comparison of the shared OTUs of the three data sets. The summary of this is shown in Table 5.4. From the results it becomes apparent that the shallow water sponge communities share more OTUs and that these OTUs make up a significant proportion of all reads obtained (45.3% of all reads obtained from the two studies were found in shared 95% similarity OTUs of these communities, distributed roughly evenly between the sample groups). The much smaller proportion of shared OTUs between the deep- and shallow-water sponge communities is striking and implies a deep-water specific community.

Table 5.4: Comparison of sequencing reads derived from 2 shallow water sponge studies with the here presented data.

S1	S2	Shared OTUs [%]		Shared Reads [%]		% of reads in S1		% of reads in S2	
		97%	95%	97%	95%	97%	95%	97%	95%
DS	**Webster**	2.2	3.7	9.4	11.5	4.8	8.1	10.2	12.3
DS	**Lee**	2.6	6.0	7.8	19.1	9.7	16.0	6.9	20.1
Webster	**Lee**	8.1	14.0	40.9	45.3	42.5	47.6	37.1	40.7

In column 1 and 2 the two samples (S1 and S2) which are compared are shown, column 3 denotes the relative abundance of shared OTUs at both 97% and 95% sequence similarity, columns 4 the relative abundance of shared reads at both 97% and 95% sequence similarity and columns 5 and 6 return the distribution of the reads among the two compared samples at both 97% and 95% sequence similarity. DS: sequences from this study (**D**eep-**S**ea); Webster: Sequences from Webster *et al.*, 2010; Lee: Sequences from Lee *et al.*, 2011.

CONCLUSION

This study presents a comprehensive analysis of the prokaryotic diversity of two deep-water sponge species and shows that the microbiota of deep-water sponges shares many features with their shallow water counterparts. They can exhibit a tremendous microbial diversity, which in large part appears to be sponge specific, but can also be dominated by one group of organisms and have a relatively low microbial diversity.

The sponge *S. normani* was found to host a very diverse and spatially distributed prokaryotic community. Furthermore, most of this community was shown to have a close, potentially symbiotic relationship with the sponge because the dominant OTUs found in this sponge were not present in the water column and often most closely related to GenBank entries derived from other sponge studies. The spherical sponge *S. normani*, which has clearly delineated tissues, gives host to microbes potentially involved in nitrification (*Crenarchaeota*), denitrification (*Nitrospira*, *Acidobacteria* and *Ectothiorodospiraceae*), sulphate reduction (*Desulfovibrio*) and secondary metabolite production (*Poribacteria*, *Chloroflexi* and *Actinobacteria*). This suggests a complex relationship between sponge and microbes in which the microorganisms involved in ammonium oxidation could initially use the sponge waste products to generate energy and provide nitrite and nitrate to microorganisms

involved in denitrification. The sponge in turn could benefit from "bacterial farming" (Hoffmann et al., 2005) and secondary metabolite production. Spatial distribution of microbes was also demonstrated implying a distinct function of several microbial taxa in different sponge sections. The three dominant OTUs in the cortex can be linked to microbes potentially involved in nitrification and denitrification, suggesting a metabolic relationship (see Figure 5.12). Contrastingly, the leaf shaped sponge *L. diversichela* does not harbour such a functionally diverse microbial community but one which almost exclusively comprised of a single organism potentially belonging to the aerobic genus *Granulosicoccus.* The different community profiles imply different lifestyles for the two investigated sponges. A transcriptomic analysis of the sponge samples could bring a better understanding of the metabolic processes involved in the sponge and the functions carried out by the microbes present.

Last but not least, the bacterial and archaeal phyla associated with deep- and shallow water sponges turned out to be broadly similar but on the lower taxonomic levels, striking differences were observed. The OTU level comparison with two shallow water sponge studies implies a deep-water sponge-specific diversity.

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SUPPLEMENTARY TABLES

Supplementary table 5.1: Primer design including Multiplex Identifier (MID)

Primer	Sample	Adapter	Multiplex identifier (MID)	template specific primer
f	Water	CGTATCGCCTCCCTCGCGCCATCAG	AGACGCACTC	TAGATACCCSSGTAGTCC
r		CTATGCGCCTTGCCAGCCCGCTCAG	AGACGCACTC	CTGACGRCRGCCATGC
f	L. diversichela	CGTATCGCCTCCCTCGCGCCATCAG	ATATCGCGAG	TAGATACCCSSGTAGTCC
r		CTATGCGCCTTGCCAGCCCGCTCAG	ATATCGCGAG	CTGACGRCRGCCATGC
f	*S. normani* choanosome	CGTATCGCCTCCCTCGCGCCATCAG	CGTGTCTCTA	TAGATACCCSSGTAGTCC
r		CTATGCGCCTTGCCAGCCCGCTCAG	CGTGTCTCTA	CTGACGRCRGCCATGC
f	*S. normani* cortex	CGTATCGCCTCCCTCGCGCCATCAG	CTCGCGTGTC	TAGATACCCSSGTAGTCC
r		CTATGCGCCTTGCCAGCCCGCTCAG	CTCGCGTGTC	CTGACGRCRGCCATGC

Supplementary Table 5.2: Classification of all sequencing reads.

domain	phylum	class	order	family	genus	Water	LD	SNCH	SNCO
Archaea	Crenarchaeota	Thermoprotei	Acidilobales	Caldisphaeraceae	Caldisphaera	0.02	-	-	-
Archaea	Crenarchaeota	Thermoprotei	Desulfurococcales	Desulfurococcaceae	unclassified	-	-	0.02	0.02
Archaea	Crenarchaeota	Thermoprotei	Desulfurococcales	unclassified	unclassified	0.01	-	0.04	0.04
Archaea	Crenarchaeota	Thermoprotei	Thermoproteales	Thermofilaceae	Thermofilum	-	-	-	0.02
Archaea	Crenarchaeota	Thermoprotei	unclassified	unclassified	unclassified	18.52	3.36	8.96	21.20
Archaea	Euryarchaeota	Methanopyri	Methanopyrales	Methanopyraceae	Methanopyrus	0.02	-	-	-
Archaea	Euryarchaeota	Halobacteria	Halobacteriales	Halobacteriaceae	unclassified	0.05	-	-	-
Archaea	Euryarchaeota	Methanomicrobia	Methanosarcinales	Methermicoccaceae	Methermicoccus	0.02	-	0.01	-
Archaea	Euryarchaeota	Methanomicrobia	unclassified	unclassified	unclassified	0.06	-	0.02	0.05
Archaea	Euryarchaeota	Thermoplasmata	Thermoplasmatales	Ferroplasmaceae	unclassified	0.03	-	-	-
Archaea	Euryarchaeota	Thermoplasmata	Thermoplasmatales	incertae sedis	Thermogymnomonas	0.27	-	-	-
Archaea	Euryarchaeota	Thermoplasmata	Thermoplasmatales	Thermoplasmataceae	Thermoplasma	0.01	-	-	-
Archaea	Euryarchaeota	Thermoplasmata	Thermoplasmatales	unclassified	unclassified	9.42	-	-	-
Archaea	Euryarchaeota	unclassified	unclassified	unclassified	unclassified	2.70	0.10	0.07	0.37
Archaea	unclassified	unclassified	unclassified	unclassified	unclassified	7.68	0.51	0.63	3.34
Bacteria	Acidobacteria	Acidobacteria GP1	-	-	unclassified	-	-	0.01	-
Bacteria	Acidobacteria	Acidobacteria GP10	-	-	GP10	-	-	0.24	1.40
Bacteria	Acidobacteria	Acidobacteria GP11	-	-	GP11	-	-	0.85	0.62
Bacteria	Acidobacteria	Acidobacteria GP2	-	-	GP2	-	-	0.01	-
Bacteria	Acidobacteria	Acidobacteria GP21	-	-	GP21	0.11	0.03	1.88	1.34
Bacteria	Acidobacteria	Acidobacteria GP22	-	-	GP22	-	-	-	0.01
Bacteria	Acidobacteria	Acidobacteria GP26	-	-	GP26	0.05	-	0.02	-
Bacteria	Acidobacteria	Acidobacteria GP3	-	-	Bryobacter	-	-	1.02	0.72
Bacteria	Acidobacteria	Acidobacteria GP4	-	-	unclassified	-	-	0.32	0.27
Bacteria	Acidobacteria	Acidobacteria GP5	-	-	GP5	-	-	0.01	0.01
Bacteria	Acidobacteria	Acidobacteria GP6	-	-	GP6	0.40	0.08	2.64	1.69
Bacteria	Acidobacteria	Acidobacteria GP9	-	-	GP9	-	-	0.12	0.24
Bacteria	Acidobacteria	Holophagae	Holophagales	Holophagaceae	Geothrix	-	-	0.09	0.06
Bacteria	Acidobacteria	Holophagae	Holophagales	Holophagaceae	Holophaga	-	-	0.11	0.01
Bacteria	Acidobacteria	Holophagae	Holophagales	Holophagaceae	unclassified	0.03	-	0.17	0.11

domain	phylum	class	order	family	genus	Water	LD	SNCH	SNCO
Bacteria	Acidobacteria	Holophagae	unclassified	unclassified	unclassified	-	-	0.02	-
Bacteria	Acidobacteria	unclassified	unclassified	unclassified	unclassified	0.45	0.05	4.83	3.50
Bacteria	Actinobacteria	Actinobacteria	Acidimicrobiales	Acidimicrobiaceae	Ferrithrix	-	-	0.01	-
Bacteria	Actinobacteria	Actinobacteria	Acidimicrobiales	Acidimicrobiaceae	unclassified	-	-	0.03	-
Bacteria	Actinobacteria	Actinobacteria	Acidimicrobiales	incertae sedis	Aciditerrimonas	0.14	0.02	0.17	0.17
Bacteria	Actinobacteria	Actinobacteria	Acidimicrobiales	lamiaceae	lamia	-	-	0.18	1.91
Bacteria	Actinobacteria	Actinobacteria	Acidimicrobiales	unclassified	unclassified	0.14	0.02	0.49	0.52
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae	Corynebacterium	-	0.03	-	-
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Mycobacteriaceae	Mycobacterium	-	0.07	-	-
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Propionibacterium	0.02	-	-	-
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	unclassified	unclassified	-	0.03	0.10	0.09
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	unclassified	unclassified	-	0.02	-	-
Bacteria	Actinobacteria	Actinobacteria	Bifidobacteriales	Bfidobacteriaceae	Gardnerella	-	0.03	-	-
Bacteria	Actinobacteria	Actinobacteria	Coriobacteriales	Coriobacteriaceae	Paraeggerthella	-	-	0.01	-
Bacteria	Actinobacteria	Actinobacteria	Coriobacteriales	Coriobacteriaceae	unclassified	-	-	0.03	-
Bacteria	Actinobacteria	Actinobacteria	Solirubrobacterales	Solirubrobacteraceae	Solirubrobacter	-	-	0.02	-
Bacteria	Actinobacteria	Actinobacteria	Solirubrobacterales	unclassified	unclassified	-	-	0.01	-
Bacteria	Actinobacteria	Actinobacteria	unclassified	unclassified	unclassified	-	0.02	0.81	0.36
Bacteria	Actinobacteria	Actinobacteria	unclassified	unclassified	unclassified	-	-	0.06	0.02
Bacteria	Actinobacteria	Actinobacteria	unclassified	unclassified	unclassified	-	-	-	-
Bacteria	Aquificae	Aquificae	Aquificales	incertae sedis	Thermosulfidibacter	-	-	0.01	-
Bacteria	Bacteroidetes	Flavobacteria	Flavobacteriales	Cryomorphaceae	Brumimicrobium	0.02	-	-	-
Bacteria	Bacteroidetes	Flavobacteria	Flavobacteriales	Cryomorphaceae	Fluviicola	-	0.02	-	-
Bacteria	Bacteroidetes	Flavobacteria	Flavobacteriales	Cryomorphaceae	unclassified	0.08	0.10	-	0.01
Bacteria	Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	Algibacter	-	0.03	-	-
Bacteria	Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	Gilvibacter	-	0.02	-	-
Bacteria	Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	Meridianimaribacter	-	0.03	-	-
Bacteria	Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	Tamlana	-	0.07	-	-
Bacteria	Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	Ulvibacter	-	0.02	-	-
Bacteria	Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	unclassified	0.07	0.16	-	-
Bacteria	Bacteroidetes	Flavobacteria	Flavobacteriales	unclassified	unclassified	-	0.07	-	-
Bacteria	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Chitinophagaceae	Sediminibacterium	-	0.02	-	-
Bacteria	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Chitinophagaceae	unclassified	-	0.03	-	-
Bacteria	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Cytophagaceae	Ekhidna	-	0.02	-	-

domain	phylum	class	order	family	genus	Water	LD	SNCH	SNCO
Bacteria	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Flammeovirgaceae	Fabibacter	-	0.02	-	-
Bacteria	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Flammeovirgaceae	Fulvivirga	-	-	-	0.27
Bacteria	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Flammeovirgaceae	Reichenbachiella	-	-	-	0.02
Bacteria	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Flammeovirgaceae	Roseivirga	-	0.03	-	-
Bacteria	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Flammeovirgaceae	unclassified	0.01	-	-	0.01
Bacteria	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Saprospiraceae	Haliscomenobacter	-	0.03	-	-
Bacteria	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Saprospiraceae	Lewinella	0.01	0.02	-	-
Bacteria	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Saprospiraceae	unclassified	-	0.02	-	-
Bacteria	Bacteroidetes	Sphingobacteria	Sphingobacteriales	unclassified	unclassified	0.05	-	0.10	0.26
Bacteria	Bacteroidetes	unclassified	unclassified	unclassified	unclassified	-	-	0.10	0.47
Bacteria	Chlamydiae	Chlamydiaea	Chlamydiales	Parachlamydiaceae	unclassified	-	0.02	-	-
Bacteria	Chlamydiae	Chlamydiaea	Chlamydiales	Simkaniaceae	Simkania	-	0.03	-	-
Bacteria	Chlamydiae	Chlamydiaea	Chlamydiales	unclassified	unclassified	-	0.03	-	-
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	Levilinea	-	-	0.02	-
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	unclassified	0.07	-	0.12	0.06
Bacteria	Chloroflexi	Caldilineae	Caldilineales	Caldilineaceae	Caldilinea	-	-	0.01	0.01
Bacteria	Chloroflexi	Dehalococcoidetes	de	de	Dehalogenimonas	0.06	-	0.04	-
Bacteria	Chloroflexi	Ktedonobacteria	unclassified	unclassified	unclassified	-	-	0.01	-
Bacteria	Chloroflexi	unclassified	unclassified	unclassified	unclassified	0.14	-	3.39	2.05
Bacteria	Cyanobacteria/Chloroplast	Chloroplast	Chloroplast	Chloroplast	Bacillariophyta	-	0.03	-	-
Bacteria	Cyanobacteria/Chloroplast	Chloroplast	Chloroplast	Chloroplast	unclassified	0.02	0.02	-	-
Bacteria	Cyanobacteria/Chloroplast	Cyanobacteria	cy	II	GpIIa	-	0.02	-	-
Bacteria	Cyanobacteria/Chloroplast	unclassified	unclassified	unclassified	unclassified	-	0.02	-	-
Bacteria	Deinococcus-Thermus	Deinococci	Deionococcales	Trueperaceae	Truepera	0.01	-	0.20	0.09
Bacteria	Deinococcus-Thermus	Deinococci	Deionococcales	unclassified	unclassified	-	-	0.01	-
Bacteria	Deinococcus-Thermus	Deinococci	Thermales	Thermaceae	Thermus	0.01	-	-	-
Bacteria	Deinococcus-Thermus	Deinococci	unclassified	unclassified	unclassified	-	-	0.01	-
Bacteria	Firmicutes	Bacilli	Bacillales	Pasteuriaceae	Pasteuria	-	0.07	-	-
Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	Lysinibacillus	-	0.02	-	-
Bacteria	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	-	0.02	-	-
Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	-	0.02	-	-
Bacteria	Firmicutes	Bacilli	unclassified	unclassified	unclassified	-	-	-	0.01
Bacteria	Firmicutes	Clostridia	Clostridiales	unclassified	unclassified	0.11	0.02	1.23	0.46
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Selenomonas	0.01	-	-	-

domain	phylum	class	order	family	genus	Water	LD	SNCH	SNCO
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	unclassified	-	0.03	0.09	-
Bacteria	Firmicutes	Clostridia	Thermoanaerobacterales	Thermoanaerobacteraceae	Thermoanaeromonas	-	-	0.01	-
Bacteria	Firmicutes	Clostridia	Thermoanaerobacterales	Thermodesulfobiaceae	Thermodesulfobium	0.02	-	0.03	0.01
Bacteria	Firmicutes	Clostridia	Thermoanaerobacterales	unclassified	unclassified	-	-	0.01	-
Bacteria	Firmicutes	Clostridia	unclassified	unclassified	unclassified	0.15	0.02	0.56	0.29
Bacteria	Firmicutes	unclassified	unclassified	unclassified	unclassified	0.13	0.02	0.28	0.58
Bacteria	Lentisphaerae	Lentisphaeria	unclassified	unclassified	unclassified	-	-	-	-
Bacteria	Nitrospira	Nitrospira	Nitrospirales	Nitrospiraceae	Nitrospira	-	-	0.01	0.02
Bacteria	Nitrospira	Nitrospira	Nitrospirales	Nitrospiraceae	unclassified	-	-	0.24	0.56
Bacteria	Planctomycetes	Phycisphaerae	Phycisphaerales	Phycisphaceae	Phycisphaera	0.26	0.36	0.01	-
Bacteria	Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	Blastospirellula	0.15	0.43	0.01	0.04
Bacteria	Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	Isosphaera	-	-	-	0.01
Bacteria	Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	Planctomyces	0.06	-	-	-
Bacteria	Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	Rhodopirellula	0.01	0.05	-	-
Bacteria	Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	Schlesneria	0.05	-	-	-
Bacteria	Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	unclassified	0.14	0.21	-	0.02
Bacteria	Planctomycetes	unclassified	unclassified	unclassified	unclassified	0.15	0.26	-	0.06
Bacteria	Proteobacteria	unclassified	unclassified	unclassified	unclassified	3.45	1.47	5.12	10.43
Bacteria	Proteobacteria	α-proteobacteria	Caulobacterales	Caulobacteraceae	Brevundimonas	-	0.02	-	-
Bacteria	Proteobacteria	α-proteobacteria	Caulobacterales	Caulobacteraceae	Caulobacter	-	0.05	-	-
Bacteria	Proteobacteria	α-proteobacteria	Parvularculales	Parvularculaceae	Parvularcula	-	-	-	0.01
Bacteria	Proteobacteria	α-proteobacteria	Rhizobiales	Bradyrhizobiaceae	Bradyrhizobium	-	0.02	-	-
Bacteria	Proteobacteria	α-proteobacteria	Rhizobiales	incertae sedis	Bauldia	-	-	0.01	-
Bacteria	Proteobacteria	α-proteobacteria	Rhizobiales	Methylocystaceae	Terasakiella	0.03	0.02	-	-
Bacteria	Proteobacteria	α-proteobacteria	Rhizobiales	Phyllobacteriaceae	Chelativorans	-	0.02	-	-
Bacteria	Proteobacteria	α-proteobacteria	Rhizobiales	Phyllobacteriaceae	unclassified	0.03	-	-	-
Bacteria	Proteobacteria	α-proteobacteria	Rhizobiales	Rhodobiaceae	unclassified	-	-	0.01	-
Bacteria	Proteobacteria	α-proteobacteria	Rhizobiales	unclassified	unclassified	-	0.02	0.09	0.06
Bacteria	Proteobacteria	α-proteobacteria	Rhodobacterales	Rhodobacteraceae	Pelagicola	0.02	0.03	-	-
Bacteria	Proteobacteria	α-proteobacteria	Rhodobacterales	Rhodobacteraceae	Pseudoruegeria	0.01	-	-	-
Bacteria	Proteobacteria	α-proteobacteria	Rhodobacterales	Rhodobacteraceae	Pseudovibrio	-	0.02	-	-
Bacteria	Proteobacteria	α-proteobacteria	Rhodobacterales	Rhodobacteraceae	Ruegeria	0.01	-	-	-
Bacteria	Proteobacteria	α-proteobacteria	Rhodobacterales	Rhodobacteraceae	Shimia	0.02	-	-	-
Bacteria	Proteobacteria	α-proteobacteria	Rhodobacterales	Rhodobacteraceae	unclassified	0.16	0.07	0.02	0.06

domain	phylum	class	order	family	genus	Water	LD	SNCH	SNCO
Bacteria	Proteobacteria	α-proteobacteria	Rhodospirillales	Acetobacteraceae	Stella	0.01	0.02	-	-
Bacteria	Proteobacteria	α-proteobacteria	Rhodospirillales	Rhodspirillaceae	Oceanibaculum	-	0.02	0.07	0.05
Bacteria	Proteobacteria	α-proteobacteria	Rhodospirillales	Rhodspirillaceae	Pelagibius	-	-	0.02	-
Bacteria	Proteobacteria	α-proteobacteria	Rhodospirillales	Rhodspirillaceae	Skermanella	-	-	-	0.01
Bacteria	Proteobacteria	α-proteobacteria	Rhodospirillales	Rhodspirillaceae	unclassified	0.10	0.10	0.85	0.89
Bacteria	Proteobacteria	α-proteobacteria	Rhodospirillales	unclassified	unclassified	1.01	0.25	0.08	0.19
Bacteria	Proteobacteria	α-proteobacteria	Rickettsiales	Rickettsiaceae	Rickettsia	-	0.15	-	-
Bacteria	Proteobacteria	α-proteobacteria	Rickettsiales	Rickettsiaceae	unclassified	-	0.03	-	0.01
Bacteria	Proteobacteria	α-proteobacteria	Sneathiellales	Sneathiellaceae	Sneathiella	0.01	0.13	-	-
Bacteria	Proteobacteria	α-proteobacteria	Sphingomonadales	Erythrobacteraceae	Porphyrobacter	0.01	-	-	-
Bacteria	Proteobacteria	α-proteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	-	0.05	-	0.01
Bacteria	Proteobacteria	α-proteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingopyxis	0.03	-	-	-
Bacteria	Proteobacteria	α-proteobacteria	Sphingomonadales	unclassified	unclassified	-	0.02	-	-
Bacteria	Proteobacteria	α-proteobacteria	unclassified	unclassified	unclassified	0.48	0.52	0.73	0.47
Bacteria	Proteobacteria	β-proteobacteria	Burkholderiales	Alcalicenaceae	Oligella	-	-	-	0.01
Bacteria	Proteobacteria	β-proteobacteria	Burkholderiales	Comamonadaceae	Pelomonas	0.01	0.02	-	-
Bacteria	Proteobacteria	β-proteobacteria	Burkholderiales	incertae sedis	Aquabacterium	0.01	0.02	-	-
Bacteria	Proteobacteria	β-proteobacteria	Methylophilales	Methylophilaceae	Methylotenera	0.05	0.03	-	-
Bacteria	Proteobacteria	β-proteobacteria	unclassified	unclassified	unclassified	-	-	-	0.02
Bacteria	Proteobacteria	γ-proteobacteria	Aeromonadales	Aeromonadaceae	Aeromonas	-	0.02	-	-
Bacteria	Proteobacteria	γ-proteobacteria	Alteromonadales	Alteromonadaceae	Aliagarivorans	0.01	-	-	-
Bacteria	Proteobacteria	γ-proteobacteria	Alteromonadales	Alteromonadaceae	Alteromonas	0.09	-	-	-
Bacteria	Proteobacteria	γ-proteobacteria	Alteromonadales	Alteromonadaceae	Glaciecola	0.02	0.05	-	-
Bacteria	Proteobacteria	γ-proteobacteria	Alteromonadales	Alteromonadaceae	Marinobacter	0.01	-	-	-
Bacteria	Proteobacteria	γ-proteobacteria	Alteromonadales	Alteromonadaceae	Melitea	-	0.03	-	0.01
Bacteria	Proteobacteria	γ-proteobacteria	Alteromonadales	Alteromonadaceae	Microbulbifer	-	0.02	-	-
Bacteria	Proteobacteria	γ-proteobacteria	Alteromonadales	Alteromonadaceae	unclassified	0.14	-	-	-
Bacteria	Proteobacteria	γ-proteobacteria	Alteromonadales	Colwelliaceae	Colwellia	0.08	0.07	-	-
Bacteria	Proteobacteria	γ-proteobacteria	Alteromonadales	Colwelliaceae	Thalassomonas	-	0.03	-	-
Bacteria	Proteobacteria	γ-proteobacteria	Alteromonadales	Moritellaceae	Moritella	0.01	-	-	-
Bacteria	Proteobacteria	γ-proteobacteria	Alteromonadales	Pseudoalteromonadaceae	Pseudoalteromonas	42.94	-	-	-
Bacteria	Proteobacteria	γ-proteobacteria	Alteromonadales	unclassified	unclassified	0.01	-	-	-
Bacteria	Proteobacteria	γ-proteobacteria	Chromatiales	Chromatiaceae	unclassified	-	-	0.02	-
Bacteria	Proteobacteria	γ-proteobacteria	Chromatiales	Ectothiorhodospiraceae	unclassified	-	0.02	0.21	0.51

domain	phylum	class	order	family	genus	Water	LD	SNCH	SNCO
Bacteria	Proteobacteria	γ-proteobacteria	Chromatiales	Granulosicoccaceae	Granulosicoccus	-	34.64	-	-
Bacteria	Proteobacteria	γ-proteobacteria	Chromatiales	unclassified	unclassified	0.01	37.16	0.13	0.14
Bacteria	Proteobacteria	γ-proteobacteria	Enterobacteriales	Enterobacteriaceae	Pantoea	-	0.02	-	-
Bacteria	Proteobacteria	γ-proteobacteria	incertae sedis	incertae sedis	Arenicella	0.02	-	-	0.01
Bacteria	Proteobacteria	γ-proteobacteria	incertae sedis	incertae sedis	Eionea	0.02	-	-	-
Bacteria	Proteobacteria	γ-proteobacteria	incertae sedis	incertae sedis	Spongiibacter	0.01	-	-	-
Bacteria	Proteobacteria	γ-proteobacteria	incertae sedis	incertae sedis	Thiohalobacter	0.02	-	0.01	-
Bacteria	Proteobacteria	γ-proteobacteria	incertae sedis	incertae sedis	Thioprofundum	-	-	0.02	-
Bacteria	Proteobacteria	γ-proteobacteria	incertae sedis	unclassified	unclassified	0.05	-	0.54	0.53
Bacteria	Proteobacteria	γ-proteobacteria	Legionellales	Coxiellaceae	Coxiella	-	0.02	-	-
Bacteria	Proteobacteria	γ-proteobacteria	Oceanospirillales	Alcanivoracaceae	Alcanivorax	0.01	-	-	-
Bacteria	Proteobacteria	γ-proteobacteria	Oceanospirillales	Hahellaceae	Endozoicomonas	-	0.10	-	-
Bacteria	Proteobacteria	γ-proteobacteria	Oceanospirillales	Hahellaceae	unclassified	-	0.02	-	-
Bacteria	Proteobacteria	γ-proteobacteria	Oceanospirillales	Halomodaceae	Cobetia	0.91	-	-	-
Bacteria	Proteobacteria	γ-proteobacteria	Oceanospirillales	Halomodaceae	Halomonas	0.08	-	-	-
Bacteria	Proteobacteria	γ-proteobacteria	Oceanospirillales	Halomodaceae	unclassified	-	-	0.01	0.01
Bacteria	Proteobacteria	γ-proteobacteria	Oceanospirillales	Oceanospirillaceae	Aphritea	0.01	-	-	-
Bacteria	Proteobacteria	γ-proteobacteria	Oceanospirillales	Oceanospirillaceae	Nitrincola	0.08	-	-	-
Bacteria	Proteobacteria	γ-proteobacteria	Oceanospirillales	Oceanospirillaceae	Oceanospirillum	0.01	-	-	-
Bacteria	Proteobacteria	γ-proteobacteria	Oceanospirillales	Oceanospirillaceae	unclassified	0.02	0.05	0.01	0.05
Bacteria	Proteobacteria	γ-proteobacteria	Oceanospirillales	Oleiphilaceae	Oleiphilus	-	-	0.01	-
Bacteria	Proteobacteria	γ-proteobacteria	Oceanospirillales	unclassified	unclassified	0.07	0.07	0.34	0.56
Bacteria	Proteobacteria	γ-proteobacteria	Pasteurellales	Pasteurellaceae	Haemophilus	0.01	-	-	-
Bacteria	Proteobacteria	γ-proteobacteria	Pseudomonadales	incertae sedis	Dasania	0.07	0.02	-	-
Bacteria	Proteobacteria	γ-proteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	0.01	0.16	-	-
Bacteria	Proteobacteria	γ-proteobacteria	Pseudomonadales	Moraxellaceae	Enhydrobacter	-	0.02	-	-
Bacteria	Proteobacteria	γ-proteobacteria	Pseudomonadales	Moraxellaceae	Psychrobacter	0.96	0.03	-	-
Bacteria	Proteobacteria	γ-proteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	0.05	0.02	-	0.02
Bacteria	Proteobacteria	γ-proteobacteria	Pseudomonadales	Pseudomonadaceae	unclassified	0.08	-	0.01	-
Bacteria	Proteobacteria	γ-proteobacteria	Thiotrichales	incertae sedis	Fangia	-	0.08	-	0.01
Bacteria	Proteobacteria	γ-proteobacteria	unclassified	unclassified	unclassified	1.34	16.47	2.93	4.87
Bacteria	Proteobacteria	γ-proteobacteria	Vibrionales	Vibrionaceae	Lucibacterium	0.01	-	-	-
Bacteria	Proteobacteria	γ-proteobacteria	Vibrionales	Vibrionaceae	unclassified	0.05	-	-	-
Bacteria	Proteobacteria	γ-proteobacteria	Vibrionales	Vibrionaceae	Vibrio	0.47	-	-	-

domain	phylum	class	order	family	genus	Water	LD	SNCH	SNCO
Bacteria	Proteobacteria	γ-proteobacteria	Xanthomonadales	Sinobacteraceae	Steroidobacter	-	-	**0.22**	**0.24**
Bacteria	Proteobacteria	γ-proteobacteria	Xanthomonadales	Sinobacteraceae	unclassified	-	-	0.01	-
Bacteria	Proteobacteria	γ-proteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotophomonas	-	0.08	-	-
Bacteria	Proteobacteria	δ-proteobacteria	Bdellovibrionales	Bacteriovoracaceae	unclassified	0.02	-	-	-
Bacteria	Proteobacteria	δ-proteobacteria	Desulfovibrionales	unclassified	unclassified	-	-	0.01	-
Bacteria	Proteobacteria	δ-proteobacteria	Desulfurellales	Desulfurellaceae	Hippea	-	-	0.01	-
Bacteria	Proteobacteria	δ-proteobacteria	Desulfuromonadales	Geobacteraceae	Geothermobacter	-	0.02	0.01	-
Bacteria	Proteobacteria	δ-proteobacteria	Desulfuromonadales	Geobacteraceae	unclassified	-	-	0.04	0.01
Bacteria	Proteobacteria	δ-proteobacteria	Desulfuromonadales	unclassified	unclassified	-	0.02	0.03	-
Bacteria	Proteobacteria	δ-proteobacteria	Myxococcales	Haliangiaceae	Haliangium	-	-	-	0.01
Bacteria	Proteobacteria	δ-proteobacteria	Syntrophobacterales	Syntrophobacteraceae	Desulfoglaeba	-	-	0.01	0.01
Bacteria	Proteobacteria	δ-proteobacteria	Syntrophobacterales	Syntrophobacteraceae	unclassified	-	-	0.10	0.19
Bacteria	Proteobacteria	δ-proteobacteria	Syntrophobacterales	unclassified	unclassified	-	-	0.01	0.02
Bacteria	Proteobacteria	δ-proteobacteria	unclassified	unclassified	unclassified	0.15	0.13	0.81	0.31
Bacteria	Spirochaetes	Spirochaetes	Spirochaetales	incertae sedis	Exilispira	-	-	0.01	-
Bacteria	Spirochaetes	Spirochaetes	Spirochaetales	unclassified	unclassified	-	-	0.05	0.01
Bacteria	Thermotogae	Thermotogae	Thermotogales	Thermotogaceae	Thermococcoides	-	-	0.03	-
Bacteria	Thermotogae	Thermotogae	Thermotogales	Thermotogaceae	unclassified	-	-	0.02	-
Bacteria	TM7	incertae sedis	incertae sedis	incertae sedis	incertae sedis	0.01	-	0.01	-
Bacteria	Verrucomicrobia	Optitutae	Optitutales	Optitutaceae	Alterococcus	-	-	0.01	0.02
Bacteria	Verrucomicrobia	Optitutae	Optitutales	Optitutaceae	Optitutus	-	-	0.01	-
Bacteria	Verrucomicrobia	Optitutae	Optitutales	Optitutaceae	unclassified	-	0.02	0.02	0.01
Bacteria	Verrucomicrobia	Optitutae	unclassified	unclassified	unclassified	-	-	0.03	0.01
Bacteria	Verrucomicrobia	Subdivision 3	incertae sedis	incertae sedis	incertae sedis	0.07	0.02	-	-
Bacteria	Verrucomicrobia	unclassified	unclassified	unclassified	unclassified	0.05	-	-	-
Bacteria	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Rubritaleaceae	Rubritalea	0.02	0.18	-	-
Bacteria	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	unclassified	unclassified	-	0.03	-	-
Bacteria	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	unclassified	0.03	0.03	-	-
Bacteria	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Verrucomicrobium	-	0.02	-	-
Bacteria	Unclassified	Unclassified	Unclassified	Unclassified	Unclassified	4.53	0.51	56.82	35.37
unclassified	unclassified	unclassified	unclassified	unclassified	unclassified	0.01	0.08	0.09	1.32

Supplementary Table 5.3: Composition of the most dominant OTUs from all 4 samples.

OTU	reads	SNCH	SNCO	LD	W	GenBank Entry	Organism	Similarity
1	5242	-	-	85.82	-	JN596757.1	Uncultured gamma proteobacterium clone XD2032	96
361	3103	8.45	19.48	-	-	FJ487504.1	Uncultured marine group I crenarchaeote SPG11_H2O_A34	98
212	3030	-	-	-	34.64	HE655441.1	Pseudoalteromonas sp. RT8	99
24	1135	-	0.01	1.92	11.63	FJ150797.1	Uncultured marine group I crenarchaeote clone 061exp4	99
323	1113	0.80	11.99	-	0.01	JQ612268.1	Uncultured bacterium clone GBc143	98
367	833	3.60	2.22	-	-	JQ612256.1	Uncultured bacterium clone GBc160	99
74	763	-	-	0.46	8.40	HM103748.1	Uncultured marine group I crenarchaeote clone Ma101_1A_61	99
359	719	0.40	8.02	-	-	EU237479.1	Contamination! (Cinachyrella kuekenthali mitochondrion complete genome)	89
218	626	-	-	-	7.16	EU935099.1	Pseudoalteromonas sp. P55	98
228	566	-	-	-	6.47	DQ156458.1	Uncultured marine group II euryarchaeote HF500_24F01	99
424	453	0.87	3.66	-	-	JF802708.1	Uncultured Nitrospira sp. clone TsNOB5	99
452	408	1.98	0.61	-	-	JQ612176.1	Uncultured bacterium clone GBc110	99
381	313	1.30	0.96	-	-	EU071668.1	Uncultured Poribacteria bacterium clone S10	99
674	281	1.34	0.47	-	-	JN596673.1	Uncultured Chloroflexi bacterium clone XC1041	98
421	265	0.96	1.13	-	-	FJ560485.1	Uncultured Chloroflexi bacterium	99
695	253	1.02	0.84	-	-	JQ612274.1	Uncultured bacterium clone GBc041	99
725	250	1.00	0.84	-	-	AB453757.1	Uncultured bacterium gene for 16S rRNA, clone: GM-WBS-cloneBB	99
414	240	1.10	0.50	-	-	JN596655.1	Uncultured Chloroflexi bacterium clone XC1018	98
464	214	0.97	0.47	-	-	JQ844348.1	Uncultured bacterium clone Pori39	99
448	206	0.73	0.92	-	-	FJ900580.1	Uncultured bacterium clone AncE9	99
377	205	0.39	1.65	-	0.01	FJ900564.1	Uncultured bacterium clone AncC16	99
411	183	0.57	0.99	-	-	JQ612379.1	Uncultured bacterium clone GBc286	98
157	181	-	-	0.39	1.79	JQ712431.1	Uncultured bacterium clone AV08-BC-36	99

OTU	reads	SNCH	SNCO	LD	W	GenBank Entry	Organism	Similarity
711	168	0.85	0.17	-	-	EF076111.1	Uncultured bacterium clone GBc183	98
662	168	0.83	0.21	-	-	JQ612188.1	Uncultured Chloroflexi bacterium clone PK064 16S	98
399	165	0.47	0.99	-	-	EU071654.1	Uncultured Poribacteria bacterium clone F7	98
473	157	0.10	1.71	-	-	FJ543186.1	Uncultured actinobacterium clone OPM48	98
501	155	0.65	0.46	-	-	JQ612281.1	Uncultured bacterium clone GBc128	99
426	147	0.65	0.32	0.02	0.02	GQ351195.1	Uncultured bacterium clone J8P41000_1H01	97
461	144	0.41	0.86	-	-	EU035924.2	Uncultured bacterium clone KspoA7	99
716	137	0.66	0.21	-	-	JQ612229.1	Uncultured bacterium clone GBc130	99
471	136	0.17	1.30	-	-	FJ269307.1	Uncultured Acidobacteria bacterium clone XA3H11F	99
1391	134	0.57	0.37	-	-	JQ612260.1	Uncultured marine archaeon clone SHZY711	97
362	134	0.02	1.59	-	0.02	JQ227905.1	Uncultured bacterium clone GBc170	99
746	132	0.63	0.21	-	-	JN596673.1	Uncultured Chloroflexi bacterium clone XC1041	99
122	123	-	0.04	0.54	0.99	JQ225890.1	Uncultured marine archaeon clone SGSZ650	100
384	100	0.21	0.76	-	-	JQ612356.1	Uncultured bacterium clone GBc038	98
282	98	-	-	-	1.12	HQ163277.1	Uncultured marine group III euryarchaeote clone SHZW623	99
113	94	-	-	0.10	1.01	EF106813.1	Uncultured marine group I crenarchaeote clone ALOHA200m_0050	97
223	89	-	-	0.10	0.95	HM103765.1	Uncultured marine group I crenarchaeote clone Ma120_1A_69	99
215	86	-	-	0.03	0.96	JQ218882.1	Uncultured proteobacterium clone ncTAUSP4	98
291	80	-	-	-	0.91	JQ670742.1	Cobetia marina strain KW30-8-3	99
436	76	0.03	0.86	0.03	-	JQ227905.1	Uncultured marine archaeon clone SHZY711	98
433	75	0.05	0.82	-	-	EU237479.1	Contamination! (Cinachyrella kuekenthali mitochondrion complete genome)	89
284	75	-	-	0.13	0.77	JN833435.1	Uncultured archaeon clone A_NY_1F01	99
1099	74	-	-	0.03	0.82	GU234953.1	Uncultured marine bacterium clone SHFB543	99
134	72	-	-	0.11	0.74	FJ150797.1	Uncultured marine group I crenarchaeote clone 061exp4	98
28	72	-	-	0.49	0.48	HQ242110.1	Uncultured SAR11 cluster alpha proteobacterium clone F9P4_10A06	99
303	70	-	-	-	0.80	FJ457156.1	Pseudoalteromonas sp. S1660	98

OTU	reads	SNCH	SNCO	LD	W	GenBank Entry	Organism	Similarity
252	69	-	-	0.13	0.70	HQ674139.1	Uncultured bacterium clone F9P261000_S_H07	98
488	68	0.04	0.76	-	-	JN655243.1	Uncultured bacterium clone AF10-3-7_C30	97
565	67	-	-	1.10	-	JN596757.1	Uncultured gamma proteobacterium clone XD2032	96
326	49	-	-	-	0.56	FJ002860.1	Uncultured marine group II euryarchaeote clone A3_75_A1-2	99
137	49	-	-	0.03	0.54	AB194005.1	Uncultured archaeon gene for 16S rRNA clone: Sd-EA08	99
214	46	-	-	-	0.53	JQ907344.1	Vibrio sp. BSw21846	99
316	45	-	-	-	0.51	DQ156462.1	Uncultured marine group II euryarchaeote HF500_47D04	99
183	27	-	-	0.39	0.03	HM798666.1	Uncultured planctomycete clone PRTAB7735	98
123	21	-	-	0.34	-	JN596669.1	Uncultured gamma proteobacterium clone XC1009	92
637	14	-	-	0.23	-	EU350894.1	Uncultured Verrucomicrobia bacterium clone HAL-SW-5	98

Given are the OTU number, the reads represented by each OTU, the relative abundance of reads assigned to each sample (as percentage of the total number of reads in the according sample), the gene bank entry of the closest BLAST hit of a representative sequence of the OTU and the sequence similarity to the closest BLAST hit. SNCH: *S. normani* choanosome, SNCO: *S. normani* cortex, LD: *L. diversichela*, W: Water.

Chapter 6

General Discussion

GENERAL DISCUSSION

Marine sponges are drawing increased levels of attention by researchers from all over the world mainly due to two reasons. Firstly they have been shown to harbour the largest diversity of microbial symbionts known in any marine invertebrate coupled with the fact that they are the most prolific marine source for novel chemical entities, including bioactive compounds.

Early work on marine sponges proposed many of the still widely held theories about sponge-microbe associations. The presence of sponge specific microbial organisms (Wilkinson, 1978a), the existence of sponges with more or less microbial symbionts (Vacelet and Donadey, 1977), the role of symbionts in nitrogen-metabolism (Wilkinson, 1978a) and photosynthesis (Wilkinson and Vacelet, 1979; Wilkinson, 1983), the presence of similar sponge-associated microbes in sponges from different parts of the world (Wilkinson and Fay, 1979; Wilkinson, 1978b) and the vertical transfer of microbial organisms (Gaino et al., 1987) have been discussed as early as the 1970s and 1980s. The method of choice in most of those studies was microscopy. Even though it was obvious by that time that sponges harbour different phenotypes of microbial cells in their mesohyle, the degree of diversity was difficult and time consuming to assess. Molecular methods as a mean of phylogenetic classification were introduced in the late 1980s/ early 1990s (Gutell et al., 1985; Lane, 1991; Medlin et al., 1988) and subsequently revolutionized the field of microbial ecology by providing a means to rapidly classify microbial isolates and to assess microbial diversity in metagenomes by preparing clone libraries of amplified metagenomic 16S/18S-rRNA. The application of this technique improved our understanding of the diversity of sponge associated microbes and revealed that microbes from all three domains of life including 17 described bacterial phyla and 12 candidate phyla are present in marine sponges (Simister et al., 2012). The introduction of high-throughput sequencing technologies, especially 454 pyrosequencing, improved our opportunities to assess microbial diversity even further and enabled us to study the so called “rare biosphere” (Sogin et al., 2006). Through the application of the latest DNA-sequencing technologies the diversity of microbial organisms in association with sponges has now been estimated to exceed 3000 genus-level OTUs in certain

sponges (Webster et al., 2010) compared to up to seven phenotypes observed in 1977 (Vacelet and Donadey, 1977). Additionally, more than 30 bacterial phyla have been detected in marine sponges (Schmitt et al., 2011). A very important discovery, only made possible through the application of culture independent methods, was the abundant presence of microbes belonging to phyla which are not readily culturable. The most notable example is the sponge specific candidate phylum *Poribacteria* (Fieseler et al., 2004). Members of the phyla *Acidobacteria*, *Nitrospirae* and *Chloroflexi* as well as *Thaumarchaeota* have also often been observed in studies employing culture independent methods but have not or have seldom been obtained in pure culture (Simister et al., 2012; Taylor et al., 2007). To date one *Chloroflexi* isolate (Brück et al., 2010) and one *Nitrospirae* isolate (Off et al., 2010) have been obtained from a sponge, whereas *Poribacteria* or the sponge symbiont *Cenarchaeum symbiosum* have never been isolated. Since it is very difficult or even impossible to cultivate members of many of the most often encountered sponge-associated microbial phyla, the opportunities offered by molecular methods are very valuable. Not only can the diversity of unculturable microbes be assessed, it is also possible to analyze the metabolic potential of such organisms with molecular methods, such as, for example, has been done for *Poribacteria* (Siegl et al., 2011), *Chloroflexi* (Siegl and Hentschel, 2010) and *Cenarchaeum symbiosum* (Hallam et al., 2006). In this way information can be obtained about the possible roles of the uncultured symbiont in the sponge such as the possible involvement of *Cenarchaeum symbiosum* in nitrogen metabolism as well as the production of secondary metabolites by *Poribacteria* and *Chloroflexi*.

The ability of marine sponges to produce secondary metabolites with bioactivity is the second reason for the enormous interest of the international research community in marine sponges. Marine sponges are not only the most prolific source of secondary metabolites in marine environments (Blunt et al., 2012, 2011; Leal et al., 2012) but they are also the source for a plethora of bioactive compounds of which three are approved drugs; namely eribulin mesylate, Ara-A and Ara-C number, while a number are currently in preclinical trials (Nastrucci et al., 2012). The increasing threat of antibiotic resistant microbes both in hospital settings and the food industry as well as the fact that many sponge associated microbes were shown to produce bioactive compounds in general (e.g. Kennedy et al., 2009) and sometimes are thought to produce compounds previously attributed to their sponge host (Piel, 2002;

Piel et al., 2004) further increased the research community's interest in marine sponges.

The aim of the work presented in this thesis was to study marine sponges collected from Irish waters for their associated microbes employing both culture dependent and culture independent methods. Additionally, the bacterial and fungal isolates obtained were to be studied for their potential as producers of antimicrobial compounds using bioassays and molecular tools for the analysis of secondary metabolite genes in the genome of bioactive isolates. Additionally, bioactive compounds produced by isolates were to be purified if possible and subsequently characterized using LC-MS and NMR.

In Chapter 2 the isolation of over 200 bacteria from two sponges, *Suberites carnosus* and *Leucosolenia* sp., is described. The two sponges represented different classes of *Porifera,* one demosponge (*S. carnosus*) and one calcareous sponge (*Leucosolenia* sp.) and the microbes isolated from the two sponges differed as did their antimicrobial activity profiles. The isolates obtained from *S. carnosus* were predominantly *Pseudovibrio,* while the isolates from *Leucosolenia* sp. were predominantly *Vibrio* and *Pseudoalteromonas*. The isolates from *S. carnosus* displayed mainly antibacterial activity, whereas the isolates from *Leucosolenia* sp. displayed only antifungal activity. The antibacterial activity observed was mostly from *Pseudovibrio* and *Spongiobacter* isolates, while the majority of the antifungal activity was observed from *Pseudoalteromonas, Bacillus* and *Vibrio* isolates. The differences in the activity profiles of sponge associated bacteria could partially be explained due to the different isolates obtained from each sponge (only 11 out of the 89 OTUs obtained in total were isolated from both sponges) but also inter-genus differences in bioactivity were observed. That is, isolates of the genera *Arthrobacter, Aquimarina, Bacillus, Microbulbifer, Psychrobacter, Shewanella, Staphylococcus* and *Vibrio* showed antibacterial activity if obtained from *S. carnosus* but inhibited the fungal test strains or did not show any inhibition at all when obtained from *Leucosolenia* sp. Of the bioactive isolates obtained the most interesting were from the *Spongiobacter, Pseudovibrio* and *Aquamarina* genera because not much had been known to that point about the antimicrobial compounds produced by these genera. Other isolates, mostly isolated from *Leucosolenia* sp., were not bioactive but

were only distantly related to their closest BLAST hit (97% or less sequence identity). Thus overall, the two sponges both proved to be a rich source of diverse and bioactive bacterial isolates, some of which have only seldomly been shown to inhibit the growth of microbial test strains and some of which are likely to represent novel species or genera. Also, the differences in bioactivity obtained from the two sponges were striking.

In Chapter 3 the isolation and bioactivity of fungi from 12 sponges collected in Lough Hyne is reported. The isolated fungi were diverse with 22 OTUs observed at an 18S-rRNA sequence identity of 98.5%. The isolates mostly belonged to orders often found in marine sponges (*Pleosporales, Hypocreales* and *Eurotiales*) but some belonged to orders seldomly isolated from sponges (*Cystofilobasidiales, Sporidiobolales, Erythrobasidiales, Glomerellales, Pseudogymnoascus* and *Apiospora*) and a large number displayed antibacterial and/or antifungal activity. The isolate W9F6, probably a *Fusarium* sp., which showed broad range antibacterial and antifungal activity, was also analysed for the presence of NRPS genes and 5 different genes were identified, some of which displayed low levels of similarity to publicly available NRPS gene sequences, indicating the potential that this isolate may produce novel bioactive metabolites.

The bacterial and fungal isolates obtained from the sponges isolated from Lough Hyne proved to be a rich source of antimicrobial compounds. Interestingly, in three additional studies (Jackson et al., 2012; Margassery et al., 2012; O'Halloran et al., 2011) various *Pseudovibrio* isolates were obtained from sponges collected at the same time and the same location, many of which inhibited the growth of various bacterial test strains. In addition a novel *Pseudovibrio* sp. has been described, *Pseudovibrio axinellae* from the same sampling site (O'Halloran et al., 2012). It is worthwhile commenting that while none of the *Pseudovibrio* isolates obtained from either of the studies (including Flemer et al., 2012) inhibited the growth of fungal test strains, many phylogenetically closely related isolates had varying inhibition profiles versus bacterial test strains. O'Halloran and colleagues thus analysed the *Pseudovibrio* isolates using random amplification of polymorphic DNA (RAPD) and identified 33 different RAPD types amongst the 73 *Pseudovibrio* isolates (3 of the isolates were from a sponge collected at a different location and time, but also from Irish waters). The bioactivity profiles for replicate members of the different groups

were more closely correlated to the RAPD types (i.e. isolates of one RAPD type mostly had the same bioactivity profile). The presence of several PKS genes in the genome of some of the *Pseudovibrio* isolates (O'Halloran et al., 2011) further highlighted the potential of *Pseudovibrio* for the production of bioactive compounds. The bacterial diversity in the water column at the same location and time was also assessed (Jackson et al., 2012) and it is interesting to note that, whereas other genera of bacteria also frequently isolated from the sponges, e.g. *Vibrio* and *Pseudoalteromonas*, were discovered in high abundances in the water column, sequences classified as *Pseudovibrio* were only found in the sponge *Raspailia ramosa* and not in the water column. The species *Pseudovibrio denitrificans* has been previously shown to be a facultative anaerobe which can reduce nitrate, nitrite or nitrous oxide (Shieh et al., 2004). A potential role of *Pseudovibrio* in nitrogen metabolism as a sponge symbiont is therefore conceivable. And indeed isolates closely related to *Pseudovibrio denitrificans s*have also been obtained from seven other sponges and vertical transmission has also been shown (Enticknap et al., 2006). The diverse antibacterial activities of the *Pseudovibrio* isolates could likewise be attributed to a potential role of *Pseudovibrio* in host defence mechanisms within marine sponges. Considering the clustering of *Pseudovibrio*-related sequences into the sponge specific cluster SC84 (Simister et al., 2012) a symbiotic relationship of marine sponges with *Pseudovibrio* spp. is likely. It would be interesting to add the sequences obtained from the sponges collected in Irish waters to the alignment from Simister and colleagues in order to analyse any potential relationship to SC84. Overall, demosponges collected at Lough Hyne were found to be an extraordinarily rich source of *Pseudovibrio* spp. with antibacterial activity and the isolates obtained could be a good source of diverse and possibly novel antibacterial compounds. An interesting observation can be made when comparing the amount of fungi obtained from the sponges *S. carnosus* (W13) and *Leucosolenia* sp. (W15) with the bioactivities of the bacterial isolates from each of the sponges. The bacterial isolates from *S. carnosus* exhibited mainly antibacterial activity and only one fungal isolate was obtained from this sponge. Contrastingly, the bacterial isolates from *Leucosolenia* sp. exhibited mainly antifungal activity and the sponge was one of the best sources for fungi as described in Chapter 3. Whether this is chance or has an ecological significance remains speculation but e.g. W15 could have been colonized by pathogenic fungi at the time of sampling and bacteria with the ability to fend of

fungi through the production of antifungal compounds could have been present in larger abundances than in the relatively fungus-free sponge sample W13.

In Chapter 4 the isolation, bioactivity and chemical analysis of a potentially novel compound from a *Micromonospora* isolate is described. A *Micromonospora* species was isolated from the marine sponge *Haliclona simulans* and was found to exhibit bioactivity in the deferred antagonism assay. Subsequent experiments also found the cell free supernatant of a liquid culture of this isolate to be active, however initial chemical analysis of chemical extracts of the supernatant was unable to identify the compound of activity. The production of the bioactive compound(s) was optimized by testing several production media and eventually a large scale extract was obtained for the isolation of a pure bioactive compound. A relatively pure compound was obtained through the application of preparative thin layer chromatography and the LC-MS analysis implied the presence of a quinocycline compound in the extract. Subsequent NMR studies could not verify the presence of such a compound but implied that a different, potentially novel compound with strong bioactivity vs. Gram positive test strains was present. Further spectroscopic analysis of this compound will be necessary in order to characterize it fully.
The genus *Micromonospora* has been described in 1923 and to date 44 species are known (Xie et al., 2012). *Micromonospora* spp. are frequently isolated from marine sponges (Abdelmohsen et al., 2012; Selvin et al., 2009; Xi et al., 2012; Zhang et al., 2012) and other marine sources (Jensen et al., 2005; Tanasupawat et al., 2010; Xie et al., 2012) and their bioactivity has been shown as well with 740 *Micromospora* derived bioactive compounds known by 2005 (Bérdy, 2005). Due to the genus' potential for the production of bioactive secondary metabolites and its frequent association with marine sponges a role of *Micromonospora* spp. in the defence of their host sponge against microbial pathogens is likely. The compound(s) found to be produced by the here presented isolate FMC8 had very strong activity and could thus play a significant role in the defence of its host sponge *Haliclona simulans* against Gram positive bacteria.

In Chapter 5 the diversity of prokaryotic (both archaeal and bacterial) diversity in two deep sea sponges from a depth of 1348m and a water sample collected close to the sponges was assessed by the application of 454 pyrosequencing. A primer pair

which targeted both archaeal and bacterial 16S-rRNA was employed. Bacteria have been shown to be very diverse in marine sponges from shallow waters but little is known about the diversity of bacteria in deep sea sponges. *Archaea* have recently been proposed to be present in vast abundance in deep water marine sponges and are also thought to be involved in nitrogen metabolism (Hoffmann et al., 2009; Radax et al., 2012). Thus this study set out to analyse both the bacterial and archaeal diversity associated with the studied sponges. Additionally, the spatial distribution of microbial symbionts in the sponge *S. normani* was analyzed. The two studied sponges played host to a remarkably different bacterial and archaeal diversity. Whereas the sponge *S. normani* harboured more than 3,000 genus level OTUs which were relatively homogenous in their abundance and completely different from the seawater community the sponge *L. diversichela* harboured only ~200 genus level OTUs which were more often related to sequences also obtained from the water column, while one of the gammaproteobacterial OTUs made up ~80% of all sequences derived from the sponge. Furthermore, the bacterial and archaeal symbionts of *S. normani* were often spatially distributed with several OTUs being predominantly present in the outer sponge, the cortex, especially microbes which have previously been associated with nitrogen and sulphur metabolism. A comparison of the obtained sequences from this deep water sampling site with two other studies from shallow sites implied a closer relationship between the two shallow water sites with each other than to the deep water site.

It has to be stressed that the results obtained from this study are very interesting but that due to the lack of replicate samples analysed, the observation pertaining to spatial distribution of microbial populations in the sponge *S. normani* remains qualitative at best. A second sample at least and ideally a third sample would need to have been analysed, in order to confirm such findings. The retrieval of replicate samples for marine sponges from such depths is unfortunately very expensive and often not possible. During the last 4 years three similar cruises to the one in May 2010 from which these deep sea sponges were obtained have been carried out but each of them was less successful in the retrieval of sponge samples than the May 2010 cruise. One of the cruises encountered the loss of the R.O.V. due to a torn cable, in another cruise the weather conditions were in most parts too harsh to carry out any sampling with the R.O.V and in yet another cruise the biodiversity encountered on the sea floor was very scarce. However, when comparing the results

with a similar study on sponges obtained during the same research cruise it is interesting that two more sponges (*Poecilastra compressa* and *Inflatella pellicula*) had a roughly similar microbial cohort with relatively little diversity (200-300 genus level OTUs), an abundant proteobacterial OTU closely related to that of the sponge symbiont in *L. diversichela*, and more OTUs common with the seawater than did the microbiota associated with *S. normani* (Stephen Jackson, personal communication). Even though abundance data on the microbial community in all of the sponges was not obtained, a differentiation of the sponges *S. normani* and *L. diversichela* into HMA sponges and LMA sponges, respectively, is likely possible. Similar results as obtained here for deep water sponges, i.e. a more diverse and sponge specific microbiota in some sponges and a microbiota often similar to that of the surrounding sea water and dominated by relatively few organisms, have previously been reported from other sponges (Schmitt et al., 2011; Vacelet and Donadey, 1977; Weisz et al., 2007). From the same sponges which were analysed for their associated microbiota via pyrosequencing bacterial isolates were also obtained. The most abundant isolates were classified as either *Pseudoalteromonas* or *Psychrobacter* (Stephen Jackson, personal communication). Sequences classified as *Pseudoalteromonas* were the most abundant water column derived reads in the pyrosequencing study (~43% of all reads) and *Psychrobacter* reads were still abundant (~1%) whereas both genera were (almost) absent from both sponge derived amplicon libraries. This, together with similar results when comparing the culture independent assessment of the water column's microbiota in Lough Hyne with the bacteria obtained from various sponges collected there, yet again highlights the fact that many of the microbes associated with marine sponges are not readily culturable as for example has been shown for whole phyla (e.g. *Poribacteria*). The development of alternative culturing methods (e.g. Sipkema et al., 2011) will therefore be an important part of sponge-microbiology research in the future. It also stresses the point that metatranscriptomics as well as single-cell genomic approaches to identify the potential of unculturable important sponge associates will become increasingly important tools in deciphering the bacterial-sponge relationships both from a phylogenetic and functional perspective. The application of these techniques to the sponges analysed in Chapter 5 could therefore prove particularly fruitful, especially the sponge *L. diversichela* which could be an interesting model sponge due to the apparent "simplicity" of its associated microbiota. In particular the dominant

gammaproteobacterial OTU in this sponge's amplicon library would be an interesting target for single cell genomics in order to obtain information on this symbiont's potential ecological role in the sponge. The sponge *S. normani* on the other hand could be an interesting target for the application of metatranscriptomics in order to analyse the active bacteria and genes in the metagenome of this sponge.

The currently most comprehensive study of sponge microbiota by Schmitt and colleagues (Schmitt et al., 2012) implied the presence of a tropical sponge microbiota. Likewise, the data presented here suggests a different microbiota in deep water than in shallow water sponges. The number of samples collected however is unfortunately not sufficient to make this assumption more than speculation at this point, but it will be interesting to see in the future whether in larger scale studies such as the Schmitt et al. study this can be confirmed. Another possible scenario is that a deep water specific microbial diversity is not in fact present in sponges, but that the diversity dependence of sponge associated microbes is extended to a tropical-subtropical-cold-water gradient. Again, larger scale studies involving various sponges from different environments such as the tropics, subtropics, temperate waters and cold waters will have to be undertaken in order to test for this hypothesis. It should be noted that future studies, even if carried out in different research groups, should ideally employ primer pairs targeting the same region of the 16S-rRNA in order to make an OTU level comparison of different studies possible. Studies comparing different primer sets could be employed in order to determine the ideal target region as for example those which have been carried out for the analysis of the gut microbiome (for which the V4 region was the ideal target; Claesson et al., 2009). The higher sequencing depths of Illumina sequencing could also be applied in the future in order to obtain even more detailed insights into sponge microbiota than currently possible through 454 pyrosequencing. When for example considering the rarefaction curve of the sponge microbiota of *S. normani* it becomes clear that a plateau is not reached and many more organisms await discovery. It has to be noted though that the current read length of Illumina sequencing platforms are not long enough for a robust classification of sequencing reads to the genus level as shown by Claesson and colleagues (Claesson et al., 2010). The extraordinarily high level of unclassified sequencing reads in the choanosome and cortex derived amplicon library of *S. normani* is also worth discussing. The absence of candida phyla from the RDP classifier makes it likely that at least some of the unclassified reads are

indeed members of such phyla, as has been shown previously for sponge associated microbes (Webster et al., 2010). The application of a BLAST search has already identified several of the more abundant OTUs derived from both the cortex and the choanosome as *Poribacteria,* while other analysis tools which have only recently become available to our research group should be applied in order to analyze the obtained sequence data, such as CloVR (http://clovr.org/) and/or Qiime (http://qiime.org/).

In summary several sponges and their associated microbes collected in Irish waters have been shown to be promising sources of potentially novel secondary metabolites and in addition deep water sponges have been shown to harbour a unique microbiota which should be the subject of further more in depth analysis.

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