Universidade de Aveiro Departamento de Biologia

2015

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Potencial ecológico e biotecnológico do microbioma da esponja

Ecological and biotechnological potential of sponge microbiome

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Ecological and biotechnological potential of sponge microbiome

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica do Doutor Francisco José Riso da Costa Coelho, investigador de Pós-Doutoramento do Departamento de Biologia e do Centro de Estudos do Ambiente e do Mar da Universidade de Aveiro, e co-orientação científica do Doutor Daniel Francis Richard Cleary, investigador principal do Departamento de Biologia e do Centro de Estudos do Ambiente e do Mar da Universidade de Aveiro.

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palavras-chave

Esponjas marinha, microbioma, potencial biotecnológico, metagenómica

resumo

As esponjas marinhas abrigam comunidades microbianas de grande importância ecológica e biotecnológica. Recentemente, estas têm recebido maior atenção devido ao grande número de compostos com actividade biológica, com potencial aplicação, particularmente, nas indústrias química, cosmética e farmacêutica. No entanto, a ecologia e o potencial biotecnológico dos seus microrganismos ainda permanecem largamente desconhecidos. O desenvolvimento de tecnologias de sequenciação de alta resolução deu origem a novo grupo de abordagens que nos podem ajudar a explorar o potencial biotecnológico das esponjas com um detalhe sem precedentes. As abordagens metagenómicas, em particular, tem poder para revolucionar a produção de compostos com actividade biológica produzidos por microrganismos não cultiváveis, ao permitir a identificação de genes ou *clusters* de genes biosintéticos com capacidade para serem expressos heterologamente num organismo hospedeiro adequado e cultivável. Esta revisão foca particularmente a exploração do potencial biotecnológico dos microrganismos associados a esponjas, e a integração de abordagens moleculares, cuja eficiência crescente pode desempenhar um papel essencial no desenvolvimento de uma fonte sustentável de produtos naturais.

keywords

Marine sponges, microbiome, biotechnological potential, metagenomics

abstract

Marine sponges harbor microbial communities of immense ecological and biotechnological importance. Recently, they have been focus of heightened attention due to the wide range of biologically active compounds with potential application, particularly, in chemical, cosmetic and pharmaceutical industries. However, we still lack fundamental knowledge of their microbial ecology and biotechnological potential. The development of high-throughput sequencing technologies has given rise to a new range of tools that can help us explore the biotechnological potential of sponges with incredible detail. Metagenomics, in particular, has the power to revolutionize the production of bioactive compounds produced by unculturable microorganisms. It can offer the identification of biosynthetic genes or gene clusters that can be heterologously expressed on a cultivable and suitable host. This review focus on the exploration of the biotechnological potential of sponge-associated microorganisms, and integration of molecular approaches, whose increasing efficiency can play an essential role on achieving a sustainable source of natural products.

Contents

List of publications

Cleary DFR, Polónia ARM, Becking LE, de Voogd NJ, Purwanto, Gomes H, Gomes NCM (in press) Compositional analysis of bacterial communities in seawater, sediment and high and low microbial abundance sponges in the Misool coral reef system, Indonesia. *Austral Ecology.*

List of tables

List of figures

CHAPTER 1

INTRODUCTION

Introduction

Marine sponges (Phylum *Porifera*) are ancient aquatic metazoans with a fossil record dating back more than 580 million years to the Precambrian (van Soest et al., 2012). Today, the majority of the 8500 formally described living species belong to the class *Demospongia* (demosponges) (Borchiellini et al., 2001; van Soest et al., 2012) (Figure 1). The remaining species represent the classes *Hexactinellida* (glass sponges) and *Calcarea* (calcareous sponges) (Hentschel et al., 2006). Sponges are placed among the oldest metazoan and the simplest multicellular animals, possessing little tissue differentiation and coordination (Li et al., 1998; Lee et al., 2009). Their aptitude to survive over time appears to be closely related to the adaptability of their body plan to dramatic environmental shifts (Palumbi, 1986; Müller & Müller, 2003). This has proven advantageous in sub-optimal conditions where sponges may increase their chances of survival through physiological or morphological adaptations (McDonald et al., 2002). Sponges have successfully colonized a wide range of habitats from tropical to polar seas, shallow to deep waters, and marine to freshwater habitats (Rützler, 2004). They can be voracious space competitors, overgrowing sessile reef organisms (Diaz & Rützler, 2001) and, due to their efficient filter feeding ability, even live in nutrient-poor habitats (Cuvelier et al., 2014). Sponges are structurally important components of coral reefs where they contribute to reef stabilization and regeneration by connecting pieces of rubble to the reef frame and creating a stable foundation for hard corals to settle on (Wulff, 2001; Ilan et al., 2004). They have been shown to provide shelter for numerous organisms (Ilan et al., 2004). Villamizar and Laughlin (1991) reported 139 and 53 species of crustaceans, ophiuroids, mollusks and fishes inhabiting only two reef sponge species (*Aplysina lacunosa* and *Aplysina archeri*, respectively) in a study conducted in a reef on the coast of Venezuela. Nonetheless, despite their role in marine ecosystems, we still lack fundamental knowledge regarding their spatial distribution. Environmental parameters, including depth, light, tidal amplitude, water flow rate, pollution level and content of dissolved organic matter, are known to affect sponge distribution (de Voogd et al., 2006; Santos-Gandelman et al., 2014).

Sponges have arisen in an era when bacteria were already strongly established in the oceans. Interestingly, what could have become a dangerous 'playground' for sponges turned out to be one of the most promising symbiotic associations ever known. Sponges can harbor dense and complex communities of bacteria, archaea and eukaryotes in an association whose ecologic and evolutionary importance is tightly connected to the increasing perception that microorganisms may be the ones responsible for the production of most of the compounds found in their tissues (Haygood et al., 1999). The production of bioactive compounds by sponges has become focus of heightened interest in 1950 when Bergmann and Feeney (Santos-Gandelman et al., 2014) (and references therein) discovered nucleosides spongothymidine and spongouridine in the marine sponge *Cryptotethya crypta*, that would become the basis for the synthesis of Ara-A and Ara-C, an antiviral compound and the first anticancer agent derived of marine organisms, respectively. Since then, besides the production of bioactive compounds they have been used in a number of different fields such as environmental monitoring and bioremediation processes, partially due to their role in nutrient cycling, water filtering, bio-erosion, spatial competition and production of biologically active compounds (Faulkner, 2002; Bell, 2008).

Figure 1 Sponges *Stylissa massa* (Image by Ana R. M. Polónia) and *Xestospongia testudinaria* (Image by Rossana Freitas).

The increase perception of sponge biotechnological potential, and the development of molecular biology technologies, has fostered the research and understanding of their associated microbial community structure and function. The development of high-throughput sequencing technologies, stimulated by the Human Genome Project has made it feasible to sequence the DNA from complex communities; thus revealing the sponge associated microbial community structure and ecology with an unprecedented detail. On the other hand, these technologies have created a new range of tools that is helping us to explore the sponge biotechnological potential. While several symbionts are culturable, providing open access to test their capacity to synthesize natural products, the greatest share of them appear to be obligate symbionts, not possessing the mechanisms required to live away from the sponge. Recent advances in molecular techniques now enable us to uncover their biosynthetic genes through metagenomics and single-cell genomics approaches getting us one step closer to secure a nearly unlimited, environmentally conscious and improved production source of biologically active compounds (Schirmer et al., 2005).

The aim of this work is to review the current knowledge of sponge-associated microbial ecology and biotechnology, focusing recent advances in molecular biology and their potential to improve our ability to explore the sponge biotechnological potential.

References

Bell, J. J. (2008). The functional roles of marine sponges. *Estuarine, Coastal and Shelf Science*, *79*, 341–353. doi:10.1016/j.ecss.2008.05.002

Borchiellini, C., Manuel, M., Alivon, E., Boury-Esnault, N., Vacelet, J., & Le Parco, Y. (2001). Sponge paraphyly and the origin of Metazoa. *Journal of Evolutionary Biology*, *14*, 171–179. doi:10.1046/j.1420-9101.2001.00244.x

Cuvelier, M. L., Blake, E., Mulheron, R., McCarthy, P. J., Blackwelder, P., Thurber, R. L. V., & Lopez, J. V. (2014). Two distinct microbial communities revealed in the sponge Cinachyrella. *Frontiers in Microbiology*, *5*(November), 1–12. doi:10.3389/fmicb.2014.00581

de Voogd, N. J., Cleary, D. F. R., Hoeksema, B. W., Noor, A., & van Soest, R. W. M. (2006). Sponge beta diversity in the Spermonde Archipelago, SW Sulawesi, Indonesia. *Marine Ecology Progress Series*, *309*, 131–142. doi:10.3354/meps309131

Diaz, M. C., & Rützler, K. (2001). Sponges: an essential component of caribbean coral reefs. *Bulletin of Marine Science*, *69*(2), 535–546.

Faulkner, D. J. (2002). Marine natural products. *Natural Product Reports*, *19*, 1–48. doi:10.1039/b009029h

Haygood, M. G., Schmidt, E. W., Davidson, S. K., & Faulkner, D. J. (1999). Microbial symbionts of marine invertebrates: opportunities for microbial biotechnology. *Journal of Molecular Microbiology and Biotechnology*, *1*(1), 33–43.

Hentschel, U., Usher, K. M., & Taylor, M. W. (2006). Marine sponges as microbial fermenters. *FEMS Microbiology Ecology*, *55*, 167–77. doi:10.1111/j.1574-6941.2005.00046.x

Ilan, M., Gugel, J., & van Soest, R. W. M. (2004). Taxonomy, reproduction and ecology of new and known Red Sea sponges. *Sarsia*, *89*, 388–410. doi:10.1080/00364820410002659

Lee, O. O., Chui, P. Y., Wong, Y. H., Pawlik, J. R., & Qian, P.-Y. (2009). Evidence for vertical transmission of bacterial symbionts from adult to embryo in the caribbean sponge Svenzea zeai. *Applied and Environmental Microbiology*, *75*(19), 6147–56. doi:10.1128/AEM.00023-09

Li, C.-W., Chen, J.-Y., & Hua, T.-E. (1998). Precambrian Sponges with Cellular Structures. *Science*, *279*, 879–82.

McDonald, J. I., Hooper, J. N. A., & McGuinness, K. A. (2002). Environmentally influenced variability in the morphology of Cinachyrella australiensis (Carter 1886) (Porifera: Spirophorida: Tetillidae). *Marine Freshwater Research*, *53*, 79–84. doi:10.1071/MF00153

Müller, W. E. G., & Müller, I. M. (2003). Analysis of the sponge [Porifera] gene repertoire: implications for the evolution of the metazoan body plan. In W. E. G. Müller (Ed.), *Sponges (Porifera)* (p. 258). Heidelberg: Springer-Verlag. doi:10.1007/978-3-642-55519-0_1

Palumbi, S. R. (1986). How Body Plans Limit Acclimation: Responses of a Demosponge to Wave Force. *Ecology*, *67*(1), 208–214.

Rützler, K. (2004). Sponges on coral reefs: a community shaped by competitive cooperation. *Bollettino Dei Musei E Degli Instituti Biologici dell'Universita Di Genova*, *68*, 85–148.

Santos-Gandelman, J., Giambiagi-deMarval, M., Oelemann, W. M. R., & Laport, M. S. (2014). Biotechnological potential of sponge-associated bacteria. *Current Pharmaceutical Biotechnology*, *15*(2), 143–155. doi:10.2174/1389201015666140711115033

Schirmer, A., Gadkari, R., Reeves, C. D., Ibrahim, F., DeLong, E. F., & Hutchinson, C. R. (2005). Metagenomic analysis reveals diverse polyketide synthase gene clusters in microorganisms associated with the marine sponge Discodermia dissoluta. *Applied and Environmental Microbiology*, *71*, 4840–4849. doi:10.1128/AEM.71.8.4840-4849.2005

van Soest, R. W. M., Boury-Esnault, N., Vacelet, J., Dohrmann, M., Erpenbeck, D., de Voogd, N. J., Santodomingo, N., Vanhoorne, B., Kelly, M., &Hooper, J. N. A. (2012). Global diversity of sponges (Porifera). *PLoS ONE*, *7*(4), e35105. doi:10.1371/journal.pone.0035105

Villamizar, E., & Laughlin, R. A. (1991). Fauna associated with the sponges Aplysina archeri and Aplysina lacunosa in a coral reef of the Archipiélago de Los Roques, National Park, Venezuela. In J. Reitner & H. Keupp (Eds.), *Fossil and Recent Sponges* (pp. 522–542). Berlin: Springer-Verlag.

Wulff, J. (2001). Assessing an monitoring coral reef sponges: why and how? *Bulletin of Marine Science*, *69*(2), 831–846.

CHAPTER 2

SPONGE AND MICROORGANISMS, A COMPLEX INTERDEPENDENT RELATIONSHIP

Sponge and microorganisms, a complex interdependent relationship

Sponges are known to be important members of both shallow- and deep-water communities, hosting large and diverse communities of microbial symbionts of extraordinary ecological and biotechnological importance (Taylor et al. 2007a)(Freeman & Thacker, 2011). Numerous marine invertebrates engage in long-term, and sometimes highly specialized, associations with microorganisms representing an ancient evolutionary relationship that results from a fusion of multiple host colonization events and co-speciation (Thacker & Starnes, 2003). The abundance and composition of sponge-associated microorganisms can vary greatly among sponge species, including those inhabiting the same habitat (Cleary et al., 2013). Microbes inhabit the mesophyll matrix of numerous demosponges (Hentschel et al., 2003), known as high-microbial-abundance (HMA) sponges, to which the great majority of the described living species belong (Borchiellini et al., 2001; van Soest et al., 2012). High-microbial-abundance sponges can contain around 10^{10} bacterial cells g^{-1} wet weight of sponge (2 to 4 orders of magnitude higher than concentrations in sea water) (Hentschel et al., 2006), while low-microbial abundance (LMA) sponges contain around 10^{6} cells g^{-1} (similar to concentrations in sea-water) (Kamke et al., 2010). LMA sponges less diverse bacterial community can however still harbor interesting diversity (Izumi et al., 2013).

Relatively little is known about the ecological relationship between symbionts and sponges, or the role played by symbionts on the host (Taylor et al., 2007b; Webster & Taylor, 2012). Phylogenetic inference suggests that associated bacteria and archaea can conduct a wide range of metabolic processes such as ammonium- and nitrite-oxidation, nitrogen fixation, sulfate reduction and photosynthesis (Taylor et al., 2007b). Nevertheless, sponge-associated microorganisms may establish a commensal or even parasitic relationship with their hosts as opposed to a strictly mutualistic one. Thacker (2005) demonstrated, that sponge hosts rely on symbiont nutrition to varying degrees. Shading experiments revealed that while some host sponge species appeared unaffected by the experiments and managed to compensate for reduced symbiont nutrition, other species experienced a drastic reduction in growth rates (Thacker, 2005). This suggests a

direct relationship between the presence of internal symbiotic microorganisms and sponge growth. Kamke and colleagues (2010) would later publish a study comparing 16S rRNA- and 16S rRNA gene-derived sequences from different sponge species revealing what Thacker's study suggested: most of the microbial community is metabolically active within their host. Indeed, even though the main via of carbon acquisition is heterotrophic filter feeding, sponges can get up to 50% of their energy and 80% of their carbon requirements from their photosymbionts (Cheshire & Wilkinson, 1991; Cheshire et al., 1997) .

Sponges have been shown to be unique and highly selective environments for bacteria (Hentschel et al., 2002; Hentschel et al., 2006). These associations may be due to highly selective conditions within the sponge mesohyl, as suggested by low detection levels or even complete absence of sponge-specific phylotypes from the surrounding environments (Hentschel et al., 2006). Such relations are often very specific with many of the microorganisms inhabiting exclusively sponge hosts (Webster & Taylor, 2012) or even inhabiting utterly particular sponge species throughout large geographical areas (Flatt et al., 2005). Hentschel and colleagues (2002) performed the first study on biogeographical variability spanning hundreds of thousands of kilometers. They encompassed a comprehensive phylogenetic analysis of bacteria associated with sponges *Theonella swinhoei* and *Aplysina aerophoba*, taxonomically distantly related species from the western Pacific Ocean and the Mediterranean Sea, respectively. The study comprised 190 sponge-derived 16S rRNA gene sequences and suggested highly consistent microbial communities, regardless of host or location (Hentschel et al., 2002). As another example, Taylor and colleagues (2005) examined the geographic variability of the bacterial community of sponge *Cymbastela concentrica* from the eastern Australian coast. The study revealed the composition of the community remained similar throughout a 500 kilometers area. Webster et al. (2004) further characterized the whole microbial community (archaeal, bacterial and eukaryotic) from five Antarctic sponges throughout a 10 kilometers transect. Phylogenetic analysis reported highly consistent communities among co-specific hosts. Additionally, the bacterial communities differed among host species, but even at different locations conserved the referred consistency.

Further than through geographical areas, these associations seem to be maintained through time. Friedrich and colleagues (2001) incubated organisms of sponge specie of *Aplysina aerophoba* in untreated water for 11 days and observed no significant changes in the microbial population within the sponge tissues. In 2004, Taylor and colleagues carried out a study to understand whether co-occurring sponge species *Cymbastela concentrica*, *Callyspongia sp.* and *Stylinos sp.* harbored particular microbial communities. The study uncovered the existence of apparently stable bacterial communities over the course of one year (Taylor et al., 2004). Interestingly, in *Callyspongia sp.* and *Stylinos sp.*, the sponges whose bacterial communities were most similar to seawater, the variations registered occurred independently of those that occurred in the surrounding seawater. Such data further highlights the structural differences between bacterial communities of both biotopes, even considering LMA sponges.

Sponge-associated microbial communities seem to be stable within individuals and through time. Nonetheless, specific subsets of the overall community can occur consistently within the same sponge species from different locations. This phenomenon, in which specific microbial communities of different sponge species may be the result of highly evolved relations maintained by vertical transmission within the sponge specie, was first proposed in the early 60's. Since then, vertical transmission of bacterial symbionts through embryos has been explored for several sponge species (Lee et al., 2009). Recently, Sharp and colleagues (2007) published strong evidences that even complex bacterial communities can be vertically transmitted in the tropical sponge *Corticium sp.*. Their research has revealed that bacteria can be acquired by sponge embryos during embryogenesis indicating a specialized mechanism of transfer during embryonic development.

References

Borchiellini, C., Manuel, M., Alivon, E., Boury-Esnault, N., Vacelet, J., & Le Parco, Y. (2001). Sponge paraphyly and the origin of Metazoa. *Journal of Evolutionary Biology*, *14*, 171–179. doi:10.1046/j.1420-9101.2001.00244.x

Cheshire, A. C., & Wilkinson, C. R. (1991). Modelling the photosynthetic production by sponges on Davies Reef, Great Barrier Reef. *Marine Biology*, *109*, 13–18.

Cheshire, A. C., Wilkinson, C. R., Seddon, S., & Westphalen, G. (1997). Bathymetric and seasonal changes in photosynthesis and respiration of the phototrophic sponge Phyllospongia lamellosa in comparison with respiration by the heterotrophic sponge Ianthella basta on Davis Reef, Great Barrir Reef. *Marine Freshwater Research*, *48*, 589–99. doi:10.1071/MF96070

Cleary, D. F. R., Becking, L. E., de Voogd, N. J., Pires, A. C. C., Polónia, A. R. M., Egas, C., & Gomes, N. C. M. (2013). Habitat- and host-related variation in sponge bacterial symbiont communities in Indonesian waters. *FEMS Microbiology Ecology*, *85*, 465–82. doi:10.1111/1574-6941.12135

Freeman, C. J., & Thacker, R. W. (2011). Complex interactions between marine sponges and their symbiotic microbial communities. *Limnology and Oceanography*, *56*(5), 1577–1586. doi:10.4319/lo.2011.56.5.1577

Friedrich, A. B., Fischer, I., Proksch, P., Hacker, J., & Hentschel, U. (2001). Temporal variation of the microbial community associated with the mediterranean sponge Aplysina aerophoba. *FEMS Microbiology Ecology*, *38*, 105–115. doi:10.1111/j.1574-6941.2001.tb00888.x

Hentschel, U., Hopke, J., Horn, M., Friedrich, A. B., Wagner, M., Hacker, J., & Moore, B. S. (2002). Molecular Evidence for a Uniform Microbial Community in Sponges from Different Oceans. *Applied and Environmental Microbiology*, *68*(9), 4431–40. doi:10.1128/AEM.68.9.4431-4440.2002

Hentschel, U., Fieseler, L., Wehrl, M., Gernert, C., Steinert, M., Hacker, J., & Horn, M. (2003). Microbial diversity of marine sponges. In W. E. Muller (Ed.), *Sponges (Porifera)* (Springer-V., pp. 59–88). Heidelberg.

Hentschel, U., Usher, K. M., & Taylor, M. W. (2006). Marine sponges as microbial fermenters. *FEMS Microbiology Ecology*, *55*, 167–77. doi:10.1111/j.1574-6941.2005.00046.x

Izumi, H., Sagulenko, E., Webb, R. I., & Fuerst, J. A. (2013). Isolation and diversity of planctomycetes from the sponge Niphates sp., seawater, and sediment of Moreton Bay, Australia. *Antonie van Leeuwenhoek*, *104*, 533–546. doi:10.1007/s10482-013-0003-5

Kamke, J., Taylor, M. W., & Schmitt, S. (2010). Activity profiles for marine sponge-associated bacteria obtained by 16S rRNA vs 16S rRNA gene comparisons. *The ISME Journal*, *4*, 498–508. doi:10.1038/ismej.2009.143

Lee, O. O., Chui, P. Y., Wong, Y. H., Pawlik, J. R., & Qian, P.-Y. (2009). Evidence for vertical transmission of bacterial symbionts from adult to embryo in the caribbean sponge Svenzea zeai. *Applied and Environmental Microbiology*, *75*(19), 6147–56. doi:10.1128/AEM.00023-09

Sharp, K. H., Eam, B., Faulkner, D. J., & Haygood, M. G. (2007). Vertical transmission of diverse microbes in the tropical sponge Corticium sp. *Applied and Environmental Microbiology*, *73*(2), 622–9. doi:10.1128/AEM.01493-06

Taylor, M. W., Schupp, P. J., Dahllof, I., Kjelleberg, S., & Steinberg, P. D. (2004). Host specificity in marine sponge-associated bacteria, and potential implications for marine microbial diversity. *Environmental Microbiology*, *6*(2), 121–130. doi:10.1046/j.1462-2920.2003.00545.x

Taylor, M. W., Schupp, P. J., de Nys, R., Kjelleberg, S., & Steinberg, P. D. (2005). Biogeography of bacteria associated with the marine sponge Cymbastela concentrica. *Environmental Microbiology*, *7*(3), 419–433. doi:10.1111/j.1462-2920.2004.00711.x

Taylor, M. W., Hill, R. T., Piel, J., Thacker, R. W., & Hentschel, U. (2007a). Soaking it up: the complex lives of marine sponges and their microbial associates. *The ISME Journal*, *1*, 187–90. doi:10.1038/ismej.2007.32

Taylor, M. W., Radax, R., Steger, D., & Wagner, M. (2007b). Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. *Microbiology and Molecular Biology Reviews*, *71*(2), 295–347. doi:10.1128/MMBR.00040-06

Thacker, R. W. (2005). Impacts of shading on sponge-cyanobacteria symbioses: a comparison between host-specific and generalist associations. *Integrative and Comparative Biology*, *45*, 369–376.

Thacker, R. W., & Starnes, S. (2003). Host specificity of the symbiotic cyanobacterium Oscillatoria spongeliae in marine sponges, Dysidea spp. *Marine Biology*, *142*, 643–8. doi:10.1007/s00227-002-0971-x

van Soest, R. W. M., Boury-Esnault, N., Vacelet, J., Dohrmann, M., Erpenbeck, D., de Voogd, N. J., Santodomingo, N., Vanhoorne, B., Kelly, M., &Hooper, J. N. A. (2012). Global diversity of sponges (Porifera). *PLoS ONE*, *7*(4), e35105. doi:10.1371/journal.pone.0035105

Webster, N. S., Negri, A. P., Munro, M. M. H. G., & Battershill, C. N. (2004). Diverse microbial communities inhabit Antarctic sponges. *Environmental Microbiology*, *6*(3), 288–300. doi:10.1111/j.1462-2920.2004.00570.x

Webster, N. S., & Taylor, M. W. (2012). Marine sponges and their microbial symbionts: love and other relationships. *Environmental Microbiology*, *14*(2), 335–346. doi:10.1111/j.1462-2920.2011.02460.x

CHAPTER 3

RECENT ADVANCES IN METAGENOMIC APPROACHES - A NEW TOOL FOR EXPLOITING THE SPONGE-ASSOCIATED MICROORGANISMS BIOTECHNOLOGY

Recent advances in metagenomic approaches- a new tool for exploiting the sponge-associated microorganisms biotechnology

The introduction of DNA sequencing technologies revealed that cultured microorganisms are a mere fraction of the total diversity; only an estimate 1% of microorganisms can grow under laboratory conditions (Reid & Buckley, 2011). With the development of community genomics (metagenomics), we have now access to the uncultured majority and its biotechnological potential (Wilson & Piel, 2013). Metagenomic approaches to discover novel natural products have provided valuable insights into the chemistry of uncultivable organisms. Typical steps of a metagenomic workflow are illustrated in Figure 2. In the following sections we will focus two different approaches; the traditional construction of metagenomic libraries and the recent advances in high-throughput sequencing technologies and its potential.

Construction of metagenomic libraries

The construction of a library of environmental DNA in a suitable host can be a powerful tool for investigating the biosynthetic potential of unculturable microorganisms. High molecular weight environmental DNA, isolated from the environmental sample, is the starting point, although enrichment of specific cell populations can be performed prior to isolation to enhance the representation of target organisms in the sample and target them for distinct analysis. In the construction of a genomic library, the choice of host and vector are absolutely critical. Its success can depend on this step. *Escherichia coli* is the most common host, although specific function-based screening protocols can require particular DNA recipients (Brady et al., 2009), while cosmid and fosmid vectors are the most common ones. Partially due to their high stability, capacity to load fragments of great dimension (35-45 kb), and ability to be easily transfected into *E. coli* cells (Piel, 2011). Here, the library can be screened for its function or homology. Directed functional-based screening (i.e. directed for modified phenotypes or specific enzymatic function) is, actually, a common and efficient method for identifying metabolites, biocatalysts or even

novel biochemical mechanisms (Wilson & Piel, 2013). Furthermore, this screening can be based on color, zones of inhibition on bacterial or fungal lawns, and specific reporter genes which are activated when a specific target from the DNA sample is expressed (Piel, 2011).

High-throughput sequencing, a game-changing technology

The complexity of microbial assemblages and the technical constrains associated to measure its components have limited our understating of the structure and function of sponge-associated microbes. However, despite the minor percentage of sponge-associated cultivable microorganisms, recent advances in molecular techniques, such as next generation high-throughput sequencing DNA technologies, now enable us to uncover microbial communities at an unprecedented level of detail.

The development of next-generation technologies was greatly stimulated by the largest collaborative biological project to date- the Human Genome project. This project started in 1990 with the first draft genome being published in 2001; costing approximately US$3 billion (van Dijk et al., 2014). In 2004 the National Human Genome Research Institute launch a funding program to reduce the cost of human sequencing, establishing a goal that seemed almost fictional; the reduction of the human genome sequencing to US$1000 in ten years (Schloss, 2008). This stimulated the development of high-throughput next generation sequencing, with the Illumina HiSeq X Ten breaking the barrier of the US$1000 genome in 2014. A quick overview of the price evolution is presented in Figure 3. This substantial cost reduction and the increase in throughput has made it technically and economically feasible to sequence community DNA, without previous cloning or cultivation. Although initially it was limited to DNA, it is now possible to sequence RNA; thus obtaining a snapshot of what part of the genetic capability of the microbial community function is being actually used (Reid, 2011).

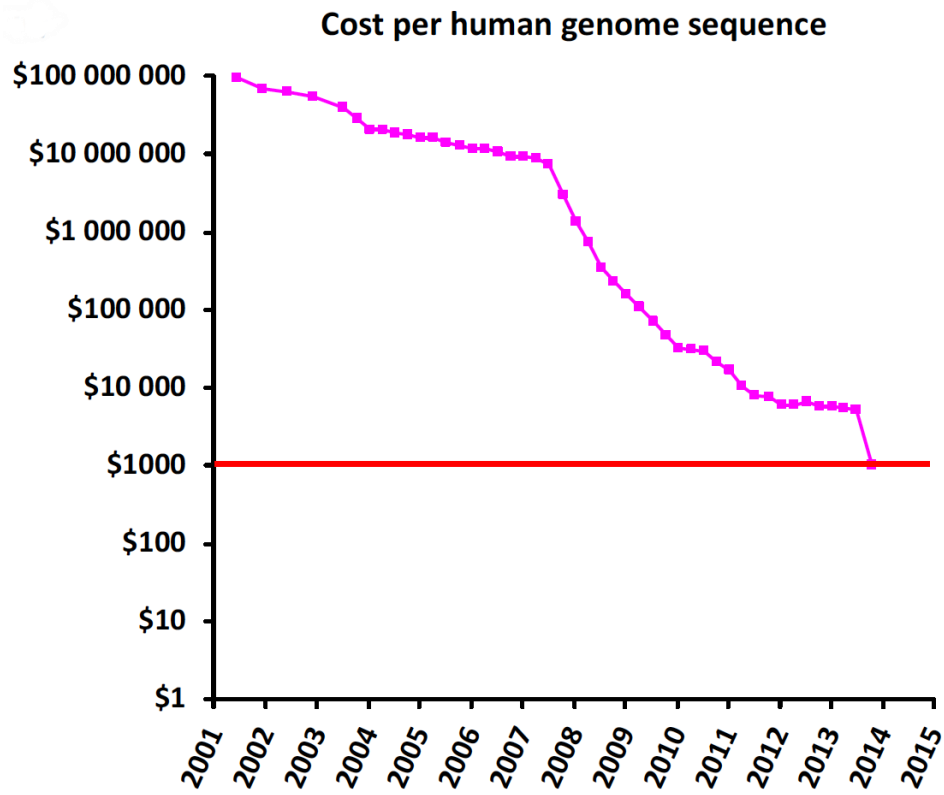

Figure 3 Evolution of the cost of sequencing a human genome from 2001 to 2014. Adapted from van Dijk et al. (2014).

This revolution in sequencing technologies has allow deeper insights into sponge-associated microbiome and the sponge itself, at several orders of magnitude higher than it was previous possible, shifting our perception of the microbial structure and function in sponges (Webster et al., 2010; Jackson et al., 2012). Furthermore, besides the ecological insights, by using a variety of bioinformatics screening methods the discovery of novel biocatalysts and biosynthetic genes can be identified at higher speed.

The use of metagenomics to isolate biosynthetic genes and gene clusters can be particularly useful when chasing after compounds produced by unculturable symbionts. Genome mining approaches could provide the foundation for development of new natural products based on gene clusters expressed from uncultured symbionts (Piel, 2011).

Gene homology-based screening does not require expression of biosynthetic genes for detection. Instead, it relies on sequence similarity to previously known biosynthetic genes. Natural products are often synthesized by gene clusters whose dimension exceed the insert size limit of cosmid, fosmid or BAC vectors (Wilson & Piel, 2013). Here, degenerated primers from conserved regions of biosynthetic targets can be used to PCR amplify particular gene fragments, from which specific PCR primers can be generated for screening a metagenomic clone library for genes or pathways hard to detect in functional screens (Brady et al., 2009), such as modular polyketide synthesis (PKS) or nonribosomal peptide synthesis (NRPS) pathways. In the end, the identification of a natural product biosynthetic gene cluster will lead to heterologous expression of the entire cluster and production of its compound. Metagenomics' potential to revolutionize large-scale and sustainable production of bioactive compounds produced, in particular, by yet uncultivated microorganisms is unquestionable (Wilson & Piel, 2013). Nonetheless, assembly can become very challenging. PKS and NRPS pathways can be particularly hard to assembly and complex systems which houses thousands of different species of microorganisms with several PKS and NRPS genes that can rarely be attributed to a specific producer (Piel, 2011). But every problem has a solution. Single-cell genomics has been developed to fulfill this gap. Through it, the genome of an isolated cell from a complex microbial community can be amplified and sequenced or screened in order to identify particular genes or pathways (Wilson & Piel, 2013) (and references therein).

The greatest technologies often carry the greatest technical challenges. Next-generation sequencing technologies are improving as fast as its price is dropping. Nowadays, the amount of data generated by a single unit can be incredibly hard to analyze without the help of genome mining tools. However, these approaches grounded on homology-based queries limit greatly the identification of novel compounds. The solution may lay on new strategies for bioinformatic genome mining. Nonetheless, cultivation should remain an important piece in the discovery of natural products. Recently, advances in cocultivation and environmental simulation have shown that cultivation of (so far) unculturable bacteria can be achieved (Stewart, 2012). Application of semiporous culture chambers, where organisms can grow independently but exchange small molecules, such as nutrients and growth factors, identification of signaling molecules that regulate

microbial growth and creation of appropriate conditions are proving to be successful (Nichols et al., 2008).

Integration of metagenomics with single-cell analysis, metatranscriptomics and proteomics can further enhance our understanding on how to express biosynthetic genes heterologously and grow uncultivated microorganisms under artificial conditions (Wilson & Piel, 2013).

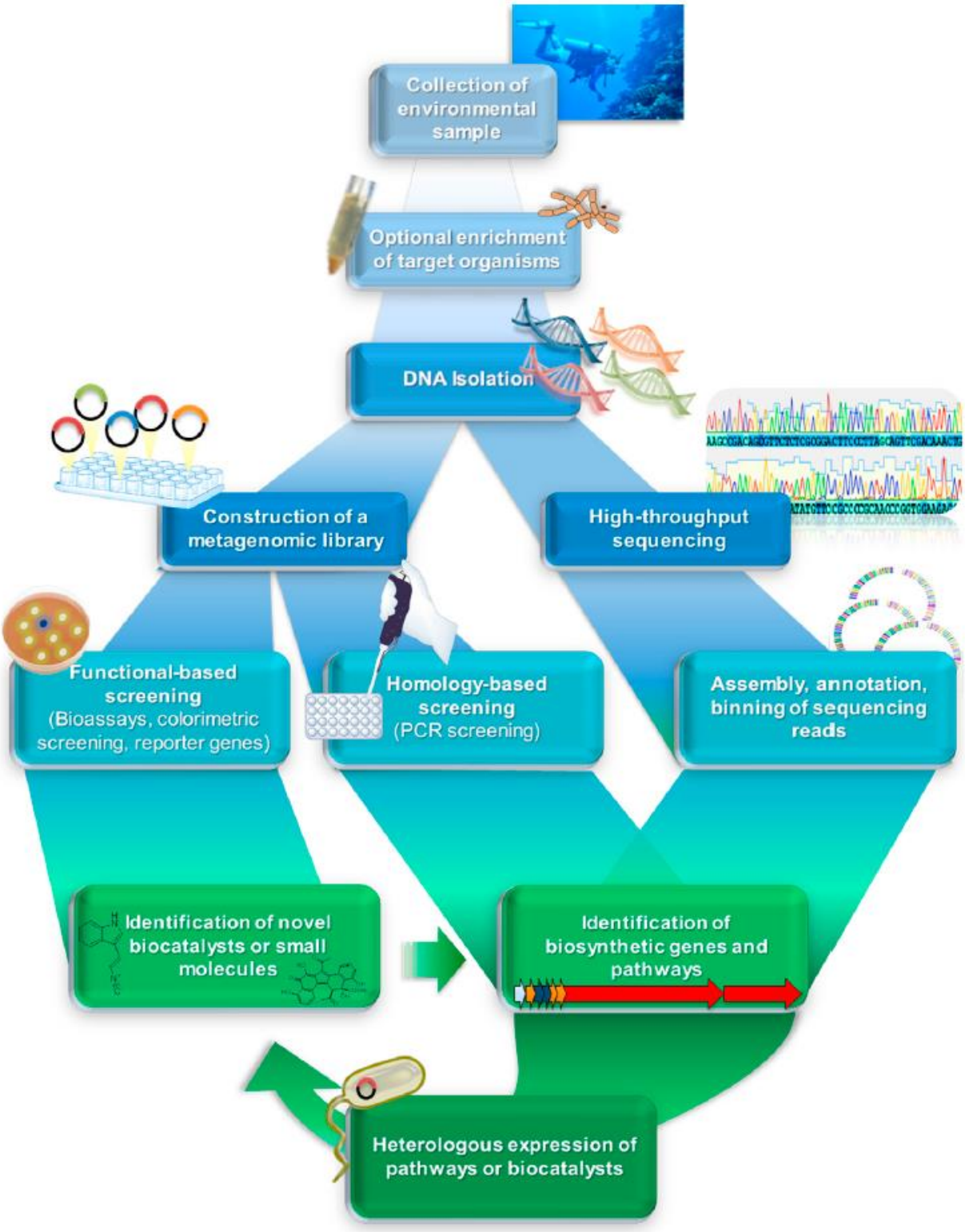

Figure 2 Typical steps of a metagenomic workflow (Wilson and Piel, 2013).

References

Brady, S. F., Simmons, L., Kim, J. H., & Schmidt, E. W. (2009). Metagenomic approaches to natural products from free-living and symbiotic organisms. *Natural Product Reports*, *26*(11), 1488–1503. doi:10.1039/b817078a.Metagenomic

Jackson, S. A., Kennedy, J., Morrissey, J. P., O'Gara, F., & Dobson, A. D. W. (2012). Pyrosequencing reveals diverse and distinct sponge-specific microbial communities in sponges from a single geographical location in Irish waters. *Microbial Ecology*, *64*, 105–16. doi:10.1007/s00248-011-0002-x

Nichols, D., Lewis, K., Orjala, J., Mo, S., Ortenberg, R., O'Connor, P., Zhao, C., Vouros, P., Kaeberlein, T., & Epstein, S. S. (2008). Short peptide induces an "uncultivable" microorganism to grow in vitro. *Applied and Environmental Microbiology*, *74*(15), 4889–4897. doi:10.1128/AEM.00393-08

Piel, J. (2011). Approaches to capturing and designing biologically active small molecules produced by uncultured microbes. *Annual Review of Microbiology*, *65*, 431–53. doi:10.1146/annurev-micro-090110-102805

Reid, A. (2011). *Incorporating microbial processes into climate models*. *A report from the American Society of Microbiology*.

Reid, A., & Buckley, M. (2011). *The Rare Biosphere*. *A report from the American Academy of Microbiology*.

Schloss, J. A. (2008). How to get genomes at one ten-thousandth the cost. *Nature Biotechnology*, *26*(10), 1113–1115.

Stewart, E. J. (2012). Growing unculturable bacteria. *Journal of Bacteriology*, *194*, 4151–4160. doi:10.1128/JB.00345-12

van Dijk, E. L., Auger, H., Jaszczyszyn, Y., & Thermes, C. (2014). Ten years of next-generation sequencing technology. *Trends in Genetics*, *30*(9), 418–426.

Webster, N. S., Taylor, M. W., Behnam, F., Lücker, S., Rattei, T., Whalan, S., Horn, M., & Wagner, M. (2010). Deep sequencing reveals exceptional diversity and modes of transmission for bacterial sponge symbionts. *Environmental Microbiology*, *12*(8), 2070–2082. doi:10.1111/j.1462-2920.2009.02065.x

Wilson, M. C., & Piel, J. (2013). Metagenomic approaches for exploiting uncultivated bacteria as a resource for novel biosynthetic enzymology. *Chemistry & Biology*, *20*, 636–647. doi:10.1016/j.chembiol.2013.04.011

CHAPTER 4

BIOTECHNOLOGICAL POTENTIAL OF SPONGE-ASSOCIATED MICROORGANISMS

Biotechnological potential of sponge-associated microorganisms

Marine sponges are the sea's greatest producers of novel compounds (Taylor et al., 2007a). The occurrence of similar compounds, known exclusively from microorganisms, in unrelated sponges prompted the hypothesis that they could be of microbial origin (Piel, 2004), especially when chemical synthesis of natural products can be both hard and expensive (Sipkema et al., 2005b). These compounds have been shown to be important in conferring resistance to microbial infections and in overcoming predation and competition (Thacker, 2005). Broad competition for nutrients and space in the marine environment can be a powerful driving force leading to the development of effective strategies of colonization and growth of marine microorganisms. In fact, part of the secondary metabolites produced by them aim to antagonize the growth of other microorganisms (Burgess et al., 1999). The awareness that these compounds can be produced by associated microbes, integrally or partially, has rendered them heightened interest as they can represent a potentially unlimited supply of compounds for biomedical, pharmaceutical and biotechnological applications.

In order to be encompassed in drug development pipelines, compounds must combine a unique pharmacological profile (i.e. new mechanism of action against a specific target), a controlled and effective action in human system and a commercially-accessible source of large-scale supply to solve the 'supply problem' early-on the development process (Sipkema et al., 2005b). Despite their potential, however, none of the sponge-derived compounds discovered so far has been approved as a clinical treatment agent. Ara-A and Ara-C remain in a grey area: while they were commercialized as antiviral and anticancer agents, respectively, they are actually synthetic derivatives based on nucleosides spongothymidine and spongouridine found in sponge *Cryptotethia crypta,* not compounds isolated straight from the sponge (Santos-Gandelman et al., 2014) (and references therein).

The aforementioned stability of microbial communities over space and time relies on the increased genomic flexibility these communities often present in order to remain

able to adapt constantly to changing environmental conditions (McDaniel et al., 2010). Such capacity largely increases the potential of enzymes produced by sponge-associated microorganisms for industrial application. These have been shown to produce amylases, cellulases, lipases and proteases (Kiran et al., 2008; Shanmughapriya et al., 2008, 2009, 2010) suggested to play a role in the conversion of the filtered organic matter into nutrients for the sponge. The remarkable habitat these microorganisms inhabit gives them distinct features that enables them to produce distinct enzymes with unique properties. Amylases and proteases in particular, are among the most important industrial enzymes (Burhan et al., 2003; Shanmughapriya et al., 2009).

Pharmacologically relevant compounds

Sponge-associated bacteria have also been shown to produce enzymes with potential application in the treatment of neurodegenerative diseases such as Alzheimer and Parkinson. This potential lies on its capacity to inhibit acetylcholinesterase (AChE) activity. AChE inhibitors (AChEIs) increase the concentration of acetylcholine (ACh), essential in cholinergic brain synapses and neuromuscular junctions, by reducing the rate of its breakdown by AChE. Zhou and colleagues (2011) reported the first brominated ene-tetrahydrofuran, isolated from *Xestospongia testudinaria*, displaying AChE inhibitory activity close to that of tacrine, mutafuran H. Tacrine is the active ingredient of one of the first approved drugs for the treatment of Alzheimer's symptoms. More recently, Pandey et al. (2014) identified a potent AChEI produced by *Bacillus subtilis* strain M18SP4Q(ii), isolated from the marine sponge *Fasciospongia cavernosa*. There's no cure for Alzheimer's disease yet but, today, most of the approved drugs are AChEIs (Pandey et al., 2014). Recent inclusion of AChEIs in the treatment for symptoms of the early stages of Alzheimer's disease has encouraged a crescent interest into finding natural products with biotechnological potential (Williams et al., 2011).

Curiously, giant barrel sponge *X. testudinaria* has been shown to produce compounds with potential clinical applications. Akiyama and colleagues (2013) reported the production of a new brominated acetylenic fatty acid, testufuran A, capable of increasing adiponectin secretion, a protein involved in regulation of diabetes mellitus and

atherosclerosis. *X. testudinaria* has also been shown to be a source a new brominated polyunsaturated lipid whose activity was shown to be similar to that of Orlistat®, an approved anti-obesity drug clinically used for years (Liang et al., 2014).

Members from Actinobacteria, Proteobacteria and Firmicutes phyla are the major producers of pharmacologically relevant compounds but, despite the great potential for drug development, so far none resulted in commercial medication (Santos-Gandelman et al., 2014). However some may be 'close'. Salinosporamide A, produced by strains of the *Salinispora* genus, is currently in clinical trials as NPI-0052 (Marizomib) for treatment of leukemia, multiple myeloma and cancer, for its proteasome inhibition and anticancer potential (Feling et al., 2003; Fenical et al., 2009; Gulder & Moore, 2010; Niewerth et al., 2014). In fact, *Salinispora* species are prolific producers of natural products particularly due to the gene clusters involved in the biosynthesis of natural products shared among them (Miyanaga et al., 2011). Bose and colleagues (2014) identified at least 57 new compounds potentially produced by *S. arenicola* and *S. pacifica* isolated from sponges *Dercitus xanthus*, *Cinachyrella australiensis* and *Hyattella intestinalis* from the Great Barrier Reef. Furthermore, *S. arenicola* strains have been found to produce antimycobacterial rifamycins (Kim et al., 2006), clinically relevant antibacterial agents previously observed only in the terrestrial soil actinobaterium *Amycolatopsis mediterranei*, broadening the scope of new natural products to be found and studied.

Manzamine A produced by sponge *Acanthostrongylophora sp.*'s associated actynomycete *Micromonospora sp.* is among the most promising compounds since its antibacterial, anti-malarial, anti-HIV, anti-tumor, insecticidal and anti-inflammatory activities were revealed (Radwan et al. 2012). Although, growth of manzamine-producing *Micromonospora sp.* has been achieved in large scale through fermentation with the conservation of manzamine production, further research is needed in order to uncover its true potential and advance into clinical trials.

Sometimes, the most harmful compounds are the most promising solutions. Brominated aliphatic hydrocarbons, such as ene-tetrahydrofurans, found in *X. testudinaria* are extremely toxic. Since AChE inhibition is considered one of the most important mechanisms of chemical defense of marine organisms, it is plausible to

speculate that brominated aliphatic hydrocarbons are probably involved in the sponge's chemical defense (Key & Fulton, 2006). Metagenomic analysis of *Theonella swinhoei* revealed the bacterial production of potent cytotoxins, onnamides (Piel et al., 2004), closely related to an highly active antitumor polyketide pederin produced by a bacterial symbiont of terrestrial rove beetles *Paederus fuscipes* with the closest relationship to *Pseudomonas aeruginosa*. Pederin's antitumor activity lies on its capacity to block the synthesis of proteins in the ribosomes of eukaryotic cells and, thus, inhibit mitose (Kellner & Dettner, 1996), but its potential can go beyond that. The way biosynthetic genes from *T. swinhoei* are clustered together (Figure 4) and can be isolated makes it plausible to believe that gene cloning and heterologous expression in a culturable host can help reach otherwise inaccessible potential new bioactive compounds (Piel et al., 2005).

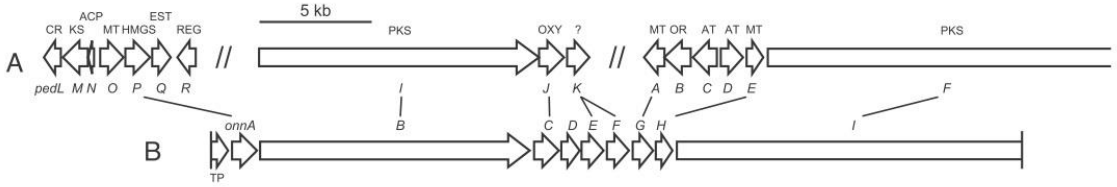

Figure 4 Comparison of the onnamide (onn) gene cluster with the pederin (ped) system. **A.** Map of the pederin gene cluster from *P. fuscipes* symbiont. Double slashes divide the three genomic regions. **B.** Map of the onnamide gene cluster and its correlation to pederin homologs. Adapted from Piel et al. (2004).

Notwithstanding the antitumoral potential of pederin, such relation raised the question of what evolutionary forces may have led to the production of similar substances in symbionts of dissimilar hosts. Interestingly, metabolites highly similar to pederin, produced by the above referred bacterial symbiont of terrestrial rove beetles, have been found in several marine sponges (Bewley & Faulkner, 1998) getting both phyla closer. Phospholipase A2 (PLA2), produced by *Dendrilla nigra*'s associated bacterium *Streptomyces dendra sp.* nov. MSI051, is a hydrolytic enzyme with recognized biotechnological potential in the hydrolysis of the sn-2 acyl ester bond of phospholipases resulting in the release of a free fatty acid and a lysophospholipid, products encompassed in the generation of important second messengers that play important physiological roles.

Before its key functional role in the defense of sponges against predators was recognized, its ubiquitous defense action was only described in snake and bee venoms (Selvin, 2009).

Isolation and growth of these symbionts in laboratorial conditions can allow the synthesis of a wide range of potentially and known bioactive products but there are other ways: metagenomics and genetic engineering. The use of metagenomics to isolate biosynthetic genes and gene clusters can be particularly useful when chasing after compounds produced by unculturable symbionts. Genome mining approaches could provide the foundation for development of new natural products based on gene clusters expressed from uncultured symbionts (Piel, 2011). Nonetheless, engineering of completely new compounds via gene recombination holds great potential and can actually be the future. Li and Piel (2002) performed it applying an established genetic system (*Streptomyces lividans)* and a novel integrative cosmid vector in order to clone, sequence and heterologously express the griseorhodin biosynthesis gene cluster of griseorhodin A. This molecule integrates a group of rubromycins that have been shown to inhibit telomerase activity (Yunt et al., 2009), known to be stimulated by cancer cells in order to control the indefinite growth capacity maintained by their telomeres (Saretzki, 2003). Hence, griseorhodin A has the potential to include the group of new anticancer drugs whose cancer reversal approach is based on telomerase inhibition.

Amylases

Amylases are applied in food, brewing, distilling, detergent, textile, paper, leather, cosmetic, chemistry and medical industries (Burhan et al., 2003; Shanmughapriya et al., 2009). They can be produced by plants and animals but the productivity and thermostability of microbial amylases made of them the most used in industry (Shanmughapriya et al., 2009). The specificity of the processes where they are involved requires thermophilic and thermostable enzymes active and stable at elevated temperatures (80-110 ºC, according to the processes) to achieve a sustainable use (Lévêque et al., 2000). Therefore, there has been an ongoing interest in the discovery of new bacterial strains capable of producing such amylases to new industrial applications (Singh et al., 2014). In a study conducted by Mohapatra et al. (1998) a novel acidic amylase

was isolated from *Mucor* sp. associated with sponge *Spirastrella* sp.. Its maximum activity (41.84 U/ml) was reached at pH 5.0. More recently, Shanmughapriya and colleagues (2009) discovered a novel alkaline amylase produced by *Halobacterium sp.* strain MMD047 isolated from sponge *Fasciospongia cavernosa*. The strain has been shown to be capable of reaching its maximum amylase production yield (75.27 U/mg) in only 18h, whereas *Bacillus cereus* and *Bacillus subtilis* have been shown to need over 48h to reach its maximum production (Anto et al., 2006; Asgher et al., 2007). Additionally, *Halobacterium sp.* strain MMD047 has been shown to be capable of producing amylase with a specific activity of 206.53 U/mg after purification process, which represents a 2.74-fold increase (Shanmughapriya et al., 2009).

Proteases

Proteases play a key role in the regulation of metabolic processes such as protein catabolism, blood coagulation, inflammation, zymogenes activation, hormones releasing, cell development, morphogenesis and tumor growth and metastasis (Rao et al., 1998).

In 2003, a group of researchers investigated the capacity of bacteria associated with eight marine sponges to produce great levels of protease. Here, strains of *Alcaligenes*, *Alteromonas*, *Bacillus*, *Corynebacterium*, *Flavobacterium*, *Micrococcus*, *Vibrio* and one unidentified bacterium revealed pronounced protease activity ranging from 0.5 to 0.971 U/ml (Mohapatra et al., 2003). Shanmughapriya and colleagues (2008) isolated an endosymbiotic *Roseobacter sp.* strain MMD040 from marine sponge *Fasciospongia cavernosa* capable of producing high yields of protease (1.125 U/mg) under optimal conditions. Suzuki et al. (1997) reported ATP-dependent proteases Clp and FtsH conserved between bacteria and eukaryotes believed to act as chaperones and mediate the insertion of proteins into membranes and the disassembly or oligomerization of protein complexes. Curiously, Chung & Goldberg (1981) reported years before an ATP-dependent protease, product of *lon* gene, which plays a critical role in the hydrolysis of abnormal proteins in *Escherichia coli*. This mechanism becomes increasingly interesting as we consider that accumulation of abnormal proteins can be directly related to several human degenerative diseases such as frontotemporal dementia (FTD), Alzheimer's

disease and amyotrophic lateral sclerosis (ALS) (Huang & Mucke, 2012; Ash et al., 2013). The development of a highly-specific, controlled and effective treatment based on proteolysis of abnormal proteins could have a wide application in medicine.

Proteases seem to play on both sides of the fence. Further than being a product of a gene they can modulate gene expression in a way that proteolysis of a repressor, by an ATP-dependent protease, can result in a derepression of the gene (Roberts et al., 1977). This inactivation runs on the same mechanism that works during induction of SOS response to DNA damage. In fact, specific gene products of *recA* and *lexA* genes may be directly involved in the referred inactivation, as mutations *tif-1* and *spr-51* responsible for expression of SOS functions seem to map these genes. In this line, the study conducted by Isono et al. (1978) revealed that 'enzymes' can modify ribosomal proteins and play a crucial role in the assembly, structure and function of the ribosome, and thus in the regulation of translation.

Proteases currently are applied in the detergent, silver, food, leather, chemical and pharmaceutical industries (Shanmughapriya et al., 2008). They account for approximately 60% of the total worldwide enzyme market (Rao et al., 1998), expected to be worth US$2767 million by 2019 (Markets and Markets, 2014). Proteases of microbial source account for approximately 40% of the total worldwide production of enzymes (Rao et al., 1998). Furthermore, over 50% of the industrially important enzymes are produced from genetically engineered microorganisms (Rao et al., 1998). This manipulation is carried out either to study their properties, structure and role in the pathogenicity of the microorganism, since the virulence of bacteria is often related to the secreted extracellular proteases, or to fuel their overproduction to meet the market demands (Rao et al., 1998). The aforementioned specificity of the industrial processes require target-specific enzymes active and stable within a narrow range of pH and temperature values. Unfortunately, most of the enzymes also require specific substrates, incubation period, and pH and temperature values in order to reach productive yields. Over the last decades, enzyme engineering has been proved to be a plausible solution to enhance the productivity of those processes. Qin et al. (2008) improved the optimum pH and catalytic efficiency of *Trichoderma reesei* endo-beta-1,4-glucanase II through combination of saturation and random mutagenesis, and DNA shuffling techniques. In the end, they

managed to rise the optimum pH value of one mutant in 1 unit without the loss of efficiency, and achieve a 4.5-fold higher activity of another mutant against the wild-type at the same pH value. Jaouadi and colleagues (2010) achieved the production of a mutant protease whose optimum temperature is 10. ºC higher than that of the wild type in *Escherichia coli*, through the introduction of disulfide bonds by site-directed mutagenesis (SDM). Furthermore, the researchers also achieved a 31-fold enhancement of the catalytic efficiency of the same protease. Industrial processes often require specific substrates that can differ from the natural substrates of enzymes. Here, substrate-specificity of protease could become a problem, but introduction of point mutations into the substrate-binding site of proteases has been shown to improve its specificity (Graham et al., 1993).

Proteases are truly a unique class of enzymes with an enormous physiological and commercial importance in the modern world. Especially, those produced by microorganisms. Their rapid growth, small space requisition and great accessibility for genetic manipulation has allowed the production of 'tailor-made' products with novel properties and heightened their successful application in industry. Furthermore, enzymes such as proteases have been key targets for the development of therapeutic agents against fatal diseases such as cancer, malaria and AIDS (Joyce et al., 2004; Rosenthal, 2004; Yanchunas et al., 2005).

Joyce and colleagues (2004) performed an experiment in which cathepsin cysteine proteases, upregulated in pancreatic islet tumors, were knocked out using a broad-spectrum cysteine cathepsin inhibitor. Interestingly, the inhibition resulted in a decrease in the progression to invasive carcinoma and did not present any kind of toxicity. Proteolytic enzymes, particularly cysteine proteases, play critical roles in the life cycle of malaria parasites, in hemoglobin hydrolysis and erythrocyte rupture and invasion. Inhibition of these enzymes has been proved to exhibit antimalarial effects. Falcipain cysteine protease inhibitors have been shown to prevent hemoglobin hydrolysis, block parasite development and cure murine malaria (Rosenthal, 2003), in an accurate action mechanism.

Remarkably, it is in AIDS treatment that proteolytic enzymes, principally HIV-1 and HIV-2, have been prime targets for the development of drug therapy. Here, HIV protease inhibitors bind to the active site of the enzyme blocking its maturation into infectious virions (Brik & Wong, 2003). There are, in fact, several protease inhibitors licensed for HIV therapy (Amprenavir®, Atazanavir®, Darunavir®, Fosamprenavir®, Indinavir®, Lopinavir®, Nelfinavir®, Ritonavir®, Saquinavir® and Tipranavir®), including five second-generation protease inhibitors that work against HIV variants resistant to older drugs (Atazanavir®, Darunavir®, Fosamprenavir®, Lopinavir® and Tipranavir®).

However, the long-term application of protease inhibitors is being compromised by the emergence of drug-resistant variants (Yanchunas et al., 2005). Retroviruses have particularly high mutation rates. Under the selective pressure of replication-inhibiting drugs, substitutions of only a few amino acid residues within the protease can change dramatically the active site of the enzyme and block the action of the inhibitor (van Maarseveen & Boucher, 2006). The combination of complementary drugs that inhibit key points of the HIV replication cycle simultaneously, rather than one drug at a time, helps minimize the development of drug-resistance, but so far no cure has been established.

Considering the great number of human cancers that express high levels of cathepsin activity, the increasing resistance of malaria parasites to available drugs and the elevated mutation rate of retroviruses, the investment on the study of protease inhibition mechanisms and the development of new protease-targeting approaches may actually lead to the creation of promising strategies to fight cancer, malaria and AIDS. It is important that studies such as the one performed by Bose and colleagues (2014) involving chemometric approaches combined with metabolic profiling techniques start playing a major role in the screening of sponge bacterial natural products.

References

Akiyama, T., Takada, K., Oikawa, T., Matsuura, N., Ise, Y., Okada, S., & Matsunaga, S. (2013). Stimulators of adipogenesis from the marine sponge Xestospongia testudinaria. *Tetrahedron*, *69*(32), 6560–6564. doi:10.1016/j.tet.2013.06.007

Anto, H., Trivedi, U., & Patel, K. (2006). Alpha Amylase Production by Bacillus cereus MTCC 1305 Using Solid-State Fermentation. *Food Technology and Biotechnology*, *44*(2), 241–245.

Asgher, M., Asad, M. J., Rahman, S. U., & Legge, R. L. (2007). A thermostable α-amylase from a moderately thermophilic Bacillus subtilis strain for starch processing. *Journal of Food Engineering*, *79*, 950–955. doi:10.1016/j.jfoodeng.2005.12.053

Ash, P. E. A., Bieniek, K. F., Gendron, T. F., Caulfield, T., Lin, W.-L., DeJesus-Hernandez, M., van Blitterswijk, M. M., Jansen-West, K., Paul III, J. W., Rademakers, R., Boylan, K. B., Dickson, D. W., & Petrucelli, L. (2013). Unconventional translation of C9ORF72 GGGGCC expansion generates insoluble polypeptides specific to c9FTD/ALS. *Neuron*, *77*, 639–646. doi:10.1016/j.neuron.2013.02.004

Bewley, C. A., & Faulkner, D. J. (1998). Lithistid Sponges: Star Performers or Hosts to the Stars. *Angewandte Chemie International Edition*, *37*, 2162–2178. doi:1433-7851/98/3715-2163

Bose, U., Hewavitharana, A. K., Vidgen, M. E., Ng, Y. K., Shaw, P. N., Fuerst, J. A., & Hodson, M. P. (2014). Discovering the recondite secondary metabolome spectrum of Salinispora species: a study of inter-species diversity. *PLoS One*, *9*(3), 1–10. doi:10.1371/journal.pone.0091488

Brik, A., & Wong, C.-H. (2003). HIV-1 protease: mechanism and drug discovery. *Organic & Biomolecular Chemistry*, *1*, 5–14. doi:10.1039/b209248a

Burgess, J. G., Jordan, E. M., Bregu, M., Mearns-Spragg, A., & Boyd, K. G. (1999). Microbial antagonism: a neglected avenue of natural products research. *Journal of Biotechnology*, *70*, 27–32. doi:10.1016/S0079-6352(99)80094-0

Chung, C. H., & Goldberg, A. L. (1981). The product of the lon (capR) gene in Escherichia coli is the ATP-dependent protease, protease La. *Proceedings of the National Academy of Sciences USA*, *78*(8), 4931–4935.

Feling, R. H., Buchanan, G. O., Mincer, T. J., Kauffman, C. A., Jensen, P. R., & Fenical, W. (2003). Salinosporamide A: a highly cytotoxic proteasome inhibitor from a novel microbial source, a marine bacterium of the new genus Salinospora. *Angewandte Chemie International Edition*, *42*(3), 355–357.

Fenical, W., Jensen, P. R., Palladino, M. A., Lam, K. S., Lloyd, G. K., & Potts, B. C. (2009). Discovery and development of the anticancer agent Salinosporamide A (NPI-0052). *Bioorganic & Medicinal Chemistry*, *17*(6), 1–13. doi:10.1016/j.bmc.2008.10.075.Discovery

Graham, L. D., Haggett, K. D., Jennings, P. A., Le Brocque, D. S., Whittaker, R. G., & Schober, P. A. (1993). Random mutagenesis of the substrate-binding site of a serine protease can generate enzymes with increased activities and altered primary specificities. *Biochemistry*, *32*, 6250–6258. doi:10.1021/bi00075a019

Gulder, T. A. M., & Moore, B. S. (2010). Salinosporamide natural products: potent 20S proteasome inhibitors as promising cancer chemotherapeutics. *Angewandte Chemie International Edition*, *49*, 9346–9367. doi:10.1002/anie.201000728.Salinosporamide

Huang, Y., & Mucke, L. (2012). Alzheimer mechanisms and therapeutic strategies. *Cell*, *148*, 1204–1222. doi:10.1016/j.cell.2012.02.040

Isono, S., Isono, K., & Hirota, Y. (1978). Mutations affecting the structural genes and the genes coding for modifying enzymes for ribosomal proteins in Escherichia coli. *Molecular & General Genetics*, *165*, 15–20. doi:10.1007/BF00270371

Jaouadi, B., Aghajari, N., Haser, R., & Bejar, S. (2010). Enhancement of the thermostability and the catalytic efficiency of Bacillus pumilus CBS protease by site-directed mutagenesis. *Biochimie*, *92*, 360–9. doi:10.1016/j.biochi.2010.01.008

Joyce, J. A., Baruch, A., Chehade, K., Meyer-Morse, N., Giraudo, E., Tsai, F.-Y., Greenbaum, D. C., Hager, J. H., Bogyo, M., & Hanahan, D. (2004). Cathepsin cysteine proteases are effectors of invasive growth and angiogenesis during multistage tumorigenesis. *Cancer Cell*, *5*, 443–453. doi:10.1016/S1535-6108(04)00111-4

Kellner, R. L. L., & Dettner, K. (1996). Differential efficacy of toxic pederin in deterring potential arthropod predators of Paederus (Coleoptera: Staphylinidae) offspring. *Oecologia*, *107*, 293–300. doi:10.1007/BF00328445

Key, P. B., & Fulton, M. H. (2006). Correlation between 96-h mortality and 24-h acetylcholinesterase inhibition in three grass shrimp larval life stages. *Ecotoxicology and Environmental Safety*, *63*, 389–392. doi:10.1016/j.ecoenv.2005.10.015

Kim, T. K., Hewavitharana, A. K., Shaw, P. N., & Fuerst, J. A. (2006). Discovery of a new source of rifamycin antibiotics in marine sponge actinobacteria by phylogenetic prediction. *Applied and Environmental Microbiology*, *72*, 2118–25. doi:10.1128/AEM.72.3.2118-2125.2006

Kiran, G. S., Shanmughapriya, S., Jayalakshmi, J., Selvin, J., Gandhimathi, R., Sivaramakrishnan, S., … Natarajaseenivasan, K. (2008). Optimization of extracellular psychrophilic alkaline lipase produced by marine Pseudomonas sp. (MSI057). *Bioprocess and Biosystems Engineering*, *31*, 483–92. doi:10.1007/s00449-007-0186-0

Lévêque, E., Janecek, S., Haye, B., & Belarbi, A. (2000). Thermophilic archaeal amylolytic enzymes. *Enzyme and Microbial Technology*, *26*, 3–14.

Li, A., & Piel, J. (2002). A gene cluster from a marine Streptomyces encoding the biosynthesis of the aromatic spiroketal polyketide griseorhodin A. *Chemistry & Biology*, *9*, 1017–1026. doi:10.1016/S1074-5521(02)00223-5

Liang, L.-F., Wang, T., Cai, Y.-S., He, W.-F., Sun, P., Li, Y.-F., Huang, Q., Taglialatela-Scafati, O., Wang, H.-Y., & Guo, Y.-W. (2014). Brominated polyunsaturated lipids from the Chinese sponge Xestospongia testudinaria as a new class of pancreatic lipase inhibitors. *European Journal of Medicinal Chemistry*, *79*, 290–297. doi:10.1016/j.ejmech.2014.04.003

Markets and Markets. (2014). *Protein Hydrolysis Enzymes Market by Sources (Microorganisms, Animals, Plants), Applications (Detergent Industry, Pharmaceuticals, Food Industry and Others), & Geography (North America, Europe, Asia-Pacific & ROW) - Global Trends & Forecasts to 2019*. doi:FB 2392

McDaniel, L. D., Young, E., Delaney, J., Ruhnau, F., Ritchie, K. B., & Paul, J. H. (2010). High frequency of horizontal gene transfer in the oceans. *Science*, *330*, 50. doi:10.1126/science.1192243

Miyanaga, A., Janso, J. E., Mcdonald, L., He, M., Liu, H., Barbieri, L., Eustáquio, A. S., Fielding, E. N., Carter, G. T., Jensen, P. R., Feng, X., Leighton, M., Koehn, F. E., & Moore, B. S. (2011). Discovery and assembly-Line biosynthesis of the lymphostin pyrroloquinoline alkaloid family of mTOR inhibitors in Salinispora bacteria. *Journal of the American Chemical Society*, *133*, 13311–13313.

Mohapatra, B. R., Banerjee, U. C., & Bapuji, M. (1998). Characterization of a fungal amylase from Mucor sp. associated with the marine sponge Spirastrella sp. *Journal of Biotechnology*, *60*, 113–117. doi:10.1016/S0168-1656(97)00197-1

Niewerth, D., Jansen, G., Riethoff, L. F. V., van Meerloo, J., Kale, A. J., Moore, B. S., Assaraf, Y. G., Anderl, J. L., Zweegman, S., Kaspers, G. J. L., & Cloos, J. (2014). Antileukemic activity and mechanism of drug resistance to the marine Salinispora tropica proteasome inhibitor salinosporamide A (Marizomib). *Molecular Pharmacology*, *86*, 12–9. doi:10.1124/mol.114.092114

Pandey, S., Sree, A., Sethi, D. P., Kumar, C. G., Kakollu, S., Chowdhury, L., & Dash, S. S. (2014). A marine sponge associated strain of Bacillus subtilis and other marine bacteria can produce anticholinesterase compounds. *Microbial Cell Factories*, *13*(1), 24. doi:10.1186/1475-2859-13-24

Piel, J. (2004). Metabolites from symbiotic bacteria. *Natural Product Reports*, *21*, 519–538.

Piel, J. (2011). Approaches to capturing and designing biologically active small molecules produced by uncultured microbes. *Annual Review of Microbiology*, *65*, 431–53. doi:10.1146/annurev-micro-090110-102805

Piel, J., Butzke, D., Fusetani, N., Hui, D., Platzer, M., Wen, G., & Matsunaga, S. (2005). Exploring the chemistry of uncultivated bacterial symbionts: antitumor polyketides of the pederin family. *Journal of Natural Products*, *68*(3), 472–479. doi:10.1021/np049612d

Qin, Y., Wei, X., Song, X., & Qu, Y. (2008). Engineering endoglucanase II from Trichoderma reesei to improve the catalytic efficiency at a higher pH optimum. *Journal of Biotechnology*, *135*, 190–195. doi:10.1016/j.jbiotec.2008.03.016

Radwan, M., Hanora, A., Khalifa, S., & Abou-El-Ela, S. H. (2012). Manzamines: a potential for novel cures. *Cell Cycle (Georgetown, Tex.)*, *11*(9), 1765–1772. doi:10.4161/cc.20135

Rao, M. B., Tanksale, A. M., Ghatge, M. S., & Deshpande, V. V. (1998). Molecular and Biotechnological Aspects of Microbial Proteases †. *Microbiology and Molecular Biology Reviews*, *62*, 597–635.

Roberts, J. W., Roberts, C. W., & Mountt, D. W. (1977). Inactivation and proteolytic cleavage of phage delta repressor in vitro in an ATP-dependent reaction. *Proceedings of the National Academy of Sciences USA*, *74*(6), 2283–2287.

Rosenthal, P. J. (2003). Antimalarial drug discovery: old and new approaches. *The Journal of Experimental Biology*, *206*, 3735–3744. doi:10.1242/jeb.00589

Rosenthal, P. J. (2004). Cysteine proteases of malaria parasites. *International Journal for Parasitology*, *34*, 1489–99. doi:10.1016/j.ijpara.2004.10.003

Santos-Gandelman, J., Giambiagi-deMarval, M., Oelemann, W. M. R., & Laport, M. S. (2014). Biotechnological potential of sponge-associated bacteria. *Current Pharmaceutical Biotechnology*, *15*(2), 143–155. doi:10.2174/1389201015666140711115033

Saretzki, G. (2003). Telomerase inhibition as cancer therapy. *Cancer Letters*, *194*, 209–219. doi:10.1016/S0304-3835(02)00708-5

Selvin, J. (2009). Exploring the Antagonistic Producer Streptomyces MSI051: Implications of Polyketide Synthase Gene Type II and a Ubiquitous Defense Enzyme Phospholipase A2 in the Host Sponge Dendrilla nigra. *Current Microbiology*, *58*, 459–63. doi:10.1007/s00284-008-9343-1

Shanmughapriya, S., Krishnaveni, J., Selvin, J., Gandhimathi, R., Arunkumar, M., Thangavelu, T., Kiran, G. S., & Natarajaseenivasan, K. (2008). Optimization of extracellular thermotolerant alkaline protease produced by marine Roseobacter sp. (MMD040). *Bioprocess and Biosystems Engineering*, *31*, 427–433. doi:10.1007/s00449-007-0179-z

Shanmughapriya, S., Kiran, G. S., Selvin, J., Gandhimathi, R., Baskar, T. B., Manilal, A., & Sujith, S. (2009). Optimization, production, and partial characterization of an alkalophilic amylase produced by sponge associated marine bacterium Halobacterium salinarum MMD047. *Biotechnology and Bioprocess Engineering*, *14*, 67–75. doi:10.1007/s12257-008-0060-1

Shanmughapriya, S., Kiran, G. S., Selvin, J., Thomas, T. A., & Rani, C. (2010). Optimization, purification, and characterization of extracellular mesophilic alkaline cellulase from sponge-associated Marinobacter sp . MSI032. *Applied Biochemistry and Biotechnology*, *162*, 625–640. doi:10.1007/s12010-009-8747-0

Singh, S., Singh, S., Bali, V., Sharma, L., & Mangla, J. (2014). Production of fungal amylases using cheap, readily available agriresidues, for potential application in textile industry. *BioMed Research International*, *2014*, 1–9. doi:10.1155/2014/215748

Sipkema, D., Osinga, R., Schatton, W., Mendola, D., Tramper, J., & Wijffels, R. H. (2005). Large-scale production of pharmaceuticals by marine sponges: sea, cell, or synthesis? *Biotechnology and Bioengineering*, *90*(2), 201–222. doi:10.1002/bit.20404

Suzuki, C. K., Rep, M., van Dijl, J. M., Suda, K., Grivell, L. A., & Schatz, G. (1997). ATP-dependent proteases that also chaperone protein biogenesis. *Trends in Biochemical Sciences*, *22*(4), 118–123.

Taylor, M. W., Radax, R., Steger, D., & Wagner, M. (2007b). Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. *Microbiology and Molecular Biology Reviews*, *71*(2), 295–347. doi:10.1128/MMBR.00040-06

Thacker, R. W. (2005). Impacts of shading on sponge-cyanobacteria symbioses: a comparison between host-specific and generalist associations. *Integrative and Comparative Biology*, *45*, 369–376.

van Maarseveen, N., & Boucher, C. (2006). Resistance to protease inhibitors. In A. M. Geretti (Ed.), *Antiretroviral Resistance in Clinical Practice*. London: Mediscript.

Williams, P., Sorribas, A., & Howes, M.-J. R. (2011). Natural products as a source of Alzheimer's drug leads. *Natural Product Reports*, *28*, 48–77. doi:10.1039/c0np00027b

Yanchunas, J., Langley, D. R., Tao, L., Rose, R. E., Friborg, J., Colonno, R. J., & Doyle, M. L. (2005). Molecular basis for increased susceptibility of isolates with atazanavir resistance-conferring substitution I50L to other protease inhibitors. *Antimicrobial Agents and Chemotherapy*, *49*(9), 3825–32. doi:10.1128/AAC.49.9.3825-3832.2005

Yunt, Z., Reinhardt, K., Li, A., Engeser, M., Dahse, H.-M., Gütschow, M., Bruhn, T., Bringmann, G., & Piel, J. (2009). Cleavage of four carbon-carbon bonds during biosynthesis of the griseorhodin A spiroketal pharmacophore. *Journal of the American Chemical Society*, *131*, 2297–2305.

Zhou, X., Lu, Y., Lin, X., Yang, B., Yang, X., & Liu, Y. (2011). Brominated aliphatic hydrocarbons and sterols from the sponge Xestospongia testudinaria with their bioactivities. *Chemistry and Physics of Lipids*, *164*(7), 703–706. doi:10.1016/j.chemphyslip.2011.08.002

CHAPTER 5

CHALLENGES IN EXPLORING THE SPONGE-ASSOCIATED MICROORGANISMS BIOTECHNOLOGY

Challenges in exploring the sponge-associated microorganisms biotechnology

An important threat to the exploration of sponge-associated microorganisms biotechnology are the changes to sponge habitat due to anthropogenic action. Human activities such as fossil fuel combustion, fertilizer use, destructive fishing and industrial activities are fundamentally changing the ocean chemistry at a global scale and at rates that exceed the historical records (Doney, 2010). In fact, nitrogen-rich agricultural runoffs can fuel extensive and toxic phytoplankton blooms. Beman et al. (2005) predict that in 2050 the global expansion of industrialized agriculture will lead to the increase of the use of nitrogen-based fertilizer two or three times, particularly, close to nitrogen-vulnerable marine areas. Here, 27 to 59% of all nitrogen fertilizer will probably be applied in regions upstream of nitrogen-deficit marine areas, increasing dramatically the vulnerability and disturbing the structure and function of the surrounding marine ecosystems (Beman et al., 2005).

Destructive fishing, such as the use of fine mesh nets, and blast, dynamite or toxic fishing, further than target organisms, threatens the sustainability of the entire ecosystem, disrupting trophic relations, damaging species composition and richness, and ultimately declining the ecosystem dynamics (Bacalso & Wolff, 2014; and references therein). Moreover, destructive fishing is predicted to result in gradually lower catches and, consequently, less income through time (Bailey & Sumaila, 2015). Contrariwise, (Bailey & Sumaila, 2015) have recently anticipated the elimination of explosive and toxic approaches could result in higher and consistent productivity through time. Destructive fishing has been shown in the past to provoke severe and long-term damages to coral-dependent fish populations via destruction of coral reefs essential for refuge (Ainsworth et al., 2008).

Climate change and environmental stress-related disruption of the microbial symbiosis may have a significant impact on the development and protection of marine sponges against contamination, predation and, particularly, diseases (Santos-Gandelman et al., 2014). Sponge disease has been reported for over a century (Carter, 1878). In fact, one of the most dramatic examples was the epidemic in the Caribbean in 1938, described

by Smith (1941), in which 70-95% of sponges specimens disappeared. However, over the last years, disease and commercial sponge harvesting have taken numerous sponge populations close to extinction (Gaino et al., 1992). Disease in marine organisms can be easily circumscribed to key elements: rise of seawater temperature, nutrient enrichment, introduction of new species and anthropogenic pollution. In fact, Bruno et al., (2003) reported an increase in the impact of coral disease after increased nutrient exposure strengthening the idea that elevated concentration of inorganic nitrogen and phosphorus can affect disease dynamics and increase pathogen virulence and fitness.

Nevertheless, global climate change will play the most crucial role on the future of marine organisms. Anthropogenic emissions of carbon dioxide (CO_2) have increased from approximately 280 ppm (parts per million) to nearly 397 ppm between the preindustrial era and 2015 (Indermühle et al., 1999; NOAA, 2015). Consequently, the increasing atmospheric CO_2 concentration is leading to net air-to-sea flux of CO_2 that is reducing seawater pH and modifying the chemical balance among inorganic carbon species (Coelho et al., 2013). Oceanic pH will decline 0.3 to 0.4 units by the end of this century, and up to 0.7 units in 2300 (Caldeira & Wickett, 2003) affecting dramatically organisms whose skeleton or shells contain calcium carbonate, such as those composing coral reefs. For example, a recent survey along a natural CO_2 gradient has highlighted the potential effect of ocean acidification in sponge composition, in particular in demosponge species (Goodwin et al., 2014). Interesting, also along a natural CO_2 gradient, Morrow et al. (2015) found that increase CO_2 lead to an increase in photosynthetic microorganisms that potentially provides more nutrients to the sponge and, therefore, may increase their growth under these conditions (Morrow et al. 2015). Furthermore, organisms establishing symbiotic relationships with microorganism can also be disturbed, as microbial-mediated processes such as carbon and nitrogen cycles may also be affected by this decline. Moreover, the Intergovernmental Panel on Climate Change (IPCC) has predicted a rise of 1-5 ºC in seawater temperature by 2100 (Webster, 2007) that can be just enough to increase the impact of disease outbreaks. Temperature rising can enhance prevalence and virulence of pathogens, and virulence of sponge disease has already been linked to high seawater temperature in the past (Sutherland et al., 2004) (and references therein). Webster et al. (2008) demonstrated that when temperature rise above 33ºC, there is a

marked change in the structure of microbial symbionts of the marine sponge *Rhopaloeides odorabile*; favoring microbes that had a high similarity with sequences retrieved from disease and bleached corals (Webster et al., 2008; Pantile & Webster, 2011). Overall, these examples illustrate the potential that changes in global environment could have in the ecology of sponge-associated microorganisms, compromising the exploration of their full biotechnological potential.

Another well know challenge for the exploration of the sponge-associated microorganisms biotechnology is the supply of the sponge biomass necessary to extract sufficient amount of bioactive compounds for preclinical development. The investment in the synthesis route for complex molecules is generally done after the efficiency of the bioactive compound has been proven (Schippers et al., 2012). The isolation and cultivation of associated microorganisms is not always possible or desirable. Metabolic and nutritional interdependences between the microorganisms and the sponge host are extremely difficult to obtain; even if the microorganisms can be cultivated it may stop producing the compound of interested due to unknown dependencies with the sponge host (Taylor et al., 2007). A possible solution for this problem may come from the development of aquaculture techniques that cultivate both the invertebrate host and the associated microbial community (Leal et al., 2014).

Ex situ aquaculture allows the manipulation of environmental conditions in order to maximize the biomass of target species. The use of controlled, optimized and stable conditions enhances the compound production rate to impressively higher levels than that for *in situ* aquaculture. Furthermore, *ex situ* systems prevent the risk of genetic contamination of natural populations commonly related to mass culture of single genotypes in the wild (Cognetti et al., 2006). Nonetheless, it requires a high investment in building and operating the culture facilities, and strong control of effluents loaded with nutrients, drugs and chemicals to minimize its environmental impact. *Ex situ* cultures have already been shown to increase the growth of cultured species, regulate the presence of symbionts known to be involved in the production of target compounds, and enhance the yields of target compounds (Leal et al., 2014) (and references therein). They may actually represent a sustainable solution, particularly, when several sponges presenting potential for drug discovery can't be found in the required volume in nature. Halichondrin B,

isolated from sponge *Halichondria okadai*, is behind the discovery of Halaven®'s active compound anticancer activity. The compound is found in nature in such minimal amounts (less than 1 mg kg^{-1} of wet sponge biomass) that its extraction becomes unsustainable. The synthesis of a chemically synthesized halichondrin B analogue, with similar activity, was only achieved through controlled *ex situ* aquaculture (Munro et al., 1999).

In situ aquaculture, or mariculture, on the other hand relies completely on natural conditions for the growth of target species (Figure 5). It requires no adaptation to an artificial system but exposes target species to potentially limiting environmental conditions that can restrict their development, as manipulation of culture conditions of *in situ* aquaculture is greatly limited to the selection of the production site (Page et al., 2011). Still, this decision plays a critical role on the success of the aquaculture as each area may present particular environmental conditions.

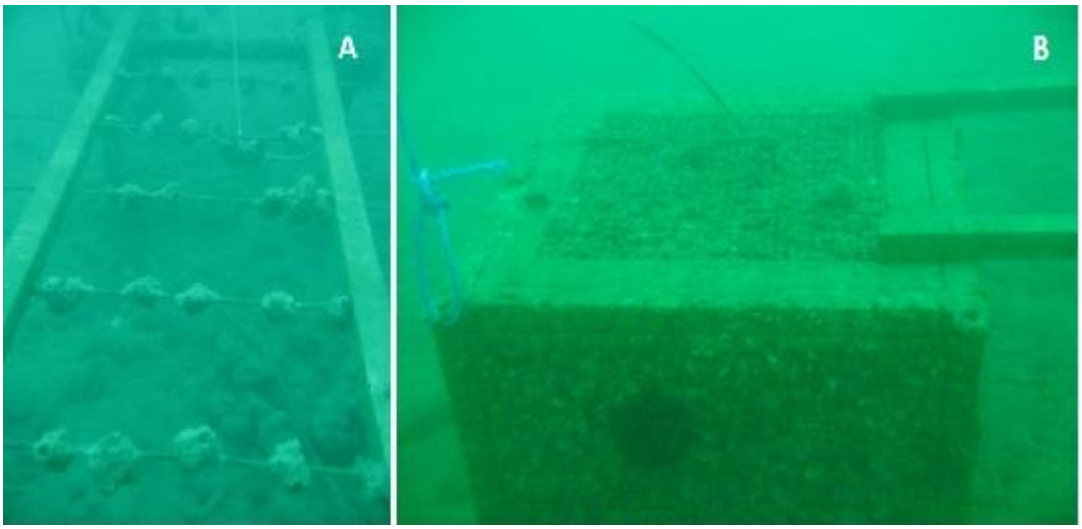

Figure 5 Mediterranean sponges in sea-based aquacultures. **A.** Culture frame with spike-cultures of *Dysidea avara*. Adapted from Leal et al. (2014). **B.** Culture cage with grid-cultures of *Chondrosia reniformis*. Adapted from Osinga et al. (2010).

Nevertheless, sponges have been shown to preserve, or even enhance, their production yields of bioactive compounds in mariculture (Osinga et al., 2010; Ruiz et al., 2013). Cultured explants of *Discodermia dissoluta* presented higher levels of discodermolide than wild specimens after 6 months of mariculture (Ruiz et al., 2013). Nevertheless, the overall compound concentration was 27 mg kg^{-1} of wet sponge. This

ratio would require the harvesting of 38 kg of sponge in order to extract 1 g of target compound. Mariculture of *Dysidea avara* can reach higher yields of avarol. Here, even the target compound concentration similar to that displayed by wild sponges (2 000 mg kg^{-1} of wet sponge) would require the harvesting of only 0.5 kg of sponge in order to extract the same amount of target compound (Sipkema et al., 2005b).

In fact, low weight of bioactive compounds in marine invertebrates is fairly common. However, in clinical trials and, once the compound proceeds through them, in drug production this limitation can impair its commercialization. To supply pharmaceutical development, the extraction of kilograms of the target compound may represent the harvesting of tones of the invertebrate (Munro et al., 1999), triggering supply problems. Pharmaceutical industry is currently prompting the development of synthetic or hemisynthetic analogues, heterologous gene expression, and design of similar, but less complex, molecules (Piel, 2011; Radjasa et al., 2011). Nonetheless, the synthesis of artificial molecules in large scale can become incredibly difficult, long and expensive (Sipkema et al., 2005). Furthermore, our lack of knowledge on the role played by the remaining microbial community or the host on the expression of target genes still defies our ability to exploit the full potential of heterologous gene expression (Piel, 2011).

Here, the *ex situ* aquaculture of the holobiont under controlled environmental parameters can stabilize the host and the microbial community, and emerge as a promising approach to achieve higher levels of target compound production, particularly, when these are found in rare invertebrate hosts.

Remarkably, as previously referred, advances in metagenomic and molecular biology techniques not only allow understanding the ecology of sponge-associated microorganism, but could also provide an interesting tool to help solve the “supply problem”. Enzyme production can be taken as example. Its elevated costs can be a major obstacle in the crescent application of proteases in industry. In order to overcome it, protease production yields have been improved, essentially, through screening for hyperproducing strains, overexpression of proteases and genetic manipulation of both enzymes and microorganisms. Indeed, transfer of the biosynthetic genetic machinery from the invertebrate host to an easily cultivable microorganism can be the solution to solve the ‘supply problem’ of natural products. The possibility to safely genetically

manipulate microorganisms to overexpress desired genes and reach higher yields without harming more organisms seems to be a promising e attainable pathway. However, significant improvements in the current knowledge regarding the molecular biology and chemistry of these products are required.

References

Ainsworth, C. H., Varkey, D. A., & Pitcher, T. J. (2008). Ecosystem simulations supporting ecosystem-based fisheries management in the Coral Triangle, Indonesia. *Ecological Modelling*, *214*, 361–374. doi:10.1016/j.ecolmodel.2008.02.039

Bacalso, R. T. M., & Wolff, M. (2014). Trophic flow structure of the Danajon ecosystem (Central Philippines) and impacts of illegal and destructive fishing practices. *Journal of Marine Systems*, *139*, 103–118. doi:10.1016/j.jmarsys.2014.05.014

Bailey, M., & Sumaila, U. R. (2015). Destructive fishing and fisheries enforcement in eastern Indonesia. *Marine Ecology Progress Series*, *530*, 195–211. doi:10.3354/meps11352

Beman, J. M., Arrigo, K. R., & Matson, P. A. (2005). Agricultural runoff fuels large phytoplankton blooms in vulnerable areas of the ocean. *Nature*, *434*, 211–214. doi:10.1038/nature03370

Bruno, J. F., Petes, L. E., Drew Harvell, C., & Hettinger, A. (2003). Nutrient enrichment can increase the severity of two Caribbean coral diseases. *Ecology Letters*, *6*, 1056–1061. doi:10.1046/j.1461-0248.2003.00544.x

Caldeira, K., & Wickett, M. E. (2003). Anthropogenic carbon and ocean pH. *Natural Product Reports*, *425*, 365.

Carter, H. J. (1878). XVIII - Parasites of the Spongida. *Annals and Magazine of Natural History: Series 5*, *2*(8), 157–172. doi:10.1080/00222937808682402

Coelho, F. J. R. C., Santos, A. L., Coimbra, J., Almeida, A., Cunha, A., Cleary, D. F. R., Calado, R., & Gomes, N. C. M. (2013). Interactive effects of global climate change and pollution on marine microbes: the way ahead. *Ecology and Evolution*, *3*(6), 1808–1818. doi:10.1002/ece3.565

Cognetti, G., Maltagliati, F., & Saroglia, M. (2006). The risk of “genetic pollution” in Mediterranean fish populations related to aquaculture activities. *Marine Pollution Bulletin*, *52*, 1321–1323. doi:10.1016/j.marpolbul.2006.09.016

Doney, S. C. (2010). The growing human footprint on coastal and open-ocean biogeochemistry. *Science*, *328*(5985), 1512–1516.

Gaino, E., Pronzato, R., Corriero, G., & Buffa, P. (1992). Mortality of commercial sponges: incidence in two mediterranean areas. *Bolletino Di Zoologia*, *59*, 79–85. doi:10.1080/11250009209386652

Goodwin, C., Rodolfo-Metalpa, R., Picton, B., & Hall-Spencer, J. M. (2014). Effects of ocean acidification on sponge communities. *Marine Ecology*, *35*, 41–49.

Indermühle, A., Stocker, T. F., Joos, F., Fischer, H., Smith, H. J., Wahlen, M., Deck, B., Mastroianni, D., Tshumi, J., Meyer, R., & Stauffer, B. (1999). Holocene carbon-cycle dynamics based on CO_2 trapped in ice at Taylor Dome, Antarctica. *Nature*, *398*, 121–126.

Leal, M. C., Sheridan, C., Osinga, R., Dionísio, G., Rocha, R. J. M., Silva, B., Rosa, R., & Calado, R. (2014). Marine microorganism-invertebrate assemblages: perspectives to solve the "supply problem" in the initial steps of drug discovery. *Marine Drugs*, *12*(7), 3929–3952. doi:10.3390/md12073929

Morrow C. & Cárdenas P. (2015). Proposal for a revised classification of the Demospongiae (Porifera). Frontiers in Zoology, 12:7

Munro, M. H. G., Blunt, J. W., Dumdei, E. J., Hickford, S. J. H., Lill, R. E., Li, S., Battershill, C. N., & Duckworth, A. R. (1999). The discovery and development of marine compounds with pharmaceutical potential. *Journal of Biotechnology*, *70*, 15–25. doi:10.1016/S0168-1656(99)00052-8

NOAA Earth System Research Laboratory (2015) Available at http://www.esrl.noaa.gov/gmd/ccgg/trends/

Osinga, R., Sidri, M., Cerig, E., Gokalp, S. Z., & Gokalp, M. (2010). Sponge aquaculture trials in the East-Mediterranean Sea: new approaches to earlier ideas. *The Open Marine Biology Journal*, *4*, 74–81.

Page, M. J., Handley, S. J., Northcote, P. T., Cairney, D., & Willan, R. C. (2011). Successes and pitfalls of the aquaculture of the sponge Mycale hentscheli. *Aquaculture*, *312*, 52–61. doi:10.1016/j.aquaculture.2010.12.006

Pantile, R., & Webster, N. S. (2011). Strict thermal threshold identified by quantitative PCR in the sponge Rhopaloeides odorabile. *Marine Ecology Progress Series*, *431*, 97–105.

Piel, J. (2011). Approaches to capturing and designing biologically active small molecules produced by uncultured microbes. *Annual Review of Microbiology*, *65*, 431–53. doi:10.1146/annurev-micro-090110-102805

Radjasa, O. K., Vaske, Y. M., Navarro, G., Vervoort, H. C., Tenney, K., Linington, R. G., & Crews, P. (2011). Highlights of marine invertebrate-derived biosynthetic products: their biomedical potential and possible production by microbial associants. *Bioorganic & Medicinal Chemistry*, *19*(22), 6658–6674. doi:10.1016/j.bmc.2011.07.017.

Ruiz, C., Valderrama, K., Zea, S., & Castellanos, L. (2013). Mariculture and natural production of the antitumoural (+)-discodermolide by the Caribbean marine sponge Discodermia dissoluta. *Marine Biotechnology*, *15*, 571–583. doi:10.1007/s10126-013-9510-7

Santos-Gandelman, J., Giambiagi-deMarval, M., Oelemann, W. M. R., & Laport, M. S. (2014). Biotechnological potential of sponge-associated bacteria. *Current Pharmaceutical Biotechnology*, *15*(2), 143–155. doi:10.2174/1389201015666140711115033

Schippers, K. J., Sipkema, D., Osinga, R., Smidt, H., Pomponi, S. A., Martens, D. E., & Wijffels, R. H. (2012). Cultivation of sponges, sponge cells and symbionts: achievements and future prospects. In M. J. U. M. M. M. A. Becerro & T. Xavier (Eds.), *Advances in Marine Biology* (pp. 273–337). Academic Press.

Sipkema, D., Osinga, R., Schatton, W., Mendola, D., Tramper, J., & Wijffels, R. H. (2005). Large-scale production of pharmaceuticals by marine sponges: sea, cell, or synthesis? *Biotechnology and Bioengineering*, *90*(2), 201–222. doi:10.1002/bit.20404

Smith, F. G. W. (1941). Sponges disease in British Honduras, and its transmission by water currents. *Ecology*, *22*(4), 415–421. doi:10.2307/1930719

Sutherland, K. P., Porter, J. W., & Torres, C. (2004). Disease and immunity in Caribbean and Indo-Pacific zooxanthellate corals. *Marine Ecology Progress Series*, *266*, 273–302.

Taylor, M. W., Radax, R., Steger, D., & Wagner, M. (2007b). Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. *Microbiology and Molecular Biology Reviews*, *71*(2), 295–347. doi:10.1128/MMBR.00040-06

Webster, N. S. (2007). Sponge disease: a global threat? *Environmental Microbiology*, *9*(6), 1363–1375. doi:10.1111/j.1462-2920.2007.01303.x

Webster, N. S., Coob, R. E., & Negri, A. P. (2008). Temperature thresholds for bacterial symbiosis with a sponge. *The ISME Journal*, *2*(8), 830–842.

CHAPTER 6

CONCLUDING REMARKS

Concluding remarks

Marine sponges are among the richest sources of pharmacologically active chemical compounds isolated from marine organisms but there's an urgent need for understanding their symbiont ecology as the biotechnological potential of their microorganisms remains little investigated and discussed (Santos-Gandelman et al., 2014). There are distinct uncultured apparently obligate symbionts associated with sponges that, so far, seem to be attainable only via culture-independent genome and gene retrieval strategies. However, biotechnology has grown dramatically over the years. Advances in microbiology and biotechnology have created a favorable atmosphere for the development of new compounds and will continue to contribute to a sustainable environment and an improvement of the quality of life of mankind as long as we bet on them. Despite this progress, extensive and focused approaches aided by genomic, metabolomics and biomedical analysis could lead to a greater understanding of sponge-associated microorganisms interaction, relationship and potential, and to the discovery of new bioactive compounds (Santos-Gandelman et al., 2014).

History of drug development has shown that, in the discovery of natural products, the native molecule rarely becomes the active ingredient of the approved drug. Instead, it becomes the starting point for the derivatization of functional analogues (Sipkema et al., 2005b). This is partially due to the enormous volume required to the production of pharmaceuticals or to the high complexity of most of the natural compounds. Here, the 'supply problem' has been a major limiting factor. The amount of pure compound required increases from milligrams to kilograms as the compound progresses in the drug development pipeline (Leal et al., 2014). In the later stages, the required volume of pure compound can comprise the capture, or production, of several tones of the producing organisms. Hence, the pharmaceutical industry has been prompting the development of synthetic or hemisynthetic analogues, expression of heterologous genes, design of less complex molecules with similar bioactive potential (Piel, 2011; Radjasa et al., 2011). However, high complexity of particular natural molecules, requirement of an affordable large-scale production, and our lack of understanding on the effect of symbiosis, with the

host or community members, on the production of interesting molecules remains a barrier impairing drug discovery.

Still, combination of different approaches such as genetic modification of source genes, bacterial fermentation to produce precursor molecules, and post-fermentation chemical synthesis can become the top strategy for the production of drugs (Sipkema et al., 2005b).

References

Leal, M. C., Sheridan, C., Osinga, R., Dionísio, G., Rocha, R. J. M., Silva, B., Rosa, R., & Calado, R. (2014). Marine microorganism-invertebrate assemblages: perspectives to solve the "supply problem" in the initial steps of drug discovery. *Marine Drugs*, *12*(7), 3929–3952. doi:10.3390/md12073929

Piel, J. (2011). Approaches to capturing and designing biologically active small molecules produced by uncultured microbes. *Annual Review of Microbiology*, *65*, 431–53. doi:10.1146/annurev-micro-090110-102805

Radjasa, O. K., Vaske, Y. M., Navarro, G., Vervoort, H. C., Tenney, K., Linington, R. G., & Crews, P. (2011). Highlights of marine invertebrate-derived biosynthetic products: their biomedical potential and possible production by microbial associants. *Bioorganic & Medicinal Chemistry*, *19*(22), 6658–6674. doi:10.1016/j.bmc.2011.07.017.

Santos-Gandelman, J., Giambiagi-deMarval, M., Oelemann, W. M. R., & Laport, M. S. (2014). Biotechnological potential of sponge-associated bacteria. *Current Pharmaceutical Biotechnology*, *15*(2), 143–155. doi:10.2174/1389201015666140711115033

Sipkema, D., Osinga, R., Schatton, W., Mendola, D., Tramper, J., & Wijffels, R. H. (2005). Large-scale production of pharmaceuticals by marine sponges: sea, cell, or synthesis? *Biotechnology and Bioengineering*, *90*(2), 201–222. doi:10.1002/bit.20404

APPENDIX

Appendix

Compositional analysis of bacterial communities in seawater, sediment and high and low microbial abundance sponges in the Misool coral reef system, Indonesia

Cleary DFR, Polónia ARM, Becking LE, de Voogd NJ, Purwanto, Gomes H, Gomes NCM

Abstract

Sponge species have been deemed "high microbial abundance" (HMA) or "low microbial abundance"(LMA) based on the composition and abundance of their microbial symbionts. In the present study we evaluated the richness and composition of bacterial communities associated with one HMA (*Xestospongia testudinaria*), one LMA (*Stylissa carteri*) and one sponge with a hitherto unknown microbial community (*Aaptos suberitoides*) inhabiting the Misool coral reef system in the West Papua province, Indonesia. The bacterial communities of these sponge species were also compared with bacterioplankton and sediment bacterial communities from the same open coastal coral reef habitat. Using a 16S rRNA gene barcoded pyrosequencing approach we showed that the most abundant phylum overall was Proteobacteria. The biotope (sponge species, sediment or seawater) explained almost 84% of the variation in bacterial composition with highly significant differences in composition among biotopes and a clear separation between bacterial communities from (1) seawater and *S. carteri*; (2) *X. testudinaria* and *A. suberitoides* and (3) sediment. The Chloroflexi classes SAR202 and Anaerolineae were most abundant in *A. suberitoides* and *X. testudinaria* and both of these species shared several OTUs that were largely absent in the remaining biotopes. This indicates that *A. suberitoides* is a HMA sponge. Compositional similarities were also observed between *S. carteri* and seawater. These results confirm compositional differences between sponge and non-sponge biotopes and between HMA and LMA sponges.

Introduction

Coral reefs are highly diverse, but also globally threatened ecosystems. Local perturbations including overfishing and pollution combined with global phenomena such as coral bleaching have had a sometimes disastrous effect on coral reefs (Jackson et al. 2001, Pandolfi et al. 2003, Bruno & Selig 2007, De'ath et al. 2012). Coral cover in certain reefs has virtually disappeared leading to the loss of important ecosystem services such as renewable resources (e.g., fisheries), protection against erosion, and nutrient cycling (Moberg and Folke, 1999). The loss of coral cover also leads to the loss of dependent species such as numerous fish species and shifts in composition to reefs dominated by non-coral taxa such as algae or sponges (Bellwood et al. 2004).

Sponges (Phylum *Porifera*) are ancient metazoans (van Soest et al., 2012) with a fossil record dating back to the Precambrian (Li et al., 1998). The majority of the 8500 formally described living species belong to the class Demospongia (demosponges) (Borchiellini et al., 2001; van Soest et al., 2012). The remaining species represent the classes Hexactinellida (glass sponges) and Calcarea (calcareous sponges) (Hentschel et al., 2006). They have successfully colonised a range of aquatic environments from tropical to polar, shallow to deep water, and marine and freshwater (Rützler, 2004). They are also structurally important components of coral reefs where they provide shelter for numerous organisms and contribute to reef regeneration and stabilisation (de Voogd et al., 2006). In addition to the above, they are one of the most important marine sources of promising pharmaceutical compounds (Faulkner, 2002; Taylor et al., 2007).

Sponges are known to host large communities of microbial symbionts of known ecological and biotechnological importance (Freeman & Thacker, 2011). They are also unique and, depending on the species, highly selective environments for microbes (Hentschel et al., 2006; Freeman & Thacker, 2011; Cleary et al., 2013). The prokaryote metabolism requires nitrogen and carbon, and sponges produce both, often in ample quantities, by releasing ammonia, as an end product of their metabolism, and carbohydrates and amino acids, as a consequence of phagocytosis (Hentschel et al., 2006). Microbial symbionts in turn provide sponges with important nutrients (Flatt et al.,

2005), process metabolic waste, improve host defense and stabilise the host skeleton (Hentschel et al., 2006). Some sponge species also house specific microbial communities that may be similar over large geographical distances (Flatt et al., 2005).

Microbes inhabit the mesophyll matrix of most demosponges (Hentschel et al., 2003). The abundance and composition of sponge-associated microorganisms can vary greatly among sponge species, including those inhabiting the same habitat (Cleary et al., 2013, Cleary et al. 2015, de Voogd et al. 2015). High-microbial-abundance (HMA) sponges can contain around 10^{10} bacterial cells g^{-1} wet weight of sponge (2 to 4 orders of magnitude higher than concentrations in sea water) (Hentschel et al., 2006), while low-microbial abundance (LMA) sponges contain around 10^{6} cells g^{-1} (similar to concentrations in sea water) (Kamke et al., 2010).

HMA sponges also tend to host more diverse bacterial communities including Proteobacteria, Chloroflexi, Acidobacteria, Actinobacteria (Gloeckner et al. 2014). Previously, the determination of HMA or LMA status was made on the basis of electron microscopy and morphotype with HMA sponges containing more densely packed microbial communities and smaller canals and choanocyte chambers compared to LMA sponges (Vacelet and Donadey 1977; Schlappy et al., 2010) Gloeckner et al. (2014), however showed that electron microscopy was not always sufficient to determine HMA or LMA status and that sponges existed with intermediate microbial abundances. They, therefore, suggested combining electron microscopy with 16S rRNA gene sequence data. The latter may in fact be a better determinant given the sometimes ambiguous results obtained by Gloeckner et al. (2014).

In the present study, we compared the richness and composition of bacteria in three sponge species inhabiting open coastal habitat, sediment and seawater from a coral reef system in South East Misool, Raja Ampat, Indonesia. Located on the northwestern tip of Papua, eastern Indonesia, the Raja Ampat region consists of nearly 1500 islands, and is considered an area with a global priority for conservation (Roberts et al. 2002). It is among the most biodiverse regions on Earth possessing over 75% of the world's coral species and almost a thousand species of reef fish (e.g., McKenna et al. 2002, Allen 2008, Allen & Erdmann 2009, Mangubhai et al. 2012). The reefs and mangrove systems in SE Misool encompass an area of outstanding marine biological diversity (e.g., McKenna et al. 2002,

Allen 2008, Mangubhai et al. 2012, Becking et al. 2014) and harbour some of the most pristine reefs in Indonesia (McKenna et al. 2002, Mangubhai et al. 2012, Grantham et al. 2013). As a result, a Marine Protected Area of 343 200 ha was established in SE Misool in 2009 (KKPD Misool Timur-Selatan).

We focused on bacterial communities of the low microbial abundance (LMA) sponge *Stylissa carteri* Dendy, 1889 and the high microbial abundance (HMA) giant barrel sponge *Xestospongia testudinaria* Lamarck, 1815 in addition to the sponge *Aaptos suberitoides* Brøndsted, 1934. We compared the bacterial communities of these sponges with bacterioplankton and sediment bacterial communities. This is the first study to assess the bacterial community of *A. suberitoides*.

Stylissa carteri is a common Indo-Pacific bright orange flabelliform sponge that occurs from the Red Sea to Taiwan (de Voogd & Cleary, 2008, Giles et al., 2015). Numerous bromopyrolle alkaloids with promising antiviral, antibacterial and anticancer properties have been isolated from species belonging to the genus Stylissa (Rohde et al., 2012, Ebada et al., 2015). *Xestospongia testudinaria* (Lamarck, 1813) is one of the largest known sponges. It usually has an erect and barrel-shaped structure that can measure up to 2.4 meters in height and width. The surface texture varies from smooth to highly digitate or lamellate (Swierts et al., 2013). It also has an incredible life span that may exceed 2000 years (McMurrey et al., 2008). It can be found from the Red Sea to the Great Barrier Reef (Pham et al., 1999; de Voogd et al., 2006; Moitinho-Silva et al., 2014) and can be locally abundant in coral reefs, usually at depths greater than 10 meters. *Xestospongia* species are among the richest sources of pharmacologically active chemical compounds isolated from marine organisms. *Xestospongia testudinaria* has been shown to produce compounds with potential applications in the treatment of obesity, diabetes mellitus, arteriosclerosis and Alzheimer's disease (Zhou et al., 2011b; Akiyama et al., 2013; Liang et al., 2014). *Aaptos suberitoides* (Brøndsted, 1934) occurs in shallow coral reefs in the coral triangle (de Voogd & Cleary, 2008). It forms thick irregular lobate masses that can occupy large parts of the reef. Its exterior is dark brown, but the interior is canary yellow and stains dark brown after preservation. It has been shown to produce compounds with antitumor, antimicrobial and antiviral activity (Aoki et al., 2006, Larghi et al., 2008,

Tsukamoto et al. 2010, Jin et al., 2011, Liu et al., 2012, Pham et al., 2013). Our specific goals were to (1) identify the most abundant higher bacterial taxa; (2) compare bacterial richness and composition among sponge hosts and non-sponge biotopes (sediment and seawater); (3) identify dominant bacterial OTUs and their closest known relatives.

Material and methods

Study site

Samples of *S. carteri*, *A. suberitoides* and *X. testudinaria* were collected by snorkeling and SCUBA from the 13th to the 18th of September 2013 in South East Misool, Raja Ampat region, West Papua province in Indonesia (Fig. 1). SE Misool is part of a marine protected area of 343,200 ha established in 2009 (KKPD Misool Timur-Selatan). The equatorial location of Misool means that the main seasonal influence is driven by monsoons (Prentice and Hope, 2007). Misool is most influenced by the the southeast monsoon from May to October which is characterized by cooler sea surface temperatures (SSTs), persistent winds and strong ocean swell. The annual rainfall in Papua averages 2500–4500 mm with inter-annual variability in rainfall due to the El Niño Southern Oscillation (ENSO; Prentice and Hope, 2007). There are seasonal differences in SSTs with an average SST of 29.0°C, ranging from 19.3 to 36.0 °C (Mangubhai et al. 2012)

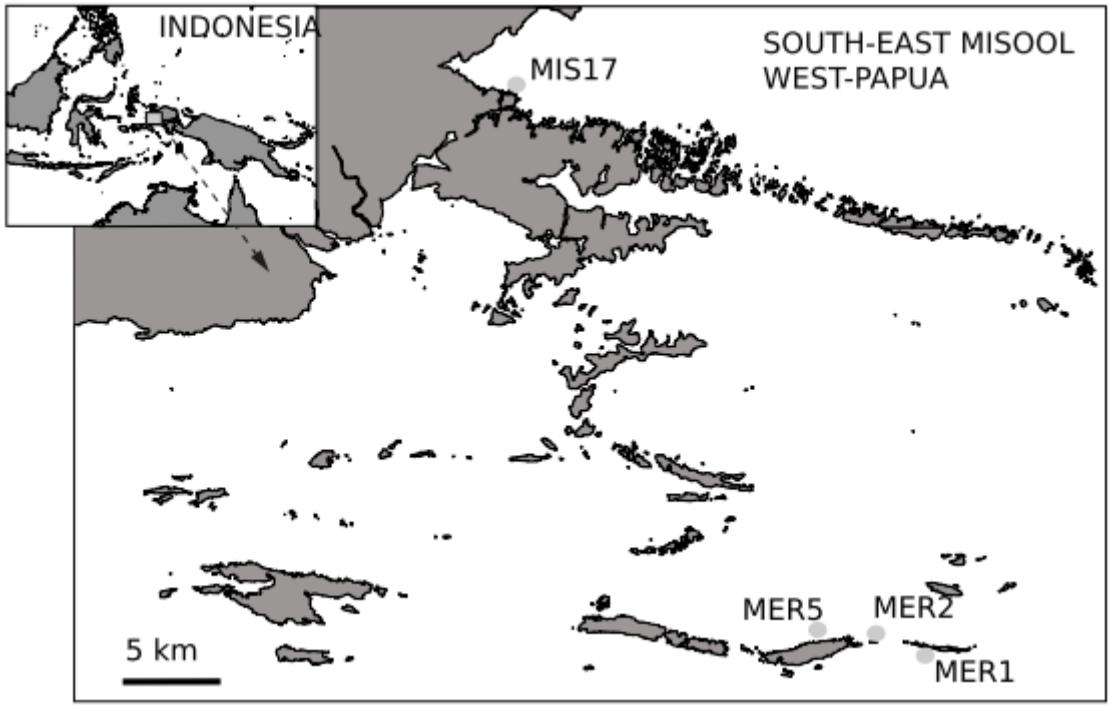

Figure A- 1 Map of study area showing the location of the study sites.

Data collection

In the study area, small fragments of the interior and exterior parts of the sponges *S. carteri* (Demospongiae: Scopalinida - Scopalinidae), *A. suberitoides* (Demospongiae: Suberitida: Suberitidae) and *X. testudinaria* (Demospongiae: Haplosclerida: Petrosiidae) were sampled in order to sample, as much as possible, the whole bacterial community. Specimens were collected from shallow water reefs (depth range: 7-17 m) and identified in the field. Voucher specimens of sponges have been deposited in the sponge collection of the Naturalis Biodiversity Center (RMNH Porifera). Sediment samples were taken using mini cores; this consisted of sampling the top 5 cm of sediment with a plastic disposable syringe from which the end had been cut in order to facilitate sampling (Capone et al., 1992). Seawater samples were collected by filtering one liter of seawater through a Millipore® White Isopore Membrane Filter (GTTP04700, 47 mm diameter, 0.22 µm pore size). Samples were stored in 96% EtOH. After sampling, tubes containing the samples were frozen or carried in ice during travel between fieldwork lodging and the Netherlands

and Portugal, where the samples were stored at-80. ºC until processing. For the present study, three samples each of the sponges *S. carteri*, *A. suberitoides* and *X. testudinaria*, sediment and seawater were assessed for bacterial community analysis.

DNA extraction and pyrosequencing

Total community-DNA extraction and 16S rRNA gene barcoded-pyrosequencing.

We isolated PCR-ready total community DNA (TC-DNA) from sediment, seawater and sponge samples using the FastDNA® SPIN Kit (MP Biomedicals) following the manufacturer's instructions. Briefly, we prepared sediment samples by centrifuging each one for 30 min at 4400 rpm and 4 ºC; the membrane filter (seawater sample) and sponge samples were each cut into small pieces. The whole membrane filter and 500 mg of sediment and sponge were transferred to Lysing Matrix E tubes containing a mixture of ceramic and silica particles. The microbial cell lysis was performed in the FastPrep® Instrument (Q Biogene) for 80 seconds at the speed of 6.0. Extracted DNA was eluted into DNase/Pyrogen-Free Water to a final volume of 50 µl and stored at-20°C until use. Prior to pyrosequencing, the amplicons of the bacterial 16S rRNA gene were obtained using bacterial specific primers 27F and 1494R (Gomes et al. 2001). After a denaturation step at 94°C for 5 min, 25 thermal cycles of 45 sec at 94°C, 45 sec at 56°C and 1:30 min at 72°C were carried out followed by an extension step at 72°C for 10 min. Using the amplicons of the bacterial 16S rRNA gene as template, the V3V4 region was amplified, using barcoded fusion primers with the Roche-454 A Titanium sequencing adapters, a six-base barcode sequence, forward V3 primer 5'-ACTCCTACGGGAGGCAG-3' (Wang and Qian 2009) and V4 reverse degenerate primer 5'-TACNVRRGTHTCTAATYC-3' (Ribosomal Database Project (RDP) (Release 10, Update 20) (http://rdp.cme.msu.edu/).

Sequence analyses was performed using previously described methods (Cleary et al., 2015, de Voogd et al. 2015). Briefly, in QIIME, fasta and qual files were used as input for the split_libraries.py script. OTUs were selected using UPARSE with usearch7 (Edgar 2013). Chimera checking was performed using the UCHIME algorithm, which is the fastest and most sensitive chimera checking algorithm currently available (Edgar et al. 2011). OTU

clustering was performed using the-cluster_otus command (cut-off threshold at 97%). (see Online Resource 1 for a detailed description). Closely related organisms of numerically abundant OTUs (≥ 200 sequences) were identified using the NCBI Basic Local Alignment Search Tool (BLAST) command line 'blastn' tool with the-db argument set to nt (Zhang et al., 2000). The DNA sequences generated in this study can be downloaded from the NCBI SRA.

Phylogenetic tree

Selected 16S rRNA gene sequences of the most dominant OTUs and representative cultured and uncultured closest relatives in GenBank [http://www.ncbi.nlm.nih.gov/] were aligned using ClustalW and a phylogenetic analysis conducted using MEGA 6 software (http://www.megasoftware.net/; last checked 2014 07 09) (Tamura et al., 2013). A phylogenetic tree was constructed according to the Neighbour-Joining method and the evolutionary distances were computed using the Maximum Composite Likelihood method with a discrete Gamma distribution (2). In the results, we present a bootstrap consensus tree based on 1000 replicates. The bootstrap value is shown next to each branch when this exceeds 49%. This value represents the percentage of replicate trees in which the associated taxa clustered together.

Statistical analysis

A table containing the presence and abundance of all OTUs per sample was imported into R using the read.table() function. Plant organelles, mitochondria or sequences not classified as Bacteria (e.g., Archaea) were removed prior to statistical analysis.

Richness and higher taxon abundance

We used a self-written function in R (Gomes et al., 2010) to estimate rarefied OTU richness for each sample. Care, however, should be taken in the interpretation of richness estimates based on sequence data given the prevalence of sequencing errors (Edgar 2013). We tested for significant differences in the relative abundance of selected higher taxon groups (phyla, classes and orders) among habitats with an analysis of deviance using the glm() function in R. As data were proportional, we first applied a GLM with the family argument set to binomial. However, the ratio of residual deviance to residual d.f. in the models substantially exceeded one so we set family to 'quasibinomial'. In the 'quasibinomial' family, the dispersion parameter is not fixed at one so that it can model over-dispersion. Using the GLM model, we tested for significant variation among habitats using the anova() function in R with the F test, which is most appropriate when dispersion is estimated by moments as is the case with quasibinomial fits.

Composition

The OTU abundance matrix was $\log_e (x + 1)$ transformed (in order to normalise the distribution of the data) and a distance matrix was constructed using the Bray-Curtis index with the vegdist() function in the VEGAN package (Oksanen et al., 2009) in R. The Bray-Curtis index is one of the most frequently applied (dis)similarity indices used in ecology (Legendre & Gallagher, 2001; Cleary, 2003; Polónia et al., 2015). Variation in OTU composition among biotopes (*S. carteri*, *A. suberitoides*, *X. testudinaria*, sediment and seawater) was assessed with Principal Coordinates Analysis (PCO) using the cmdscale() function in R with the Bray-Curtis distance matrix as input. Variation among locations was tested for significance using the adonis() function in VEGAN. In the adonis analysis, the Bray–Curtis distance matrix of species composition was the response variable with the biotope as independent variable. The number of permutations was set at 999; all other arguments used the default values set in the function. Weighted averages scores were computed for OTUs on the first two PCO axes using the wascores() function in the vegan package. Detailed descriptions of the functions used here can be found in R (e.g.,

?cmdscale) and online in reference manuals (http://cran.r-project.org/web/packages/vegan/index.html; 2015/05/29).

Results

The sequencing effort yielded 50223 sequences, which were assigned to 6400 OTUs after quality control, OTU picking and removal of chimeras, chloroplasts, mitochondria and sequences not assigned to the Bacteria domain. OTU richness was by far highest in the sediment biotope and lowest in *S. carteri* (supp Fig. 1). The most abundant phylum overall was *Proteobacteria* where mean relative abundance ranged from 23.6 ± 2.9% in *X. testudinaria* to 80.3 ± 1.8% in *S. carteri* (supp Fig. 2). Chloroflexi were most abundant in *A. suberitoides* (30.5 ± 10.6%) and *X. testudinaria* (33.0 ± 6.0%), but represented less than 1% of sequences in *S. carteri*, sediment and seawater. The same held for Actinobacteria, which represented 16.0 ± 7.8 of *A. suberitoides* sequences and 22.7 ± 3.2% of *X. testudinaria* sequences. The mean abundance of Actinobacteria in the other biotopes ranged from 0.1 ± 0.1 in *S. carteri* to 2.4 ± 1.5 in sediment. Bacteroidetes were most abundant in sediment (8.2 ± 2.4) and seawater (20.8 ± 26.6) but represented less than 1% of sequences in all sponge biotopes. Although highly variable within biotope samples, Cyanobacteria abundance was highest in *S. carteri* (9.0 ± 6.9) and seawater (9.9 ± 11.9) and much lower in sediment (1.5 ± 0.1), *A. suberitoides* (0.7 ± 0.6) and *X. testudinaria* (1.5 ± 1.5).

Higher taxon abundance

In line with the phylum-level results, there were marked differences in the abundance of classes and orders (Fig. 2). Sequences assigned to the Chloroflexi classes SAR202 and Anaerolineae and the order Caldilineales were largely restricted to *A. suberitoides* and *X. testudinaria*. The abundance of Gammaproteobacteria was greatest in *S. carteri*, largely due to the prevalence of OTUs assigned to the order Chromatiales. Deltaproteobacteria were most abundant in *S. carteri* and sediment, largely due to the order NB1−j in both biotopes and Desulfobacterales in sediment. Dominance was by far greatest in *S. carteri* with a mean of 47.4 ± 15.5 sequences assigned to a single OTU. In

contrast, dominance was lowest in sediment with a mean of only 4.4 ± 1.7 sequences assigned to a single OTU.

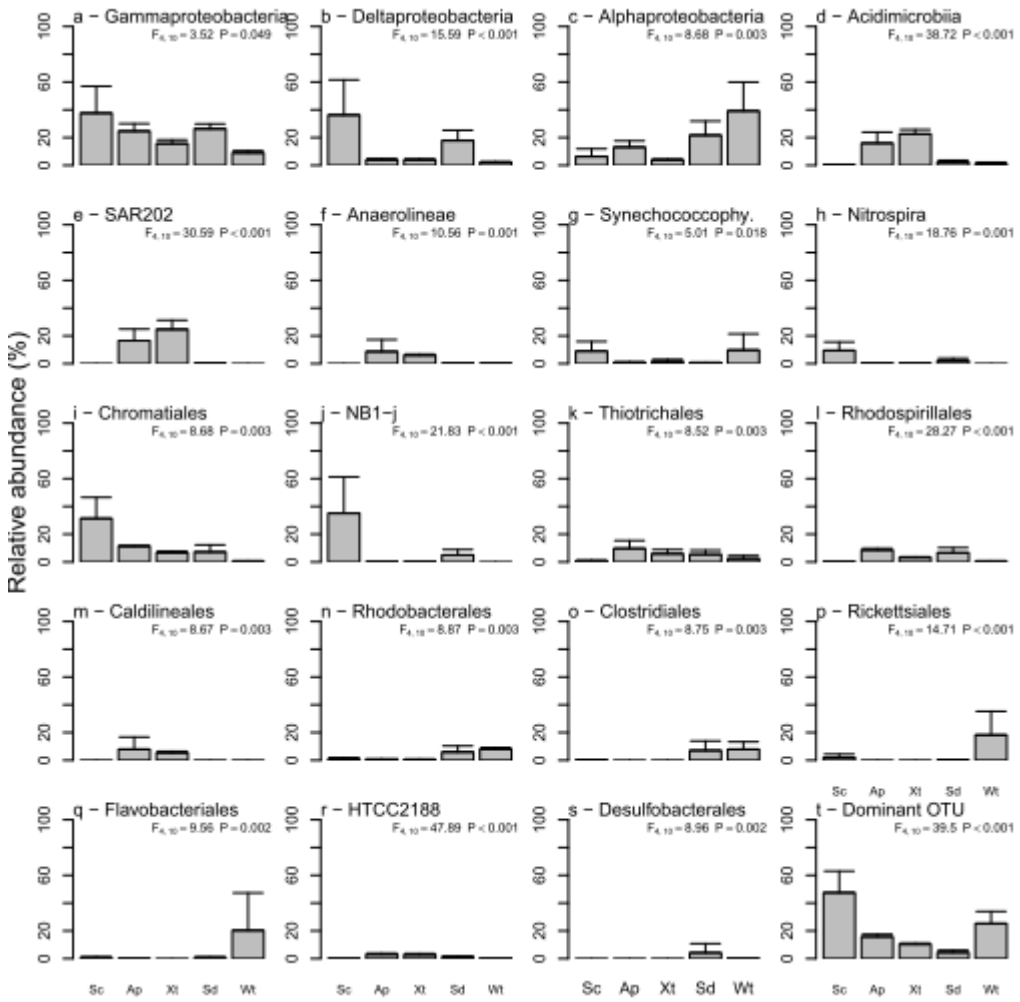

Figure A-2 Mean (error bars represent a single standard deviation) relative abundance of the most abundant bacterial classes and orders and the most abundant OTU (dominant OTU) for samples from S. carteri (Sc), A. suberitoides (Ap), X. testudinaria (Xt), sediment (Sd) and seawater (Wt). Note that the abundance of the dominant OTU refers to the abundance of the most abundant OTU per sample and thus not the most abundant OTU overall. **a** Gammaproteobacteria, **b** Deltaproteobacteria, **c** Alphaproteobacteria, **d** Acidimicrobiia, **e** SAR202, **f** Anaerolineae, **g** Synechococcophysidae, **h** Nitrospira, **i** Chromatiales, **j** NB1–j, **k** Thiotrichales, **l** Rhodospirillales, **m** Caldilineales, **n** Rhodobacterales, **o** Clostridiales, **p** Rickettsiales, **q** Flavobacteriales, **r** HTCC2188, **s** Desulfobacterales and **t** the dominant OTUs. Results of the GLM analyses for each taxon are presented in the top right of each subfigure.

Importance of biotopes in structuring composition

There was a highly significant difference in bacterial composition among biotopes ($F_{3,8}$ = 13.07, P < 0.001, R^2 = 0.839). Variation among biotopes thus explained almost 84% of the variation in composition. In the PCO ordination of the first two axes (Fig. 3), there are three distinct clusters, namely: a cluster of samples from *A. suberitoides* and *X. testudinaria*, a cluster of samples from sediment and a cluster of samples from *S. carteri* and seawater. The main axis (axis 1) separates samples of *A. suberitoides* and *X. testudinaria* from samples of *S. carteri* and seawater. The second axis (axis 2) separates all these samples from samples of sediment. For the purposes of this study, a total of 36 OTUs were considered abundant (≥ 200 sequences). Only two abundant OTUs were associated with sediment. OTU-143 was restricted to sediment samples and closely related (>99% sequence similarity) to an organism obtained from marine sediment in the Philippines (Table 1). OTU-145 was mainly found in sediment in this study but shared 100% sequence similarity with an organism previously obtained from the sponge *Rhabdastrella globostellata* in Guam. A number of OTUs were mainly found in seawater and *S. carteri*. This included OTU-45 related to an organism previously found in bottom seawater of the Atlantic abyss and OTU-811 previously found in Croatian marine lake water. Most of the abundant OTUs were associated with *A. suberitoides* and *X. testudinaria* and were often absent in other biotopes. This mainly included OTUs previously isolated from sponges and corals (e.g., OTUs 12 and 24). Most of these OTUs were shared between both sponge species although one (OTU-54) was restricted to *A. suberitoides* and three to *X. testudinaria* (OTUs 45, 48 and 658). OTUs 40 (restricted to *X. testudinaria*) and 54 (restricted to *A. suberitoides*) were both assigned to the phylum Gemmatimonadetes.

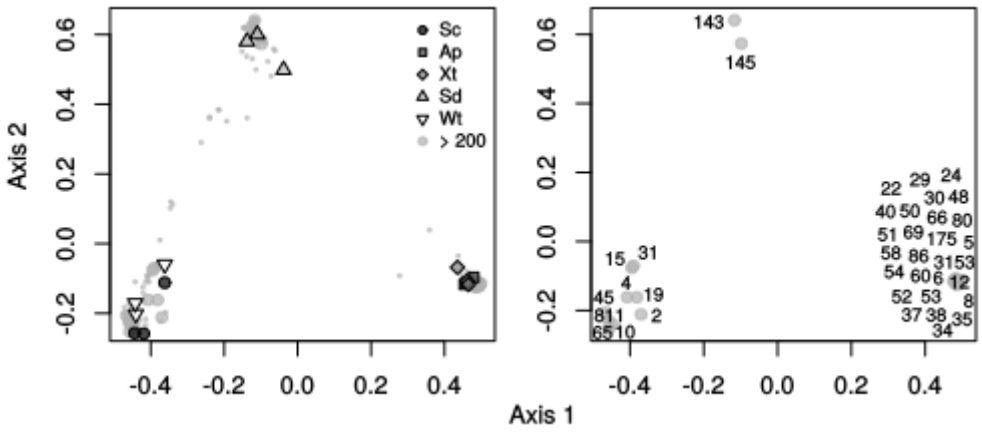

Figure A-3 Ordination showing the first two axes of the PCO analysis. a. Symbols represent samples from S. carteri (Sc), A. suberitoides (Ap), X. testudinaria (Xt), sediment (Sd) and seawater (Wt). b. Ordination showing only the most abundant OTUs. Numbers represent dominant (≥ 200 sequence reads) OTUs referred to in Table 1. Small circles represent OTUs < 200 sequence reads.

The third PCO axis mainly separated *S. carteri* samples from seawater samples. OTUs associated with and restricted to *S. carteri* include OTUs 4, 10 and 65 all of which were related to organisms previously obtained from *S. carteri* in the Red Sea (Fig. 4 and Table 1). OTUs 15 and 31, which were associated with seawater samples were related to organisms previously obtained from shrimp pond and seawater samples in China. The fourth axis mainly separated samples of *A. suberitoides* and *X. testudinaria*.

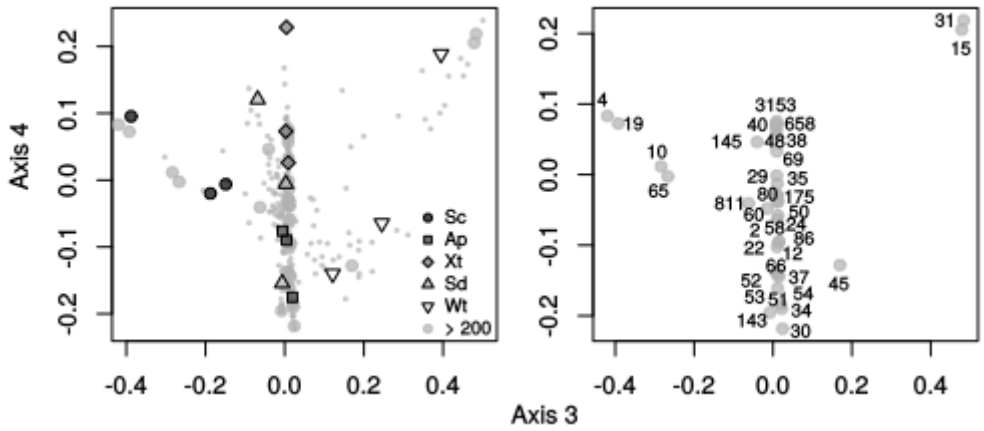

Figure A-4 Ordination showing the third and fourth axes of the PCO analysis. a. Symbols represent samples from S. carteri (Sc), A. suberitoides (Ap), X. testudinaria (Xt), sediment (Sd) and seawater (Wt). b. Ordination showing only the most abundant OTUs. Numbers represent dominant (≥ 200 sequence reads) OTUs referred to in Table 1. Small circles represent OTUs < 200 sequence reads.

In general, most of the sponge OTUs analysed here were either detected in more than one sponge biotope or were closely related to bacterial phylotypes associated with different sponge host species (Table 1 and Fig. 5). For example, *A. suberitoides* and *X. testudinaria* shared dominant bacterial symbionts (ApXt) closely related to bacterial phylotypes previously identified in a range of sponge hosts (*Xestospongia muta*, *Aplysina cauliformis*, *Ircinia* spp., *Rhopaloeides odorabile*, *Astrosclera willeyana* and *Vaceletia crypta*) in different geographical locations. Most abundant sponge OTUs were closely related to sponge bacterial symbionts from other studies from different sponge species. However, a few abudant OTUs, selectively enriched in *X. testudinaria* or *S. carteri* (OTUs 4, 10, 48, 65 and 658) appear to represent sponge species specific associations.

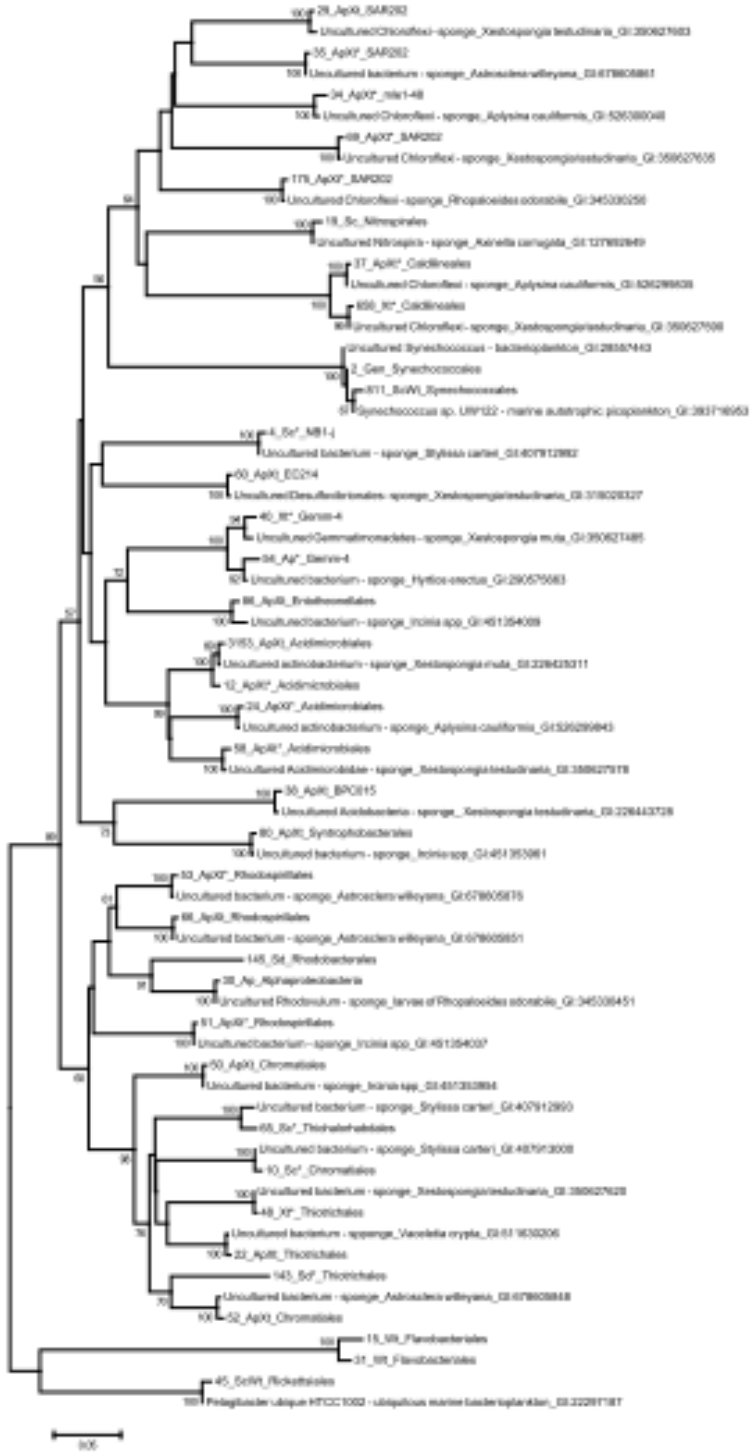

Figure A-5 Phylogenetic tree of the bacterial 16S rRNA gene sequences recovered from S. carteri, A. suberitoides, X. testudinaria, sediment and seawater from Misool coral reef system. Bootstrap values lower than 50% were omitted. The number of each OTU is indicated

Table A-2 List of abundant (≥ 200 sequence reads) OTUs and closely related organisms identified using BLAST search. OTU: OTU number; Sum: number of sequence reads; GI: GenInfo sequence identifiers of closely related organisms identified using BLAST; Seq: sequence similarity of these organisms with our representative OTU sequences and their source; Source: isolation source of organisms identified using BLAST; Location: sampling location of of organisms identified using BLAST; *restricted to group

OTU	Sum	Group	Phylum	Class	Order	Family	Genus	GI	Seq	Source	Location
2	480	Gen	Cyanobacteria	Synechococcophycideae	Synechococcales	Synechococcaceae	Synechococcus	2,9E+07	99,76	Oligotrophic oceanic water	*
4	4110	Sc*	Proteobacteria	Deltaproteobacteria	NB1-j	NB1-j	Unassigned	4,1E+08	99,54	Sponge: Stylissa carteri	Saudi Arabia
10	2742	Sc*	Proteobacteria	Gammaproteobacteria	Chromatiales	Unassigned	Unassigned	4,1E+08	100	Sponge: Stylissa carteri	Saudi Arabia
12	2579	ApXt*	Actinobacteria	Acidimicrobiia	Acidimicrobiales	wb1_P06	Unassigned	7,7E+08	99,77	Coral: Porites lutea	China: Hainan
15	287	Wt	Bacteroidetes	Flavobacteriia	Flavobacteriales	Cryomorphaceae	Unassigned	3,5E+08	100	Seawater	China: Fujian
19	1145	Sc	Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	Unassigned	6E+08	100	Sponge: Axinella sp	China
22	1487	ApXt	Proteobacteria	Gammaproteobacteria	Thiotrichales	Piscirickettsiaceae	Unassigned	5,1E+08	99,55	Sponge: Vaceletia crypta	Australia:Great Barrier Reef
24	871	ApXt*	Actinobacteria	Acidimicrobiia	Acidimicrobiales	TK06	Unassigned	7,7E+08	99,53	Coral: Porites lutea	China: Hainan
29	1447	ApXt	Chlorofilexi	SAR202	Unassigned	Unassigned	Unassigned	3,5E+08	99,29	Sponge: Xestospongia testudinaria	Indonesia: Manado
30	642	Ap	Proteobacteria	Alphaproteobacteria	Unassigned	Unassigned	Unassigned	3,5E+08	99,76	Sponge: Rhopaloeides odorabile	Australia: Great Barrier Reef
31	439	Wt	Bacteroidetes	Flavobacteriia	Flavobacteriales	Cryomorphaceae	Unassigned	5,9E+08	100	Shrimp pond	China: Fujian Province
34	488	ApXt*	Chlorofilexi	TK17	mle1-48	Unassigned	Unassigned	5,3E+08	98,82	Sponge: Aplysina cauliformis	Belize: Carrie Bow Cay
35	501	ApXt*	Chlorofilexi	SAR202	Unassigned	Unassigned	Unassigned	6,8E+08	99,76	Sponge: Astrosclera willeyana	Australia:Great Barrier Reef
37	615	ApXt*	Chlorofilexi	Anaerolineae	Caldilineales	Caldilineaceae	Unassigned	5,3E+08	99,76	Sponge: Aplysina cauliformis	Belize: Carrie Bow Cay
38	504	ApXt	Acidobacteria	Acidobacteria-6	BPC015	Unassigned	Unassigned	4,5E+08	99,33	Sponge: Ircinia strobilina	Bahamas:Sweetings Cay
40	430	Xt*	Gemmatimonadetes	Gemm-4	Unassigned	Unassigned	Unassigned	4E+08	99,55	Sponge: Xestospongia testudinaria	Australia: Great Barrier Reef
45	829	ScWt	Proteobacteria	Alphaproteobacteria	Rickettsiales	Pelagibacteraceae	Unassigned	7,3E+08	100	Bottom seawater	Atlantic sedimentary abyssal plain
48	284	Xt*	Proteobacteria	Gammaproteobacteria	Thiotrichales	Piscirickettsiaceae	Unassigned	3,5E+08	99,78	Sponge: Xestospongia testudinaria	Indonesia: Manado
50	609	ApXt	Proteobacteria	Gammaproteobacteria	Chromatiales	Ectothiorhodospiraceae	Unassigned	4,5E+08	99,78	Sponge: Ircinia strobilina	Bahamas:Exumas
51	286	ApXt*	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	Unassigned	4,5E+08	99,76	Sponge: Ircinia felix	Bahamas:Exumas
52	484	ApXt	Proteobacteria	Gammaproteobacteria	Chromatiales	Unassigned	Unassigned	6,8E+08	99,33	Sponge: Astrosclera willeyana	Australia:Great Barrier Reef
53	432	ApXt*	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	Unassigned	6,8E+08	99,53	Sponge: Astrosclera willeyana	Australia:Great Barrier Reef
54	288	Ap*	Gemmatimonadetes	Gemm-4	Unassigned	Unassigned	Unassigned	2,9E+08	98,66	Sponge: Theonella swinhoei	China:South China Sea
58	512	ApXt*	Actinobacteria	Acidimicrobiia	Acidimicrobiales	Unassigned	Unassigned	3,5E+08	99,29	Sponge: Xestospongia testudinaria	Indonesia: Manado
60	317	ApXt	SBR1093	EC214	Unassigned	Unassigned	Unassigned	3,2E+08	99,54	Sponge: Xestospongia testudinaria	Indonesia: Manado
65	278	Sc*	Proteobacteria	Gammaproteobacteria	Thiohalorhabdales	Unassigned	Unassigned	4,1E+08	97,91	Sponge: Stylissa carteri	Saudi Arabia
66	552	ApXt	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	Unassigned	6,8E+08	99,76	Sponge: Astrosclera willeyana	Australia:Great Barrier Reef
69	267	ApXt*	Chlorofilexi	SAR202	Unassigned	Unassigned	Unassigned	3,5E+08	99,53	Sponge: Xestospongia testudinaria	Indonesia: Manado
80	289	ApXt	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophobacteraceae	Unassigned	4,5E+08	99,55	Sponge: Ircinia felix	Bahamas:Exumas
86	209	ApXt	Proteobacteria	Deltaproteobacteria	(Entotheonellales)	Unassigned	Unassigned	4,5E+08	98,44	Sponge: Ircinia strobilina	Bahamas:Sweetings Cay
143	248	Sd*	Proteobacteria	Gammaproteobacteria	Thiotrichales	Thiotrichaceae	Unassigned	2,7E+08	99,77	Marine sediments	Philippines: Bolinao
145	244	Sd	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Unassigned	5,8E+08	100	Sponge: Rhabdastrella globostellata	Guam
175	289	ApXt*	Chlorofilexi	SAR202	Unassigned	Unassigned	Unassigned	3,5E+08	99,53	Sponge: Rhopaloeides odorabile	Australia: Great Barrier Reef
658	376	Xt*	Chlorofilexi	Anaerolineae	Caldilineales	Caldilineaceae	Unassigned	3,5E+08	99,76	Sponge: Xestospongia testudinaria	Indonesia: Manado
811	748	ScWt	Cyanobacteria	Synechococcophycideae	Synechococcales	Synechococcaceae	Synechococcus	7E+08	100	Saline lake water	Croatia
3158	250	ApXt	Actinobacteria	Acidimicrobiia	Acidimicrobiales	wb1_P06	Unassigned	2,3E+08	99,53	Sponge: Xestospongia muta	USA: Key Largo, Florida

Discussion

In line with previous studies (Cleary et al. 2015, de Voogd et al. 2015), the sediment biotope proved to be the richest. OTUs in sediment were mainly assigned to the Proteobacteria, but there were substantial numbers of OTUs assigned to other taxa including Actinobacteria, Bacteroidetes and Acidobacteria among others. OTUs assigned to the proteobacterial order Desulfobacterales were also restricted to sediment. The Desulfobacterales order consists of anaerobic bacteria known to use sulphate as terminal electron acceptor in order to oxidate H_2 and a wide range of organic compounds. They also have been shown to play an important role in degrading organic contaminants and appear to be sensitive to predicted shifts in ocean pH (Muyzer and Stams 2008, Zhou et al. 2011a, Coelho et al. 2015).

Proteobacteria also proved to be the most abundant phylum in *S. carteri* and two of the three seawater and *A. suberitoides* samples. In contrast, Chloroflexi and Actinobacteria were more prevalent in samples of *X. testudinaria*. The dominance of Proteobacteria in *S. carteri* is in line with previous studies of the closely related *S. massa* in Jakarta (de Voogd et al. 2015) and *S. carteri* in Makassar (Cleary et al. 2015), Indonesia. LMA sponges, such as *S. carteri*, have been shown to host bacterial communities with limited phylum-level diversity when compared to HMA species. They are also known to filter large volumes of water, which can explain the presence of large numbers of OTUs found in water and the higher similarity between bacterioplankton and *S. carteri* bacterial communities (Weisz et al. 2008, Giles et al. 2013).

The prevalence of Chloroflexi in *X. testudinaria* in the present study agrees with previous studies of the species in other regions including the Red Sea, North Sulawesi and Great Barrier Reef where Acidobacteria, Actinobacteria and particularly Chloroflexi were the most abundant phyla in terms of OTUs and sequences (Lee et al., 2011; Montalvo & Hill, 2011; Montalvo et al., 2014). It, however, contrasts with two of our previous studies (de Voogd et al. 2015, Cleary et al. 2015) where Proteobacteria were more abundant in terms of OTUs and sequence reads although both of these studies, also contained a large number of OTUs assigned to the Chloroflexi. Previous studies have also identified Chloroflexi as a consistent component of the bacterial communities of HMA sponges (Gloeckner et al. 2014; Schmitt et al., 2011).

In the present study, OTUs assigned to the Chloroflexi were mainly assigned to the orders SAR202 and Anaerolineae. Members of the SAR202 order have been found to be relatively abundant in bathypelagic waters (Varela et al. 2008). Anaerolinae members have been found in a wide range of habitats from artic permafrost to tropical marine sediment and the mammalian gastrointestinal tract (Hug et al. 2013, Campbell et al. 2014). Cambell et al (2014) have proposed that Anaerolinae in humans occupy an ecological niche where they scavage material from lysed bacterial cells and human tissue. In sponges, they may occupy a similar niche whereby they exploit the high loss of sponge biomass due to rapid sponge tissue turnover (de Goeij et al. 2013).

Seawater samples mainly consisted of OTUs assigned to Proteobacteria, Bacteroidetes and Cyanobacteria. Alphaproteobacteria reached its greatest abundance in seawater samples, and mainly consisted of OTUs assigned to the orders Rickettsiales and Rhodobacterales. The order Flavobacteriales was also mainly found in seawater samples. This result follows numerous studies that have found a prevalence of Proteobacteria (mainly Alphaproteobacteria) and Bacteroidetes in the bacterioplankton (Glöckner et al. 1999).

There were significant differences in composition among biotopes. *S. carteri* and seawater biotopes on the one hand and *A. suberitoides* and *X. testudinaria* biotopes on the other were compositionally similar. With respect to *S. carteri* and seawater, this confirms previous studies highlighting the similarity of LMA sponges and seawater (Weisz et al. 2008, Giles et al. 2013). However, it should be noted that, although similar, samples of *S. carteri* were still distinct from seawater samples as seen in the ordination of the third and fourth axes. *Stylissa carteri* also contained highly abundant OTUs that were either much more abundant in or restricted to *S. carteri*. These OTUs were also closely related to organisms previously obtained from *S. carteri* samples in Saudi Arabia or *Axinella*, previously placed in the same order (Halichondrida) as *S. carteri*.

The similarity in bacterial symbiont composition between *A. suberitoides* and *X. testudinaria* is interesting. These species are otherwise very different, e.g., their outer morphology, skeletal architecture and phylogenetic relationship. For instance, *A. suberitoides* has a massive to lobate growth form, whereas *X. testudinaria* is barrel

shaped. However, the skeleton of both sponges, is composed of high densities of silicious spicules, albeit of different size dimensions and morphologies. The similarity in the composition of their bacterial symbionts is thus in line with the theory that the internal sponge morphology is an important determinant whether a sponge hosts a HMA- or LMA-type bacterial community (Vacelet and Donadey, 1977; Weisz et al., 2008; Gloeckner et al. 2014).

This is, to the best of our knowledge, the first study of the bacterial community of *A. suberitoides*, a relatively abundant, widespread and easily recognisable sponge species. Recently, the higher classication of sponges has been altered and some sponge orders have been abandoned (Morrow & Cárdenas, 2015). Previously the genus *Aaptos* (order Suberitida, family Suberitidae) belonged to the order Hadromerida, which contained HMA sponges, such as *Speciospongia vesparium* (currently order Clionaida; family Clionaidae) and LMA sponges such as *Suberites domuncula* (currently order Suberitida, family Suberitidae) and *Suberites* diversicolor (Cleary et al. 2013). Gloeckner et al (2014) previously showed that although certain sponge taxa such as the orders Agelasida and Verongida only consisted of HMA species and the Poecilosclerida of LMA species, other taxa such as the Haplosclerida, Homoscleromorpha and Dictyoceratida consisted of both LMA and HMA species.

In addition, to *Aaptos* and *Suberites*, the family Suberitidae also contains the the distinct genus *Terpios*. The bacterial communities of species belonging to both of these taxa differed greatly from that found in *A. suberitoides*. The 'cyanosponge' *Terpios hoshinota* was found to mainly (61-98%) consist of cyanobacteria followed by smaller alpha- and gammaproteobacterial components (Tang et al. 2011). In the Berau region of Indonesia, Cleary et al. (2013) showed that *Suberites diversicolor* maintained a low diversity bacterial community dominated by a few OTUs assigned to the alphaproteobacterial order Kiloniellales.

The present study shows that the bacterial community of *A. suberitoides* is very similar to that of *X. testudinaria* and both sponge species share numerous OTUs assigned to the Chloroflexi and Actinobacteria that were largely absent in other biotopes including the LMA sponge *S. carteri* and sediment and seawater. This finding is similar to a previous study of ours (Cleary et al. 2015) where we showed that the presumed HMA sponge *H.*

erectus also contains a bacterial community very similar to *X. testudinaria* (Cleary et al. 2015).

Conclusion

The present study confirms previous studies (Lee et al. 2011, Cleary et al,. 2015; 57. Polónia et al. 2015, de Voogd et al. 2015) showing that sponges harbour microbial communities that are distinct from communities in sediment and the surrounding seawater. This finding extends to LMA sponges as demonstrated by *S. carteri* in this study. Although the bacterial communities of *S. carteri* and seawater were similar, they were still distinct with *S. carteri* hosting a small number of highly abundant OTUs including OTUs restricted to this sponge. Our study also showed that the hitherto unknown bacterial community of *A. suberitoides* is very similar to the known HMA sponge *X. testudinaria* providing strong evidence that *A. suberitoides* is also a HMA sponge. Although both *A. suberitoides* and *X. testudinaria* shared numerous OTUs, they also hosted what may be species-specific OTUs.

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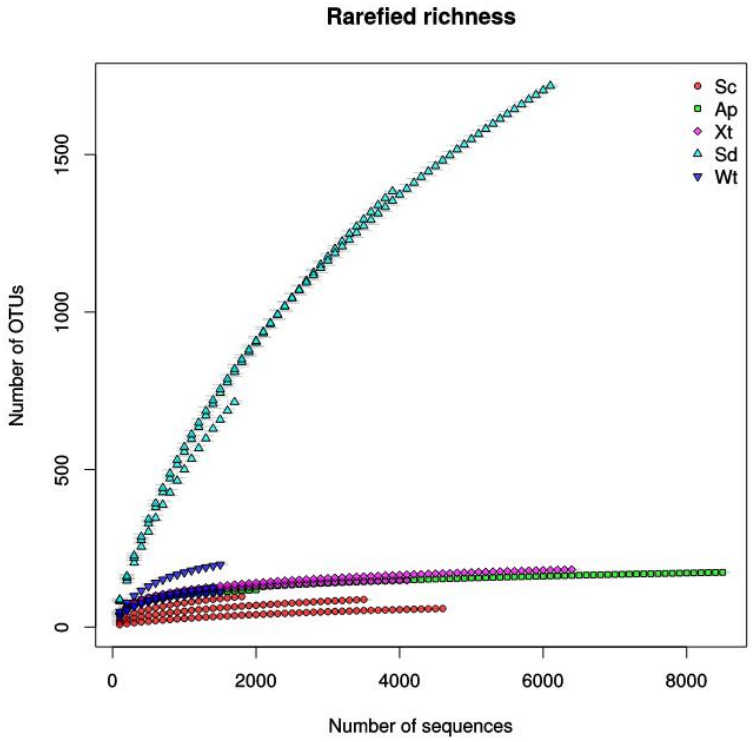
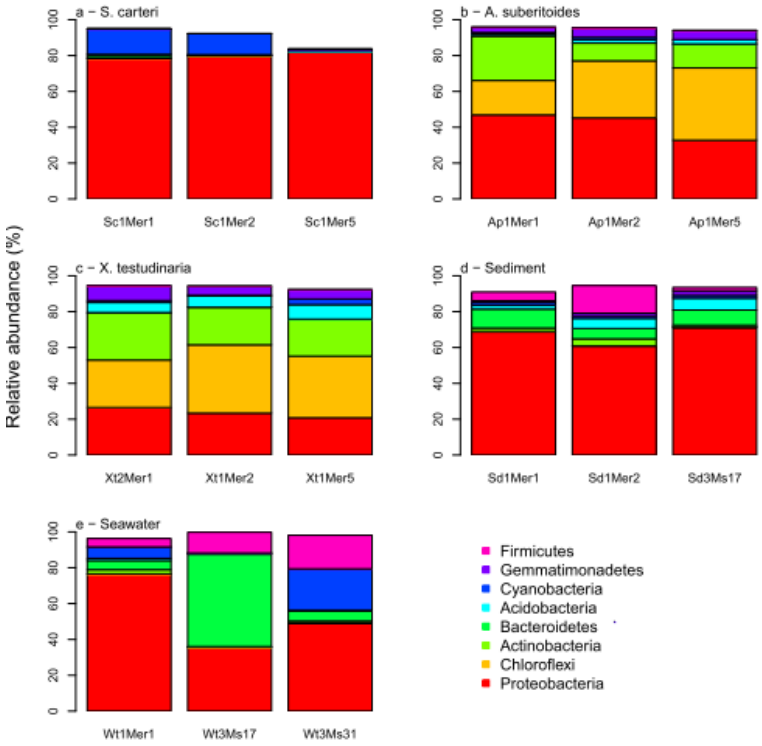
Supplemental figures



Supplemental Figure A-1 Species accumulation curves as a function of the number of sequences using resampling of bacterial 16S rRNA gene sequences from *S. carteri* (Sc), *A. suberitoides* (Ap), *X. testudinaria* (Xt), sediment (Sd) and seawater (Wt).



Supplemental Figure A-2 Stacked barplots showing the relative abundance of the 8 most abundant phyla sampled from the five biotopes. (a) *S. carteri*, (b) *A. suberitoides*, (c) *X. testudinaria*, (d) sediment and (e) seawater. The samples codes (X-axis) represent samples sampling sites Mer1, Mer2, Mer5, Ms17 and Ms31.

References

Akiyama, T., Takada, K., Oikawa, T., Matsuura, N., Ise, Y., Okada, S., & Matsunaga, S. (2013). Stimulators of adipogenesis from the marine sponge *Xestospongia testudinaria*. *Tetrahedron*, *69*(32), 6560–6564. doi:10.1016/j.tet.2013.06.007.

Allen GR (2008) Conservation hotspots of biodiversity and endemism for Indo-Pacific coral reef fishes. Aquat Conserv: Mar Freshwater Ecosystems 18:541–556.

Allen GR & Erdmann MV (2009). Reef fishes of the bird's head peninsula, West Papua, Indonesia. Check List *5*: 587-628.

Aoki, S., Kong, D., Suna, H., Sowa, Y., Sakai, T., Setiawan, A., & Kobayashi, M. (2006). Aaptamine, a spongean alkaloid, activates p21 promoter in a p53-independent manner. *Biochemical and Biophysical Research Communications*, *342*, 101–106. doi:10.1016/j.bbrc.2006.01.119

Becking LE, de Leeuw C, Vogler C (2014) Newly discovered "jellyfish lakes" in Misool, Raja Ampat, Papua, Indonesia. Marine Biodiversity, 1-2.

Bellwood DR, Hughes TP, Folke C, Nyström M (2004) Confronting the coral reef crisis. Nature 429: 827-833.

Borchiellini, C., Manuel, M., Alivon, E., Boury-Esnault, N., Vacelet, J., & Le Parco, Y. (2001). Sponge paraphyly and the origin of Metazoa. *Journal of Evolutionary Biology*, *14*, 171–179. doi:10.1046/j.1420-9101.2001.00244.x.

Bruno JF, Selig ER (2007) Regional decline of coral cover in the Indo-Pacific: Timing, extent, and subregional comparisons. PLoS ONE 2: e711.

Campbell AG, Schwientek P, Vishnivetskaya T, Woyke T, Levy S, Beall CJ, Griffen A, Leys E, Podar M. 2014. Diversity and genomic insights into the uncultured Chloroflexi from the human microbiota. Environ Microbiol. 16:2635-2643. doi: 10.1111/1462-2920.12461.

Capone DG, Dunham SE, Horrigan SG, Duguay LE (1992) Microbial nitrogen transformations in unconsolidated coral reef sediments. Mar Ecol-Prog Ser 80: 75–88.

Cleary, D. F. R. (2003). An examination of scale of assessment, logging and ENSO-induced fires on butterfly diversity in Borneo. *Oecologia*, *135*, 313–321. doi:10.1007/s00442-003-1188-5

Cleary, D. F. R., Becking, L. E., de Voogd, N. J., Pires, A. C. C., Polónia, A. R. M., Egas, C., & Gomes, N. C. M. (2013). Habitat- and host-related variation in sponge bacterial symbiont communities in Indonesian waters. *FEMS Microbiology Ecology*, *85*, 465–482. doi:10.1111/1574-6941.12135.

Cleary DFR, de Voogd NJ, Polónia ARM, Freitas R, Gomes NCM. (2015). Composition and predictive functional analysis of bacterial communities in seawater, sediment and sponges in an Indonesian coral reef environment. Microbial Ecology. In Press.

de Goeij JM, van Oevelen D, Vermeij MJ, Osinga R, Middelburg JJ, de Goeij AF, Admiraal W. 2013. Surviving in a marine desert: the sponge loop retains resources within coral reefs. Science 342: 108-10. doi: 10.1126/science.1241981.

de Voogd NJ, Cleary DFR. 2008. An analysis of sponge diversity and distribution at three taxonomic levels in the Thousand Islands/Jakarta Bay reef complex, West-Java, Indonesia. Marine Ecology 29: 205–215.

de Voogd, N. J., Cleary, D. F. R., Hoeksema, B. W., Noor, A., & van Soest, R. W. M. (2006). Sponge beta diversity in the Spermonde Archipelago, SW Sulawesi, Indonesia. *Marine Ecology Progress Series*, *309*, 131–142. doi:10.3354/meps309131.

de Voogd, N. J., Cleary, D. F. R., Polónia, A. R. M., & Gomes, N. C. M. (2015). Bacterial community composition and predicted functional ecology of sponges, sediment and seawater from the thousand islands reef complex, West Java, Indonesia. *FEMS Microbiology Ecology*, *91*, 1–12. doi:10.1093/femsec/fiv019.

De'ath G, Fabricius KE, Sweatman H, Puotinen M (2012) The 27-year decline of coral cover on the Great Barrier Reef and its causes. Proc Natl Acad Sci USA 109:17995–17999.

Ebada SS1, Linh MH, Longeon A, de Voogd NJ, Durieu E, Meijer L, Bourguet-Kondracki ML, Singab AN, Müller WE, Proksch P (2015) Dispacamide E and other bioactive bromopyrrole alkaloids from two Indonesian marine sponges of the genus Stylissa. Nat Prod Res. 29: 231-238. doi: 10.1080/14786419.2014.947496.

Edgar R, Haas B, Clemente J, Quince C, Knight R (2011) UCHIME Improves Sensitivity and Speed of Chimera Detection. Bioinformatics 27: 2194–2200.

Edgar RC (2013) UPARSE: Highly Accurate OTU Sequences from Microbial Amplicon Reads. Nat Methods 10: 996–998.

Faulkner, D. J. (2002). Marine natural products. *Natural Product Reports*, *19*, 1–48. doi:10.1039/b009029h

Flatt, P. M., Gautschi, J. T., Thacker, R. W., Musafija-Girt, M., Crews, P., & Gerwick, W. H. (2005). Identification of the cellular site of polychlorinated peptide biosynthesis in the marine sponge Dysidea (Lamellodysidea) herbacea and symbiotic cyanobacterium Oscillatoria spongeliae by CARD-FISH analysis. *Marine Biology*, *147*, 761–774. doi:10.1007/s00227-005-1614-9

Freeman, C. J., & Thacker, R. W. (2011). Complex interactions between marine sponges and their symbiotic microbial communities. *Limnology and Oceanography*, *56*(5), 1577–1586. doi:10.4319/lo.2011.56.5.1577.

Giles EC, Kamke J, Moitinho-Silva L, Taylor MW, Hentschel U, Ravasi T, Schmitt S (2013) Bacterial community profiles in low microbial abundance sponges. FEMS Microbiol Ecol 83: 232-241.

Giles EC, Saenz-Agudelo P, Hussey NE, Ravasi T, Berumen ML. 2015. Exploring seascape genetics and kinship in the reef sponge *Stylissa carteri* in the Red Sea. Ecol Evol. 5: 2487–2502, doi: 10.1002/ece3.1511.

Glöckner FO, Fuchs BM & Amann R (1999) Bacterioplankton compositions of lakes and oceans: A first comparison based on fluorescence in situ hybridization. Applied Environmental Microbiology 65: 3721-3726.

Grantham HS, Agostini VN, Wilson J, Mangubhai S, Hidayat N, Muljadi A, et al. (2013). A comparison of zoning analyses to inform the planning of a marine protected area network in Raja Ampat, Indonesia. Marine Policy 38: 184-194.

Hentschel, U., Fieseler, L., Wehrl, M., Gernert, C., Steinert, M., Hacker, J., & Horn, M. (2003). Microbial diversity of marine sponges. In W. E. Muller (Ed.), *Sponges (Porifera)* (Springer-Verlag, pp. 59–88). Heidelberg.

Hug LA, Castelle CJ, Wrighton KC, Thomas BC, Sharon I, Frischkorn KR, Williams KH, Tringe SG, Banfield JF. 2013. Community genomic analyses constrain the distribution of metabolic traits across the Chloroflexi phylum and indicate roles in sediment carbon cycling. Microbiome 1:22. doi: 10.1186/2049-2618-1-22.

Jackson JBC, Kirby MX, Berger WH, Bjorndal KA, Botsford LW, Bourque BJ, Bradbury RH, Cooke R, Erlandson J, Estes JA, Hughes TP, Kidwell S, Lange CB, Lenihan HS, Pandolfi JM, Peterson CH, Steneck RS, Tegner MJ, Warner RR (2001) Historical overfishing and the recent collapse of coastal ecosystems. Science 293:629-638.

Jin, M., Zhao, W., Zhang, Y., Kobayashi, M., Duan, H., & Kong, D. (2011). Antiproliferative effect of aaptamine on human chronic myeloid leukemia K562 cells. *International Journal of Molecular Sciences*, *12*, 7352–7359. doi:10.3390/ijms12117352

Kamke, J., Taylor, M. W., & Schmitt, S. (2010). Activity profiles for marine sponge-associated bacteria obtained by 16S rRNA vs 16S rRNA gene comparisons. *The ISME Journal*, *4*, 498–508. doi:10.1038/ismej.2009.143

Larghi, E. L., Obrist, B. V., & Kaufman, T. S. (2008). A formal total synthesis of the marine alkaloid aaptamine. *Tetrahedron*, *64*, 5236–5245. doi:10.1016/j.tet.2008.03.036

Lee, O. O., Wang, Y., Yang, J., Lafi, F. F., Al-Suwailem, A., & Qian, P.-Y. (2011). Pyrosequencing reveals highly diverse and species-specific microbial communities in sponges from the Red Sea. *The ISME Journal*, *5*, 650–664. doi:10.1038/ismej.2010.165

Legendre, P., & Gallagher, E. (2001). Ecologically meaningful transformations for ordination of species data. *Oecologia*, *129*, 271–280. doi:10.1007/s004420100716

Li, C.-W., Chen, J.-Y., & Hua, T.-E. (1998). Precambrian Sponges with Cellular Structures. *Science*, *279*, 879–882.

Liang, L.-F., Wang, T., Cai, Y.-S., He, W.-F., Sun, P., Li, Y.-F., Huang, Q., Taglialatela-Scafati, O., Wang, H.-Y., & Guo, Y.-W. (2014). Brominated polyunsaturated lipids from the Chinese sponge Xestospongia testudinaria as a new class of pancreatic lipase inhibitors. *European Journal of Medicinal Chemistry*, *79*, 290–297. doi:10.1016/j.ejmech.2014.04.003

Liu, C., Tang, X., Li, P., & Li, G. (2012). Suberitine A-D , four new cytotoxic dimeric aaptamine alkaloids from the marine sponge Aaptos suberitoides. *Organic Letters*, *14*(8), 1994–1997. doi:10.1021/ol3004589

Mangubhai S, Erdmann MV, Wilson JR, Huffard CL, Ballamu F, Hidayat NI, Hitipeuw C, Lazuardi ME, Muhajir, Pada D, Purba G, Rotinsulu C, Rumetna, Sumolang K, Wen W. (2012). Papuan Bird's Head Seascape: Emerging threats and challenges in the global center of marine biodiversity. Marine pollution bulletin 64: 2279-2295.

McKenna SA, Boli P, Allen GR (2002) Condition of coral reefs at the Raja Ampat Islands, Papua Province, Indonesia. In: A Marine Rapid Assessment of the Raja Ampat Islands, Papua Province, Indonesia, Bulletin of the Rapid Assessment Program, Vol. 22 (eds McKenna SA, Allen GR, Suryadi S), pp. 66–78. Conservation International, Washington, DC.

Moitinho-Silva L, Bayer K, Cannistraci CV, Giles EC, Ryu T, Seridi L, Ravasi T, Hentschel U (2014). Specificity and transcriptional activity of microbiota associated with low and high microbial abundance sponges from the Red Sea. *Molecular Ecology*, *23*, 1348–1363. doi:10.1111/mec.12365

Montalvo, N. F., & Hill, R. T. (2011). Sponge-associated bacteria are strictly maintained in two closely related but geographically distant sponge hosts. *Applied and Environmental Microbiology*, *77*(20), 7207–16. doi:10.1128/AEM.05285-11.

Morrow C. & Cárdenas P. (2015). Proposal for a revised classification of the Demospongiae (Porifera). Frontiers in Zoology, 12:7

Muyzer G. Stams AJM. 2008. The ecology and biotechnology of sulphate-reducing bacteria. Nature reviews. Microbiology 6, 441.

Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Simpson, G. L., Solymos, P., Wagner, H. (2009). vegan: Community Ecology Package. R Package version 1.15-4. Retrieved from http://www.cran.r-project.org/package=vegan

Pandolfi JM, Bradbury RH, Sala E, Hughes TP, Bjorndal KA, Cooke RG, McArdle D, McClenachan L, Newman MJH, Paredes G, Warner RR, Jackson JBC (2003) Global trajectories of the long-term decline of coral reef ecosystems. Science 301:955–958.

Pham, C.-D., Hartmann, R., Müller, W. E. G., de Voogd, N. J., Lai, D., & Proksch, P. (2013). Aaptamine derivatives from the indonesian sponge Aaptos suberitoides. *Journal of Natural Products*, *76*, 103–106. doi:10.1021/np300794b

Polónia, A. R. M., Cleary, D. F. R., Freitas, R., de Voogd, N. J., & Gomes, N. C. M. (2015). The putative functional ecology and distribution of archaeal communities in sponges, sediment and seawater in a coral reef environment. *Molecular Ecology*, *24*, 409–423. doi:10.1111/mec.13024

Prentice, M. L., & Hope, G. S. (2007). Climate of Papua. In A. J. Marshall & B. M. Beehler (Eds.), *The Ecology of Papua: Part One* (pp. 177–196). Singapore: Periplus.

Roberts CM, McClean CJ, Veron JE, Hawkins JP, Allen GR, McAllister DE, et al. (2002) Marine biodiversity hotspots and conservation priorities for tropical reefs. Science 295:1280-1284.

Rohde S, Gochfeld D, Ankisetty S, Avula B, Schupp P, Slattery M (2012) Spatial variability in secondary metabolites of the indo-pacific sponge Stylissa massa. J Chem Ecol 38:463–475. doi: 10.1007/s10886-012-0124-8.

Rützler, K. (2004). Sponges on coral reefs: a community shaped by competitive cooperation. *Bollettino dei Musei e degli Instituti Biologici dell'Universita Di Genova*, *68*, 85–148.

Schlappy, M. L., S. I. Schottner, G. Lavik, M. M. Kuypers, D. de Beer, and F. Hoffmann. 2010. Evidence of nitrification and denitrification in high and low microbial abundance sponges. Mar. Biol. 157: 593–602.

Schmitt, S., Deines, P., Behman, F., Wagner, M., Taylor, M.W. (2011) Chloroflexi bacteria are more diverse, abundant, and similar in high than in low microbial abundance sponges. FEMS Microbiology and Ecology 78 (3): 497-510.

Swierts, T., Peijnenburg, K. T. C. A., de Leeuw, C., Cleary, D. F. R., Setiawan, E., Wörheide, G., Erpenbeck, D., & de Voogd, N. J. (2013). Lock, stock and two different barrels: comparing the genetic composition of morphotypes of the Indo-Pacific sponge Xestospongia testudinaria. *PLoS ONE*, *8*(9), 1–12. doi:10.1371/journal.pone.0074396

Tang SL, Hong MJ, Liao MH, Jane WN, Chiang PW, Chen CB, Chen CA. 2011. Bacteria associated with an encrusting sponge (Terpios hoshinota) and the corals partially covered by the sponge. Environ Microbiol. 13: 1179-91. doi: 10.1111/j.1462-2920.2010.02418.x.

Taylor, M. W., Radax, R., Steger, D., & Wagner, M. (2007). Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. *Microbiology and Molecular Biology Reviews*, *71*(2), 295–347. doi:10.1128/MMBR.00040-06.

Tsukamoto, S., Yamanokuchi, R., Yoshitomi, M., Sato, K., Ikeda, T., Rotinsulu, H., Mangindaan, R. E. P., de Voogd, N. J., van Soest, R. W. M., & Yokosawa, H. (2010). Aaptamine, an alkaloid from the sponge *Aaptos suberitoides*, functions as a proteasome inhibitor. *Bioorganic & Medicinal Chemistry Letters*, *20*, 3341–3343. doi:10.1016/j.bmcl.2010.04.029

van Soest, R. W. M., Boury-Esnault, N., Vacelet, J., Dohrmann, M., Erpenbeck, D., de Voogd, N. J., Santodomingo, N., Vanhoorne, B., Kelly, M., & Hooper, J. N. A. (2012). Global Diversity of Sponges (Porifera). *PLoS ONE, 7*, 1-23.

Vacelet, J., and C. Donadey. 1977. Electron microscope study of the association between some sponges and bacteria. J. Exp. Mar. Biol. Ecol. 30: 301–314.

Varela MM, van Aken HM, Herndl GJ. 2008. Abundance and activity of Chloroflexi-type SAR202 bacterioplankton in the meso- and bathypelagic waters of the (sub)tropical Atlantic. Environ Microbiol. 10: 1903-11. doi: 10.1111/j.1462-2920.2008.01627.x.

Weisz JB, Lindquist N, Martens CS (2008) Do associated microbial abundances impact marine demosponge pumping rates and tissue densities. Oecologia 155: 367–376. doi:10.1007/s00442-007-0910-0.

Zhang, Z., Schwartz, S., Wagner, L., & Miller, W. (2000). A Greedy Algorithm for Aligning DNA Sequences. *Journal of Computational Biology*, *7*, 203–214.

Zhou J, He Q, Hemme CL, Mukhopadhyay A, Hillesland K, Zhou A, He Z, Van Nostrand JD, Hazen TC, Stahl DA, Wall JD, Arkin AP. 2011a. How sulphate-reducing microorganisms cope with stress: lessons from systems biology. Nature Reviews Microbiology 9: 452-466, doi:10.1038/nrmicro2575.

Zhou, X., Lu, Y., Lin, X., Yang, B., Yang, X., & Liu, Y. (2011b). Brominated aliphatic hydrocarbons and sterols from the sponge Xestospongia testudinaria with their bioactivities. *Chemistry and Physics of Lipids*, *164*, 703–706. doi:10.1016/j.chemphyslip.2011.08.002.