**Universidade de Aveiro**
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Departamento de Biologia

**Ana Cecília da Cruz Pires**

# Comparação das comunidades bacterianas em biótopos de esponja, sedimento e água na região do Indo-Pacífico

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Doutor Newton Carlos Marcial Gomes, Investigador Auxiliar do Centro de Estudos do Ambiente e do Mar (CESAM) e do Doutor Daniel Francis Richard Cleary, Colaborador do Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro.

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Dedico este trabalho aos meus avós e aos meus pais.

**o júri**

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**palavras-chave**

Esponjas Marinhas, Comunidades Bacterianas, Indo-Pacífico, sequenciação em massa, Porifera

**resumo**

As esponjas são uma parte importante e abundante dos recifes de coral, e os seus simbiontes microbianos contribuem para os ciclos de nutrientes assim como para a nutrição, saúde e defesa desses organismos. Contudo, as comunidades microbianas associadas às esponjas devem ser caracterizadas estrutural e funcionalmente para que a sua função simbiótica nesses hospedeiros seja verdadeiramente compreendida. Esta tese reporta, através de uma abordagem de sequenciação em massa do gene 16S rRNA, a análise da composição, filogenia e potenciais funções das comunidades bacterianas associadas às esponjas *Suberites diversicolor*, *Cinachyrella australiensis*, *Stylissa carteri*, *Stylissa massa*, *Aaptos lobata* e *Xestospongia testudinaria* assim como da água e sedimento de três sistemas diferentes (lagos marinhos Indonésios e recifes de coral de Singapura e Tioman) na região do Indo-Pacífico. A influência do habitat (lagos marinhos vs. águas abertas) na composição bacteriana e a influência da dicotomia HMA/LMA (elevada e baixa abundância microbiana) na potencial função das bactérias associadas às esponjas também foram avaliadas. Em geral, os resultados mostram o biótopo como o principal preditor da riqueza, composição, abundância e potencial função das comunidades bacterianas. Encontraram-se diferenças significativas entre as comunidades bacterianas das esponjas e da água e sedimento adjacentes. Sendo que, o habitat apenas foi considerado como melhor preditor para a riqueza bacteriana nos lagos marinhos. Foram detetadas elevadas similaridades entre as comunidades bacterianas de *X. testudinaria* e da putativa HMA *A. lobata*, sustentando a indicação prévia de que esta última deva ser uma esponja HMA. Além disso, todas as amostras apresentaram dominância de filos envolvidos em ciclos de nutrientes e na nutrição, saúde e defesa das esponjas, tais como Proteobacteria, Chloroflexi e Cyanobacteria. A um nível taxonómico mais baixo, as amostras mostraram predominância de Kiloniellales (com potencial para a desnitrificação), Chromatiales (bactérias sulfurosas roxas), Hyphomicrobiaceae (desnitrificadores metilotróficos), SRA202 (oxidantes de sulfitos), Anaerolineae (degradadores de matéria orgânica), Synechococcophycideae (fixação de carbono), Thiohalorhabdales (oxidantes de enxofre). A predição funcional por meio do programa PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) mostrou elevada similaridade dos perfis funcionais entre as espécies de *Stylissa* e entre *A. lobata* e *X. testudinaria*. Adindo, as subcategorias “Signaling Molecules and Interaction”, “Carbohydrate Metabolism” e “Excretory System” estavam enriquecidas nas esponjas HMA enquanto as subcategorias “Replication and Repair”, “Energy Metabolism”, “Metabolism of Cofactors and Vitamins” e “Environmental Adaptation” estavam enriquecidas nas esponjas LMA. Evidenciou-se a partilha de um conjunto de características funcionais entre as comunidades bacterianas associadas às esponjas HMA e LMA, porém que ambas usam diferentes estratégias para lidar com patogénicos, obter energia ou reparação de DNA contra estresse ambiental.

**keywords** Marine Sponges, Bacterial Communities, Indo-Pacific, Pyrosequencing, Porifera

**abstract**

Sponges are an important structural and abundant part of coral reefs, and their microbial symbionts contribute to nutrient cycling and host nutrition, health and defence. However, microbial communities associated to sponges have to be structurally and functionally characterized so their symbiotic function in such host can be truly understood. This thesis addresses, through the use of a 16S rRNA gene high-throughput sequencing approach, the analyses of the composition, phylogeny and putative functions of bacterial communities inhabiting the sponges *Suberites diversicolor*, *Cinachyrella australiensis*, *Stylissa carteri*, *Stylissa massa*, *Aaptos lobata* and *Xestospongia testudinaria* and both seawater and sediment from three different systems (Indonesian marine lakes and coral reefs from Singapore and Tioman) in the Indo-Pacific region. Also, the influence of habitat (e.g. marine lake vs. open water) on bacterial composition and the influence of HMA/LMA dichotomy on putative function of sponge-associated bacteria were evaluated. Results showed, in general, biotope as the main predictor of bacterial communities' richness, composition, abundance and putative function. Significant differences were found between sponges' bacterial communities and those from the surrounding seawater and sediment. Habitat only appeared to be a better predictor than biotope for bacterial richness in marine lakes. Strong similarities were detected between bacterial communities in *X. testudinaria* and the putative HMA *A. lobata*, supporting the previous indication that *A. lobata* might be indeed an HMA sponge. Moreover, all samples were dominated by phyla involved in nutrient cycling and in sponge nutrition, health and defence, such as Proteobacteria, Chloroflexi and Cyanobacteria. At a lower taxa level, samples were dominated by Kiloniellales (with potential for denitrification), Chromatiales (purple sulphur bacteria), Hyphomicrobiaceae (methylotrophic denitrifiers), SRA202 (sulphite-oxidizers), Anaerolineae (organic matter degraders), Synechococcophycideae (carbon fixation), Thiohalorhabdales (sulphur oxidizing bacteria). The predicted metagenome (PICRUSt) analysis showed high similarity of functional profiles between both *Stylissa* species and between *A. lobata* and *X. testudinaria*. Furthermore, subcategories "Signaling Molecules and Interaction", "Carbohydrate Metabolism" and "Excretory System" were enriched in HMA sponges whilst subcategories "Replication and Repair", "Energy Metabolism", "Metabolism of Cofactors and Vitamins" and "Environmental Adaptation" were enriched in LMA sponges. The results also showed that HMA and LMA associated bacterial communities shared similar core functional features, albeit they use distinct strategies to deal with pathogens, obtain energy or DNA repair against environmental stress.

## List of Publications

This thesis included results which have already been published or in phase of submission for publication:

Cleary DFR, Becking LE, de Voogd NJ, **Pires ACC**, Polónia ARM, Egas C and Gomes NCM (2013) Habitat- and host-related variation in sponge bacterial symbiont communities in Indonesian waters. FEMS Microbiology Ecology, 85(3): 465–482. DOI: 10.1111/1574-6941.12135

**Pires ACC**, Cleary DFR, Polónia ARM, Lim SC, de Voogd NJ, Oliveira V and Gomes NCM (2019). Comparison of bacterial communities associated with *Xestospongia testudinaria*, sediment and seawater in a Singaporean coral reef ecosystem. Journal of the Marine Biological Association of the United Kingdom, 99(2): 331-342.

**Pires ACC**, Oliveira V, Cleary DRF, Polónia ARM, Coelho FJRC, Waheed Z, de Voogd NJ and Gomes NCM (for submission) Bacterial composition and putative functions associated to different sponge species, sediment and seawater in Tioman coral reef system, Peninsular Malaysia

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# Chapter 1. Introduction

## Marine ecosystems

Marine ecosystems are amongst the most productive ecosystems in the world and include areas from coastal to open and deep ocean. The differences between habitats from such distinct areas rely on key environmental factors such as temperature, salinity, tides, wind, wave action, sunlight and sediment substrate, that greatly influence the functioning and diversity of those ecosystems (Levinton, 1995). Actually, the three-dimensional distribution of organisms in marine ecosystems is due to the water flow at different depths layers (Ministry of the Environment, 2011). For example, organisms with photosynthesis as primary producers are found from water surface to around 200 m depth or at the bottom of shallow coastal water, which do not occur in the deep sea due to the currents.
During their life cycle, several organisms migrate through different marine regions creating a complex system of interactions among the organisms coexisting in the same areas at the same time (Ministry of the Environment, 2011). Many marine species, ranging from planktonic organisms to large mammals, inhabit marine ecosystems that provide them both food and shelter from predators. Marine ecosystems are also crucial for the maintenance and protection of marine and terrestrial environments and can be divided into two main groups: open ocean and coastal habitats. Compared to coastal habitats the open ocean is relatively less productive due to the distance to land that leads to, for example, low temperature and low nutrients availability (Sigman and Hain, 2012; Dastrup, 2016).

The open ocean encompasses the entire water column outside of coastal areas, from the surface waters to the deep ocean, and is subdivided into five zones according to depth and population composition (Dastrup, 2016): epipelagic zone (from surface water to about 200 m depth, which also represents the limit of the photic zone), mesopelagic zone (from 200 m to 1000 m), bathypelagic zone (from 1000 m to 4000 m), abyssalpelagic zone (from 4000 m to 6000 m) and hadalpelagic zone (deeper than 6000 m, found in deep-sea trenches). Since the sunlight only penetrates to about 200 m of the sea surface, the epipelagic zone is also designated as photic zone. Thus, photosynthetic organisms, that depend on sunlight, are restricted to the photic zone – the layer where life is most abundant.

In turn, coastal habitats are found along the shoreline to the boundary of the continental shelf and include intertidal habitats, sandy shores, rocky shores, cliffs, mudflats, mangrove and salt marshes, estuaries, kelp forests, seagrass meadows and coral reefs. Whilst salt marshes and estuaries act as filters for sediment draining from land (Solomon and Forbes, 1999; McLean et al., 2001), areas as mangroves (that also act as filters), reefs and seagrass beds provide protection by reducing wave action, the impact of tsunamis and storms (Gelfenbaum et al., 2011; Zhang et al., 2012; Spalding et al., 2014) and the shoreline erosion by moderating wind-waves and promoting shoreline accretion (Shepard et al., 2011; McIvor et al., 2012; Alegria-Arzaburu et al., 2013; Ferrario et al., 2014). Marine systems as kelp forests, seagrass meadows, mangroves and coral reefs shape the ecosystems to the point where they provide habitat for other organisms.

Kelp forests are temperate ecosystems with a complex biological structure based on large brown algae that support a high diversity of invertebrate (e.g. sea urchin) and finfish (e.g. rockfish), being the kelp itself harvested for human use namely for food and additives (Dayton, 2003; Agardy et al., 2005). These ecosystems are extremely resistant to wave impacts and storms (Dayton, 2003).

The term seagrass refers to the flowering plants that colonize oceanic soft-bottom areas, being an important source of food for several coastal and marine organisms from tropical to temperate regions (Gray et al., 1996). It has a key role in retaining sediments and shoreline stabilization (Agardy et al., 2005) and, along with mangroves, these ecosystems provide essential nursery and habitat for coral reef fishes and invertebrates in the tropics (Gray et al. 1996; Heck et al., 1997).

Mangrove forests are restricted to both tropical and sub-tropical areas (Spalding et al., 1997; Valiela et al., 2001). According to Ewel et al. (1998), the goods and services provided by mangroves vary with the zone where these forests are found and the type of mangrove. Mangroves provide organic matter export, nursery and habitat to several animals, allowing the harvest of plant products and improving water quality. Consequently, these forests are highly used by coastal communities, playing an important role in the land stabilization, cycling nutrients, processing pollutants, supporting several marine organisms' lifecycle and as a great source of fuelwood, timber and fishery resources (Lacerda and Abrão, 1984; Agardy et al., 2005). Mangroves also act as barriers protecting seagrass beds and coral reefs from siltation and pollution (Wolanski, 2007) and those located in Southeast Asia, South

Asia, and Africa are exceptionally species-rich and provide food and shelter to several reef species (Mumby et al., 2004).

Coral reefs are structurally complex habitats found in shallow, tropical coastal waters formed by corals, other animals (such as fishes, echinoderms, molluscs, crustaceans, sponges and sea snakes) and algae near to the shore and can extend hundreds of kilometres in shallow offshore environments (Barbier et al., 2011; Dastrup, 2016), and can occur as barrier reefs, atolls, fringing reefs or patch reefs (Agardy et al., 2005). Coral reefs are considered as the "rainforests of the oceans" due to the high amount of species diversity (Swart, 2013; Dastrup, 2016). Such diversity relies on global, regional and local factors (Connell et al., 1997; Glynn, 1997; Pandolfi, 2002; Hughes et al., 2005). As mentioned above, coral reefs provide coastal protection, but they are also involved in nutrient cycling. Moreover, these ecosystems are important to humans by the maintenance of fisheries, providing unique and aesthetic reefscapes, suitable habitat for diverse fauna and flora which contribute for tourism, recreation, education and research in the coral reef area (Barbier et al., 2011), as well as opportunities for bioprospecting and ornamentals for aquarium trade (Ahmed et al., 2004).

When comparing coastal and terrestrial ecosystems (such as swamp and marsh, continental shelf, estuaries and tropical rainforest), coral reefs and kelp have the highest mean net primary productivity (Agardy et al., 2005). Coral reefs are known to occur in nutrient-poor waters with temperatures ranging from 18 to 30ºC with minimal sediment loading and freshwater input (Agardy et al., 2005; Swart, 2013). Theoretically, these conditions should result in a low level of productivity. However, due to the rapid and efficient nutrient cycling on reefs among all the components of the community productivity is high (Agardy et al., 2005; Swart, 2013). For example, corals can obtain energy and oxygen directly from their symbionts zooxanthellae, that, in return, receive shelter and can use the carbon dioxide produced by corals (Burke et al., 2002). When there is an excessive input of nutrients (e.g. by human source) the balance of the ecosystem is disturbed and consequently it declines (Swart, 2013). In addition, due to its high vulnerability to negative impacts from overuse and habitat degradation, the removal of certain elements of this complex ecosystem leads to negative feedbacks (Nystrom et al., 2000).

**Indo-Pacific coral reefs**

The majority of tropical reefs are found in developing countries and is where the most intensive degradation is occurring (Burke et al., 2002). Wilkinson et al. (2002), described that 58% of all the known tropical reef systems were found within 25 kilometers of major urban centers with populations of 100,000 or more. Near to these urban centers, coral reefs are subjected to severe risks caused by human activities, including coastal construction (that leads to habitat loss and the change of physical processes), destructive fishing with ornamental purpose, overfishing for both local consumption and export, inadequate sanitation and control of run-off, dumping, land use (leading to siltation), oil spills and degradation of linked habitats (e.g. seagrass, mangrove) (Wilkinson, 2000, 2002). Hughes et al. (2003) suggested that coral reefs may be the most vulnerable ecosystem to the effects of climate change. Besides human activity, coral reefs are also affected by bleaching, sometimes mortally, that is triggered by the rise of seawater temperature (Agardy et al., 2005). An additional negative effect of climate change on coral reefs holds on the change of the seawater pH as a consequence of carbon dioxide rising, leading to the decrease of calcium carbonate deposition by corals for reef-building (Hughes et al., 2003). Agardy et al. (2005) suggested that the combination of overfishing and pollution from land-based sources makes the reefs less resistant to diseases and climate change.

Indo-Pacific region contains the most diverse and endemic-rich coral reefs worldwide, which also constitute the most diverse of all known coral reefs (Fig. 1.1) (Burke et al., 2002; Veron et al., 2009). According to Burke et al. (2002), the diversity of coral reefs is not only based on coral species, but also to other animals inhabiting coral reefs, which extends to related coastal ecosystems, such as mangroves and seagrass beds. More than 60% (4,050) of the world's coral reef fish species (6,000) are found in the Indo-Pacific region (Allen, 2007), and nearly 1650 fish species were identified only in eastern Indonesia and most of them were associated with reefs (Hopley and Suharsono, 2000); six of the world's seven marine turtle species are found in the Indo-Pacific region (Pilcher and Ismail, 2000). Moreover, the 61,000 $km^2$ of mangroves described in all Southeast Asia until 2002 represented about 35% of the world's total, holding almost 75% of all known mangrove species and 45% of seagrass species (Spalding et al., 1997; Burke et al., 2002).

Besides the presence of several fish, turtle and coral species, coral reefs are home to other thousands of species, such as dolphins [17 of 36 species worldwide can be found in the Great Barrier Reef (GBR; Lawler et al., 2007)]; whales (Steele, 2003); sharks and rays [135 species of sharks, rays, skates and chimeras were described in the GBR (Chin et al., 2016)]; sea snakes (about 70 species inhabit the Indo-Pacific) (Lillywhite et al., 2018); birds [252 species nest and breed on the GBR (Steele, 2003)]; sea urchins (Barrett et al., 2018); starfish and snails [the crown-of-thorns starfish and both starfish *Drupella cornus* and *Coralliophila* sp. were described as great predators of Indo-Pacific reefs (Beeden et al., 2008)]; shrimps (Burke et al., 2002); lobsters and crabs (Barbier et al., 2011); nudibranchs [more than 2,000 species have been identified in the Indo-Pacific (Gosliner et al., 2008)]; algae [zooxanthallae have a symbiotic relationship with corals (Beeden et al., 2008)] and coralline algae [important in stabilization and cementation of the coral reef structure (Barbier et al., 2011)]; jellyfish and sponges [Indonesian sponge community may comprise almost 830 species (van Soest, 1989)].

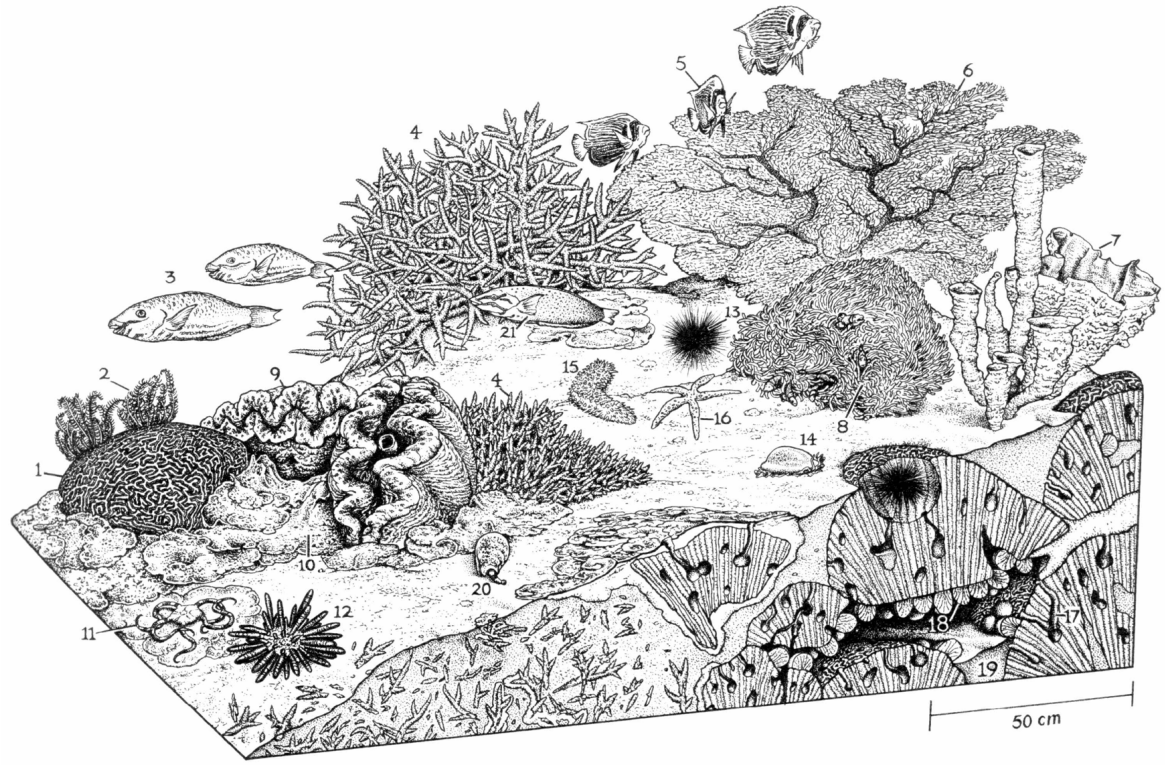


Fig. 1.1 – Reconstruction of a modern Indo-Pacific coral reef (Wood, 1999; Copyright John Sibbick).

Marine habitats also include marine lakes, which are anchialine systems of small bodies of landlocked seawater isolated from the surrounding environment (Holthuis, 1973; Hamner and Hamner, 1998; Colin, 2009; Becking et al., 2011). The connection between lakes and the adjacent sea is made by subterranean tunnels, fissures or small dissolution channels (Hamner and Hamner, 1998; Cerrano et al., 2006; Azzini et al., 2007; Becking et al., 2011). These connections confer the marine character to the lakes (varying from brackish to almost fully marine) by subjecting them to a tidal regime, although delayed and diminished compared with the surrounding sea (Hamner and Hamner, 1998; Becking et al., 2011). A study published by Dawson et al. (2009), estimated that there are only 200 marine lakes worldwide, occurring in clusters of ten or more lakes in Croatia, Bermuda, Vietnam, Palau and Indonesia. Kakaban lake (Indonesia) is one of the largest known marine lakes, with a high degree of isolation from the surrounding sea and contains many rare and endemic species (Tomascik and Mah, 1994; Becking et al., 2011). The connection to the adjacent sea widely varies within lakes of Palau and Vietnam, resulting in distinct environmental regimes (Hamner and Hamner, 1998; Cerrano et al., 2006; Azzini et al., 2007; Colin, 2009). According to Becking et al. (2011), marine lakes of Indonesia showed the same tidal dampening and salinity as marine lakes of Palau described by Hamner and Hamner (1998). Due to its marine origin, floral and faunal colonization of marine lakes depends directly on the type of their connection to the adjacent sea, which results in different species assemblages between distinct lakes. Independently on the taxa, to colonize marine lakes they have to adapt themselves to lower salinities and higher temperatures than the surrounding sea (Becking et al., 2011).

Lakes and pools of Indonesia are characterized for having algae, sponges, molluscs, ascidians and even mangrove as dominant taxa (Tomascik and Mah, 1994; Hoeksema, 2004; Becking et al., 2008; Becking et al., 2011). Many surveys have shown that sponges are one of the most dominant taxa in terms of biomass and diversity in marine lakes in Indonesia, Vietnam and Palau (Azzini et al., 2007; Colin, 2009; Becking et al., 2011, 2013).

## Sponges (phylum Porifera)

Sponges (phylum Porifera) are primitive, sessile, bottom-dweller and filter feeding organisms (Duckworth et al., 2006). Although, recently, several carnivorous sponges were identified in deep-sea habitats (Maldonado et al., 2015; Dressler-Allame et al., 2016; Hestetun et al., 2016). Sponges are asymmetrical or radially symmetrical animals that come in many different colours, sizes (varying from few mm to 2 m of height) and shapes, including cup-, tube-, ball-, fan-shaped, tree-like and shapeless (Mandal, 2018). Sponges are exclusively aquatic animals currently with 9080 extant species – 229 non-marine species and 8851 marine species (Table 1.1) and are classified into four classes (Fig. 1.2): Calcarea (calcareous sponges), Demospongia (demosponges), Hexactinellida (glass sponges) and Homoscleromorpha (Gazave et al., 2012; van Soest et al., 2018). Demospongia is the most diverse class comprehending about 83% of all extant sponge species (van Soest et al., 2018). According to Hooper and van Soest (2002), Calcarea sponges produce extracellular calcite, Hexactinellida have triaxonix silica spicules and Demospongiae hold monaxonic, tetraxonic, and/or polyaxonic silica spicules, and/or collagen-derived skeletal structures. Homoscleromorpha sponges differ morphologically from Demospongiae by having distinguishable siliceous tetractinal-like calthrop spicules (Lévi, 1956). While Calcarea and Desmospongia are mostly found on continental shelf rocky bottoms, Hexactinellida occur in oceans and seas muddy bottoms and Homoscleromorpha usually occur at shallow depths, but some specimens have been retrieved from abyssal depths (Gazave et al., 2012; Mandal, 2018).

Table 1.1 – Number of described marine and non-marine species of the four Porifera classes and total number of sponge species extracted from the World Porifera Database (Source: www.marinespecies.org/porifera, accessed 2018 October 14).

| Taxon | All species | Accepted marine species | Accepted non-marine species |
|---|---|---|---|
| **Calcarea** | 1483 | 769 | 0 |
| **Demospongiae** | 16040 | 7299 | 229 |
| **Hexaxtinella** | 1139 | 662 | 0 |
| **Homoscleromorpha** | 152 | 120 | 0 |
| **Total species** | 18814 | 8851 | 229 |

Sponges can successfully inhabit shallow to deep waters of marine and freshwater habitats from tropical to polar seas, attaching themselves to rocks, hard-shelled animals, or seaweed (Rützler, 2004). As an important structural part of coral reefs, sponges help reefs building up, contributing for their regeneration and stabilisation, providing themselves shelter and habitat for several marine species (e.g. fishes, brittle stars and shrimps) (Diaz and Rützler, 2001; de Voogd et al., 2006). Sponges' diversity and the area they occupy in coral reefs are also essential for such ecosystems, as are the diversity of sponges' microbial associations, their competition for space used in reef regeneration and their contribution to nutrient cycling by exchanging nutrients with the surrounding water (Diaz and Rützler, 2001; de Goeij and van Duyl, 2007; de Goeij et al., 2008, 2013). In addition, they are a great source of promising pharmaceutical biocompounds (Faulkner, 2002; Taylor et al., 2007).

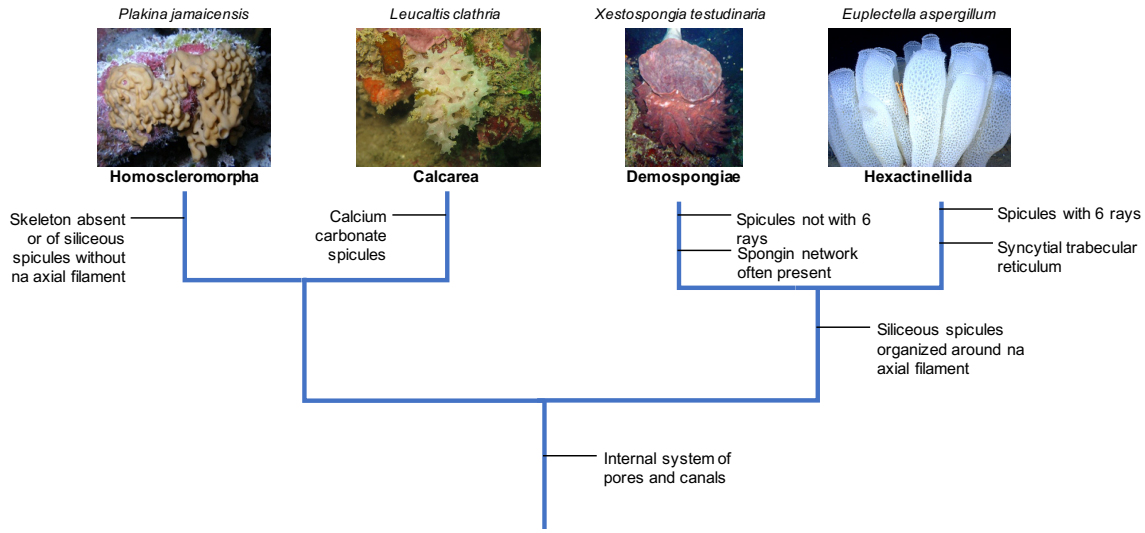


Fig. 1.2 – Cladogram of Porifera classes: Homoscleromorpha, Calcarea, Demospongiae and Hexactinellida. Photographs: *Plakina jamaicensis* taken by Susanna López-Legentil in Northern Exuma Cays – Bahamas (http://www.spongeguide.org), *Leucaltis clathria* taken by Sven Zea in Bocas del Toro – Panama (http://www.spongeguide.org), *Xestospongia testudinaria* taken by LE Becking in Misool – Indonesia (Cleary et al., 2018) and *Euplectella aspergillum* taken by NOAA researchers from the Okeanos Explorer Program in the Gulf of Mexico (http://www.seasky.org).

In a healthy coral reef, sponges associated microbial communities allow nutrients capture and recycling. The outstanding biodiversity and productivity of coral reef ecosystems are supported by all habitats constituting the three-dimensional structure of the coral reef system (Garren and Azam, 2012). As an integrant habitat, sponges are believed to support coral reefs life by increasing diversity and maintaining high productivity and to contribute

positively through to their functioning by taking dissolved organic matter (DOM) and transforming it in particulate organic matter (POM) that is consumed by other reef organisms (de Goeij et al., 2013).

Microbial symbiosis with sponges may be responsible for the host's ecological success, contributing to nutrient cycling and to sponge nutrition, health and defence, by the degradation of toxic substances and the production of antibiotics and biologically active metabolites (Sipkema et al., 2005; Taylor et al., 2007; van Soest et al., 2012; de Goeij et al., 2013; Colman, 2015). Indeed, Taylor et al. (2007) suggested that sponge-microbial symbionts can be the responsible for the biosynthesis of metabolites used in host's defence. In turn, microbial effects on sponges may influence their performance in coral reef system.

**Sponge microbial communities**

It is known that sponges harbour dense, diverse and very specific microbial communities – including bacteria, archaea, unicellular algae, fungi and viruses – that can constitute up to 35% of the host's biomass (Taylor et al., 2007; Webster and Taylor, 2012). It is also known that there are several microbial communities establishing symbiosis with sponges, forming stable clusters divergent from microbial communities in the surrounding seawater (Taylor et al., 2007). Many sponges have been shown to harbour microbial communities greatly diverse and dense, however abundance and structural composition of microbial communities can show huge differences between sponge species. Regarding the number of microbial cells per gram of tissue, sponges have been classified as high microbial abundance (HMA) and low microbial abundance (LMA) species (Table 1.2), where HMA sponges host more abundant and diverse microbial communities (up to $10^9$ cells per gram of sponge wet weight) than LMA sponges ($10^5$-$10^6$ cells per gram of sponge wet weight) (Hentschel et al., 2003; Weisz et al., 2007; Ribes et al., 2012). Although the newest insights given by metagenomics and metatranscriptomics, it still remains to reveal the reason for the differences among HMA and LMA sponges and if the interactions sponge-microbes also differ between sponge type (Webster and Thomas, 2016), especially because HMA and LMA sponges' microbiomes also share some functional features (Fan et al., 2012; Bayer et al., 2014).

The perception of taxonomy, phylogeny and metabolism of sponges' microbial symbionts has changed due to molecular studies, which led to the discovery of an unusually high phylum-level diversity and stability of sponge-microbes associations (Hentschel et al., 2006; Schmitt et al., 2012b; Thomas et al., 2016; Webster and Thomas, 2016). Hentschel et al. (2006) identified Cyanobacteria, Archaea, Proteobacteria, Actinobacteria and the candidate phylum "Poribacteria" as the main specific lineages associated to sponges. Independently on the relative abundance values, until now 52 bacterial phyla were identified associated to sponges, including candidate phyla described as belonging to the rare community (Schmitt et al., 2012b; Reveillaud et al., 2014). In a more recent study, 41 bacterial phyla were detected in 81 sponge species, where all the species hosted at least 13 different phyla (Thomas et al., 2016). Of those, Proteobacteria (mainly Gamma- and Alphaproteobacteria), Actinobacteria, Chloroflexi, Cyanobacteria, Nitrospirae and the candidate phylum "Poribacteria" were identified as the most dominant phyla (Hentschel et al., 2012; Simister et al., 2012). Kamke et al. (2010) compared 16S rRNA gene- vs 16S rRNA-derived bacterial community profiles for the first time and showed that a great part of the bacterial communities associated to sponges are metabolically active. In sponge-Cyanobacteria interaction, one of the oldest microbe-metazoan mutualistic association, cyanobacterial symbionts fix carbon and provide photosynthesis products like glycerol to the host (Wilkinson and Fay, 1979; Hentschel et al., 2006; Taylor et al., 2007; Webster and Thomas, 2016). Proteobacteria phylum includes ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) (such as *Nitrosococcus* and *Nitrosomonas*), that together might be responsible for nitrification in sponges (Davy et al., 2002). Proteobacteria also comprises sulfate-reducing bacteria (SRB), producers of carboxylic acids, and sulphur-oxidizing bacteria (SOB), that were suggested to prevent toxic accumulation of sulfide (Hoffmann et al., 2005). Actinobacteria representatives retrieved from marine sponges are important and of great interest due to their prolific production of secondary metabolites (Hentschtel et al., 2006). The candidate phylum "Poribacteria" appears to be enriched in marine sponges (Fieseler et al., 2004) and it was suggested that its members can degrade complex carbohydrates produced by the sponges (Kamke et al., 2013). In addition to the above, sponge-microbial symbionts are also known producers of polyphosphate granules that may be used as energy storage and essential vitamins such as B vitamin (Fan et al., 2012; Zhang et al., 2015; Webster and Thomas, 2016).

Sponge microbial communities' structure and composition are affected by both host and abiotic characteristics of the surrounding environment. Functional analysis indicated that in general, sponge microbial symbionts under environmental stress have acquired resistance mechanisms (Fan et al., 2012). Caporaso et al. (2011) also showed that structure and composition of sponge microbial communities reflect the differences among phylogenetic distinct host species and between host and non-host (e.g. sediment and seawater). Thus, functional and structural characterization of different sponges and the surrounding biotopes allow to a better understand of the way microbial symbionts adapt to different hosts and their interaction in coral reef ecosystems.

Some authors have shown strong evidences for an existing coevolution of sponges and their associated microorganisms. Based on sequence information (estimated rates of 16S rRNA), Taylor et al. (2007) suggested that coevolution could be evaluated through the analysis of the last common ancestor of sponge-specific microrganisms (such as the "*Ca*. Synechococcus spongiarum" cluster and the candidate phylum "Poribacteria") from different hosts. Erpenbeck et al. (2002) approached sponge-microbes coevolution through the phylogenetic analysis of the mitochondrial cytochrome oxidase subunit 1 (CO1) of diverse halichondrid sponges and six putative alphaproteobacterial symbionts. They were able to detect sequences' origin, up to a certain level, and four putative cospeciation events. However, as reviewed by Webster and Thomas (2016), additional effort has to be made in order to ascertain the extent of sponge-microbes coevolution and what driven this process. This is mainly because the symbionts community similarity observed among closely related sponges might not be due to sponge-microbes coevolution, but to a simple mutualistic relationship (Moran and Sloan, 2015).

Table 1.2 – Examples of known HMA or LMA sponge species.

| Sponge species | Reference | Sponge species | Reference |
|---|---|---|---|
| **HMA** | | **LMA** | |
| *Agelas clathroides* | Poppell et al., 2014 | *Acanthella acuta* | Gloeckner, 2014 |
| *Agelas conifera* | Poppell et al., 2014 | *Amphimedon compressa* | Angermeier, et al. 2012; Poppell et al., 2014 |
| *Agelas citrina* | Wehrl, 2006 | *Amphimedon ochracea* | Gloeckner, 2014 |
| *Agelas dilatata* | Wehrl, 2006 | *Axinella cannabina* | Gloeckner, 2014 |
| *Agelas dispar* | Gloeckner, 2014 | *Axinella corrugata* | White et al., 2012 |
| *Agelas oroides* | Björk et al., 2013 | *Axinella damicornis* | Erwin et al., 2015 |
| *Agelas tubulata* | McMurray et al., 2018 | *Axinella polypoides* | Wehrl, 2006 |
| *Agelas wiedenmayeri* | Wehrl, 2006 | *Batzella rubra* | Gloeckner, 2013 |
| *Aiolochroia crassa* | Gloeckner, 2014 | *Callyspongia plicifera* | Gloeckner, 2013 |
| *Ancorina alata* | Simister et al., 2013 | *Callyspongia* sp. | Taylor et al., 2004 |
| *Aplysina archeri* | Wehrl, 2006 | *Callyspongia vaginalis* | Schiller, 2006; Wehrl, 2006 |
| *Aplysina cauliformis* | Wehrl, 2006; Poppell et al., 2014 | *Chalinula molitba* | Schiller, 2006; Wehrl, 2006 |
| *Aplysina cavernicola* | Friedrich et al., 1999 | *Cinachyrella alloclada* | Gloeckner, 2013 |
| *Aplysina fistularis* | Wehrl, 2006; Gloecnker et al., 2013 | *Cliona varians* | Schiller, 2006 |
| *Aplysina insularis* | Wehrl, 2006 | *Crambe crambe* | Wehrl, 2006 |
| *Aplysina lacunosa* | Wehrl, 2006 | *Crella cyathophora* | Giles et al., 2013 |
| *Calyx podatypa* | Gloeckner, 2014; Poppell et al., 2014 | *Cymbastela concentrica* | Taylor et al., 2004 |
| *Chondrilla caribensis* forma *hermatypa* | Poppell et al., 2014 | *Dysidea avara* | Wehrl 2006; Björk et al., 2013; Erwin et al., 2015 |
| *Chondrilla nucula* | Thiel et al., 2007 | *Dysidea etheria* | Schiller, 2006 |
| *Chondrosia collectrix* | Gloeckner, 2014 | *Dysidea fragilis* | Moitinho-Silva et al., 2017 |
| *Chondrosia reniformis* | Wehrl, 2006; Gloecnker et al., 2013 | *Erylus formosus* | Gloeckner, 2014 |
| *Craniella zetlandica* | Schöttner et al., 2013 | *Halichondria panicea* | Wichels et al., 2006; Motinho-Silva et al., 2017 |
| *Cribrochalina vasculum* | Schiller, 2006 | *Hymeniacidon sinapium* | Cao et al., 2012 |
| *Ectyoplasia ferox* | Schmitt et al., 2008a, b; Gloeckner et al., 2013 | *Iotrochota birotulata* | Wehrl, 2006 |
| *Erylus formosus* | Moitinho-Silva et al., 2017 | *Monanchora arbuscula* | Gloeckner, 2014 |
| *Geodia atlantica* | Cárdenas et al., 2013; Schöttner et al., 2013 | *Mycale (Mycale) lingua* | Schöttner et al., 2013 |
| *Geodia barretti* | Cárdenas et al., 2013; Schöttner et al., 2013 | *Mycale hentscheli* | Anderson et al., 2010 |
| *Geodia macandrewii* | Cárdenas et al., 2013; Schöttner et al., 2013 | *Mycale laxissima* | Wehrl 2006 |
| *Geodia neptuni* | Gloeckner, 2014 | *Niphates digitalis* | Schiller, 2006; Wehrl, 2006; Poppell et al., 2014 |
| *Geodia phlegraei* | Cárdenas et al., 2013; Schöttner et al., 2013 | *Niphates erecta* | Wehrl, 2006 |
| *Ircinia campana* | Poppell et al., 2014 | *Oscarella lobularis* | Gloeckner et al., 2013 |
| *Ircinia fasciculata* | Erwin et al., 2012b | *Phakellia robusta* | Schöttner et al., 2013 |
| *Ircinia felix* | Schmitt et al., 2007 | *Phakellia ventilabrum* | Schöttner et al., 2013 |
| *Ircinia oros* | Erwin et al., 2012b | *Ptilocaulis* sp. | Wehrl, 2006 |
| *Ircinia strobilina* | Schmitt, 2007 | *Scopalina ruetzleri* | Wehrl, 2006; Gloeckner, 2013; Poppell et al., 2014 |
| *Ircinia variabilis* | Erwin et al., 2012b; Moitinho-Silva et al., 2017 | *Spheciospongia vesparium* | Poppell et al., 2014 |
| *Myrmekioderma gyroderma* | Gloeckner, 2013 | *Spirastrella cunctatrix* | Erwin et al., 2015 |
| *Pachymatisma normani* | Schöttner et al., 2013 | *Stylinos* sp. | Taylor et al., 2004 |
| *Petrosia ficiformis* | Erwin et al., 2015; Motinho-Silva et al., 2017 | *Stylissa carteri* | Giles et al., 2013 |
| *Petrosia* sp. | Gloeckner, 2014 | *Stylissa massa* | Schöttner et al., 2013; Moitinho-Silva et al., 2014 |
| *Plakortis lita* | Gloeckner, 2014 | *Suberites domuncula* | Wehrl, 2006 |
| *Plakortis* sp. | Laroche et al., 2007; Schöttner et al., 2013 | *Sympagella* sp. | Schöttner et al., 2013 |
| *Rhopaloeides odorabile* | Erwin et al., 2015; Moitinho-Silva et al., 2017 | *Tedania ignis* | Schiller 2006; Wehrl, 2006 |
| *Sarcotragus spinosulus* | Hardoim and Costa, 2014 | *Tedania klausi* | Poppell et al., 2014 |
| *Siphonodictyon coralliphagum* | Schiller 2006; Schmitt et al., 2008b | *Tethya aurantium* | Wehrl, 2006 |
| *Smenospongia aurea* | Schmitt et al., 2008b; Gloeckner, 2013 | *Tethya stolonifera* | Simister et al., 2013 |
| *Spheciospongia vesparium* | Gloeckner, 2014 | | |
| *Spongia officinalis* | Bauvais et al., 2015 | | |
| *Svenzea zeai* | Gloeckner, 2014 | | |
| *Verongula gigantea* | Wehrl, 2006 | | |
| *Xestospongia muta* | Wehrl 2006; Hentschel et al., 2006 | | |
| *Xestospongia testudinaria* | Gloeckner, 2014 | | |

**Metagenomic analysis – microbial community structure and function**

In order to disclosure and understand the roles of yet uncultivable bacteria in their communities, an increasing effort has been made in the last years and culture-independent approaches have been developed and applied to study microbial metagenomes (Rinke et al., 2013; Esteves et al., 2016).

Sanger sequencing was the first technology developed in this field, marking the beginning of the study of genomes, and was classified as the First Generation Sequencing Technology (Sanger et al., 1977; Michael, 2010). For almost 30 years first generation sequencing dominated the sequencing field, until the emergence of a new generation of sequencers, producing millions of short reads in parallel with low costs and less time consuming (Kchouk et al., 2017). Webster et al. (2001) performed the first study where "clone libraries" of 16S ribosomal RNA (rRNA) gene sequences were used to evaluate sponge-associated microbial diversity independently of microbial cultivability. Later, Schmitt et al. (2012a) targeted the same gene but using the high-throughput sequencing method pyrosequencing for amplicon sequencing. These studies only targeted the 16S rRNA gene to access microbial diversity, no functional genomic information was provided.

Metagenomics is a culture-independent method developed in the 1900's that is used to access and investigate both diversity and function of microbial communities through the information extracted directly from all microorganisms in each environmental sample. The use of next-generation sequencing (NGS) technology in metagenomics studies can either be based on a specific target gene (e.g. 16S rRNA gene) – amplicon-based approach – or on small gene fragments from entire genomes – shotgun-based approach (Mineta and Gojobori, 2016). Metagenomes have been explored and used for the identification of novel bioactive compounds (Lejon et al., 2011). For example, Heath et al. (2009) have identified an alkaliphilic esterase through functional screening of a metagenomic library from Antarctic desert soil and Waschkowitz et al. (2009) have isolated and characterized a novel domain of bacterial metalloproteases through the construction and screening for the presence of genes conferring proteolytic activity of metagenomic libraries.

Recently, several metagenomic studies have focused on marine sponge microbiome and on the comparison of microbial communities' phylogenetic composition and structure among sponge species and between host and the surrounding environment (e.g. seawater and sediment), in order to understand if sponge-associated microbes are sponge-specific and/or may derive from non-host biotopes and the functional nature of such interactions (Thomas et al., 2010; Fan et al., 2012; Polónia et al., 2014; Cleary et al., 2015; de Voogd et al., 2015; Nakashima et al., 2016; Thomas et al., 2016). Through metagenomics it was shown that microbial symbionts with distinct structures associated to different marine sponges presented similar core metabolic profiles, indicating that symbiont communities converged functionally to occupy similar niches or to fulfil common functions in different sponges (Fan et al., 2012; Li et al., 2016).

**Objectives and thesis outline**

The general objective of this thesis was to characterize the distribution, composition and putative function of sponge bacterial symbionts in coral reefs and marine lakes and investigate their relationship with environmental biotopes (seawater and sediment) in the Indo-Pacific region.

The main objectives were: 1) to understand how bacterial communities inhabiting different biotopes differ in composition, phylogeny and putative function; 2) evaluate the influence of habitat (e.g. marine lake vs. open water) on bacterial composition and 3) investigate how bacterial community structure differs among LMA and HMA sponges.

All samples for this thesis were taken in the Indo-Pacific region (Fig. 1.3), specifically in Berau – East Kalimantan Province (Indonesia), along the Southern Islands of Semakau and Kusu (Singapore) and in Tioman Island – state of Pahang (Peninsular Malaysia).

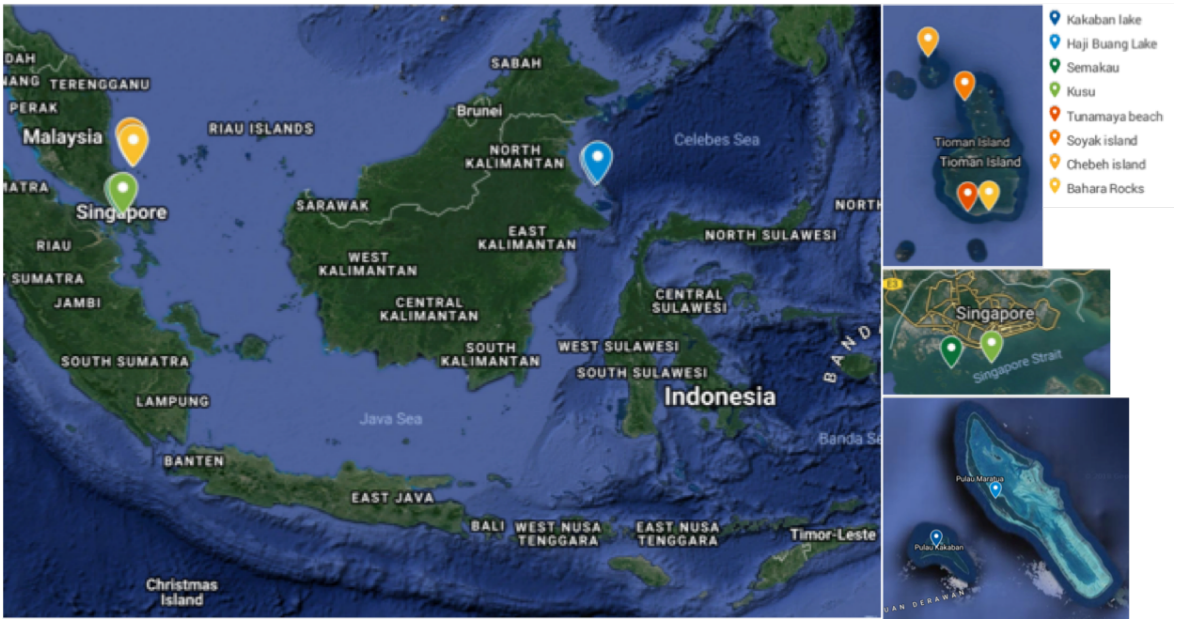


Fig. 1.3 – Map of study area showing sampling sites in the Indo-Pacific region.

A brief description of each chapter is given bellow:

**Chapter 1** – Introduction

In this chapter we present an introduction concerning coral reef systems as one of the most diverse and productive marine ecosystems (especially in the Indo-Pacific region), sponges as an integrant part of coral reefs, and the use of metagenomic analysis in the study of sponge-microbial communities' structure and function.

**Chapter 2** – Habitat- and host-related variation in sponge bacterial symbiont communities in Indonesian waters

In this chapter we access the bacterial richness and composition of the marine sponges *Suberites diversicolor* and *Cinachyrella australiensis* inhabiting enclosed marine lakes and surrounding open coastal habitats in the islands of Kakaban and Maratua in the Berau Delta barrier reef system (Indonesia). Moreover, we evaluated to what extent host species and habitat (marine lake vs. open water) influence sponge bacterial composition.
This chapter was published in the journal FEMS Microbiology Ecology – DOI: 10.1111/1574-6941.12135

This chapter is included in this thesis due to the contribution of the PhD student, as a co-author, in samples' DNA extraction, statistical analysis and in the manuscript writing.

**Chapter 3** – Comparison of bacterial communities associated with *Xestospongia testudinaria*, sediment and seawater in a Singaporean coral reef ecosystem

In this chapter we use 16S rRNA gene barcoded pyrosequencing to characterize and compare the bacterial communities from the sponge *Xestospongia testudinaria* (commonly found and widespread in Singapore), sediment and seawater in a Singaporean coral reef ecosystem. We also identified dominant bacterial symbionts of *X. testudinaria*.
This chapter was published in the Journal of the Marine Biological Association of the United Kingdom – DOI: 10.1017/S0025315418000188

**Chapter 4** – Composition and putative function of bacterial communities associated to different sponge species in Tioman coral reef system, Peninsular Malaysia

In this chapter we investigate to what extent bacterial community structure differs among LMA and HMA sponges and between host and non-host biotopes. Moreover, we evaluated how structural differences of bacterial communities are related to their predicted function. We used high-throughput pyrosequencing of the 16S rRNA gene and the bioinformatic tool PICRUSt to characterize the diversity, composition and predict metagenomic gene content of bacterial communities of the LMA sponges *Stylissa carteri* and *Stylissa massa*, the well-known HMA sponge *Xestospongia testudinaria*, the putative HMA sponge *Aaptos lobata* and non-host biotopes (sediment and seawater) in coral reef ecosystem around Tioman Island.
This chapter is in phase of submission.

**Chapter 5** – Conclusions

In this chapter we summarize the main conclusions of the work.

**Chapter 6** – Appendix

In this chapter we include the list of the publications produced during the PhD and information about the database of the biological samples and bacterial isolates whose field and/or laboratory work (sampling, DNA extraction and amplification, sequencing) were financed and supported by the Project EcoTech-SPONGE (Assessing the ECOlogical functions and potential bioTECHnological applications of plasmid assemblages from microbial symbionts of marine SPONGEs) created and managed by the PhD student.

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## Chapter 2. Habitat- and host-related variation in sponge bacterial symbiont communities in Indonesian waters

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**Abstract**

Marine lakes are unique ecosystems that contain isolated populations of marine organisms. Isolated from the surrounding marine habitat, many lakes house numerous endemic species. In this study, microbial communities of sponges inhabiting these lakes were investigated for the first time using barcoded pyrosequencing of 16S rRNA gene amplicons. Our main goals were to compare the bacterial richness and composition of two sponge species (*Suberites diversicolor* and *Cinachyrella australiensis*) inhabiting both marine lakes and adjacent open coastal systems. Host species and habitat explained almost 59% of the variation in bacterial composition. There was a significant difference in composition between both host species. Within *S. diversicolor*, there was little discernible difference between bacterial communities inside and outside lakes. The bacterial community of this species was, furthermore, dominated (63% of all sequences) by three very closely related alphaproteobacterial taxa identified as belonging to the recently described order Kiloniellales. *Cinachyrella australiensis*, in contrast, hosted markedly different bacterial communities inside and outside lakes with very few shared abundant taxa. *Cinachyrella australiensis* in open habitat only shared 9.4% of OTUs with *C. australiensis* in lake habitat. Bacteria were thus both highly species specific and, in the case of *C. australiensis,* habitat specific.

**Keywords**

Borneo; community composition; mangroves; marine lakes; pyrosequencing; Porifera

## Introduction

Marine lakes, a very rare and unique habitat, are anchialine systems, which are small bodies of landlocked seawater isolated to varying degrees from the surrounding marine environment (Holthuis, 1973; Hamner and Hamner, 1998; Colin, 2009; Becking et al., 2011); they contain brackish to almost fully marine waters. The marine character of these systems is maintained by subterranean tunnels, fissures or small dissolution channels in the surrounding rock, connecting the lakes to the adjacent sea, and as such display a wide variety in the degree of connection to the sea and environmental regimes within the lakes (Hamner

and Hamner, 1998; Cerrano et al., 2006; Azzini et al., 2007; Becking et al., 2011). These landlocked pools of water are subjected to a tidal regime, which is typically delayed and dampened compared with the adjacent sea (Hamner and Hamner, 1998; Becking et al., 2011). The number of marine lakes worldwide is estimated at only 200 with clusters of ten or more lakes occurring in areas with a karstic limestone landscape in Croatia, Bermuda, Vietnam, Palau and Indonesia (Dawson et al., 2009). This enclosed environment has set the stage for small, isolated, rapidly evolving populations and endemic (sub)species (Holthuis, 1973; Maciolek, 1983; Tomascik and Mah, 1994; Massin and Tomascik, 1996; Dawson and Hamner, 2005). Kakaban lake, for example, one of the largest marine lakes presently known to science and highly isolated from the adjacent sea (Tomascik and Mah, 1994; Becking et al., 2011), contains many rare and endemic species across a variety of taxa including a crab (*Orcovita saltatrix* Ng and Tomascik, 1994), two holothurians (*Holothuria* (*Lessonothuria*) *cavans* Massin and Tomascik, 1996 and *Synaptula spinifera* Massin and Tomascik, 1996) and an ascidian (*Styela complexa* Kott, 1995). Surveys of marine lakes in Indonesia, Vietnam and Palau have shown sponges to be one of the most dominant taxa in terms of biomass and diversity (Azzini et al., 2007; Colin, 2009; Becking et al., 2011, 2013). However, to the best of our knowledge, no studies have been conducted on the diversity and composition of symbiont microbial communities associated with these unique and unexploited environments.

Sponges (phylum Porifera) are exclusively aquatic animals with currently 8553 extant species and with an estimated 25 000 species (Appeltans et al., 2012; van Soest et al., 2012). They are successful colonisers of a wide range of habitats, from tropical to polar seas and shallow to deep waters, and are found in marine and freshwater habitats, where they are involved in a host of ecological processes (Rutzler, 2004). Sponges have been shown to be unique and highly selective environments for bacteria; the bacterial assemblages they host are, furthermore, of substantial ecological, biotechnological and pharmaceutical importance (Hentschel et al., 2002, 2003, 2006; Taylor et al., 2007; Webster et al., 2010; Jackson et al., 2012; Webster and Taylor, 2012). In many cases, the bacterial symbionts either are the source or contribute significantly to the production of bio-active secondary metabolites found in sponges (Lee et al., 2001; Piel, 2004; Erpenbeck and van Soest, 2007; Taylor et al., 2007). As a result, there is heightened interest in these bacteria. Different sponge species cohabiting the same habitat can greatly differ in the abundance of their associated

microorganisms. High-microbial abundance sponges can contain around $10^{10}$ bacterial cells $g^{-1}$ wet weight of sponge (orders of magnitude higher than concentrations in seawater); low-microbial abundance sponges contain densities of around $10^6$ cells $g^{-1}$ (similar to densities in seawater) (Hentschel et al., 2006; Kamke et al., 2010). Only a minute percentage of bacteria found in sponges are cultivable (Hentschel et al., 2003; Jackson et al., 2012). Recent advances, however, in molecular techniques, such as pyrosequencing, now enable us to assess bacterial communities at an unprecedented level of detail (Webster et al., 2010; Lee et al., 2011; Jackson et al., 2012; Schmitt et al., 2012b; White et al., 2012).

In the present study, we compare the richness and composition of bacteria in two sponge species inhabiting enclosed marine lakes and surrounding open coastal habitat in the islands of Kakaban and Maratua in the Berau Delta barrier reef system. The species selected were *Suberites diversicolor* (Becking and Lim, 2009) (Demospongiae: Hadromerida: Suberitidae) and *Cinachyrella australiensis* (Carter, 1886) (Demospongiae: Spirophorida: Tetillidae). These sponges were selected because they were relatively abundant inside and outside marine lakes. Specimens of both species were sampled in marine lakes located in the islands of Kakaban and Maratua in the Berau Delta barrier reef system, East Kalimantan, Indonesia (Fig. 2.1). In addition to this, we also sampled sponges from marine shallow open water habitat surrounding both islands.

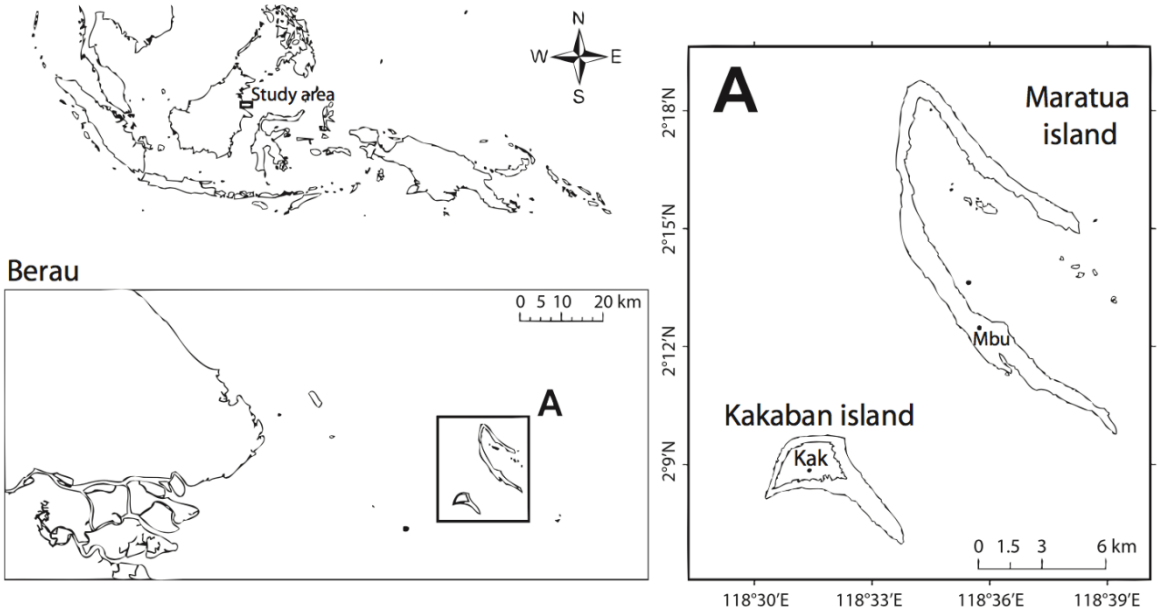


Fig. 2.1 – Map of study area showing Indonesia in the upper left inset. The bottom left and right insets show the location of the marine lakes sampled during this study: Kakaban lake (Kak) and Haji Buang lake (Mbu).

Our specific goals were to (1) compare bacterial richness between host species and among habitats; (2) identify the most abundant higher bacterial taxa; (3) assess to what extent host species and habitat (marine lake vs. open water) structure sponge bacterial composition; and (4) identify dominant (≥500 sequences) bacterial OTUs and their closest known relatives using BLAST and assess the relationships among taxa using phylogenetic analysis.

## Materials and methods

### Sampling

Sampling was performed by snorkelling and SCUBA diving from 10 August to 10 September 2008 inside and outside (Opn) the marine lakes of Berau, East Kalimantan Province, Indonesia (Fig. 2.1). The Berau Delta barrier reef system in East Kalimantan, Indonesia, is an intricate coastal system with a variety of coastal landforms and associated ecosystems such as coral reefs and mangroves. The offshore islands of the barrier reef system, Kakaban and Maratua, contain marine lakes. Kakaban lake (Kak), a large marine lake of c. 4 $km^2$ fringed by mangroves, is located in Kakaban island (N02°08′57.3″ E118°31′26.4″). Kakaban lake is one of the largest marine lakes presently known and strongly disconnected from the adjacent sea (Tomascik and Mah, 1994; Becking et al., 2011). Haji Buxang Lake (Mbu), a smaller marine lake of c. 0.14 $km^2$, is located in Maratua Island (N02°12′31.2″ E118°35′46.8″). A detailed description of the lakes of Kakaban and Maratua is provided by Tomascik and Mah (1994), Tomascik et al. (1997) and Becking et al. (2011).

Cores of the sponge species *C. australiensis* (Demospongiae: Spirophorida: Tetillidae) and *S. diversicolor* (Demospongiae: Hadromerida: Suberitidae) were sampled including segments of surface and interior to sample, as much as possible, the whole bacterial community; these were stored in 96% EtOH for microbial analysis. All specimens were collected from shallow water habitat (< 5 m depth). Specimens were identified to species by LE Becking and NJ de Voogd. Voucher specimens were preserved in 70% EtOH and deposited in the sponge collection of the Naturalis Biodiversity Center (RMNH Porifera). In the Berau region, *S. diversicolor* occurs predominantly in marine lakes, but can also be found

in sheltered areas outside the marine lakes. The species has also been found in Singapore and Northern Australia in sheltered habitats. The external coloration of *S. diversicolor* varies greatly between and within localities from green to red, while being bright to dark yellow internally. The variable external coloration is thought to be due to the presence of photosynthesising symbionts (Becking and Lim, 2009). *Cinachyrella australiensis* is a globular yellow sponge that occurs in a great variety of habitats across the Indo-Pacific. Both species tolerate and thrive in extreme or perturbed environments including marine lakes and intertidal areas subject to fluctuations in salinity, sediment loads and exposure to air during low tide (McDonald et al., 2002; Becking and Lim, 2009; de Voogd et al., 2009; Becking et al., 2013). Four specimens were collected per location; both species were collected from the marine lakes in Kakaban and Haji Buang, and four additional samples were collected from open waters surrounding the islands of Kakaban and Maratua (Fig. 2.1).

**DNA extraction and pyrosequencing**

Based on the work of Hardoim et al. (2009), genomic DNA was extracted using 0.5 g of tissue from each individual. After cutting the tissue samples into small slices, DNA was extracted with the FastDNA® Spin Kit for Soil (MP Biomedicals). To optimise DNA extraction, we followed the standard MP Biomedicals protocol. After DNA extraction, the community 16S rRNA gene was amplified for the V3V4 hypervariable region with barcoded fusion primers containing the Roche-454 A and B Titanium sequencing adapters, an eight-base barcode sequence in adaptor A and specific sequences for the ribosomal region.
Two replicate PCRs were performed for each sample using the primer pair V3F (5′–ACTCCTACGGGAGGCAG-3′) and V4R (5′– TACNVRRGTHTCTAATYC-3′) (Wang and Qian, 2009), 1X Advantage 2 Polymerase Mix (Clontech, Mountain View, CA), 1X Advantage 2 PCR Buffer, 0.2 µM of each PCR primer, 0.2 mM dNTPs (Bioron, Ludwigshafen am Rhein, Germany), 5% DMSO (Roche Diagnostics GmbH, Mannheim, Germany) and 2 µL of genomic DNA template in a total volume of 25 µL. The PCR conditions involved a 4-min denaturation at 94ºC, followed by 30 cycles of 94°C for 30 s, 44°C for 45 s and 68°C for 60 s and a final extension at 68°C for 10 min. Negative controls were included for all amplification reactions. Electrophoresis of duplicate PCR products was undertaken on a 1% (w/v) agarose gel, and the 470 bp amplified fragments were purified

using AMPure XP beads (Agencourt, Beckman Coulter) or, if more than the expected fragment was amplified, gel-purified using High-Pure PCR Product Purification Kit (Roche Diagnostics GmbH), according to manufacturer's instructions. The amplicons were quantified by fluorimetry with PicoGreen dsDNA quantitation kit (Invitrogen, Life Technologies, Carlsbad, CA), pooled at equimolar concentrations and sequenced in the A direction with GS 454 FLX Titanium chemistry, according to manufacturer's instructions (Roche, 454 Life Sciences, Branford, CT) at Biocant (Cantanhede, Portugal). The sequences generated in this study can be downloaded from the NCBI Short Read Archive, accession number: SRA049887.1.

**Sequence analyses of 16S rRNA gene fragments**

In this study, the barcoded pyrosequencing libraries were analysed using the Quantitative Insights Into Microbial Ecology (QIIME; Caporaso et al., 2010) software package (http://www.qiime.org/, last accessed 19 November 2012) on a computer running the BioLinux operating system (http://nebc.nerc.ac.uk/tools/bio-linux/bio-linux-6.0, last accessed 19 November 2012). In QIIME, fasta and qual files were used as input for the split_libraries.py script. Default arguments were used except for the minimum sequence length, which was set at 218 bp after removal of forward primers and barcodes, backward primers were removed using the 'truncate only' argument, and a sliding window test of quality scores was enabled with a value of 50 as suggested in the QIIME description for the script. In addition to user-defined cut-offs, the split_libraries.py script performs several quality-filtering steps (http:// qiime.org/scripts/split_libraries.html). OTUs were selected using the pick_otus.py script in QIIME with the usearch_ref method, default sequence similarity threshold of 0.97 and minimum cluster size of 1, and OTUs were selected using the most recent Greengenes release (Greengenes 12_10; http://qiime.wordpress.com/2012/10/ 16/greengenes-12_10-is-released/) as reference database. Reference-based OTU picking using the 12_10 release led to a large increase in the number of reads assigned to the reference database of soil and human microbiome data when compared to an earlier Greengenes release (release 4feb2011; http://qiime.wordpress.com/, last accessed 19 November 2012). The usearch sequence analysis tool (Edgar, 2010) implemented in QIIME provides clustering, chimera checking

and quality filtering on demultiplexed sequences. Chimera checking was performed using the UCHIME algorithm, which is the fastest and most sensitive chimera-checking algorithm currently available (Edgar et al., 2011). In the present study, we used de novo checking and reference-based chimera checking using a reference fasta file ('99_otus.fasta') from the Greengenes 12_10 release. The quality filtering as implemented in usearch filters noisy reads and preliminary results suggest it gives results comparable to other denoisers such as AmpliconNoise, but is much less computationally expensive (http://drive5.com/usearch/features.html, last accessed 19 November 2012). Representative sequences were selected using the pick_rep_set.py script in QIIME using the 'most_abundant' method. Reference sequences of OTUs were assigned taxonomies using default arguments in the assign_taxonomy.py script in QIIME with the rdp method (Wang et al., 2007). In the assign_taxonomy.py function, we used a fasta file containing reference sequences from the Greengenes 12_10 release as training sequences for the rdp classifier. We used a modified version of the taxonomy file supplied with the Greengenes 12_10 release to map sequences onto the assigned taxonomy. Finally, we used the make_otu_ table.py script in QIIME to generate a square matrix of OTUs by samples. This was subsequently used as input for further analyses using the R package (http://www. r-project.org/, last accessed 19 November 2012).

**Statistical analysis**

A square matrix containing the abundance of all OTUs per sample was imported into R using the read.table() function. Plant organelles, mitochondria and sequences not classified as Bacteria (e.g., Archaea) were removed prior to statistical analysis. Samples with <100 sequences were also removed prior to analysis. After importing into R, we used a self-written function (Gomes et al., 2010) to estimate total rarefied OTU richness for pooled samples belonging to each sponge host in each habitat.

The OTU abundance matrix was log10 (x + 1) transformed (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 and Gallagher, 2001; Cleary, 2003; Cleary and Genner, 2004; Cleary et al., 2004; Becking

et al., 2006; de Voogd et al., 2009). Variation in sponge composition among habitats (Opn, Kak and Mbu) and sponge hosts (*C. australiensis* and *S. diversicolor*) was assessed with principal coordinates analysis (PCO) using the cmdscale() function in R with the Bray–Curtis distance matrix as input. Variation among habitats and sponge hosts 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 habitat and sponge host as independent variables; the strata argument was set to habitat so that randomisations were constrained to occur within each habitat and not across all habitats. 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.

**Description of phylogenetic analysis**

Sequence identifiers of closely related taxa of numerically dominant OTUs (≥ 500 sequences) were downloaded using the NCBI Basic Local Alignment Search Tool (BLAST) command line 'BLASTN' tool with the -db argument set to nt (Zhang et al., 2000). BLAST identifies locally similar regions between sequences, compares sequences to extant databases and assesses the significance of matches; functional and evolutionary relationships can subsequently be inferred. Each run produces a list of hits based on significant similarity between pairs of sequences, that is, the target sequence and taxa present in the database (or no hits if no significantly similar sequences are found). A discussion of how significance is determined can be found at http://www.ncbi.nlm.nih.gov/BLAST/tutorial/Altschul-1.html. We used the BLASTN command line tool in a Linux environment to query representative sequences of selected taxa including all the dominant OTUs (≥ 500 sequences) against the online NCBI nucleotide database. We then generated a vector containing sequence identifiers (GIs) of the 10 top hits of all representative sequences and used the Entrez.efetch function in BioPython (Cock et al., 2009) with the rettype argument set to 'gb' to download GenBank information of afore- mentioned top hits including the isolation source of the organism and the host. From the list of hits, we selected taxa with the highest maximum sequence identity score (and total score) to our target representative sequences and included these in a phylogenetic analysis of the dominant OTUs. Fasta files of the closely related

organisms identified with BLAST were downloaded using the Entrez.efetch function with the rettype argument set to 'fasta'.

We constructed a phylogenetic tree including all dominant taxa (≥ 500 sequences), some additional taxa, for example the most dominant poribacterial OTU, and their closest relatives identified using BLAST as previously described. The phylogenetic tree was built using the MEGA5 program (http://www.megasoftware.net/, last accessed 20 November 2012; Tamura et al., 2011) with the neighbour-joining method (Saitou and Nei, 1987) based on the Kimura 2-parameter method (Kimura, 1980). In the results, we present a bootstrap consensus tree based on 1000 replicates (Felsenstein, 1985). Branches reproduced in < 50% of the bootstrap replicates are collapsed. The bootstrap value is shown next to each branch when this exceeds 50%. This value represents the percentage of replicate trees in which the associated taxa clustered together. In the results, the tree presented is drawn to scale; branch lengths are measured in the number of substitutions per site. Fifty-nine nucleotide sequences were involved in the analysis. Codon positions included were 1st + 2nd + 3rd + Noncoding. Positions with gaps and missing data were eliminated. There were a total of 1569 positions in the final data set.

**Results**

The sequencing effort yielded 53 683 sequences, which were assigned to 3064 OTUs after quality control, OTU picking and removal of chimera (Table S2.1). The assign taxonomy script, however, failed to assign 890 OTUs to the kingdom 'Bacteria'; 47 OTUs were identified as either chloroplasts or mitochondria. After removal of these OTUs, chloroplasts and mitochondria, our sequencing effort yielded 50 892 sequence reads and 2127 OTUs. OTUs were assigned to a total of 29 phyla. These included Proteobacteria (975 OTUs), Bacteroidetes (104), Actinobacteria (71), Firmicutes (60), Chloroflexi (52), Cyanobacteria (37), Acidobacteria (26), Gemmatimonadetes (17), Spirochaetes (13), Nitrospirae (8), ZB3 (7), Verrucomicrobia (7), Chlamydiae (5) and Poribacteria (4). 716 OTUs remained unclassified at the phylum level. 773 OTUs from 14 150 sequence reads were identified from *S. diversicolor* hosts, while 1504 OTUs from 36 742 sequence reads were identified from *C. australiensis* hosts. A total of 22 OTUs were dominant, that is, were represented by more

than 500 sequences. The most dominant OTU overall was OTU-1733, an Alphabacterium only found in *S. diversicolor* hosts and represented by 5631 sequences. As can be seen in the heat map (Fig. 2.2), the distribution of dominant OTUs suggests pronounced clustering. Taxa were largely restricted to *S. diversicolor* hosts and *C. australiensis* hosts in open vs. lake habitat. Only one OTU was found in every sponge, OTU-218.

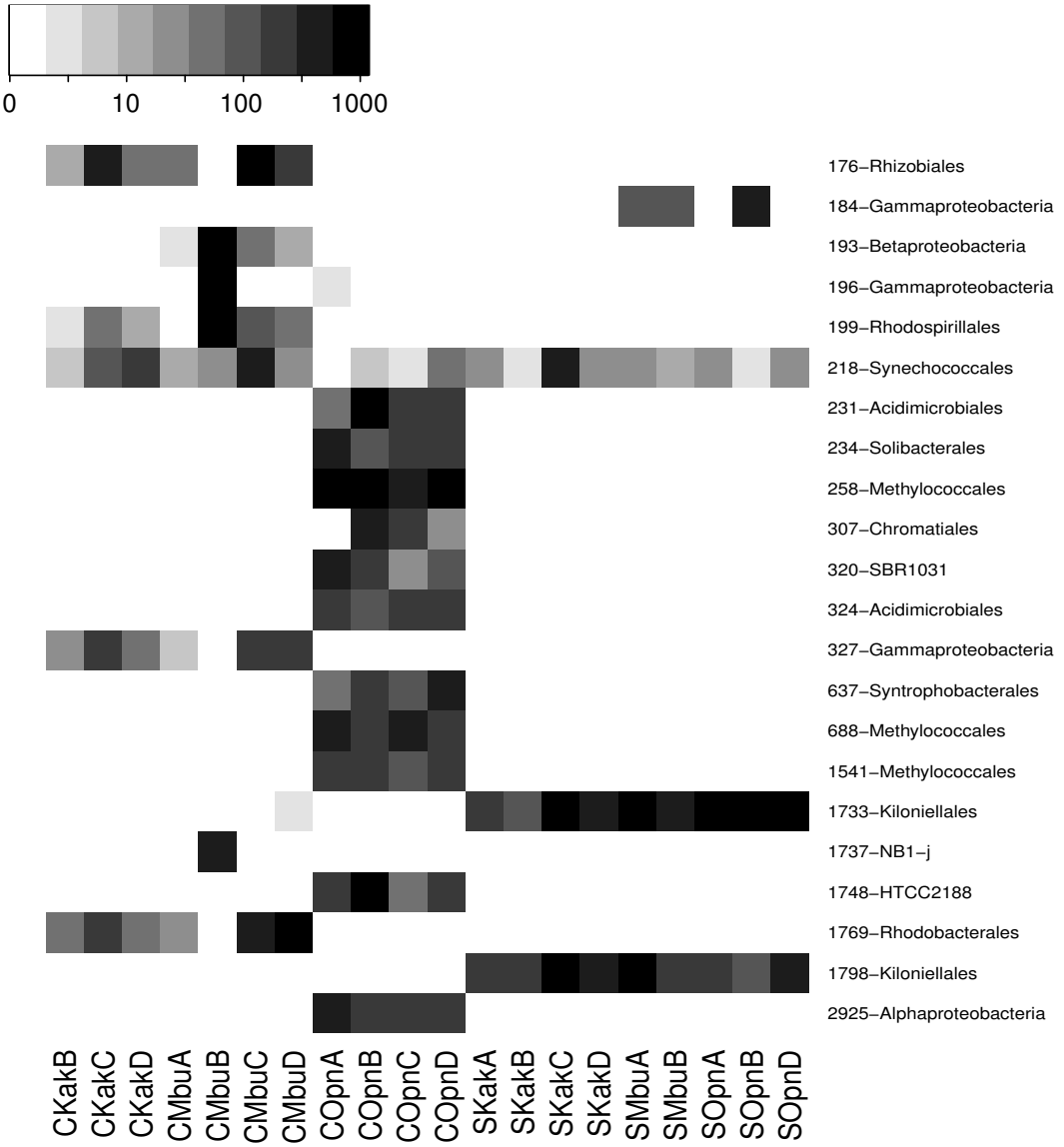


Fig. 2.2 – Heat map showing the abundance of 16S rRNA gene sequence reads of bacterial OTUs with ≥ 500 sequence reads. All samples are indicated along the x-axis: CKak: *Cinachyrella australiensis* in Kakaban, CMbu: *C. australiensis* in Haji Buang lake, Maratua, COpn: *C. australiensis* in open habitat, SKak: *Suberites diversicolor* in lake Kakaban, SMbu: *S. diversicolor* in Haji Buang lake, Maratua, SOpn: *S. diversicolor* in open habitat. OTUs are indicated along the y-axis by numbers and the lowest taxonomic classification by QIIME. The abundance of each OTU is indicated by colours ranging from black (low abundance or absent) to white (high abundance). The scale bar indicates abundance using a logarithmic base-10 scale.

**OTU richness**

Rarefied bacterial OTU richness was highest in *C. australiensis* and *S. diversicolor* hosts in Haji Buang lake, Maratua, intermediate in open water habitat and lowest in Kakaban lake. In each habitat, *C. australiensis* harboured more diverse bacterial assemblages than *S. diversicolor* (Fig. 2.3). Despite this, habitat appears to be a better predictor of bacterial richness than host. OTU richness approached 800 OTUs when more than 20 000 sequences were sampled for the most abundant host–habitat combination, namely *C. australiensis* hosts in open habitat. There was no evidence of an asymptote for any host–habitat combination, which indicates that true richness is higher than that reported here.

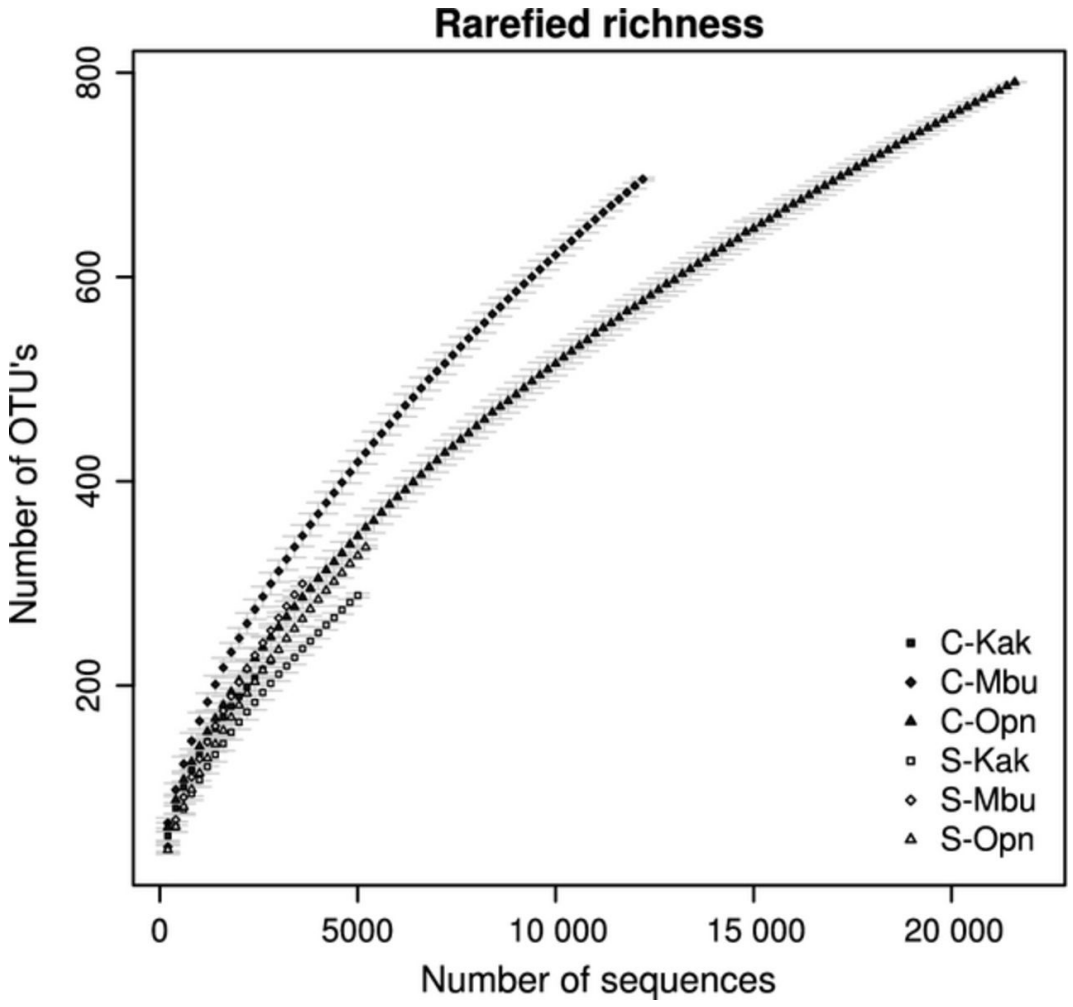


Fig. 2.3 - Species accumulation curves as a function of the number of sequences using resampling of bacterial 16S rRNA gene sequences from *Suberites diversicolor* and *Cinachyrella australiensis* hosts in lake Kakaban (Kak), Haji Buang lake Maratua (Mbu) and open habitat (Opn). Samples are pooled per treatment.

**Higher taxon abundance**

There were marked differences in the abundance of higher bacterial taxa between host species and among habitats (Fig. 2.4). The Acidimicrobidae, Gammaproteobacteria and Deltaproteobacteria were markedly more abundant in *C. australiensis* than in *S. diversicolor* hosts. In contrast, Alphaproteobacteria were more abundant in *S. diversicolor* than in *C. australiensis* hosts. This was, however, largely due to the dominance of taxa belonging to the Kiloniellales order in *S. diversicolor* hosts. Alphaproteobacteria in *C. australiensis* hosts were more abundant in lake habitat than in open habitat. Taxa belonging to the Anaerolineae and Methylococcales were largely restricted to *C. australiensis* hosts in open habitat. Of the 1061 sequences identified as Anaerolineae, only three were not found in *C. australiensis* hosts in open habitat. Of the 6402 sequences identified as Methylococcales, only two (both from a single specimen of *C. australiensis* in Haji Buang Lake) were not found in *C. australiensis* hosts in open habitat. Betaproteobacteria were largely restricted to *C. australiensis* hosts in lake habitat. Of the 1149 sequences identified as Betaproteobacteria, only two (both from a single specimen of *S. diversicolor* in open habitat) were not found in *C. australiensis* hosts in lake habitat. Likewise, taxa belonging to the Rhizobiales and Rhodobacterales were much more abundant in *C. australiensis* hosts in lake habitat than in open habitat or *S. diversicolor* hosts in all habitats. Taxa belonging to the Synechococcophycideae were found in all host–habitat combinations but reached their greatest abundance in both sponge hosts from Kakaban lake. Finally, dominance as indicated by the relative abundance of the most dominant OTU in each sponge was higher in *S. diversicolor* than in *C. australiensis* hosts (Fig. 2.4). In samples from *C. australiensis* hosts, the average abundance of the most dominant OTU varied from 12.5% to 34.6% in all habitats; these OTUs included OTU-258 in open habitat and OTUs 176, 1769, 193 and 218 in lake habitats. In samples from *S. diversicolor* hosts, average abundance of the dominant OTU varied from 22.4% to 56.1% in all habitats with the dominant OTU invariably OTU-1733 or OTU-1798.

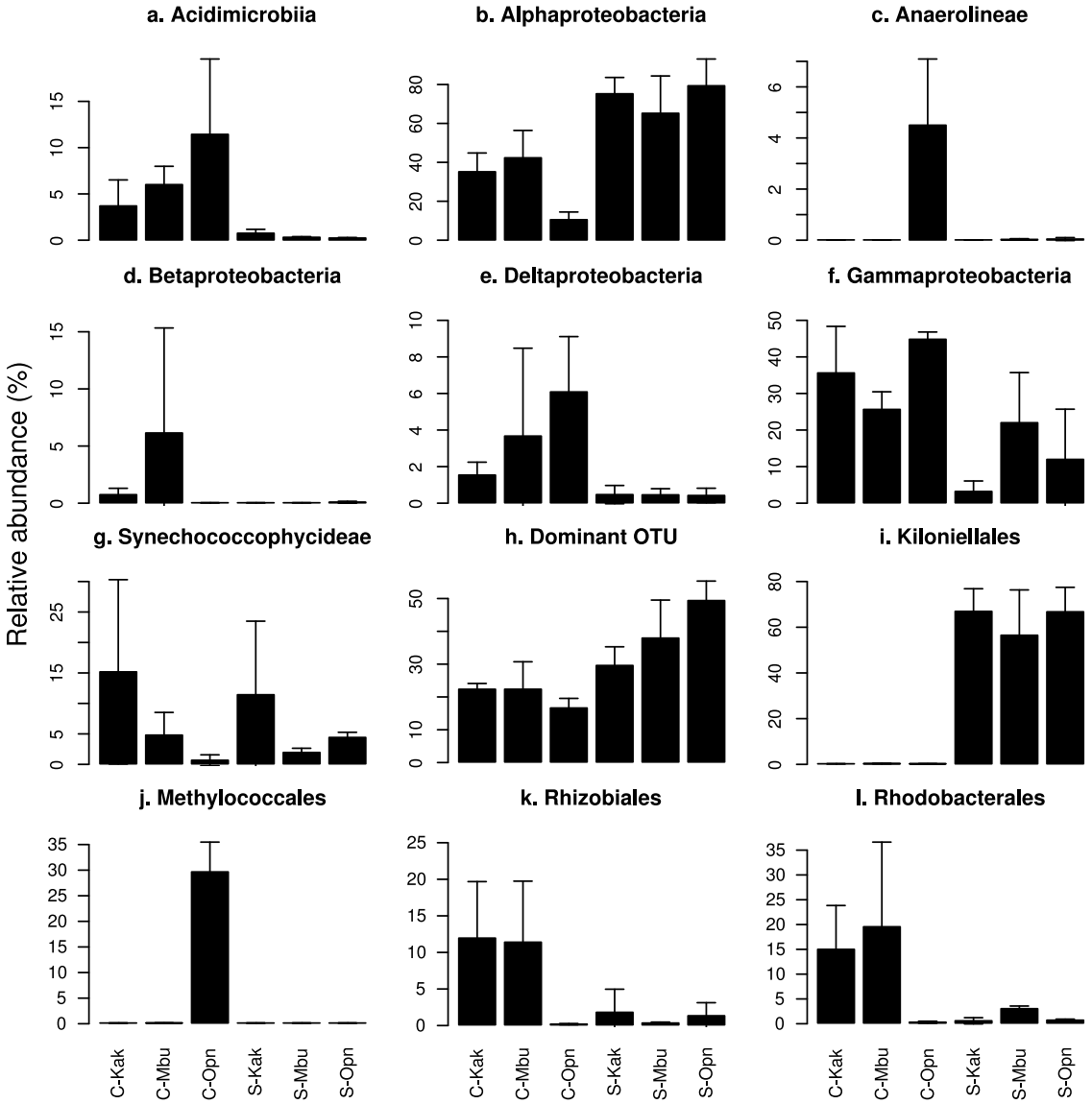


Fig. 2.4 – Relative abundance of the most abundant bacterial classes and the dominant OTU for samples from *Suberites diversicolor* and *Cinachyrella australiensis* hosts in lake Kakaban (Kak), Haji Buang lake Maratua (Mbu) and open habitat (Opn). Error bars represent a single standard deviation. The dominant OTU represents the mean abundance for the single most abundant OTU in each sample, thus not necessarily the same OTU.

## Importance of host and habitat in structuring composition

There was a highly significant difference in bacterial composition among sponge hosts ($F_{1,14}$ = 7.66, P < 0.001, $R^2$ = 0.225), habitat ($F_{2,14}$ = 3.51, P < 0.001, $R^2$ = 0.206) and a significant interaction between host species and habitat ($F_{2,14}$ = 2.70, P < 0.001, $R^2$ = 0.158). Together, host and habitat explained almost 59% of the variation in bacterial composition. A PCO

ordination of the first two axes is presented in Fig. 2.5. There are three distinct clusters: (1) samples from *C. australiensis* hosts in open water habitat; (2) samples from *S. diversicolor* hosts in open water and lake habitats; and (3) samples from *C. australiensis* hosts in lake habitats. Axis 1 separates samples from *C. australiensis* hosts in open water habitat from *S. diversicolor* hosts in open water and lake habitats and *C. australiensis* hosts in lake habitats. Axis 2 separates samples from *C. australiensis* hosts and *S. diversicolor* hosts. 80.6– 86.6% of OTUs were restricted to these clusters (Fig. 2.6; *S. diversicolor*: 80.6%, i.e. 623 of 773 OTUs; *C. australiensis* lakes: 81.3%; *C. australiensis* open: 86.6%). Less than 2% of OTUs were found in all three clusters (37 of 2127). *Suberites diversicolor* shared 14.2% of OTUs (110 of 773) with *C. australiensis* in lake habitat and 10.0% (77 of 773) with *C. australiensis* in open habitat. *Cinachyrella australiensis* in open habitat shared 9.4% of OTUs (74 of 791) with *C. australiensis* in lake habitat.

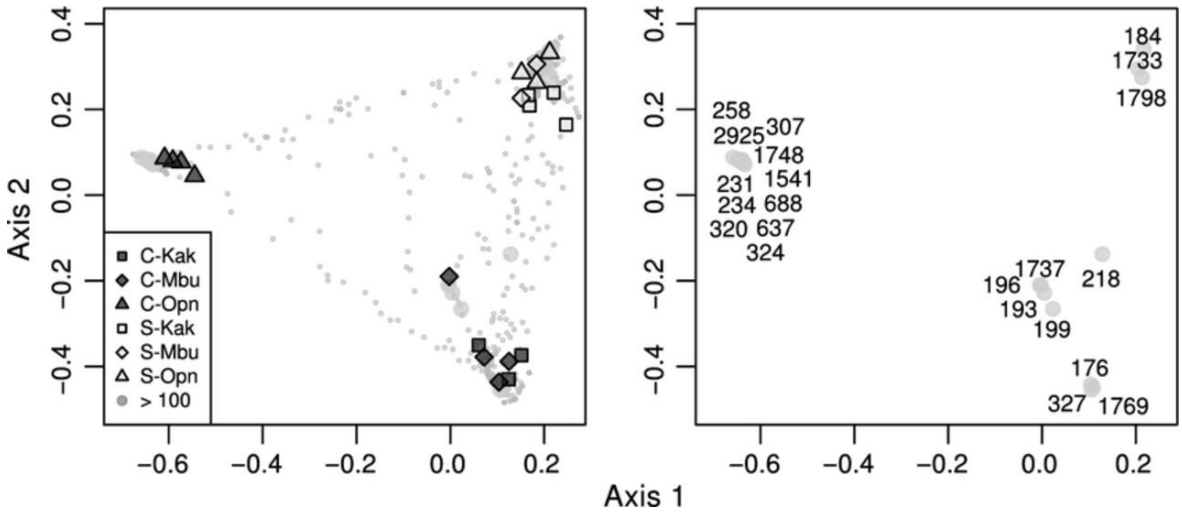


Fig. 2.5 - Ordination showing the first two axes of the PCO analysis. Symbols represent host–habitat combinations for *Suberites diversicolor* and *Cinachyrella australiensis* hosts in lake Kakaban (Kak), Haji Buang lake Maratua (Mbu) and open habitat. Numbers represent dominant (≥ 500 sequence reads) OTUs. Very small circles represent OTUs < 500 sequence reads.

There does not appear to be a pronounced difference in composition among samples from *S. diversicolor* in lake or open water habitats. *Suberites diversicolor* hosted three dominant OTUs (184, 1733 and 1798). OTU-184 was identified as a Gammaproteobacterium, and its closest relatives included an organism isolated from an oceanic dead zone environment and

mussel gill tissue, but with maximum identities of only c. 93% (Table 2.1). OTUs 1733 and 1798 were both identified as belonging to the recently described alphaproteobacterial order Kiloniellales. *Suberites diversicolor* in fact hosted three closely related and abundant alphaproteobacterial taxa (OTUs 1733, 1798 and 1850). Together, these three OTUs made up 63.4% of the total bacterial community.

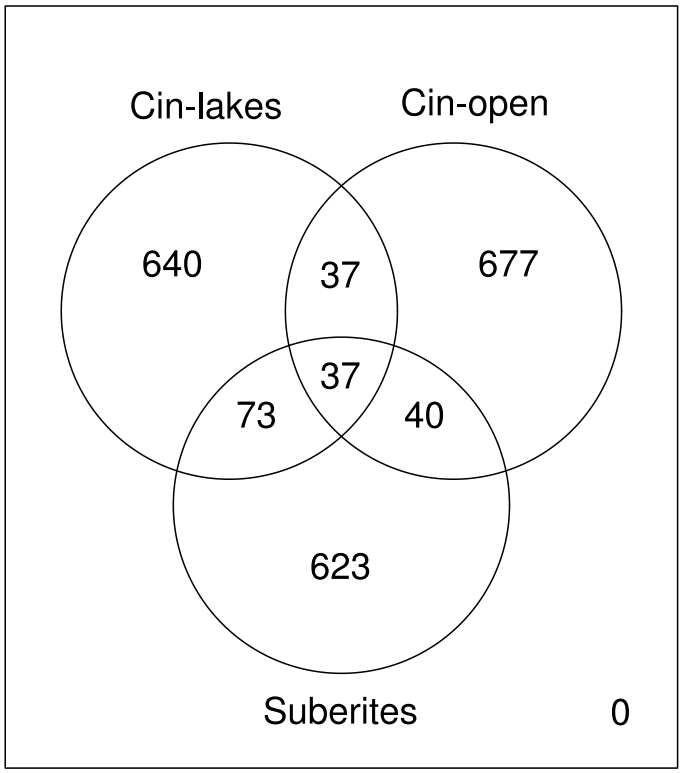


Fig. 2.6 – Venn diagram showing the number of OTUs restricted to given host–habitat combinations, namely *Suberites diversicolor* hosts in open and lake habitat, *Cinachyrella australiensis* hosts in lake habitat (Cin-Lak) and *C. australiensis* hosts in open habitat (Cin-Opn). Overlapping circles indicate shared OTUs.

All three of these taxa clustered together with organisms isolated from sandy reef sediment and two sponges (*Hymeniacidon heliophila* and *Halichondria* sp.; Fig. 2.7). They form a strongly supported (bootstrap value = 100) cluster and are distinct from other (putative) members of the Kiloniellales including the type species *Kiloniella laminariae* and may represent a novel family (or families) within the order.

In contrast to samples obtained from *S. diversicolor* hosts, there was a pronounced difference in bacterial composition of samples obtained from *C. australiensis* hosts in open vs. lake

habitat, which would explain the significant interaction in the adonis analysis. *Cinachyrella australiensis* sponges in lake habitat thus host unique bacterial assemblages compared with sponges in open habitat. Most of the dominant Alpha- and Betaproteobacteria were restricted to lake habitat. OTUs 199 and 1769 are related to organisms recently isolated from sponges in the Great Barrier Reef. The closest known relative of OTU-193, a Betaproteobacterium, was isolated from the sponge *Xestospongia muta* in Florida, but the sequence identity was only 91% (Table 2.1). The only dominant Alphaproteobacterium isolated from *C. australiensis* sponges in open habitat was OTU-2925, whose closest relative was isolated from carbonate sediments in the SouthWest Indian Ridge. Dominant OTUs from *C. australiensis* identified as belonging to the Gamma- or Deltaproteobacteria were all closely related to organisms isolated from sponges. Methylococcales taxa were very closely related (sequence identities > 99%) to organisms isolated from sponges in the Great Barrier Reef and Florida. Interestingly, two OTUs from lake habitat (196 and 327) form a distinct cluster, but this cluster clusters together with that of the Methylococcales taxa and is distinct from other Gammaproteobacteria. All other OTUs identified as belonging to other phyla were closely related to taxa isolated from sponges with the exception of OTU- 218 (Fig. 2.7). OTU-218 was the only OTU found in all sponge specimens and was identified as belonging to the family Synechococcaceae (Table 2.1). It was very closely related (maximum identity = 100%) to an organism isolated from seawater in the Mediterranean Sea. Only four OTUs were identified as belonging to the proposed phylum Poribacteria, and these were all only found in *C. australiensis* hosts in open habitat. The closest relative (sequence identity = 93.72%) of the most abundant of these (OTU-226) was an organism isolated from the sponge *Ircinia variabilis* in the Mediterranean Sea.

Table 2.1 – List of abundant OTUs and closely related organisms identified using BLAST search.

| OTU | Sum | Host-habitat | Class | Order | Family | GI | Sq ident | Source | Location | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| 1733 | 5631 | *Suberites* | Alphaproteobacteria | Kiloniellales | Unclassified | 226880021 | 96,31 | marine reef sandy sediment | Hawaii: Oahu, Kilo Nalu reef | Gao et al. 2011 |
| 1733 | 5631 | | | | | 335060676 | 94,33 | sponge *Hymeniacidon heliophila* | USA: Alabama, Dauphin Island | Erwin et al. 2011 |
| 258 | 3285 | *Cinachyrella* open | Gammaproteobacteria | Methylococcales | Methylococcaceae | 345330439 | 99,53 | sponge *Rhopaloeides odorabile* | Australia: Rib Reef, GBR | Webster et al. 2011 |
| 1798 | 2895 | *Suberites* | Alphaproteobacteria | Kiloniellales | Unclassified | 226880021 | 96,31 | marine reef sandy sediment | Hawaii: Oahu, Kilo Nalu reef | Gao et al. 2011 |
| 176 | 1528 | *Cinachyrella* lakes | Alphaproteobacteria | Rhizobiales | Hyphomicrobiaceae | 148732264 | 98,77 | site S25 near Coco's Island | Costa Rica | Rojas-Jimenez et al. Unpub |
| 1769 | 1496 | *Cinachyrella* lakes | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | 400269119 | 95,54 | sponge *Coelocarteria singaporensis* | Australia: Orpheus Island, GBR | Webster et al. Unpub |
| 231 | 1476 | *Cinachyrella* open | Acidimicrobiia | Acidimicrobiales | wb1_P06 | 82470225 | 98,52 | sponge *Corticium candelabrum* | Palau | Sharp et al. 2007 |
| 688 | 1246 | *Cinachyrella* open | Gammaproteobacteria | Methylococcales | Methylococcaceae | 126033048 | 99,07 | sponge *Agelas dilatata* | Bahamas: Little San Salvador Island | Taylor et al. 2007 |
| 218 | 1192 | Ubiquitous | Synechococcophycideae | Synechococcales | Synechococcaceae | 407728972 | 100 | seawater | Spain: Catalunya | Erwin et al. 2012 |
| 2925 | 1086 | *Cinachyrella* open | Alphaproteobacteria | Unclassified | Unclassified | 364524658 | 97,78 | carbonate sediments | South West Indian Ridge | Li et al. Unpub |
| 193 | 1052 | *Cinachyrella* lakes | Betaproteobacteria | Unclassified | Unclassified | 134290589 | 90,99 | sponge *Xestospongia muta* | USA: Key Largo, FL | Schmitt et al. 2008 |
| 1748 | 1017 | *Cinachyrella* open | Gammaproteobacteria | HTCC2188 | HTCC2089 | 110265023 | 96,28 | sponge larva *Ircinia felix* | USA: Key Largo, FL | Schmitt et al. 2007 |
| 234 | 830 | *Cinachyrella* open | Solibacteres | Solibacterales | Solibacteraceae | 400269018 | 99,26 | sponge *Cymbastella coralliophila* | Australia: Orpheus Island, GBR | Webster et al. Unpub |
| 199 | 821 | *Cinachyrella* lakes | Alphaproteobacteria | Rhodospirillales | Rhodospirillaceae | 400269153 | 99,26 | sponge *Cinachyra* sp. | Australia: Orpheus Island, GBR | Webster et al. Unpub |
| 1541 | 786 | *Cinachyrella* open | Gammaproteobacteria | Methylococcales | Methylococcaceae | 126033057 | 100 | sponge *Agelas dilatata* | Bahamas: Little San Salvador Island | Taylor et al. 2007 |
| 327 | 668 | *Cinachyrella* lakes | Gammaproteobacteria | Unclassified | Unclassified | 110265053 | 96,77 | sponge larva *Ircinia felix* | USA: Key Largo, FL | Schmitt et al. 2007 |
| 324 | 652 | *Cinachyrella* open | Acidimicrobiia | Acidimicrobiales | wb1_P06 | 158342512 | 98,28 | sponge *Rhopaloeides odorabile* | Australia: Pelorus island | Webster et al. 2008 |
| 196 | 636 | *Cinachyrella* lakes | Gammaproteobacteria | Unclassified | Unclassified | 110265053 | 95,58 | sponge larva *Ircinia felix* | USA: Key Largo, FL | Schmitt et al. 2007 |
| 320 | 602 | *Cinachyrella* open | Anaerolineae | SBR1031 | A4b | 126033013 | 97,54 | sponge *Agelas dilatata* | Bahamas: Little San Salvador Island | Taylor et al. 2007 |
| 307 | 563 | *Cinachyrella* open | Gammaproteobacteria | Chromatiales | Unclassified | 110265084 | 96,28 | sponge larva *Ircinia felix* | USA: Key Largo, FL | Schmitt et al. 2007 |
| 184 | 552 | *Suberites* | Gammaproteobacteria | Unclassified | Unclassified | 260069446 | 93,02 | Saanich Inlet, 120 m depth | 48.5883 N 123.5037 W | Walsh et al. 2009 |
| 184 | 536 | | | | | 187473670 | 92,79 | *Bathymodiolus* sp. mussel gill tissue | Papua New Guinea: Manus basin | Won et al. 2008 |
| 1737 | 512 | *Cinachyrella* lakes | Deltaproteobacteria | NB1-j | Unclassified | 400269180 | 99,07 | sponge *Cinachyra* sp. | Australia: Orpheus Island, GBR | Webster et al. Unpub |
| 637 | 666 | *Cinachyrella* open | Deltaproteobacteria | Syntrophobacterales | Syntrophobacteraceae | 326372184 | 97,91 | Zhongyuan oil field | China | Wu et al. Unpub |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 637 | 666 | | | | | 379771488 | 96,75 | sponge *Geodia barretti* | Norway | Radax et al. 2012 |
| 203 | 257 | *Cinachyrella* open | Alphaproteobacteria | Rhodospirillales | Rhodospirillaceae | 22797718 | 98,52 | sponge *Aplysina aerophoba* | Mediterranean: Banyuls sur Mer | Hentschel et al. 2002 |
| 174 | 225 | *Cinachyrella* open | Nitrospira | Nitrospirales | Nitrospiraceae | 210161954 | 99,05 | sponge *Corallistes* sp. | ? | Lopez et al. Unpub |
| 187 | 158 | *Cinachyrella* lakes | Nitrospira | Nitrospirales | Nitrospiraceae | 62944578 | 99,05 | sponge *Cymbastela concentrica* | Australia | Taylor et al. 2005 |
| 226 | 87 | *Cinachyrella* open | Unclassified | Unclassified | Unclassified | 407728880 | 93,72 | sponge *Ircinia variabilis* | Spain: Catalunya | Erwin et al. 2012 |
| 1850 | 456 | *Suberites* | Alphaproteobacteria | Kiloniellales | Unclassified | 226880021 | 98,28 | marine reef sandy sediment | Hawaii: Oahu, Kilo Nalu reef | Gao et al. 2011 |
| 1850 | 456 | | | | | 335060688 | 95,07 | sponge *Halichondria* sp. | USA: Alabama, Dauphin Island | Erwin et al. 2011 |
| 1850 | 456 | | | | | 343202360 | 89 | marine alga *Saccharina latissima* | Germany: Baltic Sea | Wiese et al. 2009 |
| 1850 | 456 | | | | | 74136944 | 89 | mature marine biofilm | ? | Kwon et al. unpub |
| 1850 | 456 | | | | | 158537152 | 86 | oil-polluted saline soil | China: Xianhe, Shangdong | Zhao et al. 2010 |

OTU number of dominant OTUs and selected other OTUs; GI, GenInfo sequence identifier of closest related organisms identified using BLAST; Sq ident, sequence identity; Sq len, length of representative sequence used to construct phylogenetic tree; Source, isolation source; Location, location where organism was sampled; ?, No location.

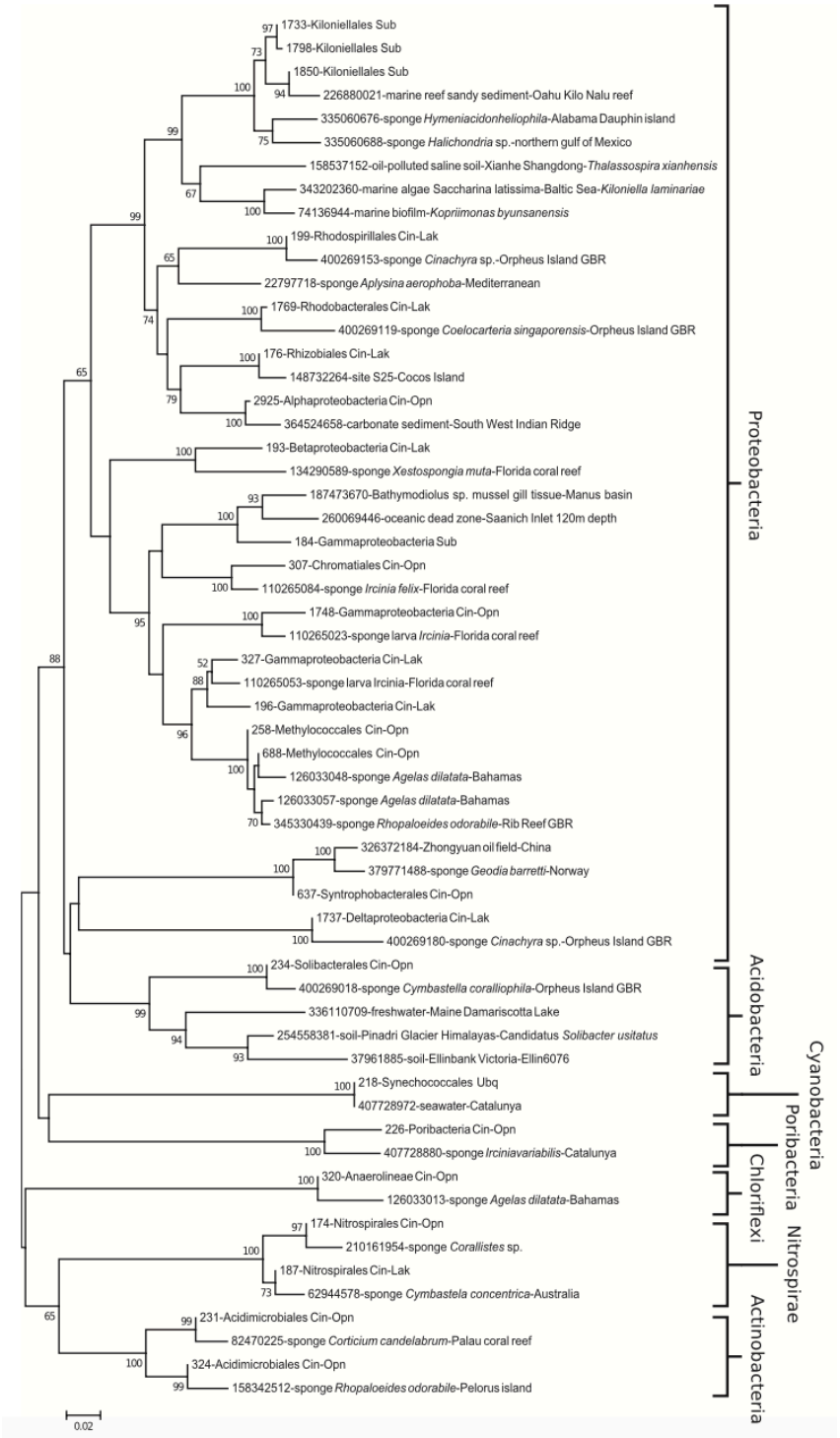


Fig. 2.7 – Phylogenetic tree of the bacterial 16S rRNA gene sequences recovered from *Suberites diversicolor* and *Cinachyrella australiensis* hosts in lake Kakaban, Haji Buang lake, Maratua and open habitat; bootstrap values lower than 50% were omitted. The number of each OTU is indicated as are GenInfo sequence identifiers of sequences obtained using BLAST. Classes of bacteria are indicated. OTUs are assigned to the following clusters: Sub: found in *S. diversicolor* hosts, Cin-Opn: found in *C. australiensis* hosts in open habitat, Cin-Lak: found in *C. australiensis* hosts in lake habitat, Ubq: ubiquitous, found in all host individuals. For organisms found using BLAST, we include the host and/or habitat from which the organism was isolated as well as the geographical locality where the organism was isolated.

## Discussion

In our study, Proteobacteria dominated both sponge species. In contrast to Schmitt et al. (2012b), where Poribacteria were relatively abundant and present in a variety of sponge species, they were only a minor component of the bacterial flora in this study and were, furthermore, restricted to *C. australiensis* hosts in open habitat. A large number of OTUs remained unassigned at phylum level in the present study. This high number of unassigned sequences is similar to those reported by White et al. (2012), a pyrosequencing study of bacterial symbionts within *Axinella corrugata* sponges; in their study, 36% of 16S rRNA gene fragments were unassigned OTUs. In addition to the above, we recorded a large number of OTUs that were unassigned to kingdom, even after quality control and removal of chimera. These included some moderately abundant OTUs. We did not, however, include these OTUs in subsequent analyses, but their presence is noteworthy.

With respect to bacterial OTU richness, there appeared to be both a habitat and species effect. OTU richness was highest in Haji Buang lake, Maratua, intermediate in open habitat and lowest in Kakaban. In each habitat, however, *C. australiensis* hosted more OTUs than *S. diversicolor*. None of the rarefaction curves, though, approached an asymptote indicating that true richness is higher. Other studies of microbial communities of sponges also failed to sample till saturation (Webster et al., 2010; Lee et al., 2011; Jackson et al., 2012; Schmitt et al., 2012b; White et al., 2012).

There were marked differences in the abundance of bacterial classes among sponge hosts and habitats. These results are in accordance with Lee et al. (2011). They reported pronounced differences in the abundance of bacterial phyla in different sponge species (*Hyrtios erectus*, *Stylissa carteri*, *Xestospongia testudinaria*) from different locations. Likewise, Erwin et al. (2011) reported differences in the relative abundances and presence/absence of OTUs between the sponges *Hymeniacidon heliophila* and *Haliclona tubifera*.

Both sponge species exhibited pronounced dominance with respect to the relative abundance of single taxa in a given sponge individual. This was most pronounced with *S. diversicolor* in open habitats where, on average, close to 50% of the sponge community was dominated by a single OTU. In comparison, the mean relative abundance of the dominant OTU in mangrove rhizosphere and sediment in Brazil was < 2% for all microhabitats (Gomes et al.,

2010). The only study we know of where dominance approached the level reported in this study was another sponge study by Webster et al. (2010). In most of their samples, the bulk of bacterial diversity consisted of rare OTUs with only one or a few tags with a relatively low proportion of highly abundant taxa. The sponge *Lanthella basta*, however, exhibited pronounced dominance.

Most of the OTUs identified in this study were restricted to a single host (*S. diversicolor* vs. *C. australiensis*) or a single habitat (lake vs. surrounding water). Only 37 OTUs (of 2127, thus, 1.7% of OTUs) were found in both host species in open and lake habitat. The only OTU found in all samples was assigned to the family Synechococcaceae. This sequence had 100% sequence similarity to a number of organisms isolated from seawater and various sponge species including *Crella cyathophora* and *S. carteri* (Giles et al., 2013) in Saudi Arabian waters and a large number of cultured organism identified as *Synechococcus* spp. including an organism isolated from the Sargasso Sea (Ahlgren and Rocap, 2012). *Synechococcus* is a widespread Cyanobacterium that can be very abundant in the marine euphotic zone. It is also an important component of the autotrophic plankton community (Waterbury et al., 1979).

Most of the dominant OTUs identified during this study were closely related to organisms isolated from sponges, also called Plus-OTUs by other authors as opposed to Minus-OTUs, which are OTUs assigned to a non-sponge-derived sequence (Schmitt et al., 2012a, b). Minus-OTUs in the present study included OTU-1733, whose closest relative was isolated from marine reef sandy sediment, OTU-2925, whose closest relative was isolated from carbonate sediment, OTU-184, whose closest relative was isolated from an oceanic dead zone, and OTU-637, whose closest relative was isolated from an oil field. Given the relatively low sequence similarities (Table 2.1), however, it is likely that closer, possibly sponge-derived, relatives will be found in the future.

The pronounced dominance of Kiloniellales OTUs in *S. diversicolor* hosts is intriguing. Three OTUs identified as belonging to the Kiloniellales made up more than 63% of the total bacterial community in *S. diversicolor*. Previous studies have shown that *Suberites* species and their endosymbiotic bacteria produce strong antimicrobial compounds suggesting that the sponge host is a strongly selective environment for bacteria (Thakur et al., 2003; Wiens et al., 2011; Flemer et al., 2012). In addition to this, the type species of the order

Kiloniellales, *K. laminariae*, was first isolated by selecting active antibiotic producers on agar plates (Wiese et al., 2009).

*Kiloniella laminariae* is a mesophilic, chemoheterotrophic aerobe with the potential for denitrification and exhibits a typical marine growth response. In its natural environment, it was found in association with the brown alga *Laminaria saccharina*. Although the dominant OTUs inhabiting *S. diversicolor* were identified as Kiloniellales, sequence similarity values were below 90% to other (putative) members of the Kiloniellales including the type species *K. laminariae*. They did, however, cluster together with these species forming a well-supported cluster in our phylogenetic analysis. The results of the BLAST analysis and our phylogenetic tree, however, indicate the existence of a distinct cluster within the Kiloniellales, possibly a new family (or families). This cluster includes organisms isolated from the sponges *H. heliophila* and *Halichondria* sp. in Alabama and an organism isolated from sandy reef sediment in Hawaii (Erwin et al., 2011; Gao et al., 2011). The sheer dominance of the three Kiloniellales OTUs in *S. diversicolor* thus indicates that they are well adapted to any antimicrobial substances produced by the host sponge and/or produce strong antimicrobial substances themselves. Either way, the sponge *S. diversicolor* is probably an interesting candidate to explore for novel bioactive compounds, particularly in relation to antimicrobial activity.

In *C. australiensis* hosts, we recorded almost twice as many OTUs (1504 vs. 773 in *S. diversicolor*) and less pronounced dominance. The most abundant OTU (258) was assigned to the family Methylococcaceae. Despite being assigned to the Methylococcaceae, its closest relative (99% sequence similarity) was identified as a *Nitrosococcus* species (order: Chromatiales) isolated from the sponge *Rhopaloeides odorabile* in the Great Barrier Reef (Webster et al., 2011). In a phylogenetic study of the Gammaproteobacteria, Gao et al. (2009) noted that species from the Thiotrichales, Cardiobacteriales, Legionellales, Chromatiales, Methylococcales and Xanthomonadales revealed deeper branching in trees and unresolved relative branching positions. In the tree, they present in Fig. 1 of their article, Chromatiales and Methylococcales taxa clustered together. Likewise, Cutiño-Jiménez et al. (2010) noted that several distinctive insertions found in most gammaproteobacterial orders were absent from groups including the Xanthomonadales, Legionellales, Chromatiales, Methylococcales, Thiotrichales and Cardiobacteriales.

Other dominant taxa in *C. australiensis* hosts included OTU-176 (family: Hyphomicrobiaceae) and OTU-1769 (family: Rhodobacteraceae), both restricted to lake habitat. The Hyphomicrobiaceae are ubiquitous in terrestrial and freshwater habitats, but only a few have been recorded from marine environments (Huo et al., 2012). Species belonging to the Hyphomicrobiaceae have been identified as important methylotrophic denitrifiers (Liessens et al., 1993; Osaka et al., 2006). OTU-1769 was an Alphaproteobacterium with 93% sequence similarity to an organism identified as *Rhodovulum imhoffii* isolated from a marine aquaculture pond (Srinivas et al., 2007). The dominance of OTUs related to taxa known to be involved in nutrient cycling, namely nitrogen fixation (*Synechococcus*), ammonia oxidation (*Nitrosococcus*), denitrification (Hyphomicrobiaceae), possible denitrification (Kiloniellales) and sulphur oxidation (*Rhodovulum*), suggests that sponges in this study may play an important role in reef and lake nutrient dynamics. In Floridian reefs, Southwell et al. (2008) demonstrated that the majority of benthic nitrification occurred within sponges and that sponge composition and abundance probably had a strong influence on the concentration and speciation of dissolved inorganic nitrogen in the reef water column. Increased nitrate concentrations near the benthos may in turn affect coral reef community structure. Sponges are found in much greater densities in marine lakes than in either adjacent coral reefs or mangroves (Becking et al., 2013). They often completely cover the roots of mangrove trees that fringe the marine lakes. Given the generally much higher densities of bacteria in sponges than in the surrounding water, sponges may play a crucial role in nutrient dynamics within the lake ecosystem.

**Importance of host and habitat in structuring composition**

In the present study, we have demonstrated the importance of both host species and habitat in structuring bacterial composition. Almost 59% of the variation in composition was explained by the combination of both factors. We were able to discern three distinct clusters in the PCO ordination: (1) a cluster representing assemblages hosted in *S. diversicolor*; (2) a cluster representing assemblages hosted in *C. australiensis* sponges sampled in open water; and (3) a cluster representing assemblages hosted in *C. australiensis* sponges sampled in lake habitat. The ordination confirms the findings of the heat map and demonstrates that *C.*

*australiensis* hosts very different bacterial communities inside and outside marine lake habitat. That this is not the case with *S. diversicolor* may be due to pronounced selective pressure of the sponge on its microbial community, possibly through the production of antimicrobial proteins or antimicrobial activity of the microorganisms themselves. In addition to this, the main populations of *S. diversicolor* are found within lake habitat in contrast to *C. australiensis*, which maintains large and extensive populations outside the lakes.

Our study provides the first assessment of bacterial communities inhabiting sponges in marine lakes. Bacterial composition also differed strongly between sponge host species. The widespread *C. australiensis* showed very pronounced variation in composition between lake and open water habitat, whereas the recently described *S. diversicolor* showed very little difference. Much yet remains to be studied in marine lake environments including bacteria and other microorganisms such as Archaea in other sponge species, other organisms, water and sediment. Given the unique nature of the marine lakes, it is probable that sponges and other lake organisms host unique bacteria with potentially valuable pharmaceutical and/or biotechnological properties.

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**Supporting Information**

Habitat- and host-related variation in sponge bacterial symbiont communities in Indonesian waters

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Table S2.1 – Total abundance (Sum) of each OTU, abundance of each OTUs per sample of all OTUs and the taxonomic assignment of each OTU.

| Kingdom | Phylum | Class | Cin Lak sqs | Cin Opn sqs | Sub Sqs | Cin Lak OTUs | Cin Opn OTUs | Sub OTUs |
|---|---|---|---|---|---|---|---|---|
| Archaea | Unclassified | Unclassified | 1 | 0 | 3 | 1 | 0 | 3 |
| Bacteria | Acidobacteria | Acidobacteria-2 | 1 | 0 | 0 | 1 | 0 | 0 |
| Bacteria | Acidobacteria | Acidobacteria-6 | 0 | 5 | 0 | 0 | 4 | 0 |
| Bacteria | Acidobacteria | AT-s2-57 | 0 | 1 | 0 | 0 | 1 | 0 |
| Bacteria | Acidobacteria | AT-s54 | 0 | 3 | 0 | 0 | 3 | 0 |
| Bacteria | Acidobacteria | RB25 | 0 | 2 | 0 | 0 | 2 | 0 |
| Bacteria | Acidobacteria | Solibacteres | 0 | 976 | 0 | 0 | 5 | 0 |
| Bacteria | Acidobacteria | Sva0725 | 4 | 8 | 0 | 4 | 6 | 0 |
| Bacteria | Actinobacteria | Acidimicrobiia | 826 | 2411 | 56 | 18 | 31 | 18 |
| Bacteria | Actinobacteria | Actinobacteria | 35 | 4 | 83 | 4 | 3 | 4 |
| Bacteria | Actinobacteria | KIST-JJY010 | 0 | 0 | 1 | 0 | 0 | 1 |
| Bacteria | Actinobacteria | Thermoleophilia | 1 | 2 | 5 | 1 | 2 | 3 |
| Bacteria | Actinobacteria | Unclassified | 0 | 0 | 1 | 0 | 0 | 1 |
| Bacteria | Bacteroidetes | Bacteroidia | 3 | 0 | 2 | 3 | 0 | 2 |
| Bacteria | Bacteroidetes | Flavobacteriia | 395 | 13 | 63 | 31 | 9 | 23 |
| Bacteria | Bacteroidetes | Sphingobacteriia | 58 | 10 | 40 | 24 | 8 | 9 |
| Bacteria | Bacteroidetes | Unclassified | 6 | 2 | 52 | 5 | 2 | 5 |
| Bacteria | Caldithrix | Caldithrixae | 0 | 0 | 0 | 0 | 0 | 0 |
| Bacteria | Chlamydiae | Chlamydiia | 2 | 0 | 3 | 2 | 0 | 3 |
| Bacteria | Chlorobi | Ignavibacteria | 0 | 1 | 0 | 0 | 1 | 0 |
| Bacteria | Chlorobi | OPB56 | 0 | 0 | 1 | 0 | 0 | 1 |
| Bacteria | Chloroflexi | Anaerolineae | 0 | 1058 | 3 | 0 | 17 | 3 |
| Bacteria | Chloroflexi | Ellin6529 | 0 | 1 | 0 | 0 | 1 | 0 |
| Bacteria | Chloroflexi | SAR202 | 101 | 1121 | 0 | 4 | 26 | 0 |
| Bacteria | Chloroflexi | TK17 | 0 | 41 | 0 | 0 | 1 | 0 |
| Bacteria | Chloroflexi | Unclassified | 0 | 0 | 1 | 0 | 0 | 1 |
| Bacteria | Cyanobacteria | 4C0d-2 | 1 | 1 | 4 | 1 | 1 | 3 |
| Bacteria | Cyanobacteria | Chloroplast | 412 | 24 | 463 | 27 | 12 | 32 |
| Bacteria | Cyanobacteria | Oscillatoriophycideae | 9 | 4 | 1 | 3 | 3 | 1 |
| Bacteria | Cyanobacteria | S15B-MN24 | 0 | 0 | 4 | 0 | 0 | 1 |
| Bacteria | Cyanobacteria | Synechococcophycideae | 945 | 125 | 1065 | 20 | 10 | 17 |
| Bacteria | Firmicutes | Bacilli | 7 | 0 | 3 | 4 | 0 | 3 |
| Bacteria | Firmicutes | Clostridia | 96 | 38 | 28 | 34 | 14 | 17 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Bacteria | Firmicutes | Unclassified | 0 | 2 | 0 | 0 | 2 | 0 |
| Bacteria | Fusobacteria | Fusobacteria | 1 | 0 | 0 | 1 | 0 | 0 |
| *Bacteria* | *Gemmatimonadetes* | *Gemm-2* | *1* | *729* | *1* | *1* | *13* | *1* |
| *Bacteria* | *Gemmatimonadetes* | *Gemm-4* | *0* | *2* | *1* | *0* | *2* | *1* |
| *Bacteria* | *GN02* | *BD1-5* | *0* | *2* | *0* | *0* | *1* | *0* |
| *Bacteria* | *Nitrospirae* | *Nitrospira* | *170* | *236* | *0* | *2* | *7* | *0* |
| *Bacteria* | *PAUC34f* | *Unclassified* | *0* | *88* | *0* | *0* | *2* | *0* |
| *Bacteria* | *Planctomycetes* | *ODP123* | *0* | *0* | *1* | *0* | *0* | *1* |
| *Bacteria* | *Planctomycetes* | *OM190* | *2* | *0* | *0* | *2* | *0* | *0* |
| *Bacteria* | *Poribacteria* | *Unclassified* | *0* | *101* | *0* | *0* | *4* | *0* |
| *Bacteria* | *Proteobacteria* | *Alphaproteobacteria* | *5722* | *2342* | *10728* | *113* | *87* | *168* |
| *Bacteria* | *Proteobacteria* | *Betaproteobacteria* | *1147* | *0* | *2* | *12* | *0* | *2* |
| *Bacteria* | *Proteobacteria* | *Deltaproteobacteria* | *675* | *1247* | *50* | *65* | *59* | *38* |
| *Bacteria* | *Proteobacteria* | *Epsilonproteobacteria* | *20* | *11* | *24* | *6* | *1* | *5* |
| *Bacteria* | *Proteobacteria* | *Gammaproteobacteria* | *4040* | *9708* | *1391* | *126* | *203* | *162* |
| *Bacteria* | *Proteobacteria* | *Unclassified* | *333* | *65* | *89* | *37* | *20* | *23* |
| *Bacteria* | *SAR406* | *AB16* | *11* | *2* | *0* | *2* | *2* | *0* |
| *Bacteria* | *SBR1093* | *A712011* | *0* | *1* | *0* | *0* | *1* | *0* |
| *Bacteria* | *SBR1093* | *EC214* | *5* | *92* | *0* | *2* | *1* | *0* |
| *Bacteria* | *Spirochaetes* | *Spirochaetes* | *1* | *532* | *5* | *1* | *11* | *2* |
| *Bacteria* | *Synergistetes* | *Unclassified* | *1* | *0* | *2* | *1* | *0* | *2* |
| *Bacteria* | *Tenericutes* | *Mollicutes* | *1* | *0* | *1* | *1* | *0* | *1* |
| *Bacteria* | *TM6* | *SJA-4* | *0* | *0* | *0* | *0* | *0* | *0* |
| *Bacteria* | *TM6* | *Unclassified* | *0* | *0* | *2* | *0* | *0* | *1* |
| *Bacteria* | *TM7* | *TM7-3* | *0* | *0* | *1* | *0* | *0* | *1* |
| *Bacteria* | *Unclassified* | *Unclassified* | *485* | *617* | *581* | *243* | *224* | *265* |
| *Bacteria* | *Verrucomicrobia* | *[Pedosphaerae]* | *8* | *1* | *0* | *2* | *1* | *0* |
| *Bacteria* | *Verrucomicrobia* | *Opitutae* | *2* | *0* | *0* | *2* | *0* | *0* |
| *Bacteria* | *Verrucomicrobia* | *Verrucomicrobiae* | *3* | *0* | *0* | *2* | *0* | *0* |
| *Bacteria* | *WPS-2* | *Unclassified* | *0* | *0* | *2* | *0* | *0* | *1* |
| *Bacteria* | *WS3* | *PRR-12* | *0* | *0* | *1* | *0* | *0* | *1* |
| *Bacteria* | *WS6* | *SC72* | *0* | *0* | *1* | *0* | *0* | *1* |
| *Bacteria* | *ZB3* | *BS119* | *19* | *0* | *0* | *7* | *0* | *0* |
| *Unclassified* | *Unclassified* | *Unclassified* | *456* | *773* | *702* | *310* | *282* | *315* |
| *Total* | | | *16006* | *22402* | *15467* | *1125* | *1085* | *1142* |

## Chapter 3. Comparison of bacterial communities associated with *Xestospongia testudinaria*, sediment and seawater in a Singaporean coral reef ecosystem

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**Abstract**

Despite alterations caused by anthropogenic activities in Singaporean coral reefs, the sponge communities are quite diverse and *Xestospongia testudinaria* is one of the most common sponge species. In the present study, we used 16S rRNA gene barcoded pyrosequencing to characterize and compare bacterial communities from different biotopes (sponge, seawater and sediment) and to identify dominant bacterial symbionts of *X. testudinaria* in a Singaporean coral reef ecosystem. Our results showed that biotope appears to affect the richness, composition and abundance of bacterial communities. Proteobacteria was the most abundant phylum in sediment and seawater whilst Chloroflexi was more abundant in *X. testudinaria*. Members of the order Caldilineales (fermentation of organic substrates), Chromatiales (purple sulphur bacteria), Rhodospirillales (purple non-sulphur bacteria) and Syntrophobacterales (sulphate-reducing bacteria) were relatively more abundant in *X. testudinaria* samples.

**Keywords**

Community composition, coral reefs, pyrosequencing, Singapore, *Xestospongia testudinaria*

## Introduction

Sponges are an integral part of coral reefs and play an important role in these ecosystems as suspension feeders (Wilkinson, 1983, 1987; Wilkinson and Cheshire, 1990) whereby they contribute to nutrient cycling (de Goeij and van Duyl, 2007; de Goeij et al., 2008, 2013). They also provide habitat for several marine species (e.g. fish, brittle stars and shrimps) (Diaz and Rützler, 2001). The ecological success of sponges may be due to their close association with highly diverse symbiont microbial communities, which contribute to sponge nutrition, health (e.g. production of antibiotics and degradation of toxic substances) and nutrient cycling (Sipkema et al., 2005; de Goeij et al., 2013; Colman, 2015). Although many sponge species have been shown to host diverse and dense microbial communities, microbial abundance can vary greatly among different sponge species. Depending on the magnitude of the number of microbial cells per gram of tissue, sponges have been classified

as high microbial abundance (HMA) or low microbial abundance (LMA) species. HMA sponges harbour more abundant and more diverse microbial communities than LMA sponges (Hentschel et al., 2003; Ribes et al., 2012).

The sponge *Xestospongia testudinaria* (Lamarck, 1815) is a well-known HMA sponge, which has been studied due to its ecological importance and potential for pharmacological use (Vogel, 2008; Lee et al., 2011). Previous studies have showed that *X. testudinaria* tends to harbour similar bacterial communities dominated by the phyla Acidobacteria, Actinobacteria, Chloroflexi and Proteobacteria (Lee et al., 2011; Montalvo and Hill, 2011; Montalvo et al., 2014; Cleary et al., 2015; de Voogd et al., 2015). Previous studies have assessed the bacterial community of *X. testudinaria* in a number of locations including the Red Sea, north and south Sulawesi, NW Java and Indonesia (Lee et al., 2011; Montalvo and Hill, 2011; Bayer et al., 2014; Cleary et al., 2015; de Voogd et al., 2015). In the present study, we assess the bacterial communities of *X. testudinaria*, sediment and seawater in the coral reefs of Singapore. According to Corlett (1992), the marine flora and fauna of Singapore has been studied since the 19$^{th}$ century. The first comprehensive study of Singaporean sponges was, however, only in 2009 (de Voogd and Cleary, 2009). de Voogd and Cleary (2009) showed that the Singaporean sponge communities appeared to be composed by low-light resistant species and were quite diverse, despite the pronounced disturbance to Singaporean coral reefs caused by anthropogenic activities. This study also revealed that *X. testudinaria* was one of the most common species in Singaporean coral reefs. In line with these findings, Lim et al. (2012) reported that *X. testudinaria* was one of 13 sponge species commonly found and widespread in Singapore. In the present study, our main goals were to (1) characterize and compare the bacterial communities from different biotopes (*X. testudinaria*, seawater and sediment) and (2) identify dominant bacterial symbionts of *X. testudinaria* in a Singaporean coral reef ecosystem.

## Materials and methods

### Study site and sampling

According to de Voogd and Cleary (2009), extensive land reclamation and busy port activities have had a great impact on the Singaporean coral reef ecosystem. The Republic of Singapore, at the southern tip of the Malayan Peninsula, consists of a major island (separated from Malaysia by the narrow Johor Strait) and some 50 islets to the south (separated from the Riau Islands Province in Indonesia by the Singapore Strait). In this study, all samples were collected in waters surrounding these small islets.

Sediment, seawater and *X. testudinaria* were collected using scuba in August 2013. Three samples of *X. testudinaria* (from different sponge individuals, collected at 4–7 m depth) and sediment and one seawater sample were taken. The samples were collected from the Singapore Southern Islands of Semakau (01º12.744′N 103º45.378′E) and Kusu (01º13.566′N 103º51.531′E) – one sample of each biotope was collected in the area of Kusu island and two samples of *X. testudinaria* and sediment were taken in the area of Semakau island. Plastic disposable syringes with the end cut were used to sample minicores of sediment (top 5 cm were collected; ~5 g) (Capone et al., 1992; Polónia et al., 2014; Cleary et al., 2015). One litre of seawater was filtered using a Millipore® White Isopore Membrane Filter (0.22 mm pore size) as previously described (Sogin et al., 2006; Bowen et al., 2012). About 1 g of segments from surface to interior containing pinacoderm and choanoderm tissue of *X. testudinaria* were sampled as previously described (Polónia et al., 2014). After sampling, all samples were stored in 96% EtOH and placed at 4 ºC. Once in the laboratory all the samples were stored at -20 ºC until DNA extraction.

### DNA extraction and pyrosequencing

Total community DNA was extracted from seawater (material retained in membrane filter) and subsamples of sediment (0.5 g) and sponge tissue (0.5 g; comprising approximately equal amounts of both pinacoderm and choanoderm layers). DNA extraction was performed using the FastDNA® SPIN Kit for soil (MP Biomedicals), following the manufacturer's instructions. Bacterial cell lysis was performed using the FastPrep® Instrument (Q Biogene)

for 40 s at speed 6.0 m $s^{-1}$ twice. This method has frequently been used for DNA extraction from sponge samples (Costa et al., 2013; Polónia et al., 2014). DNA obtained was stored at -20 ºC until use.

Pyrosequencing was performed as previously described (Cleary et al., 2015). Briefly, before pyrosequencing, a nested PCR (25 cycles) approach was employed to increase the yield of PCR fragments of 16S rRNA genes and to standardize 16S rRNA gene amplification from all DNA samples. For this goal, bacterial specific primers 27F and 1494R were used (Gomes et al., 2001). A previous study from Fan et al. (2009) showed that nested PCR produces congruent results with those obtained with reconditioning PCR for molecular analysis of microbial communities. Cleary et al. (2012) also showed that a range of nested PCR-DGGE approaches were highly congruent with barcoded pyrosequencing analysis. For pyrosequencing, samples were amplified individually using barcoded fusion primers specific for the target region (V3/V4). The primers contained the Roche-454 A and B Titanium sequencing adapters, an eight-based barcode sequence in adaptor A and specific sequences for the ribosomal region, forward V3 primer 5′-ACTCCTACGGGAGG CAG-3′ (Yu et al., 2005) and V4 reverse degenerate primer 5′ -TANVRRGTHTCTAATYC-3′ (Vaz-Moreira et al., 2011). The resulting amplicons were quantified and reunited in equimolar pools to perform the emulsion-PCR (30 cycles) needed for the pyrosequencing.

Barcoded pyrosequencing libraries were analysed using Quantitative Insights Into Microbial Ecology (QIIME; Caporaso et al., 2010) software package (http://www.qiime.org/) using fasta and qual files as input for the split_libraries.py script. Default arguments were used except for the minimum sequence length, which was set at 218 bp after removal of forward primers and barcodes, backward primers were removed using the 'truncate only' argument, and a sliding window test of quality scores was enabled with a value of 50 as suggested in the QIIME description for the script (http://qiime.org/scripts/split_libraries.html). The resulting sequences had an average length of 420 bp.

OTUs were selected using UPARSE with USEARCH7 (Edgar, 2013) and the UCHIME algorithm was used for chimera checking, which is the fastest and most sensitive chimera-checking algorithm currently available (Edgar et al., 2011). With a cut-off threshold at 97%, the – cluster_otus command was used to perform OTU clustering. For the OTU picking and taxonomic assignment, we used the most recent Greengenes database (http://greengenes.secondgenome.com/downloads/database/13_5). The relative abundance

of the bacterial groups in each biotope and the representative sequences of the most abundant OTUs (≥100 sequences) were determined using the RDP method (Wang et al., 2007). Closely related organisms to numerically abundant OTUs (≥100 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 were submitted to the NCBI SRA: accession number SRP078745.

**Statistical analysis**

A square matrix containing the abundance of all OTUs per sample was imported into R (R Core Team, 2013) using the read.table() function. After importing into R, sequences not classified as Bacteria, or classified as mitochondria or chloroplast were removed prior to statistical analysis and total rarefied OTU richness (estimated number of OTUs for a given number of sequences) for all the samples was estimated using a self-written function (Gomes et al., 2010). After the imported matrix was log10(x + 1) transformed (to normalize the distribution of the data), we used the Bray–Curtis index with the vegdist() function in the vegan package (Oksanen et al., 2009) to construct a distance matrix. This index is one of the most applied (dis)similarity indices used in ecology (Legendre and Gallagher, 2001; Cleary, 2003; Cleary and Genner, 2004; Becking et al., 2006; de Voogd et al., 2009; Cleary et al., 2013; Polónia et al., 2014, 2015).

With the Bray–Curtis distance as input and using the cmdscale() function in R (R Core Team, 2013), a principal coordinates analysis (PCO) was used to assess the variation in bacterial composition among biotopes. Weighted averages scores were computed for OTUs on the first two PCO axes using the wascores() function in the vegan package. A heatmap was constructed to visualize the distribution of the dominant OTUs (≥100 sequence reads). The heatmap was generated using the function heatmap2() in the R package gplots (http://www.cran.r-project.org/).

## Results

Barcoded pyrosequencing analysis yielded 32473 sequences assigned to 1884 OTUs after quality control, OTU picking and removal of chimera, chloroplasts and mitochondria. OTUs were assigned to 37 phyla, 88 classes and 117 orders. Richness was highest in sediment and lowest in *X. testudinaria* samples (Fig. 3.1). In order to compare samples of unequal abundances we calculated the rarefied richness for a fixed number of sequences (N = 1000; based on the number of sequences presented by the least abundant sample Xt011), OTU richness was 205.66 ± 3.58 in seawater, and varied from 95.86 ± 4.69 to 127.19 ± 1.55 in *X. testudinaria* and from 308.10 ± 10.68 to 404.65 ± 9.6 in sediment. Qualitatively, the Chao1 richness estimators revealed a pattern similar to that for rarefied richness, with the highest expected number of OTUs occurring in sediment, followed by seawater and *X. testudinaria* (Table S3.1). However, the estimated Chao 1 richness was substantially higher than the observed OTU richness.

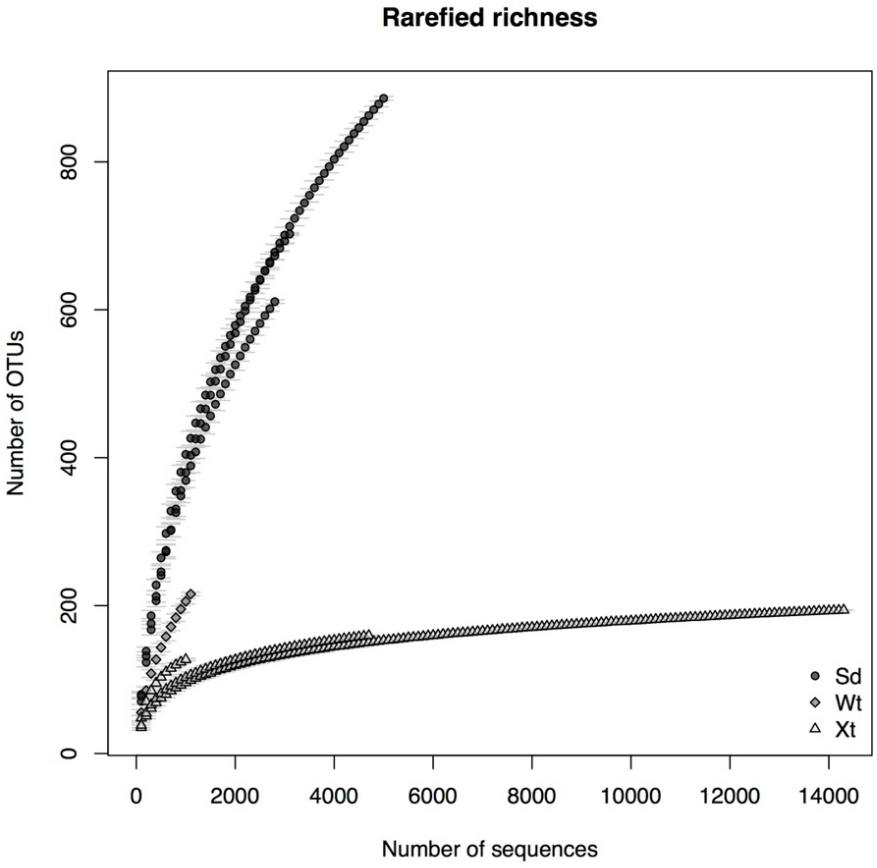


Fig. 3.1 – Rarefaction curve of bacterial communities as a function of the number of 16S rRNA gene sequences obtained from *Xestospongia testudinaria* (Xt), sediment (Sd) and seawater (Wt) samples.

The taxa composition analysis showed that at phylum level, Proteobacteria was by far the most abundant phylum (Fig. 3.2). The phylum Chloroflexi was markedly more abundant in *X. testudinaria* than in other biotopes. Proteobacteria was the most abundant phylum in sediment and seawater (Fig. 3.2).

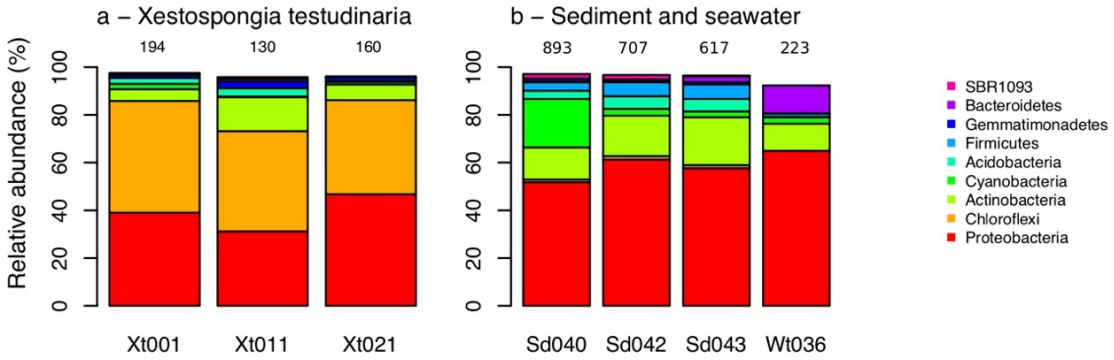


Fig. 3.2 – Relative abundance of the most abundant bacterial phyla in *Xestospongia testudinaria* (Xt), sediment (Sd) and seawater (Wt) biotopes. Total number of OTUs per sample is indicated on the top of each column.

The composition analysis of lower taxa (Fig. 3.3) showed that, at the class level, Gammaproteobacteria was the most abundant across all biotopes, and that the class Anaerolineae was most abundant in *X. testudinaria*. At the order level (Fig. 3.3) the Caldilineales, Chromatiales, Rhodospirillales, HTCC2188 and Syntrophobacterales were more abundant in *X. testudinaria* while the orders Rhizobiales and Alteromonadales were more abundant in sediment.

In line with the richness and taxa analysis, the PCO ordination (Fig. 3.4) showed compositional differences among bacterial communities from different biotopes. While the first PCO axis separated samples of *X. testudinaria* from samples of sediment and seawater, the second PCO axis separated seawater samples from *X. testudinaria* and sediment samples. However, the use of only one seawater sample precludes the use of F-test to evaluate the statistical significance of the differences observed between this biotope, *X. testudinaria* and sediment samples. A PCO ordination analysis comprising only *X. testudinaria* and sediment

samples (Fig. S1) showed that the compositional differences among these two biotopes were statistically significant ($F_{1,4}$ = 39.411, P = 0.107, $R^2$ = 0.91) and explained 91% of the variation in OTU composition.

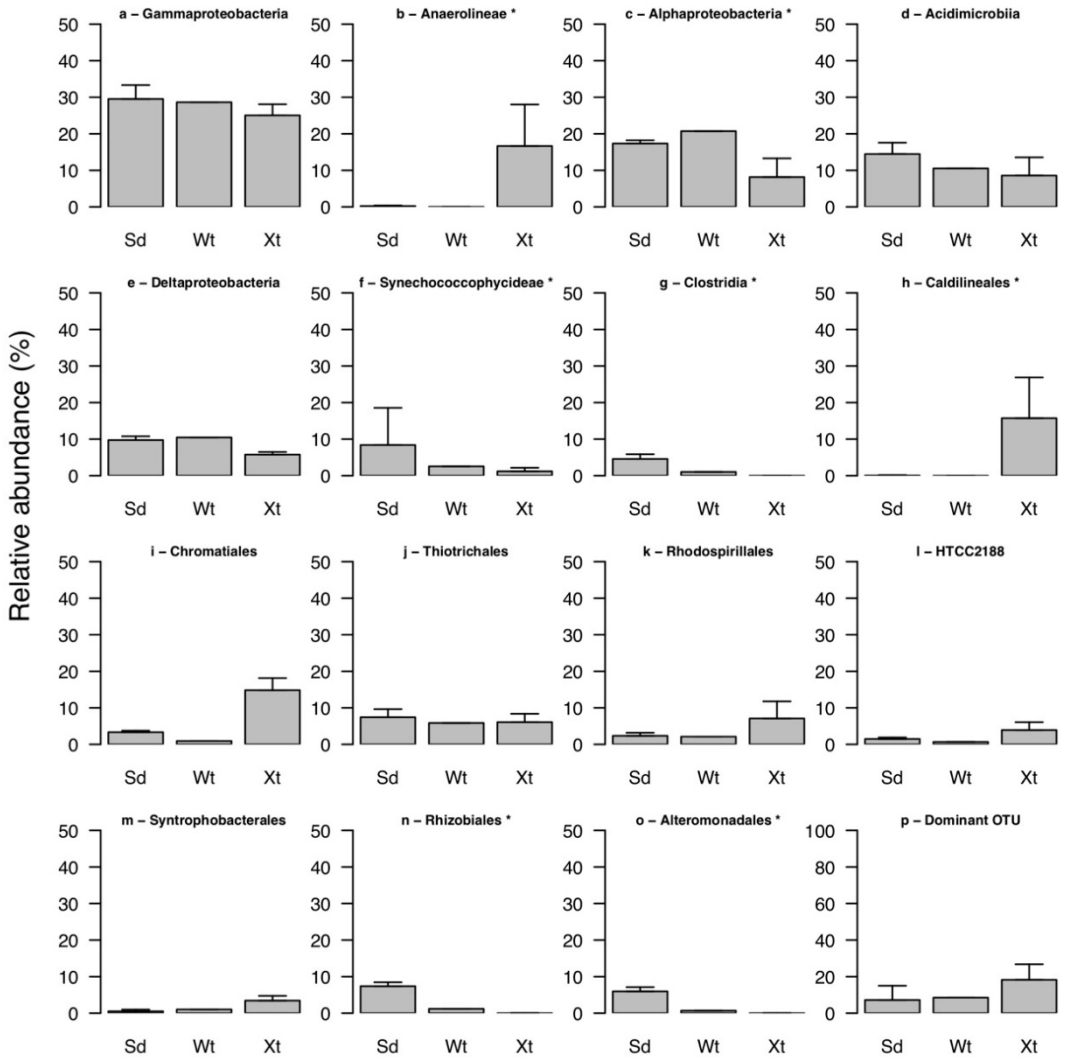


Fig. 3.3 – Relative abundance of the most abundant bacterial classes (a-g) and orders (h-o) and the most abundant OTU (p) in *Xestospongia testudinaria* (Xt), sediment (Sd) and seawater (Wt) biotopes. The bars represent a single standard deviation. The dominant OTU represents the mean abundance for the single most dominant OTU in each biotope, thus not necessarily the same OTU. ∗indicate classes/orders with significant differences among sediment and *X. testudinaria* samples.

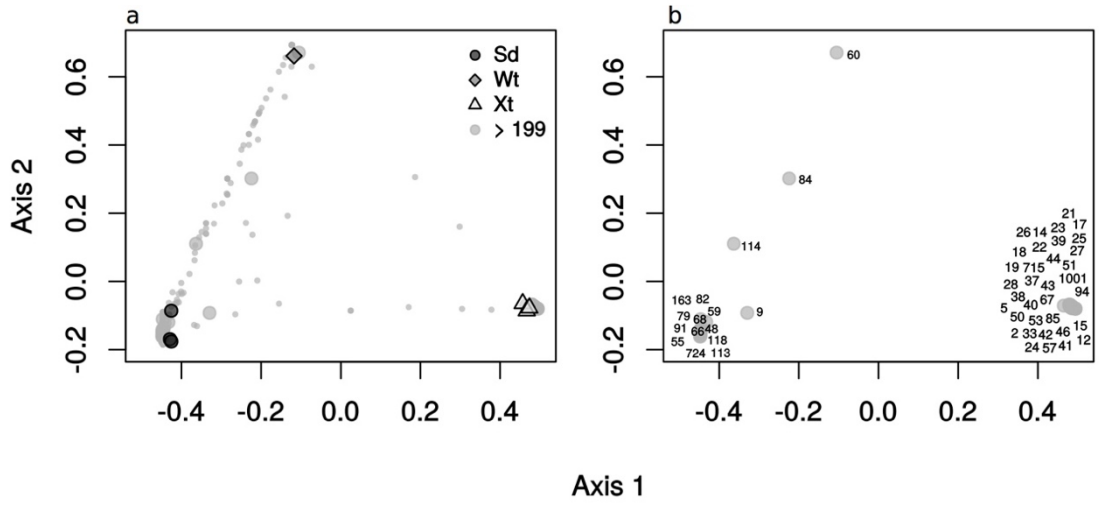


Fig. 3.4 – First two axes of the principal coordinates analysis (PCO) showing the variation of bacterial community composition in different biotopes. (a) Symbols represent samples from *Xestospongia testudinaria* (Xt), sediment (Sd) and seawater (Wt). Each grey dot represents a single OTU, where big dots represent OTUs ≥100 sequence reads and smaller dots represent OTUs <100 sequence reads. (b) Numbers represent the code of dominant OTUs referred to in Table 3.1; each dot represents a dominant OTU (≥ 100 sequence reads).

The in-depth bacterial composition analysis detected a total of 51 abundant OTUs (≥100 sequence reads) (Table 3.1). Of these, 29 OTUs were enriched (i.e. exclusive and highly abundant) in *X. testudinaria* and 9 in sediment biotopes. None of the most abundant OTUs were restricted to the seawater biotope (Fig. 3.5, Table 3.1). OTU-84 was the only OTU detected in all three biotopes but was more abundant in sediment and seawater (Fig. 3.5, Table 3.1). Closely related organisms to the most abundant OTUs were identified with BLAST (Table 3.1). OTU-84 was assigned to the OCS155 family (order Acidimicrobiales) and was closely related to two uncultured marine bacteria previously detected in coral mucus in Curaçao (accession number – Acc KU243315) and in seawater in Marquesas Islands (Acc KM223709; French Polynesia). The two most abundant OTUs overall were OTUs 2 and 5 (4445 and 2608 sequences, respectively), both assigned to the Chloroflexi phylum and restricted to *X. testudinaria* (Table 3.1). OTU-15 was the third most abundant OTU (1190 sequences) and was also found exclusively in *X. testudinaria* and was assigned to the family Ectothiorhodospiraceae (order Chromatiales) (Fig. 3.5, Table 3.1). Eight abundant OTUs were restricted to the sediment biotope (OTUs 48, 66, 68, 82, 91, 113, 118, 163 and 724; Table 3.1); most of these were closely related to organisms found in sediment samples (Table

3.1). The heatmap (Fig. 3.5) showed a clear taxa restriction to *X. testudinaria* and suggests an evident clustering in the distribution of dominant OTUs.

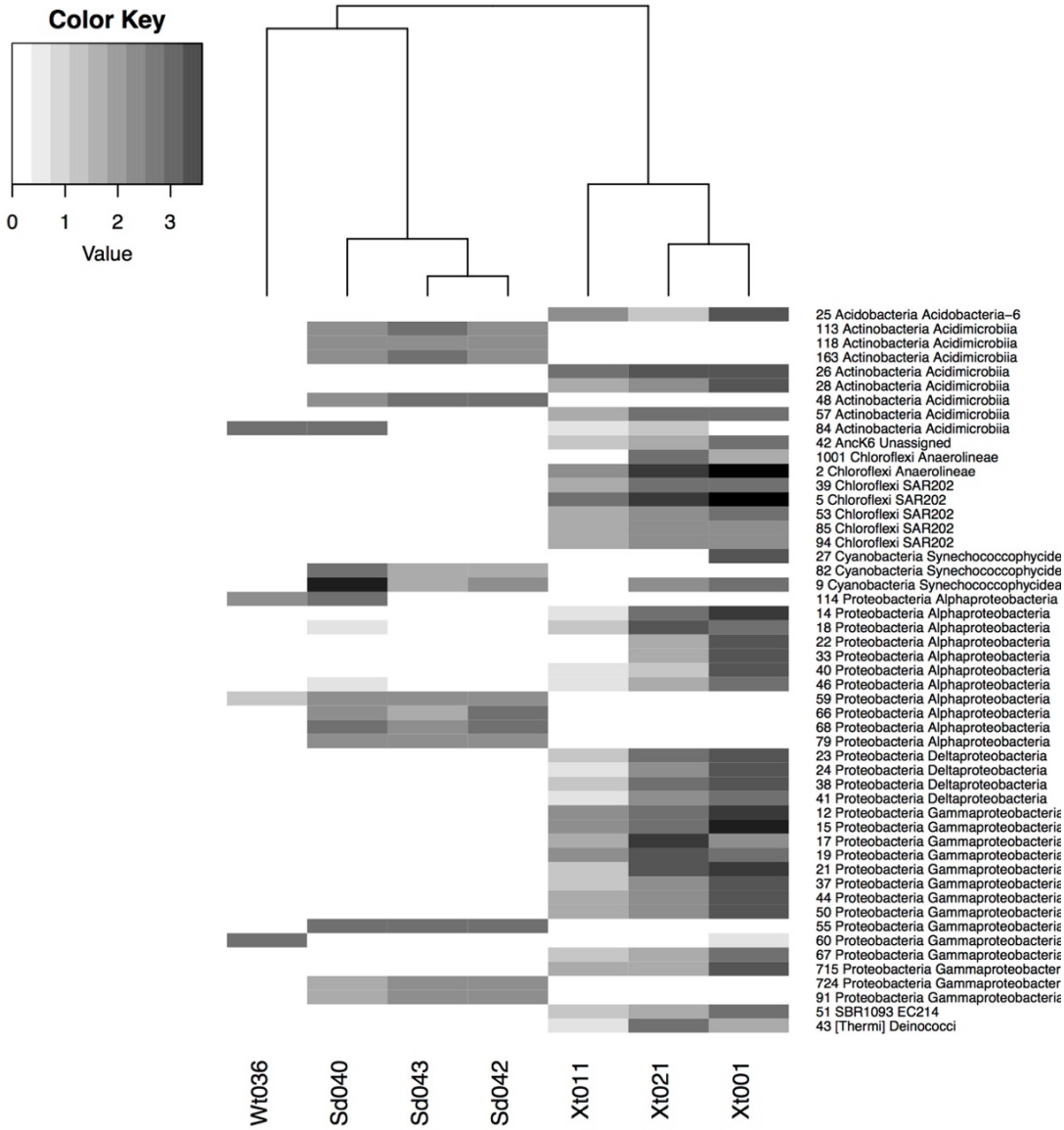


Fig. 3.5 – Heatmap showing the abundance of 16S rRNA gene sequence reads of dominant OTUs (≥100 sequence reads). Samples are indicated on x-axis: Xt – *Xestospongia testudinaria*, Sd – sediment and Wt – seawater. Abundant OTUs are indicated on y-axis by OTU numbers, phyla and class assignment. The abundance of each OTU is indicated in greyscale ranging from white (low abundance or absent) to black (high abundance). The scale bar indicates abundance using a logarithmic base 10 scale.

Table 3.1 – List of the most abundant OTUs (≥ 100 sequence reads) and closely related organisms identified using BLAST search.

| OTU | Biotope | Sum | Phylum | Class | Order | Family | Accession | Sq ident | Organism | Source | Location | DOI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **29 OTUs exclusive to *Xestospongia testudinaria*** | | | | | | | | | | | | |
| 2 | Xt | 4445 | Chloroflexi | Anaerolineae | Caldilineales | Caldilineaceae | JN596668<br>FJ481369 | 99<br>99 | Uncultured Chloroflexi<br>Uncultured *Chloroflexus* sp. | *Xestospongia testudinaria*<br>*Xestospongia muta* | Indonesia: Manado<br>USA: Key Largo, Florida | 10.1128/AEM.05285-11 |
| 5 | Xt | 2608 | Chloroflexi | SAR202 | | | JN210609 | 99 | Uncultured Chloroflexi | *Rhopaloeides odorabile* | Australia: Rib Reef, Great Barrier Reef, Queensland | 10.1111/j.1758-2229.2011.00296.x |
| 12 | Xt | 765 | Proteobacteria | Gammaproteobacteria | Thiotrichales | Piscirickettsiaceae | HE817820 | 99 | Uncultured bacterium | *Vaceletia crypta* | Australia: Great Barrier Reef, Yonge Reef | |
| 15 | Xt | 1190 | Proteobacteria | Gammaproteobacteria | Chromatiales | Ectothiorhodospiraceae | JN210811 | 99 | Uncultured *Nitrosococcus* sp. | *Rhopaloeides odorabile* | Australia: Rib Reef, Great Barrier Reef, Queensland | 10.1111/j.1758-2229.2011.00296.x |
| 17 | Xt | 470 | Proteobacteria | Gammaproteobacteria | Chromatiales | | HQ270372 | 99 | Uncultured gamma proteobacterium | *Xestospongia testudinaria* | Indonesia: Manado | 10.1128/AEM.05285-11 |
| 21 | Xt | 532 | Proteobacteria | Gammaproteobacteria | HTCC2188 | HTCC2089 | HE985154 | 99 | Uncultured bacterium | *Astrosclera willeyana* | Australia; Great Barrier Reef, Yonge Reef | |
| 22 | Xt | 298 | Proteobacteria | Alphaproteobacteria | Rhodospirillales | Rhodospirillaceae | JN210865 | 99 | Uncultured Rickettsiales | *Rhopaloeides odorabile* | Australia: Rib Reef, Great Barrier Reef, Queensland | 10.1111/j.1758-2229.2011.00296.x |
| 23 | Xt | 326 | Proteobacteria | Deltaproteobacteria | Entotheonellales | | HG423455 | 100 | Uncultured bacterium | *Astrosclera willeyana* | Egypt: Red Sea, Dahab | |
| 24 | Xt | 280 | Proteobacteria | Deltaproteobacteria | Syntrophobacterales | Syntrophobacteraceae | JX280312 | 98 | Uncultured bacterium | *Ircinia felix* | Bahamas: Sweeting's Cay | 10.1007/s00248-013-0215-2 |
| 25 | Xt | 300 | Acidobacteria | Acidobacteria-6 | BPC015 | | JX455331 | 100 | Uncultured bacterium | *Luffariella variabilis* | Australia: Orpheus Island, Great Barrier Reef, Queensland | 10.3389/fmicb.2012.00444 |
| 26 | Xt | 567 | Actinobacteria | Acidimicrobiia | Acidimicrobiales | wb1_P06 | HG764282 | 99 | Uncultured endophytic bacterium | *Holoxea* sp. | China: Yongxing Island, South China Sea | |
| 27 | Xt | 220 | Cyanobacteria | Synechococcophycideae | Synechococcales | Synechococcaceae (Synechococcus) | KF286179<br>KJ174471 | 99<br>99 | Uncultured bacterium<br>Candidatus *Synechococcus spongiarum* SH4 | *Aplysina cauliformis*<br>*Carteriospongia foliascens* | Bahamas: Lee Stocking Island<br>Red Sea | |
| 28 | Xt | 250 | Actinobacteria | Acidimicrobiia | Acidimicrobiales | TK06 | KF286009 | 99 | Uncultured actinobacterium | *Aplysina cauliformis* | Belize: Carrie Bow Cay | 10.1111/1574-6941.12222 |
| 33 | Xt | 262 | Proteobacteria | Alphaproteobacteria | Rhodospirillales | Rhodospirillaceae | FM160867 | 99 | Uncultured bacterium | *Aplysina fulva* | Brazil: Rio de Janeiro | 10.1128/AEM.02101-08 |
| 37 | Xt | 205 | Proteobacteria | Gammaproteobacteria | Thiotrichales | Piscirickettsiaceae | JX280282 | 99 | Uncultured bacterium | *Ircinia strobilina* | Bahamas: Sweeting's Cay | 10.1007/s00248-013-0215-2 |
| 38 | Xt | 255 | Proteobacteria | Deltaproteobacteria | Syntrophobacterales | Syntrophobacteraceae | HQ270338 | 99 | Uncultured delta proteobacterium | *Xestospongia testudinaria* | Indonesia: Manado | 10.1128/AEM.05285-11 |
| 39 | Xt | 245 | Chloroflexi | SAR202 | | | KT975136.1 | 98 | Uncultured prokaryote | endolithic community of Mono Island | Puerto Rico | 10.5194/bg-2016-254 |
| 40 | Xt | 170 | Proteobacteria | Alphaproteobacteria | | | HG764257 | 99 | Uncultured endophytic bacterium | *Holoxea* sp. | China: Yongxing Island, South China Sea | |
| 41 | Xt | 169 | Proteobacteria | Deltaproteobacteria | Syntrophobacterales | Syntrophobacteraceae | KM389590 | 100 | Uncultured bacterium | *Plakortis halichondrioides* | Bahamas | 10.3390/md12115425 |
| 42 | Xt | 162 | AncK6 | | | | JX280250 | 99 | Uncultured bacterium | *Ircinia strobilina* | Bahamas: Exumas | 10.1007/s00248-013-0215-2 |
| 43 | Xt | 131 | Thermi | Deinococci | Deinococcales | Trueperaceae (B-42) | HQ270396 | 100 | Uncultured *Truepera* sp. | *Xestospongia muta* | USA: Key Largo, Florida | 10.1128/AEM.05285-11 |
| 44 | Xt | 197 | Proteobacteria | Gammaproteobacteria | Chromatiales | Ectothiorhodospiraceae | KM389592 | 99 | Uncultured bacterium | *Plakortis halichondrioides* | Bahamas | 10.3390/md12115425 |
| 50 | Xt | 256 | Proteobacteria | Gammaproteobacteria | Thiotrichales | Piscirickettsiaceae | JN596737 | 99 | Uncultured gamma proteobacterium | *Xestospongia testudinaria* | Indonesia: Manado | 10.1128/AEM.05285-11 |
| 51 | Xt | 141 | SBR1093 | EC214 | | | HQ270374 | 99 | Uncultured Desulfovibrionales | *Xestospongia testudinaria* | Indonesia: Manado | 10.1128/AEM.05285-11 |
| 53 | Xt | 143 | Chloroflexi | SAR202 | | | KF286187 | 99 | Uncultured Chloroflexi | *Aplysina cauliformis* | Belize: Carrie Bow Cay | 10.1111/1574-6941.12222 |
| 67 | Xt | 111 | Proteobacteria | Gammaproteobacteria | Chromatiales | Ectothiorhodospiraceae | EU816812 | 99 | Uncultured gamma proteobacterium | *Neofibularia nolitangere* | Carribbean Sea | |
| 85 | Xt | 105 | Chloroflexi | SAR202 | | | JN596681 | 99 | Uncultured Chloroflexi | Sponge tissue *Xestospongia testudinaria* | Indonesia: Manado | 10.1128/AEM.05285-11 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 715 | Xt | 242 | Proteobacteria | Gammaproteobacteria | Chromatiales | Ectothiorhodospiraceae | HE985114 | 99 | Uncultured bacterium | *Astrosclera willeyana* | Australia: Great Barrier Reef, Yonge Reef | |
| 1001 | Xt | 112 | Chloroflexi | Anaerolineae | Caldilineales | Caldilineaceae | FJ481230 | 99 | Uncultured *Chloroflexus* sp. | *Xestospongia testudinaria* | Indonesia: Manado | 10.1128/AEM.05285-11 |
| **9 OTUs exclusive to sediment** | | | | | | | | | | | | |
| 48 | Sd | 204 | Actinobacteria | Acidimicrobiia | Acidimicrobiales | | KR086685 | 99 | Uncultured actinobacterium | Surface layer sediments | East China Sea | |
| 66 | Sd | 139 | Proteobacteria | Alphaproteobacteria | Rhizobiales | Hyphomicrobiaceae | GQ301490 | 100 | Uncultured bacterium | Juvenile Acroporid coral | Australia: Nelly Bay, Magnetic Island | 10.1111/j.1365-294X.2010.04620.x |
| 68 | Sd | 224 | Proteobacteria | Alphaproteobacteria | | | FJ175562 | 100 | Uncultured bacterium | Hydrocarbon seep sediment | Australia: Timor Sea | 10.1111/j.1574-6941.2009.00667.x |
| 82 | Sd | 145 | Cyanobacteria | Synechococcophycideae | Synechococcales | Synechococcaceae (Synechococcus) | AY712368 | 100 | Uncultured *Prochlorococcus* sp. | Surface water from salt marsh | USA: Georgia, Sapelo Island | |
| 91 | Sd | 100 | Proteobacteria | Gammaproteobacteria | Thiohalorhabdales | | JF261918 | 99 | Uncultured bacterium | Biofilm glass | Australia: Daydream Island, Whitsundays, Great Barrier Reef | 10.1111/j.1574-6968.2011.02374.x |
| 113 | Sd | 176 | Actinobacteria | Acidimicrobiia | Acidimicrobiales | koll13 | KR092737 | 99 | Uncultured actinobacterium | Coral reef sediment | India: Coral Reef Ecosystem of Gulf of Mannar | |
| 118 | Sd | 143 | Actinobacteria | Acidimicrobiia | Acidimicrobiales | koll13 | EU702800 | 99 | Uncultured actinobacterium | Benthic marine sediment | China: Jiaozhou Bay | |
| 163 | Sd | 196 | Actinobacteria | Acidimicrobiia | Acidimicrobiales | | KC607771 | 99 | Uncultured actinomycete | Marine sediment | China: The South China Sea | |
| 724 | Sd | 106 | Proteobacteria | Gammaproteobacteria | Thiotrichales | Piscirickettsiaceae | KR921393 | 98 | Uncultured bacterium | Bulk soil associated with *Rhizophora mangle* root zones at Acarau mangrove | Brazil: Northeast, Ceara | |
| **6 OTUs common to *Xestospongia testudinaria* and sediment** | | | | | | | | | | | | |
| 9 | Xt; Sd | 997 | Cyanobacteria | Synechococcophycideae | Synechococcales | Synechococcaceae (Synechococcus) | KX581283 | 99 | Uncultured *Synechococcus* sp. | Arabian Sea - Open ocean | India: Kerala | |
| 14 | Xt; Sd | 466 | Proteobacteria | Alphaproteobacteria | Rhodospirillales | Rhodospirillaceae | KF585152 | 99 | Uncultured bacterium | *Holoxea* sp. | China: Yongxing Island, South China Sea | |
| 18 | Xt; Sd | 420 | Proteobacteria | Alphaproteobacteria | Rhodospirillales | Rhodospirillaceae | KF585152 | 99 | Uncultured bacterium | *Astrosclera willeyana* | Australia; Great Barrier Reef, Yonge Reef | |
| 19 | Xt; Sd | 421 | Proteobacteria | Gammaproteobacteria | Chromatiales | | HQ270351 | 99 | Uncultured gamma proteobacterium | *Xestospongia testudinaria* | Indonesia: Manado | 10.1128/AEM.05285-11 |
| 46 | Xt; Sd | 113 | Proteobacteria | Alphaproteobacteria | Rhodospirillales | Rhodospirillaceae | KF585179 | 100 | Uncultured bacterium | *Holoxea* sp. | China: Yongxing Island, South China Sea | |
| 57 | Xt; Sd | 166 | Actinobacteria | Acidimicrobiia | Acidimicrobiales | | KF286075 | 99 | Uncultured actinobacterium | *Aplysina cauliformis* | Belize: Carrie Bow Cay | 10.1111/1574-6941.12222 |
| **2 OTUs common to *Xestospongia testudinaria* and seawater** | | | | | | | | | | | | |
| 60 | Xt; Wt | 103 | Proteobacteria | Gammaproteobacteria | Oceanospirillales | Halomonadaceae (Candidatus Portiera) | KT731832 | 100 | Uncultured gamma proteobacterium | surface seawater from Changjiang estuary and adjacent areas | China | |
| 94 | Xt; Wt | 108 | Chloroflexi | SAR202 | | | KC861145 | 99 | Uncultured bacterium | *Cinachyra* sp. | India: Coral Reef Ecosystem of Gulf of Mannar | 10.1371/journal.pone.0123222 |
| **4 OTUs common to sediment and seawater** | | | | | | | | | | | | |
| 55 | Sd; Wt | 224 | Proteobacteria | Gammaproteobacteria | Thiotrichales | Piscirickettsiaceae | KP016594 | 99 | Uncultured gamma proteobacterium | Surface layer sediment | China: Jiaojiang Estuary | |
| 59 | Sd; Wt | 112 | Proteobacteria | Alphaproteobacteria | Rhizobiales | Hyphomicrobiaceae | KP016099 | 99 | Uncultured Rhodobiaceae | Surface layer sediment | China: Jiaojiang Estuary | |
| 79 | Sd; Wt | 108 | Proteobacteria | Alphaproteobacteria | Rhizobiales | Hyphomicrobiaceae (Hyphomicrobium) | FJ175548 | 100 | Uncultured bacterium | Hydrocarbon seep sediment | Australia: Timor Sea | 10.1111/j.1574-6941.2009.00667.x |
| 114 | Sd; Wt | 121 | Proteobacteria | Alphaproteobacteria | Rickettsiales | Pelagibacteraceae | KU173670 | 99 | Uncultured SAR11 cluster alpha proteobacterium | surface seawaters from the East China sea | China | |
| **1 OTU common to all biotopes** | | | | | | | | | | | | |
| 84 | Xt; Sd; Wt | 156 | Actinobacteria | Acidimicrobiia | Acidimicrobiales | OCS155 | KU243315<br>KM223709 | 100<br>100 | Uncultured bacterium<br>Uncultured marine bacterium | Surface Mucus of Caribbean Corals<br>Seawater from 40 m depth | Curaçao<br>Marquesas Island in the South Pacific Ocean | 10.1371/journal.pone.0144702<br>10.3389/fmicb.2016.00234 |

OTU: OTU number; Biotope: biotope or biotopes where the OTUs were found; Sum: number of sequence reads; Accession: accession number of closely related organisms identified using BLAST; Sq ident: sequence similarity of these organisms with our representative OTU sequences; Source: isolation source of organisms identified using BLAST; Location: sampling location of organisms identified using BLAST.

## Discussion

Overall, the richness analysis indicated a relatively low number of OTUs observed in *X. testudinaria* samples when compared with most recent results obtained with Illumina (e.g. Thomas et al., 2016). Probably, such a difference may be explained due to the higher yield of sequence reads obtained with this technology when compared with pyrosequencing. Luo et al. (2012) compared Roche 454 and Illumina sequencing technologies on the same DNA sample and concluded that the higher number of reads (sequencing effort) obtained with Illumina resulted in a significantly higher number of OTUs. Nevertheless, in agreement with our results, a previous study showed that sponges (*Stylissa carteri*, *Aaptos suberitoides* and *X. testudinaria*) from the Misool coral reef system (Indonesia) had a much lower OTU richness than sediment. With a sequencing effort of 50,223 sequences (assigned to 3797 OTUs, for all biotopes), sponge richness was in general less than 250 OTUs (Cleary et al., 2018). Bayer et al. (2014) also showed that *X. testudinaria* from the Red Sea, at the minimum sampling effort (8925 sequences per sample), had less than 250 OTUs.

In the present study, bacterial communities associated with sediment and seawater biotopes were compositionally distinct from bacterial communities found in *X. testudinaria*. Proteobacteria was the most abundant phylum in sediment and seawater whereas Chloroflexi was most abundant in *X. testudinaria*. Previous studies showed that Chloroflexi is one of the most common and diverse bacterial phyla in sponges with numerous sponge-specific lineages (Brück et al., 2010; Schmitt et al., 2011). Some other studies suggested that members of this phylum may provide synergistic survival advantages to the host in nutrient-poor environments (Brock et al., 1984; Brück et al., 2010). For example, Brück et al. (2010) suggested that Chloroflexi might be able to provide organic photosynthates (e.g. glycerol) to sponge hosts in shallow waters, due to the ability of these bacteria to fix atmospheric $CO_2$ in these environments. Members of the Chloroflexi phylum may also be able to synthesize polyketides (bioactive compounds) in marine sponges (Siegl and Hentschel, 2010), indicating that they might also play a role in sponge defence against predators.

At lower taxonomic levels, our results showed a specific enrichment of Anaerolineae (Chloroflexi) populations in *X. testudinaria*. Members of this family have been previously detected in a range of environments such as marine and freshwater sediments, marine sponges and human microbiota and are commonly described as versatile carbohydrate

fermenters (Yamada et al., 2006; Cleary et al., 2018; Campbell et al., 2014; de Voogd et al., 2015; Xia et al., 2016). In addition to Anareolineae, other members of the Chloroflexi cluster 'subphylum IV' (SAR202) were specifically enriched in *X. testudinaria*. Representatives of the SAR202 cluster have been found in a variety of habitats, including deep terrestrial subsurface, soil, marine sponges, seawater and freshwater environments (Dunbar et al., 2002; Hentschel et al., 2002; Morris et al., 2004). However, so far, no cultured representatives have been isolated and no information about their ecophysiology is known (Morris et al., 2004; Thrash et al., 2016). Our results also showed that the order Caldilineales (Chloroflexi) was almost exclusively found in *X. testudinaria*. While Anaerolineae members are obligately anaerobic, Caldilineae members are facultative aerobes (Yamada et al., 2006). In general, despite the relatively high abundance and putative functional importance of members of Chloroflexi in sponge biotopes, there is limited information available on cultured representative strains belonging to this phylum (Brück et al., 2010; Schmitt et al., 2011).

The in-depth bacterial community analysis showed that OTU-84, assigned to family OCS155, was the only abundant OTU common to all three biotopes with high dominance in seawater and sediment samples. According to Fan et al. (2012) and references therein, members of this family are frequently detected in seawater worldwide. The family OCS155 belongs to the order Acidimicrobiales, a group of planktonic photo-heterotrophic free-living microorganisms commonly inhabiting tropical and temperate areas (Ghai et al., 2013; Mizuno et al., 2015). Members of the Acidimicrobiales order include obligatory acidophilic organisms capable of ferrous iron oxidation, sulphur oxidation and ferric iron reduction (Hardoim et al., 2012). OTU-15 was specifically enriched in *X. testudinaria* and was assigned to the order Chromatiales (family Ectothiorhodospiraceae) and showed high sequence similarity with an uncultured clone of *Nitrosococcus* sp. (Acc JN210811) previously detected in the sponge *Rhopaloeides odorabile* in Rib Reef (Great Barrier Reef, Queensland; Pollock et al., 2010). Members of this order, known as purple sulphur bacteria, can perform photosynthesis under anoxic conditions using hydrogen sulphide as electron donor and are able to fix molecular nitrogen (Proctor, 1997; Imhoff, 2005). Such functions might contribute to the sponge nitrogen metabolism and may explain why members of this group are consistently found in marine sponges (Pollock et al., 2010; Montalvo and Hill, 2011; Hardoim et al., 2012; Pita et al., 2013; Della Sala et al., 2014). Other dominant OTUs,

belonging to the orders Entotheonellales (OTU-23) and Syntrophobacterales (OTUs 24, 38 and 41), were also specifically enriched in *X. testudinaria*. The known bacterial lineages related to the Entotheonellales are widely distributed in marine sponges and have a diverse metabolism; they are able to produce chemically diverse bioactive products of medical interest (Wilson et al., 2014). All OTUs assigned to the Syntrophobacterales were classified within the Syntrophobacteraceae family (Table 3.1). Members of this family are strictly anaerobic sulphate-reducing bacteria, mainly found in freshwater, sewage sludge and marine habitats (Kuever, 2014). However, they have also previously been detected in sponges (Fiore et al., 2013; Cleary et al., 2015; Wang and Wu, 2017).

Overall, dominant OTUs belonging to the Alphaproteobacteria class were detected in all three biotopes, however, OTUs belonging to the Rhodospirillaceae family were mainly found in *X. testudinaria* and sediment. All the dominant OTUs assigned to this family (OTUs 14, 18, 22, 33 and 46) showed high similarity with uncultured bacteria previously detected in a diverse range of sponge species (*Rhopaloeides odorabile*, *Aplysina fulva*, *Holoxea* sp. and *Astrosclera willeyana*) located across a broad geographic range (Table 3.1). OTUs 22 and 33 were exclusively detected in *X. testudinaria* (Table 3.1). Members of the Rhodospirillaceae are purple non-sulphur bacteria with diverse metabolic and nutritional properties, comprising lineages able to grow heterotrophically, photoheterotrophically under anoxic conditions in the light and chemoheterotrophically in the dark (Baldani et al., 2014). A number of OTUs (OTUs 12, 37 and 50) assigned to the order Thiotrichales were also enriched in *X. testudinaria*. These OTUs were assigned to the Piscirickettsiaceae family and were similar (99%) to organisms previously detected in different sponge species including *Vaceletia crypta*, *Ircinia strobilina* and *X. testudinaria* across a broad geographic range (Table 3.1). The Piscirickettsiaceae family comprises metabolically diverse organisms, including chemoorganotrophs, methylotrophs and chemolithotrophic sulphur-oxidizers (Garrity et al., 2005).

OTUs assigned to the order Rhizobiales were exclusively or predominantly found in sediment and were not detected in *X. testudinaria*. Members of this order can be found in several environments such as freshwater, soils, seawater, brackish water and sediment (Gliesche et al., 2005; Oren and Xu, 2014). Only one abundant OTU (OTU-114) was assigned to the order Rickettsiales (family Pelagibacteraceae) and was dominant in both sediment and seawater biotopes. Its closest relative was an uncultured bacterium previously

detected in surface seawaters of the East China sea (Acc KU173670). Previous studies have reported that members of this family are adapted to low nutrient environments and may be one of the most abundant Alphaproteobacteria in seawater (Campbell et al., 2015; Angly et al., 2016; Frank et al., 2016).

In general, our results showed that the structure of the bacterial communities associated with *X. testudinaria* in the Singaporean coral reef ecosystem followed a profile similar to other communities associated with *X. testudinaria* from the Red Sea, north and south Sulawesi, NW Java and Misool Indonesia (Lee et al., 2011; Montalvo and Hill, 2011; Bayer et al., 2014; Cleary et al., 2015, 2018; de Voogd et al., 2015). These studies revealed that *X. testudinaria* bacterial communities were also dominated by the phyla Chloroflexi, Proteobacteria and Actinobacteria.

## Conclusion

In this study, we have provided an in-depth compositional analysis of bacterial communities inhabiting distinct biotopes in a Singaporean coral reef ecosystem. Our data revealed that biotope affects the richness, composition, abundance and putative function of bacterial communities. Overall, bacterial communities of *X. testudinaria* diverged greatly from the surrounding sediment and seawater bacterial communities. Our results also showed that Proteobacteria was the most abundant phylum in sediment and seawater while Chloroflexi was more abundant in *X. testudinaria*. Members of the order Caldilineales (fermentation of organic substrates), Chromatiales (purple sulphur bacteria), Rhodospirillales (purple non-sulphur bacteria) and Syntrophobacterales (sulphate-reducing bacteria) were enriched in *X. testudinaria* samples.

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**Supporting Information**

Comparison of bacterial communities associated with *Xestospongia testudinaria*, sediment and seawater in a Singaporean coral reef ecosystem

Pires ACC, Cleary DFR, Polónia ARM, Lim SC, de Voogd NJ, Oliveira V, Gomes NCM
Journal of the Marine Biological Association of the United Kingdom, 99(2): 331-342.

Table S3.1 – Species Chao1 richness estimation for a controlled sample size (100 - 1000 sequences) using resampling of bacterial 16S rRNA gene sequences from *Xestospongia testudinaria* (Xt), sediment (Sd) and seawater (Wt) samples.

| Groups | Sample | Sample size | Chao1 | SD |
|---|---|---|---|---|
| Xt | BSin13Xt001 | 100 | 74,66 | 34,59 |
| | BSin13Xt011 | | 102,03 | 38,71 |
| | BSin13Xt021 | | 80,89 | 35,52 |
| Sd | BSin13Sd040 | | 350,54 | 192,10 |
| | BSin13Sd042 | | 343,35 | 140,91 |
| | BSin13Sd043 | | 289,77 | 100,85 |
| Wt | BSin13Wt036 | | 140,79 | 51,61 |
| Xt | BSin13Xt001 | 200 | 86,35 | 22,87 |
| | BSin13Xt011 | | 116,83 | 21,57 |
| | BSin13Xt021 | | 95,53 | 26,35 |
| Sd | BSin13Sd040 | | 421,90 | 105,68 |
| | BSin13Sd042 | | 432,65 | 97,67 |
| | BSin13Sd043 | | 374,61 | 80,69 |
| Wt | BSin13Wt036 | | 187,66 | 46,30 |
| Xt | BSin13Xt001 | 300 | 96,45 | 19,73 |
| | BSin13Xt011 | | 125,74 | 18,25 |
| | BSin13Xt021 | | 105,97 | 22,42 |
| Sd | BSin13Sd040 | | 503,84 | 98,83 |
| | BSin13Sd042 | | 501,16 | 85,98 |
| | BSin13Sd043 | | 436,99 | 71,40 |
| Wt | BSin13Wt036 | | 230,58 | 48,23 |
| Xt | BSin13Xt001 | 400 | 106,09 | 22,61 |
| | BSin13Xt011 | | 133,75 | 16,97 |
| | BSin13Xt021 | | 112,80 | 21,24 |
| Sd | BSin13Sd040 | | 574,52 | 97,56 |
| | BSin13Sd042 | | 557,75 | 75,69 |
| | BSin13Sd043 | | 491,38 | 69,52 |
| Wt | BSin13Wt036 | | 266,86 | 49,34 |
| Xt | BSin13Xt001 | 500 | 114,18 | 22,02 |
| | BSin13Xt011 | | 138,12 | 15,90 |
| | BSin13Xt021 | | 119,13 | 20,69 |
| Sd | BSin13Sd040 | | 627,48 | 95,64 |
| | BSin13Sd042 | | 600,76 | 72,56 |
| | BSin13Sd043 | | 538,73 | 69,89 |
| Wt | BSin13Wt036 | | 296,09 | 49,83 |
| Xt | BSin13Xt001 | 600 | 119,48 | 20,57 |
| | BSin13Xt011 | | 143,47 | 14,49 |
| | BSin13Xt021 | | 124,62 | 19,62 |
| Sd | BSin13Sd040 | | 669,87 | 86,95 |
| | BSin13Sd042 | | 635,34 | 71,76 |
| | BSin13Sd043 | | 569,46 | 66,14 |
| Wt | BSin13Wt036 | | 316,34 | 47,89 |
| Xt | BSin13Xt001 | 700 | 125,32 | 21,05 |
| | BSin13Xt011 | | 148,40 | 13,16 |
| | BSin13Xt021 | | 130,46 | 19,89 |
| Sd | BSin13Sd040 | | 716,53 | 89,10 |
| | BSin13Sd042 | | 670,65 | 69,24 |
| | BSin13Sd043 | | 606,19 | 64,00 |
| Wt | BSin13Wt036 | | 331,69 | 42,88 |
| Xt | BSin13Xt001 | 800 | 130,52 | 21,96 |
| | BSin13Xt011 | | 152,21 | 12,36 |
| | BSin13Xt021 | | 136,30 | 20,34 |
| Sd | BSin13Sd040 | | 748,11 | 84,63 |
| | BSin13Sd042 | | 698,74 | 67,52 |
| | BSin13Sd043 | | 636,00 | 67,08 |
| Wt | BSin13Wt036 | | 344,31 | 38,82 |
| Xt | BSin13Xt001 | 900 | 135,35 | 23,07 |
| | BSin13Xt011 | | 156,17 | 10,40 |
| | BSin13Xt021 | | 140,32 | 19,99 |
| Sd | BSin13Sd040 | | 785,36 | 83,97 |

| | | | | |
|---|---|---|---|---|
| | BSin13Sd042 | | 724,72 | 64,75 |
| | BSin13Sd043 | | 658,84 | 61,30 |
| Wt | BSin13Wt036 | | 357,15 | 35,85 |
| Xt | BSin13Xt001 | 1000 | 139,04 | 23,02 |
| | BSin13Xt011 | | 159,02 | 8,18 |
| | BSin13Xt021 | | 145,07 | 20,70 |
| Sd | BSin13Sd040 | | 811,05 | 79,67 |
| | BSin13Sd042 | | 751,43 | 64,14 |
| | BSin13Sd043 | | 681,39 | 60,62 |
| Wt | BSin13Wt036 | | 368,22 | 29,85 |

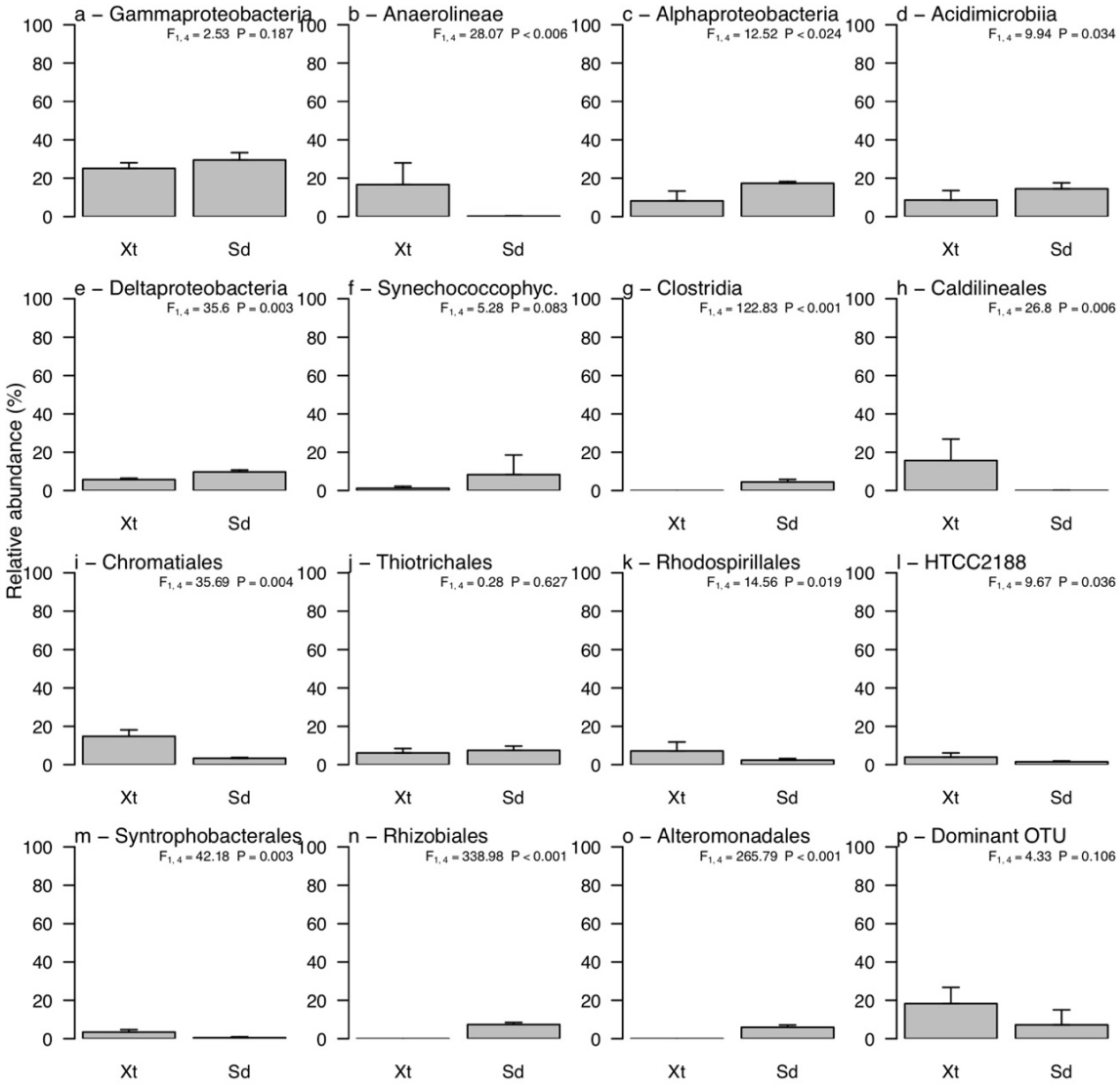


Fig. S3.1 – Relative abundance of the most abundant bacterial classes (a-g) and orders (h-o) and the most abundant OTU (p) for samples from *Xestospongia testudinaria* (Xt) and sediment (Sd). Error bars represent a single standard deviation. The dominant OTU represents the mean abundance for the single most dominant OTU in each biotope, thus not necessarily the same OTU.

# Chapter 4. Bacterial composition and putative functions associated with different sponge species, sediment and seawater in Tioman coral reef system, Peninsular Malaysia

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(for submission)

**Abstract**

Studying the ecology of microbial communities associated with sponges is essential to understand their role in sponge health, defence and nutrient dynamics in coral reef systems. Here, we used a 16S rRNA gene high-throughput sequencing approach in order to test the compositional and predictive functional differences among bacterial communities associated with two LMA sponges (*Stylissa carteri* and *Stylissa massa*), one well-known HMA (*Xestospongia testudinaria*), one putative HMA sponge species (*Aaptos lobata*), sediment and seawater in a coral reef ecosystem around Tioman Island.

Our results showed that biotope (sponge species, sediment and seawater) was the main predictor of richness, composition and abundance of bacterial communities. Proteobacteria was the dominant phylum in *S. carteri*, seawater, sediment and *A. lobata* biotopes, whilst bacterial communities of *S. massa* and *X. testudinaria* were dominated by Cyanobacteria and Chloroflexi, respectively. The predicted metagenome revealed differentially enriched pathways in HMA and LMA sponges. Differentially enriched subcategories in HMA sponges included "Signaling Molecules and Interaction", "Carbohydrate Metabolism" and "Excretory System". Subcategories enriched in LMA sponges included "Replication and Repair", "Energy Metabolism", "Metabolism of Cofactors and Vitamins" and "Environmental Adaptation". Overall, the predicted functional analysis indicated that, although HMA and LMA associated bacterial communities shared core functional features, they use different strategies to defend against pathogens, obtain energy or repair against stress.

**Keywords**

Community composition, coral reefs, pyrosequencing, PICRUSt Tioman, HMA/LMA dichotomy

## Introduction

The study of the ecology of sponge-associated microbial communities is critical for a better understanding of the role of microbes in the maintenance of sponge health and nutrient

cycling in coral reef systems (Hentschel et al., 2002; Taylor et al., 2007; van Soest et al., 2012). Sponge microbial symbionts can be highly specific and contribute to host defence by the production of biologically active metabolites, and to sponge metabolic functions (Wilkinson and Fay, 1979; Unson et al., 1994; Thakur et al., 2003; Hoffmann et al., 2005; Taylor et al., 2007; Bayer et al., 2008). Taylor et al. (2007) provided evidence for the existence of diverse sponge-specific microorganisms, which form clusters and establish an association with specific sponges. Microbial communities in such clusters appear to be compositionally stable in space and time and to diverge from microbial communities in the surrounding seawater. In 2002, the hypothesis of widespread sponge-specific microbial communities was raised by Hentschel et al. (2002). Taylor et al. (2007) also suggested that biosynthesis of metabolites can be induced by microbial symbionts that are, in turn, used in sponge defence. Hentschel et al. (2006) identified the main specific lineages associated with sponges as including Cyanobacteria, Archaea, Proteobacteria, Actinobacteria and the candidate phylum "Poribacteria". According to the authors, molecular studies have allowed the perception of the taxonomy and phylogeny of sponge microbial symbionts, as well as their metabolism when associated to such hosts. The interaction sponge-Cyanobacteria is believed to be one of the oldest microbe-metazoan mutualistic associations. This phylum is mainly represented by the *Synechococcus*/*Prochlorococcus* clade, where *Synechococcus* associated to sponges may be considered sponge specific – a "sponge ecotype". Hentschel et al. (2006) considered that some sponges, such as aplysinid sponges, may be considered a great source for dehalogenating microorganisms (mainly Deltraproteobacteria and Chloroflexi), whilst Actinobacteria within marine sponges represent a are prolific producers of secondary metabolites. Webster and Thomas (2016) reviewed the developments in sponge microbiology by the use of next-generation sequencing technologies, which have allowed to explore not only sponge microbial rare biosphere but also microbial function in such hosts.

The abundance and structural composition of microbial communities can be highly variable between sponge species. For example, whilst high microbial abundance (HMA) sponges have associated dense microbial communities, low microbial abundance (LMA) contain less abundant and less diverse microbial communities (Hentschel et al., 2003; Weisz et al., 2007). As mentioned by Webster and Thomas (2016), despite the newest insights given by metagenomics and metatranscriptomics the reason for such differences between HMA and

LMA sponges is still unclear. However, HMA and LMA sponges may also share structural and functional similarities (Bayer et al., 2014). Fan et al. (2012) had already shown that six sponge species with distinct phylogenetic and functional composition shared some core functions, diverging from the microbial communities found in seawater. Recently, Cleary et al. (2018) showed that *Aaptos suberitoides* [recently reclassified as *Aaptos lobata* (Calcini et al, 2017)], *X. testudinaria* and *S. carteri* harboured species specific microbial communities distinct from sediment and the surrounding seawater. The authors also observed similarities among bacterial communities of *A. suberitoides* and the well-known HMA sponge *X. testudinaria*. This could be an indication that *A. suberitoides* might also be an HMA sponge. *Aaptos suberitoides* is an abundant organism of ecological and biotechnological importance, typically found in shallow coral reefs located in the Asia-Pacific's Coral Triangle (de Voogd and Cleary, 2009; Pham et al., 2013).

With respect to sponge microbial community function, previous studies highlighted the role of sponge associated microbiota in the biogeochemical cycles of nitrogen, carbon, sulphur and phosphate and to essential vitamin biosynthesis (Wilkinson and Fay, 1979; Hoffmann et al., 2005; Bayer et al., 2008). Wilkinson and Fay (1979) were pioneers in this field by showing cyanobacterial nitrogenase activity in sponges inhabiting Harvey Reef in the Red Sea and, therefore, linking for the first-time nitrogen fixation to sponge-symbiotic cyanobacteria. More recently, Bayer et al. (2008) reported, in vivo activity of putative bacterial (*Nitrosospira* cluster 1) and archaeal (Crenarchaeota group I.1A) nitrifiers in the marine sponge *Aplysina aerophoba*. Hoffmann et al. (2005) demonstrated that carboxylic acids produced by sulfate-reducing bacteria (SRB) were transferred to the host sponge *Geodia barretti* and suggested that sulphur-oxidizing bacteria (SOB) act to prevent toxic accumulation of sulphide. More recently, metagenomic studies have shown that some microbial symbionts belonging to different species will converge functionally to occupy similar niches or to fulfil common functions in different sponges. For example, Li et al. (2016) compared the shallow-water sponge *Theonella swinhoei* (from the South China Sea) and the deep-sea sponge *Neamphius huxleyi* (from the Indian Ocean) and showed that both sponges had different microbial community structures but with similar core metabolic profiles. In addition to the host effects on the sponge microbial communities, abiotic characteristics of the surrounding environment may also influence the structure and function of these communities. The differences among host species and between host and non-host

biotopes (e.g., sediment and seawater) are reflected in the structural and functional diversity of the associated microbial communities (Caporaso et al., 2011). Therefore, the characterization of microbial communities from phylogenetically distinct sponges and their environment (seawater and sediment) contributes to unravelling core features generally relevant for microbial adaptation to different host species and contributes to a better understand of sponge symbiotic interactions in coral reef ecosystems.

In this study, we aimed to characterize the diversity, composition and predict metagenomic gene content of bacterial communities of two LMA sponges [*Stylissa carteri* (Dendy, 1889) and *Stylissa massa* (Carter, 1887)], one well known HMA [*Xestospongia testudinaria* (Lamarck, 1815)], one putative HMA sponge [*Aaptos lobata* (Calcini et al, 2017)] and non-host biotopes (sediment and seawater) in a coral reef ecosystem around Tioman Island. Our results highlight the influence of HMA/LMA dichotomy on sponge microbiome putative function.

## Materials and methods

### Study site and sampling

All the samples were taken from Tioman Island, an island located in the South China Sea, 40 km off the southeast coast of Peninsular Malaysia in the state of Pahang. Along with nine other islands, Tioman Island forms the Palau Tioman Marine Park; whose coral reefs are part of the "Coral triangle", an area identified as having the highest diversity of coral species anywhere in the world (Area, A. M. P. EXPEDITION REPORT).

Three samples of each sponge species, sediment and seawater were collected around Tioman Island using SCUBA diving in August 2013, near Tunamaya beach (02°43'12.4"N 104°09'07.7"E), in the area of Soyak island (02°52'24.1"N 104°08'52.7"E), in Chebeh island (02°56'04.3"N 104°05'50.8"E) and near the Bahara Rocks (02°43'17.8"N 104°10'53.4"E). The sponge species *S. carteri, S. massa* (order Halichondrida: family Dictyonellidae), *A. lobata* (order Hadromerida: family Suberitidae) and *X. testudinaria* (order Haplosclerida: family Petrosiidae) were collected at 12 - 18 m depth. Sediment samples were taken using

mini-cores (a plastic disposable syringe from which the end has been cut to facilitate sampling; Capone et al., 1992; Cleary et al., 2015; Polónia et al., 2015). Seawater samples (1 l) were filtered through a Millipore® White Isopore Membrane Filter (0.22 µm pore size) (Bowen et al., 2012). After sampling, all samples were stored in 96% EtOH and kept at temperatures lower than 4 °C. In the laboratory, all the samples were stored at -20 ºC until DNA extraction.

**DNA extraction and pyrosequencing**

Total genomic DNA was extracted from seawater (membrane filter) and 0.5 g of sediment and sponge tissue with the FastDNA® SPIN Kit for soil (MP Biomedicals), following the manufacturer's instructions. This extraction method has been frequently used for DNA extraction from sponge samples (Cleary et al., 2013; Polónia et al., 2014; Polónia et al., 2015; Polónia et al., 2017; Cleary et al., 2018; Pires et al., 2019). Briefly, samples were transferred to Lysing Matrix E tubes containing a mixture of ceramic and silica particles and cell lysis was performed using the FastPrep® Instrument (Q Biogene) for 40 s at speed 6.0 m/s twice. Extracted DNA was eluted to a final volume of 50 µl and stored at -20 ºC until use. Prior to pyrosequencing, the amplicons of the bacterial 16S rRNA gene were amplified by PCR using the specific primers 27F and 1494R (Gomes et al., 2001). Amplicons were sequenced using barcoded fusion primers with Roche-454 A Titanium sequencing adapters, a six-base barcode sequence, with 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/]). Barcoded pyrosequencing libraries were analysed using QIIME (Quantitative Insights Into Microbial Ecology; Caporaso et al., 2010) software package following previously described methods (Pires et al., 2012; Cleary et al., 2013; Polónia et al., 2014). 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) and 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 97%). Closely related taxa of numerically dominant OTUs (>100 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). DNA sequences generated in this study can be downloaded from the NCBI SRA: PRJNA327694.

**Statistical analysis**

A square matrix generated in QIIME was used as input for further analysis using the R package (R core Team, 2013). The table containing the counts of all OTUs per sample was used to assess the relative abundance of selected higher taxa, estimate richness and compare community composition. Significant differences in the relative abundance of selected higher taxa and dominance (the relative abundance of the most abundant OTU in each sample) among biotopes (sediment, seawater, *S. carteri*, *S. massa*, *X. testudinaria* and *A. lobata*) was tested with an analysis of deviance using the glm() function in R (R core Team, 2013). Because the data was 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 1, 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. A self-written function in R to estimate total rarefied OTU richness was used (Gomes et al., 2010). Variation in bacterial composition among biotopes was assessed with principal coordinates analysis (PCO) using the cmdscale() function in R on Bray-Curtis distance matrix obtained with the vegdist() function in vegan on log10 (x+1) transformed. Variation among biotopes was tested for significance using the adonis() function in vegan package. More details descriptions of the functions used here can be found in R (e.g. ?cmdscale) and online in the reference manuals (e.g. http://cran.r-project.org/web/packages/vegan/index.html).

**Predictive metagenome analysis**

PICRUSt was used to predict metagenome gene functional content of each sample, using 16S rRNA (Langille et al., 2013). We used linear discriminant analysis (LDA) effect size (LEfSe) method, where results are presented hierarchically using cladograms (LEfSe) and histograms (LDA scores) (Segata et al., 2011). The output table of PICRUSt contains the functional gene counts known as KEGG Orthologs (KOs), which are sets of homologous

sequences assigned to specific molecular functions. They were arranged hierarchically and grouped into biological pathways. In order to assess the variation in the distribution of KOs among biotopes, we performed a PCO using the cmdscale() function in R. In addition to this, we obtained weighted nearest sequenced taxon index (NSTI) scores for each sample, which provide the average branch length between a given OTU and a reference OTU (Cleary et al., 2013; Langille et al., 2013; Polónia et al., 2015).

## Results and Discussion

### Biotope bacterial community analysis

After quality control, OTU picking and removal of chimera, chloroplasts and mitochondria, a total of 112684 sequences were recovered, which were assigned to 3224 OTUs. In turn, those OTUs were assigned to 39 phyla, 95 classes and 128 orders. Bacterial richness was markedly higher in sediment and lowest in both *Stylissa* species (Fig. 4.1). On the basis of 800 sequences (based in the number of sequences in the least abundant sample Wt012), OTU richness varied from 352.48 ± 9.73 to 409.23 ± 10.68 in sediment, followed by seawater (from 118.29 ± 1.53 to 132.84 ± 4.56), *X. testudinaria* (from 114.47 ± 4.88 to 122.19 ± 4.42), *A. lobata* (from 97.79 ± 4.33 to 113.69 ± 4.78) and finally by *S. carteri* (from 25.07 ± 2.95 to 117.41 ± 7.31) and *S. massa* (from 27.48 ± 3.21 to 94.12 ± 6.38). PCO ordination (Fig. 4.2) showed significant compositional differences among biotopes ($F_{5,12} = 6.54$, $P < 0.001$, $R^2 = 0.731$), explaining 73% of the variation in bacterial composition. PCO ordination of the first two axes of variation showed four different clusters, one represented by *A. lobata* and *X. testudinaria* samples, another by sediment samples, a third cluster represented by both *Stylissa* species and a cluster represented by seawater samples close to the *Stylissa* spp. samples. Fig. 4.2 also showed that most dominant OTUs (> 100 sequences reads) were exclusively or mainly found in specific biotopes. Our results indicated that the bacterial communities of sponges were markedly different from the surrounding environment (sediment and seawater), highlighting their importance as reservoirs of microbial diversity in coral reef ecosystems (Thomas et al., 2016; Polónia et al., 2016; Cleary et al., 2018). The putative HMA *A. lobata* and the known HMA *X. testudinaria* hosted similar bacterial

communities (richness, OTU dominance and structural composition), therefore, supporting the initial indication that *A. lobata* is also an HMA sponge (Cleary et al., 2018). *Stylissa* species hosted the least diverse bacterial communities when compared with all other biotopes and showed higher similarity to seawater. Such a trend may be explained by their ability to filter larger amount of seawater than HMA sponges, which may result in the increase of bacterioplankton retention in their tissue (Cleary et al., 2015).

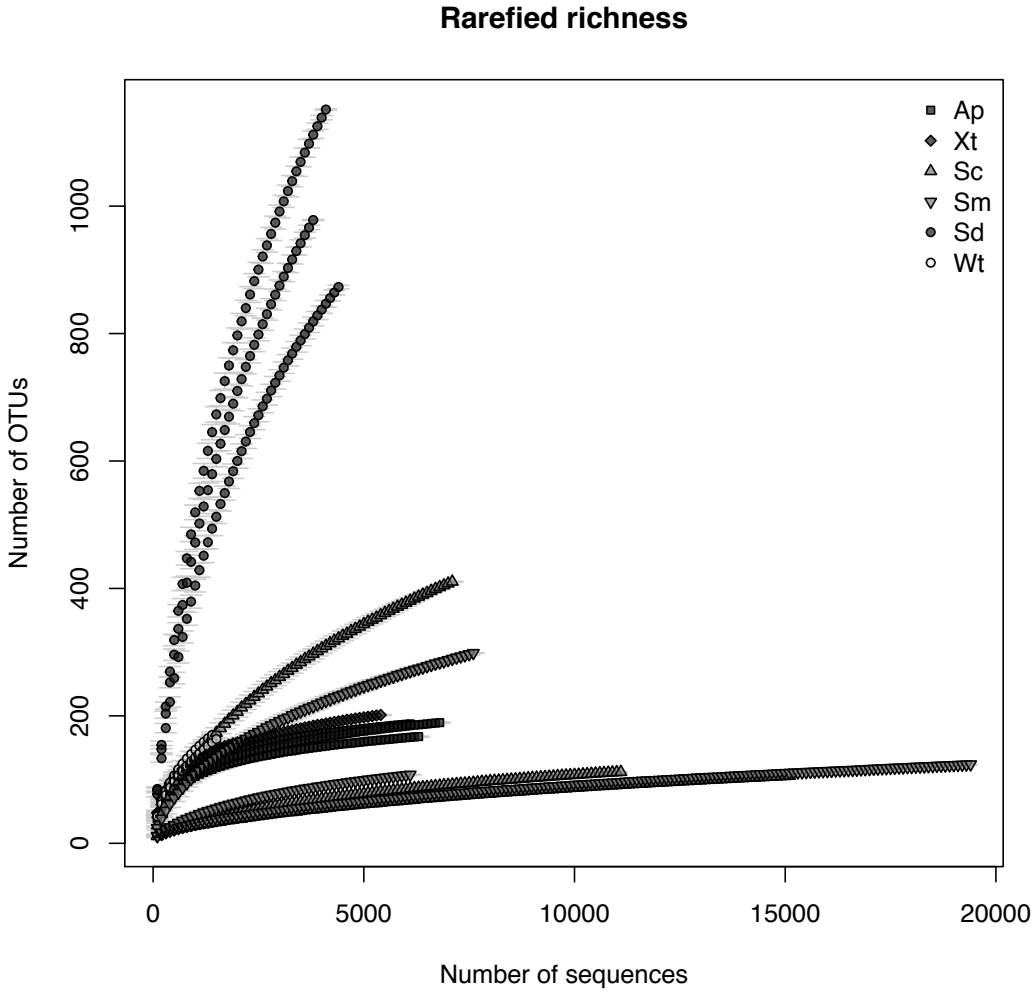


Fig. 4.1 – Species accumulation as a function of the number of sequences using resampling of bacterial 16S rRNA sequences from samples of *Aaptos lobata* (Ap), *Xestospongia testudinaria* (Xt), *Stylissa carteri* (Sc), *Stylissa massa* (Sm), sediment (Sd) and seawater (Wt) samples.

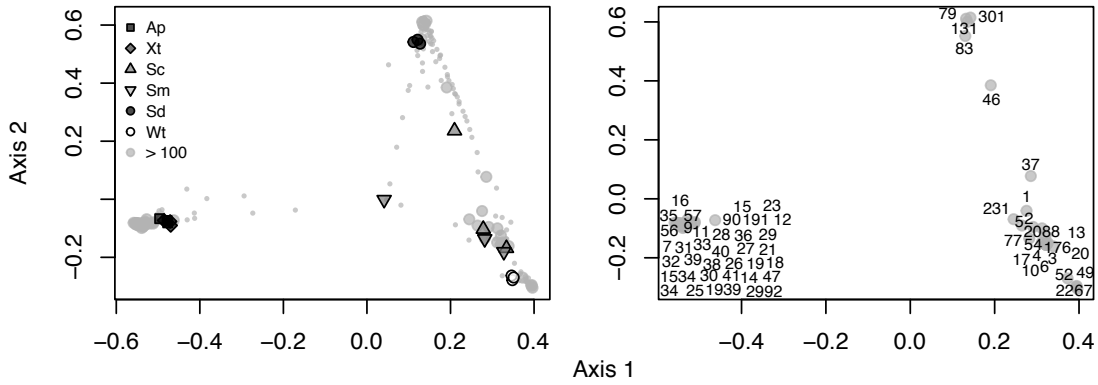


Fig. 4.2 – Principal coordinates analysis (PCO) showing the variation of bacterial community composition in different biotopes (first two axes). Symbols represent samples *Aaptos lobata* (Ap), *Xestospongia testudinaria* (Xt), *Stylissa carteri* (Sc), *Stylissa massa* (Sm), sediment (Sd) and seawater (Wt) samples. Numbers represent dominant OTUs (> 100 sequence reads).

The compositional analysis showed that at phylum level, Proteobacteria was abundant in all biotopes and by far the most abundant (relative abundance) phylum in *S. carteri*, seawater, sediment and *A. lobata* samples (86% ± 6.14, 80% ± 6.22, 60% ± 2.81 and 42% ± 1.52, respectively) (Fig. 4.3). On the other hand, Proteobacteria represented 36% ± 38.95 in *S. massa* and 26% ± 3.60 in *X. testudinaria*. The higher abundance of this phylum in *S. carteri* is in line with previous studies of this species in the Red Sea, Korea and Indonesia (Lee et al., 2011; Moitinho-Silva et al., 2013; Cleary et al., 2015; de Voogd et al., 2015; Jeong et al., 2015; Polónia et al., 2016; Cleary et al., 2018). Proteobacteria is an environmentally important group comprising key players in the process of carbon, sulphur and nitrogen cycles in marine sponges (Kersters et al., 2006; Bayer et al., 2008; Siegl et al., 2011; Webster and Taylor, 2012).

In general, Cyanobacteria were more abundant in the *Stylissa* species and seawater biotopes. However, this phylum was clearly more enriched in *S. massa* (60% ± 41.00). Chloroflexi was the most abundant phylum in *X. testudinaria* (39% ± 4.01), while it represented 16% ± 5.48 in *A. lobata* and < 1% in sediment, seawater and both *Stylissa* species (Fig. 4.3). In agreement with these findings, Schmitt et al. (2011) and Gloeckner et al. (2014) have shown that Chloroflexi is often one of the most abundant groups in HMA sponges. In general, due to the ability to convert inorganic into organic carbon, Chloroflexi may contribute to formation of microbial biomass that can be transferred to and metabolized by the host sponge (Brock et al., 1984; Brück et al., 2010). Thus, it can be assumed that Chloroflexi might play

an important role in nutrition of the sponge host. Actinobacteria abundance was much higher in *A. lobata* (27% ± 4.72) than in *X. testudinaria* (17% ± 2.02) and represented 20% ± 4.17 of sediment bacterial abundance. Actinobacteria are prolific producers of secondary metabolites and may play an important role in the chemical defence of the sponge host; studies aiming at untapping their diversity and biotechnological potential are of great interest (Hentschel et al., 2006; Lewin et al., 2016). Considering the biotechnological potential of the actinobacterial populations, the high dominance and diversity of Actinobacteria in *A. lobata* is interesting and deserves further study.

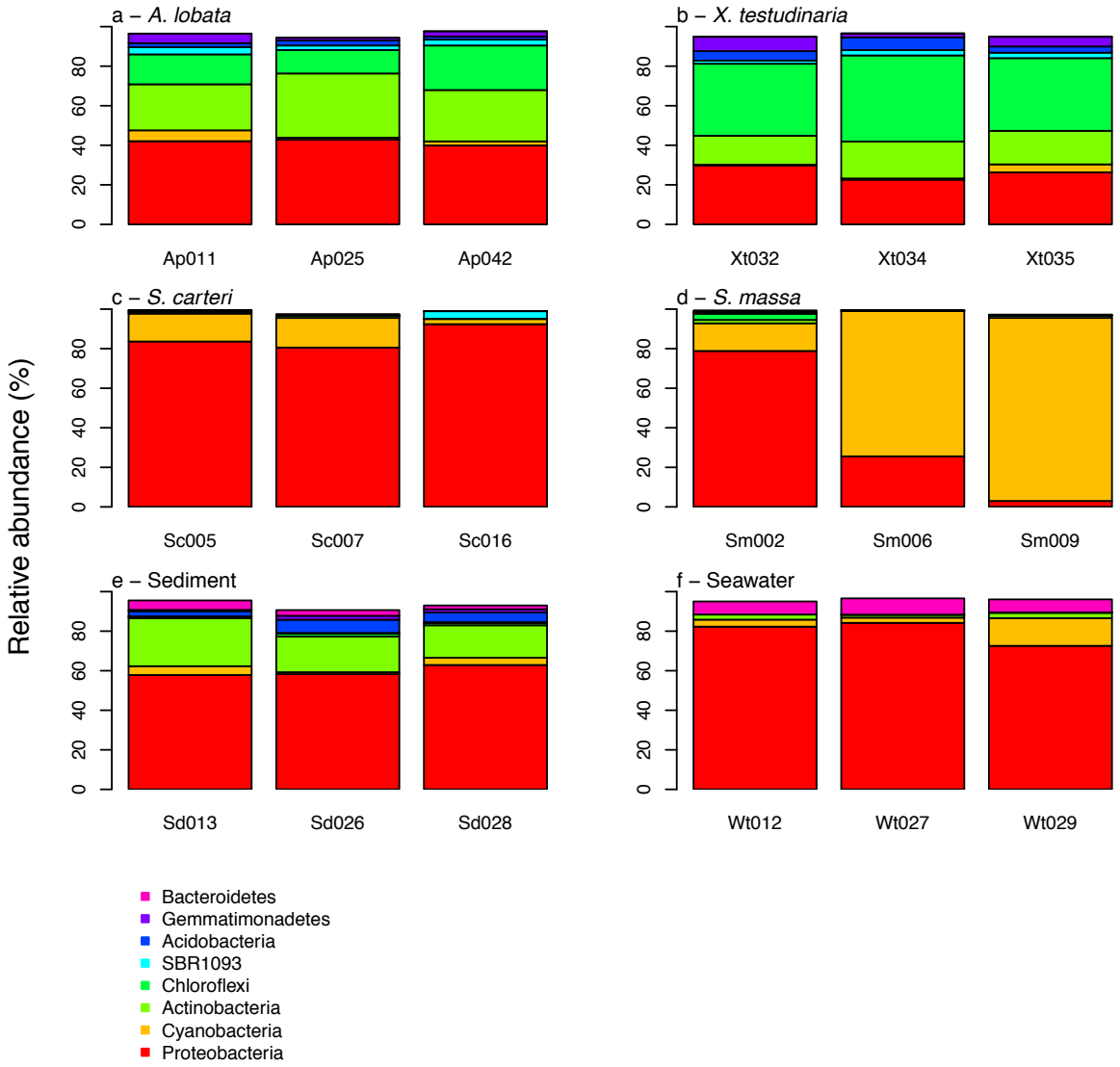


Fig. 4.3 – Relative abundance of the most dominant bacterial phyla in *Aaptos lobata* (*A. lobata*), *Xestospongia testudinaria* (*X. testudinaria*), *Stylissa carteri* (*S. carteri*), *Stylissa massa* (*S. massa*), sediment and seawater samples.

The compositional analysis of lower taxa (Fig. 4.4) showed that at class level Acidimicrobiia (Actinobacteria) was the most abundant in *A. lobata*, *X. testudinaria* and sediment. A specific enrichment of the classes SAR202 and Anaerolineae (both within Chloroflexi) was observed in *A. lobata* and *X. testudinaria* (Fig. 4.3). Members of the SAR202 cluster have been found in the deep terrestrial subsurface, soil, marine sponges, seawater and freshwater environments and in the deep-sea (Dunbar et al., 2002; Hentschel et al., 2002; Morris et al., 2004). Recently, Mehrshad et al. (2018) suggested that SAR202 are sulphite-oxidizers and, thus, have important roles in the sulphur cycle in the deep marine environment. Members of this Anaerolineae are strictly anaerobic organisms that have been found in anaerobic digesters, hot springs, artic permafrost, tropical marine sediment and the mammalian gastrointestinal tract (Hug et al., 20013; Campbell et al., 2014; Hanada, 2014). de Goeij et al. (2013) suggested that Anaerolineae might use sponges as a niche to access the high loss of their host's biomass, as they were identified as organic matter degraders (Hug et al., 2013). In contrast to the enrichment of SAR202 and Anaerolineae in HMA sponges, Synechococcophycideae (Cyanobacteria) was enriched in *S. massa*, which is in accordance with Cleary et al. (2015) and Coelho et al. (2018), however contradicting the de Voogd et al. (2015) study. *Synechococcus* is the type genus of Synechococcophycideae and along with *Prochlorococcus* are responsible for up to 50% of the carbon fixation in marine systems, representing the main photosynthetic bacteria in oceanic waters (Partensky et al., 1999). LMA sponges, such as *Stylissa* species can filter great amounts of seawater (Weisz et al., 2007), which can explain the presence of large numbers of OTUs assigned to the *Synechococcus* genus. However, despite the fact that *S. carteri* and seawater biotopes showed similar relative abundance values for Synechococcophycideae, this group was particularly enriched in *S. massa*. Erwin and Thacker (2008) and Freeman and Thacker (2011), suggested that members of this class may contribute to improve host sponge growth rates. Moreover, Gammaproteobacteria (Proteobacteria) were highly abundant in *S. carteri*, which is in agreement with Bayer et al. (2014) and Moitinho-Silva et al. (2013).

At the order level (Fig. 4.4) Chromatiales and Thiohalorhabdales (both within Gammaproteobacteria) were more abundant in both *Stylissa* species whilst Caldilineales (Chloroflexi) was the most abundant in *A. lobata* and *X. testudinaria*. In addition to this, Fig. 4.4 showed that *S. carteri* and *S. massa* were the biotopes with a higher percentage of dominant OTUs.

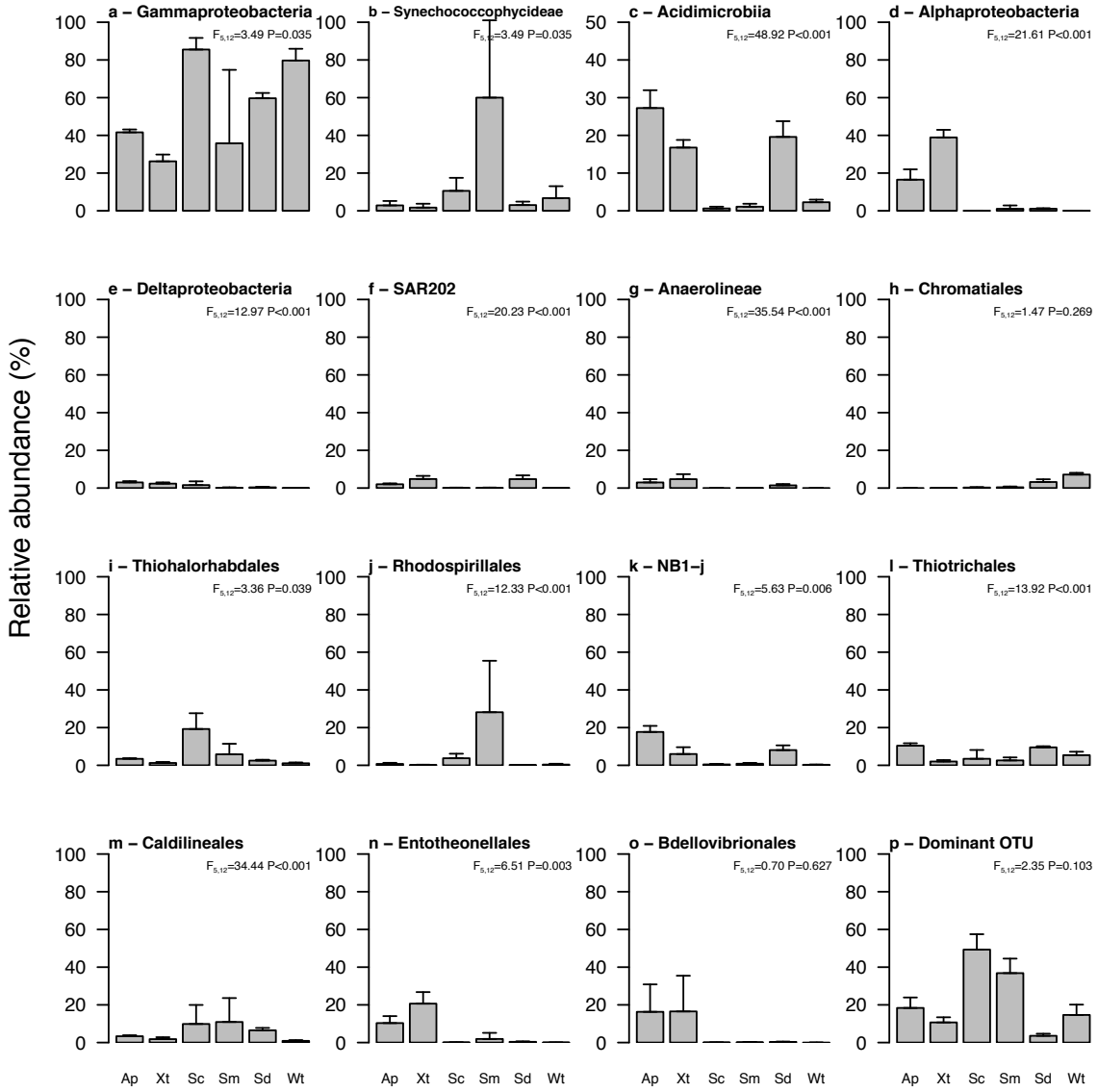


Fig. 4.4 – Relative abundance of the most abundant bacterial classes (a-g) and orders (h-o) and the most abundant OTU (p) for samples from *Aaptos lobata* (Ap), *Xestospongia testudinaria* (Xt), *Stylissa carteri* (Sc), *Stylissa massa* (Sm), sediment (Sd) and seawater (Wt) samples. Error bars represent a single standard deviation. The dominant OTU represents the mean abundance for the single most dominant OTU in each biotope, thus not necessarily the same OTU.

Members of Chromatiales, also known as purple sulphur bacteria, are frequently found associated with marine sponges and were suggested to play an important role in host survival due to their ability to perform the fixation of molecular nitrogen (Proctor, 1997; Imhoff, 2005). Sorokin et al. (2008) isolated Thiohalorhabdales from hypersaline lakes and considered them as sulphur oxidizing bacteria (SOB); more recently, members of this order were suggested to play a role in nitrogen cycling (Frank et al., 2016). Caldilineales is a non-

photosynthetic and ubiquitous class detected in soils (Breuker et al., 2011), marine sediments (Schmitt et al., 2011), activated sludge (Kindaichi et al., 2012), and marine animals, such as sponges (Schmitt et al., 2011; de Voogd et al., 2018; Pires et al., 2019).

**OTU composition analysis**

An in-depth analysis of bacterial composition detected 109 abundant OTUs (> 100 sequence reads) (Table S4.1). Where, 44 OTUs were identified in *S. carteri*, 67 in *S. massa*, 66 in *A. lobata*, 65 in *X. testudinaria*, 36 in sediment and 19 in seawater (Table S4.1). Eighteen had an abundance higher than 800 sequences, of which OTUs 1 (18582 sequences) and 2 (10321 sequences) were restricted to both *Stylissa* hosts. The closest relative of OTU-1 (order Chromatiales) was an uncultured bacterium previously detected in the sponge *Axinella* sp. (Acc KJ007847), OTU-2 (order Thiohalorhabdales) was closely related to an uncultured bacterium detected in the sponge *Phakellia fusca* (Acc HQ877729), both from China. Overall, most OTUs detected in both *Stylissa* species were assigned to the orders Chromatiales and Thiohalorhabdales, with higher abundance in *S. carteri*.

Twelve of the 109 abundant OTUs were restricted to specific biotope (Table S4.1); 2 (OTUs 42 and 45) were exclusively found in *S. carteri*, 5 (OTUs 6, 10, 17, 20 and 53) in *S. massa*, 3 (OTUs 38, 61 and 2280) in *A. lobata* and 2 (OTUs 301 and 2460) in sediment, whilst no abundant OTU was exclusively found in seawater and *X. testudinaria*. Thus, in accordance with Cleary et al. (2019), this study shows that most of the abundant OTUs are shared by sponges and the surrounding environment (sediment and/or seawater).

OTUs 20, 42 and 45 were closely related to an uncultured deltaproteobacterium (Acc JN596600) collected from the sponge *Xestospongia muta* from the Key Largo in Florida (USA) and two uncultured bacteria (Acc HQ877728 and JQ359622) detected in the sponges *P. fusca* from the Red Sea and *X. muta* from the Bahamas, respectively (Table S4.1). All three OTUs were assigned to the genus *Bdellovibrio*, whose members were described as a parasite of cyanobacteria associated with coral reef sponges (Wilkinson, 1979 and the references therein). Of the 109 abundant OTUs, only OTUs 20, 42 and 45 were assigned to

the genus *Bdellovibrio*. The high abundance of those OTUs may be due to the higher abundance of Cyanobacteria in both *Stylissa* species.

OTUs 6, 17 and 61 were assigned to the order Chromatiales and their closest relatives were an uncultured prokaryote (Acc KT979752) detected in the marine endolithic community of Mono Island from Puerto Rico and an uncultured bacterium (Acc JX455299) detected in the sponge *Cymbastella coralliophila* from the Orpheus Island GBR (Australia), respectively (Table S4.1). As described above, members of this order are frequently associated with marine sponges and were suggested as important players in sponge survival (Proctor, 1997; Imhoff, 2005).

OTU-10 was assigned to the family Entotheonellaceae (order Entotheonellales) and was closely related to an uncultured bacterium (Acc JF809701) detected in Medea hypersaline basin from the Mediterranean Sea (Table S4.1). According to Wilson et al. (2014), Entotheonellales bacterial lineages are commonly distributed in marine sponges and are able to produce chemically diverse bioactive products of medical interest.

OTU-38 was classified as Gemm-4 class (phylum Gemmatimonadetes), whose closest relative was an uncultured Gemmatimonadetes (Acc JN210644) detected in the sponge *Rhopaloeides odorabile* from the Gulf of Mexico (Table S4.1). Zhang et al. (2003) described Gemmatimonadetes as a hypersaline microbial phylum involved in biogeochemical transformations.

OTU-53 was assigned to the family Rhodospirillaceae and was closely related to an uncultured bacterium (Acc JN410047) detected in water from Puerto Rico (Table S4.1). Members of this family are known as purple non-sulphur bacteria with diverse metabolic and nutritional properties, which are able to grow heterotrophically, photoheterotrophically under anoxic conditions in the light and chemoheterotrophically in the dark (Baldani et al., 2014).

OTU-2460 was assigned to the family Piscirickettsiaceae and its closest relative was an uncultured bacterium (Acc GQ412880) detected in marine sediment from the Medas Islands, Mediterranean Sea (Table S4.1). This family includes chemoorganotrophs, methylotrophs, and chemolithotrophic sulphur-oxidizers organisms (Garrity et al., 2005).

OTUs 301 and 2280 were assigned to the order Acidimicrobiales (Actinobacteria) and were closely related to an uncultured *Ilumatobacter* sp. (Acc KC817043) detected in marine sediment from the Red Sea, Dahab (Egypt) and an uncultured Acidimicrobidae (Acc

JN210648) detected in the sponge *R. odorabile* from Bolinao (Philippines), respectively (Table S4.1). The order Acidimicrobiales comprises organisms involved in ferrous iron oxidation, sulfur oxidation and ferric iron reduction (Hardoim et al., 2012). Of all the abundant OTUs assigned to Actinobacteria (all belonged to Acidimicrobiales order), only OTUs 301 and 2280 were restricted to one biotope.

Phylogenetic analysis of the abundant OTUs classified as Actinobacteria (Fig. S4.1), revealed a clear cluster formed by OTUs 50, 91, 129 and 301, all belonging to the family C111 and restricted or mainly found in sediment samples. Urbach et al. (2001) suggested that members of this family may be involved in carbon and nutrient recycling. Besides these, OTUs 79 (family koll13), 88 (unassigned family) and 164 (JdFBGBact) were also mainly detected in sediment samples. With the exception of OTU-77 (family OCS155) that was mainly detected in both *Stylissa* hosts, all other abundant OTUs (OTUs 7, 11, 47, 57, 58 and 2280) classified as Actinobacteria were restricted or mainly detected in *A. lobata* and *X. testudinaria*. Of these, OTUs 47 and 2280 clustered together and were closely related to two uncultured Acidimicrobidae bacteria previously detected in the sponges *X. testudinaria* from Manado (Indonesia) and *R. odorabile* from the Rib Reef GBR (Australia) (Table S4.1), which might indicate that both OTUs represent a sponge-specific actinobacterial cluster. According to Hentschel et al. (2006), since Actinobacteria are prolific producers of secondary metabolites their identification is of great interest in marine sponges. Despite this, their ecological functions in association with such hosts have yet to be elucidated (Webster and Taylor, 2012). Lewin et al. (2016) suggested that Actinobacteria act in mutualistic defence of marine sponges as has been demonstrated for several other eukaryotic hosts.

**Predictive functional analysis**

In order to analyse the functional gene content of each sample, functional profiles were predicted using PICRUSt software. The analysis of the availability of nearby genome representatives for the samples studied here resulted in NSTI values of 0.18 ± 0.01 for sediment, 0.28 ± 0.01 for *X. testudinaria*, 0.23 ± 0.01 for *A. lobata*, 0.14 ± 0.04 for *S. carteri*, 0.08 ± 0.07 for *S. massa* and 0.14 ± 0.00 for seawater. Low NSTI values (roughly <0.06) indicate that sequences obtained are closely related with reference genomes. The highest NSTI values (~ >0.15) might be due to unclassified OTUs at the order level or assigned to

barely known taxa (e.g. orders NB1-j, classes EC214 and SAR202, families OCS155, PAUC26f and HTCC2089).

PCO ordination of bacterial KO composition (Fig. 4.5) showed significant differences among biotopes explaining almost 80% ($F_{5,12} = 9.38$, $P < 0.001$, $R^2 = 0.796$) of the variation. Axis 1 separated host (all sponge biotopes) from non-host biotopes (sediment and seawater), while axis 2 separated both *Stylissa* species (LMA) from all other biotopes. HMA sponges (*X. testudinaria* and putative HMA *A. lobata*) formed a clear cluster together. In contrast to the ordination of the bacterial communities (Fig. 4.2), seawater showed low similarity to *Stylissa* species and was similar to the sediment biotope (Fig. 4.5).

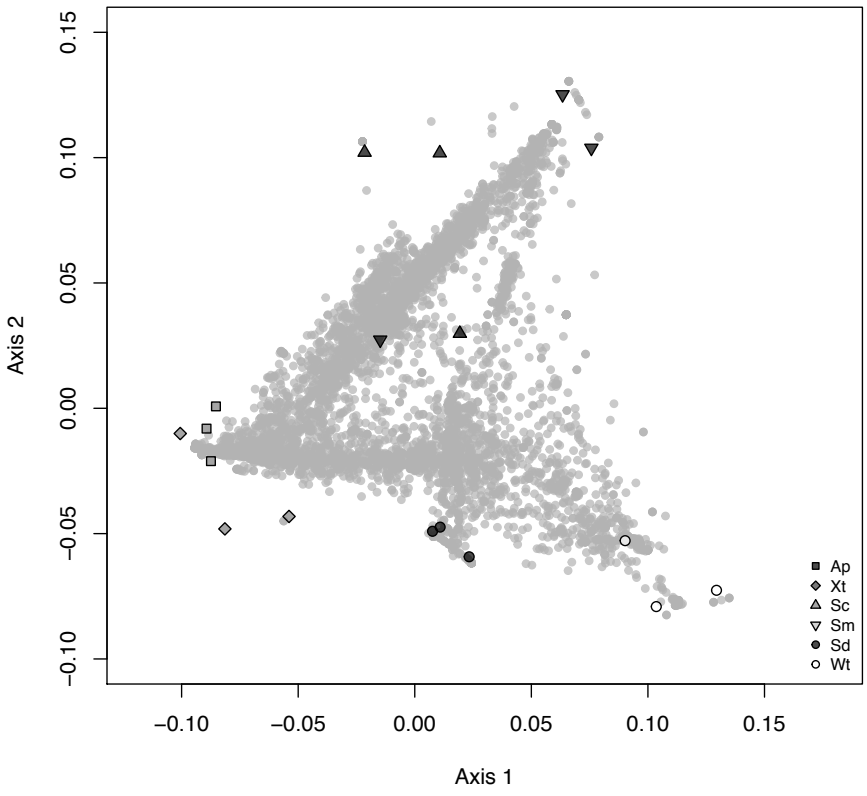


Fig. 4.5 – Principal coordinates analysis (PCO) showing the variation of bacterial KO composition in different biotopes (first two axes). Symbols represent samples *Aaptos lobata* (Ap), *Xestospongia testudinaria* (Xt), *Stylissa carteri* (Sc), *Stylissa massa* (Sm), sediment (Sd) and seawater (Wt) samples.

In line with the PCO analysis, LEfSe (Fig. 4.6) revealed significant differences in the relative abundance of bacterial functional categories and subcategories. At KEGG level 2 category, LEfSe showed that seawater was enriched in "Cardiovascular Diseases", "Infection Diseases", "Amino Acid Metabolism" and "Glycan Biosynthesis and Metabolism"

subcategories whilst sediment was enriched in "Transcription" and "Lipid Metabolism" subcategories. In HMA sponges, "Signaling Molecules and Interaction", "Carbohydrate Metabolism" and "Excretory System" were differentially enriched. In LMA we detected 9 differentially enriched subcategories. These included "Folding, Sorting and Degradation", "Replication and Repair", "Translation", "Energy Metabolism", "Metabolism of Cofactors and Vitamins", "Environmental Adaptation", "Immune System", "Genetic information processing" and "Metabolism".

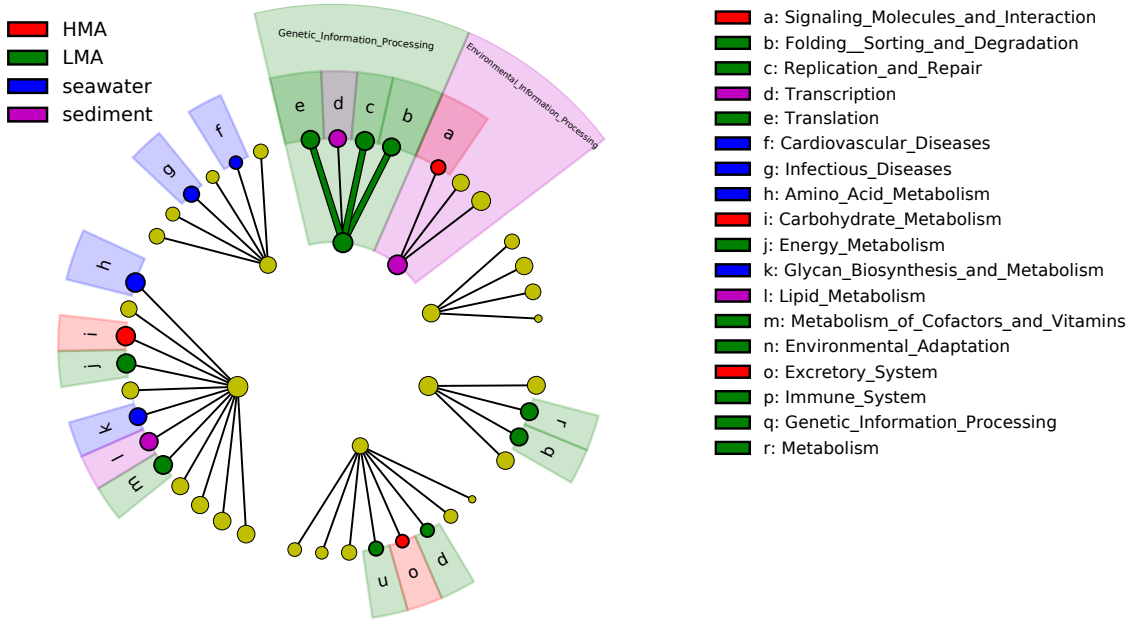


Fig. 4.6 – Cladogram generated using LEfSe showing significant differences in KEGG categories and subcategories between the bacterial communities of HMA (*Aaptos lobata* and *Xestospongia testudinaria*) and LMA (*Stylissa carteri* and *Stylissa massa*) sponges, seawater and sediment. KEGG categories and subcategories were coloured as follow: red indicates significant enrichment in HMA (*A. lobata* and *X. testudinaria*) sponges; green indicates significant enrichment in LMA (*S. carteri* and *S. massa*) sponges; blue indicates significant enrichment in seawater; purple indicates significant enrichment in sediment; gold indicates no significant difference between biotopes.

In HMA sponges, the enrichment in level 2 KEGG category "Signaling Molecules and Interaction" was related with "bacterial toxins" level 3 KEGG pathway (Fig. S4.2). Overall, these results are consistent with a recent metagenomic analysis of a sponge microbiome that found a significant enrichment of genes related to bacterial defence, including toxin-antitoxin systems (Slaby et al., 2017). The authors suggested that toxin-antitoxin systems are possible defence systems against the continuous exposure to free DNA from the sponge

filtration and phagocytosis activity. "Carbohydrate Metabolism" was also significantly enriched in HMA sponges. This subcategory comprises diverse processes involved in the formation, breakdown and interconversion of carbohydrates. Bayer et al. (2018) suggested that members of Anaerolinae and Caldilineae (Chloroflexi phylum) are metabolically specialized in carbohydrate uptake and degradation. The enrichment of "Carbohydrate Metabolism" subcategory could be related to the high abundance of Chloroflexi in HMA sponges (Schmitt et al., 2011; Gloeckner et al., 2014). HMA sponges were also enriched in other individual pathways such as "chlorocyclohexane and chlorobenzene degradation" and "xylene degradation" (Fig. S4.2), both within "Xenobiotics Biodegradation and Metabolism" subcategory. In line with previous studies, these results indicate that this sponge species may play a role in xenobiotics degradation in coral reef ecosystems (Polónia et al., 2014; Polónia et al., 2017; Polónia et al., 2018). Enrichment of these pathways in the *X. testudinaria* associated prokaryotic community appears to be a consistent trend, detected in members of this species in different geographical areas.

Regarding LMA sponges, the enrichment in "Replication and Repair" mechanisms is also noteworthy. In a previous work, we found that the associated bacterial community of the LMA sponge *Hymeniacidon* sp. retrieved from a hydrothermal vent was also enriched in "Replication and Repair" orthologs. We suggested that this could be related to the harsh conditions of this species' habitat and may enable the host to deal with the effects of environmental disturbance (Coelho et al., 2018). Other differentially abundant subcategories in LMA sponges included "Energy Metabolism" and "Metabolism of Cofactors and Vitamins". The high abundance of Cyanobacteria in LMA sponges (mainly in *S. massa*) could be responsible for the higher abundances of the "Energy Metabolism" subcategory which includes "carbon fixation in photosynthetic organisms", "photosynthesis", "photosynthesis - antenna proteins" and "photosynthesis proteins" pathways (Fig. S4.2). The enrichment of "Metabolism of Cofactors and Vitamins" subcategory was probably related to the fact that sponges retrieve the needed vitamins through their microbial symbionts, since animals are unable to synthesize essential vitamins (Webster and Thomas, 2016 and the references therein). Moreover, LMA sponges were enriched in "atrazine degradation" pathway (Fig. S4.2), within "Xenobiotics Biodegradation and Metabolism" subcategory. The enrichment of this pathway in LMA sponges could be explained by the high abundance

of Cyanobacteria in these hosts. Atrazine is a selective herbicide environmentally prevalent and persistent used to control broadleaf and grassy weeds by photosystem II inhibition, that leads to physiological alterations in cyanobacteria, as lower growth and greening effect (Cohen et al., 1984; Koenig, 2001). Polónia et al. (2014) also detected a significant enrichment of "atrazine degradation" pathway in prokaryotic community associated with *S. massa* from the Kepulauan Seribu Reef System (Indonesia). Along with *X. testudinaria*, *S. massa* also seems to play a role in xenobiotics degradation.

Overall and corroborating other studies, our results showed that HMA and LMA sponges shared some core functional features (Fan et al., 2012; Bayer et al., 2014), although with different relative abundances. And, as observed, both HMA and LMA sponges were enriched with different pathways involved in defence as well as in pathways involved in energy balance, suggesting that HMA and LMA sponges use different strategies to defend themselves against pathogens and to obtain energy. The reason for such differences among HMA and LMA sponges, however, remains elusive (Webster and Thomas, 2016).

## Conclusion

Our study provided an in-depth analysis of bacterial communities inhabiting four sponge species (*A. lobata*, *S. carteri*, *S. massa* and *X. testudinaria*) and two non-host biotopes (sediment and seawater) in Tioman coral reef ecosystem. We showed that bacterial community richness, composition and abundance are affected by different types of biotope. In addition to this, our data showed that LMA sponges (*S. carteri* and *S. massa*) host significantly distinct communities when compared to HMA sponges (*A. lobata* and *X. testudinaria*) and that bacterial communities associated with sponges differed from the surrounding environment (sediment and seawater). Furthermore, our results also showed the same trend for KO compositional analysis. This study also corroborated previous studies showing that bacterial symbionts with distinct structural composition associated to different sponge species had similar core metabolic profiles. The differential enrichment in pathways involved in host defence, energy balance or DNA repair, suggests that HMA and LMA

bacterial communities use different strategies to deal with pathogens, to obtain energy or to improve their resilience against environmental stress.

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**Supporting Information**

Bacterial composition and putative functions associated with different sponge species, sediment and seawater in Tioman coral reef system, Peninsular Malaysia

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(for submission)

Table S4.1 – List of the most abundant OTUs (> 100 sequence reads) and closely related organisms identified using BLAST search. OTU: OTU number; Sum: number of sequence reads; Biotope: biotope or biotopes where the OTUs were found, with the respective number of sequence reads per biotope; Accession: accession number of closely related organisms identified using BLAST; Sq ident: sequence similarity of these organisms with our representative OTU sequences; Source: isolation source of organisms identified using BLAST; Location: sampling location of organisms identified using BLAST.

| OTU | Sum | Biotope | | | | | | Phylum | Class | Order | Family | Accession | Sq ident | Organism | Source | Location |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Sc | Sm | Ap | Xt | Sd | Wt | | | | | | | | | |
| 1 | 18582 | 15427 | 3155 | 0 | 0 | 0 | 0 | Proteobacteria | Gammaproteobacteria | Chromatiales | Unassigned | KJ007847 | 100 | Uncultured bacterium | *Axinella* sp. | China |
| 2 | 10321 | 8953 | 1368 | 0 | 0 | 0 | 0 | Proteobacteria | Gammaproteobacteria | Thiohalorhabdales | Unassigned | HQ877729 | 100 | Uncultured bacterium | *Phakellia fusca* | China: South China Sea around Xisha Yongxing Island |
| 3 | 9654 | 1381 | 8157 | 55 | 29 | 28 | 4 | Cyanobacteria | Synechococcophycideae | Synechococcales | Synechococcaceae | KX581285 | 100 | Uncultured *Synechococcus* sp. | Arabian Sea-Open ocean region-Cochin Transect | India: Kerala |
| 54 | 7907 | 747 | 6882 | 11 | 20 | 25 | 222 | Cyanobacteria | Synechococcophycideae | Synechococcales | Synechococcaceae | AY125383 | 100 | Uncultured *Synechococcus* sp. | oceanic waters | Arabian Sea |
| 4 | 5333 | 404 | 4911 | 3 | 6 | 7 | 2 | Cyanobacteria | Synechococcophycideae | Synechococcales | Synechococcaceae | KP638857 | 100 | Uncultured bacterium | water | East China Sea |
| 7 | 4603 | 3 | 8 | 3566 | 1021 | 5 | 0 | Actinobacteria | Acidimicrobiia | Acidimicrobiales | wb1_P06 | KT974046 | 100 | Uncultured prokaryote | marine endolithic community of Mono Island | Puerto Rico |
| 5 | 2432 | 1875 | 557 | 0 | 0 | 0 | 0 | Proteobacteria | Deltaproteobacteria | NB1-j | NB1-i | JQ062836 | 96 | Uncultured bacterium | *Stylissa carteri* | Saudi Arabia |
| 6 | 1945 | 0 | 1945 | 0 | 0 | 0 | 0 | Proteobacteria | Gammaproteobacteria | Chromatiales | Unassigned | KT979752 | 96 | Uncultured prokaryote | marine endolithic community of Mono Island | Puerto Rico |
| 9 | 1647 | 0 | 27 | 1310 | 308 | 2 | 0 | Proteobacteria | Gammaproteobacteria | Thiotrichales | Piscirickettsiaceae | KT974066 | 100 | Uncultured prokaryote | marine endolithic community of Mono Island | Puerto Rico |
| 18 | 1335 | 0 | 0 | 539 | 796 | 0 | 0 | Chloroflexi | SAR202 | Unassigned | Unassigned | FJ481364 | 95 | Uncultured *Chloroflexus* sp. | *Xestospongia muta* | USA: Key Largo, Florida |
| 10 | 1256 | 0 | 1256 | 0 | 0 | 0 | 0 | Proteobacteria | Deltaproteobacteria | Entotheonellales | Entotheonellaceae | JF809701 | 95 | Uncultured bacterium | Medea hypersaline basin, Mediterranean Sea | |
| 11 | 1212 | 3 | 0 | 908 | 301 | 0 | 0 | Actinobacteria | Acidimicrobiia | Acidimicrobiales | TK06 | KF286009 | 100 | Uncultured actinobacterium | *Aplysina cauliformis* | Belize: Carrie Bow Cay |
| 12 | 1141 | 0 | 0 | 157 | 978 | 6 | 0 | Chloroflexi | Anaerolineae | Caldilineales | Caldilineaceae | FJ481369 | 100 | Uncultured *Chloroflexus* sp. | *Xestospongia muta* | USA: Key Largo, Florida |
| 23 | 879 | 0 | 19 | 712 | 147 | 1 | 0 | Proteobacteria | Alphaproteobacteria | Rhodospirillales | Rhodospirillaceae | KF585152 | 100 | Uncultured bacterium | *Holoxea* sp. | Yongxing Island, South China Sea |
| 191 | 874 | 2 | 0 | 871 | 1 | 0 | 0 | Chloroflexi | Anaerolineae | Caldilineales | Caldilineaceae | KF286001 | 100 | Uncultured Chloroflexi | *Aplysina cauliformis* | Belize: Carrie Bow Cay |
| 29 | 817 | 0 | 44 | 302 | 469 | 2 | 0 | Proteobacteria | Gammaproteobacteria | Chromatiales | Ectothiorhodospiraceae | JN210811 | 100 | Uncultured *Nitrosococcus* sp. | *Rhopaloeides odorabile* | Australia: Rib Reef, Great Barrier Reef, Queensland |
| 15 | 816 | 0 | 4 | 568 | 244 | 0 | 0 | SBR1093 | EC214 | Unassigned | Unassigned | HQ270374 | 100 | Uncultured Desulfovibrionales | *Xestospongia testudinaria* | Indonesia: Manado |
| 14 | 803 | 0 | 13 | 681 | 109 | 0 | 0 | Proteobacteria | Alphaproteobacteria | Rhodospirillales | Rhodospirillaceae | HE985126 | 100 | Uncultured bacterium | *Astrosclera willeyana* | Australia:Great Barrier Reef, Yonge Reef |
| 20 | 729 | 0 | 729 | 0 | 0 | 0 | 0 | Proteobacteria | Deltaproteobacteria | Bdellovibrionales | Bdellovibrionaceae | JN596600 | 96 | Uncultured delta proteobacterium | *Xestospongia muta* | USA: Key Largo, Florida |
| 13 | 681 | 665 | 16 | 0 | 0 | 0 | 0 | SBR1093 | EC214 | Unassigned | Unassigned | EF092164 | 97 | Uncultured Desulfohalobiaceae | *Axinella corrugata* | |
| 176 | 661 | 75 | 540 | 1 | 1 | 0 | 44 | Cyanobacteria | Synechococcophycideae | Synechococcales | Synechococcaceae | KT361396 | 100 | Uncultured *Synechococcus* sp. | sea water | Arabian Sea |
| 19 | 638 | 6 | 0 | 217 | 415 | 0 | 0 | Acidobacteria | Acidobacteria-6 | BPC015 | Unassigned | FJ269344 | 100 | Uncultured Acidobacteria | *Xestospongia testudinaria* | Indonesia: Manado |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 17 | 633 | 0 | 633 | 0 | 0 | 0 | 0 | Proteobacteria | Gammaproteobacteria | Chromatiales | Unassigned | JX455299 | 95 | Uncultured bacterium | *Cymbastella coralliophila* | Australia: Orpheus Island, Great Barrier Reef, Queensland |
| 2088 | 632 | 585 | 47 | 0 | 0 | 0 | 0 | Proteobacteria | Gammaproteobacteria | Chromatiales | Unassigned | JQ062818 | 100 | Uncultured bacterium | *Stylissa carteri* | Saudi Arabia |
| 16 | 561 | 21 | 0 | 468 | 72 | 0 | 0 | Cyanobacteria | Synechococcophycideae | Synechococcales | Synechococcaceae | JN596629 | 100 | Uncultured *Cyanobacterium* sp. | *Xestospongia muta* | USA: Key Largo, Florida |
| 21 | 548 | 0 | 7 | 97 | 444 | 0 | 0 | Chloroflexi | SAR202 | Unassigned | Unassigned | KF286187 | 100 | Uncultured Chloroflexi | *Aplysina cauliformis* | Belize: Carrie Bow Cay |
| 231 | 530 | 360 | 170 | 0 | 0 | 0 | 0 | Proteobacteria | Gammaproteobacteria | Thiohalorhabdales | Unassigned | HQ877729 | 100 | Uncultured bacterium | *Phakellia fusca* | China: South China Sea around Xisha Yongxing Island |
| 47 | 518 | 1 | 5 | 262 | 250 | 0 | 0 | Actinobacteria | Acidimicrobiia | Acidimicrobiales | Unassigned | JN596695 | 100 | Uncultured Acidimicrobidae | *Xestospongia testudinaria* | Indonesia: Manado |
| 27 | 455 | 0 | 0 | 453 | 2 | 0 | 0 | Proteobacteria | Gammaproteobacteria | Chromatiales | Unassigned | KF881019 | 100 | Uncultured Pseudoalteromonadaceae | *Spongia officinalis* | |
| 22 | 454 | 0 | 1 | 0 | 0 | 0 | 453 | Proteobacteria | Gammaproteobacteria | Pseudomonadales | Moraxellaceae | KY218847 | 100 | *Acinetobacter pittii* | International Space Station environmental microbiota | China |
| 52 | 442 | 26 | 64 | 0 | 0 | 0 | 352 | Proteobacteria | Alphaproteobacteria | Rickettsiales | Pelagibacteraceae | KU937451 | 100 | Uncultured marine bacterium | sea water Eastern Mediterranean Sea | |
| 2992 | 435 | 0 | 0 | 393 | 42 | 0 | 0 | Proteobacteria | Gammaproteobacteria | Chromatiales | Unassigned | HQ270412 | 100 | Uncultured gamma proteobacterium | *Xestospongia muta* | South of Thailand |
| 25 | 419 | 0 | 0 | 417 | 2 | 0 | 0 | Proteobacteria | Alphaproteobacteria | Unassigned | Unassigned | JN210813 | 100 | Uncultured *Rhodovulum* sp. | *Rhopaloeides odorabile* | Israel |
| 1939 | 363 | 0 | 0 | 10 | 353 | 0 | 0 | Chloroflexi | Anaerolineae | Caldilineales | Caldilineaceae | JN596707 | 100 | Uncultured Chloroflexi | *Xestospongia testudinaria* | USA: Key Largo, Florida |
| 26 | 352 | 0 | 24 | 275 | 53 | 0 | 0 | Proteobacteria | Alphaproteobacteria | Rhodospirillales | Rhodospirillaceae | JX280344 | 100 | Uncultured bacterium | *Ircinia felix* | Australia: Rib Reef, Great Barrier Reef, Queensland |
| 41 | 341 | 0 | 17 | 253 | 71 | 0 | 0 | Proteobacteria | Gammaproteobacteria | HTCC2188 | HTCC2089 | KT974009 | 100 | Uncultured prokaryote | marine endolithic community of Mono Island | Indonesia: Manado |
| 28 | 327 | 0 | 0 | 264 | 63 | 0 | 0 | Chloroflexi | Anaerolineae | Caldilineales | Caldilineaceae | JN596651 | 100 | Uncultured Chloroflexi | *Xestospongia testudinaria* | Bahamas:Exumas |
| 1534 | 327 | 0 | 0 | 258 | 69 | 0 | 0 | Proteobacteria | Gammaproteobacteria | Chromatiales | Unassigned | HQ270412 | 100 | Uncultured gamma proteobacterium | *Xestospongia muta* | Puerto Rico |
| 30 | 326 | 0 | 0 | 229 | 97 | 0 | 0 | Chloroflexi | TK17 | mle1-48 | Unassigned | KF286207 | 100 | Uncultured Chloroflexi | *Aplysina cauliformis* | Indonesia: Manado |
| 36 | 322 | 0 | 1 | 262 | 59 | 0 | 0 | Spirochaetes | Spirochaetes | Spirochaetales | Spirochaetaceae | JN596734 | 100 | Uncultured Spirochaetes | *Xestospongia testudinaria* | USA: Key Largo, Florida |
| 34 | 313 | 0 | 2 | 248 | 63 | 0 | 0 | Proteobacteria | Deltaproteobacteria | Entotheonellales | Unassigned | HG423455 | 100 | Uncultured bacterium | *Astrosclera willeyana* | Belize: Carrie Bow Cay |
| 83 | 311 | 5 | 30 | 0 | 0 | 276 | 0 | Proteobacteria | Alphaproteobacteria | Rhodospirillales | Rhodospirillaceae | KX035234 | 100 | Uncultured Rhodospirillaceae | rhizosphere soil Stellera chamaejasme | Indonesia: Manado |
| 301 | 292 | 0 | 0 | 0 | 0 | 292 | 0 | Actinobacteria | Acidimicrobiia | Acidimicrobiales | C111 | KC817043 | 100 | Uncultured *Ilumatobacter* sp. | marine sediment | Egypt:Red Sea, Dahab |
| 67 | 289 | 10 | 8 | 0 | 0 | 0 | 271 | Proteobacteria | Gammaproteobacteria | Oceanospirillales | Halomonadaceae | KF786856 | 100 | Uncultured gamma proteobacterium | oil sheen | Tibet |
| 131 | 283 | 2 | 8 | 0 | 0 | 273 | 0 | Proteobacteria | Gammaproteobacteria | Thiotrichales | Piscirickettsiaceae | KC009913 | 100 | Uncultured bacterium | shallow fluidized muds | Thailand: Bann-Klong-Ta-Guan, Rayong Province |
| 77 | 268 | 70 | 170 | 0 | 3 | 0 | 25 | Actinobacteria | Acidimicrobiia | Acidimicrobiales | OCS155 | KT318695 | 100 | Uncultured bacterium | ocean water | Gulf of Mexico |
| 32 | 262 | 0 | 9 | 239 | 14 | 0 | 0 | Proteobacteria | Deltaproteobacteria | Syntrophobacterales | Syntrophobacteraceae | KM389590 | 100 | Uncultured bacterium | *Plakortis halichondrioides* | French Guiana coast |
| 38 | 246 | 0 | 0 | 246 | 0 | 0 | 0 | Gemmatimonadetes | Gemm-4 | Unassigned | Unassigned | JN210644 | 100 | Uncultured Gemmatimonadetes | *Rhopaloeides odorabile* | Gulf of Mexico |
| 39 | 243 | 0 | 4 | 206 | 33 | 0 | 0 | Proteobacteria | Gammaproteobacteria | Chromatiales | Unassigned | JN596730 | 100 | Uncultured gamma proteobacterium | *Xestospongia testudinaria* | Bahamas |
| 31 | 240 | 0 | 0 | 208 | 32 | 0 | 0 | Proteobacteria | Gammaproteobacteria | Thiotrichales | Piscirickettsiaceae | HE817801 | 100 | Uncultured bacterium | *Vaceletia crypta* | Australia: Rib Reef, Great Barrier Reef, Queensland |
| 49 | 235 | 5 | 5 | 0 | 0 | 0 | 225 | Proteobacteria | Gammaproteobacteria | Oceanospirillales | Halomonadaceae | KF972687 | 100 | Uncultured bacterium | shrimp pond | Indonesia: Manado |
| 46 | 222 | 103 | 20 | 0 | 0 | 98 | 1 | Proteobacteria | Alphaproteobacteria | Rhizobiales | Phyllobacteriaceae | KR303588 | 100 | Uncultured bacterium | Sepiella maindroni (cuttlefish) | Australia:Great Barrier Reef, Yonge Reef |
| 37 | 218 | 43 | 118 | 0 | 0 | 57 | 0 | Firmicutes | Clostridia | Clostridiales | Clostridiaceae | LC020514 | 100 | *Clostridium* sp. | Biologically-disinfected soil incorporated with Brassica plants | |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 33 | 217 | 0 | 0 | 1 | 216 | 0 | 0 | Gemmatimonadetes | Gemm-4 | Unassigned | Unassigned | JN596602 | 100 | Uncultured Gemmatimonadetes | *Xestospongia muta* | |
| 56 | 216 | 0 | 0 | 169 | 47 | 0 | 0 | Proteobacteria | Gammaproteobacteria | HTCC2188 | HTCC2089 | KT976077 | 100 | Uncultured prokaryote | marine endolithic community of Mono Island | Japan: Tokushima |
| 79 | 213 | 0 | 3 | 0 | 0 | 210 | 0 | Actinobacteria | Acidimicrobiia | Acidimicrobiales | koll13 | KT973265 | 100 | Uncultured prokaryote | marine endolithic community of Mono Island | USA: Key Largo, Florida |
| 40 | 210 | 1 | 4 | 161 | 44 | 0 | 0 | Proteobacteria | Alphaproteobacteria | Rhodospirillales | Rhodospirillaceae | EF159799 | 100 | Uncultured bacterium | *Smenospongia aurea* | Puerto Rico |
| 90 | 207 | 0 | 31 | 101 | 75 | 0 | 0 | Chloroflexi | SAR202 | Unassigned | Unassigned | JN210622 | 100 | Uncultured Chloroflexi | *Rhopaloeides odorabile* | Puerto Rico |
| 57 | 204 | 0 | 0 | 130 | 73 | 1 | 0 | Actinobacteria | Acidimicrobiia | Acidimicrobiales | Unassigned | KF286119 | 100 | Uncultured bacterium | *Aplysina cauliformis* | USA: Key Largo, Florida |
| 35 | 203 | 0 | 0 | 132 | 70 | 1 | 0 | Acidobacteria | Solibacteres | Solibacterales | PAUC26f | JN596743 | 100 | Uncultured *Acidobacterium* sp. | *Xestospongia testudinaria* | Australia: Rib Reef, Great Barrier Reef, Queensland |
| 62 | 176 | 0 | 35 | 0 | 0 | 141 | 0 | Proteobacteria | Alphaproteobacteria | Rhodospirillales | Rhodospirillaceae | KJ007965 | 100 | Uncultured bacterium | *Terpios hoshinota* | Belize: Carrie Bow Cay |
| 58 | 175 | 0 | 0 | 132 | 43 | 0 | 0 | Actinobacteria | Acidimicrobiia | Acidimicrobiales | Unassigned | JN596670 | 100 | Uncultured Acidimicrobidae | *Xestospongia testudinaria* | Indonesia: Manado |
| 112 | 175 | 0 | 1 | 69 | 105 | 0 | 0 | Proteobacteria | Gammaproteobacteria | Chromatiales | Ectothiorhodospiraceae | EU816812 | 100 | Uncultured gamma proteobacterium | *Neofibularia nolitangere* | China |
| 63 | 163 | 1 | 9 | 75 | 78 | 0 | 0 | Proteobacteria | Gammaproteobacteria | Chromatiales | Ectothiorhodospiraceae | KM389592 | 100 | Uncultured bacterium | *Plakortis halichondrioides* | Indonesia: Manado |
| 43 | 161 | 2 | 0 | 0 | 0 | 159 | 0 | Proteobacteria | Alphaproteobacteria | Rhizobiales | Unassigned | DQ351777 | 100 | Uncultured alpha proteobacterium | marine sediments | Carribbean Sea |
| 45 | 160 | 160 | 0 | 0 | 0 | 0 | 0 | Proteobacteria | Deltaproteobacteria | Bdellovibrionales | Bdellovibrionaceae | JQ359622 | 94 | Uncultured bacterium | *Xestospongia muta* | Bahamas |
| 48 | 159 | 1 | 4 | 0 | 0 | 0 | 154 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | KX890260 | 100 | Uncultured Rhodobacteraceae | water | Belgian continental plate |
| 51 | 151 | 0 | 0 | 106 | 44 | 1 | 0 | Thermi | Deinococci | Deinococcales | Trueperaceae | HQ270413 | 100 | Uncultured *Truepera* sp. | *Xestospongia muta* | China: South China Sea, Yongxing Island |
| 86 | 149 | 0 | 11 | 62 | 76 | 0 | 0 | Chloroflexi | TK17 | TK18 | Unassigned | FJ481340 | 96 | Uncultured *Chloroflexus* sp. | *Xestospongia muta* | Arabian Sea-Shelf region-Cochin Transect |
| 106 | 149 | 82 | 7 | 0 | 0 | 59 | 1 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | KM079049 | 100 | Uncultured marine bacterium | *Porites* sp. | USA: Key Largo, Florida |
| 102 | 147 | 0 | 68 | 11 | 68 | 0 | 0 | Chloroflexi | SAR202 | Unassigned | Unassigned | JQ844425 | 100 | Uncultured bacterium | *Geodia barretti* | USA: Key Largo, Florida |
| 55 | 144 | 0 | 3 | 18 | 122 | 1 | 0 | Proteobacteria | Deltaproteobacteria | Syntrophobacterales | Syntrophobacteraceae | HQ270338 | 100 | Uncultured delta proteobacterium | *Xestospongia testudinaria* | Southern Taiwan |
| 129 | 143 | 4 | 0 | 0 | 0 | 139 | 0 | Actinobacteria | Acidimicrobiia | Acidimicrobiales | C111 | KR303486 | 100 | Uncultured bacterium | *Sepiella maindroni* | Norway: Korsfjord |
| 1742 | 141 | 42 | 84 | 1 | 0 | 1 | 13 | Cyanobacteria | Synechococcophycideae | Synechococcales | Synechococcaceae | KX581285 | 100 | Uncultured *Synechococcus* sp. | Arabian Sea-Open ocean region-Cochin Transect | Indonesia: Manado |
| 68 | 140 | 0 | 0 | 99 | 40 | 1 | 0 | Proteobacteria | Alphaproteobacteria | Rhodospirillales | Rhodospirillaceae | KT975541 | 100 | Uncultured prokaryote | marine endolithic community of Mono Island | |
| 70 | 140 | 2 | 1 | 0 | 0 | 136 | 1 | Proteobacteria | Alphaproteobacteria | Rhodospirillales | Rhodospirillaceae | FR851542 | 100 | Uncultured bacterium | permeable coral reef sands | India: Kerala |
| 74 | 137 | 0 | 1 | 46 | 90 | 0 | 0 | Chloroflexi | SAR202 | Unassigned | Unassigned | JN596681 | 100 | Uncultured Chloroflexi | *Xestospongia testudinaria* | Puerto Rico |
| 42 | 136 | 136 | 0 | 0 | 0 | 0 | 0 | Proteobacteria | Deltaproteobacteria | Bdellovibrionales | Bdellovibrionaceae | HQ877728 | 100 | Uncultured bacterium | *Phakellia fusca* | Red Sea |
| 50 | 136 | 3 | 0 | 0 | 0 | 133 | 0 | Actinobacteria | Acidimicrobiia | Acidimicrobiales | C111 | KT979339 | 100 | Uncultured prokaryote | marine endolithic community of Mono Island | Indonesia: Manado |
| 1703 | 136 | 5 | 130 | 0 | 0 | 0 | 1 | Cyanobacteria | Synechococcophycideae | Synechococcales | Synechococcaceae | JQ421031 | 100 | *Synechococcus* sp. | water | China: South China Sea around Xisha Yongxing Island |
| 53 | 135 | 0 | 135 | 0 | 0 | 0 | 0 | Proteobacteria | Alphaproteobacteria | Rhodospirillales | Rhodospirillaceae | JN410047 | 96 | Uncultured bacterium | water | Puerto Rico |
| 164 | 135 | 0 | 18 | 0 | 0 | 117 | 0 | Actinobacteria | Acidimicrobiia | Acidimicrobiales | JdFBGBact | KC817083 | 100 | Uncultured *Iamia* sp. | marine sediment | Sargasso Sea |
| 92 | 133 | 0 | 26 | 46 | 61 | 0 | 0 | Chloroflexi | SAR202 | Unassigned | Unassigned | KC200514 | 100 | Uncultured Chloroflexi | *Chondrosia reniformis* | Svalbard: Ny-Alesund, Spitsbergen |
| 103 | 133 | 0 | 6 | 75 | 52 | 0 | 0 | Chloroflexi | SAR202 | Unassigned | Unassigned | FJ900578 | 100 | Uncultured bacterium | *Ancorina alata* | Thailand: Bann-Klong-Ta-Guan, Rayong Province |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2460 | 132 | 0 | 0 | 0 | 0 | 132 | 0 | Proteobacteria | Gammaproteobacteria | Thiotrichales | Piscirickettsiaceae | GQ412880 | 100 | Uncultured bacterium | marine sediments | Mediterranean Sea: Medas Islands |
| 71 | 131 | 0 | 1 | 112 | 17 | 1 | 0 | Proteobacteria | Gammaproteobacteria | Chromatiales | Unassigned | JX280288 | 100 | Uncultured bacterium | *Ircinia felix* | New Zealand: northeastern, Jones Bay |
| 2280 | 127 | 0 | 0 | 127 | 0 | 0 | 0 | Actinobacteria | Acidimicrobiia | Acidimicrobiales | Unassigned | JN210648 | 100 | Uncultured Acidimicrobidae | *Rhopaloeides odorabile* | Philippines: Bolinao |
| 94 | 124 | 0 | 0 | 82 | 42 | 0 | 0 | Chloroflexi | Anaerolineae | Caldilineales | Caldilineaceae | JX280280 | 100 | Uncultured bacterium | *Ircinia strobilina* | Bahamas:Sweeting's Cay |
| 2479 | 124 | 0 | 0 | 0 | 122 | 2 | 0 | Proteobacteria | Deltaproteobacteria | Syntrophobacterales | Syntrophobacteraceae | HF912445 | 100 | *Alteromonas* sp. | *Isops phlegraei* | Australia: Rib Reef, Great Barrier Reef, Queensland |
| 59 | 119 | 0 | 0 | 20 | 98 | 1 | 0 | AncK6 | Unassigned | Unassigned | Unassigned | JX455625 | 100 | Uncultured bacterium | *Xestospongia testudinaria* | Bahamas:Sweeting's Cay |
| 100 | 119 | 15 | 13 | 0 | 1 | 0 | 90 | Proteobacteria | Gammaproteobacteria | Oceanospirillales | Halomonadaceae | KJ007869 | 100 | Uncultured bacterium | *Axinella* sp. | |
| 91 | 117 | 1 | 0 | 0 | 0 | 116 | 0 | Actinobacteria | Acidimicrobiia | Acidimicrobiales | C111 | DQ200570 | 100 | Uncultured actinobacterium | *Montastraea annularis* | Australia: Orpheus Island, Great Barrier Reef, Queensland |
| 88 | 115 | 0 | 0 | 0 | 1 | 114 | 0 | Actinobacteria | Acidimicrobiia | Acidimicrobiales | Unassigned | KX100064 | 100 | Uncultured actinobacterium | sediment | China |
| 61 | 114 | 0 | 0 | 114 | 0 | 0 | 0 | Proteobacteria | Gammaproteobacteria | Chromatiales | Unassigned | HQ270201 | 95 | Uncultured gamma proteobacterium | *Xestospongia testudinaria* | Netherlands: Antilles, Curacao |
| 64 | 113 | 0 | 21 | 0 | 88 | 4 | 0 | Proteobacteria | Gammaproteobacteria | Thiotrichales | Piscirickettsiaceae | JN596737 | 100 | Uncultured gamma proteobacterium | *Xestospongia testudinaria* | Arabian Sea, Cochin Transect |
| 85 | 113 | 0 | 2 | 53 | 58 | 0 | 0 | PAUC34f | Unassigned | Unassigned | Unassigned | JN596732 | 100 | Uncultured Deferribacteres | *Xestospongia testudinaria* | Indonesia: Manado |
| 283 | 111 | 2 | 5 | 0 | 0 | 0 | 104 | Proteobacteria | Alphaproteobacteria | Rickettsiales | Pelagibacteraceae | LN850157 | 100 | Candidatus *Pelagibacter* sp. | sea water | Indonesia: Manado |
| 98 | 110 | 0 | 0 | 92 | 18 | 0 | 0 | Gemmatimonadetes | Gemm-2 | Unassigned | Unassigned | HG764251 | 100 | Uncultured endophytic bacterium | *Holoxea* sp. | Indonesia: Manado |
| 138 | 110 | 0 | 13 | 26 | 71 | 0 | 0 | Chloroflexi | SAR202 | Unassigned | Unassigned | JN596744 | 97 | Uncultured Chloroflexi | *Xestospongia testudinaria* | Saudi Arabia:Farasan Banks |
| 111 | 109 | 0 | 0 | 37 | 72 | 0 | 0 | Proteobacteria | Gammaproteobacteria | HTCC2188 | HTCC2089 | JX206625 | 100 | Uncultured bacterium | *Ircinia oros* | China: Yongxing Island, South China Sea |
| 96 | 108 | 0 | 25 | 28 | 55 | 0 | 0 | Chloroflexi | SAR202 | Unassigned | Unassigned | KF286163 | 100 | Uncultured Chloroflexi | *Aplysina cauliformis* | Indonesia: Manado |
| 84 | 107 | 5 | 1 | 1 | 0 | 100 | 0 | Proteobacteria | Alphaproteobacteria | Rhizobiales | Cohaesibacteraceae | JQ217285 | 100 | Uncultured *Tepidamorphus* sp. | marine sediment | Spain: Catalunya |
| 69 | 104 | 4 | 0 | 0 | 0 | 100 | 0 | Proteobacteria | Alphaproteobacteria | Rhodospirillales | Unassigned | JF789377 | 100 | Uncultured bacterium | marine sediment | Bahamas: Lee Stocking Island |
| 147 | 104 | 2 | 8 | 0 | 0 | 0 | 94 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | KF786682 | 100 | Uncultured Rhodobacteraceae | oil sheen | Nansha Bay |
| 1653 | 104 | 0 | 7 | 88 | 9 | 0 | 0 | Proteobacteria | Gammaproteobacteria | Chromatiales | Ectothiorhodospiraceae | JX455230 | 100 | Uncultured bacterium | *Coscinoderma matthewsi* | salmon farming |
| 1809 | 104 | 1 | 0 | 103 | 0 | 0 | 0 | Chloroflexi | TK17 | mle1-48 | Unassigned | KF286207 | 100 | Uncultured Chloroflexi | *Aplysina cauliformis* | Gulf of Mexico |
| 76 | 103 | 4 | 3 | 0 | 0 | 0 | 96 | Proteobacteria | Alphaproteobacteria | Unassigned | Unassigned | KR269619 | 100 | Uncultured alpha proteobacterium | sea water | Australia: Orpheus Island, Great Barrier Reef, Queensland |
| 146 | 103 | 0 | 0 | 80 | 23 | 0 | 0 | Proteobacteria | Gammaproteobacteria | HTCC2188 | HTCC2089 | KT973119 | 100 | Uncultured prokaryote | marine endolithic community of Mono Island | Belize: Carrie Bow Cay |
| 81 | 102 | 0 | 0 | 6 | 96 | 0 | 0 | Chloroflexi | SAR202 | Unassigned | Unassigned | JN596750 | 100 | Uncultured Chloroflexi | *Xestospongia testudinaria* | India |

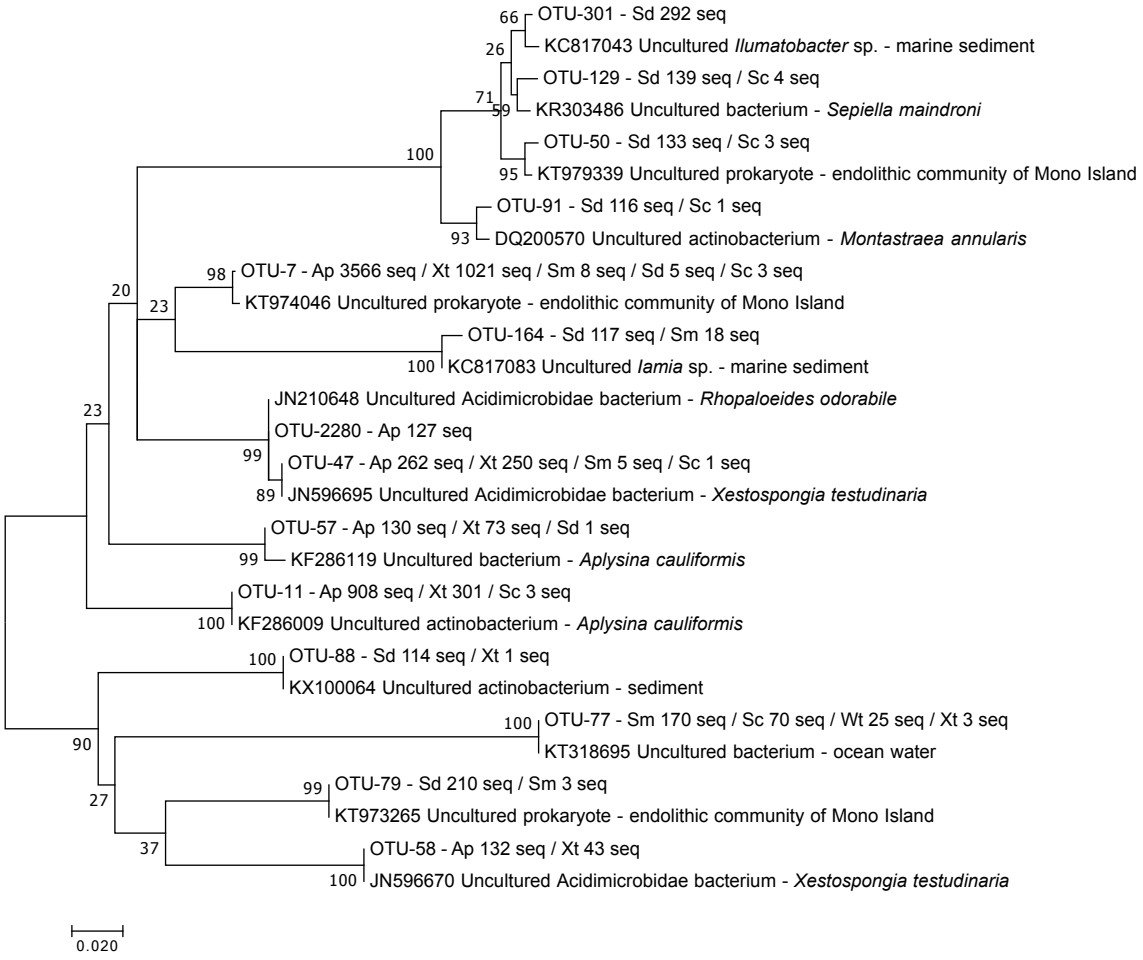


Fig. S4.1 – Phylogenetic tree of the Actinobacterial 16S rRNA gene sequences recovered from *Aaptos lobata* (Ap), *Xestospongia testudinaria* (Xt), *Stylissa carteri* (Sc), *Stylissa massa* (Sm), sediment (Sd) and seawater (Wt). The number of each OTU is indicated followed by the biotope and the number of sequences per biotope where they were detected. Accession numbers of sequences obtained using BLAST were also included as are the name of the organisms and the host/habitat where they were recovered.

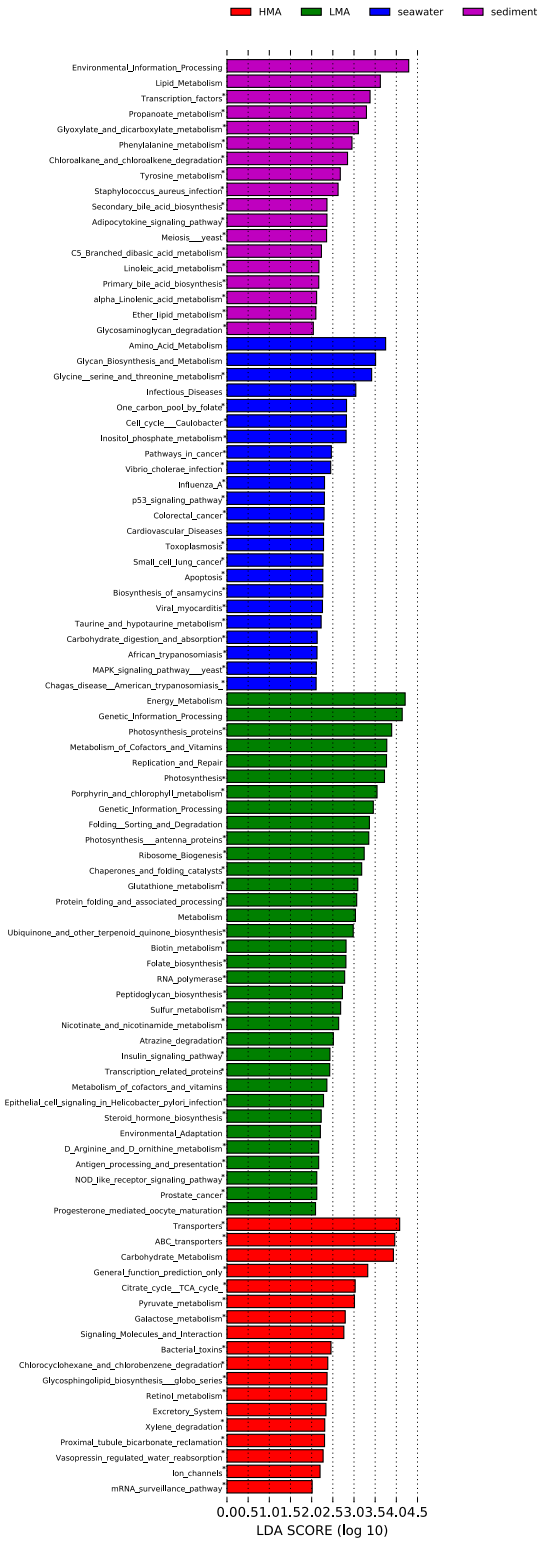


Fig. S4.2 – Histogram of the LDA scores ranking the statistically and biologically different categories, subcategories and individual pathways according to the effect size. KEGG categories, subcategories and pathways coloured: red indicates significant enrichment in HMA (*A. lobata* and *X. testudinaria*) sponges; green indicates significant enrichment in LMA (*S. carteri* and *S. massa*) sponges; blue indicates significant enrichment in seawater; purple indicates significant enrichment in sediment. *indicates individual pathways.

# Chapter 5. Conclusions

Sponges colonise habitats from tropical to polar seas and can be found in marine and freshwater environments. These animals represent an important structural part of coral reefs and are abundant in such ecosystems. Sponge's microbial symbionts contribute to nutrient cycling and to sponge nutrition, health and defence (Sipkema et al., 2005; de Goeij et al., 2013; Colman, 2015). However, in order to truly understand symbionts function in such associations, sponges' microbial communities have to be characterized at structural and functional level, considering different host species at distinct locations. Thus, using a 16S rRNA gene high-throughput sequencing approach, this study provides new insights on composition, phylogeny and putative functions of bacterial communities inhabiting a large range of sponge hosts (*Suberites diversicolor*, *Cinachyrella australiensis*, *Stylissa carteri*, *Stylissa massa*, *Aaptos lobata* and *Xestospongia testudinaria*) and non-host (seawater and sediment) biotopes in coral reefs and marine lakes from the Indo-Pacific region (Indonesian marine lakes and coral reefs from Singapore and Tioman). In addition, we explored the influence of habitat (e.g. marine lake vs. open water) on bacterial composition and the difference between structure and putative function of bacterial communities associated to LMA and HMA sponges, highlighting the influence of HMA/LMA dichotomy on putative function of sponge-associated bacteria. Microbial abundance and structural composition can vary greatly between HMA and LMA sponges (Hentschel et al., 2003; Weisz et al., 2007). And, despite the new insights given by metagenomics and metatranscriptomics, the reason for such differences remains unclear (Webster and Thomas, 2016). Thus, much more is still to unveil, even because HMA and LMA sponges may also present structural and functional similarities, sharing some core functions divergent from microbial communities found in seawater (Fan et al., 2012; Bayer et al., 2014).

In general, this thesis supports previous studies suggesting biotope as the main predictor of bacterial communities' richness, composition, abundance and putative function. Overall, sponges' bacterial communities were significantly divergent from the surrounding sediment and seawater bacterial communities, which shows the importance of biotopes in structuring bacterial communities' composition in marine ecosystems.

Here, is provided the first study of sponges' bacterial communities in marine lakes, showing that both host species (*S. diversicolor* and *C. australiensis*) and habitat (marine lakes and

open water) influence bacterial structural richness and composition. Despite *C. australiensis* harboured more diverse bacterial assemblages than *S. diversicolor*, habitat appears to be a better predictor of bacterial richness than host. In turn, highly significant differences were observed in bacterial composition among sponge hosts and among habitats, and together, host and habitat, explained almost 59% of the variation in bacterial composition. The samples studied here formed three distinct clusters: (1) samples from *C. australiensis* hosts in open water habitat; (2) samples from *S. diversicolor* hosts in open water and lake habitats; and (3) samples from *C. australiensis* hosts in lake habitats. Contrariwise to *C. australiensis*, whose bacterial communities' composition greatly varied in marine lake and open water habitats, bacterial communities in *S. diversicolor* did not show a pronounced difference among habitats. Moreover, bacterial composition was strongly different between sponge species, and overlapped in bacterial samples from both lakes. More recently, Cleary et al. (2018a) studied bacterial communities associated to seawater, sediment and sponge *Biemna fortis* in marine lakes of the islands Kakaban and Maratua and the surrounding marine environment (Berau region, East Kalimantan, Indonesia). They showed that biotope was the main predictor for bacterial composition variation, despite samples from all biotopes presented compositional differences among biotopes.

In agreement with Cleary et al. (2018) and de Voogd et al. (2018), this thesis shows that sponges *X. testudinaria*, *A. lobata*, *S. carteri* and *S. massa* had a much lower OTU richness than sediment. Concerning samples from Singapore coral reef ecosystem, we observed that samples were grouped by biotope, in three distinct clusters in PCO ordination. However, since we used only one seawater sample, we were not able to evaluate the statistical significance with F-test. Thus, an additional PCO was performed using only *X. testudinaria* and sediment samples showing significant differences between these two biotopes, explaining 91% of the variation in OTU composition. This is in accordance with Polónia et al. (2017), whose results showed significant differences in bacterial composition between *X. testudinaria* and sediment from the barrier reef system of Berau, East Kalimantan (Indonesia).

In turn, PCO ordination of samples from Tioman coral reef ecosystem showed significant compositional differences among biotopes, explaining 73% of the variation in bacterial composition. In this case, PCO ordination showed four different clusters: (1) samples from *A. lobata* and *X. testudinaria*; (2) samples from sediment; (3) samples from both *Stylissa*

species and (4) samples from seawater. Our results indicated that bacterial communities of sponges had pronounced differences from the surrounding environment (sediment and seawater), and strong similarities between bacterial communities in *X. testudinaria* and the putative HMA *A. lobata*, supporting the indication that *A. lobata* might be indeed an HMA sponge (Cleary et al., 2018). Moreover, comparing to other biotopes, *Stylissa* species hosted the least diverse bacterial communities and showed higher similarity to seawater, which can be explained by the great amount of seawater filtered by LMA sponges (Cleary et al., 2015). Moitinho-Silva et al. (2013) also showed that microbial composition of *S. carteri* had more similarity to that of seawater than to microbial communities of *X. testudinaria*. In both cases (Singapore and Tioman coral reef ecosystems), variation between biotopes has been shown as the main predictor of bacterial composition. All PCO ordination analyses showed that abundant OTUs (≥100 sequences reads for Singaporean samples and >100 sequences reads for samples from marine lakes and Tioman) were exclusively or mainly detected in specific biotopes. In samples from Singapore the only OTU common to all biotopes was assigned to the family OCS155 (Actinobacteria), while in samples from the marine lakes and Tioman the only OTU common to all biotopes was assigned to the family Synechococcaceae (Cyanobacteria).

The compositional analysis at the phylum level showed that across all samples studied here, Proteobacteria was the most abundant phylum in both non-host biotopes and in sponges *S. diversicolor*, *C. australiensis*, *S. carteri* and *A. lobata*, whilst *X. testudinaria* was dominated by Chloroflexi and *S. massa* by Cyanobacteria. Recently, prokaryotic communities of *Suberites* sp. and five other sponge species from a Taiwanese coral reef and shallow hydrothermal vent ecosystems were accessed and revealed that Proteobacteria was the most abundant phylum in this sponge host (Coelho et al., 2018). With the purpose to describe the baseline microbial community of *Cinachyrella* to use the sponge as a future experimental model, Cuvelier et al. (2014) showed that all the 58 samples were dominated by Proteobacteria. A more recent study accessing bacterial and archaeal communities of *Cinachyrella* spp. collected from two different marine lakes from eastern Indonesia also revealed Proteobacteria as the most abundant phylum (Cleary et al., 2018b). In agreement with several studies on samples from the Red Sea, Korea and Indonesia, *S. carteri* had higher relative abundance of Proteobacteria (Lee et al., 2011; Moitinho-Silva et al., 2013; Cleary et

al., 2015; de Voogd et al., 2015; Jeong et al., 2015; Polónia et al., 2016). Bacterial community of *A. lobata* was studied for the first time by Cleary et al. (2018), revealing the dominance of Proteobacteria and Chloroflexi, which is corroborated by our results. Previous studies have shown that Chloroflexi is a highly common and diverse phylum in sponges comprising several sponge-specific lineages, and oftentimes is one of the most abundant phyla in HMA sponges (Schmitt et al., 2011; Gloeckner et al., 2014). This thesis corroborates previous studies that showed a prevalence of this phylum in bacterial communities of *X. testudinaria* from the Red Sea, Celebes Sea, and Great Barrier Reef (Lee et al., 2011; Montalvo and Hill, 2011; Moitinho-Silva et al., 2013; Montalvo et al., 2014), however contrasting with three more recent studies (Cleary et al., 2015; de Voogd et al., 2015; Polónia et al., 2017), where Proteobacteria was the most abundant phylum in *X. testudinaria*.

Cyanobacteria dominated *S. massa*, however also had high abundances in *S. carteri* and seawater biotopes. This is in accordance with the general assumption that LMA sponges have a low diversity at phylum level, with prevalence of Proteobacteria and Cyanobacteria (Hentschel et al., 2006; Giles et al., 2013; Moitinho-Silva et al., 2014). Actinobacteria abundance was much higher in *A. lobata*, *X. testudinaria* and sediment. This is in agreement with several studies that showed that, with Proteobacteria, Chloroflexi, Nitrospirae, Cyanobacteria and candidatus phylum Poribacteria, Actinobacteria is one of the most dominant phyla associated to sponge hosts (Hentschel et al., 2012; Simister et al., 2012; Webster and Thomas, 2016).

The compositional analysis of lower taxa showed in a clearer way that samples studied here were dominated by taxa involved in nutrient cycling and in sponge nutrition, health and defence (Sipkema et al., 2005; de Goeij et al., 2013; Colman, 2015). For example, *S. diversicolor* was dominated by three OTUs (63.4%) assigned to order Kiloniellales. Its type species *Kiloniella laminariae* was first isolated by selecting active antibiotic producers on agar plates (Wiese et al., 2009), and its members have the potential for denitrification. And, considering the production of strong antimicrobial compounds by *S. diversicolor* symbionts, this sponge species may be of great interest to search for novel bioactive compounds. In turn, the most abundant OTU detected in *C. australiensis* was assigned to the family Methylococcaceae whose closest relative (99% sequence similarity) was identified as a

*Nitrosococcus* species (order Chromatiales) isolated from the sponge *Rhopaloeides odorabile* in the Great Barrier Reef, whose members are involved in ammonia oxidation (Webster et al., 2011). Another dominant taxon in *C. australiensis* was Hyphomicrobiaceae. Species belonging to the family Hyphomicrobiaceae have been identified as important methylotrophic denitrifiers (Liessens et al., 1993; Osaka et al., 2006). The classes SAR202 and Anaerolineae (both within Chloroflexi) were specifically enriched in *A. lobata* and *X. testudinaria*. As sulphite-oxidezers, SRA202 members are important players in the deep marine environment (Mehrshad et al., 2018), while Anaerolineae were identified as organic matter degraders that might use sponges as a niche to access the high loss of their host's biomass (de Goeij et al., 2013; Hug et al., 2013). In agreement with Cleary et al. (2015) and Coelho et al. (2018), although contradicting the study of de Voogd et al. (2015), Synechococcophycideae (Cyanobacteria) was enriched in *S. massa*. Its type genus *Synechococcus* is a widespread genus with high abundance in marine euphotic zone and, along with *Prochlorococcus*, is responsible for up to 50% of the carbon fixation in marine systems (Partensky et al., 1999). The high abundance of OTUs assigned to this genus in LMA sponges as *Stylissa* species can be due to the great amounts of seawater they are able to filter (Weisz et al., 2007). In accordance with Bayer et al. (2014) and Moitinho-Silva et al. (2013), the class Gammaproteobacteria (Proteobacteria) was highly abundant in *S. carteri*. Within this class, the orders Chromatiales and Thiohalorhabdales were more abundant in both *Stylissa* species whilst Caldilineales (Chloroflexi) was the most abundant in *A. lobata* and *X. testudinaria*. Also known as purple sulphur bacteria, Chromatiales are commonly found in marine sponges, playing an important role in their survival through the fixation of molecular nitrogen (Proctor, 1997; Imhoff, 2005). Thiohalorhabdales are considered as sulphur oxidizing bacteria (SOB) with a role in nitrogen cycling (Sorokin et al., 2008; as Frank et al., 2016). Caldilineales are non-photosynthetic previously detected in soils (Breuker et al., 2011), marine sediments (Schmitt et al., 2011), activated sludge (Kindaichi et al., 2012), and marine animals, such as sponges (Schmitt et al., 2011; de Voogd et al., 2018). The dominance of OTUs related to taxa known to be involved in nutrient cycling suggests that all sponges studied here may play an important role in reef and lake nutrient dynamics.

A prediction on functional profiles using PICRUSt software is given in Chapter 4 for all biotopes, i.e. *S. carteri*, *S. massa*, *A. lobata*, *X. testudinaria*, sediment and seawater. PCO ordination revealed biotope as the main predictor of bacterial KO composition variation, showing high similarity, and thus putative functions, between both *Stylissa* species and between *A. lobata* and *X. testudinaria*. In addition, our results corroborate previous studies showing similar core metabolic profiles between HMA and LMA sponges (Fan et al., 2012; Bayer et al., 2014), although with different relative abundances. HMA sponges, for example, were enriched in pathways related to bacterial defence as "bacterial toxins", to carbohydrate uptake and degradation which processes are included in "Carbohydrate Metabolism" subcategory and to "Xenobiotic Biodegradation and Metabolism". In turn, LMA sponges were enriched in "Replication and Repair" mechanisms which can be related to the way host sponge deal with environmental stress. LMA sponges were also enriched in "Energy Metabolism", "Metabolism of Cofactors and Vitamins" and in pathways within "Xenobiotic Biodegradation and Metabolism". Both HMA or LMA sponges were enriched differently in pathways related to host defence, energy balance or DNA repair, suggesting that LMA and HMA sponges studied here use distinct strategies to defend themselves against pathogens, to obtain energy or to deal with environmental stress.

# Chapter 6. Appendix

**Other outputs achieved during the execution of the PhD thesis**

During my PhD in Biology (specialization in Microbiology), I participated in several studies that resulted in the following list of publications:

1. **Pires ACC**, Cleary DFR, Polónia ARM, Lim SC, de Voogd NJ, Oliveira V and Gomes NCM (2019). Comparison of bacterial communities associated with *Xestospongia testudinaria*, sediment and seawater in a Singaporean coral reef ecosystem. Journal of the Marine Biological Association of the United Kingdom, 99(2): 331-342.
2. Martins PT, Coelho FJRC, Cleary DFR, **Pires ACC**, Marques B, Rodrigues AM, Quintino V and Gomes NCM (2018) Seasonal patterns of bacterioplankton composition in a semi-intensive European seabass (*Dicentrarchus labrax*) aquaculture system. Aquaculture, 490: 240-250.
3. Boaventura CM, Coelho FJRC, Martins PT, **Pires ACC**, Duarte LN, Uetanabaro APT, Cleary DFR and Gomes NCM (2018) Micro-eukaryoticplankton diversity in an intensive aquaculture system for production of *Scophthalmus maximus* and *Solea senegalensis*. Aquaculture, 490: 321-328.
4. Gomes NCM, Cleary DFR, **Pires ACC**, Almeida A, Cunha A, Mendonça-Hagler LCS and Smalla K (2014) Assessing variation in bacterial composition between the rhizospheres of two mangrove tree species. Estuarine, Coastal and Shelf Science, 139: 40-45.
5. Martins PT, Cleary DFR, **Pires ACC**, Rodrigues AM, Quintino V, Calado R and Gomes NCM (2013) Molecular Analysis of Bacterial Communities and Detection of Potential Pathogens in a Recirculating Aquaculture System for *Scophthalmus maximus* and *Solea senegalensis*. PLoS ONE, 8 (11): e80847.
6. Cleary DFR, Becking LE, de Voogd NJ, **Pires ACC**, Polónia ARM, Egas C and Gomes NCM (2013) Habitat- and host-related variation in sponge bacterial symbiont communities in Indonesian waters. FEMS Microbiology Ecology, 85 (3): 465-482.
7. Coelho FJRC, Rocha RJM, **Pires ACC**, Ladeiro B, Castanheira JM, Costa R, Almeida A, Cunha A, Lillebø AI, Ribeiro R, Pereira R, Lopes I, Marques

C, Moreira-Santos M, Calado R, Cleary DF and Gomes NC (2013) Development and validation of an experimental life support system for assessing the effects of global climate change and environmental contamination on estuarine and coastal marine benthic communities". Global Change Biology, 19 (8): 2584-2595.

8. Gomes NCM, Manco SC, **Pires ACC**, Gonçalves SF, Calado R, Cleary DFR and Loureiro S (2013) Richness and composition of sediment bacterial assemblages in an Atlantic port environment. Science of The Total Environment, 452-453: 172-180.

Development of the EcoTech-SPONGE databank.

During my activities within the scope of the Project EcoTech-SPONGE (Assessing the ECOlogical functions and potential bioTECHnological applications of plasmid assemblages from microbial symbionts of marine SPONGEs; PTDC/BIAMIC/6473/2014 – POCI-01-0145-FEDER-016531), I built a database, organized and managed all the metadata generated in the project (including the outputs of this thesis). Metadata for biological samples included: sample code; fixation method; sampling location, coordinates, depth and time; type of habitat and biotope; bacterial isolation; sequencing technology, Sequence Read Archive – SRA; publication title, journal and DOI. Metadata for isolates include: bacterial isolate code; origin sample for the isolation; sponge species from which isolates were obtained; isolation date and medium (including selective agents for transconjugants and endogenous plasmid isolation); selective compound; 16S rRNA gene sequence, RDP classification and its closest relative (NCBI-BLAST with similarity %) and accession number; GenBank accession numbers and sequencing technology; publication title, journal and DOI.

This database is available in the project page: http://www.cesam.ua.pt/index.php?menu=200&language=eng&tabela=projectosdetail&projectid=658

## Chapter 7. Literature cited (in this thesis)

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