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Macro- and micro -symbioses involving sponges: Ecological roles in the marine benthos

Marta Turon Rodrigo

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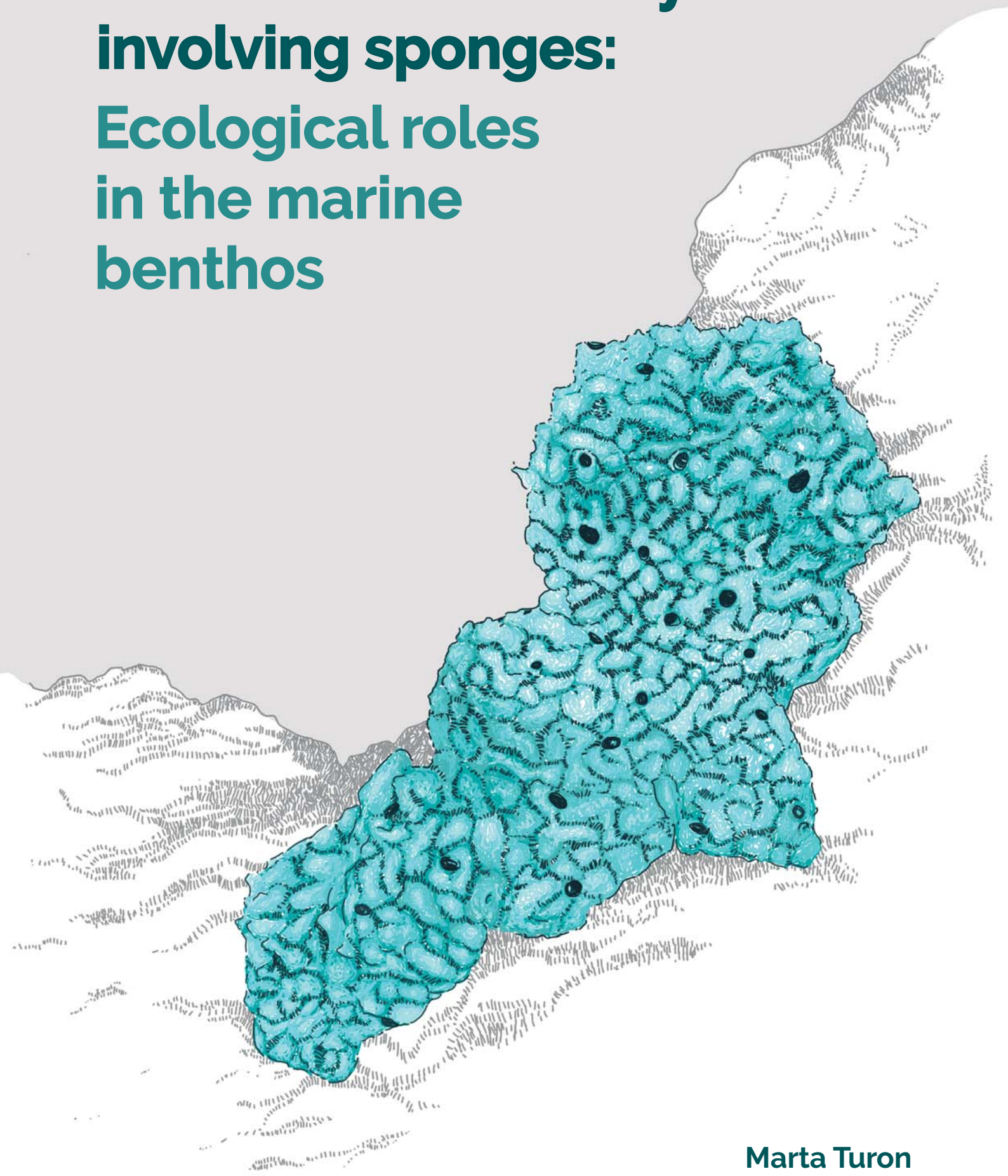

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Macro- and micro- symbioses involving sponges: ecological roles in the marine benthos

Macro- i micro simbiosis en esponges: funcions ecològiques en el bentos marí

Memòria presentada per Marta Turon Rodrigo per obtar al Grau de Doctor per la Universitat de Barcelona

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Als meus pares,
Al Raül,
i als amics que heu fet d'aquests anys
una experiència inoblidable...

"Life did not take over the globe by combat,
but by networking"
Lynn Margulis, Microcosmos.

Agraïments

Per fi ha arribat el moment d'escriure les últimes línies d'aquesta tesis i agrair a tots els que heu fet possible que arribés fins aquí, ja sigui a nivell científic com a nivell personal. Espero que això només sigui un punt i seguit a totes les experiències i amistats viscudes durant aquests anys, aquesta tesis no hauria estat el mateix sense totes vosaltres.

Primer de tot a la persona més important que ha fet possible aquesta tesis, Iosune, ha estat una sort tenir-te com a directora! Quan vaig marxar de l'ICM em van dir que vigilés, que no tots els *jefes* eren iguals i que això de poder entrar al despatx sense avisar per parlar de qualsevol cosa no era una cosa tant habitual. Doncs sort que s'equivocaven! Tot i que a vegades m'he sentit un "márchate que me estás estresando", en general sempre has estat disposada a ajudar-me en tot. Has fet que aquesta relació directora-estudiant fos fàcil, agradable i enriquidora. Gràcies per ensenyar-me tant, la confiança que m'has transmès i deixar-me créixer, tant de bo algun dia pugui saber-ne tant com tu, de taxonomia, però sobretot de viure la vida!

També vull agrair a en Dani com a codirector de tesis, especialment per l'ajuda amb els mostrejos a Vietnam, les identificacions de poliquets i les teves classes de Photoshop.

I a la meva tutora, la Creu, que sempre ha estat predisposada a ajudar-me en qualsevol cosa que he necessitat encara que fos a última hora, gràcies per posar les coses tant fàcils!

Si penso on va començar tot, penso en els anys a l'ICM. Vosaltres sou els responsables d'iniciar-me en aquest món i motivar-me a seguir endavant. És per això que primer de tot vull agrair a la meva primera *jefa*, l'Esther, la teva proximitat i energia van ser claus per endinsar-me en la ciència. També a la Eli, que sempre vas ser el meu exemple a seguir, a l'Albert, que tot i ser de poques paraules, sempre estaves allà, i al Jordi, pels teus bons consells. Tots vosaltres em vau fer veure que per sobre de científics, sou grans persones. Tant de bo hi haguessin més grups així! A part de la ciència, l'ICM va estar ple d'amistats i sobretot de bones festes amb tots vosaltres: Soto, Lau, Royo, Raül, Pablo, Pau, Gas, Maria, Sílvia, les nits a

Enfants sempre mereixien un bon cafè a la nostra *Peixera*. Royo, ens vam conèixer demanant beques com unes desesperades fa 6 anys i recordo com vam saltar d'alegria quan ens van donar la FPI. Tot i ser en centres separats, hem anat seguint camins paral·lels que espero que es tornin a creuar aviat, va que ja som a la recta final! Lau, definitivament mereixes un agraïment especial, moltes gràcies per fer que aquesta tesis tingui una mica del teu art, per escoltar tota la xapa i saber-ho plasmar tant bé! Sílvia, per tot el temps de confidències i sobretot dels millors atacs de riure escoltant-te parlar. Y Gas, que suerte fue tenerte por aquí y que gran viaje el de Argentina, nos volveremos a ver pronto!

I ara sí, a tots els indispensables del CEAB que heu fet que aquests anys fossin molt més divertits: Jana, per ser-hi des de el principi i evidentment sempre estar disposada a una bona conversa, la teva alegria és contagiosa! Roger, des de que ens vam conèixer al Màster sabia que això aniria per llarg, perquè amb una mirada ens entenem i perquè poder compartir aquests 4 anys amb tu ha estat tot un regal, encara ens queden unes quantes sortidetes de bici pendents. Mateuet, vas arribar a la meitat del meu doctorat però sembla que hi hagis estat sempre, crec q mai m'has abandonat a cap cafè ni partit de pàdel, i m'has escoltat sempre (per això saps recitar el nom de totes les meves amigues), prepara't que encara em queda rollo per un temps. Juanito, crec q t'endús la palma d'aguantar-me, a part d'ajudar-me en gran part d'aquesta tesis, mai has tingut un no per resposta, gràcies per fer divertits els viatges de Girona a Blanes i en general, els dies al CEAB. Xavi, definitivament les hores de desconnexió jugant a pàdel te les dec a tu, sempre insistint per fer el partidet a l'hora que fos, espero compartir algun torneig més amb tu. Jorjito, si ya sé que ahora compartimos pareja y casi no te dejas ver por el CEAB, pero aun así, gracias por las cenas, las cervecitas y las esquiades, seguro que vienen muchas más! Arnau, perquè tot i que ja no estàs al CEAB t'has motivat a organitzar sortidetes per desconnectar i sempre que vens em fas passar una bona estona. Gràcies també a tots els que hi éreu des de l'inici, Boadilla, per la teva alegria i optimisme, Xevi, per la teva ajuda amb la tesis i ensenyar-me a parlar una mica millor, a la Maria, la Marta, la Cris i la Cèlia, per sempre trobar algun moment per allargar el dinar i explicar-nos la vida, Lei, por tus consejos

y horas de charla en Holanda, Rudi y Vicente, por haber compartido gran parte de este trayecto. I als que us heu incorporat més recentment, Dani i Jan, pel bon rotllo que porteu al grup. Als meus compis de despatx, a la Maria, perquè vam començar i acabarem juntes, i en especial a l'Héctor, sempre has estat disposat a ajudar-me. En general, gràcies a tota aquesta gent del centre que feu del CEAB un lloc agradable, ja sigui a les hores dels cafès, allargant les converses al despatx o muntant partidets de pàdel o esquiades fora de la feina!

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No m'oblido d'un dels anys més especials, el Màster, on us vaig conèixer

a tots vosaltres: Anna, Pol, Sílvia i Roger, les nostres retrobades de dinars que s'allarguen amb gin tònics o acaben a *Diverfiestas* sempre ens deixen amb ganes de repetir més sovint. Polete, un agraïment especial per l'ajuda en passar aquesta tesis a Latex, m'has salvat!

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I a tu Raül, res d'això hagués estat possible sense tu. Gràcies per fer que aquests 4 anys passessin de la millor manera, entre pedalades, esquiades, excursions, sopars, viatges, escapades amb furgo i sorpreses, gràcies per la teva confiança cega en mi i animar-me incansablement, amb tu sempre és tot possible.

GRÀCIES A TOTS,

aquest tesis porta una mica de tots vosaltres...

Informe dels directors

La Dra. M. Jesús Uriz Lespe i el Dr. Daniel Martin Sintes, directors de la Tesi Doctoral elaborada per la candidata Marta Turon Rodrigo i que porta per títol "Macro- and micro- symbioses involving sponges: ecological roles in the marine benthos",

INFORMEN

Que els treballs de recerca duts a terme per Marta Turon com a part de la seva formació pre-doctoral i inclosos a la seva Tesi Doctoral han donat lloc a cinc capítols, tres dels quals ja estàn publicats i dos estan a punt per ser enviats a revistes d'àmbit internacional. A continuació es detalla la llista d'articles, així com els índex d'impacte (segons la ISI Web of Science) de les revistes on han estat publicats.

1. **Turon, M**., Cáliz, J., Garate, L., Casamayor, E.O., Uriz, M.J.(2018) Showcasing the role of seawater in bacteria recruitment and microbiome stability in sponges *Scientific Reports*, 8: 15201. `DOI: 10.1038/s41598-018-33545-1`. Multidisciplinary Sciences (Rank: 15/69) **Q1. Impact Factor: 4.01**

2. **Turon, M**. Cáliz, J., Triadó-Margarit, X., Casamayor, E.O., Uriz, J.M. (2019) Sponges and their microbiomes show similar community metrics across impacted and well-preserved reefs. *Fron. Microbiol.* 10:1961. `doi:10.3389/fmicb.2019.01961`. Microbiology (Rank: 32/133) **Q1. Impact Factor: 4.25**

3. **Turon, M**., Uriz, M.J., Martin, D. (2019) Multipartner symbiosis across biological domains: looking at the Eukaryotic associations from a Microbial perspective *mSystems*, 4(8): e00148-19. `10.1128/mSystems.00148-19`. Microbiology (Rank: 15/133) **Q1. Impact Factor: 6.51**

Addicionalment, es preveu enviar a publicar 2 articles de taxonomia corresponents al primer capítol i un de microbiologia (arqueas) corresponent al cinquè capítol:

4. **Turon, M**. and Uriz, M.J. Description of the shallow sponge fauna from Nha Trang Bay (Vietnam). Preparat per enviar a *Zootaxa*. Zoology (Rank 101/170) **Q3. Impact Factor: 0.99**

5. **Turon, M**. and Uriz, M.J. New sponge species of Vietnam and their phylogenetic relationships. Preparat per enviar a *Zoological Journal of the Linnean Society*. Zoology (Rank 10/170) **Q1. Impact Factor: 2.9**

6. **Turon, M**. and Uriz, M.J. Insights into the archaeal consortium of sponge species from Vietnam. Preparat per enviar a *Frontiers in Marine Science*. Marine and freshwater biology sciences (Rank: 13/108) **Q1. Impact Factor: 3.086**

Alhora CERTIFIQUEN

Que Marta Turon Rodrigo ha participat activament en el desenvolupament del treball de recerca associat a cadascun d'aquests articles, així com en la seva elaboració. En concret, la seva participació en cadascun dels articles ha estat la següent:

- Plantejament dels objectius.
- Planificació i execució dels experiments, tant pel què fa a feina de camp com al laboratori.
- Processat i anàlisi de les mostres obtingudes, a més dels anàlisis de dades obtingudes.
- Redacció dels articles i seguiment del procés de revisió dels mateixos.

Finalment, certifiquen que cap dels coautors dels articles presentats a continuació i que formen part de la Tesi Doctoral de Marta Turon Rodrigo utilitzarà implícitament o explícitament aquests treballs per a l'elaboració d'una Tesi Doctoral.

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Abstract

The symbiotic lifestyle represents a fundamental contribution to the diversity of marine ecosystems. Sponges are ideal models to study symbiotic relationships from evolutionary and ecological points of view since they are the most ancient metazoans on Earth, are ubiquitous in the marine benthos, and establish complex symbiosis with both prokaryotes and animals, which in turn harbour their own bacterial communities.

In this thesis, we aim to go deeper into the mechanisms by which sponges establish symbiotic associations with members of the three domains of life, combining taxonomical, ecological, and molecular approaches. We study how sponges acquire their symbiotic microbes and whether these microbes contribute to shape the ecological distribution of their hosts. Moreover, we use the sponge-polychaete relationship as an example of multi-partner symbiosis and study the eukaryotic association from the microbial perspective. Finally, we focus on the less studied domain of life, the archaea, to gain insights into the composition and stability of these symbionts in sponges.

To assess these goals, we characterized the sponge assemblages in two contrasting environments (well-preserved and impacted) of Nha Trang Bay (Vietnam) and selected the most abundant species for the study of their microbiomes. Additionally, four sponge species harbouring thousands of polychaetes were sampled to analyse the relationships sponge-microbes-polychaetes. Sponges and polychaetes were identified and their respective microbiones, and the seawater bacterial communities were analysed by high-throughput sequencing of the 16S rRNA gene (V4 region).

Our results show that sponge assemblages were more diverse and rich in the well-preserved reefs. Similar ecological metrics were shown by the sponge microbiomes according to the type of habitat. Species-specificity and stability were a rule for the majority of the symbioses studied, regardless of the environment where the sponges were living. The high overlap in bacteria composition between sponges and seawater suggest microsymbiont acquisition from the environment. Polychaetes were also able to specifically

select and enrich some bacteria from their food sponge. Overall, most sequences were shared between biotypes, but at differential abundances leading to highly specific and stable invertebrate microbiomes, acquired from the environment. Our results support the tenet "*Everything is everywhere, but the environment selects*".

Contents

Chapter 1

General Introduction

This thesis is planned to improve knowledge on multiple associations involving simultaneously organisms of the three domains of life, Archaea, Bacteria and Eukarya, which establish complex interactions in the holobiont and are widespread in the marine realm. A symbiotic mode of life has obvious benefits for one or more of the partners, increasing their success. Moreover, it also generates ecological niches, facilitating many species to coexist in the same space and thus, favouring biodiversity.

The function of some associated bacteria in the holobiont metabolism has been nicely demonstrated in many terrestrial symbioses, which have inspired similar approaches in marine ecosystems. In fact, several metabolic interactions among marine microbial and animal partners have been proposed, but rarely demonstrated.

1.1 Symbiotic lifestyle

1.1.1 From individuals to holobionts

Since the postulation of the endosymbiotic origin of the eukaryotic cell (Margulis, 1981), the research on symbioses has experienced a rapid growth, involving the study of the interplay between organisms in the three domains of life, from molecular to ecological perspectives (Mcfall-Ngai, 2008). Nowadays, symbiosis is seen as a rule, rather than an exception, and as a general principle in Eukaryotic evolution (Douglas, 2014). Historically, biologists have studied animals, plants and fungi as individuals. However, the recognition of symbiotic relationships changed our perception of individuality. The *holobiont* concept, which takes into account the multicellular eukaryotes plus their populations of persistent symbiont populations (Margulis, 1991) gained attention in the last decades. The hologenome evolutionary theory Zilber-Rosenberg and Rosenberg (2008) proposes the holobiont as

unit of selection, considering the sum of the genetic information of the host and its microbiota (the hologenome). This theory was based on four basic principles: (1) all animals and plants are holobionts as they establish symbiotic relationships, at least, with microorganisms, (2) the holobiont is a distinct biological entity anatomically, metabolically and immunologically, as well as during development and evolution, (3) a significant fraction of the microbiome genome is transmitted across generations together with the host genome and, thus, can propagate unique properties of the holobiont, and (4) variation in the hologenome can be brought by change in either the host or the microbiota genomes. Moreover, the microbiome genome may adjust more rapidly to environmental dynamics than the host genome and thus, playing fundamental roles in the adaption and evolution of the holobiont (Rosenberg and Zilber-Rosenberg, 2018; Zilber-Rosenberg and Rosenberg, 2008). Accordingly, natural selection would not act at the species level but at the holobiont (hologenome) level (Dittami et al., 2019). However, this theory has also been criticized, as it neglects all the broad range of interactions occuring in a host-microbiome system, the different modes of transmission and the levels of fidelity among partners (Douglas and Werren, 2016; Moran and Sloan, 2015). Basically, these systems have to be viewed from a community perspective, where all members are independent evolutionary entities rather than a "superorganism", as selective interests among microbial partners and between the microbiota and the host might be different (Douglas and Werren, 2016).

With this context in mind, it is difficult to study the evolution of species without considering their symbiotic companions. Indeed, studying microbial symbiosis could be crucial to understand the origin of species, as microbes play important roles in host behaviour and speciation (Shropshire and Bordenstein, 2016). The advent of new sequencing technologies (early 90's), allowed adressing the taxonomic identification of bacterial species associated with multicellular organisms, both animals and plants (McFall-Ngai et al., 2013), which reflected their strong interdependencies. However, the specific functional traits provided by these microbes to the holobiont were difficult to predict only from their taxonomic composition (Douglas, 2014). Moreover, the symbiotic nature of these symbiotic associations prevents in most cases

the culture-independent analysis of the microbial communities without their hosts. That is why the best-studied symbioses involve just one or few microbial partners (i.e; aphids, squid-*Vibrio*. Only in these cases, the costs/benefit analysis, at least for the host, is feasible (Clay, 2014). In multi-partner symbioses (i.e. microbial consortia), the specific functionalities of each microbe are hard to elucidate.

The "nested ecosystems" concept is nowadays used to refer to holobionts as complex ecosystems interacting and responding at multiple scales to environmental changes (McFall-Ngai et al., 2013; Pita et al., 2018). The contribution of a single microbe to a particular holobiont would thus have a cascading effect beyond the holobiont itsef, even generating responses at community or ecosystem levels. A paradigmatic example of the nested-ecosystems concept is the reef "sponge loop" (Goeij et al., 2013), in which, sponge-associated microbes assimilate Dissolved Organic Matter (DOM) to make it available to other reef fauna as particulate organic matter (POM). Other microbial functions, such as photosynthesis, nitrification, defence, or competition, might also have cascading effects at the community or ecosystem scale (Pita et al., 2018).

1.1.2 Costs and benefits

Symbiosis was described as the intimate association of two or more organisms of different species, usually to the benefit of one of them (de Bary, 1879). Symbiotic microorganisms usually provide benefits to its host, such as complementary metabolic capabilities that spread its ecological niche (Douglas, 2014), or production of secondary metabolites that confer resistance in front of parasites and pathogens (Oliver et al., 2008) and signal molecules used in potential mate recognition (Venu et al., 2014). However, the symbiotic associations may be mutualistic, commensal or parasitic and thus, host-microbe interactions are not necessarily beneficial to the host all the time (Relman, 2008). In some cases, symbiotic associations come with a cost for the host when environmental or physiological conditions change. Some examples of shifts in the nature of the associations are the pathogenic infection resulting from interactions in the in plant microbial rhizosphere (Morgan et al., 2005), the loss of defensive behaviour in aphids

harbouring symbionts (Polin et al., 2014), the reduction of salinity tolerance of the brine shrimp by its symbiotic gut microbiota (Nougué et al., 2015), or the increased risk of cancer in humans modulated by the coevolution with *Helicobacter pylori* (Kodaman et al., 2014).

1.2 Symbiosis in marine environments: the invertebrates

Marine environments offer a great opportunity to study symbiosis with bacteria and archaea, as the marine organisms are in permanent contact with the seawater microorganisms, enhancing the chances for the establishment of beneficial associations but also, pathogenic invasions (Dubilier et al., 2008). Moreover, microbial shifts can occur faster in marine than in terrestrial habitats due to the lower dispersal barriers in the former environments (Dittami et al., 2019). The main proposed benefits involve nutrition protection immunity and development, among others.

Chemosynthetic nutritive symbioses allow hosts to live in otherwise inhospitable environments (e.g., hydrothermal vents). They have been largely studied since the discovery of the association between the tubeworm *Riftia pachyptila* Jones, 1981 and chemolithoautotrophic bacteria in the early 1980s (Cavanaugh et al., 1981). At least seven animal phyla have been later reported to host highly diverse chemosynthetic bacteria that make nutrients available to the host in a wide range of marine habitats, including hydrothermal vents, whale and wood falls, cold seeps, mud volcanoes and shallow-water coastal sediments (Dubilier et al., 2008).

Defensive symbioses, through the interplay of secondary metabolites, are indirect interactions that involve at least three partners (host, symbiont and enemy). In the presence of predators, they provide the host with a higher fitness (protection) than in the absence of symbiotic partner (Clay, 2014; Lopanik, 2014). They have been of particular interest due to the applicability of the defensive chemical compounds to a wide range of medical, industrial, agricultural and bioremediation of the defensive chemical compounds (Rizzo and Lo Giudice, 2018; Sipkema, 2017). Besides these human applications, defensive symbiosis also plays a profound role in the structuring and dynam-

ics of communities and ecosystems in marine environments (Garate et al., 2015; Lopanik, 2014).

The vast majority (97%) of living animals on Earth are invertebrates, "the spinless majority" (May, 1988). However, most microbiome studies focus on vertebrates (Petersen and Osvatic, 2018), which generally appear to harbour much more complex microbiomes than invertebrates. Nevertheless, invertebrates may offer singular opportunities to understand the host-microbe interactions, leading to insights of broader relevance (Petersen and Osvatic, 2018). This postulated simplicity of invertebrate microbiomes, together with the high diversity of their virtually known types of beneficial interactions with hosts, make them ideal models for animal-microbe cross talk studies (O'Brien et al., 2019; Petersen and Osvatic, 2018). In particular, filter-feeder invertebrates are constantly exposed to water micro-organisms, being thus particularly interesting to assess the immunological mechanisms allowing them to differentiate between food, pathogens, and symbionts. Moreover, sessile marine invertebrates, such as sponges, cnidarians, bryozoans and tunicates, offer good opportunities to study defensive symbiosis mediated by the release of bioactive (allelochemicals and deterrents) compounds (Lopanik, 2014; Rizzo and Lo Giudice, 2018).

1.3 Sponges as models of multi-partner symbioses

Sponges play key functional roles in marine habitats. They modify the substrate (i.e., reef creation, bioerosion), participate in the bentho-pelagic coupling (i.e., releasing propagula, exchanging carbon, nitrogen and other nutrients from the plankton to the benthos and *vice-versa*), and provide niches to other species in such a way that the biodiversity per square cm is increased notably (Bell, 2008; Diaz and Rützler, 2001; Wulff, 2001).

In this thesis, we focus on sponges as ideal targets to improve our understanding on the symbiotic relationships from evolutionary and ecological points of view, since 1) they are most ancient metazoans on Earth, 2) are ubiquitous in the marine benthos, and 3) establish complex associations with both macro- and microorganisms.

Descriptive studies of the sponge microbiomes have proliferated in the last ten years (Hentschel et al., 2012; Lee et al., 2011; Schmitt et al., 2012; Thomas et al., 2016; Webster and Thomas, 2016), providing consistent messages as, for instance, their species-specificity, the existence of SC clusters (Box 1), and the dichotomy between HMA and LMA sponges (Box 2). However, further research is still required to better understand sponge- microbial interactions. For instance, differentiate the between "true" (permanent) symbionts and temporally variable (transient) microbes (Blanquer et al., 2013) may help to better focus on the symbiotic interactions. This is a particularly relevant issue in terms of evolution, as resilient microbes likely contribute (more than those occasional), to the holobiont success and may have co-evolved with the host.

On the other hand, sponges live in association with many other invertebrates, including annelid polychaetes (Martin and Britayev, 2018), which in turn must have their own associated microbiomes. Nevertheless, how and when the sponges and their symbiotic invertebrates acquire their true symbionts is not well understood. The acquisition mechanisms reported in the literature ranged from vertical transmission from progenitors (as the only mode of acquiring a true symbiont), to the more recent and substantiated idea of horizontal transmission of selected microbes from the water bacterial community (Box 3). Which is the more relevant mechanism, which one has more evolutionary sense, or whether both transmission ways contribute to the microbiome composition, are still under discussion topics. Another inconclusive issue is how microbiomes respond to environmental changes. For example, many transplant experiments place sponges under contrasting conditions having as working hypothesis that microbiomes will change together with the environment, while the most frequents result is that microbiomes are highly resilient (Erwin et al., 2012; Luter et al., 2014; Pita et al., 2013b; Strand et al., 2017).

The question arises on whether researchers have followed always the right approach. If a tight association with particular microbes is species-specific and has co-evolved with the host, would one expect rapid (i.e., for weeks or months) changes in these microbes other than those resulting from harmful stress and thus, leading to the holobiont to dysbiosis? As

an extreme example, would it be logical to expect human mitochondria to change when travelling from the Atacama Desert to the Antarctic Larsen territory or to a tropical forest? Or, conversely, would it be more reliable that human very long-term associated mitochondria evolved to successfully resist major environmental changes?

In this thesis, we try to clarify some aspects of the above outlined issues in five auto-explanatory study chapters that all together help to progress in our knowledge on the sponge symbioses topic.

Box 1: Sponge-Specific Clusters (SC)

Sponges are known to habour large amounts of bacteria in their tissues that can represent up to 40% of their biomass and are different from those in the surrounding seawater (Vacelet and Donadey, 1977). The phylogenetic study of 190 sponge-derived 16S rRNA gene sequences revealed the widespread presence of monophyletic sponge-specific clusters (SC) (Hentschel et al., 2002). These clusters are defined as groups of at least three 16S rRNA gene sequences that:

- i) are more similar to each other than to sequences from other (non-sponge) sources
- ii) are derived from two or more sponge species, or from the same species but from different locations
- iii) are supported by three independent phylogenetic tree-building approaches (neighbour-joining, maximum parsimony, maximum likelihood).

In the first study, 14 monophyletic SC of at least 7 different bacterial phyla were identified and 70% of the 190 sponge-derived sequences analysed fell into one of these clusters (Hentschel et al., 2002). With the increased availability of sponge-derived sequences (∼7500 in 2010), further revisions of these clusters were made identifying several SC in at least 14 different bacterial phyla, as well as in archaea and fungi (Simister et al., 2012b; Taylor et al., 2007). Later, deep sequencing allowed the detection of some of the SC clusters as rare members of the biosphere, questioning the transmission modes of such bacteria and the widespread diversity and distribution of these sequences outside the hosts (Taylor et al., 2013; Webster et al., 2010).

Box 2: HMA and LMA dichotomy

Sponges can be classified as High Microbial Abundance (HMA) or Low Microbial Abundance (LMA), regarding their bacterial composition (Gloeckner et al., 2014; Hentschel et al., 2003). Bacterial densities in HMA are two to four orders of magnitude higher than in LMA sponges (Hentschel et al., 2003) and also harbour richer and more diverse microbial communities (Moitinho-Silva et al., 2017c). These differences have been related to contrasting structural and physiological traits of the two species groups, with HMA sponges having a denser mesohyle and more complex aquiferous systems than LMA sponges (Blanquer et al., 2013), but with no relation to sponge phylogeny (Gloeckner et al., 2014). Moreover, certain microbial taxa (Phyla, Classes and OTUs) have been found differentially abundant in either group, and have been used to describe HMA and LMA indicators in the predictions made by a machine learning study (Moitinho-Silva et al., 2017c).

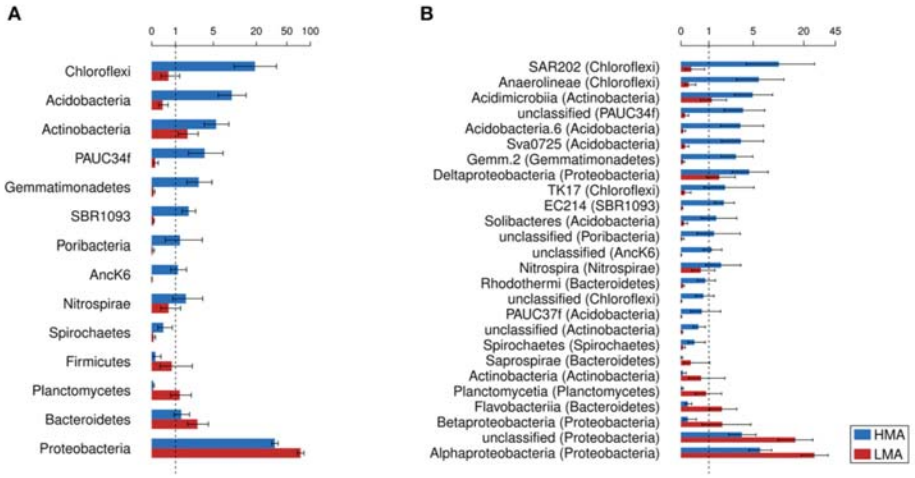
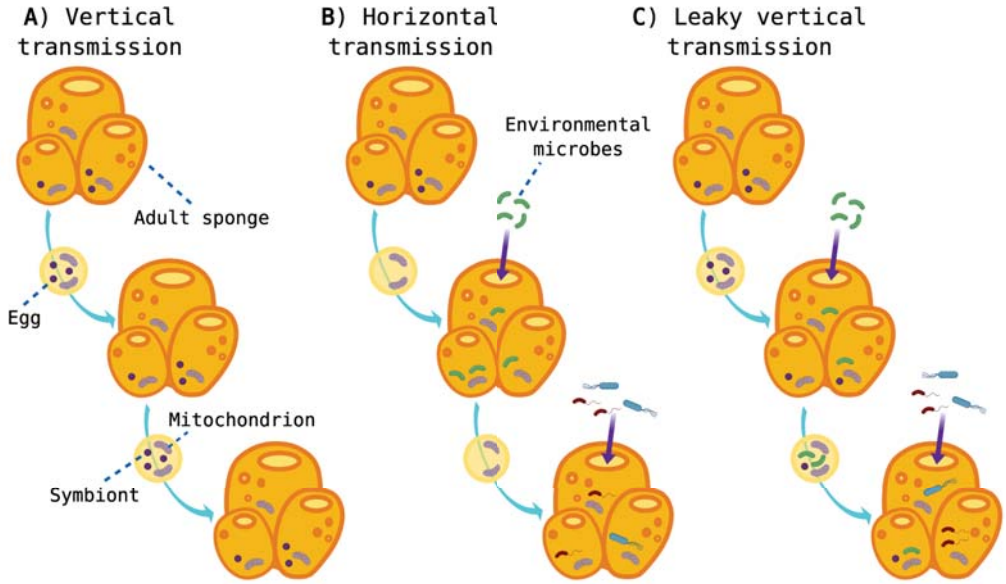


Figure from Moitinho-Silva et al. (2017c). Selection of differentially abundant bacterial and archaeal taxa in the microbiomes of HMA and LMA sponge species at Phyla (A) and class (B) level.

Also see Figure B.4 for differentially abundant taxa found in our study sponges.

Box 3: Transmission modes

Acquisition of microbes by the progeny may occur via two primary modes, vertical and horizontal transmission, but also by a combination of both. Under obligate vertical transmission (A), the symbionts are inherited maternally along with the mitochondria via gametes or embryos. Thus, the offspring symbionts are identical to parental ones. In this case, coevolution between microbes and their hosts is more likely to occur (O'Brien et al., 2019). Under horizontal transmission, symbionts are acquired environmentally in each generation, usually implying a free-living stage of the symbiont. Finally, "leaky vertical transmission" (C) mixes the two primary transmission modes, so that symbionts are both transmitted vertically via gametes and acquired from the surrounding environment.



The illustration is adapted from (Thacker and Freeman, 2012).

1.4 Objectives

This thesis aims to improve our knowledge on the symbiotic lifestyle in marine benthic ecosystems, using sponges as models of a multi-partner symbiosis that encompass the three domains of life: Bacteria, Archaea and Eukaryota. To comply our objectives, we first prospected quantitatively the shallow sponge fauna of the Nha Trang Bay (Vietnam) to ensure we were working with the most representative species in the area. Then, we identified the species recorded to the lower taxonomical level possible. The most representative sponge species in the area (most abundant) were used as models to particularly address the study of their micro, and macro-symbiosis. The thesis goals are dealt in five chapters (Figure 1.1) detailed below:

- Chapter 2: **Taxonomy of the common shallow sponges from Nha Trang Bay (Vietnam).** This chapter aims firstly at providing a taxonomical basis to study the sponge-associated organisms in the following chapters by identifying all the sponge species collected. Additionally, we aim to complement previous studies on the shallow sponge fauna of the area by providing an illustrated sponge identification guide, which complements the scarce previous studies in the area. As expected, some undescribed sponge species arose, and we fully described them. However, their formal descriptions as new species and the assignation of holotypes require to be published in a specialised journal, which exceeds the objectives of the present thesis.

- Chapter 3: **Bacteria stability and acquisition modes in sponge microbiomes:** showcasing the role of seawater. In this chapter, we intend to describe the composition and structure of the sponge associated bacteria communities and to explore the main microbiome differences between High Microbial Abundance (HMA) and Low Microbial Abundance (LMA) sponges. Moreover, we pay particular interest to the bacteria transmission mechanisms that allow maintaining stable bacterial communities across species replicates. In this sense, we aim to reveal the degree of overlap between sponge core bacterial communities and seawater bacteria communities, to gain insights into the possible role of seawater as a seed bank for the sponge microbiome.

- Chapter 4: **Sponges and their microbiomes show similar community metrics across impacted and well-preserved reefs.** The main aim of this chapter is to determine the role of the sponge-associated bacteria in the ecological distribution of sponges by comparing their microbiomes in assemblages inhabiting two contrasting environments. Moreover, we attempt to assess whether the study microbiomes changed under these contrasting conditions or they depend on the sponge species able to proliferate in each environment. We quantify the differences in composition and diversity of the sponge assemblages between habitats and explore whether differences on sponge community metrics, according to the degree of environmental preservation, are mirrored or not by their sponge microbiomes.

- Chapter 5: **Multipartner symbiosis across biological domains: looking at the eukaryotic associations from a microbial perspective.** The main aim of this chapter is to explore whether the bacterial communities of sponge-associated polychaetes resemble those of their hosts. First, we determine the specificity of the relationships by identifying the polychaete species associated to four different sponge species. Then, we examine the bacterial communities of the two eukaryotic partners to assess the degree of overlap of their respective microbiomes by comparing the relative abundances of the shared bacterial taxa. We expected to gain information on the purported source of the bacteria conforming the polychaete microbiome.

- Chapter 6: **Insights into the archaeal consortium of sponge species from Vietnam.** In this last chapter, we want to cast some light on the still poorly known Archaea domain associated to sponges. We aim to describe the composition and structure of these particular microbial communities and to compare several parameters (i.e., stability, diversity and specificity) between them and the corresponding associated bacteria. We finally want to assess whether or not the most abundant Archaea sequences are sponge specific and thus, belong to some of the sponge-specific clusters (SC) described to date.

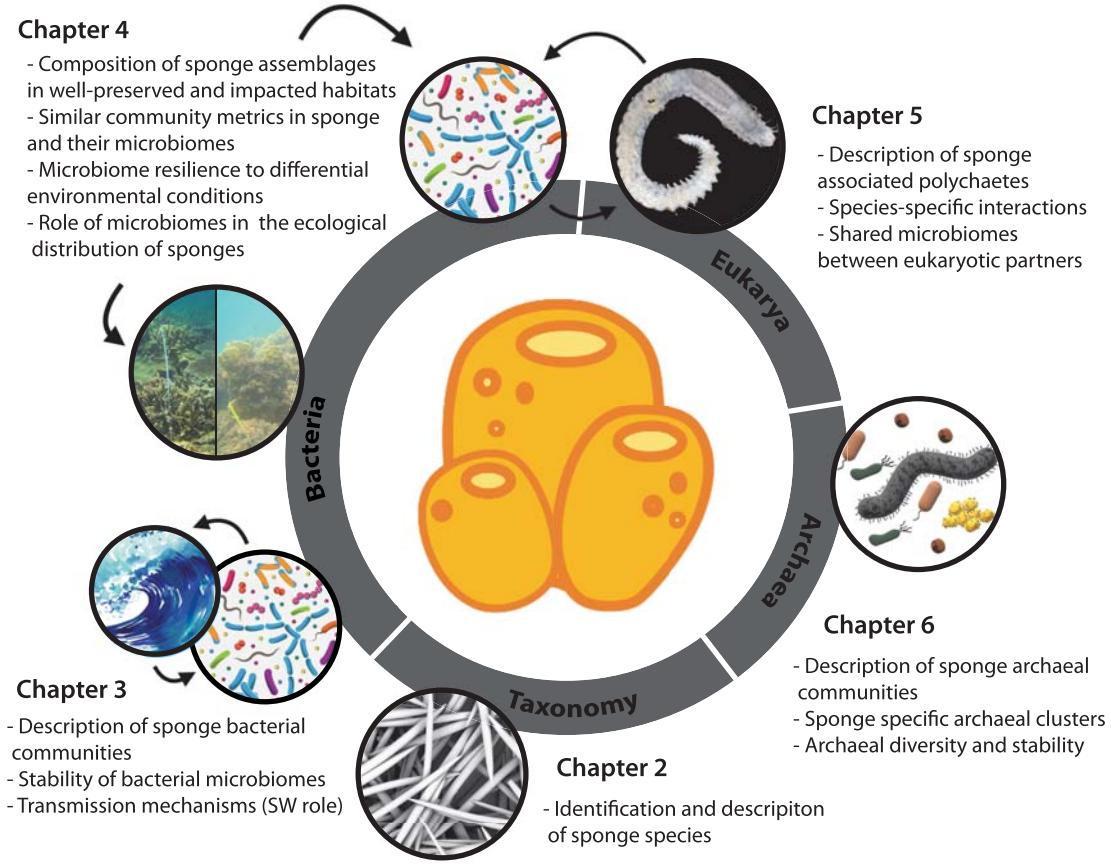
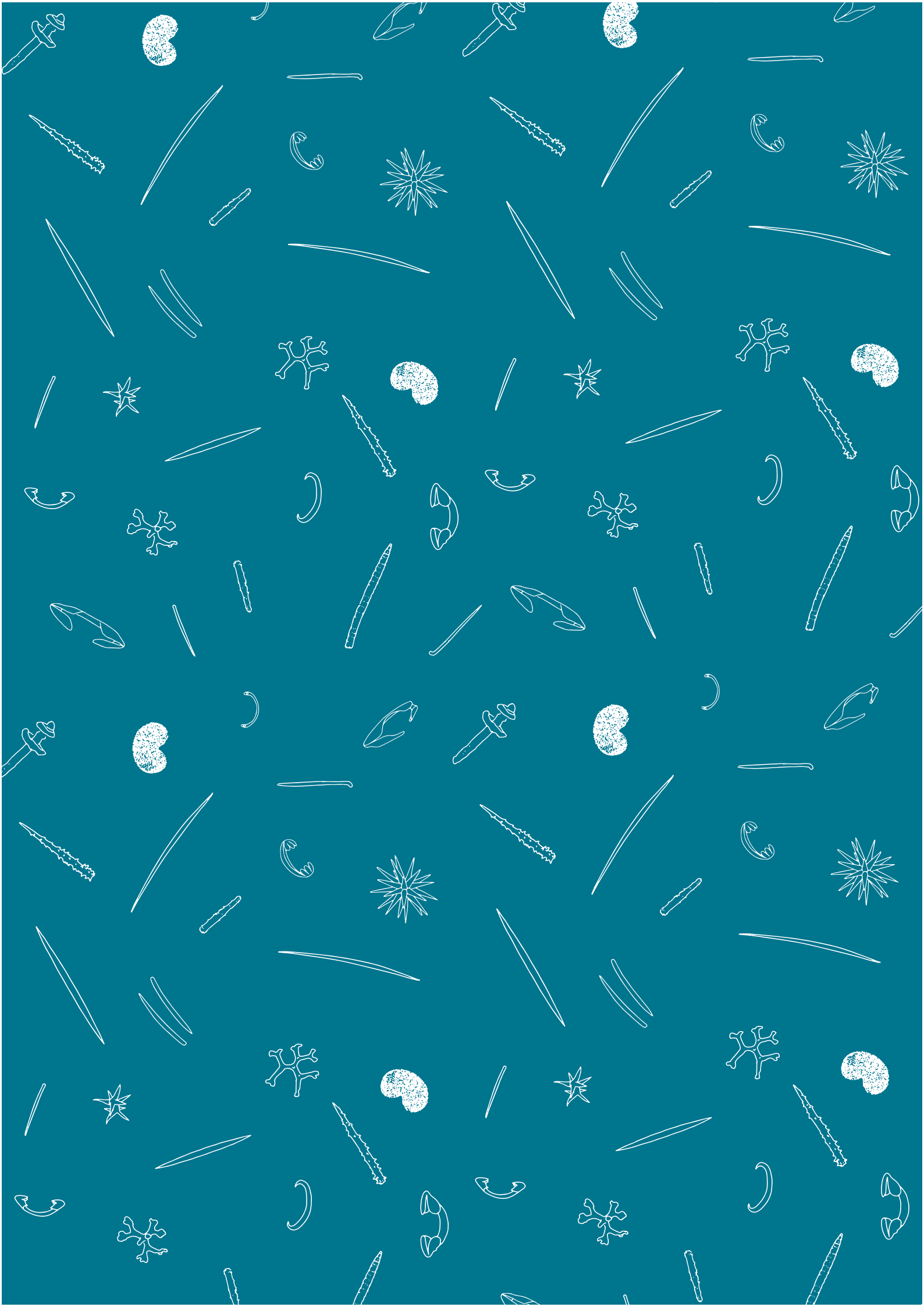


Figure 1.1: Schematic representation of the thesis structure and objectives of each chapter.



Turon, M. and Uriz, M.J. Description of Sponges from Nha Trang Bay (central Vietnam).

Aquest capítol s'està preparant per enviar en forma de dos articles a les revistes *Zootaxa* i *Zoological Journal of the Linnean Society*.

Imatge capítol: Espícules de diferents espècies d'esponja.

Autora coberta: Laura López.

Chapter 2

Taxonomy of shallow common sponges from Nha Trang Bay (Vietnam)

2.1 Abstract

We have characterized the shallow (3-9 m deep) sponge assemblages of geographically close well-preserved and polluted areas in Nha Trang Bay (Vietnam). Sampling was performed by displaying 13, 25 m long horizontal line transects and collecting all the sponges crossed by the transect tape. Species identification was in principle performed on the basis of *in situ* external characteristics and light and SEM skeleton observation. LSU, SSU, and COI gene partitions were amplified and sequenced (whenever possible) to help with the morphological identifications. Sponge species were described and illustrated to facilitate further taxonomic and faunistic studies in the area. Samples belonged to 60 species (9 orders, 22 families, and 36 genera) of demosponges. A total of 24 species were added to the known sponge fauna of Vietnam, from which, 11 species likely represent new species to science. The most abundant sponges in *Acropora* (well-preserved) reefs were *Neofibularia* sp. and *Aaptos suberitoides*, while *Monanchora unguiculata*, *Antho (Antho)* sp., and *Amphimedon sulcata* predominated in rocky habitats. *Millepora* (eutrophic) reefs were dominated by *Clathria reinwardti* and *Amphimedon paraviridis*. Thee described species represent an increase of 8% in the already known sponge list of Vietnam. The study also contributed to improve the molecular characterization of several species, which may facilitate further phylogenetic studies.

2.2 Introduction

Nha Trang Bay offers one of the highest coral diversity in Vietnam (Latypov, 2011). However, the inventory of sponges from Vietnam is still largely incomplete. A few studies have dealt with the sponges of this area (Dawydoff, 1952; Lévi, 1961; Lindgren, 1898; Quang, 2013) recording a total of 299 species, which is a relatively low number compared with those compiled in the sponge inventory (388 species) from other close areas, such as South China or Indonesia (Lim et al., 2016). Many species in those previous studies were not identified and were published as undetermined. In the current study, we attempt to improve the knowledge of the Vietnam shallow sponge fauna and particularly that of the Nha Trang Bay, from well-preserved environments (rocky walls and reefs dominated by *Acropora* spp) to habitats subjected to the harmful influence of the numerous cage cultures in close areas, which resulted in highly polluted reefs dominated by *Millepora* spp (Latypov, 2006).

Contrasting environments can be found in relatively close areas of the Nha Trang Bay. The islands located closer to the city and the port of Nha Trang show a higher degree of anthropogenic impact and also receive pollution inputs from the important mariculture activities in the bay, but the ones located further from the coast line are well-preserved (Latypov, 2006; Tkachenko et al., 2016). We have explored the shallow (3-9 m deep) assemblages of the Nha Trang Bay with the aim of, once characterized the sponge communities in well-preserved and polluted areas of the bay and to disclose the most relevant (abundant) sponge species in both types of habitats to study their microbiomes (chapters from 3 to 6). The sampled sponges are fully described and illustrated to facilitate further taxonomic and faunistic studies in the area.

2.3 Materials and methods

Sampling was performed in April 2015 by SCUBA diving along 13 transects, 25m long each, randomly placed on the rocky vertical walls, well-preserved coral reefs and polluted coral reefs of Nha Trang Bay. This method is explained in detail in Chapter 3. Fragments of ca. 3 cm^3 of the 203 sponges

crossed by the metric tape were collected and preserved in 100% ethanol. We also took *in situ* pictures and measured the diameter of all the specimens.

Species identification followed standard procedures as for spicule and skeleton preparations and observation through light and Scanning electron microscopes (see 3). DNA from all the sponges was extracted using the DNeasy Blood & Tissue kit (Qiagen) following the standard protocol and, whenever possible (not all the species amplified for the three genes assayed), fragments of the nuclear genes encoding the 18S rRNA (∼1700 bp) and 28S rRNA (∼650 bp), as well as the cytochrome c oxidase subunit I (COI ∼680 bp) were amplified and sequenced to assess genetic identification of species. PCR conditions and primers used for the genes amplification are summarized in Table A.1. Alignments of the obtained sequences with related sequences available in GenBank were done using MAFFT v.6 (Katoh et al., 2002) and manually checked with Geneious v 9.0.2.We inferred the phylogenetic relationships using maximum-likelihood (ML) and Bayesian inference methods. GTR nucleotid substitution model was used on PhyML (Guindon et al., 2010) and Bootstrap ML analysis was done with 100 pseudoreplicates. The accession numbers of the sequences obtained are included in a separate table (Table A.2) and phylogenetic trees were added, in the cases they are informative, as supplementary material.

Descriptions are illustrated with pictures of the specimens *in situ* and of their skeleton and/or spicules (light and SEM). A "remarks" section was added to each description whenever necessary to explain the particularities of the specimens collected and the taxonomical decisions taken.

Even in the cases in which we were not able to attribute our specimen to a known species, we performed a complete description of the specimens of known genera to facilitate posterior identification of these undetermined species. Some species are very likely new, but they have not been named in this thesis waiting for their formal publication in a taxonomical journal.

2.4 Results

The identification of the sponges of Nha Trang Bay yielded a total of 60 species, belonging to 9 orders within the class Demospongiae. Here,

we present the systematic descriptions of all these species following the order of the World Porifera Database. Species of the genera *Haliclona* and *Callyspongia* remain unidentified, as their identification requires a deeper, time-consuming taxonomical work, which is beyond the thesis objectives.

2.4.1 Systematic descriptions

Phylum Porifera Grant, 1836

Class Demospongiae Sollas, 1885

Subclass Verongimorpha Erpenbeck, Sutcliffe, De Cook, Dietzel, Maldonado, Van Soest, Hooper and Worheide, 2012

Order Verongiida Bergquist, 1978

Family Aplysinellidae Carter, 1875

Genus *Suberea* Bergquist, 1995

Suberea fusca (Carter, 1880)

Syn.: *Aplysina fusca* Carter, 1880

Material examined: Individuals S13.13, S13.16, S10.6, Vietnam (Nha Trang). Rocky shore of Hun Mun Island (South) and well-preserved coral reef, close to Dambay. Depth 6-9 m. 8th. Sampling date: April 10th,2015.

Description: thick encrusting to massive sponges, less than 4 cm in diameter. Colour brownish outside, yellow inside when alive (pinkish in alcohol). Conulous surface, with irregularly separated, slightly marked conules. Compressible, fleshy consistency. Thick ectosome, difficult to separate from the choanosome (Figure 2.1A).

Skeleton: Few, dendritic irregularly branching fibres without differentiation between primary and secondary elements. Fibres orange to brown in colour, 360-500 μm in diameter, easy to break, with large pith (ca. 3/4 of the total diameter) and thin laminated bark (Figure 2.1B,C).

Distribution and ecology: Indian Ocean: Seychelles Islands, India and Sri Lanka.

Remarks: The individuals from NhaTrang showed the typical colour, morphological, and skeletal characteristics of the species but their skeletal fibres are wider than those of the holotype. The phylogenetic tree based on LSU sequences (Figure A.1), placed *S. fusca* close to *Porphyria flintae* in a clade also containing *Suberea laboutei*, far from *Suberea creba*, suggesting that the genus *Suberea* is polyphyletic. The clades containing *Verongula* plus *Aplysina*, *Porphyria* and two *Subera* species were strongly supported (98%) as it was the subclade containing the three later genera (100%). The genus *Aplysina* was also well-supported (93%). However, the sequenced species available of the Family *Aplysinidae* are clearly insufficient to generate a consistent phylogeny for the genera *Suberea*, *Porphyria*, and *Aiolochrota*. The COI based phylogeny did not generate any consistent tree (Figure A.2).

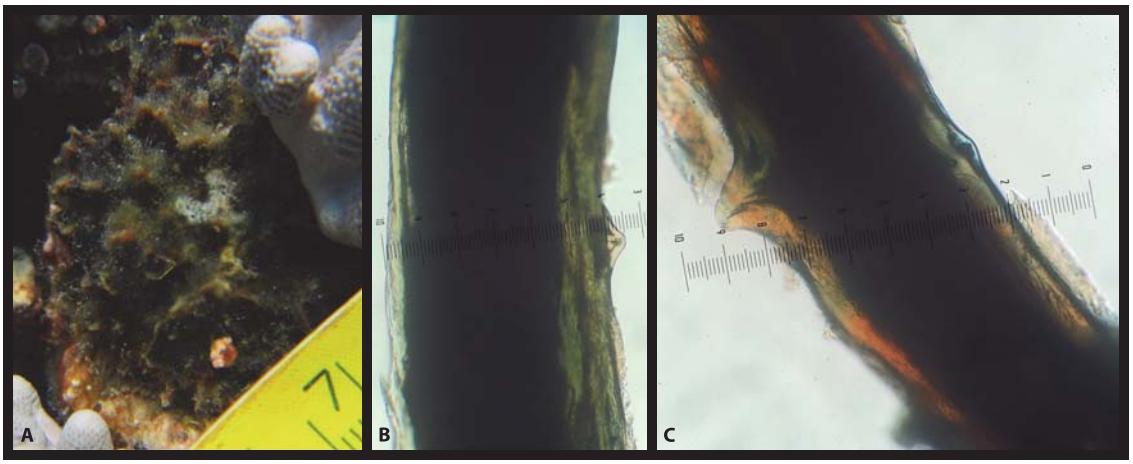


Figure 2.1: *Suberea fusca*. A) *In situ* specimen. B, C) Light microscopic image of pithed fibres. Scale bar in μm.

Family Ianthellidae Hyatt, 1875

Genus *Hexadella* Topsent, 1896

Hexadella indica Dendy, 1905

Material examined: Individual 10.3, Vietnam (Nha Trang). Well-preserved coral reef close to Dambay. Depth 6 m. Sampling date: April 8th, 2015.

Description: Thin encrusting sponge, 3 cm of diameter. Bright yellow in colour. Surface smooth. Small orifices irregularly scattered on the surface (Figure 2.2).

Skeleton: Without fibres with a collagen rich surface. Thick ectosome separable in flakes.

Distribution and ecology: Indian-Ocean: Sri Lanka

Remarks: This species, together with *H. purpurea* Dendy are the only ones of the genus known from the Indo-Pacific area. They are easily distinguishable by the colour purple of the later, and yellow in *H. indica*.

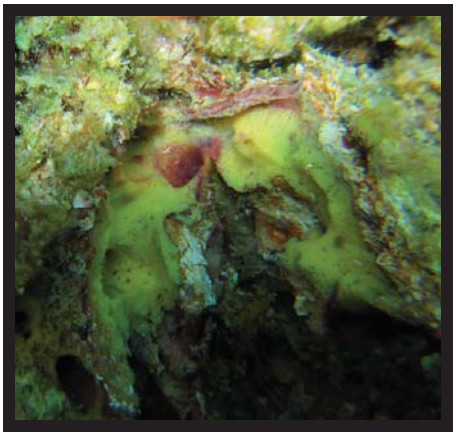


Figure 2.2: *In situ* specimen of *Hexadella indica*.

Subclass Keratosa Grant, 1861

Order Dictyoceratida Minchin, 1900

Family Dysideidae Gray, 1867

Genus *Dysidea* Johnston, 1842

Dysidea sp1

Material examined: Individuals 1.8, 1.14, 1.24, Vietnam (Nha Trang). Rocky vertical, low lighted wall (crack) of Hun Mun Island Depth 10 m. Sampling date: March 28th 2015.

Description: Encrusting sponge, between 3-6 cm in diameter, with a strongly conulous surface with algal epibionts on the ridges between conules.

Pronounced conules, 4-5 apart. A reticulation of ectosomal (collagen-made) ridges between conules. Soft consistency. Inhalant orifices in concavities between conules. Several sparse oscules of a cloacal type at the end of an ectosome-made short tube. Pinkish to purple colour in life (Figure 2.3A).

Skeleton: Reticulated network of primary and secondary fibres both full of foreign material: sand grains and foreign spicules (Figure 2.3B,C). The primary fibres end in the surface conules and can divide at the distal part.

Remarks: The Nha Trang individuals differ from the several species recorded in the Indo-Pacific and represent a new species, which will be formally described separetely. The LSU sequences are close to those available of *Dysidea frondosa* and *D. fragilis* but the clade was poorly supported in the phylogenetic tree (Figure A.3). The COI place the species among the *Dysidea* clade, which form a 100% supported clade with *Euryspongia lobata* (Figure A.4). The SSU sequence also place the study species in a *Dysidea* clade but it forms a separed cluster from the one formed by *D. frondosa* and *D. fragilis* (Figure A.5). According to the LSU phylogenetic tree, the genus *Dysidea* would be polyphyletic since Different *Dysidea* species clustered in several subclades with *Euryspongia*, *Lamellodysidea*, and *Faciospongia*, but the support of the subclades is also low likely due to a insufficient number of representatives of *Dysideidae* genera available from the NCBI database, except for *Dysidea*.

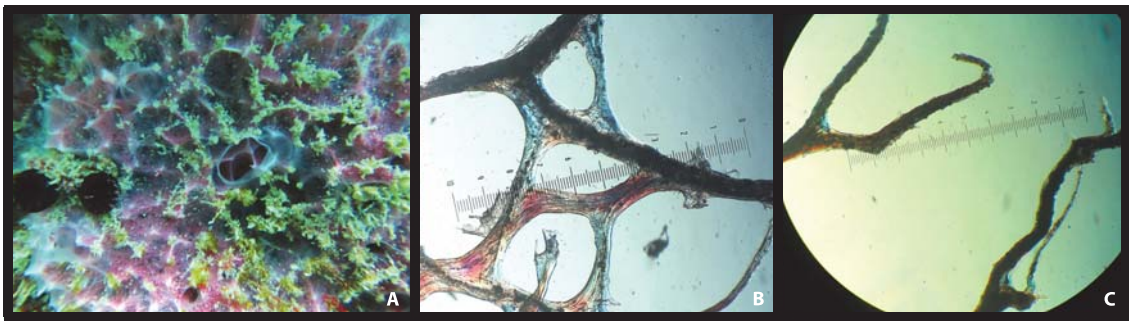


Figure 2.3: *Dysidea* sp1, A) *In situ* specimen. B, C) Light microscopic image of skeleton of fibres. Scale bar in μm.

Dysidea sp2

Material examined: Individual 3.3, Vietnam (Nha Trang). Rocky semi-vertical shore of Hun Mun Island (North). Depth 8 m. Sampling date: March 30th 2015.

Description: Surface smooth and even with conules up to 2mm high, 3-5 mm apart. Soft consistency. Ectosome difficult to separate from the choanosome. Oscules 5-7 mm wide. Plain white colour in life. Dirty white in alcohol(Figure 2.4A).

Skeleton: Reticulate, with primary (22-54 μm in diameter) and secondary fibres (22- 10 16 μm in diameter) filled with abundant foreign bodies. Primary fibres end in the surface conules (Figure 2.4B).

Remarks: This individual likely represents a new *Dysidea* species, as no similar species have been found among those described from the eastern Pacific and Indian-Ocean

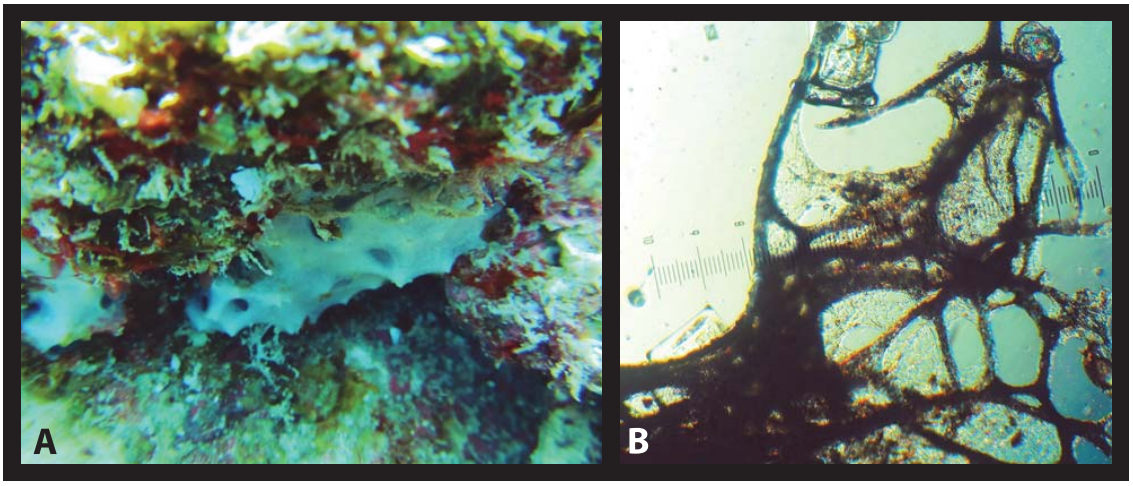


Figure 2.4: *Dysidea* sp2, A) *In situ* specimen. B) Light microscopic image of skeleton of fibres. Scale bar in μm.

Genus *Euryspongia* Row, 1911

Euryspongia lobata Bergquist, 1965

Material examined: Individual 4.21, Vietnam (Nha Trang). Rocky vertical wall of Nock Island. Depth 9 m. Sampling date: April 1st 2015.

Description: Soft compressible consistency. Conulous surface, with spread short conules connected by a network of ectosomal ridges and two,

2-3 mm wide, elevated oscules at the end of a short ectosomal projection. Blue greyish colour in life, greyish in alcohol (Figure 2.5A).

Skeleton: A reticulated network of primary and secondary fibres. Primary fibres run perpendicular to the surface forming the conules. They range from 400 to 500 μm in diameter and are filled with abundant foreign material. Secondary fibres free of foreign material, measured from 80-430 μm in diameter (Figure 2.5B).

Distribution and ecology: Pacific: Palau, West Caroline Islands

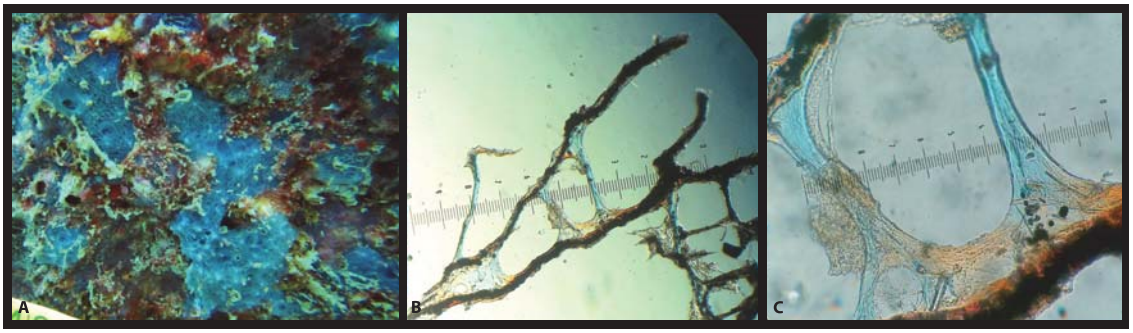


Figure 2.5: *Euryspongia lobata*, A) *In situ* specimen. B) Light microscopic image of skeleton of fibres. Scale bar in μm.

Family Thorectidae Bergquist, 1978

Genus *Dactylospongia* Bergquist, 1965

Dactylospongia elegans (Thiele, 1899)

Syn.: *Luffariella elegans* (Thiele, 1899)

Material examined: Individual 3.7 Vietnam (Nha Trang). Rocky semi-vertical shore of Hun Mun Island (North). Individual 4.18, rocky vertical walls of Nock Island. Depth 8-9 m. 30th March and 1st April 2015.

Description: Thick encrusting, very hard sponges, 7 to 10 cm long axis, with irregular surface. Colour brownish outside and dirty yellowish inside, in life; brownish in alcohol (Figure 2.6A).

Skeleton: Ectosomal irregular network formed by fibres containing sand grains and another foreign material. Choanosomal skeleton consisting of dense reticulated hard fibres 9-18 μm in diameter, free of foreign debris forming polygonal meshes of 45 to 500 μm in diameter (Figure 2.6B,C).

Distribution and ecology: Sulawesi Sea/ Makassar Strait, Banda Sea, Eastern Philippines, Malacca Strait and West Caroline Islands. The specimens collected were inhabited by high amounts of polychaetes.

Remarks: The species shows habitually an erect or ramified growth habit. Our encrusting individuals may correspond to first growth stages.

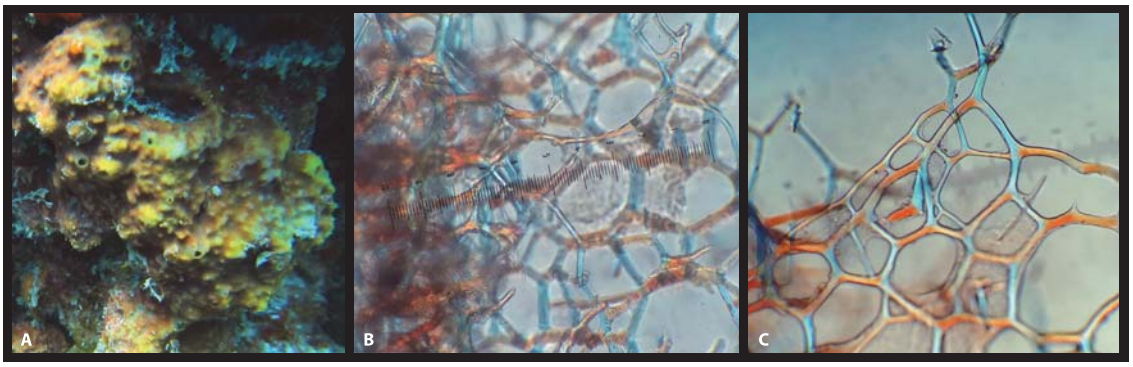


Figure 2.6: *Dactylospongia elegans*, A) *In situ* specimen. B, C) Light microscopic image of skeleton of fibres. Scale bar in μm.

Genus *Hyrtios* Duchassaing and Michelotti, 1864

Hyrtios spinifer (Poléjaeff, 1884)

Syn. *Cacospongia spinifera* Polejaeff, 1884.

Material examined: Individual 12.3, Vietnam (Nha Trang). Rocky shore of Hun Mun Island (South). Depth 10 m. 10th April 2015.

Description: Small encrusting specimen of 2-3 cm diameter, with consistency somewhat hard but fragile and surface highly conulouse with conules up to 3mm high. Surface, relatively free of foreign particles. Colour dark grey outside in life (Figure 2.7A). Grey in alcohol.

Skeleton: Irregular reticulation of primary (65-90 μm in diameter) and secondary 20-50 μm in diameter) fibres full of foreign material. Primary fibres converge at the surface level with secondary in a reticulate manner to shape the surface conules (Figure 2.7B,C).

Distribution and ecology: Indonesia. The species has been cited only once Polejaeff (1984).

Remarks: The skeletal characteristics are common to the other species of *Hirtyos* from the Indo-Pacific and thus this species is difficult to differen-

tiate from the species *H. reticulata* and *H. erectus*. It cannot be completely rule out that this species is cospecific and represents a young individual (initial stages of growth) of *H. erectus*.

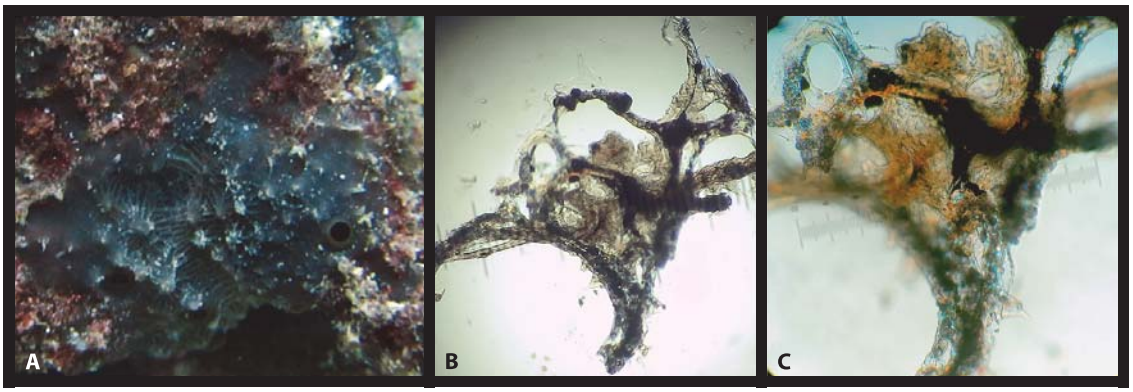


Figure 2.7: *Hyrtios spinifera*, A) *In situ* specimen. B, C) Light microscopic image of skeleton of fibres. Scale bar in μm.

Order Haplosclerida Topsent, 1928

Family Callyspongidae de Laubenfels, 1936

Genus *Callyspongia* Duchassaing and Michelotti, 1864

Callyspongia sp1

Material examined: Individuals 12.7, 12.11, Vietnam (Nha Trang). Rocky shore of Hun Mun Island (South). Depth 10 m. April 10th 2015.

Description: Very soft thick encrusting creeping sponge. Surface with a clear reticular pattern with minute conules at the confluence of meshes. Oscula slightly elevated at the end of somewhat globular projections. Blue-greyish colour with a narrow white ring surrounding the oscula in life (Figure 2.8A,B).

Skeleton: Plurispicular reticulated tracks in the choanosome. Bidimensional to three dimensional (in some places) ectosomal skeleton. Oxeas 105-130 x 3-4 μm (Figure 2.8C).

Remarks: The LSU phylogenetic tree place this species in the clade of *Callyspongia pseudoreticulada*, an unidentified *Callyspongia* species and an unidentified *Siphonochalina species* (Figure A.6).

Callyspongia sp2

Material examined: Individual 7.15, Vietnam (Nha Trang). Impacted coral reef (dominated by Millepora) of Dambay region. Depth 2-4 m. April 3th, 2015.

Description: Massive, ca. 2 cm in diameter globulous individual with a single large oscule cloacal of ca. 1cm of diameter at the central zone. Conulous surface (spiny appearance) surface. Soft consistency, yellowish colour in life (Figure 2.8D).

Skeleton: Choanosomal skeleton reticulated with multispicular tracks surrounded by spongin. Three dimensional ectosomal network, with primary multispiculate meshes, secondary paucispicular meshes and tertiary unispicular meshes (Figure 2.8E,F). Oxeas of 75-93 x 3-4 μm.

Remarks: the LSU tree place this species in a clade together with *Callyspongia fallax*, *C. multiformis*, *C. ramosa* and *Arenosclera heroni*, with a bootstrap support of 85% (Figure A.6).

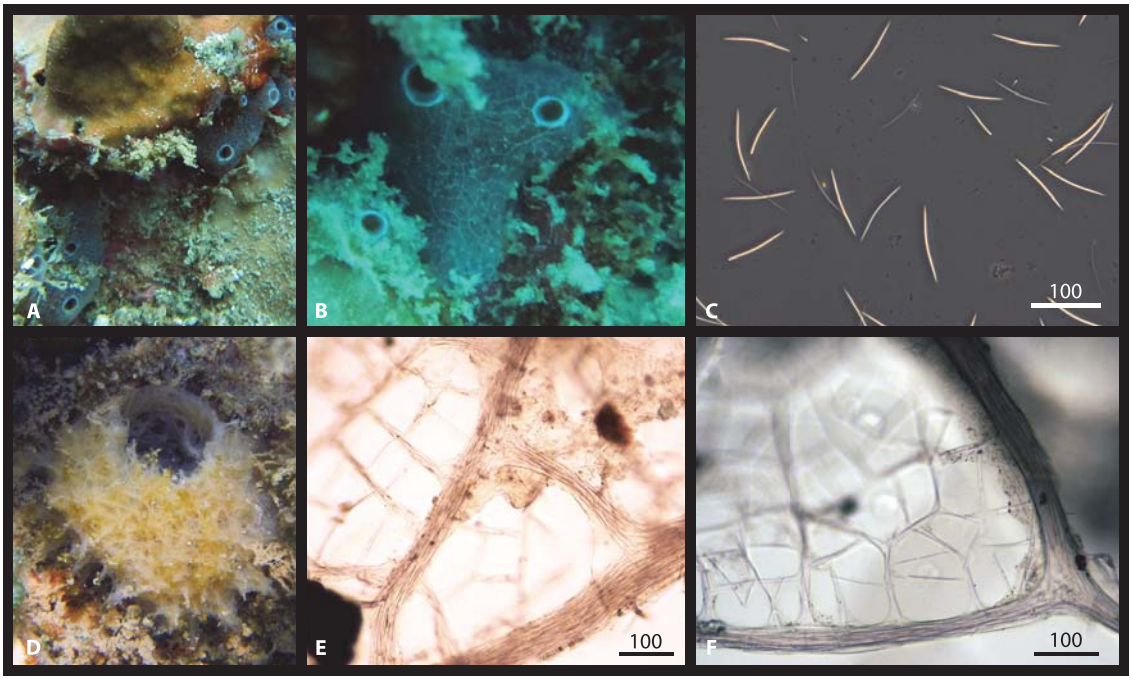


Figure 2.8: *Callyspongia* sp1 (A-C) and sp2 (D-F). A,B,D) *In situ* specimens. C,E,F) Light microscopic images of oxeas (C) and skeleton (E-F). Scale bar in μm.

Genus *Dactylia* Carter, 1885

Dactylia sp.

Material examined: Individual 1.28, Vietnam (Nha Trang). Rocky vertical wall (crack) of Hun Mun Island. Low light. Depth 10 m. Sampling date: March 28th 2015.

Description: Sponge 6 cm diameter, encrusting with soft consistency. Surface slightly conulose, with abundant, randomly distributed 2-3m in diameter oscules over the entire surface. Blue-purple colour with a whitish semi-transparent membrane. Net-like drawing in the surface (Figure 2.9A).

Skeleton: Ectosomal skeleton formed by an irregular reticulation of foreign debris. Choanosomal skeleton reticulated formed by spongin fibres containing oxeas. Primary and secondary fibres differentiated, the former containing more than 10 spicules in cross-section. Secondary fibres with only one spicule in a line. Oxeas of 103-127 μm. (Figure 2.9B-D).

Distribution and ecology: Australia, Catham Island.

Remarks: This individual belongs to a new species of *Dactylia*, which will be formally described in a separate paper. The closest species is *Dactylia repens* (Carter, 1986). However, our specimen differs from the Carter species by the colour (bluish instead of brown) and the smaller size of the oscules.

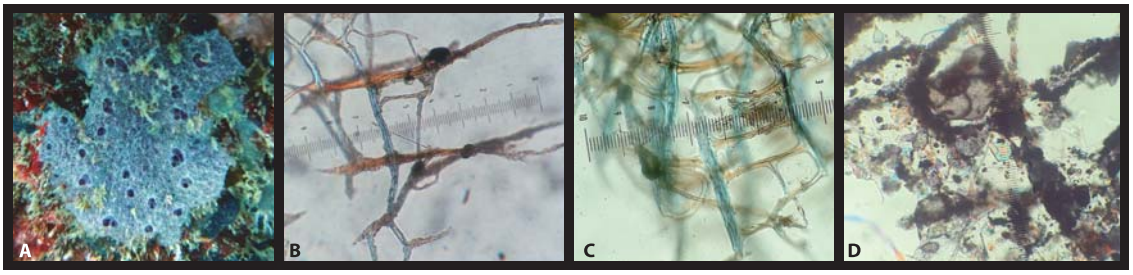


Figure 2.9: *Dactylia* sp. A) *In situ* specimen. B, C, D) Light microscopic image of skeleton. Scale bar in μm.

Family Chalinidae Gray, 1867

Genus *Haliclona*Grant, 1835

Haliclona (Reniera) Schmidt, 1862

Haliclona (Reniera) sp1

Material examined: Individual 1.9. Vietnam (Nha Trang). Rocky vertical wall (crack) of Hun Mun Island. Low light. Depth 10 m. Sampling date: March 28th 2015.

Description: Encrusting with numerous 3-4 mm in diameter oscules surrounded by an ectosomal ring, placed at the end of short protuberances. Ostia clustered in small inhalant areas spread among the oscula. Sky blue in life (Figure 2.10A).

Skeleton: 160-190 x 3-4 μm strongyles. Ectosomal isodictyal network of unispiculated meshes (Figure 2.10B,C).

Haliclona (Reniera) sp2

Material examined: Individual 4.16. Vietnam (Nha Trang). Rocky vertical wall of Nock Island. Depth 9 m. 1st April 2015.

Description: Encrusting with 5mm in diameter depressed oscules. Ostia clustered in small inhalant areas spread among the oscula. Consistency shoft. Blue with a purple tinge in life (Figure 2.10D).

Skeleton: Oxeas of 135-210 x 4-5 μm. Thinner spicules 120-145 x 2 μm can be observed, likely representing young spicules. Ectosomal isodictyal network of unispiculated meshes (Figure 2.10E,F).

Haliclona (Reniera) sp3

Material examined: Individual 9.20. Vietnam (Nha Trang). Coral reef semi-good conditions close to Hun Mun Island (north). Depth 6 m. 6th April 2015.

Description: An encrusting individual, likely death without conspicuous oscula. Consistency very shoft. Translucid colour with a mauve tinge (Figure 2.11A).

Skeleton: Oxeas of 145-185 x 3 μm. Ectosomal isodictyal network of unispiculated meshes (Figure 2.11B,C).

Haliclona (Reniera) sp4

Material examined: Individuals 6.2, 6.8, 11,8.Well-preserved reef (Acropora dominated) close to Dambay region. Depth 4-8 m. 2nd and 8th April 2015.

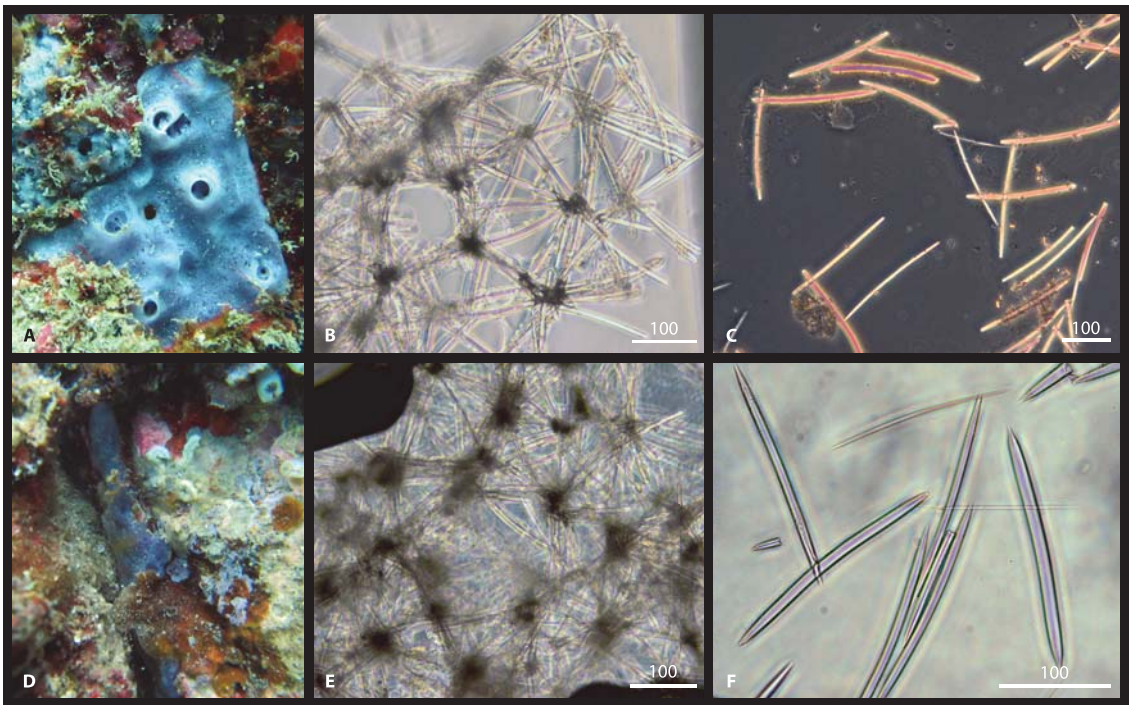


Figure 2.10: *Haliclona (Reniera)*sp1 (A-C) and sp2 (D-E). A, D) *In situ* specimens. B, C, E, F) Light microscopic image of skeleton (B,E), strongyles (C) and oxeas (F). Scale bar in μm.

Description: Thick encrusting individuals, with protuberances ended in oscula across the whole surface. Inhalant areas among oscula. Oscula 2-3 mm in diameter. Consistency soft. Colour from dirty yellow to orange and red in life (Figure 2.11D).

Skeleton: Ectosomal isodictyal network of unispiculated meshes. Oxeas of 120-147 μm (Figure 2.11E,F).

Haliclona (Reniera) sp5

Material examined: Individual 8.14. Vietnam (Nha Trang). Impacted coral reef (Millepora dominated) of Dambay region. Depth 2-4 m. 4th of April 2015.

Description: Thick encrusting rampant individual with numerous oscules of different size (1-4 mm diameter). Consistency soft and slightly compressible. Colour mauve pale (Figure 2.12A).

Skeleton: Oxeas 130-175 x 2-3 μm. Ectosomal isodictyal network of triangular meshes, 1 spicule long per side (Figure 2.12B,C).

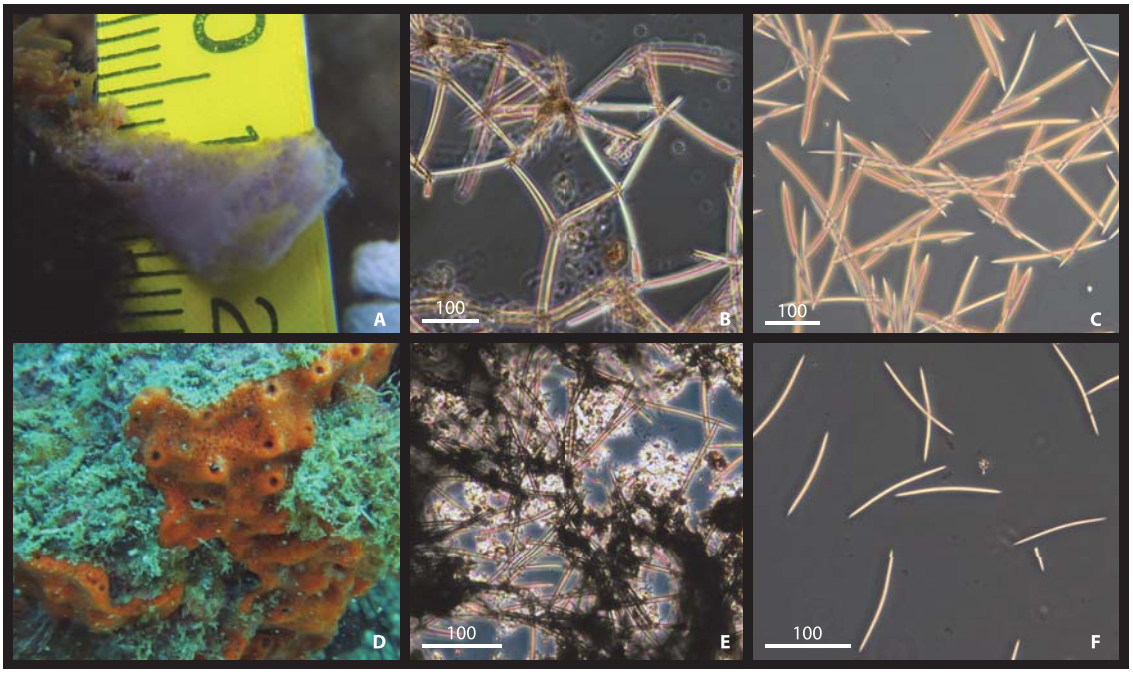


Figure 2.11: *Haliclona (Reniera)* sp3 (A-C) and sp4 (D-E). A, D) *In situ* specimens. B, C, E, F) Light microscopic image of skeleton (B,E) and oxeas (C,F). Scale bar in μm.

Haliclona (Reniera) sp6

Material examined: Individual 9.28. Vietnam (Nha Trang). Coral reef semi-good conditions close to Hun Mun Island (north). Depth 6 m. 6th April 2015.

Description: Encrusting individual without oscula visible. Consistency soft. Colour blue-greyish (Figure 2.12D).

Skeleton: Ectosomal isodictyal network of triangular meshes, 1 spicule long per side. Oxeas 140.180 x 2-3 μm. Sigmas 45-60 μm chord (Figure 2.12E,F).

Haliclona (Reniera) sp7

Material examined: Individual 1.4. Vietnam (Nha Trang). Rocky vertical wall (crack) of Hun Mun Island. Low light. Depth 10 m. 28th March 2015.

Description: Thin encrusting individual with superficial sinuous aquiferous canals converging in small (less than 1mm in diameter) oscula. Colour turquoise blue.

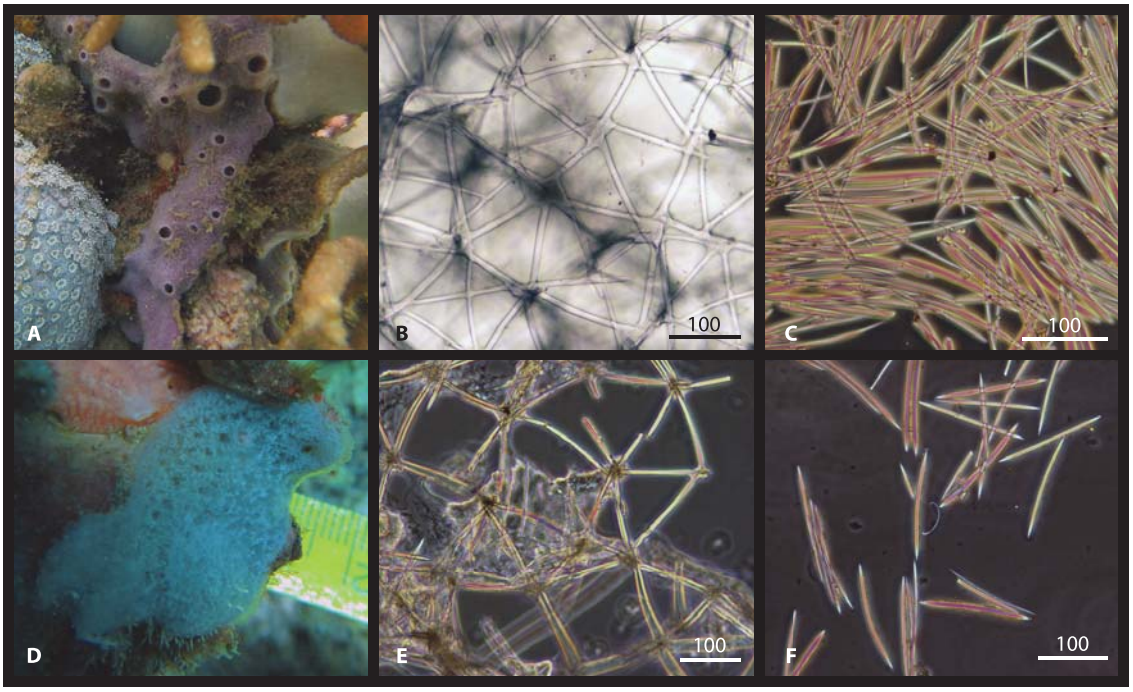


Figure 2.12: *Haliclona (Reniera)* sp5 (A-C) and sp6 (D-E). A, D) *In situ* specimens. B, C, E, F) Light microscopic image of skeleton (B,E) and oxeas (C,F). Scale bar in μm.

Skeleton: Ectosomal isodictyal network of triangular meshes, 1 spicule long per side. Oxeas 140.180 x 2-3 μm. Sigmas 45-60 μm of chord.

Haliclona (Gellius) Gray, 1867

Haliclona (Gellius) toxia (Topsent, 1897)

Syn.: *Gellius toxius* Topsent, 1897; *Toxadocia toxius* (Topsent, 1897); *Toxiclona toxius* (Topsent, 1897).

Material examined: Individual 4.5, 4.11, 4.17, Vietnam (Nha Trang). Rocky vertical wall of Nock Island. Depth 9 m. 1st April 2015.

Description: Thick encrusting, branching, repent sponges, between 2 and10 cm long. Surface smooth or hispid in some zones. Consistency compressible and soft. Oscules of 1.5-2 mm wide, at the end of short protuberances. Inhaling areas visible in shallow depressions. Colour light blue-greenish in life. Whitish in alcohol (Figure 2.13A).

Skeleton: Network of uni-spiculate meshes with spongin almost reduced to the mesh nodes. Some oxeas also disarranged outside the fibres.

Spicules: Oxeas: 100-135 μm X 5-8, slightly curved, with blunt ends (180 x9 μm in the holotype (Topsent 1987). Some oxeas thinner and smaller (grow forms?). Toxas 14-53 μm with a strong rounded curvature (up to 90 μm in the holotype (Topsent 1897) (Figure 2.13B-E).

Distribution and ecology: Red sea: southern zone. Indian and Pacific oceans: Banda Sea, East African Coral Coasts, Seychelles Islands, South India and Sri Lanka. Southern China.

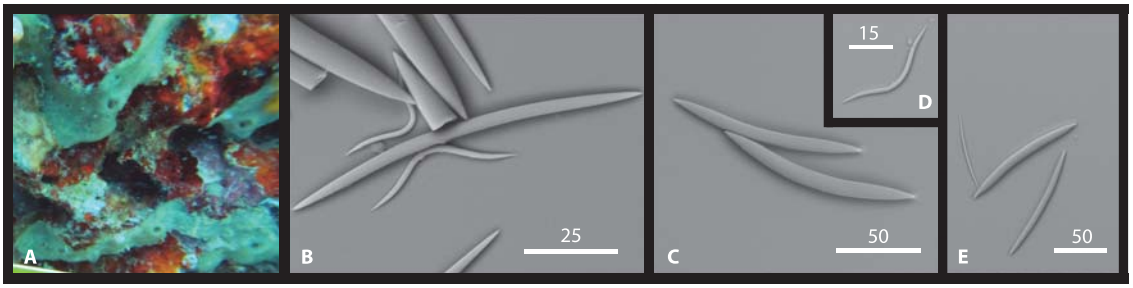


Figure 2.13: *Haliclona (Gellius) toxia*. A) *In situ* specimen. B-E) SEM images of oxeas (B,C,E) and toxas (B,D). Scale bar in μm.

Haliclona (Gellius) amboinensis (Lévi, 1961)

Syn.: *Gellius amboinensis* Lévi, 1961; *Sigmadocia amboinensis* Lévi, 1961

Material examined: Individuals 9.10, 10.4, Vietnam (Nha Trang). Coral reef semi-good conditions close to Hun Mun Island (north) and well-preserved reef (*Acropora* dominated) close to Dambay region. Depth 6 m. April 6th and 8th, 2015.

Description: Sponge thick encrusting 3-13 cm in size, with and irregular shape and short protuberances ended in oscula of sizes between 1.5 and 3 mm. Irregular, sulcate surface with small polygonal depressed areas. Colour blue with purple and whitish tinges (Figure 2.15A).

Skeleton: Reticulation anisotropic with multispicular and paucispicular tracks and additional spicules disarranged. Ectosomal skeleton formed by irregularly arranged tangential oxeas. Oxeas 185-260 x 3-5μm. Sigmas 13-17 μm of chord (Figure 2.15B).

Distribution and ecology: Indian Ocean, Mozambique Canal, East Africa Coral coast; Pacific Ocean, Banda Sea, Southern Vietnam.

Genus *Dendroxea* Griessinger, 1971

Dendroxea sp.

Material examined: Individuals 4.12, 4.14, Vietnam (Nha Trang). Rocky vertical wall of Nock Island. Depth 9 m. April 1st, 2015.

Description: Encrusting sponge up to 10 cm in diameter, with a uniformly hispid surface and soft consistency. Black colour in life. Evenly distributed oscula over the entire surface (Figure 2.14A).

Skeleton: Perpendicular to the surface plumose spicule tracks that cross slightly the sponge surface producing a uniform hispidation. Oxeas 115-145 x 2-3 μm (Figure 2.14B,C).

Remarks: The skeletal arrangement is typical of the genus *Dendroxea*, a Mediterranean genus. This is the first time that a *Dendroxea* species is recorded form the Indo-Pacific area.

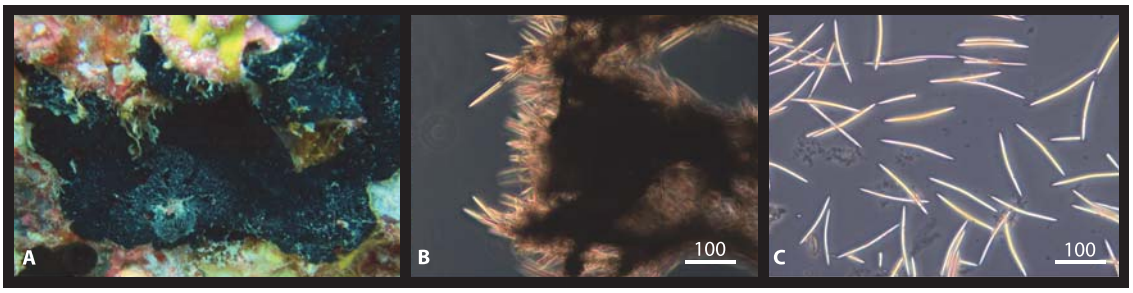


Figure 2.14: *Dendroxea* sp. A) *In situ* specimens. B,C) Light microscopic image of skeleton (B) oxeas (C). Scale bar in μm.

Genus *Chalinula* Schmidt, 1868

Chalinula nematifera (de Laubenfels, 1954)

Syn. *Nara nematifera* de Laubenfels, 1954; *Haliclona nematifera* (de Laubenfels, 1954)

Material examined: Individual 6.10, Vietnam (Nha Trang). Well-preserved reef (dominated by *Acropora* spp.) close to Dambay region. Depth 4-6 m. April 2nd 2015.

Description: Thin encrusting sponges growing on corals. Typical surface smooth with white lines, which resemble small worms. Thin ectosome

distinguishable but difficult to separate from the choanosome. Purple colour outside in life (Figure 2.15C).

Skeleton: Reticulated spongin fibres including a few oxeas. Primary and secondary fibres are distinguishable. The spongin meshes are large and contain more than one spicule long per side. Oxeas small and straight or slightly curved. They measured 55-110 x 2-5μm (Figure 2.15D).

Distribution and ecology: Indic Ocean. Marshall Islands, Central and Southern Great Barrier reef, Micronesia.

Remarks: This species is common across the Indian and Pacific oceans and it is easily recognisable because the worm-like external white lines. The colour may vary between purple, blueish, and or pink.

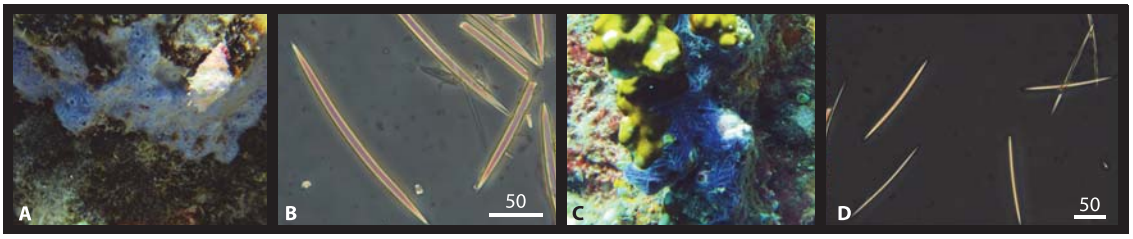


Figure 2.15: *Haliclona (Gellius) amboinensis*(A-B) *Chalinula nematifera*(C-D). A,C) *In situ* specimens. B,D) Light microscopic image of oxeas. Scale bar in μm.

Amphimedon paraviridis Fromont, 1993

Material examined: Individual S7.10, S5.6, S7.2, S7.3, S7.13, S7.16, S7.18, S7.20, S7.21, S7.23, S8.1, S8.4, S8.5, S8.7, S8.9, S8.12, S7.7, Vietnam (Nha Trang). Impacted coral reef (*Millepora* dominated) of Dambay region. Depth 2-4 m. 3th of April 2015. This is one of the sponges more abundant in the polluted areas of Nha Trang.

Description: Massive, thick encrusting, irregularly branching, up to 20cm long sponge. Consistency firm but slightly compressible. Even surface to the touch, microhispid through the steromicroscope. Oscules, 2-3 mm in diameter, on top of short protuberances. Olive green or bright green colour alive, sometimes dirty because of the sediment incorporated (Figure 2.16A).

Skeleton: Dense, irregularly reticulated spongin fibres, cored by a variable number of spicules. Primary fibres and secondary fibres distinguishable. Ectosomal skeleton formed by a tangential spicule network with spicules perpendicular to the surface at the mesh nodes. Oxeas of 150-185 x 3-4 μm.

Small spicules measure 70-115 x 1-2 μm, likely representing growth forms (Figure 2.16C,D).

Distribution and ecology: Australia, Central and Southern Great Barrier Reef. Highly abundant in areas highly eutrophicated from Vietnam where it is always associated to polychaetes of the genus *Haplosyllis*.

Remarks: The species is morphologically identical to *A. viridis* from the Caribbean. The LSU sequence is also identical in both species. These similarities and the extraordinary abundance of *A. paraviridis* in areas with strong aquaculture activities, suggest that both species might be conspecific, the later introduced in the Pacific (or vice-versa) trough human activities.

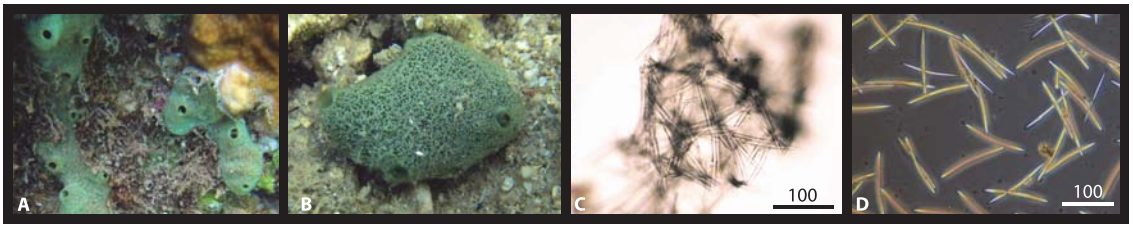


Figure 2.16: *Amphimedon paraviridis* A,B) *In situ* specimens. C,D) Light microscopic image of skeleton (C) oxeas (D). Scale bar in μm.

Amphimedon sulcata Fromont, 1993

Material examined: Individuals S1.11, S1.15, S1.21, S2.2, S2.5, S3.2, S5.4, S6.4, S6.7, S6.11, S7.14, S8.3, S8.6, S9.14, S9.31, S11.2, S11.10, S12.10, S13.4, S13.6, S13.8, S13.10, S13.14, S13.15, Vietnam (Nha Trang). Widely distributed in most of the transects examined in the current study. Mostly in rocky areas.

Description: Thick encrusting to massive, somewhat rounded sponge, Consistency hard but compressible. Grooved appearance, with meandering ridges interspersed with sulcus.. Small oscules between the protruding ridges. Ostia visible on the grooves. The individuals examined measured 2-3cm in diameter. Bright light blue colour, to greyish-mauve in individuals form the polluted habitats (Figure 2.17A,D).

Skeleton: Plumo-reticulate network of spongin fibres cored by few spicules with meshes 95-230 μm in diameter. Primary and secondary fibres differentiated. Primary fibres, 70-85 μm in diameter cored by a few spicules slightly surpass the sponge surface. Secondary fibres 20-50 μm wide.

Additional spicules disarranged between the meshes. Ectosome skeleton formed by tangential oxeas.

Spicules: oxeas straight or slightly curved with blunt ends, 122-153 x 3-5 μm in size. C-shaped sigmes 25-30 μm of chord (Figure 2.17B,C,E,F).

Distribution and ecology: Arafura Sea, Indonesia, Palau, West Caroline Islands. In the study area, the species lives preferentially in well-preserved reefs but it can also be occasionally found in polluted sites of Nha Trang Bay.

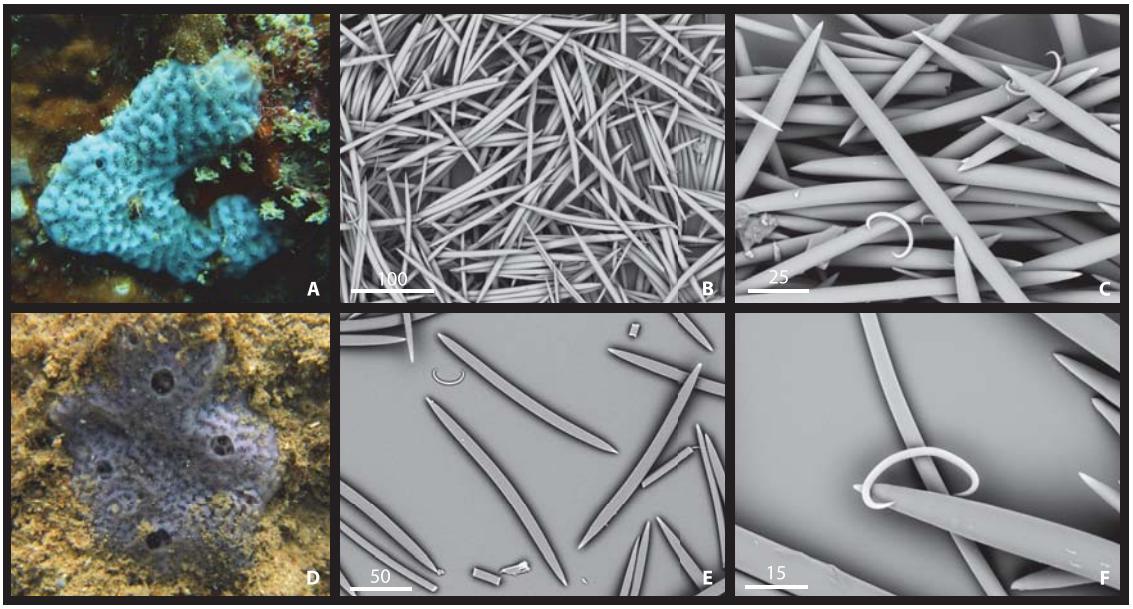


Figure 2.17: *Amphimedon sulcata*. A,D) *In situ* specimens. B,C,E,F) SEM images of oxeas and sigmes. Scale bar in μm.

Genus *Gelliodes* Ridley, 1884

Gelliodes fibulata (Carter, 1881)

Material examined: Individual 7.4, Vietnam (Nha Trang). Impacted coral reef (dominatedby Millepora spp.) of Dambay region. Depth 2-4 m. Sampling: April 3th , 2015.

Description: Branching sponge with relatively thin, long branches. Surface highly colunulose (conules of ca. 5 mm long). Consistency hard but compressible. Light blue colour alive (Figure 2.18A).

Skeleton: Fibro-reticulate with primary 300-450 um wide fibres cored by oxeas, densely packed. Secondary tracks across the primary tracks 80-150

μm wide. Primary tracks form the conules at the sponge surface. Spicules spread out of the spongin tracks.

Spicules: oxeas straight or slightly curved, with blunt points, 200-285 x 3-4 μm; Sigmas C-shaped 13-17 μm of chord (Figure 2.18B-D).

Distribution and ecology: Bassian, Andaman and Nicobar Islands, Arafura Sea, Banda Sea, Bismarck Sea, Central and Southern Great Barrier Reef, Gulf of Aden, Malacca Strait, New Caledonia, Singapore, Southern Vietnam, Torres Strait Northern Great Barrier Reef.

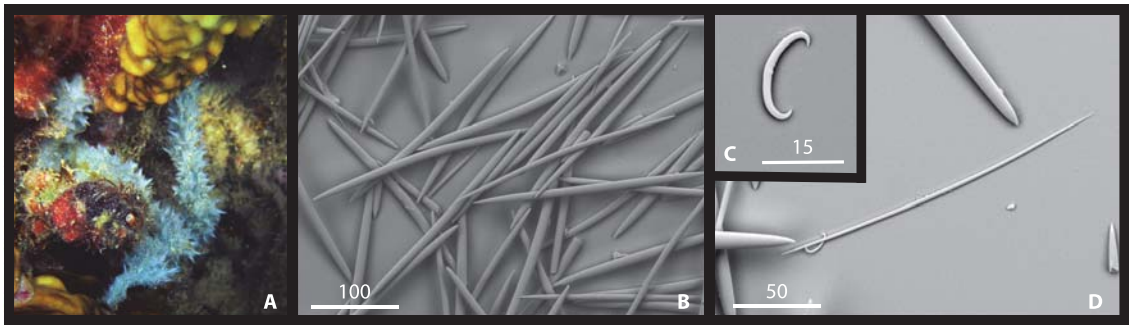


Figure 2.18: *Gelliodes fibulata.* A)*In situ* specimen. B-D) SEM images of oxeas and sigmas. Scale bar in μm.

Gelliodes sp.

Material examined: Individual 9.3, Vietnam (Nha Trang). Depth 6 m. Sampling: 6th April 2015.

Description: Individual mauve pale thick encrusting irregular shape with irregular oscules of different sizes, some seem to result from fusion o two smaller soscule. The surface is largely covered by sediment ant epibiotic microalgae (Figure 2.19A).

Skeleton: Polyspicular thick tracks irregularly reticulated (Figure 2.19B). Oxeas slightly curved of 230-285 x 4-5 μm and sigmas of 13-17 μm (Figure 2.19C-F).

Genus *Niphates* Duchassaing and Michelotti, 1864

Niphates olemda (De Laubenfels, 1954)

Syn.: *Cribochalina olemda* De Laubenfles, 1954

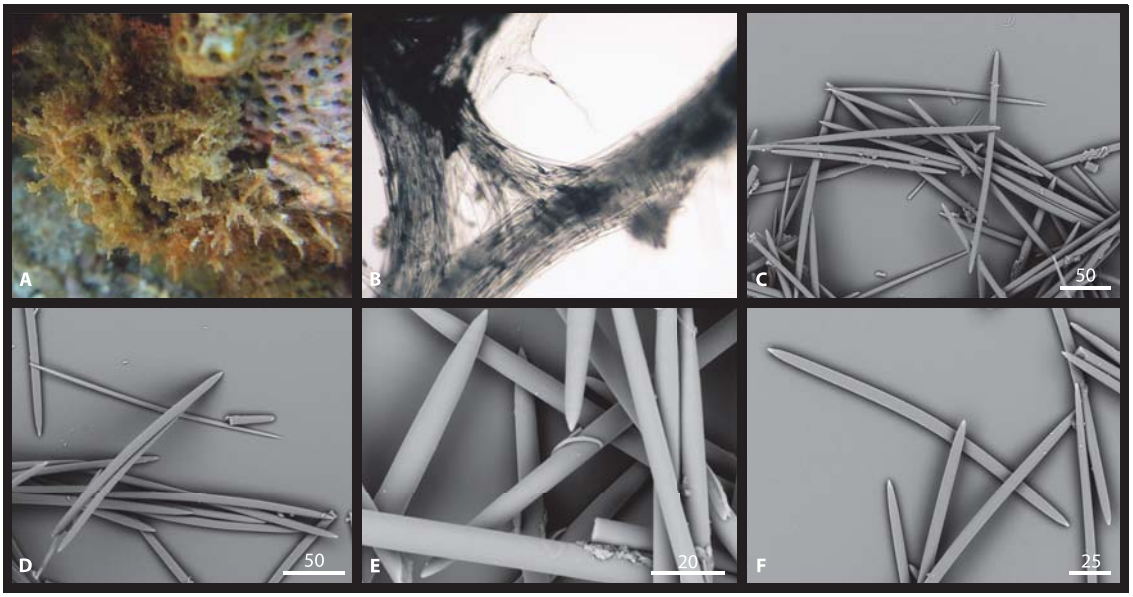


Figure 2.19: *Gelliodes sp* A)*In situ* specimen. B) Light microscopic image of skeleton C-F) SEM images of oxeas and sigmas. Scale bar in μm.

Material examined: Individual 4.4 and 9.26, Vietnam (Nha Trang). Several additional individuals photographed. Rocky vertical wall of Nock Island. Depth 9 m. 1st April 2015.

Description: Massive sponge which form tubes of ca. 10 cm long, 1.5 cm wide tubes. Consistency, hardt and compressible. Typical light blue colour. The specimen was found associated to polychaetes (Figure 2.20A).

Skeleton: reticulated network with thick primary and secondary fibres differentiated. Choanosomal network formed by thick spongin fibres cored by spicules (Oxeas of 60-80 μm). Ectosomal skeleton formed by a network of mono or paucispicular network (Figure 2.20B-D).

Distribution and ecology: Banda Sea, Indonesia, Palau Island, West Caroline Islands.

Remarks: The species has been repeatedly confounded with *Haliclona fascigera* because of its similar external tubular shape and sometimes also colour. The differences are with respect to the choanosomal skeleton. Moreover, *H. fascigera* has its external surface much more uniform than *N. olemda* can present external irregularities or conical projections.

Niphates sp.

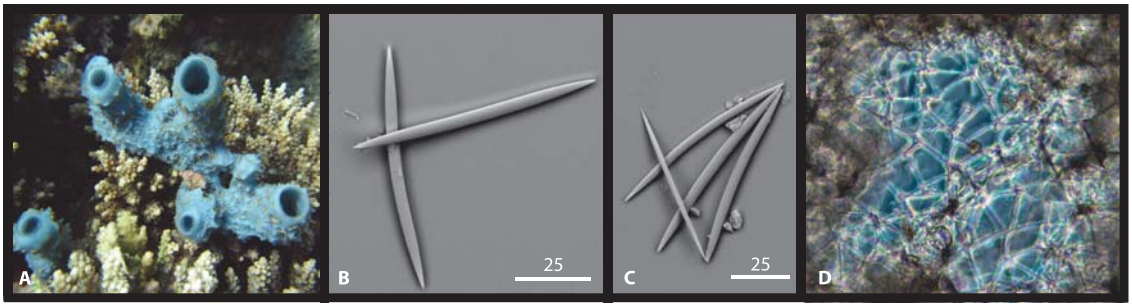


Figure 2.20: *Niphates olemda*. A) *In situ* specimen. B,C) SEM images of oxeas. D) Light microscopic image of skeleton. Scale bar in μm.

Material examined: Individual 8.2, Vietnam (Nha Trang). Impacted coral reef (dominates by Millepora spp) of Dambay region. Depth 2-4 m. Sampling: April 4th 2015.

Description: Encrusting sponge of irregular shape. Oscules of different sizes (from 2 to 5 mm) usually on the top of short protuberances, irregularly distributed on the sponge surface. Surface smooth and slightly brilliant and a little translucid. Consistency firm but slightly compressible. Outside colour difficult to define, as it moves from brown close to the oscula to bluish greenish purple a to the basal zones (Figure 2.21A).

Skeleton: Conanosomal skeleton formed by a reticulated spongin fibres cored by oxeas, which form an irregular network with a general direction toward the sponge surface. Secondary fibres form an ectosomal, tangential, irregular network slightly crossed by the end of the primary fibres. Primary and secondary fibres are differentiated. Oxeas short and thick (cigar shape). They measure 90-120 x 4 μm; Toxas with moderately marked angle and ends slightly curved back. They measure 13-50 μm. Sigmas in C-shape, slightly asymmetric of 30-45 μm of chord (Figure 2.21B-D).

Order Axinellida Lévi, 1953

Family Heteroxydae Dendy, 1905

Genus *Didiscus* Dendy, 1912

Didiscus aceratus (Ridley and Dendy, 1886)

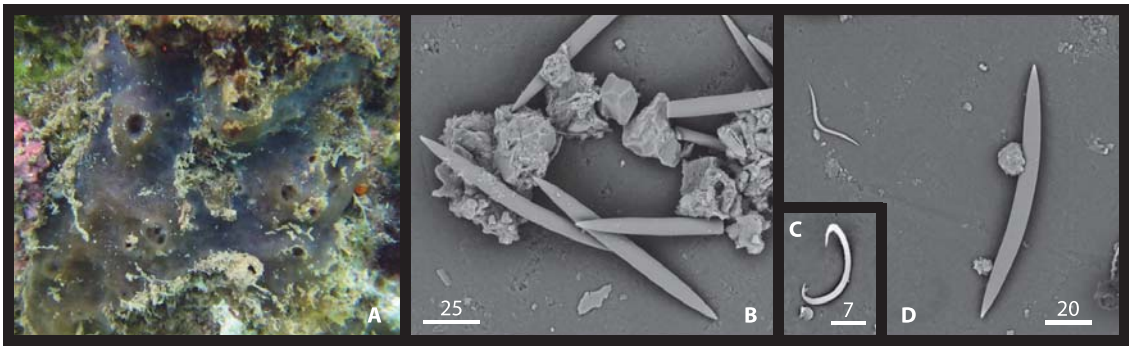


Figure 2.21: *Niphates* sp. A) *In situ* specimen. B,C,D) SEM images of oxeas, sigmas and toxas. Scale bar in μm.

Syn. *Latrunculia acerata* Ridley and Dendy, 1886; *D. clavigerus* (Kirkpatrick, 1900); *Latrunculia clavigera* Kirkpatrick, 1900

Material examined: Individual 1.20, Vietnam (Nha Trang). Rocky, semidark vertical wall (crack) of Hun Mun Island. Sampling date: March 28th 2015.

Description: Massive individual. Surface difficult to observe because of the epibionts. Ectosome thick translucid, separable in flakes allowing to see a bright yellow choanosome. Consistency soft. Oscula inconspicuous. Coulour yellow inside, and outside in the rare parts free from foreign debris and epibiotic organisms, in life (Figure 2.22A).

Skeleton: Spicule tracts irregularly arranged in the choanosome, mainly formed by oxeas. Tangential oxeas in the choanosome.

Spicules: oxeas two class sizes mainly differentiable by the thickness. Strongyles abundant mainly placed in the ectosome but also present in the choanosome. Spicules: oxeas I: 650-990 x 15-25 μm, oxeas II 270-512 x 8-13 μm and strongyles 330-480 x 8-13 μm. Dischorhabdes with rounded spiny ends and more or less spiny shaft, of two size classes, one with a shaft longer and slightly narrower than the other, but with similar in size discs. Short robust dischorabds: 16-19 x 3-3.5 μm; Longer dischorhabs 22-34 x 2.5-3.5μm in size; the small discs 7-8 μm in diameter, and large discs 25-35 μm of diameter in both types. Discs are in a plane with a regular rim (Figure 2.22B-D).

Distribution and ecology: Indian and Pacific oceans: western and northern Madacascar, Sheychelles I. Banda Sea; South Pacific Ellis I.

Remarks: the Strongyles are much shorted than those described in the type (Ridely and Dendy, 1986) and these authors neither described two types of dischorhads. However, we do not know the exact variability of the species and that is because we do not considered this individual different from *D. aceratus*.

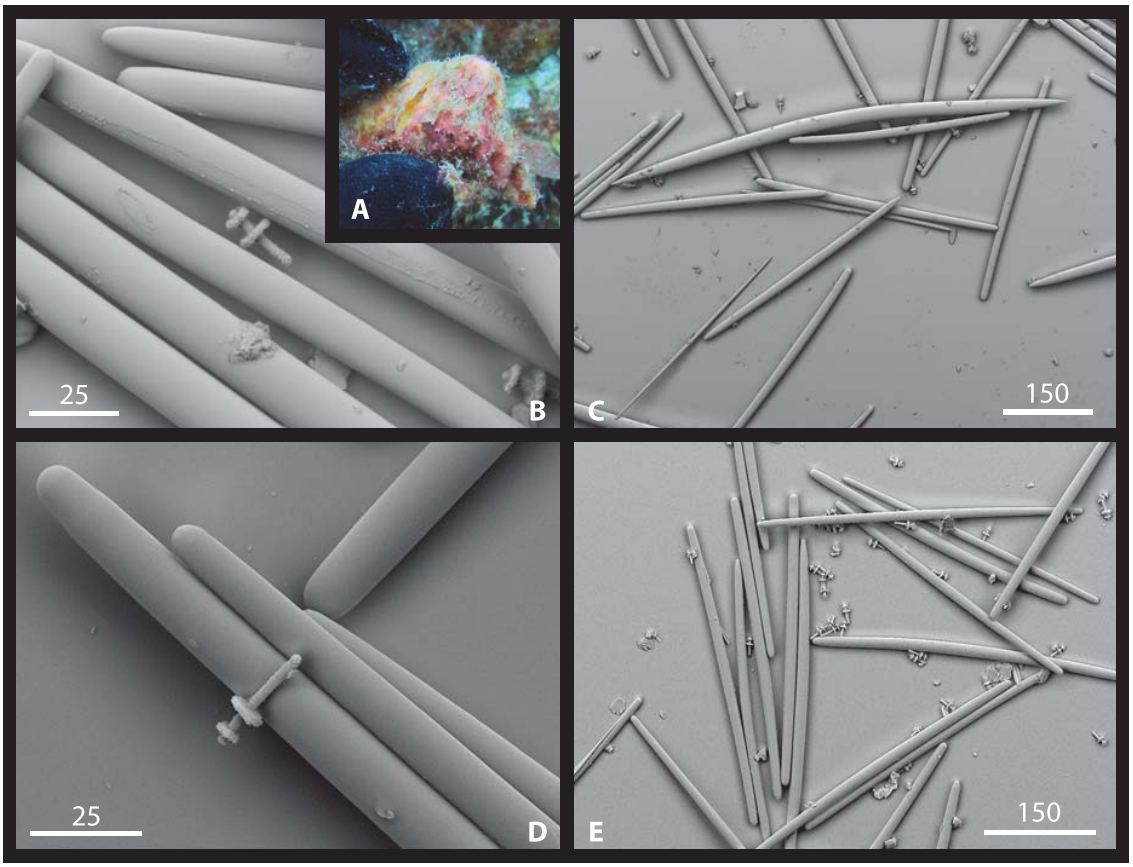


Figure 2.22: *Didiscus aceratus*. A) *In situ* specimen. B-D) SEM images of oxeas, strongyles and dischorhabdes. Scale bar in μm.

Didiscus sp.

Material examined: Individual 6.5, Vietnam (Nha Trang). Well-preserved reef (dominated by *Acropora* spp.) close to Dambay region. Depth 4-10 m. Sampling date: April 2nd 2015.

Description: Thick encrusting to massive irregular individual. Surface difficult to observe as it is covered by numerous epibionts and sediment. Ectosome thick. Consistency soft. Oscula numerous 4-9 μm in diameter oscula. Coulour yellow inside, and masked by epibionts outside. A yellow

ring around the oscula is visible, as this perioscular area is free from epibionts (Figure 2.23A).

Skeleton: Spicule tracks divided or reticulated in the choanosome with spicules spread across the sponge body. Ectosomal skeleton formed by tangential large oxeas. Coanosomal skeketon formed by plumous oxea tracks irregularly reticulated and additional spicules spread. Oxeas I: 750-999 x 22-31 μm; oxeas II 400-450 x 9-11 μm, some of them (very few) modified into styles 400-650 μm in size. Dischorhabds with rounded spiny ends 15-33 x 2.5-3.5 μm in size, with the small discs 7-8 μm in diameter, and the large discs 25-30 μm of diameter (Figure 2.23B-E).

Remarks: The dischorhabds are similar in shape and size to those of *D. aceratus* (indiv. 1.20) However, the rest of the spicule complement are clearly different. The large oxeas are present in the ectosomal zone but most of the choanosomal spicules are also oxeas and not strongyles (as in *D. aceratus*). Some oxeas can be transformed into styles and this approaches this species to *D. anisodiscus* Vacelet and Vasseur, 1971, from Madagascar, although the styles of our specimen are notably shorter. This specimen corresponds to an undescribed species.

Family Raspailiidae Nardo, 1833

Genus *Thrinacophora* Ridley, 1885

Thrinacophora cervicornis Ridley and Dendy 1986

Material examined: Individual 1.1, 13.2, Vietnam (Nha Trang). Rocky vertical wall (crack) of Hun Mun Island. Low light. Depth 8-10 m. 28th March 2015 and 10th April 2015.

Description: Thick-encrusting to massive sponge with conules ending in a long spicule, which renders the surface hispid. Consistency very soft. Several oscules, l4-5mm wide on the top of small protuberances. Bright orange in colour alive (Figure 2.24).

Skeleton: Plumose tracks that arose from a basal spongin plate 2-3 mm thick and cross the sponge ectosome, ending in a large spicule that surpass the sponge surface ca. 5mm, surrounded by bundles of 4-6 styloids. Oxeas in criss-cross in the ectosome and choanosome.

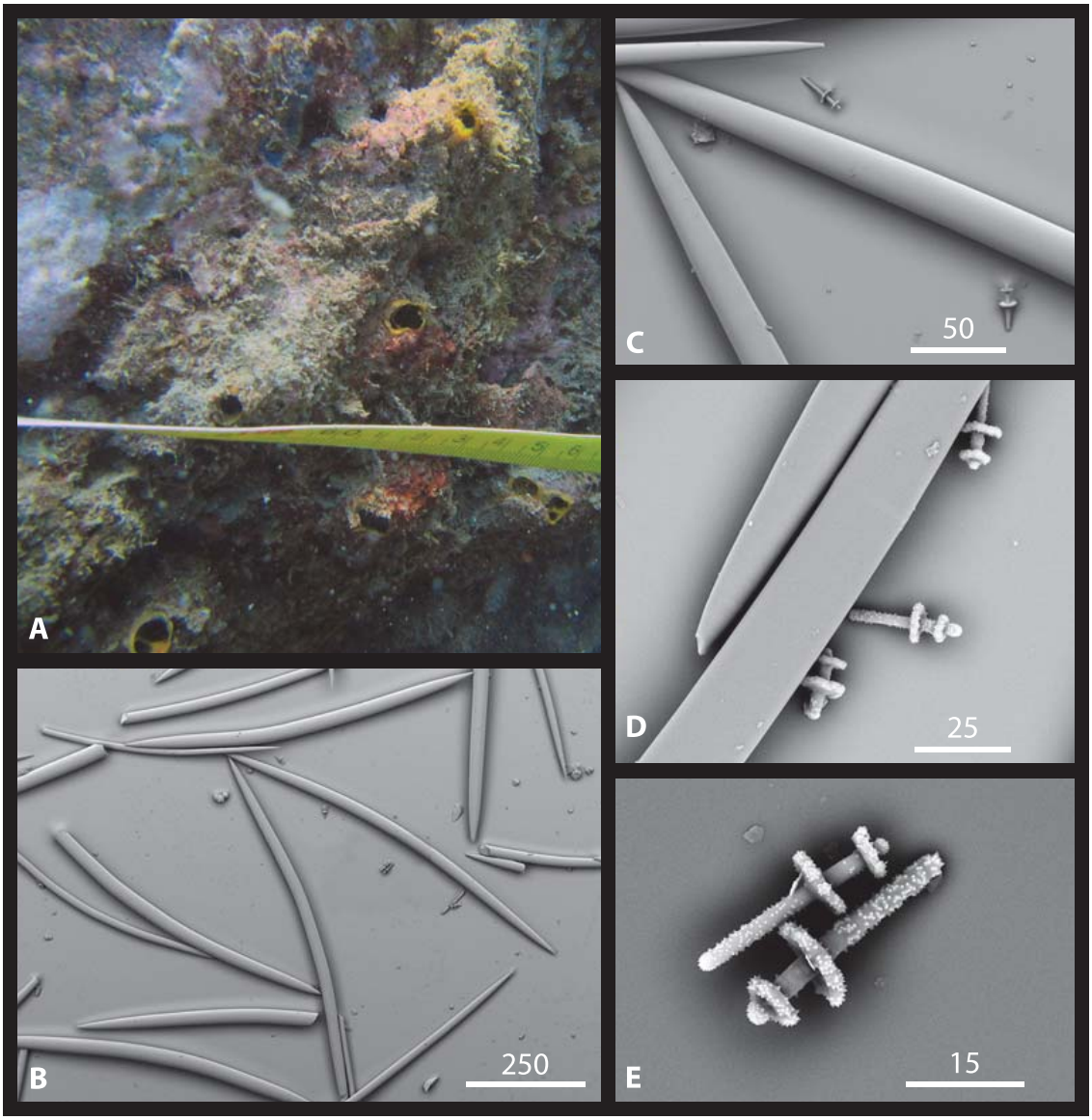


Figure 2.23: *Didiscus aceratus*. A) *In situ* specimen. B-E) SEM images of oxeas, styles and dischorhabdes. Scale bar in μm.

Spicules: Long, ectosomal styles-subtylostyles, 3800-4175 x 18-33 μm; Oxeas with blunt points, 200-330 x 2-12 μm; Estyloids 445-625 x 2,5-4 μm: rhaphides of variable abundance as a function of the specimen 70-150 μm long (Figure 2.24).

Distribution and ecology:Indian Ocean: western India. Pacific ocean: Philipines, Northwestern Australia.

Remarks: The individuals studied are thick encrusting and did not form erect projections or dichotomized branches, such in *R. raphidophora* and *R. cervicornis*. Thus the skeletal axial condensation, which differentiates the genus *Thricanophora* from *Dragmacidon*, is not conspicuous, likely because of an early growth stage. However, the conulous surface and the skeletal arrangement at the conules with long projected styles surrounded by styloids is similar to *Tricanophora*. The molecular study showed that both our specimens and *R. cervicormis* have exactly the same COI sequences, which indicates that they simply are different growth forms of the same species. Our species might also belong to *R. incrustans* Kieschnick, 1896, from Ternate, which has a similar growth-habit, but our individuals lack the cladostrongyles and their styles are longer than in the Kieschnick' species (Ridley and Dendy 1096). The phylogenetic tree based on the LSU gen place this species in the same clade that other T. cervicornis, together with Eurypon hispidum, but the clade support is only of 75% (Figure A.7), whereas the COI tree place these individuals with *T. cervicormis* and *Raspailia elegans* , also with a low bootstrapping support (Figure A.8).

Order Biemnida Morrow in Redmond et al., 2013

Family Biemnidae Hentschel, 1923

Genus *Biemna*Gray, 1867

Biemna fistulosa (Topsent, 1897)

Material examined: Individual 7.24, Vietnam (Nha Trang). Impacted coral reef (Millepora dominated) of Dambay region. Depth 2-4 m. 3th of April 2015.

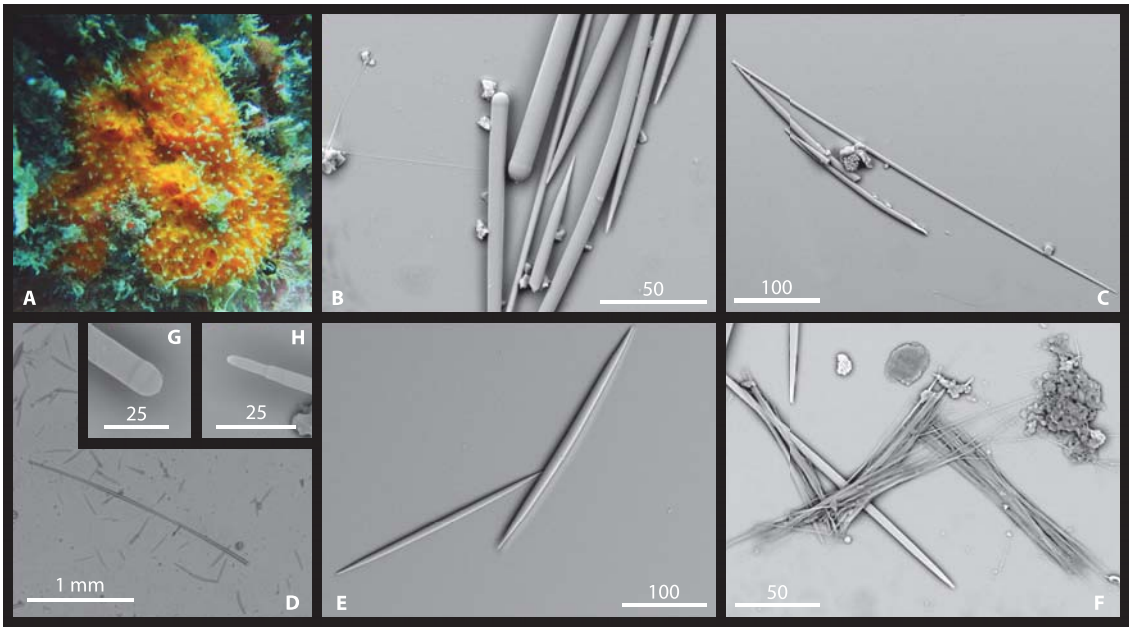


Figure 2.24: *Thrinacophora cervicornis*. A) *In situ* specimen. B-F) SEM images of long styles (D), oxeas, styloids and rhaphids (B,C,E,F) and detail of blunt points (G,H). Scale bar in μm.

Description: Fistulose sponge formed by three wide fistulas with a 6-8 mm wide pseudo-oscules, representing the atrial cavities of the tubes and thin tube walls which become larger at the end. Surface hispid, uneven with entrapped sediment. Consistency soft and breakable. Colour yellow pale in life.

Skeleton: Plumose tracks of styles, that run up perpendicular to the sponge surface.

Spicules: Styles 240-330 x 5.5-7 μm; Microxeas fusiform of two class sizes; I 75-100 x 2-5 μm in the middle and II: 20-30 x 0.4-1 μm; Rhafides in bundles, straight or ondulated, very thin, 75-115 x 0.1 μm long; Sigmas I: 40-45 μm of chord, 0.2-0.4 μm, sigmas II 13-18 μm x 0.1 μm of chord; Comata, rare, 14-15 μm long, 13-19 μm (Figure 2.25).

Distribution and ecology: South India and Sri Lanka, Greater Antilles, Seychelles, Southern Caribbean, Southwestern Caribbean, Western Caribbean, Western India.

Remarks: The species may be confounded with *Biemna tubulata* Dendy but differs from the later in some spicule particularities, such as the two sigma categories, different sizes of the two microxeas categories, and the

smaller styles.

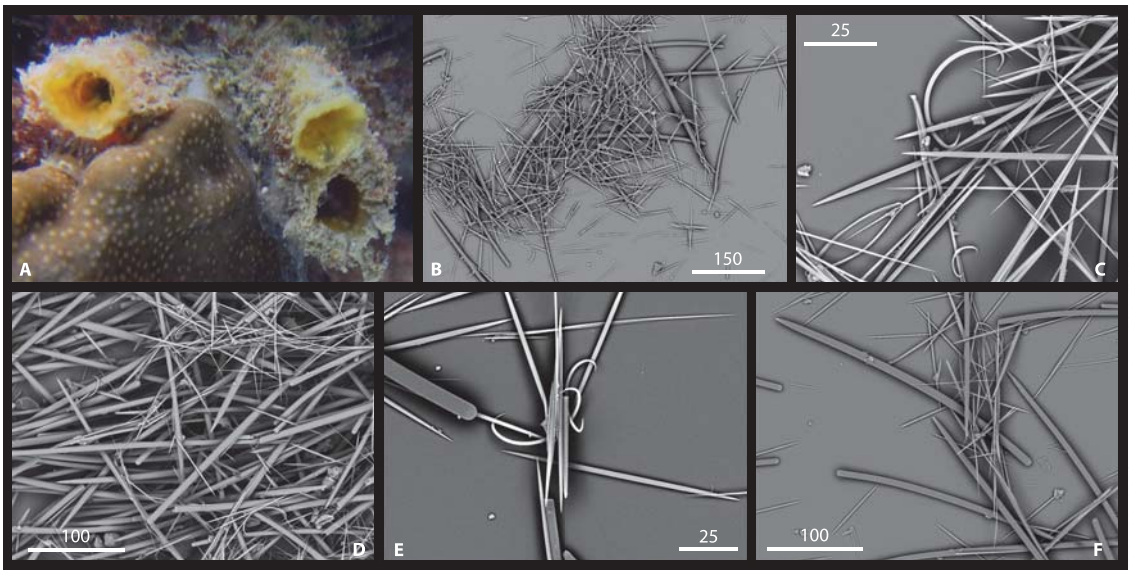


Figure 2.25: *Biemna fistulosa*. A) *In situ* specimen. B-F) SEM images of styles, microxeas, rhafides and sigmas. Scale bar in μm.

Biemna trirhaphis (Topsent, 1897)

Syn. *Desmacella peachi var. thrirhaphis* Tosent, 1897 *Desmacella thrirhaphis* Topsent, 1897

Material examined: Individual 11.5 . Vietnam (Nha Trang). Well-preserved reef (Acropora dominated) close to Dambay region. Depth 6 m. Sampling date: 8th April 2015.

Description: Massive individual of 2 cm in size, surface irregular, consistency firm. Colour grey brownish outside, cream yellowish inside, in life.

Skeleton: Plumose spicule tracks run toward the sponge surface. Sigmas microxeas and raphides are abundant among the tracks.

Spicules: Styles robust, slightly curved, 265-355 x 9-22 μm in size; Sigmas with a flagellated shape, of three size categories, measuring: I 80-105 μm chord, II 30-45 μm chord, and III 10-15 μm chord. The two larger sizes show microserrated ends. Microxeas of two categories: I 100-135 x 4-5; II 30-45 μm chord; raphides in tricodragma 90-120 μm long, very thin (Figure 2.26).

Distribution and ecology: Pacific: Banda Sea (type locality). Indian Ocean: Seychelles I. East African coral coasts, Zanzibar. Red Sea: south coasts.

Remarks: Topsent (1897) described in the type species three categories of thricodragma, as for *B. fistulosa*, which indeed correspond to the two types of microxeas plus the raphides.

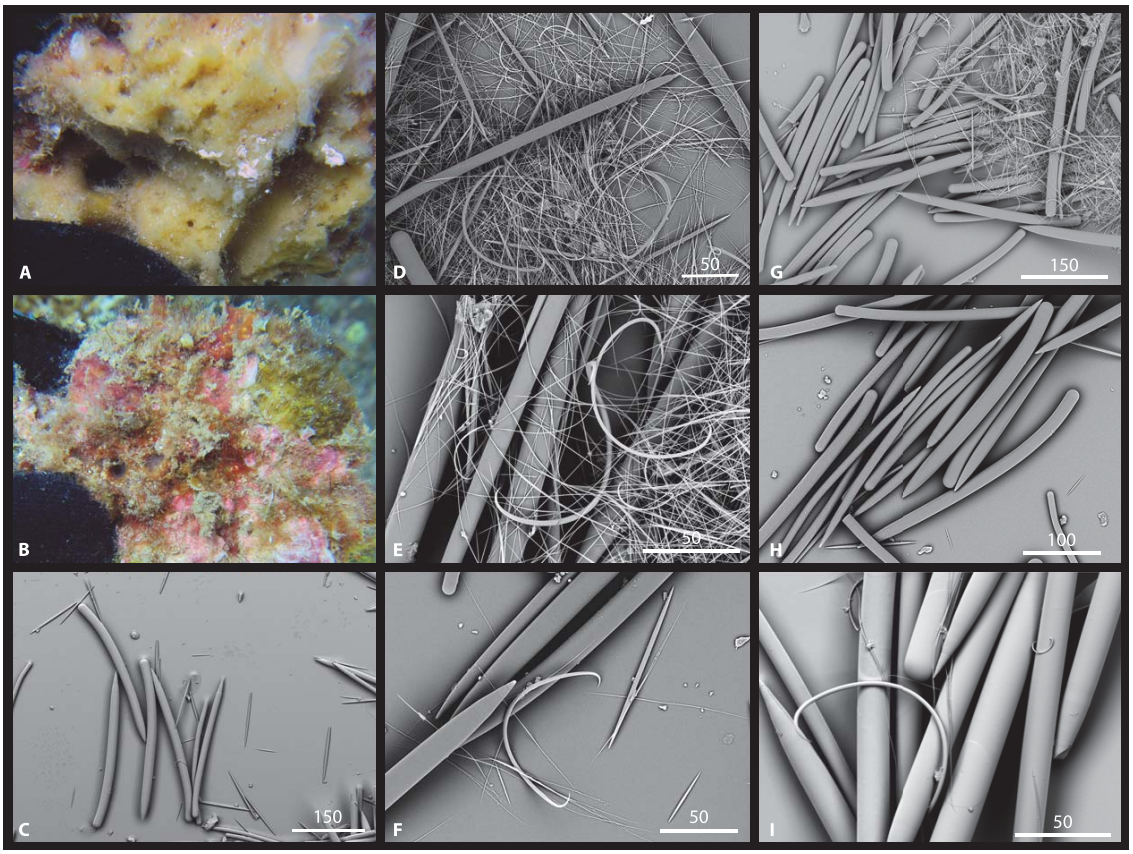


Figure 2.26: *Biemna trirhaphis*. A,B) *In situ* specimen. C-I) SEM images of styles, microxeas, rhafides and sigmas. Scale bar in μm.

Genus *Neofibularia* Hechtel, 1965

***Neofibularia* sp (Topsent, 1897)** **Material examined**: Individuals 5.1, 5.3, 6.12, 10.8, 11.3, 11.7, 11.9, Vietnam (Nha Trang) .Well-preserved reef (Acropora dominated) close to Dambay region. Depth 4-6 m. Sampling dates: 2nd and 8th April 2015.

Description: Thick encrusting sponge with the surface covered by small conulose protrusions, uniformly distributed across the surface ending

in oscula. Surface irregular. Oscula 2-3 mm wide. Inhalant depressed areas visible across the surface. Consistency relatively soft, easy to separate from the substrate. Colour from dirty yellow to duly orange with brownish tinges (Figure 2.27A).

Skeleton: Reticulated arrangement of spicule tracks.

Spicules: Styles slightly curved with blunt point, 260-355 x 9-22 μm. Microxeas I 100-135 x 3,5-4,5, II 30-40 μm x 0.2-0.3 μm; Sigmas I 80-100 μm chord; II 30-40 μm chord. No raphides could be observed (Figure 2.27B-G).

Remarks: These individuals have a spicule complement similar to that of of *B. trirhaphids* but spicules form reiticulate tracks, which are characteristic of *Neofibularia* instead of plumous tracks, typical of *Biemna*. These individuals represent a new species.

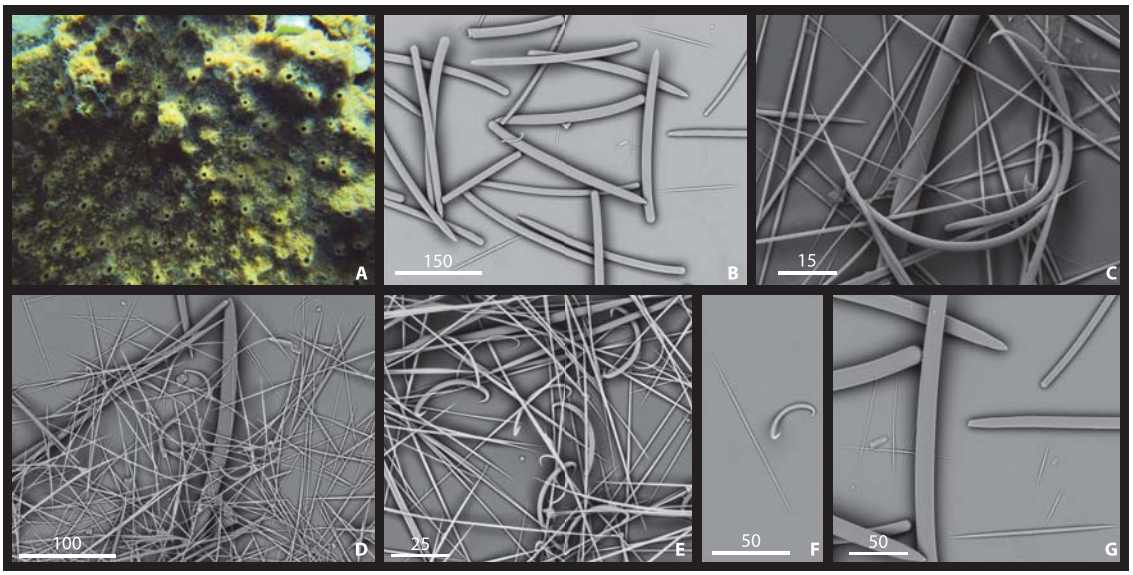


Figure 2.27: *Neofibularia* sp. A) *In situ* specimen. B-G) SEM images of styles, microxeas and sigmas. Scale bar in μm.

Family Rhabderemiidae Topsent, 1928

Genus *Rhabderemia* Topsent, 1890

Rhabderemia acanthostyla Thomas, 1968

Material examined: Individual 2.10, Vietnam (Nha Trang). Rocky semi-vertical shore of Hun Mun Island (north). Depth 9 m. 30th March 2015.

Description: Thin encrusting sponge firmly attached to the substrate. Surface smooth, Oscula small (1-1.5 mm in diameter) spread on the surface (Figure 2.28A).

Skeleton: Hymedesmoid arrangement: rhabdostyles form a layer of short spicule bundle with their base on the spongin later that cover the substrate.

Spicules: Spiny rhabdostyles (the young forms less spiny or almost smooth) of two size categories: I 105-135 x 6-7 μm and II 220-310 x 9-10 μm. Contorted spiny sigmas of two types: I smaller and more contorted of 8-12 x 0.5-1 μm and II larger and less contorted, 18-20 x 2 μm in size. Spiny microstyles very thin, 20-28 x 0.5 μm in size (Figure 2.28B-H).

Distribution and ecology: Pacific Ocean: Indonesia, Vietnam

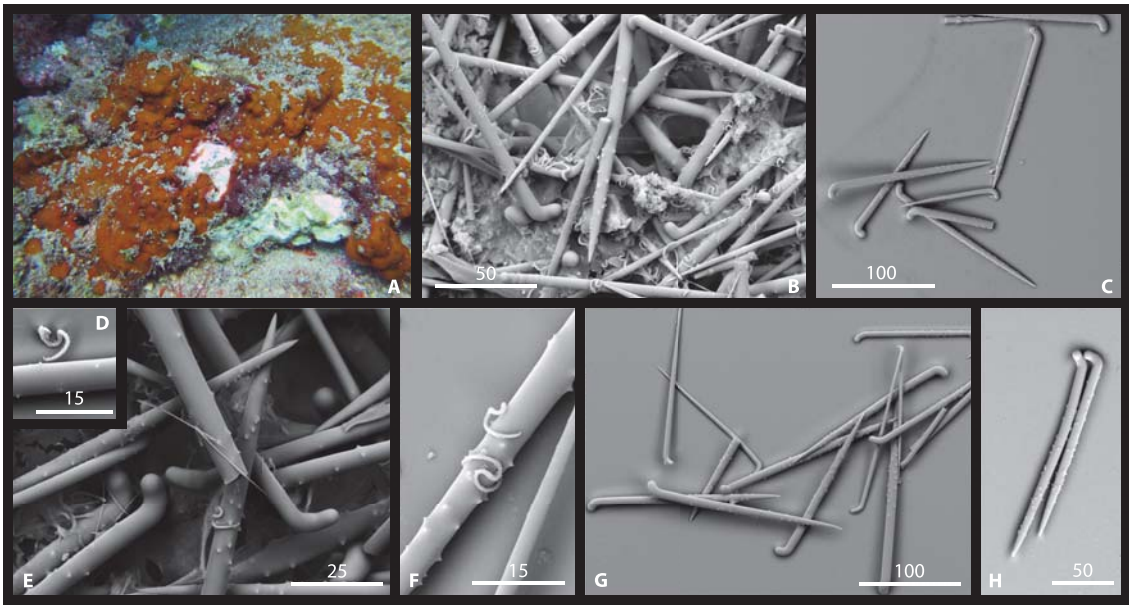


Figure 2.28: *Rhabderemia acanthostyla*. A) *In situ* specimen. B-H) SEM images of spinny rhabdostyles and microstyles. Detailed image of spinny sigmas (D,F). Scale bar in μm.

Order Tetractinellida Marshall, 1876

Family Ancorinidae Schmidt, 1870

Genus *Stelletta* Schmidt, 1862

Stelletta herdmani (Dendy, 1905)

Material examined: Individual 10.5. Vietnam (Nha Trang) Well-preserved reef (Acropora dominated) close to Dambay region. Depth 6 m. 8th April 2015.

Description: Sponge massive, irregular, with epibionts growing on its surface. Surface irregularly hispid. No oscula visible. Consistency dense. Colour yellowish outside, whitish inside, in life (Figure 2.29B-F).

Skeleton: spicule tracks both radial and irregularly arranged, crossing the surface in in some areas.

Spicules: Oxeas of two class sizes: I, choanosomal, 1300-1900 x 40-85 μm and II, ectosomal, 850-1100 x 25-30 μm; Small plagiotriaenes with short clades: rhabodme 550-850 x 13-30 μm, clades 28-80 x 15-25 μm; No anatriaenes present; oxyasters smooth or rugose 10-12 μm in diameter; spiny chiasters with a thick centrum 6-8 μm in diameter (Figure 2.29B-F).

Distribution and ecology: Indian Ocean: South India and Sri Lanka, East coasts of Africa (Natal and Coral Coast), western Arabia Sea

Remarks: *S. herdmani* matches the best our specimen among the Stelletta species recorded in the Indo-Pacific. However, the Nha Trang individual differs from the type specimen of *S. herdmani* in the smaller size their spicules in general. In particular the oxyasters are much smaller in the Vietnamese individual, which have the size of the individual recorded from the Mozambique Channel (Thomas, 1979). It has also similarities with *S. kundukensis* Sim, 1996, from Korea, but lacks the large type of oxyaster and also differs in spicule sizes. The COI phylogenetic tree place this species in the same clade as *S. dorsigera* and *S. grubei* with a high (98%) of bootstrapping support (Figure A.9).

Order Agelasida Hartman, 1980

Family Agelasidae Verrill, 1907

Genus *Agelas* Duchassaing and Michelotti, 1864

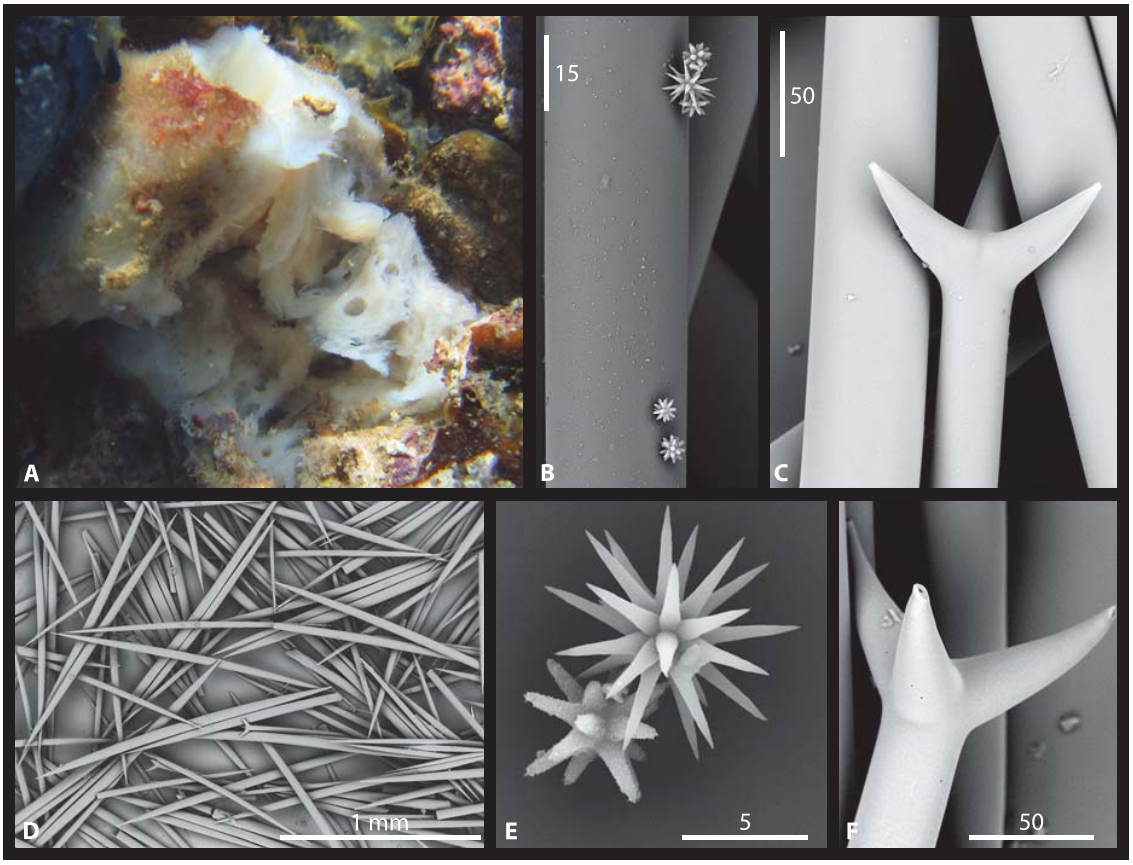


Figure 2.29: *Stelleta herdmani*. A) *In situ* specimen. B-F) SEM images of oxeas, plagiotriaenes, oxyasters and chiasters. Scale bar in μm.

Agelas bispinata Vacelet, Vasseur and Lévi, 1976

Material examined: Individual 12.5, Rocky shore of Hun Mun Island (South). Depth 10 m. 10th April 2015.

Description: Massive, with a large area attached to the substrate and forming two wide protrusions. Large oscula, ca. 1cm wide, on the top of protrusions. Surface uneven but not hispid, covered in places by epibionts. Consistency firm but compressible, difficult to tear. Colour grey in life (Figure 2.30A).

Skeleton: Skeleton formed by a reticulated spongin network with the acanthostyles hispidating the fibres.

Spicules: Acanthostyles totally covered by spines, less marked in the young spicules. They can be classed in two size categories: I. 265-300 x 10-18 μm with 23-26 whorls of spines and II 90-140 x 6-10 μm, with 16-18 spine whorls (Figure 2.30B,C).

Distribution and ecology: Indian Ocean, Madagascar

Remarks: The larger acanthostyles reach a smaller size than in the type specimen (Vacelet, Vasseur, Lévi, 1971). The species clusters together with *A. dispar* and *A. conifera* in a poorly supported clade, in the phylogenetic LSU tree of family Agelasidae.

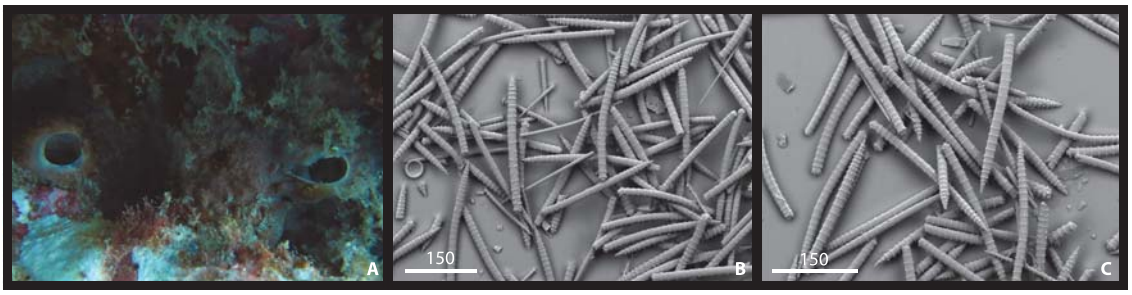


Figure 2.30: *Agelas bispinata*. A) *In situ* specimen. B,C) SEM images of acanthostyles. Scale bar in μm.

Order Poecilosclerida Topsent, 1928

Family Crambeidae Lévi, 1963

Genus *Crambe* Vosmaer, 1880

Crambe sp.

Material examined: Individual 1.26, Vietnam (Nha Trang). Rocky vertical wall (crack) of Hun Mun Island. Low light. Depth 10 m. 28th March 2015.

Description: Irregular, thick encrusting individual of 4 cm acroos the largest diameter. Surface smooth. Consistence fleshy. Oscula from1.5 to 3 mm wide. Colour bright red-orange in life (Figure 2.31A).

Spicules: two size categories subtylostyles, mainly differentiated by their thickness: I, 245-450 x 9-20 μm round marked head, and II, 150-240 x 2,5-5 μm oval less marked head; Pseudoastrose desmoids with dichotomized or trichotomized branches in the same plane, and a few spines on the superior zone. They are abundant and measure: protoclades 20-30 μm long and deuteroclades 30-45 μm long. Isanchorae 30-36 μm long with three

well-formed teeth (not anguiferous), reaching up to 30% of the total spicule length (Figure 2.31B-H).

Remarks: The closest species to this individual is *C. acuata* from the South Atlantic, Indian Ocean and red Sea, but the differences are enough to consider the Vietnamese sponge as a new species. The main differences are: subtylostyles I with a more marked base (rather tylostyles), desmoids more complex with dichotomized branches, isochelae of a sole category, with longer well-formed teeth. The formal description and holotype designation of this new species will be published, together with the descriptions of the other new species from Nha Trang, in a separate paper.

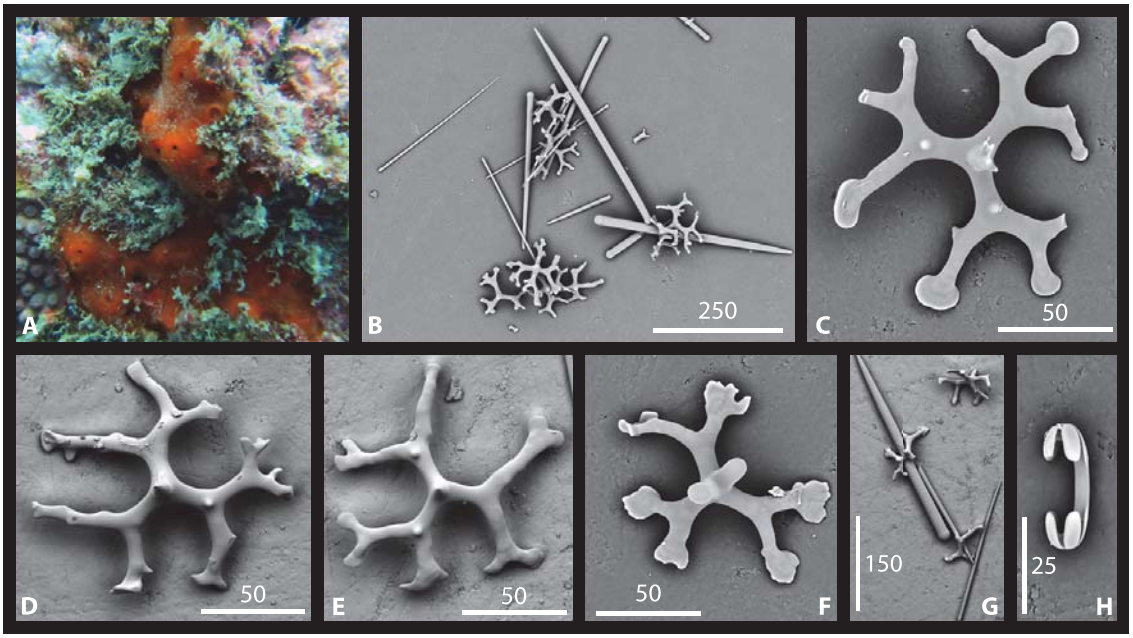


Figure 2.31: *Crambe* sp. A) *In situ* specimen. B-H) SEM images of subtylostyles (B,G), pseudoastrose desmoids (C-G) and isanchorae (H). Scale bar in μm.

Genus Monanchora Carter, 1883

Monanchora unguiculata (Dendy, 1922)

Material examined: Individual S1.5, S1.6, S1.7, S1.9, S1.12, S1.22, S1.23, S1.25, S1.27, S3.8, S4.1, S4.3, S4.6, S4.8, S4.13, S4.19, S12.2, S12.6, S12.8, S12.9, S12.12. Vietnam (Nha Trang). Rocky vertical wall (crack) of Hun Mun and Nock Island. Low light. Depth 9-10 m. 28th March 2015, 1st and 10th April 2015.

Description: Massive sponge with rounded edges, the small specimens are globulous but larger forms are more irregular. Consistency firm. Oscula very conspicuous, 3-4 mm wide on the top of prominent lobes. Thich ectosome from translucid to white colour. Exhalant canals leading into the oscula visible trough the translucid ectosome. Surface with typical perforated areas (inhalant) concentrated in one or two concrete zones, which give to the sponge a particular appearance. The several individuals observed showed a more or less whitish appearance. Some of them are totally white as if some white (calcareous) material is accumulated at the ectosomal layer (Figure 2.32A,B).

Skeleton: Plumose bundles of subtylostyles in the choanosome. Ectosomal skeleton basically formed by isanchorae.

Spicules: Subtylostyles 208-340 x 4-5.5 μm; isanchorae, 16-34 μm long, with shafts 2-4 μm wide and four teeth that occupy 1/5 of the total microsclera length (Figure 2.32C-H).

Distribution and ecology: India Ocean: Chagos, Seychelles I., Madagascar; Pacific Ocean: Vietnam. In the study area, it lives preferentially on rocky semidark walls.

Remarks: There are not differences in the COI sequence between *M. clathrata* and *M. unguiculata*, but morphological differences, in particular with respect their respective growth habit, are noticeable. Our sequenced specimens (4.3, 4.8, and 4.19) matched the LSU partition with *M. unguiculata* sequences deposited in the NCBI database (Figure A.10), and appeared as a sister clade of *M. arbuscula* in the SSU based tree (Figure A.11). Both species *M. clathrata* and *M. unguiculata* have been reported from Vietnam (Davidoff 1952). The whitish look of many individuals of *M. unguiculata* has not been reported for *M. clathrata* and remember the look of specimens of several species, which accumulate calcareous spherules produced by bacteria at the sponge periphery (Garate et al., 2015; Uriz et al., 2012). This species is one of the most abundant sponges on rocky substrates of the study area.

Family Hymedesmiidae Topsent, 1928

Genus *Hymedesmia* Bowerbank, 1864

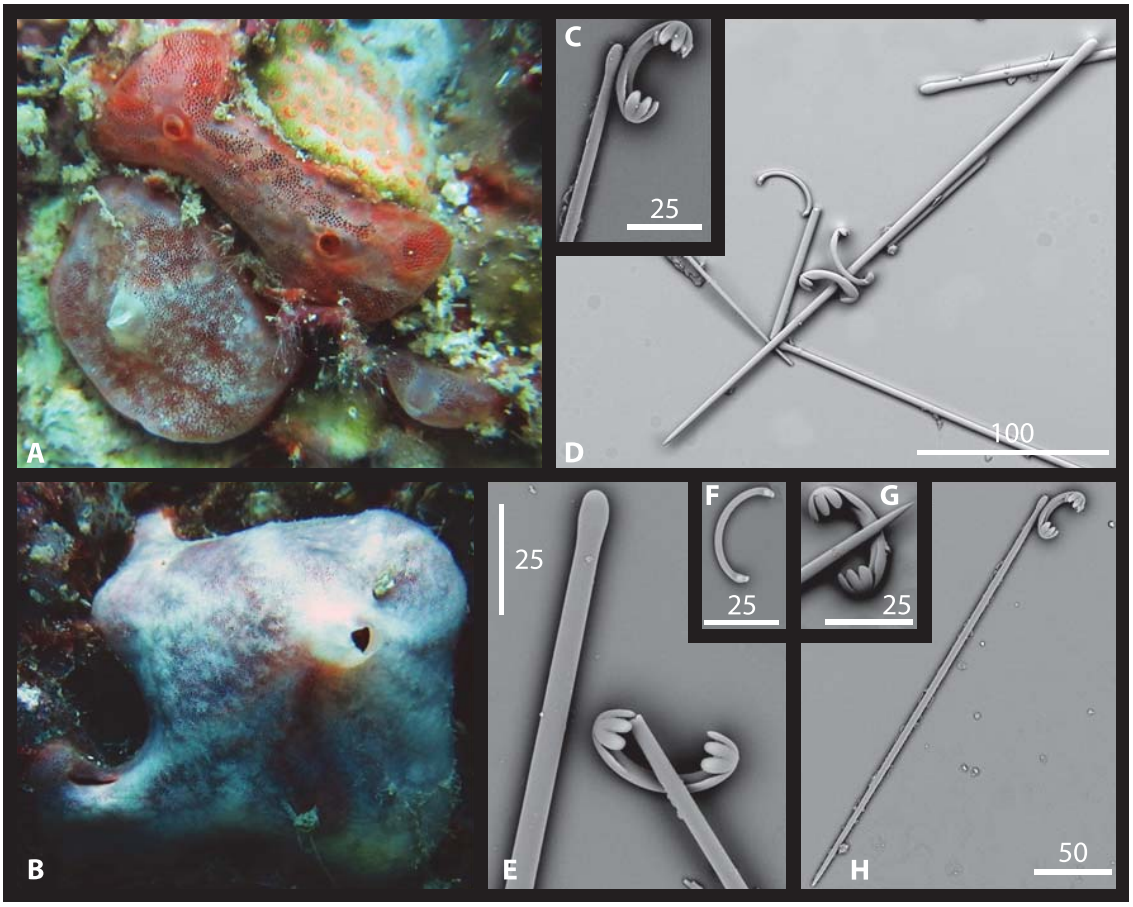


Figure 2.32: *Monanchora unguiculata* A,B) *In situ* specimen. C-H) SEM images of subtylostyles and isanchorae. Scale bar in μm.

Hymedesmia sp.

Material examined: Individual 9.29, Vietnam (Nha Trang).

Description: Small encrusting individual. Surface smooth with excurrent aquiferous canals visible. Only one osculum at the end of a ectosomal protuberance visible. Ectosome not differentiable. Consistency soft. Colour salmon in life (Figure 2.33A).

Skeleton: Short plumose spicule bundles ramified in the choanosome. Tangential tornotes and isochelae in the ectosome.

Spicules: Acanthostyles of two size classes: I, slightly curved completely spiny, 130-140 x 7-8 μm in size and II, straight, 72-100 x 6-7 μm; tornotes 180-230 x 5-6 μm in size; Isochelae in two size categories: I, 25-30 μm long , 3.5-5 μm wide shaft and II, 13-14 μm long, shaft 1.5-2 μm wide (Figure 2.33B-G).

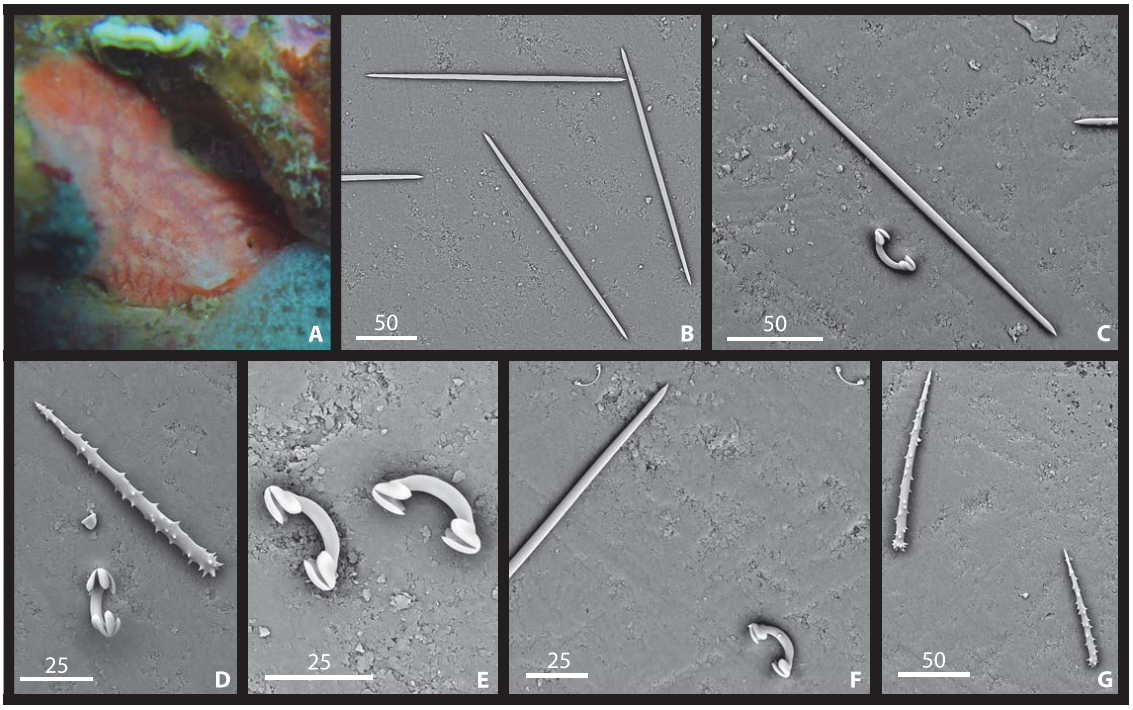


Figure 2.33: *Hymedesmia* sp1. A) *In situ* specimen. B-G) SEM images of acanthostyles (D,G), tornotes (B,C) and isaochelae (C-F). Scale bar in μm.

Genus *Phorbas* Duchassaing and Michelotti, 1864

Phorbas sp1

Material examined: Individuals 13.12, 12.4, 13.7, Vietnam (Nha Trang). Rocky shore of Hun Mun Island (South). Depth 8-9 m. 10th April 2015.

Description: Encrusting sponges with irregular shape spreading on the substrate with rounded edges. Oscules 3-4 mm wide on the top of protuberances. Excurrent conducts leading into the oscules are visible through the ectosome. Consistence soft. Surface smooth at a naked eye but microhispid through the light microscope. Colour variable in the different specimens, from grey reddish to grey yellowish (Figure 2.34A).

Skeleton: Plumose spicule bundles in the choanosome, tangential tornote tracks and isochelae form the ectosomal skeleton.

Spicules: straight tornotes with blunt points 170-240 x 3.5-5 μm; acanthostyles in two size categories: I, with the spines concentrated in the basal zone, 130-290 x 5.5-12 μm and II, completely spiny, 58-75 x 3.5-5.5μm;

isochelae in two size categories: I, 15-25 μm long with 3 μm wide shaft and II, 11-13 μm long with 1-1.5 μm wide shaft (Figure 2.34B-I).

Remarks: This sponge is abundant in the study area, but the spicule complement does not match that of the described Phorbas species in the area.

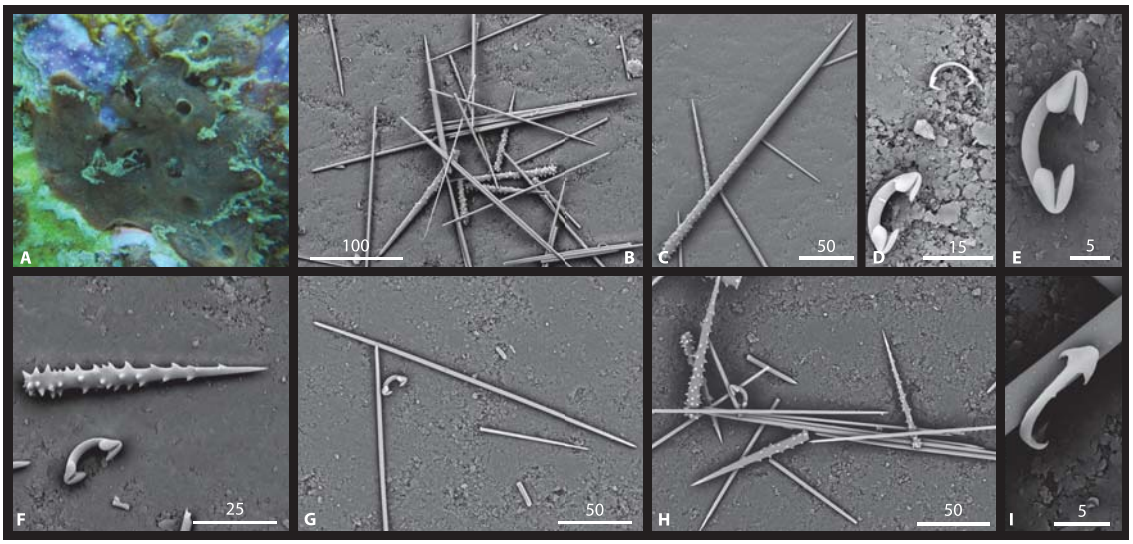


Figure 2.34: *Phorbas* sp. A) *In situ* specimen. B-I) SEM images of acanthostyles, tornotes and isaochelae. Scale bar in μm.

Phorbas sp2

Material examined: Individual 4.15, Vietnam (Nha Trang). Vertical rocky wall of Nock Island. Depth 8-9 m. 1st April 2015.

Description: Encrusting individual with irregular surface spreading on the substrate. Small oscules spread on the sponge surface. Consistence soft. Colour blue greyish.(Figure 2.35A).

Skeleton: Plumose spicule bundles in the choanosome, tangential tornote tracks and isochelae form the ectosomal skeleton.

Spicules: straight tornotes with blunt points 170-240 x 3.5-5 μm; acanthostyles in two size categories: I, with the spines concentrated in the basal zone, 130-290 x 5.5-12 μm and II, completely spiny, 58-75 x 3.5-5.5μm; isochelae in two size categories: I, I, 24-33 μm long, 4 μm wide shaft and II, 11-19 μm long, shaft 1-2 μm wide um long (Figure 2.35B-I).

Remarks: The skeletal arrangement suggest this individual belongs to the genus *Phorbas*, but the spicule complement does not match that of the

described *Phorbas* species in the area.

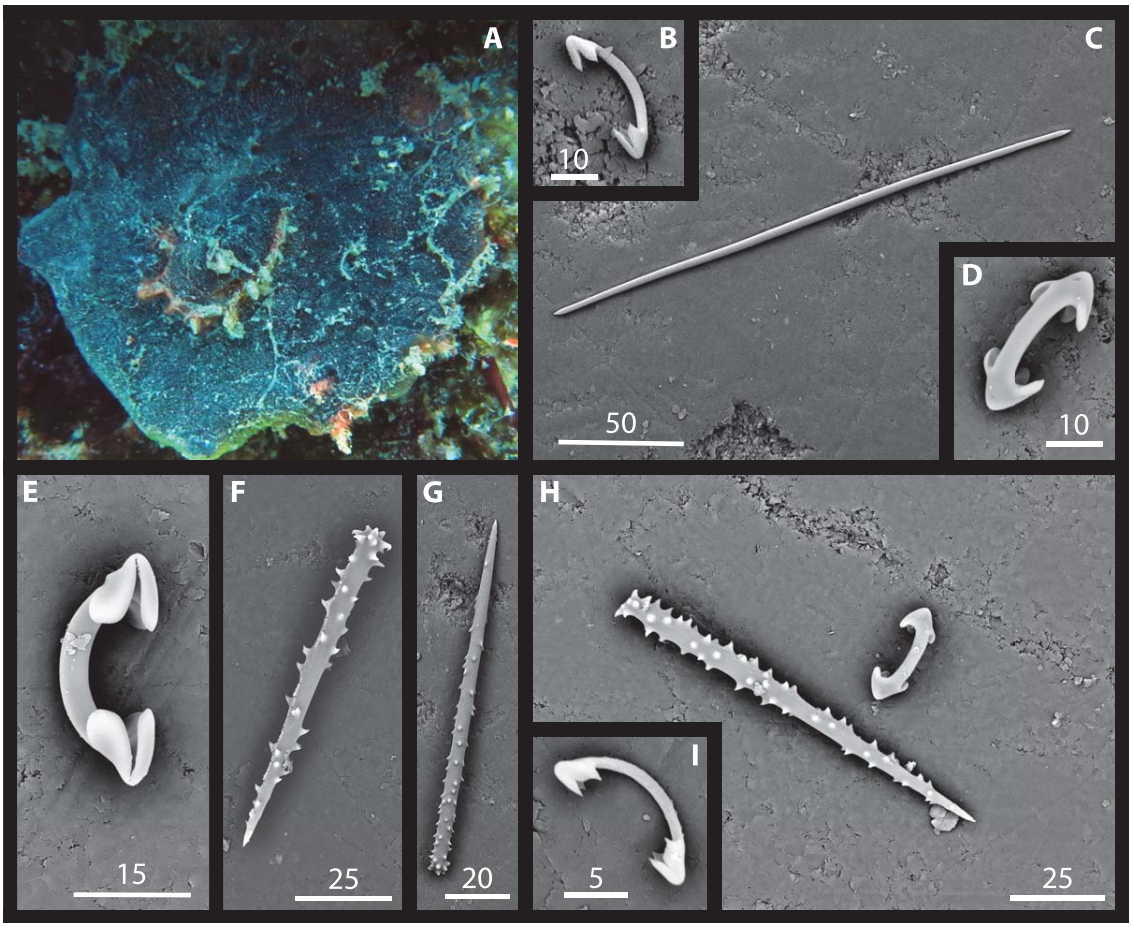


Figure 2.35: *Phorbas* sp. A) *In situ* specimen. B-I) SEM images of acanthostyles, tornotes and isaochelae. Scale bar in μm.

Family Microcionidae Carter, 1875

Genus *Antho* Gray, 1867

Antho (Antho) sp.

Material examined: Individuals 2.1, 2.3, 2.4,2.6, 2.7,3.1, 3.4, 3.6, Vietnam (Nha Trang). Rocky semi-vertical shore of Hun Mun Island (north). Depth 8-9 m. 30th March 2015.

Description: Encrusting individuals spreading several cm on the substrate. Consistency soft. Surface minutely hispid. Oscules, 2-3 mm wide, with the exhalant conducts visible under the ectosome. Colour garnet red,

in life, with a whitish pattern in several zones likely due to the retention of carbonated fine sediment (Figure 2.36A).

Skeleton: choanosomal sodictyal network of tylostyles, echinated in the nodes by acanthostyles. Ectosomal skelton of subtylostyles troughly tangential to the sponge surface causing a slight hispidation.

Spicules: Choanosomal tylostyles, slightly curved, wider at the central zone that below the inflated base (typical shape of Microcionidae), 200-370 x 11-13 μm; Ectosomal subtylostyles straight longer but narrower than the tylostyles, in two size categories : I, 430-480 x 5-7 μm and II, 230.280 x 2-4 μm, the larger may show some spines at the base; Acanthostyles of a singles category with the spines distributed along the shaft but more concentrated at the basal zone, 110-130 x 4-8 μm; Palmate isochelae 10-18 μm long; strongly curved toxas with slightly spiny ends, 50-60 μm (Figure 2.36B-I).

Remarks: The species, which belongs to the subgenus *Antho* because the spicules that form the choanosomal network are acanthostyles and not acantostrongyles, did not match any known species from the area and likely represents a new species, which will be formally described elsewhere. The phylogenetic tree based on the LSU gene place this species with the other species of *Antho* available from the NCBI database forming a monophyletic clade, separate from the other genera of Microcionidae (Figure A.12).

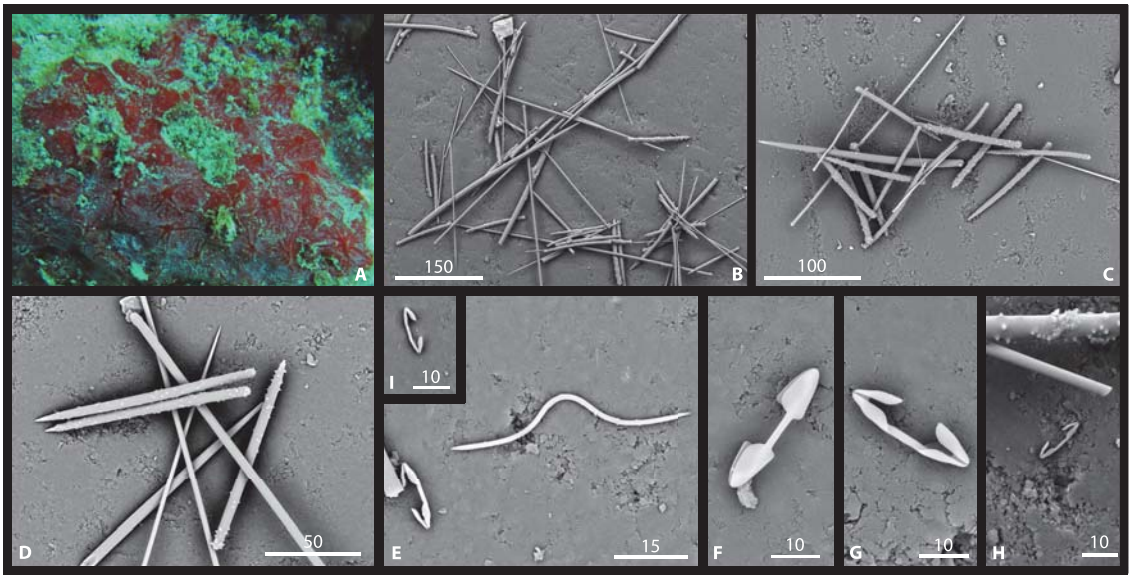


Figure 2.36: *Antho* sp. A) *In situ* specimen. B-I) SEM images of acanthostyles, tylostyles, subtylostyles toxas (E) and palmate isaochelae (E-I). Scale bar in μm.

Genus *Clathria* Schmidt, 1862

Clathria (Axosuberites) Topsent, 1893

Clathria (Axosuberites) sp.

Material examined: Individual 7.6, Vietnam (Nha Trang). Impacted coral reef (Millepora dominated) of Dambay region. Depth 2-4 m. 3th of April 2015.

Description: Digitiform sponge arising from a thick encrusting basis. Surface smooth. Consistency slightly compressible. Oscula inconspicuous. Ectosome not separable from the choanosome. Colour bright orange in life (Figure 2.37A).

Skeleton: Characteristic of the subgenus *Axosuberites*: a central compressed axis formed by a dense reticulate network and an extra-axial laxer zone.

Spicules: styles slightly bent, of two size classes: I, 335-430 x 9-18 μm and II, 265-300 x 5.3-13 μm; Ectosomal subtylostyles straight, hardly separable in two size categories: I, 300-330 x 3.5-5.3 μm and II, 240-275 x 2.2-3 μm; Palmate isochelae 13-22 μm long; toxas with a shallow curvature, differentiable in two size categories: I, 177-270 x 3.5-5 μm and II 100-120 x 0.8-1.6 μm (Figure 2.37B-G).

Remarks: The subgenus *Axosuberites* is characterized by the absence of echinating spicules and the clear differentiation of a compressed axis and an extra-axial zone. These characteristics are showed by the Vietnamese specimen, but the spicule sizes do not match any known species of *Clathria (Axosuberites)* in the area. It may be a new species, but a more in depth study is necessary to confirm this issue.

Clathria (Isociella) Hallman, 1929

Clathria (Isociella) skia Hooper, 1996

Material examined: Individuals 7.11, 7.17, 8.13, 8.15, Vietnam (Nha Trang). Impacted coral reef (Millepora dominated) of Dambay region. Depth 2-4 m. 3th and 4th of April 2015.

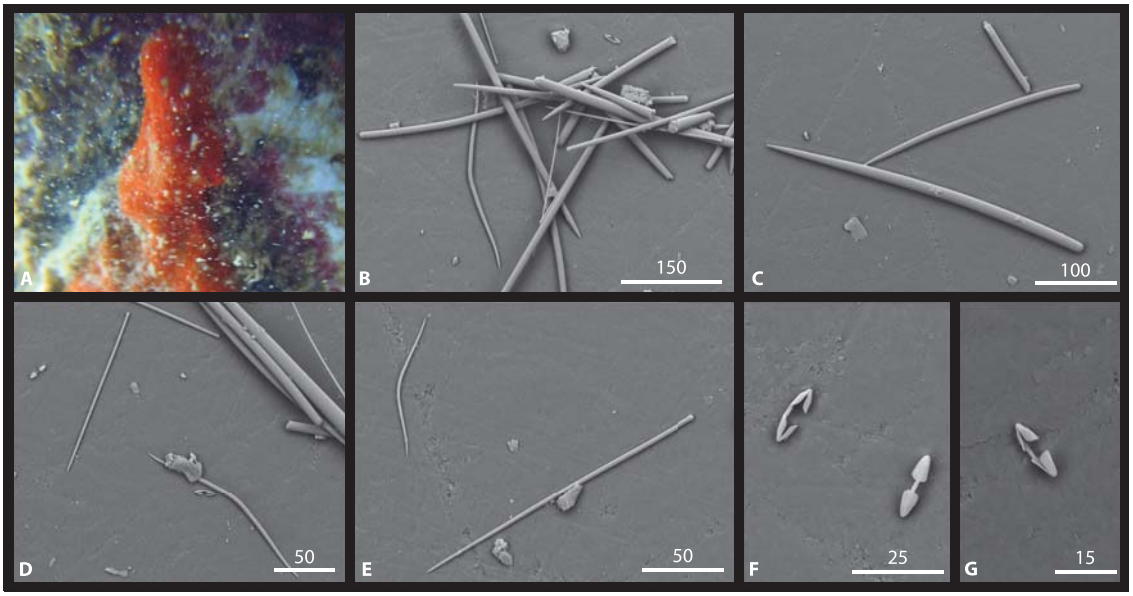


Figure 2.37: *Clathria (Axosuberites)* sp. A) *In situ* specimen. B-G) SEM images of styles, subtylostyles, palmate isochelae and toxas. Scale bar in μm.

Description: Sponge massive-irregular that form protuberances and digitations ususlly finishing in a cloacal 4.5 mm wide oscule. Surface hispid that traps abundant sediment. Consistency firm. Colour bright red in life (Figure 2.38A).

Skeleton: Similar irregular reticulation of fibres 4-8 μm wide with few spongin and some plumose appearance in some zones. Some styles widespread across the sponge with spicules in both the central zone and in the species periphery. Two zones differentiated, the inner zone or core and the external zone or peripheral. Reticulation formed by spongin fibres, in both the inner and the external zones, which contained the styles. Some additional style and the microscleres widespread across the sponge choanosome, the later particularly abundant in the ectosome.

Spicules: Choanosomal styles, smooth, 220-311 x 2.2-7 μm; Ectosomal subtylostyles, 164-222 x 2.3-2.7 μm; Palmate isochelae in two size categories; I, 22-24 μm long and II, 14-18 μm long; Toxas in two size categories: I, 88-100 μm (chord) and II, 20-25 μm chord. Raphides lineal, 45-90 μm long (Figure 2.38B-E).

Distribution and ecology: Pacific Ocean, the whole Australian coasts and Great Barrier Reef.

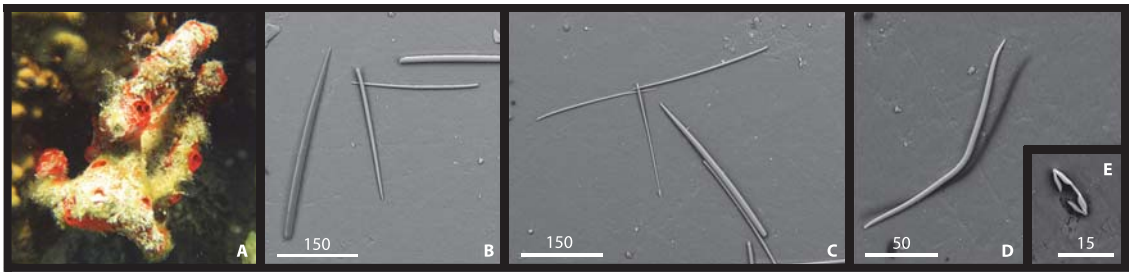


Figure 2.38: *Clathria (Isociella) skia* A) *In situ* specimen. B-E) SEM images of styles, subtylostyles, palmate isochelae and toxas. Scale bar in μm.

Clathria (Microciona) Bowerbank, 1862

Clathria (Microciona) cf. lizardensis Hooper, 1996

Material examined: Individual 13.11, Vietnam (Nha Trang). Rocky shore of Hun Mun Island (South) Depth 9 m. 10th April 2015.

Description: Encrusting slightly lobulated sponge with smooth surface and consistency. Colour bright orange (Figure 2.39A).

Skeleton: Plumose bundles of smooth styles perpendicular to the sponge base, echinated by acanthostyles. Ectosomal subtylostyles tangential or inclined.

Spicules: Choanosomal styles with smooth shafts and spiny bases, and a wide range of sizes, 178-360 x 8-20 μm in size; Ectosomal subtylostyles, in two size classes, hard to distinguish: I, 180-335 x 2-5 μm, with spiny heads, and II, 230-350 x 2-2.5 μm, completely smooth and with a slightly inflated base; Echinating acanthostyles, 80-150 x 4-7 μm in size with spines stronger and more concentrated at the spicule base. Toxas with "Oxhorn" shape (with a marked curve and the ends directed outwards, in two categories: I, 150-160 x 5-6 μm and II, 35-40 μm. Palmate isochelae 10-18 μm long are extremely in our slides and might represent contamination (Figure 2.39B-H).

Distribution and ecology: Pacific Ocean: all around the Australia coasts, including Great barrier Islands.

Clathria (Thalysias) Duchassaing and Michelotti, 1964

Clathria (Thalysias) reinwardti Vosmaer, 1880

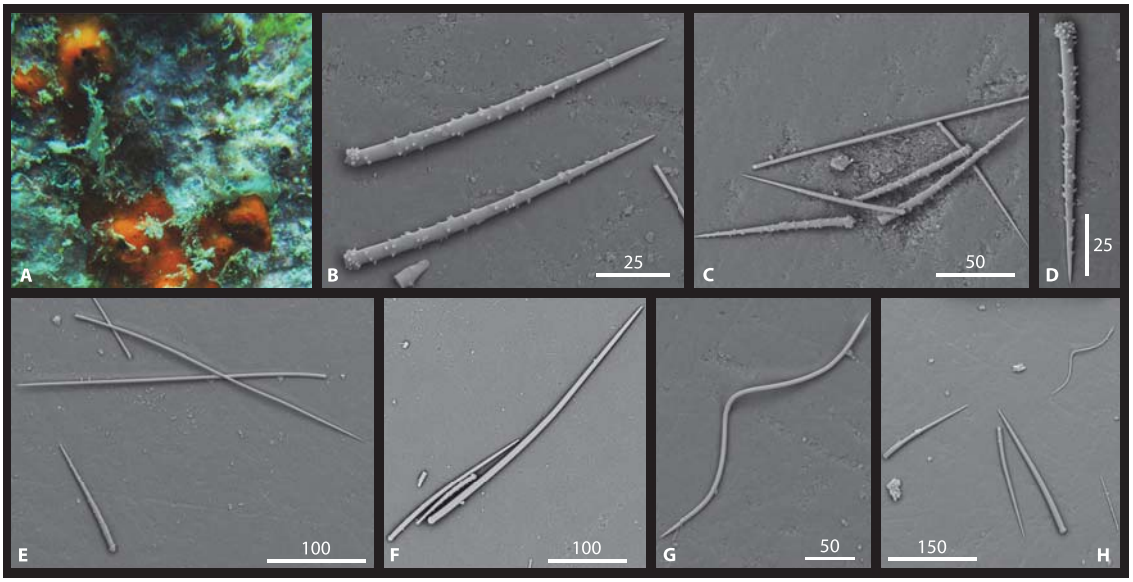


Figure 2.39: *Clathria (Microciona) cf. lizardensis* A) *In situ* specimen. B-H) SEM images of styles, subtylostyles, acanthostyles and toxas. Scale bar in μm.

Material examined: Individuals S7.1, S7.5, S7.12, S7.19, S7.22, S8.10, S8.11, S8.16, S9.1, S9.2, S9.4, S9.5, S9.6, S9.7, S9.8, S9.9, S9.12, S9.15, S9.16, S9.17, S9.18, S9.19, S9.21, S9.22, S9.25, Vietnam (Nha Trang). Impacted coral reef (Millepora dominated) of Dambay region and coral reef semi-good conditions close to Hun Mun Island (north). Depth 2-6 m. 4th and 6th of April 2015.

Description: Polymorphic sponge with different shapes from massive to digitate or branching with branches of variable length and thickness. Branches measure from 1 cm to more than 3 cm of length and ca. 1 cm of diameter. Surface smooth to the touch but covered by dense rounded small projections, conferring to the sponge a granulate pattern. Ocula, 1-5 mm in diameter, scattered across the surface generally on the upper surface. Consistency firm but flexible. Colour variable from grey pinkish, yellow pinkish to bright pink outside but is bright pink in side, in live (Figure 2.40A).

Spicules: Choanosomal styles, 158-261/146-24 μm in two different undividuals; acanthostyles 63-83/56.77μm (two individuals), Toxas almost straight (like raphides) 29-62 μm and toxas curved, 12-13 μm (chord); Palmate Isochelae 12-13 μm long (Figure 2.40B-H).

Distribution and ecology: The species is widely distributed in the

Pacific central, Indonesia, around the Australia coasts, Banda Sea, Ararufa sea, East Caroline I.

Remarks: This species dominates, together with Amphimedon paraviridis the polluted reefs of Nha Trang Bay. The identity of this species is confirmed by its LSU sequence, which is identical to that deposited for *C. reinwardi* en the NCBI database (Figure A.12).

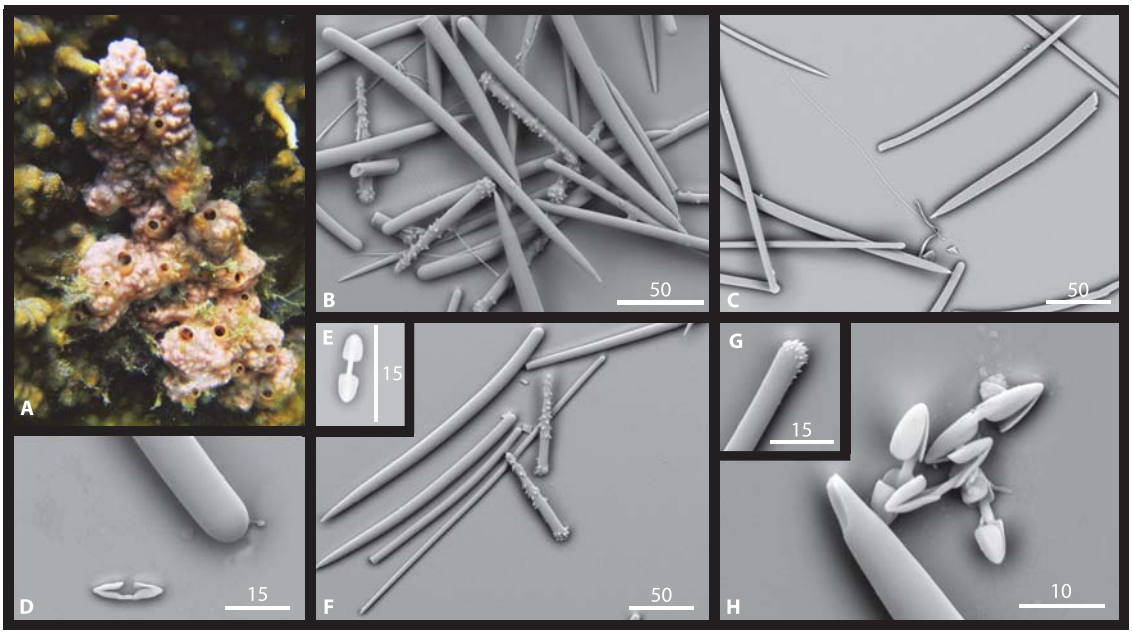


Figure 2.40: *Clathria reinwardti* A) *In situ* specimen. B-H) SEM images of styles, subtylostyles, acanthostyles palmate isochelae and toxas. Scale bar in μm.

Genus *Mycale* Gray, 1867

Mycale (Carmia) Gray, 1867

Mycale (Carmia) phylophila Hentschel, 1911

Material examined: Individual 2.8, Vietnam (Nha Trang). Rocky semi-vertical shore of Hun Mun Island (north). Depth 9 m. 30th March 2015.

Description: Thick encrusting sponge. One oscula 3mm in diameter is visible, Consistency unknown, Surface micro-hispida; Blue-greyish in colour, in life (Figure 2.41A).

Skeleton: Polyspiculated tracks of mycalostyles irregularly arranged, plumose toward the sponge surface. The ecotosome is perforeated by the terminations of the choanosomal plumose tracks and contains abundant sigmas but not anisochelae in rosettes.

Spicules: subtylostyles (mycalostyles) thin straight with a somewhat fusiform shaft and an slightly inflated head, 231-271 x 4-5 μm; anisochelae in two size classes hardily distinguishable: I, 17-21 μm long and II, 11-13 μm long; sigmas 30-36 μm of chord (Figure 2.41B-F).

Distribution and ecology: Pacific Central Indonesia, Vietnam and Australia. Previously recorded from Nha Trang (Lévi 1961).

Remarks: the individual of Nha Trang clustered with the species *M. phylophyila* in a clade with *M. fistulifera*, *M. microsigmatosa*, *M. cecilia*, and *M. adhaerens* in a phylogenetic tree based in the LSU partition (Figure A.13).

Mycale (Arenochalina) Lendenfeld, 1887

Mycale (Arenochalina) sp.

Material examined: Individuals 4.9 (three individuals), 7.8, 8.17, Vietnam (Nha Trang). Impacted coral reef (dominated by *Millepora* spp.) and well-preserved reef (dominated by *Acropora* spp) of Dambay region. Depth 2-9 m. Sampling dates: April 1st and 3th, 2015.

Description: thick encrusting forms, highly compressible with a smooth clean surface, conulous in some places. One 4-5mm large oscule and several smaller per specimen. Round or elongated perforations concentrated in areas, which might represent inhalant zones, but the holes are 1-2 mm hard and rather seem surface cribose areas covering subectomal cavities. Colour bright yellow or clean orange, in life (Figure 2.42A).

Skeleton: Choanosomal skeleton formed by polispiculated tracks with spongin irregularly reticulated. Ectosonal skeleton formed by tangential mycalostyles without forming reticulation.

Spicules: Mycalostyles straight with a slightly inflated base, 210-260 x 2.2-10 μm; Anisochaele, 11-20 μm. Sigmas in two size classes: I, 22-25 μm chord and II, 11-13 μm chord; Rhaphides undulated wit a toxiform shape,

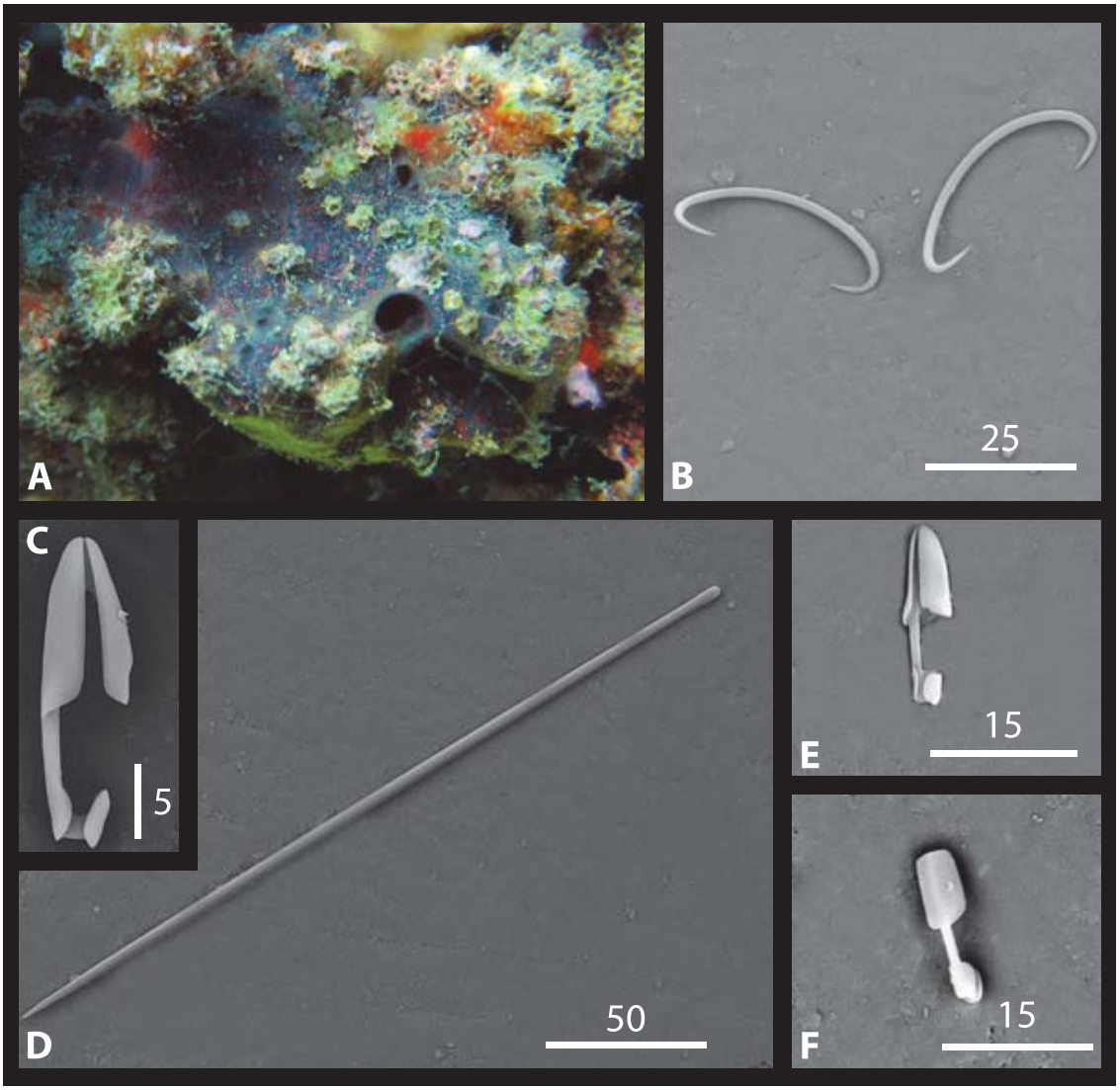


Figure 2.41: *Mycale (Carmia) phylophila* A) *In situ* specimen. B-F) SEM images of sigmas (B), mycalostyles (D) and anisochelae (C,E,F). Scale bar in μm.

forming toxodragmata of 29-43 x 6-9 μm (thicknes of the toxodragmata measured in the middle). Spinny microxeas rare, 5-7 μm long (Figure 2.42B-I).

Remarks: The species correspond to a new Mycale species, that will be formally described in a separate taxonomical paper. The only *Mycale* species from the central Indo-Pacific previously described with toxodramata is *M (Arenosclera) tenuityla* (Pulitzer-Finali 1982) but the spicules characteristics other than the presence of toxodragmata are different in the Vietnamese species.

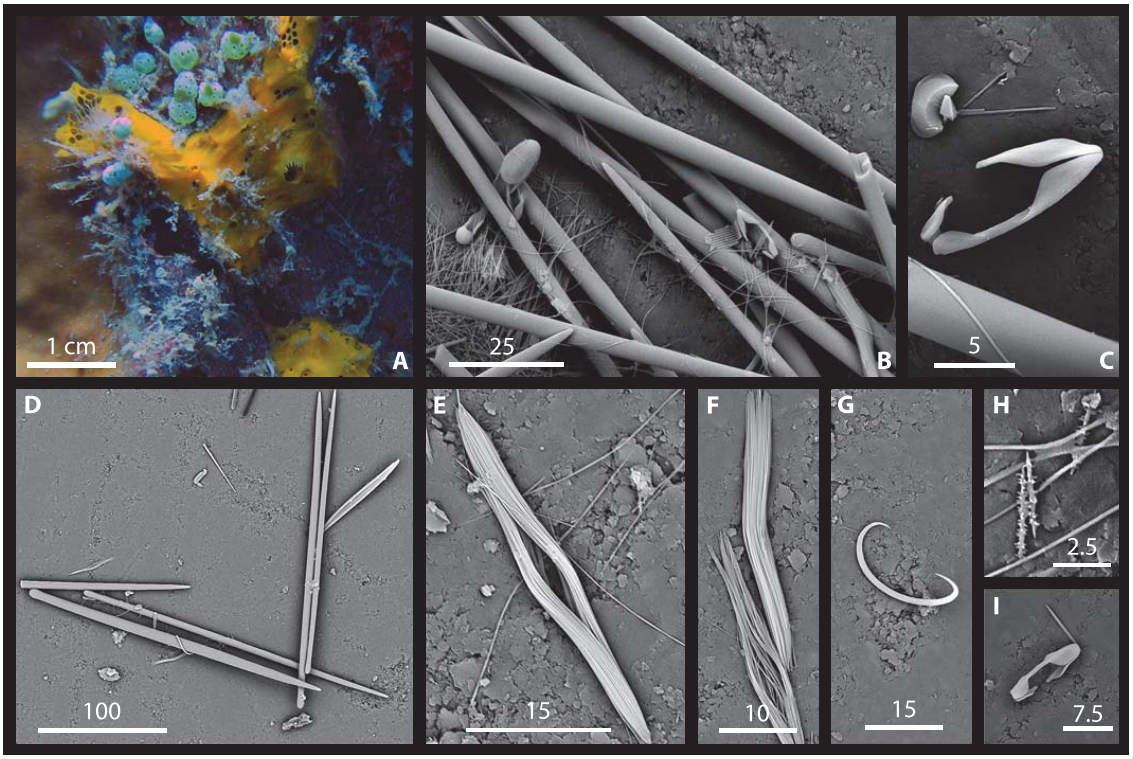


Figure 2.42: *Mycalea(Arenochalina)* sp. A) *In situ* specimen. B-I) SEM images of mycalostyles (B,D), anisochaele (B,C,I), toxodragmata (E,F), sigmas (G) and spinny microxeas (H). Scale bar in μm.

Mycale (Zigomycale) Topsent, 1930

Mycale (Zigomycale) parishii (Bowerbank, 1875)

Syn.: *Raphiodesma parishi* Bowerbank, 1875

Material examined: Individual 3.5, Vietnam (Nha Trang). Rocky semi-vertical shore of Hun Mun Island (North). Depth 8 m. Sampling date; March 30th 2015.

Description: Massive soft sponge soft. Surface smooth. Several oscules from 2 to 5 mm in diameter visible. Colour, grey yellowish in life (Figure 2.43A).

Skeleton: Plumoreticulated spicule tracks in the choanosome and dense tangential mycalostyles without forming a clear reticulation, in the ectosome.

Spicules: Subtylostyles (mycalostyles) with a neck slightly bent and and slightly inflated head, 208-290 x 3.5-9 μm; Anisochaele in two size categories: I, 35-55 μm long and II, 13-22 μm long; Palmate isochaele, 9-11 μm long. Sigmas in two size classes: I, 68-90 x 2.2-7 μm and II, 26-36 x 1

μm; Toxes of two size classes: I, 70-124 x 1.3-1.5 μm and II, 25-30 x 0.5 μm (Figure 2.43B-G).

Distribution and ecology: Malacca strait, Australia, Bight of Sofala, East African Coral Coast, Eastern Philippines, Gulf of Thailand, Hawaii, Houtman, Maldives, Natal, Seychelles, Shark Bay, Singapore, South India and Sri Lanka, Western India, Western and Northern Madagascar, North Pacific Ocean. The species has been previously recorded from Vietnam.

Remarks: These Vietnamese individuals are attributed here to *M. (Zygomycale) parishi*, which seems to be a widely distributed species in the Pacific. However, some characteristics of our individuals such as the absence of raphides cast some doubt about its correct identification. Only three species of *M. (Zygomycale)* are presently known from the Pacific (*M. (Z.) pectinicola, M. (Z.) parishi, M. and M. (Z) ramulosa*, and our specimens might also be a new species.

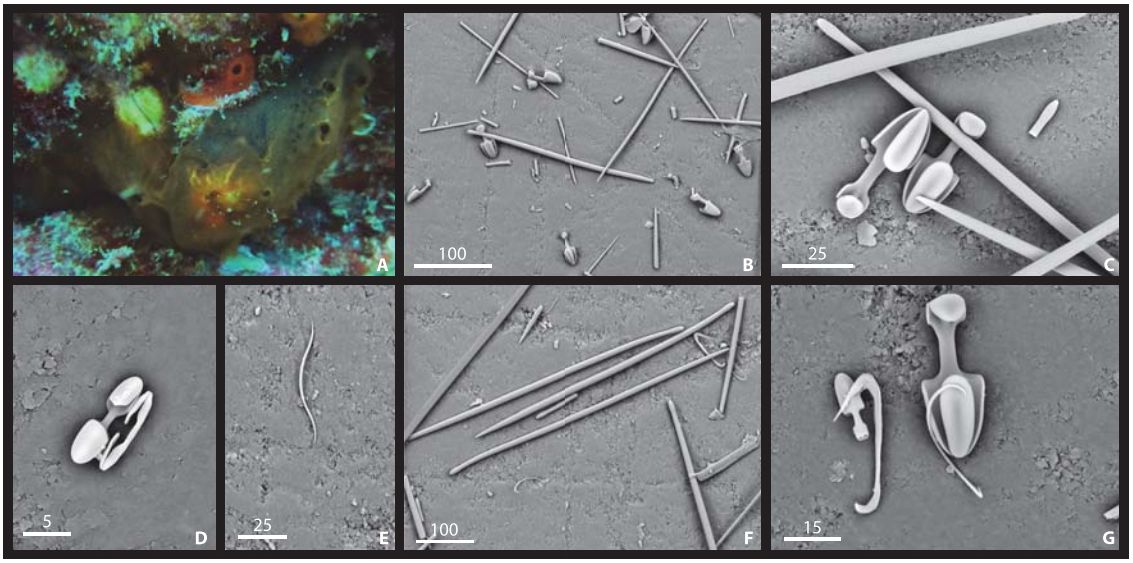


Figure 2.43: *Mycale (Zigomycale) parishii.* A) *In situ* specimen. B-G) SEM images of mycalostyles (B,F), anisochaele (B,C,G), isochaele (D), sigmas (G) and toxas (E). Scale bar in μm.

Mycale (Naviculina) Gray, 1867

Mycale (Naviculina) sp.

Material examined: Individual 6.9, Vietnam (Nha Trang). Well-preserved reef (Acropora dominated) close to Dambay region. Depth 4-6 m. Sampling date: April 2nd 2015.

Description: Delicate encrusting sponge attached to a death coral. Very soft consistency. Surface smooth with a reticulate skeletal network visible from outside. A 1mm wide oscule visible. Colour light translucid blue green.

Skeleton: Choanosomal and ectosomal reticulation of spicule tracks of few spicules forming delicate meshes, in particular in the ectosome.

Spicules: Tylostrongyles, 335-510 x 2.5-13 μm. Naviculichelae in two size categories: I, 22-27 μm long and II, 15-18 μm long. Sigmas, 12-15 μm chord.

Remarks: This species does not correspond to any of those previously described with naviculichelae microscleres and thus, it likely represents a new species that will be formally described elsewhere.

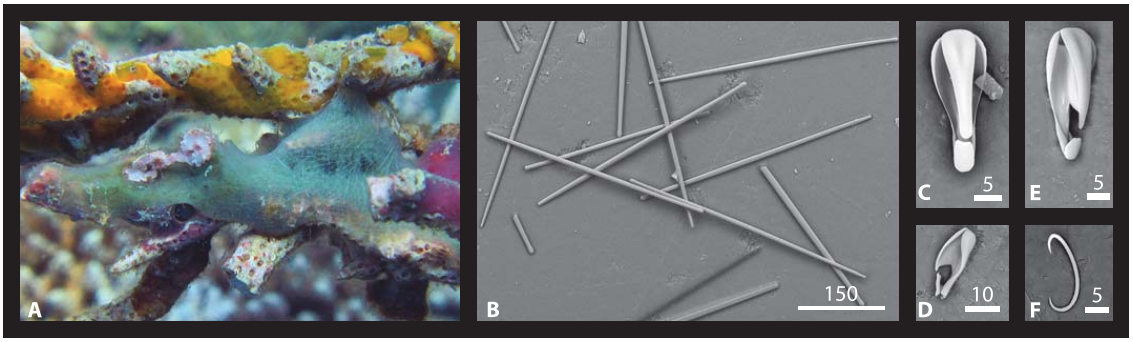


Figure 2.44: *Mycale (Naviculina)* sp. A) *In situ* specimen. B-F) SEM images of Tylostrongyles (C), naviculichelae (E) and sigmas (F). Scale bar in μm.

Family Tedaniidae Ridley and Dendy 1922

Genus *Tedania* Liberkühn, 1859

Tedania (Tedania) Gray, 1867

Tedania (Tedania) panis (Selenka, 1867)

Syn.: *Suberites panis* Selenka, 1867

Material examined: Individual 9.27, Vietnam (Nha Trang). Coral reef semi-good conditions close to Hun Mun Island (north). Depth 6 m. 6th April 2015.

Description: Thick ectosome, separable from the choanosome. Surface smooth. Consistency soft. Colour bright red in life (Figure 2.45A).

Skeleton: Ectosomal skeleton formed by ramified bundles of tylotes and tangential onychaetes. Choanosomal skeleton formed by substylostyle tracks.

Spicules: Subtilostyles smooth, 180-240 x 7-8 μm; Tylotes 256-330 x 3,3-4.5 μm; Onychaetes straight, spiny in a unique size category of 130-165 x 2.2-2.4 μm (Figure 2.45B,C).

Distribution and ecology: South Pacific; South coast of Australia

Remarks: This is one of the few *Tedania* species of the Pacific with the subtylostyles shorter than the tylotes. The species appears in the same clade as *T. tubulifera* and *T. cf. ferrolensis* in the tree constructed with the LSU gene but this and the other tree clades are poorly supported.

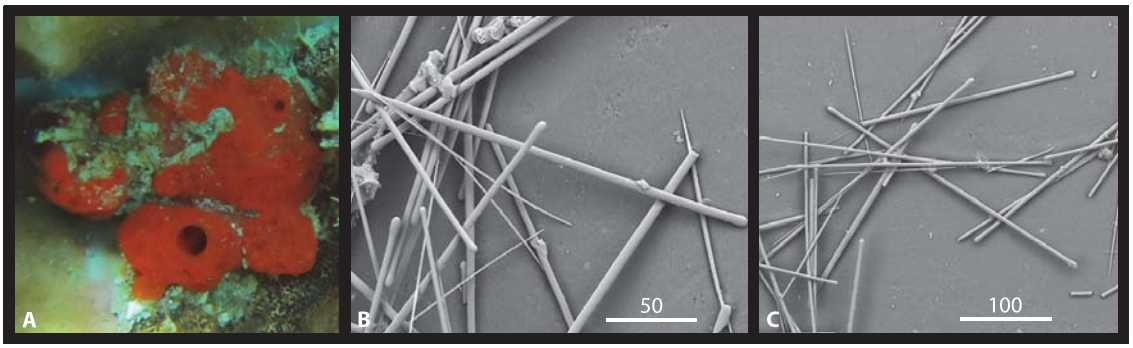


Figure 2.45: *Tedania (Tedania) panis*. A) *In situ* specimen. B-C) SEM images of subtylostyles, tylostyles and onychaetes. Scale bar in μm.

Family Placospongiidae Grant, 1867

Genus *Placospongia* Grant, 1826

Placospongia sp.

Material examined: Individual 13.9, Vietnam (Nha Trang). Rocky shore of Hun Mun Island (South) Depth 9 m. 10th April 2015.

Description: Thick encrusting individual with the typical parchment surface pattern leaving grooves between plates, but covered by corallinaceus encrusting algae. Consistency hard but easy to broke. One 6 mm wide oscula visible. Colour whitish green (Figure 2.46A).

Skeleton: Choanosomal skeleton formed by tylostyle bundles from the substratum to the species surface. The ectosome skeleton is formed by densely arranged selenarsters.

Spicules: Tylostyles in two size categories: I, 800-1000 x 11-18 μm and II, 170-295 x 2.2-4.5 μm. Selenasters of different sizes as a function of the stage of development: completely developed 70-80 x 35-40 μm (two main diameters), immature 16-30 μm long axis; streptasters with few long spines, some of them completely smooth, some others with a twisted axis, 17-30 μm in diameter. No spiny microrhabds, no comate, no spherasters (Figure 2.46B-I).

Remarks: Among the 6 species of *Placospongia* recorded from the Indo-Pacific region, only *P. carinata* and *P. melobesioides* lack spherasters (Becking 2013). The closest species to our smecimen is *P. carinata* because it also lacks spherasters. However, the Vietnamese sponge differs by the absence of microrhabs and the reduced spination of the streptasters. This individual likely represents a new species, which will be formally described in a taxonomical paper.

Order Suberitida Chombard and Boury-Esnault, 1999

Family Halichondriidae Gray, 1867

Genus *Axynissa* Lendenfeld, 1897

Axinyssa variabilis (Lindgren, 1897)

Syn.: *Halichondria (Halichondria) variabilis* Lindgreen, 1897

Material examined: Individual 5.5, Vietnam (Nha Trang). Well-preserved reef (Acropora dominated) close to Dambay region. Depth 4-6 m. 2nd April 2015.

Description: Thick encrusting specimen with two 4-5 mm wide oscula. Ectosome not separable from the choanosome. Surface hispid, uniformly

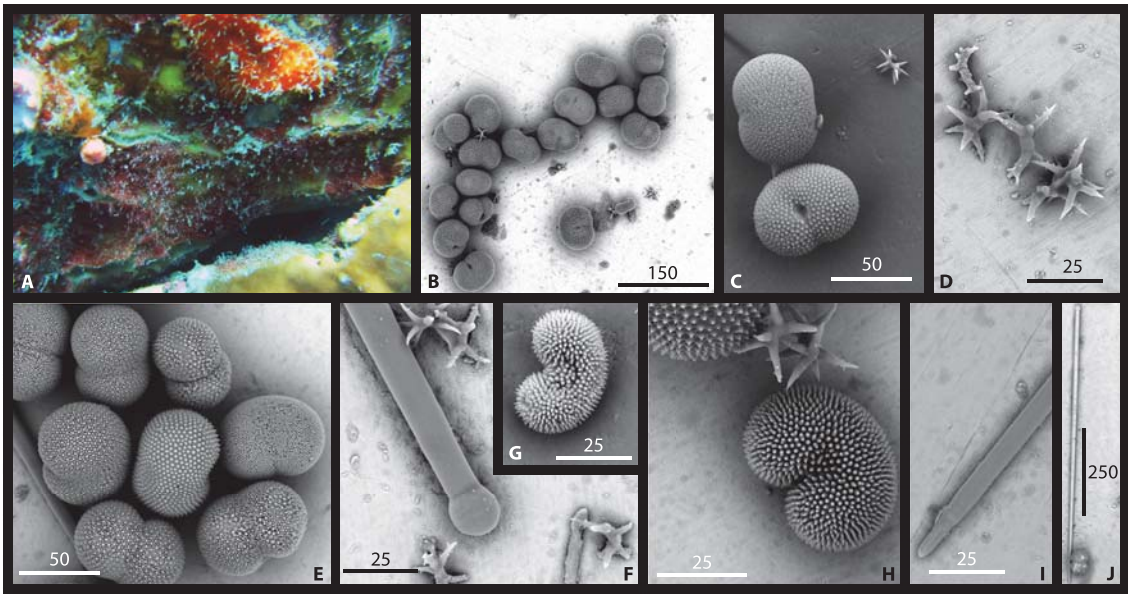


Figure 2.46: *Placospongia* sp. A) *In situ* specimen. B-J) SEM images of tylostyles, selenasters and streptasters. Scale bar in μm.

covered by small conules except in the zone close to the oscula. Consistency compressible but resistant. Colour intense yellow, in life (Figure 2.47A).

Skeleton: Disarranged choanosomal skeleton except at the sponge periphery where the spicule tracks are arranged with the spicules perpendicular to the sponge surface. These tracks cross the surface forming the characteristic small conules. No particular ectosomal skeleton is visible.

Spicules: oxeas slightly curved, with blunt points,620-800 x 11-20 μm (Figure 2.47B,C).

Distribution and ecology: Indonesia, Vietnam, Java Sea

Remarks: This individual has similarities with A. aplisinoides, but the oxeas of this species are longer and thicker (Dendy 1992). The individual from Nha Trang is placed with other *Axinyssa* species in a well-supported clade of the LSU tree of family Halichondriidae (Figure A.14).

Genus *Topsentia* Berg, 1899

Topsentia cf. halichondroides (Dendy 1905)

Syn.: *Trachyopsis halichondroides* Dendy, 1905

Material examined: Individual 9.23, Vietnam (Nha Trang). Coral reef semi-good conditions close to Hun Mun Island (north). Depth 6 m. 6th

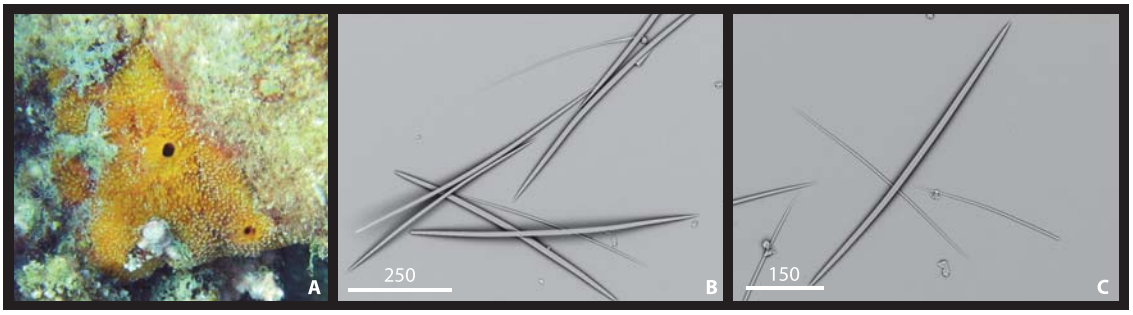


Figure 2.47: *Axynissa variabilis* A) *In situ* specimen. B-C) SEM images of oxeas. Scale bar in μm.

April 2015.

Description: Massive sponge, grey dark outside, whitish-cream inside, in life. Ectosome conspicuous as a layer but difficult to separate from the choanosome. Consistency firm but breakable. Colour mauve translucid in life (Figure 2.48A).

Skeleton: Choanosomal skeleton formed by oxeas densely inter-crossed without any specific orientation. Ectosomal skeleton formed by dense tangential and oblique oxeas (Figure 2.48D).

Spicules: oxeas slightly curved with blunt points, in two size-classes hardly differentiable: I, ranging from 450-965 x 10-31 μm and II, 320.382 x 5-9 μm (Figure 2.48B,C).

Distribution and ecology: Indian Ocean: Madagascar , Sheychelles, Chatan I. East Africa, south India, Sri Lanka.

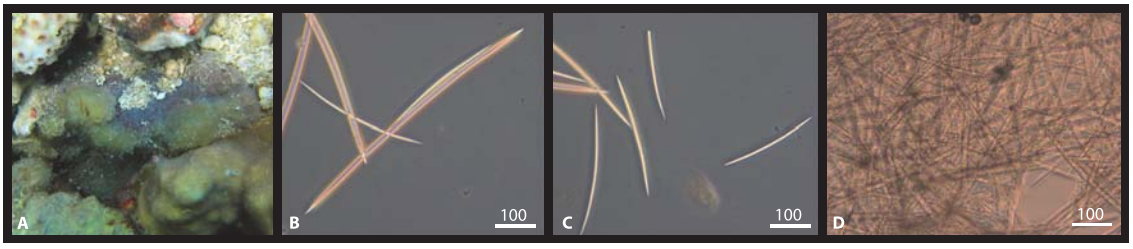


Figure 2.48: *Topsentia cf. halichondroides* A) *In situ* specimen. B-D) Light microscopic images of oxeas (B-C) and skeleton (D). Scale bar in μm.

Genus *Halichondria* Fleming, 1828

Halichondria (Halichondria) Fleming, 1828

Halichondria (Halichondria) cf. cartilaginea (Esper, 1794)

Material examined: Individual 10.1, Vietnam (Nha Trang). Well-preserved reef (Acropora dominated) close to Dambay region. Depth 6 m. 8th April 2015.

Description: Encrusting sponge is hardly visible among the protuberances of a calcareous substratum and lives in close association to a green filamentous seaweed. The ectosome is separable from the choanosome as in typical Halichondria spp. Consistency soft. No oscules are distinguishable. Colour translucid greenish in live (Figure 2.49A).

Skeleton: Choanosomal skeleton disarranged. Ectosomal skeleton formed by some tangential spicules enclosed in the ectosomal layer.

Spicules: oxeas of 245-340 x 3.5-15 μm (Figure 2.49B,C).

Distribution and ecology: Pacific Ocean, China Sea, Vietnam. Singapur. Malaka strait; Indian Ocean: African Coral coasts, living in association with a green algae. It had been previously recorded from Nha Trang Bay (Lévi 1961).

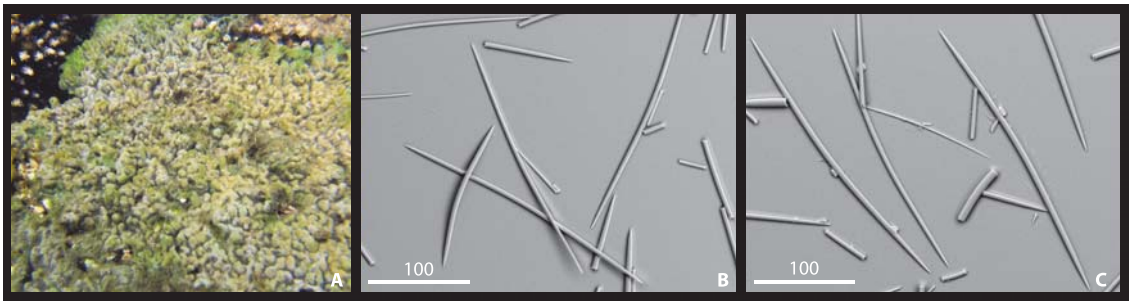


Figure 2.49: *Halichondria (Halichondria) cf. cartilaginea*. A) *In situ* specimen. B-C) SEM images of oxeas. Scale bar in μm.

Family Suberitidae Schmidt, 1870

Genus *Aaptos* Gray, 1867

Aaptos suberitoides (Brondsted, 1934)

Syn.: *Stylotella suberitoides* Brondsted, 1934

Material examined: Individual S5.7, S6.1, S12.1B, S3.10, S6.3, S11.6, S12.1A, S1.16, S10.2, S11.1, Vietnam (Nha Trang). Well-preserved reefs

(*Acropora* dominated) close to Dambay region and rocky vertical walls of Hun Mun Island (North and South) Depth 4-9 m. 30th March, 2nd 8th and 10th April 2015.

Description: Massive sponge that form globulose wide protuberances with one oscule on top. Surface smooth to the naked eye or forming a short granulation, depending on the specimen. Consistency firm and gummy. Ectosome inconspicuous and difficult to separate from the choanosome. Oscules numerous, 2-4 um in diameter. Colour brownish yellow-orange outside, bright yellow inside, in life (Figure 2.50A,B).

Skeleton: Choanosomal skeleton consisting in dense spicule tracks without the typical radial arrangement of the genus. Only close to the periphery the small spicules are placed perpendicular to the sponge surface forming a palisade and crossing the ectosome slightly as in Suberites.

Spicules: strongyloxeas, in two categories with intermediate sizes 275-450 x 7-9 μm and II, 600-1500 x 12-18 μm (Figure 2.50C-F).

Distribution and ecology: Pacific Ocean: Banda Sea, Malaka Street, Vietnam, East Caroline I.

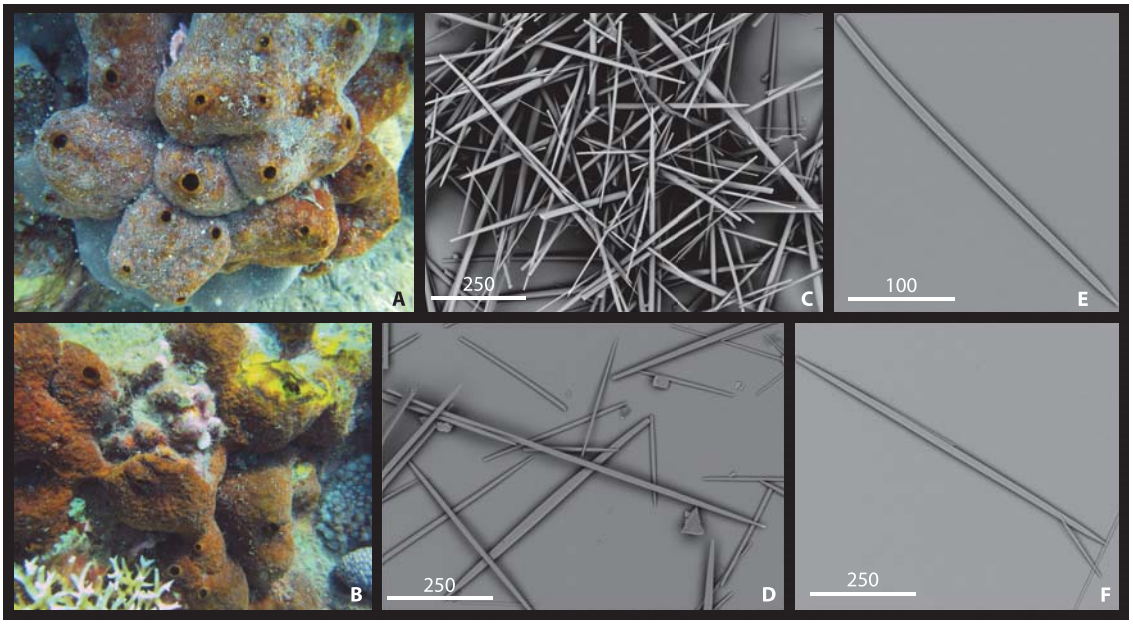


Figure 2.50: *Aaptos suberitoides*. A,B) *In situ* specimen. C-F) SEM images of strongiloxeas. Scale bar in μm.

Genus *Protosuberites* Swartschewsky, 1905

Protosuberites proteus (Hentschel, 1909)

Syn.: *Laxosuberites proteus* Hentschel, 1909

Material examined: Individual 1.10, 1.17, Vietnam (Nha Trang). Rocky vertical wall (crack) of Hun Mun Island. Low light. Depth 10 m. 28th March 2015.

Description: Thin encrusting sponge. One osculum slightly elevated. Ectosome not differentiable. Surface smooth under the naked eye, but minutely hispid under a stereomicroscope. Consistency soft. Bright red colour (Figure 2.51A).

Skeleton: Skeleton consisting in spicule bundles from the susbtrate to the sponge surface, which they cross, conferring to the sponge a short hispidation Figure 2.51B-D).

Spicules: tylostyles in two categories mainly differentiated because of their thickness: 180-315 x 5-12 μm and 230-500 x 17-20 μm thickness.

Distribution and ecology: Indo-Pacific: Australia coasts, Indochina, Sri-Lanka and India.

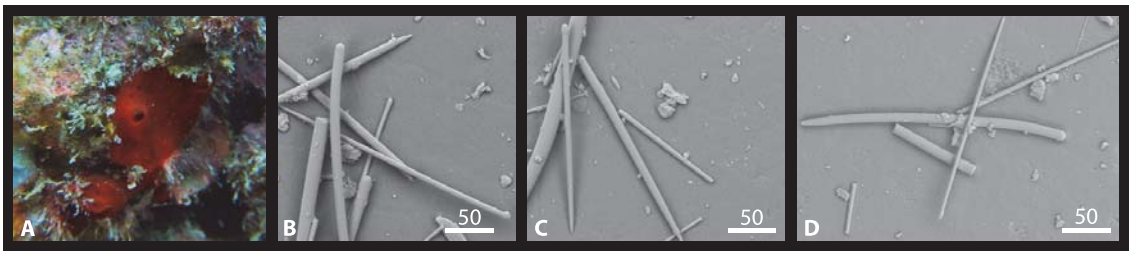


Figure 2.51: *Protosuberites proteus*. A) *In situ* specimen. B-D) SEM images of strongiloxeas. Scale bar in μm.

Genus *Terpios* Duchassaing and Michelotti, 1864

Terpios sp.

Material examined: individual 1.13 Vietnam (Nha Trang). Rocky vertical wall (crack) of Hun Mun Island. Low light. Depth 10 m. 28th March 2015.

Description: encrusting individual with a characteristic star-like pattern of excurrent canals. Very small oscula slightly visible in the middle of excurrent canals. Dark grey-greenish (Figure 2.52).

Remarks: The whole sample was used for the microbiome and DNA study, so than not sample remained for spicule measurement and microscope observation.

Figure 2.52: *In situ* specimen of *Terpios* sp.

Terpios cruciatus (Dendy, 1905)

Syn.: *Suberites cruciatus* Dendy, 1905

Material examined: Individual 4.7, Vietnam (Nha Trang). Rocky vertical wall of Nock Island. Depth 9 m. 1st April 2015.

Description: Encrusting individual with inhalant orifices visible but without anyconspicuous oscula. Smooth to the touch. Cosistency soft. Ectosome not separable. Colour bright orange in life (Figure 2.53A).

Skeleton: Tylostyles directly with their base on the substratum and the point outwards in an hymedesmoid arrangement.

Spicules: Tylostyles with a characteristic base formed by four swellings flat on the exterior side, in two size categories but with some intermediated forms: I, 110-170 x 2,5-9 μm and II, 225-350 x 4.5-9 μm (Figure 2.53B-F).

Distribution and ecology: India Ocean: Madagascar Sheychelles, India and Sri Lanka.

2.5 Discussion and Conclusions

A total of 203 sponge individuals were recorded along the 13 horizontal transects displayed between 3 and 9 m of depth on rocky bottoms and reefs

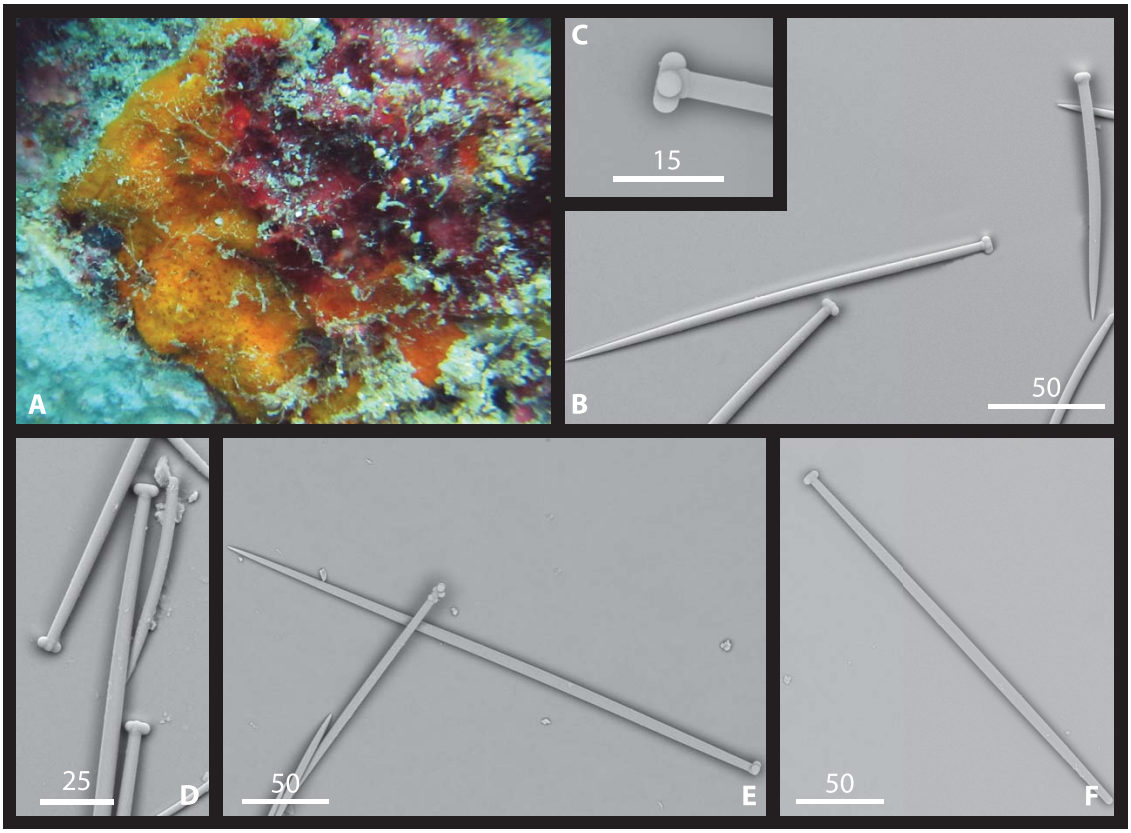


Figure 2.53: *Terpios cruciatus*. A) *In situ* specimen. B-F) SEM images of tylostyles. Scale bar in μm.

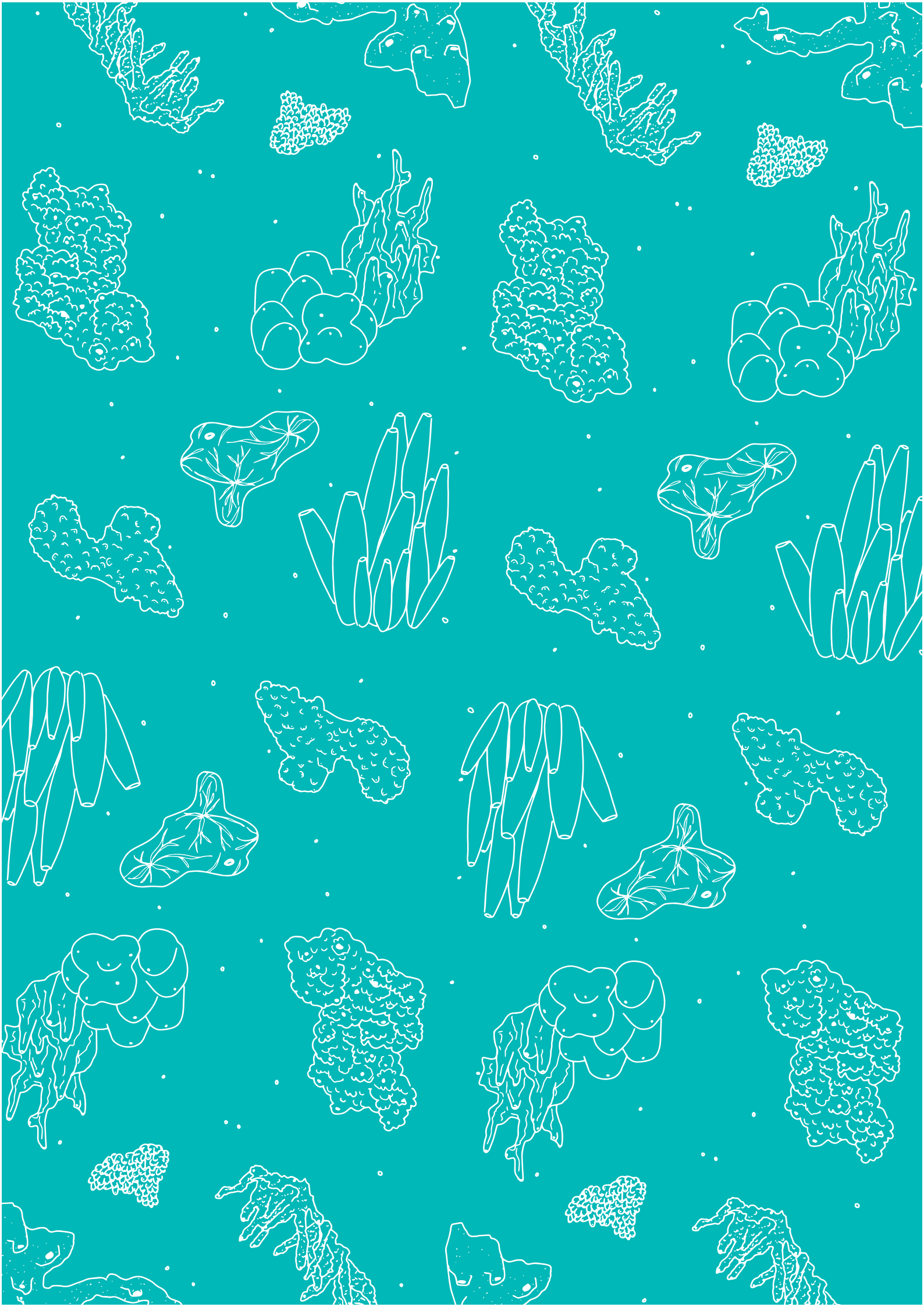
from Nha Trang Bay, which belonged to 9 orders, 22 families, 36 genera, and 60 species of demosponges. From these, 17 species were identified only to a genus level.

Within Heteroscleromorpha, Orders Poecilosclerida and Haplosclerida were well represented, followed by Orders Suberitida, Verongiida, and Dictyoceratida. Families better represented were Chalinidae, Microcionidae and Niphatidae and the genera *Haliclona* and *Clathria* contained the highest number of representatives.

The most abundant species in *Accropora* (well-preserved) reefs were *Neofibularia* sp. and *Aaptos suberitoides*, while *Monanchora unguiculata*, *Antho (Antho)* sp., and *Amphimedon sulcata* predominated in rocky bottoms. *Millepora* (eutrophic) reefs were dominated by *Clathria reinwardti* and *Amphimedon paraviridis*, which represented >50% of the total species recorded in these impacted areas. 24 species were added to the Vietnam fauna, from which 11 species could not be attributed to any known species and likely

represent new species to science. These belonged to genera *Dysidea* (2 species), *Dactylia* (1 species), *Didiscus* (1 species), *Neofibularia* (1 species), *Crambe* (1 species), *Antho* (1 species), *Clathria (Axosuberites)* (1 species), *Mycale (Arenochalina)* (1 species), *Mycale (Naviculina)* (1 species) and *Placospongia* (1 species). Other more species differed from the type species to which they were ascribed in some particular traits such as colour or spicule sizes but they were attributed to the already known species despite the small differences, since more individuals need to be examined to fix the intra-species variation in morphological or spicule characteristics.

To summarize, this chapter describe external and skeletal characteristics of 60 sponge species of Nha Trang, which represent an increase of 8% in the already known fauna of Vietnam. The study also contributed to the general sponge knowledge by recording 11 new sponge species, and improve the molecular characterization of several species by adding their LSU (53 species), SSU (11 species) and COI (11 species) sequences to the NCBI database (see Table A.2).



Turon, M., Cáliz, J., Garate, L., Casamayor, E.O., Uriz, M.J.(2018) Showcasing the role of seawater in bacteria recruitment and microbiome stability in sponges *Scientific Reports*, 8: 15201. DOI:10.1038/s41598-018-33545-1.
Authors affiliations: Centre d'Estudis Avançats de Blanes (CEAB) CSIC
La publicació d'aquest capítol es troba en l'Annex de la tesis *Published chapters*. Els apartats i distribució del capítol estan presentats en l'ordre que estableix l'editorial de la revista. Únicament s'ha editat la forma i mida de lletra per unificar el format de tesis. La bibliografia del capítol es troba en l'apartat *Bibliography*.
Imatge capítol: Dibuix d'esponges característiques de la zona de mostreig.
Autora coberta: Laura López

Chapter 3

Bacteria stability and acquisition modes in sponge microbiomes: showcasing the role of seawater

3.1 Abstract

We studied the core bacterial communities of 19 sponge species from Nha Trang Bay (Central Vietnam), with particular emphasis on the contribution of planktonic seawater bacteria to the sponge core microbiomes. To ensure consistent sponge-microbe associations and accurate identification of planktonic bacteria transmitted from seawater, we were very restrictive with the definition of the sponge core microbiomes (present in all the replicates), and with the identification of valid biological 16S rRNA gene sequences (100% sequence identity) that belonged to potentially different bacterial taxa. We found a high overlap ($\geq$ 50% relative abundance) between the sponge species core microbiome and the seawater bacterial core in ca. a half of the studied species, including representatives of both, HMA and LMA sponges. From our restrictive analysis, we point to horizontal transmission as a relevant way of symbiont acquisition in sponges. Some species-specific recognition mechanisms may act in sponges to enrich specific seawater bacteria in their tissues. These mechanisms would allow the maintenance of bacterial communities in a species across geographical ranges. Moreover, besides contrasting preferences in bacteria selection from seawater, divergent physiological traits may also account for the different microbiomes in species of HMA and LMA sponges.

3.2 Introduction

The first step to study multi-microbial symbionts within animals is to focus on permanent symbionts by ruling out the background noise produced by transient microbes (Ainsworth et al., 2015). In other words, to concentrate on those bacteria, which have established tight associations with the host through several evolutionary time scales (Douglas, 2014), independently of potential, mutual benefits or costs for the partners involved (Moran and Sloan, 2015). In this context, the core microbiota concept was adopted to ascertain the consistent associations of a metaorganism (Bäckhed et al., 2012; Shade and Handelsman, 2012), but also allows to study the core metabolic functions provided by the host–microbe interaction to the system (Shafquat et al., 2014). The core concept was first applied to differentiate host–microbe interactions of the mammalian gut and in plant root systems (Shade and Handelsman, 2012; Shafquat et al., 2014). Further, it was extended to marine animals with the aim of understanding the consistent contributions of the microbial symbionts to the host ecology, success, or decay (Ainsworth et al., 2015).

In marine habitats, studies on coral and sponge microbiomes have proliferated in the last 10 years. For instance, the persistent microbial symbionts (core) of several coral species from a reef were identified (Hernandez-Agreda et al., 2016) through spatial and temporal scales, concluding that the complexity of the reef habitat, and the life coral history traits likely influence the coral core microbiomes. Thus, more in deep research to explore accurately the core microbiome of many invertebrates is needed to overcome the constraints associated to their complex habitats. Although there is some controversial in the literature about how the core microbiota should be defined (Ainsworth et al., 2015; Hernandez-Agreda et al., 2016), the most reasonable definition always depends on the question approached (Astudillo-García et al., 2017). For instance, more or less restrictive criteria (i.e. present from 7 % to 100 % of the replicates) have been used for marine invertebrates (Ainsworth et al., 2015; Schmitt et al., 2012; Shade and Handelsman, 2012; Thomas et al., 2016).

Sponges are a diverse group of sessile filtering invertebrates that play important ecological roles in benthic marine ecosystems (Bell, 2008; Goeij

et al., 2013). They harbour the highest diversity of microsymbionts among marine invertebrates (Simister et al., 2012a; Taylor et al., 2007). A wide range of studies using massive sequencing methods have retrieved thousands of microbial 16S rRNA gene sequences for each targeted sponge species (Thomas et al., 2016), which stressed the difficulty to unveil the mechanisms underlying these associations due to the high diversity of the partners involved. Indeed, some of the 16S rRNA gene sequences belonged to transient bacteria captured from the environment by the sponges while filtering seawater, and do not represent permanent symbionts (Thomas et al., 2016). Consequently, indirect analytical methods have been implemented trying to split stable symbionts from transient planktonic bacteria in sponge-microbial systems. The core microbiota concept was adopted to focus on permanent bacteria in a sponge species (Thomas et al., 2016) or phylum (Schmitt et al., 2012), notwithstanding the host geographical and ecological origins, or temporal scales (Bjork et al., 2018). The rationale underlying the core concept is that stable symbionts should represent tight biological associations and thus, they are expected to be present in most, or all, host individuals.

Substantial differences in the resulting diversity metrics related to the core definition applied (from 12% to 100% occurrence), have been reported recently (Astudillo-García et al., 2017). Several studies have proposed that stable sponge microsymbionts should be present in the sponges but not (at least not in high abundance) in the surrounding seawater (Thomas et al., 2016). But, recording the presence of a sponge bacterium in seawater greatly depends on the abundance threshold used to include or exclude sequences from downstream analysis, the sequencing depth and the number of samples. Moreover, it has been proposed that stable symbionts are mainly inherited by the progeny from their parent sponges (Enticknap et al., 2006; Lee et al., 2009; Schmitt et al., 2007; Webster et al., 2010). However, vertical and horizontal transmission of the same bacteria have been described (Sipkema et al., 2015), and more recent investigations reported different microbiomes in adults and larvae of the same species, which highlights the role of seawater bacteria in the structural composition of sponge microbiomes (Fieth et al., 2016; Webster et al., 2010).

Differences in microbial diversity between High Microbial Abundance

(HMA) and Low Microbial Abundance (LMA) sponges have been reported (Hentschel et al., 2002; Moitinho-Silva et al., 2014). These differences have been related to contrasting structural and physiological traits of the two species groups (Blanquer et al., 2013). HMA sponges have a denser mesohyle and a more complex aquiferous system, with smaller choanocyte chambers, than LMA sponges (Blanquer et al., 2013). Also a partial trophic niche separation for HMA and LMA sponges has been proposed (Morganti et al., 2017). Comparisons of the core microbiomes of these two groups, which consisted in bacterial T-RFLPs that were present in all species replicates across seasons and study years, showed that HMA sponges had a larger number of core bacterial groups with a higher overlap with seawater than LMA hosts (Erwin et al., 2015).

In this study, we attempted to cast some light on the acquisition modes of microbial symbionts in sponges by i) unveiling the permanent microbiomes within a large number of sponge species, and ii) estimating the contribution of seawater bacteria to the sponge core microbiomes in representatives of both HMA and LMA sponges. To address these goals, we analysed the sponge microbiomes of the 19 most abundant sponge species inhabiting a small geographical area in Nha Trang Bay (Central Vietnam), as well as the bacterial assemblages of the surrounding seawater. We applied a restrictive approach to core community concept in terms of bacteria occurrence across species replicates and to OTU definition. Sequence identity thresholds <99 % for the 16S rRNA V4 region have been proved to be inaccurate for bacterial species delimitation (Edgar, 2017), in particular for short reads obtained from Next Generation Sequencing (NGS). Instead, a 100 % of sequence identity has been proposed to obtain true biologically informative sequences, which can underlie metabolic and ecological particularities (Edgar, 2017). To ensure that the 16S rRNA gene sequences recovered from the seawater samples were identical to those recovered from the sponges, we clustered OTUs (Operational Taxonomic Units) at 100 % identity (Zero radius OTUS or ZOTUs (Edgar, 2017)), which has only been recently applied in a couple of studies of sponge microbiomes (Glasl et al., 2018; Moitinho-Silva et al., 2017b). Clustering sequences at 100 % identity and restricting the core to microbes present in 100 % of the analysed samples seem particularly

relevant when trying to elucidate horizontal symbiont acquisition.

3.3 Results

3.3.1 Specificity of sponge bacterial communities

The main factor structuring the sponge microbiomes was the sponge species (Permanova: R^2 0.56, p-value <0.01), which means that replicates from the same species were more closely related to each other than to any other species. However, dispersion within replicates greatly varied depending on the sponge species (Permutest F: 6.9 p-value <0.01). A dichotomy (Permanova: R^2 0.11, p-value <0.01) between the so-called HMA and LMA sponges regarding their bacterial composition was detected (Figure B.2). Moreover, dispersion within the three HMA species (*Aaptos suberitoides*, *Neofibularia hartmani* and *Suberea cf. laboutei*) was much lower than the dispersion within the 16 LMA species (Permutest F: 60.8 p-value < 0.01, Figure B.2).

3.3.2 Core communities and species specific ZOTUs

The ZOTU richness of the core communities (ZOTUs present in all replicates of the same sponge species) varied from 54 in *Clathria reinwardti* to 600 in *Thrinacophora cf. raphidophora* (Table 3.1). The number of species replicates influences the number of ZOTUs forming the core community, the more replicates taken into account, the lowest the number of core ZOTUs (RS= -0.83, p-value < 0.01, Figure B.3A). However, ZOTU abundance of the sponge species core did not depend on the number of species replicates since no correlation was found between both variables (RS= -0.21, p-value > 0.05, Figure B.3B). This means that the abundant ZOTUs are the major contributors to the core of a species, and that the variable fraction is represented by the low abundance ZOTUs. Thus, we have considered the comparisons based on the relative abundances of the core ZOTUs.

The core community represented more than 75% of the reads from the total microbiome in most species (Figure 3.1). *N. hartmani* was the species with the largest core microbiome, representing up to 94% of relative

Table 3.1: Mean, core, species-specific, and SW ZOTUs of all species studied. Values are given in number and relative abundance (%) of ZOTUs. HMA species are marked with an ∗ (n= number of replicates per species). (a): Number and percentages of abundances of species-specific ZOTUs are calculated for the core community of each species (b): Number and percentages of abundances of SW ZOTUs are calculated for the core community of each species. Values correspond to the comparison with the SW core ZOTUs (cosmpolitan).

Species	n	Mean ZOTUs ± SD	Core ZOTUs	% Ab. Core ± SD	Sp-sp ZOTUs (a)	% Ab. Sp-sp ZOTUs	SW ZOTUs (b)	% Ab. SW ZOTUs
Aaptos suberitoides∗ (Brondsted, 1934)	13	740 ± 139	134	77.6 ± 6.2	6	1.1	53	48.79
Neofibularia hartmani∗ (Hooper and Lévi, 1993)	10	883 ± 182	167	94.2 ± 1.6	32	4.4	67	71.59
Suberea cf.laboutei∗ (Bergquist, 1995)	3	812 ± 133	206	79.2 ± 7.8	65	20.6	61	26.67
Amphimedon paraviridis (Fromont, 1993)	10	587 ± 223	57	61.9 ± 22.1	1	0.0	52	99.54
Antho sp.	3	1587 ± 872	404	88.2 ± 5.1	71	2.0	107	9.90
Callyspongia sp.	2	850 ± 507	229	83.9 ± 2	30	1.0	76	38.55
Clathria reinwardti (Vosmaer, 1880)	15	769.1 ± 171	54	88.4 ± 7.3	0	0.0	36	90.40
Clathria sp.	4	838 ± 420	144	69.6 ± 19.9	2	0.3	50	33.57
Dendroxea sp.	2	795 ± 118	310	77.7 ± 13.9	50	11.6	99	33.99
Dysidea sp.	3	1811 ± 902	453	84.6 ± 6.8	66	3.7	131	27.35
Gellioides cf.gracilis (Hentchel, 1912)	9	647 ± 123	138	82.9 ± 5.3	1	0.4	69	53.03
Gellioides sp.	4	456 ± 87	96	49.3 ± 27.5	8	25.7	83	93.22
Haliclona sp.	3	1343 ± 611	365	79.5 ± 9.7	44	3.1	123	16.06
H. (Gellius) toxotes (Hentchel, 1912	3	892 ± 327	249	74.3 ± 4.2	15	0.2	88	60.34
Monanchora unguiculata (Dendy, 1922)	3	622 ± 115	254	86 ± 7.8	12	0.3	93	58.71
Mycale sp.	4	1720 ± 1576	80	54 ± 7.3	2	0.7	42	77.51
Phorbas sp.	3	905 ± 349	214	86.6 ± 6.6	14	0.6	77	52.20
Pseudosuberites sp.	2	1791 ± 1205	579	86.4 ± 9.4	171	4.3	125	10.97
Thrinacophora rhaphidophora (Hentschel, 1912)	2	1392 ± 231	600	89.1 ± 5.2	195	9.6	169	22.67

abundance (Table 1) and *Gellioides* sp. had the smaller core microbiome representing $< 50\%$ of relative abundance. The number of species-specific ZOTUs (those present in all replicates of a species and absent from the core of any other study sponge species) varied from 0 (in *C. reinwardti*) to 195 (in *T. cf. rhaphidodophora*). However, the abundance of species–specific ZOTUs did not surpass 25% in any case, and most often were below 5% of relative abundance in the respective core communities. *Gellioides* sp. and *S. cf. laboutei* were the species with the highest relative abundances of species-specific ZOTUs (25% and 20%, respectively).

Similar values of core communities and species-specific bacteria were obtained for the dataset analysed with OTUs clustered at 97% sequence similarity (Table B.1).

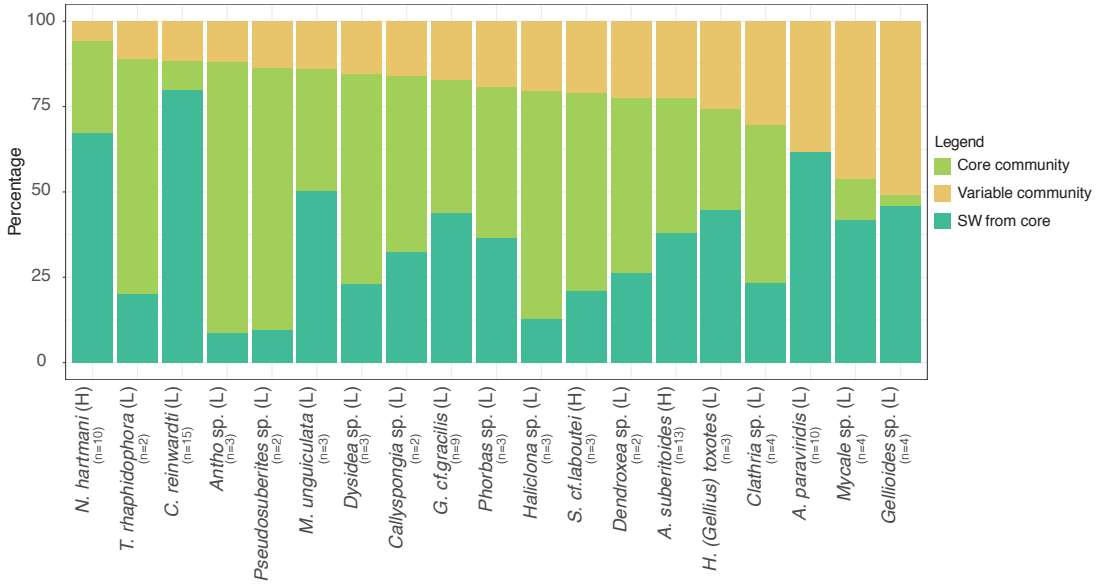


Figure 3.1: Mean relative abundances for the core (green) and the variable fraction (yellow) of the sponge microbiomes. Blue lines delimit the percentage of the sponge bacterial core shared with the seawater bacterial community. H= High Microbial Abundance sponge, L= Low Microbial Abundance sponge.

3.3.3 Composition of the bacterial core communities

Specific associations between certain bacteria (phylum and class level) and HMA or LMA species were detected with the Indval analysis (Figure B.4). These indicator bacteria are present in the core communities of the studied sponges (Figure 3.2). The three HMA species presented an alike core microbial composition and abundances at phylum level with specific bacterial phyla overrepresented, compared to LMA species and seawater (SW). Phyla associated with LMA sponges also showed a similar core microbial composition but with contrasting abundances in the several species. Seawater samples had their own core bacterial composition with representatives of bacterial phyla shared with either HMA or LMA sponges. Mean Shannon diversity indices of the species core communities were significantly higher (*Kruskal-Wallis* <0.01) for HMA species (3.8 $\pm$ 0.24) than for LMA species (2.3 $\pm$ 0.61) (Figure B.5).

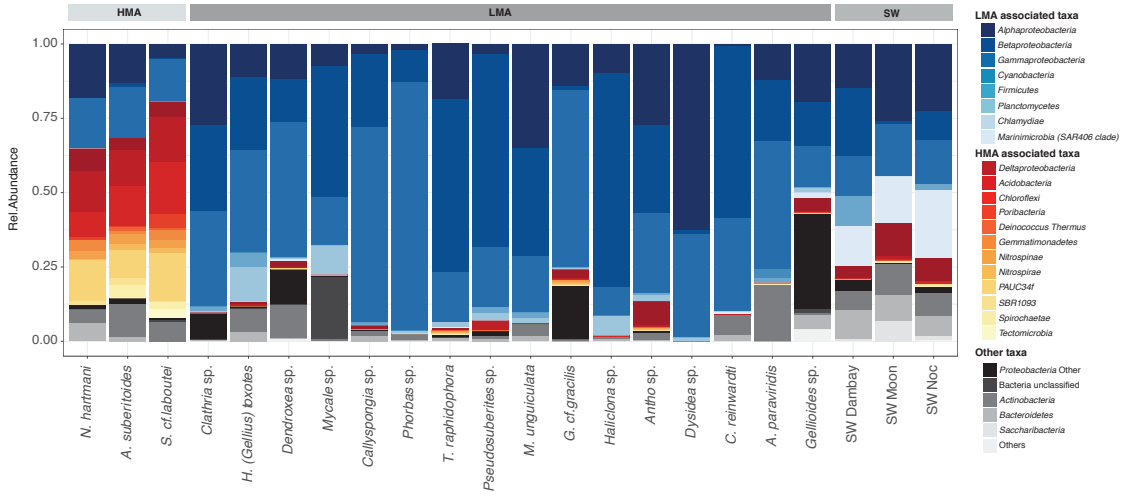


Figure 3.2: Mean relative abundance of core microbial taxa at a phylum level (class for *Proteobacteria*) within each sponge species and seawater samples. Bacterial taxa with significant Indval values (Supplementary Fig S3) associated to HMA species are marked in reddish colours and the ones associated to LMA species are marked in bluish colours.

3.3.4 Seawater (SW) ZOTUs

The relative abundances of the shared core SW ZOTUs (detailed in the Experimental procedures section) with the sponge core microbiomes varied between 9% and 99% depending on the sponge species (Figure 3.3). The sponges with the highest contribution of core SW ZOTUs to their core microbiome were A. paraviridis (99.5%), *Gellioides* sp. (93.2%) and *C. reinwardti* (90.4%), while *Antho* sp. (9.9%) and *Pseudosuberites* sp. (10.9%) showed the lowest overlap between the sponge and the SW core bacteria (Figure 3.3a, Table 1). In some species (i.e.: *A. suberitoides*, *C. reinwardti*, *Mycale sp.*), rare SW ZOTUs (> 0.01 % of relative abundance) were abundant in the core of the sponge species. This is particularly visible in the example of *C. reinwardti*, which harboured ZOTUs that represented just a 5% of the SW core community and 90% of the sponge core. The opposite occurred in other sponge species such as *Pseudosuberites* sp. and *T. cf. rhaphidophora*, which had highly abundant (> 1% of relative abundance) SW ZOTUs poorly represented in their core.

In addition, when the most abundant SW ZOTUs were considered (abundances higher than 0.01%), instead of the SW core ZOTUs, the relative abundances of bacteria shared with SW were drastically reduced in most of

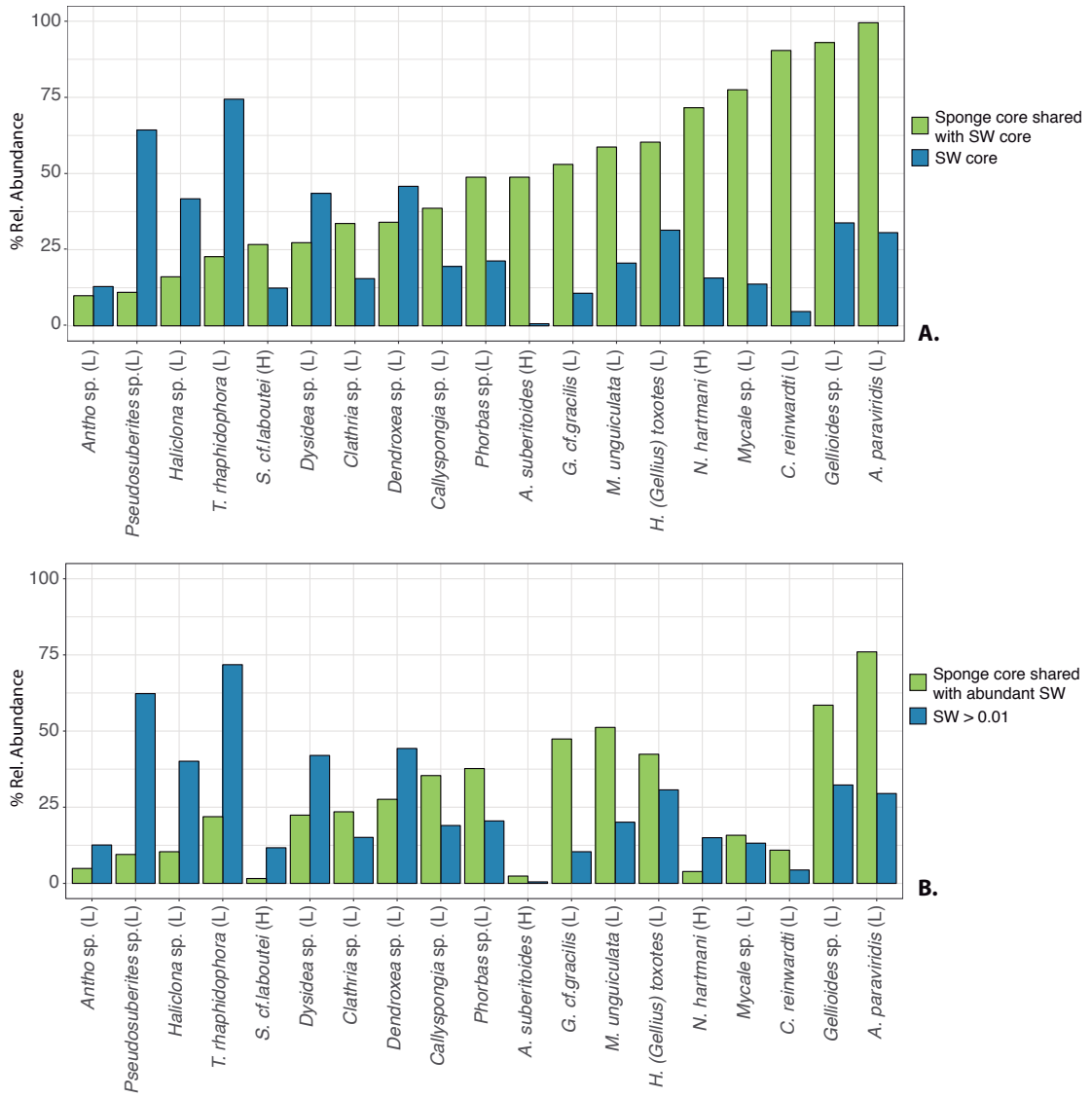


Figure 3.3: Mean relative abundances of shared bacteria between the SW and the core microbiomes for each sponge species when comparisons were made with the A) SW core (cosmopolitan bacteria) and B) the abundant (>0.01%) SW bacteria. Bars represent percentages of relative abundances of the shared bacteria in both, the sponge (green colour) and the SW (blue colour). H= High Microbial Abundance sponge, L= Low Microbial Abundance sponge.

sponge core microbiomes, with most values below 40% (Figure 3.3b). This reduction was notably relevant in some species, such as *Gellioides* sp. *N. hartmani*, *Mycale* sp. *A. suberitioides*, *C. reinwardti*, and *S. cf. labutei* (Figure 3.4).

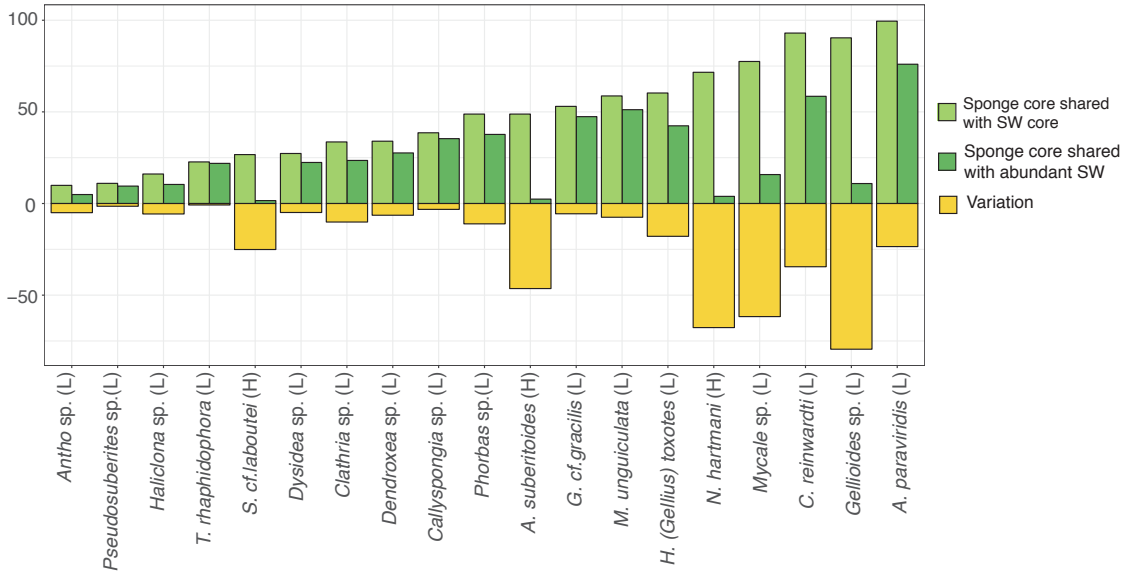


Figure 3.4: Variation in relative abundance of SW ZOTUs in the sponge cores according to the method used for comparisons. Light green bars show ZOTUs shared with the SW core. Dark green bars represent ZOTUs shared with the abundant SW ZOTUs. Negative bars (yellow) represent the differences in shared ZOTUS between methodologies. H= High Microbial Abundance sponge, L= Low Microbial Abundance sponge.

Results of both comparisons for OTUs at 97% sequence identity are shown in Table B.2. Differences between OTUs and ZOTUs were more remarkable when considering the abundant SW ZOTUs. For instance, in the case of *C. reinwardti*, the proportion of SW OTUs changed from 10% (for ZOTUs) to 73% (for OTUs). Also remarkable were the differences for *A. paraviridis*, *Gellioides* sp., and *Phorbas sp.*

The abundance and distribution of the SW core ZOTUs in the sponge core microbiomes is shown as a heatmap representation (Figure 3.5). Two sponge clusters (A and B) were differentiated in the dendrogram. Cluster A contained sponge species that shared two highly abundant SW ZOTUs (ZOTU1, ZOTU2 at mean abundances of $\sim$ 10%), and some ZOTUs (10, 37) at abundances higher than 1%. Cluster B comprised species harbouring different SW bacteria at contrasting abundances. Two different ZOTUs of

Candidatus Branchiomonas (*Betaproteobacteria*) accounted for more than 30% and 50% of the *Mycale* sp. and *A. paraviridis* core microbiomes, respectively. Similarly, ZOTUs belonging to several *Endozoicomonas* (*Gammaproteobacteria*) were present at different relative abundances in the four species with the highest proportion of SW ZOTUs (*Gellioides* sp, *A. paraviridis*, *Mycale* sp. and *C. reinwardti*). Two main groups (C, D) were also differentiated according to the bacterial classes. The first group (C) corresponded to bacterial classes associated to LMA sponges, with high abundances of *Alpha-*, *Beta-*, and *Gamma- Proteobacteria*. The second group (D) showed bacterial classes associated to HMA sponges. ZOTUs belonging to PAUC34f, *Chloroflexi*, *Acidobacteria*, *Actinobacteria*, and *Nitrospinae* were almost exclusively found in the three HMA species (*N. hartmani*, *S. cf. laboutei* and *A. suberitioides*).

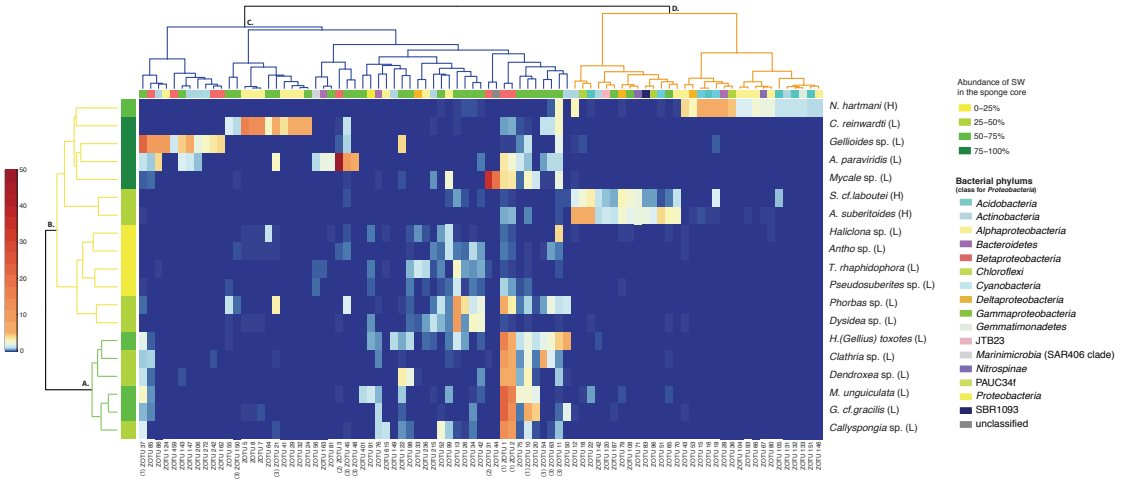


Figure 3.5: Variation in relative abundance of SW ZOTUs in the sponge cores according to the method used for comparisons. Light green bars show ZOTUs shared with the SW core. Dark green bars represent ZOTUs shared with the abundant SW ZOTUs. Negative bars (yellow) represent the differences in shared ZOTUS between methodologies. H= High Microbial Abundance sponge, L= Low Microbial Abundance sponge.

3.4 Discussion

The sponge-associated bacteria were species specific for the 19 study species, as previously reported for sponges from other geographical areas (Blanquer et al., 2013; De Mares et al., 2017; Easson and Thacker, 2014; Lee et al.,

2011; Reveillaud et al., 2014; Schmitt et al., 2012; Webster et al., 2010). Selection of specific bacteria and competition among the selected bacteria (Easson and Thacker, 2014) may converge to shape the species-specific patterns of sponge microbiomes, which used to be similar in geographically distant individuals of the same genus (Montalvo and Hill, 2011).

However, differentiated patterns of bacterial composition are observed according to the affiliation to the HMA or LMA groups, as previously reported (Giles et al., 2013; Schmitt et al., 2011). Indicator bacteria at class level for both groups are found, which match the indicator classes inferred from differential abundance analysis (Moitinho-Silva et al., 2017b). It has been reported that HMA sponges show higher microbiome similarity among species than LMA sponges (Erwin et al., 2015). Moreover, bacterial diversity is also claimed to be higher in HMA (Erwin et al., 2015; Gerçe et al., 2011; Hentschel et al., 2002; Schmitt et al., 2008; Taylor et al., 2007). These aspects are confirmed in our study when considering the core members of both HMA and LMA sponges, although the number of representatives of each group is unbalanced.

It has been assumed (Gerçe et al., 2011; Weisz et al., 2007), but also questioned (Blanquer et al., 2013; Moitinho-Silva et al., 2014), that LMA sponges contain mainly transient seawater bacteria. Representatives of both HMA and LMA study sponges contained high percentages of SW core bacteria (>50% of core relative abundance). However, species of each group acquire different SW bacteria that are indicators of either HMA or LMA sponges (Figure 3.2 and Figure B.4), suggesting contrasting bacteria selection mechanisms in each group. Differences might also be enhanced by particular traits of the respective physiology of the two sponge types (Ribes et al., 2012).

To define species core and species-specific bacteria, we clustered sequences at 100% identity (ZOTUs). Only recently, the sponge microbiomes have been analysed at the ZOTU level (Glasl et al., 2018; Moitinho-Silva et al., 2017b). By recording ZOTUs, we were able to identify closely related bacteria (Edgar, 2017), which may inhabit sponge and seawater biomes making comparisons more reliable. Analysing the bacterial core at species level provides information about stable, purportedly fixed associations, which

may be involved in sponge-bacteria interaction patterns. To define the species core, an appropriate percentage of bacteria occurrence across species replicates has to be selected depending on the study aims (Astudillo-García et al., 2017). For example, 85% occurrence was used for a species with more than 47 replicates to conduct an interaction network analysis (Thomas et al., 2016). In our study, as we wanted to focus on the truly symbiotic bacterial community of each sponge species, we choose a restrictive approach to the species core by only considering bacteria that were present in all the replicates of each species (100% occurrence), disregarding whether they were present among the SW core or not. In this way we ensured that we were focusing on persistent symbionts rather than on transient microbes.

The size of the bacterial core, which represents the permanent part of the sponge microbiome, seems to be intrinsic of each sponge species (Bjork et al., 2018). A high stability of the microbiome across sponge replicates can be indicative of the strength of the sponge-bacteria associations, whereas the opposite would indicate the presence of facultative /transient bacteria (Thomas et al., 2016). Most of the associations among bacteria and the studied species appeared to be highly constant, thus, suggesting a strongly fixed relationship, although we are aware that this might depend on the number of replicates analysed.

The contribution of the species-specific ZOTUs to the core communities, in terms of relative abundance, was surprisingly low. Those values may be influenced by the number of sponge species taken into account in the study, the similarity between their microbiomes, and, more strongly, by the restrictions associated to the way species-specific OTUs are defined, whether being part of the species core community (our study) or not (Reveillaud et al., 2014; Schmitt et al., 2012). Schmitt et al. (2012) suggested that the species-specific bacteria would probably be vertically inherited. If this assumption is true, the percentage of vertically inherited symbionts in our sponges would be rather low, since we have found a few species-specific microbes and the majority of the sponge bacterial taxa are found in more than one species.

Both, sponge microbiomes and seawater communities are in close contact because of the filtering activity of sponges, which can result in occasional

bacteria transfer from one source to another (Moitinho-Silva et al., 2014). To avoid this potential contamination, we used the 100% occurrence core approach in both the sponges and seawater, because it is unlikely that a ZOTU contaminates all the replicates of the same source. Moreover, we took into account the differential abundances that a ZOTU can be present in both, the sponge and the seawater. In this way, to be conservative, we could only suspect of SW bacteria contamination in the cases in which abundant SW bacteria are in low abundance in a sponge for which only few replicates are available. These cases would merit further investigation.

Our results comparing the bacterial core of sponges and seawater showed that all the studied sponges contained SW bacteria, as previously reported for other sponge species (Moitinho-Silva et al., 2014; Reveillaud et al., 2014). However, the relative abundance of SW bacteria in the sponge microbiomes was species dependent, ranging from almost 100% in *A .paraviridis* to less than 10% in *Antho* sp.

The quantification of the relative abundance of SW bacteria in the sponge microbiomes is particularly relevant to address seasonal or geographical changes and species specific traits (Moitinho-Silva et al., 2014), but also allows inference about bacterial transmission modes. Thus, we estimated the relative abundance of the SW- sponge shared bacteria in each biome core trying to differentiate SW microbes that may represent stable symbionts from transient contaminant bacteria. We postulated that enrichment of seawater bacteria in the sponge occurred when low abundance SW core bacteria were found as the main components of the sponge microbiomes (Figure 3.3). This can be proposed for 11 out of 19 sponge species analysed and in particular, for *C. reinwardti*, *A. paraviridis*, *Mycale* sp., *Gellioides* sp. *N. hartmani*, and *A. suberitoides*. No pattern related to the HMA and LMA dichotomy could be withdrawn here, as representatives of both groups showed enrichment of seawater bacteria in their microbiomes. The proportion of the sponge core bacteria shared with SW is reduced drastically in many species when comparisons are performed with the abundant SW bacteria, instead of with the SW core bacteria. Among these species, the three HMA species (*N. hartmani*, *A. suberitioides*, and *S. cf. laboutei*), reduced their relative abundance of SW ZOTUs from 50%, 70% and 25%,

respectively, to values below 5% (see Figure 3.4). Reduction occurs in sponges that harbour low-abundance SW bacteria that would be ignored when only abundant SW bacteria are used for comparisons. Therefore, the study HMA sponges contain in their microbiomes SW bacteria that are at low-abundance in the water. For species that harbour both abundant and SW core bacteria in their microbiomes, similar percentages of SW bacteria were obtained with both methodologies. With this comparison, we like to point out how different approaches may influence the results on the overlap between the sponge and SW microbiomes. In particular, we emphasize the importance of the approach used for studies aiming to elucidate the dichotomy between HMA and LMA sponges.

We considered relevant the contribution of SW bacteria to the formation of the sponge microbiome when they were present across all species replicates. In contrast, some authors (Thomas et al., 2016) considered that abundant (>0.01%) SW OTUs likely represent environmental contaminants and should be removed from the sponge samples, independently of their abundance in the sponge. Conversely, we propose, that only highly abundant SW bacteria that are rare in the sponge-species core are potential candidates to represent SW bacteria contamination, especially when only few replicates are available. Two sponge species from our dataset (e.g. *Pseudosuberites* sp. and *T. raphidophora*) are examples of possible contamination.

Overall, we consider the comparisons using the SW core bacteria as a more accurate way to assess the true sponge-SW shared bacteria, as it considers bacteria that would be available across locations to be incorporated in the sponge microbiome. Our results support that "sponge-specific" bacteria are rather "sponge-enriched" bacterial clusters, and that seawater acts as a seed bank for sponge microbiomes, as suggested by Webster et al. (2010),Webster and Thomas (2016) and Moitinho-Silva et al. (2014). We detected widespread but rare (Taylor et al., 2013) SW bacteria forming part of sponge core microbiomes. Whether these taxa are metabolically active in the water column or represent dormant stages that reactivate after being incorporate to the sponge (Moitinho-Silva et al., 2014) remains to be elucidated.

We find a high specificity of the associations between sponges and

seawater bacteria. Each sponge species seems to incorporate different bacteria from the seawater in its microbiome. This suggests that some species-specific mechanisms have been fixed in the sponges to select some seawater bacteria and not others. Recognition mechanisms have been proposed to explain horizontal acquisition of microbes from the surrounding environment (Fieth et al., 2016). Taking into account the high percentages of seawater bacteria detected in some of our sponge species and the species-specificity of many of them, we propose that environmental acquisition would play a major role in the establishment of species-specific sponge microbiomes.

To summarize, sponge species is the main factor structuring microbiomes of the most common sponges from Nha Trang bay (Vietnam). By using a very restrictive approach of the "core species" concept and ZOTUs with 100% sequence identity for defining bacterial species, we proved that intra-species microbiome stability is the rule for most sponges. A high percentage of SW bacteria shaped the core microbiome in many study species. Our results point to horizontal transmission, as an ubiquitous mechanism of symbiont acquisition in sponges, while vertical transmission would represent a rather complementary acquisition way. Apparently, some highly specific recognition mechanisms may be acting in sponges to specifically enrich some SW bacteria in their tissues, and not others. Moreover, contrasting preferences in bacteria selection may account for differences in the microbiomes of HMA and LMA sponges and some physiological traits such as contrasting filtration rates might also contribute to enhance the differences. These mechanisms would allow the maintenance of stable bacterial communities disregarding environment conditions and geographical distance and merits to be confirmed by analysing in the same way as in the current study a larger number of sponge and water samples from different geographical regions.

3.5 Experimental procedures

3.5.1 Sponge and seawater sampling and DNA extraction

Sponge samples were collected in April 2015 by SCUBA diving along 13 transects, 25 m long each, randomly placed between 3 and 9 m deep in

three neighbouring locations ∼ 2 km apart (i.e.; Dambay, Hun Mun and Nock Island) within Nha Trang Bay (central Vietnam). This quantitative sampling method allowed us to detect the most representative sponges in the study area but not to collect the same number of replicates for all the species. For instance, only three HMA sponges were found in the whole sampling but two of them were present at high abundances. Overall, we collected 203 sponge samples, from which we only considered for this study the ones that were found at least twice. Thereby, 98 samples belonging to 19 sponge species with between 2 and 15 replicates each (Table 3.1) were analysed.

Each sponge individual fitting within a transect was photographed and a piece of ca. 3 cm^3 was collected in a 50 mL Falcon tube in seawater. Seawater was immediately replaced by 100 % ethanol once on board. Back in the lab, the ethanol was replaced twice again with fresh absolute ethanol for a good sample preservation. DNA from those sponges was extracted following the protocol of DNeasy Blood & Tissue Kit (Qiagen).

Triplicate plankton samples were taken from the three sampling locations where the ecological transects were performed (i.e.; Dambay, Hun Mun and Nock Island). Two litres of water were collected and sequentially filtered throughout 5 μm, to remove undesired plankton components, and then throughout 0.22 μm polycarbonate membranes. The size fraction between 5 and 0.22 μm was processed for DNA extraction. Membranes were enzymatically digested with lysozyme, proteinase K and sodium dodecylsulfate and afterwards, DNA was extracted with phenol:chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) and chloroform:isoamyl alchohol (24:1, vol/vol). Purification and concentration of the DNA was carried out with Amicon® Ultra 4 Centrifugal Filter Units – 100000 NMWL (Millipore). The extraction procedures used for sponge samples and SW were the most appropriated according to their respective preservation.

3.5.2 Sponge identification

We identified sponge species to the best possible taxonomic resolution by molecular markers and morphological features. Fragments of the nuclear genes encoding the 18S rRNA (∼1700 bp) and 28S rRNA (∼650 bp), as well

as the cytochrome c oxidase subunit I (COI ∼680 bp) were amplified and sequenced. Primers 1F and 1795R (Medlin et al., 1988) were employed to amplify 18S rRNA, Por28S-830F and Por28S-1520R (Morrow et al., 2012) primers were used to amplify the D3-D5 partition of the 28S rRNA and LCO1490 and HCO2198 (Folmer et al., 1994) were used for COI. PCR amplifications were conducted in 50 μl reactions containing 1 ng of template genomic DNA, 5 μl of 10x PCR buffer (containing 1.5 mM $MgCl_2$), 2 μl of dNTP mix (10 mM), 2 μl of bovine serum albumin, 1 μl pf each primer (10 mM) and 0.4 μl of Taq DNA polymerase (5 U μl-1). The temperature profile for the 18S rRNA was as follows: 94°C/5 min; (94°C/1 min, 50°C/1 min, 72°C/1 min) x 35 cycles; 72°C/5 min; for 28S rRNA: 94°C/5 min; (94°C/30 s, 53°C/30 s, 72°C/30 s) x 30 cycles; 72°C/5 min; and for COI: 94°C/2min; (94°C/1 min, 45°C/1 min, 72°C/1 min) x 35 cycles; 72°C/7 min. Purification and sequencing were carried out by an external service (Macrogen, Netherlands). The obtained sequences were manually edited in Geneious v 9.0.2 and blasted against NCBI databaste (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm the morphological identification of the sponges at the lowest taxonomic level possible.

Preparation of spicules and histological sections were made from specimens' subsamples and observed under both, light and scanning electron microscopes. Morphological characters such as spicule types, shape, length, and width, as well as skeletal arrangement (Hooper and Van Soest, 2002) were used in combination with individual sequences and phylogenetic reconstructions to obtain, the most accurately possible, taxonomic identifications. Sponges were classified as HMA or LMA on the basis of their pertinence to genera already known to belong to any of these two groups, what was additionally confirmed by looking to the characteristics and structure of the sponge aquiferous system: small, relatively few (HMA) vs. large abundant choanocyte chambers (LMA), and mesohyle (dense vs. lax mesohyle, respectively) (Blanquer et al., 2013).

3.5.3 16S rRNA gene amplification, sequencing and analysing

PCR and high-speed multiplexed SSU rRNA gene Illumina MiSeq sequencing (NGS) were carried out following the genomic core facilities and methods of the MrDNA Lab (Texas, USA) (http://www.mrdnalab.com/). The variable V4 region of the 16S rRNA gene (c.a. 250 nt) was amplified using the primers 564F (5'AYTGGGYDTAAAGNG3') and 785R (5'TACNVGGGTATCTAATCC3) (Klindworth et al., 2013). Raw rRNA gene sequences were processed using the UPARSE pipeline (Edgar, 2013). A quality check and de-replication were applied to our dataset. Denoising (error-correction) of amplicons was performed to identify all correct biological sequences following the UNOISE pipeline (Edgar, 2016). This algorithm removed chimeras, reads with sequencing errors, PhiX, and low complexity sequences due to Illumina artifacts, and generates ZOTUs ("zero-radius" OTUs) consisting of sequences of 100% identity. For comparison purposes, sequences were also clustered at 97% threshold (Supplementary information). For this analysis, reads were dereplicated and clustered into operational taxonomic units (OTUs) at cut-off 0.03% identity after chimera removal (UCHIME) and excluding the singletons.

Taxonomic assignment was done with SINA v1.2.11 (Pruesse et al., 2012) using SILVA 128 database. SINA uses Lowest Common Ancestor method (LCA). We configurated a "Min identity" of 0.7 and a maximum number of search results of 1 per sequence results in "best match" type. Sequences with low alignment quality (<75%) and sequences identified as mitochondria or chloroplasts were removed from the analysis. In order to minimize biased effects for differences in sampling effort, the original ZOTU table was rarefied (Figure B.1) at a minimum reads threshold of 41000 (Caporaso et al., 2010).

Raw sequences are available in the SRA archive under the project number PRJNA453898.

3.5.4 Defining core and species-specific ZOTUs

We identified the ZOTUs that were present in all replicates to define the core microbiome of each sponge species. The ZOTUs that did not meet this

requirement were assigned to the variable community. Moreover, we considered species-specific ZOTUs those belonging to a single core microbiome for a particular sponge species, compared to the remaining collected sponges.

3.5.5 Seawater (SW) ZOTUs

We combined two approaches to estimate the real contribution of the seawater (SW) bacteria to the sponge core microbiomes. First, we looked for bacteria in each sponge species that were already present in the core SW community. We considered the core community of the SW as the community formed by the ZOTUs present in all water replicates. With this approach, we attempted to identify bacteria that commonly inhabited in the SW and that its presence was not merely circumstantial. Therefore, they could represent a potential source for the formation of the sponge microbiome over time. In the second approach, we made the comparison with the most abundant bacteria of the SW. We identified the ZOTUs with relative abundances higher than 0.01% in average across all water samples. This threshold was chosen since it has been used previously to remove from the sponge microbiome samples the OTUs that were likely to represent environmental contaminants (Thomas et al., 2016). With this approach, we aimed to gain insight on the SW bacteria that are more likely detected in the sponge microbiomes just because of their high abundance in SW and may represent transient (environmental) contaminants (Thomas et al., 2016). On the other hand, we considered rare ZOTUs those with a relative abundance $<0.01\%$ and highly abundant ZOTUs those with relative abundance $>1\%$ (Reveillaud et al., 2014).

3.5.6 Statistical analyses

We carried out a distance-based multivariate analysis at ZOTU level of the microbial communities of the sponges and seawater samples using the vegan package (Oksanen et al., 2018) in R. A cluster dendrogram was built using the Bray-Curtis dissimilarity distance matrix of samples to visualize patterns of bacterial community structure in sponges and seawater. We tested the effect of host identity (species) as well as the effect of the HMA/LMA

identity, on the structure of microbial communities with non-parametric Permutational Analysis of Variance (PERMANOVA). PERMUTEST was applied to detect differences in the dispersion between groups. A bias correction (Stier et al., 2013) for the unequal sample size of HMA and LMA groups was applied in the betadisper function of the vegan package (Oksanen et al., 2018). P-values of PERMANOVA and PERMUTEST were calculated using 999 permutations and significance cut-off for p-values was 0.05. The mean relative abundance of the bacterial phyla and classes was calculated for both, HMA and LMA groups. We applied an IndVal analysis using the labdsv package (Roberts and Roberts, 2016) in R to detect potential associations of certain bacterial phyla and classes to any of these two groups. We fixed an IndVal threshold of 0.6 (p-value < 0.01) to consider a bacterial taxon strongly associated to (or Indicator of) HMA or LMA sponges.

3.6 Acknowledgements

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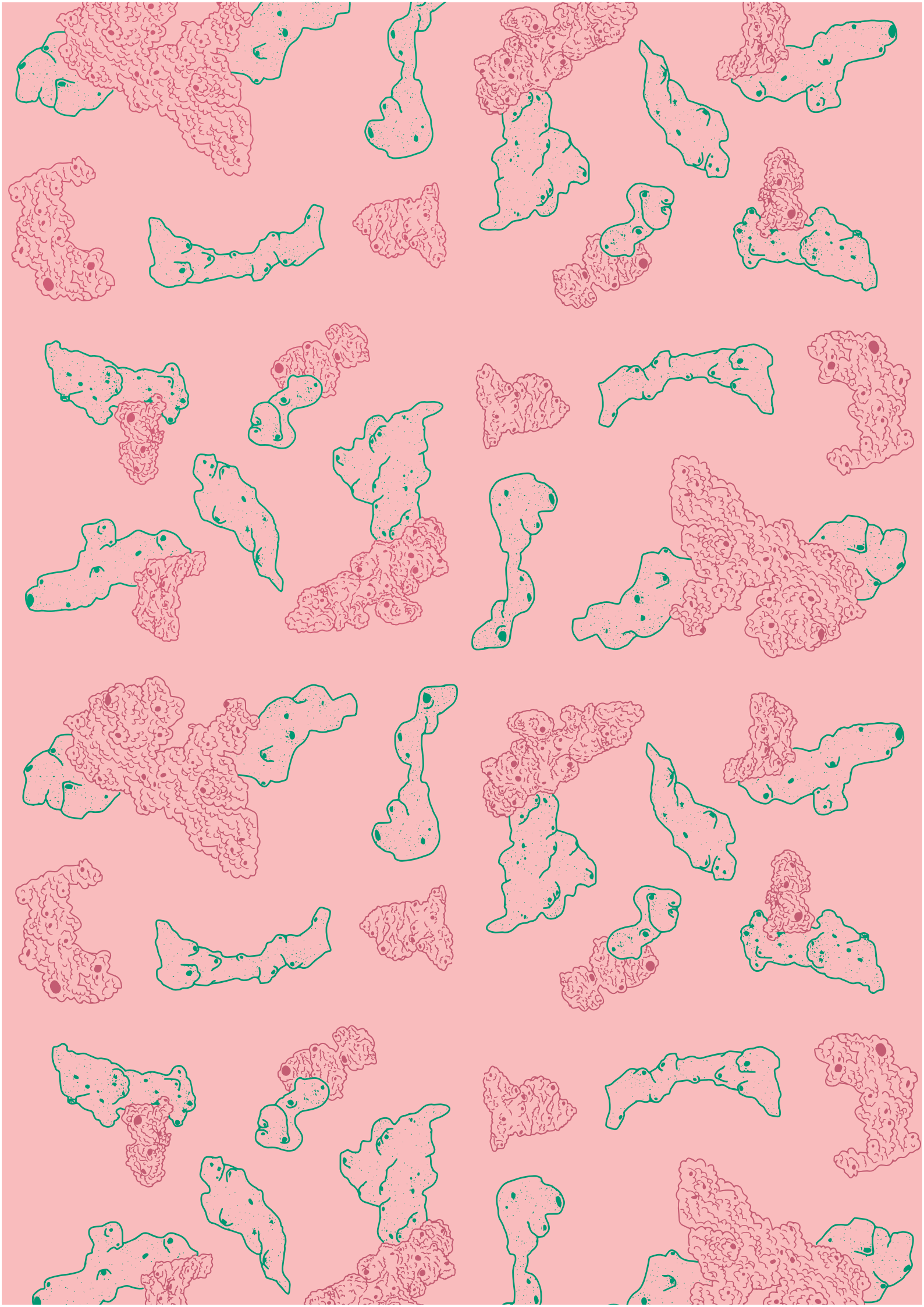

Turon, M. Cáliz, J., Triadó-Margarit, X., Casamayor, E.O., Uriz, J.M. (2019) Sponges and their microbiomes show similar community metrics across impacted and well-preserved reefs. *Fron. Microbiol.* 10:1961. doi: 10.3389/fmicb.2019.01961

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La publicació d'aquest capítol es troba en l'Annex de la tesis *Published chapters*. Els apartats i distribució del capítol estan presentats en l'ordre que estableix l'editorial de la revista. Únicament s'ha editat la forma i mida de lletra per unificar el format de tesis. La bibliografia del capítol es troba en l'apartat *Bibliography*.

Imatge capítol: Dibuix de les esponges dominants en els ambients impactats de la zona de mostreig.

Autora coberta: Laura López

Chapter 4

Sponges and their microbiomes show similar community metrics across impacted and well-preserved reefs

4.1 Abstract

Sponge diversity has been reported to decrease from well-preserved to polluted environments, but whether diversity and intra-species variation of their associated microbiomes also change as function of environmental quality remains unknown. Our study aimed to assess whether microbiome composition and structure are related to the proliferation of some sponges and not others under degraded conditions. We characterized the most frequent sponges and their associated bacteria in two close areas (impacted and well-preserved) of Nha Trang Bay (Indo-Pacific). Sponge assemblages were richer and more diverse in the well-preserved reefs, but more abundant (individuals/ m. transect) in the impacted environments, where two species (*Clathria reinwardti* and *Amphimedon paraviridis*) dominated. Sponge microbiomes from the polluted zones had, in general, lower bacterial diversity and core size and consequently, higher intra-species dispersion than microbiomes of sponges from the well-preserved environments. Microbial communities reflect the reduction of diversity and richness shown by their host sponges. In this sense, sponges with less complex and more variable microbiomes proliferate under degraded environmental conditions, following the ecological paradigm that negatively correlates community diversity and environmental degradation. Thereby, the diversity and structure of sponge microbiomes might indirectly determine the presence and proliferation of sponge species in certain habitats.

4.2 Introduction

Sponges are key invertebrates in marine benthic ecosystems where they play essential functions in many ecological processes. Besides increasing benthic diversity by supplying ecological niches to other organisms, they contribute to benthic–pelagic coupling by exchanging particulate and dissolved organic matter with the water column (Morganti et al., 2017; Richter et al., 2001). Sponges also participate in marine biogeochemical fluxes and some species may also show detoxifying potential by transforming noxious products present in polluted waters through the interplay of symbiotic bacteria (Loredana et al., 2017).

Microbes have been intimate partners of sponges since the Pre-Cambrian (Wilkinson, 1984). They can represent up to ca. 50% in volume of the microbial-sponge holobiont in some sponge species (Uriz et al., 2012) and are taxonomically and metabolically diverse in most cases (Thomas et al., 2016; Weisz et al., 2007). Thus, it is hard to envisage the causes of sponge success or failure without considering its accompanying bacterial microbiome. Indeed, unbalancing of their microbial symbioses has been considered to trigger extensive mass mortalities of sponges in the Mediterranean (Cebrian et al., 2011; Webster et al., 2008), and Red Sea (Gao et al., 2014). In addition, some purported benefits that sponges may obtain from their associations with microbes have also been proposed such as, antifungal activity, production of bioactive compounds against predation, roles in nitrogen and carbon cycle and vitamin biosynthesis (Freeman and Thacker, 2011; Freeman et al., 2013; Hentschel et al., 2012; Schmidt et al., 2000), but rarely have been experimentally demonstrated (de Voogd et al., 2015; Garate et al., 2015).

In contrast to the spatial and temporal variations reported for microbial communities in seawater (Glasl et al., 2017; Zeglin, 2015), the structure of the sponge microbiome does not vary substantially in the same species along geographical and bathymetrical ranges or over temperature, eutrophication or irradiance shifts (Erwin et al., 2012; Hentschel et al., 2002; Luter et al., 2014; Pita et al., 2013a,b; Strand et al., 2017). Although several experimental studies reported microbiome shifts under strong environmental changes in some host species (Fan et al., 2013; Glasl et al., 2018; Lesser et al., 2016;

Mohamed et al., 2008; Pineda et al., 2017; Ramsby et al., 2018; Webster and Thomas, 2016; Weigel and Erwin, 2017) they only inform us about short term changes, which might reflect temporal responses to environmental stresses.

Sponges usually show a high species diversity in well-preserved ecosystems (Van Soest et al., 2012), which has been suggested to decrease under anthropogenic pressures (Easson et al., 2015). Shifts in nutrient cycling and ecosystem functioning, which occur in degraded reef systems (Bell et al., 2013, 2018; Easson et al., 2015), are considered to be responsible for decreases in sponge biodiversity. However, a few, likely oportunistic, sponge species have been reported to inhabit and even dominate degraded coral reefs (Maliao et al., 2008). How these sponges cope with the potentially noxious products of polluted waters and whether particular sponge-associated bacteria are involved or not in their ecological success are challenging issues poorly understood (Douglas, 2014). Indeed, little is known about the role (if any) that symbiotic bacteria may play in shaping the ecological distribution of sponge species and whether the decrease of sponge diversity observed in perturbed assemblages also involves a lower diversity of their associated bacterial communities.

In this study, we explored whether microbiomes of sponges inhabiting degraded environments show differential characteristics, in such a way that they might influence the sponge distribution and proliferation in those adverse conditions. With this goal, we characterized the most frequent sponges and their associated bacteria in two close areas of Nha Trang Bay (Indo-Pacific) subjected to contrasting environmental conditions: well-preserved and impacted coral systems. In particular, we looked for sponge-associated bacteria in the few sponge species able to proliferate in the polluted zones.

Nha Trang bay is located in central Vietnam and harbors one of the highest coral diversities in the area (Latypov, 2011). Unfortunately, the health of the local ecosystems is being threatened by an increase of human activities leading to an alarming degradation of the bay in some zones (Latypov, 2015). The islands located closer to the city and the port of Nha Trang show a higher degree of anthropogenic impact, with higher

sedimentation fluxes and lower water transparency compared to the ones located farther from the coast line (Latypov, 2006; Tkachenko et al., 2016). Moreover, Vietnam has been ranked the world's third largest producer of farmed food (Nguyen et al., 2016) and certain areas of Nha Trang bay are highly impacted by mariculture activities, which also produce eutrophication and release of xenobiotics to the water (Nguyen et al., 2016). Tkachenko et al. (2016) reported the concentration of nutrients in areas close to our sampling sites. The nutrient values were 2.63 μM $\pm$ 0.21 for dissolved inorganic nitrogen and 0.29 μM $\pm$ 0.11 for phosphorus at the impacted sites (culture cages) and 2.41 μM $\pm$ 0.2 and 0.28 μM $\pm$ 0.08 at the unperturbed sites. As a result, the native Acropora coral assemblages have been replaced by the more resistant to silting Millepora communities in these perturbed areas (Latypov, 2015; Tkachenko et al., 2016). Conversely, several outer areas of Nha Trang Bay, such as Hun Mun, receive a low anthropogenic impact and still present well-developed Acropora communities with high coral coverage (ca. 60-70%) (Tkachenko et al., 2016).

4.3 Materials and Methods

4.3.1 Sample collection and DNA extraction

Quantitative sponge sampling was approached by SCUBA diving by randomly placing a total of 13, 25 m-long transect lines between 3-9 m depth along both well-preserved and impacted areas of Nha Trang Bay (Figure C.1). This quantitative method has been traditionally used for biodiversity studies in coral reefs and other structurally complex habitats (Loya, 1978) and provides a good approach on the abundance (density or coverage) of the non cryptic fraction of the reef benthos. Specimens that were crossed by the metric tape along the line transect were sampled. The well-preserved areas were coral reefs and rocky shores of the eastern part of Hun Mun Island and the southern part of Hun Tre Island, considered to be the most well preserved areas of the bay (Tkachenko et al., 2016). The impacted targeted zones were 2 km apart from the well-preserved areas, next to Dambay region, which harbors an intensive mariculture system (lobster caging) that causes chronic eutrophication in the area (Tkachenko et al.,

2016). Overall, the quantitative sampling provided 203 sponge samples, from which 71 individuals were from the impacted areas and 132 individuals from the well-preserved environments.

Each sponge individual fitting within a line transect was photographed and a piece of ca. 3 cm^3 (whenever possible) was collected in a 50mL Falcon tube with seawater. Seawater was immediately replaced by 100% ethanol once on board. Back in the lab, the ethanol was replaced twice with fresh absolute ethanol again for a good sample preservation. DNA was extracted following the protocol of DNeasy Blood and Tissue Kit (Qiagen). Triplicate plankton samples were taken from the three sampling locations where the ecological transects were performed (i.e.; Dambay, Hun Mun and Nock Island). Two litres of water were collected and sequentially filtered throughout 5-μm, to remove undesired plankton components, and then throughout 0.22 μm polycarbonate membranes. The size fraction between 5 and 0.22 μm was processed for DNA extraction. Membranes were enzymatically digested with lysozyme, proteinase K and sodium dodecylsulfate and afterwards, DNA was extracted with phenol:chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) and chloroform:isoamyl alchohol (24:1, vol/vol).

Sponge species were previously identified in Turon et al. (2018) by morphological features (Hooper and Van Soest, 2002) and molecular markers. Preparation of spicules and histological sections were made from specimens' subsamples and observed under both light and scanning electron microscopes.

4.3.2 16S rRNA gene sequencing and processing

Only representative sponge species (n=18) at each environment, which appeared replicated in the quantitative sampling, were used for the study of microbial symbionts. Replicates varied from 2 to 9, depending on the species abundance (n. individuals) and material availability (sponge fragment size), as the sampling was intended to reflect the sponge diversity and abundance at each environment (Table 4.1). PCR and high-throughput multiplexed 16S rRNA gene amplicon Illumina MiSeq sequencing, were carried out following the genomic core facilities and methods of the MrDNA Lab (Texas, USA) (http://www.mrdnalab.com/). The variable V4 region of the bacterial 16S rRNA gene was amplified using the primers 564F

(5'AYTGGGYDTAAAGNG-3') and 785R (5'TACNVGGGTATCTAATCC-3') (c.a. 250 nt) (Klindworth et al., 2013). Raw rRNA gene sequences were processed separately using the UPARSE pipeline (Edgar, 2017). A quality check was applied to our dataset with the fastq_filter command and the arguments -fastq_trunclen 208 –fastq_maxee 0.25. Sequences were then dereplicated with the -derep_fulllength command and sorted by size (-sortbysize command) in usearch 9.2 version. Denoising (error-correction) of amplicons was performed following the UNOISE pipeline (Edgar, 2016) using the -unosie2 command. This algorithm removed chimeras, reads with sequencing errors, PhiX, and low complexity sequences due to Illumina artefacts, and generates ZOTUs ("zero-radius" OTUs) with 100% identity sequences. Finally, -usearch_global command with identity threshold set at 0.97 was applied to our dataset. Taxonomic assignment was done with SINA v1.2.11 (Pruesse et al., 2012) using SILVA 128 database. Sequences with low identity (<75%) and sequences identified as mitochondria or chloroplasts were removed from the analysis. In order to minimize biased effects for differences in sampling effort, the original bacterial ZOTU table was rarefied at a minimum reads threshold of 41000, using QIIME (Caporaso et al., 2010).

4.3.3 Sponge and bacterial community analysis

Statistical analyses were run in the R environment (R core Team, 2013). Community ecology related parameters were calculated using the vegan v2.5-1 (Oksanen et al., 2017) and iNEXT v2.0.15 packages (Hsieh et al., 2018), and figures were drawn with ggplot2 3.0.0 (Wickham, 2009). We determined sponge species composition on impacted and well-preserved environments: the number of sponge individuals was standardised per meter of sampled transect to minimise the bias of the sampling effort in the two habitats. Shannon diversity indices and species richness were calculated for both environments using the *ChaoEntropy* and *ChaoSpecies* functions, respectively, and integrated curves that smoothly link rarefaction (interpolation) and prediction (extrapolation) were computed for both variables (Chao et al., 2014) to facilitate their comparison of between habitats (Figure C.2). Additionally, we used the speacaccum function with 1000 permutation

Table 4.1: Species replicates used for the microbiome study.

Species	N. Impacted Env.	N. W-P Env.
Aaptos suberitoides	0	7
Amphimedon paraviridis	5	0
Antho (Antho) sp.	0	3
Callyspongia sp.	0	2
Clathria reinwardti	9	0
Clathria (Isociella) skia	4	0
Dendroxea sp.	0	2
Dysidea sp.	0	3
Amphimedon sulcata	3	6
Haliclona (Reniera) sp.	0	3
Monanchora unguiculata	0	3
Mycale (Arenochalina) sp.	2	1
Neofibularia sp.	0	4
Phorbas sp.	0	3
Protosuberites proteus	0	2
Suberea fusca	0	3
Thrinacophora cervicornis	0	2

of the vegan package to represent the accumulated number of species per sampled meter in each habitat (Figure C.3).

A Venn diagram of total species richness in both environments was generated using the *eulerr* package (Larson et al., 2018) and *t-tests* were conducted to assess differences in community metrics between sites. We calculated the Bray-Curtis dissimilarity of species composition of all transects to assess the beta diversity patterns between environments.

We performed a hierarchical cluster analysis (Ward method) based on Bray-Curtis dissimilarity matrix using the rarefied ZOTU table to determine whether sponge bacterial communities were more similar among replicates from the same species than between impacted and well-preserved environments. To test the effects of host identity and environment on structuring the sponge bacterial communities, we used PERMANOVA (Anderson, 2001) based on 999 permutations as implemented in *adonis* function. A heatmap was generated for the most abundant bacterial families (>1% relative abun-

dance in any of the samples). We performed and Indicator Value (IndVal) (Dufrêne and Legendre, 1997) analysis to detect if particular microbial taxa were differentially found at each site using the *labdsv* package (Roberts and Roberts, 2016) in R. We set the Indval threshold to 0.7 and the p-value for significance at 0.01.

We compared three main bacterial community features between the sponge assemblages from impacted and well-preserved environments: i) intra-species dispersion (beta diversity), ii) Shannon diversity and iii) core size (explained below). For these comparisons, we included *Amphimedon sulcata* and *Mycale (Arenochalina)* sp., for which we had replicates in the impacted and well-preserved environments, within the category of sponges able to survive in impacted sites.

Only two species inhabited both environments (*A. sulcata* and *Mycale (Arenochalina)* sp.). However, only one individual of *Mycale (Arenochalina)* sp. was found in the well-preserved habitats, preventing statistically based comparisons between environments for this species. *Betadisper* and *Shannon* functions were used to calculate beta and alpha diversity measures, respectively. We defined the bacterial core of each sponge species considering the ZOTUs present across all species replicates. The core size of each individual represented the percentage of core bacteria (of the sponge species) with respect to the total bacteria present in that individual. *T-test* and *Kruskal-Wallis* test were used to detect significant differences between sponge species from impacted and well-preserved environments. *Pearson* correlation was used to detect the relationship between intra-species dispersion and bacterial core size for each sponge species.

4.4 Results

4.4.1 Sponge assemblages in two contrasting environments

A total of 71 sponge species were identified in the overall sampling area (Figure 4.1). The impacted and well-preserved environments showed strong differences in terms of sponge richness, diversity and density (Figure 3.1, Figure C.2). Sponge richness and diversity were much lower in the impacted sites than in the well-preserved environments (t-test: p-val= 0.039, p-val=

0.037, respectively), although the respective rarefaction curves per number of individuals sampled did not reach the saturation point, in particular for species richness (Figure C.2). The rarefaction curves of accumulated species per sampled area neither reached the saturation point (Figure C.3).

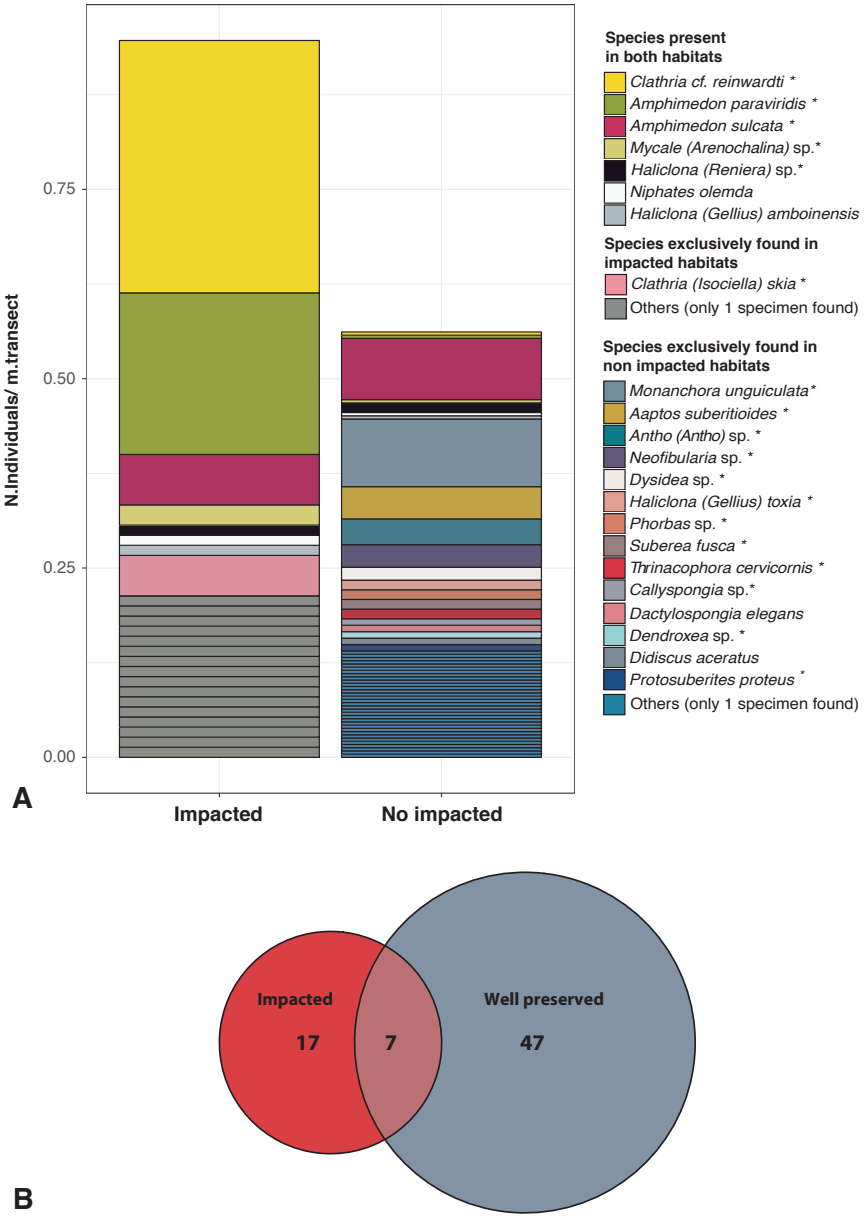

Figure 4.1: A) Bar plots of sponge species composition and abundance at the well-preserved and impacted habitats. Y-axis represents the numbers of individuals per meter of transect. B) Venn diagram of total species richness and the species overlap between well-preserved (grey) and impacted (red) environments. ∗Indicates species for which microbiome has been analysed.

Conversely, the overall sponge density was higher (t-test: p-val= 0.05) in

the impacted (0.94 individuals/m) than in the well-preserved environments (0.56 individuals/m). Most species were environment-specific with only seven species found in both environments, though all of them but *Amphimedon sulcata* were much more abundant at the polluted sites (Figure 4.1). The well-preserved habitats harboured a more diverse and evenly distributed sponge assemblage with *Monanchora unguiculata*, *A. sulcata*, *Antho (Antho)* sp., *Aaptos suberitoides*, and *Neofibularia* sp. (Figure 4.1, Figure 4.2) being the most abundant species. Conversely, the impacted habitats showed a community mostly dominated by *Clathria reinwardti* and *A. paraviridis* (Figure 4.1, Figure 4.2) (>50% of the specimens sampled). Moreover, significant differences in beta diversity for species composition were found between sites (*adonis*, F= 2.72, df = 1, R^2= 0.19, P= 0.01).

4.4.2 Sponge-associated bacterial communities

We obtained a total of 15,712 high-quality ZOTUs (Zero-radius Operational Taxonomic Unit, 100% identity) corresponding to 48 bacterial phyla, with *Proteobacteria* (52.9%), *Actinobacteria* (2.8%), *Acidobacteria* (2.4%), *Chloroflexi* (2%), and *Planctomycetes* (1.7%) being the most abundant taxa. Bacterial communities of HMA sponges (*Neofibularia* sp., *Aaptos suberitoides*, and *Suberea fusca* clearly differed from those of LMA sponges (Figure 4.3). *Chloroflexi*, PAUC34f, *Caldilineaceae* and Sva0996 marine group were consistently associated to the HMA sponges.

The cluster analysis showed that the sponge-associated bacterial communities were closely related to their host-species regardless of the environment (impacted vs. well-preserved) where the sponges were living, with, in general, low variation among replicates of a species (Figure 4.3). Indeed, we found a strong effect of host identity on the composition of the sponge microbiomes (*adonis*: Pseudo-F: 4.46, $R^2 = 0.593$, $p < 0.001$), but a negligible effect of the environment (adonis: Pseudo-F: 1.07, $R^2 = 0.008$, $p > 0.05$). Thus, each sponge species presented a unique microbiome that differed from that of other sponges and from that of the surrounding seawater (Figure C.4), even if they were living in the same environment (Figure 4.3). Planktonic communities showed significant differences in bacterial composition between types of habitats (*adonis*: Pseudo-F: 2.2, $R^2 = 0.23$, $p < 0.05$, Figure C.4A).

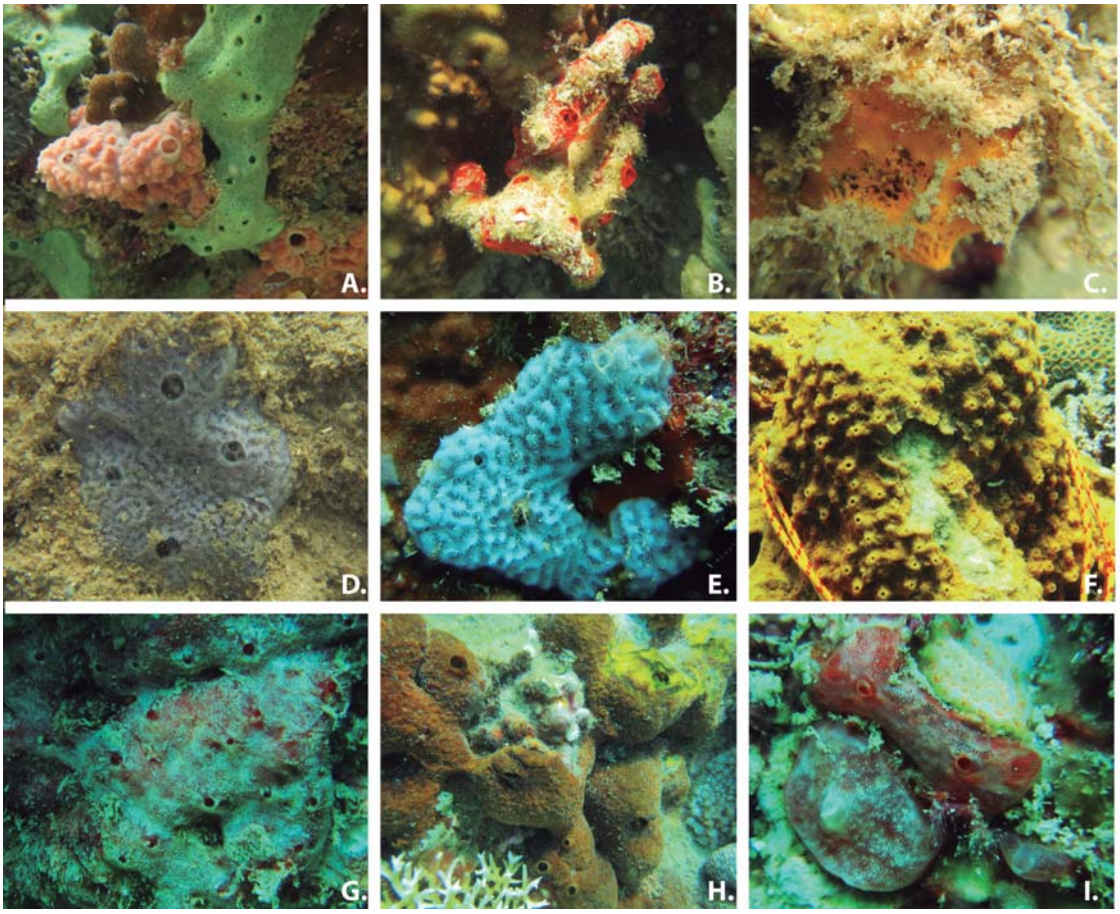

Figure 4.2: Pictures of the most common sponges of the impacted (A, B, C, D) and the well-preserved (E, F, G, H, I) habitats: A) *Amphimedon paraviridis* (greenish) and *Clathria reinwardti* (pinkish), B) *Clathria (Isociella) skia*, C) *Mycale (Arenochalina)* sp., D) *Amphimedon sulcata* (in the impacted environment) E) *Amphimedon sulcata* (in the well-preserved environment), F) *Neofibularia* sp., G) *Antho (Antho)* sp., H) *Aaptos suberitioides* and I) *Monanchora unguiculata*.

Shannon diversity was higher for the seawater bacterial communities in the well-preserved habitat than in the impacted habitat, but differences were only statistically significant at an alpha value of 0.07 (*Kruskal-Wallis* test: p-val = 0.07), likely due to the unbalanced design (3 vs. 6 replicates) and the large variation in the Shanon diversity Index across replicates at the polluted environment (Figure C.4B).

We looked in further detail into the bacterial composition of the two purportedly opportunistic species that dominate the perturbed (polluted) environments (*Clathria reinwardti* and *Amphimedon paraviridis*). A common feature of these two species was that their bacterial communities were dominated by a few taxa such as *Candidatus Branchiomonas* (42.85% ± 12.5%)

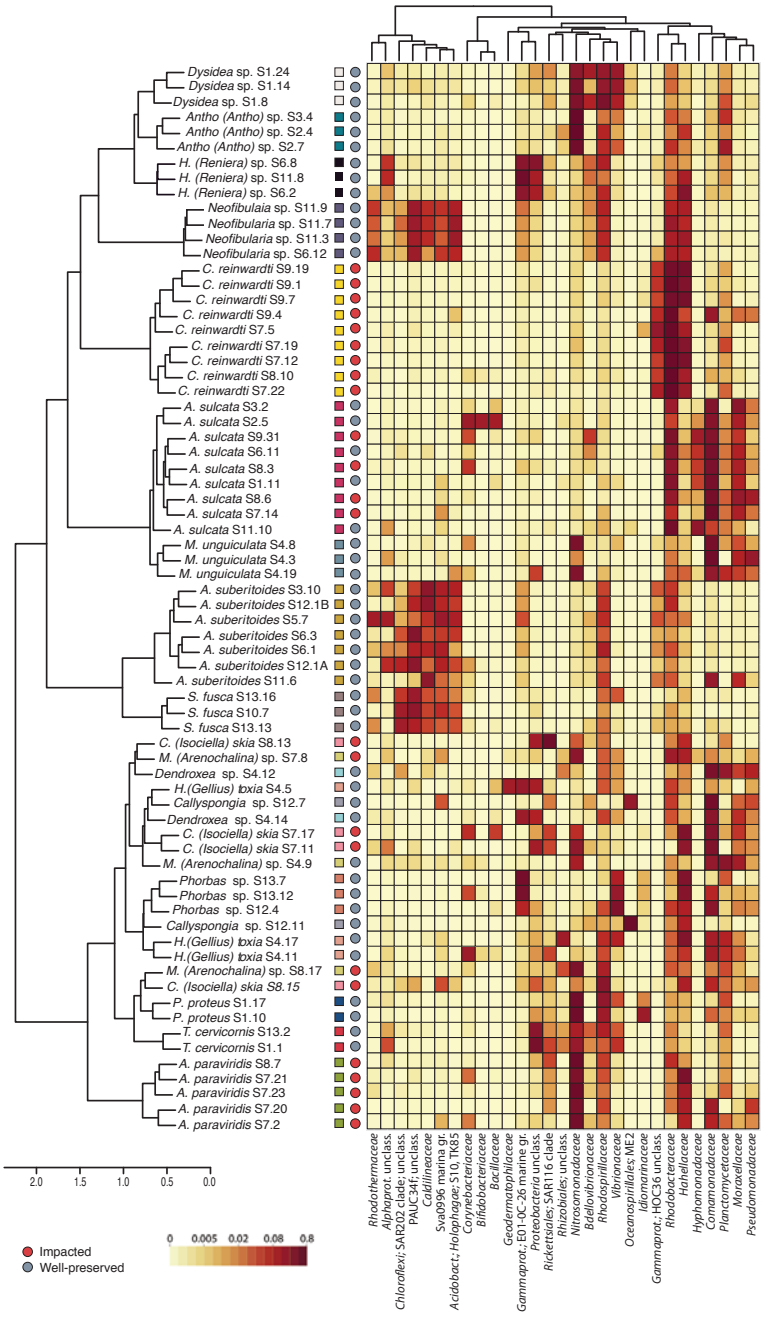

Figure 4.3: Heatmap of the sponge bacterial composition at the family level. Only taxa with relative abundances higher than 1% in any of the samples are shown, with abundance represented in the colour temperature bar. Sponge samples (y-axis) and bacterial taxa (x-axis) are organized according to a hierarchical clustering based on Bray-Curtis dissimilarity matrices (at the level of ZOTU and family, respectively). Square colours correspond to different sponge species, and circle colours indicate the site of collection: impacted (red) or well-preserved (grey).

and *Endozoicomonas* (12.74% ± 12.1%) in *A. paraviridis*, and members of the family *Rhodobacteraceae* (58.54% ± 22%) and *Endozoicomonas* (21.63% ± 12.9%) in *C. reinwardti* (Figure 4.3). In both sponges, only two bacterial taxa achieved > 60% of the whole microbiome. Moreover, these two taxa were consistently found across all species replicates (i.e. they belong to the sponge core). A single *C. Branchiomonas* ZOTU made up 56% of the total core reads of *A. paraviridis* and 6 ZOTUs belonging to *Rhodobacteraceae* family made up 64% of the total core reads in *C. reinwardti*. Moreover, both species had in their core communities ZOTUs belonging to *Endozoicomonas* and *Shewanella* at abundances higher than 10% and 1%, respectively. Indval analysis (Figure C.5) showed that some bacteria were significantly associated to the impacted environment (Indval > 0.7, p-val < 0.01) but only *Rhodobacteraceae* and *Shewanella* were found at high abundances in the sponges of that habitat.

4.4.3 Between environment comparisons: general microbiome ecological descriptors

To test for microbiome differences among replicates of the same species living in the two environments, we focused on the unique species equally found in both habitats: *Amphimedon sulcata*. No influence of the environment could be detected for inter-individual variation (PERMANOVA: $R^2 = 0.128$, $p > 0.05$) and no differences (*t-test*, p-val > 0.05) were detected for intra-species dispersion, core size and Shannon diversity of the *A. sulcata* bacterial communities between individuals from both environments.

Intra-species dispersion of the sponge microbiomes was species-specific in all the species tested, with *Neofibularia* sp., *Thrinacophora cervicornis*, and*A. suberitoides* having the lowest dispersions and *Mycale (Arenochalina)* sp., *Clathria (Isociella) skia*, and *A. paraviridis* having the highest (Figure C.6A). Interestingly, intra-species dispersions were higher in impacted than in well-preserved environments (*t-test*: p-val < 0.001; Figure 4.4A). That is, the bacterial communities of sponge replicates from well-preserved environments are more similar to each other than seen for those sponge replicates from impacted environments. Consequently, intra-species dispersion was negatively correlated to the size of the sponge bacterial core

(Rp= -0.65, p-val < 0.01: Figure C.7): the higher the intra-species dispersion, the lower the size of the core and the larger the variable bacterial community of the sponge species. *C. reinwardti*, *Mycale (Arenochalina)* sp. and *A. paraviridis* had the smallest core sizes (10.55%, 17.34% and 18.44%, respectively), whereas *T. cervicornis*, *Protosuberites proteus*, *Monanchora unguiculata*, and *Neofibularia* sp. (43.71%, 41.78%, 41.73% and 41.72%, respectively) had the largest ones (Figure C.6B). Overall, the bacterial core sizes of the sponge species living in the impacted environments were lower (*Kruskal-Wallis* test: p-val < 0.001) than those of the sponges living in the well-preserved environments (Figure 4.4B). Shannon diversity indices (H') of the sponge microbiomes were also species-specific and ranged from 2.7 to 4.7 (mean values for *C. reinwardti* and *S. fusca*, respectively) (Figure C.6C), which were lower in impacted than in well-preserved environments (Kruskal-Wallis test: p-val < 0.001) (Figure 4.4C).

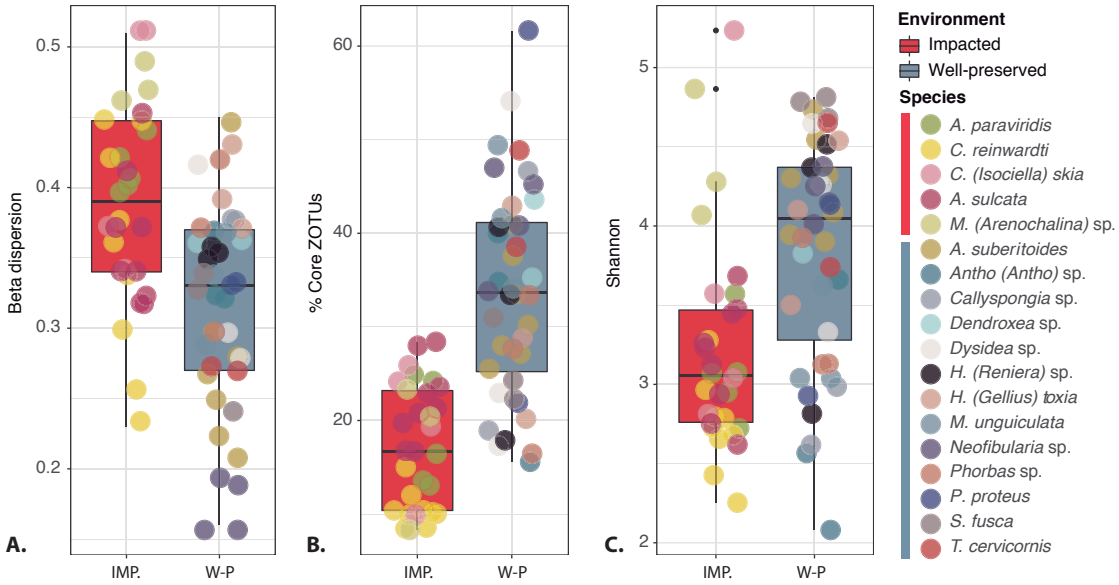

Figure 4.4: Box plots comparing the intra-species dispersion (A), core size (B) and Shannon diversity (C) of the sponge microbiomes between impacted (red) and well-preserved (grey) environments. Replicates of the same species are depicted in the same dot colour.

To sum up, microbiomes of sponge species living in the impacted environment had in general, higher intra-species dispersion, lower core size and lower bacterial diversity, than the microbiomes of sponges living in the well-preserved environments (Figure 4.5).

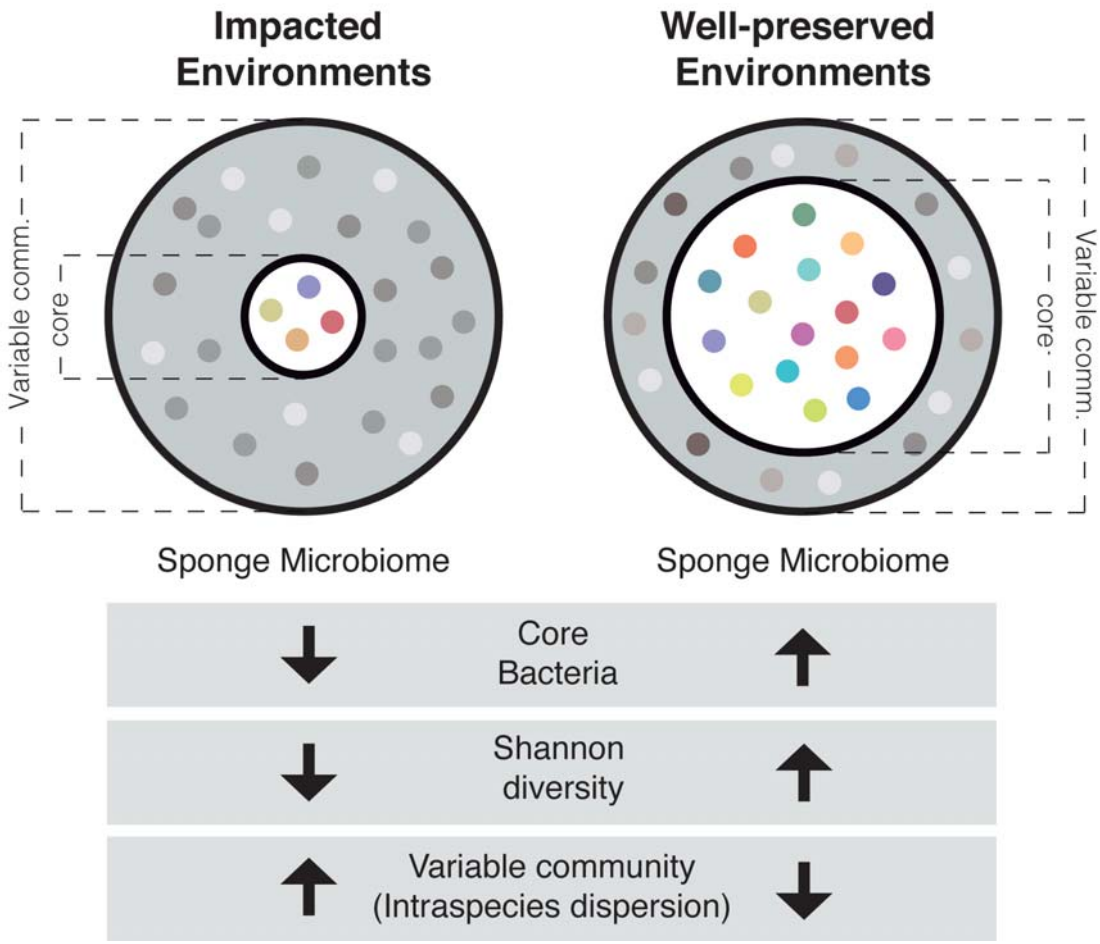

Figure 4.5: Schematic representation of the sponge microbiomes in impacted and well-preserved environments. Grey fraction represents the variable community and white fraction represents the core community. Each coloured dot corresponds to a single bacterial species.

4.5 Discussion

4.5.1 Sponge ecology: well-preserved vs. impacted environments

We have found clear differences between the sponge assemblages from well-preserved and impacted environments of Nha Trang Bay, with different species, lower sponge richness and diversity but higher sponge density (individuals/m.) in the perturbed zones. Similar decrease in species richness (S) and Shannon diversity index (H') due to anthropogenic impacts has been previously reported for the coral communities in the study area (Tkachenko et al., 2016) and for sponge diversity in different oceans (Easson et al.,

2015; Powell et al., 2014). Indeed, low diversity but high abundance with dominance of a few species is a common feature of many polluted habitats (Piola and Johnston, 2008; Powell et al., 2014). However, although our sampling captured differences in diversity between habitats quite acceptably, rarefaction curves for accumulated species predict an insufficient sampling effort to completely catch the true species richness.

In the Nha Trang region, marine cultures are producing chronic eutrophication and high sedimentation rates in some areas. As expected, the composition of sponge communities severely changes in those perturbed areas, with only a few sponge species inhabiting there. Body architecture and physiological traits, such as sediment removing mechanisms through mucus production (Bell et al., 2015; Schönberg, 2015, 2016) have been reported and allow sponges to survive under anomalous sedimentation rates (Pineda et al., 2017; Strehlow et al., 2017), as those resulting from severe eutrophication (Ralph et al., 2006). Some of these mechanisms are indeed displayed by the most abundant sponge species inhabiting the polluted habitat of Nha Trang (e.g. *Clathria reinwardti*, *Amphimedon paraviridis*, *Amphimedon sulcata*), as the sponge surfaces appear completely clean whereas a dense layer of sediment covers the remaining substrate (Figure 4.2). However, a series of xenobiotic compounds, resulting from an intense mariculture, are also released to the water at the impacted study area (Nguyen et al., 2016), so that biological/ecological sponge traits other than resistance to sedimentation, are expected to contribute to the observed abundance of a few species in those areas.

4.5.2 Sponge microbiomes: Shannon diversity, intra-species dispersion and core community

The bacterial communities of the study sponges show a high fidelity to the sponge species independently of the environmental conditions where the host species are living, as reported for many other sponge species (Bosch and Miller, 2016; Gantt et al., 2017; Glasl et al., 2018; Luter et al., 2014, 2012; Simister et al., 2012a; Webster and Thomas, 2016).

Experimental studies repeatedly show a high resilience of the sponge microbiota in species exposed to a range of environmental variables, such

as irradiance, temperature, salinity, acidification, and contrasting habitats (Cárdenas et al., 2014; Erwin et al., 2012; Glasl et al., 2017; Luter et al., 2014; Pita et al., 2013a,b; Ribes et al., 2016). A similar absence of effects on microbiome composition, and diversity was reported for the sponge *Gelliodes obtusa* under eutrophic conditions from mariculture (Baquiran and Conaco, 2018). As in the mentioned study, similar microbiomes were found in our study sponge *Amphimedon sulcata*, inhabiting either well-preserved or polluted environments. Thus, some haplosclerid sponges seem to tolerate eutrophication pressures although they can also inhabit well-preserved habitats, suggesting that they are sponges able to grow in a broad range of environmental conditions.

Most of the above-mentioned experimental studies focus on ecological adaptation of the sponge microbiomes to the assayed conditions but instead they found microbiome stability across treatments. Although surprising at first sight, microbiome resilience is indeed logical if we consider the concept of hologenome evolution (Rosenberg and Zilber-Rosenberg, 2018) and that these microbial-eukaryote symbioses have been evolutionarily fixed thousands of years ago (Sirová et al., 2018). Seemingly, structural microbiome stability has also been reported for few coral species across environmental gradients, which suggests resilience of microbiomes to environmental fluctuations or stress also in corals (Grottoli et al., 2018; Pogoreutz et al., 2018; Sawall et al., 2014).

Sponge microbiomes are species-specific in both environments, with no differences between individuals of the same species (i.e. *A. sulcata*) living at both habitats. However, significant differences in community metrics are revealed when the microbiomes of the sponge assemblages, as a whole, were compared between environments, with some particular bacterial groups proliferating in the polluted sites. Thus, even though the conditions of the perturbed environment cannot modify the evolutionarily fixed microbial communities of the sponge species (i.e. microbiomes do not change as a result of ecological adaptation to environmental conditions in species living at both habitats), they might influence the ecological distribution of the sponge species.

The sponge microbiomes of the polluted sites show a significantly lower

Shannon diversity than sponge microbiomes in well-preserved environments. This is in accordance with the agreed general loss of biodiversity in impacted environments (Rygg, 1985). However, community success does not seem to directly depend on its diversity, but on its ability to respond to particular environmental conditions (Evans et al., 2017, 2018; Glasl et al., 2018; McCann, 2000). Indeed, the few species proliferating in the polluted environments of Nha Trang Bay (i.e. *C. reinwardti* and *A. paraviridis*) show a high density and a large coverage in the area, regardless of their low diversity microbiomes.

Moreover, the microbiomes of the sponges inhabiting the polluted sites show a higher intra-species dispersion than those of the sponges from well-preserved environments. The high microbiome dispersion in the former might indicate that the sponges living in the polluted environments, despite being visibly healthy are subjected to some stress. The Anna Karenina principle (Zaneveld et al., 2017), which proposes that variability is higher in dysbiotic than in healthy individuals of the same species, might be extended to species assemblages, according to our results. That is, bacterial communities of sponges in general, would be more variable in impacted than in well-preserved reefs. Similar trends in alpha and beta diversity metrics to those found in these contrasting environments have been also observed in successional stages of bacterial communities from primary, supposedly more stressed, to late or more mature (Ortiz-Álvarez et al., 2018).

The bacterial cores (Astudillo-García et al., 2017), predicted to play crucial roles in the sponge functioning (Cárdenas et al., 2014; Pita et al., 2018), represent a low percentage of the total microbiome in the study sponges inhabiting the polluted site. Consequently, most bacteria in these species are transient and thus, may be site-variable. This is confirmed by a negative correlation between the core size and the intra-species dispersion (variable community) of sponge microbiomes. Conversely, sponges inhabiting the well-preserved study sites contain large diverse bacterial cores that might be difficult to maintain in perturbed habitats, as both, theory and empirical evidence point to the simplification of ecological communities in those habitats (Piola and Johnston, 2008). As for the seawater bacteria communities, Shannon diversity was lower and variation between replicates

was higher at the polluted habitats than at the well-preserved environments. Thus, seawater bacteria communities follow a similar pattern as for among replicates variation and alpha diversity than sponge microbiomes at both environments.

Moreover, the simplified microbial systems of the perturbed study habitats contain certain bacterial species that might play a role in the holobiont success. *Rhodobacteraceae* members and *C. Branchiomonas* are major members of core communities of the two dominant sponges in the polluted sites (*C. reinwardti*and *A. paraviridis*), pointing to a potential function of these bacteria in the sponge success by facilitating them to cope with some pollutants. The family *Rhodobacteraceae* includes key players in biogeochemical cycling (Simon et al., 2017) and several members with chemotrophic anaerobic metabolisms, which are able to oxidize the noxious hydrogen sulphide present in eutrophic environments (Zhang et al., 2013). Members of the *Nitrosomonadaceae* family (i.e. *C. Branchiomonas*) are reported to be ammonia oxidizers (Prosser et al., 2014) and have been found to be dominant symbionts in sponges, suggesting that they may represent a source of bioavailable nitrogen for their hosts (Matcher et al., 2017). Also remarkable is the high abundance of *Endozoicomonas* in these sponge species. This genus, with various marine species distributed worldwide (Neave et al., 2016), is commonly found in close associations with sponges (Nishijima et al., 2013). Functions related to sponge health (Gardères et al., 2015), bromopyrrole production (Haber and Ilan, 2014), carbohydrate fermentation/nitrate reduction (Nishijima et al., 2013), and antibiotic production (Rua et al., 2014) in sponge hosts have been proposed (Neave et al., 2016). Thus, sponge-associated *Endozoicomonas* might play biological functions in the study sponges by participating in the nitrogen and sulphur cycles, influencing the inter-species interactions of the microbial community by producing antimicrobial compounds or signalling molecules, as reported for other sponges (Morrow et al., 2015). Finally, *Shewanella* is also found in the core of the two species at relatively high abundances at the impacted habitats. Members of this genus have been reported to reduce heavy metals, sulphates, nitrates, and chromates (Fredrickson et al., 2008), which are expected to be abundant in the polluted study habitats due to the antifouling

paints containing heavy metals and residuals from industrial activities. The purported functions of the bacteria associated with the dominant sponges in the impacted habitats suggest a plausible role of the sponge microbiomes in detoxification of the seawater at a local scale. Indeed, sponges have been reported to exert a bioremediation effect (Milanese et al., 2003) in zones where they are abundant as they process high volumes of water per day (Leys et al., 2011), but further studies would be needed to confirm the real functionality of these bacteria in our study sponges.

4.6 Conclusions

Overall, the microbiomes might play a certain role in determining the presence and proliferation of sponge species in polluted environments. Low core size, low diversity, and high intra-species dispersion are microbiome features shared by the study sponges inhabiting the prospected polluted environments, which suggests that less complex microbiomes are favoured under degraded environmental conditions. Thus, the microbial communities associated with sponges mimic the reduction of diversity showed by animal or plant assemblages at the ecosystem scale.

Under the context of climate change, it has been proposed that coral reefs may change to sponge dominated reefs (Bell et al., 2013) due to the lower sensitivity of sponges to ocean acidification and eutrophication. However, few sponges are favoured by these stressful conditions and, as shown in the present study, we may expect to face a scenario with less diverse but highly abundant sponge assemblages with low complexity microbiomes.

4.7 Acknowledgements

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Autora coberta: Laura López

Chapter 5

Multi-partner symbiosis across biological domains: looking at the eukaryotic associations from a microbial perspective

5.1 Abstract

Sponges establish tight associations with both micro- and macro-organisms. However, while studies on sponge microbiomes are numerous, nothing is currently known about the microbiomes of sponge-associated polychaetes and their relationships with those of their host sponges. We analyzed the bacterial communities of symbiotic polychaetes (*Haplosyllis* spp.) and their host sponges (*Clathria reinwardti*, *Amphimedon paraviridis*, *Neofibularia hartmani*, and *Aaptos suberitioides*) to assess the influence of the sponges on the polychaete microbiomes. We identified both eukaryote partners by molecular (16S and COI genes) and morphological features, and their microbial communities by high-throughput sequencing of the 16S rRNA gene (V4 region). We unravel the existence of six *Haplosyllis* species (five likely undescribed) associated at very high densities with the study sponge species in Nha Trang Bay (Central Vietnam). A single polychaete species inhabited *A. paraviridis* and differed to the single species that inhabited *A. suberitoides*. Conversely, two different polychaete species were found in *C. reinwardti* and *N. hartmani*, depending on the two host locations. Regardless of the host sponge, polychaete microbiomes were species-specific, which is a widespread feature in marine invertebrates. More than half of the polychaete bacteria were also found in the host sponge microbiome, but at contrasting abundances. Thus, the associated polychaetes seemed to be able to select, incorporate, and enrich part of the sponge microbiome, a selection that appears to be polychaete species-specific. Moreover, the bacterial diversity is similar in both eukaryotic partners, which additionally confirms the influence of

the food (host sponge) on the structure of the polychaete microbiome.

5.1.1 Importance

Symbiotic lifestyle represents a fundamental cryptic contribution to the diversity of marine ecosystems. Sponges are ideal targets to improve understanding the symbiotic relationships from evolutionary and ecologic points of view, because they are the most ancient metazoans on Earth, are ubiquitous in the marine benthos, and establish complex symbiosis with both prokaryotes and animals, which in turn also harbor their own bacterial communities. Here, we study the microbiomes of sponge-polychaete associations and confirm that polychaetes feed on their host sponges. The study worms select and enrich part of the sponge microbiome to shape their own species-specific bacterial communities. Moreover, worm microbiome diversity runs parallel to that of its food host sponge. Considering our results on symbiotic polychaetes and previous studies on fishes and mammals, diet appears to be an important source of bacteria for animals to shape their species-specific microbiomes.

5.2 Introduction

Living in symbiosis (in its broader sense) is a general lifestyle across terrestrial and marine ecosystems (McFall-Ngai et al., 2013; Moran et al., 2005), but seems to be particularly remarkable in the latter (Dubilier et al., 2008; Levitt-Barmats and Shenkar, 2018; Porat and Chadwick-Furman, 2004). Marine sedentary invertebrates, such as sponges and corals, are engineer organisms habitually used as a refuge by diverse mobile fauna. Among them, cnidarians, crustaceans, mollusks, nematodes, and polychaetes are the most frequently reported in association with sponges in temperate, cold, and tropical oceans (García-Hernández et al., 2019; Martín and Britayev, 1998; Martin and Britayev, 2018; Martín et al., 1992; Uriz et al., 1992; Westinga and Hoetjes, 1981).

Many tropical sponges provide refuge to polychaetes. In particular, endosymbiotic species of Syllidae in sponges represent a paradigmatic model for the study of symbiosis, as thousands of individuals of the same worm

species colonize one (or a few) sponge species (Glasby et al., 2012; Lattig et al., 2010; Lopez et al., 2001; Magnino and Gaino, 1998), and all phases of the polychaete life cycle seem to occur inside the host (Lopez et al., 2001; Wulff, 2006). However, whether these associations are species-specific, symbiotic, mutualistic, or parasitic is under discussion (Lattig and Martin, 2009; Lattig and Martín, 2011; Lattig et al., 2010; Lopez et al., 2001; Martín and Britayev, 1998). While these associations are undoubtedly considered advantageous for the polychaete, because sponges represent a food source and a clear refuge against predation (Martín and Britayev, 1998), the potential benefits for the sponge are more difficult to deduce. Polychaete predation does not seem to cause detectable harm to the host sponges so that the nature of the association has been interpreted as commensalism, mutualism or "good" parasitism (Lattig and Martín, 2011; Lopez et al., 2001; Magnino and Gaino, 1998; Martín and Britayev, 1998; Martin and Britayev, 2018). Indeed, sponge-polychaete associations represent multipartner symbioses as both eukaryotes establish tight associations with multiple microbes (Mcfall-Ngai, 2008). Eukaryote partners harbor their own microbiomes, formed of hundreds of bacterial species interacting among themselves and with their respective hosts. Bacteria have been decisive protagonists in the development of the eukaryote cell (Margulis, 1981). Since then, they inhabit almost every terrestrial and aquatic niche on our planet and accompany eukaryote organisms along their complete life (McFall-Ngai, 2014). However, the potential role, if any, of microbiomes in eukaryotic symbiotic associations has not yet been explored. While studies on sponge microbiomes have proliferated in the last decades (Hentschel et al., 2012; Pita et al., 2018; Taylor et al., 2007; Webster et al., 2012; Webster and Thomas, 2016), nothing is currently known about the microbiomes of symbiotic polychaetes, including syllids.

In the field of invertebrate-microbial symbioses, how symbiotic bacteria are acquired by a host species remains under debate. Initially, the concept of true symbiont was associated to a maternal inherence (vertically transmitted). Currently, the idea of a species-specific selection of bacteria from the environment by the eukaryote host to conform its specific microbiome is gaining support (Taylor et al., 2013; Turon et al., 2018; Walke et al., 2014),

particularly since host's bacterial composition does not directly reflect that of the environment (Sullman et al., 2012; Walke et al., 2014).

Our study identified the bacterial communities of four tropical sponges, *Clathria (Thalysias) reinwardti* Vosmaer, 1880, *Amphimedon paraviridis* Fromont, 1993, *Neofibularia hartmani* Hooper and Lévi, 1993 and *Aaptos suberitoides* Br⊘ndsted, 1934, and those of their respective polychaetes of the genus *Haplosyllis* in different locations of Nha Trang Bay (central Vietnam), aimed at assessing the contribution of the host sponges to the microbiome composition of their associated polychaetes. Considering that syllid worms feed on their host sponges, and that diet is known to influence the feeder microbiome, at least in vertebrates (Heiman and Greenway, 2016; Ley et al., 2008; Nayak, 2010; Walburn et al., 2018), we hypothesized that polychaete microbiomes would reflect to some extent that of their host sponges. In this case, one would expect to find a high degree of similarity between the bacterial communities of the symbiotic partners, with the most abundant members of the sponge microbiome being also the major components of the polychaete microbiome.

5.3 Results

5.3.1 Polychaete identification and associations with host sponges

All sponge species were dominated by a single polychaete species at high abundance. Figure D.1 shows worm individuals extracted from a 3 cm^3 sponge fragment. Six species of *Haplosyllis* could be distinguished based on morphological (Figure 5.1) and molecular characters. Species identity could only be confirmed for *Haplosyllis tenhovei*, Lattig et al., (2010), while the remaining five worms likely represented undescribed species, whose formal description will be submitted to a specialized journal and thus, is out of the scope of the present study.

Both 16S and COI sequences (see Data availability for Accession numbers) differed among all identified species, except for *Haplosyllis* sp3 and *Haplosyllis* sp4, whose sequences are identical despite they show enough

morphological differences to be considered different species under traditional taxonomic criteria (Figure 5.1).

All respective replicates of *A. suberitoides* and *A. paraviridis* were constantly found in association with a single polychaete species, *Haplosyllis* sp1 and *Haplosyllis* sp2, respectively (Figure 5.1). Conversely, in *N. hartmani* and *C. reinwardti*, two different polychaete species were found in each sponge, depending on the geographical location. *Neofibularia hartmani* harboured *Haplosyllis* sp3 at Noc Island, and *Haplosyllis* sp4 at Hun Moon Island, while *C. reinwardti*, harboured *Haplosyllis* sp5 at Hun Moon Island and *H. tenhovei* at Dam Bay (Figure 5.1).

In all cases, evidence of sponge spicules inside the worms confirmed that the symbiotic polychaetes feed on the host sponges (data not shown).

5.3.2 Sponge and polychaete microbiomes

Host identity was the main factor structuring the bacterial communities of both sponges and polychaetes (Figure 5.2) (PERMANOVA: $R^2 = 0.62$, p-val < 0.001). Polychaete microbiomes had unique bacterial communities markedly different from those of their host sponges and the surrounding seawater (Figure D.2), but also differing between the worm species.

Based on Bray-curtis, bacterial communities were more similar to each other in polychaetes than in host sponges (Figure D.3). Although highly different (BC distances > 0.6), microbiome distances in specific associations (host sponge vs. its symbiotic polychaete) were significantly lower (Kruskal-Wallis: p-val < 0.001) than in non-specific associations (sponge vs. polychaetes from all other sponge species) (Figure D.2). The most similar microbiomes were found in *N. hartmani* and *Haplosyllis* sp3 and in *A. paraviridis* and *Haplosyllis* sp2 (Figure D.4), while the most distant were those of *A. suberitoides* and *Haplosyllis* sp1 (Figure D.4). Moreover, microbiomes of HMA sponges (i.e., *A. suberitoides* and *N. hartmani*) were associated to polychaete microbiomes with Shannon diversities higher than microbiomes of polychaetes associated to the LMA sponges (i.e., *C. reinwardti* and *A. paravirdis*) (Figure 5.3).

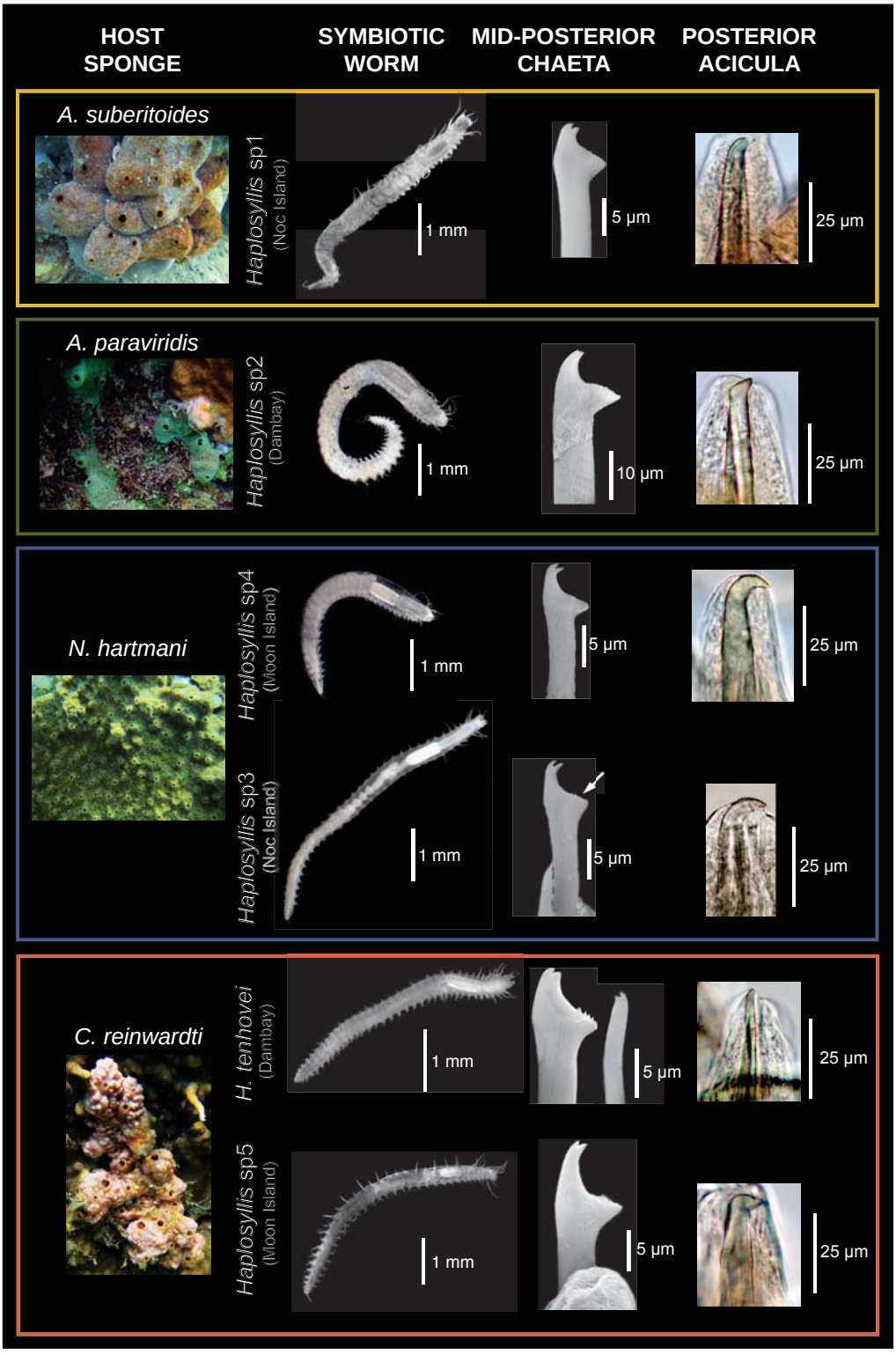

Figure 5.1: Pictures of the sponges and their associated polychaetes. Scanning electron microscopy photos of the mid-posterior chaetes and optical microscopy photos of the posterior acicula, which were considered diagnostic characters for polychaete species differentiation. Sampling region is indicated in brackets.

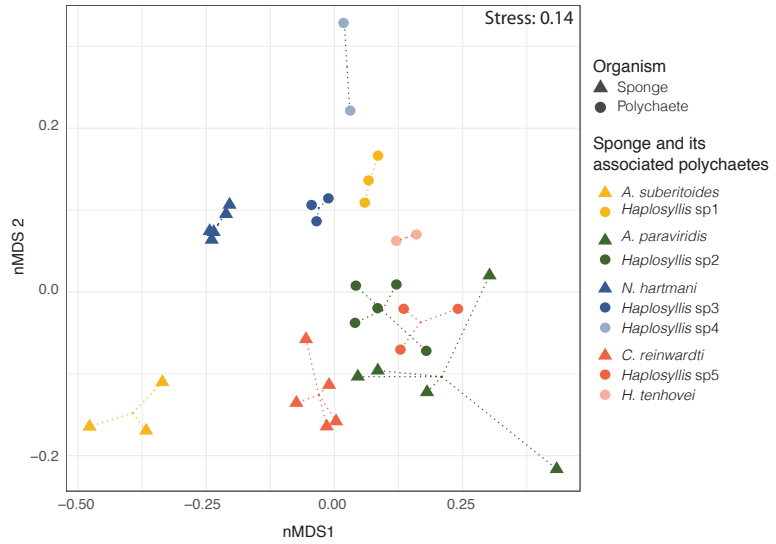

Figure 5.2: Non-metric multidimensional scaling (nMDS) ordination of the sponge (triangles) and polychaete (circles) bacterial communities based on Bray-Curtis distances. Sponge species and their associated polychaete species are depicted in the same color.

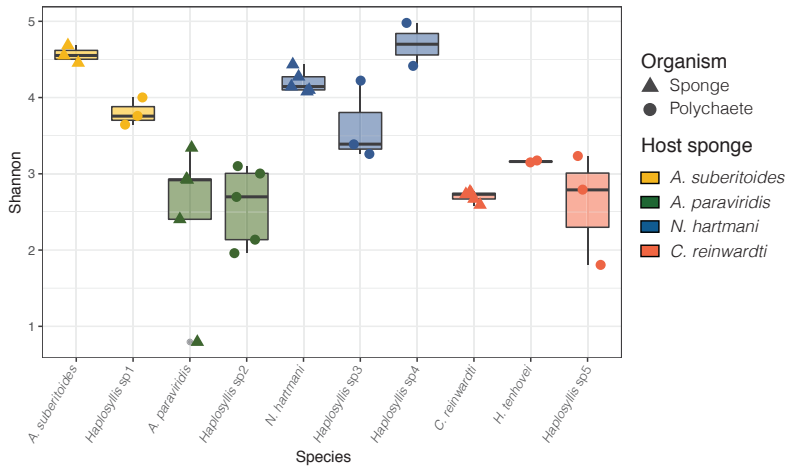

Figure 5.3: Box plots showing the Shannon diversity of microbiomes in sponge (triangles) and polychaete (circles). Sponge species and their associated polychaete species are depicted in the same color.

5.3.3 Core microbiome communities

Core bacterial communities of both, sponges and polychaetes appeared to be large and represented more than 80% of relative abundance of the total microbiome in most species (Table D.1). 44 ZOTUs were detected in all polychaete samples, with the most abundant belonging to *Vibrio*, *Litori-*

monas, *Endozoicomonas*, *Pesudoalteromonas*, *Shewanella* and *Alteromonas* (Table D.2). The results shown in the following sections are based on core bacterial communities.

5.3.4 Taxonomic profiles of sponge and polychaete bacterial communities

The most abundant orders in polychaete communities were *Vibrionales* (24.3%), *Alteromonadales* (17.7%), *Oceanospirillales* (14.3%), *Burkholderiales* (7.6%) and *Caulobacterales* (4.3%), whereas in sponges they were *Rhodobacterales* (16.29%), *Oceanospirillales* (14.9%), *Nitrosononadales* (8.9%) and PAUC34f unclassified (5.9%).

In most cases, sponges and their associated polychaetes showed highly different bacterial communities (Figure 5.4). In *Haplosyllis* sp1, the dominant *Vibrionales* and *Alteromonadales* occurred at relative abundances lower than 0.5% that those in *A. suberitoides*, while in *Haplosyllis* sp2 and *A. paraviridis*, *Oceanospirillales* were highly abundant in both partners. In *N. hartmani* and *C. reinwardti*, each polychaete species (two for each sponge from different localities) inhabiting the same host sponge presented a unique bacterial composition that also differed from the sponge bacterial community. In *N. hartmani*, *Vibrionales* and *Alteromonadales* dominated the microbiome of *Haplosyllis* sp4 (as in *Haplosyllis* sp1 from *A. suberitoides*), whereas *Burkholderiales* and *Rhodobacterales* dominated in *Haplosyllis* sp3. In *C. reinwardti*, *Rhodobacterales* were dominant whereas *Vibrionales* dominated in *H. tenhovei* and *Sphingomonadales*, *Caulobacterales* and *Alteromonadals* dominated in *Haplosyllis* sp5.

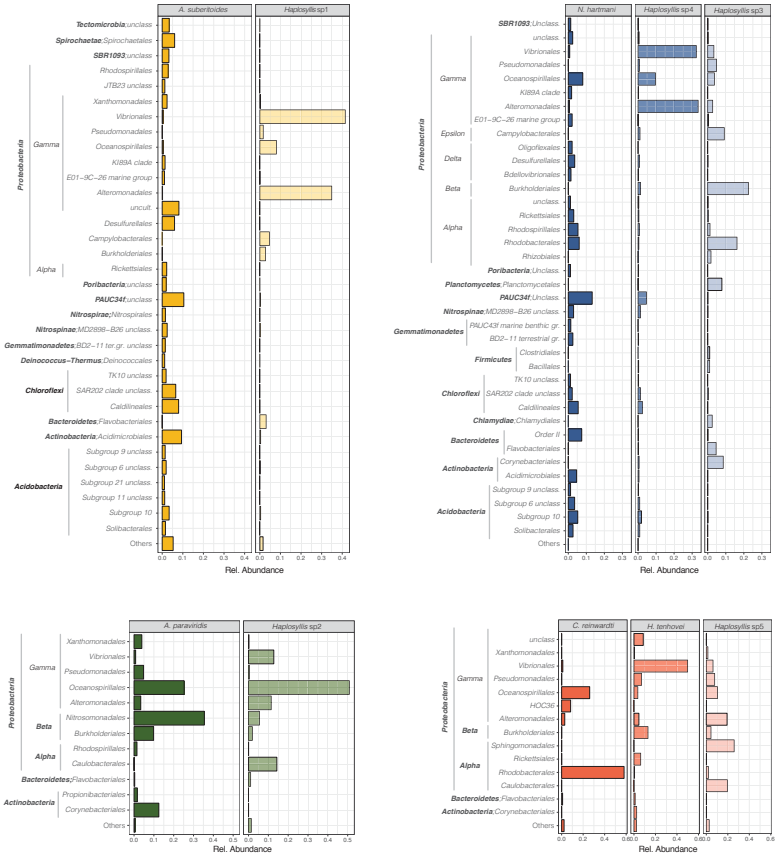

Figure 5.4: Bacterial composition (at order level) for each sponge species and its associated polychaetes. Bars represent relative abundance of each bacterial order in the sponge or polychaete core community.

5.3.5 Bacterial communities shared between the eukaryotic partners

The number of ZOTUs shared between the sponges and their polychaete symbionts varied among the studied species. More than a half of the polychaete ZOTUs were present in their host sponge microbiomes, except for *Haplosyllis* sp4 (Figure 5.5), with the most abundant polychaete ZOTU occurring at low abundances in the respective host sponges and vice versa (Figure 5.5). Indeed, the two most abundant ZOTUs of all polychaete microbiomes were found at relative abundances lower than 0.5% in the microbiomes of the respective host sponges (Figure 5.5).

Few ZOTUs showed similar relative abundances in both polychaete and its sponge host (Figure 5.5). In the case of *C. reinwardti*, ZOTU 55 belonging to *Shewanella* was also abundant in *H. tenhovei*, and ZOTU 21

(*Endozoicomonas*) and ZOTU 32 (*Rhodobactereaceae*) were both found at high abundances in *Haplosyllis* sp5. ZOTU 48 (*Endozoicomonas*), while ZOTU 81 (*Shewanella*) was abundant in both *A. paraviridis* and its associated polychaete *Haplosyllis* sp2. Finally, in the case of *N. hartmani* ZOTU 11 (*Endozoicomonas*) was highly abundant in the sponge and in its both associated polychaetes and ZOTU 19 (PAUC34f) and ZOTU 36 (*Caldilineaceae* uncult.) were also abundant in *Haplosyllis* sp3.

Haplosyllis sp1 (*A. suberitoides*) and *Haplosyllis* sp4 (*N. hartmani*) microbiomes were mainly composed by ZOTUs that were rare or absent in their host sponges (Figure 5.6). On the other hand, *Haplosyllis* sp2 (*A. paraviridis*) and *Haplosyllis* sp3 (*N. hartmani*) microbiomes had a greater proportion of ZOTUs that were either relative abundant or highly relative abundant in their respective host sponges.

In all cases, polychaetes shared more ZOTUs with the sponges than with the sea water bacterial communities (Figure D.6). Moreover, only few of the ZOTUs having a relative abundance $> 0.05\%$ in the polychaetes, were found exclusively in seawater and absent from the sponge (marked withn $*$ in the Figure D.6).

5.4 Discussion

5.4.1 The sponge-polychaete association

Observations of tropical worms associated with sponges, most of them classified as *Haplosyllis spongicola* Grube, 1855, have been widely reported (Tsurumi and Reiswig, 1997). However, very few data on the relationships between these worms and their host sponges are available (Lopez et al., 2001; Martín and Britayev, 1998; Martin and Britayev, 2018). Currently, *H. spongicola* is known to be a species complex (Martin and Britayev, 2003) that includes several misidentified species (Lattig and Martin, 2009), and new species of *Haplosyllis* are continuously discovered. Thus, it was not unexpected that five out of the six species of *Haplosyllis* living in association with the four study sponge species were also new. The Vietnam area seems to be rich in symbiotic polychaetes, according to the numerous species of *Haplosyllis* described there (Britayev and Antokhina, 2012; Martin and

Britayev, 2018), although this can be related to the large number of studies carried out in this area.

We recorded the presence of host-specific spicules in representative samples of all sponge-associated worms. This confirms a sponge-based diet for these symbiotic syllids, as previously proposed for other species (Lattig and Martín, 2011; Lopez et al., 2001; Martin and Britayev, 2018; Wulff, 2006) and suggests damage to the host. However, worms' grazing does not seem to significantly harm their host sponges, since they are among the largest and most abundant sponge species in the study area (this study). The absence of negative effects on the hosts would confirm that these associations are rather commensalistic than parasitic, or even mutualistic as it was recently proposed (Lattig and Martín, 2011; Martin and Britayev, 2018).

On the basis of the study examples, the sponge-polychaete associations appeared to be species-specific, that is all the sponge individuals of the same species in a given area are colonized by the same polychaete species. However, in two cases, the same sponge species harbored two different species of *Haplosyllis* depending on the geographic location. In accordance, *Haplosyllis nicoleae* instead of *H. tenhovei* (our study) was found associated *C. reinwardti* in Indonesia (Lattig et al., 2010). On the other hand ca. eight of the known symbiotic species of *Haplosyllis* are reported to colonize more than one sponge species (Martin and Britayev, 2018). Thus, although these associations appear to be species-specific, at first sight, they may also be ecologically modulated and depend on the geographical/ecological distribution of the involved species. Chemical metabolites, released by the host (Crocker and Reiswig, 1981; Davenport and Hickok, 1951) may represent attractant cues for more than one polychaete species (Pawlik, 1992), so that colonization by one or other might depend on the most prevalent syllid species in a particular area. Colonization by symbiotic polychaetes may be followed by rapid proliferation and complete niche occupation, which could explain the dominance of a single symbiont species in most cases (Britayev et al., 2017; Martín and Britayev, 1998; Martin and Britayev, 2018).

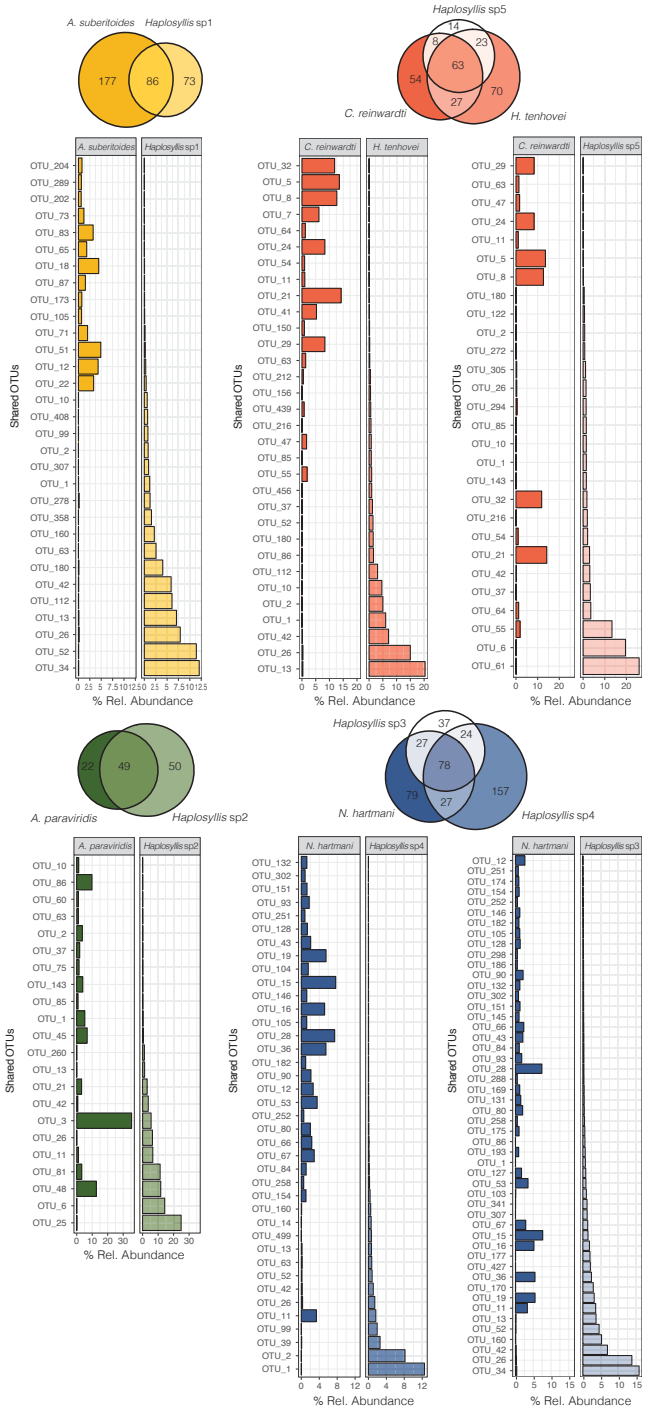

Figure 5.5: Venn diagrams showing the overlap between the bacterial core communities of each sponge species and its associated polychaete. The circle size represents that of the bacterial core (in number of ZOTUs). Bar plots represent the relative abundances (%) of the shared ZOTUs between each sponge - polychaete system. Only ZOTUs with relative abundances higher than 0.5% in any of the eukaryotic partners are shown in the bar plots.

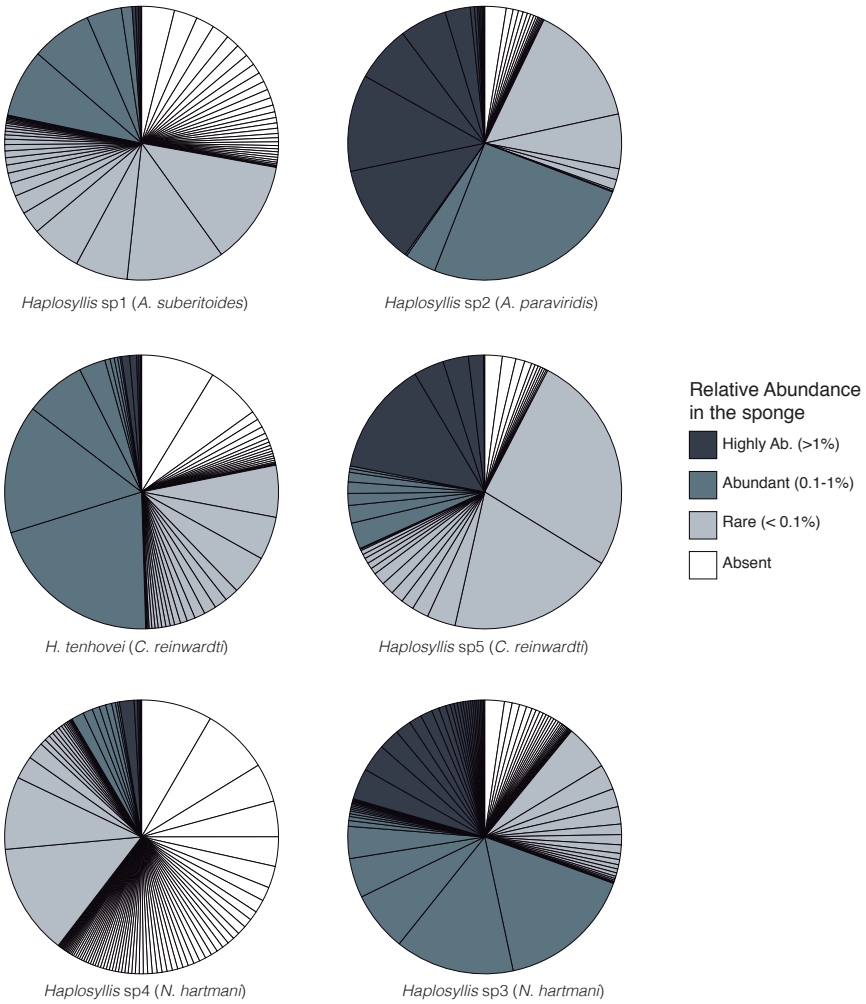

Figure 5.6: Bacterial core communities of each polychaete species. Only ZOTUs with relative abundances higher than 0.1 in the polychaete core are depicted in the pie charts. Each pie slice corresponds to a polychaete core ZOTU and its size is proportional to its relative abundance. Colors represent the categorical relative abundance that each polychaete ZOTUs is found in the sponge microbiome: highly abundant (dark grey), abundant (grey), rare (light grey) and absent (white).

5.4.2 Bacterial communities from the eukaryote partners

Sponge-polychaete symbioses involve many more than two partners, as both eukaryotes harbor particular microbiomes formed by hundreds of bacterial species establishing a tight network of potential interactions. Sponge microbiomes have been intensively investigated during the past fifteen years (Hentschel et al., 2002; Pita et al., 2018; Taylor et al., 2007; Thomas et al., 2016; Turon et al., 2018; Webster et al., 2012; Webster and Thomas, 2016). Conversely, polychaete microbiomes are still poorly known (Neave et al.,

2012; Shankar et al., 2010; Stabili et al., 2006), with most studies focusing on worms inhabiting hydrothermal vents (Rizzo et al., 2014). The microbiomes of the species of *Haplosyllis* here studied were more closely related to each other than those of their respective host sponges among them. Taking into account that all the worms belong to the same genus, while the host sponges belong to different orders (i.e., Suberitida, Poecilosclerida, Desmacellida and Haplosclerida), we suggest that this pattern may have an evolutionary component.

Polychaete microbiomes are species-specific

In general, sponge microbiomes tend to be species-specific, and the same pattern has been reported for nematodes (Derycke et al., 2016). Our results also show a high species-specificity of the polychaete bacterial communities, regardless of their host sponges. Species-specificity of microbiomes seems to be more common in invertebrates than previously thought, and suggests the existence of species-specific mechanisms of bacteria selection (Fieth et al., 2016), pointing to a relevant role of the associated microbes in the invertebrate functioning.

Since polychaete microbiomes appear to be species-specific, they may have a diagnostic value in addition to morphological traits. This could be the case of the two species of *Haplosyllis* found in *N. hartmani*, which were morphologically different but molecularly cryptic, and harbor very different bacterial communities. In this sense, microbiomes might inform on ongoing speciation processes before being detected by molecular markers (e.g. COI and 16S).

The influence of the diet (sponge) on the polychaete microbiomes

Based on previous studies with other organisms, we hypothesized that polychaete microbiomes would reflect that of their prey species of sponges. If true, two polychaete species feeding on the same sponge would have similar microbiomes. In contrast, *Haplosyllis* sp3 and *Haplosyllis* sp4 feeding on *N. hartmani* and *Haplosyllis* sp5 and *H. tenhovei* feeding on *C. reinwardti* have distinct bacterial communities. Our results suggest that each polychaete

species selectively incorporates and enriches specific bacteria, even if these bacteria are rare members of its prey microbiome. Enrichment of environmentally rare microbes has been reported for sponges (Turon et al., 2018; Webster et al., 2010), mollusks (Nyholm and McFall-Ngai, 2004), fishes (Roeselers et al., 2011; Sullman et al., 2012), and amphibians (Walke et al., 2014). Microbiome diversity positively related between polychaetes and their food source (host sponges), which has also been reported for fish larvae and their food source (Walburn et al., 2018) as well as for human gut and diet (Heiman and Greenway, 2016). Thus, our results seem to agree with those reported for other organisms, pointing to what could be a widespread pattern relating bacteria diversity of food and feeder. Recently, Cleary et al. (2019) also found a compositional similarity between certain sponge samples and sponge denizens already suggesting that sponges may influence the prokaryote composition of organisms that live on or within them or that feed on them.

The reliance of the polychaete microbiome on the sponge microbiome

When analyzing the polychaete-sponge relationship from a microbial perspective, we considered that the higher the number of bacterial ZOTUs present in polychaete and absent from the sponge, the lower the polychaete dependency on the sponge microbiome. In this sense, *Haplosyllis* sp1 (from *A. suberitoides*) and *Haplosyllis* sp4 (from *N. hartmani*) would depend less on the sponge microbiome to build up their own microbiome than the remaining polychaete/sponge partnerships studied. The worm bacteria that were not recorded in the host sponge microbiome may possibly correspond to vertically transmitted bacteria (i.e., through sexual or asexual propagula). However, we cannot fully discard some methodological constrains i.e., if bacteria in the sponge escaped our detection limits. We can also envisage some of these microbes being acquired horizontally from environmental sources other than the host tissues (e.g., from seawater (supplementary material)).

In most cases, more than a half of the bacteria from a polychaete microbiome, which probably correspond to the gut microbiome, were also

found in the sponge, but at contrasting abundances, suggesting different levels of between-partner dependency. It would be interesting to assess to what extent the polychaetes maintain their microbiomes when associated to other sponge hosts showing different bacterial communities.

The polychaete bacterial core

We have found a quite large core bacterial community in all species of *Haplosyllis* indicating that polychaete bacteria might play general metabolic or defensive roles (Rizzo et al., 2014). Among these core microbes, we found representatives of *Vibrionales*, *Caulobacterales*, *Alteromonadales*, and *Oceanospiralles*. Respresentatives of these groups have also been reported in other polychaetes such as *Vibrio* in the filter-feeding *Sabella spallanzanii* Gmelin, 1791 (Stabili et al., 2006), *Alteromonadales* and *Oceanospiralles* in deposit feeders Opheliids (Neave et al., 2012) and *Oceanospiralles* in *Osedax* bone-eating polychaetes (Goffredi et al., 2005).

Polychaetes have been proposed as bioremediation agents in polluted waters due to their ability to accumulate *Vibrio* species, which are well-known pathogens in aquaculture (Licciano et al., 2005, 2007; Stabili et al., 2006). Conversely, high abundance of non-pathogenic *Vibrio* strains have been recently reported in shrimp guts (Zoqratt et al., 2018), suggesting a possible beneficial role in the invertebrate fitness. Moreover, different members of *Vibrionaceae* are also reported to be Extracellular Polymeric Substances (EPS) producers (Rizzo and Lo Giudice, 2018), which are important cell protective agents (i.e. against environmental stressful conditions or from xenobiotic substances) and allow them to capture nutrients (Rizzo and Lo Giudice, 2018). In turn, *Alteromonadales* increased in abundance at sites affected by urbanization and eutrophication (Neave et al., 2012) due to their purported tolerance to high copper levels (Jeanthon and Prieur, 1990; Neave et al., 2012), and to other metals (Chen and Shao, 2009; Neave et al., 2012). Moreover, members of *Alteromonadales* are well-known EPS and Biosurfactant (BS) producers (Rizzo and Lo Giudice, 2018; Rizzo et al., 2014), the latter being correlated with antimicrobial activity suggesting a defensive role against pathogens (Rizzo et al., 2014). Finally, *Oceanospiralles* are well-known heterotrophic degraders of complex organic compounds

(Goffredi et al., 2005), which may also contribute to increase the fitness of the associated polychaetes.

5.5 Conclusions

To summarize, the sponge-polychaete associations seem to be basically species-specific but can be ecologically modulated, as different polychaete species inhabited the same sponge species depending on the habitat. The microbiomes of both the sponges and their associated polychaetes are also species-specific, pointing to the relevance of the microbial component on the invertebrate functioning. Our results suggest that the associated polychaetes select, incorporate, and enrich a part of the sponge microbiome to form their individual microbiomes, but the selection appears to be species-specific, possibly reflecting the specific polychaete needs. Diet appears to be an important source of bacteria for invertebrates (this study) and vertebrates (previous studies) to shape their specific microbiomes.

5.6 Materials and Methods

5.6.1 Sponge and polychaete sampling and DNA extraction

A quantitative sampling method to describe the sponge assemblages of Nha Trang Bay (central Vietnam) was carried out in April 2015 (Turon et al., 2018). During that campaign, sponge species associated with polychaetes were surveyed. Four of them were later selected for the present study due to their high abundance and density of associated polychaetes. Among the selected species, *A. suberitoides* and *N. hartmani* belonged to High Microbial Abundance (HMA) sponges whereas *C. reinwardti* and *A. paraviridis* belonged to Low Microbial Abundance (LMA) species (Turon et al., 2018). Sponges containing polychaetes were collected in April 2016 by SCUBA diving between 3 and 9 m depth in three neighboring locations $\sim$ 2 km apart (i.e.; Dam Bay and Hun Mun and Nock Islands) within Nha Trang Bay. Three replicates of *A. suberitoides* (all from Nock Is.), five of *N. hartmani* (three from Nock Is., two from Hun Mun Is.), five of *C. reinwardti* (two

from Dam Bay Is., three from Hun Mun Is.) and five of *A. paraviridis* (all from Dam Bay) were collected. Each sponge sample was kept in a 50 ml Falcon tube with native seawater from same depth and sampling point, and later replaced by 100% ethanol once the polychaetes left the host sponge (ca. 10 min). The released polychaetes were then cleaned from all remaining sponge tissues and allocated to Eppendorf tubes containing 100% ethanol. Back in the lab, sponges were examined under the microscope to extract the possible remaining polychaetes. In the case of *A. suberitoides*, only a few polychaetes left the host sponge spontaneously and thus, sponge dissection and careful examination was key to extract the sponge-associated polychaetes. Ethanol was replaced twice with fresh absolute ethanol to ensure good sample preservation. DNA from sponge and polychaete samples was extracted following the DNeasy Blood and Tissue Kit protocol (Qiagen).

Additionally, triplicate 2L water samples were taken from the three locations (ca. 50 cm apart from the sponges) and sequentially filtered throughout 5-μm and 2-μm polycarbonate membranes. The size fraction 5 to 2 μm was process for DNA extraction. Membranes were enzymatically digested with lysozyme, proteinase K and sodium dodecylsulfate and afterwards, DNA was extracted with phenol:chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) and chloroform:isoamyl alchohol (24:1, vol/vol). Purification and concentration of the DNA was carried out with Amicon® Ultra 4 Centrifugal Filter Units – 100000 NMWL (Millipore).

5.6.2 Polychaete identification

Once separated from their respective host sponges, all polychaetes were carefully identified under the microscope. Anecdotal species (i.e., other than the most abundant one, present as 1 or 2 specimens per sample) were discarded. Only the dominant symbiotic species from each sponge was considered for this study.

We identified polychaete species to the best possible taxonomic resolution by molecular markers and morphological features. Fragments of the mitochondrial small subunit 16S rRNA gene (∼650 bp) and the cytochrome c oxidase subunit I (COI ∼680 bp) were amplified and sequenced. Primer pairs 16SarL/16SbrL (Palumbi, 1996) and jgLCO1490/jgHCO2198 (Geller

et al., 2013) were employed to amplify 16S and COI, respectively. PCR amplifications were conducted in 50 μl reactions containing 1 ng of template genomic DNA, 5 μl of 10x PCR buffer (containing 1.5mM $MgCl_2$), 2 μl of dNTP mix (10 mM), 1 μl of each primer (10mM) and 0.4 μl of Taq DNA polymerase (5 U μl-1). The temperature profiles to obtain the PCR products were set following the protocols of Álvarez-Campos et al. (2017). Purification and sequencing were carried out by an external service (Macrogen, Spain).

The morphology of the dominant polychaete species, all them belonging to the genus *Haplosyllis*, was observed under light and the scanning electron microscopes following the procedures described by Martin and Britayev (2003). All relevant diagnostic morphological characters required for species identifications according to Lattig et al. (2007) were recorded and then checked against the currently existing literature.

5.6.3 Verification of polychaete feeding behavior

From each sponge sample, 25 polychaete specimens were carefully examined to ensure the absence of externally attached sponge spicules, dissolved in boiling nitric acid to totally remove organic matter and then examined under a light microscope (Leitz Axioplan) to confirm the presence of host sponge spicules in the worm.

5.6.4 Bacterial 16S rRNA gene amplification, sequencing and analysing

PCR and high-speed multiplexed 16S rRNA gene Illumina MiSeq sequencing (NGS), were carried out following the genomic core facilities and methods of the MrDNA Lab (Texas, USA) (http://www.mrdnalab.com/). The variable V4 region of the bacterial 16S rRNA gene was amplified using the primers 564F (5'AYTGGGYDTAAAGNG-3') and 785R (5'TACNVGGGTATCTAATCC-3') (c.a. 250 nt) (Klindworth et al., 2013). Raw rRNA gene sequences were processed separately using the UPARSE pipeline (Edgar, 2017). A quality check and de-replication were applied to our dataset. Denoising (error-correction) of amplicons was performed

following the UNOISE pipeline (Edgar, 2016). This algorithm removed chimeras, reads with sequencing errors, PhiX, and low complexity sequences due to Illumina artefacts, and generates ZOTUs ("Zero-radius" OTUs) with 100% identity sequences.

Taxonomic assignment was done with SINA v1.2.11 (Pruesse et al., 2012) using SILVA 128 database. Sequences with low identity (<75%) and sequences identified as mitochondria or chloroplasts were removed from the analysis. To minimize biased effects for differences in sampling effort, the original bacterial ZOTU table was rarefied at a minimum reads threshold of 40000, using QIIME (Caporaso et al., 2010). We normalized our dataset to the same reads count, which means that all data on "bacterial abundance" refers to relative abundance.

5.6.5 Bacterial community analyses of sponges and their associated polychaetes

Distance-based multivariate analysis of the sponge and polychaete bacterial communities (at ZOTU level) was carried out using the vegan package in R (Oksanen et al., 2017). An nMDS (multidimensional scaling) was used to visualize the Bray-Curtis dissimilarity matrix. PERMANOVA (non-parametric Permutation Analysis of Variance), based on 999 permutations as implemented in adonis function, was used to test the effect of host identity in the structuring of bacterial communities. We calculated the Bray-Curtis distances between microbial communities of: i) polychaete species, ii) sponge species, iii) polychaetes and their host sponge (specific) and, iv) polychaetes and non-host sponges (non-specific). Shannon diversity (Shannon, 1948) of the bacterial communities for each sponge and polychaete species was calculated in vegan. The polychaete microbiomes reported here likely reflect the polychaete gut-content bacteria more than bacteria from other body regions. However, we were not able to separate the polychaete body regions due to the small body size (> 0.5 cm).

Core microbiomes (i.e., ZOTUs present in all species replicates) according to Turon et al. (2018) were used for comparing sponge microbiomes with those of their respective polychaete partners. The mean relative abundance of bacterial orders was calculated for each sponge species and its associated

polychaete species, and the corresponding Venn diagrams of the shared core microbiomes were drawn using eulerr package in R (Larson et al., 2018). Pie charts were used to represent the relative abundant ZOTUs (> 0.1%) in the core communities of each polychaete species and their relative abundance in the core microbiome of the respective sponge hosts, categorized as highly relative abundant (> 1%), relative abundant (0.1-1%), rare (<0.1%), and absent.

Comparisons with seawater bacterial communities were made and are presented as supplementary material. An nMDS was used to visualize the Bray-Curtis dissimilarity matrix of each sponge species, its associated polychaetes and seawater. The shared microbiomes were represented by using Venn diagrams. The mean relative abundances of shared bacteria between the three biotypes or between polychaetes and seawater were represented as bar plots. Only ZOTUs with relative abundance >0.05% in the polychaete microbiome were considered for these comparisons.

5.7 Acknowledgements

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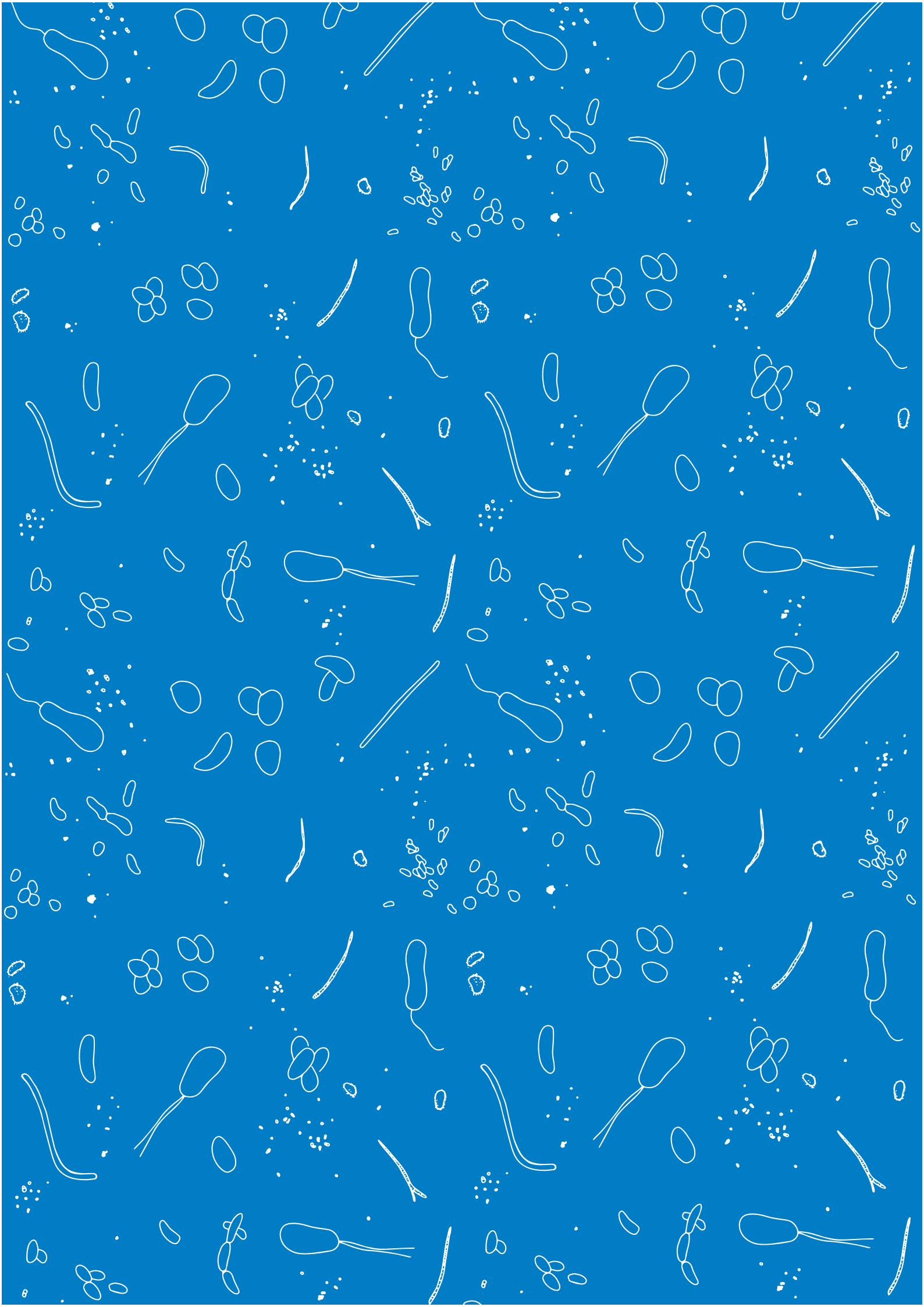

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Imatge capítol: Dibuix d'arqueas.

Autora coberta: Laura López

Chapter 6

Insights into the archaeal consortium of sponge species from Vietnam

6.1 Abstract

Archaea is the less studied domain associated with sponges. Many questions that have been addressed for bacteria still remain largely unknown for archaea. In this study, we analysed the archaeal communities of 17 tropical sponge species from Nha Trang Bay (Vietnam) using archaea specific primers. We recorded patterns of diversity and stability of these microbial communities and compared the results obtained with the bacterial communities, already reported in our previous study. In our study species, Shannon diversity was always lower for archaeal than for bacteria communities. The clear dichotomy between HMA and LMA reported for the bacterial domain could not be confirmed for the archaeal communities. Host identity was the main factor structuring the archaeal assemblage. Archaea core was formed by few but very abundant ZOTUs, which contributed with a high proportion to the relative archaea abundance. The inclusion of the obtained sequences into phylogenetic trees allowed to find out whether or not they belonged to SC clusters. Our results showed that most of the *Thaumarchaeota* and *Euryarchaeota* sequences were more closely related to environmental samples than to SC clusters, which implies that they were acquired from the seawater. However, representatives of *Woesarchaeota*, which were major members of the archaea microbiome of two sponge species, formed a monophyletic tree, distantly related to any known environmental sequence.

6.2 Introduction

Sponges and corals are paradigmatic representatives of the holobiont concept (Simon et al., 2019) in marine ecosystems. Sponges establish tight associations simultaneously with members of the 3 domains of life: Eukarya (De Mares et al., 2017; Turon et al., 2019b; Wulff, 2006), Bacteria (Pita et al., 2018; Taylor et al., 2007; Turon et al., 2018, 2019a) and Archaea (De Mares et al., 2017; Margot et al., 2002; Rodríguez-Marconi et al., 2015; Simister et al., 2012a; Zhang et al., 2014). Among the many eukaryotes reported to live in high numbers associated with sponges, crustaceans (VanSyoc et al., 2015), cnidarians (Uriz et al., 1992; Wulff, 2006) and polychaetes (Turon et al., 2019b; Wulff, 2006) predominate, but other invertebrates and fish have also been recorded within the sponge canals (Wulff, 2006). Sponge-associated bacteria have been widely analysed in the last decades due to their spectacular abundance and diversity, in particular in the so-called bacteriosponges (Vacelet and Donadey, 1977) or High Microbial Abundance sponges -HMA- (Hentschel et al., 2003). Other sponge species with lower bacteria richness, named Low Microbial Abundance -LMA- sponges (Hentschel et al., 2003), have also been deeply explored attempting to assign most of the extant sponge species to any of these two categorized groups (Alex and Antunes, 2015) and to attribute the differences in bacteria community to contrasting structural and ecological characteristics of the host sponges (Blanquer et al., 2013; Gerçe et al., 2011; Ribes et al., 2012; Weisz et al., 2007). Consistent generalizations on the composition, structure and functionality (genomes) of bacteria communities within sponges have arose from these studies (Webster and Thomas, 2016), although a black box on the complex bacteria-sponge and bacteria-bacteria multiple interactions remains to be lighted with the advent of new appropriate tools (Moitinho-Silva et al., 2017a).

Conversely, archaeal associations with sponges are largely unknown even for some basic aspects. Features that have been widely investigated for sponge bacteria such as presence of Sponge Clusters (SC), species specificity, acquisition ways, environmental resilience, HMA-LMA dichotomy, or even functionality, remain poorly explored for the archaeal domain.

Sponge specific bacterial and archaeal clusters (SC) have been reported

in sponge hosts (Simister et al., 2012a; Taylor et al., 2007). However, with the increase of sequencing depth, SC bacterial sequences were also detected outside the sponges, albeit at low abundances. This made the researchers to change the concept of SC to that of "Sponge Enriched Bacterial clusters" (Taylor et al., 2013). Whether the proposed SC for archaea (Simister et al., 2012a) are sponge specific or not after deep sequencing studies, needs to be confirmed. For instance, Simister et al. (2012a) identified 5 putative SC clusters within the *Thaumarchaeota* phylum, which were at that moment unknown from the seawater and from other (non-sponge) marine habitats. Revision of these archaea SC on the light of the new sequences deposited in the databases might confirm or discard the existence of these and others archaea SC.

On the other hand, the available studies suggest that, as for bacteria, host identity is a main driver of archaeal community structure (De Mares et al., 2017; Polónia et al., 2014). The available studies suggest that archaea are resilient to different environments (e.g. the archaeal microbiome of *B. fortis* does not change from marine lakes to open water habitats (Polónia and Cleary, 2019)), but additional species should be analysed to generalize this issue.

The research on how microsymbionts are acquired by the host sponges has run in parallel for bacteria and archaea. Archaea symbionts were first proposed to be transmitted from progenitors to their progeny, due to their presence in sponge larvae (Schmitt et al., 2012; Steger et al., 2008). However, later studies indicated that, as for bacteria microbiomes, host biogeography and environment might also shape the sponge archaeal communities (Turque et al., 2010; Zhang et al., 2014), pointing to an horizontal acquisition of these microbes from the seawater.

Comparative studies on the two microbial domains in sponges often found archaeal cells at lower abundances than bacterial cells, although some exceptions have also been reported (Jackson et al., 2013). Bayer et al. (2014) also found 4-6 orders of magnitude higher 16S rRNA gene numbers for archaea in HMA over LMA sponges, so that the dichotomy showed between these groups for bacteria also seems to apply to archaea. However, regardless the apparently lower abundance of archaea in sponge microbiomes with

respect to bacteria, some archaea have been recognized to play chief roles in the holobiont functioning (Moeller et al., 2019; Radax et al., 2012; Tian et al., 2014).

Thaumarchaetoa is the most abundant phylum reported among the sponge-associated archaea (Hoffmann et al., 2009; Margot et al., 2002; Moeller et al., 2019; Radax et al., 2012). Some members of this Phylum are key chemoautotrophic ammonia-oxidizers microorganisms in marine habitats (Pester et al., 2012) and have been suggested to play a role in ammonia detoxification within the host sponges (Hoffmann et al., 2009; Steger et al., 2008). This ability to metabolize nitrogenous waste products has been proposed to represent a common benefit for the mutualistic relationship between AOA and sponges (Steger et al., 2008; Tian et al., 2014). Indeed, adaptions to a symbiotic lifestyle have been found in the genome of the thaumarchaeal symbiont from Ianthella basta (Moeller et al., 2019). Amonia oxidation in sponges has usually been linked to the presence of archaea through the amplification of the archaeal amoA gene (Hoffmann et al., 2009; Liu et al., 2011; Radax et al., 2012) or metagenomic sequencing (Fan et al., 2012; Moeller et al., 2019), although its presence does not prove necessarily its activity (Mubmann et al., 2011).

In this study, we aimed to characterize the sponge archaeal communities of the most abundant sponges in Nha trang Bay (Vietnam), to cast some light on some of the above-mentioned issues. Bacterial communities of these sponges have been already analysed (Turon et al., 2018) thus, allowing for comparisons between the two prokaryote domains in terms of specificity, diversity, and stability.

6.3 Material and Methods

6.3.1 Sponge sampling and DNA extraction

Sponge samples used in this study are the same used in the author's previous work Turon et al. (2018) (Chapter 3) to study the sponge bacterial communities. For more information on sponge sampling, identification and DNA extraction please refer to the Methods section of the Chapters 2 and 3.

6.3.2 Archaeal 16S rRNA gene sequencing and processing

PCR and high-speed multiplexed SSU rRNA gene Illumina MiSeq sequencing (NGS) were carried out following the genomic core facilities and methods of the MrDNA Lab (Texas, USA) (http://www.mrdnalab.com/). From the 68 samples sent for amplification and sequencing, only 52 yielded proper results. The variable V4 region of the archaeal 16S rRNA gene was amplified using the primers 349F (5' GYGCASCAGKCGMGAAW-3') and 806R (5' GGACTACVSGGGTATCTAAT-3') (c.a. 380 nt). These archaea- specific primer pairs were chosen according to annealing temperatures, overall coverage of V4 region, and amplicon length (Klindworth et al., 2013). UPARSE pipeline (Edgar, 2017) was used to process the raw rRNA gene sequences. The fastq filter command with the arguments -fastq_trunclen 380 –fastq_maxee 0.25 was applied to our dataset for quality checking. After this filtering, we kept 67% of the raw original sequences. Sequences were then dereplicated with the –derep_fulllength command and sorted by size (-sortbysize command) in usearch 10.0.240 version. Denoising (error-correction) of amplicons was performed following the UNOISE pipeline (Edgar, 2016) using the –unosie3 command. This algorithm removed chimeras, reads with sequencing errors, PhiX, and low complexity sequences due to Illumina artefacts, and generates ZOTUs ("zero-radius" OTUs) with 100% identity sequences. Finally, -usearch_global command with identity threshold set at 0.97 was applied to our dataset. Taxonomic assignment was done with SINA v1.2.11 (Pruesse et al., 2012) using SILVA 132 database. Sequences with low identity (<75%) and sequences identified as mitochondria or chloroplasts were removed from the analysis. In order to minimize biased effects for differences in sampling effort, the original bacterial ZOTU table was rarefied at a minimum reads threshold of 5000, using QIIME (Caporaso et al., 2010). Samples with less than 5000 reads were removed from the analysis. From the 68 sponge samples, 52 could be amplified and only 43 samples belonging to 17 sponge species met the quality requirements.

6.3.3 Archaeal community analysis

A distance-based multivariate analysis of the rarefied ZOTU table was carried out using the vegan package (Oksanen et al., 2017) in R (R core Team, 2013). The Bray-Curtis dissimilarity distance matrix of sponge archaeal communities was used to build a hierarchical cluster dendogram (Ward method). PERMANOVA (Anderson, 2001) based on 999 permutations as implemented in adonis function was used to test the effect of host identity (species) on structuring the sponge archaeal communities. Archaeal composition at the lowest taxonomic level and the relative abundance of the most abundant ZOTU were represented for each sponge sample. Moreover, Shannon diversity (*vegan* package) and the abundance-based species richness estimator Chao 1, as implemented in the iNEXT package (Hsieh et al., 2018), with the dissimilarity level set to 0.05, were calculated for sponge archaeal communities. Shannon diversity indices of archaeal communities were compared with those obtained for bacterial communities (already reported in Turon et al. (2018)).

We defined the archaeal sponge core as those ZOTUs present across all samples and the archaeal species core as those ZOTUs present across all replicates of each species. For sponge species with replicates, we calculated the relative abundance of the core ZOTUs to the overall species archaeal community.

6.3.4 Phylogenetic analysis

The most abundant ZOTU sequences ($> 0.1\%$ relative abundance) were added into the original tree containing the previously identified archaeal clusters (Simister et al., 2012a) by using the Parsimony Interactive tool in the ARB program (Ludwig et al., 2004). The SILVA database and alignment of the archaeal sequences, used for the identification of SC clusters, was kindly provided by the authors of the study (Simister et al., 2012a). Only ZOTUs belonging to *Thaumarchaetoa* and *Euryarchaeota* were included in those trees. *Woesarchaetoa* representative sequences ($> 0.1\%$ rel. abundance) were imported into the SILVA SSU Ref NR 132 database (Quast et al., 2013) and inserted in the original tree keeping the overall tree topology by using

the Parsimony Interactive tool implemented in ARB (Ludwig et al., 2004).

6.3.5 Functional predictions of archaeal sponge community

Tax4Fun2 (Abhauer et al., 2015; Wemheuer et al., 2018) was used to predict the metabolic pathways of the sponge archaeal communities. This software predicts the functional profiles of 16S rRNA gene data by aligning 16S sequences with reference genomes and subsequently calculating the functional predictions based on KEGG pathways. Our data was first aligned with the reference dataset provided (275 archaeal genomes available through NCBI RefSeq database) with the *runRefBlast* function in the *tax4fun* R package, and the functional predictions were then calculated with the *makefunctionalprediction* function (Wemheuer et al., 2018). Only the samples for which at least 75% of the reads were used in the prediction (close genome available) were used for further analysis and interpretation.

6.4 Results

6.4.1 Sponge archaeal communities

Nine sponge samples had less than 5000 archaea reads and were excluded from the analysis. Thereby, we present the results based on 43 sponge samples belonging to 17 sponge species with replicates varying from 1 to 8 (see Table 6.1).

We obtained a total of 316 archaeal high-quality ZOTUs belonging to 4 different phyla *Thaumarchaeota* (∼85%), *Nanoarchaeota* (∼10%), *Euryarchaeota* (∼4%) and *Diapherotrites* (> 0.5%). The most dominant genus were *Candidatus Nitrosopumilus* (∼60%), *Nitrosopumilaceae* unclassified (∼16%), *Woesearchaeia* unclassified (∼10%), *Cenarchaeum* (∼8%), *Thermoplasmata* Marine group II (∼4%) and *Candidatus Nitrosopelagicus* (∼1.5%). Of the 316 ZOTUs, 119 ZOTUs were found at relative abundances higher than 0.01% in at least one sample but only 39 ZOTUs were kept when the relative abundance threshold was set to 0.1%.

Clustering of sponge host species as a function of similarity among their archaeal communities showed that, in most cases, species replicates

Table 6.1: Number of replicates, total ZOTUs, number and relative abundance of core ZOTUs, and diversity indices of the study species.

Species	n	Total ZOTUs	Core ZOTUs	% Rel. Ab. Core	Chao1 obs. ± SD	Chao1 est. ± SD	Shannon ± SD
Aaptos suberitoides	6	111	15	95.86	47.16 ± 5.42	109.46 ± 17.92	0.95 ± 0.51
Antho (Antho) sp.	1	74			74	107.06	2.22
Callyspongia sp.	2	102	32	84.26	67 ± 33.94	101.29 ± 20.08	1.16 ± 1.18
Clathria (Isociella) skia	2	72	28	98.16	50 ± 8.49	81.62 ± 7.95	0.86 ± 0.44
Dendroxea sp.	2	87	26	59.3	56.5 ± 27.58	104.05 ± 31.02	1.33 ± 0.79
Dysidea sp.	2	156	77	88.22	116.5 ± 36.06	136.52 ± 38.06	3.14 ± 0.47
Amphimedon sulcata	8	135	18	97.54	56.25 ± 7.44	98.01 ± 28.86	0.85 ± 0.33
Haliclona sp.	3	93	28	98.62	55.66 ± 8.5	160.09 ± 48.86	0.78 ± 0.31
H. (Gellius) toxia	2	104	44	76.4	74 ± 9.9	121.61 ± 57.1	1.77 ± 0.33
Mycale sp.	3	84	17	95.6	47.66 ± 10.50	123.11 ± 40.17	0.91 ± 0.54
Neofibularia sp.	4	110	33	96.72	65.25 ± 4.27	113.26 ± 37.73	1.21 ± 0.29
Niphates sp.	1	85			85	146.53	1.59
Phorbas sp.	1	113			113	158.12	2.55
Prosuberites proteus	2	140	45	58.02	92.5 ± 43.13	170.65 ± 93.92	2.54 ± 1.34
Terpios sp1.	1	65			65	135.07	1.3
Terpios cruciatus	1	190			190	239.49	4.33
Thrinacophora cervicornis	2	125	67	91.42	96 ± 8.49	130.14 ± 28.48	2.56 ± 0.68

were more similar to each other than to other sponge species, with the few exceptions of *Protosuberites proteus*, *Dendroxea* sp. and *Haliclona (Gellius) toxia* (Figure 6.1). Overall, host identity was the main factor structuring the sponge archaeal communities (*Adonis*, Pseudo-F: 4.67, R^2=0.74, p-val < 0.001), indicating a species-specific component of sponge archaeal composition.

Most of the species analysed were dominated by different genera of the *Thaumarchaeota* phylum. Among them, *Candidatus Nitrosopumilus* was the dominant genus in the majority of the species analysed (i.e: *Amphimedon sulcata*, *Aaptos suberitoides*, *Neofibularia* sp., *Terpios* sp.1 and *Antho* sp.) (Figure 6.1). *Cenarchaeum* was dominant only in the three replicates of *Haliclona* sp. with more than 50% of its relative abundance due to a single ZOTU (ZOTU2). Unclassified members of the *Nitrosopumilaceae* family were the major components of some replicates of *Dendroxea* sp., *Callyspongia* sp. and *Haliclona (Gellius) toxia*. Among the *Nanoarchaetoa* phylum, unclassified members of *Woesarchaeota* were the major components of *Mycale* sp. and *Clathria (Isociella) skia*.

6.4.2 The dominant ZOTU

The relative abundance of the most abundant archaeal ZOTU per sponge species varied from 2% (*Terpios cruciatus*) to 98% (*Aaptos suberitoides*). Some microbiomes were dominated by a single ZOTU, while in some others, the most abundant archaeal ZOTU did not reach 15% of relative abundance. Intraspecies variation in the dominant ZOTU occurred in *Amphimedon sulcata*, *A. suberitoides*, *Mycale* sp. and *Clathria (Isociella) skia*, but consistency among replicates was found for some species, such as *Neofibularia* sp. (ZOTU 4) and *Haliclona* sp. (ZOTU2) (Figure 6.1). Most of the dominant ZOTUs belonged to *Thaumarchaeota*, except for those of *Mycale* sp. and *C. (Isociella) skia*, which belonged to *Woesarchaeia* and ZOTU104 (*Euryarchaeota*), which dominated in a replicate of *Prosuberites proteus*.

6.4.3 The core community

We detected 5 ZOTUs present across all sponge individuals (ZOTU 10, 4, 26, 17, 2), which constituted the archaeal core community. All of them belonged to the phylum *Thaumarchaeota*, family *Nitrosopumilaceae*. ZOTU 4, 26 and 17 belonged to *Candidatus Nitrosopumilus*, ZOTU 10 was an unclassified member of the *Nitrosopumilaceae*, and ZOTU 2 belonged to *Cenarchaeum*.

The number of ZOTUs that constituted the archaeal core community of each sponge species varied from 15 (*Aaptos suberitoides*) to 77 (*Dysidea* sp) (Table 6.1). However, the relative abundance contribution of those core ZOTUs to the overall community was relatively high in most cases, accounting for more than 60% in the majority of the species. Thus, although the number of replicates analysed influenced the number of ZOTUs that constitute the core community, the relative abundance of the core ZOTUs remained stable regardless of the number of replicates. In this sense, the sponges with a higher number of replicates (*A. suberitoides* and *Amphimedon sulcata*), had a lower number (15 and 17, respectively) of core ZOTUs, but these ZOTUs accounted for more than 95% of the archaea relative abundance in the species. Thus, the small variable fraction was formed by low abundance ZOTUs, which indicated a high contribution of the few core ZOTUs to the overall archaeal community (Table 6.1).

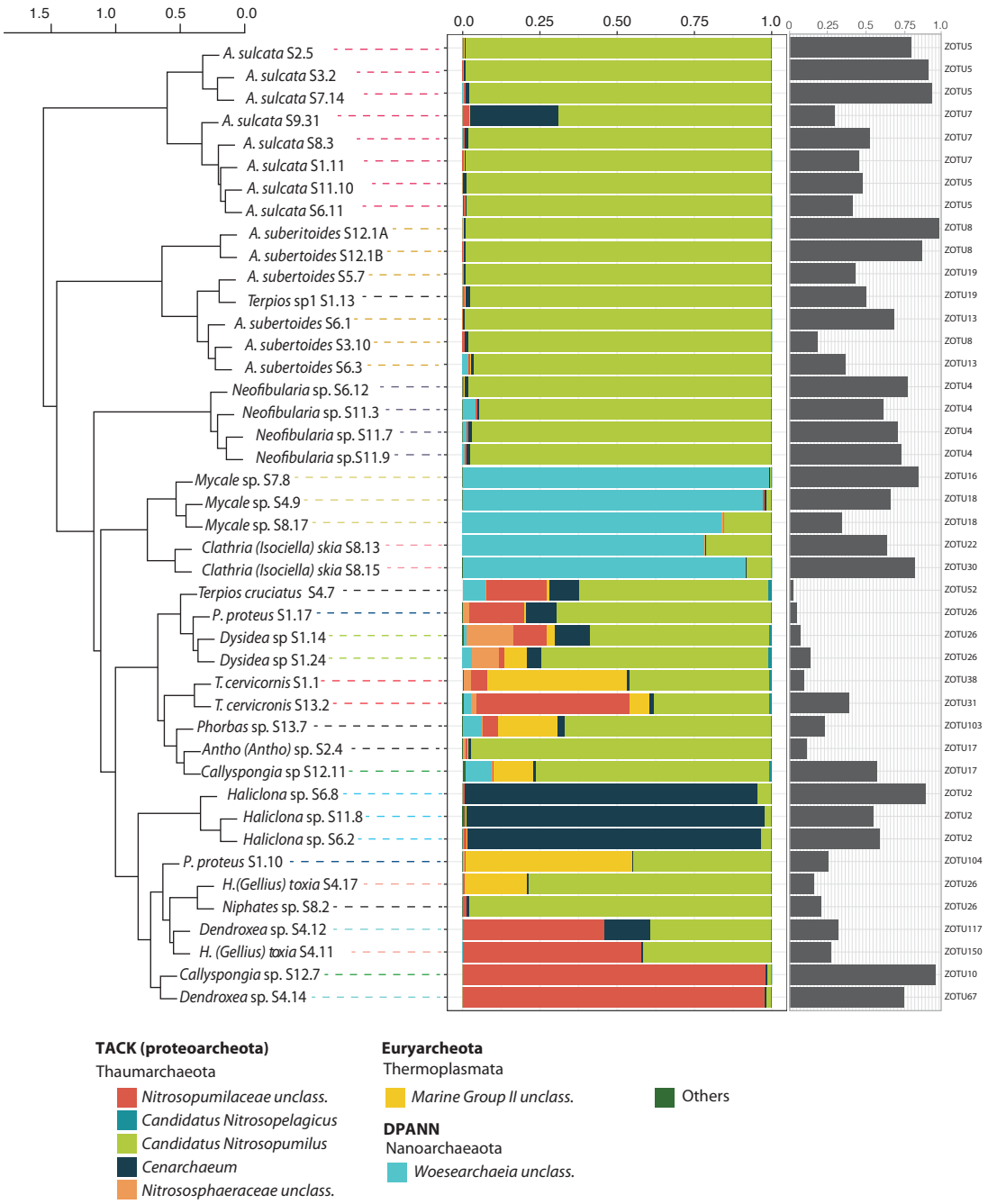

Figure 6.1: Relative abundance of archaeal community at the lowest taxonomic level for each sponge sample. Grey bars represent the relative abundance of the most dominant ZOTU for each sample. The dominant ZOTU number is indicated. Sponge samples are organized according to a hierarchical clustering (Ward method) based on Bray-Curtis dissimilarity matrix (at the level of ZOTU).

6.4.4 Diversity metrics

The Shannon diversity of sponge archaeal communities was in general low compared to the values obtained for bacterial communities (Figure 6.2), except for the species *Terpios cruciatus* that showed similar diversity for both biological domains. Most archaeal Shannon diversity ranged between 1 and 2, whereas values of Shannon diversity of the sponge bacterial communities ranged from 3 to 4. The Chao estimates showed that most species had values between 100 and 150 archaeal ZOTUs, except *T. cruciatus* with more than 200 ZOTUs (Table 6.1).

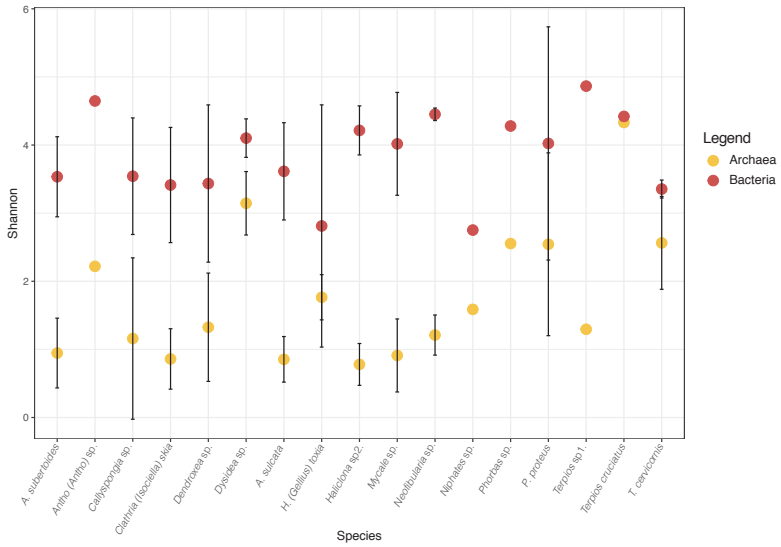

Figure 6.2: Mean Shannon diversity for Bacteria (red) and Archaea (yellow) of each sponge species. Bars represent Standard deviation. Bacteria data is obtained from Turon et al. (2018).

6.4.5 Phylogenetic analysis

Only 3 ZOTUs (ZOTU 19, 58 and 150) of the 39 ZOTUs added to the phylogenetic trees fell into monophyletic sponge clusters within the *Thaumarchaeota* phylum (Figure 6.3). ZOTU19, the dominant ZOTU in the species *Aaptos suberitoides* and *Terpios* sp1 fell, together with ZOTU58, within the SC174 while ZOTU150, dominant in a replicate of *H. (Gellius) toxia*, fell within the SC175 (Figure 6.3). None of the 5 ZOTUs of the sponge core belonged to a SC cluster. However, ZOTU2 (*Cenarchaeum*), was related (96.5% identity) to the *Cenarchaeum symbiosum* cluster, formed

by clones derived from *Axinella mexicana*. Blast search of the other core ZOTUs (ZOTUs 4, 26, 17, and 10) revealed that they were more similar (> 96% identity) to archaeal clones retrieved from environmental samples (data not shown) than to other sponge derived clones (Figure 6.3). Whilst some of the *Thaumarchaeota* abundant ZOTUs were also similar to other archaeal sequences retrieved from sponges, they did not fell within SC clusters (Figure 6.3). None SC existed within *Euryarchaeota* phylum (Simister et al., 2012a) and the 4 abundant *Euryarchaeota* ZOTUs from our dataset were distantly related to other sponge derived sequences (Figure E.1).

Noteworthy, all the *Woesarchaeota* ZOTUs, highly abundant in the species *Mycale* sp. and *Clathria (Isociella) skia*, fell all together and were far related to any existing *Woesarchaeota* sequence available in SILVA132 database (Figure 6.4). Additionally, blast search of these ZOTUs against NCBI database did not retrieve any sequence with higher identity than 85% (data not shown).

6.4.6 Functional prediction

The amount of sequences used in the prediction of functionalities was fairly high in the majority of our species (Figure E.2). Most of the species had a total fraction of at least 75% of reads that matched a "known" genome in the reference database. However, *Haliclona* sp., *Mycale* sp. and *Clathria* sp. had to be removed from the analysis as more than 80% of their reads could not be predicted (Figure E.2). This poor prediction rate was likely due to the dominant archaea in these sponges, *Woesarchaeota* and *Crenarchaetota*, for which no genomes were available in the reference database used. Several members of *Thaumarchaetoa*, for which genomes were available, dominated in all other sponge species and most of their reads could be used in the functional predictions.

All the study sponge species showed a similar pattern of archaea functionalities. The predicted metagenomes of archaeal communities revealed that pathways related to the metabolism (carbohydrate, cofactors and vitamins, amino acid and energy), environmental processing (membrane transport), translation and cellular processes (cellular community-prokaryotes) were the most abundant (Figure 6.5). However, functions related with defence

and competition mechanisms such as metabolisms related to biosynthesis of secondary metabolites (terpenoids and polyketides) and drug resistance (antimicrobial) functions were also present, and are purportedly involved in microbe-microbe interactions.

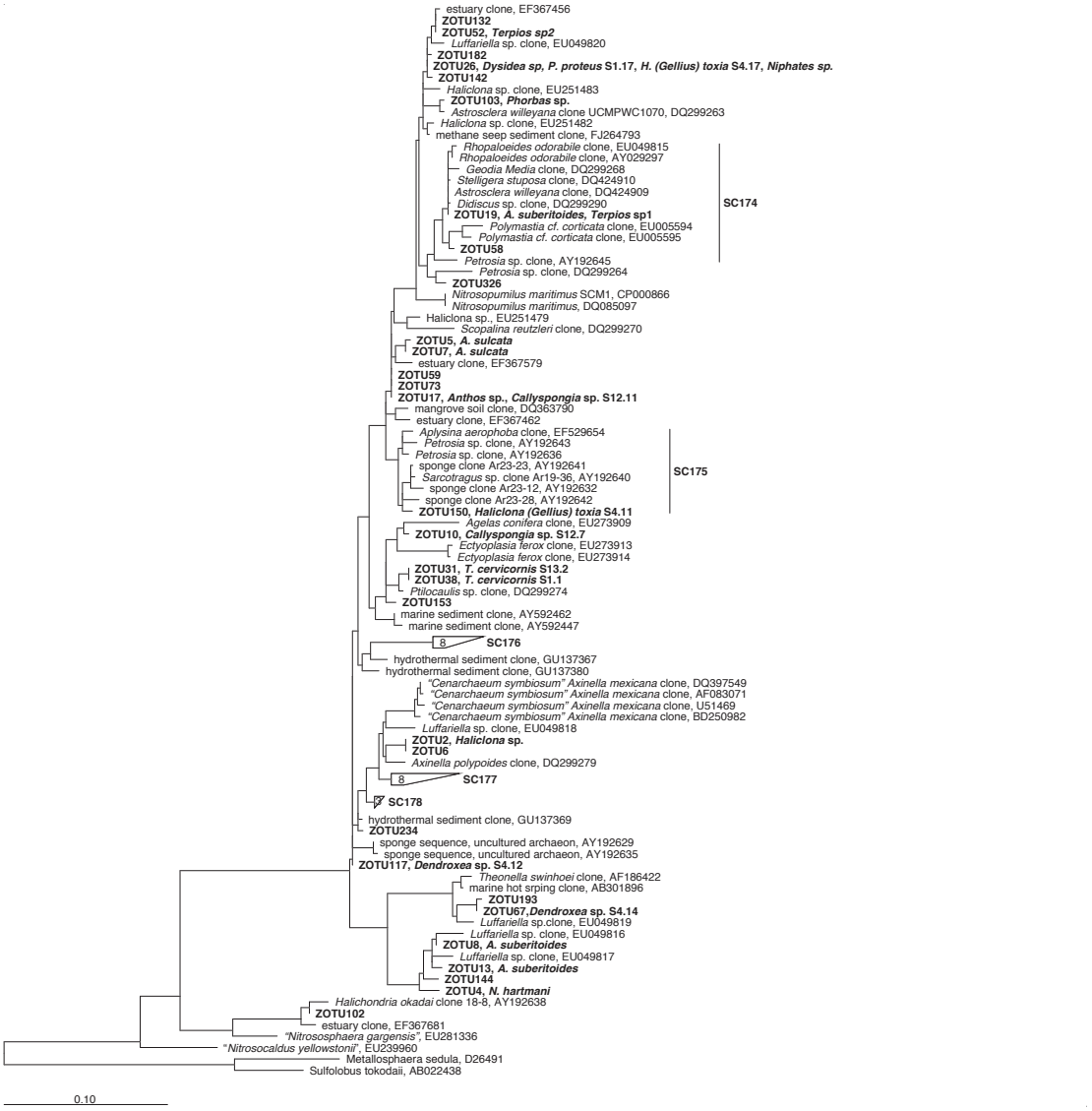

Figure 6.3: 16S rRNA-based phylogeny of sponge associated *Thaumarchaeota* obtained in Simister et al. (2012a). The displayed tree is a maximum likelihood tree constructed based on long sequences only (>1200 bp). Sponge-specific monophyletic clusters are indicated (SC num). ZOTUs obtained in this study were added using the parsimony interactive tool in ARB and are indicated in Bold. Sponge species replicate is indicated next to the ZOTU if that ZOTU is the dominant in this sponge.

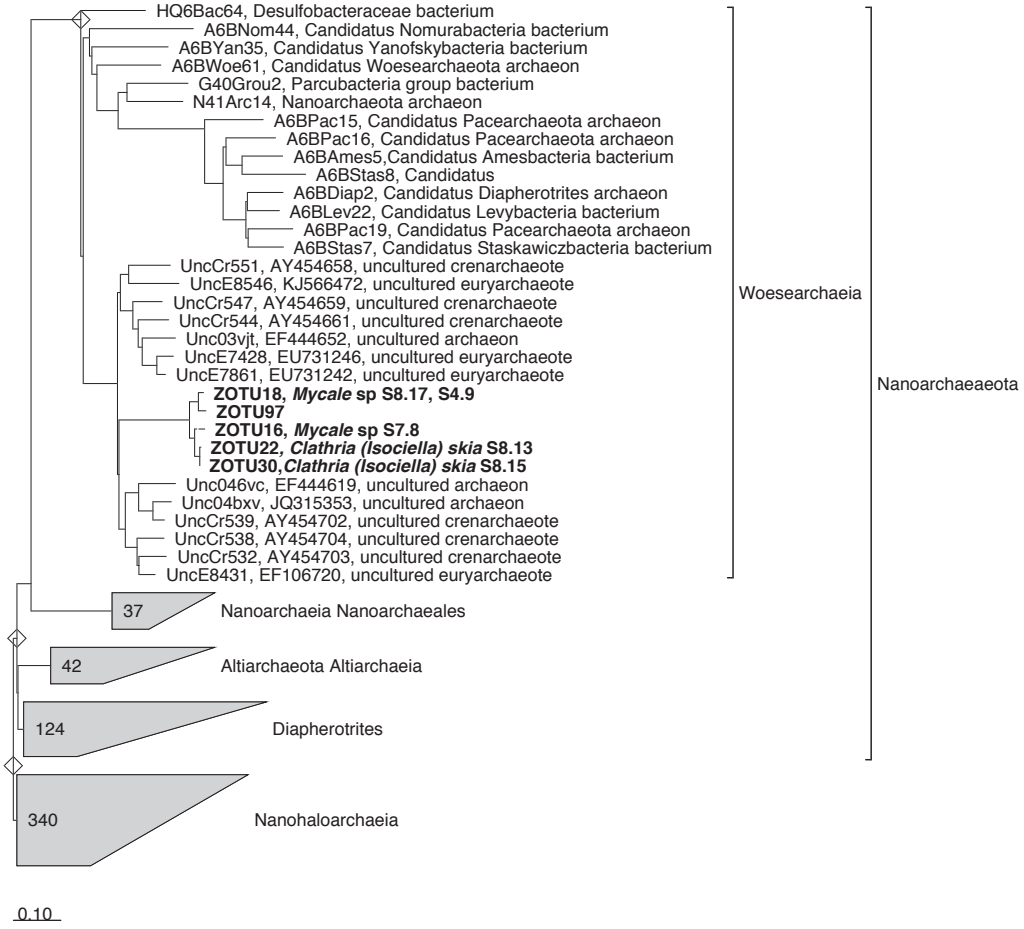

Figure 6.4: 16S rRNA *Woesarchaetoa* tree imported from ARB (SILVA SSU Ref NR 132 database). ZOTUs obtained in this study were added using the parsimony interactive tool in ARB and are indicated in Bold. Sponge species replicate is indicated next to the ZOTU if that ZOTU is the dominant in this sponge.

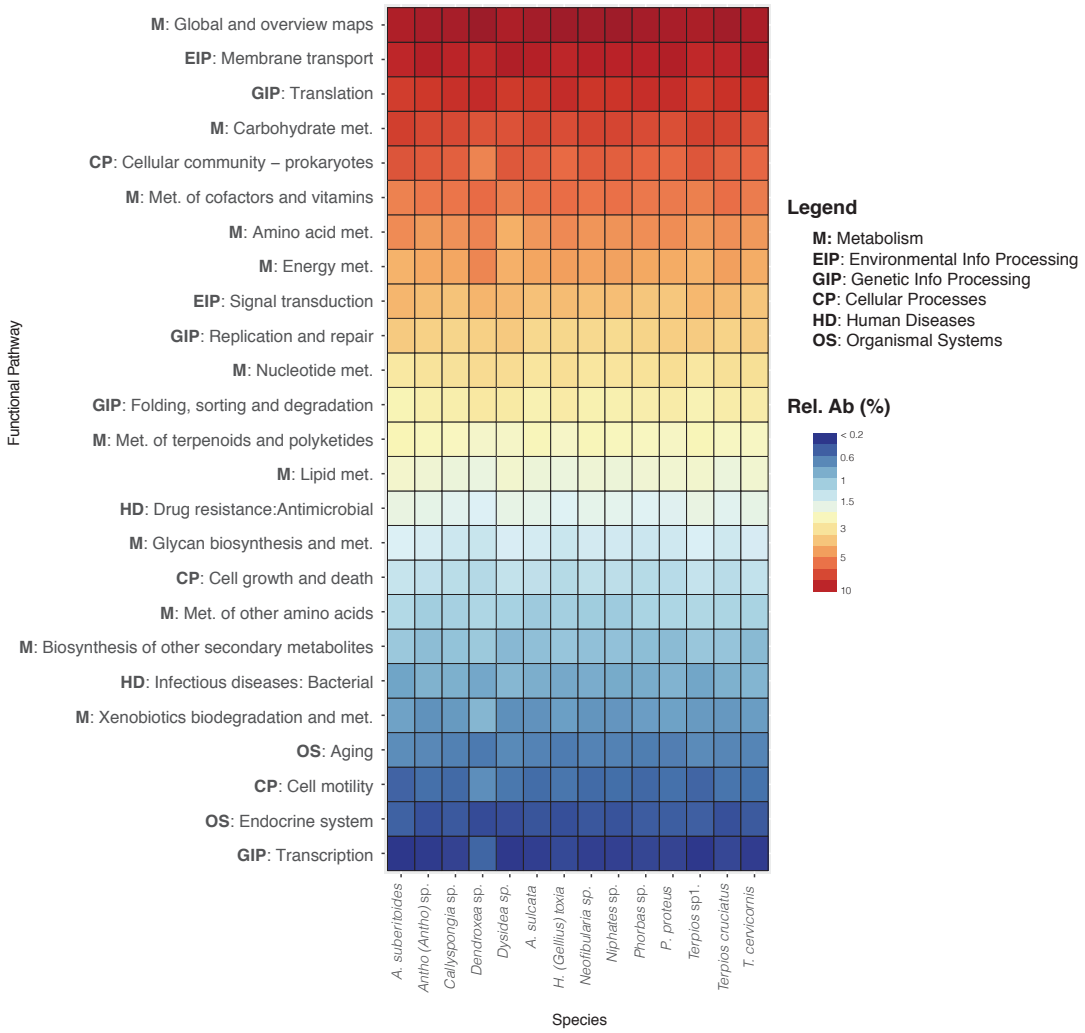

Figure 6.5: Heatmap of the most abundant functional pathways obtained from Tax4fun for each sponge species. Abundance is represented in the colour temperature bar.

6.5 Discussion

6.5.1 Archaea amplification constrains

A critical step for accurate rDNA amplicon analysis is still the choice of appropriate primers (Klindworth et al., 2013), which has been proven particularly difficult for Archaea (Dupont et al., 2013). The use of inappropriate primer pairs, can lead to under-representation or selection against single species or even whole groups. Thus, between studies comparison should be taken with care as they could lead to equivoque conclusions, in particular

when archaea composition and abundance are inferred from massive amplicon sequencing using primers primarily designed for bacteria (Klindworth et al., 2013) and *vice-versa*. The study outcome may also vary as a function of the targeted region of the 16S rRNA gene. The use of two couple of primers, one for bacteria amplification exclusively and the other for bacteria plus archaea identification has been recommended (Dupont et al., 2013) to obtain a more complete microbiome landscape.

Amplification can be problematic even with archaea directed primers, as it occurred with our study sponges. Only 52 out from 68 samples belonging to 17 sponge species provided good-quality archaea sequences and from these, only 43 species retrieved over 5000 reads. However, the Archaea sequences obtained, despite possible primer constrains, may provide some useful information on the Kingdom characteristics.

6.5.2 Sponge archaeal communities

The archaeal communities of our study sponges showed a relatively high species-specific component, as previously reported for other sponge species from other locations (De Mares et al., 2017; Kennedy et al., 2014; Polónia et al., 2014; Rodríguez-Marconi et al., 2015; Zhang et al., 2014) and for sponge-associated bacteria (Lee et al., 2011; Schmitt et al., 2012; Turon et al., 2018). However, a higher intra-species variability for both domains was found in the sponges *Dendroxea* sp. and *Haliclona (Gellius) toxia*, with some replicates that did not cluster together (also see Fig 3 in (Turon et al., 2019a), for comparison with bacteria). Conversely, *Clathria (Isociella) skia* and *Mycale* sp., showed a much higher species-specific component for the archaea than for bacteria communities. The opposite was reported for sponges from the Red Sea (Lee et al., 2011) in which high species specificity was observed for bacterial communities but not for archaeal communities (Lee et al., 2011).

Taxonomic affiliation of the archaeal community showed that *Candidatus Nitrosopumilus* was the most abundant taxon in the majority of the study species, especially, in *Amphimedon sulcata*, *Aaptos suberitoides* and *Neofibularia* sp., where a few ZOTUs of this species accounted for most of the archaeal relative abundance. Dominance of this species has also been

recorded in a calcareous sponge (Lopes et al., 2019), in Antarctic sponges (Rodríguez-Marconi et al., 2015), and in three *Ircinia* species and *Mycale laxissima* from the Mediterranean and Caribbean seas (Zhang et al., 2014). *C. Nitrosopumilus* was also highly abundant in other 18 sponges from the same region in Vietnam amplified with bacteria primers (Dat et al., 2018).

These authors found many aerobic nitrifiers among both symbiotic bacteria and archaea taxa (*C. Nitrosopumilus* (AOA), *Nitrosococcus*, *Nitrosomonadaceae* (AOB), and *Nitrospira* (NOB)) and pointed out the putative relevance of aerobic nitrification in those sponges. In addition to *C. Nitrosopumilus*, we also retrieved members of *Cenarchaeaum*, which had not been previously detected in the Vietnamese species (Dat et al., 2018). Particulary, ZOTU2, affiliated to *Cenarchaeaum*, was consistently found in all the sponge samples studied, although it was solely abundant in *Haliclona* sp., where it accounted for more than 50% of the archaea relative abundance. Members of *Cenarchaeaum* have been widely reported to be associated with sponges. Usually, few archaeal OTUs dominated the archaeal community of sponges from deep-sea environments (Jackson et al., 2013; Kennedy et al., 2014), polluted estuarine waters (Turque et al., 2010), arctic deep-waters (Pape et al., 2006) and Indonesian reefs (Polónia et al., 2014). Indeed, the description of *Cenarchaeum symbiosum* associated to the marine sponge *Axinella mexicana* represented the first described symbiosis between sponges and archaea (Preston et al., 1996). Since then, many other sequences retrieved from sponge samples had been found to be identical or similar to that of *C. symbiosum*. The closest sequence retrieved by blast search of our ZOTU2 (*Cenarchaeum*) had 96.5% identity to the archaeal clone (Ar20-3) found in an unidentified marine sponge from Korea (AY192631.1), which was 100% identical to *C. symbiosum* (Lee et al., 2003). Thus, although different phylotypes of this archaea might exist (Margot et al., 2002), its presence in all sponge samples gives support to the cosmopolitan distribution of *Cenarchaeum* in sponges. However, the exclusive presence of *C. symbiosum* in some sponges such as *Petromica citrina* (Turque et al., 2010) and *A. mexicana* (Preston et al., 1996) is rather an exceptional pattern in our samples.

Interestingly, few ZOTUs belonging to unclassified members of order

Woesearchaeia dominated the microbiomes of *Mycale* sp. and *Clathria (Isociella) skia*. To our knowledge, this linage has been only reported previously in two sponge species from the Mediterranean and two species from Caribbean Sea (De Mares et al., 2017). These authors detected up to 8 archaeal phyla, with *Crenarchaetoa*, *Euryarchaeota*, *Thaumarchaeota*, and *Woesarchaeota* being present in all samples. However, *Cenarchaeaceae* family was by far, the most dominant in their study species (De Mares et al., 2017). Blast search of these *Woesearchaeia* abundant ZOTUs did not retrieve any sequence with similarity higher than 85%, emphasizing the novelty of this recently described group (Castelle et al., 2015). Moreover, the dominant *Woesarchaeia* ZOTUs found in our sponges clustered together into the same monophyletic group. This group was far from any environmental archaeal clone sequence, pointing the putative existence of a SC cluster in the *Woesarchaeota* group. Members of *Woesarchaetoa* have only been detected in sponges with the advent of new sequencing technologies (Illumina), and thus only short amplicon reads are available. More studies are required to obtain long sequences of this group to be used in phylogenetic reconstructions to ascertain whether or not a SC cluster in the *Woesarchaetoa* exists.

Finally, *euryarchaeotas* have been typically reported in seawater (Alex and Antunes, 2015; Jackson et al., 2013; Lee et al., 2011) and are usually absent (Jackson et al., 2013; Turque et al., 2010) or detected at low abundances in sponges (Dupont et al., 2013; Holmes and Blanch, 2007; Kennedy et al., 2014; Polónia and Cleary, 2019; Webster et al., 2001). Only 10 *Euryarchaeota* sequences were retrieved from sponges in the revision from 2010 data in Simister et al. (2012a), and none SC cluster was detected for this group (Simister et al., 2012a; Taylor et al., 2007). However, the first pyrosequencing study of sponge archaeal communities (Lee et al., 2011) contributed to increase the number of *Euryarchaeota* sequences from sponges, and since then, they have been more frequently reported (De Mares et al., 2017). In our study sponges, *Euryarchaeota* of the *Marine Group* II were detected in several sponges being particularly abundant in *Thrinacophora cervicornis*, *Phorbas* sp., *Haliclona (Gellius) toxia* and *Protosuberites proteus*.

6.5.3 Archaeal diversity

Many of the studied species presented an archaeal community mostly dominated by just few ZOTUs, as already reported for other sponge species (Jackson et al., 2013; Kennedy et al., 2014; Polónia and Cleary, 2019; Polónia et al., 2014). However, some other species such as *Terpios cruciatus*, *Protosuberites proteus*, *Dysidea* sp. and *Thricanophora cervicornis*, presented the highest Shannon diversity indices, with equal contribution of many ZOTUs to the overall archaeal community. The number of observed ZOTUs per sample varied from 47 to 190, which agrees with the number of archaea OTUs reported for the Red Sea sponges *Hyrtios erectus*, *Stylissa carteri* and *Xestospongia muta* (Lee et al., 2011), but it was lower than that of two *Dysidea* and *Aplysina* species, the latter *A. cauliformis* with up to 370 archaeal OTUs (De Mares et al., 2017).

In our the study species, Shannon diversity was always higher for bacteria than for archaeal communities. The dichotomy between HMA and LMA, which was clear for the bacterial domain (Turon et al., 2018), could not be confirmed for the archaeal communities. In contrast to what has been reported for sponge associated bacteria, which show contrasting bacteria composition and specific bacterial phyla in HMA and LMA species, neither specific archaeal phyla nor Shannon diversity differentiated HMA and LMA species in our study. However, only two HMA species (*Aaptos suberitoides* and *Neofibularia* sp.) could be analysed in the present study, and thus, the withdrawn conclusions should be taken with care. Bayer et al. (2014) found up to 4 orders of magnitude higher archaeal gene numbers in HMA than in LMA sponges, although they also found an exception, as *S. carteri*, a LMA sponge, showed archaeal numbers within the range of HMA sponges. Moreover, the correspondence between low abundance of microorganisms and low phylum-level diversity found for the bacterial communities of LMA sponges was not confirmed for the archaeal and eukaryote domains in the two *Dysidea* (LMA) species (De Mares et al., 2017).

6.5.4 The archaeal core community

The archaeal core community of sponge species was similar to the bacterial core community in terms of relative abundance, however, it was mostly dominated by a few abundant symbionts. The existence of only 5 ZOTUs in the archaeal core community of the 17 study sponge species suggests a potential functional importance of these microbes for the holobiont functioning, although none of them belonged to the known SC archaeal clusters. The ubiquity of *C. symbiosum* in sponges has been long recognized (Jackson et al., 2013; Kennedy et al., 2014; Preston et al., 1996; Turque et al., 2010) and ammonia oxidation within the sponge, which could play an important role in ammonia detoxification, has been related to this *Cenarchaeal* symbiont (Steger et al., 2008).

The presence of AOA in sponge larvae suggested the vertical inherence of *C. symbiosum* (Steger et al., 2008). However, we found 4 other ZOTUs belonging to *Nitrosopumilales* in the sponge core, whose closest sequences were clones derived from environmental samples, which points to a purported horizontal acquisition of these archaea from the surrounding seawater. Overall, a combination of both, vertical and horizontal transmission of archaeal symbionts might exist in sponges, as already reported for the bacterial domain (Björk et al., 2019; Sipkema et al., 2015).

6.5.5 Predictive metagenomic analysis

Functional prediction of sponges dominated by *Woesarchaeota* and *Cenarchaeum* could not be made, as sequences of those groups did not match any close genome in the database used. This fact puts into relevance the lack of knowledge of the archaeal domain, and specifically for the newly described *Woesarchaetoa* group, for which no genome is available yet.

Most of our Archaea ZOTUs showed similar functional patterns, which is likely due to a similar archaeal composition in the species analysed, as archaea functional capabilities have been predicted from closely related metagenomes. Similar approaches have been used to predict functionalities of sponge archaeal communities in the sponges *Biemna fortis* (Polónia and Cleary, 2019) and *P. magna* (Lopes et al., 2019). Members of the *Thau-*

marchaeota phylum also dominated those species and the most abundant pathways were related to metabolisms of nitrogenated compounds, energy metabolism, and cellular maintenance (Lopes et al., 2019). Our results also showed a high abundance of heteretrophic metabolisms, followed by environmental and genetic information processing, including metabolism of secondary metabolites likely involved in allopatric interactions among microorganisms.

6.6 Conclusions

The analysis of the archaeal communities of 17 tropical sponge species allowed us to record patterns of diversity and stability, which, as for bacterial communities, depended on the sponge species. Other traits such as high relative abundance of core communities run in parallel for archaea and bacteria in sponge microbiomes. In contrast, the number of ZOTUs that formed the core species community was lower for archaea than for bacteria and the dichotomy between HMA and LMA, which was clear for the bacterial domain could not be confirmed for the archaeal communities. Many *Thaumarchaeota* and *Euryarchaeota* sequences obtained from the study sponges were close to sequences from environmental samples and, thus, they are presumable acquired from the surrounding seawater. However, a few ZOTUs belonging to unclassified members of order *Woesearchaeia*, which dominated the microbiomes of two poecilosclerid species analysed, formed a monophyletic group far from any environmental archaeal clone sequence. This group might correspond to a SC cluster within the *Woesarchaeota*, although long sequences of these ZOTUs are required to be used in phylogenetic reconstructions to confirm its monophyly.

Chapter 7

General Discussion

In this thesis, we have surveyed the shallow sponge assemblages from an Indo-Pacific tropical bay, which have well-preserved and impacted habitats in a small geographical area, with the aim of selecting representative models to study sponge symbioses in both types of habitats. Since the area was still poorly explored, in particular for the sponge fauna, we inevitably found a high percentage of likely new species, which have been described and illustrated in detail. Species diversity and composition of sponges changed as function of the environmental quality, as previously reported for other organisms in the same area (Tkachenko et al., 2016) and for sponges in other geographical areas (Easson et al., 2015; Powell et al., 2014). We particularly focus on the possible role that, together with sponge biology, physiology and reproduction, some sponge-associated bacteria might play in colonization and success of sponges in *a priori* stressful environments, and consequently, in their final ecological distribution.

7.0.1 The sponge as holobiont: Symbiosis

The studies of different aspects of the sponge symbioses with members of bacteria, archaea, and polychaetes conducted in this thesis have deepened our understanding on these complex associations. The thesis results, when taken all together, can withdraw some general features of the symbiotic lifestyle, which are dealt with in the five thesis chapters.

Species-specificity

An important species-specificity has been revealed in all the sponge symbioses studied, with both macro and microsymbionts, which indicates that natural selection and evolution forces operated in the establishment of these associations. We detected a high species-specific component in the microbial

communities of both, sponges (bacteria and archaea) and polychaetes (bacteria). This specificity has been largely reported for sponge microbiomes (Lee et al., 2011; Reveillaud et al., 2014; Schmitt et al., 2012) and for other invertebrates (Ainsworth and Gates, 2016; Derycke et al., 2016; Erwin et al., 2014), suggesting that it might be a widespread trend in marine invertebrates and pointing to relevant roles of these microbes in the holobiont functioning. However, the environment appears to modulate to some extent the species-specificity of symbiotic eukaryotes (sponge-polychaete) more than that of the prokaryote-eukaryote associations. This may be explained by a differential dispersion of macro- and micro-organisms. Polychaetes might have a more limited dispersion range than water microbes and thus, sponges hosting a polychaete species may not encounter the same worm species in other locations. Conversely, ecological niches use to be large for microbes. Indeed, the Baas Becking old theory that states, "*Everything is everywhere, but the environment selects*" (Baas-Becking, 1934) may facilitate understanding of the establishment of highly specific microbe symbiosis across sponge species from distant geographic regions, provided that mechanisms of bacteria selection are specifically fixed. In the frame of this hypothesis, the assumption of horizontal transmission of microbes gains support.

The symbiont transmission

Sponge-polychaete associations may depend on the geographical/ecological distribution of the involved species (Martin and Britayev, 2018). The way by which polychaetes select a given sponge species is unknown for our study associations but chemical metabolites, released by the host, specific morphologies, or niche selection are reported to play a role in host selection (Crocker and Reiswig, 1981; Davenport and Hickok, 1951). Moreover, these chemical cues might attract more than one polychaete species, in particular when they are taxonomically close, leading to a less species-specific symbiosis.

The maintenance of a uniform symbiont microbial community has been thought to lie on the efficient transmission of symbionts from parents to offspring (Sharp et al., 2007). However, our data (Turon et al., 2018) and previous studies (Björk et al., 2019; Fieth et al., 2016) point to the acquisition of selected microbes from the seawater. Thus, we propose that recognition

mechanisms, allowing the selection of specific symbionts from the seawater, may be widespread to conform sponge microbiomes, rather than (or besides) proper symbiont transmission from parents to offspring. A species-specific microbiome might be shaped in parallel to the host speciation process by fixation of specific bacteria selection mechanisms. Once host speciation has occurred and selection mechanisms fixed, posterior species dispersion would generate similar microbiomes in geographically far individuals of the same species despite different environmental conditions (Montalvo and Hill, 2011). In this context, sponge bacteria coevolution only would occur at the species level and this might explain the strong differences found in microbiomes of congeneric species, which has been repeatedly defined as a lack of phylogenetic signal (Blomberg et al., 2003). However, further genomic studies may illuminate the particular host genes involved in the animal-bacteria crosstalk (Fieth et al., 2016).

We assessed microbe acquisition in sponges by comparing the permanent (the core) bacteria present in a sponge species with the seawater bacteria surrounding the sponges (Turon et al., 2018). We came to this method because, with the advent of new sequencing technologies, sequences that were first considered exclusive of sponges (SC clusters) were also detected, albeit at low abundances, in seawater (Taylor et al., 2013; Webster et al., 2010). This finding changed the widespread perception that such stable species-specific bacterial communities could only be maintained across generations through vertical transmission (Sharp et al., 2007). When focusing on the overlap between sponge and water sequences, we found differential relative abundances of shared sequences in both environments with the particularity that microbes present at low relative abundances in seawater were significantly enriched in the sponge tissues (Turon et al., 2018). We used the same approach to assess the differences in relative abundances of shared microbes between the host sponge and the polychaetes feeding on the host, as food has been reported to be an importance source of potential symbiotic microbes in other animals (Heiman and Greenway, 2016; Walburn et al., 2018). Differential relative abundances of the shared bacteria between polychaetes and their food (the sponge) were found, as it occurs in other biological models (Nyholm and McFall-Ngai, 2004; Roeselers et al., 2011;

Sullman et al., 2012; Walke et al., 2014).

Our results support the above-mentioned tenet that states: "*Everything is everywhere, but the environment selects*" (EiE, BES), as recently supported by other studies on microbial communities (Cleary et al., 2019; Troussellier et al., 2017). Sponges and polychaetes represent different environments that may specifically select certain microbes from their respective surroundings: seawater and sponge host. Indeed, Troussellier et al. (2017) postulated an integrated view of interactions between macro and microorganisms that was named "*Sustaining the rare*" (StR), which suggests that macroorganisms favour the maintenance of diversity of the rare microbial biosphere. The highly differential relative abundances found in our study systems between "sponge-seawater" and "sponge-polychaete" bacterial communities certainly matches this theory, as rare microbes in the surrounding habitat are usually found at high abundances in the studied hosts (Figure 7.1).

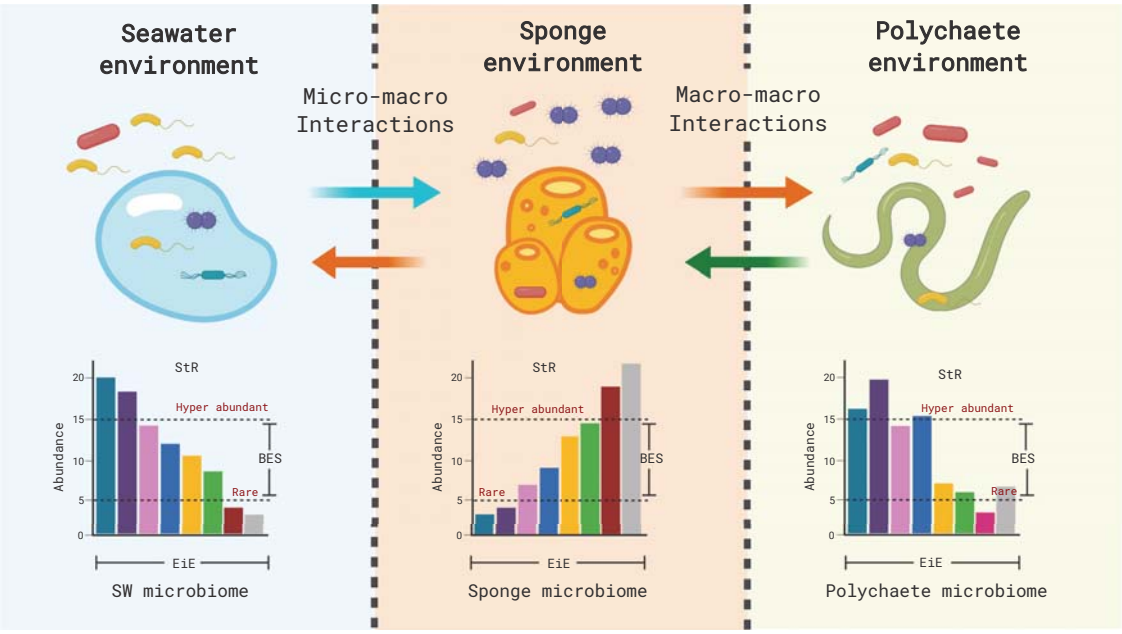

Figure 7.1: Schemtatic representation of the shared microbes of our study systems involving Micro-Macro interactions (SW-Sponge) and Macro-Marco interactions (Sponge-Polychaete). EiE "Everything is Everywhere, BES "But the Environment Selects". StR "Sustaining the Rare". Figure adapted from Troussellier et al. (2017). Note that the sponge enriches rare bacteria from the SW and the polychaete enriches rare bacteria from the sponge.

In the case of the archaeal domain, we used a different methodology to approach theories of archaea transmission in sponges due to the lack

of data on seawater archaea composition. The Sponge-Specific clusters (SC) represent clades of microbes that are absent from the environment and are specifically adapted to live as sponge symbionts (Taylor et al., 2007), and thus, they are likely vertically transmitted. Although deep sequencing allowed the detection of some of these SC clusters as rare members of the marine environments (Taylor et al., 2013; Webster et al., 2010), little research has been conducted in this regard for the archaea domain. We used a phylogenetic approach to include the most abundant sequences retrieved from our study into the phylogenetic trees of the archaeal domain containing the SC clusters (Simister et al., 2012b). Our results showed that the majority of our sequences were more closely related to environmental than to sponge derived sequences, with the exception of only 3 ZOTUs. This suggests that horizontal transmission is also occurring for many representative of the archaeal domain. However, new groups of archaea are being discovered (Castelle et al., 2015), and thus, new SC clusters might arise from these new linages. To date, we cannot withdraw consistent conclusions regarding the *Woesarchaeaota* in sponges, as although some representatives of our *Woesarchaeaota* seem to form a SC group, these sequences could be detected in a near future in environmental samples by increasing sequencing deep. Certainly, the use of proper primers and deep sequencing will allow clarifying whether new SC clusters exist in the archaea linages that are being discovered.

To summarize, our results perfectly match and, thus, support the hypothesis formulated by Cleary et al. (2019) that states "*All microbes are found everywhere due to the immensity and persistence of this seed bank, and apparent local or host-associated endemism is merely a result of insufficient sequencing. Community structure is, thus, a function of relative abundance rather than the presence or absence of certain microbial taxa*".

True *vs.* transient symbionts: core concept

A key issue in symbiosis field is disentangling which ones among the associated organisms represent true or transient symbionts, especially in filter-feeder invertebrates inhabiting marine environments, which are in permanent contact with potential symbionts. Recently, some genomic studies

have addressed this issue by analysing the putative genomic features that may be indicative of free-living or host-associated life strategies (Burgsdorf et al., 2019; Karimi et al., 2019). In the absence of genomic information, on our target symbioses, we have restricted the core concept (Astudillo-García et al., 2017) to those microbes that were consistently present across all replicates of a given host species. With this restrictive core concept we attempted to gain insights into the permanent stable symbionts associated to either sponges or polychaetes. In the analysed species, we only considered core bacteria those that were consistently present across all replicates of a given host species. The core approach proved to be robust (Astudillo-García et al., 2017) in disentangling the microbiome fraction that is constantly associated to a host sponge and thus, likely mutualistic, commensalistic or symbiotic, from that formed by merely transient bacteria. By focusing on the core communities, we analyze constant specific relationships between certain bacteria and their eukaryotic hosts (whether mutualistic or not).

Overall, the bacterial and archaeal core communities of the study sponge species and the bacterial core communities of polychaete species were relatively large and represented a high proportion of the overall microbiome in most cases, pointing to a functional relevance of these purportedly "true" symbionts in the study hosts (Turon et al., 2018, 2019b). However, the core of all the sponges analyzed was null or really small: none bacterial ZOTU and only 5 archaeal ZOTUs were found across all sponge samples. Conversely, in the case of polychaetes, 44 bacterial ZOTUs were shared by all samples (belonging to 6 polychaete species). These differences in the microbial core size between sponges and polychaetes may be related to the much larger genetic distances among the study sponges belonging to different Orders and families than among the polychaetes, which belonged to the same genus.

The benefits and costs

Although it was not the scope of the present thesis, with some of the results obtained, we may indirectly hypothesize on some plausible benefits of the micro-macro interactions for one or more partners as well as from the sponge-polychaete associations. Certain bacteria that are associated to

the dominant sponges in the adverse habitats may facilitate the holobiont success by helping sponges to cope with some pollutants or by reducing heavy metals, sulfates, nitrates and chromates to less toxic chemical compounds (Fredrickson et al., 2008). Moreover bacteria might represent a source of nutrients for the sponges as they can make sulphur or nitrogen available for their hosts through the participation in the biogeochemical cycles (Goeij et al., 2013). However, instead of focusing on functions of specific bacteria, which we cannot demonstrate with the present data, we suggest that bacteria community structure itself might provide some benefits for the holobiont success. In this sense, low diversity and low core size microbiomes might represent an advantage to live under stressful environmental conditions, where only variable, low diversity assemblages can proliferate. Thus, sponges with lower microbial diversity appear to be more resilient to perturbations.

For the archaeal communities, most of the studies pointed to the nitrifying capability of archaea as the main benefit for the sponge holobiont. Basically, the ability to metabolize nitrogenous waste products from the sponge has been proposed to represent a mutual benefit for the archaea-sponge relationship (Steger et al., 2008; Tian et al., 2014). In our study, we assessed the predicted functional capabilities of the archaeal consortium pointing to a potential role of these microbes in different metabolic pathways or in the biosynthesis of secondary metabolites with putative implications in microbe interactions.

Finally, as for the polychaete-sponge associations, we have proven the commensalistic nature of these interactions, as sponge spicules were found inside the polychaetes. Other benefits for polychaetes, besides feeding on the host, such as protection from predators could be proposed but remain to be demonstrated. On the other hand, the benefits for the sponge host are unknown. The polychaete presence in the sponge did not seem to harm the host suggesting that these interactions might not be parasitic, but whether they represent a mutualistic relationship or not remains to be elucidated.

The nested ecosystems: ecological roles in the marine benthos

Finally, we would like to place our results on mulipartner symbioses into an ecological perspective in the frame of the nested-ecosystems theory which

states that, the patterns observed in the holobiont might have an impact at community and ecosystem scales (McFall-Ngai et al., 2013; Pita et al., 2018). We particularly focus on the holobiont success in the face of anthropogenic impacts, such as eutrophication derived from mariculture. Sponge species were completely different at the well-preserved and impacted sites. Only *A. sulcata* was equally abundant in the two contrasting habitats explored, and its microbiome did not show differences between replicates present in the two habitats, which indicates that microbiomes are resilient to changes in the environmental conditions.

In our study sponges, bacterial communities were highly stable regardless of the environment, whereas some of their associated polychaetes varied depending on the sampling location. Environmental resilience to different habitat conditions was certainly true for bacterial communities of *A. sulcata*, the solely species that was found equally abundant in the two contrasting habitats explored.

Moreover, the Anna Karenina concept, which states that intraspecific variability is higher in dysbiotic than in healthy individuals (Zaneveld et al., 2017), has also been applied to coral species (Ahmed et al., 2019; Pollock et al., 2019). In this study (Turon et al., 2019a), we propose that the same principle can be applied at community level. The sponge microbiomes of the perturbed habitats are in general, more variable (their cores are smaller) than microbiomes of well-preserved areas.

The "sponge" holobiont interacts with the biotic and abiotic factors of the benthic community it inhabits by exchanging propagula, microbes and metabolites with its surroundings. These activities modify the environment and can produce indirect effects on the benthic assemblages through facilitation or competition processes, contributing to the final structure and functioning of the ecosystem.

7.1 General Conclusions

Chapter 2 Taxonomy

- Accurate host taxonomy is fundamental for a deep understanding of their symbiotic relationships with macro-and microorganisms. However, as the expertise necessary for species identification of some marine invertebrates such as sponges, is time consuming, this has not been always performed. The true sponge biodiversity in Nha Trang Bay is still far to be known and many new species are still being discovered, such as the ones presented in this thesis.

Chapter 3 Sponge microbiomes: general concepts

- Sponge microbiomes are species-specific, being host identity the most relevant factor structuring the bacterial communities of sponges.
- Sponge species have a large bacterial core, mainly formed by few abundant ZOTUs present across all species replicates
- There is a high percentage of overlap between seawater bacteria communities and sponge bacteria, which points to horizontal transmission as a relevant acquisition mechanism of those microsymbionts in sponges.
- Specific recognition mechanisms may be acting in sponges to specifically enrich some SW bacteria in their tissues and not others. These mechanisms may be species-specific and between species differences in bacteria selection would account for differences in their microbiomes. Shared selection mechanisms might also contribute to explain microbiome differences between HMA and LMA sponges.

Chapter 4 Sponge microbiomes and ecological distribution

- Sponge assemblages differ depending on the environmental conditions, being more diverse in the study well-preserved reefs and dominated by few abundant species in the impacted reefs.
- Sponge microbiomes do not change as function of the environment, so that the microbiomes of species present in two contrasting conditions remains stable.

- Microbiomes of the sponges inhabiting impacted reefs have higher intra-species dispersion, lower core size and lower bacterial diversity than the microbiomes of sponges living in the well-preserved environments.
- Sponge microbiome might play a role in determining the presence and proliferation of sponge species in certain habitats.
- Microbial communities associated with sponges mimic the reduction of diversity showed by animal or plant assemblages at the ecosystem scale.

Chapter 5 Sponge and polychaete relationship

- The sponge-polychaete associations are species-specific but can be ecologically modulated.
- Microbiomes of polychaetes are species specific but can be modified to a certain extent by their diet (the sponge microbiome).
- Polychaetes select, incorporate, and enrich a part of the sponge microbiome to conform their specific microbiome.

Chapter 6 Archaeal communities

- The sponge archaeal communities were also species-specific with a few ZOTUs accounting for the majority of the species core community.
- Most of the obtained archaeal sequences were closer to environmental sequences than to sequences belonging to the previously described SC clusters, and thus, presumably acquired from the surrounding seawater.
- *Woesearchaeia* were found to be major members of two sponge species, and formed a monophyletic group far from any environmental clone sequence, pointing to the putative existence of a SC cluster in this group.

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Appendix A

Chapter 2 Supporting Information

Table A.1: PCR conditions conducted in 50 μl reactions containing 1 ng of template genomic DNA, 5 μl of 10x PCR buffer (containing 1.5 mM $MgCl_2$), 2 μl of dNTP mix (10 mM), 2 μl of bovine serum albumin, 1 μl pf each primer (10 mM) and 0.4 μl of Taq DNA polymerase (5 U μl-1).

Gene	Primers	Temperature profile
18S rRNA (∼1700 bp)	1F and 1795R (Medlin et al., 1988)	94°C/5 min 35cycles x (94°C/1 min, 50°C/1 min, 72°C/1 min) 72°C/5 min
28S rRNA D3-D5 region (∼650 bp)	Por28S-830F and Por28S-1520R (Morrow et al., 2012)	94°C/5 min 30 cycles x (94°C/30 s, 53°C/30 s 72°C/30 s) 72°C/5 min
COI (∼680 bp)	LCO1490 and HCO2198 (Folmer et al., 1994)	94°C/2min 35 cycles x (94°C/1 min, 45°C/1 min, 72°C/1 min) 72°C/7 min

Table A.2: NCBI Accession numbers for the sponge sequences obtained in this study.

Family	Species	LSU	COI	SSU
Aplysinellidae	*Suberea fusca*	MH731296	MH784612	
Inathellidae	*Hexadella indica*	MN386039		
Dysideidae	*Dysidea* sp1	MH731286	MH784606	MH731301
	Dysidea sp2	MN386030		
	Euryspongia lobata	MN386031		
Thorectidae	*Dactylospongia elegans*	MN386027		
	Hyrtios spinifer	MN386041		
Callyspongidae	*Callyspongia* sp1	MH731282		
	Callyspongia sp2	MN386023		
Chalinidae	*Haliclona (Reniera)* sp1	MN386034		
	Haliclona (Reniera) sp2	MN386035		
	Haliclona (Reniera) sp3	MN386036		
	Haliclona (Reniera) sp4	MH731289		MH731302
	Haliclona (Reniera) sp5	MN386037		
	Haliclona (Reniera) sp6	MN386038		
	Haliclona (Gellius) toxia	MH731290		MH731303
	Dendroxea sp	MH731285		MH731300
	Chalinula nematifera	MN386024		
Niphatidae	*Amphimedon paraviridis*	MH731280		
	Amphimedon sulcata	MH731287	MH784607	
	Gelliodes fibulata	MH731288		
	Gellius sp	MN386032		
	Niphates olmeda	MN386043		
	Niphates sp	MN386044		
Heteroxydae	*Didiscus aceratus*	MN386028		
	Didiscus sp	MN386029		
Raspailiidae	*Thrinacophora cervicornis*	MH731297	MH784613	MH731308
Biemnidae	*Biemna fistulosa*	MN386021		
	Biemna trirhaphis	MN386022		
	Neofibularia sp	MH731293	MH784610	MH731306
Rhabderemiidae	*Rhabderemia acanthostyla*	MN386047		
Ancorinidae	*Stelleta herdmani*	MN386048		
Agelasidae	*Agelas bispiculata*	MN386019		
Crambeidae	*Monanchora unguiculata*	MH731291	MH784608	MH731304
Hymedesmidae	*Hymedesmia* sp1	MN386040		
	Phorbas sp.	MH731294	MH784611	MH731307
	Phorbas sp S4.15	MN386045		
Microcionidae	*Antho (Antho)* sp	MH731281	MH784604	MH731299
	Clathria (Axosuberites) sp.	MN386025		
	Clathria (Isociella) skia	MH731284		
	Clathria (Microciona) cf lizardensis	MN386026		
	Clathria (Thalysias) reinwardti	MH731283	MH784605	
Mycalidae	*Mycale (Carmia) phylophila*	MN386042		
	Mycale (Arenochalina) sp	MH731292	MH784609	MH731305
Tedaniidae	*Tedania (Tedania) panis*	MN386049		
Placospongiidae	*Placospongia* sp	MN386046		
Halichondridae	*Axynissa variabilis*	MN386020		
	Topsentia cf. Halichondrides	MN386052		
	Halichondria (Halichondria) cartilaginea	MN386033		
Suberitidae	*Aaptos suberitioides*	MH731279	MH784603	MH731298
	Prosuberites proteus	MH731295		
	Terpios cruciatus	MN386051		
	Terpios sp	MN386050		

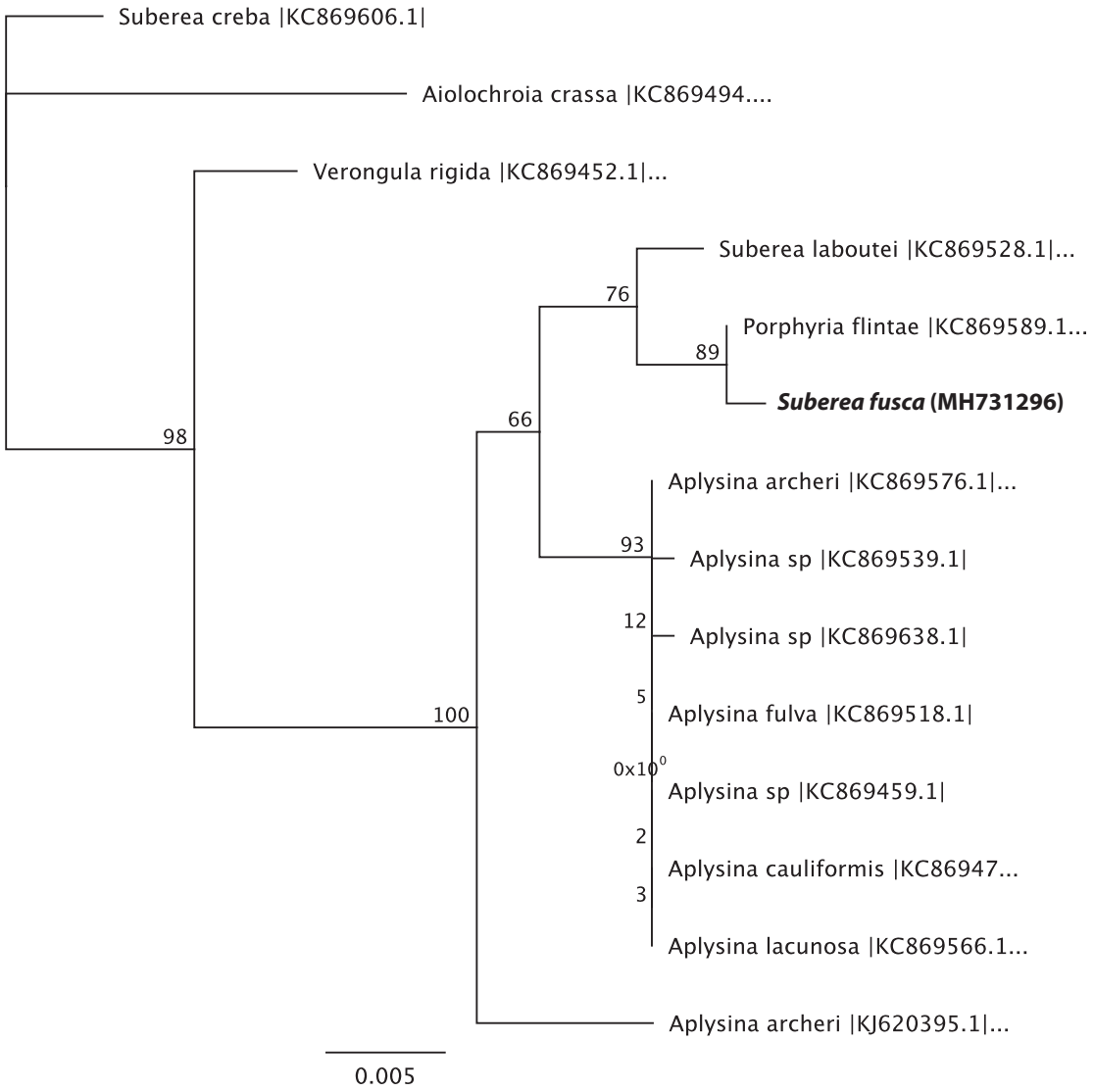

Figure A.1: Tree output from PHYML analysis of 28S rRNA (D3-D5 region) barcoding fragment of the family *Aplysinellidae*.

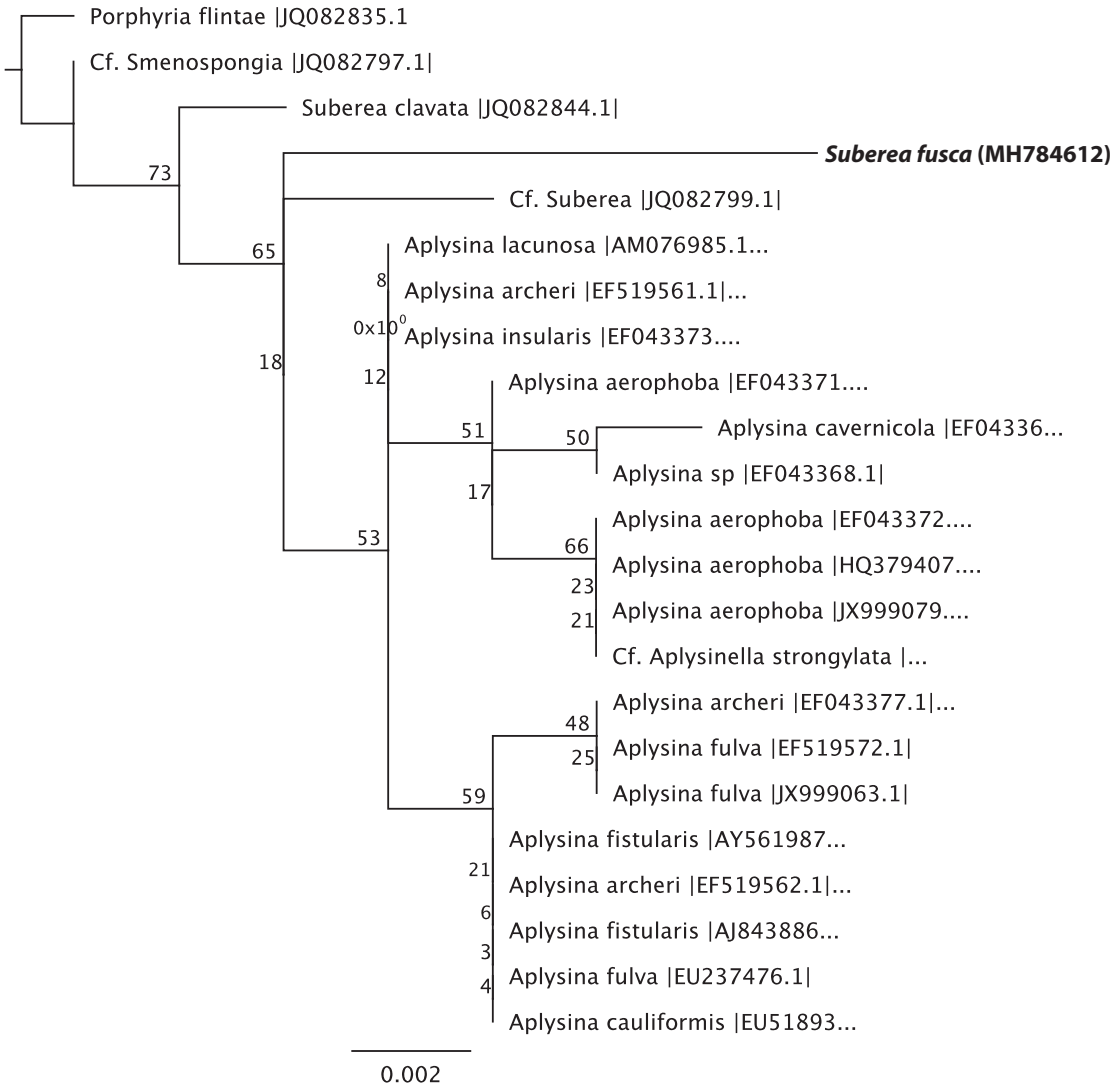

Figure A.2: Tree output from PHYML analysis of mitochondrial COI barcoding fragment of the family *Aplysinellidae*.

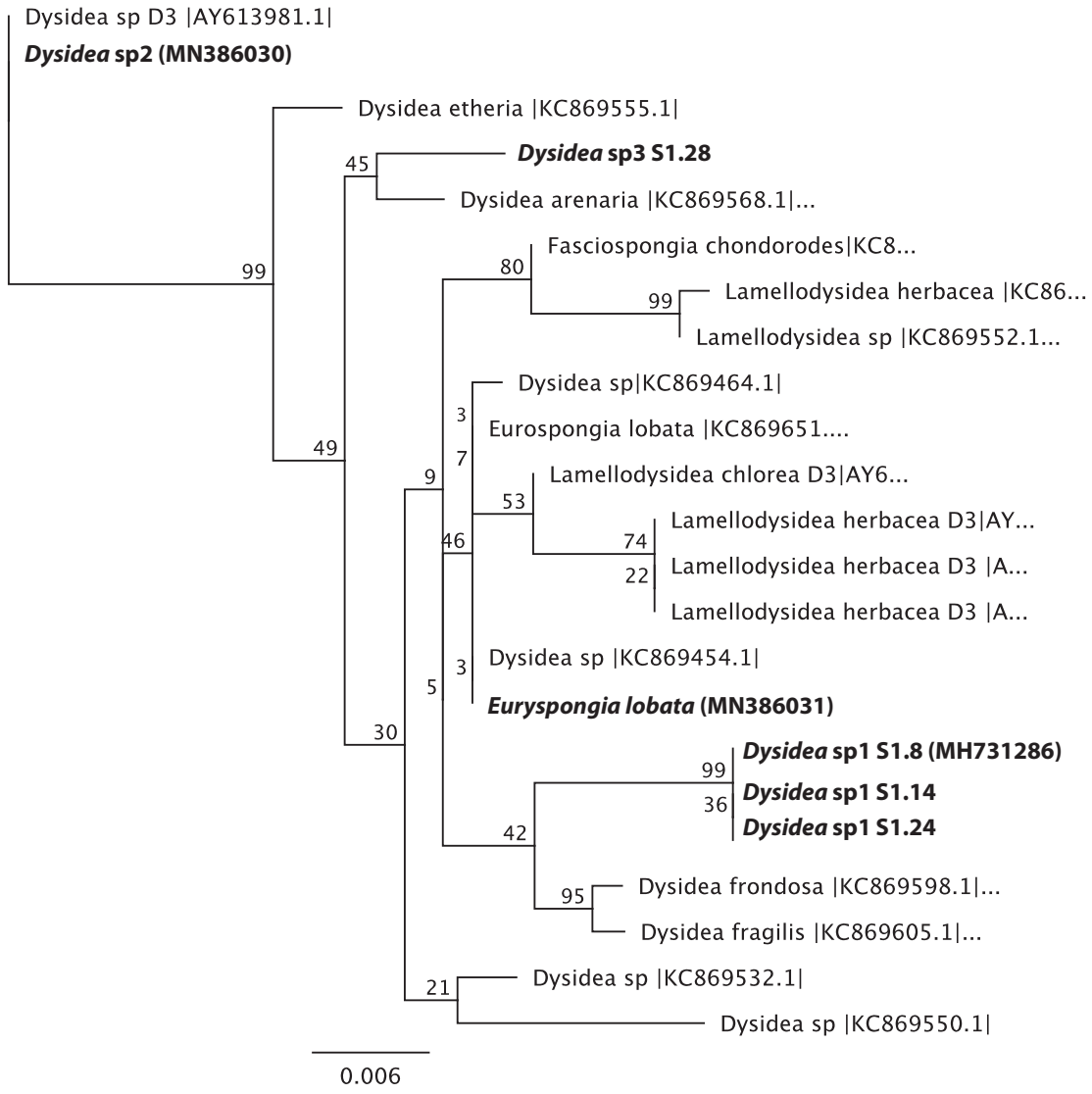

Figure A.3: Tree output from PHYML analysis of 28S rRNA (D3-D5 region) barcoding fragment of the family *Dysideidae*.

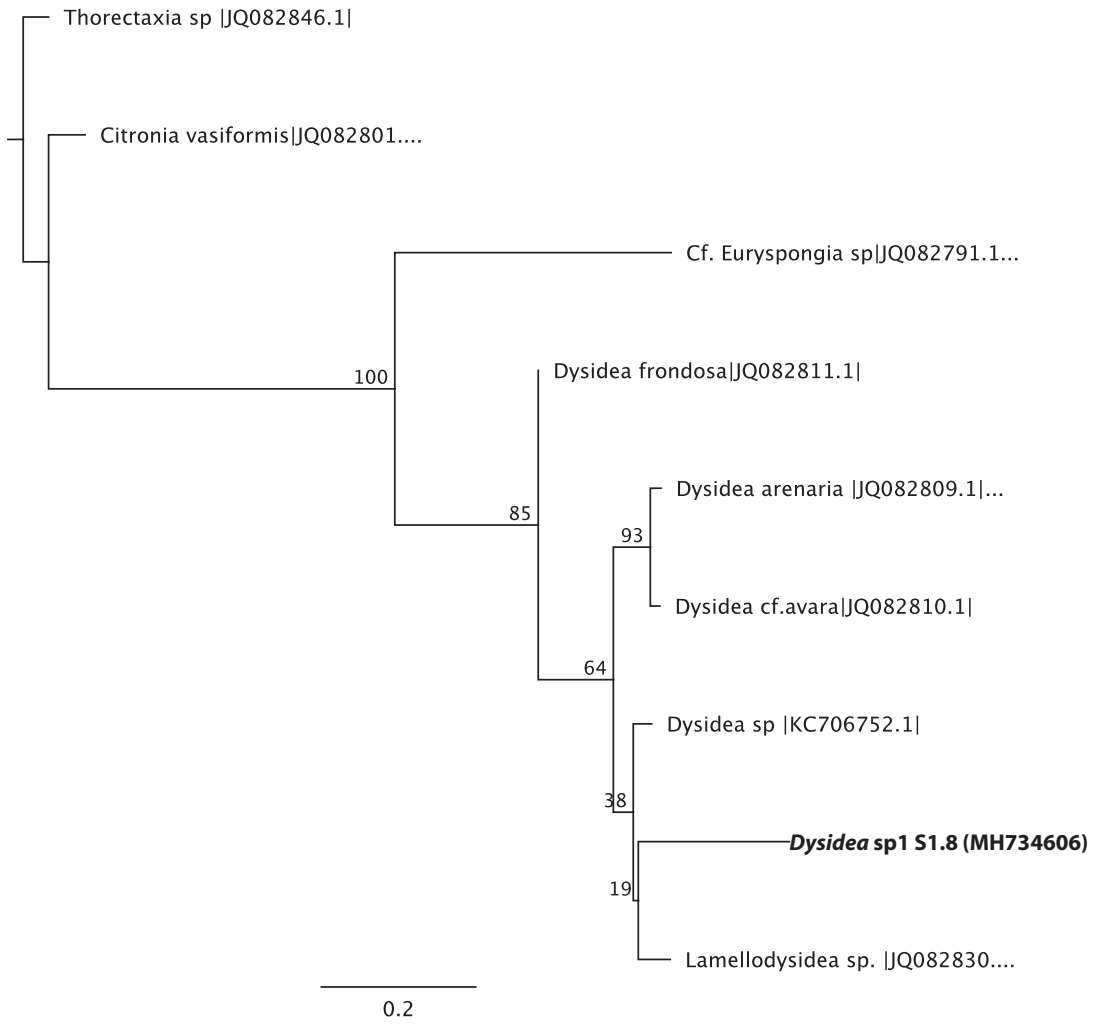

Figure A.4: Tree output from PHYML analysis of mitochondrial COI barcoding fragment of the family *Dysideidae*.

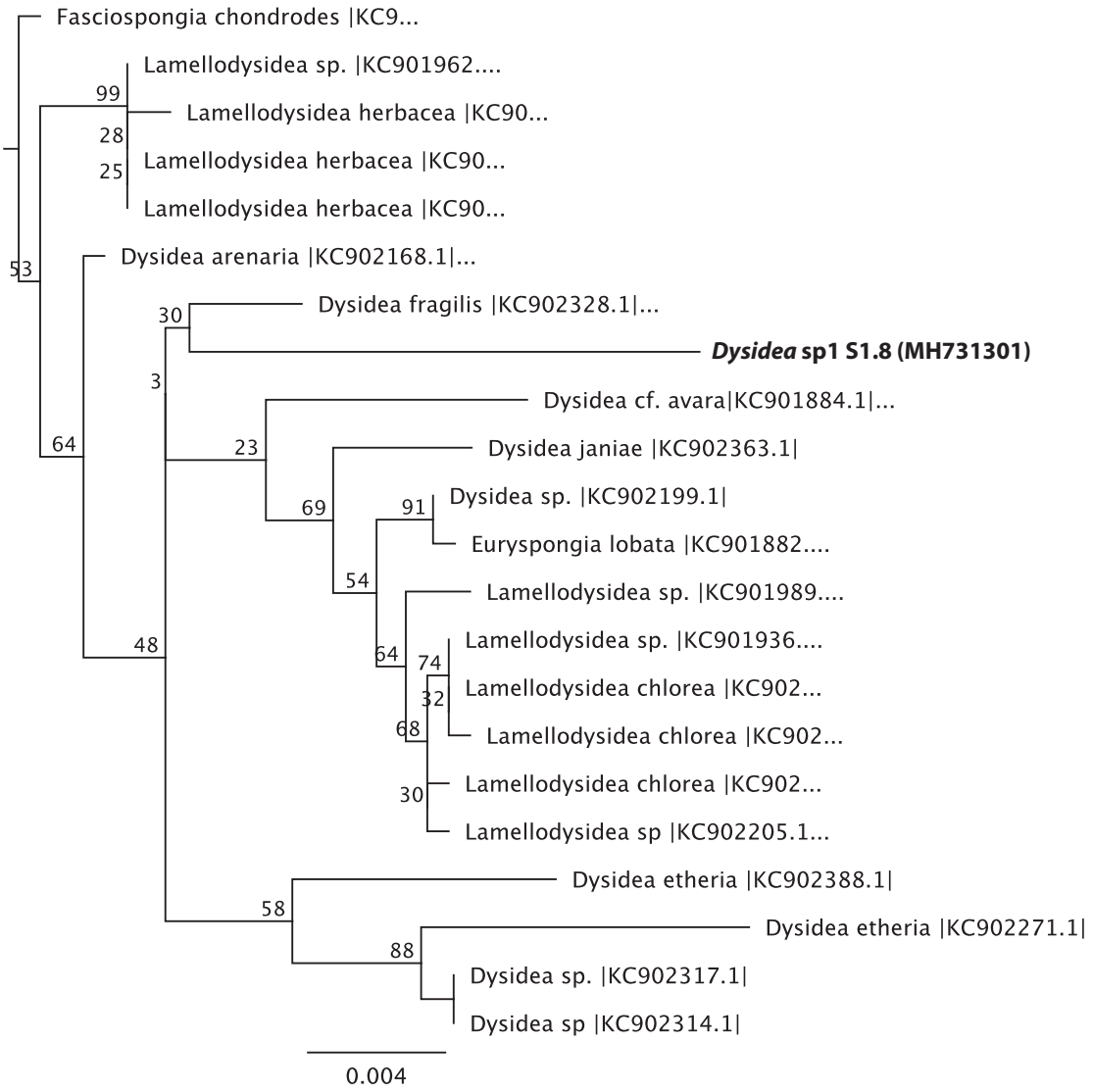

Figure A.5: Tree output from PHYML analysis of the full-length 18S rRNA barcoding fragment of the family *Dysideidae*.

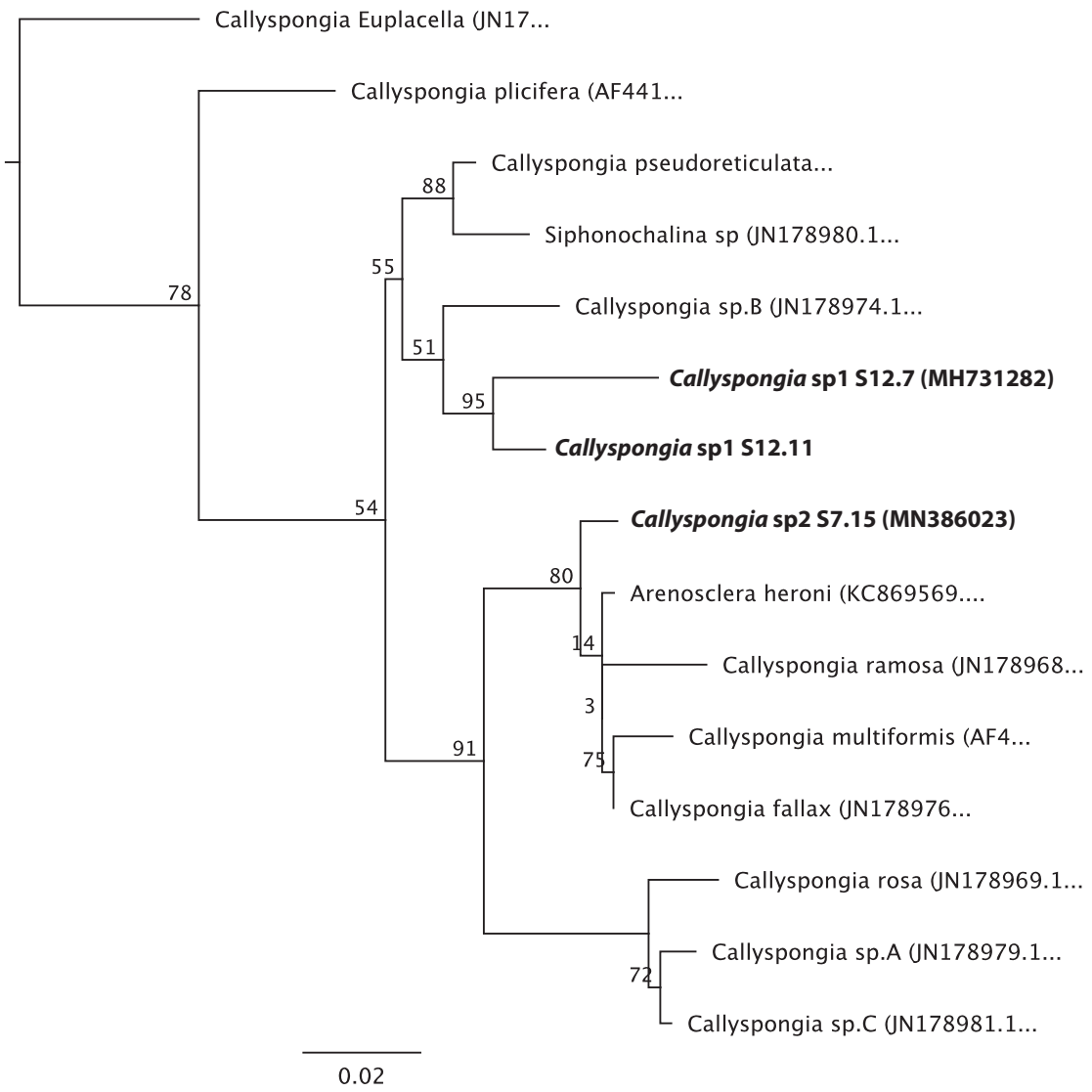

Figure A.6: Tree output from PHYML analysis of 28S rRNA (D3-D5 region) barcoding fragment of the family *Callyspongidae*.

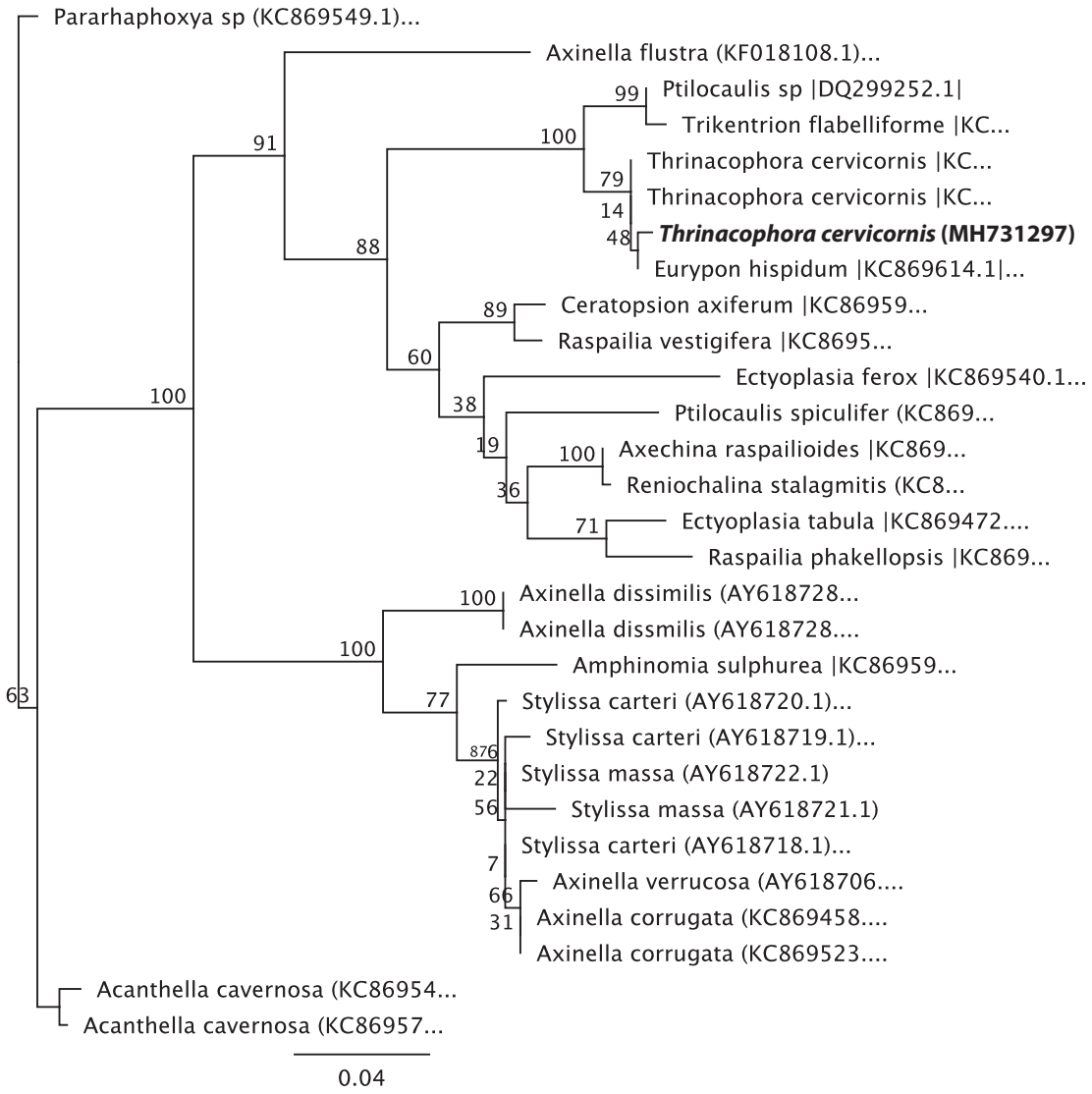

Figure A.7: Tree output from PHYML analysis of 28S rRNA (D3-D5 region) barcoding fragment of the family *Raspailiidae*.

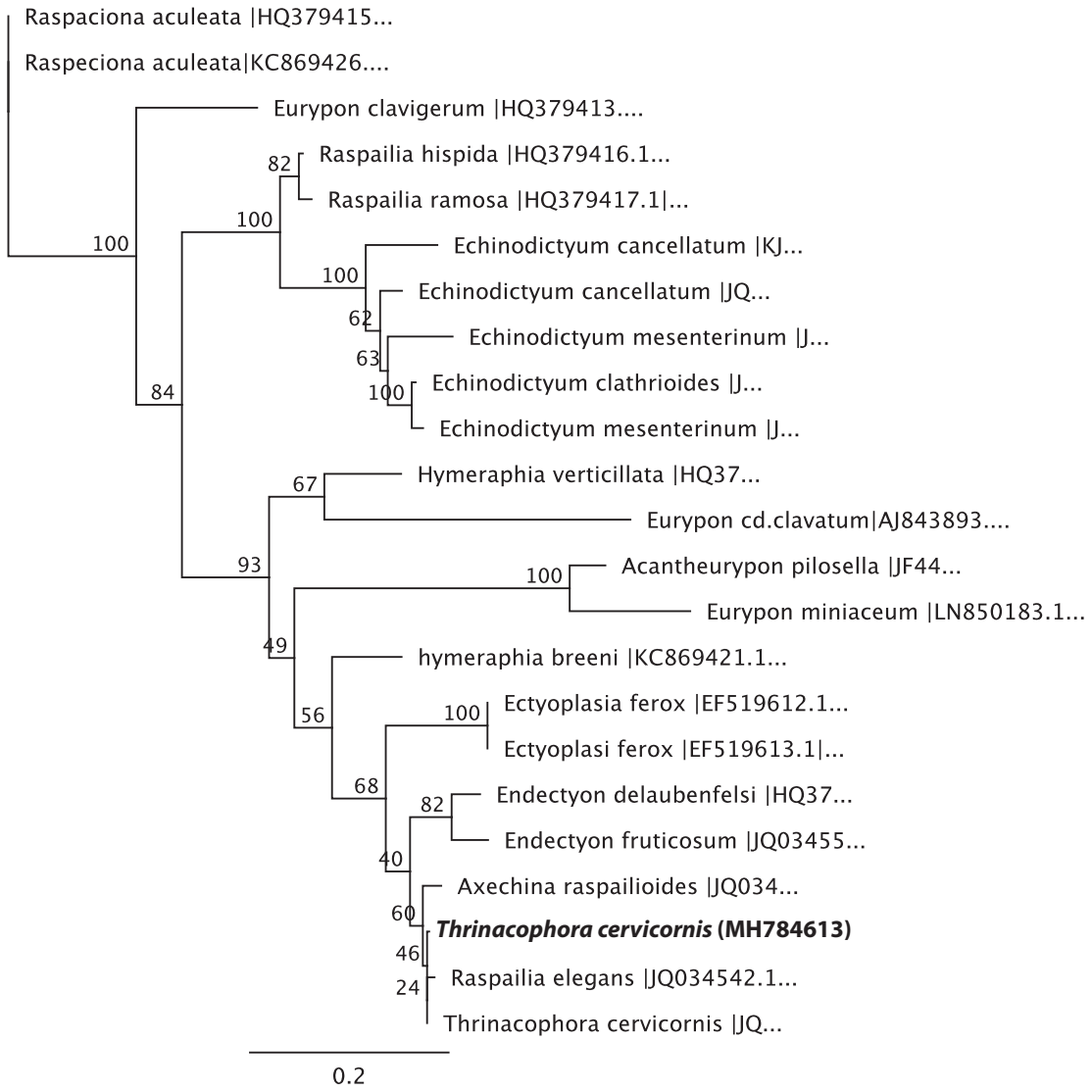

Figure A.8: Tree output from PHYML analysis of mitochondrial COI barcoding fragment of the family *Raspailiidae*.

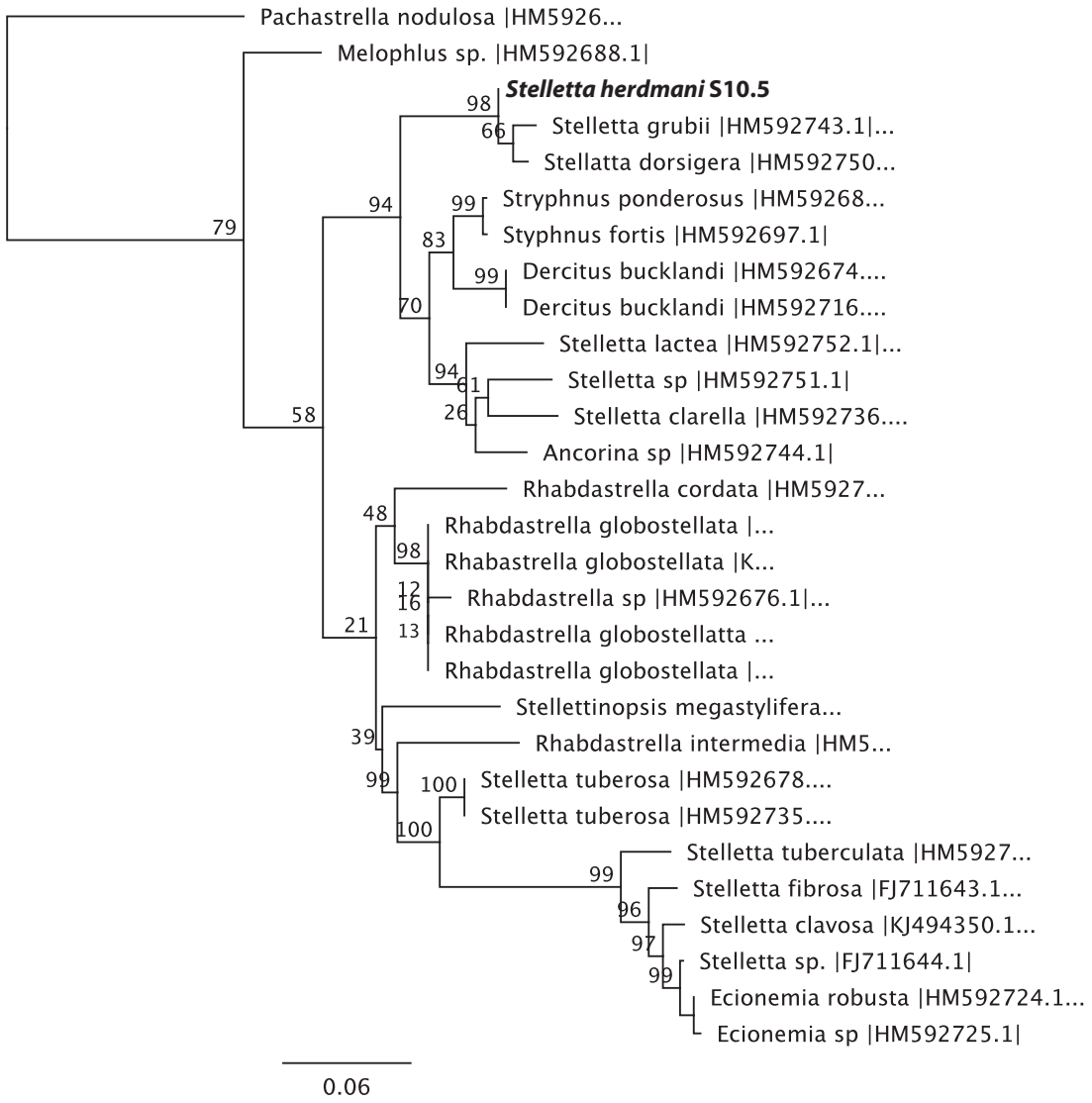

Figure A.9: Tree output from PHYML analysis of mitochondrial COI barcoding fragment of the family *Ancorinidae*.

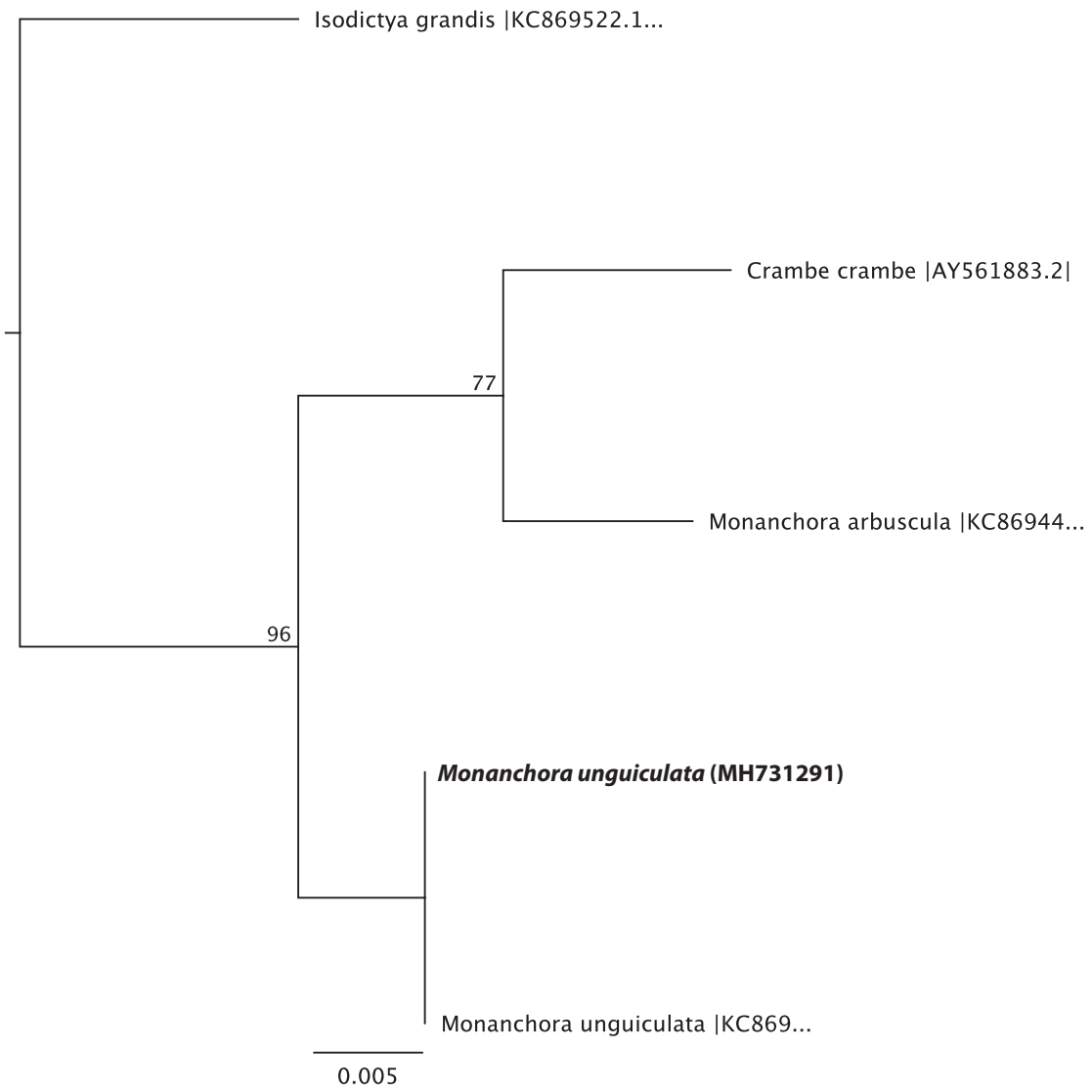

Figure A.10: Tree output from PHYML analysis of 28S rRNA (D3-D5 region) barcoding fragment of the family *Crambeidae*.

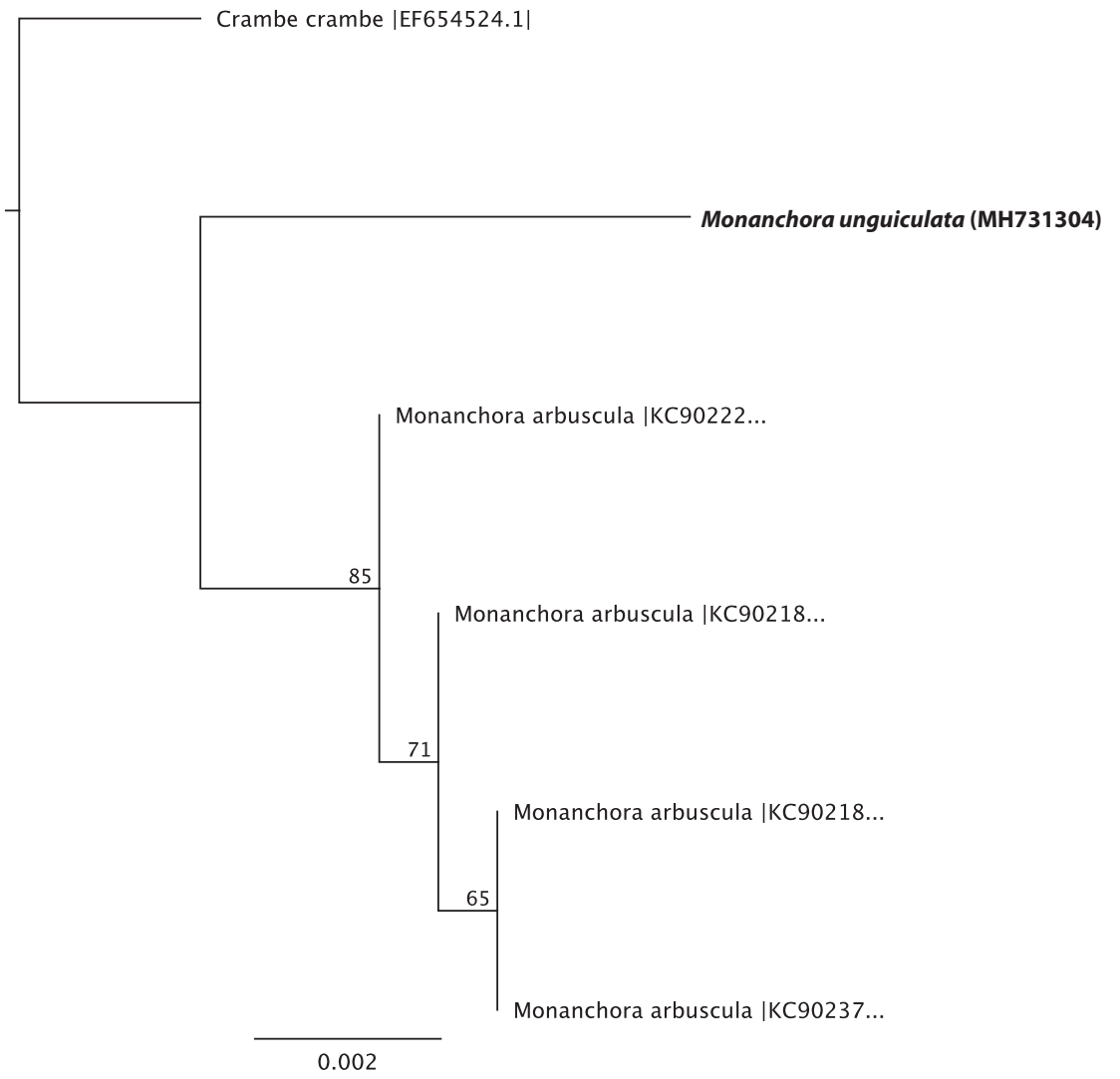

Figure A.11: Tree output from PHYML analysis of the full-length 18S rRNA barcoding fragment of the family *Crambeidae*.

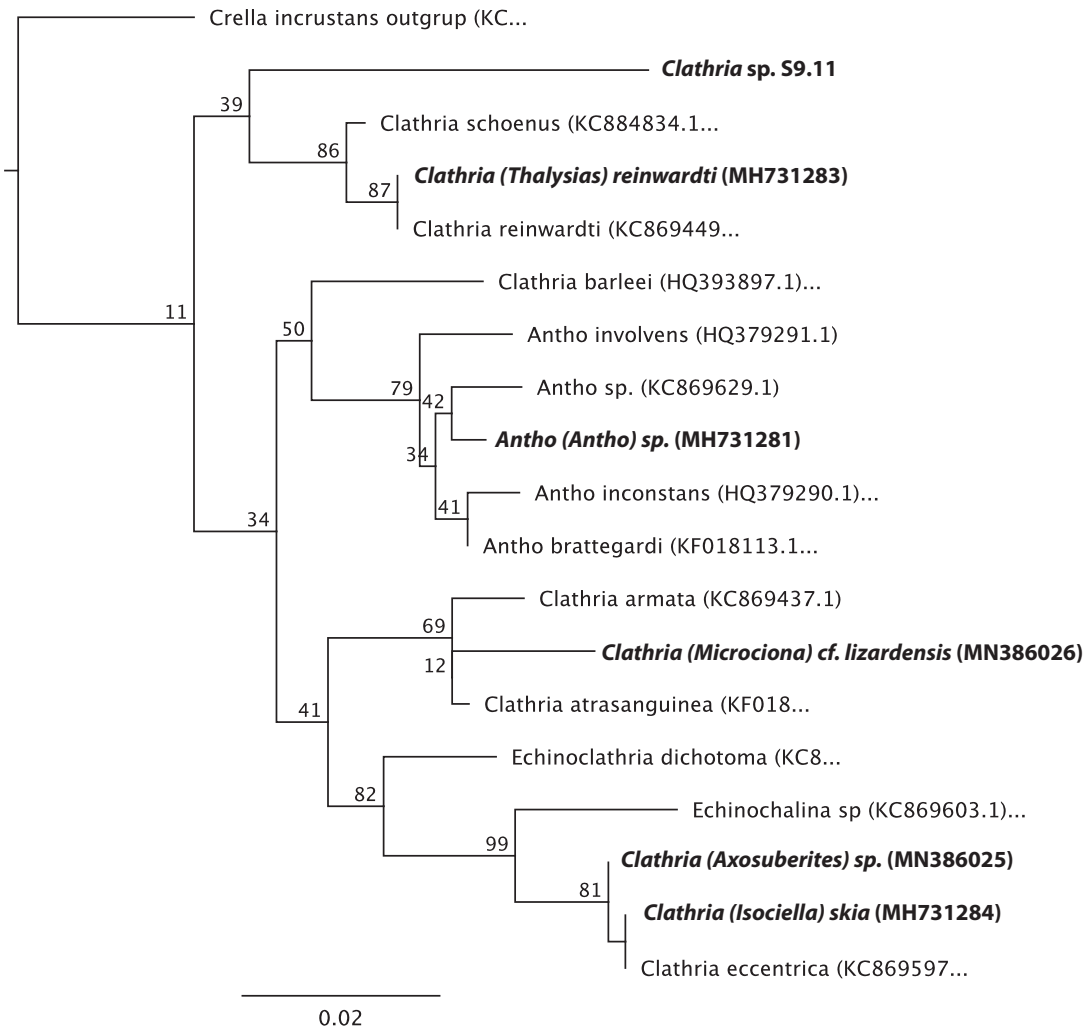

Figure A.12: Tree output from PHYML analysis of 28S rRNA (D3-D5 region) barcoding fragment of the family *Microcionidae*.

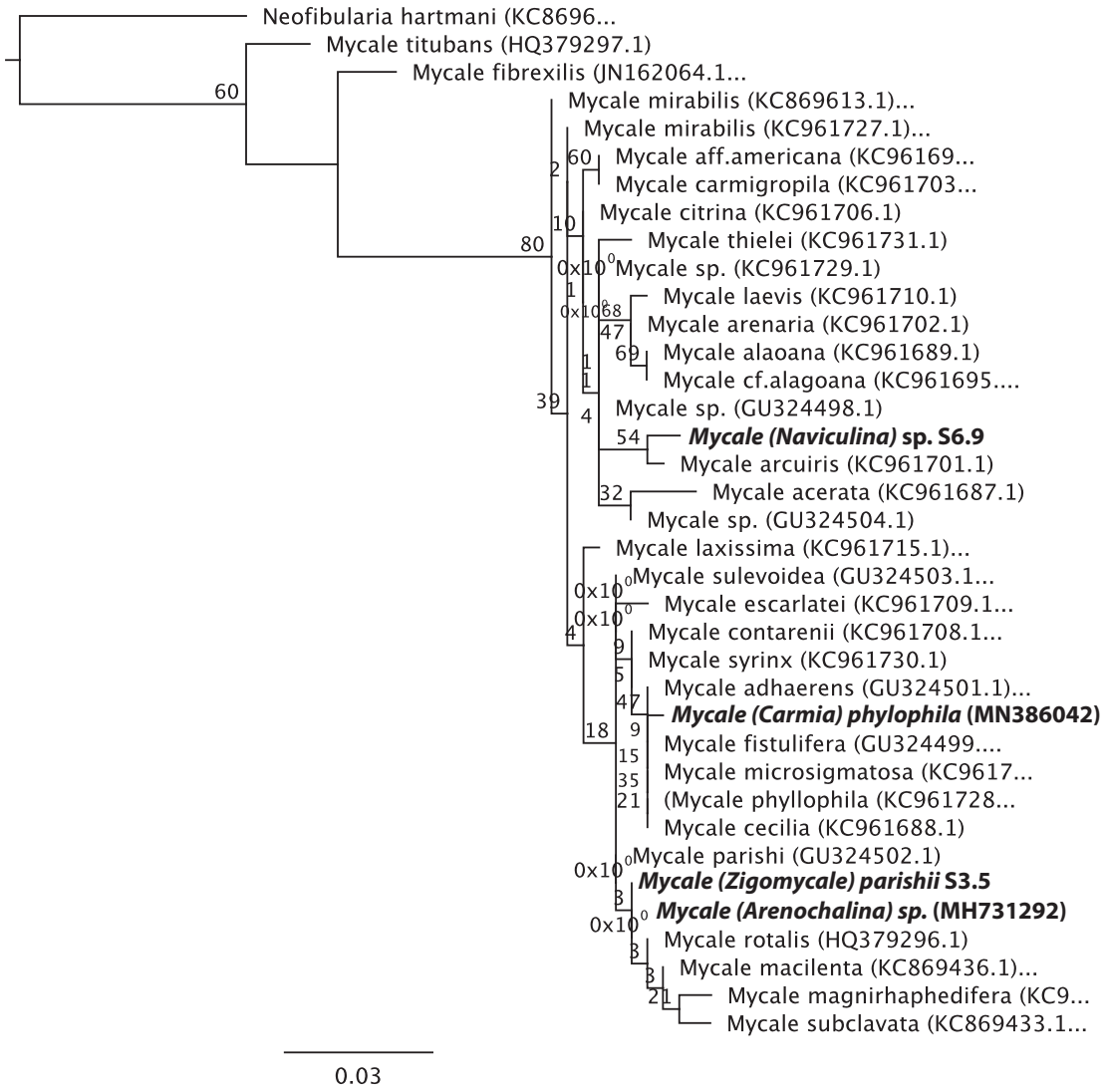

Figure A.13: Tree output from PHYML analysis of 28S rRNA (D3-D5 region) barcoding fragment of the family *Mycalidae*.

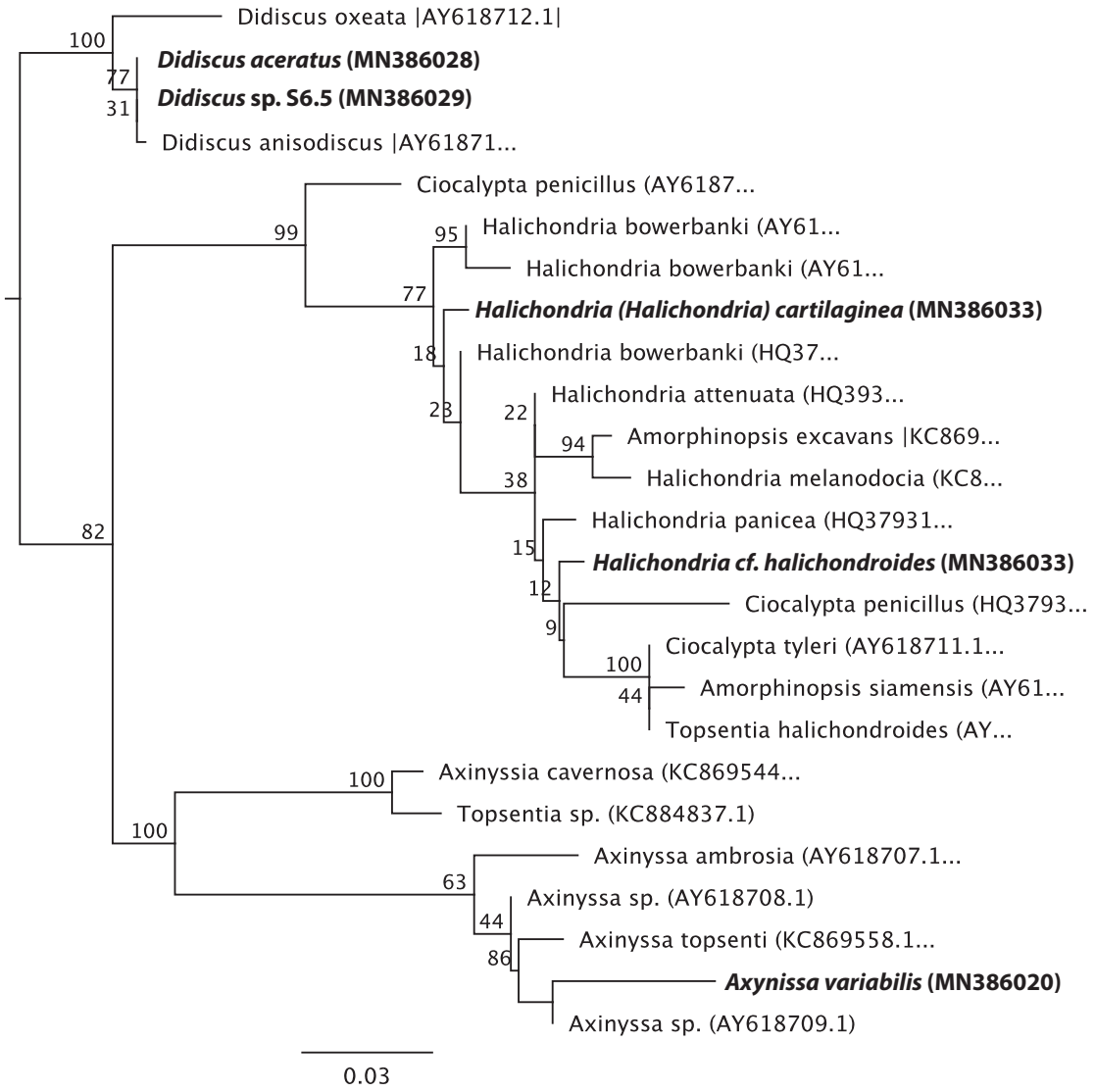

Figure A.14: Tree output from PHYML analysis of 28S rRNA (D3-D5 region) barcoding fragment of the family *Halichondridae*.

Appendix B

Chapter 3 Supporting Information

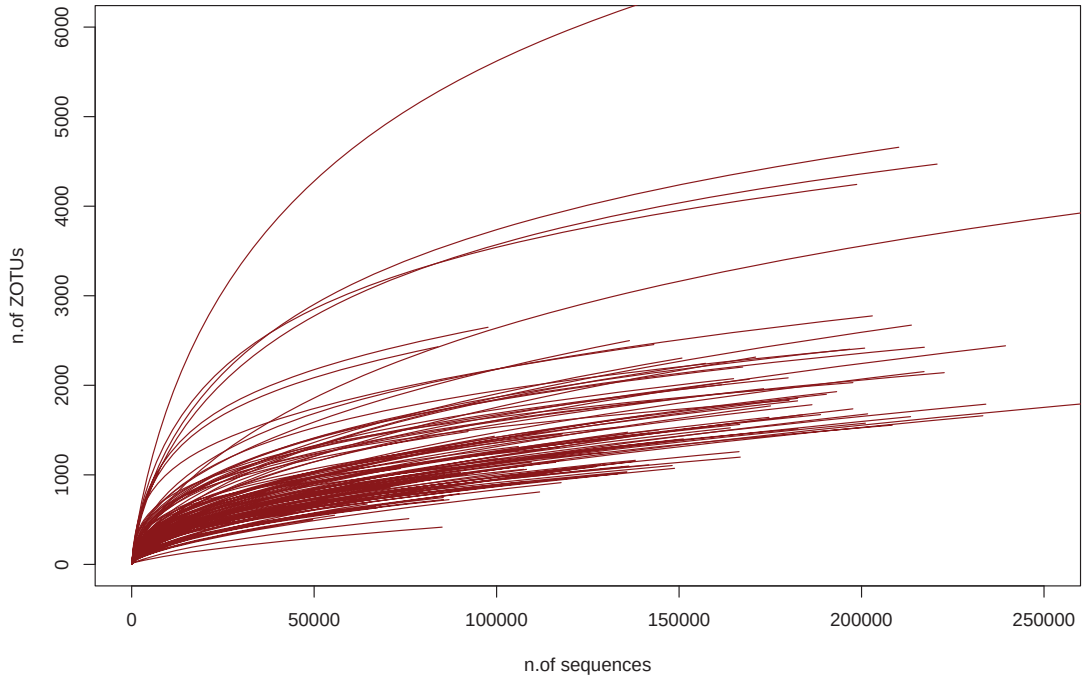

Figure B.1: Rarefaction curves of all the samples used in this study. Y axis represents the number of ZOTUs and X axis represents the number of sequences. Rarefactions were performed at a minimum reads treshold of 41000.

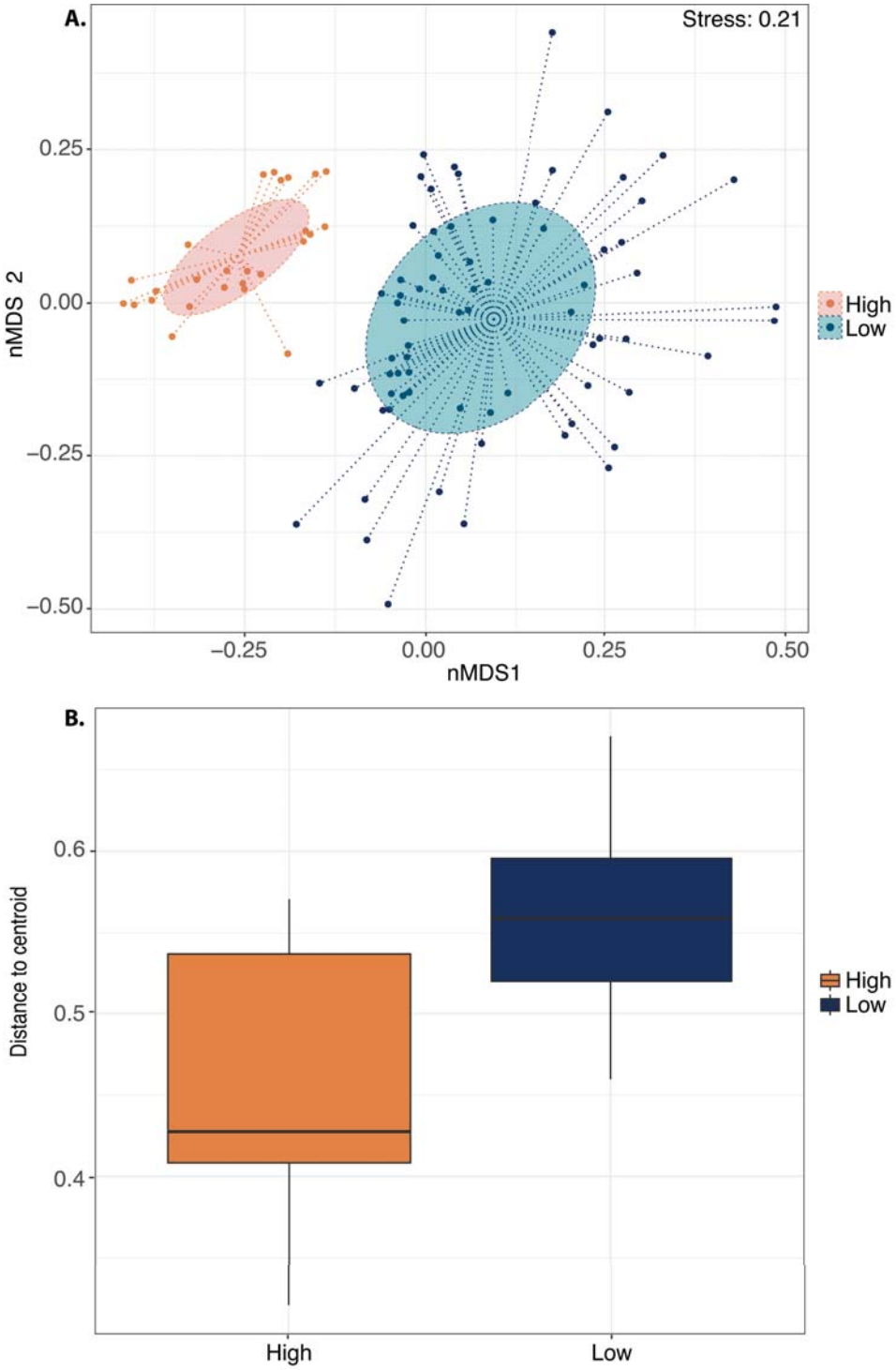

Figure B.2: (A) Non-metric multidimensional scaling (nMDS) ordination of the sponge bacterial communities based on Bray-Curtis distances coloured by their belongingness to HMA (orange) or LMA (blue) species. (B) Comparison of the beta dispersion between groups of HMA and LMA species, values represent the distance to centroid of the replicates for each group.

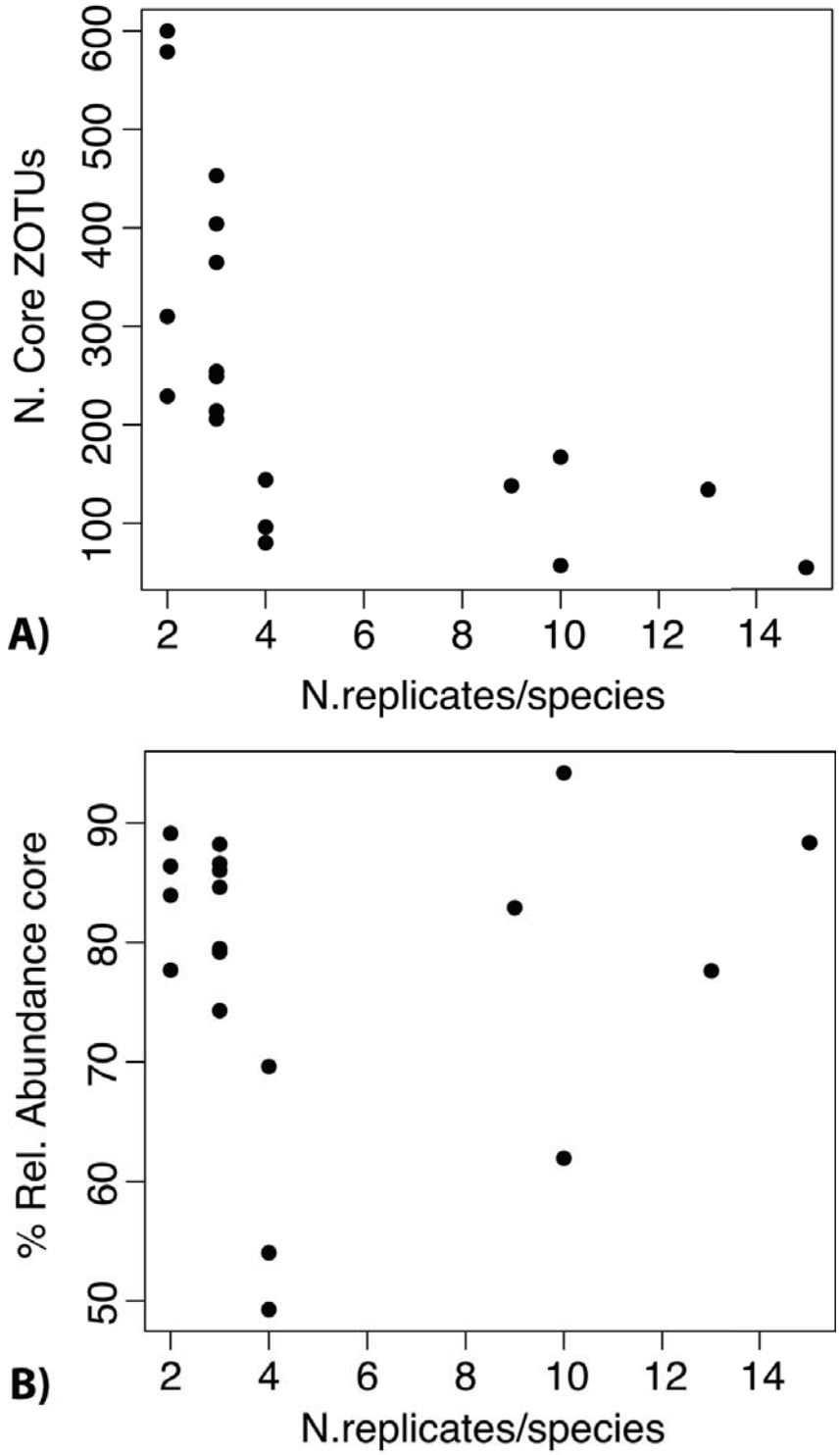

Figure B.3: (A) Relation between the number of species replicates (from 2 to 15) and the number of ZOTUs that constitute the core community of an species. (B) Relation between the number of species replicates (from 2 to 15) and the percentage of relative abundance that the core ZOTUs represent to the overall microbiome of each species.

Figure B.4: Indval analysis at phylum (A) and class (B) levels for bacterial taxa assignation to either HMA or LMA species groups. Only those taxa with Indval values higher than 0.6 for any of the two groups are represented. Phyla and classes significantly associated to HMA species are represented in orange and the associated to LMA species are marked in blue. Size of circles refers to the Indval value. Only the indicator classes that had abundances higher than 0.1% in each group are shown.

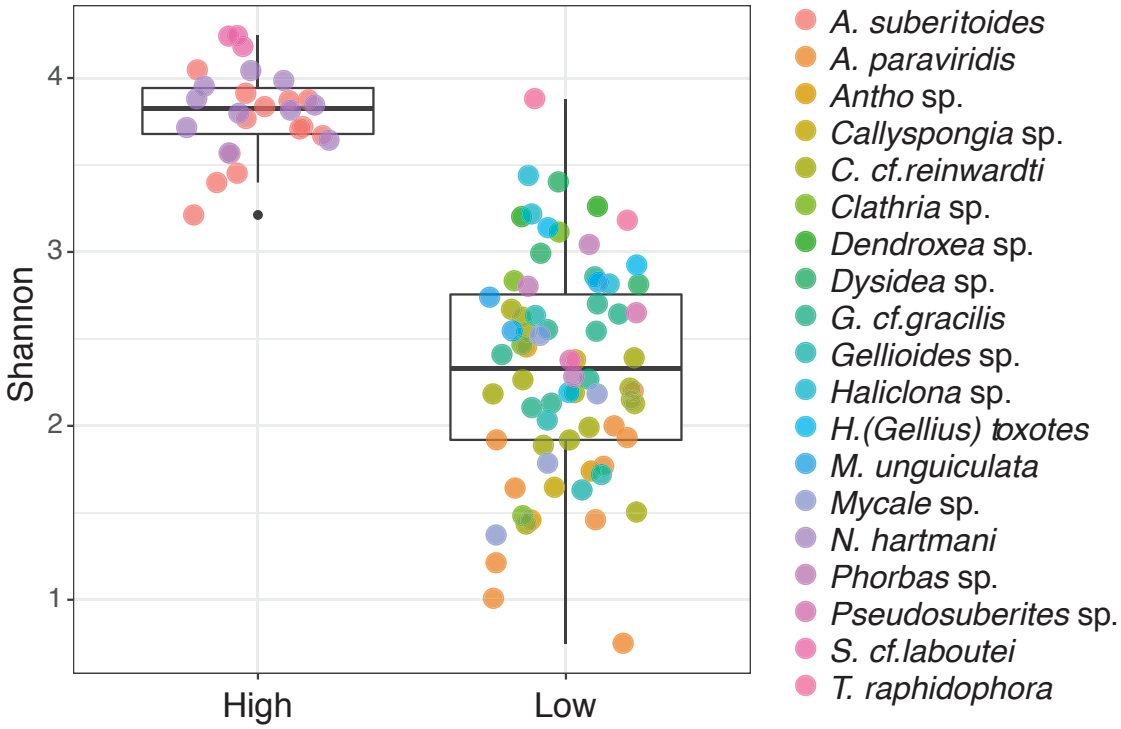

Figure B.5: Boxplot showing the Shannon diversity indices of the core microbial communities for each sponge species belonging to either HMA or LMA sponge groups. Each colour corresponds to one species. Replicates of the same species are painted in the same colour.

Table B.1: Mean, core, species-specific, and SW OTUs of all species studied. Values are given in number and relative abundance (%) of OTUs. HMA species are marked in dark grey and LMA species are marked in light grey. (n= number of replicates per species). (a): Number and percentages of abundances of species-specific OTUs are calculated for the core community of each species (b): Number and percentages of abundances of SW OTUs are calculated for the core community of each species. Values correspond to the comparison with the SW core OTUs (cosmpolitan).

Species	n	Mean ZOTUs ± SD	Core ZOTUs	% Ab. Core ± SD	Sp-sp ZOTUs (a)	% Ab. Sp-sp ZOTUs	SW ZOTUs (b)	% Ab. SW ZOTUs
Aaptos suberitoides (Brondsted, 1934)	13	776 ± 100	119	81.2 ± 4	4	0.5	47	56.6
Neofibularia hartmani (Hooper and Lévi, 1993)	10	969 ± 101	164	93.2 ± 1.4	45	4.7	68	75.4
Suberea cf.laboutei (Bergquist, 1995)	3	709 ± 50	213	91.7 ± 3.1	64	14.1	67	33.5
Amphimedon paraviridis (Fromont, 1993)	10	524 ± 192	48	58.6 ± 20.8	0	0	47	100
Antho sp.	3	1214 ± 562	369	89.8 ± 4.3	63	2.1	109	11.2
Callyspongia sp.	2	663 ± 379	203	88.5 ± 1.8	21	1.5	69	36.6
Clathria reinwardti (Vosmaer, 1880)	15	712 ± 146	52	85.3 ± 14.4	8	0.4	30	96.1
Clathria sp.	4	679 ± 265	143	73.8 ± 18.2	10	0.5	52	35.5
Dendroxea sp.	2	614 ± 89	265	76.7 ± 11.7	30	11.7	88	37.5
Dysidea sp.	3	1366 ± 640	409	88.1 ± 5.8	45	1.5	135	28.8
Gellioides cf.gracilis (Hentchel, 1912)	9	539 ± 93	122	82.6 ± 6.8	1	0.1	65	62.8
Gellioides sp.	4	421 ± 109	100	56.8 ± 11.5	4	2.7	83	99.4
Haliclona sp.	3	1055 ± 368	229	82.3 ± 8.2	17	1.9	120	19.6
H. (Gellius) toxotes (Hentchel, 1912	3	718 ± 235	353	82.3 ± 3	38	3.2	91	57.6
Monanchora unguiculata (Dendy, 1922)	3	538 ± 111	228	86.6 ± 11.3	14	1.3	87	62.1
Mycale sp.	4	1236 ± 998	95	56.8 ± 6.8	8	0.2	46	79
Phorbas sp.	3	713 ± 251	184	87.8 ± 7.4	18	0.4	75	51.4
Pseudosuberites sp.	2	1297 ± 816	501	89.9 ± 8.4	129	3.6	125	11.6
Thrinacophora rhaphidophora (Hentschel, 1912)	2	1007 ± 160	473	92.6 ± 3.5	125	7.8	151	24.1

Table B.2: Mean, core, species-specific, and SW OTUs of all species studied. Values are given in number and relative abundance (%) of ZOTUs. HMA species are marked in dark grey and LMA species are marked in light grey. (n= number of replicates per species). (a): Number and percentages of abundances of species-specific OTUs are calculated for the core community of each species (b): Number and percentages of abundances of SW OTUs are calculated for the core community of each species. Values correspond to the comparison with the SW core OTUs (cosmpolitan).

Methodologies	Sponge core shared with SW core community			Sponge core shared with abundant SW ZOTUs		
Species	ZOTUs	OTUs	Variation	ZOTUs	OTUs	Variation
Aaptos suberitoides (n= 13)	48.8	56.6	7.8	2.4	3	0.6
Neofibularia hartmani (n= 10)	71.6	75.4	3.8	3.9	5.1	1.2
Suberea cf.laboutei (n= 3)	26.7	33.5	6.8	1.6	1.7	0.1
Amphimedon paraviridis (n= 10)	99.5	100	0.4	76	84	8
Antho sp. (n= 3)	9.9	11.2	1.3	4.9	8.8	3.9
Callyspongia sp. (n= 2)	38.6	36.6	-1.9	35.4	34.4	-0.9
Clathria reinwardti (n= 15)	90.4	96.1	5.7	10.9	73.6	62.7
Clathria sp. (n= 4)	33.6	35.5	1.9	23.5	24.5	1.1
Dendroxea sp. (n= 2)	34	37.5	3.6	27.6	31.5	3.9
Dysidea sp. (n= 3)	27.3	28.8	1.4	22.4	26.1	3.6
Gellioides cf.gracilis (n= 9)	53	62.8	9.7	47.4	45.9	-1.5
Gellioides sp. (n= 4)	93.2	99.4	6.2	58.5	65.8	7.3
Haliclona sp. (n= 3)	16.1	19.6	3.5	10.4	12.1	1.7
H. (Gellius) toxotes (n= 3)	60.3	57.6	-2.7	42.4	41.9	-0.5
Monanchora unguiculata (n= 3)	58.7	62.1	3.4	51.2	51	-0.2
*Mycale*sp. (n= 4)	77.5	79	1.5	15.8	19.5	3.7
Phorbas sp. (n= 3)	48.7	51.4	2.7	33.3	45.6	12.3
Pseudosuberites sp. (n= 2)	11	11.6	0.7	9.5	10.5	1
Thrinacophora raphidophora (n= 2)	22.7	24.1	1.5	21.9	23.5	1.6

Appendix C

Chapter 4 Supporting Information

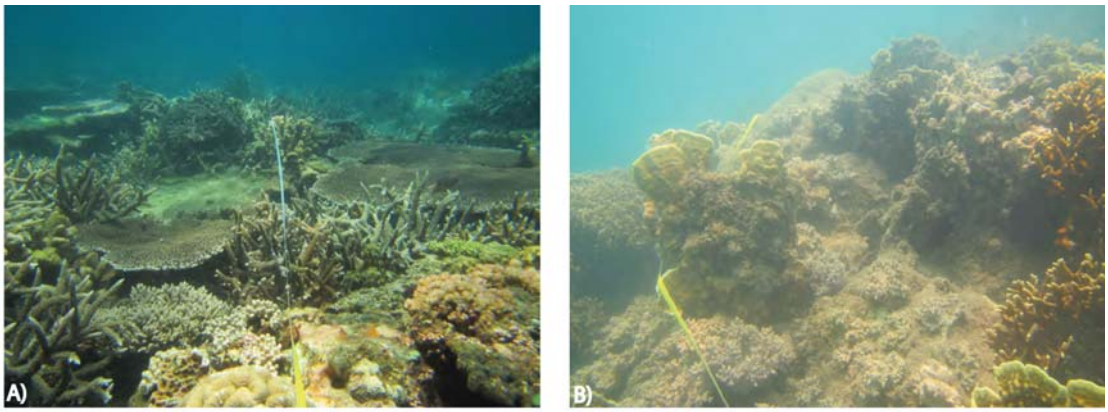

Figure C.1: Pictures of representative transects from the well-preserved (A) and impacted (B) environments.

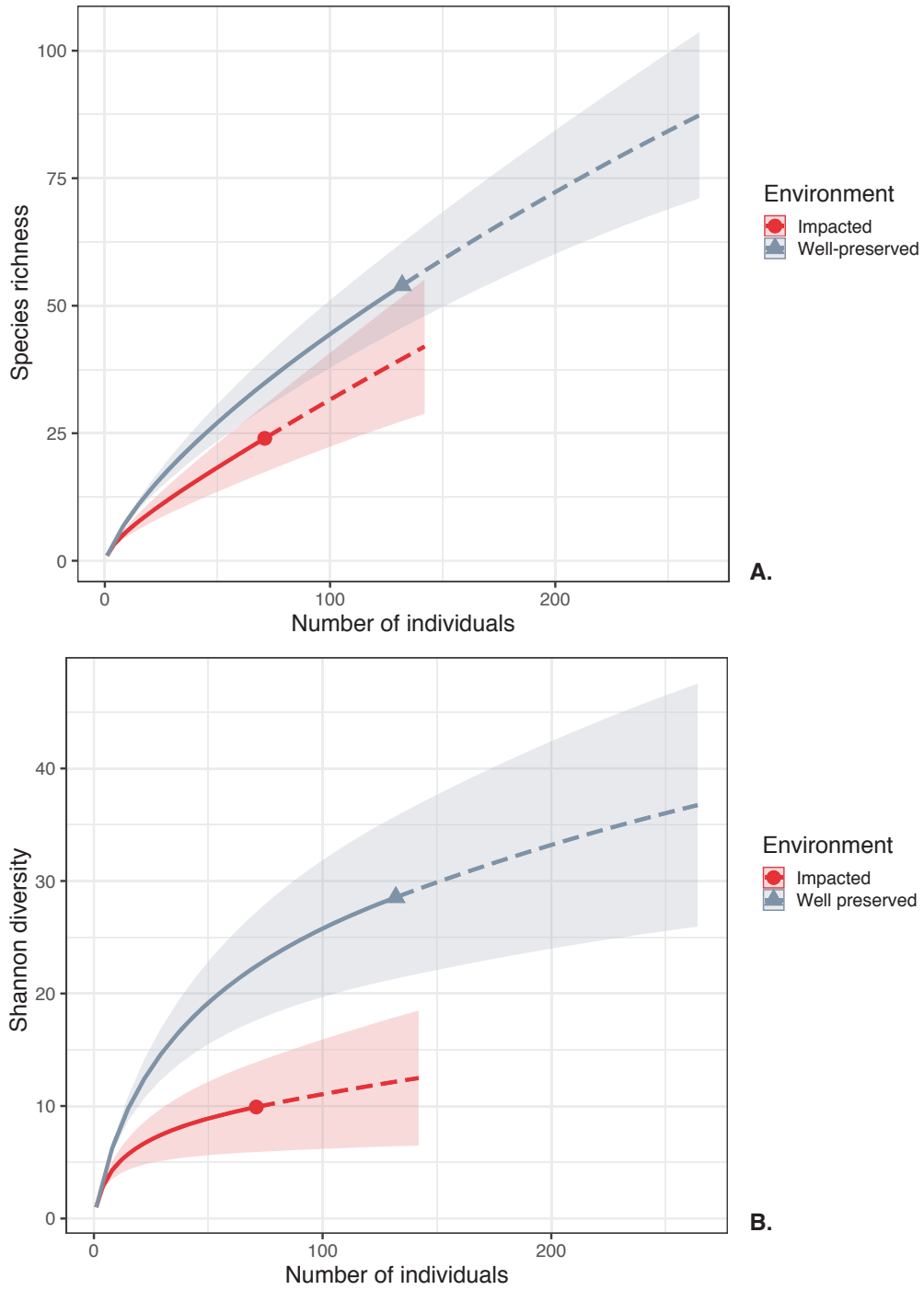

Figure C.2: Sponge species richness (A) and Shannon diversity (B) for a given number of individuals in impacted (red) and well-preserved (grey) environments. Continuous lines represent interpolated values from the observed data and discountinuous lines represent extrapolated data.

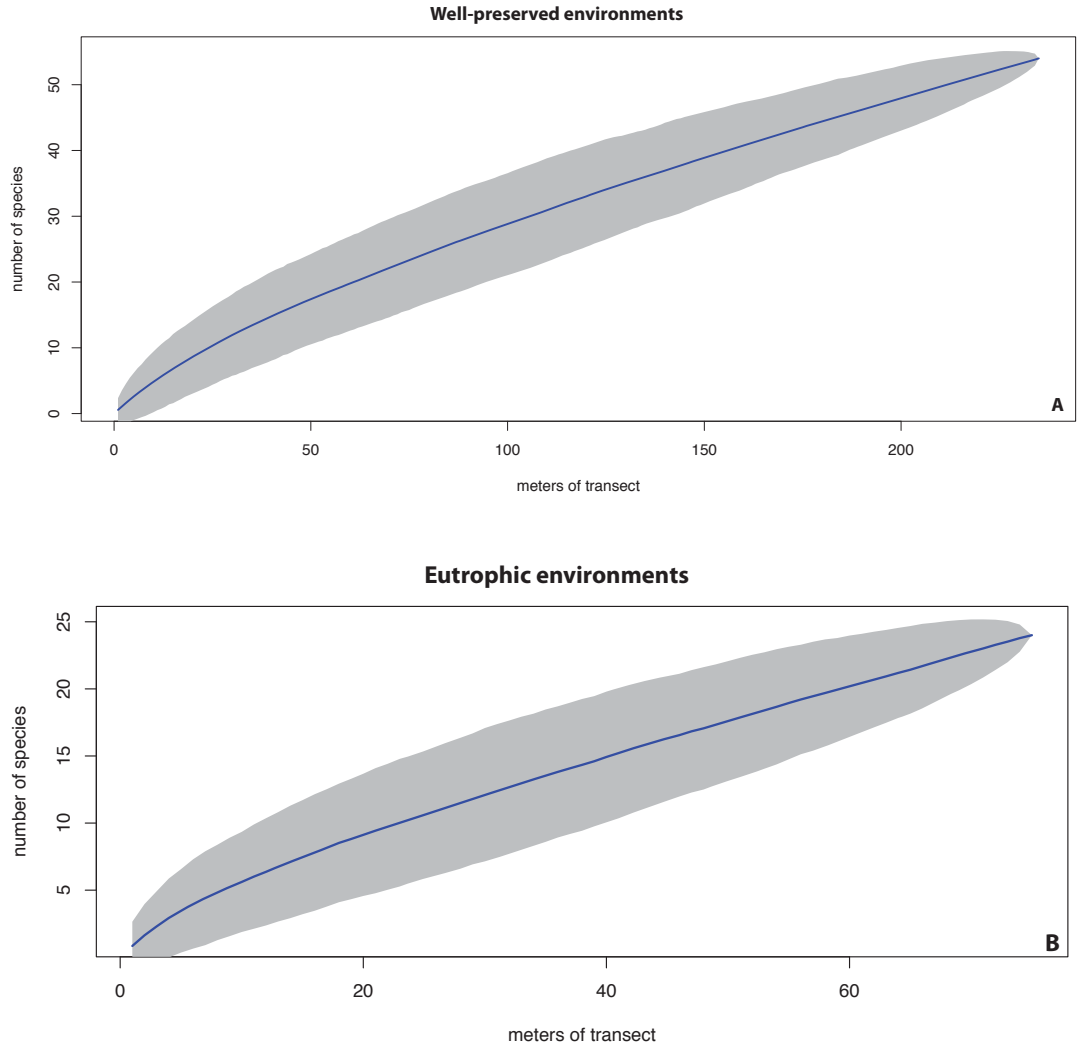

Figure C.3: Species accumulation curves for well-preserved (A) and Impacted/Eutrophic sites (B). Blue line indicates the mean species accumulation at each sampled meter and grey area indicates the standard deviation.

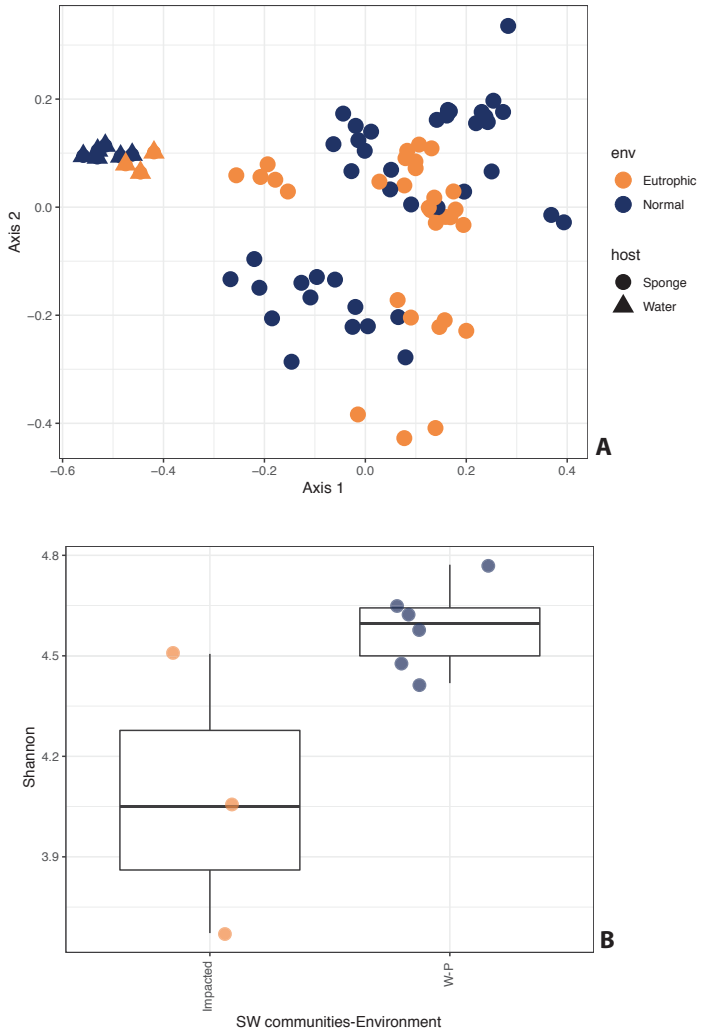

Figure C.4: A) Non-metric multidimensional scaling (nMDS) ordination of the sponge bacterial communities ans sea-water bacterial samples based on Bray-Curtis distances coloured by their belonginess to impacted (orange) or well-preserved (blue) environment. B) Box plot of the Shannon diversity indices of sea water bacterial communities from the impacted (orange) or well-preserved (blue) environments. Water samples were collected during the same sampling dates as the study sponges (see Turon et al. (2018) for raw data).

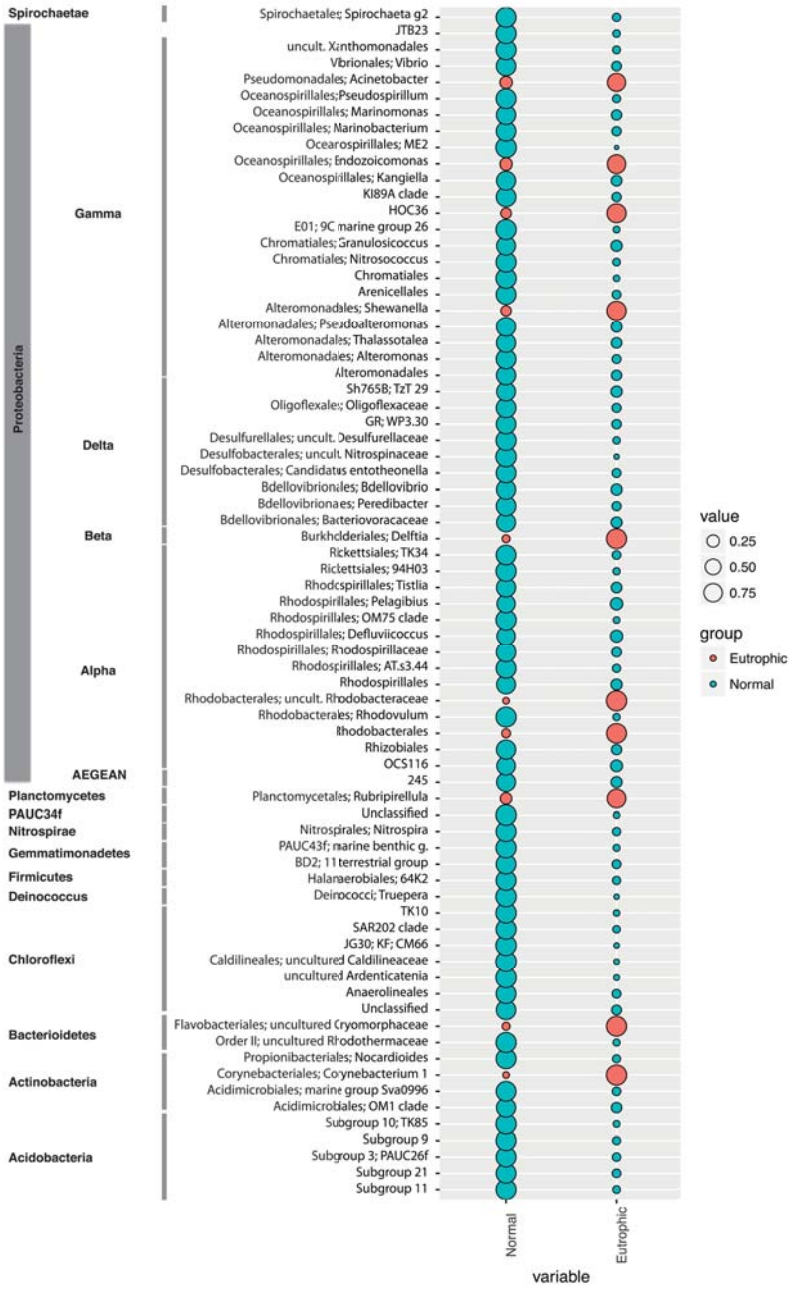

Figure C.5: Results of the Indval analysis. Bacterial taxa represented were indicators of impacted (red) or well-preserved (green) environment (p-val < 0.01).

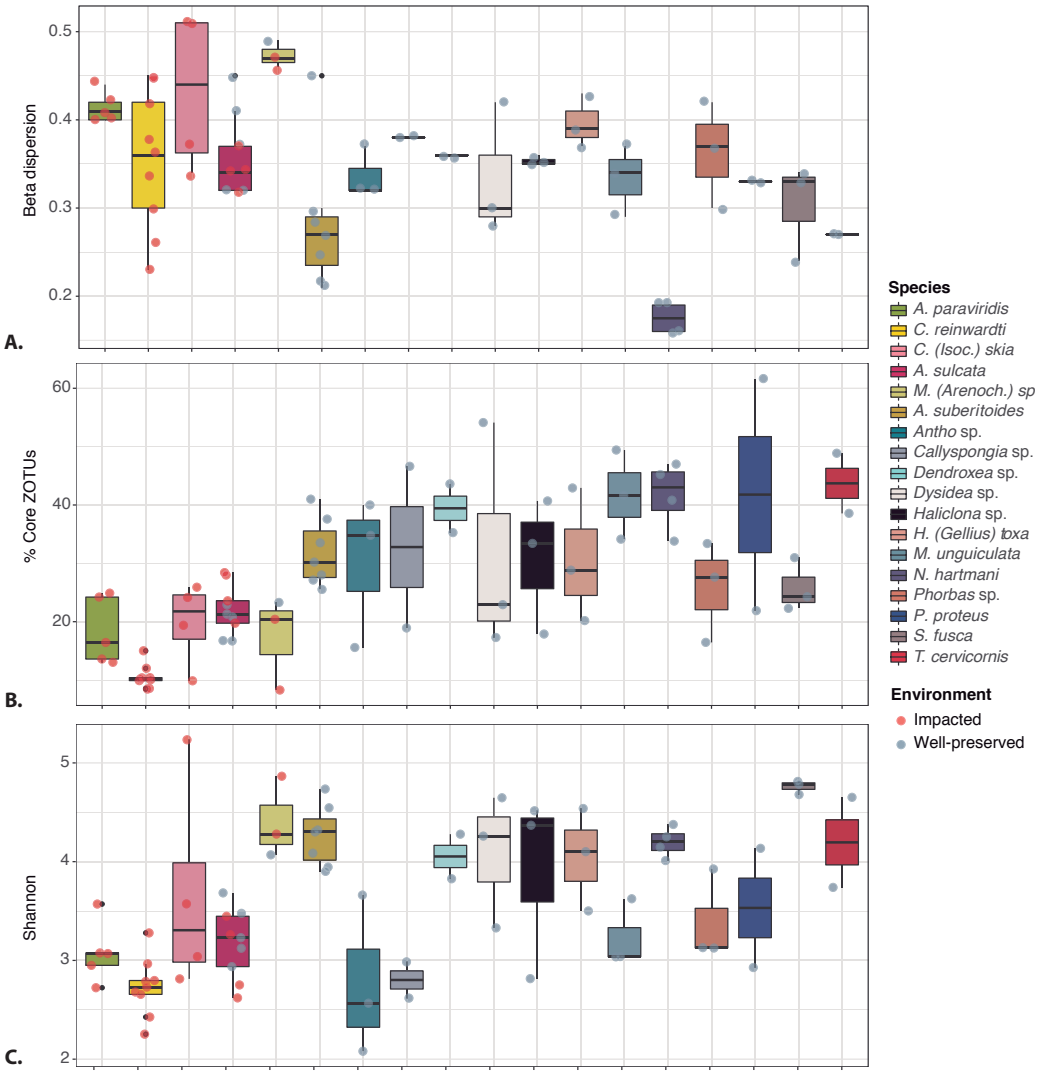

Figure C.6: Box plots showing the intra-species dispersion (A), core size (B) and Shannon diversity (C) of each sponge species microbiome. Species replicates from impacted and well-preserved habitats are indicated by red and grey dots, respectively.

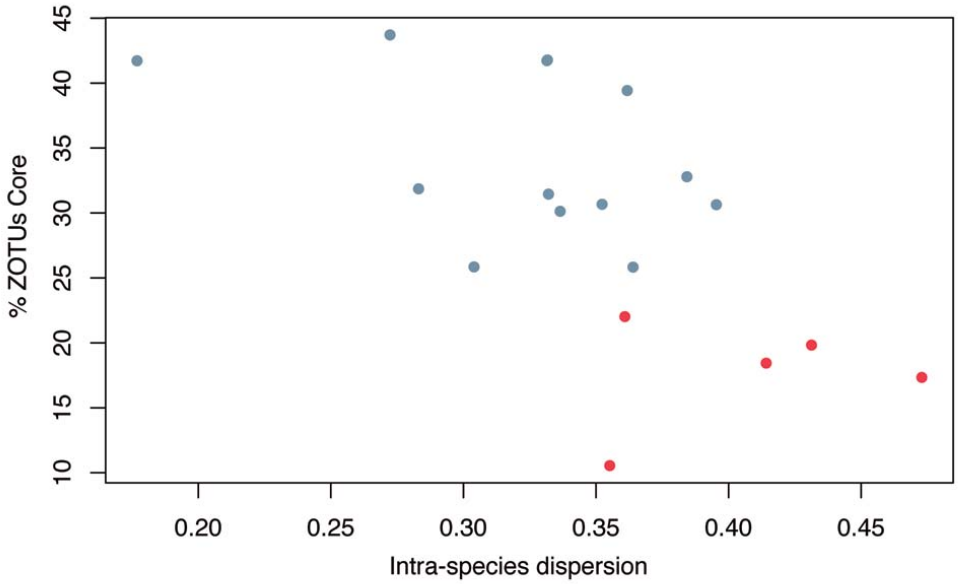

Figure C.7: Relation between the intra-species dispersion and core size of the sponge microbiomes. Grey dots correspond to species from well-preserved habitats and red dots correspond to species from the impacted habitats.

Appendix D

Chapter 5 Supporting Information

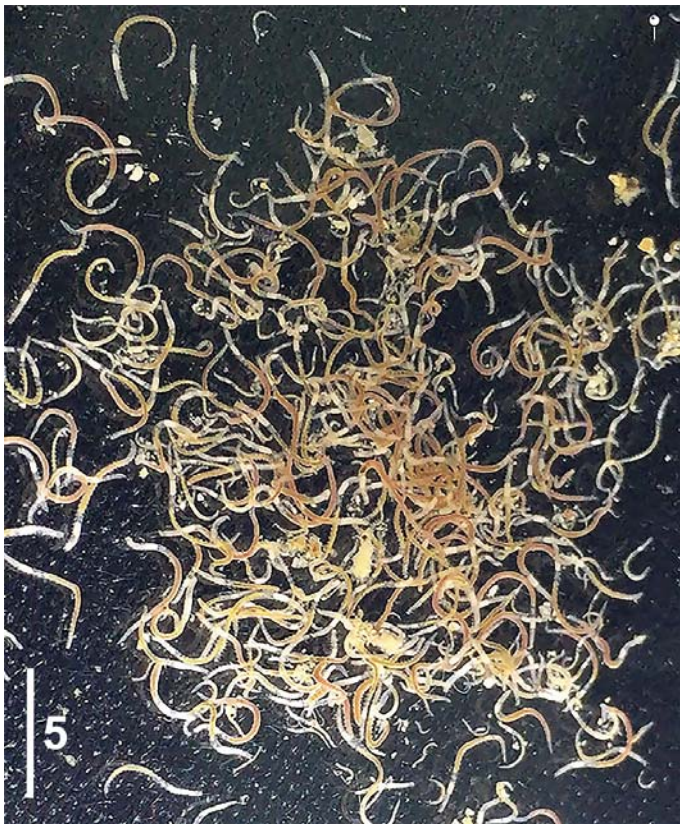

Figure D.1: Photo of *Haplosyllis* sp. extracted from a sponge fragment of *Neofibularia hartmani*. Scale bar is represented in mm. Photo by: Dr. Daniel Martin.

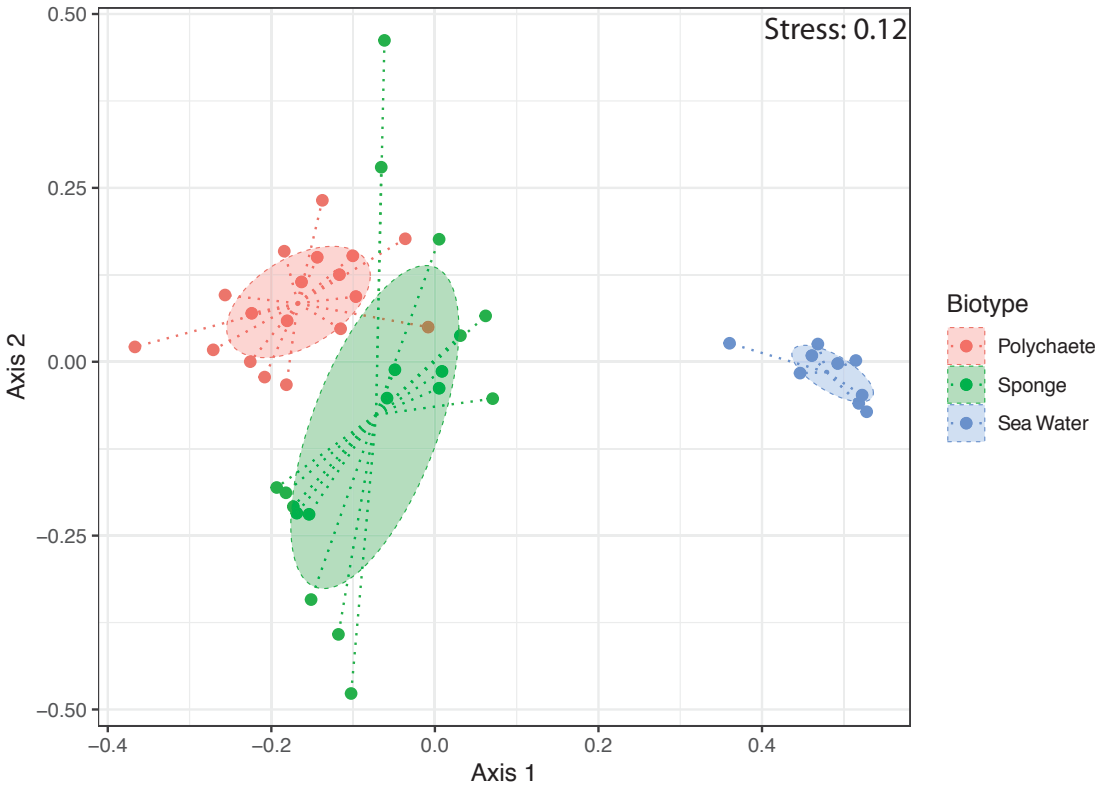

Figure D.2: Non-metric multidimensional scaling (nMDS) ordination of the sponge (green) polychaete (red) and sea water (blue) bacterial communities based on Bray-Curtis distances.

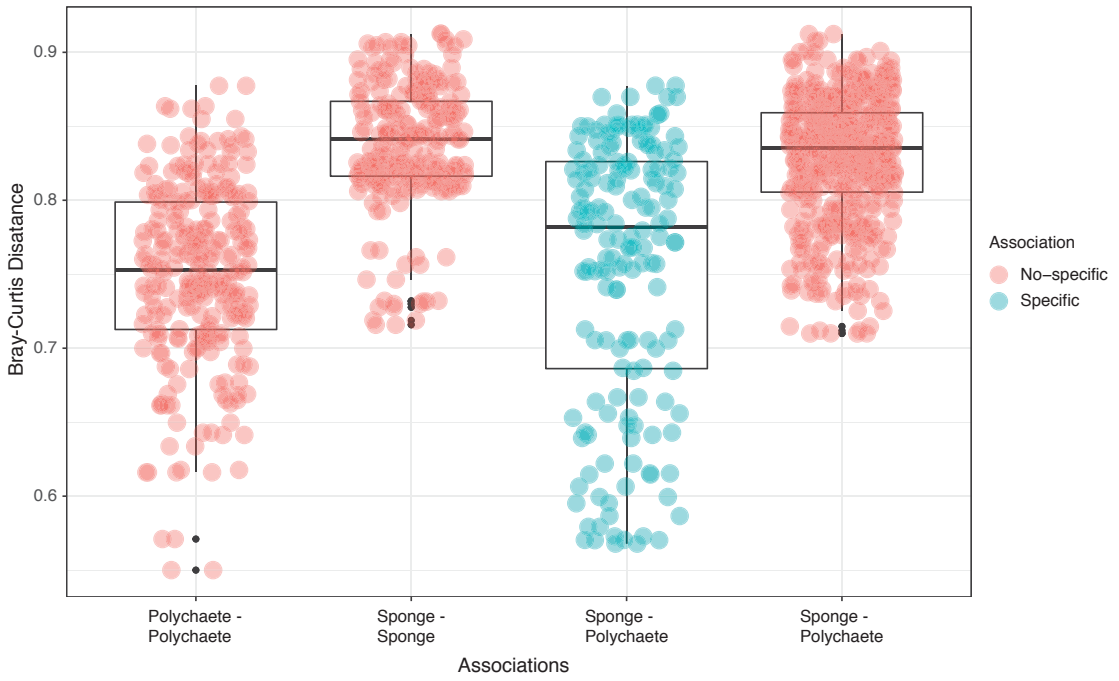

Figure D.3: Box plot of the Bray-Curtis distances of the bacterial communities between: i) polychaete species, ii) sponge species, iv) polychaetes and their host sponge (specific) and, v) polychaetes and non-host sponges (non-specific). The specific associations are depicted in green and the non-specific associations are depicted in red.

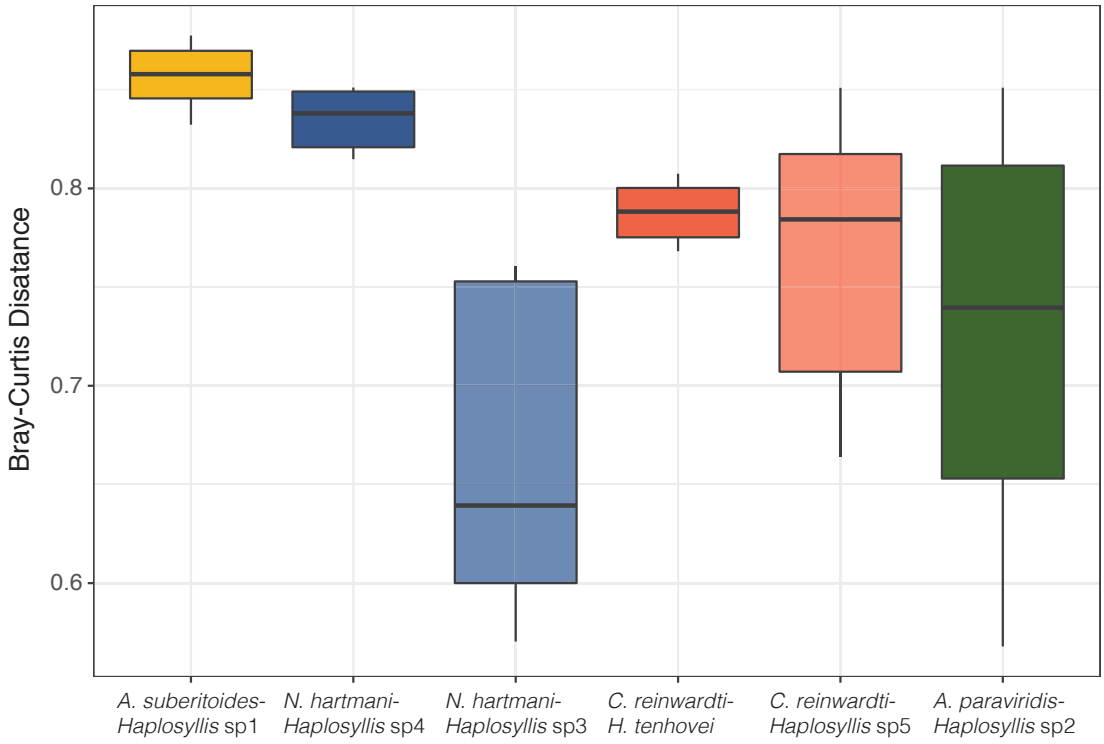

Figure D.4: Box plot of the Bray-Curtis distances of the bacterial communities between each polychaete species and its host sponge (specific associations). Polychaete species associated to the same sponge host are depicted in a similar colour.

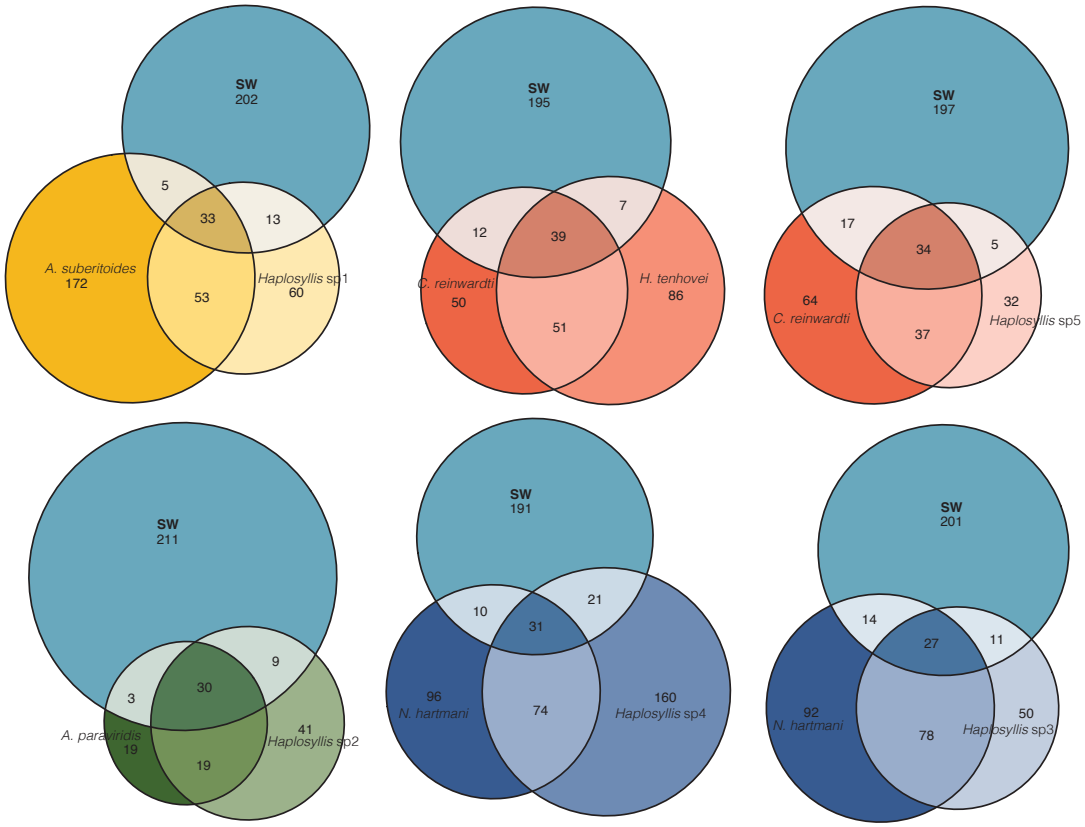

Figure D.5: Venn diagrams showing the overlap between the bacterial core communities of each sponge species, its associated polychaete and sea water. The circle size represents that of the bacterial core (in number of ZOTUs).

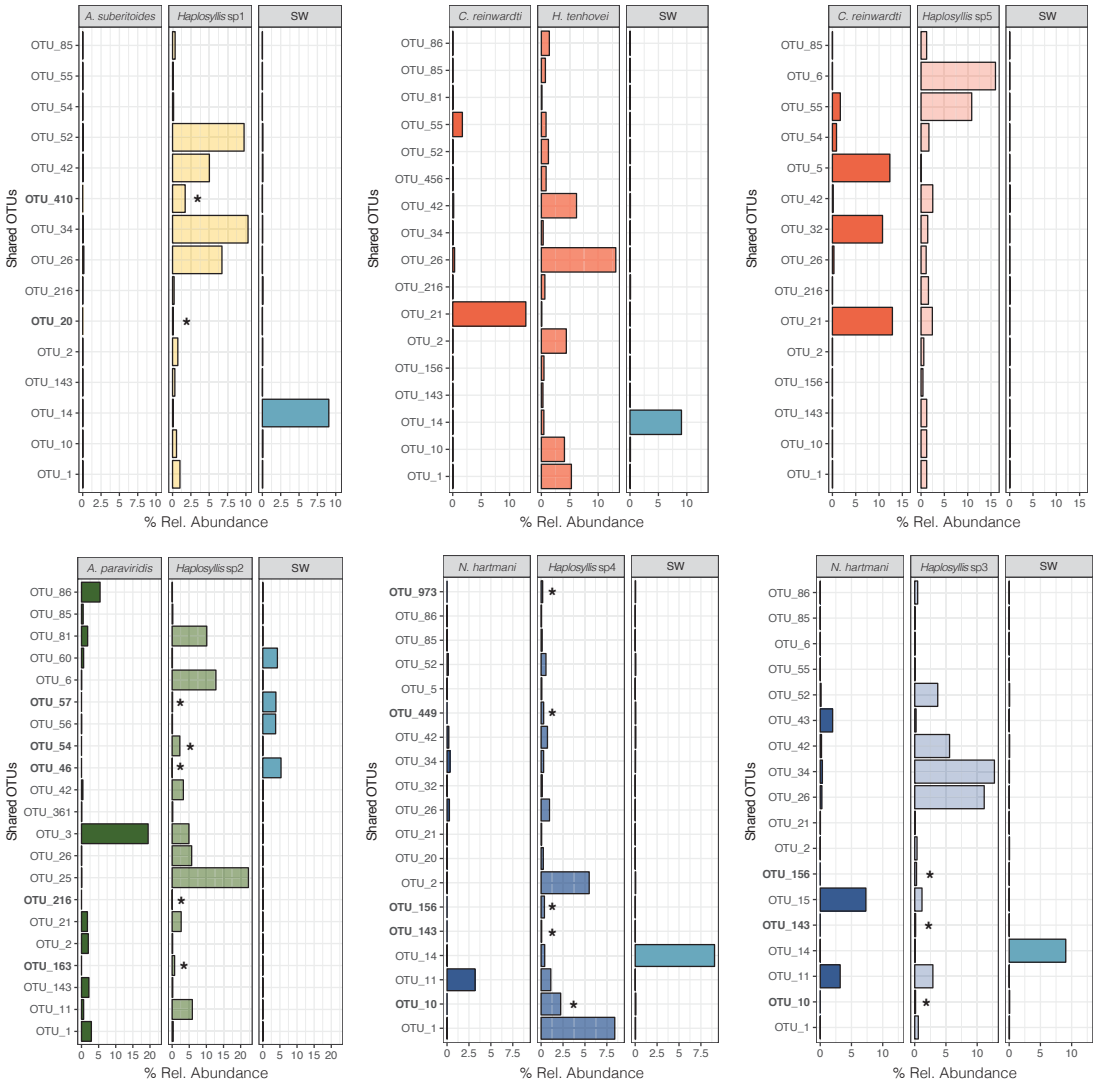

Figure D.6: Bar plots represent the relative abundances (%) of the shared ZOTUs between each polychaete species, its host sponge and seawater. Only ZOTUs with relative abundances higher than 0.5% in the polychaete are shown in the bar plots.

Table D.1: Number of replicates, total ZOTUs, core ZOTUs, core reads and relative abundance of core ZOTUs for each sponge and polychaete species.

Species	Num. replicates	Num. ZOTUs total	Num. ZOTUS Core	Num. reads Core	Rel. Abundance Core ZOTUS (%)
A. suberitoides	3	1222	263	36196	90.49
A. paraviridis	5	1268	71	21979	54.94
C. reinwardti	5	2525	152	36221	90.55
N. hartmani	5	2096	211	38113	95.28
Haplosyllis sp1	3	1101	159	34138	85.34
Haplosyllis sp2	5	1242	99	35851	89.62
H. tenhovei	2	699	183	35008	87.52
Haplosyllis sp5	3	837	108	32619	81.54
Haplosyllis sp4	2	1343	286	26918	67.29
Haplosyllis sp3	3	985	166	32229	80.57

Table D.2: List of the 44 core ZOTUs present in all polychaete species. The mean relative abundance (%) and the affiliation at all taxonomic levels are shown for each ZOTU.

ZOTU	Mean Rel. Abundance (%)	Phylum	Class	Order	Family	Genus
OTU26	6.3	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio
OTU6	6.23	Proteobacteria	Alphaproteobacteria	Caulobacterales	Hyphomonadaceae	Litorimonas
OTU25	6.2	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Hahellaceae	Endozoicomonas
OTU34	3.93	Proteobacteria	Gammaproteobacteria	Alteromonadales	Pseudoalteromonadaceae	Pseudoalteromonas
OTU13	3.91	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrionaceae-unclass
OTU42	3.86	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrionaceae-unclass
OTU48	2.93	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Hahellaceae	Endozoicomonas
OTU81	2.85	Proteobacteria	Gammaproteobacteria	Alteromonadales	Shewanellaceae	Shewanella
OTU52	2.45	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Alteromonas
OTU11	2.26	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Hahellaceae	Endozoicomonas
OTU1	2.07	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonadaceae-unclass
OTU55	1.94	Proteobacteria	Gammaproteobacteria	Alteromonadales	Shewanellaceae	Shewanella
OTU2	1.42	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonas
OTU3	1.39	Proteobacteria	Betaproteobacteria	Nitrosomonadales	Nitrosomonadaceae	Candidatus Branchiomonas
OTU112	1.29	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Photobacterium
OTU21	1.17	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Hahellaceae	Endozoicomonas
OTU160	1.09	Proteobacteria	Gammaproteobacteria	Alteromonadales	Pseudoalteromonadaceae	Pseudoalteromonas
OTU10	1.02	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter
OTU180	0.93	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Photobacterium
OTU170	0.79	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Oceanospirillaceae	Marinomonas
OTU37	0.75	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
OTU63	0.52	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Hahellaceae	Endozoicomonas
OTU64	0.49	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Hahellaceae	Endozoicomonas
OTU85	0.41	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Delftia
OTU358	0.37	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio
OTU143	0.37	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas
OTU16	0.26	Acidobacteria	Holophagae	Subgroup 10	TK85	TK85-unclass
OTU32	0.26	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Rhodobacteraceae-unclass
OTU294	0.23	Proteobacteria	Gammaproteobacteria	Alteromonadales	Shewanellaceae	Shewanella
OTU15	0.2	PAUC34f	PAUC34f-unclass	PAUC34f-unclass	PAUC34f-unclass	PAUC34f-unclass
OTU122	0.18	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Escherichia-Shigella
OTU14	0.14	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonas
OTU45	0.13	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Hahellaceae	Endozoicomonas
OTU53	0.13	Proteobacteria	Deltaproteobacteria	Desulfurellales	Desulfurellaceae	G55
OTU47	0.11	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Hahellaceae	Endozoicomonas
OTU12	0.05	Actinobacteria	Acidimicrobiia	Acidimicrobiales	Sva0996 mar. gr.	Sva0996 mar. gr. unclass
OTU43	0.04	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Albidovulum
OTU31	0.04	Proteobacteria	Betaproteobacteria	Nitrosomonadales	Nitrosomonadaceae	Candidatus Branchiomonas
OTU8	0.03	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	uncultured
OTU60	0.03	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	AEGEAN-169 marine group
OTU5	0.03	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	uncultured
OTU33	0.01	Proteobacteria	Deltaproteobacteria	SAR324 clade (Marine group B)	SAR324 clade (M.gr.B)-unclass	SAR324 clade (M. gr. B)-unclass
OTU115	0.01	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus
OTU118	0.01	Proteobacteria	Alphaproteobacteria	SAR11 clade	Surface 4	Surface 4-unclass

Appendix E

Chapter 6 Supporting Information

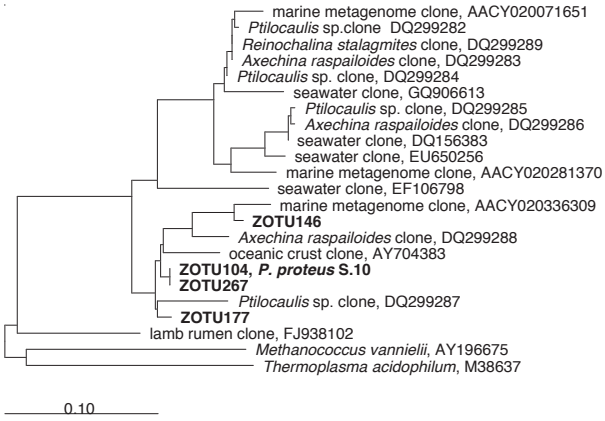

Figure E.1: 16S rRNA-based phylogeny of sponge associated *Euryarchaeota* obtained in Simister et al. (2012a). The displayed tree is a maximum likelihood tree constructed based on long sequences only (>1200 bp). ZOTUs obtained in this study were added using the parsimony interactive tool in ARB and are indicated in Bold. Sponge species replicate is indicated next to the ZOTU if that ZOTU is the dominant in this sponge.

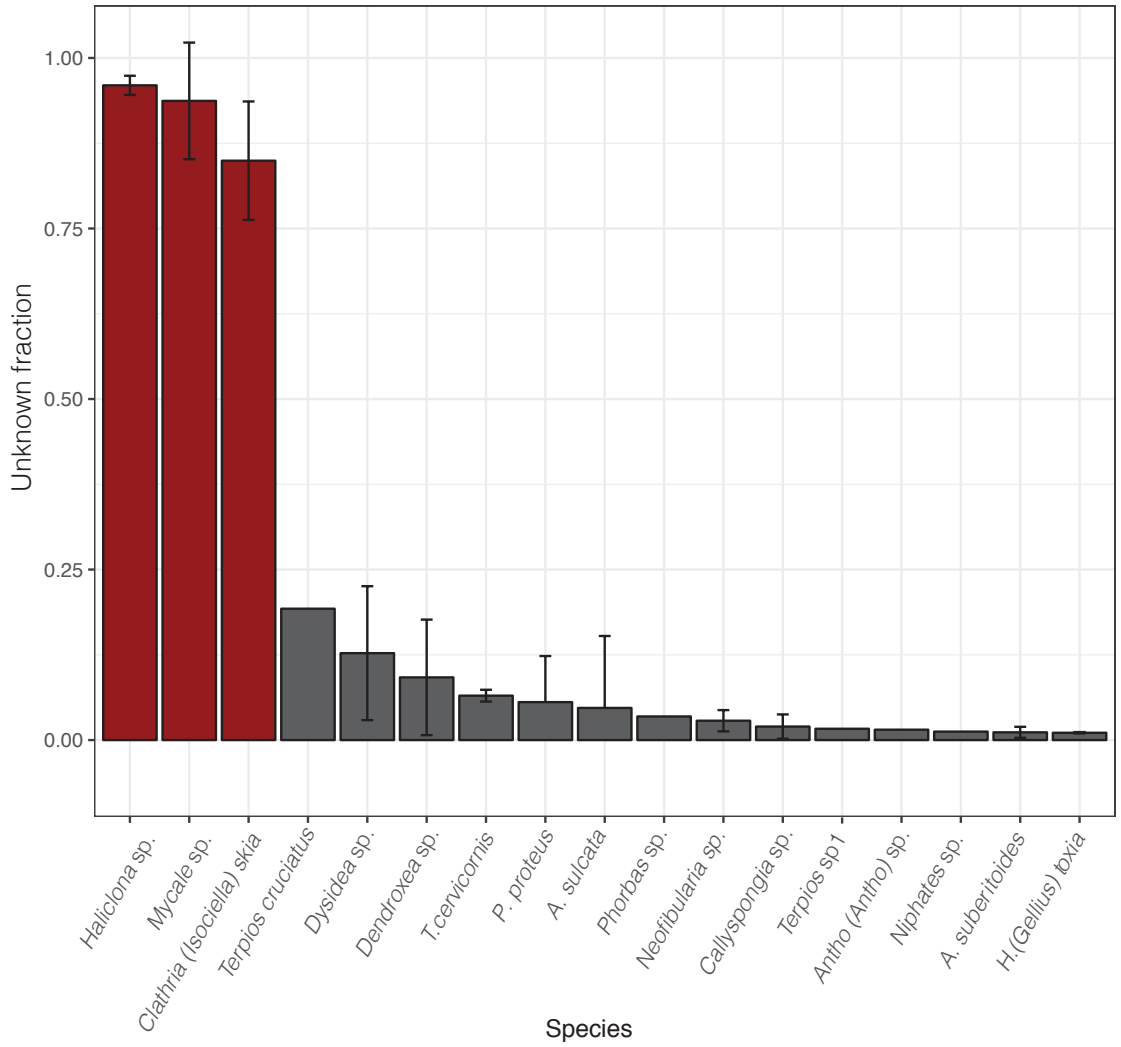

Figure E.2: Percentage of reads unused in the *Tax4Fun* prediction for each sponge species. In red, species discarded for further analysis. In grey, species used for the functional prediction analysis. SD bars are represented.

Appendix F

Published Chapters

SCIENTIFIC REPORTS

OPEN

Showcasing the role of seawater in bacteria recruitment and microbiome stability in sponges

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Marta Turon, Joan Cáliz, Leire Garate, Emilio O. Casamayor & Maria J. Uriz

We studied the core bacterial communities of 19 sponge species from Nha Trang Bay (Central Vietnam), with particular emphasis on the contribution of planktonic seawater bacteria to the sponge core microbiomes. To ensure consistent sponge-microbe associations and accurate identification of planktonic bacteria transmitted from seawater, we were very restrictive with the definition of the sponge core microbiomes (present in all the replicates), and with the identification of valid biological 16S rRNA gene sequences (100% sequence identity) that belonged to potentially different bacterial taxa. We found a high overlap (>50% relative abundance) between the sponge species core microbiome and the seawater bacterial core in ca. a half of the studied species, including representatives of both, HMA and LMA sponges. From our restrictive analysis, we point to horizontal transmission as a relevant way of symbiont acquisition in sponges. Some species-specific recognition mechanisms may act in sponges to enrich specific seawater bacteria in their tissues. These mechanisms would allow the maintenance of bacterial communities in a species across geographical ranges. Moreover, besides contrasting preferences in bacteria selection from seawater, divergent physiological traits may also account for the different microbiomes in species of HMA and LMA sponges.

The first step to study multi-microbial symbionts within animals is to focus on permanent symbionts by ruling out the background noise produced by transient microbes[1]. In other words, to concentrate on those bacteria, which have established tight associations with the host through several evolutionary time scales[2], independently of potential, mutual benefits or costs for the partners involved[3]. In this context, the core microbiota concept was adopted to ascertain the consistent associations of a metaorganism[4,5], but also allows to study the core metabolic functions provided by the host–microbe interaction to the system[6]. The core concept was first applied to differentiate host–microbe interactions of the mammalian gut and in plant root systems[5,6]. Further, it was extended to marine animals with the aim of understanding the consistent contributions of the microbial symbionts to the host ecology, success, or decay[1].

In marine habitats, studies on coral and sponge microbiomes have proliferated in the last 10 years. For instance, the persistent microbial symbionts (core) of several coral species from a reef were identified[7] through spatial and temporal scales, concluding that the complexity of the reef habitat, and the life coral history traits likely influence the coral core microbiomes. Thus, more in deep research to explore accurately the core microbiome of many invertebrates is needed to overcome the constraints associated to their complex habitats. Although there is some controversial in the literature about how the core microbiota should be defined[1,8], the most reasonable definition always depends on the question approached[9]. For instance, more or less restrictive criteria (i.e. present from 7% to 100% of the replicates) have been used for marine invertebrates[1,5,10,11].

Sponges are a diverse group of sessile filtering invertebrates that play important ecological roles in benthic marine ecosystems[12,13]. They harbour the highest diversity of microsymbionts among marine invertebrates[14,15]. A wide range of studies using massive sequencing methods have retrieved thousands of microbial 16S rRNA gene sequences for each targeted sponge species[11], which stressed the difficulty to unveil the mechanisms underlying these associations due to the high diversity of the partners involved. Indeed, some of the 16S rRNA gene sequences belonged to transient bacteria captured from the environment by the sponges while filtering seawater, and do not represent permanent symbionts[11]. Consequently, indirect analytical methods have been implemented trying to split stable symbionts from transient planktonic bacteria in sponge-microbial systems. The core

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microbiota concept was adopted to focus on permanent bacteria in a sponge species[11] or phylum[10], notwithstanding the host geographical and ecological origins, or temporal scales[16]. The rationale underlying the core concept is that stable symbionts should represent tight biological associations and thus, they are expected to be present in most, or all, host individuals.

Substantial differences in the resulting diversity metrics related to the core definition applied (from 12% to 100% occurrence), have been reported recently[9]. Several studies have proposed that stable sponge microsymbionts should be present in the sponges but not (at least not in high abundance) in the surrounding seawater[11]. But, recording the presence of a sponge bacterium in seawater greatly depends on the abundance threshold used to include or exclude sequences from downstream analysis, the sequencing depth and the number of samples. Moreover, it has been proposed that stable symbionts are mainly inherited by the progeny from their parent sponges[17–20]. However, vertical and horizontal transmission of the same bacteria have been described[21] and more recent investigations reported different microbiomes in adults and larvae of the same species, which highlights the role of seawater bacteria in the structural composition of sponge microbiomes[17,22].

Differences in microbial diversity between High Microbial Abundance (HMA) and Low Microbial Abundance (LMA) sponges have been reported[23,24]. These differences have been related to contrasting structural and physiological traits of the two species groups[25]. HMA sponges have a denser mesohyle and a more complex aquiferous system, with smaller choanocyte chambers, than LMA sponges[25]. Also a partial trophic niche separation for HMA and LMA sponges has been proposed[26]. Comparisons of the core microbiomes of these two groups, which consisted in bacterial T-RFLPs that were present in all species replicates across seasons and study years, showed that HMA sponges had a larger number of core bacterial groups with a higher overlap with seawater than LMA hosts[27].

In this study, we attempted to cast some light on the acquisition modes of microbial symbionts in sponges by i) unveiling the permanent microbiomes within a large number of sponge species, and ii) estimating the contribution of seawater bacteria to the sponge core microbiomes in representatives of both HMA and LMA sponges. To address these goals, we analysed the sponge microbiomes of the 19 most abundant sponge species inhabiting a small geographical area in Nha Trang Bay (Central Vietnam), as well as the bacterial assemblages of the surrounding seawater. We applied a restrictive approach to core community concept in terms of bacteria occurrence across species replicates and to OTU definition. Sequence identity thresholds $<$99% for the 16S rRNA V4 region have been proved to be inaccurate for bacterial species delimitation[28], in particular for short reads obtained from Next Generation Sequencing (NGS). Instead, a 100% of sequence identity has been proposed to obtain true biologically informative sequences, which can underlie metabolic and ecological particularities[28]. To ensure that the 16S rRNA gene sequences recovered from the seawater samples were identical to those recovered from the sponges, we clustered OTUs (Operational Taxonomic Units) at 100% identity (Zero radius OTUS or ZOTUs[28]), which has only been recently applied in a couple of studies of sponge microbiomes[29,30]. Clustering sequences at 100% identity and restricting the core to microbes present in 100% of the analysed samples seem particularly relevant when trying to elucidate horizontal symbiont acquisition.

Results

Specificity of sponge bacterial communities. The main factor structuring the sponge microbiomes was the sponge species (Permanova: R^2 0.56, p-value $<$ 0.01), which means that replicates from the same species were more closely related to each other than to any other species. However, dispersion within replicates greatly varied depending on the sponge species (Permutest F: 6.9 p-value $<$ 0.01). A dichotomy (Permanova: R^2 0.11, p-value $<$ 0.01) between the so-called HMA and LMA sponges regarding their bacterial composition was detected (Supplementary Fig. S1). Moreover, dispersion within the three HMA species (*Aaptos suberitioides, Neofibularia hartmani* and *Suberea cf. laboutei*) was much lower than the dispersion within the 16 LMA species (Permutest F: 60.8 p-value $<$ 0.01, Supplementary Fig. S1).

Core communities and species specific ZOTUs. The ZOTU richness of the core communities (ZOTUs present in all replicates of the same sponge species) varied from 54 in *Clathria reinwardti* to 600 in *Thrinacophora cf. raphidophora* (Table 1). The number of species replicates influences the number of ZOTUs forming the core community, the more replicates taken into account, the lowest the number of core ZOTUs ($R_S = -0.83$, p-value $<$ 0.01, Supplementary Fig. S2A). However, ZOTU abundance of the sponge species core did not depend on the number of species replicates since no correlation was found between both variables ($R_S = -0.21$, p-value $>$ 0.05, Supplementary Fig. S2B). This means that the abundant ZOTUs are the major contributors to the core of a species, and that the variable fraction is represented by the low abundance ZOTUs. Thus, we have considered the comparisons based on the relative abundances of the core ZOTUs.

The core community represented more than 75% of the reads from the total microbiome in most species (Fig. 1). *N. hartmani* was the species with the largest core microbiome, representing up to 94% of relative abundance (Table 1) and *Gellioides* sp. had the smaller core microbiome representing $<$50% of relative abundance.

The number of species-specific ZOTUs (those present in all replicates of a species and absent from the core of any other study sponge species) varied from 0 (in *C. reinwardti*) to 195 (in *T. cf. raphidodophora*). However, the abundance of species–specific ZOTUs did not surpass 25% in any case, and most often were below 5% of relative abundance in the respective core communities. *Gellioides* sp. and *S. cf. laboutei* were the species with the highest relative abundances of species-specific ZOTUs (25% and 20%, respectively).

Similar values of core communities and species-specific bacteria were obtained for the dataset analysed with OTUs clustered at 97% sequence similarity (Supplementary Table S1).

Composition of the bacterial core communities. Specific associations between certain bacteria (phylum and class level) and HMA or LMA species were detected with the *Indval* analysis (Supplementary Fig. S3).

Species	n	Mean ZOTUs ±SD	Core ZOTUs	% Ab. Core ±SD	Sp-sp ZOTUs[a]	% Ab. Sp-sp ZOTUs	SW ZOTUs[b]	% Ab. SW ZOTUs
**Aaptos suberitoides* (Brondsted, 1934)	13	740 ± 139	134	77.6 ± 6.2	6	1.1	53	48.79
**Neofibularia hartmani* (Hooper & Lévi, 1993)	10	883 ± 182	167	94.2 ± 1.6	32	4.4	67	71.59
**Suberea cf.laboutei* (Bergquist, 1995)	3	812 ± 133	206	79.2 ± 7.8	65	20.6	61	26.67
Amphimedon paraviridis (Fromont, 1993)	10	587 ± 223	57	61.9 ± 22.1	1	0.0	52	99.54
Antho sp.	3	1587 ± 872	404	88.2 ± 5.1	71	2.0	107	9.90
Callyspongia sp.	2	850 ± 507	229	83.9 ± 2	30	1.0	76	38.55
Clathria reinwardti (Vosmaer, 1880)	15	769.1 ± 171	54	88.4 ± 7.3	0	0.0	36	90.40
Clathria sp.	4	838 ± 420	144	69.6 ± 19.9	2	0.3	50	33.57
Dendroxea sp.	2	795 ± 118	310	77.7 ± 13.9	50	11.6	99	33.99
Dysidea sp.	3	1811 ± 902	453	84.6 ± 6.8	66	3.7	131	27.35
Gellioides cf.gracilis (Hentchel, 1912)	9	647 ± 123	138	82.9 ± 5.3	1	0.4	69	53.03
Gellioides sp.	4	456 ± 87	96	49.3 ± 27.5	8	25.7	83	93.22
Haliclona sp.	3	1343 ± 611	365	79.5 ± 9.7	44	3.1	123	16.06
H. (Gellius) toxotes (Hentchel, 1912)	3	892 ± 327	249	74.3 ± 4.2	15	0.2	88	60.34
Monanchora unguiculata (Dendy, 1922)	3	622 ± 115	254	86 ± 7.8	12	0.3	93	58.71
Mycale sp.	4	1720 ± 1576	80	54 ± 7.3	2	0.7	42	77.51
Phorbas sp.	3	905 ± 349	214	86.6 ± 6.6	14	0.6	77	52.20
Pseudosuberites sp.	2	1791 ± 1205	579	86.4 ± 9.4	171	4.3	125	10.97
Thrinacophora rhaphidophora (Hentschel, 1912)	2	1392 ± 231	600	89.1 ± 5.2	195	9.6	169	22.67

Table 1. Mean, core, species-specific, and SW ZOTUs of all species studied. Values are given in number and relative abundance (%) of ZOTUs. HMA species are marked in dark grey and LMA species are marked in light grey. (n = number of replicates per species). *HMA species. [a]Number and percentages of abundances of species-specific ZOTUs are calculated for the core community of each species. [b]Number and percentages of abundances of SW ZOTUs are calculated for the core community of each species. Values correspond to the comparison with the SW core ZOTUs (cosmpolitan).

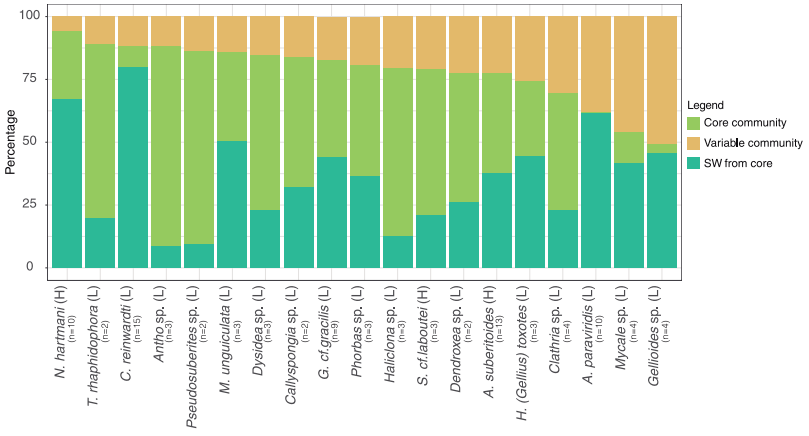

Figure 1. Mean relative abundances for the core (green) and the variable fraction (yellow) of the sponge microbiomes. Blue lines delimit the percentage of the sponge bacterial core shared with the seawater bacterial community. H = High Microbial Abundance sponge, L = Low Microbial Abundance sponge.

These indicator bacteria are present in the core communities of the studied sponges (Fig. 2). The three HMA species presented an alike core microbial composition and abundances at phylum level with specific bacterial phyla overrepresented, compared to LMA species and seawater (SW). Phyla associated with LMA sponges also showed a similar core microbial composition but with contrasting abundances in the several species. Seawater samples had their own core bacterial composition with representatives of bacterial phyla shared with either HMA

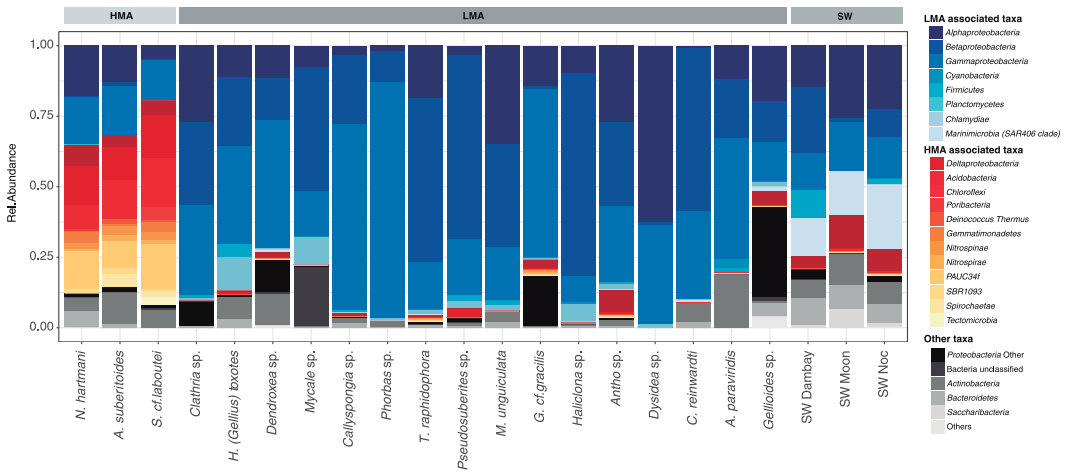

Figure 2. Mean relative abundance of core microbial taxa at a phylum level (class for *Proteobacteria*) within each sponge species and seawater samples. Bacterial taxa with significant Indval values (Supplementary Fig. S3) associated to HMA species are marked in reddish colours and the ones associated to LMA species are marked in bluish colours.

or LMA sponges. Mean Shannon diversity indices of the species core communities were significantly higher (Kruskal-Wallis <0.01) for HMA species (3.8 ± 0.24) than for LMA species (2.3 ± 0.61) (Supplementary Fig. S4).

Seawater (SW) ZOTUs. The relative abundances of the shared core SW ZOTUs (detailed in the Experimental procedures section) with the sponge core microbiomes varied between 9% and 99% depending on the sponge species (Fig. 3A). The sponges with the highest contribution of core SW ZOTUs to their core microbiome were *A. paraviridis* (99.5%), *Gellioides* sp. (93.2%) and *C. reinwardti* (90.4%), while *Antho* sp. (9.9%) and *Pseudosuberites* sp. (10.9%) showed the lowest overlap between the sponge and the SW core bacteria (Fig. 3A, Table 1). In some species (i.e.: *A. suberitoides, C. reinwardti*, *Mycale* sp.), rare SW ZOTUs (>0.01% of relative abundance) were abundant in the core of the sponge species. This is particularly visible in the example of *C. reinwardti*, which harboured ZOTUs that represented just a 5% of the SW core community and 90% of the sponge core. The opposite occurred in other sponge species such as *Pseudosuberites* sp. and *T. cf. raphidophora*, which had highly abundant (>1% of relative abundance) SW ZOTUs poorly represented in their core.

In addition, when the most abundant SW ZOTUs were considered (abundances higher than 0.01%), instead of the SW core ZOTUs, the relative abundances of bacteria shared with SW were drastically reduced in most of sponge core microbiomes, with most values below 40% (Fig. 3B). This reduction was notably relevant in some species, such as *Gellioides* sp. *N. hartmani, Mycale* sp. *A. suberitioides, C. reinwardti*, and *S. cf. labutei* (Fig. 4).

Results of both comparisons for OTUs at 97% sequence identity are shown in Table S2. Differences between OTUs and ZOTUs were more remarkable when considering the abundant SW ZOTUs. For instance, in the case of *C. reinwardti*, the proportion of SW OTUs changed from 10% (for ZOTUs) to 73% (for OTUs). Also remarkable were the differences for *A. paraviridis*, *Gellioides* sp., and *Phorbas* sp.

The abundance and distribution of the SW core ZOTUs in the sponge core microbiomes is shown as a heatmap representation (Fig. 5). Two sponge clusters (A and B) were differentiated in the dendrogram. Cluster A contained sponge species that shared two highly abundant SW ZOTUs (ZOTU1, ZOTU2 at mean abundances of ~10%), and some ZOTUs (10, 37) at abundances higher than 1%. Cluster B comprised species harbouring different SW bacteria at contrasting abundances. Two different ZOTUs of *Candidatus Branchiomonas* (*Betaproteobacteria*) accounted for more than 30% and 50% of the *Mycale* sp. and *A. paraviridis* core microbiomes, respectively. Similarly, ZOTUs belonging to several *Endozoicomonas* (*Gammaproteobacteria*) were present at different relative abundances in the four species with the highest proportion of SW ZOTUs (*Gellioides* sp, *A. paraviridis*, *Mycale* sp. and *C. reinwardti*). Two main groups (C, D) were also differentiated according to the bacterial classes. The first group (C) corresponded to bacterial classes associated to LMA sponges, with high abundances of *Alpha-, Beta-*, and *Gamma- Proteobacteria*. The second group (D) showed bacterial classes associated to HMA sponges. ZOTUs belonging to PAUC34f, *Chloroflexi, Acidobacteria, Actinobacteria*, and *Nitrospinae* were almost exclusively found in the three HMA species (*N. hartmani*, *S. cf. laboutei* and *A. suberitioides*).

Discussion

The sponge-associated bacteria were species specific for the 19 study species, as previously reported for sponges from other geographical areas[10,17,25,31–34]. Selection of specific bacteria and competition among the selected bacteria[31] may converge to shape the species-specific patterns of sponge microbiomes, which used to be similar in geographically distant individuals of the same genus[35].

However, differentiated patterns of bacterial composition are observed according to the affiliation to the HMA or LMA groups, as previously reported[36,37]. Indicator bacteria at class level for both groups are found, which match the indicator classes inferred from differential abundance analysis[38]. It has been reported that HMA sponges show higher microbiome similarity among species than LMA sponges[27]. Moreover, bacterial diversity is

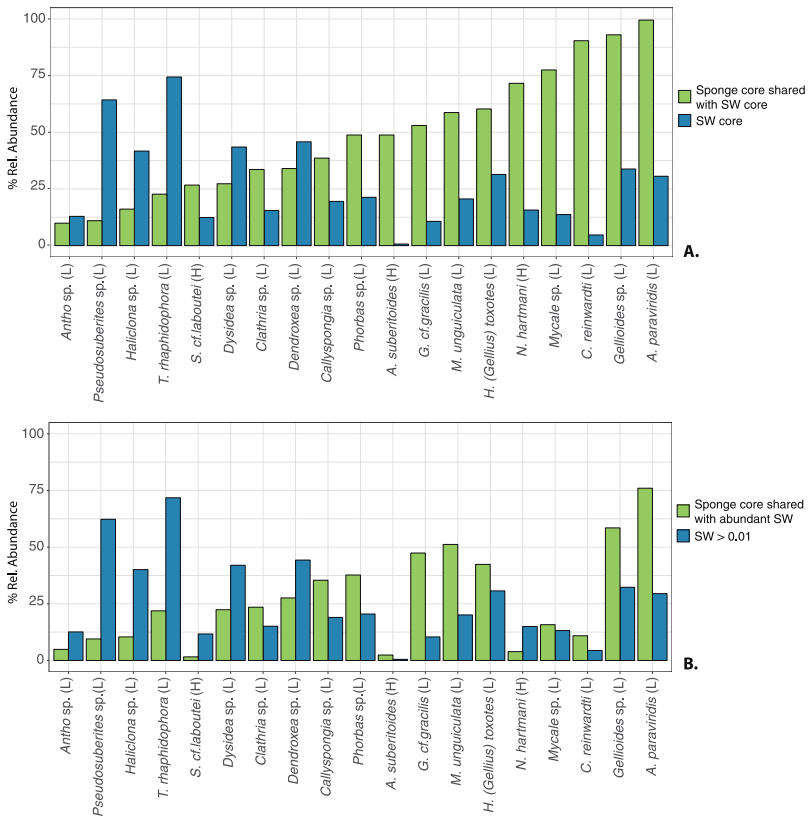

Figure 3. Mean relative abundances of shared bacteria between the SW and the core microbiomes for each sponge species when comparisons were made with the (**A**) SW core (cosmopolitan bacteria) and (**B**) the abundant (>0.01%) SW bacteria. Bars represent percentages of relative abundances of the shared bacteria in both, the sponge (green colour) and the SW (blue colour). H = High Microbial Abundance sponge, L = Low Microbial Abundance sponge.

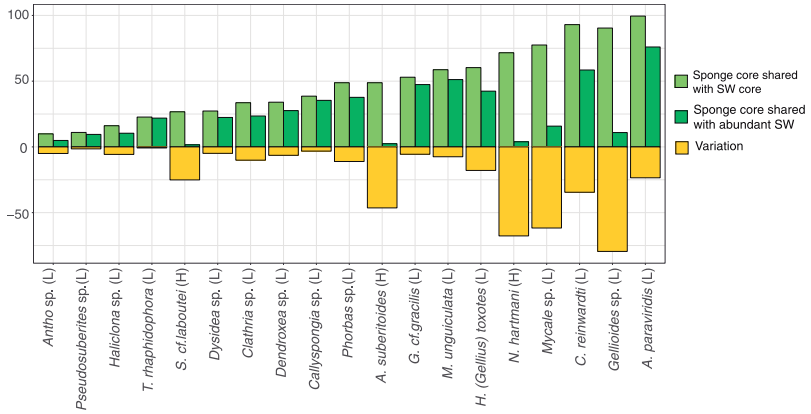

Figure 4. Variation in relative abundance of SW ZOTUs in the sponge cores according to the method used for comparisons. Light green bars show ZOTUs shared with the SW core. Dark green bars represent ZOTUs shared with the abundant SW ZOTUs. Negative bars (yellow) represent the differences in shared ZOTUS between methodologies. H = High Microbial Abundance sponge, L = Low Microbial Abundance sponge.

also claimed to be higher in HMA[14,23,27,39,40]. These aspects are confirmed in our study when considering the core members of both HMA and LMA sponges, although the number of representatives of each group is unbalanced.

It has been assumed[40,41], but also questioned[24,25], that LMA sponges contain mainly transient seawater bacteria. Representatives of both HMA and LMA study sponges contained high percentages of SW core bacteria (>50% of core relative abundance). However, species of each group acquire different SW bacteria that are indicators of either HMA or LMA sponges (Fig. 2 and Supplementary Fig. S3), suggesting contrasting bacteria selection

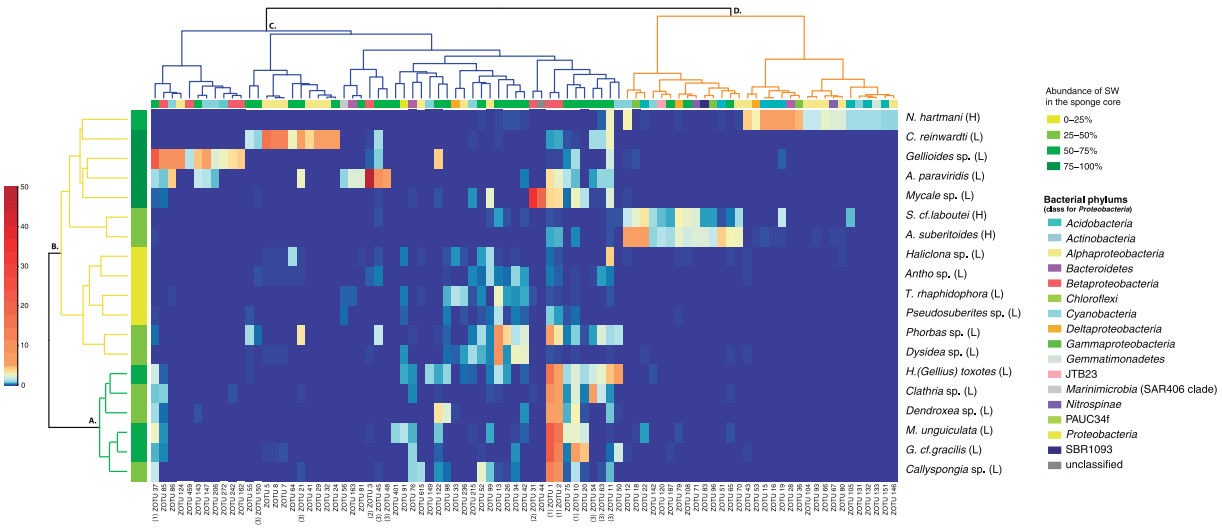

Figure 5. Heatmap showing core (cosmopolitan) SW ZOTUs with relative abundances higher than 1% across sponge species (listed on the right side). On the left, hierarchical clustering using Bray-Curtis dissimilarity matrix of the sponge species (colours on the vertical stripe represent the relative abundance of SW ZOTUs). On top, hierarchical clustering using Bray-Curtis dissimilarity matrix of the bacterial ZOTUs (colours on the horizontal stripe indicate ZOTU taxonomy at a Phylum level (class level for *Proteobacteria*). ZOTUs abundance is represented in the colour temperature bar on the left. Letters A, B, C, D indicate the different clusters. ZOTU numbers between parenthesis means: (1) ZOTUs shared by cluster A, (2) *Candidatus Branchiomonas* ZOTUs, (3) *Endozoicomonas* ZOTUs. H = High Microbial Abundance sponge, L = Low Microbial Abundance sponge.

mechanisms in each group. Differences might also be enhanced by particular traits of the respective physiology of the two sponge types[42].

To define species core and species-specific bacteria, we clustered sequences at 100% identity (ZOTUs). Only recently, the sponge microbiomes have been analysed at the ZOTU level[29,30]. By recording ZOTUs, we were able to identify closely related bacteria[28], which may inhabit sponge and seawater biomes making comparisons more reliable.

Analysing the bacterial core at species level provides information about stable, purportedly fixed associations, which may be involved in sponge-bacteria interaction patterns. To define the species core, an appropriate percentage of bacteria occurrence across species replicates has to be selected depending on the study aims[9]. For example, 85% occurrence was used for a species with more than 47 replicates to conduct an interaction network analysis[11]. In our study, as we wanted to focus on the truly symbiotic bacterial community of each sponge species, we choose a restrictive approach to the species core by only considering bacteria that were present in all the replicates of each species (100% occurrence), disregarding whether they were present among the SW core or not. In this way we ensured that we were focusing on persistent symbionts rather than on transient microbes.

The size of the bacterial core, which represents the permanent part of the sponge microbiome, seems to be intrinsic of each sponge species[16]. A high stability of the microbiome across sponge replicates can be indicative of the strength of the sponge-bacteria associations, whereas the opposite would indicate the presence of facultative /transient bacteria[11]. Most of the associations among bacteria and the studied species appeared to be highly constant, thus, suggesting a strongly fixed relationship, although we are aware that this might depend on the number of replicates analysed.

The contribution of the species-specific ZOTUs to the core communities, in terms of relative abundance, was surprisingly low. Those values may be influenced by the number of sponge species taken into account in the study, the similarity between their microbiomes, and, more strongly, by the restrictions associated to the way species-specific OTUs are defined, whether being part of the species core community (our study) or not[10,32]. Schmitt *et al.*[10] suggested that the species-specific bacteria would probably be vertically inherited. If this assumption is true, the percentage of vertically inherited symbionts in our sponges would be rather low, since we have found a few species-specific microbes and the majority of the sponge bacterial taxa are found in more than one species.

Both, sponge microbiomes and seawater communities are in close contact because of the filtering activity of sponges, which can result in occasional bacteria transfer from one source to another[24]. To avoid this potential contamination, we used the 100% occurrence core approach in both the sponges and seawater, because it is unlikely that a ZOTU contaminates all the replicates of the same source. Moreover, we took into account the differential abundances that a ZOTU can be present in both, the sponge and the seawater. In this way, to be conservative, we could only suspect of SW bacteria contamination in the cases in which abundant SW bacteria are in low abundance in a sponge for which only few replicates are available. These cases would merit further investigation.

Our results comparing the bacterial core of sponges and seawater showed that all the studied sponges contained SW bacteria, as previously reported for other sponge species[24,32]. However, the relative abundance of SW

bacteria in the sponge microbiomes was species dependent, ranging from almost 100% in *A. paraviridis* to less than 10% in *Antho* sp.

The quantification of the relative abundance of SW bacteria in the sponge microbiomes is particularly relevant to address seasonal or geographical changes and species specific traits[24], but also allows inference about bacterial transmission modes. Thus, we estimated the relative abundance of the SW- sponge shared bacteria in each biome core trying to differentiate SW microbes that may represent stable symbionts from transient contaminant bacteria. We postulated that enrichment of seawater bacteria in the sponge occurred when low abundance SW core bacteria were found as the main components of the sponge microbiomes (Fig. 3). This can be proposed for 11 out of 19 sponge species analysed and in particular, for *C. reinwardti*, *A. paraviridis, Mycale* sp., *Gellioides* sp. *N. hartmani*, and *A. suberitoides*. No pattern related to the HMA and LMA dichotomy could be withdrawn here, as representatives of both groups showed enrichment of seawater bacteria in their microbiomes. The proportion of the sponge core bacteria shared with SW is reduced drastically in many species when comparisons are performed with the abundant SW bacteria, instead of with the SW core bacteria. Among these species, the three HMA species (*N. hartmani, A. suberitioides*, and *S. cf. laboutei)*, reduced their relative abundance of SW ZOTUs from ~50%, ~70% and ~25%, respectively, to values below 5% (see Fig. 4). Reduction occurs in sponges that harbour low-abundance SW bacteria that would be ignored when only abundant SW bacteria are used for comparisons. Therefore, the study HMA sponges contain in their microbiomes SW bacteria that are at low-abundance in the water. For species that harbour both abundant and SW core bacteria in their microbiomes, similar percentages of SW bacteria were obtained with both methodologies. With this comparison, we like to point out how different approaches may influence the results on the overlap between the sponge and SW microbiomes. In particular, we emphasize the importance of the approach used for studies aiming to elucidate the dichotomy between HMA and LMA sponges.

We considered relevant the contribution of SW bacteria to the formation of the sponge microbiome when they were present across all species replicates. In contrast, some authors[11] considered that abundant (>0.01%) SW OTUs likely represent environmental contaminants and should be removed from the sponge samples, independently of their abundance in the sponge. Conversely, we propose, that only highly abundant SW bacteria that are rare in the sponge-species core are potential candidates to represent SW bacteria contamination, especially when only few replicates are available. Two sponge species from our dataset (e.g. *Pseudosuberites* sp. and *T. raphidophora*) are examples of possible contamination.

Overall, we consider the comparisons using the SW core bacteria as a more accurate way to assess the true sponge-SW shared bacteria, as it considers bacteria that would be available across locations to be incorporated in the sponge microbiome. Our results support that "sponge-specific" bacteria are rather "sponge-enriched" bacterial clusters, and that seawater acts as a seed bank for sponge microbiomes, as suggested by Webster *et al.*[17], Webster and Thomas[43] and Moitinho-Silva *et al.*[24]. We detected widespread but rare[44] SW bacteria forming part of sponge core microbiomes. Whether these taxa are metabolically active in the water column or represent dormant stages that reactivate after being incorporate to the sponge[24] remains to be elucidated.

We find a high specificity of the associations between sponges and seawater bacteria. Each sponge species seems to incorporate different bacteria from the seawater in its microbiome. This suggests that some species-specific mechanisms have been fixed in the sponges to select some seawater bacteria and not others. Recognition mechanisms have been proposed to explain horizontal acquisition of microbes from the surrounding environment[22]. Taking into account the high percentages of seawater bacteria detected in some of our sponge species and the species-specificity of many of them, we propose that environmental acquisition would play a major role in the establishment of species-specific sponge microbiomes.

To summarize, sponge species is the main factor structuring microbiomes of the most common sponges from Nha Trang bay (Vietnam). By using a very restrictive approach of the "core species" concept and ZOTUs with 100% sequence identity for defining bacterial species, we proved that intra-species microbiome stability is the rule for most sponges. A high percentage of SW bacteria shaped the core microbiome in many study species. Our results point to horizontal transmission, as an ubiquitous mechanism of symbiont acquisition in sponges, while vertical transmission would represent a rather complementary acquisition way. Apparently, some highly specific recognition mechanisms may be acting in sponges to specifically enrich some SW bacteria in their tissues, and not others. Moreover, contrasting preferences in bacteria selection may account for differences in the microbiomes of HMA and LMA sponges and some physiological traits such as contrasting filtration rates might also contribute to enhance the differences. These mechanisms would allow the maintenance of stable bacterial communities disregarding environment conditions and geographical distance and merits to be confirmed by analysing in the same way as in the current study a larger number of sponge and water samples from different geographical regions.

Experimental Procedures

Sponge and seawater sampling and DNA extraction. Sponge samples were collected in April 2015 by SCUBA diving along 13 transects, 25 m long each, randomly placed between 3 and 9 m deep in three neighbouring locations ~2 km apart (i.e.: Dambay, Hun Mun and Nock Island) within Nha Trang Bay (central Vietnam). This quantitative sampling method allowed us to detect the most representative sponges in the study area but not to collect the same number of replicates for all the species. For instance, only three HMA sponges were found in the whole sampling but two of them were present at high abundances. Overall, we collected 203 sponge samples, from which we only considered for this study the ones that were found at least twice. Thereby, 98 samples belonging to 19 sponge species with between 2 and 15 replicates each (Table 1) were analysed.

Each sponge individual fitting within a transect was photographed and a piece of ca. 3 cm^2 was collected in a 50 mL Falcon tube in seawater. Seawater was immediately replaced by 100% ethanol once on board. Back in the

lab, the ethanol was replaced twice again with fresh absolute ethanol for a good sample preservation. DNA from those sponges was extracted following the protocol of DNeasy Blood &Tissue Kit (Qiagen).

Triplicate plankton samples were taken from the three sampling locations where the ecological transects were performed (i.e.; Dambay, Hun Mun and Nock Island). Two litres of water were collected and sequentially filtered throughout 5 μm, to remove undesired plankton components, and then throughout 0.22 μm polycarbonate membranes. The size fraction between 5 and 0.22 μm was processed for DNA extraction. Membranes were enzymatically digested with lysozyme, proteinase K and sodium dodecylsulfate and afterwards, DNA was extracted with phenol:chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) and chloroform:isoamyl alchohol (24:1, vol/vol). Purification and concentration of the DNA was carried out with Amicon® Ultra 4 Centrifugal Filter Units – 100000 NMWL (Millipore). The extraction procedures used for sponge samples and SW were the most appropriated according to their respective preservation.

Sponge identification. We identified sponge species to the best possible taxonomic resolution by molecular markers and morphological features. Fragments of the nuclear genes encoding the 18S rRNA (~1700 bp) and 28S rRNA (~650 bp), as well as the cytochrome c oxidase subunit I (*COI* ~680 bp) were amplified and sequenced. Primers 1 F and 1795R[45] were employed to amplify 18S rRNA, Por28S-830F and Por28S-1520R[46] primers were used to amplify the D3-D5 partition of the 28S rRNA and LCO1490 and HCO2198[47] were used for *COI*. PCR amplifications were conducted in 50 μl reactions containing 1 ng of template genomic DNA, 5 μl of 10x PCR buffer (containing 1.5 mM $MgCl_2$), 2 μl of dNTP mix (10 mM), 2 μl of bovine serum albumin, 1 μl pf each primer (10 mM) and 0.4 μl of Taq DNA polymerase (5 U μl^{-1}). The temperature profile for the 18S rRNA was as follows: 94 °C/5 min; (94 °C/1 min, 50 °C/1 min, 72 °C/1 min) × 35 cycles; 72 °C/5 min; for 28S rRNA: 94 °C/5 min; (94 °C/30 s, 53 °C/30 s, 72 °C/30 s) × 30 cycles; 72 °C/5 min; and for COI: 94 °C/2 min; (94 °C/1 min, 45 °C/1 min, 72 °C/1 min) × 35 cycles; 72 °C/7 min. Purification and sequencing were carried out by an external service (Macrogen, Netherlands). The obtained sequences were manually edited in Geneious v 9.0.2 and blasted against NCBI databaste (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm the morphological identification of the sponges at the lowest taxonomic level possible.

Preparation of spicules and histological sections were made from specimens' subsamples and observed under both, light and scanning electron microscopes. Morphological characters such as spicule types, shape, length, and width, as well as skeletal arrangement[48] were used in combination with individual sequences and phylogenetic reconstructions to obtain, the most accurately possible, taxonomic identifications. Sponges were classified as HMA or LMA on the basis of their pertinence to genera already known to belong to any of these two groups, what was additionally confirmed by looking to the characteristics and structure of the sponge aquiferous system: small, relatively few (HMA) *vs*. large abundant choanocyte chambers (LMA), and mesohyle (dense *vs*. lax mesohyle, respectively)[25].

16S rRNA gene amplification, sequencing and analysing. PCR and high-speed multiplexed SSU rRNA gene Illumina MiSeq sequencing (NGS) were carried out following the genomic core facilities and methods of the MrDNA Lab (Texas, USA) (http://www.mrdnalab.com/). The variable V4 region of the 16S rRNA gene (c.a. 250 nt) was amplified using the primers 564F (5′AYTGGGYDTAAAGNG3′) and 785R (5′TACNVGGGTATCTAATCC3)[49]. Raw rRNA gene sequences were processed using the UPARSE pipeline[50]. A quality check and de-replication were applied to our dataset. Denoising (error-correction) of amplicons was performed to identify all correct biological sequences following the UNOISE pipeline[51]. This algorithm removed chimeras, reads with sequencing errors, PhiX, and low complexity sequences due to Illumina artifacts, and generates ZOTUs ("zero-radius" OTUs) consisting of sequences of 100% identity. For comparison purposes, sequences were also clustered at 97% threshold (Supplementary information). For this analysis, reads were dereplicated and clustered into operational taxonomic units (OTUs) at cut-off 0.03% identity after chimera removal (UCHIME) and excluding the singletons.

Taxonomic assignment was done with SINA v1.2.11[52] using SILVA 128 database. SINA uses Lowest Common Ancestor method (LCA). We configurated a "Min identity" of 0.7 and a maximum number of search results of 1 per sequence results in "best match" type. Sequences with low alignment quality (<75%) and sequences identified as mitochondria or chloroplasts were removed from the analysis. In order to minimize biased effects for differences in sampling effort, the original ZOTU table was rarefied (Supplementary Fig. S5) at a minimum reads threshold of 41000[53].

Raw sequences are available in the SRA archive under the project number PRJNA453898.

Defining core and species-specific ZOTUs. We identified the ZOTUs that were present in all replicates to define the core microbiome of each sponge species. The ZOTUs that did not meet this requirement were assigned to the variable community. Moreover, we considered species-specific ZOTUs those belonging to a single core microbiome for a particular sponge species, compared to the remaining collected sponges.

Seawater (SW) ZOTUs. We combined two approaches to estimate the real contribution of the seawater (SW) bacteria to the sponge core microbiomes. First, we looked for bacteria in each sponge species that were already present in the core SW community. We considered the core community of the SW as the community formed by the ZOTUs present in all water replicates. With this approach, we attempted to identify bacteria that commonly inhabited in the SW and that its presence was not merely circumstantial. Therefore, they could represent a potential source for the formation of the sponge microbiome over time. In the second approach, we made the comparison with the most abundant bacteria of the SW. We identified the ZOTUs with relative abundances higher than 0.01% in average across all water samples. This threshold was chosen since it has been used previously to remove from the sponge microbiome samples the OTUs that were likely to represent environmental

contaminants[11]. With this approach, we aimed to gain insight on the SW bacteria that are more likely detected in the sponge microbiomes just because of their high abundance in SW and may represent transient (environmental) contaminants[11]. On the other hand, we considered rare ZOTUs those with a relative abundance <0.01% and highly abundant ZOTUs those with relative abundance >1%[32].

Statistical analyses. We carried out a distance-based multivariate analysis at ZOTU level of the microbial communities of the sponges and seawater samples using the *vegan* package[54] in R. A cluster dendrogram was built using the Bray-Curtis dissimilarity distance matrix of samples to visualize patterns of bacterial community structure in sponges and seawater. We tested the effect of host identity (species) as well as the effect of the HMA/LMA identity, on the structure of microbial communities with non-parametric Permutational Analysis of Variance (PERMANOVA). PERMUTEST was applied to detect differences in the dispersion between groups. A bias correction[55] for the unequal sample size of HMA and LMA groups was applied in the *betadisper* function of the *vegan* package[54]. P-values of PERMANOVA and PERMUTEST were calculated using 999 permutations and significance cut-off for p-values was 0.05.

The mean relative abundance of the bacterial phyla and classes was calculated for both, HMA and LMA groups. We applied an IndVal analysis using the *labdsv* package[56] in R to detect potential associations of certain bacterial phyla and classes to any of these two groups. We fixed an IndVal threshold of 0.6 (p-value < 0.01) to consider a bacterial taxon strongly associated to (or Indicator of) HMA or LMA sponges.

Data Availability Statement

The raw prokaryotic sequences analysed during the current study are available in the SRA archive under the project number PRJNA453898 (https://www.ncbi.nlm.nih.gov/sra/?term = PRJNA453898). SSU and LSU Sponge sequences are available under the accession numbers MH731279 to MH731308. COI sponge sequences are available under the accession numbers MH784603 to MH784613. Moreover, the results of the dataset analysed with OTUs clustered at 97% sequence similarity are available in the Supplementary material of the current paper.

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Author Contributions

M.T. and M.J.U. conceived the study. M.T. and J.C. performed the analyses. M.T. and M.J.U. wrote the manuscript. L.G., E.O.C. and J.C. commented on later versions of the manuscript.

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Sponges and Their Microbiomes Show Similar Community Metrics Across Impacted and Well-Preserved Reefs

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Sponge diversity has been reported to decrease from well-preserved to polluted environments, but whether diversity and intra-species variation of their associated microbiomes also change as function of environmental quality remains unknown. Our study aimed to assess whether microbiome composition and structure are related to the proliferation of some sponges and not others under degraded conditions. We characterized the most frequent sponges and their associated bacteria in two close areas (impacted and well-preserved) of Nha Trang Bay (Indo-Pacific). Sponge assemblages were richer and more diverse in the well-preserved reefs, but more abundant (individuals/m. transect) in the impacted environments, where two species (*Clathria reinwardti* and *Amphimedon paraviridis*) dominated. Sponge microbiomes from the polluted zones had, in general, lower bacterial diversity and core size and consequently, higher intra-species dispersion than microbiomes of sponges from the well-preserved environments. Microbial communities reflect the reduction of diversity and richness shown by their host sponges. In this sense, sponges with less complex and more variable microbiomes proliferate under degraded environmental conditions, following the ecological paradigm that negatively correlates community diversity and environmental degradation. Thereby, the diversity and structure of sponge microbiomes might indirectly determine the presence and proliferation of sponge species in certain habitats.

Keywords: ecology, sponges, microbiomes, diversity, resilience, contrasting environments, eutrophication

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INTRODUCTION

Sponges are key invertebrates in marine benthic ecosystems where they play essential functions in many ecological processes. Besides increasing benthic diversity by supplying ecological niches to other organisms, they contribute to benthic–pelagic coupling by exchanging particulate and dissolved organic matter with the water column (Richter et al., 2001; Morganti et al., 2017). Sponges also participate in marine biogeochemical fluxes and some species may also show detoxifying potential by transforming noxious products present in polluted waters through the interplay of symbiotic bacteria (Loredana et al., 2017).

Microbes have been intimate partners of sponges since the Pre-Cambrian (Wilkinson, 1984). They can represent up to ca. 50% in volume of the microbial-sponge holobiont in some sponge

species (Uriz et al., 2012) and are taxonomically and metabolically diverse in most cases (Weisz et al., 2007; Thomas et al., 2016). Thus, it is hard to envisage the causes of sponge success or failure without considering its accompanying bacterial microbiome. Indeed, unbalancing of their microbial symbioses has been considered to trigger extensive mass mortalities of sponges in the Mediterranean (Webster et al., 2008; Cebrian et al., 2011), and Red Sea (Gao et al., 2014). In addition, some purported benefits that sponges may obtain from their associations with microbes have also been proposed such as, antifungal activity, production of bioactive compounds against predation, roles in nitrogen and carbon cycle and vitamin biosynthesis (Schmidt et al., 2000; Freeman and Thacker, 2011; Hentschel et al., 2012; Freeman et al., 2013), but rarely have been experimentally demonstrated (de Voogd et al., 2015; Garate et al., 2015).

In contrast to the spatial and temporal variations reported for microbial communities in seawater (Zeglin, 2015; Glasl et al., 2017), the structure of the sponge microbiome does not vary substantially in the same species along geographical and bathymetrical ranges or over temperature, eutrophication or irradiance shifts (Hentschel et al., 2002; Erwin et al., 2012; Pita et al., 2013a,b; Luter et al., 2014; Strand et al., 2017). Although several experimental studies reported microbiome shifts under strong environmental changes in some host species (Mohamed et al., 2008; Fan et al., 2013; Lesser et al., 2016; Webster et al., 2016; Pineda et al., 2017; Weigel and Erwin, 2017; Glasl et al., 2018; Ramsby et al., 2018) they only inform us about short term changes, which might reflect temporal responses to environmental stresses.

Sponges usually show a high species diversity in well-preserved ecosystems (Van Soest et al., 2012), which has been suggested to decrease under anthropogenic pressures (Easson et al., 2015). Shifts in nutrient cycling and ecosystem functioning, which occur in degraded reef systems (Bell et al., 2013, 2018; Easson et al., 2015), are considered to be responsible for decreases in sponge biodiversity. However, a few, likely opportunistic, sponge species have been reported to inhabit and even dominate degraded coral reefs (Maliao et al., 2008). How these sponges cope with the potentially noxious products of polluted waters and whether particular sponge-associated bacteria are involved or not in their ecological success are challenging issues poorly understood (Douglas, 2014). Indeed, little is known about the role (if any) that symbiotic bacteria may play in shaping the ecological distribution of sponge species and whether the decrease of sponge diversity observed in perturbed assemblages also involves a lower diversity of their associated bacterial communities.

In this study, we explored whether microbiomes of sponges inhabiting degraded environments show differential characteristics, in such a way that they might influence the sponge distribution and proliferation in those adverse conditions. With this goal, we characterized the most frequent sponges and their associated bacteria in two close areas of Nha Trang Bay (Indo-Pacific) subjected to contrasting environmental conditions: well-preserved and impacted coral systems. In particular, we looked for sponge-associated bacteria in the few sponge species able to proliferate in the polluted zones.

Nha Trang bay is located in central Vietnam and harbors one of the highest coral diversities in the area (Latypov, 2011). Unfortunately, the health of the local ecosystems is being threatened by an increase of human activities leading to an alarming degradation of the bay in some zones (Latypov, 2015). The islands located closer to the city and the port of Nha Trang show a higher degree of anthropogenic impact, with higher sedimentation fluxes and lower water transparency compared to the ones located farther from the coast line (Latypov, 2006; Tkachenko et al., 2016). Moreover, Vietnam has been ranked the world's third largest producer of farmed food (Nguyen et al., 2016) and certain areas of Nha Trang bay are highly impacted by mariculture activities, which also produce eutrophication and release of xenobiotics to the water (Nguyen et al., 2016). Tkachenko et al. (2016) reported the concentration of nutrients in areas close to our sampling sites. The nutrient values were 2.63 μM $\pm$ 0.21 for dissolved inorganic nitrogen and 0.29 μM $\pm$ 0.11 for phosphorus at the impacted sites (culture cages) and 2.41 μM $\pm$ 0.2 and 0.28 μM $\pm$ 0.08 at the unperturbed sites. As a result, the native *Acropora* coral assemblages have been replaced by the more resistant to silting *Millepora* communities in these perturbed areas (Latypov, 2015; Tkachenko et al., 2016). Conversely, several outer areas of Nha Trang Bay, such as Hun Mun, receive a low anthropogenic impact and still present well-developed *Acropora* communities with high coral coverage (ca. 60–70%) (Tkachenko et al., 2016).

MATERIALS AND METHODS

Sample Collection and DNA Extraction

Quantitative sponge sampling was approached by SCUBA diving by randomly placing a total of 13, 25 m-long transect lines between 3–9 m depth along both well-preserved and impacted areas of Nha Trang Bay (**Supplementary Figure S1**). This quantitative method has been traditionally used for biodiversity studies in coral reefs and other structurally complex habitats (Loya, 1978) and provides a good approach on the abundance (density or coverage) of the non-cryptic fraction of the reef benthos. Specimens that were crossed by the metric tape along the line transect were sampled. The well-preserved areas were coral reefs and rocky shores of the eastern part of Hun Mun Island and the southern part of Hun Tre Island, considered to be the most well preserved areas of the bay (Tkachenko et al., 2016). The impacted targeted zones were 2 km apart from the well-preserved areas, next to Dambay region, which harbors an intensive mariculture system (lobster caging) that causes chronic eutrophication in the area (Tkachenko et al., 2016). Overall, the quantitative sampling provided 203 sponge samples, from which 71 individuals were from the impacted areas and 132 individuals from the well-preserved environments.

Each sponge individual fitting within a line transect was photographed and a piece of ca. 3 cm^2 (whenever possible) was collected in a 50 mL Falcon tube with seawater. Seawater was immediately replaced by 100% ethanol once on board. Back in the lab, the ethanol was replaced twice with fresh absolute ethanol again for a good sample preservation. DNA was extracted

following the protocol of DNeasy Blood & Tissue Kit (Qiagen). Triplicate plankton samples were taken from the three sampling locations where the ecological transects were performed (i.e., Dambay, Hun Mun and Nock Island). Two liters of water were collected and sequentially filtered throughout 5-μm, to remove undesired plankton components, and then throughout 0.22 μm polycarbonate membranes. The size fraction between 5 and 0.22 μm was processed for DNA extraction. Membranes were enzymatically digested with lysozyme, proteinase K and sodium dodecylsulfate and afterward, DNA was extracted with phenol:chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) and chloroform:isoamyl alcohol (24:1, vol/vol).

Sponge species were previously identified in Turon et al. (2018) by morphological features (Hooper and Van Soest, 2002) and molecular markers. Preparation of spicules and histological sections were made from specimens' subsamples and observed under both light and scanning electron microscopes.

16S rRNA Gene Sequencing and Processing

Only representative sponge species ($n = 18$) at each environment, which appeared replicated in the quantitative sampling, were used for the study of microbial symbionts. Replicates varied from 2 to 9, depending on the species abundance (n. individuals) and material availability (sponge fragment size), as the sampling was intended to reflect the sponge diversity and abundance at each environment (**Table 1**). PCR and high-throughput multiplexed 16S rRNA gene amplicon Illumina MiSeq sequencing, were carried out following the genomic core facilities and methods of the MrDNA Lab (Texas, United States)[1]. The variable V4 region of the bacterial 16S rRNA gene was amplified using the primers 564F (5′AYTGGGYDTAAAGNG-3′) and 785R (5′TACNVGGGTATCTAATCC-3′) (c.a. 250 nt) (Klindworth et al., 2013). Raw rRNA gene sequences were processed separately using the UPARSE pipeline (Edgar, 2017). A quality check was applied to our dataset with the fastq_filter command and the arguments –fastq_trunclen 208 –fastq_maxee 0.25. Sequences were then dereplicated with the – derep_fulllength command and sorted by size (-sortbysize command) in Usearch 9.2 version. Denoising (error-correction) of amplicons was performed following the UNOISE pipeline (Edgar, 2016) using the - unosie2 command. This algorithm removed chimeras, reads with sequencing errors, PhiX, and low complexity sequences due to Illumina artifacts, and generates ZOTUs ("zero-radius" OTUs) with 100% identity sequences. Finally, -usearch_global command with identity threshold set at 0.97 was applied to our dataset. Taxonomic assignment was done with SINA v1.2.11 (Pruesse et al., 2012) using SILVA 128 database. Sequences with low identity (< 75%) and sequences identified as mitochondria or chloroplasts were removed from the analysis. In order to minimize biased effects for differences in sampling effort, the original bacterial ZOTU table was rarefied at a minimum reads threshold of 41000, using QIIME (Caporaso et al., 2010).

[1]http://www.mrdnalab.com/

TABLE 1 | Species replicates used for the microbiome study.

Species	N. Impacted Env.	N. W-P Env.
Aaptos suberitoides	0	7
Amphimedon paraviridis	5	0
Antho (*Antho*) sp.	0	3
Callyspongia sp.	0	2
Clathria reinwardti	9	0
Clathria (Isociella) skia	4	0
Dendroxea sp.	0	2
Dysidea sp.	0	3
Amphimedon sulcata	3	6
Haliclona (*Reniera*) sp.	0	3
Monanchora unguiculata	0	3
Mycale (*Arenochalina*) sp.	2	1
Neofibularia sp.	0	4
Phorbas sp.	0	3
Protosuberites proteus	0	2
Suberea fusca	0	3
Thrinacophora cervicornis	0	2

Sponge and Bacterial Community Analysis

Statistical analyses were run in the R environment (R Core Team, 2013). Community ecology related parameters were calculated using the vegan v2.5-1 (Oksanen et al., 2017) and iNEXT v2.0.15 packages (Hsieh et al., 2018), and figures were drawn with ggplot2 3.0.0 (Wickham, 2009).

We determined sponge species composition on impacted and well-preserved environments: the number of sponge individuals was standardized per meter of sampled transect to minimize the bias of the sampling effort in the two habitats. Shannon diversity indices and species richness were calculated for both environments using the *ChaoEntropy* and *ChaoSpecies* functions, respectively, and integrated curves that smoothly link rarefaction (interpolation) and prediction (extrapolation) were computed for both variables (Chao et al., 2014) to facilitate their comparison of between habitats (**Supplementary Figure S2**). Additionally, we used the specaccum function with 1000 permutation of the vegan package to represent the accumulated number of species per sampled meter in each habitat (**Supplementary Figure S3**).

A Venn diagram of total species richness in both environments was generated using the *eulerr* package (Larson et al., 2018) and *t*-tests were conducted to assess differences in community metrics between sites. We calculated the Bray–Curtis dissimilarity of species composition of all transects to assess the beta diversity patterns between environments.

We performed a hierarchical cluster analysis (Ward method) based on Bray–Curtis dissimilarity matrix using the rarefied ZOTU table to determine whether sponge bacterial communities were more similar among replicates from the same species than between impacted and well-preserved environments. To test the effects of host identity and environment on structuring the sponge bacterial communities, we used PERMANOVA (Anderson, 2001) based on 999 permutations as implemented in *adonis* function. A heatmap was generated for the most

abundant bacterial families (>1% relative abundance in any of the samples). We performed and Indicator Value (IndVal) (Dufrêne and Legendre, 1997) analysis to detect if particular microbial taxa were differentially found at each site using the *labdsv* package (Roberts and Roberts, 2016) in R. We set the Indval threshold to 0.7 and the *p*-value for significance at 0.01.

We compared three main bacterial community features between the sponge assemblages from impacted and well-preserved environments: (i) intra-species dispersion (beta diversity), (ii) Shannon diversity and (iii) core size (explained below). For these comparisons, we included *Amphimedon sulcata* and *Mycale* (*Arenochalina*) sp., for which we had replicates in the impacted and well-preserved environments, within the category of sponges able to survive in impacted sites.

Only two species inhabited both environments [*A. sulcata* and *Mycale (Arenochalina)* sp.]. However, only one individual of *Mycale* (*Arenochalina*) sp. was found in the well-preserved habitats, preventing statistically based comparisons between environments for this species. *Betadisper* and *Shannon* functions were used to calculate beta and alpha diversity measures, respectively. We defined the bacterial core of each sponge species considering the ZOTUs present across all species replicates. The core size of each individual represented the percentage of core bacteria (of the sponge species) with respect to the total bacteria present in that individual. *T*-test and *Kruskal–Wallis* test were used to detect significant differences between sponge species from impacted and well-preserved environments. *Pearson* correlation was used to detect the relationship between intra-species dispersion and bacterial core size for each sponge species.

RESULTS

Sponge Assemblages in Two Contrasting Environments

A total of 71 sponge species were identified in the overall sampling area (**Figure 1**). The impacted and well-preserved environments showed strong differences in terms of sponge richness, diversity, and density (**Figure 1** and **Supplementary Figure S2**). Sponge richness and diversity were much lower in the impacted sites than in the well-preserved environments (*t*-test: p-value = 0.039, p-value = 0.037, respectively), although the respective rarefaction curves per number of individuals sampled did not reach the saturation point, in particular for species richness (**Supplementary Figure S2**). The rarefaction curves of accumulated species per sampled area neither reached the saturation point (**Supplementary Figure S3**).

Conversely, the overall sponge density was higher (*t*-test: p-value = 0.05) in the impacted (0.94 individuals/m) than in the well-preserved environments (0.56 individuals/m). Most species were environment-specific with only seven species found in both environments, though all of them but *A. sulcata* were much more abundant at the polluted sites (**Figure 1**). The well-preserved habitats harbored a more diverse and evenly distributed sponge assemblage with *Monanchora unguiculata, A. sulcata, Antho (Antho)* sp., *Aaptos suberitoides,* and *Neofibularia* sp. (**Figures 1**, **2**) being the most abundant species. Conversely, the impacted habitats showed a community mostly dominated by *Clathria reinwardti* and *A. paraviridis* (**Figures 1**, **2**) (>50% of the specimens sampled). Moreover, significant differences in beta diversity for species composition were found between sites (adonis, $F = 2.72$, $df = 1$, $R^2 = 0.19$, $P = 0.01$).

Sponge-Associated Bacterial Communities

We obtained a total of 15,712 high-quality ZOTUs (Zero-radius Operational Taxonomic Unit, 100% identity) corresponding to 48 bacterial phyla, with *Proteobacteria* (52.9%), *Actinobacteria* (2.8%), *Acidobacteria* (2.4%), *Chloroflexi* (2%), and *Planctomycetes* (1.7%) being the most abundant taxa. Bacterial communities of HMA sponges (*Neofibularia* sp., **A. suberitoides**, and *Suberea fusca* clearly differed from those of LMA sponges (**Figure 3**). *Chloroflexi,* PAUC34f, *Caldilineaceae* and Sva0996 marine group were consistently associated to the HMA sponges.

The cluster analysis showed that the sponge-associated bacterial communities were closely related to their host-species regardless of the environment (impacted vs. well-preserved) where the sponges were living, with, in general, low variation among replicates of a species (**Figure 3**). Indeed, we found a strong effect of host identity on the composition of the sponge microbiomes (*adonis*: Pseudo-*F*: 4.46, $R^2 = 0.593$, $p < 0.001$), but a negligible effect of the environment (*adonis*: Pseudo-*F*: 1.07, $R^2 = 0.008$, $p > 0.05$). Thus, each sponge species presented a unique microbiome that differed from that of other sponges and from that of the surrounding seawater (**Supplementary Figure S4**), even if they were living in the same environment (**Figure 3**). Planktonic communities showed significant differences in bacterial composition between types of habitats (*adonis*: Pseudo-*F*: 2.2, $R^2 = 0.23$, $p < 0.05$, **Supplementary Figure S4A**). Shannon diversity was higher for the seawater bacterial communities in the well-preserved habitat than in the impacted habitat, but differences were only statistically significant at an alpha value of 0.07 (*Kruskal–Wallis* test: p-value = 0.07), likely due to the unbalanced design (3 vs. 6 replicates) and the large variation in the Shannon diversity Index across replicates at the polluted environment (**Supplementary Figure S4B**).

We looked in further detail into the bacterial composition of the two purportedly opportunistic species that dominate the perturbed (polluted) environments (*C. reinwardti* and *Amphimedon paraviridis*). A common feature of these two species was that their bacterial communities were dominated by a few taxa such as *Candidatus Branchiomonas* (42.85 ± 12.5%) and *Endozoicomonas* (12.74 ± 12.1%) in *A. paraviridis,* and members of the family *Rhodobacteraceae* (58.54 ± 22%) and *Endozoicomonas* (21.63 ± 12.9%) in *C. reinwardti* (**Figure 3**). In both sponges, only two bacterial taxa achieved >60% of the whole microbiome. Moreover, these two taxa were consistently found across all species replicates (i.e., they belong to the sponge core). A single *C. Branchiomonas* ZOTU made up 56% of the total core reads of *A. paraviridis* and 6 ZOTUs belonging to *Rhodobacteraceae* family made up 64% of the total core reads in *C. reinwardti.* Moreover, both

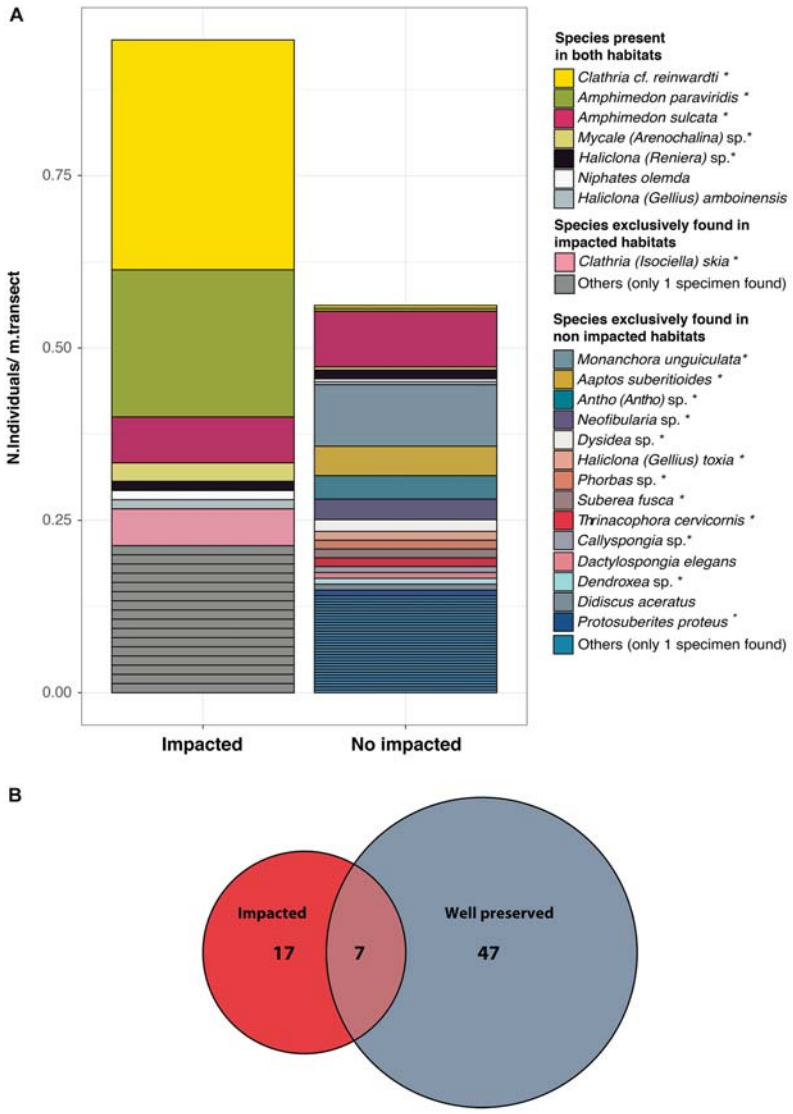

FIGURE 1 | (A) Bar plots of sponge species composition and abundance at the well-preserved and impacted habitats. *Y*-axis represents the numbers of individuals per meter of transect. **(B)** Venn diagram of total species richness and the species overlap between well-preserved (gray) and impacted (red) environments.
* Indicates species for which microbiome has been analyzed.

species had in their core communities ZOTUs belonging to *Endozoicomonas* and *Shewanella* at abundances higher than 10 and 1%, respectively. Indval analysis (**Supplementary Figure S5**) showed that some bacteria were significantly associated to the impacted environment (Indval > 0.7, p-value < 0.01) but only *Rhodobacteraceae* and *Shewanella* were found at high abundances in the sponges of that habitat.

Between Environment Comparisons: General Microbiome Ecological Descriptors

To test for microbiome differences among replicates of the same species living in the two environments, we focused on the unique species equally found in both habitats: *Amphimedon sulcata*. No influence of the environment could be detected for inter-individual variation (PERMANOVA: $R^2 = 0.128$, $p > 0.05$) and no differences (t-test, p-value > 0.05) were detected for intra-species dispersion, core size and Shannon diversity of the *A. sulcata* bacterial communities between individuals from both environments.

Intra-species dispersion of the sponge microbiomes was species-specific in all the species tested, with *Neofibularia* sp., *Thrinacophora cervicornis,* and *A. suberitoides* having the lowest dispersions and *Mycale (Arenochalina)* sp., *Clathria* (*Isociella*) *skia*, and *A. paraviridis* having the highest (**Supplementary Figure S6A**). Interestingly, intra-species dispersions were higher in impacted than in well-preserved environments (t-test: p-value < 0.001; **Figure 4A**). That is, the bacterial communities of sponge replicates from well-preserved environments are more

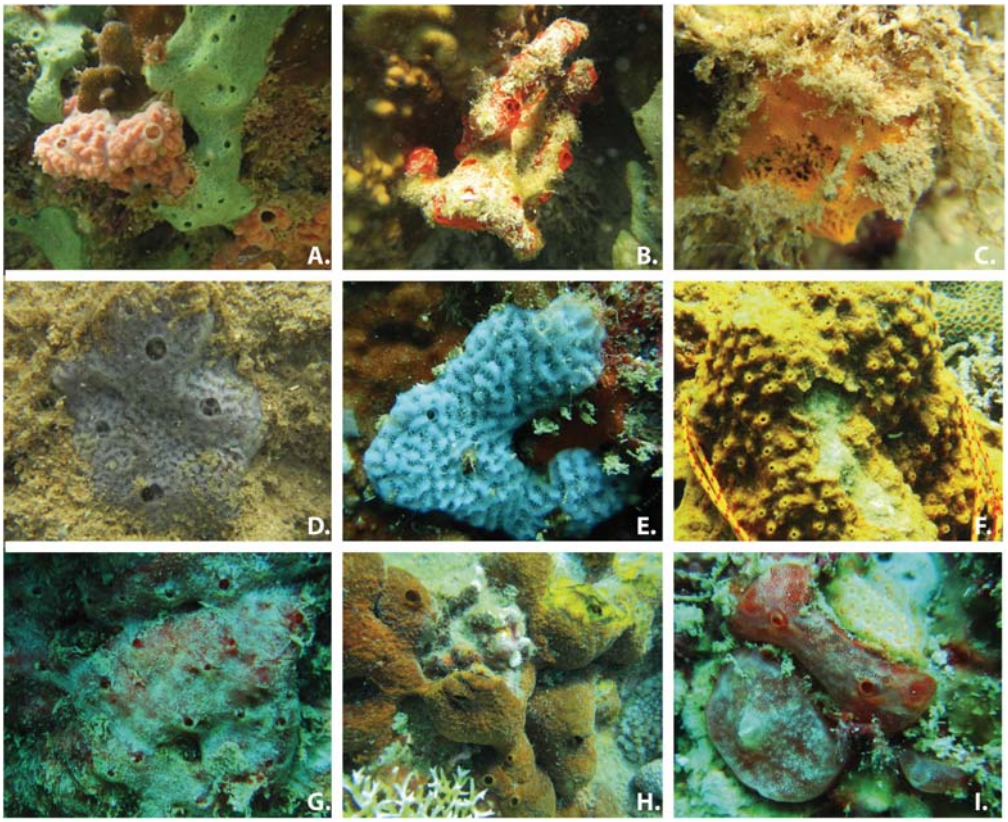

FIGURE 2 | Pictures of the most common sponges of the impacted **(A–D)** and the well-preserved **(E–I)** habitats: **(A)** *Amphimedon paraviridis* (greenish) and *Clathria reinwardti* (pinkish), **(B)** *Clathria* (*Isociella*) *skia*, **(C)** *Mycale* (*Arenochalina*) sp., **(D)** *Amphimedon sulcata* (in the impacted environment), **(E)** *A. sulcata* (in the well-preserved environment), **(F)** *Neofibularia* sp., **(G)** *Antho* (*Antho*) sp., **(H)** *Aaptos suberitioides* and **(I)** *Monanchora unguiculata*.

similar to each other than seen for those sponge replicates from impacted environments. Consequently, intra-species dispersion was negatively correlated to the size of the sponge bacterial core ($R_p = -0.65$, p-value < 0.01: **Supplementary Figure S7**): the higher the intra-species dispersion, the lower the size of the core and the larger the variable bacterial community of the sponge species. *C. reinwardti, Mycale* (*Arenochalina*) sp. and *A. paraviridis* had the smallest core sizes (10.55, 17.34 and 18.44%, respectively), whereas *T. cervicornis, Protosuberites proteus, Monanchora unguiculata*, and *Neofibularia* sp. (43.71, 41.78, 41.73, and 41.72%, respectively) had the largest ones (**Supplementary Figure S6B**). Overall, the bacterial core sizes of the sponge species living in the impacted environments were lower (*Kruskal–Wallis* test: p-value < 0.001) than those of the sponges living in the well-preserved environments (**Figure 4B**). Shannon diversity indices (H′) of the sponge microbiomes were also species-specific and ranged from 2.7 to 4.7 (mean values for *C. reinwardti* and *S. fusca,* respectively) (**Supplementary Figure S6C**), which were lower in impacted than in well-preserved environments (*Kruskal–Wallis* test: p-value < 0.001) (**Figure 4C**).

To sum up, microbiomes of sponge species living in the impacted environment had in general, higher intra-species dispersion, lower core size and lower bacterial diversity, than the microbiomes of sponges living in the well-preserved environments (**Figure 5**).

DISCUSSION

Sponge Ecology: Well-Preserved vs. Impacted Environments

We have found clear differences between the sponge assemblages from well-preserved and impacted environments of Nha Trang Bay, with different species, lower sponge richness and diversity but higher sponge density (individuals/m.) in the perturbed zones. Similar decrease in species richness (S) and Shannon diversity index (H′) due to anthropogenic impacts has been previously reported for the coral communities in the study area (Tkachenko et al., 2016) and for sponge diversity in different oceans (Powell et al., 2014; Easson et al., 2015). Indeed, low diversity but high abundance with dominance of a few species is a common feature of many polluted habitats (Piola and Johnston, 2008; Powell et al., 2014). However, although our sampling captured differences in diversity between habitats quite acceptably, rarefaction curves for accumulated species predict an insufficient sampling effort to completely catch the true species richness.

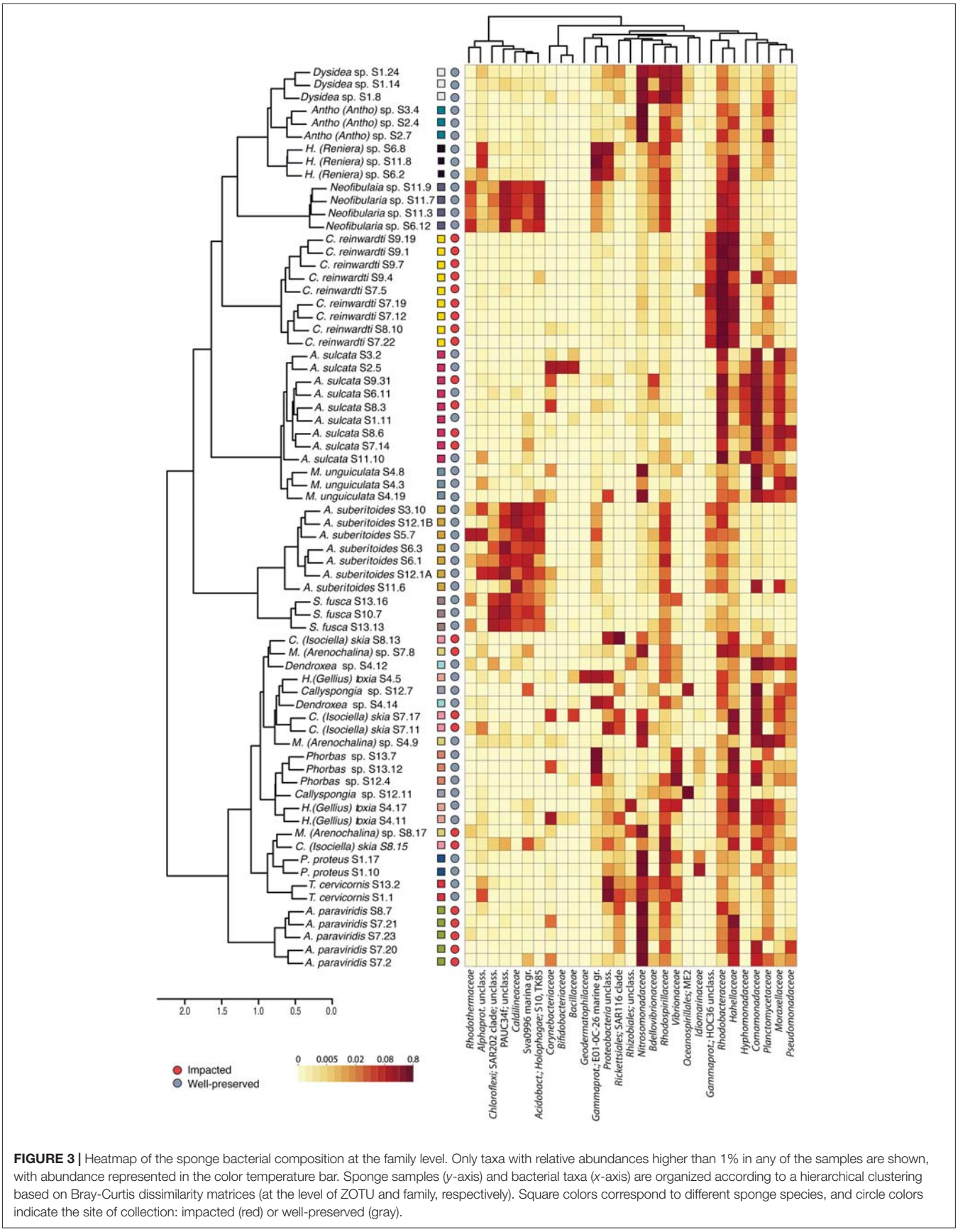

FIGURE 3 | Heatmap of the sponge bacterial composition at the family level. Only taxa with relative abundances higher than 1% in any of the samples are shown, with abundance represented in the color temperature bar. Sponge samples (*y*-axis) and bacterial taxa (*x*-axis) are organized according to a hierarchical clustering based on Bray-Curtis dissimilarity matrices (at the level of ZOTU and family, respectively). Square colors correspond to different sponge species, and circle colors indicate the site of collection: impacted (red) or well-preserved (gray).

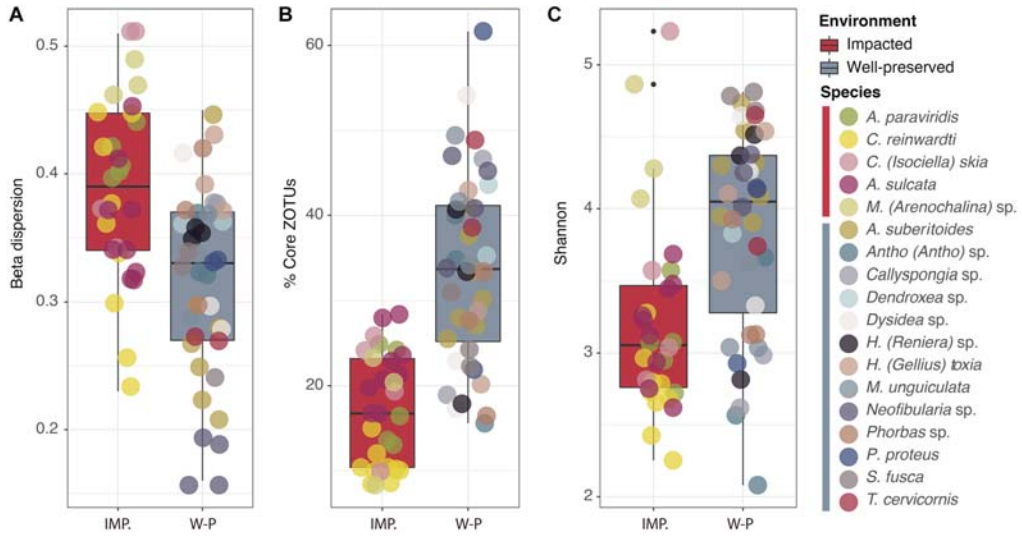

FIGURE 4 | Box plots comparing the intra-species dispersion **(A)**, core size **(B)** and Shannon diversity **(C)** of the sponge microbiomes between impacted (red) and well-preserved (gray) environments. Replicates of the same species are depicted in the same dot color.

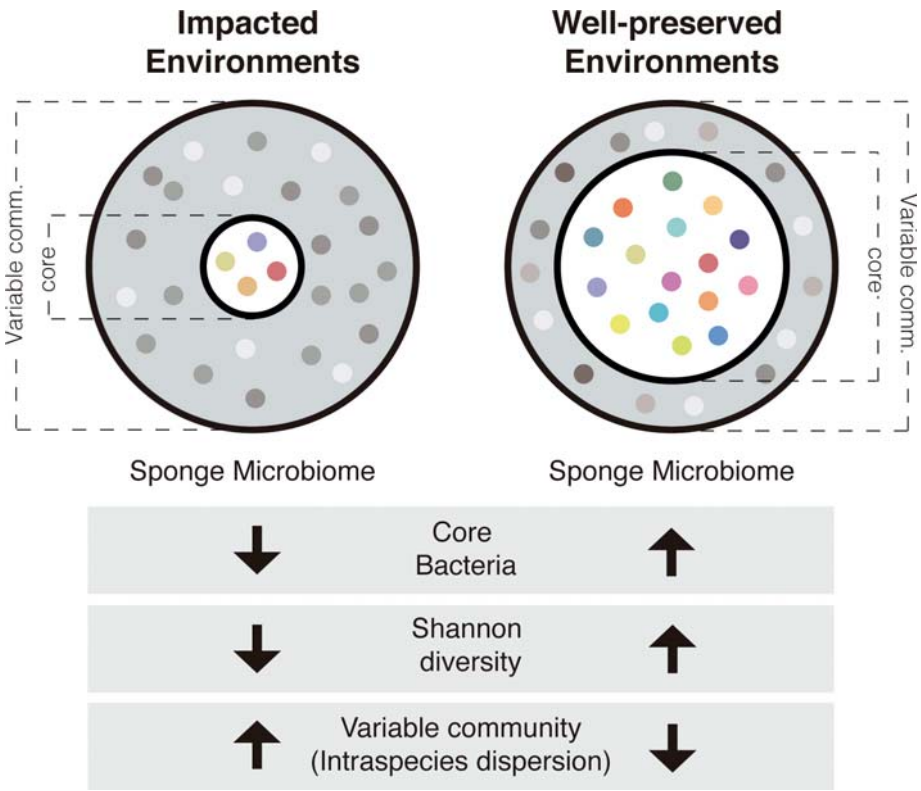

FIGURE 5 | Schematic representation of the sponge microbiomes in impacted and well-preserved environments. Gray fraction represents the variable community and white fraction represents the core community. Each colored dot corresponds to a single bacterial species.

In the Nha Trang region, marine cultures are producing chronic eutrophication and high sedimentation rates in some areas. As expected, the composition of sponge communities severely changes in those perturbed areas, with only a few sponge species inhabiting there. Body architecture and physiological traits, such as sediment removing mechanisms through mucus production (Bell et al., 2015; Schönberg, 2015, 2016) have been reported and allow sponges to survive under anomalous

sedimentation rates (Pineda et al., 2017; Strehlow et al., 2017), as those resulting from severe eutrophication (Ralph et al., 2006). Some of these mechanisms are indeed displayed by the most abundant sponge species inhabiting the polluted habitat of Nha Trang (e.g., *C. reinwardti*, *A. paraviridis*, *A. sulcata*), as the sponge surfaces appear completely clean whereas a dense layer of sediment covers the remaining substrate (**Figure 2**). However, a series of xenobiotic compounds, resulting from an intense mariculture, are also released to the water at the impacted study area (Nguyen et al., 2016), so that biological/ecological sponge traits other than resistance to sedimentation, are expected to contribute to the observed abundance of a few species in those areas.

Sponge Microbiomes: Shannon Diversity, Intra-Species Dispersion and Core Community

The bacterial communities of the study sponges show a high fidelity to the sponge species independently of the environmental conditions where the host species are living, as reported for many other sponge species (Luter et al., 2012, 2014; Simister et al., 2012; Bosch and Miller, 2016; Webster and Thomas, 2016; Gantt et al., 2017; Glasl et al., 2018).

Experimental studies repeatedly show a high resilience of the sponge microbiota in species exposed to a range of environmental variables, such as irradiance, temperature, salinity, acidification, and contrasting habitats (Erwin et al., 2012; Pita et al., 2013a,b; Cárdenas et al., 2014; Luter et al., 2014; Ribes et al., 2016; Glasl et al., 2017). A similar absence of effects on microbiome composition, and diversity was reported for the sponge *Gelliodes obtusa* under eutrophic conditions from mariculture (Baquiran and Conaco, 2018). As in the mentioned study, similar microbiomes were found in our study sponge *A. sulcata,* inhabiting either well-preserved or polluted environments. Thus, some haplosclerid sponges seem to tolerate eutrophication pressures although they can also inhabit well-preserved habitats, suggesting that they are sponges able to grow in a broad range of environmental conditions.

Most of the above-mentioned experimental studies focus on ecological adaptation of the sponge microbiomes to the assayed conditions but instead they found microbiome stability across treatments. Although surprising at first sight, microbiome resilience is indeed logical if we consider the concept of hologenome evolution (Rosenberg and Zilber-Rosenberg, 2018) and that these microbial-eukaryote symbioses have been evolutionarily fixed thousands of years ago (Sirová et al., 2018). Seemingly, structural microbiome stability has also been reported for few coral species across environmental gradients, which suggests resilience of microbiomes to environmental fluctuations or stress also in corals (Sawall et al., 2014; Grottoli et al., 2018; Pogoreutz et al., 2018).

Sponge microbiomes are species-specific in both environments, with no differences between individuals of the same species (i.e., *A. sulcata*) living at both habitats. However, significant differences in community metrics are revealed when the microbiomes of the sponge assemblages, as a whole, were compared between environments, with some particular bacterial groups proliferating in the polluted sites. Thus, even though the conditions of the perturbed environment cannot modify the evolutionarily fixed microbial communities of the sponge species (i.e., microbiomes do not change as a result of ecological adaptation to environmental conditions in species living at both habitats), they might influence the ecological distribution of the sponge species.

The sponge microbiomes of the polluted sites show a significantly lower Shannon diversity than sponge microbiomes in well-preserved environments. This is in accordance with the agreed general loss of biodiversity in impacted environments (Rygg, 1985). However, community success does not seem to directly depend on its diversity, but on its ability to respond to particular environmental conditions (McCann, 2000; Evans et al., 2017, 2018; Glasl et al., 2018). Indeed, the few species proliferating in the polluted environments of Nha Trang Bay (i.e., *C. reinwardti* and *A. paraviridis)* show a high density and a large coverage in the area, regardless of their low diversity microbiomes.

Moreover, the microbiomes of the sponges inhabiting the polluted sites show a higher intra-species dispersion than those of the sponges from well-preserved environments. The high microbiome dispersion in the former might indicate that the sponges living in the polluted environments, despite being visibly healthy are subjected to some stress. The Anna Karenina principle (Zaneveld et al., 2017), which proposes that variability is higher in dysbiotic than in healthy individuals of the same species, might be extended to species assemblages, according to our results. That is, bacterial communities of sponges in general, would be more variable in impacted than in well-preserved reefs. Similar trends in alpha and beta diversity metrics to those found in these contrasting environments have been also observed in successional stages of bacterial communities from primary, supposedly more stressed, to late or more mature (Ortiz-Álvarez et al., 2018).

The bacterial cores (Astudillo-García et al., 2017), predicted to play crucial roles in the sponge functioning (Cárdenas et al., 2014; Pita et al., 2018), represent a low percentage of the total microbiome in the study sponges inhabiting the polluted site. Consequently, most bacteria in these species are transient and thus, may be site-variable. This is confirmed by a negative correlation between the core size and the intra-species dispersion (variable community) of sponge microbiomes. Conversely, sponges inhabiting the well-preserved study sites contain large diverse bacterial cores that might be difficult to maintain in perturbed habitats, as both, theory and empirical evidence point to the simplification of ecological communities in those habitats (Piola and Johnston, 2008). As for the seawater bacteria communities, Shannon diversity was lower and variation between replicates was higher at the polluted habitats than at the well-preserved environments. Thus, seawater bacteria communities follow a similar pattern as for among replicates variation and alpha diversity than sponge microbiomes at both environments.

Moreover, the simplified microbial systems of the perturbed study habitats contain certain bacterial species that might play a role in the holobiont success.

Rhodobacteraceae members and *C. Branchiomonas* are major members of core communities of the two dominant sponges in the polluted sites (*C. reinwardti* and *A. paraviridis*), pointing to a potential function of these bacteria in the sponge success by facilitating them to cope with some pollutants. The family *Rhodobacteraceae* includes key players in biogeochemical cycling (Simon et al., 2017) and several members with chemotrophic anaerobic metabolisms, which are able to oxidize the noxious hydrogen sulfide present in eutrophic environments (Zhang et al., 2013). Members of the *Nitrosomonadaceae* family (i.e., *C. Branchiomonas*) are reported to be ammonia oxidizers (Prosser et al., 2014) and have been found to be dominant symbionts in sponges, suggesting that they may represent a source of bioavailable nitrogen for their hosts (Matcher et al., 2017). Also remarkable is the high abundance of *Endozoicomonas* in these sponge species. This genus, with various marine species distributed worldwide (Neave et al., 2016), is commonly found in close associations with sponges (Nishijima et al., 2013). Functions related to sponge health (Gardères et al., 2015), bromopyrrole production (Haber and Ilan, 2014), carbohydrate fermentation/nitrate reduction (Nishijima et al., 2013), and antibiotic production (Rua et al., 2014) in sponge hosts have been proposed (Neave et al., 2016). Thus, sponge-associated *Endozoicomona*s might play biological functions in the study sponges by participating in the nitrogen and sulfur cycles, influencing the inter-species interactions of the microbial community by producing antimicrobial compounds or signaling molecules, as reported for other sponges (Morrow et al., 2015). Finally, *Shewanella* is also found in the core of the two species at relatively high abundances at the impacted habitats. Members of this genus have been reported to reduce heavy metals, sulfates, nitrates, and chromates (Fredrickson et al., 2008), which are expected to be abundant in the polluted study habitats due to the antifouling paints containing heavy metals and residuals from industrial activities. The purported functions of the bacteria associated with the dominant sponges in the impacted habitats suggest a plausible role of the sponge microbiomes in detoxification of the seawater at a local scale. Indeed, sponges have been reported to exert a bioremediation effect (Milanese et al., 2003) in zones where they are abundant as they process high volumes of water per day (Leys et al., 2011), but further studies would be needed to confirm the real functionality of these bacteria in our study sponges.

CONCLUSION

Overall, the microbiomes might play a certain role in determining the presence and proliferation of sponge species in polluted environments. Low core size, low diversity, and high intra-species dispersion are microbiome features shared by the study sponges inhabiting the prospected polluted environments, which suggests that less complex microbiomes are favored under degraded environmental conditions. Thus, the microbial communities associated with sponges mimic the reduction of diversity showed by animal or plant assemblages at the ecosystem scale.

Under the context of climate change, it has been proposed that coral reefs may change to sponge dominated reefs (Bell et al., 2013) due to the lower sensitivity of sponges to ocean acidification and eutrophication. However, few sponges are favored by these stressful conditions and, as shown in the present study, we may expect to face a scenario with less diverse but highly abundant sponge assemblages with low complexity microbiomes.

DATA AVAILABILITY

Raw 16S rRNA gene amplicon sequences are available in the SRA archive under the project number PRJNA453898 (https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA453898). Accession numbers for sponge eukaryotic sequences are reported in the author's previous publication (Turon et al., 2018).

AUTHOR CONTRIBUTIONS

MT and MU conceived the study. MT, JC, and XT-M performed the analyses. MT and MU wrote the manuscript. JC, XT-M, and EC commented on later versions of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01961/full#supplementary-material

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RESEARCH ARTICLE
Host-Microbe Biology

Multipartner Symbiosis across Biological Domains: Looking at the Eukaryotic Associations from a Microbial Perspective

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ABSTRACT Sponges establish tight associations with both micro- and macroorganisms. However, while studies on sponge microbiomes are numerous, nothing is currently known about the microbiomes of sponge-associated polychaetes and their relationships with those of their host sponges. We analyzed the bacterial communities of symbiotic polychaetes (*Haplosyllis* spp.) and their host sponges (*Clathria reinwardti*, *Amphimedon paraviridis*, *Neofibularia hartmani*, and *Aaptos suberitoides*) to assess the influence of the sponges on the polychaete microbiomes. We identified both eukaryote partners by molecular (16S and COI genes) and morphological features, and we identified their microbial communities by high-throughput sequencing of the 16S rRNA gene (V4 region). We unravel the existence of six *Haplosyllis* species (five likely undescribed) associated at very high densities with the study sponge species in Nha Trang Bay (central Vietnam). A single polychaete species inhabited *A. paraviridis* and was different from the single species that inhabited *A. suberitoides*. Conversely, two different polychaete species were found in *C. reinwardti* and *N. hartmani*, depending on the two host locations. Regardless of the host sponge, polychaete microbiomes were species specific, which is a widespread feature in marine invertebrates. More than half of the polychaete bacteria were also found in the host sponge microbiome but at contrasting abundances. Thus, the associated polychaetes seemed to be able to select, incorporate, and enrich part of the sponge microbiome, a selection that appears to be polychaete species specific. Moreover, the bacterial diversity is similar in both eukaryotic partners, which additionally confirms the influence of food (host sponge) on the structure of the polychaete microbiome.

IMPORTANCE The symbiotic lifestyle represents a fundamental cryptic contribution to the diversity of marine ecosystems. Sponges are ideal targets to improve understanding the symbiotic relationships from evolutionary and ecological points of view, because they are the most ancient metazoans on earth, are ubiquitous in the marine benthos, and establish complex symbiosis with both prokaryotes and animals, which in turn also harbor their own bacterial communities. Here, we study the microbiomes of sponge-polychaete associations and confirm that polychaetes feed on their host sponges. The study worms select and enrich part of the sponge microbiome to shape their own species-specific bacterial communities. Moreover, worm microbiome diversity runs parallel to that of its food host sponge. Considering our results on symbiotic polychaetes and previous studies on fishes and mammals, diet appears to be an important source of bacteria for animals to shape their species-specific microbiomes.

KEYWORDS invertebrate-microbe interactions, marine microbiology, symbiosis

Living in symbiosis (in its broader sense) is a general lifestyle across terrestrial and marine ecosystems (1, 2), but it seems to be particularly remarkable in the latter (3–5). Marine sedentary invertebrates, such as sponges and corals, are engineer organ-

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isms habitually used as a refuge by diverse mobile fauna. Among them, cnidarians, crustaceans, mollusks, nematodes, and polychaetes are the most frequently reported in association with sponges in temperate, cold, and tropical oceans (6–11).

Many tropical sponges provide refuge to polychaetes. In particular, endosymbiotic species of Syllidae in sponges represent a paradigmatic model for the study of symbiosis, as thousands of individuals of the same worm species colonize one (or a few) sponge species (12–15), and all phases of the polychaete life cycle seem to occur inside the host (13, 16). However, whether these associations are species specific, symbiotic, mutualistic, or parasitic is under discussion (8, 13, 14, 17, 18). While these associations are undoubtedly considered advantageous for the polychaete because sponges represent a food source and a clear refuge against predation (8), the potential benefits for the sponge are more difficult to deduce. Polychaete predation does not seem to cause detectable harm to the host sponges so that the nature of the association has been interpreted as commensalism, mutualism, or "good" parasitism (8, 10, 12, 13, 19).

Indeed, sponge-polychaete associations represent multipartner symbioses as both eukaryotes establish tight associations with multiple microbes (20). Eukaryote partners harbor their own microbiomes, formed of hundreds of bacterial species interacting among themselves and with their respective hosts. Bacteria have been decisive protagonists in the development of the eukaryote cell (21). Since then, they inhabit almost every terrestrial and aquatic niche on our planet and accompany eukaryote organisms along their complete life cycle (22). However, the potential role, if any, of microbiomes in eukaryotic symbiotic associations has not yet been explored. While studies on sponge microbiomes have proliferated in the last decades (23–26), nothing is currently known about the microbiomes of symbiotic polychaetes, including syllids.

In the field of invertebrate-microbe symbioses, how symbiotic bacteria are acquired by a host species remains under debate. Initially, the concept of true symbiont was associated with a maternal inherence (vertically transmitted). Currently, the idea of a species-specific selection of bacteria from the environment by the eukaryote host to form its specific microbiome is gaining support (27–29), particularly since the host's bacterial composition does not directly reflect that of the environment (28, 30).

Our study identified the bacterial communities of four tropical sponges, *Clathria* (*Thalysias*) *reinwardti* Vosmaer 1880, *Amphimedon paraviridis* Fromont 1993, *Neofibularia hartmani* Hooper and Lévi 1993, and *Aaptos suberitoides* Brøndsted 1934, and those of their respective polychaetes of the genus *Haplosyllis* in different locations of Nha Trang Bay (central Vietnam), aimed at assessing the contribution of the host sponges to the microbiome composition of their associated polychaetes. Considering that syllid worms feed on their host sponges and that diet is known to influence the feeder microbiome, at least in vertebrates (31–34), we hypothesized that polychaete microbiomes would reflect to some extent the microbiomes of their host sponges. In this case, one would expect to find a high degree of similarity between the bacterial communities of the symbiotic partners, with the most abundant members of the sponge microbiome also being major components of the polychaete microbiome.

RESULTS

Polychaete identification and associations with host sponges. All sponge species were dominated by a single polychaete species at high abundance. Figure S1 shows individual worms extracted from a 3-cm^3 sponge fragment. Six species of *Haplosyllis* could be distinguished based on morphological (Fig. 1) and molecular characteristics. Species identity could be confirmed only for *Haplosyllis tenhovei* Lattig, Martin, and Aguado 2010, while the remaining five worms likely represented undescribed species, whose formal description will be submitted to a specialized journal and thus, is out of the scope of the present study.

Both 16S and COI sequences (see "Data availability" below for accession numbers) differed among all identified species, except for *Haplosyllis* species 3 (sp3) and *Haplosyllis* sp4, whose sequences are identical despite showing enough morphological

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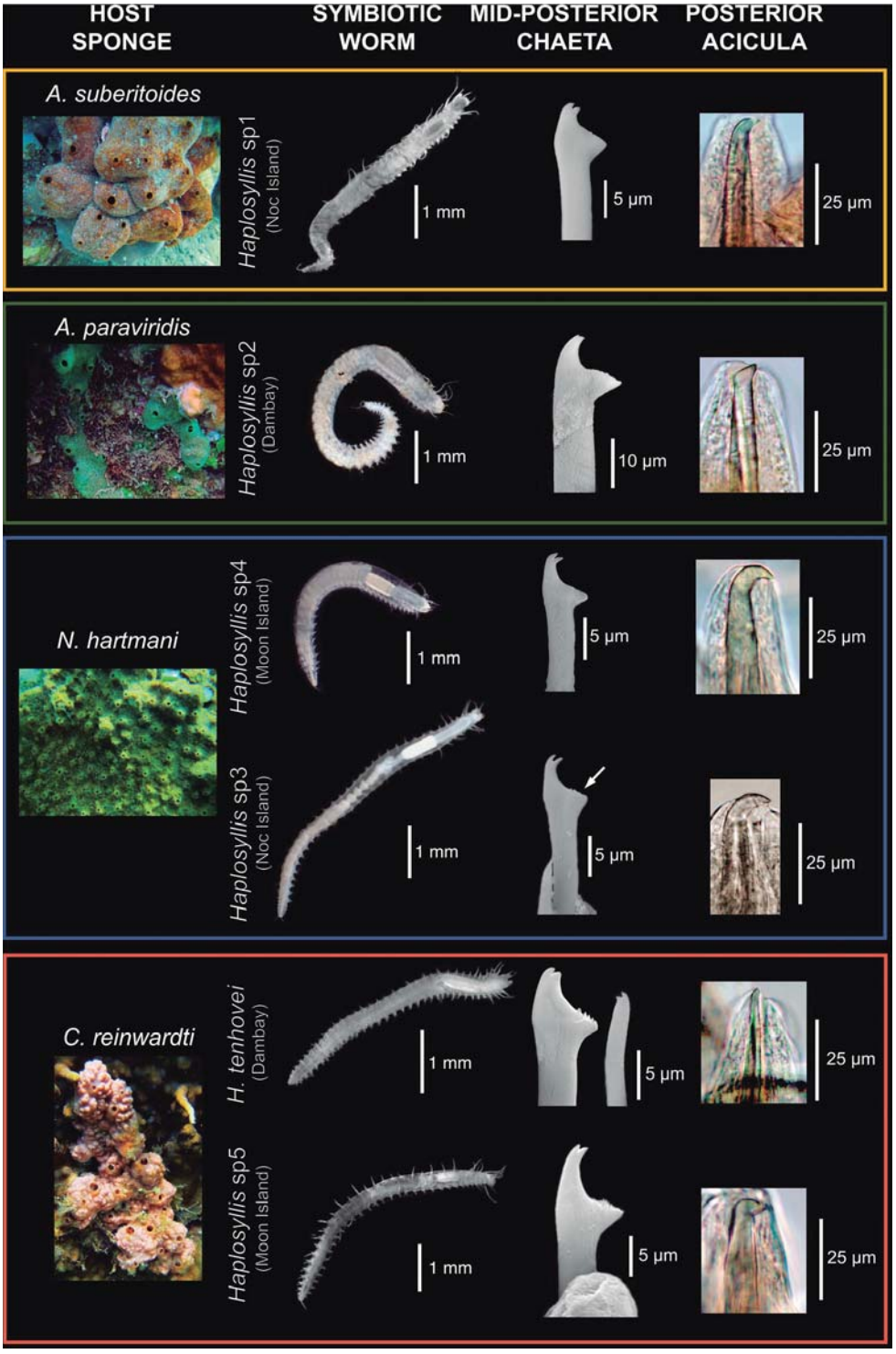

FIG 1 Pictures of the sponges and their associated polychaetes. Scanning electron microscopy photos of the mid-posterior chaetae and optical microscopy photos of the posterior acicula, which were considered diagnostic characteristics for polychaete species differentiation. The locations where the sponges and associated polychaetes were found are indicated in parentheses. Six *Haplosyllis* spp. (five likely undescribed [species 1 {sp1} to 5 {sp5}]) are shown.

differences to be considered different species under traditional taxonomic criteria (Fig. 1).

All respective replicates of *Aaptos suberitoides* and *Amphimedon paraviridis* were constantly found in association with a single polychaete species, *Haplosyllis* sp1 and

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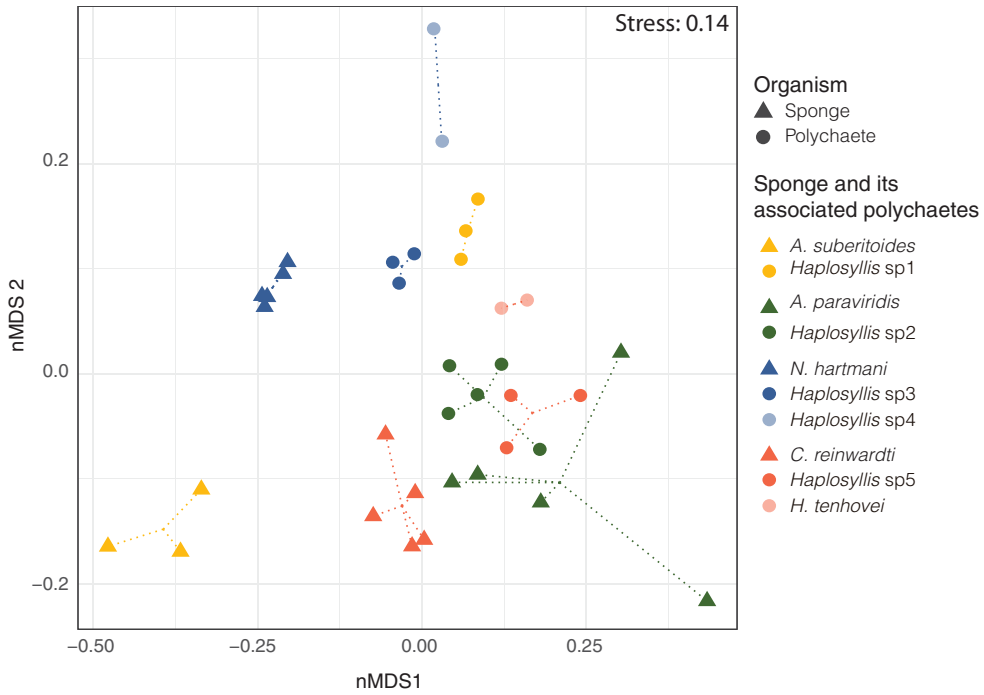

FIG 2 Nonmetric multidimensional scaling (nMDS) ordination of the sponge (triangles) and polychaete (circles) bacterial communities based on Bray-Curtis distances. Sponge species and their associated polychaete species are depicted in the same color.

Haplosyllis sp2, respectively (Fig. 1). Conversely, in *Neofibularia hartmani* and *Clathria reinwardti*, two different polychaete species were found in each sponge, depending on the geographical location. *N. hartmani* harbored *Haplosyllis* sp3 at Noc Island and *Haplosyllis* sp4 at Hun Moon Island, while *C. reinwardti* harbored *Haplosyllis* sp5 at Hun Moon Island and *H. tenhovei* at Dam Bay (Fig. 1).

In all cases, evidence of sponge spicules inside the worms confirmed that the symbiotic polychaetes feed on the host sponges (data not shown).

Sponge and polychaete microbiomes. Host identity was the main factor structuring the bacterial communities of both sponges and polychaetes (Fig. 2) (R^2 = 0.62 and $P < 0.001$ by PERMANOVA [nonparametric permutation analysis of variance]). Polychaete microbiomes had unique bacterial communities markedly different from those of their host sponges and the surrounding seawater (Fig. S2), but they also differed between the worm species.

On the basis of Bray-Curtis distances, bacterial communities were more similar to each other in polychaetes than in host sponges (Fig. S3). Although highly different (Bray-Curtis distances $>$ 0.6), microbiome distances in specific associations (host sponge versus its symbiotic polychaete) were significantly lower ($P < 0.001$ by Kruskal-Wallis test) than in nonspecific associations (sponge versus polychaetes from all other sponge species) (Fig. S3). The most similar microbiomes were found in *N. hartmani* and *Haplosyllis* sp3 and in *A. paraviridis* and *Haplosyllis* sp2 (Fig. S4), while the most distant were those of *A. suberitoides* and *Haplosyllis* sp1 (Fig. S4). Moreover, the microbiomes of high-microbial-abundance (HMA) sponges (i.e., *A. suberitoides* and *N. hartmani*) were associated with polychaete microbiomes with Shannon diversities higher than the microbiomes of polychaetes associated with the low-microbial-abundance (LMA) sponges (i.e., *C. reinwardti* and *A. paraviridis*) (Fig. 3).

Core microbiome communities. The core bacterial communities of both sponges and polychaetes appeared to be large and represented more than 80% of the relative abundance of the total microbiome in most species (Table S1). A total of 44 ZOTUs ("zero-radius" operational taxonomic units) were detected in all polychaete samples,

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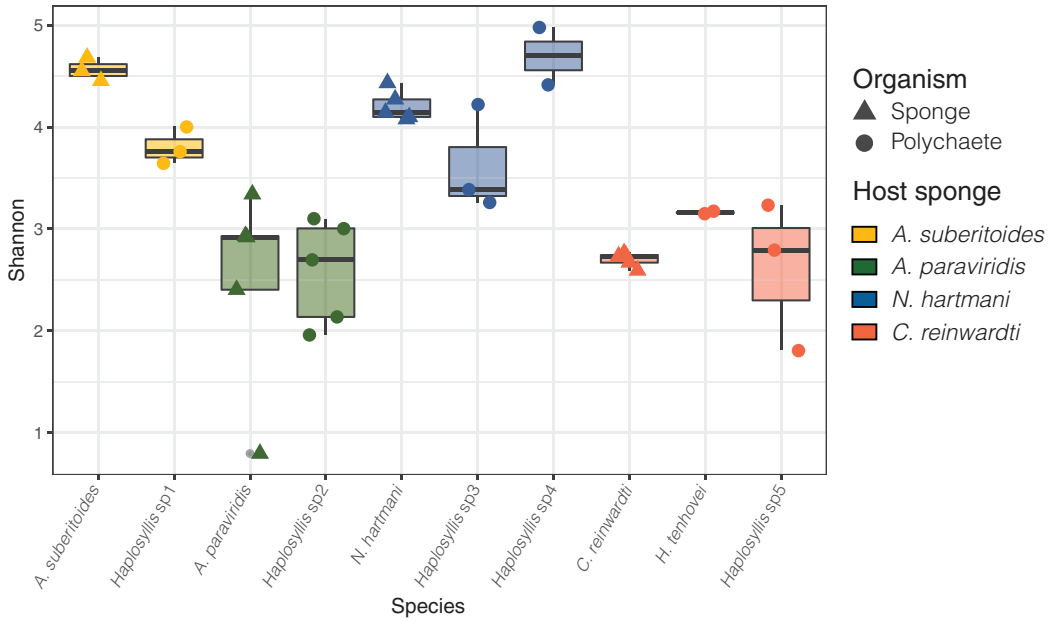

FIG 3 Box plots showing the Shannon diversity of microbiomes in a sponge (triangles) and polychaete (circles). Sponge species and their associated polychaete species are depicted in the same color.

with the most abundant belonging to *Vibrio*, *Litorimonas*, *Endozoicomonas*, *Pseudoalteromonas*, *Shewanella*, and *Alteromonas* (Table S2). The results shown in the following sections are based on core bacterial communities.

Taxonomic profiles of sponge and polychaete bacterial communities. The most abundant orders in polychaete communities were *Vibrionales* (24.3%), *Alteromonadales* (17.7%), *Oceanospirillales* (14.3%), *Burkholderiales* (7.6%), and *Caulobacterales* (4.3%), whereas in sponges, they were *Rhodobacterales* (16.29%), *Oceanospirillales* (14.9%), *Nitrosononadales* (8.9%), and PAUC34f unclassified (5.9%).

In most cases, sponges and their associated polychaetes showed highly different bacterial communities (Fig. 4). In *Haplosyllis* sp1, the dominant *Vibrionales* and *Alteromonadales* occurred at relative abundances lower than 0.5% than those in *A. suberitoides*, while in *Haplosyllis* sp2 and *A. paraviridis*, *Oceanospirillales* were highly abundant in both partners. In *N. hartmani* and *C. reinwardti*, each polychaete species (two for each sponge from different localities) inhabiting the same host sponge presented a unique bacterial composition that also differed from the sponge bacterial community. In *N. hartmani*, *Vibrionales* and *Alteromonadales* dominated the microbiome of *Haplosyllis* sp4 (as in *Haplosyllis* sp1 from *A. suberitoides*), whereas *Burkholderiales* and *Rhodobacterales* dominated in *Haplosyllis* sp3. In *C. reinwardti*, *Rhodobacterales* were dominant, whereas *Vibrionales* dominated in *H. tenhovei* and *Sphingomonadales*, *Caulobacterales*, and *Alteromonadales* dominated in *Haplosyllis* sp5.

Bacterial communities shared between the eukaryotic partners. The number of ZOTUs shared between the sponges and their polychaete symbionts varied among the studied species. More than half of the polychaete ZOTUs were present in their host sponge microbiomes, except for *Haplosyllis* sp4 (Fig. 5), with the most abundant polychaete ZOTUs occurring at low abundances in the respective host sponges and vice versa (Fig. 5). Indeed, the two most abundant ZOTUs of all polychaete microbiomes were found at relative abundances lower than 0.5% in the microbiomes of the respective host sponges (Fig. 5).

Few ZOTUs showed similar relative abundances in both the polychaete and its sponge host (Fig. 5). In the case of *C. reinwardti*, ZOTU 55 belonging to *Shewanella* was also abundant in *H. tenhovei*, and ZOTU 21 (*Endozoicomonas*) and ZOTU 32 (*Rhodobacteraceae*) were both found at high abundances in *Haplosyllis* sp5 ZOTU 48 (*Endozoicomonas*), while ZOTU 81 (*Shewanella*) was abundant in both *A. paraviridis* and its

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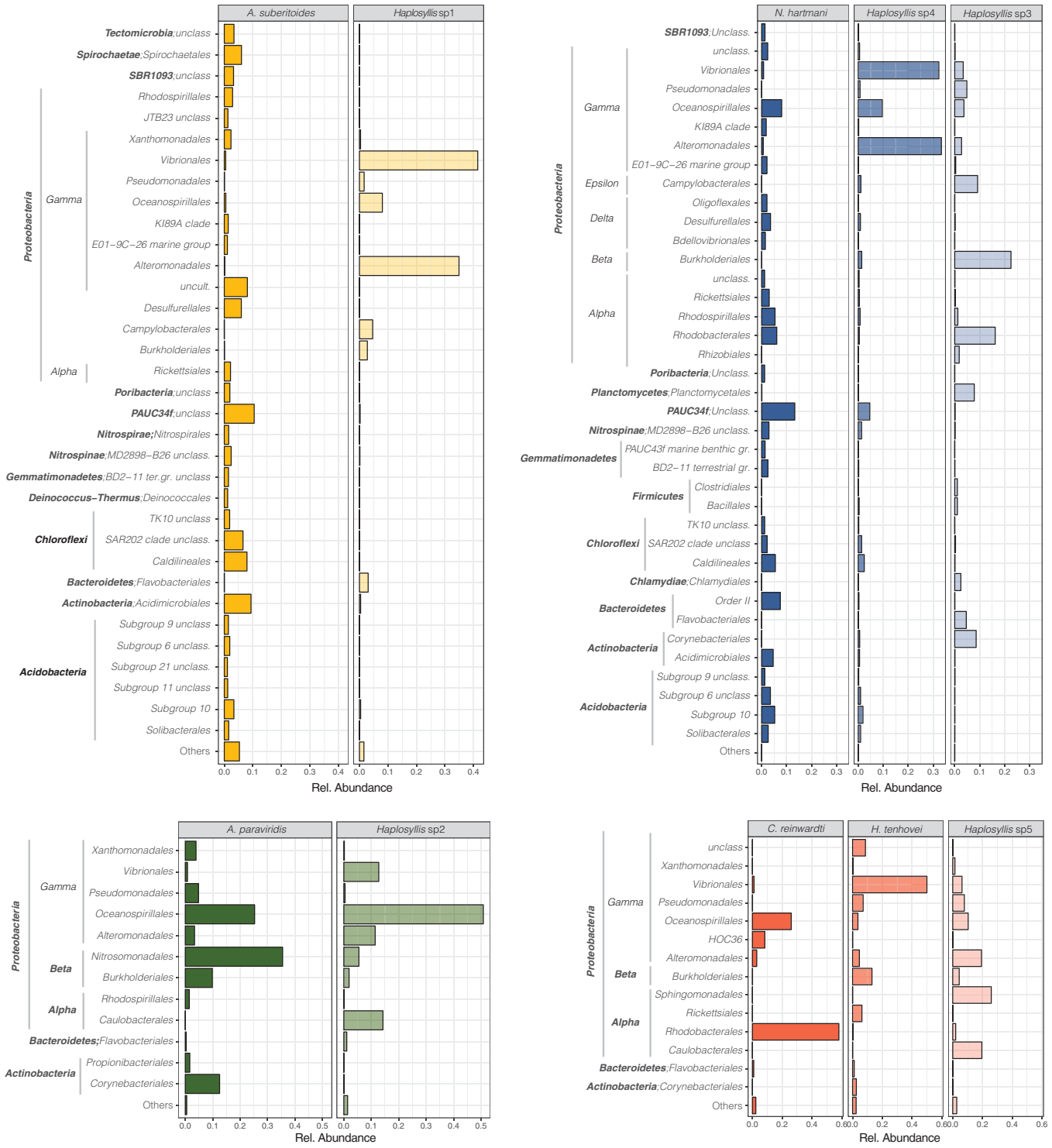

FIG 4 Bacterial composition (at the order level) for each sponge species and its associated polychaetes. The bars represent the relative abundance of each bacterial order in the sponge or polychaete core community. unclass, unclassified; uncult., uncultivated; gr., group.

associated polychaete *Haplosyllis* sp2. Finally, in the case of *N. hartmani*, ZOTU 11 (*Endozoicomonas*) was highly abundant in the sponge and in both of its associated polychaetes, and ZOTU 19 (PAUC34f) and ZOTU 36 (*Caldilineaceae* uncultivated) were also abundant in *Haplosyllis* sp3.

Haplosyllis sp1 (*A. suberitoides*) and *Haplosyllis* sp4 (*N. hartmani*) microbiomes were mainly composed by ZOTUs that were rare or absent in their host sponges (Fig. 6). On

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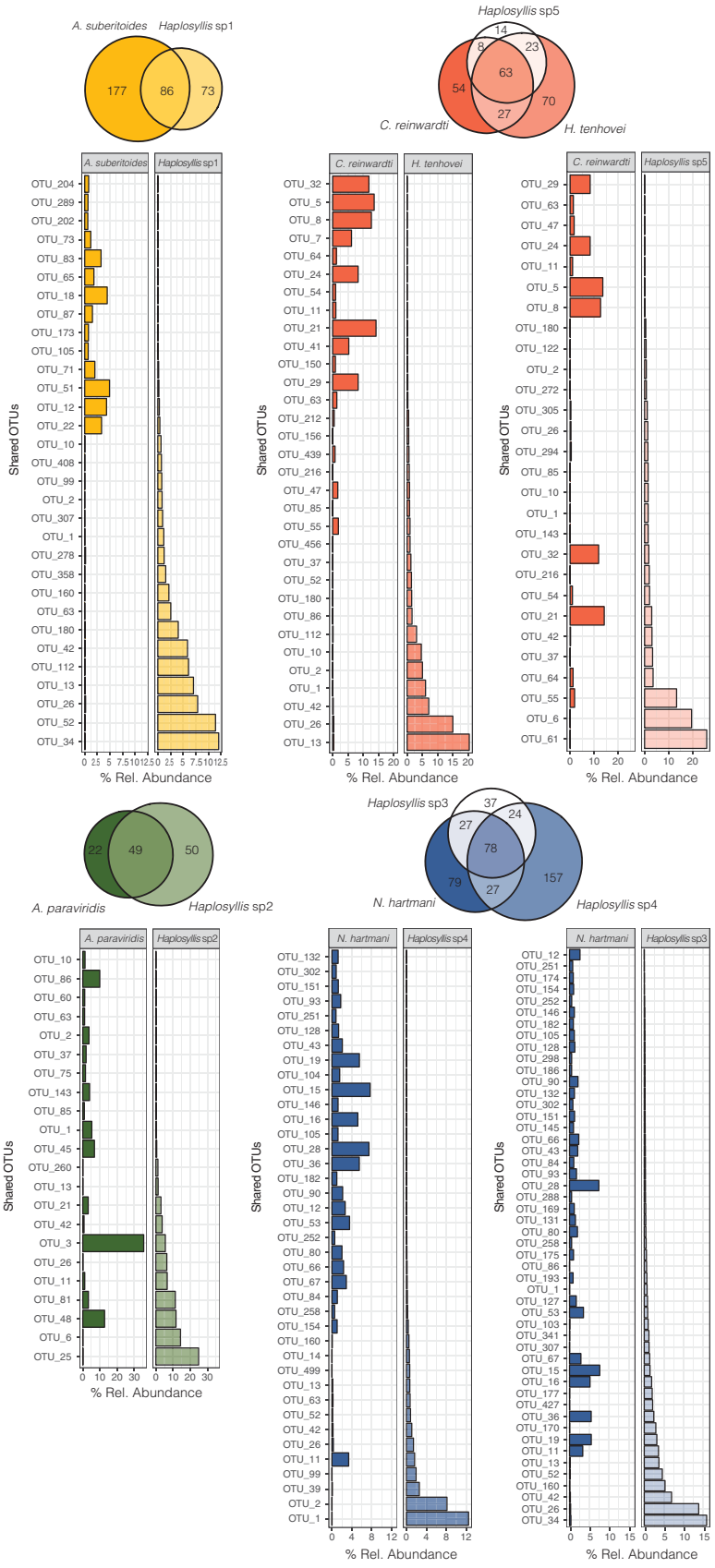

FIG 5 Venn diagrams showing the overlap between the bacterial core communities of each sponge species and its associated polychaete. The size of the circle represents the size of the bacterial core (number of ZOTUs). Bar plots represent the relative abundances (as a percentage) of the shared ZOTUs

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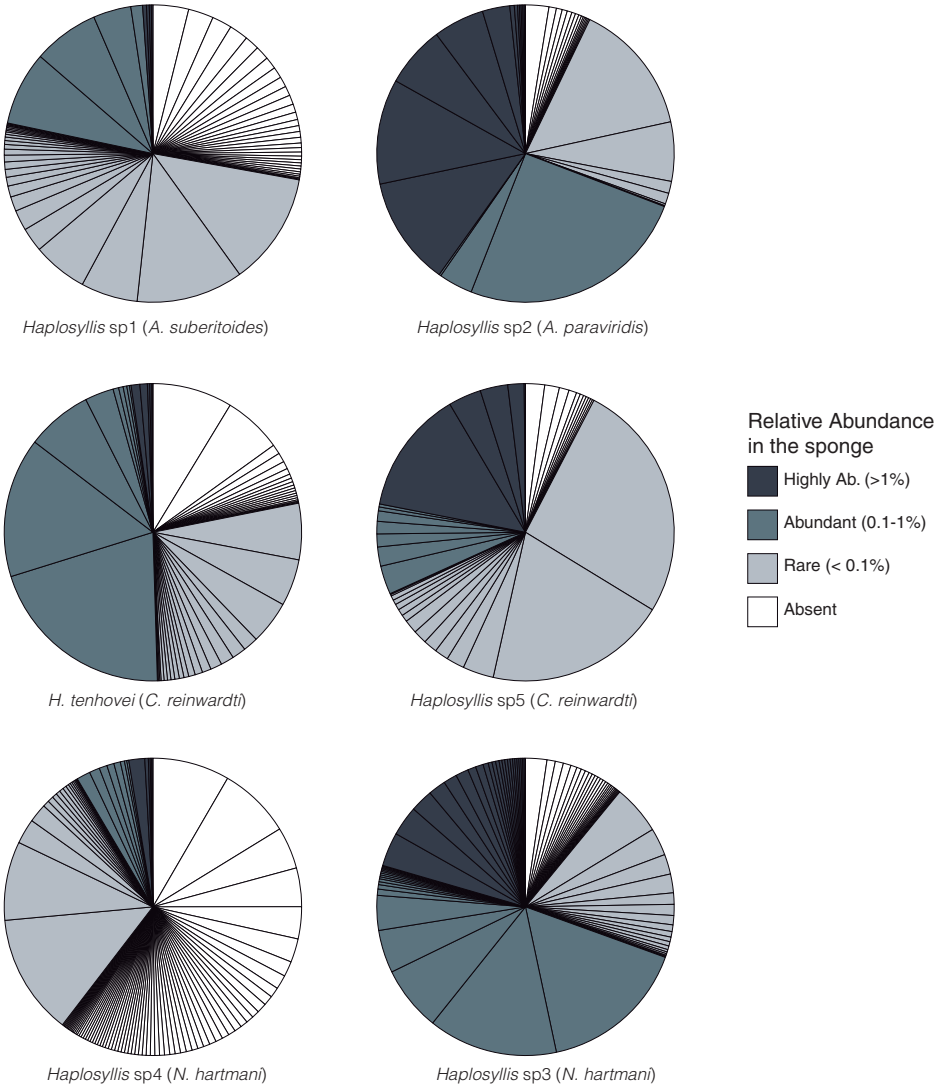

FIG 6 Bacterial core communities of each polychaete species. Only ZOTUs with relative abundances higher than 0.1 in the polychaete core are depicted in the pie charts. Each pie slice corresponds to a polychaete core ZOTU, and its size is proportional to its relative abundance. Colors represent the categorical relative abundance that each polychaete ZOTU is found in the sponge microbiome: highly abundant (dark gray), abundant (gray), rare (light gray), and absent (white).

the other hand, *Haplosyllis* sp2 (*A. paraviridis*) and *Haplosyllis* sp3 (*N. hartmani*) microbiomes had a greater proportion of ZOTUs that were either relatively abundant or highly relatively abundant in their respective host sponges.

In all cases, polychaetes shared more ZOTUs with the sponges than with the seawater bacterial communities (Fig. S5). Moreover, only a few of the ZOTUs having a relative abundance of >0.05% in the polychaetes were found exclusively in seawater and absent from the sponge (marked with an asterisk in Fig. S6).

DISCUSSION

The sponge-polychaete association. Observations of tropical worms associated with sponges, most of them classified as *Haplosyllis spongicola* Grube 1855 have been widely reported (35). However, there is little data available on the relationships be-

FIG 5 Legend (Continued)
between each sponge-polychaete system. Only ZOTUs with relative abundances higher than 0.5% in any of the eukaryotic partners are shown in the bar plots.

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tween these worms and their host sponges (8, 10, 13). Currently, *H. spongicola* is known to be a species complex (36) that includes several misidentified species (17), and new species of *Haplosyllis* are continuously discovered. Thus, it was not unexpected that five out of the six species of *Haplosyllis* living in association with the four study sponge species were also undescribed. The Vietnam area seems to be rich in symbiotic polychaetes, according to the numerous species of *Haplosyllis* present there (10, 37), although this can be related to the large number of studies carried out in this area.

We recorded the presence of host-specific spicules in representative samples from all sponge-associated worms. This confirms a sponge-based diet for these symbiotic syllids, as previously proposed for other species (10, 13, 16, 19) and suggests damage to the host. However, the worms' grazing does not seem to significantly harm their hosts, since they are among the largest and most abundant sponge species in our study area. The absence of negative effects on the hosts would confirm that these associations are commensalistic rather than parasitic, or even mutualistic as recently proposed (10, 19).

On the basis of the study examples, the sponge-polychaete associations appeared to be species specific, that is, all the sponge individuals of the same species in a given area are colonized by the same polychaete species. However, in two cases, the same sponge species harbored two different species of *Haplosyllis* depending on the geographical location. One example is that *Haplosyllis nicoleae* (instead of *H. tenhovei* in our study) was found associated with *C. reinwardti* in Indonesia (14). On the other hand, ca. 8 of over the more than 30 known symbiotic species of *Haplosyllis* are reported to colonize more than one sponge species (10). Thus, although these associations appear to be species specific at first sight, they may also be ecologically modulated and depend on the geographical/ecological distribution of the species involved. Chemical metabolites released by the host (38, 39) may represent attractant cues for more than one polychaete species (40), so that colonization by one or other might depend on the most prevalent syllid species in a particular area. Colonization by symbiotic polychaetes may be followed by rapid proliferation and complete niche occupation, which could explain the dominance of a single symbiont species in most cases (8, 10, 41).

Bacterial communities from the eukaryote partners. Sponge-polychaete symbioses involve many more than two partners, as both eukaryotes harbor particular microbiomes formed by hundreds of bacterial species establishing a tight network of potential interactions. Sponge microbiomes have been intensively investigated during the past 15 years (23–25, 29, 42–44). Conversely, polychaete microbiomes are still poorly known (45–47), with most studies focusing on worms inhabiting hydrothermal vents (48). The microbiomes of the *Haplosyllis* species studied here were more closely related to each other than those of their respective host sponges. Taking into account that all the worms belong to the same genus, while the host sponges belong to different orders (i.e., Suberitida, Poecilosclerida, Desmacellida, and Haplosclerida), we suggest that this pattern may have an evolutionary component.

Polychaete microbiomes are species specific. In general, sponge microbiomes tend to be species specific, and the same pattern has been reported for nematodes (49). Our results also show a high species specificity of the polychaete bacterial communities, regardless of their host sponges. Species specificity of microbiomes seems to be more common in invertebrates than previously thought and suggests the existence of species-specific mechanisms of bacterial selection (50), pointing to a relevant role of the associated microbes in invertebrate functioning.

Since polychaete microbiomes appear to be species specific, they may have a diagnostic value in addition to morphological traits. This could be the case of the two species of *Haplosyllis* found in *N. hartmani*, which were morphologically different but molecularly cryptic, and harbor very different bacterial communities. In this sense, microbiomes might inform on ongoing speciation processes even before being detected by molecular markers (e.g., COI and 16S).

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Influence of diet (sponge) on the polychaete microbiomes. On the basis of previous studies with other organisms, we hypothesized that polychaete microbiomes would reflect those of their prey sponge species. If this were true, two polychaete species feeding on the same sponge would have similar microbiomes. In contrast, *Haplosyllis* sp3 and *Haplosyllis* sp4 feeding on *N. hartmani* and *Haplosyllis* sp5 and *H. tenhovei* feeding on *C. reinwardti* have distinct bacterial communities. Our results suggest that each polychaete species selectively incorporates and enriches specific bacteria, even if these bacteria are rare members of its prey's microbiome. Enrichment of environmentally rare microbes has been reported for sponges (29, 51), mollusks (52), fishes (30, 53), and amphibians (28). Microbiome diversity is positively related between polychaetes and their food source (host sponges), which has also been reported for fish larvae and their food source (34) as well as for the human gut and diet (33). Thus, our results seem to agree with those reported for other organisms, pointing to what could be a widespread pattern relating bacterial diversity of food and feeder. Recently, Cleary et al. (54) also found a compositional similarity between certain sponge samples and sponge denizens, suggesting that sponges may influence the prokaryote composition of organisms that live on or within them or that feed on them.

Reliance of the polychaete microbiome on the sponge microbiome. When analyzing the polychaete-sponge relationship from a microbial perspective, we considered that the higher the number of bacterial ZOTUs in the polychaete and absent from the sponge, the lower the polychaete dependence on the sponge microbiome. In this sense, *Haplosyllis* sp1 (from *A. suberitoides*) and *Haplosyllis* sp4 (from *N. hartmani*) would depend less on the sponge microbiome to build up their own microbiome than the remaining polychaete/sponge partnerships studied. The worm bacteria that were not recorded in the host sponge microbiome may possibly correspond to vertically transmitted bacteria (i.e., through sexual or asexual propagula). However, we cannot fully discard some methodological constrains, i.e., if bacteria in the sponge escaped our detection limits. We can also envisage some of these microbes being acquired horizontally from environmental sources other than the host tissues (e.g., from seawater [but see supplemental material]).

In most cases, more than half of the bacteria from a polychaete microbiome, which probably correspond to the gut microbiome, were also found in the sponge, but at contrasting abundances, suggesting different levels of between-partner dependency. It would be interesting to assess to what extent the polychaetes maintain their microbiomes when associated with other sponge hosts with different bacterial communities.

The polychaete bacterial core. We have found a quite large core bacterial community in all species of *Haplosyllis*, indicating that polychaete bacteria might play general metabolic or defensive roles (48). Among these core microbes, we found representatives of *Vibrionales*, *Caulobacterales*, *Alteromonadales*, and *Oceanospiralles*. Representatives of these groups have also been reported in other polychaetes such as *Vibrio* in the filter-feeding *Sabella spallanzanii* Gmelin 1791 (47), *Alteromonadales* and *Oceanospiralles* in deposit feeders Opheliids (45) and *Oceanospiralles* in the bone-eating *Osedax* (55).

Polychaetes have been proposed as bioremediation agents in polluted waters due to their ability to accumulate *Vibrio* species, which are well-known pathogens in aquaculture (47, 56, 57). Conversely, high levels of nonpathogenic *Vibrio* strains have been recently reported in shrimp guts (58), suggesting a possible beneficial role in the invertebrate fitness. Moreover, different members of *Vibrionaceae* are also reported to be extracellular polymeric substances (EPS) producers (59), which are important cell protective agents (i.e., against environmental stressful conditions or from xenobiotic substances) and allow them to capture nutrients (59). In turn, *Alteromonadales* increased in abundance at sites affected by urbanization and eutrophication (45) due to their purported tolerance to high copper levels (45, 60) and to other metals (45, 61). Moreover, members of *Alteromonadales* are well-known EPS and biosurfactant (BS) producers (48, 59), the latter being correlated with antimicrobial activity suggesting a

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defensive role against pathogens (48). Finally, *Oceanospiralles* are well-known heterotrophic degraders of complex organic compounds (55), which may also contribute to increase the fitness of the associated polychaetes.

Conclusions. To summarize, the sponge-polychaete associations seem to be basically species specific but can be ecologically modulated, as different polychaete species inhabited the same sponge species depending on the habitat. The microbiomes of both the sponges and their associated polychaetes are also species specific, pointing to the relevance of the microbial component on the invertebrate functioning. Our results suggest that the associated polychaetes select, incorporate, and enrich a part of the sponge microbiome to form their individual microbiomes, but the selection appears to be species specific, possibly reflecting the specific polychaete needs. Diet appears to be an important source of bacteria for invertebrates (this study) and vertebrates (previous studies) to shape their specific microbiomes.

MATERIALS AND METHODS

Sponge and polychaete sampling and DNA extraction. A quantitative sampling method to describe the sponge assemblages of Nha Trang Bay (central Vietnam) was conducted in April 2015 (29). During that campaign, sponge species associated with polychaetes were surveyed. Four of them were later selected for the present study due to their high abundance and density of associated polychaetes. Among the selected species, *A. suberitoides* and *N. hartmani* belonged to high-microbial-abundance (HMA) sponges, whereas *C. reinwardti* and *A. paraviridis* belonged to low-microbial-abundance (LMA) species (29). Sponges containing polychaetes were collected in April 2016 by SCUBA diving between a depth of 3 and 9 m in three neighboring locations ~2 km apart (i.e., Dam Bay and Hun Mun and Nock Islands) within Nha Trang Bay. Three samples of *A. suberitoides* (all from Nock Island), five samples of *N. hartmani* (three from Nock Island, two from Hun Mun Island), five samples of *C. reinwardti* (two from Dam Bay Island, three from Hun Mun Island), and five samples of *A. paraviridis* (all from Dam Bay) were collected. Each sponge sample was kept in a 50-ml Falcon tube with native seawater from same depth and sampling point and later replaced by 100% ethanol once the polychaetes left the host sponge (ca. 10 min). The released polychaetes were then cleaned from all remaining sponge tissues and allocated to Eppendorf tubes containing 100% ethanol. Back in the lab, sponges were examined under the microscope to extract any possible remaining polychaetes. In the case of *A. suberitoides*, only a few polychaetes left the host sponge spontaneously, and thus, sponge dissection and careful examination were key to extracting the sponge-associated polychaetes. Ethanol was replaced twice with fresh absolute ethanol to ensure good sample preservation. DNA from sponge and polychaete samples was extracted by following the DNeasy Blood & Tissue kit protocol (Qiagen).

Additionally, triplicate 2-liter water samples were taken from the three locations (ca. 50 cm apart from the sponges) and sequentially filtered throughout 5-μm and 2-μm polycarbonate membranes. The size fraction (5 to 2 μm) was processed for DNA extraction. The membranes were enzymatically digested with lysozyme, proteinase K, and sodium dodecyl sulfate, and afterwards, DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) and chloroform-isoamyl alcohol (24:1, vol/vol). Purification and concentration of the DNA was performed with Amicon Ultra 4 centrifugal filter units with 100,000 nominal molecular weight limit (NMWL) (Millipore).

Polychaete identification. Once the polychaetes were separated from their respective host sponges, all polychaetes were carefully identified using a microscope. Anecdotal species (i.e., species other than the most abundant one, present as 1 or 2 specimens per sample) were discarded. Only the dominant symbiotic species from each sponge was considered for this study.

We identified polychaete species to the best possible taxonomic resolution by molecular markers and morphological features. Fragments of the mitochondrial small subunit 16S rRNA gene (~650 bp) and the cytochrome *c* oxidase subunit I (*COI* ~680 bp) were amplified and sequenced. Primer pairs 16SarL/16SbrL (62) and jgLCO1490/jgHCO2198 (63) were employed to amplify 16S rRNA and COI, respectively. PCR amplifications were conducted in 50-μl reaction mixtures containing 1 ng of template genomic DNA, 5 μl of 10× PCR buffer (containing 1.5 mM $MgCl_2$), 2 μl of dNTP mix (10 mM), 1 μl of each primer (10 mM), and 0.4 μl of *Taq* DNA polymerase (5 U μl^{-1}). The temperature profiles to obtain the PCR products were set by following the protocols of Álvarez-Campos et al. (64). Purification and sequencing were conducted by an external service (Macrogen, Spain).

The morphology of the dominant polychaete species, all them belonging to the genus *Haplosyllis*, was observed by using light and scanning electron microscopes following the procedures described by Martin et al. (36). All relevant diagnostic morphological characteristics required for species identification according to Lattig et al. (65) were recorded and then checked against the currently existing literature.

Verification of polychaete feeding behavior. From each sponge sample, 25 polychaete specimens were carefully examined to ensure the absence of externally attached sponge spicules, dissolved in boiling nitric acid to totally remove organic matter, and then examined with a light microscope (Leitz Axioplan) to confirm the presence of host sponge spicules in the worm.

Bacterial 16S rRNA gene amplification, sequencing, and analyzing. PCR and high-speed multiplexed 16S rRNA gene Illumina MiSeq sequencing (next-generation sequencing [NGS]), were performed following the methods of the genomic core facilities and the methods of MrDNA lab (Shallowater, TX, USA). The variable V4 region of the bacterial 16S rRNA gene was amplified using the primers 564F

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(5′-AYTGGGYDTAAAGNG-3′) and 785R (5′-TACNVGGGTATCTAATCC-3′) (ca. 250 nucleotides [nt]) (66). Raw rRNA gene sequences were processed separately using the UPARSE pipeline (67). A quality check and dereplication were applied to our data set. Denoising (error correction) of amplicons was performed by using the UNOISE pipeline (68). This algorithm removed chimeras, reads with sequencing errors, PhiX, and low-complexity sequences due to Illumina artefacts, and generates ZOTUs ("zero-radius" operational taxonomic units [OTUs]) with 100% identity sequences.

Taxonomic assignment was done with SINA v1.2.11 (69) using SILVA 128 database. Sequences with low alignment quality (<75%) and sequences identified as mitochondria or chloroplasts were removed from the analysis. To minimize biased effects for differences in sampling effort, the original bacterial ZOTU table was rarefied at a minimum read threshold of 40,000, using QIIME (70). We normalized our data set to the same read count, which means that all data on "bacterial abundance" refer to relative abundance.

Bacterial community analyses of sponges and their associated polychaetes. Distance-based multivariate analysis of the sponge and polychaete bacterial communities (at the ZOTU level) was conducted using the *vegan* package in R (71). An nMDS (nonmetric multidimensional scaling) was used to visualize the Bray-Curtis dissimilarity matrix. PERMANOVA (nonparametric permutation analysis of variance), based on 999 permutations as implemented in *adonis* function, was used to test the effect of host identity in the structuring of bacterial communities. We calculated the Bray-Curtis distances between the following microbial communities: (i) polychaete species, (ii) sponge species, (iii) polychaetes and their host sponge (specific), and (iv) polychaetes and nonhost sponges (nonspecific). Shannon diversity (72) of the bacterial communities for each sponge and polychaete species was calculated in *vegan*. The polychaete microbiomes reported here likely reflect the polychaete gut content bacteria more than bacteria from other body regions. However, we were not able to separate the polychaete body regions due to the small body size (>0.5 cm).

Core microbiomes (i.e., ZOTUs present in all species replicates) according to Turon et al. (29) were used for comparing sponge microbiomes with those of their respective polychaete partners. The mean relative abundance of bacterial orders was calculated for each sponge species and its associated polychaete species, and the corresponding Venn diagrams of the shared core microbiomes were drawn using *eulerr* package in R (73). Pie charts were used to represent the relative abundant ZOTUs (>0.1%) in the core communities of each polychaete species and their relative abundance in the core microbiome of the respective sponge hosts, categorized as highly relatively abundant (>1%), relatively abundant (0.1 to 1%), rare (<0.1%), and absent.

Comparisons with seawater bacterial communities were made and are presented as supplemental material. An nMDS was used to visualize the Bray-Curtis dissimilarity matrix of each sponge species, its associated polychaetes, and seawater. The shared microbiomes were represented by using Venn diagrams. The mean relative abundances of shared bacteria between the three biotypes or between polychaetes and seawater were represented as bar plots. Only ZOTUs with a relative abundance of >0.05% in the polychaete microbiome were considered for these comparisons.

Data availability. The raw prokaryotic sequences analyzed during the current study are available in the SRA archive under the project number PRJNA453898. Polychaete sequences are available under the GenBank accession numbers MK532398 to MK532403 for the 16S rRNA gene and MK524577 to MK524582 for the COI mitochondrial gene.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mSystems.00148-19.

FIG S1, JPG file, 1.5 MB.
FIG S2, PDF file, 0.2 MB.
FIG S3, PDF file, 1.9 MB.
FIG S4, PDF file, 0.1 MB.
FIG S5, EPS file, 2 MB.
FIG S6, EPS file, 2.4 MB.
TABLE S1, PDF file, 0.03 MB.
TABLE S2, PDF file, 0.04 MB.

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