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Study of the community structure and functional features of the *Haliclona fulva* associated microbiome and possible relationships with its composite holobiont metabolome

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Study of the community structure and functional features of the *Haliclona fulva* associated microbiome and possible relationships with its composite holobiont metabolome

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A mi padre, quien siempre ha estado en mi corazón y me acompaña en cada paso de mi vida

A mi madre, quien con su amor, paciencia y fortaleza me ayudo en estos largos 4 años...eres el mejor ejemplo

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Abstract

Haliclona fulva is a marine sponge species from the Mediterranean coralligenous producing original secondary metabolites with biotechnological potential. I am reporting the first detailed description of its microbiome composition by 16S rRNA gene amplicon sequencing and metagenome shotgun sequencing, and the development and evaluation of sponge cultures in aquaria examined as a model holobiont system. I tested the possible effects on microbiome and metabolome content and stability of environmental variables related to human-induced global climate change, temperature and light. I had determined consistently and reproducibly that *H. fulva* has a unique, stable and highly enriched microbial community dominated by two symbionts in sponge specimens in the wild or cultured in aquaria: Nitrosomonadales (Uncultured Betaproteobacteria named HF1) and Thaumarchaeota (*Cenarchaeum symbiosum*) representing a remarkable ~70% of the total symbiotic bacterial community. Stressors tested on sponge cultures did not evidence drastic changes on microbiome composition of abundant groups, only minor shifts of rare groups at 1h or 24h after disturbances. Light and temperature did not affect idiosyncratic *H. fulva* metabolites renierins and fulvynes, while temperature (31° C) caused a significant decrease in peptides after 1 h of disturbance. Sequencing-based metagenomics showed sequences mainly associated with metabolism and information storage and processing, and a high percent of reads (39%) are classified as virus, pointing out a link of this component with the microbiome maintenance in *H. fulva*. In conclusion, this work provides a comprehensive baseline about *H. fulva* as a suitable marine holobiont model for studying basic and environmental aspects and for biotechnological applications.

Keywords: *Haliclona fulva*, symbionts, Nitrosomonadales, Cenarchaeales, climate change, metabolome, virus.

Resumen

Haliclona fulva es una esponja marina que hace parte del coralígeno Mediterráneo y se caracteriza por la producción de metabolitos secundarios con potencial biotecnológico. Este estudio reporta la primera descripción detallada de su composición microbiana por secuenciación de amplicones del gen 16Sr ARN y secuenciación del metagenoma, y la evaluación de cultivos de esponja en acuarios como un modelo de holobionte. También se determinó el posible efecto de variables asociadas al cambio climático como luz y temperatura sobre el microbioma y metaboloma. Los resultados demostraron consistentemente que *H. fulva* mantiene una comunidad microbiana estable y altamente enriquecida, tanto en su hábitat natural como en acuario. La comunidad estuvo dominada por dos simbiosomas Nitrosomonadales (Uncultured Betaproteobacteria llamado HF1) y Thaumarchaeota (*Cenarchaeum symbiosum*), los cuales representaron ~70% del total de la comunidad. Los estresores ambientales evaluados sobre cultivos de esponja no generaron cambios significativos sobre grupos microbianos abundantes, únicamente se observaron cambios en grupos minoritarios a 1h o 24h después del disturbio. Luz y temperatura no afectaron los metabolitos de *H. fulva* como renierinas y fulvinas, mientras que la temperatura generó una disminución en los péptidos a 1h del disturbio. Finalmente, el análisis del metagenoma demostró funciones principalmente asociadas a metabolismo y almacenamiento y procesamiento de la información. A nivel taxonómico, un alto porcentaje de secuencias (39%) fueron clasificadas como virus, sugiriendo que este componente tiene alguna función en el mantenimiento del microbioma en *H. fulva*. En conclusión, este estudio proporciona un conocimiento claro y concreto de línea base sobre *H. fulva*, como un modelo holobionte marino para estudiar aspectos básicos y ambientales y con aplicaciones biotecnológicas.

Palabras clave: *Haliclona fulva*, simbiosomas, Nitrosomonadales, Cenarchaeales, cambio climático, metaboloma, virus.

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Introduction

Biological exploration of marine ecosystems has been always a rich source of knowledge and key resources for mankind. In the last years, thanks to the great advances in concepts and technologies for biological and chemical characterization, it was possible to realize the vast dimension of the biological potential of this ecosystem, allowing us to find novel molecular structures that, while evolved for a function on its original environmental context and having obvious interests for basic science advances, have bioactivities that could in turn be used for practical applications, such as industrial and medical purposes. This potential is derived of an enormous and unique biodiversity of micro and macroorganisms that have been evolved in the marine world. Exist associations between organisms/habitats and microbial communities, suggesting there is a specific symbiotic interaction between them in order to thrive and colonize different environments found in the ocean.

That biological diversity (prokaryotic and eukaryotic) and derived chemical potential have gained attention in the last years from scientific community, as evidence in the marked increase in publications numbers. According to Scopus analysis (using keywords as marine sponges AND microorganism) three papers were published in 1997 and 46, in 2016. In relation to chemical diversity (using keywords as marine sponges AND chemical compounds), 15 were found for 1997, and 75 for 2016. In general, the leading countries in scientific production are USA, Germany, Japan, France, Germany, Australia, China and India.

Among this huge diversity, the microbial component has gained special attention due to their vast adaptation to extreme environmental conditions (e.g. halophilic, hyperthermal, oligotrophic habitats) and their wide physiological and biochemical versatility. It is known that some ocean niches could reach microbial densities up to 10^{12} cells/ml such as sediments and microbial mats [1] and in open planktonic conditions, the average is 10^6 . Despite of the enormous amount of microbial cells (10^{23}) contained on Earth and oceans, the knowledge about them is still relatively scarce. Up to now, the majority of research studies on marine micro and macro-organisms derived natural products are reporting the discovery of new chemical structures and bioactivities, but the understanding about its functional role in this ecosystem and in symbiotic or antagonist interactions is still on its early steps. The main model organisms to study these aspects are marine sponges, as they are considered as one of the most prolific chemical factories of bioactive compounds in the marine environment, they represent an important fraction of benthic fauna and are an example of most ancestral symbiosis in animal kingdom.

Marine sponges (phylum Porifera), represent an ancient and primitive lineage of metazoans, with earliest fossil records detected in Precambrian formations [2]. These multicellular organisms had been inhabiting earth for at least 590 million years, and are adapted to thrive in a diverse range of aquatic environments (from shallow coastal waters to very deep zones to freshwater) [3] and are found across different latitudes varying from tropical to arctic regions [4, 5]. Their ecological roles are very likely not completely discerned and understood out of the large amount of complex biotic and abiotic interactions and its biodiversity. Bell (2008) [6] and Wulff (2006, 2008) [7,8] recall the most recognized functions of sponges, such as, participants in nutrients cycling, primary

production, and as filtering systems of the water column. They can also serve as food source for others animals and as producers of secondary metabolites excreted into the environment.

Surprisingly, the fitness and adaptability to produce such repertoire of functions and thrive in different conditions could be explained to symbiosis with microorganisms. This kind of relationship is considered as a beneficial association between organisms sharing the same space, where the symbiont (including prokaryotes and eukaryotes) occupies a habitat and provides some advantage to the hosting organism (sponges). In this case, the evolved relationship of sponges and their symbionts can be interpreted as one functional symbiotic entity called "holobiont" [9, 10]. According to that, sponges can be studied as models of symbiogenesis, defined by Guerrero, Margulis and Berlanga (2013) as "*the result of the permanent coexistence of various bionts to form the holobiont (namely, the host and its microbiota). The holobiome is the sum total of the component genomes in a eukaryotic organism; it comprises the genome of an individual member of a given taxon (the host genome) and the microbiome (the genomes of the symbiotic microbiota)*".

The relative cell density of the microbial communities in sponges is variable depending of the sponge species. In some species hosts, they can represent up to 40% of sponges biomass, being classified as "bacteriosponges" or high microbial-abundance sponges (HMA) [11, 12], reaching densities up to 10^8 - 10^{10} bacteria per gram of sponge wet weight, concentrations higher four orders of magnitude than those reported for average seawater [1]. By contrast, when the density of microorganism is between 10^5 - 10^6 bacteria per gram of sponge wet weight, similar to average bacterial cell densities found in seawater, these sponges are called low-microbial-abundance-sponges (LMA) [11].

The microbial populations are mainly concentrated in the mesohyl matrix of the sponge bodies. It is a stable niche with constant supply of nutrients, appropriate for maintaining heterotrophic and autotrophic bacteria [13, 14]. A large fraction of these bacteria are highly adapted to live in this specific association, thus, they present the behavior of the major fraction of environmental microbial communities, this is, exhibiting very low culturability by standard microbiological methods [15, 16] and the recovery of the bacterial strains is useful to some extent, but not necessarily representative of the corresponding abundance in the holobiont. To overcome this issue, several studies have described the bacterial diversity present in sponges by culture independent means [5, 17–20], they have reported bacterial phyla such as *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Acidobacteria*, *Chloroflexi*, *Cyanobacteria*, *Gemmatimonadetes*, *Nitrospira*, *Verrucomicrobia*, *Chlamydia*, *Planctomyces* and *Poribacteria*. Interestingly, the last phyla had been named as it has been primarily found in sponges and it has been suggested as a new "candidate phylum", representing a rRNA gene sequence cluster that corresponds to atypical bacterial cell morphologies with membrane-bound nuclear bodies [5]. In contrast, the archaeal diversity is lower and few groups like Crenarchaeota, Thaumarchaeota and Euryarchaeota, rather ubiquitous in the sea, have been reported within microbial communities [21].

As an attempt to maintain a stable symbiosis, it is plausible the coexistence of the three mechanisms to ensure the persistence and composition of microbiome. The first one is vertical transmission, which involves sponge reproduction stages for transmitting microorganism from generation to generation [14]. This process is well documented and microorganisms have been identified in embryos, oocytes and larvae [22, 23]. The second one, is horizontal or environmental transmission, a mechanism that includes the faithful uptake microorganisms from seawater, and although many researchers propose that this process would not explain microbial communities

establishment in sponges, Maldonado and Riesgo (2009) showed that mature oocytes, spermatozoa or embryos apparently had not detectable symbionts, supporting the idea that horizontal transmission is a key factor for acquiring microbes by juvenile sponges. Finally, the third one is a combination of both strategies. Each one has its pros and cons, and so far, is still unknown how coexist those process and if they have any relationship with the hologenome evolution [24].

The distribution, shape and diversity of microbial communities for diverse sponges across geographic and environmental gradients have been widely documented; for example, Hentschel et al. (2002) [14] and Webster et al. (2004) [25] suggested that sponges have a “microbial signature” with very large and complex interactions with its host. Also, Schmitt et al. (2012) [17], reported that OTUs (Operational Taxonomic Unit) found in sponges, can be classified in a core, variable and specie-specific community; Erwin et al. (2011) [26] suggesting that generalists and host-specific symbionts are both co-inhabiting sponges, and probably these communities are maintained as a core microbiome by the host, independently of the geographic locations of the holobiont. And, Thiel et al. (2007) divided bacterial populations in specialists (present only in host), sponge-associate (present in sponges species but not in seawater) and generalists (present in sponges and seawater), but the proportion among them depends on the sponge.

While there is a significant knowledge about the diversity of microorganisms associated with sponges, little is known on the physiological interactions and functions performed by each counterpart. Is recognized that some factors as high temperature [27–29], pollutants [30, 31], pH [32], nutrients [33], etc., can produce dysbiosis process and as a consequence the sponge’s death.

Studies of the holobiome concept as defined above, also known as hologenome, has been facilitated greatly on last years thanks to advances in “omic” technologies, notably increasing in the last 7 years. We are still on the early descriptive steps to understand of dynamics of interactions sponge-microbiome and its genetic complexity. The first published genome of a marine sponge was the one of *Amphimedon queenslandica* in 2010 [34] followed by transcriptomes [35]; since then, only 6 sponges more had been studied in this regard: metagenome sequences of *Aplysina aerophoba* [36], transcriptomes of *Cliona varians* [37], metagenome and metaproteome of *Cymbastela concentrica* [38, 39], microbial metagenome of *Rhopaloides odorabile* [39, 40], *Stylissa carteri* [41] and transcriptomes of *Xestospongia muta* [42]. Overall, this relatively low publication number given the large amount of sponge species still to be deciphered in this omic aspects is due to the recent access to NGS, summed up to the difficulty of DNA/RNA extraction process by the presence of inhibitors or secondary metabolites, the scarce information available in database to analyze the results and the difficulties of identifying the structure and function of genes in species that are phylogenetically distant from traditional model organisms [43].

As mentioned previously, marine sponges are generally divided in HMA and LMA groups. So far, there are a significant number of studies focused on HMA sponges versus LMA because of their enormous microbial and chemical diversity. However, LMA sponges may enrich or harbour specialist microorganisms with particular metabolic pathways, which make these organisms of high biotechnological potential and ecological relevance [44]. In this study, we focused on the *Haliclona fulva* (Topsent, 1893), for several reasons explained below. This is a sponge species belonging to a genus of the LMA group, but in this specific case it remains to be elucidated. *H. fulva* dwells in the Mediterranean sea in semi-dark benthic communities, in the coralligenous or at the entrance of underwater caves, between 5 and 50 m depth. Up to now and to the best of our

knowledge, the microbiome, and metagenomic and metabolomic information about *Haliclona fulva* sponge is still scarce, despite it has a well identified remarkable chemical potential according to a recent study, where comparatively evaluating the chemical diversity in different species inside order Haplosclerida [45].

Haliclona spp. genus is known as producer of compounds with a broad structural diversity: alkaloids, isoquinones, terpenoids, steroids, polyketide, fatty acids and peptides [46, 47]. Some of them have shown a remarkable pharmaceutical potential, for example, haligramide A and B, isolated from *Haliclona nigra* exhibited a cytotoxic effect [48] and manzamine A obtained from *Haliclona* sp., exhibited antitumor activity [49]. In the case of *H. fulva*, there are few but insightful reports about the secondary metabolites it produces and its bioactivities. Chemical studies of this species have one report in 1977, when Cimino and De Stefano [50] reported polyacetylene compounds. Then, Ortega et al. (1996) [47] found a diacetylenic metabolite with cytotoxic activity against tumoral cells; in 1993, Casapullo *et al.* [46] identified paniceins (sesquiterpenes), which showed cytotoxic activity. In 2012, Nuzzo et al., [51] reported linear polyoxygenated acetylenes called fulvynes, which had antimicrobial activity against a chloramphenicol-resistant strain of *Bacillus subtilis*. Subsequently, Genta-Jouve and Thomas (2013) [52] characterized 3-epi-cladocroic acid, and in 2014, Ciavatta and coworkers [53], isolated long-chain fulvinol-like polyacetylenes, some of which were active against melanoma cells.

The present study is reporting the first characterization, at a greater level of resolution and by means of molecular and analytical tools, of the microbiome and chemical diversity associated to *H. fulva* determined in different sponge specimens in their natural habitats and under controlled conditions (aquaria). To achieve that, the following objectives were proposed: i) To characterize the microbial core community composition associated with *H. fulva* on its natural habitat; ii) To describe general trends in metabolomes and microbiomes of *H. fulva* holobiont in cultured specimens under environmental stressors, and iii) To explore by metagenomic means the inferences of metabolic functions and microbial communities found in *H. fulva* holobiont (Figure 1).

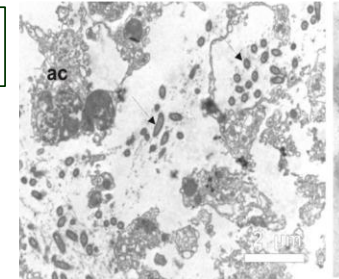
The specific objectives and results of this research are presented in chapters. In the first one, samples of the model holobiont were taken at different times and season in a location where are naturally thriving at the Mediterranean sea, and the microbial composition was inferred by means of massive NGS sequencing of 16s rRNA gene amplicons from metagenomic DNA. For the second objective, *H. fulva* was cultured in aquaria and it was submitted to stress conditions related to human-induced global climate change: light, temperature, and its combination. Sampling was performed before stress condition (time 0h), and after exposure (1h and 24h). The microbial communities were characterized by Illumina sequencing of 16s rRNA hypervariable region V4 amplicons, obtained from metagenomic DNA. Metabolites extraction and detection in the same samples were performed and identified by mass spectrometry in a UHPLC Thermo Scientific™ Dionex UltiMate 3000, linked to an ion trap mass spectrometer fitted with an electrospray ionization interface (Bruker Impact II). Mass spectra were recorded in negative and positive ion mode. Finally, in the third chapter, the main purpose was to have an integrated view about the hologenome content of the model holobiont by direct total metagenomic NGS sequencing.

The results obtained from this thesis as will be detailed in the following chapters are providing novel information and insights about the rich and original source of compounds from the metabolome, and the stable and specific microbiome composition of *H. fulva*, providing evidence

that it can be a suitable model for holobiont studies. Additionally, I am reporting the evaluation of the feasibility of culturing a LMA sponge as *H. fulva* in aquaria under controlled conditions, thus supporting its potential to be used as biological model in experiments with environmental, ecological and biotechnological applications. A wide characterization of microbial communities as well as derived functional and metabolomics analysis were generated from this model holobiont, constituting an original contribution to our current knowledge and understanding of sponge hologenomes, introducing a unique and valuable resource for such basic interests as well as the derived environmental and biotechnological applications it may offer.



Evaluate the effect of different environmental conditions on the microbial community composition and the chemical diversity in *H. fulva* - holobiont



Research Questions:

1. Does *H. fulva* have a microbial core community?, How is it constituted?, How does *H. fulva* microbiome influence the microbial community surrounding seawater?
2. What is the response of *H. fulva* and its microbiome to stress conditions? Does the response of *H. fulva* and its microbiome involve changes in chemical compounds production?
3. At genomic level, Which are the microorganisms composing the microbiome of *H. fulva* detected by metagenomic means?, What are the main functions of holobiont?

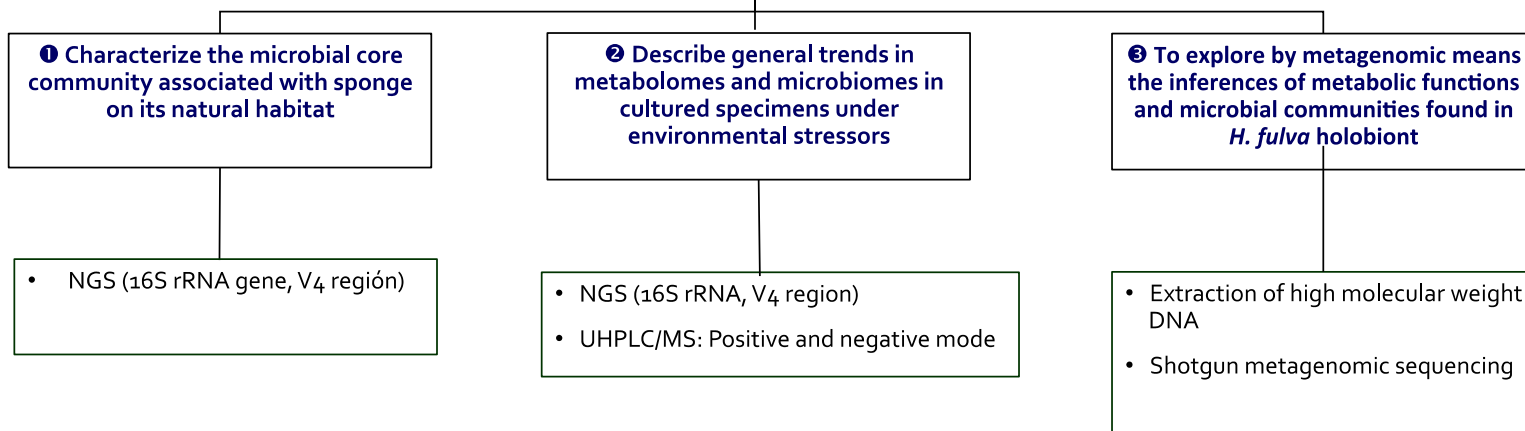


Figure 1. Research scheme representing research questions, objectives and used methodology.

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Chapter 1: Stable and enriched *Cenarchaeum symbiosum* and Uncultured Betaproteobacteria HF1 in the microbiome of the Mediterranean sponge *Haliclona fulva* (Demospongiae: Haplosclerida)

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1.1 ABSTRACT

Sponges harbor characteristic microbiomes derived from symbiotic relationships shaping their lifestyle and survival. We characterized the microbiome associated to the Mediterranean sponge *Haliclona fulva* using 16S amplicon Illumina sequencing to detect its specific microbial members and to define if there are evidences of excretion of those microorganisms influencing the seawater microbiome. We found that *H. fulva* core microbiome composition is dominated by sequences belonging to the orders Nitrosomonadales and Cenarchaeales. 70% of the reads assigned to these phylotypes grouped in a very small number of high frequency OTUs representing niche-specific species *Cenarchaeum symbiosum* and Uncultured Betaproteobacteria HF1. This bacterial composition is quite distinct to those reported in sponge species of the same *Haliclona* genus. The analysis of seawater microbial abundances exposed to sponge specimens in aquaria revealed that *H. fulva* core microbiome exerts a strong influence. This sponge is the first environmental or organismal matrix in which these Eubacterial and Archaeal groups have been

reported enriched at these very high relative abundances together. *H. fulva* can therefore be considered as a natural reservoir of such microbial types. Our data suggest that this symbiotic relationship is very stable, as it was observed in all the specimens analyzed, possibly contributing to the fitness and the metabolome, and therefore to the adaptation capabilities of the sponge.

KEYWORDS: Microbiome, Cenerchaeales, Nitrosomonadales, *Haliclona fulva*, LMA sponge, aquaria

1.2 INTRODUCTION

Porifera represent a substantial part of the benthic fauna in several marine ecosystems. Even though their ecological roles are likely to be of key importance in these ecosystems, they are not fully understood due to a large array of complex biotic interactions with micro- and macro-organisms. Bell [1] and Wulff [2, 3] reviewed some ecological functions of sponges and their associated microbes, pointing out a key position in benthic-pelagic couplings through the filtration and recycling of suspended organic materials [4]. In some cases, they can also serve as food for higher animals like sea-slugs, fishes or turtles [5]. Finally, they have been identified as a source of bioactive metabolites, most of them being suspected to be defensive traits [6]. The symbiotic relationship between sponges and microorganisms has been known for decades, and some evidences suggest that both parts are codependent to thrive and to perform its ecological role. The relative overall abundance and composition of the associated microbial communities are variable among species and depending on environmental factors. In some Demospongiae, they may represent up to 40 % of the sponges biomass, being so-called “bacteriosponges”, or high microbial-abundance sponges (HMA) [7, 8]. In contrast, when the microbial relative abundances are very low *versus* sponge cells, they are called low microbial abundance sponges (LMA) [7, 9]. Although widely reported, this classification should be taken with caution and cannot be considered as a “general rule”, once the differences at physiological, metabolic, microbial diversity and chemical level for the sponges under that classification, as well as the defined threshold and the possibility that there are sponges with microbial abundances representing a wide and continuous range not compatible with a binomial classification, are still requiring further investigation [10]. However, many studies have used it as an attempt to understand general aspects of a given sponge ecology and its symbiotic relationships with microorganisms.

Physiological, chemical and microbial differences between HMA and LMA sponges have been reported in different studies: i) Metabolic activity appears to be generally higher in HMA, exhibiting high nitrification rates, dissolved organic carbon and nitrogen in comparison with LMA [11]; ii) LMA generally use less oxygen and their energetic needs are mainly supplied by particulate organic matter; in contrast, some reports indicate that HMA can obtain nutrients and a great fraction of their energy sources from their symbionts [12]; iii) HMA harbors an enormous microbial diversity including prokaryotic and eukaryotic organisms with richness and abundances higher compared to LMA [13, 14]; iv) HMA can reach densities up to 10^8 - 10^{10} prokaryotic cells per gram of sponge wet weight, whilst, LMA have densities between 10^5 - 10^6 [15]; v) the microbial

population of LMA could be composed by a more restricted species-specific set of microorganisms, whereas HMA harbor more diverse communities composed of a larger fraction of generalist prokaryotes [16]; vi) In HMA, vertical transmission to offspring seems to be the main mechanism of symbionts acquisition, whereas LMA tend to contain a greater percentage of environmental microbes acquired by horizontal transmission through the sponge filtering activity [17].

Studies to characterize sponge microbial communities and to understand their dynamics were developed using different techniques and resolutions: electron microscopy [7], DGGE [11, 12], FISH [18], Real-time PCR [14] and next generation sequencing technologies as pyrosequencing and Illumina [19–21].

To date, members of 29 Eubacterial phyla have been reported in association with sponges [14], including Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria, Acidobacteria, Chloroflexi, Cyanobacteria, Gemmatimonadetes, Nitrospira, Verrucomicrobia, Chlamydia, Planctomyces and Poribacteria [18, 22–24]. In contrast, the archaeal diversity is lower and few groups like Crenarchaeota, Thaumarchaeota and Euryarchaeota, rather ubiquitous in the sea, have been reported within sponges microbial communities [21]. To understand the dynamics of those microbial communities, studies have also characterized microbial communities in seawater, and results have shown that those phyla are found in low abundances, where Cyanobacteria, Proteobacteria and Bacteroidetes are usually predominant. There are changes in microbial community composition related to higher depth (e.g. 150, 400 m) [25]; with phyla like Crenarchaeota, Euryarchaeota, Flavobacteria, Chloroflexi becoming predominant. At lower taxonomical levels, members of Class Oceanospirillales, Prochlorococcus, Rickettsiales, Rhodobacteriales, SAR11, ammonia oxidizing archaea (AOA) and marine group II-III families have been observed [25]. In the Mediterranean sea, Zaballos et al. (2006) [26] identified sequences belonging to *Pelagibacter ubique*, SAR11, *Pseudovibrio*, *Erythrobacter*, SAR86, *Alteromonas*, *Pedobacter*, *Polaribacter*, *Fibrobacter*, Acidobacteria and Actinobacteria. It has been suggested that due to the enormous microbial diversity observed in seawater, this medium can constitute a “rare biosphere” serving as seed to colonize sponges [27]. In general, regarding the microbial composition of seawater, studies sampling the Atlantic ocean, Pacific Ocean, Caribbean and Mediterranean Sea [28–31] in shallow or deep waters, changes were drastically observed with depth but the frequencies of abundant OTUs are relatively low, with no single predominance above 10% of the total community.

Most studies in sponge microbiology were conducted on HMA, because of their huge microbial and chemical diversity. However, LMA sponges may enrich or harbor specialist microorganisms with particular metabolic pathways, which make these organisms of high biotechnological potential and ecological relevance [32].

Members of the *Haliclona* genus analyzed so far have been described as LMA. In this genus, the characterization of the microbiome composition was recently reported for *H. tubifera* collected in Panama (2006) using 16S amplicon Illumina sequencing. In this species, the composition is dominated by Gammaproteobacteria, Cyanobacteria, Alphaproteobacteria and unclassified bacteria accounting all together for more than 60% of the microbiome, the remaining being

composed by low frequency OTUs mainly affiliated to uncultured bacterial phyla [33]. More recently, Moitinho-Silva and colleagues [10] developed a machine learning for predicting HMA and LMA status in sponges and they found through algorithms that *H. fulva* belongs to LMA group; however, no biological evidence support that classification.

At chemical level, species belonging to this genus are considered as promising sources of bioactive metabolites like terpenoids, polyacetylenes, alkaloids, peptides and polyketides [22, 34–36]. Some of these natural products showed pharmacological properties, such as the cytotoxic haligramide A and B isolated from *Haliclona nigra* [37] and the highly bioactive manzamine A from *Haliclona* sp. later isolated from a cultured microbe.

While there are numerous efforts to isolate, characterize and identify unique metabolites from this sponge with bioactivities of pharmacological or ecological interest, there are not detailed descriptions on the *H. fulva* microbial community component. The present study fills this gap, describing in detail the microbial composition of this Mediterranean LMA sponge, *H. fulva*. We report herein the precise identification of its main and stable core microbiome community members, and we show its influence on the planktonic microbial composition of the surrounding seawater setup in experimental aquaria.

1.3 MATERIALS AND METHODS

1.3.1. Sampling

Specimens of *Haliclona fulva* were sampled at different times in order to evaluate the variability of the microbial community composition within this species. Samples (n°1-2 in May 2011, 17 m depth; n°3 in February 2014, 17 m depth; n°4-9, in July 2013, 35 m depth) were collected by scuba-diving in the NW Mediterranean Sea (Grotte du Lido, Villefranche-sur-Mer France, Lat: 43°41'31.487" N; Lon: 7°19'12.186" E). After collection, all individual samples were placed in independent plastic bags. A fraction was preserved in ethanol 70% (v/v) and the rest stored at -20 °C until further analysis. Sponges samples collected were identified based on morphological characteristics by expert marine sponge taxonomist Dr. Thierry Perez.

Seawater samples exposed to healthy specimens of *H. fulva* (SW1.HF-SW6.HF) were maintained in aquaria for five days (time considered as acclimation period), and seawater samples from the same aquaria source at Villefranche-sur-Mer marine station laboratories without such exposure (SW7-SW12) were collected, filtered and further processed for microbiome composition determination (see supplementary material for further details).

1.3.2 Electron microscopy

Small sponge fragments were fixed in 2.5% glutaraldehyde in 2 M phosphate buffer and filtered sea water (1 vol: 4 vol: 5 vol), then post-fixed in 2% OsO₄ in seawater. Each fragment was embedded in AralditeTM. Semi-thin sections were stained with toluidine blue and observed under

a Leica DMBL light microscope (LM) in order to localize the best parts of the tissue to observe microbial symbionts. Then, ultra-thin sections were made using a RCMC ultramicrotome PTXL. The cuts were placed on a copper grid (3.05 mm in diameter, 300 meshes) and stained with 2% uranyl acetate for 15 min. Observations were carried out with a JEOL JEM-1400 transmission electron microscope (TEM).

1.3.3 DNA Extraction

Sponge tissue (~0.5 g per sample) were transferred to 1.5 mL Eppendorf tubes, mixed with sterile glass beads and re-suspended in 600 μ L of lysis buffer (100 mM Tris, 100 mM EDTA, 1.5 M NaCl, 1% CTAB, 2% SDS, pH 8.0) [22]. Samples were disrupted by bead beater for 2 min, then, proteinase K (10 mg/ μ L) was added and the mixture was incubated at 55 °C with occasional mixing for 1 h. This was followed by centrifugation at 13,000 rpm for 15 min, and the obtained extracts were loaded on agarose gels 1X TAE. Electrophoretic run was performed at 60 V for 80 min. DNA was purified using a QIAquick gel extraction kit (Qiagen), its quality and quantity was assessed using a NanoDrop 2000 spectrophotometer.

To extract DNA from seawater, water samples (1 L) were filtered using a 0.22 μ m polycarbonate membrane filter (Whatman – Austin, TX, USA). Filter was cut into small pieces and DNA was extracted using PowerSoil DNA isolation kit (MoBio Laboratories, CA).

1.3.4 Sequencing

16s rRNA gene amplicons comprising the hypervariable region V₄ were amplified using primers 515f and 806r, which have previously been used to successfully target, retrieve, quantify and classify simultaneously Eubacterial and Archaeal reads [38]. Libraries were constructed using paired-end technology and all samples were sequenced using the Illumina-MiSeq platform (Sequencing Facilities, Alkek Center for Metagenomics and Microbiome Research, Houston-USA).

1.3.5 Sequence analysis and taxonomic assignment

Taxonomic assignment was carried out using the pipeline, programs and tools available in QIIME package v. 1.9.1 [39]. Raw sequences were filtered to clean and maintain those over a quality score of 25. Chimeric sequences were identified, extracted and excluded out of the datasets by Usearch 6.1 [40]. Open-reference OTU picking was performed using UCLUST algorithm [41]. Sequences with more than 97% similarity were clustered into operational taxonomic units (OTUs). Representative centroid sequences of each OTU were used for alignment against SILVA database (version 128 released on September 28.2016) using the Ribosomal Data Project (RDP) classifier [42]. All figures, ecological indices, NMDS analysis and UniFrac analysis were generated using Phyloseq package version 1.10.0 in R [43]. Venn diagram was made on line in: <http://bioinfogp.cnb.csic.es/tools/venny/index.html>. Phylogenetic analysis was made from the most abundant centroid sequences using the software MEGA6 (Molecular Evolutionary Genetics

Analysis) [44]. The most abundant OTUs obtained from centroid sequences were deposited in GenBank under accession numbers KY270508-KY270557.

Statistical analysis was made to test whether the sample groupings (*H. fulva*, SW.HF and SW) were significantly different from each other. ADONIS and ANOSIM were performed on unweighted Unifrac matrix in QIIME v.1.9.1. [39]. This first one, through a permutation evaluates the differentiation between the means of two or more groups of data, to explain the percentage of variation by computing the effective size (R^2) and a p-value; whilst the second, evaluate whether the groups are significantly different by comparing the ranks of distances between the groups and within the groups. The number of permutations was set at 999.

1.4 RESULTS

TEM observations of *H. fulva* choanosome revealed a homogeneous distribution of microorganisms in the mesohyl (Figure 1). A total of 404,539 sequences were obtained with an average read length of 253 pb. A total of 1,622 OTUs were observed (97% sequence similarity), and they could be classified in well-defined taxonomic affiliations down to family level for Eubacteria or Archaea.

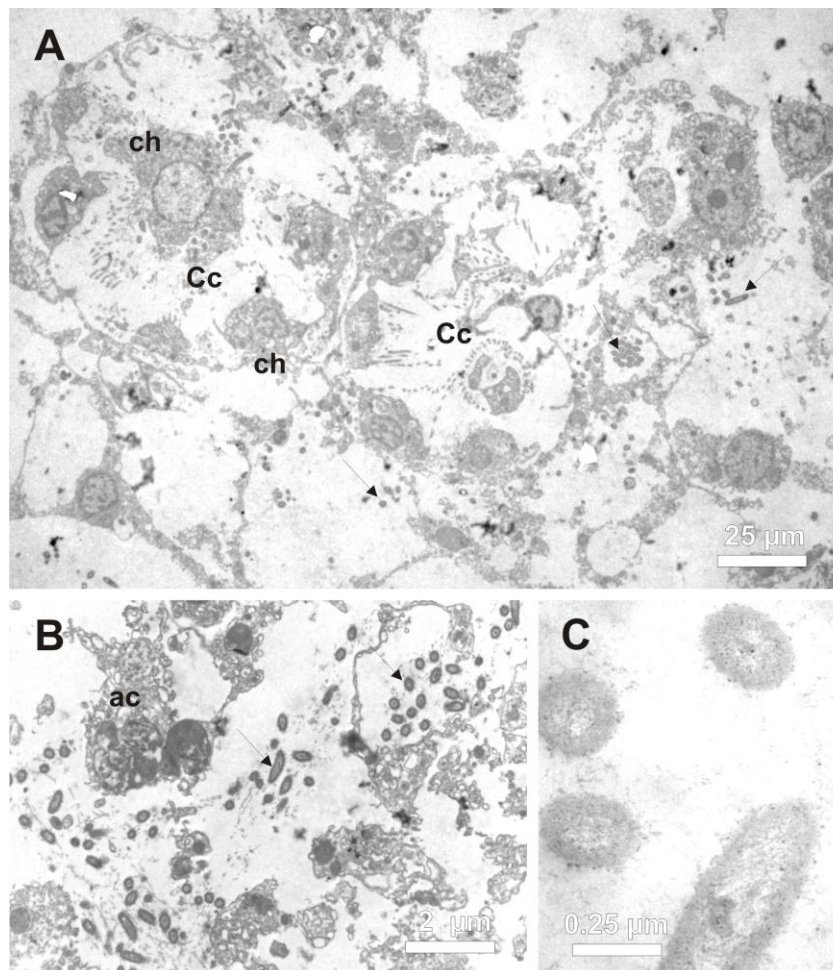


Figure 1: Transmission Electron Microscope micrographs of *Haliclona fulva* choanosome. Choanocytes chambers (Cc) are rather small composed of a little number of Choanocytes (ch). The sponge mesohyl is poorly developed and contains only few scattered cells, including Archeocytes (Ac). The prokaryotic community is composed by a low abundance of cells dominated by an elongated morphotype (Arrows). Micrographs A and C by T. Pérez and E. Garcia-Bonilla; B by courtesy of A. Ereskovsky.

Diversity plots indicated that diversity was covered near completion for all samples, with curves reaching asymptote with Good's C coverage values above 98 for all the samples (Figure 2A). Species abundance distribution plotted in logarithmic scale showed that communities exhibited a similar uneven distribution across the samples, with few OTUs dominating the community, whereas most OTUs presented lower abundances of 3 to 4 orders of magnitude (Figure 2B).

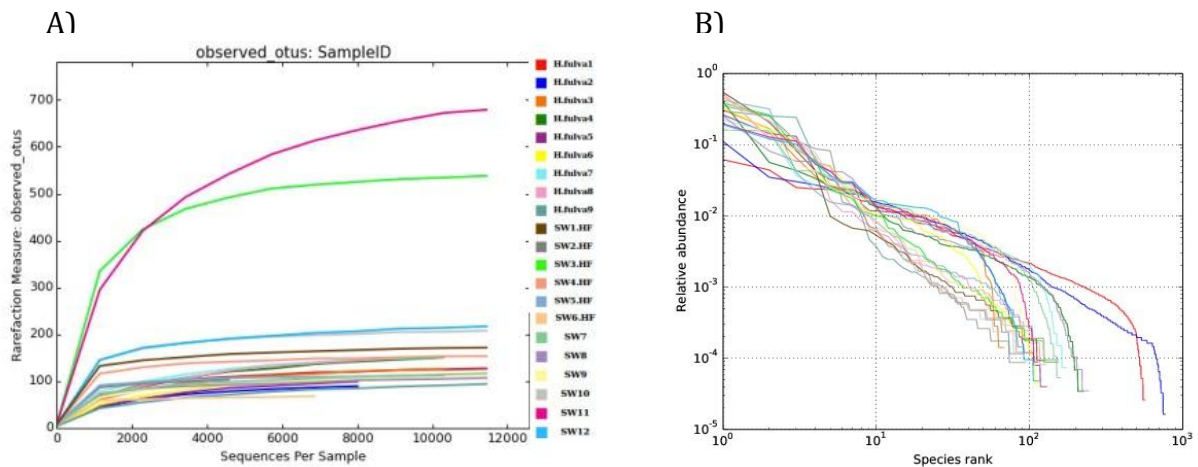


Figure 2. Diversity of microbial communities associated to *H. fulva* (1-9), in seawater surrounding the sponge (SW.HF1-6), and in seawater without sponge exposure (SW7-12). A) Rarefaction plots and B) Rank-abundance curves based on operational taxonomic units (OTUs) at a 97% similarity.

The OTU richness and alpha diversity indicators calculated between different individuals of *H. fulva* and seawater samples showed that diversity was much lower in sponges. Shannon index values ranged between 2.5 - 3.2 for sponges and 4.4 - 7.3 for seawater sets samples. Similar results were obtained for Simpson index, which showed higher values for seawater samples (>0.82) in comparison to indexes observed in sponge samples (0.65-0.79). The similarity of Eubacterial/Archaeal community composition was analyzed. The non-metric multidimensional scaling (NMDS) plot discriminated microbial communities (represented by stress value 0.06) according to their origin (sponge and seawater) (Figure 3). That separation among communities showed a significant distinction (ADONIS $P=0.001$, $R^2=0.27$ and ANOSIM $p = 0.001$, $R = 0.56$), as the UPGMA clustering shows (Figure 4). Overall, It is therefore possible to distinguish 3 clusters. The first one corresponds to the mixture of seawater with and without sponges' exposure with a high dissimilarity (~50%), in this group is possible to observe the samples with a different pattern of OTUs. Other two clusters with values below 30%, correspond the sponge microbiomes and to

the rest of seawater sets samples. The presence of common microbial groups among all sponge samples explained the clustering in one and unique group. In the core community, we identified 21 OTUs, which were present in all samples, mainly belonging to Proteobacteria (11 OTUs), followed by Thaumarchaeota (3 OTUs), Bacteroidetes (2 OTUs), unassigned (2 OTUs), Cyanobacteria (1 OTUs), SBR1093 (1 OTUs) and Actinobacteria (1 OTUs).

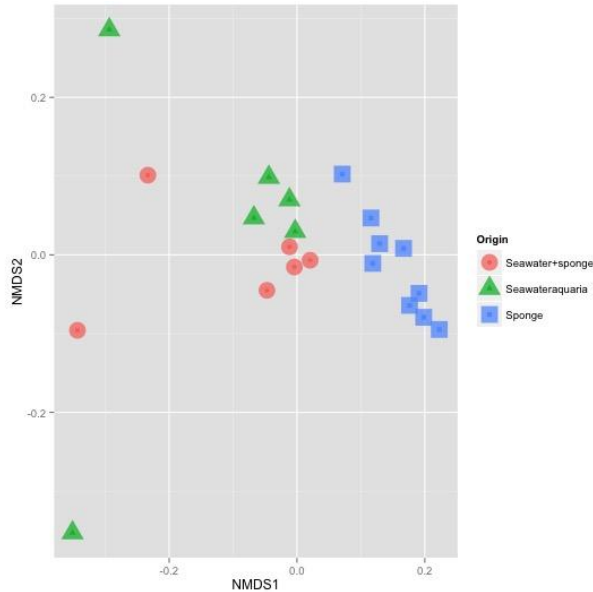


Figure 3. Non-metric multidimensional scaling plots showing the pattern of microbial communities from *H. fulva*, seawater surrounding the sponge and seawater without sponge exposure.

Haliclona fulva definitely presents traits of LMA sponges, as low microbial diversity was recorded by our molecular approach and a low microbial density observed in its tissues by electron microscopy (Figure 1) compared to what is usually observed in HMA sponges [10, 17]. At phyla level, Proteobacteria was the most abundant (223 OTUs: 64.45%), followed by other phyla like Bacteroidetes, Actinobacteria, Cyanobacteria, and SBR1093 (27: 7.80%, 20: 5.78%, 14: 4.04% and 3: 0.86% OTUs, respectively). In Archaeas, the dominant phylum was Thaumarchaeota, with 5 OTUs: 1.44%, followed by Euryarchaeota with 1 OTU: 0.28% (Figure 4).

At lower taxonomical levels, Proteobacteria was represented by many different groups and not dominated by one single group. The groups were Gamma, Alpha, Beta and Deltaproteobacteria, from high to low richness respectively. In the first group, the most abundant order was Enterobacteriales followed by Oceanospirillales and Cellvibrionales (29, 18 and 9 OTUs, respectively). The second group includes Rhodobacterales, Rhodospirillales, Rhizobiales with 23, 19 and 13 OTUs. The third group was composed mainly by Burkholderiales with up to 12 OTUs, and Nitrosomonadales (9 OTUs). The latter group had the largest number of reads and they were assigned to OTUs (New.ReferenceOTU28, New.ReferenceOTU26, New.ReferenceOTU11, New.CleanUp.ReferenceOTU663, New.ReferenceOTU14, New.CleanUp.ReferenceOTU1125,

JQ579751.1.1494, New.CleanUp.ReferenceOTU77, New.CleanUp.ReferenceOTU896). Finally, the fourth group was represented mainly by Bdellovibrionales (3 OTUs). In relation to Archaeas, Marine Group I was the single dominant class composed by 5 OTUs, and among them, the OTU AY192631.1.915, highly related to Cenarchaeum represented on average 31.46% of the total amplicon reads across the samples (Figure 4, Table 1).

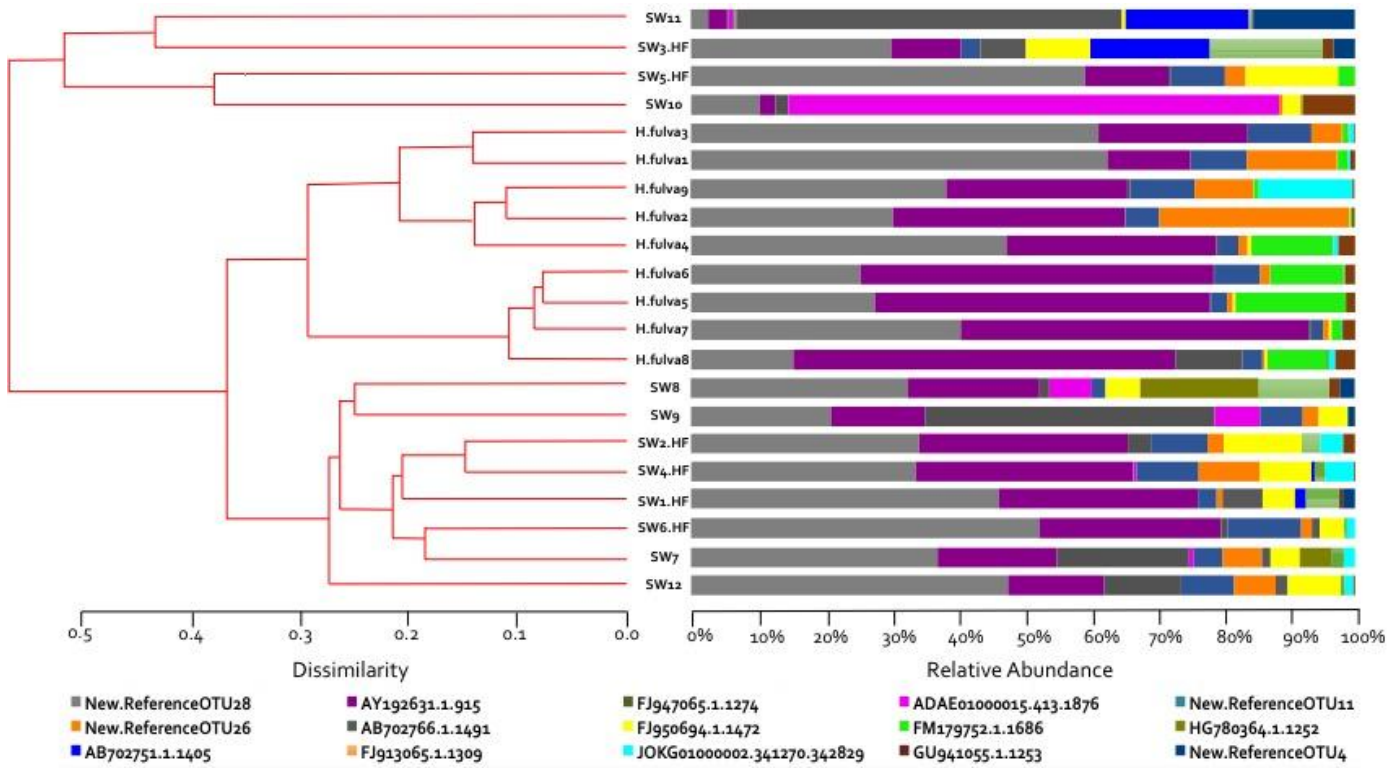


Figure 4. *H. fulva* microbiomes by UniFrac weighted UPGMA clustering and corresponding classification at Order level (Plot bar represents 15 most abundant OTUs), compared microbial communities found in seawaters.

Table1. Taxonomical classification of 15 OTUs most abundant identified in *H. fulva* microbiome

OTU ²	Phyla	Class	Order
New.ReferenceOTU28	Proteobacteria	Betaproteobacteria	Nitrosomonadales
AY192631.1.915	Thaumarchaeota	MarineGroup ³	Cenarchaeales
FJ947065.1.1274	Proteobacteria	Gammaproteobacteria	Enterobacteriales
ADAE01000015.413.1876	Proteobacteria	Alphaproteobacteria	Sphingomonadales
New.ReferenceOTU11	Proteobacteria	Betaproteobacteria	Nitrosomonadales
New.ReferenceOTU26	Proteobacteria	Betaproteobacteria	Nitrosomonadales
AB702766.1.1491	Bacteroidetes	Bacteroidia	Bacteroidales
FJ950694.1.1472	Proteobacteria	Gammaproteobacteria	Enterobacteriales
FM179752.1.1686	Proteobacteria	Gammaproteobacteria	Enterobacteriales
HG780364.1.1252	Proteobacteria	Alphaproteobacteria	Rhodobacterales
AB702751.1.1405	Bacteroidetes	Bacteroidia	Bacteroidales
FJ913065.1.1309	Proteobacteria	Gammaproteobacteria	Enterobacteriales
JOKG01000002.341270.342829	Proteobacteria	Gammaproteobacteria	Oceanospirillales
GU941055.1.1253	Cyanobacteria	Cyanobacteria	Subsection ⁴
New.ReferenceOTU4	Bacteroidetes	Bacteroidia	Bacteroidales

The OTU picking process was performed under the “open-reference” strategy, where reads not producing a hit against a reference sequence are then clustered *de novo*. In this process, representative sequences are selected as the centroid of each OTU and are denominated “New Reference OTU” [45]. In our case, 68 OTUs were assigned to this category, which suggests a lack of a closer sequences representative with a well-defined taxonomical rank in the classification system used in approximately one fifth of the *H. fulva* microbiome, what indicates its peculiarity as a microbial biodiversity niche.

In this study, most reads in all samples were assigned as belonging to Nitrosomonadales and Cenarchaeales orders. In the first case, the representative sequence for this OTU (New.ReferenceOTU28) was compared in different databases but it did not match against any reference organism, suggesting that it can be new specie inside that bacterial order. Here, it was reported as Uncultured Betaproteobacteria named HF1.

To explore in more detail the intradiversity of these predominant Archaea, we built a tree representing the distances of the centroid sequences of OTUs classified in this group and type and non-type sequences from RDP database (Ribosomal Data Project) (Supplementary Figure 2). Phylogenetic analysis showed that the AY192631.1.915, which represented on average 31.4% of reads might be classified as *Cenarchaeum symbiosium*. Konstantinidis *et al.* [46] in a work evaluating Eubacterial and Archaeal species definition, specifically discussed the particular case of *C. symbiosum* proposing that it can be considered an exception of fine-tuned niche-specific variants of the same species that are nevertheless having 16S rRNA gene sequences up to 5% dissimilar.

Considering the well-known dominant assemblages of planktonic microbial communities in Mediterranean seawaters, we wanted to assess the contribution of *H. fulva* microbiome to the biosphere members of seawater. We therefore characterized the microbial communities of seawater surrounding the sponge to determine if they are indeed modified or affected by such holobiont exudation of microbes hosted and/or enriched. We observed a strong influence of *H. fulva* microbiome on seawater microbiome (Figure 4). Surprisingly, seawater (without sponge exposition in aquaria) exhibited an atypical dominance pattern composed by a high proportion of Betaproteobacteria (specifically Nitrosomonadales order, New.ReferenceOTU28) with an average of 15.4% reads among all samples. Inside this order, other OTUs were identified with abundances was above 1%: New.ReferenceOTU11: 2.2% and New.ReferenceOTU26: 1.5%. Others orders were: Shingomonadales (OTUs: ADAE01000015.413.1876: 8.3%); Enterobacteriales (OTUs: FJ947065.1.1274: 9.2%, FJ950694.1.1472: 2.5%); Cenerchaeales (OTUs: AY192631.1.915: 7.4%); Bacteroidales (OTU: AB702766.1.1491: 2.2%) and Rhodobacterales (OTU: HG780364.1.1252: 2.3%).

Other phyla as Cyanobacteria, Firmicutes, Actinobacteria, Verrucomicrobia, Chloroflexi were also identified.

Particularly, the distinct pattern of SW11 with a clear predominance of Bacteroidetes types, is a standard indicator of human fecal contamination [47], and a common coastal marine contamination from urban domestic wastewaters.

Seawater exposed to sponge specimens in aquaria showed a similar trend with Nitrosomonadales (New.ReferenceOTU28: 16.7%) and Cenarchaeales (AY192631.1.915: 10.7%) as dominant members of its microbiome, followed by Acidobacteria (HQ598859.1.1454: 3.2%), Enterobacteriales (FJ950694.1.1472: 2.9%) and Rhizobiales (New.ReferenceOTU12: 2.1%).

To analyze the overlapping of OTUs among the three sample types analyzed (1. *H. fulva*, 2. SW, 3. SW+HF), a Venn diagram was generated (Figure 5). The majority of OTUs found were retrieved from seawater (a total of 1070 OTUs). Of these, 6.4% were shared between all samples types, including those classified as dominant orders described previously, which were abundant in all of them as well. SW+HF shared only 26.2% OTUs with SW, suggesting that sponge microbiome might be exerting an effect on microbial communities in surrounding seawater. In *H. fulva* samples, only 7% OTUs were unique and they were represented in a low proportion. Overall, the sponge only shared 10.8% OTUs (on average 176 OTUs) with SW and SW+HF samples.

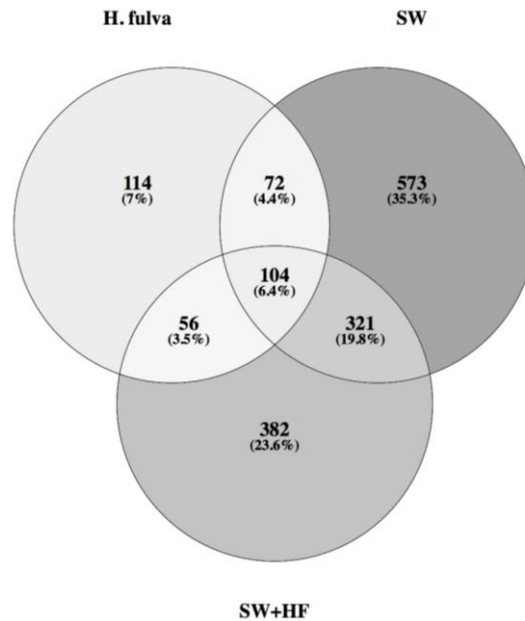


Figure 5. Venn diagram representing bacterial communities shared within the three different sample types. Numbers on each sample type (group) represent the number of OTUs and their percentages.

1.5 DISCUSSION

Our results confirm that *Haliclona fulva* belongs to low microbial abundance sponge (LMA), with key features as its low richness in microbes at phylum-level together with low bacterial densities in tissue.

The microbial community exhibited a low diversity and it was dominated by a few prokaryotic phyla (17 phyla), results consistent with previous reports [16, 48–50]. Comparing microbial communities among *Haliclona* species, the same description of low diversity at phylum-level is a

general feature. In *H. simulans*, Kennedy et al. (2008) found that the major phylum was γ -Proteobacteria, followed by α, β, δ , Planctomycetes, Verrucomicrobia, Actinobacteria and Bacteroidetes. Similar results were reported by Erwin et al. (2011) in *H. tubifera*: the predominant phylum was Proteobacteria distributed across two classes (α, γ), and others were Cyanobacteria, Actinobacteria, Verrucomicrobia and Bacteroidetes. Recently, the study of *H. cinerea* showed a high abundance of γ, β -Proteobacteria [51].

As expected from previous reports [13, 14, 16, 51, 52], the presence of dominant groups like Proteobacteria in sponge microbiomes was also evidenced for *H. fulva*. Some examples of LMA showing this trend are *Stylissa carteri*, whose community was dominated by Proteobacteria, Cyanobacteria and Nitrospira [20] just like *C. cyathophora*, *C. vaginalis*, *Niphates digitalis* and *R. topsenti* [13]. More recently, Moitinho-silva et al (2017) [10] purposed this phylum as "LMA indicators", once, its abundance is much higher than HMA. In our study, the most abundant OTU (ranging between 30-55% of total community) was assigned taxonomically to the phylum Proteobacteria and order Nitrosomonadales. Here it was reported as Uncultured Betaproteobacteria named HF1. The comparison of OTU abundance with other sponges microbial communities is difficult because little is known about its taxonomy. However, at order level, Nitrosomonadales has also been reported in sponges' microbiome as: *Clathria* sp. and E7 (sample non identified) representing a mean relative abundance of $15.9 \pm 28.7\%$ [53].

Other phyla from *H. fulva* commonly found in other sponges include Cyanobacteria, Bacteroidetes, Chloroflexi, Acidobacteria, Actinobacteria, among others. All of them belong to the common bacterial profile of HMA and LMA [18, 19, 34-36]. In this study, we could not elucidate if they are free-living or sponge-specific symbionts due to the lack of resolution on the short 16S rRNA amplicon sequence fragment. Regardless of their origin, they have a wide metabolic versatility that can be useful for sponges. For example, here, the major OTU within Cyanobacterias was classified as *Prochlorococcus* sp., and this free living organism is considered be responsible for a big part of the carbon fixation in marine environments [55]. Also, Actinobacteria are frequently related to important metabolic pathways responsible for the production of secondary metabolites [36, 56].

Remarkably no Poribacteria was identified among the most abundant members of the *H. fulva* microbial community. This phylum has been observed both in HMA and LMA, though their abundances were found to be higher in HMA [52]. A recent study described densities around 1.62×10^3 – 3.83×10^5 in LMA sponges such as *Dysidea avara*, *Stylissa carteri* and *Callyspongia vaginalis* from the Mediterranean Sea, the Red Sea and the Caribbean Sea, respectively [14]. Despite its widespread distribution in Demospongiae, this phylum may be absent as it is the case of *H. fulva* or it can be represented by a few number of reads, that together, with the sampling depth, could limit its detection. Recently, Moitinho-Silva and colleagues (2017) [10], found significant differences in the abundances of this phyla for HMA and LMA. In the first group, it was overrepresented, while in the second they were extremely low.

Giles et al. (2013) [13] who found that in 5 LMA sponges (*Crella cyathophora*, *Stylissa carteri*, *Callyspongia vaginalis*, *Niphates digitalis* and *Raspailia topsenti*), the microbial community was

dominated by clades belonging to Cyanobacteria or Proteobacteria, but curiously no Poribacteria was identified. Discrepancies between LMA sponges might indicate that the phylum Poribacteria is not a dominant member in their bacterial communities and, when present, it is usually at low abundance. This phylum strongly influences the microbiomes of HMA sponges [14] and shape their distinct microbial signatures, with an additional body of evidence as can be observed in the recent report by Thomas et al. (2016) [52].

Microbial diversity analysis of *H. fulva* showed additional interesting results for the Archaea domain. A single OTU, classified taxonomically within the phylum Thaumarchaeota, order Cenarchaeales, was predominant. In this study, phylogenetic analysis showed that it grouped with *C. symbiosum* [46, 57].

Recently, Kerou and Schleper (2017), reported that *C. Symbiosum* of the genus Candidatus Cenarchaeum has a distribution confined to marine sponges and they have a chemolithoautotrophic metabolism (oxidizing ammonia to nitrite) [58]. The presence of Archaea in LMA sponges was expected, since several studies already reported them as part of sponge microbial communities. Indeed, for LMA sponges, the archaeal 16S gene copy numbers ranged from 4.33×10^4 to 3.25×10^8 [14]. The main archaeal groups usually found in sponges are Euryarchaeota and Thaumarchaeota [48], but interestingly, they are dominant in only few species. The first sponge microbiome dominated by Archaea was *Axinella mexicana* [59], followed by *Tentorium semisuberites* [60] and *Inflatella pellicula* [61]. Our results showed that *H. fulva* could certainly be added to the list of sponge species harboring Archaeal phylotypes among its dominant microbial community. Even when the abundance of Cenarchaeales varied between samples, they reached up to 40% of the community.

As previously mentioned, the microbial community of *H. fulva* is shaped by two OTUs assigned to Uncultured Betaproteobacteria HF1 and *C. symbiosum*, which were also found in seawater samples set in high proportions. This finding suggests that seawater provides symbionts to sponge, but at the same time they are enriched and excreted by the animal, it provides habitat with a constant supply of nutrients and environmental conditions that favors their growth.

The relative abundance for Cenarchaeales (OTU AY192631.1.915) was higher in the sponge compared to SW.HF and SW (32%; 10.7%, 7.5% respectively). Similar trend was observed for Nitrosomonadales represented mainly by 3 OTUs (New.ReferenceOTU28, New.ReferenceOTU26, New.ReferenceOTU11) and in all three cases, the abundances were higher in sponges followed by SW.HF and SW; those order suggest that during sponge's pump activity one part of enriched-symbionts are released to surrounding water. This trend was also reported by Moitinho-Silva and colleagues (2014) [49] who proposed the term "sponge-enriched" to include some clades that are present in seawater in low proportions and probably they are not active metabolically, and their abundances are higher in the sponges.

Seawater exhibited a greater diversity and dominance of orders as Nitrosomonadales, Sphingomonadales, Enterobacteriales, Cenarchaeales and Bacteroidales. The presence of Archaea is in agreement with previous studies, which showed that Thaumarchaeota was an abundant phylotype in the intermediate and deep water mass and sediments of the

Mediterranean sea [25, 62] and Irish territorial seawater [48]. However, high abundances of Cenerchaeales and Nitrosomonadales in seawater at a depth 35 m very close to *H. fulva* colonies (as it was observed by geographic location in this study) suggests that the sponge can favor their enrichment and increase their densities in this specific natural habitat, where are found the wild *H. fulva* colonies analyzed in this research.

The symbiosis established between *H. fulva*-Nitrosomonadales-Cenarchaeales was identified in all samples. In this study, this association only exhibited a dissimilarity percentage of 25%, a relatively low shift considering that the sponge samples were taken in their natural environment at different times and seasons. Additionally, the low variability and abundance observed in others symbionts in *H. fulva* microbial communities (~30% of total community) might be indicative of the strength of the interaction of the predominant groups. This would support the idea that this sponge species is able to maintain a permanent and structured association with these microbial orders. A follow up observation of *H. fulva* specimens sampled at longer timescales is currently underway in order to define the extent and stability of this association.

The identification of dominant OTUs in seawater samples classified as Sphingomonadales (ADAE01000015.413.1876) is another feature to be highlighted. Interestingly, they were absent in sponge microbial communities despite their high abundances, which suggest that i) sponge generates a special and selective habitat where only few symbionts can establish, and/or ii) interaction between Sponge-Eubacteria-Archaea produce a competitive or antagonist effect excluding other microorganisms. This behavior might explain the low number of sequences found for OTU270391 in seawater surrounding the sponge vs. seawater. This defense mechanisms have been described in sponges mainly mediated by secondary metabolites with production modulated by abiotic (salinity, light, temperature) and biotic (space competition, predation, symbionts) factors [63].

On the other hand, a fraction of the sequences obtained for bacterial domains on *H. fulva* microbiome corresponds to non-identified phylotypes. In those cases, the maximum taxonomical classification precision achieved was only at phylum-level, in some cases down to order. These results are not surprising for sponge microbial communities; i.e. Lee *et al.* (2011) and Jackson *et al.* (2013) found that some sequences could not be classified at phyla level [61, 64], and White *et al.* (2012), detected around 187 OTUs which were not assigned to a bacterial phyla. Our knowledge about microbial diversity is still very limited especially regarding associations to "invertebrate" metazoans. Many bacterial phyla have yet to be uncovered and additional studies are required to fulfill a marine microbiology inventory to better understand the functional role of these associations with the sponge host. This may help to further valorize their potentials at biological and chemical levels.

Comparing to other species within the same genus (*H. simulans*, *H. tubifera* and *H. cinerea*) [16, 22, 51], this first report of the microbial community of *H. fulva* indicates a distinct and atypical Archaeal/Eubacterial composition, and this sponge also has a very distinct metabolic profile as reported by Tribalat *et al.* (2016) [65] within the order Haplosclerida. They found that inside Chalinidae sponges, *H. fulva* holobiont produces an original chemical diversity dominated by

polyacetylenes in large amounts, and long chain acetylenic products like renierins and fulvynes were identified all the samples analyzed. It is therefore likely that there is a possible contribution on metabolite biosynthesis or modification can be performed by predominant members of its associated microbiome, as a large number of polyacetylenes are polyketides or peptidic in origin, and previous studies proposed the microbial PK biogenesis of the highly oxygenated marine polyacetylenes given that polycistronic operons for PK biosynthesis are frequently found on terrestrial and marine bacterial strains [66, 67].

While for three other sponge genera with predominance of associated Archaea (*Axinella*, *Tentorium* and *Inflatella*) it has never been reported the production of acetylenic metabolites, there is high genomic and metabolomic versatility, and intraspecies variability among Archaea [68] that could explain the absence of these compounds despite this kingdom predominance. This microbial contribution or mediation on such metabolites biosynthesis remains to be elucidated.

In conclusion, this study provides evidence on the specific microbial signature that exists in the *H. fulva* sponge. This signature seems to be different even among LMA sponges. The presence of Uncultured Betaproteobacteria HF1 and *C. symbiosum* as dominant symbionts suggests that those relationships are highly specific and that the sponge might be an enrichment source for them. The stability of that association indicates that they could be of importance for sponge health, fitness and thus, for its derived metabolic activity.

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Supplementary Material

Aquarium Establishment: To evaluate effect of microbial communities of *H. fulva* and to detect if there is evidence of sponge symbionts release or if they are taken from seawater, six sponge specimens ($\sim 4 \text{ cm}^2$) were collected at 35 m depth in the NW Mediterranean Sea (Grotte du Lido, Villefranche-sur-Mer France, Lat: $43^{\circ}41'31.487'' \text{ N}$; Lon: $7^{\circ}19'12.186 \text{ E}$) and transported in plastic bags filled with seawater to aquaria at Villefranche-sur-Mer, France . They were placed into independent aquaria (4 L volume), supplied with flowing seawater pumped from 35 m depth in the facilities at Villefranche-sur-mer oceanological observatory (<http://www.obs-vlfr.fr/web/index.php>) ($43^{\circ}41'47'' \text{ N } 7^{\circ}18'24'' \text{ E}$) at a flow rate of 250 mL min^{-1} . Sponge individuals had a period of acclimatization of five days, time at which the health condition of sponges was monitored by phenotype observations such as color and shape preservation, as well as activity of the oscula.

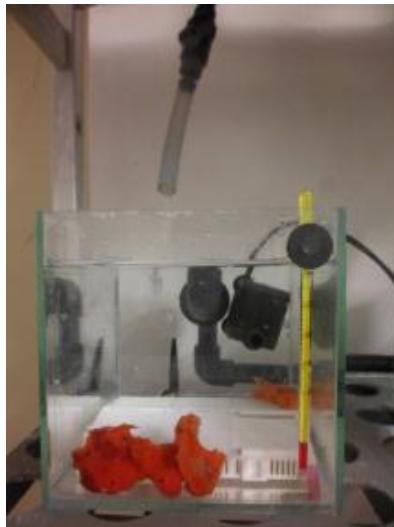
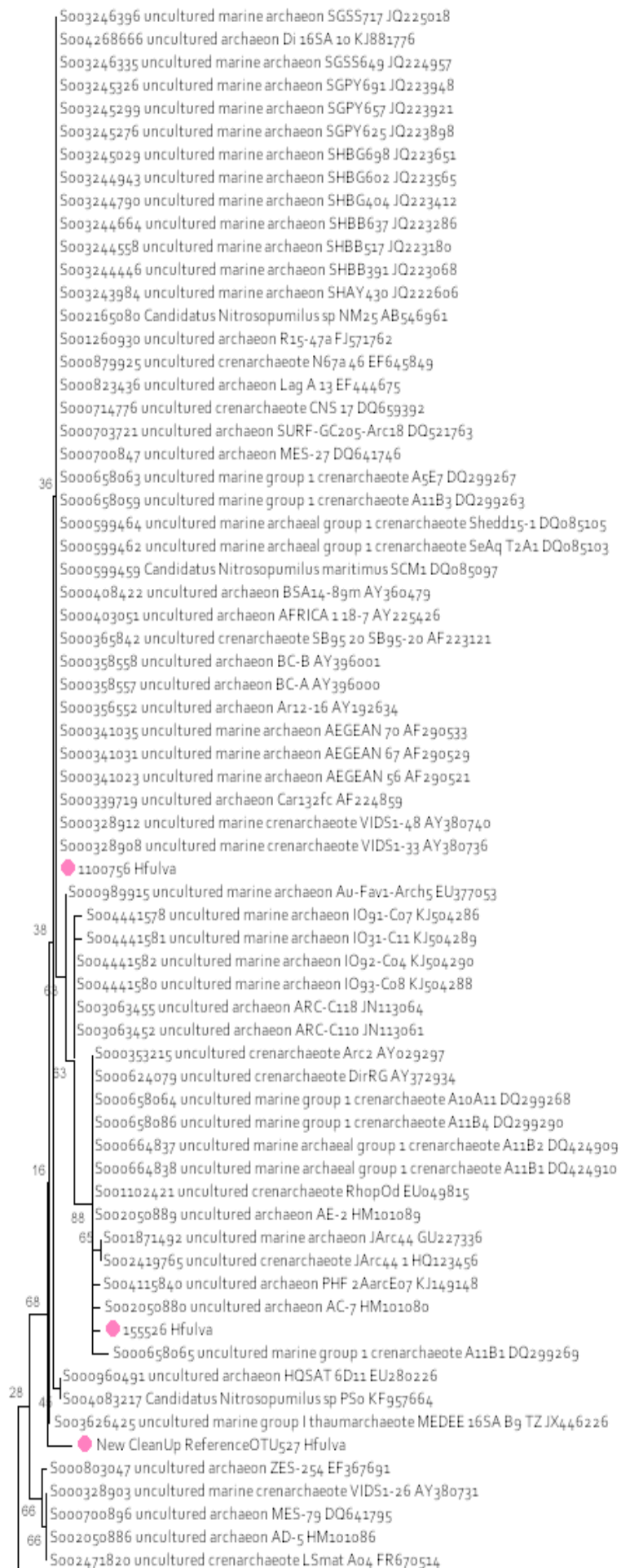
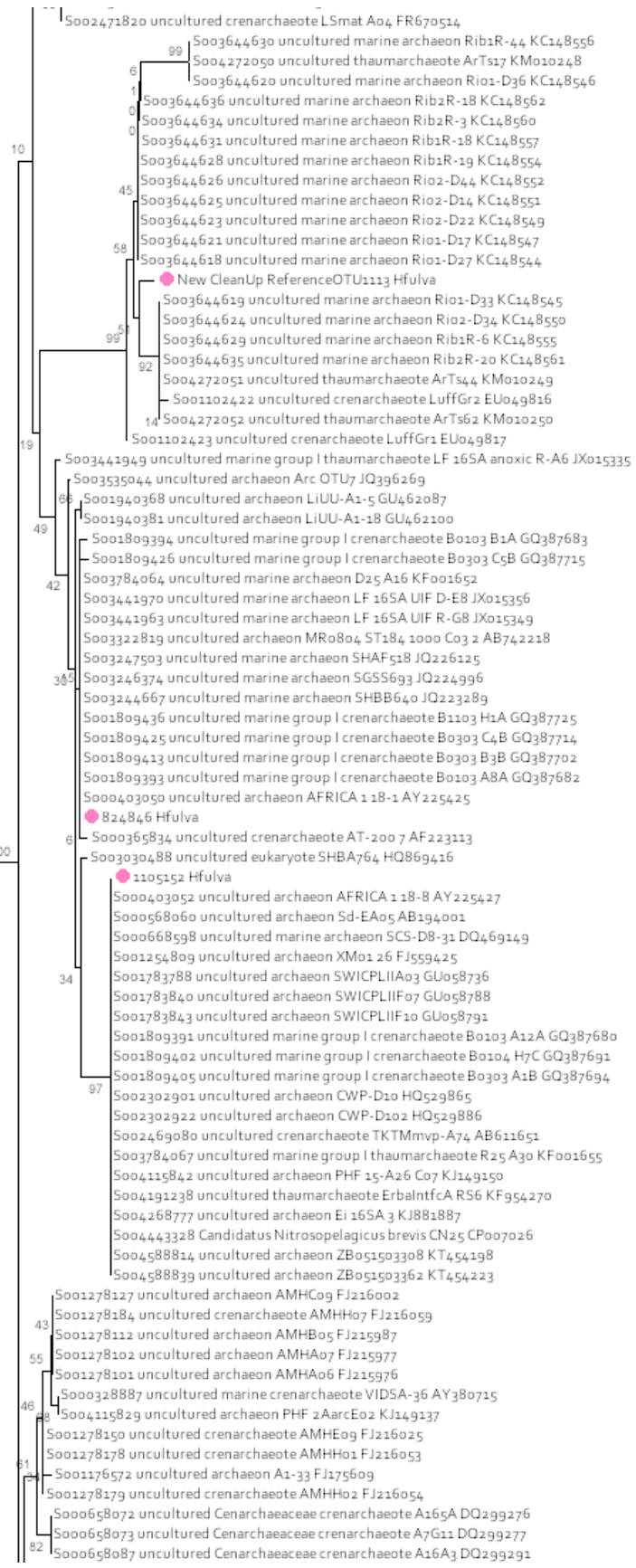


Figure SM1. Photographs of aquariums for the indoor cultivation of *Haliclona fulva* (VilleFranch-sur-Mer France Laboratories).

Figure SM2. Neighbor-joining distance tree for 16S rRNA sequences obtained from centroid sequences of Archaea and type and non-type sequences from RDP database (Ribosomal Data Project) [52]. The bootstrap consensus tree inferred from 1000 replicates. Pink circles represent centroid sequences from sponge and the red square the most abundant sequence.







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Chapter 2: Effects of key climate-change drivers in an experimental controlled assessment over the microbiome and the metabolome of a marine cultured holobiont model, the Mediterranean sponge *Haliclona fulva*

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2.1 ABSTRACT

One of the greater problems associated with climate change is its impact on biological diversity. Up to now, little is known how macro y microorganism will respond in ecological terms to new environmental conditions and how changes in their functional role would affect functioning ecosystems. In face to this scenario, there is an urgent need to better our understanding on the effects of identified climate-change drivers in selected biological models under controlled conditions. In this study, we evaluated the influence of environmental stressors (light, temperature and its combination) on metabolome and microbial communities associated with

Haliclona fulva under controlled culturing conditions in aquaria. *H. fulva* (LMA sponge) was able to thrive in aquaria, microbial communities were dominated by Proteobacteria (Nitrosomonadales) and Thaumarchaeota (Cenarchaeales) and major metabolites were renierins and fulvynes. There was not a significant effect of stressors on microbial communities at 1h or 24h after disturbance in abundant microbial groups. In contrast, they seem affect groups represented by low abundances. While light and temperature did not affect renierins and fulvynes production, temperature (31° C) caused a significant decrease in peptides just after 1 h of disturbance. To our best knowledge, this is the first report of peptides from the sponge *H. fulva*. So far, the ecological response of *H. fulva* associated microbial communities faced to adverse environmental conditions was unknown; results suggest that its communities can withstand short-term exposure to temperatures of 31°C and intense light conditions, but at that temperature changes in secondary metabolites production might alter holobiont fitness in its environment.

KEYWORDS: Climate change, temperature, peptides, *Haliclona fulva*, microbial community, metabolome

2.2 INTRODUCTION

Earth's anthropocene resulting from population growth and human activities [1] have consequences at environmental level such as shifts in biodiversity, atmospheric changes and derived climatic conditions [2]. This may also have severe impacts in human populations, and there are international agreements recognizing them and setting up priorities to achieve sustainable developments goals [3] closely linked with characteristic biological process of this epoch (i.e. defaunation) [4].

In face of this large-scale global alteration, there is the urgent need to better understand the effects of identified climate-change drivers in selected biological models under controlled experimental conditions. In this respect, aquatic holobionts, such as marine sponges, are representing a large portion of the benthic fauna in biomes providing key ecosystem services and resources. These organisms have developed a high capability for thriving in a diverse range of aquatic environments, from shallow coastal waters to very deep zones to freshwater [5] and are found across different latitudes from tropical to polar regions [6, 7].

All these adaptive advantages are accompanied and sustained by microbial symbionts associated to sponges, helping them not only to colonize all types of environments but also to participate in important ecological functions like nutrients cycling, filtering systems of the water column, and primary production. Also, they are considered one of the most prolific sources of secondary metabolites [8–10]. This symbiotic relationship of sponges occurs mainly with a microbial community of eubacteria, archaea and fungi [11], and is considered to be highly stable. Given this property, sponges can be studied and understood as an holobiont, the host and its associated microbiome [12]. To date, members of 29 Eubacterial phyla have been reported as being part of the associations with different sponges species [13], some of them being generally dominated by *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Acidobacteria*, *Chloroflexi*, *Cyanobacteria*, *Gemmatimonadetes*, *Nitrospira*, *Verrucomicrobia*, *Chlamydia*, *Planctomyces* and *Poribacteria* [7, 14–16]. The symbiont diversity is usually considered lower for archaeas and only the phyla *Thaumarchaeota* and *Euryarchaeota* have been identified in this group [17].

The distribution, shape and complexity of microbial communities have been well documented for several sponges across geographic and environmental gradients. For example, Hentschel et al. (2002) [18] and Webster et al. (2004) [19] suggested that sponges may have a “microbial

signature" with very large and complex interactions with its host. Also, Schmitt et al. (2012) [15], reported that OTUs (Operational Taxonomic Unit) found in sponges, can be classified in a core, variable and species-specific community. Erwin et al. (2011) [20] suggested that generalists and host-specific symbionts are both co-inhabiting sponges, and probably these communities are maintained as a core microbiome by the host, independently of the geographic locations of the holobiont. Finally, Thiel et al. (2007) divided bacterial populations in specialists (present only in the host), sponge-associate (present in sponge species but not in seawater) and generalists (present in sponges and seawater), but the proportion among them depends on the sponge species.

The functional roles of those symbionts have not been fully elucidated yet. In the case of secondary metabolites, some key activities linked to the syntheses or catabolism have been proposed, but that understanding is still limited to relatively few metabolites or metabolic steps, or at an ecological scale, how this associated functions encoded in the microbiome are reacting to climate-drivers or modifying the fitness of the hosting organism. For example, Piel and colleagues found that Poribacteria phylum might be the real producer of the methyl- branched mid-chain fatty acids in sponges [21]; Raederstorff et al. [22] reported that probably methyl-branched short-chain fatty acid found in demosponges as *Aplysina fistularis* are produced by symbionts; and, Sacristan-Soriano et al. [23] reported that in *A. aerophoba*, brominated compounds are produced by the sponge, however, the halogenation process only is performed by bacteria, which suggest that the symbiont can be involved during its biosynthesis.

The scarce knowledge is, to some extent due to the difficulties associated to the issue of the very low culturability of environmental bacteria [24] and the culturability of sponges, which is affected mainly by animal biomass loss [25, 26] and symbiosis stability. Some examples of successful sponge culturing under controlled conditions have been reported in species as *Rhopaloeides odorabile* [27], *Crambe crambe* [28], *Aplysina aerophoba* [26], *Xestospongia muta* [29].

Maintaining sponges in aquaria supplied with open flow seawater provides an ideal environment for assessing the effects of environmental parameters on the composition and the functionality of the microbial symbiosis, and to know the influence of shifting members on fitness, resilience or adaptive capacity of holobiont. Studies have showed that when symbiotic relationship is altered, negative effects are produced for the host sometimes leading to death. Variables as pollutants [30, 31], pH [32], high nutrient input [33], high temperature [27, 34, 35] can produce dysbiosis processes.

Currently the impact of variables associated with global warming is widely studied, once models predicted that in the year 2100, the global air temperature will increase 1.3-4.5° C (IPCC, 2001) [36]. This increase also alter others factors, such as global sea level, dissolved oxygen and CO₂ and salinity, among others, which are affecting negatively the health of marine organisms. It is recognized that coral reef are one of the most disturbed systems because the elevated temperatures cause the loss of symbiotic algae (zooxanthellae) [37], however, the magnitude and consequences in other sessile organisms remain to be elucidated.

So far, a few sponges have been used as biological models for studying stress conditions and little is known about how is the holobiont response as a whole, including how is altered the hosted microbiome and how does it affect the metabolomic profile and thus, chemical compounds biosynthesis. In this study, *Haliclona fulva*, a sponge dwells in semi-dark benthic communities, in the Mediterranean coralligenous or at the entrance of underwater caves, between 5 and 50 m depth, was selected as a model because it is a low microbial abundance sponge (LMA), and it has a particular and distinctive microbial community dominated by Nitrosomonadales and Cenarchaeales microbial orders (Garcia-Bonilla et al. 2017, *Submitted*). Furthermore, this sponge species is able to produce a wide range of secondary metabolites, many of them of original

chemical structures not found anywhere else in nature and having bioactivities of potential ecological or biotechnological applications. The first chemical studies on *H. fulva* were reported in 1977, when Cimino and de Stefano described acetylenic derivatives named renierins [38]. Then, Ortega et al. (1996) described another derivative with cytotoxic activity against tumor cells [39]. In 2012, Nuzzo *et al.*, reported several linear polyoxygenated acetylenes called fulvynes, with antimicrobial activity against a chloramphenicol-resistant strain of *Bacillus subtilis* [40]. Subsequently, Genta-Jouve and Thomas characterized the 3-epi-cladocroic acid [41], and in 2014, Ciavatta and coworkers, isolated long-chain fulvinols, some of which were active against melanoma cells [42]. In 1993, Casapullo and colleagues identified paniceins (meroterpenoids) but misidentification of the sponge species in this genus could explain this rather peculiar finding as *Haliclona mucosa* is another Mediterranean species known to produce this other family of natural products [43].

Therefore, the goal of our study was to track the stability and changes, at a finer detail of resolution by means of 16S rRNA gene amplicon sequencing, of the eubacterial and archaeal communities composition associated to the Mediterranean sponge *Haliclona fulva* that is being exposed, under controlled culturing conditions in aquaria, to environmental stressors (light, temperature and its combination), together with a determination of compositional average patterns and shifts on the metabolome, plus the pinpointed effects of such climatic drivers on selected metabolites, in order, to resolve if the fluctuation on production and concentration are shaped by those conditions. This model holobiont was selected and is tested against environmental variables related to human-induced global climate change as it is known that sponges are considered to be animals sensitive to temperature changes (see studies cited above), and considering that according to IPCC (2007) (https://www.ipcc.ch/publications_and_data/ar4/wg1/en/faq-5-1.html), the sea level is not rising uniformly in the world, in some parts rates are up significantly, while in others, it is falling, sponge communities are currently experiencing significant changes in the light intensity at their natural environments, a condition to change even more in the near future.

2.3 MATERIALS AND METHODS

2.3.1 Sampling and Experimental design

Samples of the marine sponge *Haliclona fulva* were collected by SCUBA diving in the Mediterranean Sea at the Grotte du Lido, in the bay of Villefranche-sur-Mer France (Lat : 43° 41' 31.49" N ; Lon : 7° 19' 12.19" E) at 35m depth. For each experiment, one mother sponge was cut in small pieces (in total 18 samples, each one ~5 cm³) and transported in plastic bag with seawater to aquaria at Villefranche-sur-Mer, France. To evaluate each variable, one sample was collected as an attempt to reduce the variability among replicate clones from different donor.

The experimental design included six aquaria per experiment; three were used as control and three as treatment, each one with three samples of sponges (replicates) (Figure 1) (Supplementary figure SM1). Seawater pumped at 5 m depth in the Bay [44] was supplied to the aquaria (4 L volume), at a flow rate of 250 mL/min. All aquaria were illuminated using fluorescent lamps and seawater temperature ranged between 23-24 °C.

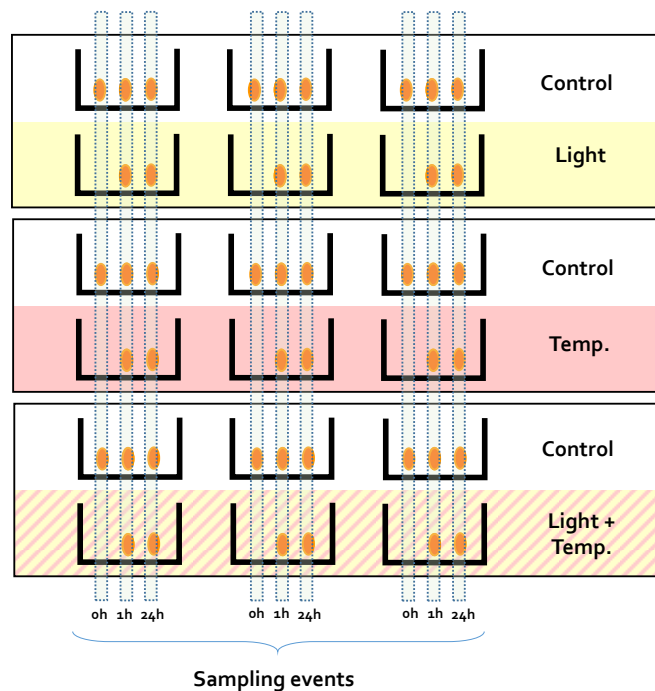


Figure 1. Diagram of experimental design

In the aquaria, the samples underwent a period of acclimatization of five days, time at which, the health condition of sponges were monitored through changes in color, shape and activity of the oscula [26].

Because our goal was to assess the effect of different parameters on the holobiont avoiding its death, the treatments were exerted for a period of 1 h. First, for a treatment evaluating the effect of light, aquaria were illuminated using a lamp, which was placed directly on glass surface of aquaria. The second treatment was a thermal stress experiment, where aquaria water temperature was increased and maintained at 31 °C, and for the third treatment both light and temperature changes were applied simultaneously. Sponge samples were randomly selected from each aquarium, treatment and controls, before stress condition at time 0h, and after stress condition (1h and 24h) for microbiome and metabolome analyses (see below). Seawater samples (1L) surrounding the sponges were also collected in all aquaria and sampling events to analyze related changes in planktonic microbiomes.

2.3.2 DNA Extraction

Sponge tissue (~0.5 g per sample) were transferred to 1.5 ml eppendorf tubes, mixed with sterile glass beads and re-suspended in 400 µl of lysis buffer (100 mM Tris, 100 mM EDTA, 1.5 M NaCl, 1% CTAB, 2% SDS, pH 8.0) [14]. Samples were disrupted by bead beater for 2 min, then, proteinase K (10 mg/µl) was added and the mixture was incubated at 55 °C with occasional mixing for 1 h. This was followed by centrifugation at 13,000 rpm for 10 min. DNA was extracted from supernatant using phenol-chloroform-isoamyl alcohol (25:24:1, pH 8.0) and chloroform/isoamyl alcohol (24:1). DNA was further cleaned using a silica based spin filter membrane, and finally it

was eluted in Tris (10 mM). Quality and quantity of DNA was measured using a NanoDrop 2000 spectrophotometer.

To extract DNA from seawater, samples were filtered through 0.22 µm polycarbonate membrane filter (Whatman – Austin, TX, USA). Filter was cut into small pieces and DNA was extracted using PowerSoil DNA isolation kit (MoBio Laboratories, CA).

2.3.3 Sequencing

16S rRNA gene amplicons comprising the hypervariable region V₄ were amplified using primers 515f and 806r, which have previously been used to successfully target, retrieve, quantify and classify simultaneously Eubacterial and Archaeal reads [45]. Libraries were constructed using paired-end technology and all samples were sequenced using the Illumina-MiSeq platform (Sequencing Facilities, Alkek Center for Metagenomics and Microbiome Research, Houston-USA).

2.3.4 Sequence Analysis and taxonomic assignment

Taxonomic assignments were carried out using the pipeline, programs and tools available in QIIME package [46]. Raw sequences were filtered to clean and maintain those over a quality score of 30. Chimeric sequences were identified, extracted and excluded out of the datasets by Usearch [47]. Open-reference OTU picking was made using UCLUST algorithm [48]. Sequences with more than 97% similarity were clustered into operational taxonomic units (OTUs). Representative centroid sequences of each OTU were used for alignment against SILVA database (version 128 released on September 28.2016) using the Ribosomal Data Project (RDP) classifier [49].

To evaluate microbial groups associated with *H. fulva* across the experiments, an initial approach to visualize similarity among them was made by using unweighted Unifrac distances. After that, a comparison among group means for each sampling event (times: 0h, 1h, 24h) was done by using non-parametric Kruskal-Wallis test in Stats package version 3.5.0 in R. For this analysis, the first 15 OTUs were selected because they had the highest abundances and represented 90.97% of reads across study. UniFrac analysis were generated using Phyloseq package version 1.10.0 in R [50].

2.3.5 Metabolomic Analysis

Reagents as methanol, dichloromethane (HPLC grade), formic acid were purchased from Sigma-Aldrich.

-Chemical extraction procedure: Samples were freeze-dried and ground to get a homogeneous mixture. Then, 100 mg were weighted from each sample and extracted three times with 5 mL of CH₂Cl₂/MeOH (1:1, v/v) in an ultrasonic bath for 10 min. Then, the solvent was evaporated and the extracts were re-dissolved in 2 mL of MeOH/CH₂Cl₂ (75:25) and it was left in the freezer at -20 °C overnight. Supernatant was filtered through a 0.2 µm PTFE syringe before UHPLC-DAD-HRMS analyses.

2.3.6 Chromatographic analysis

Analyses were carried out in UHPLC Thermo Scientific™ Dionex UltiMate 3000, connected to a qQToF mass spectrometer fit with an electrospray ionization interface (Bruker Impact II). Mass spectra were recorded in negative and positive ion mode alternatively. UHPLC separation was performed using a Nucleodur® Phenyl-Hexyl (150 mmx2 mm, 3 µm) column and an elution gradient of H₂O/CH₃CN from 95:5 (v/v isocratic from 0 to 4 min) to 60:40 (v/v gradient from 4 to 6 min) to 10:90 (v/v gradient from 6 to 16 min) at a flow rate 0.5 mL/min. To both solvents were added formic acid (0.2%) and ammonium format (0.01 M). The injection volume was set at 5 µL. Parameters for mass spectrometer analyzer were set as follows: nebulizer sheath gas, N₂ (2.1 bar); dry gas, N₂ (8 L min⁻¹); capillary temperature, 200 °C; capillary voltage, 2500 V in positive mode and 3000 in negative mode; end plate offset, 500 V; collision gas, He; collision energy, 4 eV. Data were acquired in the 50 to 1200 *m/z* range.

Quality control (QC) samples were prepared to evaluate the stability of LC-MS along the injections. It consisted of a mixture (2 µL) of each sample and it was injected at the beginning of the run and for each batch of 10 samples during the analysis.

2.3.7 Compound identification

Some ions were unequivocally identified using standards isolated from this species. Already known renierins and fulvynes were purified from a biomass of *Haliclona (Halichoelona) fulva* collected at the Grotte du Lido (Bay of Villefranche sur Mer, France) by SCUBA diving at 20 m depth. They were kept frozen at -20 °C until freeze-drying. The dry material (85 g) was extracted three times by a mixture of solvent CH₂Cl₂/MeOH (1:1) to lead 17.8 g of extract. The extract was subsequently fractionated by vacuum liquid chromatography on C₁₈ bulk powder with eluents of decreasing polarity starting from water, then MeOH, and finally CH₂Cl₂. Purification of fraction H₂O/MeOH (1:1) on a semi-preparative C₁₈ column led to unknown peptides, we were not able to identify them due to low amount available. Purification of the H₂O/MeOH (1:3) on semi-preparative Phenyl-Hexyl column led to fulvynes A-I [40] and purification of the less polar fraction MeOH on the same column led to the renierins [38].

2.3.8 Data analysis

LC/MS raw data were exported and converted to netCDF file format and processed using XCMS package in order to group, correct retention time and align peaks [51]. Subsequently, the obtained matrix was normalized using intensities of quality control pool [52]. Lastly, all data was transformed using logarithm and the final matrix was the input to statistical analysis.

2.3.9 Statistical Analyses

As a first step, mass spectrometry data were analyzed using an unsupervised clustering method as principal component analysis (PCA), but no clear separation was observed. Given the large number of metabolites (on average, 3870) across the samples (observations), a second approach was made using supervised PLS-DA (Partial least square - discriminant analysis), which indicated a separation between the samples sets and allowed the identification of markers among the groups. The model was validated using parameters as R²_Y, which refers to the variation of the

dependent variable explained by the model, and Q_2 , a measure of the variation between the predicted and original data that can be predicted by the cross-validated model. These parameters range between 0 and 1; high values indicate a model over fitting in this kind of analysis [53]. PLS-DA model was fitted using centering method. All analyses were made in SIMCA software (Version 13; Umetrics, Umeå, Sweden).

In this study, the first approach used for data analysis was non-targeted metabolomics and metabolites with a higher VIP (>1.5) were considered to have a high contribution to the discrimination between groups.

A second approach was made by using targeted metabolomics, for that, we focused on specific and known compounds of the sponge, which have been reported by Tribalat and colleagues [54]. In this stage, a filter to retain fulvynes, renierins and peptides according to their mass and retention time was made.

2.4 RESULTS

2.4.1 Microbial Community Analysis

Sponges underwent the experimental conditions in aquarium without any observable morphological changes or any other sign indicating that their health was compromised. A high coverage of the microbial communities based on 16S rRNA gene amplicon sequencing was registered with the applied methodology, with all rarefaction curves reaching the asymptote. Rank-abundances curves evidenced an uneven distribution across the samples (Supplementary Figure SM2).

A total of 8'126.669 reads were obtained for microbial communities for both seawater and sponges. The mean sequence length of the 16S amplicons reads was 253 bp. 1,547 OTUs were recovered from microbial communities in sponges and 1,696 from seawater surrounding the sponge (97% sequence similarity), belonging, respectively to 25 and 33 phyla. In general, the dominant phyla were Proteobacteria and Thaumarchaeota across all the samples.

Microbial community composition was characterized at the beginning of the experiments. The dominant orders were Nitrosomonadales (New.ReferenceOTU111) and Cenarchaeales (OTU AY192631.1.915), which represented on average 45% and 27% of the reads, respectively. Other orders representing $>2\%$ were: Nitrosomonadales (New.ReferenceOTU44 and New.ReferenceOTU 122) and Enterobacteriales (OTU FJ950694.1.1472). Comparing the abundances of phyla in sponges' samples from control aquaria across the time (Supplementary Figure SM3), it was possible to determine that there were no drastic changes in their abundances, except to Bacteroidetes, Firmicutes and unassigned (non-classified) bacterial types, who exhibited a reduction at 1h, followed by an increase at 24h; finally, they reached the initial values.

The core community with common members of the microbiome without stress conditions, e.i. OTUs, was determined. Results showed that it was composed by six OTUs belonging to phyla Proteobacteria (OTUs: FN553481.1.1542, FJ950694.1.1472, New.ReferenceOTU111, New.ReferenceOTU122, New.ReferenceOTU44, JOKG01000002.341270.342829), two to Thaumarchaeota (OTUs: AY192631.1.915, New.ReferenceOTU12) and the other classified as Actinobacteria (New.ReferenceOTU79).

2.4.2 *H. fulva* microbiome composition exposed to environmental stressors

As a first approach to know the effect on microbial communities associated with *H. fulva*, a similarity analysis was made using Unifrac (Figure 2). Results showed that the communities were similar and there was not a clear distinction between control and treatment. This indicates that likely the differences or the evaluated treatments affected mainly those microbial groups represented at low relative abundances.

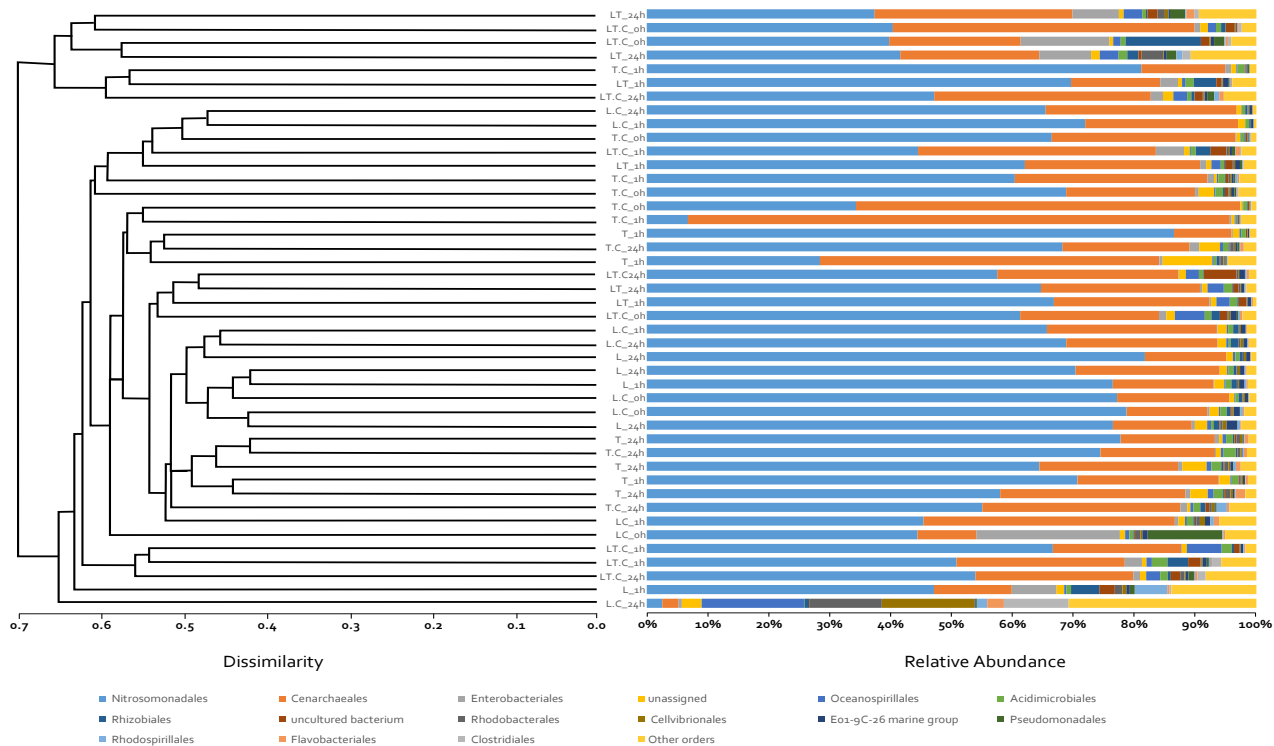


Figure 2. Similarity analysis of microbial communities associated with *H. fulva* under different stress conditions at different sampling events (oh, 1h, 24h) by UniFrac unweighted UPGMA clustering. L: Light (treatment aquaria), L.C: Light (control aquaria), T: Temperature (treatment aquaria), T.C: Temperature (control aquaria), LT: Light+temperature (treatment aquaria), LT.C: Light+temperature (control aquaria).

The microbial community under different experimental conditions (light, temperature and their combination) showed that phyla: Proteobacteria, Bacteroidetes, Firmicutes, Planctomycetes and Acidobacteria had the highest richness at OTUs level (>50 OTUs), and the most abundant phyla were Proteobacteria and Thaumarchaeota, regardless of time.

To determine the effect of all treatments on microbial communities, the abundances of the first 15 OTUs at time 1h and 24h were compared against time oh (before stress condition) (Figure 3). Statistical analysis showed that there were no significant differences for OTUs among sampling events, neither 99 nor 95% of significance level. However some patterns were observed suggesting an effect of the exerted treatments.

Results revealed that light had the strongest effect, causing a decrease in the abundances of Enterobacteriales (OTU FJ913065.1.1309) and Pseudomonadales (OTU FJ937929.1.1393), they were not capable to be recovered after the disturbance and their abundances decreased substantially after 24h. A different pattern was observed for Rhizobiales (OTU New.ReferenceOTU87), whose abundance increased 1h post stress condition.

Also, the temperature caused a negative effect on abundances of Enterobacteriales (OTU FJ913065.1.1309) and Pseudomonadales (OTU FJ937929.1.1393) (Figure 3), however at 24h, they exhibited a capacity of adaptation and their abundances were slightly higher than those reported at the beginning of the study. Interestingly, for some specific groups as Enterobacteriales (OTU FJ950694.1.1472) and Acidimicrobiales (New.ReferenceOTU79) this stress condition produced an increase in their abundances at 24h.

Finally, the condition light+temperature even though was considered the strongest; it did not had a negative effect on microbial members and, by contrast, they increased their abundances at 1h or 24h, as was it reported for the majority of phyla.

In this study, light treatment was carried out not only for analyzing microbial communities changes but also, for evaluating the behavior of Cyanobacteria phylum. OTUs GU941055.1.1253 and FJ937845.1.1374, classified as Prochlorococcus and Synechococcus, respectively, exhibited a variable behavior across the samples, and in some cases they did not register abundance data, limiting its analysis. However, a trend specifically for Prochlorococcus under temperature condition could be observed and at 24h, increasing its abundance. Contrary as expected, light treatment did not influence this phylum.

In general terms, microbial communities associated with *H. fulva* showed they are be able to withstand short-term exposure to light and temperature. The most abundant microbial groups represented by OTUs New.ReferenceOTU111, AY192631.1.915 and New.ReferenceOTU44 classified the first two as Nitrosomanadales and the third as Cenarchaeales, which did not exhibit significant changes in their abundances. Likely, environmental stressors can affect more drastically the presence of microbial groups represented by lower abundances, suggesting that those changes could be due to selective differential reproduction rates or distinct acquisition, retention or expulsion from seawater.

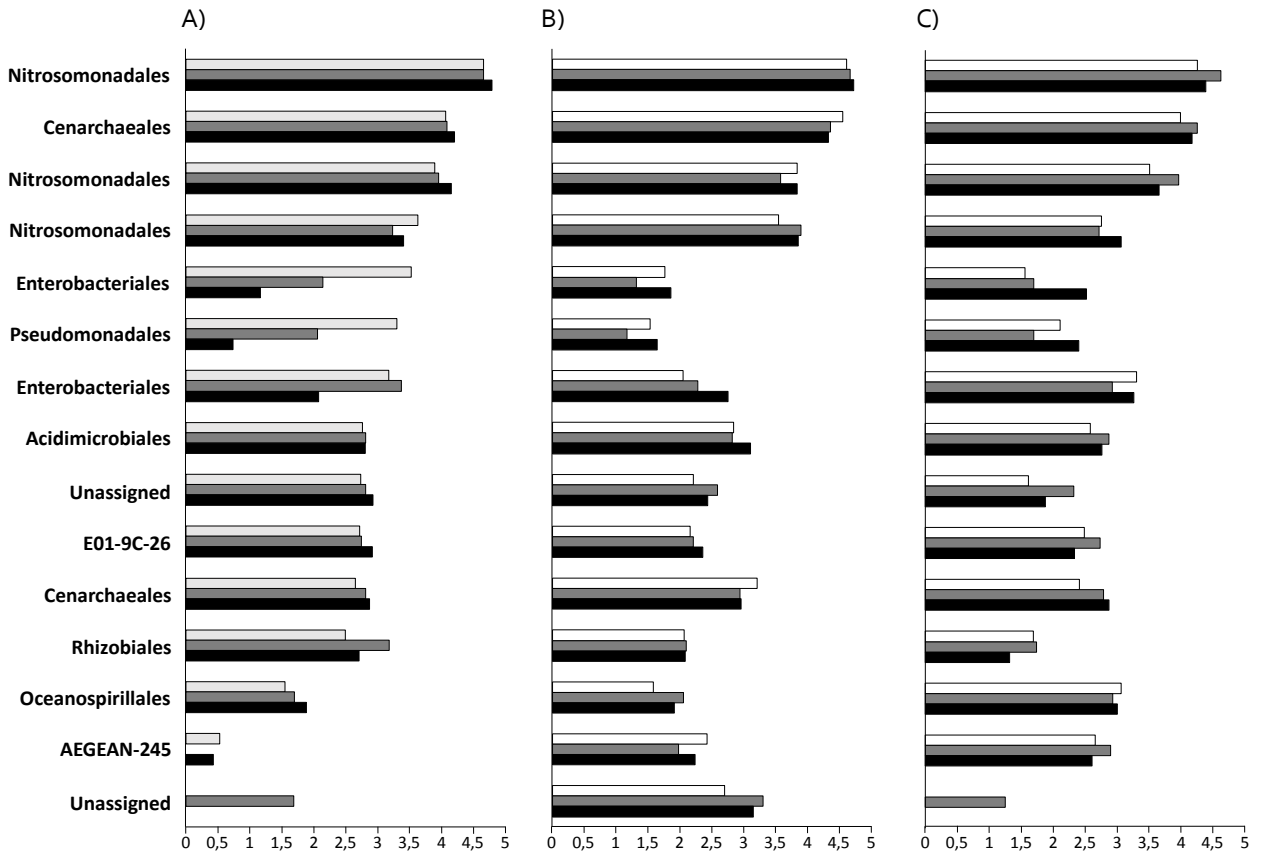


Figure 3. Relative Abundance plot of 15 most abundant OTUs associated with *H. fulva* under stress conditions: A) Light, B) Temperature and C) Light + Temperature. Each bar color represents one sampling event: oh (white), 1h (grey) and 24h (black).

2.4.3 *H. fulva* core microbiome composition and its influence on surrounding seawater

When all samples (regardless stress condition) were analyzed to know the global composition of the microbial community, a core community composed by eight OTUs was identified: OTUs (FN553481.1.1542, FJ950694.1.1472, New.ReferenceOTU111, New.ReferenceOTU122, New.ReferenceOTU44) affiliated to Proteobacteria; OTUs (AY192631.1.915, New.ReferenceOTU12) classified as Thaumarchaeota and OTU (New.ReferenceOTU79) belonging to Actinobacteria.

As for the seawater, a phyla analysis between samples under control and treatment aquaria was performed (Supplementary Figure SM4). In both cases, the influence of sponge's core microbiome on these samples could be evidenced. For seawater from control aquaria, OTUs with percentages of reads higher than 1% were: New.ReferenceOTU111: 27%; AY192631.1.915: 17%; FJ957657.1.1439: 3,9%; FJ950694.1.1472: 3,6%; New.ReferenceOTU44: 3,2%; New.ReferenceOTU122: 1,8%; FJ913065.1.1309: 1,6%; New.ReferenceOTU12: 1,1%;

FJ937929.1.1393; 1,2%; HMO22731.1.1237: 1,1%, all together accounting for 61% of the total community. While seawater from treatment were: New.ReferenceOTU111: 32%; AY192631.1.915: 17%; FJ950694.1.1472: 5,3%; New.ReferenceOTU44: 3,9%; New.ReferenceOTU122: 2,9%; FJ913065.1.1309: 2,6%; New.ReferenceOTU12: 1,3%; FJ937929.1.1393; 1,4% and HMO22731.1.1237: 1,1%, all comprising 67% of the community. The remaining OTUs in both cases had values <1%, which is considered as typical behavior of open seawater that is having low abundances represented in a high OTU richness.

In general, the same dominant pattern observed in sponge samples was observed in seawater as well, and a variable behavior for the majority of phyla over time was identified (Supplementary Figure SM4).

2.4.4 Metabolic Profiles

To better understand the effects of light and temperature on the *H. fulva* sponge, we analyzed the metabolic profiles of this holobiont under controlled conditions. Overall, raw data showed that the number of metabolites were higher in positive mode (4,521) than negative mode (3,210). A supervised partial least squares-discriminate analysis (PLS-DA) was conducted to analyze mass spectrometry data to identify differences between treatments and controls. To validate this model, values of $Q^2 \geq 0.4$ are considered to be reliable [53]. In our results, as shown in figure 4, the experiments (light and light + temperature) had good predictivity with values between 0.49-0.78, while temperature's experiment was less significative (<0.27).

Essays light and light+temperature showed a good discrimination between control and treatments in both positive and negative ion modes, indicating that their profiles were different. However, no difference was detected after stress condition at times 1h and 24h.

As for the temperature experiment, it was difficult to identify a trend from the data in negative mode and it was confirmed by cross-validation model, which values are well below of acceptable limits. The control in this case was scattered across the plane exhibiting a high variability and only in the positive ionization mode, a separation was observed for the treatment.

Variables Importance in PLS-DA (VIP) were then calculated. Surprisingly, we obtained an average of 350 and 471 VIPs, for negative and positive ion mode, respectively. As the metabolomics response was highly variable and fuzzy and large amount of VIPs were obtained, non targeted approach could not be considered. At the beginning of study, the major compounds produced by the species were characterized, so we then decided to analyze holobiont response by using target approach, quantifying changes in their concentrations under stress conditions. The compounds were produced by sponges under artificial conditions [54] and some variations could be observed under stress conditions.

Under the experimental light and light+temperature conditions, no clear and significant differences for the production of all major families of compounds was noticed in negative mode neither for the acetylenic compounds nor for the peptides.

In relation to temperature experiment, in the positive ion mode, a strong influence was observed for the peptide concentrations while no significant changes were observed for the other families of compounds (renierins and fulvynes). Six peptides were clearly underexpressed at high temperature (31° C). It is worth noticing that this decrease occurred just after 1 h of disturbance.

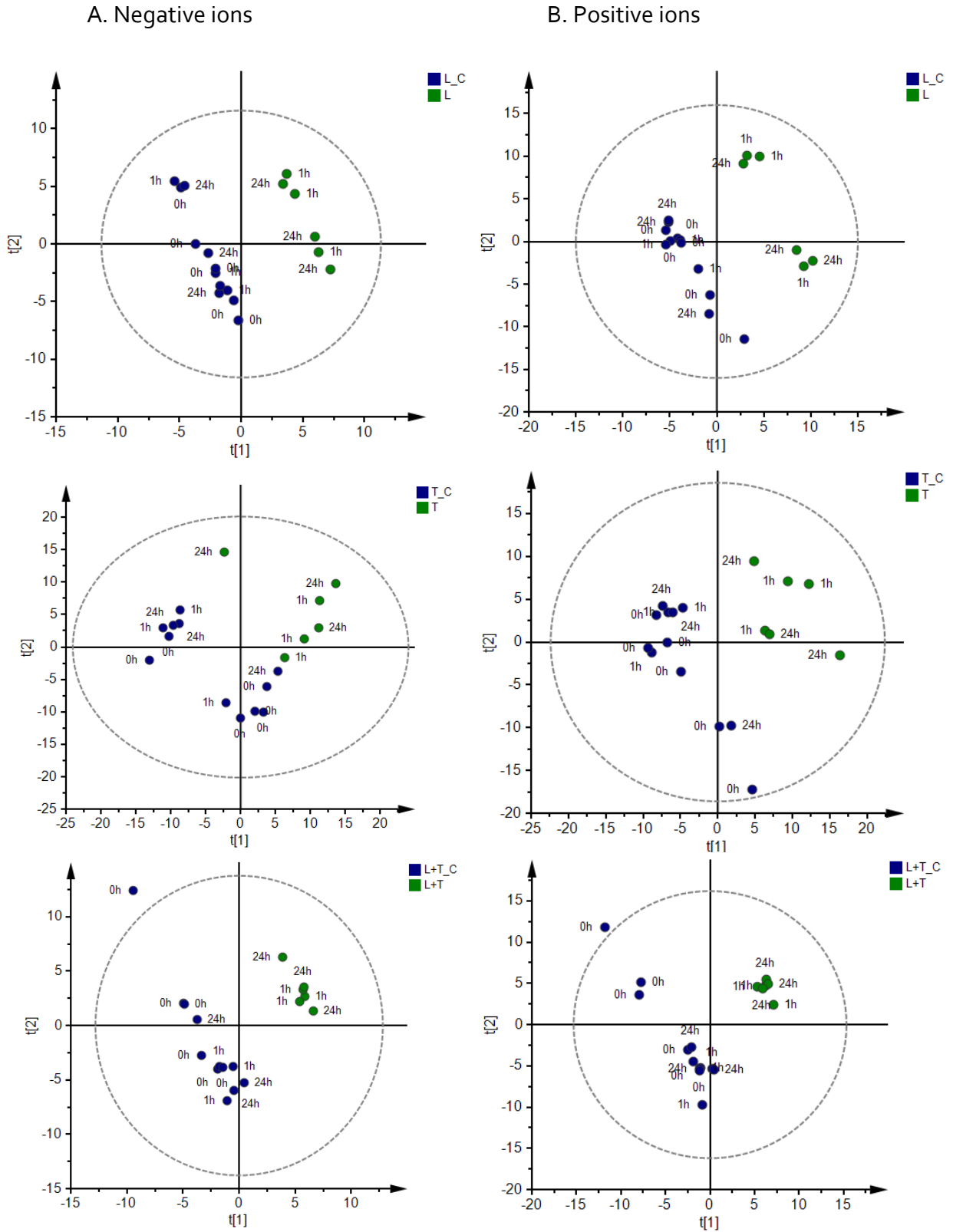


Figure 4. PLS-DA score plot for each experiment. In group A are visualized results in negative mode and group B, in positive mode. Treatments with their controls were: L=light, L_C=control

light ($R^2X = 0.165$, $R^2Y = 0.995$, $Q^2 = 0.499$ ESI- ; $R^2X = 0.29$, $R^2Y = 1$, $Q^2 = 0.774$ ESI+); T=temperature, T_C=control temperature ($R^2X = 0.385$, $R^2Y = 0.804$, $Q^2 = 0.276$ ESI- ; $R^2X = 0.248$, $R^2Y = 0.905$, $Q^2 = 0.055$ ESI+) ; L+T=light and temperature, L+T_C=control light and temperature ($R^2X = 0.211$, $R^2Y = 0.985$, $Q^2 = 0.638$ ESI- ; $R^2X = 0.296$, $R^2Y = 0.999$, $Q^2 = 0.784$ ESI+). Each point represents a sampling event (0h, 1h, 24h) (n=3), and its distance in the plot indicates the similarity between samples.

2.5 DISCUSSION

The interaction of the host sponge, microbial symbionts and seawater, despite are key aspects from a basic standpoint to understand holobiont fitness, adaptation and evolution and from an applied interest such as sustainable and fine-tuned metabolite production in blue biotechnology, still remains to be studied and elucidated in appropriate experimental models. With this work, we are providing an integrative view of the microbiome and metabolome responses to climatic stressors in controlled culturing conditions using as holobiont model the marine sponge *H. fulva*.

We found that *H. fulva* microbiome was highly conserved across all samples and maintained a similar composition as specimens living in the wild (García-Bonilla et al., 2017 - *submitted*), suggesting that it can thrive under artificial controlled conditions with a similar symbiont content. It was dominated by Proteobacteria and Thaumarchaeota phyla, reinforcing the idea that both groups establish a stable symbiotic relationship with *H. fulva*. Our study shows that both phyla are also stable after the stressing conditions tested.

H. fulva associated microbial community were dominated by Proteobacteria and Thaumarchaeota phyla. Proteobacteria are a typical and dominant symbiont in marine sponges [20, 55–58], while only a few sponges have been dominated by archaeas [59–62]. Thaumarchaeota phyla, even though has been found in marine sponges and it is considered be one of the dominant group of seawater planktonic community [63], only in a few sponges, it has been found at very high abundances [59–61]. The Eubacteria-Archaea relationship is considered to be highly specific in this study, once it has not been reported in other sponges with those particular orders; however it is still unclear whether these elevated abundances obey to a provision by the host of a particular and enriched niche or to environmental conditions of the sampled location, which favor their growth.

Other phyla found in a lesser proportion were Bacteroidetes, Acidobacteria, Verrucomicrobia, Cyanobacteria, Firmicutes, Actinobacteria, Chloroflexi, among others; these groups have been widely reported in sponges, being part of the microbial symbiont communities associated with 81 sponge species and concomitantly to the presence of phylum Porifera [56]. In this study, a low phyla number was identified for *H. fulva*, as was reported previously for this and others species within the same genus (García-Bonilla et al., 2017 - *submitted*) [14, 64, 65] a finding compatible with its classification as LMA [66–68].

2.5.1 *H. fulva* microbiome exposed to environmental stressors

Overall, microbial communities did not exhibit drastic changes under stress conditions, suggesting that they were able to withstand short-term exposure to increases in temperature, light and their combination. This biological response showed the potential for sponge-associated

symbionts to aid their host to adapt to adverse environmental conditions. In fact, Cardenas and colleagues [55] reported that highly specific core bacterial communities of *E. alata* and *T. bergquistae* were not affected by changes in environmental conditions and it was essential to maintain the health of the sponge. Also, Webster et al. [31] argued that under stress caused by copper contamination, in healthy macroorganism, symbionts are partially responsible of binding it as detoxification mechanism, so they exert a protective role for the sponge.

Besides the obvious limitations of all the techniques, even those with higher resolution [69] it is possible to conclude that no significant changes among microbial abundances for evaluated treatment were observed, indicating a very robust symbiotic relationship with the sponge, pointing out those highly abundant and stable groups could possibly be involved in metabolites production at certain steps or maintenance of essential processes for the holobiont survival. An effect of treatments could be seen on some specific and abundant microbial groups when applying a more dedicated look at them. Considering the fast changes observed in the metabolomics profiles, in the exploratory massive non targeted approach, as well as, in the more focused targeted approach, clear effects of the treatments in some metabolites were observed. This response, allow us to propose that if the microbiome is involved on this behavior, it would possibly be mostly through transient and fast shifts in the transcriptome expression profiles of the hosts and of the abundant core microbiome members, or by associated modulating biological factors triggered in response to the stress, such as protozoa or viruses.

Light conditions seem be strongest, once affected OTUs could not recover after disturbance. In contrast, under temperature and combined-stress conditions some OTUs increased their abundances. Overall, in those cases where communities were affected after disturbance, an immediate shift in their abundances was observed, followed by recover phase, where at 24h, they reached similar abundances as those reported at the beginning of study. This pattern suggest that the microbial community response in face of this stress condition was fast and its adaptive capacity was successful to respond to environmental variability, in another hand, the conditions here evaluated maybe are not representing a risk for microbial groups, suggesting the existence of a threshold, that for *H. fulva* symbiont and in the case of temperature might be close to 32-33°C, as have been reported in sponges as *R. odorabile* and some corals [34, 70, 71]. Importantly, no stress condition changed the abundance of the most dominant groups (OTUs: New.ReferenceOTU111, AY192631.1.915 and New.ReferenceOTU44), which represented around 79% of the community, it allows to infer that they were resistant (resistance defined as the degree to which a community is insensitive to a disturbance [72]), so they could adopt competitive strategies for survival in that conditions (e.g, changes at metabolic level and/or secondary metabolites production).

The case of Cyanobacteria phyla gained our attention, once a variation in factors as light can have critical effects in the population. It has been reported that its distribution in sponges is correlated with depth and decrease in light availability [73]. In our study, sponges' samples taken at 35m of depth did not show changes for this group, suggesting that it is resistant or that the light intensity or duration was not enough to cause a change in survival, reproduction, or excretion by the host, so the threshold for this factor remains to be tested in future experiments. So far, light effect has been poorly studied, it is known that it affects sponges larvae [74], and other studies reported by Cardenas et al., (2014) [55] and Thoms et al., (2003) [75] found that Cyanobacteria populations did not change their abundances besides sponges being in more light-exposed habitats. The latter

researchers argued that the absence of an exchange of Cyanobacteria in sponges transplanted is due to the existence of physical barriers or chemical defenses.

Sponges core community has been reported in diverse studies [15, 73, 76, 77] and a few have showed its stability under environmental conditions [55]. It is known that different sponge species show different degrees of stability in their microbial community depending on environmental conditions [73]. In this case, *H. fulva* associated-symbionts showed that they are resistant and resilient, under all evaluated treatments. That stability of microbial communities was deduced for its capacity for maintaining the core community. In this study, when this community between control and treatment aquaria was compared, the same pattern was observed: presence and dominance of Proteobacteria (OTUs: FN553481.1.1542, FJ950694.1.1472, New.ReferenceOTU111, New.ReferenceOTU122, New.ReferenceOTU44), Thaumarchaeota (OTUs: AY192631.1.915, New.ReferenceOTU12) and Actinobacteria (New.ReferenceOTU79). Those observations suggest that at least these OTUs could be associated with functions of the sponges metabolism or secondary metabolites production.

2.5.2 *H. fulva* core microbiome composition and influence on surrounding seawater

A dominance pattern for some microbial phyla was identified for both surrounding seawater and *H. fulva* samples, which suggest that environmental transmission could be also a mechanism used by this sponge for acquiring its symbionts. The fact of finding same OTUs classified in phyla as Proteobacteria and Thaumarchaeota both highly enriched in *H. fulva* and in a lesser proportion in seawater, confirms the “sponge-enriched” behavior proposed by Moitinho-Silva and colleagues [78], which argued that sponge-specific microbes also are found in the environment at lower abundances. Additionally, the sponge can provide a specialized niche to select and maintain a specific microbial community taken from seawater [79].

Until now, the ecological response of *H. fulva* associated microbial communities faced to adverse environmental conditions was unknown; the results presented here, suggest that its communities can withstand short-term exposure to temperatures of 31°C and intense light conditions. However, further studies are needed to improve our understanding of how microbial symbiont aid to sponge survival against those conditions, to know their physiological interactions and functional roles, and the similarities and differences of these processes and responses between HMA and LMA sponges.

Finally, by studying the stability of communities in terms of resistance and resilience provides information to understand the impact that variables, such as temperature and light, associated to climate change would have on the diversity and biomass of marine benthic fauna. Identifying a stable core community in *H. fulva* during short-term aquarium cultivation suggest that it can be used as biological model for future experiments.

2.5.3 *H. fulva* metabolomic analysis

First of all we performed a chemical analysis study on a biomass of the sponge *H. fulva* to identify the major compounds produced by this species. As expected two major families were detected and identified by comparison with literature data: a large diversity of polyacetylenic fulvynes

[38] and the less polar but highly concentrated renierins [39] (Figure 5). Interestingly, the major compound produced by this sponge was not the dihydrorenierin-1 but an analogue with a carboxylic group at the acetylenic end of the compound. Due to the high concentration of this compound, we assume that the structure should be revised and could have been misassigned due to the mode of ionization used (EI) leading to a decarboxylation during the analysis reported by Cimino et al (1977). The absence of the terminal acetylenic proton in the ^1H NMR spectrum confirmed this assumption. Therefore we propose that all renierin compounds possess a carboxyl for renierins-1 or methyl alcohol for renierins-2, this would be consistent with an oxido-reductive process linking renierins-1 and -2. The mass spectra of all compounds in negative modes were fully consistent with the presence of a carboxyl group for renierins-1.

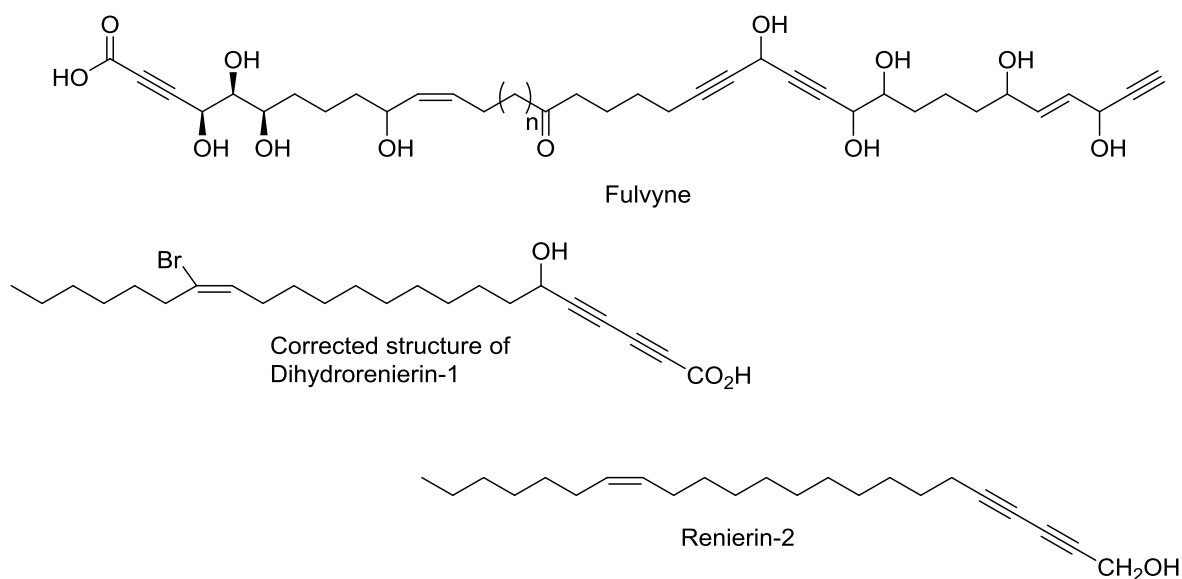


Figure 5. Chemical structure of major compounds isolated from *H. fulva*

In addition to both families of compounds mostly detected in the negative mode, we were also able to detect more polar peptides in the positive mode with mass around 3000 and triply charged. This is the first report of peptides from the sponge *H. fulva*. Even if it was not possible to identify them due to the low amount available, we could follow them during the experiments by targeted metabolomic analysis.

The effect of the light is clearly seen between the control samples and samples submitted to higher intensity of light as shown by the PLS-DA. Metabolic profiles are significantly different for samples under this condition even after only 1 h. However the three major families of compounds identified during our chemical study were not significantly affected by those changes. The VIP responsible of the changes were unidentified compounds of low intensity. They may correspond to compounds difficult to ionize in ESI in both positive and negative mode or to very minor compounds that could be produced by associated microorganisms. While fast changes in the metabolic profiles of minor compounds can be due to changes in quantities of minor microbial groups or transient shifts in gene expression of the holobiont (microbial and host components), the constant production of major compounds might be performed by those abundant member of core community and by the sponge, as it can be by cometabolism of the holobiont components, aspects remaining to be elucidated, the response in metabolome could not be observed under the sampling scheme and relatively short timing designed on this study.

The effect of temperature was less marked in general for the metabolic profiles as indicated in the PLS-DA analysis. However when looking at the VIP we could easily identify the six peptides in the positive mode. The concentrations of the six peptides clearly decrease with an increase of temperature. Because the concentration of these peptides are always lower than the concentrations of fulvynes and renierins but also because metabolic pathways involved in the synthesis of this type of compounds are mostly encountered in microbes [80–83], we suggest that these peptides are indeed produced by symbionts. Increasing in the temperature would affect the sponges' fitness or even the composition of the associated microbiome, therefore leading to a decrease in the production of these peptides, which would be participating as defense mechanism due their antibacterial and antiviral activities [84, 85].

These experiments suggest a clear link between the microbiome and the metabolomic profiles of the holobiont *H. fulva*. While the major metabolites fulvynes and renierins were not significantly affected by light and temperature, the abundant microbial groups conforming the core community were neither affected. At the same time, changes in the production of minor compounds were accompanied for changes in minor microbial groups. Those functional relationships operating in these specific cases are still unknown as no information is available about fulvynes and renierins biosynthesis or about the genomic content and prediction of the particular species predominant in *H. fulva* that may support the idea that they are encoding metabolic steps. These results provided evidences about the suitability of this sponge holobiont system, given his remarkably unique and stable microbiome, the characterized metabolome content, and the identified original metabolites that can be used as a sort of standards, facilitating from a fundamental standpoint, the tracking of the effects climatic changes impacting marine ecosystems, and how this could have environmental and biotechnological applications.

Supplementary Material



Figure SM1. Photographs of aquariums for the indoor cultivation of *Haliclona fulva* (Villefranche-sur-Mer France Laboratories)

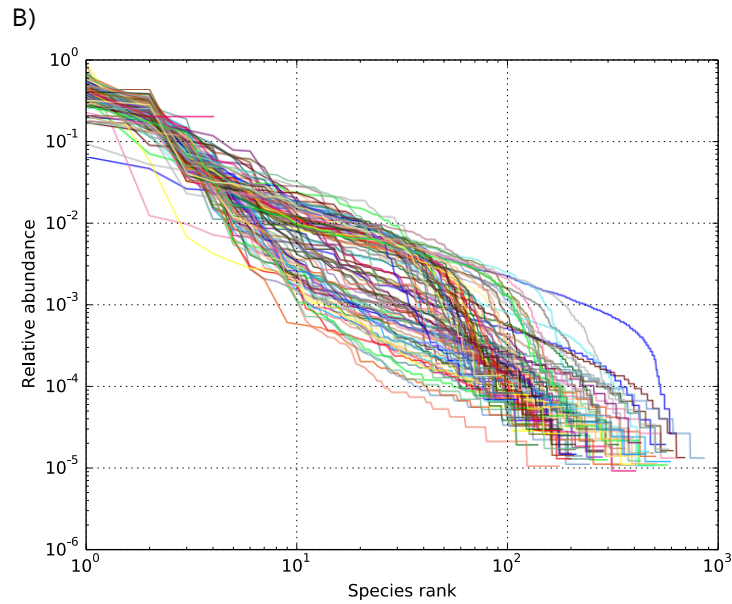
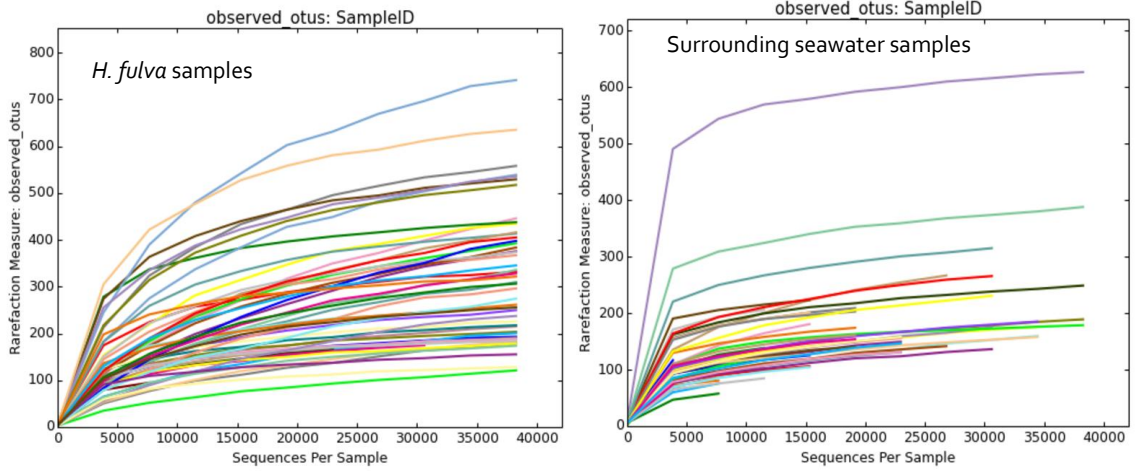


Figure SM2. Diversity of microbial communities in *H. fulva* and surrounding seawater across the study. A) Rarefaction curve and B) Rank-abundance curves based on operational taxonomic units (OTUs) at a 97% similarity.

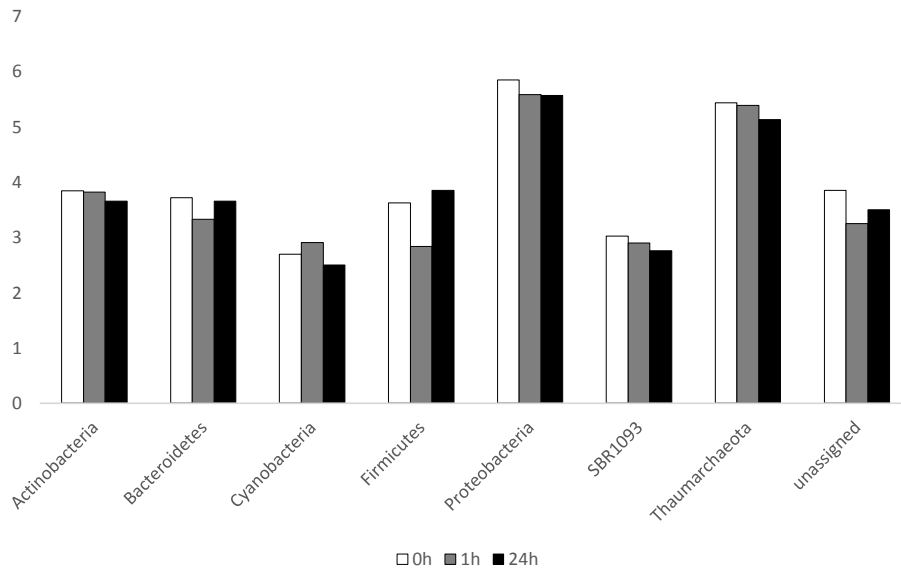


Figure SM3. Taxonomic compositions and relative abundance of the OTUs (at logarithm scale) from sponges cultured in control aquaria at different sampling events (0h, 1h, 24h). Results are from OTUs with a minimum abundance of 500 reads.

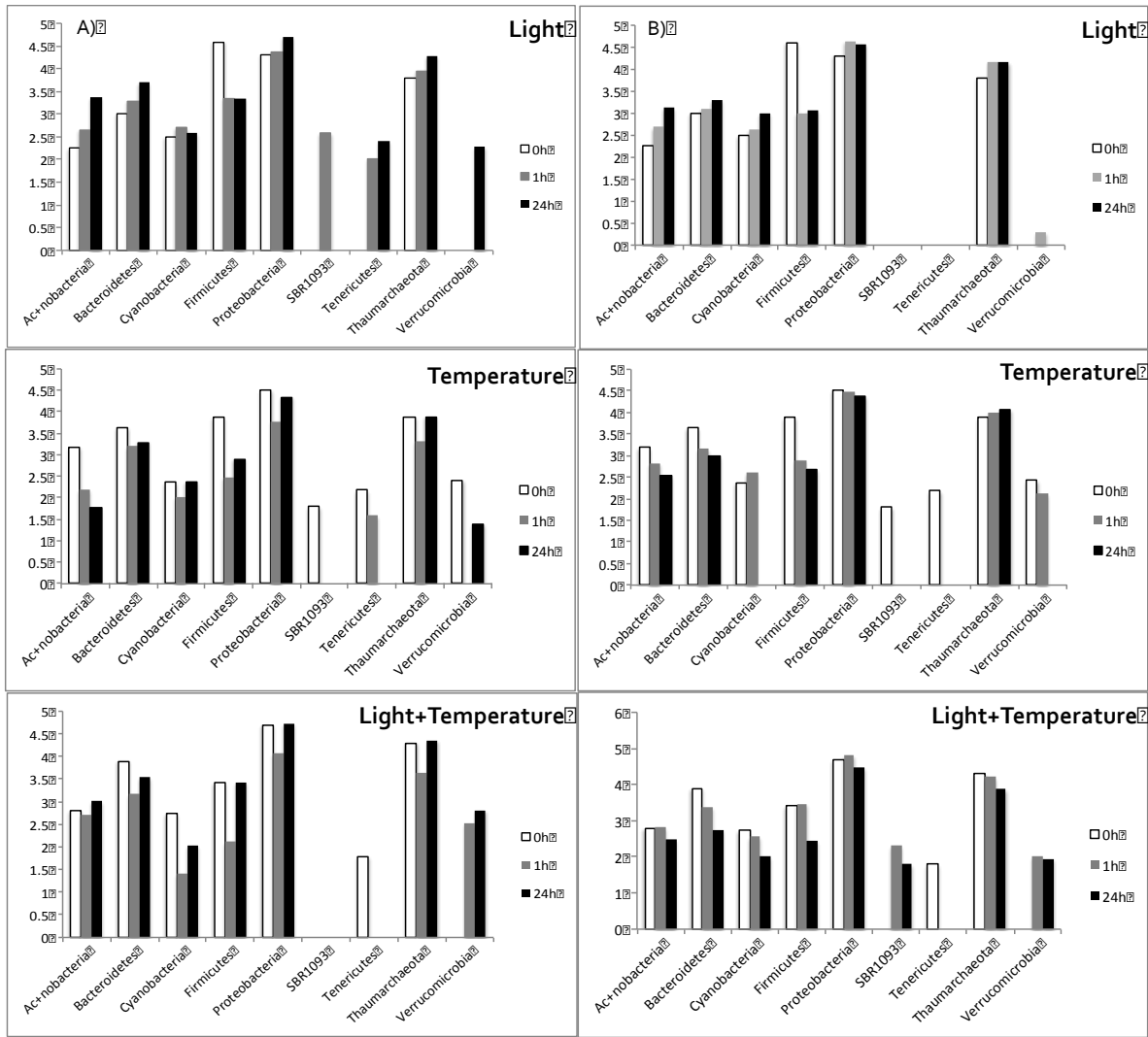


Figure SM4. Taxonomic compositions and relative abundance of the OTUs from surrounding seawater in A) control and B) treatment aquaria at different sampling events (0h, 1h, 24h). Results are from OTUs with a minimum abundance of 500 reads.

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Chapter 3: Initial hologenome probing of the *Haliclona fulva* shows a remarkable viral contribution: an overlooked symbiont component in marine sponge holobiont systems requiring thorough investigation

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3.1 ABSTRACT

Marine sponges constitute one of the biggest reservoirs of microbial communities composed by bacteria, archaeas, virus and fungi. They have established a complex symbiotic relationship where both parts are codependent to thrive and to perform ecological functions. In this study, a sequencing-based metagenomics approach was used to analyze and to have a major coverage of microbial components associated with *Haliclona fulva*, and to infer possible functional traits. Total metagenomic DNA was isolated from three sponge specimens of the same origin and species, subjected to multiple displacement amplification (MDA) technology and sequenced with Illumina technology. Bioinformatic analyses by automated taxonomical and functional annotation pipelines (i.e. MG-RAST) indicated that at defined taxonomical levels for Eubacteria and Archaea, Proteobacteria was the most abundant phylum, followed by Bacteroidetes, Actinobacteria and Planctomycetes. A high percent of reads (39%) were classified as virus, suggesting that they are a key component for maintaining ecological processes and likely, to influence bacterial symbiont composition in *H. fulva*. Single-read metagenome analyses showed that the datasets are mainly containing sequences associated with metabolism and information storage and processing. This study provides a new ecogenomic insight of *H. fulva* holobiont. A deeper analysis is required to

recover information related to viral genomes and suggest the importance of focusing further studies on tracking sponge viromes as an apparent key microbiome component.

3.2 INTRODUCTION

Among symbiotic relationships, there are beneficial associations between organisms sharing the same space, where the symbionts provide some advantage to the host. This relationship has been detected in animals across different scales of evolution, and it has originated holobiont concept, which is defined as the host and microbial genomes. Here, symbionts can be constant or inconstant, can be vertically or horizontally transmitted, and they can play a harmful, harmless, or helpful functional role [1]. Among the hologenome examples most archaic, is the one formed by marine sponges (Phylum Porifera). They represent an ancient and primitive lineage of metazoans, with earliest fossil records detected in Precambrian formations [2]. These multicellular organisms had been inhabiting Earth for at least 590 million years, and are adapted to thrive in a diverse range of aquatic environments (from shallow coastal waters to very deep zones to freshwater) [3] and are found across different latitudes varying from tropical to arctic regions [4, 5].

This complex symbiosis in sponges is responsible to achieve functions as participation in biogeochemical fluxes with releasing of nutrients; production of secondary metabolites, mechanisms providing protection against predators and epibionts, or the capacity to alter the water column, among others [6].

Thus far, microbial diversity of many sponges have been well characterized for Eubacteria and Archaea, relying on the massive sequencing and analyses of 16S rRNA gene amplicons. From these surveys it is known that this bacterial symbiotic component includes more than 40 phyla, being the most dominant Proteobacteria, Chloroflexi, Thaumarchaeota, Poribacteria, Cyanobacteria, Actinobacteria among others [7, 8]. However, while much progress has been made about diversity and patterns of sponge-associated microbial communities, information on the metabolic pathways, functional role on each counterpart and the genomic analysis as a whole is scarce.

Use of omics-sciences have become one of the most powerful strategies to unveil and to understand complex interaction between sponge and associated symbionts [9]. Among them, sequencing-based metagenomics is considered as a valuable and informative tool to attain an improved resolution on microbial community composition and expand the coverage of hologenome components that are otherwise overlooked when using a single gene marker amplicon sequencing (e.g., 16S rRNA gene) [10]. Total metagenomic DNA sequencing has been reported to detect and characterize viral populations of complex diversity and unknown (viromes) [11, 12] and currently, this technique and the viral genomes derived have been already recognized by the International Committee for Taxonomy of Viruses [12] as a standard for listing and identifying viruses.

Also, metagenomics allows the detection of genes that are associated to the synthesis or modification of secondary metabolites/natural products found in holobiont systems and for the genetic characterization of the symbionts [13]. Despite all these advantages, the sequencing-based metagenomics has some technical constraints such as the isolation of DNA, the sequence depth and coverage of abundant members, and the difficulty to annotate genes without closer functional representatives in the databases or reported in the literature. Regarding the extraction of DNA from microbial communities in natural matrices is an important limitation to overcome. The contamination of purified DNA with inhibitors usually found and coextracted from the environmental sample, such as polyphenolic compounds, and the poor quantity and quality of

genetic material are affecting the downstream analyses of this material with omics technologies [14]. While there are no reports assessing how this limitation affects the research in marine sponges, these technical difficulties could explain the relatively low number of sponge metagenomes reported.

Only few sponges have been analyzed through metagenomic and transcriptomic approaches, some of them are: *Amphimedon queenslandica* [15] [16], *Aplysina aerophoba* [10], *Cliona varians* [17], *Cymbastela concentrica* [18, 19], *Rhopaloides odorabile* [19, 20], *Stylissa carteri* [21] and *Xestospongia muta* [22].

In this study, datasets were generated out of sequencing total metagenomic DNA extracted from *H. fulva* and they were analyzed to have a more detailed picture of the microbial communities associated with *H. fulva* and to uncover other possibly overlooked members whose ecological role might be essential to maintain sponges fitness. Furthermore, this approach provides a global idea about metabolism of holobiont, whose knowledge, so far, is scarce. *H. fulva* is one of the most abundant marine sponges in the Mediterranean Sea, dwells in semi-dark benthic communities, in the coralligenous or at the entrance of underwater caves, between 5 and 50 m depth. This sponge has a stable microbial community, as we had found previously by massive 16S rRNA gene amplicon sequencing (Garcia-Bonilla et al. 2017a, *submitted*), the bacterial community is dominated by two symbionts constituting up to 70% of the total community identified as *Cenarchaeum symbiosum* and Uncultured Betaproteobacteria HF1 belonging to Cenarchaeales and Nitrosomonadales orders, respectively. It has been proposed as biological model for evaluating the impact of environmental stressors on benthic organism (Garcia-Bonilla et al. 2017b, *submitted*). To our best of knowledge, *H. fulva* hologenome information has not been retrieved or reported. We consider this research can provide new paths and ideas about microbiome components that could be playing a key role in how the holobiont balance and maintenance, is sustained to climate change drivers and how this is fine-tuning its derived metabolite production.

3.3 MATERIALS AND METHODS

3.3.1 Sample Collection

Three specimens of *Haliclona fulva* (HF1, HF2 and HF3) were collected by SCUBA diving in the Mediterranean Sea at the Grotte du Lido, in the bay of Villefranche-sur-Mer France (Lat : 43° 41' 31.49" N ; Lon : 7° 19' 12.19" E) at 35m depth. After collection, all individual samples were placed in independent plastic bags. They were preserved in ethanol 70% (v/v) and stored at -20 °C until further analysis.

3.3.2 DNA extraction

Genomic DNA was extracted from 40 mg sponge wet weight using a MagAttract® HMW DNA kit (Qiagen, Germany) following the manufacturer's instructions. Tissue lysis was made for 16 h according to protocol. Extracted DNA was eluted with 50 µl water, and concentration measured with Qubit® dsDNA BR assay kit (Thermo Fisher Scientific). Nucleic acid integrity was verified by 0.8 % agarose gel electrophoresis.

Obtained DNA was used as template to perform whole-genome amplification. This procedure was applied due to the following reasons: 1) To obtain the final concentration required for Illumina sequencing; and 2) to decrease the presence of inhibitors from *H. fulva* DNA that would restrict it to be compatible with further molecular biology downstream uses (library construction and sequencing). The amplification process was made using REPLI-g Mini kit (Qiagen, Germany), according to manufacturer's recommendations. Samples were incubated at 30 °C for 11 h followed by 3 min at 65 °C (to inactivate the polymerase). For all assays, a negative control was run for evaluating the presence of contaminants during amplification. Quantity and quality of DNA was measured as described previously.

Metagenome shotgun DNA was sequenced by Illumina platform; libraries were constructed using TruSeq DNA PCR free kit (Illumina, USA). All analysis was made in MacroGen Inc. (Seoul, South Korea).

3.3.3 Data analysis

The raw read data obtained from metagenomes were initially analyzed with FastQC 0.11.3 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) for overall quality, adapters, and length. Then, reads were filtered with Trimmomatic [23] to remove adapter sequences. Reads were assembled using IDBA-UD [24] and MegaHit [25]. Generated contig sequences were compared based on different metrics by using MetaQuast [26] (Table 1).

Fasta files obtained from each assembler were merged and uploaded for their analyses to the MG-RAST server (Meta Genome Rapid Annotation using Subsystems Technology) [27]. Functional assignment was made by SEED subsystem database. For the analysis, the parameters used were: maximum e-value of $1e-5$, a minimum identity of 60%, and a maximum alignment length of 15 bp.

Table 1. Statistics on the processing of the metagenomic samples.

Sample	Raw Data	Post- Quality Control	Contigs in IDBA assembly	Contigs in MegaHit assembly	N50 in IDBA assembly	N50 in MegaHit assembly
HF1	8'128,609	6'438,302	2,100	3,188	1,003	871
HF2	5'458,239	3'685,498	2,715	3,785	1,084	960
HF3	2'704,903	2'029,027	4,363	5,935	933	899

3.4 RESULTS

A total of 12'152,525 sequences were recovered in MG-RAST, with average read lengths of 282 bp. At taxonomic level, metagenomic data indicated that the microbial community was dominated by viruses (39.6%), followed by Bacteria (37.3%), Eukaryota (21.9%) and Archaea (0.51%) (Figure 1A). Among them, the most abundant phylum was Proteobacteria representing 18.6% of reads, followed by Cyanobacteria (13.4%), Ascomycota (5.6%), Firmicutes (1.3%), Bacteroidetes (0.93%), Actinobacteria (0.92%) and Planctomycetes (0.86%) (Figure 1B).

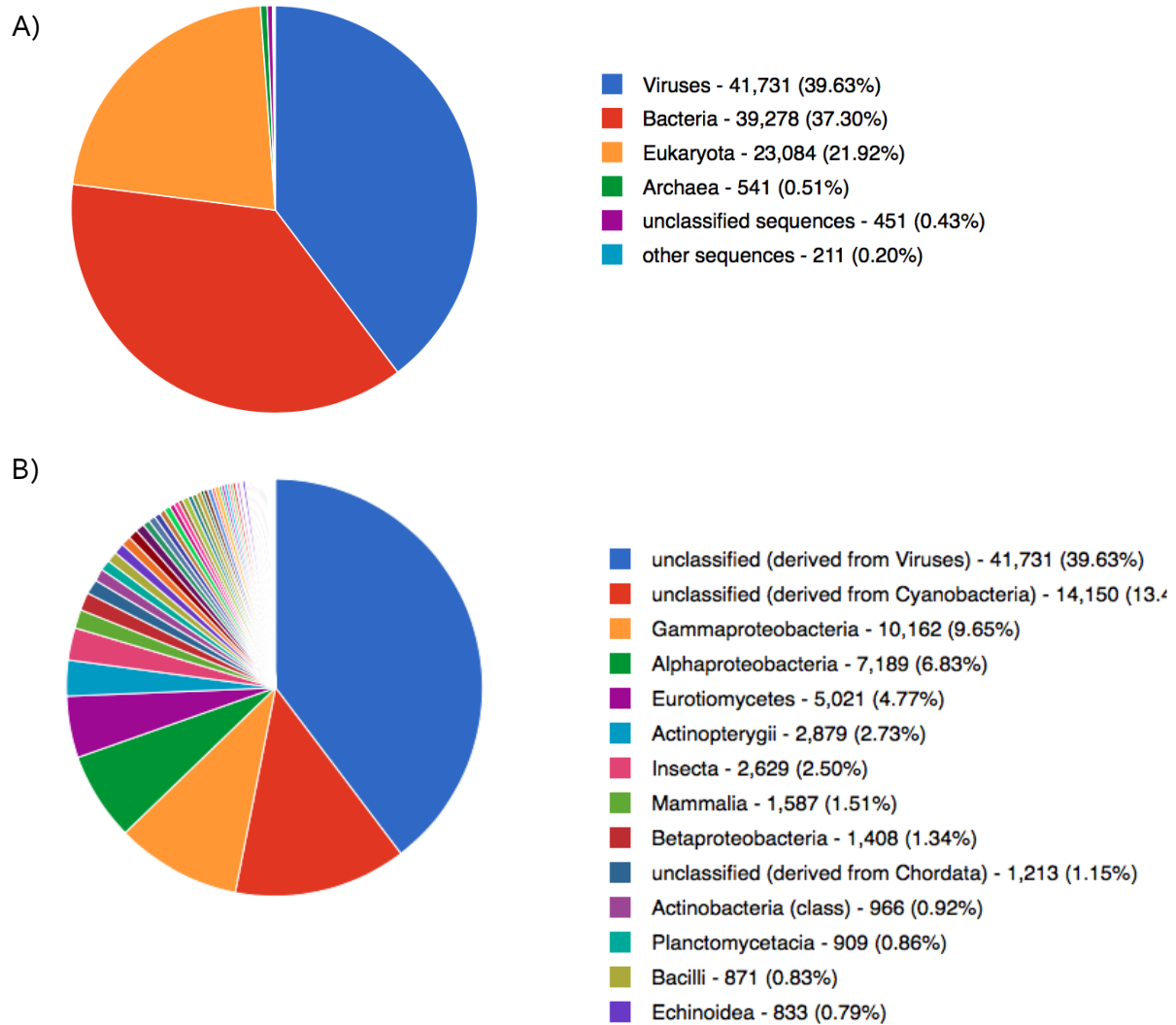


Figure 1. Taxonomical assignment of microbial community associated with *H. fulva*. A) Classification at domain level and B) Classification at class level.

Interestingly, in the case of virus, the obtained sequences did not match against known representative sequences, which is considered to be normal due to the lack of a representative database of marine viral genomes [11]. Our results indicate that we could be in front of an untapped reservoir of new virus associated to marine sponges. At Eukaryotic level, *Penicillium* and *Aspergillus* were found in a low proportion <5%.

In relation to function, sequences matched mainly genes of metabolism and information storage and processing (Figure 2). These genes are involved in carbohydrates, amino acids, proteins, cofactors, vitamins, respiration (Figure 3). Other important observation was the presence of phages, prophages, trasposable elements and plasmids, which is in agreement with the high percent of viruses found in the metagenome.

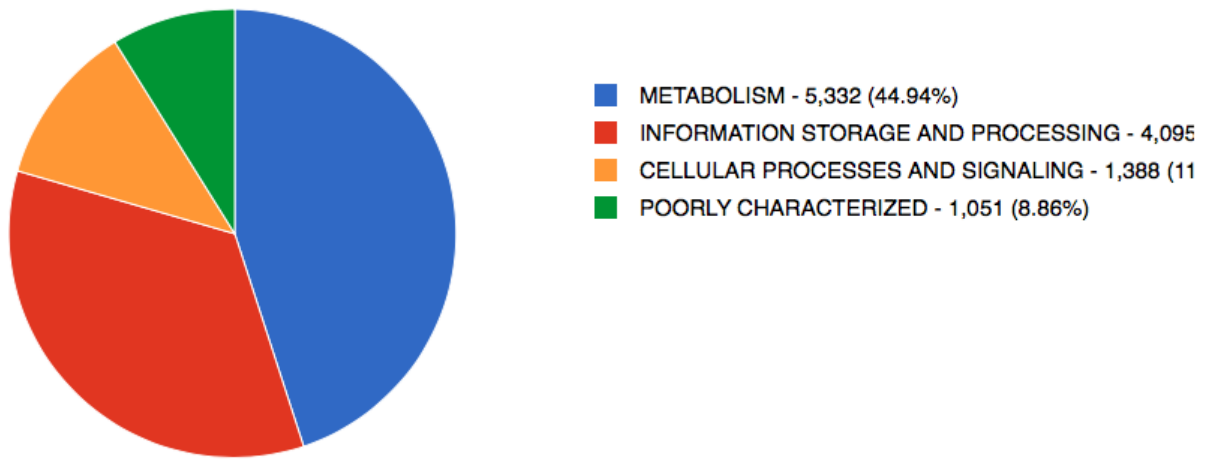


Figure 2. COG functional categories for *H. fulva* metagenome.

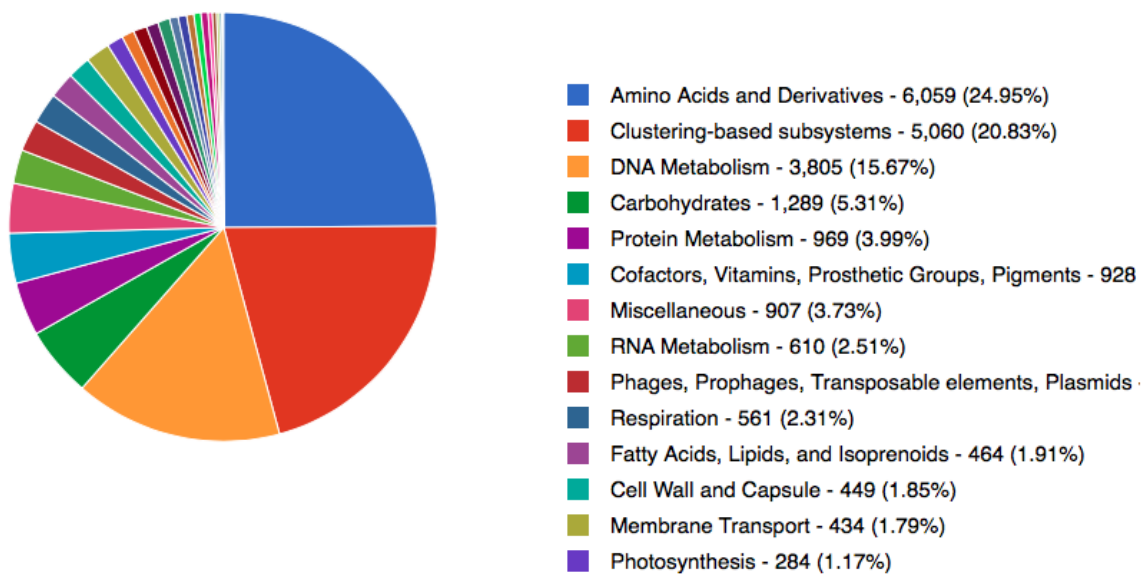


Figure 3. Functional categories for *H. fulva* metagenome (subsystems).

3.5 DISCUSSION

The use of sequencing-based metagenomics provides a broad vision of microbial communities associated with *H. fulva*, where the community not only is shaped by Eubacteria and Archaea but also by viruses and fungi. These results are not unexpected, considering the well known fact that the marine environment, where the sponges are thriving, are overpopulated in viral particles vs. microbial cells and they are recognized as being part of sponges microbial community; however,

hologenomic evidences are very scarce and their functional roles is beginning to be elucidated [8, 9].

In this study, at taxonomic level, the highest proportion of reads was assigned to virus (39%). This result on one side might be considered as a “normal pattern”, because the viruses are highly abundant in marine ecosystems. They can reach populations around 10^{30} and a mean abundance of 10^4 - 10^7 viruses/ml [28] and sponges are prone to contain big communities of bacteriophages and other viruses [29]. On the other side, this result suggests that they are a major genomic contributor of the hologenome and a key component for maintaining ecological processes in marine sponges symbiosis. Sponges have been classified as HMA (high microbial abundance) to describe a diverse and dense microbial consortia within their tissues, and LMA (low microbial abundance) refers to the lack of large and complex microbial consortia within their mesohyl [30–32], but there are a scarce number of studies about virus in sponges despite their influence potential in the symbiotic community. It is known that virus can perform mutualistic roles, as it was documented by Roossinck (2011) [33]: virus can attenuate diseases in their host, they can kill competitors and they can help their host to adapt to extreme environmental conditions.

In sponges it is recognized that due to their capacity as filters, they can capture bacteria, dissolved organic matter and virus. This capacity is known as virus predation and might allow a nutrient flow with the viroplankton in oligotrophic environments [34]. Otherwise, some studies have found that in sponges, functions related to viral defense (CRISPR-Cas system, RMS, clustered regularly interspaced short palindromic repeats) are highly enriched [19].

However, the observed high percent of reads assigned to virus should be interpreted with caution, as with any technique, there are limitations and biases derived from the ones applied. We employed a multiple displacement amplification (MDA) technology, which uses phi29 DNA polymerase for producing long fragments (12 kb average) under isothermal conditions [35]. While MDA is an effective technique for amplifying DNA samples, biases associated with it includes: preferential amplification of circular single stranded DNA (ssDNA) and quimeras formation [36, 37]. According to that, Marine and colleagues (2014) [38] argue that the frequency of reads from a viral community gDNA sample are not necessarily reflecting the true frequency of taxa or gene functions among viral populations within a sample. In particular these authors purposed that high frequency of viruses is due to modular genetic organization of phages, where the middle portions of linear phage genomes tended to be over-represented. Also, Kim and Bae (2011) [39] found that MDA has a bias to amplify sequences mainly from single-stranded DNA viruses, and in a lesser proportion from double-stranded DNA viral. However, discounting the influence this preferential overrepresentation may imply, assuming an actual deviation of 3X from the actual proportion in the original sample, it would still mean that the virome represents the remarkable amount of 10% of the hologenome extracted.

Considering the aspects previously described, we think that although virus community could be over-represented in *H. fulva* metagenome, this group is abundant. The fact that prokaryotic community in *H. fulva* is composed mainly by two symbionts classified as Nitrosomonadales (Proteobacteria-Beta) and Cenarchaeales (Thaumarchaeota-Marine Group I), both representing around 70% and stable in their natural as well as in experimental conditions (Garcia-Bonilla et al. 2017a,b -submitted), suggest the existence of a controlling factor such as viruses, responsible to maintain the dominance of the prokaryotic symbionts in our sponge. An argument in this direction was given by Thingstad and Lignell (1997) in their hypothesis “killing the winner” [40],

which consist in selectively killing the winner, it means, superior competitor populations to favor viral production (lytic or lysogenic cycles) and promote the diversity. The identification of viruses associated with specific taxa in marine environments is beginning to be elucidated. Studies reported by Philosof et al. (2017) [41] showed that the Uncultured Marine Group II Euryarchaeota, one of the most abundant groups in the oceans, is the hosts of magroviruses, and they are considered key ecological agents that apparently infect Thaumarchaeota phylum. Also, Danovaro and colleagues (2016) [42] reported the impact of viruses on members of specific clades of MarineGroup I Thaumarchaeota. Authors argued that the rates of virus-induced mortality of bacteria and archaea should be balanced by cellular turnover and that its dynamic performs a key role in global biogeochemical cycles.

Beside, no scientific reports have been published associating symbionts of *H. fulva* with specific virus. These previous findings make plausible the existence of virus for controlling archaeal population in *H. fulva*. Further studies are needed to have a complete inventory on virome in marine environments and relate possible host and functions.

Bacterial phyla found in the metagenome were: Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria and Planctomycetes. All of them are typical phylum found in marine sponges [7] and perform key ecological functions for the sponges' fitness. For example, Bacteroidetes is one of the most abundant phylum in marine ecosystems, and its role is associated with the decomposition and remineralization of phytoplankton biomass [43] and Actinobacteria is related to secondary metabolites production [44].

Here, the abundant phylum was Proteobacteria (class: Gamma, Alpha, Beta), in agreement with previous reports for this sponge species (Garcia-Bonilla et al. 2017a, *submitted*). Surprisingly, symbionts (Nitrosomonadales and Cenarchaeales) were not recovered, in this case, probably due to the milder DNA extraction method applied, quite different to the DNA extraction method used for the analyses of 16S rRNA gene amplicon sequencing (Garcia-Bonilla et al. 2017a,b, *submitted*). It was harsher in physical and chemical treatments, allowing the more complete disruption of Archaeal membranes that are more difficult to lyse [45–47], as for this technique is not requiring high molecular weight metagenomic DNA as template of 1.5 kb average, whereas for total metagenomic DNA sequencing requires average fragments above 10kb for proper library construction.

The overall functional annotation on the level of COG categories were not similar compared with other metagomes' studies [10, 48]. Our characterization of *H. fulva* metagenome was poor and only two enriched categories were identified: metabolism and information, storage and processing. Researches reported previously have described categories associated with defense mechanism and cytoskeleton in metagenomes of *Petrosia ficiformis*, *Sarcotragus foetidus*, *Aplysina aerophoba*; and reparation, secondary metabolites biosynthesis, transport and catabolism; replication and recombination in *Cymbastela concentrica*.

In conclusion, results presented here provided a new hologenomic insight about *H. fulva* holobiont. We consider that the obtained data is valuable to get a better understanding of the symbiotic dynamics that occurs inside the sponge. A deeper analysis is required to recover further information related to virus' genomes and help to fill the gaps in sponges' microbiology.

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4. GENERAL DISCUSSION

Marine sponges represent a great fraction of the benthic fauna. Over the last decades they have been increasingly studied as a model in the fields of biology, microbiology and chemistry given the following characteristics: i) They represent one of the most ancient models of symbiogenesis [1], ii) They can thrive in a diverse range of aquatic environments (from shallow coastal waters to very deep zones to freshwater) [2] and are found across different latitudes varying from tropical to arctic regions [3,4]; iii) They perform key ecological functions for aquatic ecosystems [5–7], and iv) They produce a huge variety of secondary metabolites with biotechnological potential [8].

Microbial symbiotic relationships in sponges are well-recognized since the early studies and they have been classified in two groups: HMA (high microbial abundance group) and LMA (low microbial abundance group) [10,13], and microbial diversity and the taxonomical contents of such symbiotic microbiomes have been documented in greater detail recently covering mainly Eubacteria and Archaea domains [9–12].

HMA sponges species have been studied as a promissory from a biotechnological point of view due to their metabolic versatility and wider distribution, but in recent years LMA sponges have been recognized as an interesting resource that are harboring specialized microorganisms with very particular metabolic pathways reflected in original metabolites with bioactivities of clinical interest, which make these organisms of high biotechnological potential and ecological relevance [14].

Haliclona fulva is one of the most abundant sponges in the Mediterranean Sea; members inside the same genus had been classified as LMA, however to our best knowledge there are not reports about the status of this species in this regard. The closer approach was made by Lucas-Moitinho and colleagues [15] that used algorithms predicting its classification as LMA. Our results support that prediction through transmission electron microscopy (TEM) and microbial community composition by 16S rRNA gene amplicon sequencing analyses, finding low bacterial densities on its cellular matrix and a low richness at phyla level observed. These characteristic patterns had been described in numerous studies for sponges inside this group [16–19].

Microbial community description of *H. fulva* exhibited a particular pattern with dominance of two symbionts. In general, phyla as Proteobacteria, Thaumarchaeota, Bacteroidetes, Actinobacteria, Planctomycetes, Cyanobacteria, among others, were observed. All of them, described and reported in the most recent and complete description of microbial communities associated with 81 sponges species [20]. Symbionts in *H. fulva* appear to be highly enriched, as it was found for Nitrosomonadales and Cenarchaeales orders composed by few OTUs which comprised up 70% of community. Additionally, that relationship seems to be stable, once samples for microbial community analysis were collected at different seasons and times (year) and the same pattern was observed.

In a deeper taxonomical analysis, a symbiont named as “Uncultured Betaproteobacteria HF1” where the HF1 stands for “*H. fulva* 1 specific ribotype” found in this work, was classified inside Nitrosomonadales order, no hits more specific against reference organisms or sequences with a

defined taxonomy were found, which suggest that it can be a representative of a new species specifically evolved and harboured in *H. fulva*, evidencing the limited knowledge we have about microbial diversity in “invertebrate metazoans”. Despite the relatively low diversity and that the coverage of the description by 16S rRNA gene amplicons was near complete, many of the OTUs at either high or low frequencies could not be classified to a lower taxonomic hierarchy of genus, indicating that this microbiome may contain many yet to be described bacterial species and it is expected that many bacterial phyla have yet to be uncovered. Thus, additional studies are required to fulfill such marine microbiology inventory.

In relation to the symbiont classified as “Cenarchaeum”, a phylogenetic analysis showed that it is closely related to *Cenarchaeum symbiosum* and according to Konstantinidis *et al.* [21] this archaea can be considered an exception of fine-tuned niche-specific variants of the same species that are nevertheless having 16S rRNA gene sequences up to 5% dissimilar, thus, taking into account this consideration, this is probably a representative of a *C. symbiosum* niche-specific strain.

It is known that *C. symbiosum* has a chemolithoautotrophic metabolism, it allows the ammonia oxidation to nitrite [22]. As sponge excretes ammonia as metabolic waste product [11], it is used by the archaea, underpinning the possibility of a cometabolism among them. This hypothesis linked to high abundances observed for this symbiont, suggests that nitrogen cycle is one of the major process occurring in the holobiont. These results are in agreement with functional categories found in metagenomic analysis, where aminoacids and protein metabolism were the main processes that could be inferred.

As dominant patterns were observed for microbial communities in *H. fulva*, it is possible to propose that vertical transmission is one of the mechanisms used for spreading symbionts, even though it is a process that has been reported to occur mainly in HMA sponges [23]. In this study, sponge surrounding seawater microbiome was analyzed finding that the pattern was influencing it. This suggest that while seawater provides symbionts to sponge, at the same time they are enriched and excreted by the animal, for some bacterial types it provides an habitat with a constant supply of nutrients and environmental conditions that favors their establishment and growth.

One point to consider is the fact that seawater samples came from the same site where *H. fulva* colonies were found and collected, so, enriched symbionts observed could be due in part to sponge filter-feeding activity, where a fraction of their microorganisms are released. While the two processes can be occurring simultaneously, the extent and contribution for this microbiome sharing and exchange remains to be elucidated, and how are the dynamics to shape and maintain the very peculiar microbial signature of this sponge species over time is an aspect requiring further work.

Microbial communities associated with *H. fulva*, exhibited a dominant and stable pattern in its natural habitat and also under controlled aquaria conditions in a consistent and reproducible manner assessed from several replicates tested. Analysis showed, on one side, that sponge underwent the experimental conditions in aquaria without any observable morphological changes or any other sign indicating that their health was compromised, and on the other side that symbionts Uncultured Betaproteobacteria HF1 and *C. symbiosum*, maintained their abundances and represented around 70% of community. These results suggest that the sponge can thrive under artificial conditions and reinforce the idea that this association could be of importance for sponge health, fitness and thus, for its derived metabolic activity. The culturability of sponges has showed be successful for some species, some of them *Rhopaloeides odorabile* [24], *Crambe crambe* [25], *Aplysina aerophoba* [26], *Xestospongia muta* [27]. However, factors as the difficulty to maintain associated microbial populations [28], sponges biomass loss [26, 29], cost of

maintaining aquaculture setups, simulation of environmental conditions like in the real scenario [30, 31] and sponges life cycle (once a year) [32], restrict its culture, but from a sustainable and stable production standpoint, it is very promising.

At chemical level, secondary metabolites produced by *H. fulva* have been reported and characterized [33–36]. This holobiont produces renierins, fulvynes and peptides as majority compounds. Comparing to other species within the same genus, this first report of the microbial community of *H. fulva* indicates a distinct and atypical Archaeal/Eubacterial composition, and this sponge also has a very distinct metabolic profile as reported by Tribalat et al (2016) [33] within the order Haplosclerida. They found that inside Chalinidae sponges, *H. fulva* holobiont produces an original chemical diversity dominated by polyacetylenes in large amounts, and long chain acetylenic products like renierins and fulvynes.

Regarding the results on metabolomics profiling and a very stable microbiome composition observed in this study, this stability in metabolite production could be due to the low influence in the holobiont of the microbial community given its LMA nature, or could be resulting from the remarkable predominance of a few Eubacterial and Archaeal members influencing/acting on its synthesis.

In this study, a global analysis was performed over the complete metabolome content of sponge samples analyzed in culture conditions showing stable and similar profiles, coupled to the detection of compounds previously identified from this sponge species. It is therefore likely, given the corresponding stability and nature of microbiome and metabolome components that there is a possible contribution on metabolite biosyntheses or modification by predominant members of *H. fulva* associated microbiome given that there are a large number of its polyacetylenes are of polyketides or of peptidic in origin; there are various studies backing up this possibility proposing the microbial PK biogenesis of the highly oxygenated marine polyacetylenes, given that polycistronic operons for its biosynthesis are frequently found on terrestrial and marine bacterial strains [37, 38].

While for others sponge genera with predominance of associated Archaea (*Axinella*, *Tentorium* and *Inflatella*) it has never been reported the production of acetylenic metabolites, there is high genomic and metabolomic versatility, and intraspecies variability among Archaea [39] that could explain the absence of these compounds despite this domain predominance. This microbial contribution or mediation on such metabolites biosynthesis is an aspect requiring further investigation, but our research suggest some possible implication in this metabolic feature in *H. fulva* of the abundant and characteristic symbionts found in this work.

H. fulva response at microbiological and chemical levels was evaluated under stress conditions. Here, variables related to human-induced global climate change as light, temperature and its combination were evaluated. Results revealed that there was not a significant effect on microbial communities and secondary metabolites, suggesting that they can withstand short-term exposure to those conditions. Identification of the same bacterial phyla, as they were described for the holobiont in the wild were observed. The core community was conserved regardless stress condition applied, indicating that they might be aiding to maintain the health of the sponge, as it has been reported in previous studies for other species [40] [41].

Treatments seem have an effect on minor and specific groups, for example, OTUs classified as Enterobacterales and Pseudomonadales exhibited a change in their abundances after disturbance. Generally, the majority of affected OTUs could recover and at the end of study, their abundances had similar values as those reported at the beginning of experiment. This pattern supports two hypotheses, the first, that the microbial community response in face of this stress condition was fast and its adaptive capacity was successful to respond to environmental variability. The second, the conditions here evaluated may have not been representing a risk for

microbial groups, suggesting the existence of a threshold, that for *H. fulva* symbiont and in the case of temperature might be close to 32-33°C, as have been reported in sponges as *R. odorabile* and some corals [42–44]. In response to light, there is a few research studies analyzing its effect in sponges in general, therefore a threshold for a significant change in sponge health, microbiome and metabolome can not be estimated yet.

At chemical level, the analysis was made for majority compounds as fulvynes, renierins and peptides, since *H. fulva* has been widely studied as chemical model by research group led by Dr. Olivier Thomas, and these compounds have been characterized previously. A common feature between microbiological and chemical responses was the behavior of minority groups and metabolites, respectively. Both exhibited a variable pattern and fluctuation across the study, restricting their analysis.

Stress conditions were not affecting neither fulvynes nor renierins detection and quantities, suggesting that their production is probably not modulated by the temperature and light changes tested. The fact of finding them in high proportions across the study in specimens dwelling either in natural habitats or in artificial conditions with and without stress evidenced that their production is stable. Based on the fact that the majority compounds and microbial communities were stable, it is possible to propose that abundant members of core community participate in biosynthesis pathways or modification steps of these compounds. In relation to peptides, interestingly, a negative effect caused by temperature was observed. It suggests that at 31°C, metabolomic response of holobiont begins to undergo changes. To assess those changes that are drastically affecting the sponges health under the evaluated artificial conditions and sampling events was not the main purpose of this study, as we avoid exerting treatments that would produce death or early necrosis signs in sponge specimens cultured. We are providing a detailed baseline for such further evaluations. We however could observe early indicators of such stressing conditions such as diminished peptides production that could alter defenses mechanism of the sponge against predator or bacteria, once is recognized that they exhibit antimicrobial and antiviral properties [45, 46], This would, in turn affects its fitness within the marine environment. As with the majority of reported peptides from marine sponges, it is still unknown the metazoan or microbial origin of them (nonribosomal or ribosomal synthesis), however, diverse studies have been reporting that marine sponges associated symbionts are responsible for important steps or complete synthesis by non-ribosomal pathways [47–50]. Microorganisms involved in this process include members of Actinobacteria (one of the bigger producers identified so far), Bacillus, Cyanobacteria and Myxobacteria, and fungi [51].

In this study, Actinobacteria phylum was identified and it showed a slight increase on its abundance under a transient wave of temperature increase as treatment. The observed inverse relationship could indicate that environmental stressors generate an immediate and drastic response at metabolomic level for some symbionts. In this scenario, an increase in the population does not implied a fast increase in metabolites production suggesting that the reason why some microorganism restrict their production relates to a cost-benefit balance logic. Because of the high energetic cost of producing peptides [52], bacterial enzymatic and metabolic machinery is focused on cellular biomass, therefore a large investment is done in primary metabolism and mechanisms that facilitate survival under stress conditions.

In this study, Eubacterial and Archaeal communities were well characterized. Nevertheless, others biological drivers can support the ecological dynamics in the holobiont, such as virus and fungi. Aiming to have a more complete hologenome description of *H. fulva* and to improve our

understanding about possible functions associated with this holobiont, a sequence-based total metagenomic DNA sequencing approach was performed.

Interestingly, a high proportion of virus was identified, the majority of them could not be classified neither to order nor to family, which evidence the limitation of representative diversity on reference databases [53]. In marine ecosystems, the functional role of marine virus role is being characterized, some researches have found that they help to carbon export to deep ocean [54], regulate “metabolic reprogramming” altering carbon cycles, nutrient and energy flows [55] and they has the capacity of influencing the composition of marine communities [56].

In *H. fulva*, a predominant pattern in microbial communities was observed, thus, it is possible to think that various mechanisms to control such composition from the constant exposure to the seawater microbiome, and it can be at many levels: antagonists activities, competition, metabolites production as defense mechanisms from the host, selection of resistant/producer microbial symbionts based on such features, and of course, viruses, that have been reported in many different microbial communities to be responsible to maintain the predominance of certain prokaryotic symbionts. A reasoning of this role was given by Thingstad and Lignell (1997) in their hypothesis “killing the winner” [57], which consists of how selectively killing winner means that superior competitor populations favor viral production (lytic or lysogenic cycles) and promote the diversity. While the enormous amounts of viral particles oin marine environments has been reported since many decades ago, the genomic and functional identification of viruses associated with specific taxa in marine environments is at the early steps. Studies reported by Philosof et al. (2017) [58] showed that the Uncultured Marine Group II Euryarchaeota, one of the most abundant groups in the oceans, is the hosts of magroviruses, and they are considered key ecological agents that apparently infect Thaumarchaeota phylum. Also, Danovaro and colleagues (2016) [59] reported the impact of viruses on members of specific clades of MarineGroup I Thaumarchaeota. Authors argued that the rates of virus-induced mortality of Eubacteria and Archaea should be balanced with cellular turnover and that it dynamic performs a key role in global biogeochemical cycles.

Besides no scientific reports have been published associating symbionts of *H. fulva* with specific virus, these previous findings make extremely likely the existence of virus for controlling archaeal population in *H. fulva*. Further studies are needed to have a complete inventory on virome in marine environments and relate possible host and functions.

In conclusion, this thesis provided new knowledge in sponge microbiology and chemistry, using as a model *Haliclona fulva*. Results provided evidence on the existence of a core community and specific microbial signature that exists in the sponge. This signature seems to be very particular and different even among LMA sponges. The presence of Uncultured Betaproteobacteria HF1 and *C. symbiosum* as dominant orders suggests that these symbiotic relationships are highly specific and stable.

Those characteristic features were also evidenced under stress conditions, where microbiome communities were able to withstand short-term exposure to temperatures of 31°C and intense light conditions. At metabolome level, temperature negatively affected peptides production, while fulvynes and renierins synthesis was constant across the study.

The fact that the LMA sponge *H. fulva*, was able to thrive in artificial conditions and having microbial communities stable when exposed to environmental stressors, make us to propose it as a suitable model for studying fundamental aspects of marine holobionts composition, functioning and adaptation to climate change and for sustainable stable production and valorization of marine natural products.

This study provided new information about sponge microbiology but it also remarks the importance to continue the research efforts given the many open questions and possibilities arising from studies on Porifera, a “black box” where microbiome studies may contribute to unveil, for instance, questions about microbial community function and the composite response in face of global warming. It may help us to have improved knowledge about the rate of adaptation and to predict the fate of this important and fascinating animal holobionts and the near future.

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