Investigation of biological factors that may contribute to bioactivity in *Haliclona* (Porifera, Haplosclerida)

PhD candidate: Maria Vittoria Marra (BSc, MSc)

Supervisor: Prof. Grace P. McCormack

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'*Ti porpatì sti stràtandu em betti mai*'

('Whosoever walketh his own way shall never fall'

Calabrian Greek proverb)

DECLARATION

I declare that this thesis has not been submitted, in whole or in part, to this, or any other University for any degree. Except where otherwise stated, this work is the original work of the author.

Signed ……

Date ……..

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ABSTRACT

Sponges (phylum Porifera) are considered the most prolific source of bioactive compounds of marine origin. These molecules have varied chemical nature and have been shown to possess several properties, e.g. antibacterial, antiviral, antifungal, anti-inflammatory, antitumor. For the sponges they play important ecological roles as antipathogenic, anti-predatory, antifouling and anti-overgrowth agents. Sponges are also known to host a large array of microbial symbionts such as heterotrophic bacteria, cyanobacteria, archaea, fungi, and microalgae. Based on the amount of microorganisms living within their tissues, sponges have been classified as high microbial abundance (HMA) and low microbial abundance (LMA) species, even though this classification seems to be too simplistic in some cases. For some potent bioactive compounds isolated from sponges, the associated microorganisms (especially heterotrophic and phototrophic bacteria as well as dinoflagellates) have been shown to be the true producers.

The general aim of this project was to investigate possible biological mechanisms underlying the bioactivity detected in several Irish and Mediterranean sponge species currently placed into the genus *Haliclona* (Grant, 1841), i.e. *H. oculata*, *H. mediterranea*, *H. cinerea*, *H. fulva*, *H. mucosa*, *H. simulans*, *H. sarai*, *H. viscosa* and *H. indistincta*. The nine species studied were selected as representative of the main phylogenetic clades within the order Haplosclerida with the idea that all the information gathered could also contribute to solve the challenging taxonomic classification of the species within this order via an integrative taxonomy approach.

In this sense, I firstly investigate the bacteria and archaea associated with the five Irish *Haliclona* species using transmission electron microscopy (TEM) and a next generation sequencing approach. Both methods determined all the sponges are LMA with species-specific microbial associations. However, the 16S rRNA gene data showed a high bacterial diversity that is incongruent with the sparse microbial diversity observed via TEM, suggesting that ecological factors such as diet and substrate contribute in shaping the predominant bacterial communities observed in these sponges. The TEM analysis also showed the presence of putative intercellular fungal spores, thus I have applied a cultivation-dependent method to analyse the fungi associated with all target *Haliclona* species. Also in this case the results obtained suggested a possible environmental origin for the fungi isolated and therefore a

doubtful involvement of these microorganisms in the production of any bioactive compounds.

I also used TEM to characterise cells with inclusions in the target *Haliclona* species because these types of cell have been associated with the production and/or storage of bioactive compounds in numerous other sponges. This has given me the opportunity to describe a novel type of cell with inclusions abundant in the tissue of *H. indistincta*, *H. viscosa* and *H. sarai*. These cells show a remarkably similar morphology to mucus-producing cells in higher animals and in fact all three species produce a similar type of thick sticky mucus. The attempts made to isolate these cells and characterise them chemically in order to determine if they contain the bioactive compounds have failed, thus I have chased the putative mucous nature of these cells to obtain a marker that could allow their isolation. For this reason I carried out a proteomic study of *H. indistincta* aimed at ascertaining the presence of mucins in this species. As comparison, I performed the same analysis on other two target *Haliclona* species producing mucus with different physical properties, i.e. *H. cinerea* and *H. mucosa*. The analysis showed that no true mucins are present in any of the three species, but all of them produce different mucin-like proteins. The further characterisation of these glycoproteins could give additional clues to help isolate the cells with inclusions in *H. indistincta*, *H. viscosa* and *H. sarai*, but also to investigate the possible role played by the mucin-like proteins in shaping the microbial communities hosted by the sponge species considered.

CHAPTER 1

General introduction

The review included in this chapter contributed to the published manuscript:

TRIBALAT, M.-A., MARRA, M. V., McCORMACK, G. P. & THOMAS, O. P. (2016), Does the chemical diversity of the order Haplosclerida (phylum Porifera: class Demospongia) fit with current taxonomic classification? *Planta Medica,* 82**,** 843–856.

Origin of bioactive compounds from Porifera

The present study aims at contributing to the comprehension of the biological mechanisms responsible for the chemical activity of sponges (phylum Porifera). Marine invertebrates are a prolific source of bioactive compounds with pharmacological application and sponges represent, at the moment, the taxonomic group with the highest potential (see for instance Munro et al., 1999, Sipkema et al., 2005, Anjum et al., 2016). The study of the peculiar chemical compounds isolated from sponges started in the 1950s (Mehbub et al., 2014), with the nucleosides isolated from the sponge *Tectitethya crypta* by Bergmann and Feeney (1951). However, awareness about the therapeutic potential of the compounds isolated from sponges developed more recently and probably started with the discovery of compounds with anti-inflammatory properties in the sponge *Halichondria* (*Halichondria*) *moorei* (Gregson et al., 1979). Since then, a huge number of compounds have been isolated from these invertebrates, some of which are currently active ingredients of pharmaceuticals on the market (Martins et al., 2014).

Bioactive molecules isolated from sponges are considered secondary products of metabolism (Rinehart, 1992) and, even though they are strictly not fundamental for the survival of these animals, these compounds play key ecological roles: in fact, generally sponges lack physical defences, therefore the production of bioactive compounds represents the first line of defence for these invertebrates (Johnston and Hildemann, 1982). The bioactive compounds play several roles in sponge ecology, having e.g. antipathogenic, anti-predatory, antifouling activity, and having a role in competition (Sullivan et al., 1983, Evans-Illidge et al., 1999, Garson et al., 1999, Chaves-Fonnegra et al., 2008, Rohde et al., 2015). Sometimes, in order to deter their predators or their competitors, sponges do not need to be ingested or touched, but their 'odor' is sufficient (Duque et al., 2001, Pawlik et al., 2002), because they are able to release the bioactive compounds in the surrounding seawater (Ternon et al., 2016).

Human interest in secondary metabolites with bioactive properties isolated from sponges is due to the fact that these compounds have been proved to have several different properties with pharmacological applications, mainly antibacterial, antiviral, antifungal, antimalarial, anti-inflammatory, antitumor, immunosuppressive, cardiovascular, antihelminthic, and muscle relaxant (Anjum et al., 2016). From a chemical point of view, the bioactive compounds isolated from sponges were classified by Rinehart (1992) as products of linear condensation of acetate, propionate or butyrate (i.e. polyketides, macrolides and polyethers), terpenes and steroids, modified amino acids and alkaloids.

A very important aspect of the bioactive compounds isolated from sponges is that, even when their chemical nature and their function have been clarified, much less is known about how these compounds are produced. In most cases, it is not even known whether the bioactive compounds are produced by the sponges or the organisms associated with the sponges (Bewley and Faulkner, 1998, Taylor et al., 2007, Schippers et al., 2012). In fact, due to their anatomy and physiology, porifera are well known to establish symbiosis with many other types of organisms (Wulff, 2006).

Sponges can host a large array of microorganisms such as archaea, eubacteria, fungi and microalgae (Webster and Taylor, 2012). In these associations sponges plausibly provide shelter and metabolic waste products that are used as source of nutrients by the microorganisms that, in turn, release oxygen and small organic molecules which are beneficial to the sponges (Sipkema and Blanch, 2010). The establishment of symbioses between sponges and microorganisms is not surprising also considering that these invertebrates are very efficient filter feeders and via a complex aquiferous system can retain from the surrounding seawater viruses, heterotrophic and autotrophic bacteria, yeasts, flagellates, ciliates and microalgae (Yahel et al., 2006). The water flow inwards is determined by the low pressure generated by the beating of the flagella characterising the choanocytes lining the surface of the choanoderm (Figure 1.1). The collar of microvilli surrounding the base

of the flagella acts like a trap for the organic matter contained in the water. This organic matter is phagocytised by the same choanocytes and to a lesser extent by pinacocytes and then transferred to the amebocytes in the mesohyl inside which digestion is completed (Bergquist, 1978, Simpson, 1984, Leys and Hill, 2012). The mechanism of seawater filtration appears to be controlled by the sponges, as observed by Maldonado et al. (2010) with the sponge *Hymeniacidon perlevis* that was shown to be more efficient in removing the bacterium *Escherichia coli* from the surrounding seawater than the pathogenic species *Vibrio anguillarum*. This selective ability of the sponges is of great importance for their symbiotic relationships with microorganisms. In fact, these invertebrates were found able to distinguish between food and symbiotic bacteria by Wilkinson et al. (1984) and Wehrl et al. (2007) in feeding experiments in which *Aplysina* species were supplied with a mix of bacteria containing also microbes isolated from the mesohyl of these sponges and labelled in order to be visualised. The sponges did not retain from the seawater the symbiotic microbial species which were not used for consumption. How sponges can discriminate between food and symbiotic microorganisms is not known yet, and it is supposed the involvement of an immune response (Webster and Thomas, 2016) or the presence of sponge lectins (Müller et al., 1981) or eukaryotic-like proteins on the surface of the hosted bacteria (Reynolds and Thomas, 2016).

Even though Faulkner et al. (1994) suggested that it might be more advantageous for sponges to concentrate a bioactive compound produced by a microorganism present in the surrounding seawater via filtration rather than to maintain an actual symbiotic relationship with it, it is clear that this is not the case. In many sponge species the associated microbial consortia are maintained via the transfer of symbiotic heterotrophic and photosynthetic bacteria, fungi and microalgae from maternal sponges to the next generation (vertical transmission) through eggs in oviparous sponges or through larvae in viviparous sponges

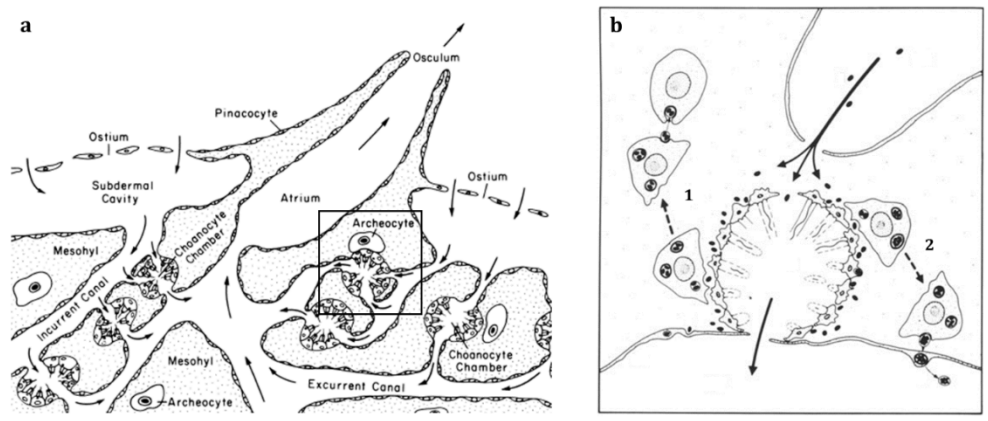

Figure 1.1. Water flow and bacteria uptake in sponges. **a**. Diagram modified from Reiswig et al. (2010) showing the water flow pattern from the ostia through the incurrent canals to the choanocyte chambers, and from there to the excurrent canals and out from the osculum. **b**. Detail of the situation marked with the black square in **a**, showing the pathway of bacteria entering the sponge tissues from the incurrent canals. Bacteria can be caught by either choanocytes or archeocytes, or they can avoid digestion and enter the sponge mesohyl where they are still susceptible to uptake by the pinacocytes lining the excurrent canals. The digestive vacuoles containing the bacteria and produced by the archeocytes (or transferred to those cells from the choanocytes) can be furtherly transferred to other archeocytes in the mesohyl to complete the digestion or to the pinacocytes to be released into the excurrent canals. Modified from Simpson (1984).

(Usher et al., 2001, Ereskovsky et al., 2005, Maldonado et al., 2005, Enticknap et al., 2006, Schmitt et al., 2008, Wang et al., 2012). In this sense, the presence of bacteria could explain chemical defences detected in some sponge larvae (Lindquist and Hay, 1996, Enticknap et al., 2006). The process of vertical transmission has also been observed to happen asexually, i.e. with zoochlorellae contained intracellularly in gemmules produced by the freshwater species *Spongilla lacustris* (Williamson, 1979) and with filamentous cyanobacteria contained in a budding specimen of *Tethya orphei* (Gaino et al., 2006).

However, in several cases sponges have been reported to acquire their microbial symbionts from the ambient seawater through a mechanism of horizontal transmission (Taylor et al., 2007, Schmitt et al., 2008, Alex et al., 2012, Sipkema et al., 2015). Therefore, vertical and horizontal symbiont transmission concur to create a microbial consortium that has been determined to be species-specific in numerous sponges (Thacker,

2005, Holmes and Blanch, 2007, Schmitt et al., 2012, Simister et al., 2012, Taylor et al., 2013, Moitinho-Silva et al., 2014, Zhang et al., 2014, Chaib De Mares et al., 2017, Steinert et al., 2017). These specialist microbial communities are maintained over time (Taylor et al., 2004) and in spite of large geographic distances among host populations (Erwin et al., 2011). They have been found to be stable also when sponges are transplanted between different habitats (Cardenas et al., 2014) or when sponges grow in polluted areas (Gantt et al., 2017).

The importance of knowing which are the actual producers of the bioactive compounds of interest between the sponge cells and the associated microorganisms is strictly related to the 'supply issue', i.e. the reliance of preclinical development studies on natural sources of compounds before synthesis routes are established (Schippers et al., 2012). In fact, once the source of the bioactive compounds has been determined, *in vitro* culture of target sponge cells or associated microorganisms could be the way to yield enough molecules of interest to supply the biodiscovery pipeline without affecting the sponge natural populations. However, despite numerous attempts having been made over the years, the establishment of a long-term, continuously dividing sponge cell line has not yet been achieved and only partial success has been obtained with primary cultures (Grasela et al., 2012). With regard to the cultivation of sponge associated microorganisms, particularly bacteria and fungi, even though successful methodologies are applied, the fraction of the sponge microbial communities that can be cultured using currently available media and fermentation technologies is only small (Proksch et al., 2003). Moreover, coupled culture-dependent and independent studies have shown that standard cultivation techniques appear to selectively foster the growth of the less abundant bacterial phylotypes, while prevalent microsymbionts escape cultivation (Hardoim et al., 2014). Efforts in order to develop alternative culturing technologies to increase the diversity of the cultivable microorganisms from sponges have been made employing multiple cultivation techniques and media (including media containing sponge extracts) and

the results are encouraging because previously uncultivable bacteria were isolated (Li et al., 2007, Sipkema et al., 2011). Proksch et al. (2003) suggested that a viable alternative to establish a sustainable production of the compounds of interest, when available, will be to transfer the natural product encoding genes isolated from sponges into controllable systems such as e.g. *Escherichia coli*.

The use of cytological approaches (e.g. involving light microscopy and ultrastructure studies) has been shown to be very informative in order to understand whether the bioactive molecules are associated with the sponge cells rather than the associated microorganisms (Becerro et al., 2001). Traditionally, the localisation of bioactive compounds in the sponge body has occurred through different methods based on the chemical characteristics of the molecules of interest (Faulkner et al., 1999). The main techniques involved include cell separation and cell fractionation using differential centrifugation or gradient density media such as Ficoll or Percoll (Flowers et al., 1998, Fuerst et al., 1998, Richelle-Maurer et al., 2001, Salomon et al., 2001, Laroche et al., 2007, Roue et al., 2010) and subsequent biological activity, chemical and microscopic analysis of the fractions obtained; natural product MALDI-TOF imaging (npMALDI-I, even though, to be informative, this technique has to be combined with investigations on the distribution of microbes within the sponge tissue (Esquenazi et al., 2008, Yarnold et al., 2012); flow cytometry (Unson and Faulkner, 1993); fluorescent *in situ* hybridization (FISH; Flatt et al., 2005); immunohistochemistry (Gillor et al., 2000, Sakai et al., 2008).

More recently, a significant contribution to determining the microbial production of bioactive compounds of interest has derived from the application of metagenomics techniques (Abe et al., 2012, Nakashima et al., 2016).

Sponge-associated microorganisms responsible for the production of bioactive compounds

Polyketides and nonribosomal peptides are two classes of compounds characterised by a huge pharmacological potential as antibiotics, cytostatics and immuno-suppressants and largely associated with fungi and several bacterial lineages (Wang et al., 2014a). The enzyme complexes that catalyse the synthesis of these compounds are broadly distributed in sponges (Pimentel-Elardo et al., 2012, Woodhouse et al., 2013, Della Sala et al., 2014). Well known examples of polyketides isolated from sponges are represented by the antiviral and cytotoxic compounds mycalamides isolated from *Mycale* species, the antitumor onnamides and theopederins from the sponge *Theonella swinhoei*, and the icadamides isolated from a *Leiosella* sp.. All these compounds are ascribed to a group of molecules with similar structure called pederins from the name of the first member of this family of molecules isolated from the beetle *Paederus fuscipes* (Narquizian and Kocienski, 2000). After construction of a genomic library from the total DNA of the beetle *P. fuscipes*, Piel (2002) used ketosynthase-specific primers to localise the putative pederin biosynthetic gene cluster. The target genomic region obtained was sequenced including adjacent nucleotides to the gene cluster in order to obtain more information about the pederin producer and most of the open reading frames obtained showed high similarity with genes of the bacterium *Pseudomonas aeruginosa*, thus the author concluded that a very close relative of this bacterium associated with the beetle is actually responsible for the production of pederin. The involvement of the gene cluster identified in *P. fuscipes* in the biosynthesis of pederin was confirmed biochemically via the methylation of the analogous compound mycalamide A by the three methyltransferase enzymes expressed from the putative pederin biosynthetic cluster (Zimmermann et al., 2009). Via the application of the same method of genomic library construction followed by identification of the putative biosynthetic cluster via PCR with the sponge *T. swinhoei*, Piel et al. (2004) confirmed a bacterial origin also for onnamides and

theopederin. The similarity of polyketides isolated from *T. swinhoei* with similar compounds obtained from distantly related sponge species and the suspicion that prokaryotic symbionts might be responsible for the production of these compounds were previously highlighted by Bewley et al. (1996). The authors determined through differential centrifugation followed by extraction and analysis by high pressure liquid chromatography (HPLC) the association of another polyketide named swinholide A isolated from the same *T. swinhoei* with the unicellular bacteria hosted by the sponge. The authors also demonstrated with the same procedure the presence of a cyclic peptide called theopalauamide (Schmidt et al., 1998) in a different bacterial fraction constituted by filamentous bacteria.

Traditional techniques of cell separation followed by chemical analysis or screening for biological activity were also applied to determine the association of specific molecules of interest to the bacteria hosted in a sponge producing multiple bioactive compounds as *Plakortis simplex* (Laroche et al., 2007) as well as in the calcarean *Leuconia johnstoni* (Quevrain et al., 2014).

The association of sponges with bacteria are extremely variable (Figure 1.2a). Sponges characterised by bacterial population densities up to 10^8–10^{10} bacteria per gram of sponge wet weight, exceeding seawater concentrations by 2–4 orders of magnitude are classified as 'High Microbial Abundance (HMA)' sponges, while 'Low Microbial Abundance (LMA)' sponges tend to have a bacterial content within the range of natural seawater, i.e. 10^5–10^6 bacteria per gram of sponge wet weight (Hentschel et al., 2006). Even though the microbial communities of LMA sponges are not necessarily less diverse than in HMA sponges (Blanquer et al., 2013, Schoettner et al., 2013), the number of 16S rRNA gene copies over 18S rRNA gene copies have been found to be much higher in HMA than in LMA sponge species via quantitative real-time PCR (qPCR; Bayer et al., 2014). Sponges host a bacterial community in part shared among phylogenetically and geographically distantly related species and in most cases clearly distinguished from the surrounding seawater and

sediments, especially with regard to the HMA sponges (Wilkinson et al., 1981, Hentschel et al., 2002, Moitinho-Silva et al., 2014, Taylor et al., 2007, Schmitt et al., 2012, Simister et al., 2012).

A total of 38 bacterial phyla of which the most common are Actinobacteria, Chloroflexi, Cyanobacteria, Nitrospira and especially α- and γ-Proteobacteria were detected in sponges, as well as 15 candidate phyla described for the first time in these invertebrates (Schmitt et al., 2011, Thomas et al., 2016). One of the most characteristic is the candidate phylum Poribacteria that has been recorded in high numbers only in sponges so far, while it appears to be rare in other environments (i.e. seawater; Moitinho-Silva et al., 2014). This novel bacterial phylum was firstly identified by Fieseler et al. (2004) and named "Poribacteria" to acknowledge its affiliation with sponges. This lineage was shown to be monophyletic and to fit in a larger clade with the PVC superphylum, comprising Planctomycetes, Verrucomicrobia and Chlamydia lines, and with other candidate phyla such as WS3 and SAUL, the latter also associated with sponges (Kamke et al., 2014). More recently Wilson et al. (2014) provided evidence that a single member of the highly diverse microbiome of *Theonella swinhoei* is the source of almost all polyketides and peptides that have been isolated from this sponge. The bacterium was described as filamentous and recalled the non-photosynthetic filamentous bacteria previously shown via TEM in this sponge species by Magnino et al. (1999) who identified them as possible sulfur bacteria belonging to the family Beggiatoaceae. The name Candidatus *Entotheonella factor* was selected to define these bacteria and, based on the evidence that their 16S rRNA gene sequences were only 82% identical to representatives from known bacterial phyla and formed a well-separated clade, the authors suggested the status of new candidate phylum for which the name "Tectomicrobia" was chosen. The closest relatives to "Tectomicrobia" are *Nitrospina* spp., which were previously proposed to belong to a new phylum, i.e. Nitrospinae. Members of the phyla Poribacteria and Tectomicrobia were found in marine sponges

from very distant geographical regions (Lafi et al., 2009, Wilson et al., 2014).

Common members of the microbial communities associated with sponges are also the Archaea. Fuerst et al. (1999) showed for the first time via TEM the occurrence of Archaea in the mesohyl of different sponges from the Great Barrier Reef, and their morphology was especially characterised by a cell wall with a structure of regular subunits that was consistent with the S-layer wall typical of the Domain Archea (Figure 1.2b). Preston et al. (1996) were the first to document a putative symbiosis between the sponge *Dragmacidon mexicanum* and a highly abundant phylotype for which the authors proposed the specific name *Cenarchaeum symbiosum*. This archaeal species was assigned to the class Thaumarchaeota within the phylum Crenarchaeota. Now this class is recognised as a separated archaeal phylum (Brochier-Armanet et al., 2008) and is the most commonly found archaeal division in sponges (Zhang et al., 2014). The archaeal community has been reported to be dominant over the bacteria in sponges collected in extreme environments, such as the deep sea (Jackson et al., 2013).

Cyanobacteria appear to be the most important group of sponge photosynthetic microsymbionts. Their presence can significantly boost sponge growth because of the photosynthates in the form of glycerol and organic phosphate that are transferred from cyanobacterial symbionts to the sponge hosts, supplying up to 50% of the sponge's energy budget and 80% of its carbon budget (Arillo et al., 1993). However, the dependence on the association with the cyanobacterial symbionts seems to vary based on the sponge species, with e.g. *Xestospongia muta* apparently unaffected (McMurray et al., 2011) and *Sarcotragus fasciculatus* fatally hindered (Cebrian et al., 2011) following the disappearance of cyanobacteria from the sponge tissue caused by the increase of the seawater temperature. In her review of ecology and phylogeny of the sponge cyanobacterial symbionts, Usher (2008) reported that the Chroococcales 'Candidatus *Synechococcus spongiarum*' represents the largest sponge-specific clade of bacterial symbionts

known. The members of this group are small coccoid cyanobacteria with a spiral thylakoid membrane around their perimeter and, before the advent of the 16S rRNA gene sequencing, they used to be considered closely related to another cyanobacterium associated with sponges i.e., *Aphanocapsa feldmannii* (Figure 1.2c). The filamentous cyanobacterium *Oscillatoria spongeliae* from the order Oscillatoriales is another common sponge symbiont, even though molecular studies have demonstrated that it represents a species complex reported over a wide geographic range. Additional cyanobacterial taxa have been found in association with a large number of sponge species (Usher, 2008, Konstantinou et al., 2018).

Several metabolites isolated from the sponge *Lamellodysidea herbacea* have been localised in the cyanobacterial symbionts *O. spongeliae*, such as a number of polychlorinated amino acid derivatives, diketopiperazines and polybrominated biphenyl ethers in (Unson and Faulkner, 1993, Unson et al., 1994, Flowers et al., 1998, Flatt et al., 2005), while the excitatory amino acid dysiherbaine isolated from the sponge *Lendenfeldia chondrodes* was found to be associated with *Synechocystis* spp. (Sakai et al., 2008). However, it has to be taken in account, as observed by Simmons et al. (2008), that a large number of heterotrophic bacteria are stuck on the cyanobacterial cell wall and it cannot be excluded that the bioactive compounds associated with cyanobacteria are actually produced by those bacteria.

To my knowledge, there are no bioactive compounds previously isolated from sponges that were later attributed to fungal production, the only exception being the 7-dehydro-sterols found in some demosponges. These sterols are characteristic of fungi and plants, thus their production by symbiotic yeasts or microalgae has been considered. However, the fact that such microsymbionts have never been observed in the sponges from which the sterols were isolated suggested that the presence of these lipids might be due to the intake of yeasts and microalgae as food by the sponges (Bergquist et al., 1980, Stoilov et al., 1987).

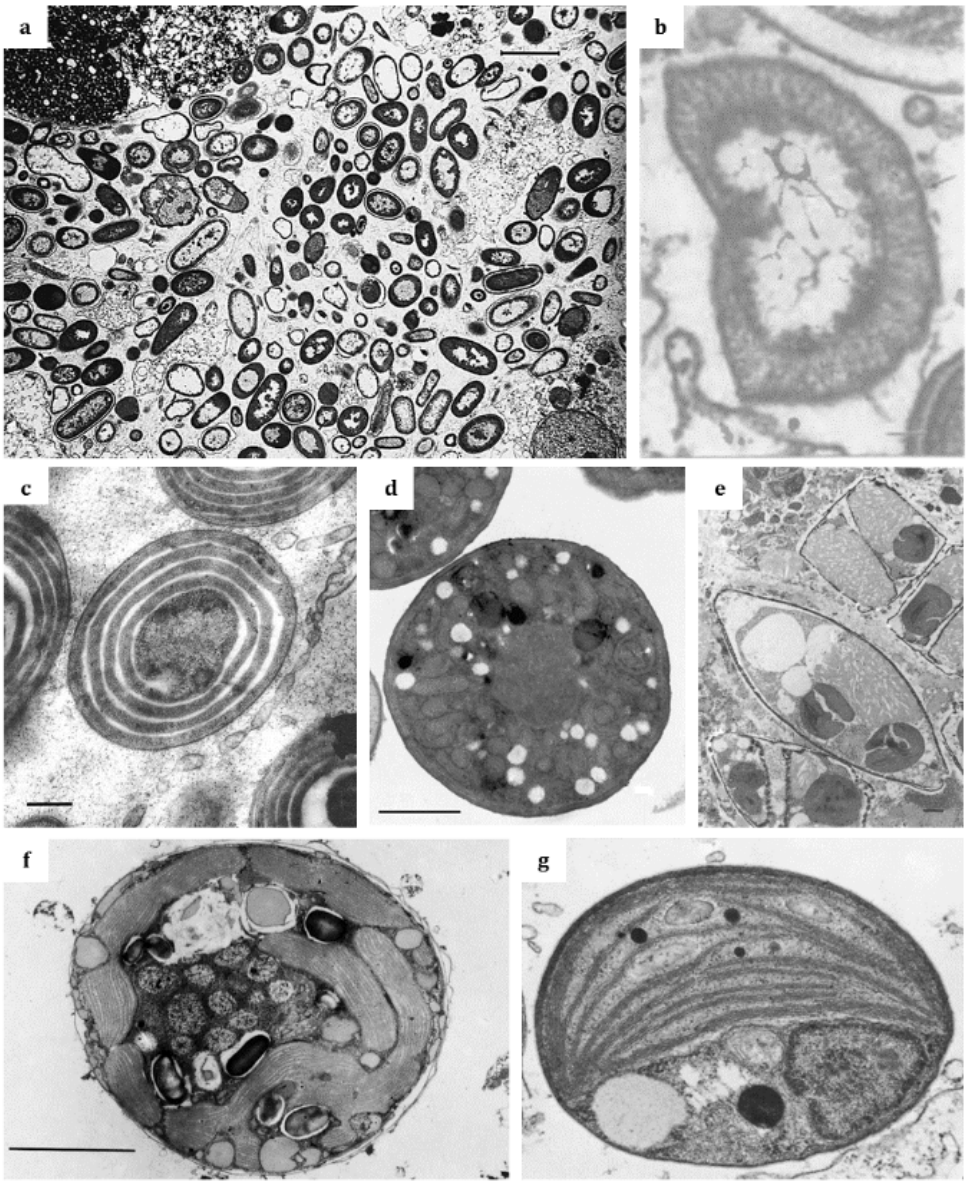

Figure 1.2. Electron micrographs of different categories of sponge microsymbionts. **a**. Several different morphotypes of heterotrophic bacteria hosted in the mesohyl of *Aplysina cavernicola* (Friedrich et al., 1999); **b**. Close-up of a D-shaped archaeal cell showing the characteristic cell wall (arrow; Fuerst et al., 1999); **c**. The cyanobacterium *Synechococcus spongiarum* with its typical spiral thylakoid (Usher et al., 2006); **d**. Cell of the marine yeast *Rhodotorula* sp. in the mesohyl of the sponge *Hymeniacidon perlevis* [image modified from Maldonado et al. (2010)]; **e**. Micrograph of differentially orientated sections of diatoms in an encrusting sponge modified from Cox and Larkum (1983); **f**. Dinoflagellate with similar morphology to *Symbodinium microadriaticum* found in the sponge *Haliclona* sp. [modified from Garson et al. (1998)]; **g**. Image of a green microalga symbiont of *Spongilla lacustris* modified from Williamson (1979). Scale bars: **a**, **d**, **e** = 1 μm; **b**, **c** = 200 nm; **f** = 2.2 μm; **g** = x 41,930.

Reports on fungi observed macroscopically or via light and electron microscopy in sponges are actually rare compared to bacteria (Figure 1.2d). The first record concerns two fungal species (*Koralionastes giganteus* and *K. violaceus*) from the Caribbean and the Australian Great Barrier Reef respectively, which were found to develop regularly in association with unidentified encrusting sponges (Kohlmeyer and Volkmannkohlmeyer, 1990). Maldonado et al. (2005) described for the first time the vertical transmission of a unicellular yeast in a Caribbean population of *Chondrilla nucula*. Gaino et al. (2014) provided the first ultrastructural evidence of the growth of fungal hyphae within both the choanodermal layer and the mesohyl matrix of the sponge *Clathrina coriacea*. The last record is considered of particular interest because it disproved the hypothesis that fungal propagules do not produce hyphae in liquid environment (Yarden, 2014).

However, in spite of the limited visual records of their actual presence in sponges, a plethora of fungi have been detected in these invertebrates through cultivation-based approaches. The sponge-associated fungi isolated so far belong mostly to the phylum Ascomycota and in particular to very common genera like *Penicillium* and *Aspergillus* (Pivkin et al., 2006, Wang et al., 2008, Li and Wang, 2009, Passarini et al., 2013, Bovio et al., 2018). Most of these fungi have been found to have significant biological activities, e.g. antibacterial, antifungal, antioxidant, cytotoxic, antitumor (Höller et al., 2000, Proksch et al., 2008, Paz et al., 2010, Zhou et al., 2011, Yu et al., 2013, Henriquez et al., 2014).

As already mentioned for the bacteria, the application of molecular techniques has highlighted a different fungal diversity compared to the cultivation-dependent approaches in most cases. A milestone in that sense is represented by the work of Gao et al. (2008) who applied molecular techniques for the first time to investigate the fungal communities of *Suberites zeteki* and *Mycale (Mycale) armata*, already studied for their cultivable associated fungi by Wang et al. (2008) and Li and Wang (2009), and found a much higher fungal diversity including five fungal orders not previously identified in sponges. However, a

limitation of the molecular exploration of sponge-associated fungi is that the number of fungal products of the polymerase chain reaction (PCR) is usually low because the primers for the target fungal genes (typically the ITS region and the 18S rRNA) amplify also the corresponding sponge host genes (Schippers et al., 2012). This issue has been partially reduced by the introduction of the next-generation sequencing (NGS) approaches (Wang et al., 2014b, Naim et al., 2017).

Sponges can also host microalgae belonging to several groups, mainly unicellular green algae, diatoms and dinoflagellates (Figure 1.2e – g). Zoochlorellae are frequently associated with freshwater sponges (Corallini and Gaino, 2001, Gaino et al., 2004), but these microalgae seem to be also present in marine species such as *Halichondria (Halichondria) panicea* (Knowlton and Highsmith, 2005). No taxonomic information is available about the sponge-associated zoochlorellae, except for those associated with *Lubomirskia baicalensis* from Lake Baikal that were isolated from the primmorphs developed from this sponge and identified as *Mychonastes* spp. (Chernogor et al., 2013).

Diatoms have been reported for being consumed by sponges as food (Gaino and Rebora, 2003, Cerrano et al., 2004a). However, cases of symbioses between sponges and diatoms are known as well, e.g. the sponge *Cymbastela concentrica*, the metabolism of which has been characterised to be interconnected with the one of a diatom and three bacterial symbionts (Moitinho-Silva et al., 2017), or the Antarctic species *Mycale (Oxymycale) acerata*, shown to host an epibiontic population of the diatom *Porannulus contentus* which probably uses the products of sponge excretion as nutrients (Cerrano et al., 2004b).

No bioactive compounds isolated from sponges have been proved to be associated with symbiotic zoochlorellae and/or diatoms, contrary to dinoflagellates. These latter microalgae (in particular species within the genus *Symbodinium*) have been long known in tropical coral reefs to be associated not only with corals, but also with burrowing sponge species in mutualistic and sometimes even parasitic relationships (Sarà and Liaci, 1964, Riesgo et al., 2014, Fang et al., 2017). There are cases of

associations between sponges and dinoflagellates that have been studied to understand whether these microalgae were responsible for the production of compounds of interest isolated from the host sponges. For instance, Garson et al. (1998) reported the association between a tropical *Haliclona* species producing potent cytotoxic and antifungal alkaloids named haliclonacyclamines and a dinoflagellate affiliated to the species *Symbodinium microadriaticum*. However, these microalgae were not found to be responsible for the production of the bioactive haliclonacycloamines after isolation via Percoll density centrifugation. Conversely, a dinoflagellate was found to be responsible for the production of okadaic acid, a cytotoxic polyether derivative of a C_{38} fatty acid which was isolated from the two congeneric sponges *Halichondria (Halichondria) okadai* and *H. (H.) melanodocia* (Tachibana et al., 1981). Given the structure of this compound, that was considered to be a possible ionophore, as well as other toxins produced by dinoflagellates such as palytoxin and ciguatoxin, the authors had already considered that the actual producer of the okadaic acid was an epiphytic microorganism. In fact, the exact same compound was successively isolated by Murakami et al. (1982) from the dinoflagellate *Prorocentrum lima*. The okadaic acid has been found to be produced also by other dinoflagellates in symbiotic relationships with sponges, such as the species *Gymnodinium sanguineum* and the sponge *L. baicalensis*, in which the compound seems to protect the sponge from the cold temperatures (Müller et al., 2007).

Sponge cells responsible for the production of bioactive compounds

Sponges possess at least 16 different cell types the function of which is known only in a few cases (Leys, 2015). After reviewing the current literature, Gaino (2011) grouped the main sponge cells into five categories. The first category is represented by the epithelial cells, namely pinacocytes and choanocytes. Pinacocytes are the cells lining the external surfaces (exopinacocytes), thus also the canals of the aquiferous

systems (endopinacocytes) as well as the region where the sponge body anchors to the substratum (basopinacocites), while the choanocytes, as mentioned already, line the choanoderm and are small cells characterised by the presence of a flagellum surrounded by an upright collar of microvillus extensions of the plasmalemma (Simpson, 1984). Porocytes are specialised contractile cells derived from the pinacocytes that generate the inhalant ostia by either shaping the canal with extensions of their cells or by several of them gathering around the ostium to create the pore (Bergquist, 1978, Leys and Hill, 2012). Porocytes are also the constitutive elements of the pinacocytic sides of the prosopyles and apopyles, while the choanocytic prosopyles and apopyles are created by pseudopodial extensions of the choanoctyes, and by ciliated cells called cone cells, respectively (Simpson, 1984, Leys and Hill, 2012).

The other four categories of sponge cells identified by Gaino (2011) are localised in the mesohyl and all have characteristics of amoeboid cells. Some of these cells are responsible for the production of the sponge skeletal elements, i.e. the sclerocytes are involved in spicule secretion, collencytes and lophocytes both release fibrillary collagen and are mostly distinguished by ultrastructural characteristics (Bergquist, 1978), while the spongocytes produce the spongin, i.e. the large collagen fibres that in the demosponges embed the spicules (or foreign particles in horny sponges; Simpson, 1984). A second type of contractile cell present in sponges are called myocytes and are characterised by the presence of numerous microtubules and microfilaments arranged in bundles parallel to the main axis of the fusiform cell (Bergquist, 1978). Sponges also possess totipotent stem cells represented by the archeocytes that are thus able to differentiate into any other type of sponge cells. From an ultrastructural point of view, archeocytes are characterised by a large nucleus and an evident nucleolus as well as the presence in the cytoplasm of numerous phagocytic vacuoles due to their function in completing the digestion of all the material transferred by the

choanocytes and pinacocytes and the bacteria and dead cells in the mesohyl (Bergquist, 1978).

The last sponge cell category reported by Gaino (2011) is represented by the so called cells with inclusions defined as all the mobile cells bearing intracellular membrane-bound vesicles. The function of these cells has been clarified only in a few cases and the inclusions they contain vary in terms of size, morphology and chemical content (Boury-Esnault, 1977, Bergquist, 1978, Simpson, 1984, Ereskovsky, 2010). A common type of cells with inclusions is represented by the spherulous cells which are characterised by very large rounded intracellular vesicles that almost occupy all the space, relegating the cytoplasm to thin strands among the vacuoles The content of their inclusions vary dramatically between sponge species (Bergquist, 1978, Gaino, 2011). Another type of cells broadly distributed in demosponges are the gray cells, so called because of the colour they show *in vivo*. These cells contain spherical or ovoid homogeneous osmophilic inclusions and are responsible for the transformation and storage of energy in the form of glycogen rosettes (Boury-Esnault, 1977). Spumeuse cells were described in *Pleraplysilla spinifera* as carrying large inclusions with finely fibrillary content which was identified as the mucus released by this sponge when handled (Donadey and Vacelet, 1977). Mucopolysaccharides are also enclosed within the rod-like inclusions of the so called rhabdiferous cells, which release their content in the mesohyl contributing to the extracellular matrix (Bergquist, 1978). A similar function is attributed also to the cystencytes, cells found only in freshwater sponges and characterised by a single large vacuole containing polysaccharides (Bergquist, 1978, Gaino, 2011). The sacculiferous cells were described for the first time by Smith (1968) in *Cyamon neon* as containing large inclusions bearing reticulating fibres and for this reason supposed to be implicated in the production of collagen fibrils (Simpson, 1984). The presence of reticular inclusions brought Gaino et al. (1986) to consider a role in collagen production also for the vacuolar cells of *Oscarella lobularis*, so called because they are characterised by the presence of large vacuoles with

aqueous content. The function of two additional types of cells with inclusions has not been determined yet, i.e. the microgranular cells, containing small dense granules sometimes with paracristalline structure, and the globoferous cells, found only in poecilosclerid sponges and characterised by a particular region of the cytoplasm called globular area where paracrystalline, hollow cylindrical rods are ordered in parallel rods (Bergquist, 1978).

Sponge cells have been shown to contain bioactive compounds, and in most cases the bioactivity has been located in the cells with inclusions, in particular the spherulous cells. For instance, the Mediterranean sponge *Crambe crambe* is known to produce a range of pentacyclic guanidine alkaloids and has been characterised as a LMA species. In fact, *C. crambe* seemed to have a stable association with a β-proteobacterium suspected to be involved in the biosynthesis of the compounds produced by the species (Croué et al., 2013). While this hypothesis has not been proved yet, the localisation of the toxic compounds in the spherulous cells of this species has been demonstrated by Uriz et al. (1996a). The authors fractionated the cells of this species through Ficoll and measured via the Microtox® bioassay that the extract obtained from the fraction enriched in spherulous cells was significantly more toxic than the other two fractions containing mainly choanocytes and archeocytes respectively. The finding was also consistent with the observation made using the same bioassay that the extract from the ectosome region of *C. crambe*, shown via light and transmission electron microscopy to be rich in spherulous cells, was more toxic than the choanosome, where the spherulous cells were scarce. Spherulous cells were also shown to contain the brominated metabolites of *Aplysina aerophoba* and *A. fistularis* as well as unconventional lipids in the latter species (Thompson et al., 1983, Lawson et al., 1988, Turon et al., 2000), lectins (Bretting and Konigsmann, 1979, Bretting et al., 1983, Dresch et al., 2011) and the alkaloids debromohymenialdisine and hymenialdisine (Song et al., 2011) from sponges of the genus *Axinella*, the furanosesquiterpenoid

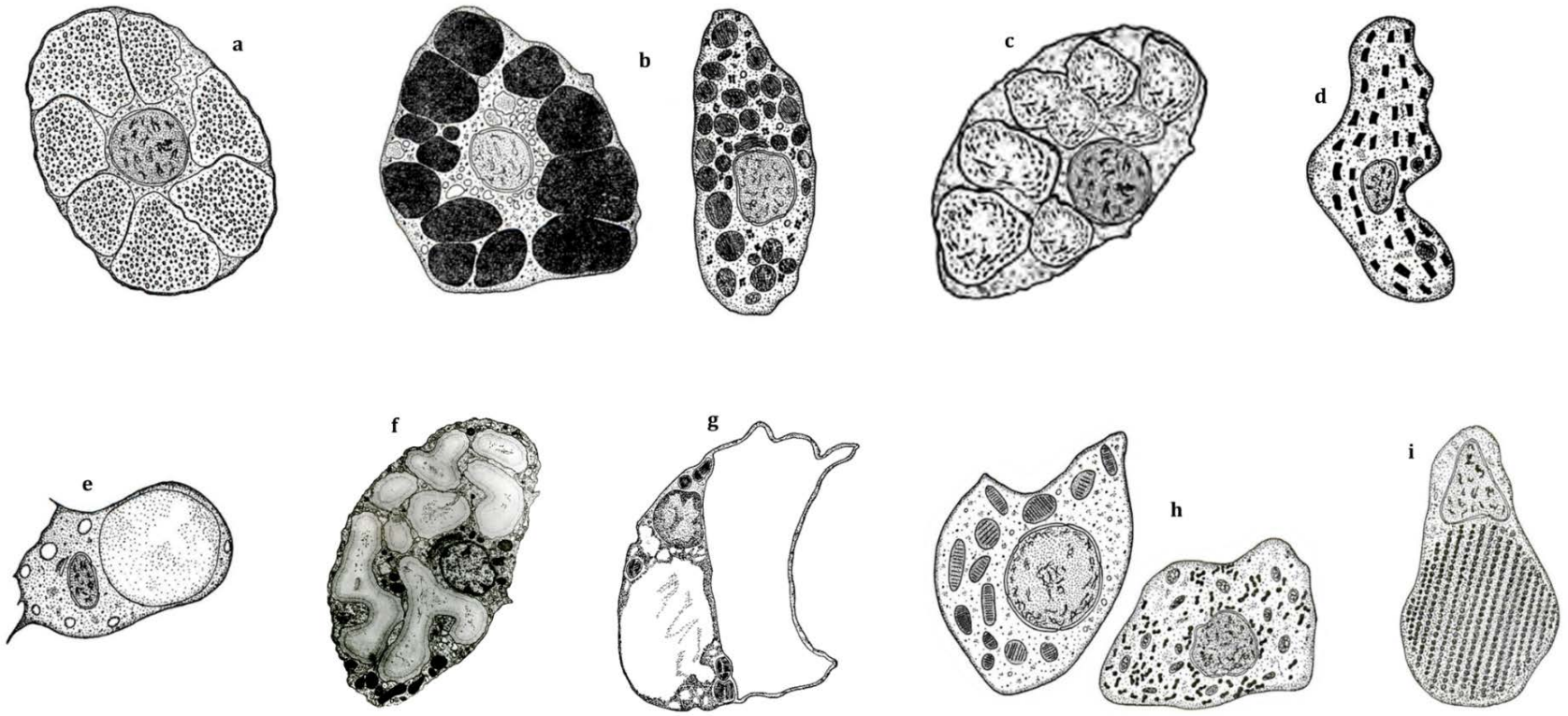

Figure 1.3. Cell with inclusions in sponges: **a**. Spheruouls cell; **b**. Two different gray cells characterised by different size and level of homogeneity of the inclusions; **c**. Spumeuse cell; **d**. Rhabdiferous cell; **e**. Cystencyte; **f**. Sacculiferous cell; **g**. Vacuolar cell; **h**. Two examples of microgranular cells; **i**. Globiferus cell. Modified after Bergquist (1978), Gaino (2011), Smith (1968) and Gaino et al. (1986).

ent-furodysin in *Dysidea fragilis* (Marin et al., 1998), the pyridoacridine alkaloid dermitamide in *Oceanapia sagittaria* (Salomon et al., 2001) and the bromopyrrole alkaloids oroidin and sceptrin in *Agelas conifera* (Richelle-Maurer et al., 2003).

Even if less common, there also reports of compounds of interest associated with different types of cells with inclusions than spherulous cells, e.g. allelogeneic cytotoxic molecules found in the grey cells of *Microciona prolifera* and *Callyspongia diffusa* (Humphreys, 1994, Yin and Humphreys, 1996) and the antimicrobial cationic peptides and proteins in the eosinophilic granular cells of *Halisarca dujardini* (Krylova et al., 2004).

Bioactive molecules of interest were determined to be localised also in different sponge cell types than cells with inclusions. Gillor et al. (2000) suggested that the production of the actin-binding 2-thiazolidinone macrolide latrunculin B occurs in the choanocytes of the Red Sea sponge *Negombata magnifica*, while the archeocytes of this species store and mobilise the compound in the sponge mesohyl. The already mentioned work by Garson et al. (1998) showed the association of the alkaloid haliclonacyclamines with sponge cells rather than the dinoflagellates associated with the sponge *Haliclona* sp., even though the authors highlighted the need for additional techniques such as flow cytometry to determine which cell type between choanocytes, spongocytes and possibly archeocytes are the actual producers of the alkaloids. Several unusual lipids have been determined to be distributed differently in more than one sponge cell type from various sponge species, especially archeocytes and choanocytes (Zimmerman et al., 1989, Zimmerman et al., 1990, Garson et al., 1992, Garson et al., 1994, Flowers et al., 1998). Archeocytes have also been shown to be the site of production of the antimicrobial (2R, 3R, 7Z)-2-aminotetradec-7-ene-1, 3-diol in *Haliclona vansoesti* (Richelle-Maurer et al., 2001). Uriz et al. (1996b) instead demonstrated that the anti-inflammatory sesquiterpene hydroquinone avarol produced by the sponge *Dysidea avara* is located in the choanocytes of this species and not in spherulous cells as previously

reported (Müller et al., 1986). The authors pointed out that *D. avara* does not actually possess true spherulous cells and the misinterpretation made by Müller et al. (1986) could be attributed to the impossibility to observe the typical morphological features of the choanocytes (e.g. the flagellum) in fixed cells. In this sense it has to be taken in account that the correct identification of the sponge cells containing the bioactive compounds of interest in experiments involving cell dissociation/fractionation can be affected by the fact that the dissociated cells in suspension acquire spherical shape, exception being the choanocytes that retain flagella and collars of microvilli (Lavrov and Kosevich, 2014). This could explain why, besides the cells with inclusions, the molecules of interest have been localised so far especially in choanocytes and archeocytes.

Biological factors implicated in the bioactivity of *Haliclona* species

In this research project I have explored the biological factors implicated in the bioactivity detected in nine marine sponges within the order of demosponges called Haplosclerida, one of the most important in the phylum Porifera for number and novelty of the compounds isolated (Mehbub et al., 2014, Tribalat et al., 2016). The sponges ascribed to this taxon are mostly characterised by diactinal megascleres (i.e. oxeas or strongyles) organised in an isodictyal anisotropic or isotropic skeleton; microscleres (i.e. microxeas, microstrongyles, sigmas, toxas, raphids and amphidiscs) can be present too (Van Soest and Hooper, 2002). The limited number of spicule types present in the sponges of this order, in spite of the very diverse architectures of the skeleton, represents a major obstacle in the classification of the haplosclerid sponges based on morphological features (Redmond et al., 2007). For this reason, the classification has been revised in recent years through different molecular studies using genetic markers targeting different regions within the rRNA gene sequences, and these studies have shown that the order cannot be considered monophyletic when it includes the

freshwater sponges (McCormack et al., 2002, Raleigh et al., 2007, Redmond et al., 2007, Redmond et al., 2011). Overall, the molecular data collected indicate the existence of four main clades within the monophylethic marine Haplosclerida plus a range of highly variable smaller clades (Redmond et al., 2011).

Within the order Haplosclerida, the genus *Haliclona* Grant, 1841 is one of the most explicative example of discrepancy between morphology– and molecular–based classifications. After a systematic revision of the species belonging to the family Chalinidae in both North–Eastern Atlantic and Caribbean shallow waters, De Weerdt (1986, 1989, 2000) grouped the species of the genus *Haliclona* in six subgenera, i.e. *Haliclona*, *Reniera*, *Soestella*, *Halichoclona*, *Gellius*, *Rhizoniera*, collapsing in some cases several pre-existing species into single species as for *H. oculata*, *H. urceolus*, *H. simulans* and *H. cinerea* (De Weerdt, 1986), even though this has not been confirmed by molecular data in cases like *H. oculata* and *H. cinerea* (Raleigh et al., 2007, Redmond and McCormack, 2009). However, the molecular studies showed the genus *Haliclona* to be dramatically polyphyletic, with most of species included in the haplosclerid clade A, but others distributed in clade B and C (Redmond et al., 2011) and even in the smaller clades.

An effective approach to solve the incongruities between morphological and molecular data in sponge classification is to take into account additional data, e.g. the bioactive compounds produced by the species studied (chemosystematics), their cytology, the microbial associations in which they are involved and their biogeographical distribution. This approach is called integrative taxonomy and it is considered to be particular useful in cases like the order Haplosclerida (Cardenas et al., 2012, Leal et al., 2017).

One of the main research focuses of the Molecular Evolution and Systematics laboratory (Zoology Department, NUI Galway) in which I have carried out this research project is to contribute to clarify the classification of the order Haplosclerida using the integrative approach.

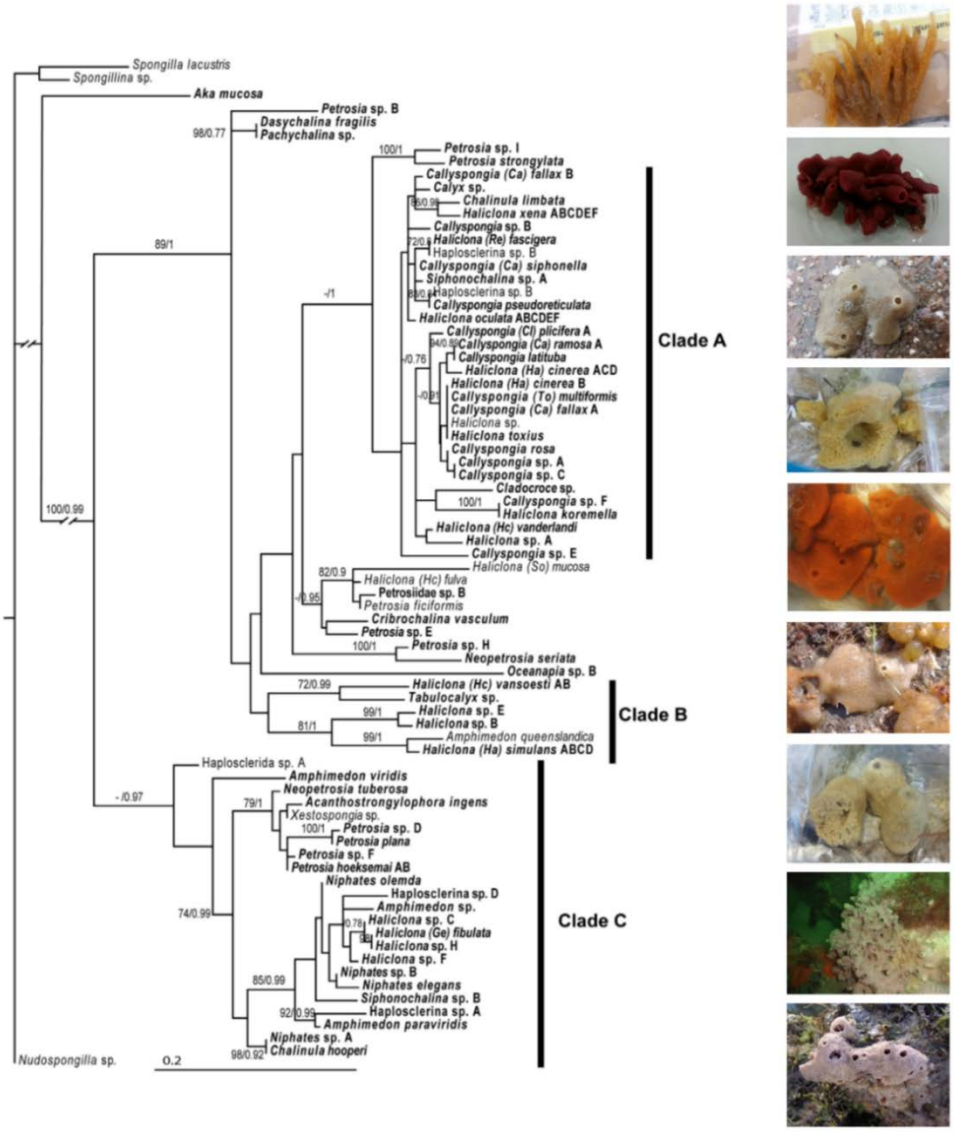

Figure 1.4. Maximum-likelihood phylogeny of the order Haplosclerida reconstructed from the D1 region of the 28S rRNA gene (from Redmond et al., 2011) and pictures of the *Haliclona* species studied in this project. From the top to the bottom: *H. oculata*, *H. mediterranea* and *H. cinerea* are all members of clade A; *H. mucosa* and *H. fulva* in the phylogeny tree show an intermediate location between clade A and B; *H. simulans* is the only clade B member considered in this project; *H. sarai*, *H. viscosa* and *H. indistincta* all belong to clade C, with *H. viscosa* and *H. indistincta* very closely related (sister species).

For this reason, the species selected for my project are currently placed into the genus *Haliclona* (as said above, one of the most challenging from a systematic point of view). More precisely, the species selected belong to different subgenera and are distantly related as shown in the phylogenetic tree in Figure 1.4, i.e. *H. (Haliclona) oculata* (Linnaeus, 1759), *H. (Haliclona) simulans* (Johnston, 1842), *H. (Reniera) cinerea* (Grant, 1826), *H. (Rhizoniera) viscosa* (Topsent, 1888) and *H. (Rhizoniera) indistincta* (Bowerbank, 1866) from the North-Eastern Atlantic, and *H. (Reniera) mediterranea* (Griessinger, 1971), *H. (Halichoclona) fulva* (Topsent, 1893), *H. (Soestella) mucosa* (Griessinger, 1971) and *H. (Rhizoniera) sarai* (Pulitzer-Finali, 1969) collected in the Mediterranean Sea. In fact, despite De Weerdt (1986) placed *H. oculata* and *H. simulans* in the same ('*oculata*') group based on the skeletal arrangement, however, the phylogenetic trees reconstructed from the D1 region of the 28S rRNA gene and Folmer (5') region of the *cox1* gene showed that the two species belong to clade A and B respectively (Redmond et al., 2011).

H. oculata has been reported to produce novel sterols (Yu et al., 2006, Findlay and Patil, 1985), but the only biological activity that has been detected so far, i.e. against the lymphatic filariasis caused by helminth parasites, was attributed to a mixture of four alkaloids previously isolated from other haplosclerid sponges (Gupta et al., 2012). However, in the latter study the collection site of the *H. oculata* samples was outside the documented distribution of this species as reported on the World Porifera Database, therefore doubts about the correct taxonomical identification of the sponges can be raised. Two additional hydroperoxide steroids were isolated from *H. oculata* here in NUI Galway by Prof. Olivier Thomas and his collaborators (Firsova, 2017).

Three steroids, of which two novel, were isolated from a specimen of *H. simulans* collected in Irish waters and determined to have antitrypanosomal and antimycobacterial properties (Viegelmann et al., 2014). The microbial associations of *H. simulans* have also been

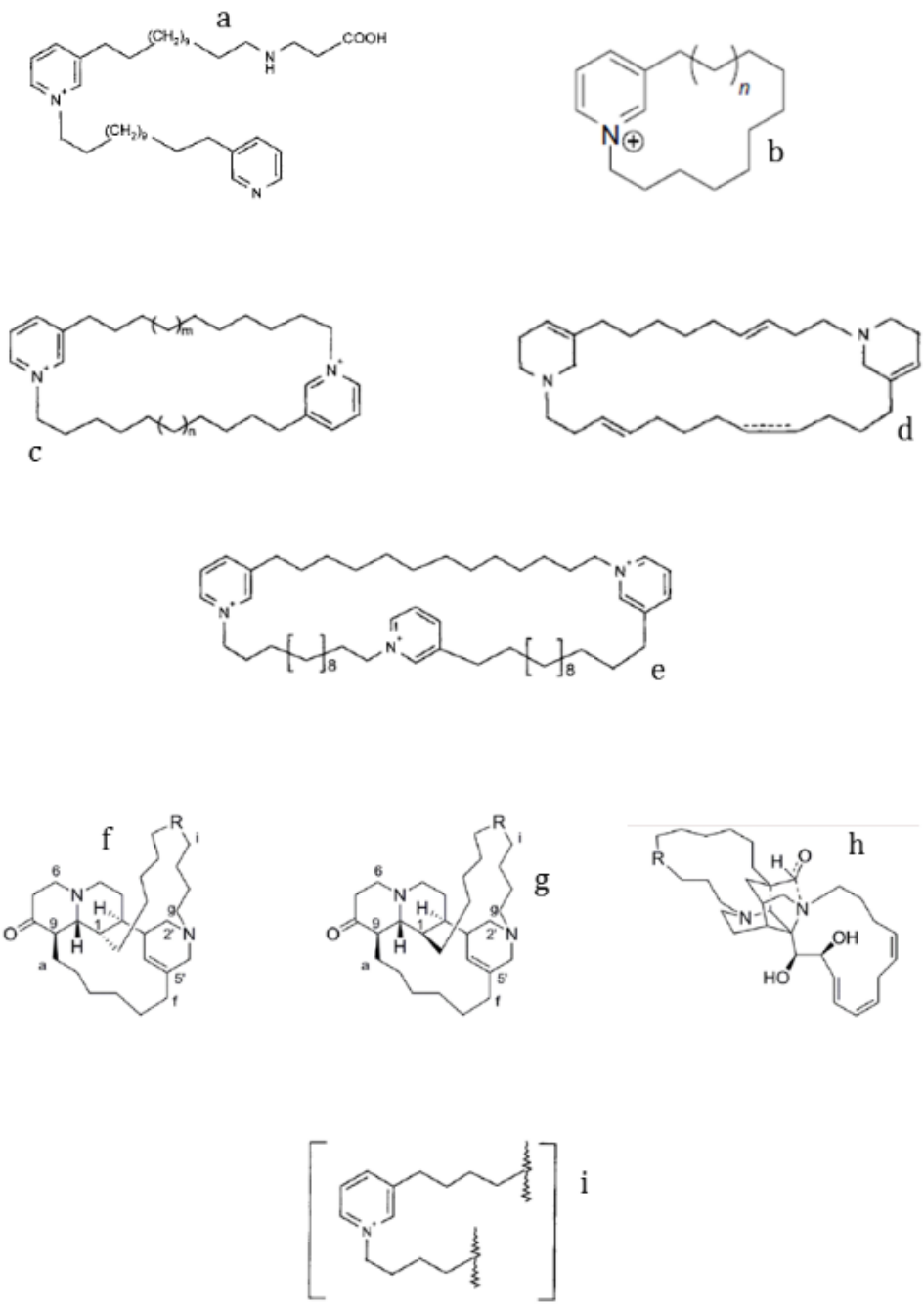

Figure 1.5. Examples of 3-alkylpyridine alkaloids from the target *Haliclona* species: **a**. Viscosaline; **b**. Haliclocyclin; **c**. Cyclostellettamine; **d**. Haliclamine, **e**. Viscosamine, **f**. Sarains (1 – 3); **g**. Isosarain; **h**. Sarains (A – C); **i**. Polymeric 3-AP salts.

extensively studied and two fungal genotypes from the orders Eurotiales and Chaetothyriales were suggested to be symbiotically associated to this sponges species (Baker et al., 2009), while seven ketosynthase (KS) sequences affiliated with PKS genes from members of the Cyanobacteria, Myxobacteria and Dinoflagellata were obtained from the metagenome of *H. simulans*, suggesting for the microbiome associated to this sponge the potential to produce this type of secondary metabolites (Kennedy et al., 2008).

No compounds described in the literature were isolated from *H. mediterranea*, but fatty acid derivatives have been isolated from this species at the Chemistry Department of the University Nice Sophia Antipolis (Marie-Aude Tribalat, personal communication). Novel fatty acids as well as sterols have been isolated also from *H. cinerea* (Joh et al., 1997, Elenkov et al., 1999), but again a discrepancy between the documented distribution of this species and the collections sites of the specimens used for these studies raises doubts about the correct identification of the species.

H. fulva and *H. mucosa* have been more intensively studied. Similar to *H. mediterranea*, they both produce fatty acid derivatives, such as the cladocroic acid and the 3-*epi*-cladocroic acid isolated from *H. fulva* (Genta-Jouve and Thomas, 2013) and the eicosanoid mucosin from *H. mucosa* (Casapullo et al., 1997). *H. fulva* also produces a number of acetylenic compounds such as the renierins (Cimino and De Stefano, 1977), fulvinol (Ortega et al., 1996) and the fulvynes (Nuzzo et al., 2012), plus six sesquiterpenes of which two were described for the first time in this species (i.e. fulvanin 1 and fulvanin 2) and the other four were known paniceins already described in *H. (H.) panicea* (Casapullo et al., 1993). The same sesquiterpenes have been isolated also from *H. mucosa*, but this sponge produces additional cytotoxic paniceins and two related cyclohexenones termed renierin A and renierin B (Zubía et al., 1994).

The bioactive compounds produced by the closely related *H. sarai*, *H. viscosa* and *H. indistincta* are 3–alkylpyridine (3–AP) alkaloids (Figure 1.5). These compounds are characteristic of haplosclerid sponges and

have been isolated from members of the family Callyspongiidae, Chalinidae, Niphatidae and Petrosiidae (Tribalat et al., 2016). 3-APs show a broad spectrum of biological activities, primarily cytotoxic, ichthyotoxic, bacteriostatic, enzyme inhibitor, transfective and antifouling (Turk et al., 2008). Two main classes of 3–APs have been described from *H. sarai*. The first gathers the macrocyclic diamine alkaloids sarains 1, 2, 3 (Cimino et al., 1986), their isomers isosarains 1, 2, 3 (Cimino et al., 1989b, Cimino et al., 1991, Guo et al., 1996) and sarains A, B, C (Cimino et al., 1989a). *H. sarai* also produces 3-AP salts that are polymers of subunits linked 'head to tail' to the nitrogen of the contiguous monomer and are characterised by potent anti-acetylcholinesterase and haemolytic action (Sepčič et al., 1997a, Sepčič, 2000). Several 3-AP compounds have been isolated also from *H. viscosa*, but the structure has been elucidated only for some of them, which can be classified in five structural classes (Köck et al., 2013): the linear dimeric viscosalines C (Volk and Kock, 2004), B_1, B_2, E_1 and E_2 (Schmidt et al., 2012) and then the macrocyclic 3–APs furtherly categorised based on the number of pyridine moieties in the monomeric haliclocyclins C and F (Schmidt et al., 2011), the dimeric cyclostellettamine C (Timm, 2007) and haliclamines C, D (Volk et al., 2004), E, F (Schmidt et al., 2009), G, H (Cychon et al., 2012), and the trimeric viscosamine C (Volk and Kock, 2003). Cyclostellettamine C was previously isolated by Fusetani et al. (1994) from the tetractinellid *Stelletta maxima*, even though it is suspected that it was contaminated by a *Haliclona* (Turk et al., 2008). It has to be mentioned that recently a different type of compound, i.e. a sphingosine derivative with antifungal properties named haliscosamine, has been isolated from *H. viscosa* (El-Amraoui et al., 2013). The first investigation of the bioactive compounds produced by *H. indistincta* was performed by Prof. Thomas and his research team. Firsova (2017) determined via nuclear magnetic resonance (NMR) that a polymeric 3–AP is a major compound within the methanolic fraction obtained through vacuum liquid chromatography of the crude extract from *H. indistincta*. However, the structure of this polymer has not been fully characterised

yet because the compound showed the tendency to stick to the column bed when HPLC was attempted in order to purify it. The author suggested that this behaviour contributes to furtherly confirm the polymeric nature of the 3–AP identified in *H. indistincta* because the same was reported by Sepčič (2000) for the polymeric 3–AP salts isolated from *H. sarai*.

The current knowledge of the bioactive compounds produced by the *Haliclona* species here considered, particularly broad for some species as e.g. *H. sarai*, is not matched by an equally detailed knowledge about either the potential microorganisms associated with these species or the presence of peculiar cell types that could be associated with the production of the compounds of interest. For this reason, the focus, and scope, of this research project was to collect data about the microbial associations and the cytology of the target *Haliclona* species. In the light of the literature review provided above, my hypothesis was that, if the target *Haliclona* species present associated microorganisms, these could be responsible of the production of the bioactive compounds, or if instead no symbiotic organisms are present, the compounds of interest could be produced by specific cell types. This aim was pursued firstly via an ultrastructural study of most of the target sponge species to determine presence of bacterial, fungal, microalgal cells and cells with inclusions. The presence of bacteria has been furtherly investigated through a NGS study of the 16S rRNA genes present in the Irish *Haliclona* species, while all the target sponge species were analysed in a cultivation-based study of their fungal associates. Finally, the cells with inclusions I found in *H. sarai*, *H. viscosa* and *H. indistincta* were examined in order to determine their function as well as their involvement in the production/storage of the bioactive compounds produced by these sponge species.

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CHAPTER 2

Exploring the microbiome of five Haplosclerid sponges (Porifera, Demospongiae)

The data included in this chapter contributed to the published manuscript:

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Introduction

Sponges (phylum Porifera) are involved in a large variety of associations with microorganisms and those with prokaryotes have been particularly well studied (for recent reviews see Webster and Taylor (2012) and Webster and Thomas (2016)). Bacteria and Archaea have been found to play several different roles in sponges including photosynthesis (Arillo et al., 1993, Freeman and Thacker, 2011), nitrogen fixation (Wilkinson and Fay, 1979), nitrification (Schlappy et al., 2010), denitrification (Han et al., 2013, Zhang et al., 2013), methane oxidation (Vacelet et al., 1996), sulfate reduction (Zhang et al., 2015), elimination of metabolic waste (Beer and Ilan, 1998) and stabilization of the sponge skeleton (Rützler, 1985). In addition to their significance for sponge biology, the symbioses between sponges and microorganisms is of great interest from a biotechnological point of view, because sponge microsymbionts have been found to be responsible for the production of bioactive compounds with pharmacological potential from some species (for recent reviews see Taylor et al., 2007; Thomas et al., 2010 and Mehbub et al., 2014).

Transmission electron microscopy (TEM) has been used for over 50 years to examine the morphology, abundance and location of the sponge microsymbionts (e.g. in the mesohyl (Wilkinson, 1978), intracellular (Vacelet and Donadey, 1977) and intranuclear (Vacelet, 1970)). This approach revealed that sponges can harbour a large number of microorganisms or, conversely, their tissues can appear largely free of bacteria introducing the differentiation between bacteriosponges and non-symbiont-harbouring “normal” sponges (Reiswig, 1981), now termed “high microbial abundance (HMA) sponges” and “low microbial abundance (LMA) sponges” (Hentschel et al., 2003, Hentschel et al., 2006, Bayer et al., 2014). HMA sponges host a more abundant and typically a more diverse bacterial community than LMA sponges (Giles et al., 2013, Moitinho-Silva et al., 2014), even though exceptions are known (e.g. Easson and Thacker, 2014; Thomas et al., 2016). Furthermore, LMA and HMA sponges seem to differ in body structure and the resulting pumping rate, with LMA sponges showing a loose mesohyl and a well-developed

aquiferous system, whereas HMA sponges are characterized by a dense mesohyl and a reduced aquiferous system (Vacelet and Donadey, 1977, Weisz et al., 2008, Schlappy et al., 2010), but again there are exceptions to this dichotomy, as observed for *Xestospongia muta* and *X. testudinaria* (Montalvo and Hill, 2011).

Over the last 20 years analyses of 16S rRNA gene sequences isolated from a large number of different Porifera species highlighted a wide diversity of bacteria hosted in sponges. Typically, sponge microsymbionts belong to the phyla Proteobacteria, Bacteroidetes, Chloroflexi, Acidobacteria, Actinobacteria or to the sponge-specific bacterial phyla such as Poribacteria and Tectomicrobia (Fieseler et al., 2004, Wilson et al., 2014). However, in the last decade the introduction of deep sequencing techniques revealed the presence of a large number of additional bacterial phyla, many of which are 'candidate' phyla, that are present only at a very low abundance (Webster et al., 2010, Schmitt et al., 2012, Thomas et al., 2016). Bacterial community analysis of several different sponge species established in many cases the presence of species-specific bacterial communities largely different from the surrounding seawater and in most cases consistent across different geographical sites (Hentschel et al., 2002, Webster et al., 2004, Lee et al., 2011, Gloeckner et al., 2013, Hardoim and Costa, 2014). To highlight the fact that these "microbial signatures" were usually undetected or very rare outside the sponge environment, Hentschel et al. (2002) introduced the term "sponge-specific 16S rRNA gene sequence clusters" to define groups of sequences that were both phylogenetically more similar to each other than to sequences from non-sponge sources and also derived from two or more sponge species or the same species from different geographic locations (Webster and Thomas, 2016). After the introduction of next-generation techniques, the detection of sponge-specific sequence clusters outside the sponges at extremely low concentrations caused the introduction of the new terminology "sponge-enriched sequence clusters" (Moitinho-Silva et al., 2014).

The presence of a microbial signature in different sponge species is possibly useful in helping to clarify the relationships among sponge species where classification may be difficult as part of an integrative taxonomy approach (Muricy et al., 1999, Ruiz et al., 2014). Indeed the observation of a highly significant relationship between bacterial community composition and sponge host phylogeny (Schoettner et al., 2013, Easson and Thacker, 2014, Liu et al., 2016) supports the hypothesis that the study of the microbial communities associated with sponges can also help to identify the biological roles of the microsymbionts. Haplosclerid sponges represent one of the most taxonomically unstable demosponge clades because of low numbers of synapomorphies, plasticity of morphological characters, large number of species, and major discrepancies between morphological and molecular data (Redmond et al., 2011). In terms of microbial associations, the order Haplosclerida is known to comprise both HMA and LMA sponges (Gloeckner et al., 2014). Interest in a deeper understanding of the microbial associations in these sponges is due also to the large diversity of novel compounds of interest that have been and will be isolated from haplosclerid sponges (Mehbub et al., 2014, Tribalat et al., 2016). Among all the Haplosclerida, the genus *Haliclona* is one of the most taxonomically difficult. The genus is polyphyletic with its members distributed broadly across the order (Redmond et al., 2011), however its members are prolific producers of bioactive compounds.

Here I present a transmission electron microscopy and deep sequencing study aimed at characterising the bacterial community associated with five Irish sponge species currently placed in the genus *Haliclona* Grant, 1841 but distributed across the three major clades in the order (i.e. the type species *H. oculata*, *H. cinerea*, *H. simulans*, *H. indistincta* and *H. viscosa*). The hypothesis behind this study was that bacterial symbionts could be responsible for the production of the bioactive compounds isolated from the target sponge species (See Chapter 1), thus determining extent and composition of the bacterial communities associated to these sponges can be useful to either assess possible

correlations with the types of compounds and/or to plan more focused studies aimed at identifying the actual producers of the bioactive compounds between the sponges and the associated bacteria. In order to characterise the bacterial communities hosted by the five *Haliclona* species studied, I (i) processed samples from each species for conventional transmission electron microscopy in order to assess the presence and the location of putative sponge bacterial symbionts and the similarities among the different sponge species, (ii) analysed 16S rRNA gene data from all the target species to explore composition of their bacterial communities and assess congruence with TEM evidence and (iii) compared the microbial communities across species, with seawater samples and between sampling sites in order to ascertain the presence of stable species-specific components of bacterial communities. The samples considered for the next-generation sequencing part of this study are included in the dataset of the Earth Microbiome Project (Moitinho-Silva et al., 2017b).

Material and methods

Species studied and sampling sites

The species investigated in this work are *Haliclona* (*Haliclona*) *oculata* (Linnaeus, 1759) (type species), *H.* (*Haliclona*) *simulans* (Johnston, 1842), *H.* (*Reniera*) *cinerea* (Grant, 1826), *H.* (*Rhizoniera*) *indistincta* (Bowerbank, 1866), *H.* (*Rhizoniera*) *viscosa* (Topsent, 1888). Details of all the sponge tissue samples considered in this study are given in Table 2.1. *H. indistincta* is an intertidal/immediately sublittoral species that can show two different morphotypes: a cushion form (collected in Corranroo, Co Clare, Ireland) and a very flat form (collected in Strangford Lough, Northern Ireland). Similarly, *H. cinerea* and *H. simulans* both showed a different morphotype when collected from intertidal (Strangford Lough) versus subtidal conditions (Kilkieran bay, Co Galway, Ireland) with an encrusting form in the first case and tall chimney-like branches in the second one. *H. viscosa* and *H. oculata* were found always

in subtidal environment showing only one morphotype represented by a massive form with volcano-like oscules for *H. viscosa* from Gurraig Sound and a branching-erect form for *H. oculata* from the subtidal environment of Strangford Lough and Saint John's Point (Co. Donegal, Ireland).

When possible, three specimens for each sponge species were collected from each collection site for each approach applied. There is some, but not complete, overlap in specimens used for electron microscopy and microbiome studies, as evident in Table 2.1. For TEM, tissue samples from the top, middle and bottom part of different specimens of the five target species were fixed immediately on collection and in 2% Gluteraldehyde + 2% Paraformaldehyde in seawater 0.1M Sodium Cacodylate buffer (pH 8). For generation of microbiome data, sponge specimens were transported in seawater within plastic bags and once in the lab 250 mg of tissue were cut from each specimen using sterile scalpels, transferred to sterile 1.5 Eppendorf tubes and kept at –80°C. Seawater samples (2L) were also collected from each sampling site (except Saint John's Point, Donegal) using autoclaved glass bottles. Each seawater sample was subsequently filtered using individual 0.22 μm Sterivex filters (Durapore, Merck Millipore, Germany) and each filter stored at –80°C before processing further.

Transmission electron microscopy

After 24 hours in the primary fixative, each sample was postfixed in 1% Osmium Tetroxide in seawater 0.1 M Sodium Cacodylate for 3 hours, dehydrated through a series of increasing percentages of ethanol and embedded in low viscosity resin (TAAB Laboratories Equipment Ltd, UK). Semi-thin sections (< 1 μm) were cut with a glass knife and stained with toluidine blue to observe the general tissue organization. Ultrathin sections of the samples (50 – 70 nm) were cut using a diamond knife and stained with uranyl acetate and lead citrate. The ultrathin sections were observed through a Hitachi H7000 Transmission Electron Microscope. A minimum of 100 micrographs were taken from each sample considered.

Table 2.1. List of the sponge and seawater samples considered in this study. The codes in bold refer to samples that were considered both for TEM and deep sequencing studies.

Species	SampleID	Collection date	Collection site	Depth	Latitude	Longitude
Haliclona indistincta	(MIIG0947, MIIG0948, MIIG0949, MIIG0950, MIIG0951, MIIG0980, MIIG0981, MIIG0982, MIIG0989, **MIIG0991**, **MIIG0993**)	16/12/2013 31/01/2014 03/02/2014 01/04/2014 04/04/2014	Corranroo, Co. Clare (ROI)	0	53.472782	-9.4282
			Portaferry, Strangford Lough (UK)	0	54.389282	-5.573406
Haliclona oculata	(MIIG0985, MIIG0986, MIIG0987)	03/04/2014	St John's Point, Co. Donegal (ROI)	22	54.3385	-8.2765
	(**MIIG1003**, **MIIG1004**, **MIIG1005**, MIIG1044)	04/04/2014 19/06/2014	Portaferry, Strangford Lough (UK)	12	54.2335	-5.3434
Haliclona cinerea	(MIIG0988, **MIIG0997**, MIIG1001, MIIG1043 MIIG1141)	04/04/2014 19/06/2014 01/04/2015	Portaferry, Strangford Lough (UK)	0	54.389282	-5.573406
			Gurraig Sound, Co. Galway (ROI)	6	53.18944	-9.4014
Haliclona simulans	(MIIG1032, MIIG1033, MIIG1014, MIIG1238, MIIG1038)	21/08/2009 22/05/2014 13/08/2015 19/06/2014	Gurraig Sound, Co. Galway (ROI)	6	53.18944	-9.4014
			Portaferry, Strangford Lough (UK)		54.389282	-5.573406
Haliclona viscosa	(MIIG1012, MIIG1025, MIIG1026 MIIG1048, MIIG1049)	21/05/2014 05/06/2014 01/07/2014	Gurraig Sound, Co. Galway (ROI)	6	53.18944	-9.4014
Sea water Environmental Control	MIIG0966a	31/03/2014	Corranroo, Co. Clare (ROI)	0	53.472782	-9.4282
Sea water Environmental Control	MIIG1003a	04/04/2014	Portaferry, Strangford Lough (UK)	12	54.2335	-5.3434
Sea water Environmental Control	MIIG1012a	21/05/2014	Gurraig Sound, Co. Galway (ROI)	6	53.18944	-9.4014

DNA extraction and amplification

DNA extraction and sequencing were performed by the Earth Microbiome Project (www.earthmicrobiome.org). The microbial DNA from each sample was extracted using the PowerLyzer® PowerSoil® DNA Extraction Kit from MoBio (https://mobio.com/products/dna-isolation/soil/powerlyzer-powersoil-dna-isolation-kit.html). The high quality DNA obtained was then used to perform a PCR in order to amplify the 16S ribosomal genes using the bacterial/archaeal primers 515F/806R (5' GTGYCAGCMGCCGCGGTAA 3' / 3' GGACTACNVGGGTWTCTAAT 5') that target the V4 region of the 16S SSU rRNA. The amplicons obtained were afterwards sequenced on the Illumina HiSeq platform (Caporaso et al., 2012). All amplicon and meta data has been made public through the Earth Microbiome Project data portal (www.microbio.me/emp).

Analysis of the 16S rRNA gene amplicons

All the analyses were performed using the QIIME pipeline (Caporaso et al., 2010b). Once extracted from the whole EMP dataset, the sequences from the Irish and Northern Irish sponge and seawater samples were subjected to open reference OTU picking using the UCLUST algorithm (Edgar, 2010) with a pairwise identity threshold of 97%. During this step the sequences were aligned with PyNAST (Caporaso et al., 2010a) and the aligned representative sequences were used as input to perform chimera checking through the ChimeraSlayer algorithm (Haas et al., 2011). Chimera and singleton sequences were then filtered out. After initial analysis of all remaining data, any sequences assigned to OTUs that were also present in the seawater samples with an abundance of > 0.1% were removed as being very likely originating from seawater contamination in the tissues of the sponge (modified from Thomas et al. 2016). Taxonomy was assigned to the representative sequences of each OTU using the most recent version of the Greengenes and RDP databases as reference, data from the former are presented here (http://greengenes.secondgenome.com/).

QIIME was further used to perform species diversity analyses of the bacterial communities focused on all the *Haliclona* plus the three seawater samples. These analyses included calculation of Chao1 richness and diversity (Shannon and Simpson) indices, and construction of rarefaction curves to assess the alpha-diversity of the microbiomes studied. Beta-diversity was assessed through principal coordinates analysis (PCoA) from unweighted and weighted UniFrac distance matrices. Jackknifing was performed to assess the robustness of the clusters in the PCoA plots (Caporaso et al., 2010b). Statistical comparisons of the alpha-diversity metrics were accomplished with two-sample t-tests, and beta-diversity differences were assessed using the adonis and the PERMDISP statistics to compare differences among species and sampling locations. A non-parametric t-test using Monte Carlo simulation was calculated to discern differences in specific taxa among species and sampling locations.

Results

Transmission Electron Microscopy

All five *Haliclona* species were determined via TEM to be LMA sponges due to the scarcity of microbes observed in the sponge tissue. Furthermore, the species also showed different microbial associations. In *H. oculata* (the type species of the genus, Figure 2.1a) the extremely large intercellular spaces in the mesohyl were almost completely free of microorganisms (Figure 2.1b-c). Bacteria were only sporadically visible in the mesohyl or inside the cells and were variable in time (Figure 2.2a). *H. cinerea* (Figure 2.1d) tissue also contained a relatively large space-cell ratio (Figure 2.1e), but for this species large rod-shaped bacteria were very evident in TEM sections (Figure 2.1f and 2.2b). The bacteria were always intact and housed within large vacuoles held in turn within individual bacteriocytes. The rounded cross sections of these bacteria were also visible in the mesohyl, but always just outside cells and slightly surrounded by the cell membranes (Figure 2.1f). The cell vacuoles were

generally found to contain up to eight-nine bacteria held in the same orientation and in many cases located around the vacuole membrane, leaving empty space in the centre of the vacuole (Figure 2.2c). The cell vacuoles hosting the bacteria were also large and in some cases leaving ample space between the microbes. We also observed smaller vacuoles containing a single bacterium.

In *H. simulans* (Figure 2.1g), the mesohyl was more dense than the species above (Figure 2.1h) and found to contain two main microbial morphotypes, the most abundant of which is an eight-arm star shaped bacterium (Figure 2.1i and 2.2d). The star shaped morphotype is a rod bacterium in cross section that has a very characteristic helical profile due to the protuberance of the bacterial wall. An additional feature of the main bacterial morphotype found in *H. simulans* was the presence of a refractive rounded organelle that recalls a typical acidocalcisome at one end of the bacterial cell (Figure 2.2e). The other bacterial morphotype hosted in the mesohyl of *H. simulans* was a rod bacterium with simple wall (Figure 2.2f). Both bacterial morphotypes in *H. simulans* were rarely observed intracellularly, but were usually distributed in the mesohyl in close association with collagen bundles (Figure 2.2f).

A single rod-shaped bacterial morphotype with simple wall similar to the one observed in *H. simulans* was visible in the mesohyl of *H. viscosa* (see Figure 2.1m–o for images of this sponge) and only rarely observed inside sponge cells. Again as observed for *H. simulans*, the bacteria hosted in *H. viscosa* were mostly located close to collagen (Figure 2.2h).

Finally, in *H. indistincta* (Figure 2.1j–l), a close sister species of *H. viscosa* (Longakit et al., unpublished), almost no intact bacteria were observed (Figure 2.1l). In this species, however, bacteria partially decomposed inside phagosomes were very apparent (Figure 2.2g). Several of these digestive vacuoles could be found in a single cell, and were present not only in amebocytes, but also in pinacocytes and choanocytes. The abundance of these phagosomes was much higher in *H. indistincta* than in the other species investigated.

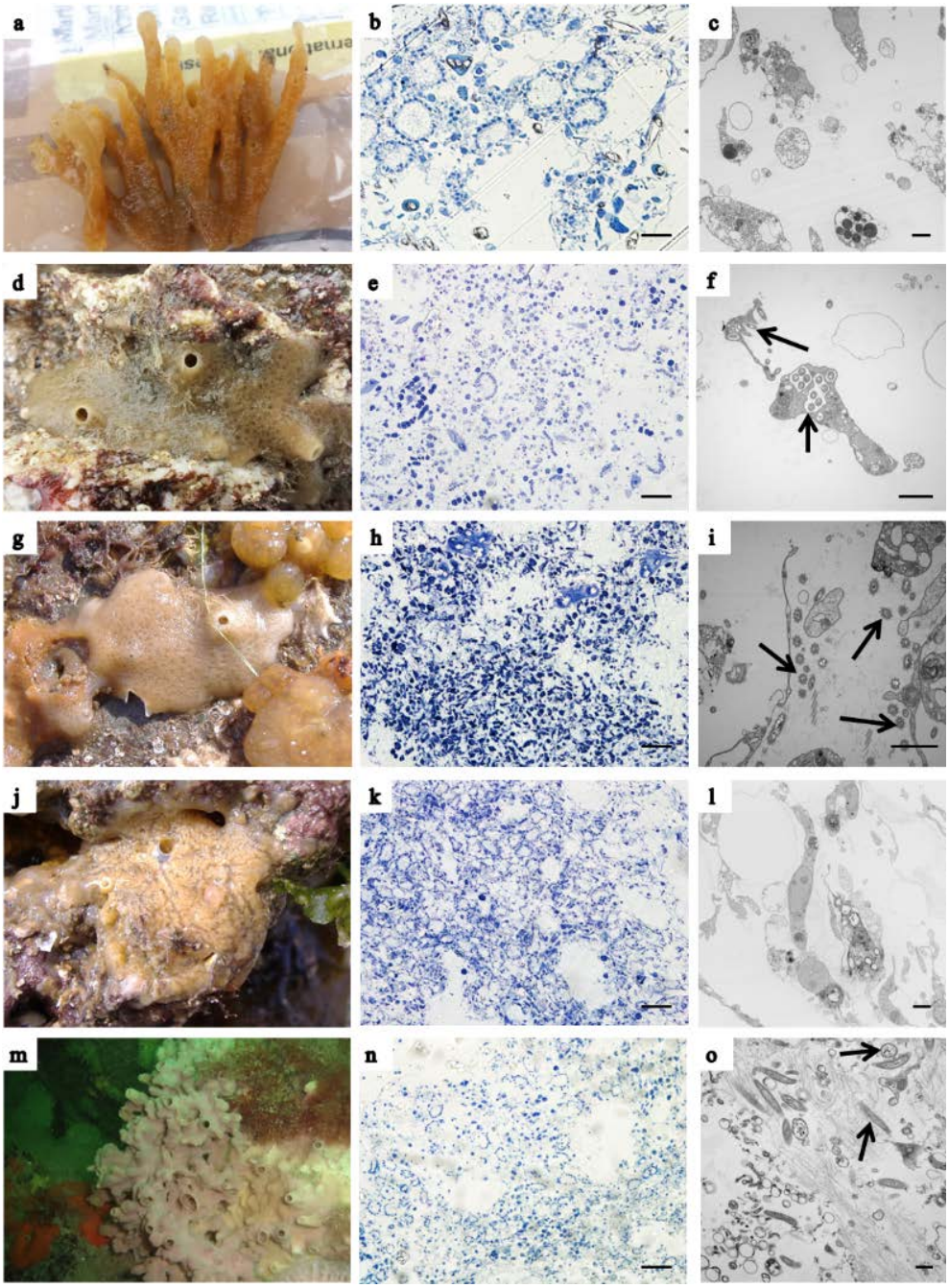

Figure 2.1. Specimens of the five target sponge species in their natural environment or right after collection, semi-thin sections of the same five species illustrating the loose mesohyl and ultrathin sections showing bacteria if present: **a–c**. *Haliclona* (*Haliclona*) oculata, **d–f**. *H.* (*Reniera*) *cinerea*, **g–i**. *H.* (*Haliclona*) *simulans*, **j–l**. *H.* (*Rhizoniera*) *indistincta* and **m–o**. *H.* (*Rhizoniera*) *viscosa*; Scale bars: **b**, **e**, **h**, **k** and **n** = 500 µm; **c**, **f**, **i** and **l** = 2 µm; **o** = 500 nm.

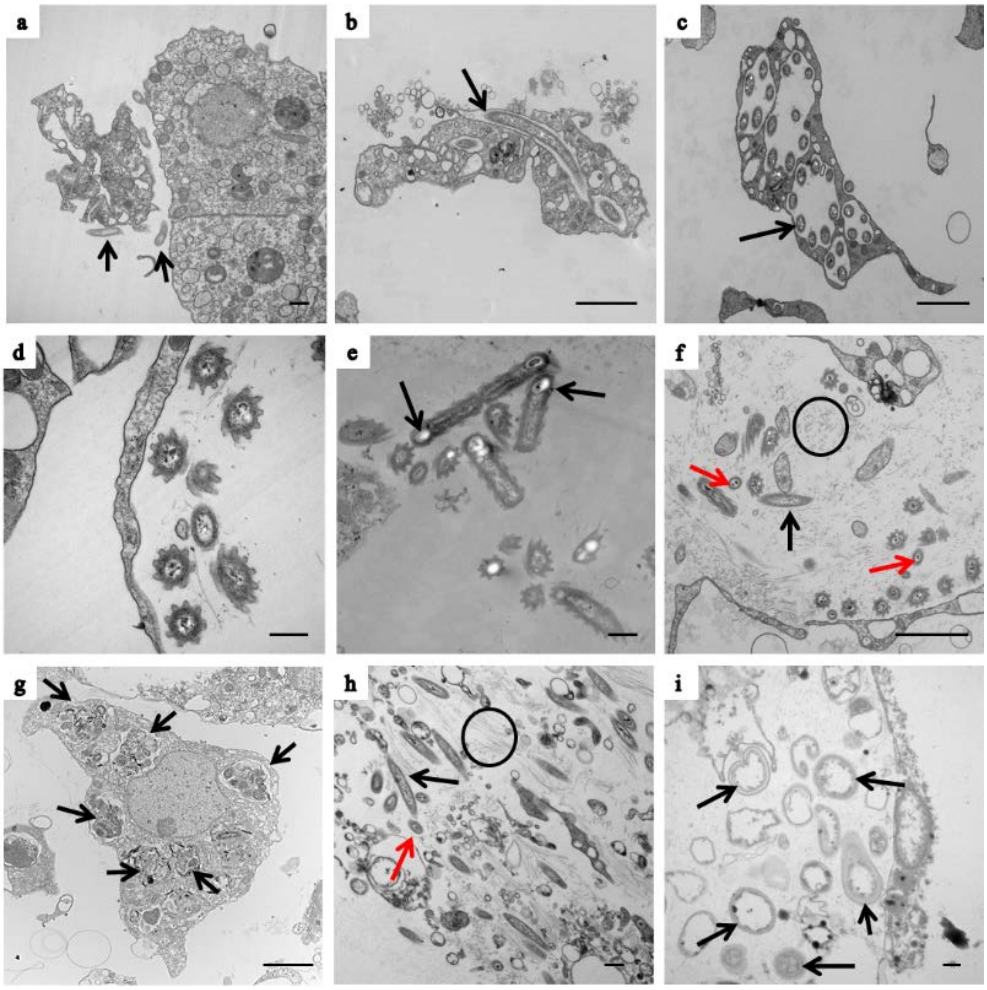

Figure 2.2. TEM micrographs of the five target *Haliclona* species showing details of their microbial associations. **a**. Bacteria rarely observed in *H. oculata* (arrows); **b**. Very long rod shaped bacterium observed inside intracellular vacuoles in *H. cinerea* (arrow); **c**. Cross sections of the rod shaped bacterium observed at the edge of intracellular vacuoles (arrow); **d**. Close-up of the eight-arm star shaped cross sections of the main bacterial morphotype in *H. simulans*; **e**. Presumtive acidocalcisome present in main bacterial morphotype in *H. simulans*; **f**. The second bacterial morphotype (rod) found in *H. simulans* (black arrow); cross sections of the same bacterium (red arrows); **g**. Bacteria in a digested state in *H. indistincta* phagosomes (arrows); **h**. Longitudinal (black arrow) and cross (red arrow) sections of the rod bacterium observed in *H. viscosa*; also showing the connection between sponge collagen and bacteria; **i**. The mesohyl of *H. sarai* showing several bacterial morphotypes (arrows). Scale bars: **a**, **d**, **e**, **h** and **i** = 500 nm; **b**, **c**, **f** and **g** = 2 μm.

Deep sequencing data

In total 3,345,481 sequences were obtained across 24 sponge and seawater samples after the initial filtering steps. When we removed chimeras, singletons and all sequences corresponding to chloroplasts and OTUs present in seawater at high abundance (> 0.1%), 2,245,739 sequences still remained from the 21 sponge tissue specimens from five species. In many cases the numbers of OTUs returned from individual sponges were similar or higher than those from seawater despite fewer numbers of sequences (Table 2.2). For seawater samples, Strangford Lough showed the lowest microbial richness (3979 OTUs), while Corranroo showed the highest OTU richness (10093) and Gurraig Sound was in between with 7179 OTUs.

Two specimens yielded very low numbers of sequences and OTUs from the microbiome data: one *H. cinerea* (MIIG0997) specimen from Strangford Lough and one *H. simulans* (MIIG1014) from the Gurraig Sound in Galway (Table 2.2), thus these samples were not considered for the downstream analyses. The rarefaction plots based on the *Haliclona* and seawater samples are shown in Figure 2.3; the sampling depth for the first plot was selected as the median of the sequence counts (122,601) (Table 2.2). The fact that most of the curves in this plot reach *plateau* suggests that sequencing and microbial diversity analyses for these samples can be considered exhaustive (Figure 2.3a). The most striking exception is the sample MIIG1033 (*H. simulans*) that in the plot shows an abrupt trend indicating for this specimen the possibility of being under-sampled (Figure 2.3b).

A large number of sequences (i.e. 152,567) were associated with the chloroplasts of eukaryotic organisms. The higher numbers of chloroplast sequences were associated to two specimens of *H. indistincta* from Corranroo (20,262 and 15,904 for MIIG0980 and MIIG0982 respectively) and one specimen of *H. oculata* from Donegal (i.e. MIIG0985 with 29,550 chloroplast sequences). These sequences were also excluded from the following analyses in order to determine a more accurate representation of the prokaryotic community in the sponges analysed.

Table 2.2. Overview of the sequence counts and OTUs observed right after quality filtering and chimera checking (original data) and after removal of singletons and sequences referring to abundant OTUs (> 1%) in the seawater samples from the sponge species.

Species	**ID sample**	**Original data**		**After removal of singletons, seawater abundant OTUs and chloroplast sequences**	
		Sequences	**OTUs**	**Sequences**	**OTUs**
Haliclona indistincta	MIIG0980	149206	10073	76312	9359
	MIIG0981	193600	11777	103907	11070
	MIIG0982	107414	7099	40286	6727
Seawater Corranroo	MIIG0966a	224064	10824	201379	10093
Haliclona oculata	MIIG0985	125514	7341	95040	6985
	MIIG0986	119613	4347	112258	4157
	MIIG0987	116719	5765	105631	5500
Haliclona oculata	MIIG1003	185653	7155	174166	6831
	MIIG1004	128939	6502	121129	6225
	MIIG1005	155315	7837	132316	7546
Haliclona cinerea	MIIG0988	205437	10351	194005	9973
	MIIG0997	1245	155	1226	146
	MIIG1001	121290	7940	111918	7718
Haliclona indistincta	MIIG0989	180751	10463	171962	10099
	MIIG0991	224748	2844	222143	2730
	MIIG0993	160592	8130	155877	7862
Seawater Strangford Lough	MIIG1003a	238414	4053	238284	3979
Haliclona simulans	MIIG1014	494	83	490	79
	MIIG1032	10490	289	8402	262
	MIIG1033	17636	976	12798	875
Haliclona viscosa	MIIG1012	174056	5119	124073	4977
	MIIG1025	158388	4593	141488	4444
	MIIG1026	148323	2991	140312	2910
Seawater Gurraig Sound	MIIG1012a	197580	7746	180639	7179

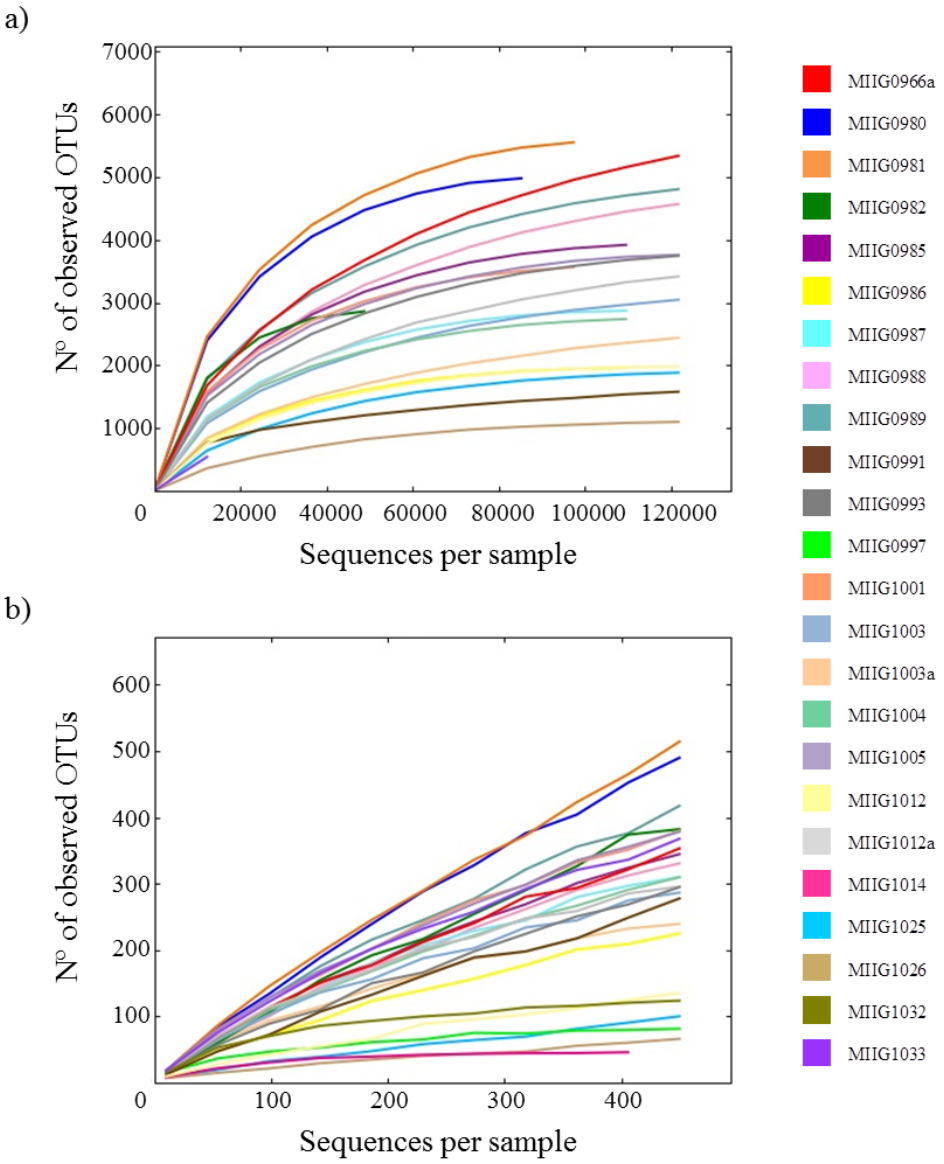

Figure 2.3. Rarefaction plots of the *Haliclona* and seawater samples: **a**. Plot constructed using the median of the sequence counts as sampling depth (122,601); **b**. Plot constructed using the lowest sequence count observed (490) as sampling depth. MIIG 0966a, MIIG1003a and MIIG1012a are the seawater samples; MIIG0980, MIIG0981 and MIIG0982 are the *H. indistincta* from Corranroo and MIIG0989, MIIG0991 and MIIG0993 are the samples of the same species form S. Lough; MIIG0985, MIIG0986, MIIG0987 and MIIG1003, MIIG1004, MIIG1005 are the *H. oculata* samples respectively from Donegal and from S. Lough; MIIG1012, MIIG1025 and MIIG1026 are the *H. viscosa* samples and MIIG1014, MIIG1032 and MIIG1033 are the *H. simulans* specimens.

Archaeal sequences were detected from all the sponge and seawater samples. In detail, members of the phylum Thaumarchaeota were more abundant across the sponge samples (1.58% ± 1.20%), while the seawater samples showed a higher presence of Euryarchaeota (0.89% ± 1.20%), exception being the sample from Strangford Lough which showed generally a low abundance of Archaea (< 0.01%). Thaumarchaeota were represented mainly by Cenarchaeaceae, in particular the genus *Nitrosopumilus* (1.32% ± 1.21%). The sponge-specific archaeon *Cenarchaeum symbiosum* was present only in extremely low numbers in the water samples from Corranroo and all the sponge species except *H. indistincta* from Corranroo and *H. simulans*. The most abundant Euryarhaeota were Termoplasmata belonging to the Marine group II (0.89% ± 1.20%). Crenarchaeota sequences were rare (low abundance and uncommon occurrence), being found only in one *H. indistincta* specimen from Corranroo (MIIG0980). Additional archaeal sequences misclassified as representatives of the phylum Parvarchaeota were present at very low abundance (< 0.01%) only in the seawater sample from Corranroo.

The bacterial sequences associated with the sponge tissue samples belong to 59 different phyla, of which 21 are established, 34 are classified as candidate phyla and 4 as *incertae sedis*. Among the candidate divisions, some have not yet been recorded for Porifera, i.e. FBP, FCPU426, H-178, LCP-89, SC4, SR1, TPD-58, WS4. In particular, FBP, FCPU426, LCP-89, SC4 and WS4, as well as LD1 and NC10 were found only in sponge samples, not in seawater. The only bacterial phylum found in seawater, but not in the sponges analysed was misclassified as Caldithrix and found specifically in the seawater sample from Corranroo. Of the two candidate phyla considered to be predominantly associated with Porifera (i.e. Poribacteria and Tectomicrobia), only Poribacteria were found in the species here analysed and always at very low abundance (< 0.1%). The bacterial phyla present at the highest concentrations in the samples studied are commonly found in seawater and Porifera: Proteobacteria, Bacteroidetes, Planctomycetes, Verrucomicrobia, Actinobacteria and

Cyanobacteria. The dominant phylum was represented by Proteobacteria (66.81% ± 17.35% of OTUs). Inside Proteobacteria, the most represented classes were γ-Proteobacteria (23.92% ± 14.05%), β-Proteobacteria (21.19% ± 21.26%), α-Proteobacteria (19.26% ± 16.11%) and finally δ-Proteobacteria (1.94% ± 2.56%). A chart showing the bacterial phyla that were present with an abundance ≥ 0.1% across all the specimens analysed in this study is shown in Figure 2.4.

The most abundant bacterial phyla found in the three seawater samples are Proteobacteria, Bacteroidetes and Verrucomicrobia, but their relative abundance is different between geographical locations with Strangford Lough showing a much higher abundance of Proteobacteria (88.26%) compared to Corranroo and Gurraig Sound (54.26% and 57.87% respectively). The only bacterial taxa present in water samples from all locations were those commonly observed across all samples, i.e. unidentified Rhodobacteraceae and Flavobacteraceae. Strangford Lough was rich in γ-Proteobacteria mostly observed only at very low concentrations in the two other water samples. Many common bacterial taxa were shared between the seawater samples from the west coast, e.g. Chryomorphaceae, Rhodobacteraceae, *Polaribacter*, *Octadecabater* and *Flavobacterium* (abundance > 5%). As might be expected given the data treatment (most seawater taxa removed), all the seawater samples showed a low similarity to the sponge samples collected in the same sampling site, as shown in the PCoA plots (Figure 2.5). Overall, the species-specific microbial communities are defined by OTUs present with low abundance (< 50%) whereas the OTUs that are present at the highest abundance vary from one specimen to the other within species (Figure 2.6). This is consistent with the significant differences found among all three alpha-diversity indices considered (see Table 2.3).

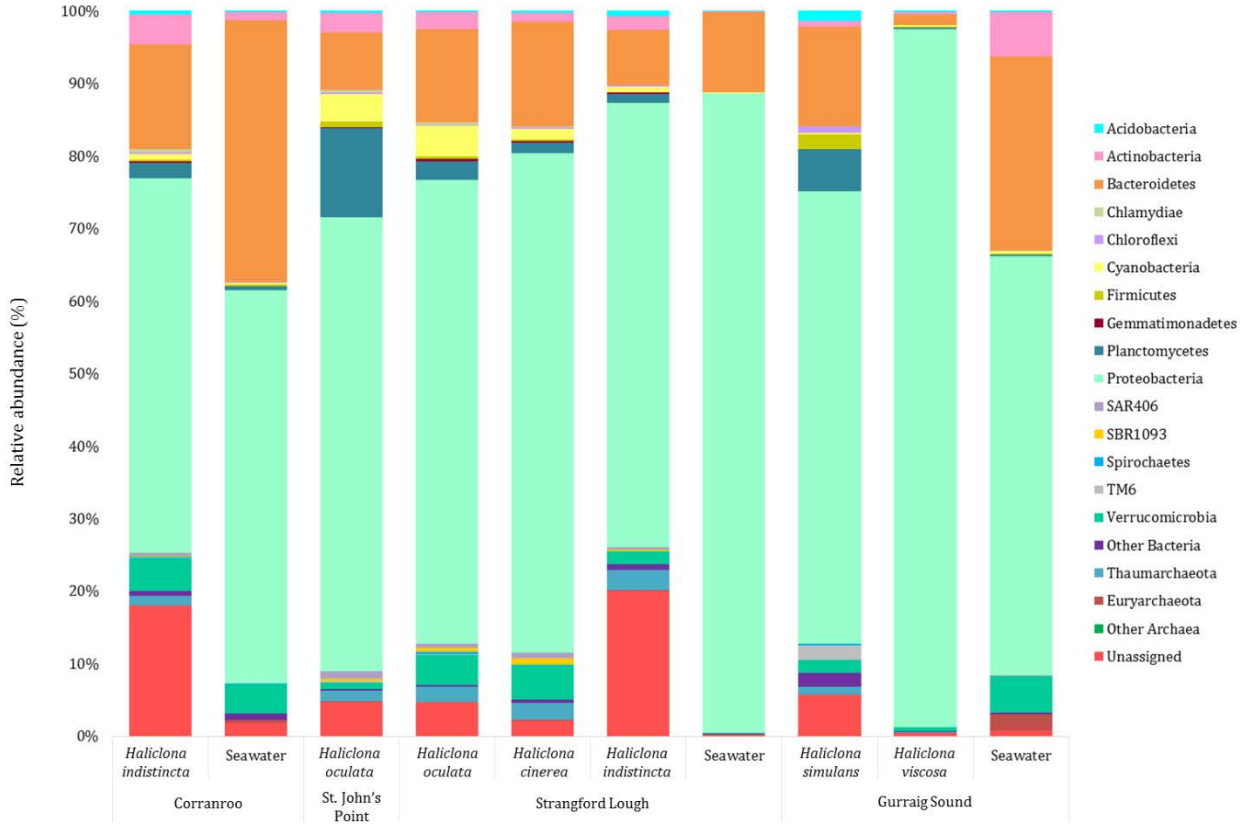

Figure 2.4. Bar chart showing the relative abundance of the bacterial phyla detected through deep sequencing in the target *Haliclona* species and in the three seawater samples.

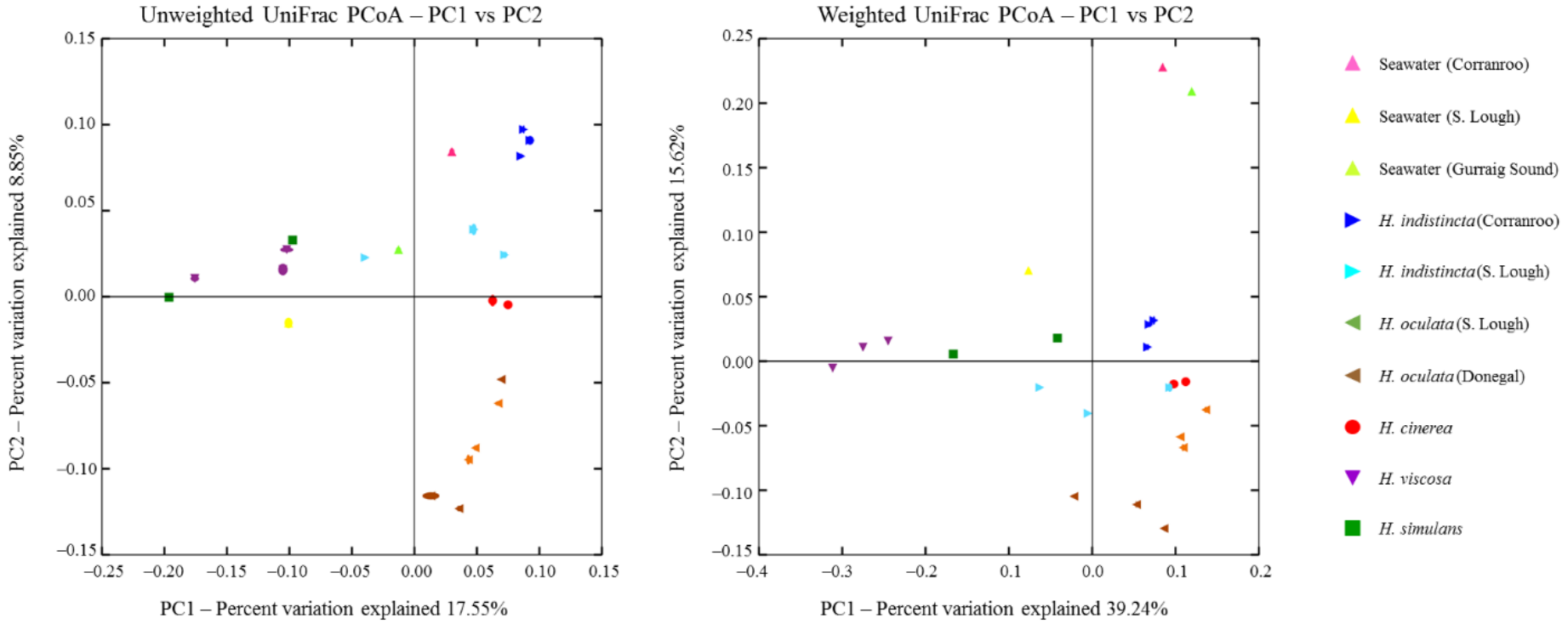

Figure 2.5. Two-dimensional PCoA plots from unweighted and weighted UniFrac distance matrices. The *x* and *y* axes are indicated by the first and second coordinates, respectively, and the values in parentheses show the percentages of the community variation explained, respectively 26.40% and 54.86% for the unweighted and weighted UniFrac.

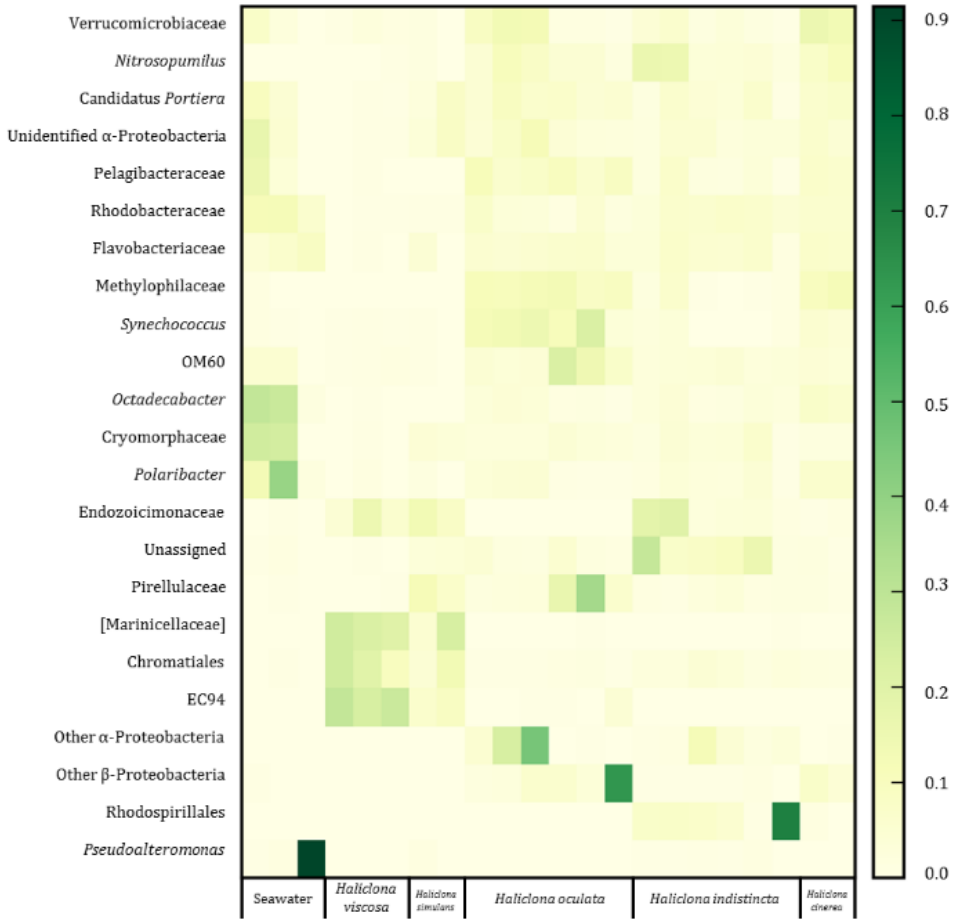

Figure 2.6. Heatmap showing the relative abundance of the more common OTUs observed in the *Haliclona* specimens and seawater samples.

Table 2.3. Diversity indices (Chao1, Simpson and Shannon) for sponge species and seawater samples from all the sampling sites considered in this study.

Sampling site	Species	Chao1	Shannon	Simpson
Corranroo	*Haliclona indistincta*	15922.95 ± 2575.73	9.56 ± 0.37	0.98 ± 0.01
	Seawater	14983.66	7.59	0.97
Saint John's Point	*Haliclona oculata*	8810.57 ± 1390.99	6.95 ± 1.52	0.93 ± 0.07
Strangford Lough	*Haliclona oculata*	12695.48 ± 388.91	7.35 ± 0.58	0.96 ± 0.01
	Haliclona cinerea	15899.92 ± 2470.63	7.79 ± 0.57	0.97 ± 0.02
	Haliclona indistincta	11702.30 ± 7219.51	6.68 ± 1.56	0.89 ± 0.08
	Seawater	4774.43	6.31	0.95
Gurraig Sound	*Haliclona simulans*	989.97 ± 619.38	6.23 ± 1.58	0.95 ± 0.03
	Haliclona viscosa	8282.40 ± 2258.22	3.08 ± 0.74	0.62 ± 0.07
	Seawater	12677.64996	7.099523403	0.969661434

Microbial diversity of the *Haliclona* species

The bacterial communities observed from the *Haliclona* tissue samples via the deep sequencing approach were extremely variable in terms of OTU diversity, with *H. oculata*, *H. indistincta* and *H. cinerea* having higher numbers of OTUs compared to *H. simulans* and *H. viscosa*. Indeed the number of OTUs returned for *H. simulans* is much lower than for the other species (Table 2.2).

H. oculata specimens were collected from two different sampling sites (Donegal on the west coast and Strangford Lough on the east coast). OTUs from the family Methylophilaceae were the dominant bacteria in *H. oculata* from both sites (15.77% ± 3.42% and 17.12% ± 0.93% of the total OTUs respectively). A large fraction of the 16S rRNA gene sequences from this species were unassigned β-Proteobacteria for the sponges from Donegal (13.33% ± 20.19%) and α-Proteobacteria for the samples from Strangford Lough (6.62% ± 5.45%). Other dominant bacterial OTUs for

the *H. oculata* specimens from Donegal belonged to the family Pirellulaceae (6.94% ± 5.27%), whereas the Rhodobacteraceae represented 5.21% ± 2.66% of the OTUs identified in *H. oculata* from Northern Ireland. However, both weighted and unweighted UniFrac comparisons of *H. oculata* from the two sampling sites showed no differences in bacterial community composition (adonis: $R^2 = 0.46$, $p = 0.1$ and $R^2 = 0.31$, $p = 0.1$ respectively; Figure 2.5). The main bacteria responsible for the differences in the bacterial communities of the *H. oculata* samples from Donegal and Northern Ireland were the γ-Proteobacterial family Piscirickettsiaceae, the α-Proteobacterial Hyphomonadaceae and the β-Proteobacterial order EC94 as well as the Bacteroidetes genera *Flavobacterium* and *Pedobacter* (non-paramentric t-test with Monte Carlo simulation).

As explained above, for *H. cinerea* we have considered only the samples MIIG0988 and MIIG1001 for the analysis of this species. The dominant bacterial taxon found in these two samples was also the β-Proteobacterial family Methylophilaceae, as observed for *H. oculata* (16.78% ± 2.22%), as well as unidentified Rhodobacteraceae (6.75% ± 0.15%).

Similarly to *H. cinerea*, for *H. simulans* we have considered only the two specimens collected in 2009, i.e. MIIG1032 and MIIG1033 which both yielded low numbers of OTUs. The main bacterial taxon found was the β-Proteobacterial order EC94 (18.08% ± 4.39%), followed by γ-Proteobacteria misclassified in the family Marinicellaceae (9.03% ± 8.17%) and unclassified members of the γ-Proteobacterial order Chromatiales (4.37% ± 3.08%).

H. indistincta from both sampling sites showed a high number of OTUs (except for sample MIIG0991, Table 2.2). A large fraction of the bacterial sequences from Corranroo samples were from Rhodobacteraceae (7.45% ± 0.57%) after the unassigned OTUs that were very abundant in all *H. indistincta* specimens (18.02% ± 6.30% for Corranroo and 20.09% ± 22.70% for Strangford Lough respectively). The other main bacterial taxa found in the *H. indistincta* samples from Corranroo were

Flavobacteriaceae (3.74% ± 0.42%) and Rhodospirillales (3.56% ± 3.15%). Members of the latter bacterial order were also the dominant OTUs found in the samples from Strangford Lough (24.59% ± 31.27%), in particular as regards the sample MIIG0991 whose 60.70% of the bacterial community was represented by Rhodospirillales. The other dominant bacterial taxa in the *H. indistincta* samples from Northern Ireland were Rhodobacteraceae (5.37% ± 2.06%), Methylophilaceae (4.90% ± 4.34%) and Endozoicimonaceae (3.89% ± 3.37%). Well represented in these samples was also the archaeal genus *Nitrosopumilus* (2.58% ± 2.24%). The results of the adonis analysis show that the differences in the microbial communities of *H. indistincta* specimens from Corranroo and Strangford Lough are not significant and they are due more to the abundance of the same taxa than the actual presence of different OTUs (adonis unweighted UniFrac: $R^2 = 0.28$, $p = 0.1$; adonis weighted UniFrac: $R^2 = 0.38$, $p = 0.1$). The OTUs that were mostly responsible for the differences between the sponges from both sampling sites belong to the Bacteroidetes families Flavobacteriaceae and NS11-12 as well as to the α-Proteobacterial genus *Octadecabacter* (non-paramentric t-test with Monte Carlo simulation).

The bacterial community of *H. viscosa* was dominated by the β-Proteobacterial order EC94 (60.06% ± 5.85%). A total of 608 different EC94 OTUs were observed across the *H. viscosa* specimens analysed, the most abundant taxon being 2330297. The same OTU was the main EC94 OTU found in the *H. simulans* specimens considered, and was present at low abundance (< 0.1%) in the seawater sample from the sampling location (Gurraig Sound).

Cross-species comparisons. Some bacterial taxa appeared to be shared between different sponge species, as e. g. the family Methylophilaceae, well represented in all the sponge species from Strangford Lough. Other bacterial taxa were present with high relative abundance in species from different localities, as the families Rhodobacteraceae, Flavobacteraceae OM60 and the genus 'Candidatus *Portiera*' which were found in the sponges from Corranroo, Donegal and Strangford Lough, or the γ-

Proteobacterial Chromatiales and Endozoicimonaceae that were shared instead between *H. indistincta* from both sampling sites and the two species collected in Gurraig Sound, i.e. *H. simulans* and *H. viscosa*. Overall, the adonis analysis showed that microbes detected were significantly different between sponge species and between sponges and seawater samples (unweighted UniFrac: $R^2 = 0.39$, $p = 0.001$; weighted UniFrac: $R^2 = 0.64$, $p = 0.001$). Furthermore, PERMDISP analysis highlighted homogeneity among the microbiomes within each species (unweighted UniFrac: $F = 2.39$, $p = 0.08$; weighted UniFrac: $F = 1.49$, $p = 0.25$). The adonis analysis of the original microbial communities before filtering out the abundant (> 0.1%) OTUs present in the seawater samples highlighted the role of host species over environment in structuring species-specific microbiomes (unweighted UniFrac: $R^2 = 0.42$, $p = 0.001$; weighted UniFrac: $R^2 = 0.53$, $p = 0.001$). Homogeneity of variance was also maintained when seawater OTUs were included, even though seawater communities at different sites appeared quite different (Figure 2.5), which further highlights species-specific microbiomes despite a superficial dissimilarity due to bacteria being present at different levels of abundance (unweighted UniFrac: $F = 1.03$, $p = 0.43$; weighted UniFrac: $F = 0.98$, $p = 0.46$). This is evidenced by the difference between unweighted and weighted UniFrac PCoA plots in Figure 2.5: when abundance is included in the latter, the species-specific pattern becomes obscured for some species such as *H. indistincta*, *H. simulans* and *H oculata*.

Discussion

Both transmission electron microscopy and next-generation sequencing analyses of the five target *Haliclona* species indicate that these sponges belong to the LMA group, confirming the prediction of LMA status that was made for *H. oculata*, *H. indistincta* and *H. viscosa* by Moitinho-Silva *et al.* (2017b) using machine learning algorithms. TEM images show the mesohyl of each species to be essentially devoid of microorganisms or to

have a few bacteria present in association with the sponge collagen or inside cell vacuoles. Usually LMA species are dominated by a large clade of Proteobacteria (specifically α-, β- or γ-) or Cyanobacteria with little overlap between bacterial communities of the different species (Giles et al., 2013; Gloeckner et al., 2013; Erwin et al., 2015). Patterns of microbial diversity observed here are consistent with this as is the nature of the sponge body structure, i.e. all the species have a loose mesohyl and a well-developed aquiferous system (Langenbruch and Jones, 1990; Kaandorp, 2009). Sequences from Poribacteria were observed only at very low concentrations in the samples considered for this study.

While TEM and microbiome investigations concur on the LMA status of the species, the microbiome data indicates a diversity of microbes that are not visible at all in the sponge tissue. This includes Archaea, which are characterized by a complex cell wall with a structure of regular subunits (Fuerst et al., 1999). As already observed for other sponge species, the archaeal communities detected via PCR/sequencing in the target sponge species were dominated by members of the family Cenarchaeaceae within the phylum Thaumarchaeota (Chaib De Mares et al., 2017). Most of the OTUs ascribed to this family were represented by members of the genus *Nitrosopumilus*, in particular in the samples from Strangford Lough, in spite of the low presence of these microorganisms in the seawater samples from the same sampling site. The enrichment of these archaeal OTUs in the sponge samples from this environment might suggest the presence of higher concentrations of ammonia in Strangford Lough compared to the sampling sites from the west coast, considering the role played by members of the genus *Nitrosopumilus* in the ammonia-oxidation process (Turque et al., 2010).

H. oculata is the type species of *Haliclona* and is shown here to have a mesohyl devoid of bacteria while a reasonable bacterial diversity was detected via PCR and deep sequencing of 16S rDNA amplicons. Giles et al. (2013) stated that it is possible that bacteria are generally missed in TEM surveys of LMA sponges because they are not equally distributed throughout the mesohyl. I mitigated against this by including multiple

specimens and tissue sections from different parts of the sponge, but it has to be considered that even this approach might not be enough to spot microbes in the tissues of LMA sponges, while the amplicon sequencing is far higher resolution and far more sensitive to detecting low abundance bacteria than is the TEM. The bacterial community associated with *H. oculata* from the North Sea was previously found to be dominated by β-Proteobacteria (Naim et al., 2014), in agreement with sequencing of specimens collected in Donegal and Strangford Lough. However, the main bacterial taxon found in the *H. oculata* samples from both west and east coast sampling sites was the β-proteobacterial family Methylophilaceae, whereas the most abundant OTU found in the specimens from the North Sea was an unclassified Proteobacterium.

The present study is the first survey of the microbiome for *H. indistincta*. The presence of so many bacterial OTUs detected in the specimens from Corranroo, despite the absence of a bacterial community by TEM, may be explained by the large number of bacteria in a decomposed stage contained in the phagosomes. This obvious feature of *H. indistincta*, not observed with the same frequency in the other target species, is an anomaly for LMA sponges that are supposed to feed predominantly on particulate organic matter (Schlappy et al., 2010). Microbiome data indicate that the dominant OTUs in two of the *H. indistincta* specimens from Corranroo were represented by Rhodophyta-associated 16S rRNA gene sequences. The occurrence of sequences from this eukaryotic phylum represents the presence of their chloroplasts (Sipkema and Blanch, 2010). The detection of so many Rhodophyta-associated sequences in *H. indistincta* is not surprising considering that this species in Corranroo was found to be associated with 48 different red algae during an annual study of the algal epibionts of this sponge species (Moniz et al., 2013). The *H. indistincta* samples collected in Strangford Lough were instead virtually free of Rhodophyta-associated sequences and in fact the removal of the chloroplasts sequences highlighted a higher homogeneity between the microbial communities of samples from the two different sampling sites, both dominated by α-

Proteobacteria Rhodobacteraceae and Rhodospirillales. However, compared to the samples from Corranroo, *H. indistincta* from Strangford Lough also features higher abundance of taxa like *Nitrosopumilus*, *Octadecabacter*, Methylophilaceae and Verrucomicrobiaceae which characterise the microbiome of the other species collected in the same sampling site. This can be explained taking in account a possible geographical/environmental impact on the deep sequencing data. In fact, *H. indistincta* can be found as two different morphotypes: the specimens from Strangford Lough were very flat and it is likely that some substrate formed part of the Strangford Lough tissue samples, a possibility also for the *H. cinerea* specimens.

However, in spite of these differences between conspecific samples from different localities, the adonis and principal coordinate analyses (Figure 2.5) highlight the existence of a host-specific microbiome as observed for other LMA species (Easson and Thacker, 2014; Erwin et al., 2015; Steinert et al., 2017). Even though the microbial communities of LMA sponges are usually dominated by few (mainly proteo)bacterial taxa (Erwin et al., 2011; Giles et al., 2013; Poppell et al., 2014), my results suggest that the less abundant OTUs mainly contribute to shape the host-specific bacterial communities, as also shown by the heatmap in Figure 2.6.

TEM pictures show that the microbial community of *H. cinerea* is comprised of a single very long rod bacterial morphotype. The shape of these microbes recalls the bacteria observed in sponge mass mortalities attributed to members of the Subclass Keratosa (Vacelet et al., 1994; Cebrian et al., 2011). The observation of the bacteria in *H. cinerea* mostly inside cell vacuoles or just outside the cell membranes, but not distributed in the sponge mesohyl, could suggest that these bacteria are pathogens against which the sponge cells act like human macrophages, but the fact that the bacteria were not observed in a decomposed stage in this sponge species as well as their consistent arrangement at the edge of the intracellular vacuoles may indicate that these bacteria play a specific role in *H. cinerea* biology. The sequencing approach suggests that

the dominant OTU in this species is an unidentified OTU (747864) belonging to the family Methylophilaceae (Class β-Proteobacteria). Representatives of this bacterial family are Gram negative rods (Doronina et al., 2014) which is consistent with the morphology detected by TEM. In *Haliclona tubifera* (a species closely related to *H. cinerea* (Redmond et al., 2011)), a single specialist γ-Proteobacteria symbiont was reported to dominate the microbial community (Erwin et al., 2011). Woollacott (1993) showed the presence of bacteria with a long rod shape morphology in the larvae of *H. tubifera* similar to those found in *H. cinerea*. However in both species the bacteria are pleomorphic, the bacteriocytes show different structure and indications are that the bacteria in *H. tubifera* are vertically transmitted (Woollacott, 1993) whereas the embryos isolated from *H. cinerea* were found to be completely free of bacteria (Marra, personal observation). More focused metagenomic and *in situ* hybridisation work is therefore needed to resolve the identity and likely functional role of this bacterium.

The pattern of bacterial morphotypes found in the *H. simulans* specimens is reminiscent of the bacteria found in *H. caerulea* (Maldonado, 2007) and *Amphimedon queenslandica* (Fieth et al., 2016), species that belong to the same haplosclerid clade (Clade B) to which *H. simulans* belongs (Redmond et al., 2011). The presence of an acidocalcisome at one end of the bacterial cell is a feature also shared between bacteria in all three species and in both *H. simulans* and *A. queenslandica* at least, the star-shaped bacteria show an affinity for collagen. Combining TEM with fluorescent *in situ* hybridization, Fieth et al. (2016) identified the star-shaped bacterial morphotype found in *A. queenslandica* as a γ-Proteobacterium Chromatiales. In the same study, this was the most abundant taxon found in *A. queenslandica* through 454 pyrosequencing of 16S rRNA gene amplicons, followed by two OTUs within the order Oceanospirillales, one OTU in Alteromonadales and further two OTUs belonging to an unknown order of β- and δ-Proteobacteria. A previous culture-independent study (PCR and Sanger sequencing of clones) of *H. simulans* from the same location, but years earlier (Kennedy et al., 2008),

showed dominance by Chromatiales followed by four other OTUs within the order Oceanospirillales. In this study, the microbiome of *H. simulans* is dominated by the β-Proteobacterial order EC94, but Chromatiales are also well represented, including the family Ectothiorhodospiraceae to which the star-shaped bacterium observed in *A. queenslandica* was shown to belong by Fieth et al. (2016).

The bacterial community of *H. viscosa* from Gurraig Sound is largely dominated by several OTUs belonging to the order EC94. These are β-Proteobacteria that have been already observed in other demosponges, including haplosclerids (Steinert et al., 2016). However, in spite of the increasing detection of the occurrence of this β-Proteobacterial order in different sponge species, still very little is known about its members, especially with regard to the potential metabolic roles that they can play in the sponge hosts (Jackson et al., 2013). In this sense, the results of the deep sequencing analysis for *H. viscosa* provide a promising opportunity to gain more insights into the association between sponges and representatives of this bacterial order. In fact, analogously to the work carried out on *Crambe crambe* by Croué et al. (2013), it would be possible to use the EC94 sequences obtained from *H. viscosa* to develop general probes for this bacterial taxon and try to localise it in the sponge body through fluorescent *in situ* hybridization in order to verify if the bacteria observed through TEM in this species actually belong to the most abundant bacterial taxon detected by the deep sequencing analysis. If this was confirmed, *H. viscosa* would represent a unique model to study the role played by EC94 bacteria in sponges as well as the presence of genes encoding for eukaryotic-like proteins in the genome of these microbes, considering their evident association with the collagen of the sponge (Hentschel et al., 2012).

Although this study shows how *H. simulans*, *H. caerulea* and *A. queenslandica* (all members of a haplosclerid subclade) host an extremely similar bacterial morphotype, which may indicate co-evolution of sponge and symbiont, no putative phylogenetic pattern was detected for the other species or clades included here. The closely related

species *H. indistincta* and *H. viscosa* (De Weerdt, 1986) host disparate bacterial communities (Figure 2.5) via both sequencing and TEM approaches. An additional relative of these, *H. sarai* (De Weerdt, 2000), has much higher numbers of bacteria in the mesohyl evident via TEM (Figure 2.2i). The microbial data shown here represent an excellent starting point towards understanding the feeding ecology of these marine sponges as the available environmental microbial communities likely significantly contribute to niche partitioning amongst filter feeding marine sponges co-occuring in a specific location.

Concluding remarks

My combined analysis of the microbiome of five target *Haliclona* species via TEM and next-generation sequencing showed how both approaches concur in attributing LMA status to these sponges. The results of PCoA and community composition statistics show an evident species-specificity for the bacterial communities present, that can be observed across different localities. However, for most of the specimens analysed the 16S rRNA gene data revealed a microbial diversity much broader than the extent of association suggested by the TEM observations. I suggest that the much diversified microbial communities identified via the sequencing approach has an environmental component, e.g. by seawater or sediment being trapped in the sponge canals or by the bacteria being used as food by the sponges. The discrepancies observed between TEM and next-generation sequencing results suggest that either approach alone is not sufficient to give a realistic representation of the microbial associations in sponges. However, as my study shows, combining TEM and next-generation sequencing with additional techniques like fluorescent *in situ* hybridization and/or real-time PCR will give an even better understanding of the nature and distribution of the associated bacteria inside the sponge body.

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CHAPTER 3

Filamentous fungi isolated from Irish and Mediterranean *Haliclona* species

Introduction

Marine fungi are known to be pathogens of different kinds of organisms like algae (Shinde and Pawar, 2013), corals (Alker et al., 2001, Geiser et al., 1998), bivalve molluscs and crustaceans (Ramaiah, 2006), fishes (Bowater et al., 2003, Marancik et al., 2011), even marine mammals (Higgins, 2000, Morris et al., 2011). However, other types of associations between fungi and other organisms also have been reported, e.g. with seaweeds (Flewelling et al., 2013, Godinho et al., 2013, Suryanarayanan and Johnson, 2014), seagrasses (Panno et al., 2013) and invertebrates (Amend et al., 2012, Moree et al., 2013, Lan et al., 2014). The roles that fungi may play in these associations have been understood only partially, but due to their ability to degrade lignocellulose (Hyde et al., 1998) marine fungi can be involved in recycling of nutrients and organic matter, energy flow, synthesis of humic enzyme-exopolysaccharide complexes (Panno et al., 2013, Wegley et al., 2007). It is difficult to define marine fungi as "parasitic" or "associate" *stricto sensu* because there are examples of fungi that are able to switch from parasite to mutualistic and *vice versa* as observed in corals (Le Campion-Alsumard et al., 1995, Moree et al., 2013) and sea fans (Toledo-Hernandez et al., 2008).

The available information about the associations between sponges (phylum Porifera) and marine fungi is still limited compared to the large literature existing on the prokaryotic microorganisms associated with these invertebrates (for recent reviews see Hentschel et al., 2012, Taylor et al., 2013 and Webster and Thomas, 2016). As generally observed with other macroorganisms, the extent and ecological significance of the associations between fungi and sponges have to be understood (Yarden, 2014). The first evidence of a fungus-sponge association was observed when ascomycetes belonging to the genus *Koralionastes* were found developing their ascomata on or within encrusting sponge hosts suggesting a possible nutritional dependence of the filamentous fungi on the sponges (Kohlmeyer and Volkmannkohlmeyer, 1990). Transmission electron microscopy (TEM) combined with immunogold labelling targeting the fungal chitin wall indicated that the yolk bodies described

by Gaino (1980) in *Chondrilla nucula* were actually yeast cells maternally transmitted through the oocytes (Maldonado et al., 2005). More recently, Gaino et al. (2014) analysed the apparently commensalistic relationship between an unidentified purple filamentous fungus and the calcareous sponge *Clathrina coriacea* and showed via electron microscopy how the fungal hyphae develop through the sponge mesohyl without affecting the functionality of the sponge. Fungi have also been found to be pathogens of sponges as part of a poly-microbial consortium responsible for the necrosis syndrome that severely affected populations of *Callyspongia* (*Euplacella*) aff *biru* in the Maldives (Sweet et al., 2015). It has also been observed that healthy specimens of different sponge species can host fungi that are pathogens of other marine invertebrates (Baker et al., 2009, Ein-Gil et al., 2009) probably because sponges trap the spores of these fungi through their water filtration system (Naim et al., 2017).

Fungi isolated from marine sponges have proven to contain interesting biological and pharmacological activities, due to their e.g. lipid-lowering, acetylcholinesterase inhibitory, cytotoxic and antimicrobial properties (Jin et al., 2016). The interest towards sponge-associated fungi is also related to the evidence that the compounds they produce often differ significantly from those of their terrestrial counterparts (Proksch et al., 2010), thus these fungi represent a still unexplored source of potentially novel interesting bioactive compounds. The current need in medicine to identify new effective antimicrobial compounds has driven a number of studies focused on assessing the antibacterial, antifungal and antiviral properties of the fungi isolated from sponges (Höller et al., 2000, Proksch et al., 2003, Meenupriya and Thangaraj, 2010, Subramani et al., 2013, Henriquez et al., 2014, Handayani et al., 2016, Lei et al., 2017). In fact, sponge-associated fungi are suggested to be an important reservoir of antimicrobial compounds despite the understanding that bacteria are responsible for the production of most of the antimicrobial compounds found in the sponge-associated microbiota (90%; Indraningrat et al., 2016). In particular, genes for polyketide synthase (PKS) and non-ribosomal peptide synthase (NRPS) compounds, known for their

antimicrobial properties, were isolated from sponge-associated fungi (Zhou et al., 2011, Lei et al., 2017), suggesting the potential of these fungi to produce bioactive metabolites with chemical (more precisely antibiotic) defence role for their sponge host (Zhou et al., 2011).

Phylogenetic taxonomy based on sequences from the ribosomal ITS (Internal Transcriber Spacer) and a partial conservative region of the 18S rRNA gene regions (Zhou et al., 2011) have been applied to identify the cultivable sponge-associated fungi isolated in bioprospecting studies which revealed a sponge fungal microbiome dominated by Ascomycota, primarily the orders Capnodiales, Eurotiales, Hypocreales and Pleosporales (Wang et al., 2008, Li and Wang, 2009, Paz et al., 2010, Wiese et al., 2011, Zhou et al., 2011, Passarini et al., 2013). The most represented genera isolated, e.g. *Penicillium*, *Aspergillus* and *Trichoderma*, are also common in terrestrial environments (Höller et al., 2000). In most recent times, the application of culture-independent techniques, including next generation sequencing (NGS) approaches, has broadened the panel of fungal taxa identified in sponges, especially for what concerns the order Malasseziales, not observed in previous culture-dependent studies but found in different sponge species (Gao et al., 2008, Passarini et al., 2015, Naim et al., 2017). However, the culture-independent studies have not always shown a larger fungal species richness compared to culture-dependent approaches (see Gao et al., 2008 and Passarini et al., 2015) nor indicated the presence of sponge-specific fungal communities compared to the seawater and among different sponge species (see Gao et al., 2008, Jin et al., 2014 and Naim et al., 2017) .

Species of the sponge genus *Haliclona* have led to an outstanding diversity of natural products, especially alkaloids (Tribalat et al., 2016), but little is known about their microbiomes. Through NGS and TEM analyses, focused on the bacterial and archeal communities, I have determined that the Irish *Haliclona* species *H. oculata*, *H. cinerea*, *H. simulans*, *H. viscosa* and *H. indistincta* can be considered 'Low Microbial

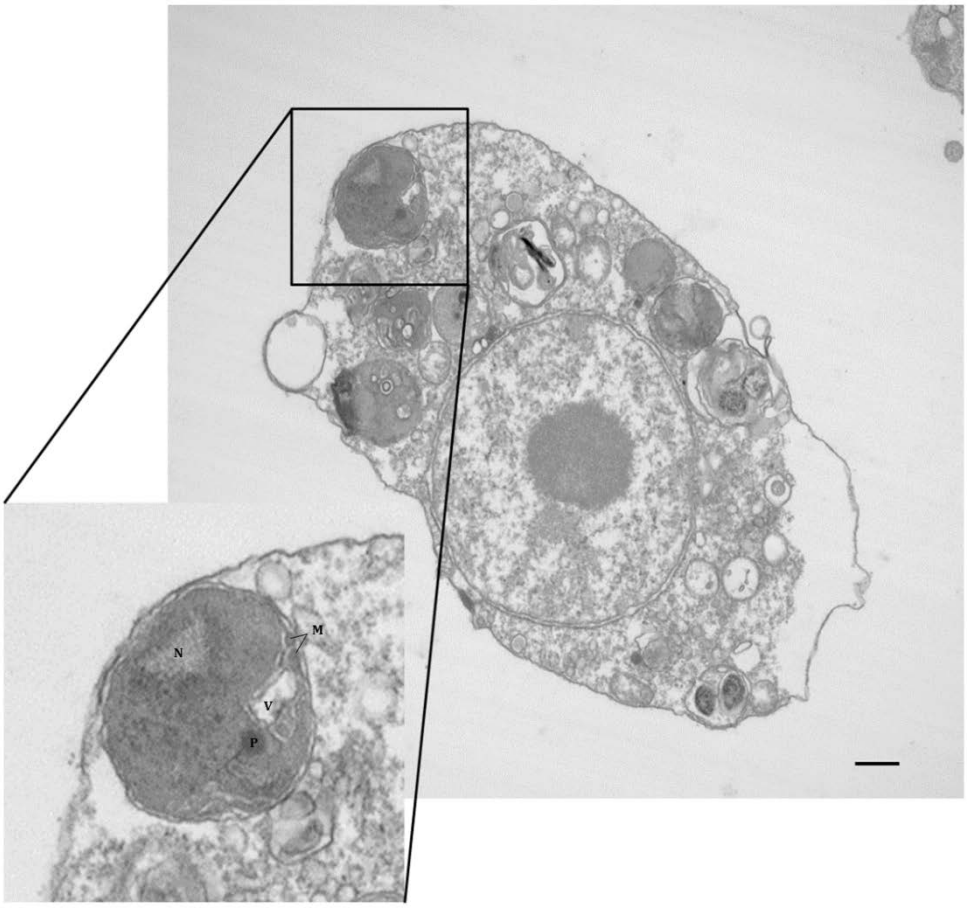

Figure 3.1. The whole cell (probable archeocyte?) of *H. oculata* containing the putative oomycete spore and close-up of the latter. By comparison with the micrographs of the yeasts in *C. nucula* described by Maldonado et al. (2005) I identified an anucleolate nucleus (N), a peroxisome (P), a putative vacuole (V) and putative mitochondria (M). Scale bar 500 nm.

Abundance' (LMA) sponge species as defined by Hentschel et al., 2006 and Giles et al., 2013 (see chapter 2). For *H. oculata* the mesohyl observed via TEM appeared almost completely free of visible bacteria, however an inclusion in some cells resembled a eukaryotic cell complete with membrane bound vacuoles and an apparent nucleus. These structures resembled the yeast cells described by Maldonado et al. (2005) in *C. nucula* (Figure 2.1; Marra et al., unpublished data). Similar intracellular inclusions characterised by internal compartmentalisation were observed also in *H. indistincta*. The TEM micrographs of these structures were analysed by mycologists who confirmed their resemblance to

spores of fungi-like organisms called Oomycota (Cavalier-Smith, 1981). To my knowledge, no previous records of possible associations between sponges and oomycetes were known. The closest case was a sponge classified as *H. montagui* (now *H. cinerea*) for which Richter (1985) hypothesised a mutualistic relationship with organisms belonging to Thraustochytriaceae, a group of fungi-like organisms currently placed into the Heterokonta as the oomycetes (Cavalier-Smith et al., 1994).

The observation of possible associations between the target sponge species and fungi-like organisms generated the hypothesis that these eukaryotic microorganisms could contribute to the production of bioactive compounds by the sponge hosts. Therefore, I set out to investigate the fungi associated to the target *Haliclona* sponge species further applying a cultivation-based method designed for isolating the intracellular fungal-like spores.

Materials and methods

Sponges studied

For the isolation of the fungi three specimens were collected for each of five *Haliclona* species from the North-Atlantic and four from the Mediterranean, i.e. *H.* (*Haliclona*) *oculata*, *H.* (*Haliclona*) *simulans*, *H.* (*Reniera*) *cinerea*, *H.* (*Rhizoniera*) *indistincta* and *H.* (*Rhizoniera*) *viscosa* from the North Atlantic and *H.* (*Reniera*) *mediterranea*, *H. (Halichoclona) fulva*, *H.* (*Soestella*) *mucosa*, *H.* (*Rhizoniera*) *sarai* from the Mediterranean. Details of the specimens collected are given in Table 3.1. Each sponge specimen was transported in individual plastic bags containing seawater from the same collection site. In the laboratory the samples were kept in refrigerated filter-sterile (0.22 μm) seawater and processed straight away or stored at –80ºC in seawater containing glycerol (20%) until processing.

H. indistincta was collected in the intertidal environment of Corranroo bay. This is a very shallow basin, characterized by very fast tidal streams although hindered by sandbanks. For these reasons the *H. indistincta*

specimens collected in this area are often covered with silt and sediment is also abundant inside the sponge canals. *H. cinerea* and *H. simulans* were collected in Roskeeda bay on a sheer rocky slope covered with a thick layer of silt. The samples of *H. viscosa* were collected in one of the diving sites of the Maharees Islands (Co. Kerry, Ireland). This site has a maximum depth of 20 meters and is exposed to tidal streams. *H. oculata* was collected in Northern Ireland, at 12 meters depth on a wreck in Strangford Lough (Portaferry, Northern Ireland). The specimens of the Mediterranean species where all collected in or around a cave located at 20 meters depth in Villefranche-Sur-Mer (Nice, France).
Additional specimens of *H. indistincta* were collected for staining with calcofluor white. These specimens were transported to the laboratory as described above. In the laboratory the specimens were fixed in 4% Paraformaldehyde in Phosphate Buffered Saline (1X, pH 7.4).

Isolation of the fungi

Eight media were used to isolate the endophytic fungi from all the sponge species, namely pure Sabouraud dextrose agar (SDA) and seven different media (labelled as M1, M2, M3, M4, M5, M6, M7) obtained by adding seven different 0.22 µm filter-sterilised extracts from both marine and terrestrial origin to the basic SDA medium. In particular, the first four extracts (used to produce the media M1, M2, M3, M4) were obtained from the brown seaweed *Ascophyllum nodosum*, the extracts to produce media M5 and M6 from tubers of *Solanum tuberosum* while the extracts used to prepare the M7 media were obtained from each of the sponge species, therefore the M7 medium was distinct for each species. Details of the extracts used are given in Table 3.2. The extracts were added at a concentration of 1% (i.e. 200 µl of extract per 20 ml of medium per plate). All the media were prepared using distilled water and chloramphenicol was added as antibiotic agent at a concentration of 50 mg/l.

Table 3.1. List of the sponge specimens collected I processed.
(*) Average seawater temperature for the sampling months and the sites (or the closest locations available) of collection from www.seatemperature.org/. Sample ID = codes given to the vouchers collected from each sponge specimens and kept at the Molecular Evolution and Systematics laboratory (Zoology Department, NUIG).

Species	Sample ID	Collection date	Collection site name	Depth (m)	Seawater temperature Cº (*)	Collection site latitude	Collection site longitude
Haliclona indistincta	MIIG1268, MIIG1269, MIIG1270	15/09/2015	Corranroo, Co. Clare (ROI)	0	14.8	53.15	-9.01
Haliclona cinerea	MIIG1235, MIIG1236, MIIG1274	13/08/2015 18/09/2015	Ail Bhuí, Co. Galway (ROI)	6	15.4 14.8	54.3385	-8.2765
Haliclona simulans	MIIG1232, MIIG1233, MIIG1234	13/08/2015	Ail Bhuí, Co. Galway (ROI)	6	15.4	53.31221	-9.6719
Haliclona viscosa	MIIG1164, MIIG1166, MIIG1167	25/08/2014	Maharees, Co. Kerry (ROI)	6	15.6	53.31221	-9.6719
Haliclona oculata	MIIG1150, MIIG1151, MIIG1155	21/05/2014	Portaferry, Strangford Lough (UK)	22	10	53.31221	-9.6719
Haliclona fulva	IRCSET385, IRCSET386, IRCSET387	28/04/2015	Villefranche-Sur-Mer, Nice (FR)	25	14.7	43.7	7.32
Haliclona mediterranea	IRCSET388, IRCSET389, IRCSET390	28/04/2015	Villefranche-Sur-Mer, Nice (FR)	25	14.7	43.7	7.32
Haliclona mucosa	IRCSET391, IRCSET392, IRCSET393	28/04/2015	Villefranche-Sur-Mer, Nice (FR)	25	14.7	43.7	7.32
Haliclona sarai	IRCSET394, IRCSET395, IRCSET396	28/04/2015	Villefranche-Sur-Mer, Nice (FR)	25	14.7	43.7	7.32

Table 3.2. List of the extracts used to prepare media M1–M7 with details of their preparation and the properties of the extracts.
(*) = The process of dyalisis determines the cut-off of all the molecules smaller than a specific molecular weight, in our case it was 12 kDa.
(**) = This kind of reaction results in the formation of Amadori products between simple reducing sugars and amino acids present in proteins and peptides.

Extract number (medium)	Organism used to produce the extract	Production process	Characteristics of the extract
I (M1)	*Ascophyllum nodosum*	Prepared by extraction of macerated seaweed in aqueous medium for 1 hour at 70°C followed by dialysis(*).	Contains soluble complex polysaccharides (alginates, fucoidans, laminarans) and some complexed phenolics or phlorotannins.
II (M2)	*Ascophyllum nodosum*	Prepared by extraction of macerated seaweed in aqueous medium for 1 hour at 70°C followed by dialysis(*).	Produced with a 2.5-fold larger volume of extractant than for extract I, contains more alginates and laminarans and less fucoidans and phenolics than extract I.
III (M3)	*Ascophyllum nodosum*	Prepared by extraction of macerated seaweed in aqueous medium for 3.5 hours at 47.5°C.	Different proportions and isotypes of complex carbohydrates plus water-soluble complex and simple phlorotannins, simple sugars and sugar alcohols, amino acids, peptides, mineral elements.
IV (M4)	*Ascophyllum nodosum*	Prepared by extraction of macerated seaweed in aqueous medium for 6 hours at 70°C followed by fractionation at 25°C.	This extract is an alginate-rich fraction with some lower residual levels of tightly-associated phenolics.
V (M5)	Potatoes (*Solanum tuberosum*)	Liquid fraction obtained by potatoes boiled in distilled water for 30 min, then filtered through multiple layers of muslin and autoclaved.	Excellent source of carbohydrates (simple sugars as glucose and sucrose as well as starch), peptides and proteins and other micronutrients (vitamins and minerals).
VI (M6)	Potatoes (*Solanum tuberosum*)	Prepared as extract V, but only filter-sterilized instead than autoclaved.	Supposed to contain some nutrients that might be lost after autoclaving because of the occurrence of maillard reactions(**) between sugars and amino acids.
VII (M7)	Sponge	Prepared mashing some sponge tissue in the same volume of sterile distilled water and then filtering the liquid obtained through syringe filter units (0.22 μm).	Not characterized chemically, but supposed to contain the main compounds produced by/present inside the sponge used.

Sponge specimens were surface-sterilised following methods modified from Schulz et al. (1993). The method chosen was stringent, attempting to remove as many loosely associated fungi as possible in efforts to reduce the number of purely environmental fungi and culture instead of fungi living within the sponge, e.g. those evident inside the cells. Each sponge was cut into small fragments (8–27 mm^3) and the fragments immersed firstly in 95% EtOH for 60 seconds, then in 2.5–5% NaClO for 30 seconds, then again in 95% EtOH for other 60 seconds and finally they were rinsed for 3–4 minutes in sterile distilled water. After that, the sponge fragments were transferred to autoclaved filter paper to reduce the moisture content and finally placed on the medium surface (partially dipped in it). One fragment per specimen was plated on each medium and the three specimens of each species were plated on the same plate (Figure 3.2). For each species two plates per medium were prepared with the same procedure. All the plates were incubated in oven at 30°C. Once the fungal structures developed from the sponge fragments they were isolated and plated again on new plates of the same media in order to obtain pure cultures that were subjected to identification.

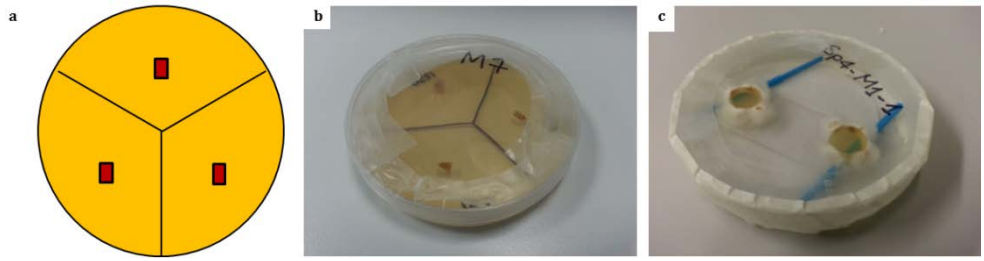

Figure 3.2. Isolation of the fungi and morphological identification of the fungal isolates. **a**. Scheme of the sponge plating; **b**. Real image of one of the plates ready to be incubated; **c**. One of the chambers prepared to obtain the growth of fungal structures on microscope slides.

Morphological and molecular identification of the fungal isolates.

For morphological identification of the sponge endophytic fungi, each fungal isolate was cultured by slide culture chamber and then stained with lactophenol cotton blue following the methods by Riddell (1950). The slides obtained were observed through an Olympus BX53 upright fluorescent microscope. The identification of the fungi was performed by the mycologists Dr Vijai K. Gupta and Dr Maria G. Tuohy (Molecular Glycobiotechnology group, Biochemistry Department, NUI Galway).

In order to extract the genomic DNA, the fungi were firstly cultured at 30ºC in sterile liquid nutrient medium containing 2% glucose for 2–4 days; during this time the flasks were kept on shaking at 200 rpm. The mycelia obtained were harvested after centrifugation decanting the medium and collecting the pellet at the bottom of the tubes; the pellet was then squeezed between muslin cloths to remove the excess liquid and ground into powder in liquid nitrogen. Genomic DNA was extracted following the method by Raeder and Broda (1985). An extraction buffer composed of 50 mM Tris-HCl, 100 mM EDTA, 100 mM NaCl and 1% w/v SDS (ph 8.0) and Proteinase K (final concentration of 100 μg/ml) were added to each sample and left to incubate in water bath at 50 °C for three hours. After centrifugation at 1,000 g for 20 minutes the supernatant was used for DNA extraction by phenol/chloroform/isoamyl alcohol (25:24:1) precipitation and purification with ethanol.

Two regions of the high-quality DNA obtained were amplified by PCR and sequenced to enable molecular identification of the fungi isolated. Two different sets of primers were selected to amplify an overlapping region of the internal spacer region between the ribosomal RNA genes, i.e. the primers ITS1/ITS4 (5’ TCCGTAGGTGAACCTGCGG 3’ / 5’ TCCTCCGCTTATTGATATGC 3’) and the primers ITS4/ITS5 (5’ TCCTCCGCTTATTGATATGC 3’ / 5’ GGAAGTAAAAGTCGTAACAAGG 3’) as described by White et al. (1990). A third PCR reaction targeted the gene encoding for the calcium-binding protein calmodulin and the primers used were Cal282F/Cal727R (5’ GAGTTCAAGGAGGCCTTCTCCC 3’ / 5’ CATCTTTCTGGCCATCATGG 3’; Carbone and Kohn, 1999). PCR

purification was carried out employing the QIAquick PCR purification kit (QIAGEN, Germany). Purified amplicons were sent for sequencing to LGC Genomics GmbH (Berlin, Germany) and chromatographs of the sequences obtained were checked for quality and trimmed using the software MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets (Kumar et al., 2016). Good quality sequences were searched against the GenBank nucleotide database (https://www.ncbi.nlm.nih.gov/genbank/?) using the algorithm Blast (Altschul et al., 1990) and the query cover, the expect (E) value and the percentage identity were noted for each sequence identified. A range of sequences identified via Blast as being similar to those generated here were downloaded from GenBank and used to build alignments with our sequences using MEGA version 7.0.

Staining of sponge tissue sections with calcofluor white.

After 24 hours in the fixative, the tissue samples from *H. indistincta* were dehydrated and embedded in paraffin wax following methods by Gupta & Pandey (2013). Tissue sections of 7 µm were cut with a Leica Jung 2035 Biocut rotary microtome and stained after dewaxing with calcofluor white mixed with an equal volume of 10% Potassium Hydroxide. The sections were observed through an Olympus BX53 upright fluorescent microscope.

Results

Morphological and molecular identification of the fungi isolated

In total 56 filamentous fungi were isolated from the nine *Haliclona* species: 23 isolates were obtained from *H. indistincta*, ten from *H. cinerea*, seven from *H. viscosa*, five from both *H. mediterranea* and *H. mucosa*, four from *H. fulva*, two from *H. sarai* and one from *H. simulans*. Unfortunately no fungi were isolated from *H. oculata* despite this species being of particular interest. The identity of the fungi isolated from the target sponge species was determined at species level for 7 isolates

based on morphology and for 33 based on PCR. The identifications are listed in Table 3.3 for *H. indistincta*, Table 3.4 for *H. cinerea*, *H. simulans* and *H. viscosa* and Table 3.5 for the Mediterranean *Haliclona*. All the fungal isolates for which it was possible to amplify good quality sequences belonged to the phylum Ascomycota, the exception being two isolates from *H. cinerea*, i.e. Sp2-3 and Sp2-M7-1 (see Appendix tables) which belonged to the phyla Basidiomycota and Mucoromycota respectively.

North-Atlantic species

Of the 23 isolates from *H. indistincta*, 14 were identified to species level (Table 3.3). One fungus isolated on medium M5 was identified to species level via morphology as *Aspergillus fumigatus* (Figure 3.3a) and the identity was confirmed by ITS sequencing. *Fimetariella rabenhorstii* (Figure 3.3b) was isolated from the SDA plates and identified from the ITS sequences. A calmodulin sequence from this fungal species is not present in GenBank and the highest hit from this gene was *Penicillium piscarium* at 92% (Table 3.4). Additional determined isolates for *H. indistincta* were *A. amstelodami*, *A. ruber* (medium M1), *A. tonophilus* (medium M4), *A. cristatus* (media M1, M5 and M6), *Sporormiella minima* (medium M2, Figure 3.3c), *Scopulariopsis brevicaulis* (Figure 3.3d), *Penicillium piceum* (medium M3), *Eurotium cristatum* (medium M7), *E. pseudoglaucum* (medium M5), *E. rubrum* (media M1 and M4). For three isolates from this species only poor quality sequences were obtained, while the remaining fungi were undetermined species of *Penicillium* and *Aspergillus* as well as representatives of the order Sordariomycetes.

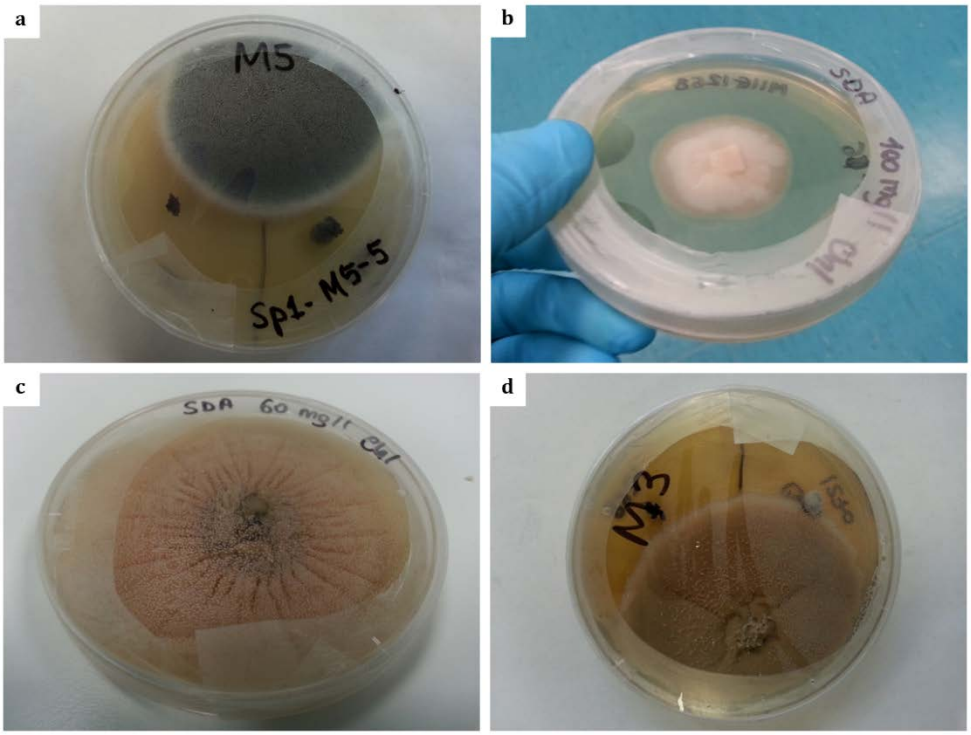

Figure 3.3. Some of the fungi isolated from *H. indistincta*: **a**. *A. fumigatus*; **b**. *F. rabenhorstii*; **c**. *Sporormiella minima*; **d**. *Scopulariopsis brevicaulis*.

Table 3.3. Identities of the fungi isolated from the *H. indistincta* based on both morphology and PCR approaches. NI = Not Identified.

Medium	Morphological identification	BLAST identification		
		ITS 1-4	**ITS 4-5**	**Calmodulin**
SDA	NI	99% *Fimetariella rabenhorstii* (KT071717)	100% *Fimetariella rabenhorstii* (KP050669)	92% *Penicillium simplicissimum* KF296379
M1	*Aspergillus* sp.	99% *Eurotium amstelodami* (LN482451)	No PCR product	No PCR product
	Aspergillus sp.	No good sequences obtained	No good sequences obtained	No good sequences obtained
	Aspergillus sp.	99% *Eurotium rubrum* (KJ766002)	100% *Aspergillus amstelodami* (KX696389)	100% *Eurotium montevidense* (LT671246)
	NI	98% Uncultured *Penicillium* (JX545192)	98% Uncultured *Eupenicillium* (JX545183)	88% *Penicillium striatisporum* (AY678534)
	NI	98% *Eurotium cristatum* (MG659640)	No PCR product	No PCR product
	NI	99% Sordariomycetes sp. (JQ758885)	99% Sordariomycetes sp. (JQ758885)	No good sequences obtained
M2	*Aspergillus* sp.	100% *Aspergillus* sp. (GU988901)	No PCR product	No PCR product
	NI	99% Sordariomycetes sp. (JQ761628)	99% Sordariomycetes sp. (JQ758885)	86% *Penicillium maximae* (KC773821)
	NI	99% Coniochaetaceae sp. (MG004797)	No PCR product	No PCR product
	NI	99% *Sporormiella minima* (KU713051)	No PCR product	No PCR product

M3	NI	99% *Scopulariopsis brevicaulis* (KP269020)	100% *Scopulariopsis flava* (LN850790)	No PCR product
	NI	99% *Scopulariopsis brevicaulis* (KP269020)	No PCR product	No PCR product
	Penicillium sp.	99% *Penicillium piceum* (JN899311)	No PCR product	No PCR product
M4	*Aspergillus* sp.	99% *Eurotium rubrum* (HM145962)	No PCR product	No PCR product
	NI	99% *Aspergillus tonophilus* (KY807659)	100 *Eurotium amstelodami* (KY828904)	99% *Eurotium montevidense* (LT671246)
M5	NI	99% *Eurotium pseudoglaucum* (KX258805)	99% *Eurotium rubrum* (KX696369)	98% *Eurotium pseudoglaucum* (KX463337)
	Aspergillus sp.	99% *Eurotium cristatum* (MG659640)	No PCR product	No PCR product
	Aspergillus fumigatus	99% *Aspergillus fumigatus* (KU321562)	99% *Aspergillus fumigatus* (MG674662)	No PCR product
	NI	No good sequences obtained	No PCR product	No PCR product
M6	NI	99% *Eurotium cristatum* (MG659639)	No PCR product	No PCR product
	NI	No good sequences obtained	No good sequences obtained	No PCR product
M7	NI	99% *Eurotium cristatum* (JQ743649)	100% *Eurotium amstelodami* (KY828904)	99% *Eurotium chevalieri* (KX463338)

Five of the fungi isolated from *H. cinerea* (Figure 3.4) were identified by Dr Gupta and Dr Tuohy as possible chytrids based on morphological features. However, DNA sequence analysis via BLAST did not support this identification (Table 3.4). In fact, besides the representative of the Basidiomycota genus *Peniophora* and the Mucoromycota *Lichtheimia corymbifera* (Figure 3.4a and 3.5a respectively), the fungi isolated from *H. cinerea* belong to the same taxa observed for *H. indistincta*, i.e. the genera *Aspergillus* and *Penicillium* and the class Sordariomycetes. The only exception is an isolate belonging to the genus *Aphanoascus* from the order Onygenales (Figure 3.5b).

The single fungus isolated from *H. simulans* had circular shape, regular margin, corrugated surface, grey colour and white margin (Figure 3.6a). The microscopic analysis showed the presence of small (< 10 μm) simple *penicillus*-like conidiophores developed from septate hyphae (Figure 3.6b-d). The sequence obtained from PCR with the primers ITS 1 and 4 identified this isolate as *Penicillium griseolum* (Table 3.4).

All the seven fungi isolated from *H. viscosa* were morphologically identified as chytrids by Dr Gupta and Dr Tuohy. Six of them had a very similar morphology characterised by irregular shape, granular surface and variable colour between yellow and light green/brown (Figure 3.7). Except for the isolates obtained from the media M1 and M6, for which no good sequences were obtained, these fungi were identified via molecular data as belonging to the genus *Aspergillus* section *Aspergillus* formerly denominated *Eurotium*. In particular, the isolate obtained on the medium M5 (Figure 3.7d) was identified as *Eurotium cristatum*. The remaining fungus isolated from *H. viscosa* on SDA with no added extract was a Sordariomycetes belonging to the species *Coniochaeta lignaria* (Figure 3.8).

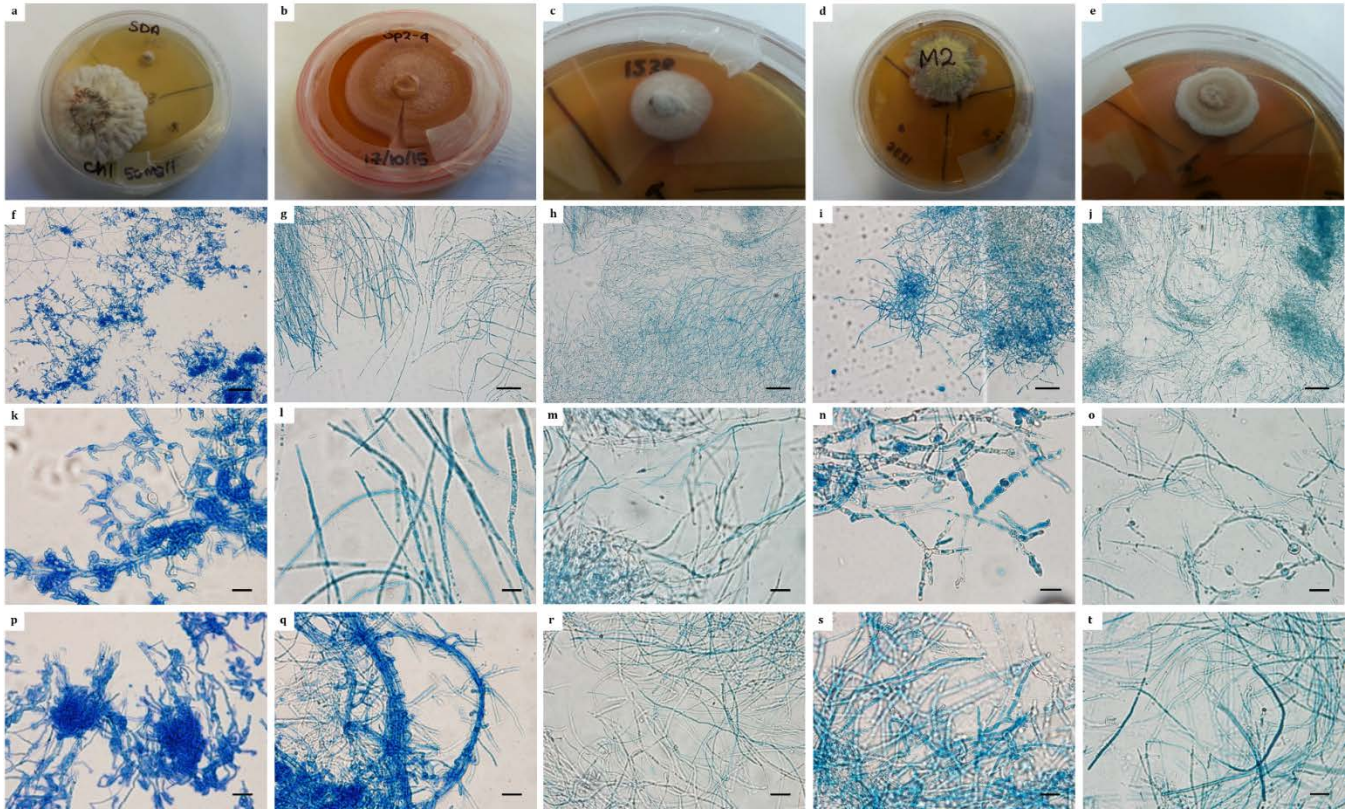

Figure 3.4. Fungi isolated from *H. cinerea* and identified as chytrids based on morphology. **a**. *Peniophora* sp.; **b**. *Penicillium nodositatum*; **c**. Sordariomycetes sp.; **d**. *Aspergillus medius*; **e**. Sordariomycetes sp.; **f – t**. Micrographs of the same fungi grown on microscope slides and stained with lactophenol cotton blue. Scale bar: **f – j**: 100 µm; **k – t**: 20 µm.

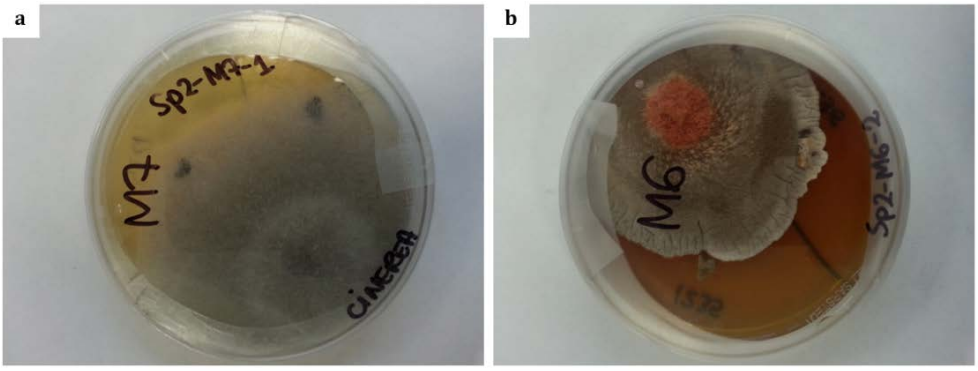

Figure 3.5. **a**. The Mucoromycota *Lichtheimia corymbifera* isolated from *H. cinerea* on medium M7 containing the sponge extract; **b**. *Aphanoascus* sp. isolated from medium M6.

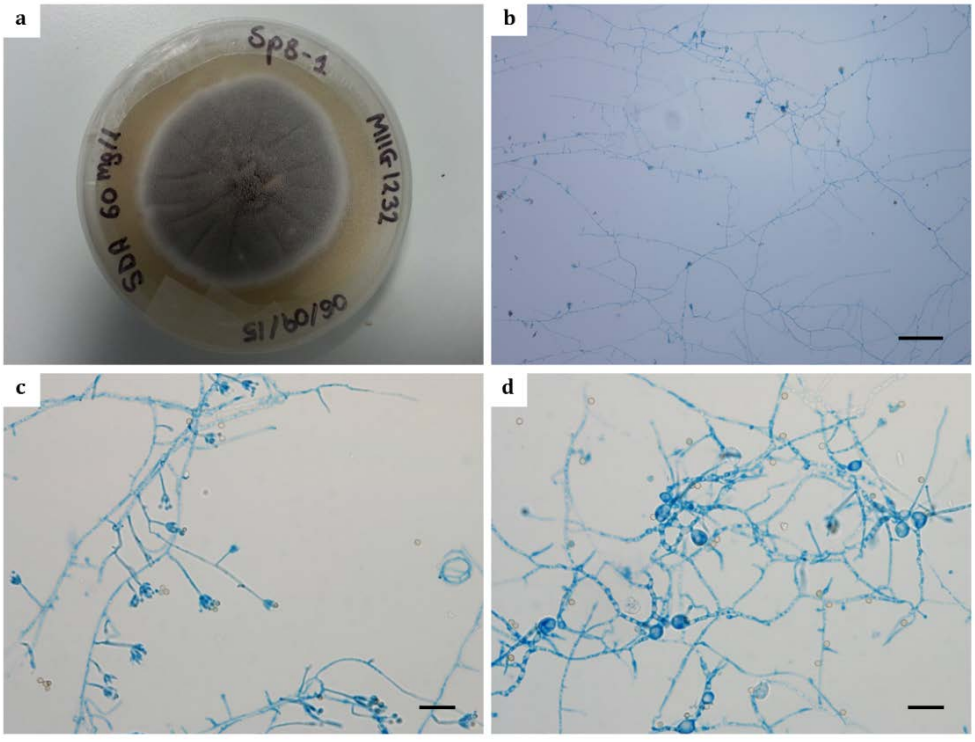

Figure 3.6. **a**. *Penicillium griseolum*, the only fungus isolated from *H. simulans* on pure Sabouraud Dextrose Agar with no additional extracts; **b – d**. Microscopic pictures of conidiophores and hyphae of *P. griseolum*. Scale bar: **b**. 100 µm; **c – d**: 20 µm.

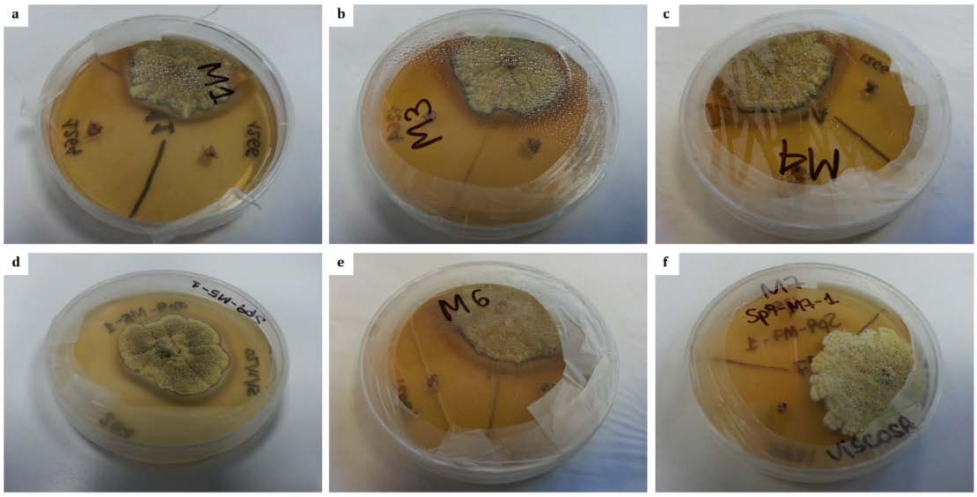

Figure 3.7. The fungi with similar morphology isolated from *H. viscosa* using medium **a**. M1, **b**. M3, **c**. M4, **d**. M5, **e**. M6 and **f**. M7.

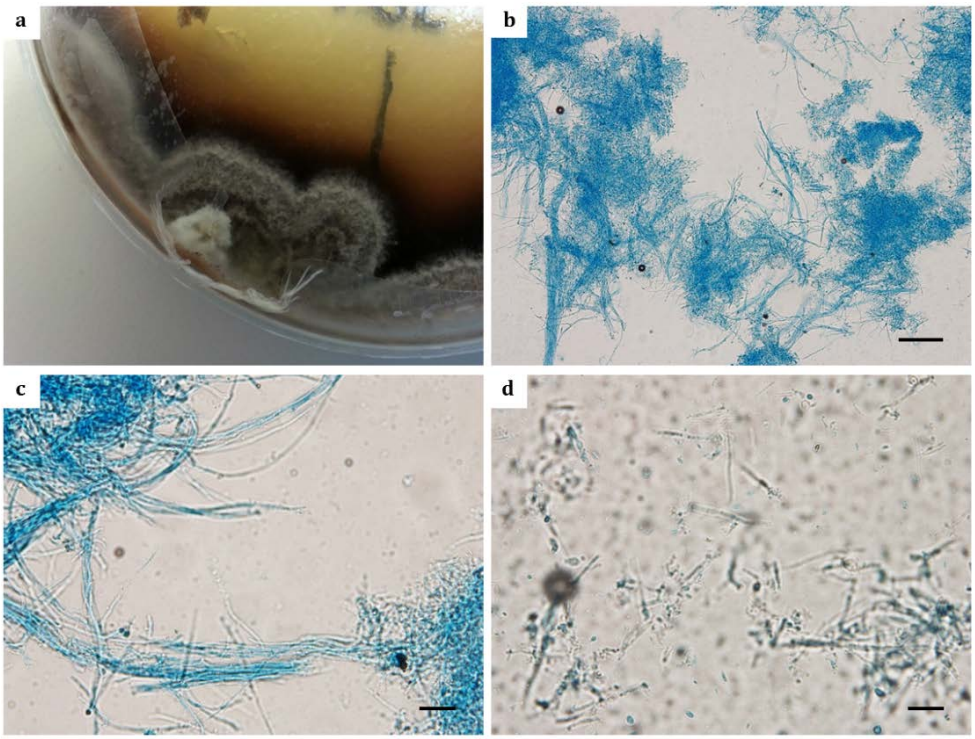

Figure 3.8. **a**. *Coniochaeta ligniaria*, isolated from *H. viscosa* on SDA. **b – d**. Microscopic pictures of *C. ligniaria*. Scale bar: **b**. 100 µm; **c – d**: 20 µm.

Table 3.4. Identities of the fungi isolated from the *H. cinerea*, *H. simulans* and *H. viscosa* based on both morphology and PCR approaches. NI = Not Identified.

Sponge species	Medium	Morphological identification	BLAST identification		
			ITS 1-4	ITS 4-5	Calmodulin
Haliclona cinerea	SDA	Chytridiomycota	98% *Peniophora* sp. (HQ607865)	No PCR product	No PCR product
		Chytridiomycota	98% *Penicillium nodositatum* (KU986771)	98% Eurotiales sp. (KF428430)	97% *Penicillium* sp. (LT899772)
	M1	Chytridiomycota	92% Sordariomycetes sp. (JQ761877)	92% Sordariomycetes sp. (JQ761877)	No PCR product
	M2	Chytridiomycota	97% *Aspergillus medius* (KT832076)	99% *Aspergillus proliferans* (KX696375)	99% *Eurotium pseudoglaucum* (KX463337)
	M3	*Aspergillus* sp	99% *Eurotium cristatum* (MG659639)	99% *Eurotium amstelodami* (KY828904)	97% *Eurotium montevidense* (LT671246)
	M6	NI	97% *Aphanoascus* sp. (KC871025)	No PCR product	No PCR product
		Chytridiomycota	92% Sordariomycetes sp. (JQ761877)	No PCR product	No PCR product
	M7	*Rhizopus oryzae*	99% *Lichtheimia corymbifera* (MF919350)	No PCR product	No PCR product
		Penicillium sp.	99% *Penicillium* sp. (LT558879)	No PCR product	No PCR product
Haliclona simulans	SDA	*Penicillium* sp.	99% *Penicillium griseolum* (NR_138277)	No PCR product	No PCR product

Haliclona viscosa	SDA	Chytridiomycota	99% *Coniochaeta ligniaria* (KX394783)	100% Sordariomycetes sp. (JQ758885)	95% *Penicillium striatisporum* (AY678534)
	M1	Chytridiomycota	No good sequences obtained	No PCR product	No PCR product
	M3	Chytridiomycota	98% *Eurotium cristatum* (MG659640)	100% *Eurotium amstelodami* (KY828904)	99% *Eurotium montevidense* (LT671246)
	M4	Chytridiomycota	98% *Eurotium amstelodami* (KF986418)	No PCR product	No PCR product
	M5	Chytridiomycota	99% *Eurotium cristatum* (JQ743649)	No PCR product	No PCR product
	M6	Chytridiomycota	No good sequences obtained	No PCR product	No PCR product
	M7	Chytridiomycota	98% *Eurotium cristatum* (KJ018708)	100% *Eurotium amstelodami* (KY828904)	99% *Eurotium montevidense* (LT671246)

Mediterranean species

The Mediterranean sponges showed the presence of a different fungal diversity. A hairy white fungus identified as a possible member of Oomycota based on morphological analysis (Figure 3.9a) was isolated from both *H. fulva* and *H. mucosa*. Unfortunately no amplicons were obtained from any of these fungi. Two of the isolates from *H. fulva* showed the same Oomycota-like morphology, while the other fungi isolated from this sponge species were *Penicillium glabrum* and a member of the order Xylariales identified as *Biscogniauxia mediterranea* (Figure 3.9b). *B. mediterranea* was also isolated from *H. mediterranea* and originally identified as Oomycota based on morphology. Four other fungi were obtained from *H. mediterranea* and two of them were identified at species level via molecular data, i.e. the Sordariomycetes Sordariales *Chaetomium globosum* and a fungus identified as *A. awamori*. The latter fungus showed circular shape, granular surface, dark green to black colour and white margin; the lactophenol cotton blue staining for this isolate showed circular conidia with wrinkled surface that did not stain, hyphae with septa and aspergillus-shaped coniophores; all these characteristics are typical of the genus *Aspergillus* section *nigri* (Silva et al., 2011; Figure 3.10). Both fungi isolated from *H. sarai* and one of the isolates from *H. mucosa* showed the same morphological characteristics as the isolate *A. awamori* from *H. mediterranea*: the isolates from *H. mucosa* and one of the two from *H. sarai* were identified as *A. niger*, the other isolate from *H. sarai* showed a similarity of 97% again with *A. niger* (Table 3.5). The only other isolate from *H. mucosa* that was possible to identify via molecular data was an additional *Aspergillus* belonging to the species *tonophilus*.

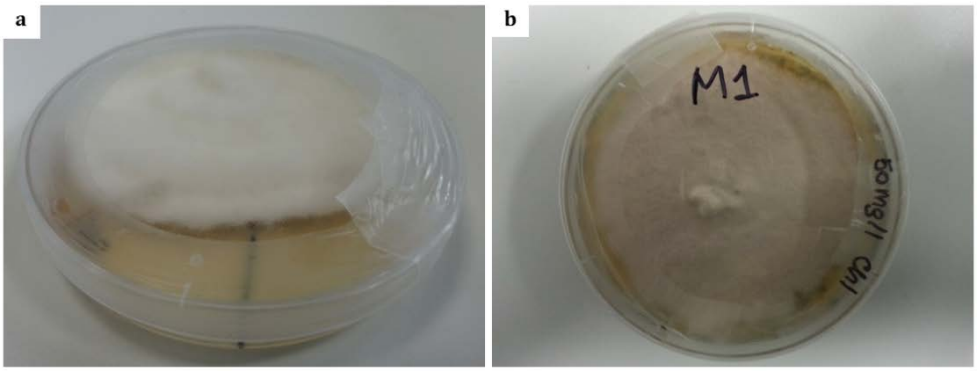

Figure 3.9 a. The hairy white fungus isolated from *H. fulva*; **b**. *Biscogniauxia mediterranea* isolated from both *H. fulva* and *H. mediterranea*.

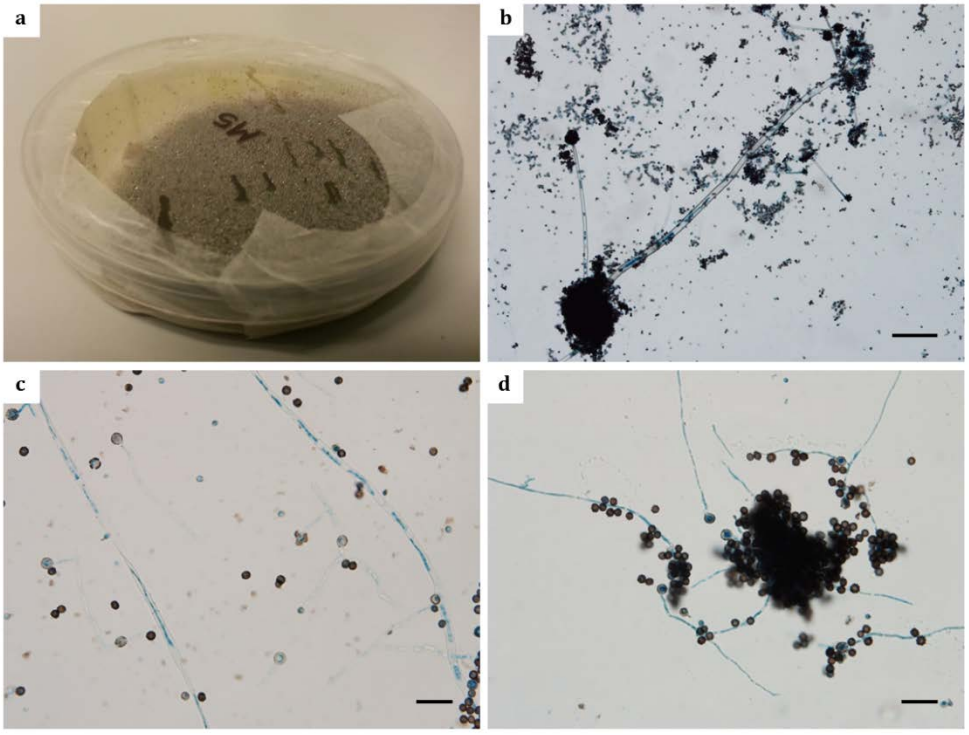

Figure 3.10. a. *Aspergillus awamori*. **b – d**. Microscopic pictures of conidiophores, hyphae and spores of *A. awamori*. Scale bar: **b**. 100 µm; **c – d**: 20 µm.

Table 3.5. Identities of the fungi isolated from the *Haliclona* species from the Mediterranean Sea based on both morphology and PCR approaches. NI = Not Identified.

Sponge species	Medium	Morphological identification	BLAST identification		
			ITS 1-4	ITS 4-5	Calmodulin
Haliclona fulva	M1	Oomycota	No PCR product	No PCR product	No PCR product
	M2	*Phaenerochaete* sp.	99% *Biscogniauxia mediterranea* (KM216776)	99% *Biscogniauxia mediterranea* (KU727203)	92% *Cylindrium elongatum* (KM231448)
	M6	*Penicillium* sp.	99% *Penicillium glabrum* (KX099660)	99% Fungal sp. (KU838981)	99% *Penicillium glabrum* (KM089147)
	M7	Oomycota	No PCR product	No PCR product	89% *Dichotomomyces cejpii* (EF669883)
Haliclona mediterranea	M1	NI *Aspergillus* sp.	No PCR product 99% Fungal sp. (KU838374)	No PCR product 100% Fungal endophyte (KT203141)	No PCR product 91% *Aspergillus striatus* (KU866759)
	M3	Oomycota	99% *Biscogniauxia mediterranea* (KM216776)	99% *Biscogniauxia* sp. (KF367566)	No PCR product
	M5	*Aspergillus niger*	99% *Aspergillus awamori* (MG576105)	No PCR product	No PCR product
	M7	*Myrothecium* sp.	99% *Chaetomium globosum* (AB511977)	100% *Chaetomium globosum* (MF461354)	99% *Chaetomium globosum* (GQ221091)

Haliclona mucosa	M3	*Aspergillus* sp.	99% *Aspergillus tonophilus* (KX858810)	99% *Eurotium chevalieri* (KX696384)	100% *Eurotium montevidense* (LT671254)
	M4	Oomycota	No PCR product	No PCR product	No PCR product
		Oomycota	No PCR product	No PCR product	No PCR product
	M5	*Aspergillus niger*	99% *Aspergillus niger* (KF358715)	100% *Aspergillus niger* (LT732555)	99% *Aspergillus awamori* (KY421637)
		Aspergillus niger	No PCR product	No PCR product	100% *Aspergillus awamori* (KJ777809)
Haliclona sarai	M5	*Aspergillus niger*	99% *Aspergillus niger* (KF706671)	99% *Aspergillus niger* (MG904945)	99% *Aspergillus awamori* (KY421633)
		Aspergillus niger	97% *Aspergillus niger* (LT732555)	No PCR product	No PCR product

Fungal isolates distribution

The number of isolates and their distribution across species, specimens and different media is shown in Table 4. The media that yielded the highest numbers of isolates were M1 (prepared with an extract from *A. nodosum*) and M5 (containing an extract from *S. tuberosum*), respectively with 22 and 20 fungal isolates. The lowest number of fungal isolates (10) was obtained from the Sabouraud dextrose agar with no extracts and the medium M4. In total the media created adding the sponge extract to SDA (labelled as M7) yielded 12 fungi, a similar number to the isolates from the media M2 and M6. Fourteen isolates were obtained from M3.

The number of fungal isolates varied across species, across media but also across specimens per species. The fungi obtained from the Irish *Haliclona* (40) were almost three times the isolates from the Mediterranean species (16). Two specimens of *H. indistincta* yielded the same number of fungi (9) while 5 isolates were obtained from the third specimen. For this sponge species the highest number of fungi (6) was isolated from the medium M1, while one isolate was obtained respectively from the medium M7 and the SDA with no added extracts. Of the nine filamentous fungi isolated from *H. cinerea* 5 were obtained from the specimen MIIG1236 and two from each of the other two samples of this species. For *H. simulans* the single fungus isolated was obtained from the sample MIIG1234 and for *H. viscosa* one of the seven fungi isolated was obtained from the specimen MIIG1264 and the remaining six from MIIG1267. The fungi isolated from the Mediterranean *Haliclona* were evenly distributed across the specimens of each sponge species (see Table 4), except for *H. mucosa* with 3 fungi isolated from specimen IRCSET392, two from IRCSET393 and zero from IRCSET391.

No *Aspergillus* species were isolated from the pure Sabouraud dextrose agar. Furthermore no fungi were isolated via this medium from any of the Mediterranean species. Across the Irish *Haliclona* this medium yielded two different species of *Penicillium*, two Sordariomycetes belonging to the species *Fimetariella rabenhorstii* and *Coniochaeta lignaria*, and the Basidiomycota *Peniophora* sp.. Different species of

Aspergillus were isolated from all of the media containing an extract, but while the species belonging to the section *Aspergillus* (i.e. *A. amstelodami*, *A. cristatus*, *A. medius*, *A. pseudoglaucus*, *A. ruber*, *A. tonophilus*) were isolated from all such media, both *A. fumigatus* (from *H. indistincta*) and the representatives of the section *nigri* (i.e. *A. awamori* from *H. mediterranea* and *A. niger* from *H. mucosa* and *H. sarai*) were isolated exclusively from the medium M5 prepared with potato extract. The only other species that was observed on more than one medium was *B. mediterranea* isolated from both *H. fulva* and *H. mediterranea* respectively from the media M2 and M3 (both containing extracts from the seaweed *A. nodosum*).

The medium produced with the sponge extract (i.e. M7) yielded the same type of fungus for both *H. indistincta* and *H. viscosa*, i.e. *A. cristatus*. Two isolates (the Mucoromycota *Lichtheimia corymbifera* and an unidentified *Penicillium*) were isolated from the medium prepared with the extract of *H. cinerea* from this sponge species, while for *H. mediterranea* the Sordariomycetes *Chaetomium globosum* was isolated from plates with medium M7. No isolates were obtained from the same type of medium for *H. oculata*, *H. simulans*, *H. mucosa* and *H. sarai*, while the fungus obtained for *H. fulva* has yet been not identified based on the molecular approach, while the morphological traits suggested its membership to the Oomycota.

Table 3.6. Number of fungi isolated from each target sponge species across specimens and different media considered.

		Medium							
Species	**Sample code**	**M1**	**M2**	**M3**	**M4**	**M5**	**M6**	**M7**	**SDA**
Haliclona indistincta	MIIG1268	4	1	1	0	2	0	0	1
	MIIG1269	1	3	1	1	1	1	1	0
	MIIG1270	1	0	1	1	1	1	0	0
Haliclona cinerea	MIIG1235	0	1	0	0	0	0	0	1
	MIIG1236	1	0	1	0	0	0	2	1
	MIIG1275	0	0	0	0	0	2	0	0
Haliclona simulans	MIIG1232	0	0	0	0	0	0	0	0
	MIIG1233	0	0	0	0	0	0	0	0
	MIIG1234	0	0	0	0	0	0	0	1
Haliclona viscosa	MIIG1264	0	0	0	0	0	1	0	0
	MIIG1266	0	0	0	0	0	0	0	0
	MIIG1267	1	0	1	1	1	0	1	1
Haliclona fulva	IRCSET385	0	0	0	0	0	0	1	0
	IRCSET386	0	1	0	0	0	1	0	0
	IRCSET387	1	0	0	0	0	0	0	0
Haliclona mediterranea	IRCSET388	1	0	0	0	0	0	1	0
	IRCSET389	0	0	1	0	0	0	0	0
	IRCSET390	1	0	0	0	1	0	0	0
Haliclona mucosa	IRCSET391	0	0	0	0	0	0	0	0
	IRCSET392	0	0	0	1	2	0	0	0
	IRCSET393	0	0	1	1	0	0	0	0
Haliclona sarai	IRCSET394	0	0	0	0	0	0	0	0
	IRCSET395	0	0	0	0	1	0	0	0
	IRCSET396	0	0	0	0	1	0	0	0

Search of fungal structures in the sponge body through calcofluor white

The histological analysis of the tissue of *H. indistincta* stained with calcofluor white did not highlight clearly the presence of fungal structures. In fact, when observed at the microscope the sections showed a diffuse blue fluorescence surrounding the pink emission of the sponge cells (Figure 3.11). The emission of blue fluorescence is expected when the stain binds the chitin present in the fungal cell walls, but in the case of the *H. indistincta* tissue the broadly emitted fluorescence of the sections was not specific in a way to suggest the presence of many hyphae scattered in the sponge tissue, nor associated with cells.

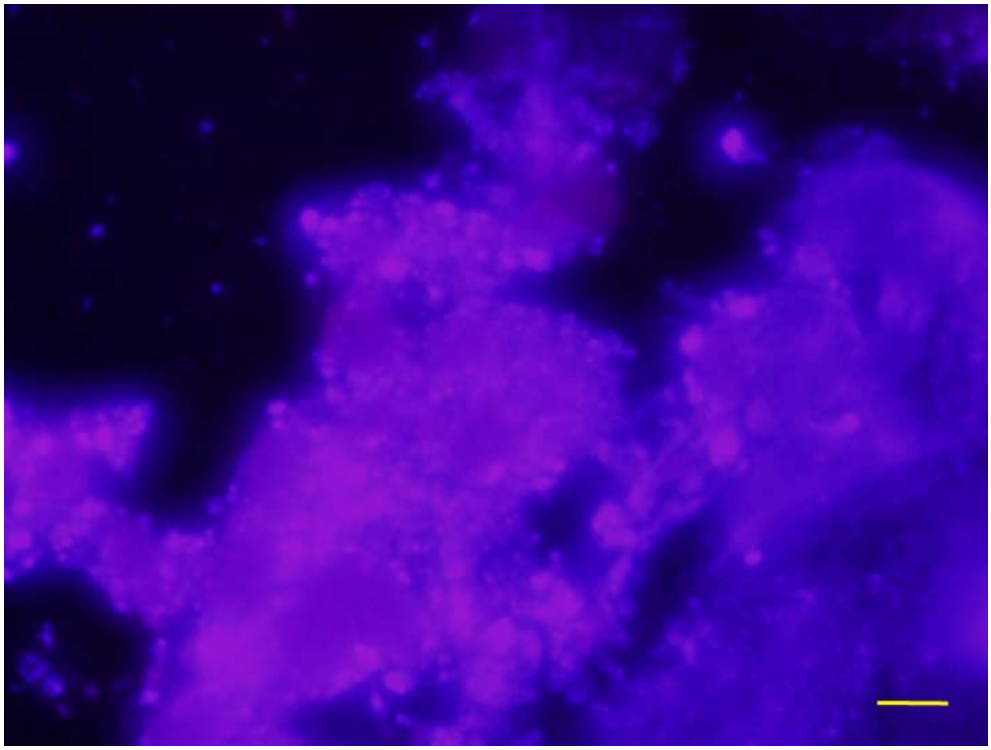

Figure 3.11. *H. indistincta* tissue section stained with calcofluor white: the pink elements are sponge cells while the blue fluorescence is possibly due to the occurrence of chitin in the sponge skeleton. Scale bar = 20 μm.

Discussion

Fungi isolated from the *Haliclona* species studied

From both the Irish and Mediterranean species considered in this study, I have isolated fungi not commonly found in sponges, such as *Fimetariella rabenhorstii* and *Sporormiella minima* from *Haliclona indistincta*, *Aphanoascus* sp. and *Lichtheimia corymbifera* from *H. cinerea*, *Coniochaeta lignaria* from *H. viscosa* and *Biscogniauxia mediterranea* from both *H. fulva* and *H. mediterranea*. *C. lignaria* has been reported as a fungus present in the marine environment in Europe (Landy and Jones, 2006), while *B. mediterranea* was previously only isolated from the deep Mediterranean Sea sediment (Wu et al., 2016). *Scopulariopsis brevicaulis,* isolated from *H. indistincta* on medium M3, was found also in marine sediment (Balabanova et al., 2018), but firstly it was isolated from the Mediterranean sponge *Tethya aurantium* and characterised for its anticancer properties (Wiese et al., 2011). *Chaetomium globosum* was found in this study in one of the *H. mediterranea* specimens on the medium containing the sponge extract and previously was isolated from two species of *Theonella* collected in the South China Sea (Jin et al., 2014). The remaining fungal isolates belong to the genera *Aspergillus* and *Penicillium* that appear to be ubiquitous based on the limited studies on few sponge species carried on so far, as observed by Suryanarayanan (2012). In particular, for both the North-Atlantic and the Mediterranean *Haliclona* species studied, fungi belonging to the genus *Aspergillus* seem to be dominating. I found representatives of three Aspergillus sections, i.e. *Fumigati* (*A. fumigatus* isolated from *H. indistincta*), *Nigri* (*A. awamori* and *A. niger* from the Mediterranean species except *H. fulva*) and *Aspergillus* (i.e. the most represented *A. cristatus* as well as *A. amstelodami*, *A. montevidensis*, *A. pseudoglaucus*, *A. medius*, *A. ruber* and *A. tonophilus*, the latter isolated from both North-Atlantic and Mediterranean sponges). All these species are known from terrestrial environment and are considered as marine-derived fungi (Chen et al., 2017). Suryanarayanan (2012) suggested that the occurrence of fungi belonging to the genus *Aspergillus* in different sponge species from

different geographical locations can imply for these fungi a role in the chemical defence of the sponge hosts against bacterial pathogens and in the selection of the endosymbiotic bacterial community via the polyketide synthase (PKS) and nonribosomal peptide synthase (NRPS) genes they are known to possess (Chiang et al., 2010). However, the widespread distribution of sponge-associated fungi as argument to support their symbiotic relationship with the invertebrates is not broadly accepted. In fact, Naim et al. (2017) considered that the occurrence of yeasts belonging to the order Malasseziales across different sponge species, geographical locations and in seawater samples is not a reason to believe that these fungi are sponge symbionts, but more likely that the sponges filter them from the surrounding seawater.

The fungi associated with three of our target *Haliclona* species (i.e. *H. mediterranea*, *H. oculata* and *H. simulans*) were previously analysed in specimens collected in the South China Sea. *H. mediterranea* was one of 11 sponge species analysed through 454 pyrosequencing by He et al. (2014) to determine their eukaryotic communities. Compared to the other 10 species considered, the fungal diversity of *H. mediterranea* was low, with only one record of an OTU belonging to the Sordariomycetes Microascales, while we isolated from this species Sordariomycetes belonging to the orders Xylariales (*B. mediterranea*) and Sordariales (*C. globosum*). Zhou et al. (2011) showed an association between *H. oculata* and *Aspergillus ochraceus* and an unidentified fungal species belonging to the Hypocreales order after plating the tissue homogenate from this sponge species on 6 different media, while we did not isolate any fungus from *H. oculata*. A culture-dependent method was also used to study the mycobiota of *H. simulans* (Liu et al., 2010) which appeared to be composed of members of the orders Capnodiales, Dothideales, Eurotiales, Hypocreales, Saccharomyceales, Xylariales, Wallemiales and Agaricostilbales. Also, the fungal consortium of *H. simulans* was already largely studied in Ireland focusing on specimens collected in the same site considered in our study (i.e. the Gurraig Sound in Kilkieran Bay; Baker et al., 2009, Baker et al., 2010, Baker et al., 2012). Baker et al.

(2009) matched culture-dependent and independent methods and identified via both types of analysis two fungal genotypes belonging to the orders Eurotiales and Chaetothyrailes, suggesting a possible symbiotic relationship with the sponge. However, not only were these two isolates not present in the Chinese sponges, but the whole set of fungal isolates were not shared between the sponges from the two geographical locations. This led Liu et al. (2010) to consider that the fungal diversity associated with sponges is more dependent on the surrounding environment than on the sponge species and there is not a species-specific fungus-sponge association in *H. simulans*. Doubts remain regarding the taxonomy of the *Haliclona* species collected in China being this area outside their geographical distribution according to the World Porifera Database (http://www.marinespecies.org/porifera). Furthermore, in the case of *H. oculata*, the statement made by Zhou et al. (2011) that the identification was made mainly looking at the spicules of this sponge is a cause for concern not being sufficient taxonomic marker to distinguish the different *Haliclona* species (Jones, 1984). However, the only fungus we isolated from *H. simulans* (i.e. *Penicillium griseolum*) was not found in the sponges from the Gurraig previously analysed by Baker et al. (2009) and this observation endorses the hypothesis prosed by Liu et al. (2010).

Possible environmental origin of the fungal isolates

Based on the evidence that in most cases we did not isolate the same fungi from all the specimens of each sponge species, while in some cases the same fungus was isolated from more than one sponge species from the same habitats, I hypothesise that all the *Haliclona* species studied here do not host species-specific fungal communities, but rather the fungi isolated have environmental origin. Even though a cultivation-indipendent approach should be applied to confirm my hypothesis, I consider that a possible factor that could have affected the diversity of the fungi obtained from the sponges studied is the surface sterilisation technique I used compared with the other studies available on the same

sponge species or different species collected in the same environment. The surface sterilisation procedure I followed was inspired by the methods used by Schulz et al. (1993) to isolate the endophytes from different plant leaves and stems. The idea behind the selection of this method was to ensure the elimination of extracellular fungal structures, supposed to be of possible environmental origin, and to isolate only the intracellular endophytes, considered as true symbionts. This may have been successful as the number of fungi isolated is less than the culture-dependent studies previously performed for some of the target species. In fact, Baker et al. (2009) plated fragments of tissue from *H. simulans* that were rinsed three times in sterile artificial seawater and in this way they yield 80 fungal isolates corresponding to 19 different genotypes, while I isolated only one fungus from a single specimen of this sponge species. However, Suryanarayanan (2012) considered that strong surface sterilization procedures as the one I applied lead to sponge disintegration, resulting in the death of their fungal symbionts. Moreover, Höller et al. (2000) have applied the same methods described by Schulz et al. (1993) to isolate fungi from 16 different sponge species and they advised against applying any of these methods to sponges because they found that even the mildest protocol damages the sponge tissue, but does not kill the superficial fungi. To avoid the occurrence of this possibility the best approach is to prepare negative controls pressing the sterilised organismal surface on the nutrient media selected for the isolation in order to assess the successful sterilisation procedure, as proposed by Kjer et al. (2010), but this was not considered for the present study.

Besides the sterilisation methods, also the type of media selected for the inoculation of the sponges seems to play an important role in determining extent and diversity of the mycobiota obtained via cultivation-based approaches. In this study I was advised by Dr Tuohy and Dr Gupta to use media prepared adding different types of extracts to Sabouraud Dextrose Agar (SDA), a medium largely used for primary fungal isolation (Scognamiglio et al., 2010). The extracts were selected

because they were considered suitable to create optimal conditions for the development of all the possible fungal taxa, also oomycetes and chytrids otherwise difficult to cultivate with standardized cultivation methods/media. In fact, *Ascophyllum nodosum* and *Solanum tuberosum* are proved to be respectively associated with and parasitized from different fungi (e.g. Beever and Bollard, 1970, Deckert and Garbary, 2005, Jermy, 2012). I also prepared a further medium (M7) adding a sponge extract obtained from each of the target *Haliclona* species to SDA following the example of Webster et al. (2001) who prepared media with three different types of sponge extract to successfully culture bacteria not previously isolated from *Rhopaloeides odorabile*. However, no particular relationships between the type of medium/extract used and the fungal isolates obtained could be observed in this study, except for the evidence that no *Aspergillus* species were obtained from SDA with no extract, while the species belonging to *Aspergillus* section *nigri* (i.e. *A. awamori* and *A. niger*) were obtained only from the medium prepared with potatoes and autoclaved (M5). Moreover, the media obtained adding the sponge extracts to SDA did not yield unique fungal isolates, exception being the Mucoromycota *Lichtheimia corymbifera* from *H. cinerea* and *Chaetomium globosum* from *H. mediterranea*.

Another aspect to consider about the media I used to isolate fungi from the nine *Haliclona* species is that they did not contain artificial seawater which should be employed to isolate the 'obligate marine fungi that grow and sporulate exclusively in a marine or estuarine habitat' compared to the 'facultative marine fungi from freshwater and terrestrial milieus [that are] able to grow and possibly also sporulate in the marine environment' based on the definition by Kohlmeyer and Kohlmeyer (1979; Kjer et al., 2010). This implies that the fungal isolates I obtained in our study can all be considered as facultative marine fungi or marine-derived fungi (Suryanarayanan, 2012). A parallel experiment of isolation of fungi using the same media prepared with artificial seawater instead of distilled water would have been useful to ascertain whether a different fungal consortium in terms of abundance and diversity could have been

obtained from the target *Haliclona* species. I was able to perform this parallel experiment only with *H. indistincta* and the fungi obtained, analysed only at morphological level in most of cases were different compared to the fungi isolated on media prepared with distilled water (data not presented here). However, Pang et al. (2016) pointed out how chytrids, and fungal-like organisms with a zoosporic stage such as oomycetes, are more susceptible to variations in salinity during sporulation, therefore the media we prepared with distilled water should have promoted the development of this type of organisms if their spores were present in the sponge cells. Unfortunately, while the morphology of the cultures indicated the likely presence of these organisms, none of the relevant isolates yielded quality sequences using the primers selected. Moreover, given time constraints, a restricted set of primers were applied to help identify the fungal cultures using molecular means. These primers were designed to amplify primarily Ascomycota and it is possible that when Oomycota-specific primers are used I will be able to identify some of these microorganisms from the cultures.

Additional factors that can affect number and diversity of the fungi isolated from sponges are the inoculation technique used and the incubation conditions. The study by Bovio et al. (2018) well summarises the effect of all the factors considered so far. The authors used two different isolation methods (plating of tissue fragments vs spreading of tissue homogenates on the medium) to isolate on four different media and at different temperatures of incubation the fungi associated with sponges collected in two of the geographical sites considered in our study, i.e. *Dysidea fragilis* and *Pachymatisma johnstonia* from Gurraig Sound and *Sycon ciliatum* from Corranroo. Significant differences were found among the fungi obtained from the different combinations of the parameters considered and based on these results the authors suggested the existence of species-specific fungal communities associated with the sponges studied. However, some fungal isolates were shared among the three sponge species and I did not isolate any of them from the *Haliclona* species from the same collection sites. This could be explained because

abundance and nature of the fungal isolates obtained from different sponges species have been correlated with the characteristics of the sponge skeleton, with less rigid sponge species hosting more fungi than stiff sponge species (Pivkin et al., 2006), and the sponges by Bovio et al. (2018) appear to be very different compared to the *Haliclona* species from this point of view. However, this observation cannot be taken in account to explain the different results observed between our study and the work of Baker et al. (2009) on *H. simulans*, thus the cultivation techniques appear to have more importance than the taxonomy of the sponges or their location to determine the fungal yield.

Uncertainties about the significance of the associations between sponges and fungi

In nearly all cases there is no evidence indicating that fungi actively grow inside the substratum from which they have been isolated (Pang et al., 2016), suggesting that their presence in sponges is in fact limited to spores (Proksch et al., 2010). However, the possibility of true fungal associations with sponges is still supported by the observation that some of the sponge mitochondrial introns are of fungal origin, perhaps as a result of horizontal gene transfer (Rot et al. 2006), while the discovery of (1→3)-β-d-glucan-binding proteins for fungal recognition were found on sponge cell surfaces (Perović-Ottstadt et al. 2004). Therefore, the question if real associations between sponges and fungi exist is still open. It has to be taken in account that the isolation of fungi from sponges via culture-dependent methods to my knowledge has never been matched with histological analyses aimed at localising fungal structures within the sponge cells/bodies. To date only Maldonado et al. (2005) have proved the presence of fungal-like structures in sponges using lectin wheat germ agglutinin–bovine serum agglutinin–gold complex to label β–1,4–N–acetyl–D–glucosamine residues of the chitin walls of the yeasts, but no cultivation neither molecular approaches were attempted to identify the yeast. Additional histological techniques could be used to visualise fungal and fungal-like structures in sponges, like the fluorescein

isothiocyanate labelled wheat germ agglutinin and/or calcofluor white, both targeting the chitin present in the fungal cell wall (Meyberg, 1988, Rasconi et al., 2009), or fluorescent *in situ* hybridisation with probes targeting unique sites on the rRNA (Takao et al., 2007). In this study I have used calcofluor white to stain tissue sections of *H. indistincta* embedded in paraffin. *H. indistincta* is the species which yielded the highest number of fungal isolates in this study; it showed via TEM the presence of compartmentalised intracellular structures resembling fungal or fungi-like spores, and it is also the most accessible among the sponge species studied here. Moreover, evidence of fungal hyphae in TEM micrographs of juveniles as well as overgrowth by a white fungus in stressed specimens have been observed for *H. indistincta* (McCormack's personal observation). The staining with calcofluor white of this sponge species did not show the presence of fungal structures in the tissue of *H. indistincta*, but instead blue fluorescence was emitted all over the sections. This result can be explained considering that the presence of chitin in the sponge skeleton has been demonstrated for some sponge species (Żółtowska-Aksamitowska et al., 2018) and perhaps *H. indistincta* shares the same characteristic. The results of this experiment suggests that probably the staining with calcofluor white is not the best technique to locate fungal structures in sponges, but the use of the lectin wheat germ agglutinin on more sponge species could allow determining the actual extensions of sponge-fungi associations.

Concluding remarks

The cultivation-dependent study of the fungal associations in the nine target Irish and Mediterranean *Haliclona* species described here was designed with the purpose to identify the intracellular putative fungal spores observed in sponges like *H. oculata* and *H. indistincta* via TEM. I did not accomplish this aim because the pattern of fungal isolation, with the same fungi obtained sometimes from multiple *Haliclona* species and no fungi isolated from all the specimens considered for each sponge

species, suggested the absence of sponge species-specific fungal associates in the sponges considered. However, I also have highlighted how the approach followed has involved a number of limitations that might have affected the fungal biodiversity obtained. Therefore, before excluding undoubtedly the existence of stable associations between fungi and the *Haliclona* species here studied, additional cultivation efforts employing different isolation techniques as well as other incubation temperatures and media mimicking as much as possible the host sponge (Bovio et al., 2018) might increase the number of cultivable fungi isolated. This could eventually lead to the identification of key fungal taxa for which developing suitable probes in order to perform *in situ* hybridisation (Ellison et al., 2016), which is probably a more reliable technique than calcofluor white to isolate fungal elements in sponges, as seen above.

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CHAPTER 4

Characterisation of 'cell with inclusions' observed in three species of *Haliclona*

Introduction

In sponge biology the definition of 'cell with inclusions' applies to all the mobile cells in the mesohyl that contain membrane-bound vesicles even if they are characterised by different morphologies and/or chemical contents (Bergquist 1978). Other typical features of these cells are the presence of a usually anucleolated nucleus, few mitochondria, reduced rough endoplasmic reticulum and no phagosomes (Simpson 1984). Cells with inclusions represent a terminal stage of differentiation (Bergquist 1978) and are typically derived from archaeocytes, even though exceptions are known (e.g. Boury-Esnault 1976; Donadey 1978; Gaino et al. 1986). The morphology of the inclusions can be extremely variable, as is their size (Cárdenas et al. 2012). Based on the size of the intracellular vesicles, Simpson (1984) distinguished between cells with larger inclusions (~ 2.0 – 2.5 µm) and cells with smaller inclusions (~ 0.1 – 1.0 µm). The function of cells with inclusions and the chemical content of their vesicles have not been fully characterised yet and this makes these cells a challenging topic in sponge biology. Cells with inclusions have been suggested to be involved in the elimination of metabolic wastes (Vacelet 1967) and in the production of the extracellular matrix (Donadey & Vacelet 1977). They were also identified as the site of storage and/or production of bioactive compounds in many sponge species, particularly those with larger inclusions (known as spherulous cells), as observed for example in the congeneric *Axinella polypoides* (Bretting et al. 1983) and *A. corrugata* (Dresch et al. 2011), *Aplysina fistularis* (Thompson et al. 1983) and *A. aerophoba* (Turon et al. 2000), *Crambe crambe* (Uriz et al. 1996), *Dysidea fragilis* (Marin et al. 1998) and *Agelas conifera* (Richelle-Maurer et al. 2003). An analogous cell type defined by Salomon et al. (2001) as an "inclusional cell" was proved to contain the pyridoacridine alkaloid dercitamide in the sponge *Oceanapia sagittaria* from the order Haplosclerida, one of the most prolific sources of bioactive compounds from the phylum Porifera (Mehbub et al. 2014). Therefore, cells with inclusions represent a very interesting topic from a bioprospecting point of view (Müller et al. 1999; Richelle-Maurer et al.

2003), in spite of the technical difficulties with their isolation (Yentsch & Pomponi 1986).

Cells with larger inclusions have been associated with the production of mucus-like material in certain sponge species, i.e. rhabdiferous (Smith 1968) and spumeuse cells (Donadey & Vacelet 1977), the latter described in the haplosclerid *Haliclona mucosa* (Donadey 1982) and in the larvae of *H. indistincta* (Stephens 2013). These cell types vary morphologically: the rhabdiferous cells are characterised by rod-like inclusions arranged parallel to the main cell axis (Gaino 2011) whereas the spumeuse cells show vesicles packed with fibrillar material (Donadey & Vacelet 1977).

Some authors consider cells with inclusions important systematic characters for groups where classification is difficult, e.g. the Homoscleromorpha genera *Oscarella* (Muricy et al. 1996; Ereskovsky et al. 2009) and *Pseudocorticium* (Boury-Esnault et al. 1995), and demosponges, e.g. *Halisarca* (Ereskovsky et al. 2011), *Hexadella* (Bergquist & de Cook 2002) and *Polymastia* (Boury-Esnault et al. 1994). Such cells may be useful for members of the order Haplosclerida, which is characterised by severe taxonomic problems due to a simple skeleton (Redmond et al. 2011; Cardenas et al. 2012). Pomponi (1976) described "granular cells" containing intensely PAS- and glycogen-positive inclusions in species from Callyspongiidae, but not in members of the Chalinidae (except for *Haliclona variabilis*), corroborating in part the distinction of the two haplosclerid families. However, no further studies of this nature have been published on sponges belonging to this order.

In this chapter I describe in detail the cells with inclusions observed in three species currently placed in *Haliclona* Grant, 1841 i.e. *H. indistincta*, *H. viscosa* and *H. sarai*. Based on their skeletal characteristics, the three species were all ascribed to the subgenus *Rhizoniera* Griessinger, 1971 (de Weerdt 2000). Employing both ribosomal and mitochondrial gene sequence data, Longakit (unpubl. data) supported the relatedness of the three *Haliclona* species showing that they belong to Clade C of the order Haplosclerida (Redmond et al. 2011); in particular *H. indistincta* and *H.*

viscosa are sister species, *H. sarai* is more distantly related. In addition, the three species produce 3 alkyl-pyridine derivatives (Cimino et al. 1986; Sepčič 2000; Defant et al 2011; Schmidt et al. 2011; Köck et al. 2013; Firsova 2017), typical compounds of haplosclerid sponges of interest due to their potential bioactivity (Tribalat et al. 2016). Based on the current knowledge about the functions of cells with inclusions in sponges reported above, I hypothesised that the cells with inclusions observed in the three target *Haliclona* species could be responsible for production and storage of both the bioactive compounds and the mucus-like material characterising these sponges. Hence, investigation of these cells in these species is of interest for helping understand their biology, as a potential source of mucus and/or compounds of interest and as a potential morphological synapomorphy in their systematics.

Methods

Species studied and sampling sites

The species investigated are *H.* (*Rhizoniera*) *indistincta* (Bowerbank 1866), *H.* (*Rhizoniera*) *viscosa* (Topsent 1888) and *H. (Rhizoniera) sarai* (Pulitzer-Finali 1969). *H. indistincta* is an intertidal/immediately sublittoral species and a population of this species is found in Corranroo (Co Clare, Ireland; 53.15 N, 9.01 W); most individuals at this location have a cushion form and a colour that varies between pinkish-brown to light purple. *H. viscosa* was always found in a subtidal environment showing a massive form with volcano-like oscules and a light pink colour; the specimens analysed for the present study were collected in Gurraig Sound, Kilkieran Bay (Co Galway, Ireland; 53.31 N, 9.67 W). *H. sarai* specimens showed a lobate-mammillated form and a beige colour and were collected in a subtidal cave in Villefranche-Sur-Mer (Nice, France; 43.70 N, 7.32 E). The three species feel tacky to the fingers when their tissues are torn or squeezed.

For TEM, three adult specimens of each sponge species were collected and for each specimen tissue samples from the top, middle and bottom

part were fixed immediately on collection in 2% Gluteraldehyde + 2% Paraformaldehyde in seawater 0.1M Sodium Cacodylate buffer (pH 8). For the histochemical and immunohistochemical stainings, one tissue sample from each of three specimens of *H. indistincta* was fixed in 4% Paraformaldehyde in seawater (pH 8).

Transmission electron microscopy of tissue samples

After 24 hours in the primary fixative, each sample was postfixed in 1% Osmium Tetroxide in seawater 0.1 M Sodium Cacodylate for 3 hours, dehydrated though a series of increasing percentages of ethanol and embedded in low viscosity resin (TAAB Laboratories Equipment Ltd, U.K.). Semi-thin sections (<1 μm) were cut with a glass knife and stained with toluidine blue to observe the general tissue organisation. Ultrathin sections of the samples (50-70 nm) were cut using a diamond knife and stained with uranyl acetate and lead citrate. The ultrathin sections were observed through a Hitachi H7000 transmission electron microscope and the measurements of all the different details considered were compared across the three replicates of each species.

Histochemistry and immunohistochemistry

After 24 hours in fixative, each sample was dehydrated as for the TEM processing and embedded in acrylic resin (Unicryl, BBI Solutions, U.K.). Semi-thin sections (<1 μm) were cut with a diamond knife and stained following procedures modified from Cerri and Sasso-Cerri (2003). For the Alcian blue-Periodic Acid-Schiff's reagent (AB-PAS) staining, the sections were stained with Alcian blue pH 2.5, then washed in distilled water and immersed firstly in 1% periodic acid and then in Schiff's reagent, lastly counterstained with Mayer's haematoxylin.

An immunhistochemical study with biotinylated lectins was performed to highlight the presence and distribution of specific sugars in the tissue of *H. indistincta*. The lectins selected were concanavalin A (Con A), wheat germ agglutinin (WGA), *Ulex europaeus* agglutinin I (UEA I), peanut agglutinin (PNA) and *Dolichos biflorus* agglutinin (DBA). The sections

were incubated firstly with the selected lectins, then with the secondary antibody (Streptavidin 488 green), finally with DAPI, and washed in between every step with Tris-buffered saline, 0.1% Tween 20 (TBST) buffer.

Both the sections stained with AB-PAS and lectins were observed through an Olympus BX53 upright fluorescent microscope.

Results

Transmission electron microscopy of tissue samples

H. indistincta: The cells with inclusions observed in *H. indistincta* were usually elongated (n = 6; length = 5.6 ± 1.2 μm, width = 3.1 ± 0.8 μm). Most of times the inclusions occupied all the cytoplasm and the nucleus of the cell was anucleolate (Figure 3.1a). The inclusions were mostly ovoid, but more rounded and more elongated inclusions were also observed (n = 10; length = 1.4 ± 0.2 μm, width = 1.0 ± 0.2 μm). The content of the intracellular vesicles was variable: it was usually made of dark granules interconnected by fibres, but it could appear more granular or, conversely, the inner part of the vesicles could be totally electron-clear. The presence of a pronounced osmiophilic edge surrounding the granules is the most characteristic feature of the cells with inclusions observed in *H. indistincta* (Figure 3.1a–b). Cells with homogenous (fine granular content) inclusions without the electron-dense edge were also observed, but rarely (Figure 3.1c). TEM did not highlight a clear pattern in the distribution of the cells with inclusions, but sometimes the target cells were observed below (or in between layers of) pinacocytes lining the canals, and in these cases the cells with inclusions appeared more flattened (Figure 3.1d). At a certain stage of development the cells with inclusions observed in *H. indistincta* appeared to disintegrate and release the vesicles into the mesohyl (Figure 3.1e); even structures resembling free nuclei were observed in the mesohyl of *H. indistincta* (Figure 3.1f, 3.2b).

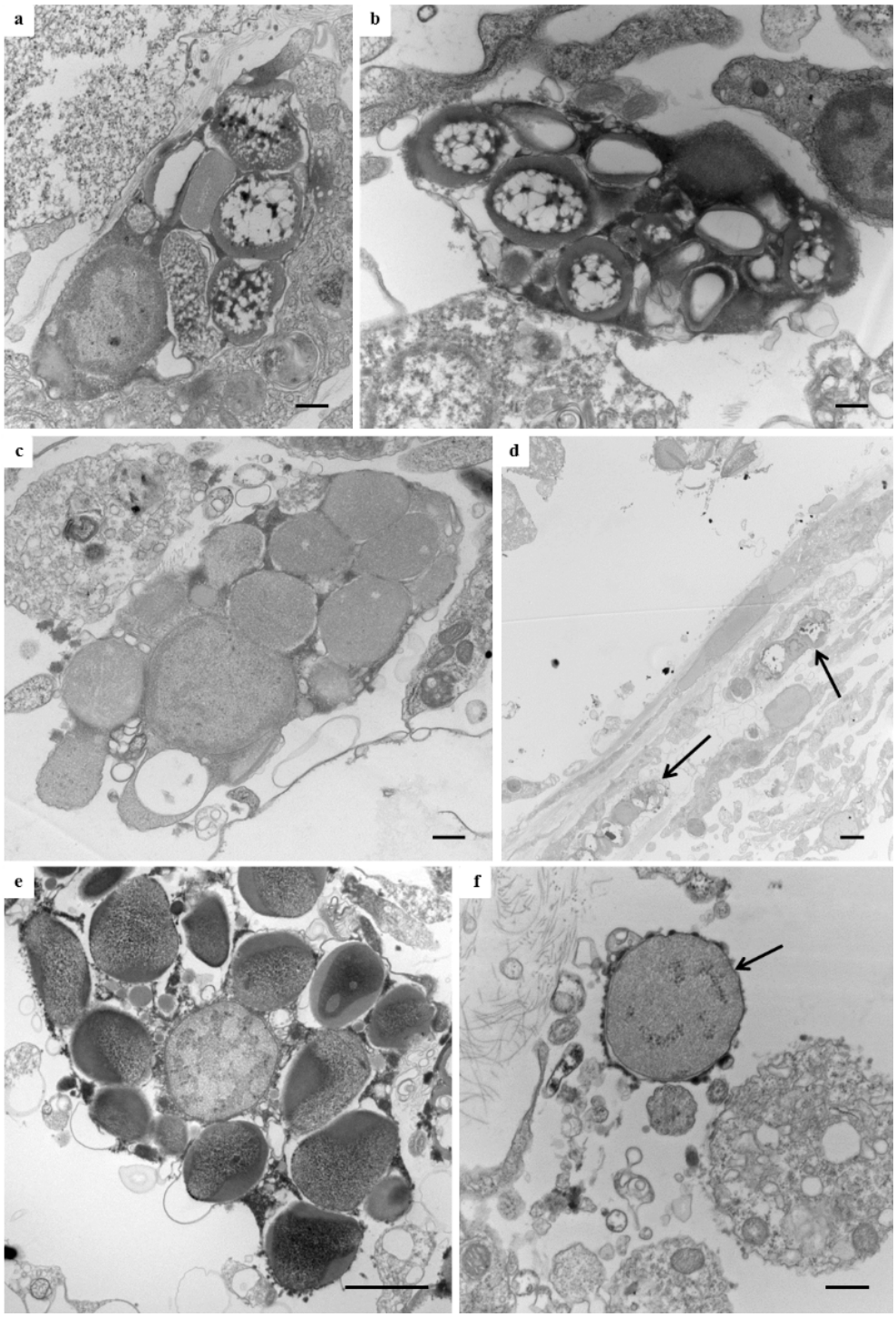

Figure 3.1. The novel type of cells with inclusions observed in *H. indistincta*. **a, b**. The cells were usually elongated, their nucleus was anucleolate and the cytoplasm packed with ovoid inclusions characterised by an extremely variable content and a distinct osmiophilic edge; **c**. Cells with inclusions with completely homogeneous vesicles, probably representing a different developmental state of the same cell type; **d**. The arrows point to cells with inclusions located between two layers of pinacocytes lining an exhalant canal; **e**. The loose inclusions and the uneven cell membrane testify the cell disruption preceding the holocrine secretion of the inclusions; **f**. An example of nucleus released in the mesohyl after the disruption of a cell with inclusions (arrow). Scale bars = **a, b, c, f**. 500 nm; **d, e**. 2 µm.

Rupture of the cells with inclusions in *H. indistincta* released into the mesohyl large ovoid/oblong vesicles (length = 4.4 ± 1.1 µm, width = 2.7 ± 0.5; Figure 3.2a). The content of the vesicles was mostly finely granular, but size, density and homogeneity of the granules appeared to vary as did the size of the vesicles with some vesicles being more fibrillar in content (Figure 3.2a–b). The osmiophilic outer layer persisted in the extracellular environment and appeared thicker on the longest sides of the vesicles becoming thinner as the vesicles increased in size (Figure 3.2). The extracellular vesicles were located close to each other forming clumps (Figure 3.2a–c) each surrounded by a membrane (Figure 3.2a). Multiple vesicles were also observed to merge with the outer rims remaining forming a 'coat' around them (Figure 3.2c, d). Extensive clumps of extracellular vesicles indicate possible fusion between vesicles derived from different cells. No specific pattern was found in the localisation of extracellular vesicles, but again proximity to pinacocytes/canals was sometimes noticed.

H. viscosa: The cells with inclusions observed in the sister species *Haliclona viscosa* were similar to the cells described in *H. indistincta* in terms of appearance and size of the inclusions (n = 10; length = 1.5 ± 0.3 µm, width = 1.0 ± 0.2 µm) as well as the characteristics of the nucleus. However, the cells with inclusions of *H. viscosa* (n = 6; length = 4.9 ± 1.0 µm, width = 4.2 ± 0.7 µm) differ in shape, being mostly rounded instead of elongated (Figure 3.3a).

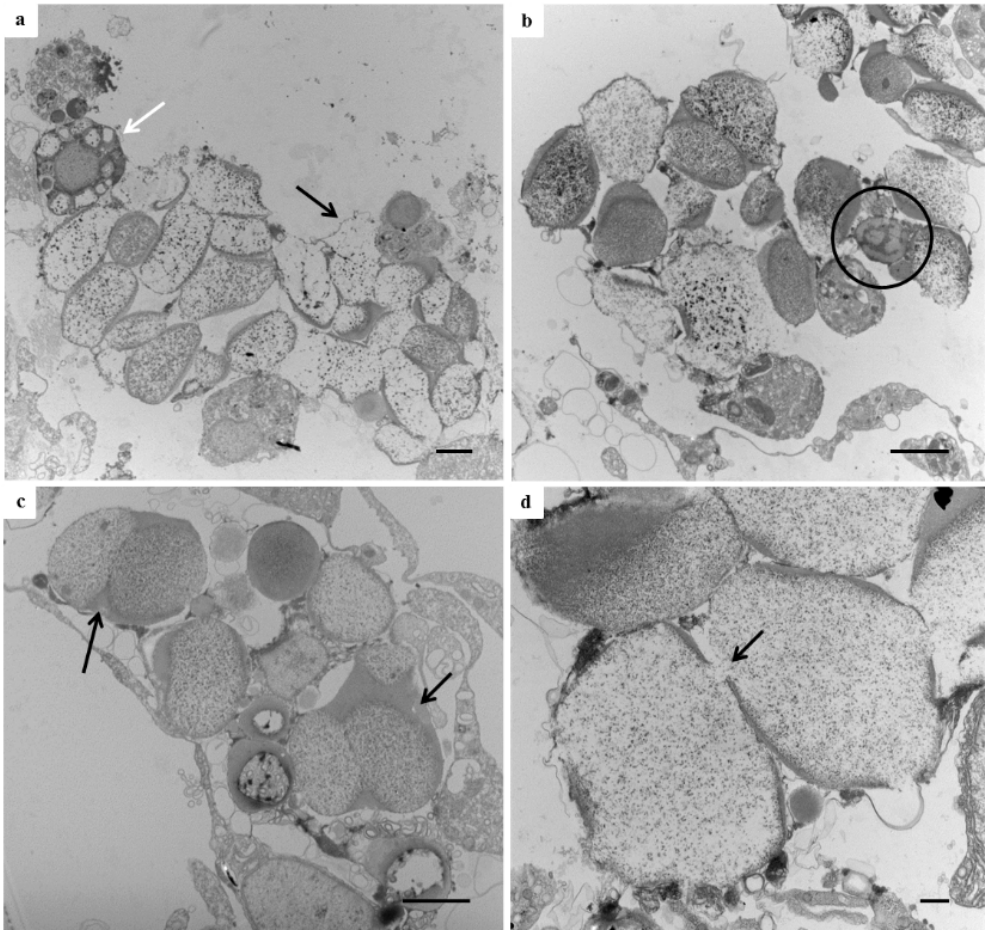

Figure 3.2. The extracellular vesicles in *H. indistincta*. **a**. The size of the vesicles in the extracellular environment is larger than within the cells (white arrow) and a loose cell membrane sometimes encloses the vesicles (black arrow); **b**. The variability of the content still persists once the inclusions are released in the sponge mesohyl (the circle highlights a released nucleus); **c**. Multiple extracellular vesicles come in touch with each other after coalescence of their osmiophilic outer rims (arrow) and eventually the fusion of the granules occurs after disaggregation of the vesicle membranes (**d**, arrow). Scale bars = **a, b, c**. 2 μm; **d**. 500 nm.

The cells containing the inclusions described in *H. viscosa* were also observed to undergo disruption and release of the inclusions in the sponge mesohyl. The extracellular vesicles found in *H. viscosa* were more abundant and slightly larger than in *H. indistincta* (n = 10; length = 5.1 ± 1.2 μm, width = 3.6 ± 0.5 μm), but they showed a similar shape, variability in terms of granularity of the content as well as the presence of the osmiophilic edge (Figure 3.3e). The extracellular vesicles were also seen touching via the electron-dense edge and to fuse with each other, sometimes releasing their granular content in to the mesohyl (Figure 3.3f).

A second type of cell with inclusions was observed in *H. viscosa* that was not seen in *H. indistincta*. These cells shared certain characteristics with the cells with inclusions described above, i.e. similar shape, a small anucleolate nucleus, large inclusions that occupy all the intracellular space (Figure 3.3a). However, the two types of cells with inclusions observed in *H. viscosa* differed in many aspects, i.e. the second cell type was larger in size (n = 7; length = 9.5 ± 1.9 μm, width = 7.3 ± 2.2 μm) as were the inclusions (n = 10; length = 3.4 ± 0.4 μm, width = 2.7 ± 0.3 μm). The inclusions also presented a more irregular shape but a more homogeneous electron-dense content, with the presence of a small core of darker colour in many (Figure 3.3b). These latter cells seemed to transdifferentiate into the first type described above. The process of transdifferentiation started with the appearance of electron-clear areas of variable diameter in the homogenous inclusions and the progressive enlargement of these areas eventually and the presence of an osmiophilic edge (Figure 3.3c-e). Usually the transdifferentation process did not involve all the inclusions in a cell at once, thus it was possible to observe cells containing both types of inclusions simultaneously (Figure 3.3d).

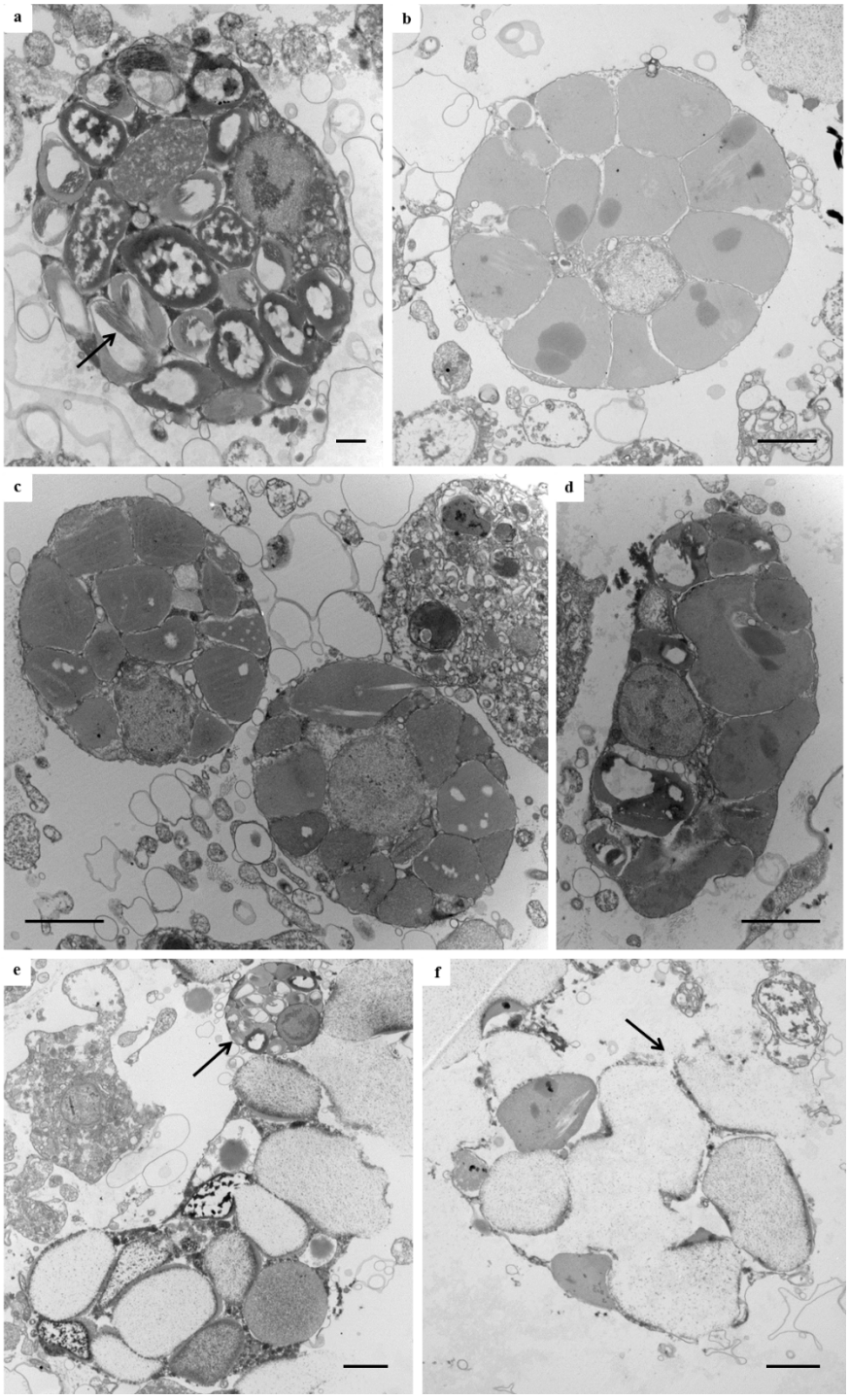

Figure 3.3. *H. viscosa*. **a**. Cell showing the characteristic inclusions, sometimes containing bundles of parallel fibres (arrow); **b**. Spherulous cell showing homogenous inclusions in some cases characterised by a darker core; **c**. The spherulous cells undergo the process of transdifferentiation in putative mucous cells starting with the comparison of electron-clear areas in the homogenous inclusions; **d**. A more advanced stage of the transdifferentiation process showing a spherulous cell containing the same type of inclusions observed in the putative mucous cells; **e**. As already seen in *H. indistincta*, the extracellular vesicles in *H. viscosa* are much bigger than within the cells (arrow) and show a very variable content; **f**. The outer rim of the extracellular vesicles persists even after rupture of the vesicles and release of their content in the mesohyl (arrow). Scale bars = **a**. 500 nm; **b, c, d, e, f**. 2 μm.

H. sarai: The cells with inclusions found in *Haliclona sarai* were slightly smaller compared to the cells observed in the other two *Haliclona* species (n = 4; length = 4.3 ± 0.2 μm, width = 3.7 ± 0.4 μm), but they were still characterised by an anucleolate nucleus (Figure 3.4a). The inclusions were the largest measured among the three sponge species and showed a more rounded shape (n = 10; length = 1.9 ± 0.6 μm, width = 1.7 ± 0.4 μm). As already seen in the other two sponge species, the inclusions displayed a variable content, spanning from finely granular vesicles to osmiophilic inclusions speckled with several rounded electron-clear areas (Figure 3.4a–b). However, inclusions surrounded by the typical electron-dense rim were also observed. Evidence of cell disruption was also observed in *H. sarai*. Once released into the mesohyl however, the vesicles were smaller and more rounded compared to the vesicles in the other two *Haliclona* species (n = 10; length = 3.6 ± 0.9 μm, width = 3.0 ± 0.6 μm), but in most cases they showed the presence of the typical but thicker outer rim (Figure 3.4c). The clumps of extracellular vesicles in this species appeared very much in contact with each other and they were observed also within choanocyte chambers (Figure 3.4d).

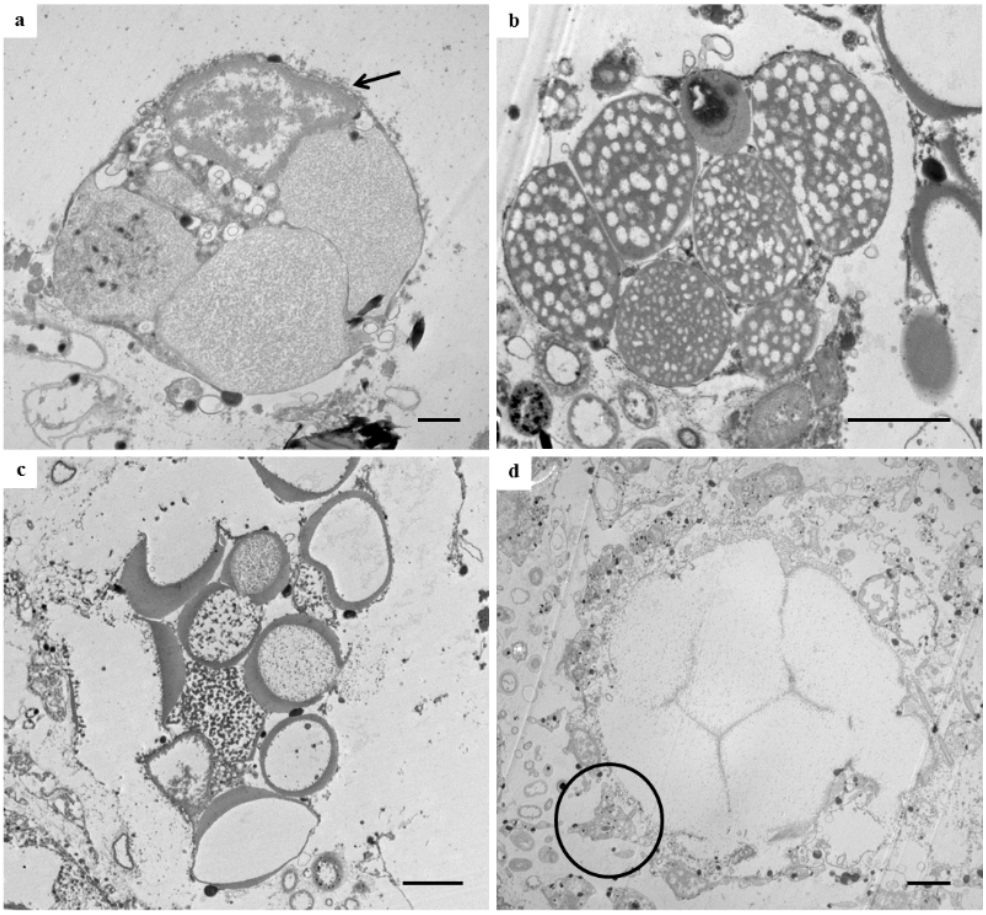

Figure 3.4. *H. sarai*. **a**. Cell containing large inclusions with finely granular content and no electron-dense outer rim (arrow = nucleus); **b**. Cell with inclusions characterised by a spotted appearance presumably due to the distributions of the (glycol)proteins within the vesicles; **c**. Extracellular vesicles in the mesohyl showing a more pronounced outer rim compared to the vesicles of the other two *Haliclona* species; **d**. A clump of extracellular vesicles located within a choanocyte chamber. Scale bars = **a**. 500 nm; **b, c, d**. 2 μm.

Light microscopy

The semi-thin sections stained with toluidine blue confirmed a significant concentration of the extracellular vesicles next to the pinacocyte layers lining the excurrent canals in all species. Inclusions were also observed in the empty space of the canals (Figure 3.5a, c, d) and extracellular vesicles were also commonly observed in the choanosome, especially in *H. viscosa* (Figure 3.5c).

The AB-PAS staining of *H. indistincta* tissues highlighted a strong positive reaction to periodic acid-Schiff's stain, indicating a broad distribution of neutral mucins in the tissue of *H. indistincta*. The staining was particularly intense in the intracellular vacuoles of cells with small nucleus but it also involved extracellular structures, such as the extracellular vesicles described here (Fig 3.5b). The Alcian blue staining was much less intense than the PAS, showing that the presence of acidic mucins is limited to the intracellular vacuoles of unidentified cell types. Some intracellular vacuoles show a purple hue, indicating a content of both neutral and acidic mucins (Fig 3.5b).

The immunohistochemical analysis of *H. indistincta* showed significant results only for the lectin Con A: α-D-mannosyl and α-D-glucosyl residues, both characteristic sugars associated with mucus, were evident in some, but not all, cells. Fluorescence was apparent in clumps of cells, and also clumped material in the mesohyl not containing nuclei, consistent respectively with the clumped appearance of these cells with inclusions, and their extracellular vesicles which tend to clump together in the mesohyl (Figure 3.6). The staining with WGA highlighted also the potential presence of N-acetylneuraminic acid in *H. indistincta* tissue, however at a much lower extent than mannose and glucose while staining with the other lectins utilised (WGA, UEA I, PNA and DBA) was not evident.

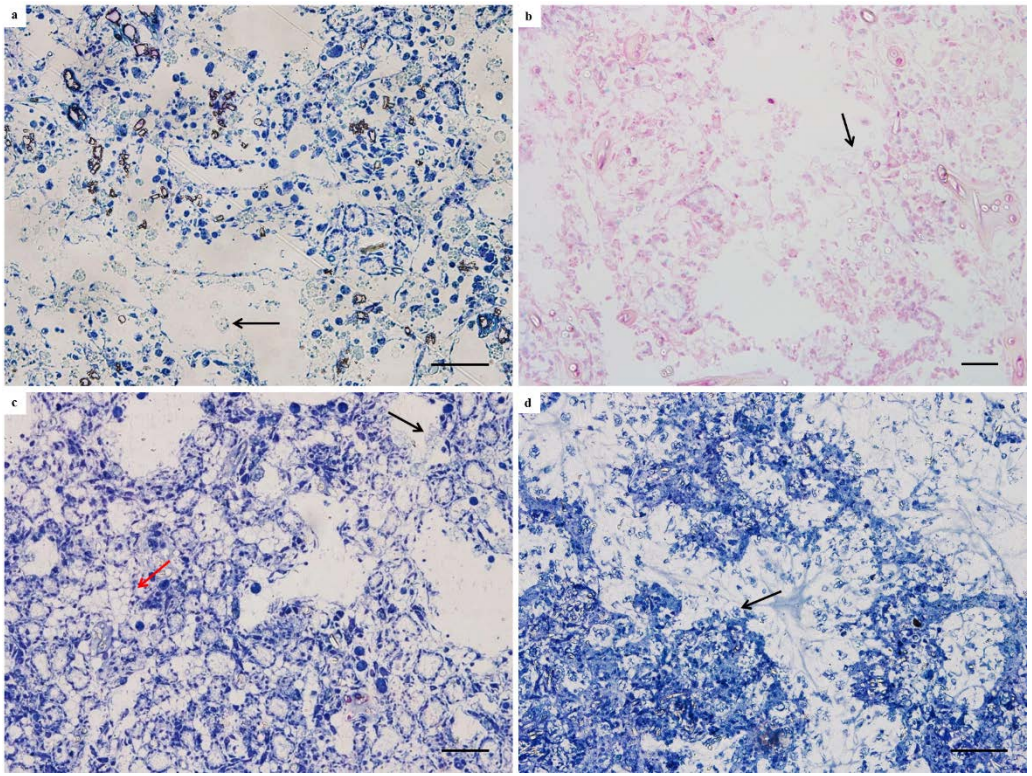

Figure 3.5. Histochemical analysis of the three sponge species. Toluidine blue staining of **a**. *H. indistincta*, **c**. *H. viscosa* and **d**. *H. sarai* showing the distribution of the extracellular vesicles in the sponge mesohyl: the vesicles are abundant between the layers of pinacocytes lining the canals where they are sometimes released (black arrows), but they are also diffuse in the choanosome (red arrow); **b**. AB-PAS staining of *H. indistincta*: the arrow points to the extracellular vesicles. Scale bars = **a**. 100 µm; **b**. 20 µm; **c**. 50 µm; **d**. 20 µm.

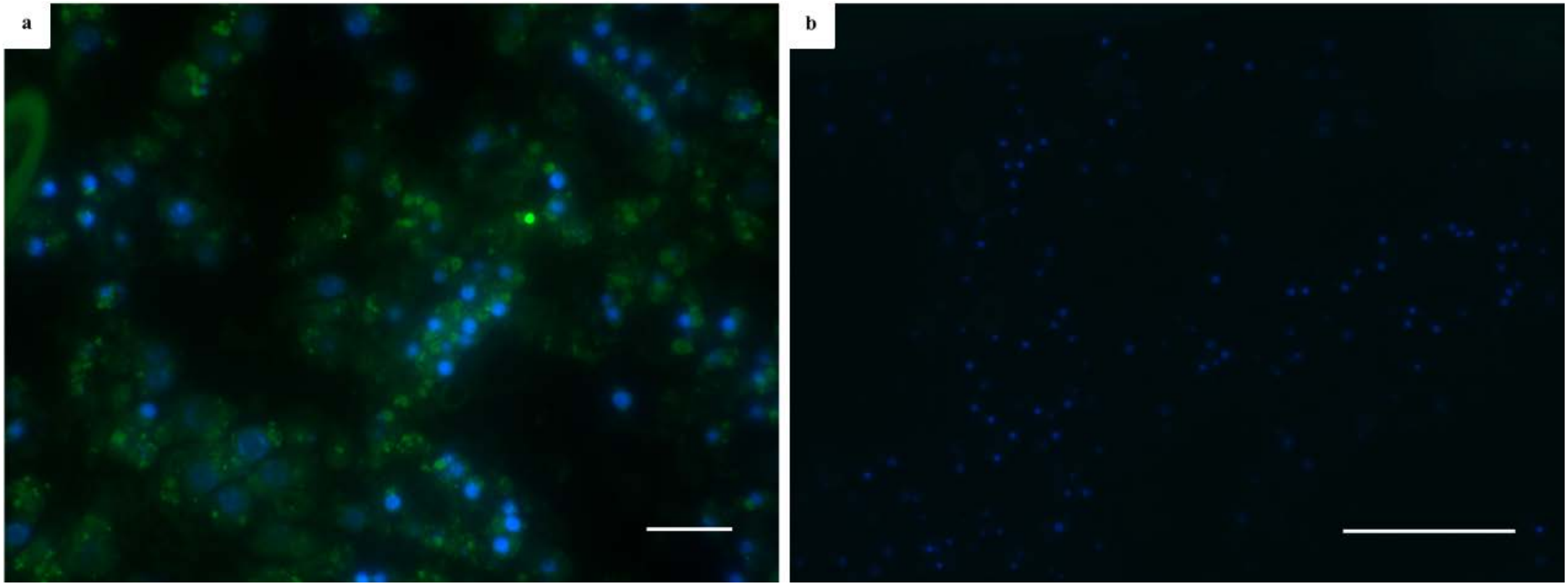

Figure 3.6. Lectin–staining of *H. indistincta*. **a**. Staining with concanavalin A (Con A): the green fluorescence shows the presence of α-D-mannosyl and α-D-glucosyl residues in intracellular vacuoles; **b**. The control slide that was stained only with the secondary antibody. In both pictures the blue dots are the nuclei of the cells stained with DAPI. Scale bars = **a**. 10 μm; **b**. 50 μm.

Discussion

The TEM micrographs of adult specimens of *H. indistincta*, *H. viscosa* and *H. sarai* show a very close similarity between the extracellular vesicles observed in the mesohyl of these species and mucous granules described in non-sponge organisms as e. g. mammals (Plopper et al. 1983), fishes (Fernandes & Perna-Martins 2001), hard corals (Goldberg 2002), reptiles (Beisser et al. 2004) and molluscs (Lobo-da-Cunha et al. 2010). The presence of mucous cells in sponges were reported more than 40 years ago (e.g. Smith 1968; John et al. 1971; Pomponi 1976); Müller et al. dedicated large attention to the 'mucoid' cells of *Geodia cydonium*, trying to assess their aggregative properties (e.g. Müller et al. 1976; 1981). However, the presence of mucous cells in sponges have been reported mainly based on the results of Alcian Blue (AB) and/or Periodic Acid-Schiff's reagent staining (PAS), which were considered as the main stain techniques for the evaluation of mucins (Mowry 1963). Ultrastructural evidence of the presence of putative mucous cells in sponges is not common. The presence of spumeuse cells with fibrillar inclusions was demonstrated in *Pleraplysilla spinifera* (Donadey & Vacelet, 1977) and with higher abundance in another *Haliclona* species, i.e. *H. mucosa* (Donadey 1982), and these cells were indicated as being responsible for the production of the mucus released by the two species. The structure of such cells and cellular content for *H. mucosa* as shown in Donadey (1982) is not reminiscent of the cells shown here and are likely to be a different type of cell. Other TEM reports refer to mucous cells and/or mucous vesicles observed in larvae of different species (e.g. Evans 1977; Leys & Degnan 2001; Maldonado et al. 2003; Ereskovsky et al. 2007; Ereskovsky 2010; Stephens 2013). The authors describe in detail the location of these cells, but as highlighted by Maldonado et al. (2003) in almost all cases the lack of cytochemical confirmation prevents any conclusions regarding their composition.

The putative mucous cells in *H. indistincta*, *H. viscosa* and *H. sarai* have the typical features of cells with larger inclusions as described by Simpson (1984), including a variable morphology even within the same

animal. Such variability is also a characteristic of mucous cells in higher organisms (Deyrup-Olsen & Luchtel 1998). The variable content of the putative mucous granules observed in the three *Haliclona* species can be the consequence of a compartmentalisation of the (glyco)proteins contained in the mucous granules (de Souza Santos 1966; Hong et al. 1997) or due to a different composition in terms of glycoproteins and this, in turn, can be reflective of a different development stage for the granules (Ottesen & Olafsen 1997). The variability of the inclusions has already been related to cell development in sponges (e.g. Bretting and Königsmann 1979; Uriz et al. 1996; Maldonado 2009; Reveillaud et al. 2012).

In healthy mucous cells of higher organisms the limiting membranes of adjacent mucous granules are normally closely apposed to each other (Neutra & Schaeffer 1977; Neutra 1982). The vesicles within the mucous cells of the sponges studied here never looked as tightly packed as observed e.g. in goblet cells of vertebrates, and in most cases were surrounded by an electron-dense homogeneous rim. It has been suggested that the presence of the outer layer could be due to differential distribution in the molecules of the mucous granules (Bowes et al. 1981). However, the presence of an outer rim in mucous granules of other organisms is not a regular observation. Moron et al. (2009) imputed the presence of an electron-dense external layer surrounding the mucous granules from the gills of two fish species to the occurrence of apocrine secretion, i.e. the extrusion from the mucous cells of discrete granules, caused by osmotic stress.

In vertebrates the secretory granules within the mucous cells are extremely small in size, but gradually fuse with each other to grow in volume before fusing with the plasma membrane (Kim et al. 1972; Takano et al. 1982). We also observed confluent vesicles in the three *Haliclona* species, but the fusion seemed to happen only in the extracellular environment and not inside the cells: in fact, we never found cells that were still intact and containing vesicles as big as those observed in the mesohyl.

The secretion modality is another difference between the putative mucous cells observed in the three target *Haliclona* species and the mucous cells in higher organisms. In fact, the standard secretion modality for mucous cells occurs through a merocrine mechanism for which the content of the granules is released after the periodic fusion of the membrane of a single apical mucous granule with the overlying cell membrane (Neutra 1982). As already mentioned, apocrine secretion has been observed as result of a variety of irritants in vertebrates (Specian & Neutra 1980; Puchelle et al. 1991), whereas it is the regular secretion modality in terrestrial slugs (Deyrup-Olsen et al. 1983). The rupture of the whole mucous cells and the release of the vesicles in the mesohyl observed in the three *Haliclona* species can be considered as a mechanism of holocrine secretion, as described by Donadey & Vacelet (1977) for the spumeuse cells of *P. spinifera* and observed by Muricy et al. (1996) with spherulous cells of *Oscarella microlobata*. However, in *P. spinifera* the release of the material contained in the inclusions of the spumeuse cells was found to occur by bursting the thin cytoplasmic border remaining around the cell. By comparison the inclusions of the three *Haliclona* species, although stuck to each other, are first released unbroken after cell disruption. This behaviour is reminiscent of the holocrine secretion of the mucin vesicles in the hagfish, for which this secretion modality assures that the granules remain intact until they come into contact with seawater in the external environment (Luchtel et al. 1991; Herr et al. 2010).

It is possible that in the target *Haliclona* species the vesicles are released in the seawater in the mesohyl in order to create a mucous layer on the sponge surface (including internal surfaces) to act as defensive barrier against pathogens; this could be achieved thanks to the physical properties of the molecules constituting the mucous vesicle content (Hansson 2012) or to the presence in the mucus of bioactive molecules (Sullivan et al. 1983), including antibacterial compounds (Muricy et al. 1993). In fact, the cells with inclusions in the three *Haliclona* species are more abundant below (and between layers of) the endopinacocytes

lining the exhalent canals, consistent with the description of spherulous cells by Bergquist (1978). Furthermore, in all three *Haliclona* species the 'mucus' vesicles were observed loose in the exhalent canals, which for *C. crambe* was proposed to represent a mechanism of chemical defence due to the presence of bioactive compounds in spherulous cells of that species (Ternon et al. 2016). The species included here are sticky to the touch indicating that mucus is prevalent in the 'tissue'. The species also produce bioactive compounds and there are no other cells present in these species that are immediate candidates for production of both substances. Poriferan cells are known to be multifunctional due to the low numbers of types of cells available therefore I propose that these cells are responsible for the production of both the mucus and bioactive compounds present. The cells are incredibly difficult to isolate based on cell fractionation methods and flow cytometry (Marra, unpubl. data) without the availability of identifying cell markers, and so proof of this hypothesis was not possible with the methods so far applied.

Cells with inclusions that are very similar to the type described here were observed in the larvae of *H. indistincta* (Stephens 2013). Here we confirm the presence of the same characteristic cells in the adults. However, some differences in shape and content were evident between the extracellular vesicles in the adults and in the larvae. In addition, the homogeneous electron-dense outer rim typically observed in the extracellular vesicles of the adults is missing in the larvae (Stephens et al. 2013). Given the abundance of these cells with inclusions in the inner cell mass of the larvae of *H. indistincta* and the sticky nature of the same larvae, a mucous nature for this type of cells with inclusions was already hypothesized (Stephens 2013). Interestingly, the same author also found cells defined as 'spumeuse cells' due to the fibrillar content of the inclusions as described in *P. spinifera* by Donadey & Vacelet (1977) in the juveniles of *H. indistincta* after 32 days post-settlement, but we never observed this cell type in the adults of this sponge species. The absence of spumeuse cells can be explained by a change of nature and/or organisation of the glycoproteins contained in the vesicles of these cells

during the development of *H. indistincta*, as discussed above. Extracellular vesicles equal to the ones observed in the adults of *H. viscosa* were also found in the embryos of this species, even though no cells with inclusions were observed (Marra, unpubl. data). This might suggest a maternal origin of the embryonic vesicles that are present, prior to differentiation of the cell responsible for mucus production, but this has not been investigated yet nor have any embryonic/larval stages in *H. sarai* been examined.

The ability of cells with inclusions in *H. viscosa* to transdifferentiate from one type to another is not an unusual observation in sponge biology. Ereskovsky (2010) stated that in adult sponges only gametes and highly developed cells are irreversibly differentiated, therefore various sponge cell types can still move, transdifferentiate and switch functions. Furthermore, transdifferentiation is also a characteristic of mucous cells in higher organisms, e.g. the transformation of the homogeneous electron-dense granules of serous cells to granules with the typical appearance of mucous cells has been observed in mammals though the reason is not yet understood (Miyazaki et al. 2002; Kikuchi et al. 2004). In addition the opposite transdifferentiation, i.e. from mucous to serous cells has also been described (Aiyama et al., 2000). Serous cells produce different types of non-mucin proteins, some of which have antibacterial properties (Basbaum et al. 1990).

Despite slight differences, the cells with inclusions described in *H. indistincta*, *H. viscosa* and *H. sarai* might represent an important synapomorphy for the subgenus grouping of de Weerdt (2000). Morphological characters that agree with molecular phylogenetic hypotheses of relationships between *Haliclona* species (and indeed the rest of this sponge Order) are rare due to a paucity of available characters. Therefore finding useful shared derived traits from ultrastructural data would significantly improve confidence in classification in this group. The possession by these three species (same subgenus and also same molecular clade C) of the same cell type with very similar ultrastructure that is not found in other *Haliclona* species

examined (e.g. *H. oculata*, *H. cinerea*, *H. simulans*, species from other subgenera and found in different molecular clades according to Redmond et al. (2011)) indicates a shared inheritance of the cell type from a more recent common ancestor. However, molecular data also place some *Niphates* and *Amphimedon* species in the same clade and show a more distant relationship between *H. indistincta/H. viscosa* and *H. sarai* (Longakit, unpubl. data). Unfortunately, very little ultrastructural information is available from both other *Haliclona* (*Rhizoniera*) and *Niphates* species. To the authors knowledge, the only available information is from Vacelet et al. (2007) who observed abundant spherulous cells in *Niphates toxifera* (7.5–12.5 µm in diameter) that contained cytoplasm almost completely filled by homogeneous subspherical or ovoid inclusions, up to 2.5 µm in diameter in mature cells. However, the authors do not mention cells with inclusions similar to the cell type described here and no DNA sequences yet exist for this species. More ultrastructural and molecular data from *N. toxifera* and other related species will be necessary to assess the level at which this type of cells with inclusions can be used as a synapomorphy.

Concluding remarks

My work describes a novel type of cells with inclusions observed in three *Haliclona* (*Rhizoniera*) species and focuses on assessing the possible mucous nature of these cells. Their ultrastructure, containing granular-fibrillar vesicles that are released in the sponge mesohyl after cell disruption, is reminiscent of mucous cells that discharge their granules with a mechanism of holocrine secretion as observed in other sponge species and higher organisms. Also the transdifferentiation from the spherulous cells described in *Haliclona viscosa* to the novel type of cells with inclusions is consistent with mucous cells, considering that mucous cells can also transdifferentiate to serous cells and *vice versa*. The light microscopy survey performed with *Haliclona indistincta* highlighted a strong positivity for PAS staining and immunostaining with lectin Con A,

further supporting the hypothesis that the novel cells with inclusions observed can produce neutral mucins rich in mannose residues.

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CHAPTER 5

Is mucus mucus? Investigating the presence of mucins in three haplosclerid species

Introduction

Mucus is a viscous complex gel-like material evolved to play different roles in organismal defence, especially to protect cells from mechanical and chemical stress factors, dehydration and pathogens (Zaretsky and Wreshner, 2013). Along with other important contributors, the main macromolecular components of mucus are high molecular weight O-glycosylated proteins called mucins (Thornton and Sheehan, 2004). These molecules are characterised by protein domains called mucin domains or PTS domains because they are primarily constituted by sequences, sometimes tandemly repeated, in which the dominant residues are proline (often > 5%), threonine and serine (> 40%, sometimes up to 90%). The last two amino acids are post-translationally modified with different types of oligosaccharide side chains that can represent up to 80% of the mucin mass (Lang et al., 2004). Glycosylation occurs via the formation of a link between an oxygen atom of serine or threonine and the N-acetylgalactosamine located at the reducing terminal of each oligosaccharide chain (O-linked glycosylation; Campbell, 1999). As result, the mucin domain acquires a 'bottle-brush' appearance that causes the polypeptide to stiffen, with consequent expansion of its volume. This feature and the capability of the oligosaccharide chains to bind water are responsible for the distinctive gel-forming properties of mucins (Campbell, 1999, Thornton and Sheehan, 2004). Mucins are usually divided into cell-surface membrane-bound and secreted gel-forming mucins, mainly differentiated by structural organisation and function (Campbell, 1999, Davies et al., 2012). Oligomeric mucins can polymerise via formation of disulphide bonds between von Willebrand factor (VWF) D domains and C-terminal cysteine knots (CK) localised respectively at the N- and C- ends of the mucin domains (Bansil and Turner, 2006).

The ability to produce mucus has been observed in several taxa of marine animals including sponges, such as sea anemones (Lubbock, 1979, Salinas et al., 1997, Stabili et al., 2015), corals (Krupp, 1984, Coffroth, 1990, Ritchie, 2006), molluscs (Davies and Hawkins, 1998), echinoderms

(Buchanan, 1963, Hennebert et al., 2015), tunicates (Flood and Fialamedioni, 1981) and fish (Cordero et al., 2015). The chemical composition of the mucus produced by marine organisms has been characterised only in a limited number of cases and sometimes no mucins were identified, thus the denomination 'slime' is often preferred (Caruana et al., 2016).

The production of mucus-like material has been observed in several different species of sponges (phylum Porifera) and this feature is considered to have taxonomic relevance (Lévi et al., 1998, Ackers et al., 2007), as observed with several *Haliclona* species from Belize (De Weerdt et al., 1991). Lévi et al. (1998) also noted how mucus production is very common in intertidal tropical sponge species, hypothesising a role of the mucus in protecting the animals from the desiccation risk occurring with low tides, but the authors attribute the main function of mucus in sponges to the protection against competitors, predators and parasites. Likely this is achieved because the mucus contains the toxic bioactive compounds produced by certain sponge species. A classic example of that is described by Sullivan et al. (1983) who reported the presence of bioactive compounds in the mucus of burrowing sponges belonging to the haplosclerid genus *Siphonodictyon* which were responsible for the death of coral polyps surrounding the oscular chimneys of the sponge buried within the coral heads. In a screening of 21 shallow-water sponges from the Southwestern Atlantic, Muricy et al. (1993) reported the presence of antibacterial and/or antifungal activities for almost all the sponges studied that produce a high amount of mucus (i.e. *Polymastia janeirensis*, *Pseudaxinella lunaecharta*, *Dictyonella ruetzleri*, *Tedania* sp., *Amphimedon viridis*, cf. *Arenosclera* sp.). Sponge mucus has been reported to also be an irritant to human skin (e.g. *Biemna tubulosa*; Govinden-Soulange et al., 2014). However, in spite of its chemical content sponge mucus seems to also have an ecological role as food for other organisms, e.g. sea cucumbers and nudibranchs (Hooper, 2008).

Mucus is also considered to play additional roles in the biology of sponges. For example, in environments characterised by heavy sedimentation, different sponge species were observed cleansing their surface by producing a copious amount of mucus that was cast off with all the sediment trapped (Leys, 2013, Pineda et al., 2017, Strehlow et al., 2017). In addition, choanocytes have been observed to capture food particles embedded in a mucus-like coating (Leys and Eerkes-Medrano, 2006) and finally sponge mucus is implicated in reproduction. In fact, oocytes and eggs were observed to be released along with nurse cells in mucous threads in *Chondrilla nucula* (Maldonado and Riesgo, 2009) while Stephens et al. (2013) reported that *Haliclona (Rhizoniera) indistincta* embryos are not held in brood chambers, but are coated in mucus, as was previously observed in corals (Brown and Bythell, 2005). Mucous cells in sponge larvae were described in detail by Leys and Degnan (2001) and Maldonado et al. (2003), and a role of mucus in facilitating larval settlement as observed in corals (Brown and Bythell, 2005) can be hypothesised.

In spite of the many records of mucus produced by sponges, no dedicated studies have hitherto been carried out on this topic, as has been done with corals (see Brown and Bythell, 2005 for a comprehensive review). In a bioinformatic analysis of all available choanoflagellate, protist and metazoan genomes aimed at studying the evolution of gel-forming mucins, Lang et al. (2016) identified genomic data from putative gel-forming mucins at early stages of animal evolution such as in Cnidaria and even Ctenophora, but not in Porifera, which instead possessed a mucin-like protein called FCGBP (Fc fragment of IgG-binding proteins) thought to be related to the gel-forming mucins. In fact, both true mucins and FCGBP present multiple Von Willebrand D (VWD) domains and the two types of glycoproteins can also be found covalently bound to each other. For instance, the link between FCGBP and the mucin 2 in the human colon has been studied by Johansson et al. (2009) and suggested to happen after pH-dependent cleavage of a glycine–aspartic acid–proline–histidine sequence that is common between 11 of the VWD

domains in the FGCBP protein and the single C-terminal VWD domain of the mucin via creation of a very reactive anhydride at the newly generated terminal aspartase. However, it has to be taken in account that for their study Lang et al. (2016) looked at only one poriferan genome from the species *Amphimedon queenslandica*.

Here I present an investigation of the 'mucus' in three haplosclerid species currently placed in the genus *Haliclona* Grant, 1841, i.e. *H.* (*Reniera*) *cinerea*, *H.* (*Soestella*) *mucosa* and *H.* (*Rhizoniera*) *indistincta*. The genus *Haliclona* is dramatically polyphyletic and the three species belong to different haplosclerid clades (Redmond et al., 2011; Longakit, unpublished data). The presence of mucous cells in *H. mucosa* is reported by Donadey (1982) whereas the mucous cells of *H. indistincta* are described in this thesis for the first time (Chapter 3); no similar cells have been observed in *H. cinerea* yet by TEM (transmission electron microscopy) analysis. The three species produce noticeably different mucus. The inner tissue of *H. indistincta*, exposed once the sponges of this species are torn apart, feels tacky on the fingers when the animal is handled and appears covered with a thin gel-like layer. *H. cinerea* produces 'slimy' mucus clearly visible as mucus strands when the sponge is pulled apart. *H. mucosa* instead releases a copious amount of mucus when stressed, as indicated by the species name.

The hypothesis behind the study here described was that the cells with inclusions observed in the *Haliclona* species considered could be responsible for the production of both the mucus and the bioactive compounds isolated by these species, as mentioned in chapter 4. Therefore, determining the composition of the mucus produced by the target species could provide useful information to characterise the mucus-producing cells via their isolation. The approach considered in this study is integrative and involves mass spectrometry and transcriptomics. As highlighted by Caruana et al. (2016), combining transcriptomic and proteomic approaches provides a more realistic picture of the protein expressed by a certain organism. Due to the difficulty of 'milking' the mucus from the target sponge species,

especially as regards *H. indistincta*, the extraction of mucins was performed following a new protocol developed combining traditional techniques of sponge cell dissociation with mucin extraction from higher organisms (Davies et al., 2012).

Materials and methods

Sample collection

Two massive specimens of *Haliclona (Rhizoniera) indistincta* (Bowerbank, 1866), coded respectively MIIG1321 and MIIG1322 (vouchers held at the Molecular Evolution and Systematics Laboratory, Zoology Department, NUI Galway), were collected in the intertidal environment of Corranroo, Co. Clare, Ireland in January 2017 and processed individually.

Haliclona (Reniera) cinerea (Grant, 1826) can be found both in intertidal and subtidal environments; the morphological features of the individuals of this species vary largely in the different habitats. In Ail Bhuí (Rosskeeda bay, Co. Galway, Ireland) a population of branched deep purple individuals of *H. cinerea* is present at 10-15 meters of depth. Several specimens of *H. cinerea* were collected in Ail Bhuí in November 2016 and combined to ensure enough biomass for further processing. The code MIIG1314 was attributed to the pool of *H. cinere*a specimens.

Both in *H. indistincta* and *H. cinerea* cases, the sponges were harvested by hand in order to reduce the handling stress and transferred immediately after collection in buckets containing seawater from the same sampling site; during the process the sponges were never exposed to the air. Immediately after collection the sponges were transferred to the laboratory keeping them constantly aerated and then subjected to cell dissociation.

Haliclona (Soestella) mucosa (Griessinger, 1971) is a subtidal sponge species characterised by a cushion form and a creamy white colour. Several individuals of *H. mucosa* were collected by Prof. Olivier Thomas (Chemistry Department, NUI Galway) in January 2017 from a cave

located at ca. 30 meters of depth in Villefranche-sur-Mer (Nice, France) and opportunistically included in this study. The sponges were kept in a plastic bag with a very small volume of seawater from the same sampling site in order to induce the release of the mucus. The sponges were then removed from the bag and the mucus preserved at -80ºC until further processing.

Sponge sample processing

The *H. indistincta* and *H. cinerea* specimens were subjected to cell dissociation. Two types of seawater were used for this purpose, i.e. natural filtered seawater (NFSW) and calcium- magnesium-free artificial seawater containing EDTA (CMF-ASW-E). The CMF-ASW-E was prepared by dissolving 26.24 g of NaCl, 0.672 g of KCl, 4.3 g of $NaHCO_3$ and 4.687 g of Na_2SO_4 in 1L of distilled water and then adding 10 ml of Tris-Cl (1M, pH 8) and 10 ml of EDTA (0.5M, pH 8). The pH of the CMF-ASW-E was adjusted to 8 before being used and both NFSW and CMF-ASW-E were filtered using 0.22 µm Sterivex filters (Durapore, Merck Millipore, Germany).

The sponge specimens were cleaned of animal and plant epi-/endobionts, rinsed three times with NFSW in order to eliminate debris and seawater from the collection site and cut into small pieces using a sterile scalpel. The sponge pieces were rinsed again with NFSW, then placed in a minimum quantity of CMF-ASW-E and shaken for 30 minutes at 200 rpm at room temperature. The mixture obtained was firstly squeezed through a sterile muslin cloth and then filtered through a sterile 40 µm nylon mesh to remove spicules and other possible debris. The filtrate was centrifuged at 600 g at 10ºC for 5 minutes using a swim bucket refrigerated centrifuge and the pellet obtained resuspended in a minimum volume of CMF-ASW-E and centrifuged again. The new pellet obtained was resuspended in an equal volume (1:1 ratio) of filtered-sterile 4M guanidinium chloride (GdmCl), whereas the supernatant obtained after the two centrifugation steps was combined and mixed with an equal volume of 4M GdmCl. All the procedures were performed

in sterile conditions in a laminar flow fume hood. The final six solutions in GdmCl (one cell and one supernatant solution from the pooled *H. cinerea* samples and each of the *H. indistincta* specimens) were kept at 4ºC until further processing.

The *H. mucosa* mucus was simply defrosted on ice at room temperature and then mixed with an equal volume of 8M GdmCl (Sigma-Aldrich, Missouri, USA) keeping the conditions sterile during all the protocol. As for the other solutions, this mixture was also stored at 4ºC.

Mucin extraction and measurements

For *H. indistincta* and *H. cinerea*, both dissociated sponge cells and supernatant were considered for the isolation of the mucins based on the assumption that these glycoproteins could be released during the cell dissociation process and then be retained in the supernatant. To extract the mucins present in both sample types from the sponge specimens as well as from the mucus of *H. mucosa* a caesium chloride (CsCl)/GdmCl isopycnic density gradient ultracentrifugation was performed. Firstly the GdmCl concentration in each solution was checked using a refractometer and adjusted to 4M when necessary, and CsCl was added to obtain a final density of 1.4 g/ml. The samples were loaded in 100 ml ultracentrifuge tube and centrifuged for 65 hours at 40.000 g at 15ºC.

From each gradient obtained after centrifugation 20 fractions (5 mls) were collected after piercing the bottom of the tubes. Density and absorbance at 280 nm of each fraction were measured: the density was considered as the weight of 1 ml of each fraction, the adsorbance was measured using a UV-Vis spectrophotometer.

A volume of 25–50 µl of each fraction was loaded on slot blots and these were stained using several techniques. First of all the periodic acid-Schiff's reagent (PAS) staining was performed with all the fractions from all the sponge samples. An anti-cystic fibrosis (CF) subunit was used to stain the slot blots prepared with the dissociated cells and supernatant fractions from *H. cinerea*. Alcian blue (AB) pH 2.5 and the lectin Concanavalin A (selected among other lectins based on the results of an

immunohistochemistry study on sponge tissue of the same species, see Chapter 3) were additionally used to stain slot blots prepared with all the fractions obtained from *H. indistincta* and *H. mucosa* due to the inconclusiveness of the PAS staining for these species and the non-availability of further CF subunit. For the PAS staining the blots were firstly incubated in 1% periodic acid and 3% acetic acid for 30 minutes, washed in sodium metabisulphite, immersed in Schiff's reagent until the bands were clearly visible and then washed in sodium metabisulphite and distilled water. For the Alcian blue staining the blots were incubated 30 minutes in AB and then washed in distilled water. For the lectin and the anti-CF subunit staining the blots were incubated in Tris-buffered saline, 0.1% Tween 20 (TBST) buffer for one hour, then overnight respectively in Concanavalin A (ConA) and anti-CF subunit and diluted 1:1000, then again 30 minutes in TBST buffer, 30 minutes in the secondary antibody Streptavidin 488 green and finally washed in TBST buffer. Images of each slot blot were taken with the image system Odyssey® CLx (LI-COR Biosciences, Nebraska, USA) and the intensity of the bands was measured using the software Image Lab™ (Bio-Rad Laboratories, California, USA) in case of the slot-blots stained with PAS and Alcian blue, and the software Image Studio™ (LI-COR Biosciences, Nebraska, USA) for the slot blots stained with antibodies.

Mass spectrometry analysis

The fractions obtained for each species were pooled depending on protein amounts indicated by the absorbance measurements and indicators of mucin-type molecules via staining of slot blots, and processed for proteomic analysis after Wisniewski et al. (2009) by Dr Caroline Ridley at the Wellcome Trust Centre for Cell-Matrix Research (University of Manchester, U. K.). The mass spectrometry analysis was also carried out at the University of Manchester by Mr Julian Selley (Biomolecular Analysis Core Research Facility, Faculty of Life Sciences). The pooled fractions were reduced with 20 mM DTT for 3 hours at 37^{o}C then alkylated with 50 mM iodoacetamide for 15 minutes at room

temperature in the dark. Subsequently the buffer of the samples was exchanged from 4M GdmCl/CsCl to 0.5M GdmCl/0.1M ammonium bicarbonate using Vivaspin® 6 5kDa MWCO spin filters (Sartorius, Germany) and then the second buffer volume was reduced through centrifugation to a volume of ~0.5 ml using the same filters. 5 μg of sequencing grade trypsin (Promega, Wisconsin, USA) were added to each sample and left to incubate at 37ºC overnight. The following day the columns were centrifuged at 4.000 g in order to separate the peptides in collection tubes whereas any larger glycopeptides was supposed to be retained in the filters. The pH of the peptides was adjusted to a value lower than 2 using formic acid and then the peptides were desalted using ZipTipC18® tips (EMD Millipore, Massachusetts, USA). The mass spectrometry analysis of the samples was performed through ion trap-liquid chromatography mass spectrometry on a Thermo Velos Pro coupled with a Thermo nanoRSLC system (Thermo Fisher Scientific, Massachusetts, USA). Data obtained from the three sponge species were searched using the software Mascot (Matrix Science, U.K.) against a database of translated sequences obtained from the transcriptomes of *H. indistincta* (Aguilar-Camacho et al., 2019) and *H. cinerea* produced previously in our laboratory. More precisely, the mass spectrometry data from *H. indistincta* and *H. cinerea* were searched against the translated sequences from the respective transcriptomes, while a database of translated sequences from both transcriptomes was used to search the mass spectrometry data from *H. mucosa*. Mascot was searched with a peptide mass tolerance of 1.2 Da and a fragment mass tolerance of 0.6 Da. The iodoacetamide derivative of cysteine and the oxidation of methionine were specified respectively as fixed and variable modifications. Finally, the protein sequences identified in the dissociated cell, supernatant and mucus fractions via mass spectrometry/transcriptome comparisons were searched against the non-redundant protein sequences in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) using the algorithm Blastp (Altschul et al., 1990) keeping the default parameters.

Results

Density gradient centrifugation

Carbohydrate analysis of the cell fractions of *H. indistincta* revealed a population with a buoyant density between 1.63 and 1.24 g/ml for the sample MIIG1321 and between 1.68 and 1.24 for the sample MIIG1322, whereas for the supernatant samples the density was comprised between 1.61 and 1.24 g/ml and between 1.65 and 1.23 g/ml respectively for MIIG1321 and MIIG1322. The values of the absorbance at 280 nm (indicating the presence of proteins), followed an overall pattern of an increase in the amount of proteins from the highest (1-10) to the lowest (11-20) buoyant density fractions for the two *H. indistincta* individuals (Figure 5.1). However, variations were observed between samples, for instance the cell fractions from MIIG1321 showed an abrupt protein increase in fraction 20 while the cell fractions from MIIG1322 showed a very irregular pattern with peaks corresponding to fractions 14 and 17 and the supernatant from MIIG1322 showed a peak corresponding to fraction 7 (Figure 5.1). PAS staining of both *H. indistincta* cell and supernatant fractions showed a similar trend to the absorbance: the band intensity was higher than 1.00×10^7 for the densest fractions (1 and 2), then a decrease in the amount of PAS positive material characterised the intermediate fractions, followed by a dramatic increase in the presence of glycoproteins in the lightest fractions, in particular for MIIG1321 of which the fraction 20 showed a band intensity > 2.00×10^7, analogously to what observed with the absorbance (Figure 5.1). The only exception to this trend is represented by the cell fractions from MIIG1322 for which the band intensity is much higher for the heavy than for the light fractions. The AB staining highlighted the presence of mucopolysaccharides in the heaviest fractions (< 8) of all the *H. indistincta* samples, but the negative results of the remaining fractions suggest an inhibition of the staining that cannot actually exclude the presence of those macromolecules (Figure 5.2). Finally, the staining with Con A, a lectin that binds mannose, showed the presence of molecules containing this sugar enriched in the light

fractions of all the samples, especially in the cell samples, for which the intensity of the bands in the slot blots is > 2.00 x 10^6 in fractions 18 and 20 both for MIIG1321 and MIIG1322 (Figure 5.3).

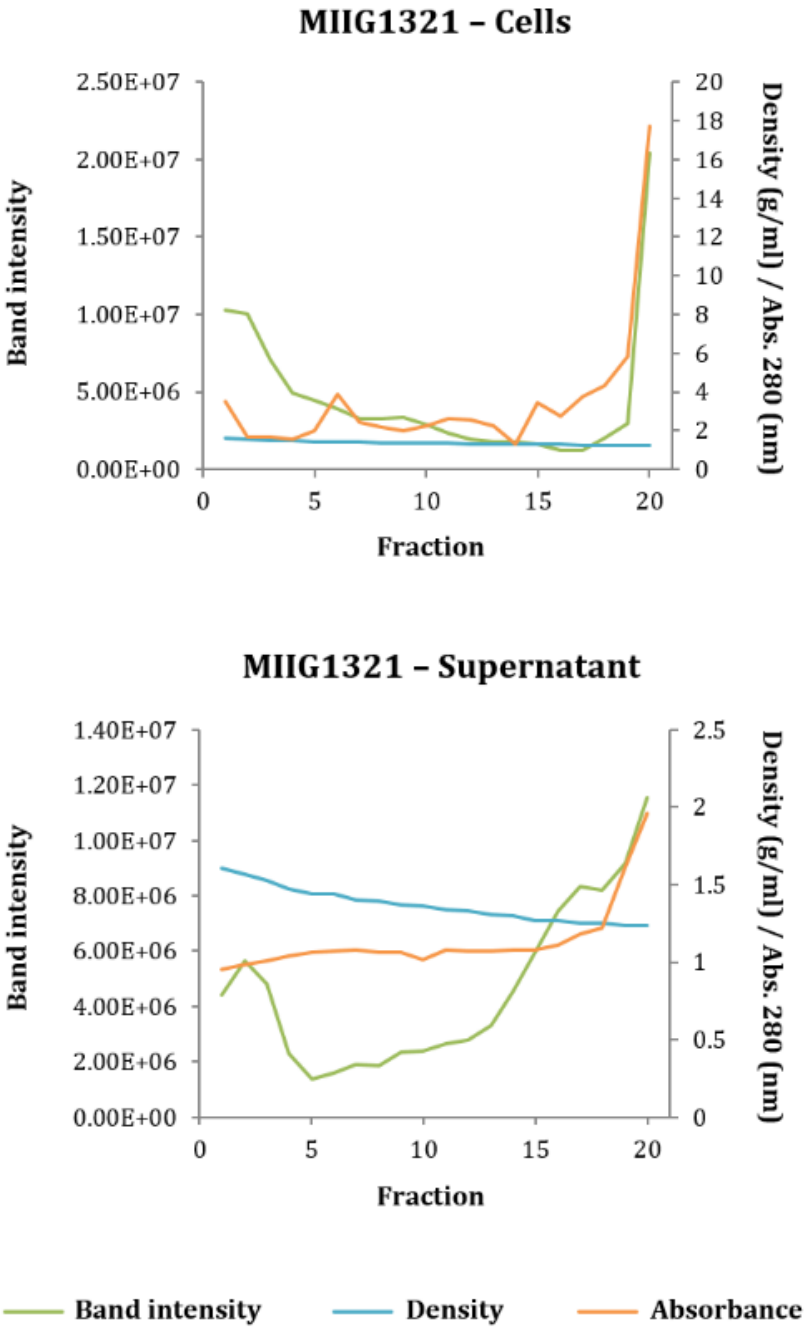

Figure 5.1. PAS staining of the fractions obtained from the sample MIIG1321 of *H. indistincta*. The graph shows also the trend of density and absorbance for the fractions considered which are both plotted on the second y-axis.

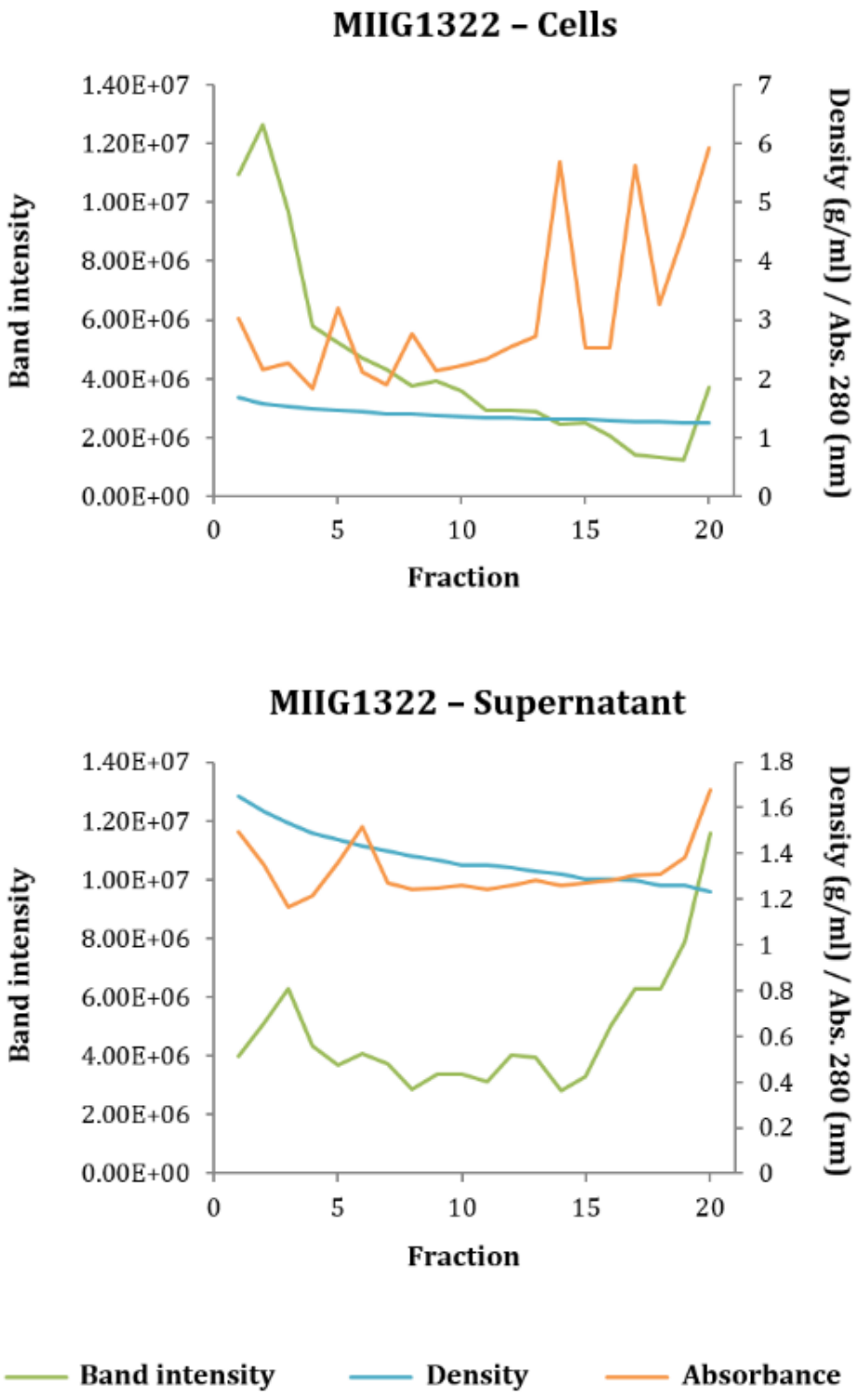

Figure 5.2. PAS staining of the fractions obtained from the sample MIIG1322 of *H. indistincta*.

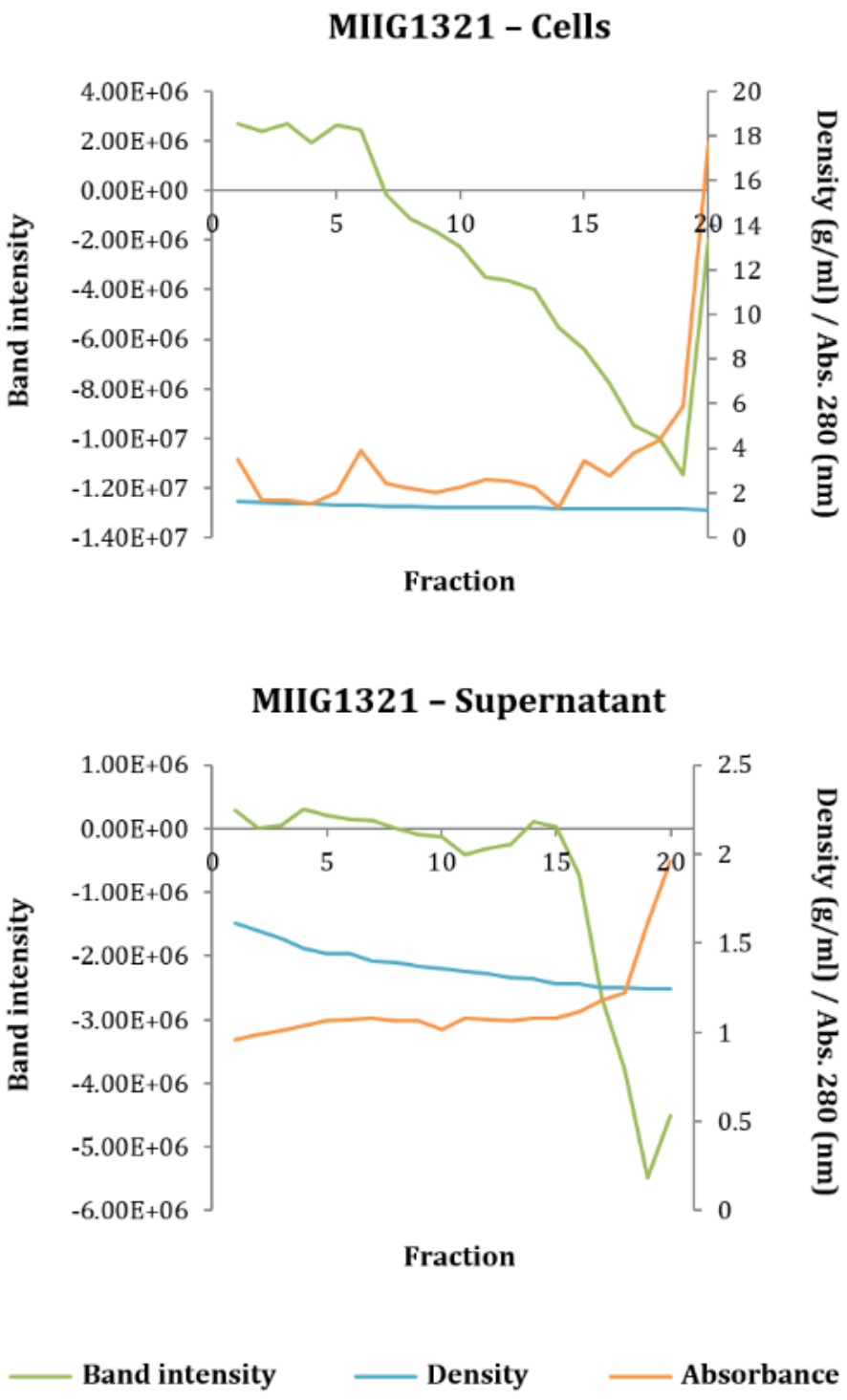

Figure 5.3. Alcian blue staining of the fractions obtained from the sample MIIG1321 of *H. indistincta*. In all cases the band intensity showed negative values, caused by the inhibition of the staining for the corresponding fractions (the origin of the negative results has to be found in the fact that the Alcian blue stained lightly the filter paper: this color was read by the machine as background color with value 0, thus the slots corresponding to the fractions where the staining was inhibited had a lighter color compared to the background, and their intensity was considered by the machine as lower than 0).

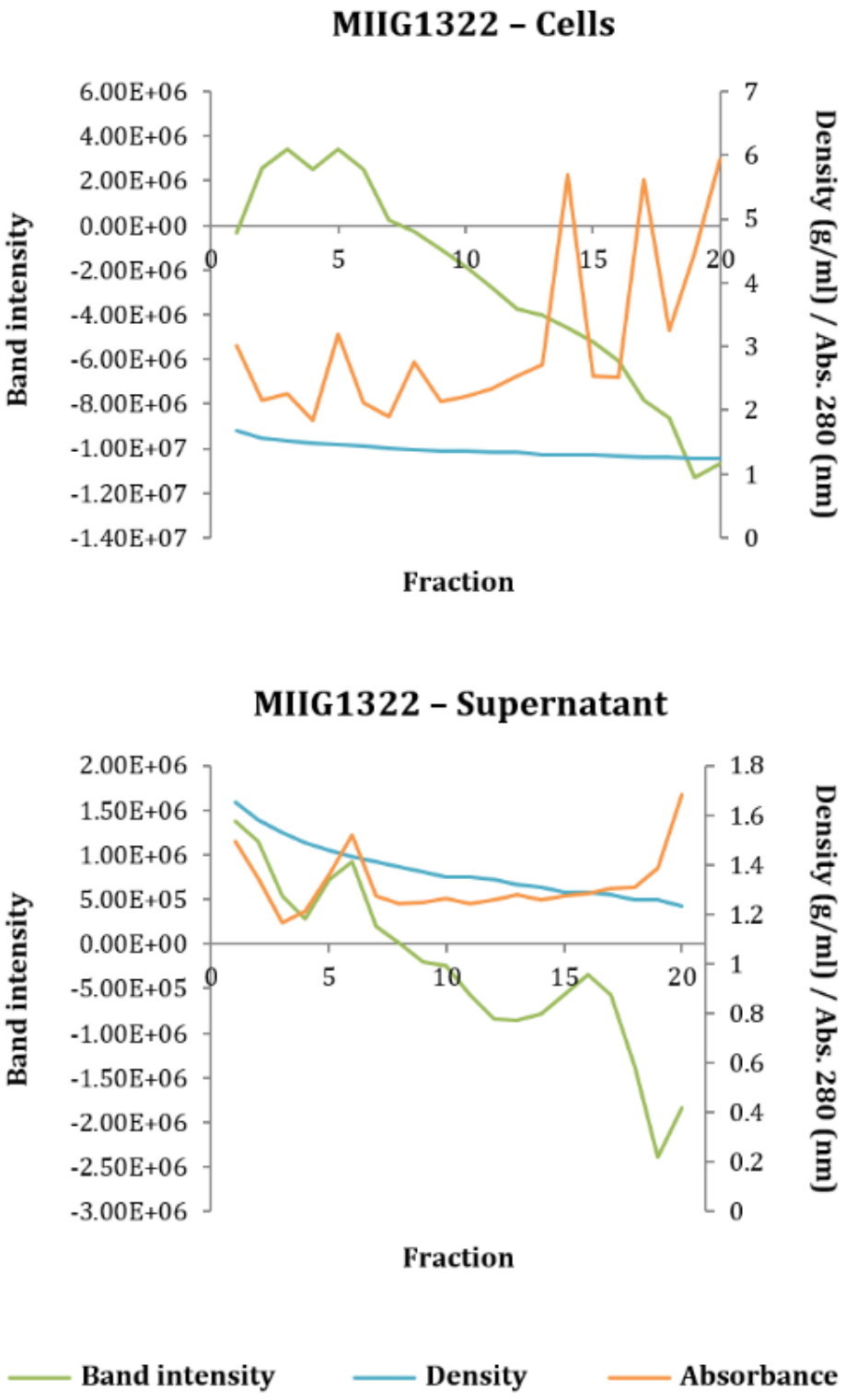

Figure 5.4. Alcian blue staining of the fractions obtained from the sample MIIG1322 of *H. indistincta*.

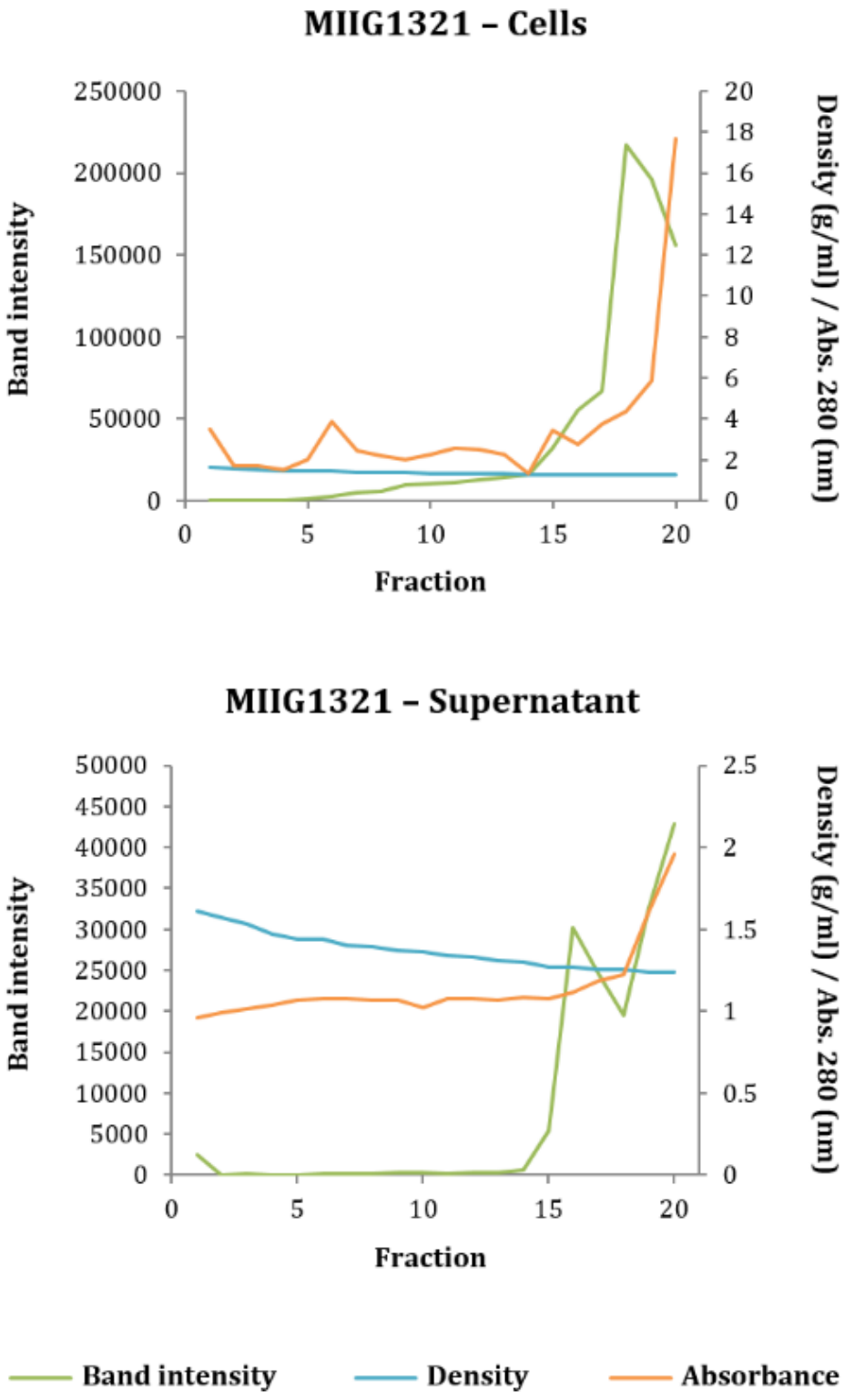

Figure 5.5. Fractions of the sample MIIG1321 of *H. indistincta* stained with the lectin Concanavalin A. The lightest fractions of all the samples showed a band intensity significantly brighter than the heavy fractions.

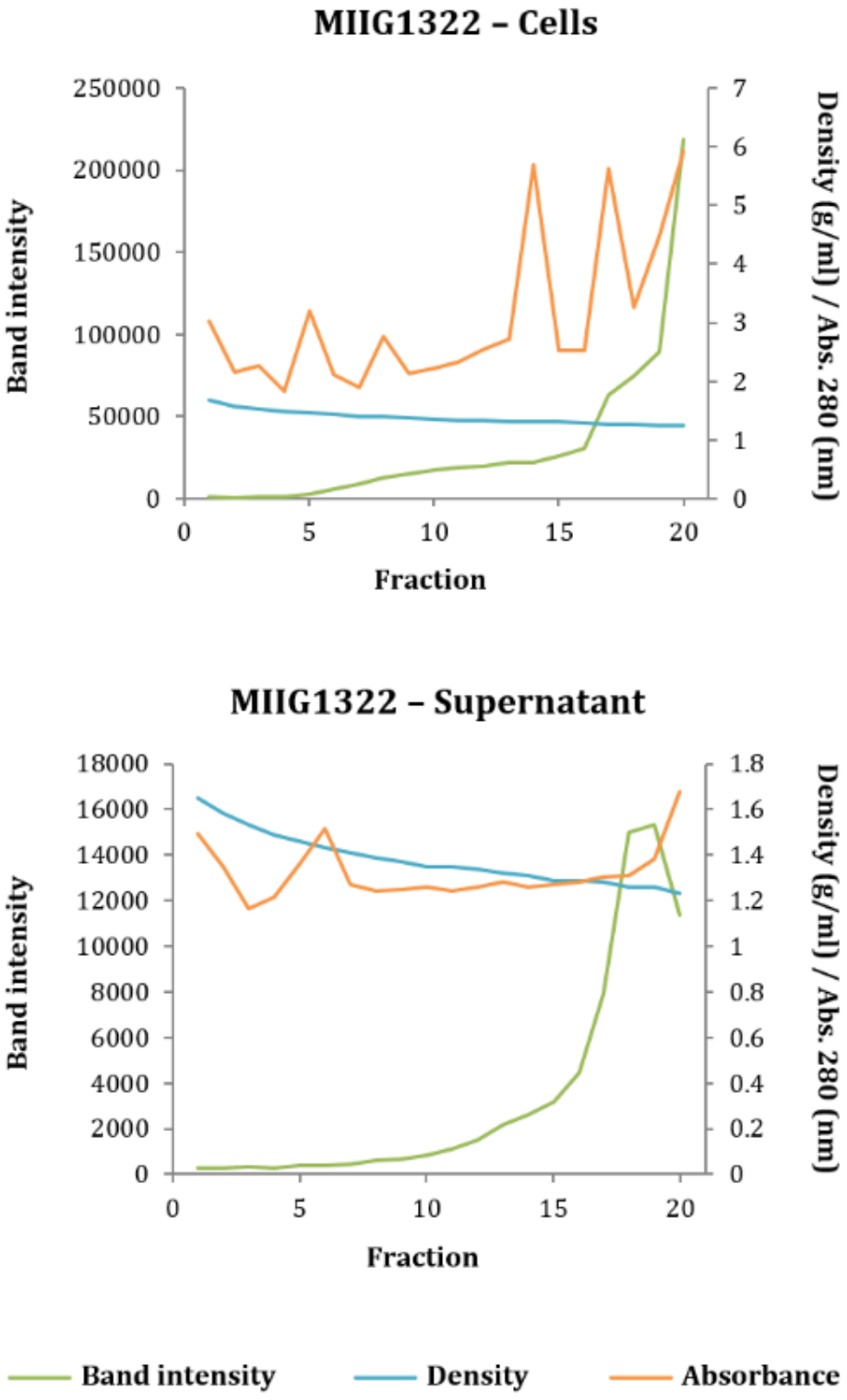

Figure 5.6. Fractions of the sample MIIG1322 of *H. indistincta* stained with the lectin Concanavalin A.

For the *H. cinerea* specimens the density measured between 1.57 and 1.26 g/ml for the cell fractions and between 1.59 and 1.28 g/ml for the supernatant, thus the density range was in both cases smaller than for *H. indistincta*. The distribution of proteins for both cell and supernatant fractions showed a parabolic trend with the absorbance values decreasing from the heaviest fractions to the medium and then rising again for the lightest fractions (Figure 5.4). The PAS staining of the cells resulted in intense bands corresponding to the heaviest fractions, whereas another peak in band intensity corresponded to fractions 15-17. The supernatant fractions from this species again showed an almost parabolic trend in terms of band intensity, with the most intense bands corresponding to the fractions 1–5 and 18–20 (Figure 5.4). Conversely, the staining of the cell slot blot with the anti-CF subunit showed a series of peaks with decreasing amplitude, the highest of which corresponded to fractions 1–5, while the supernatant fractions showed a high increase of band intensity (up to 5522) at fraction 14 (Figure 5.5).

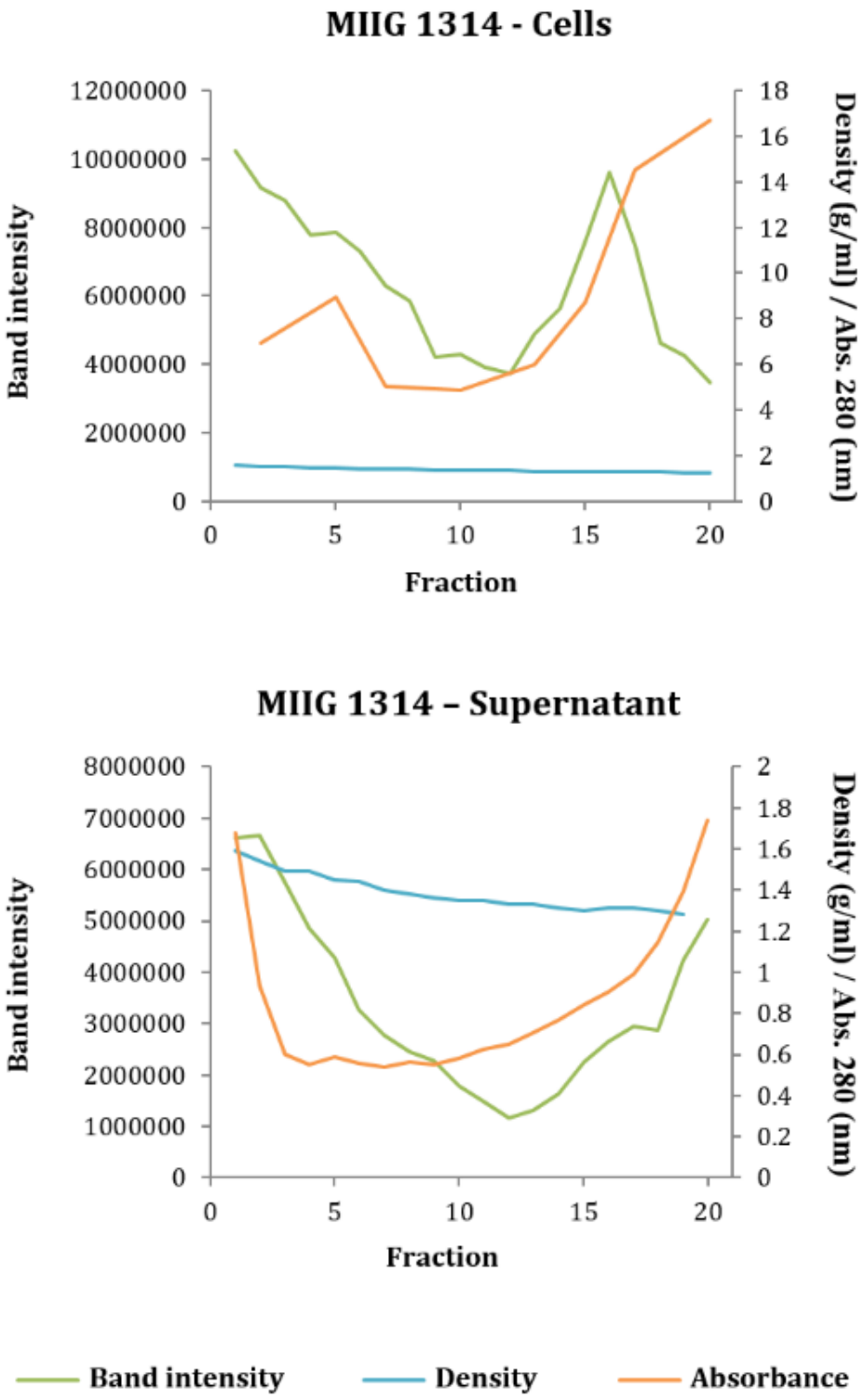

Figure 5.7. PAS staining of the fractions obtained from *H. cinerea*. As seen already for *H. indistincta*, the graphs show also the trend of density and absorbance for the fractions obtained.

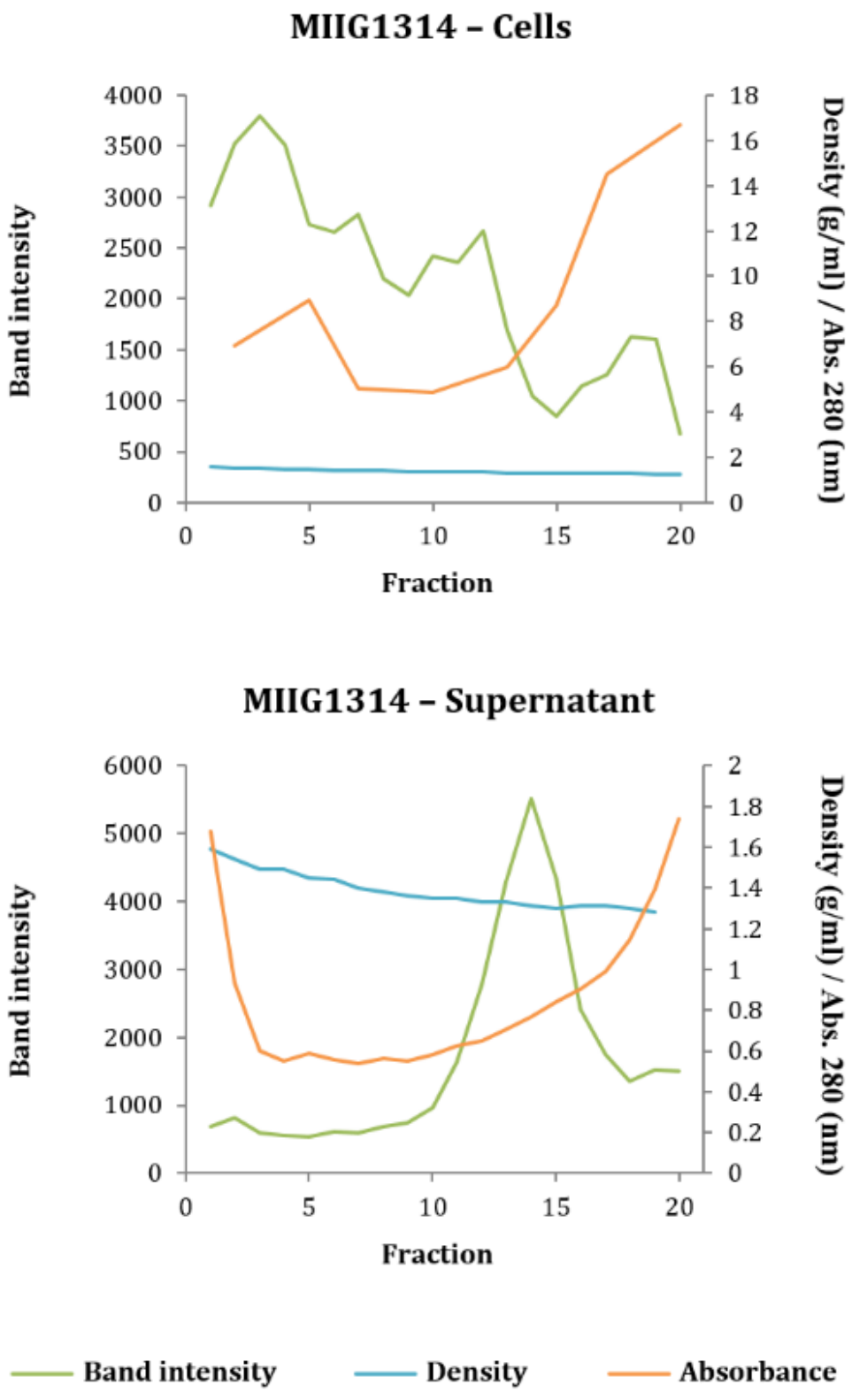

Figure 5.8. Fractions of *H. cinerea* stained with the anti-CF subunit. The band intensity for the cell fractions shows a trend completely different than for the supernatant fractions, being much higher for the heavy than for the light fractions, while it reaches its peak for the fractions 10 – 15 in the supernatant.

The density values for the fractions obtained from *H. mucosa* mucus were between 1.66 and 1.24 g/ml, again higher than observed for *H. cinerea*. An increase in protein content for the light fractions characterised the mucus as already observed for *H. indistincta*, but the trend in *H. mucosa* is less pronounced (Figure 5.6). The presence of glycoproteins highlighted by the PAS staining is generally low (< 0.4) for all the fractions up to 16 and suddenly it showed a dramatic increase reaching the top at fraction 19. The same trend was observed with the AB staining, except for fraction 1 that showed an intensity of 19975, whereas the Con A staining for this sponge species showed high band intensity (> 1) for fractions 16-20 (Figure 5.6).

Overall the density, absorbance and slot blot results did not single out any specific fraction as the one that contained any mucins that might be present for all the sponge species included. This was particularly evident for *H. indistincta* and *H. mucosa*, for which all the fractions were included for the mass spectrometry analysis. For these species equal volumes of the fractions from 1 to 10 and from 11 to 20 were pooled in each case for both cell and supernatant sample types. For *H. cinerea*, however, the indications of mucin presence were clearer for this species and thus fractions 3–5 and 15–17 of the cells as well as fractions 3–5, 13–15 and 18–20 of the supernatant were selected for the mass spectrometry processing and pooled as indicated.

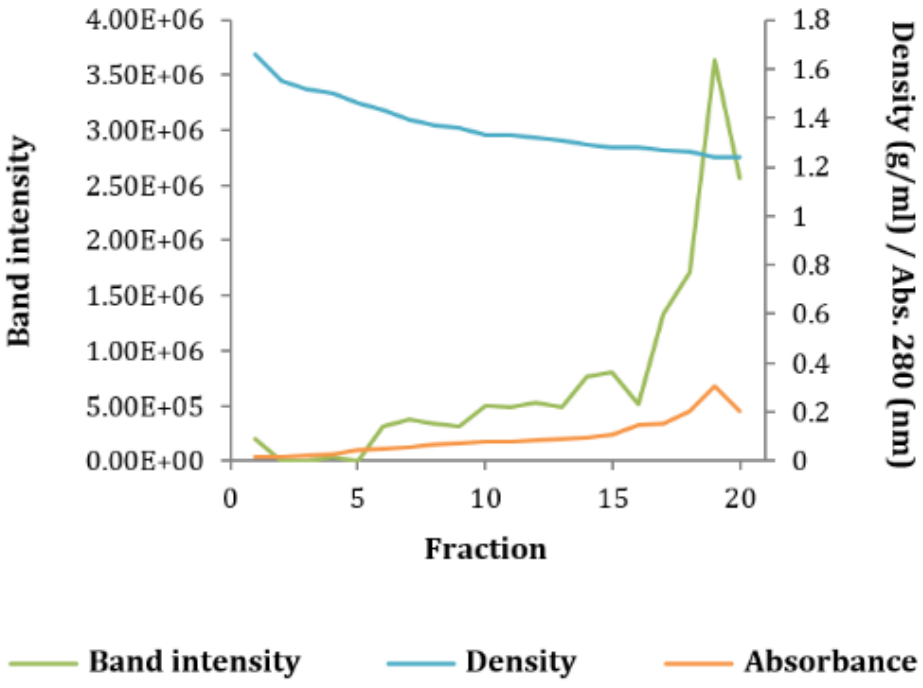

Figure 5.9. *H. mucosa* fractions stained with PAS staining. As seen for the other target sponge species, the graphs include the trend of density and absorbance for the fractions obtained from the mucus of *H. mucosa*.

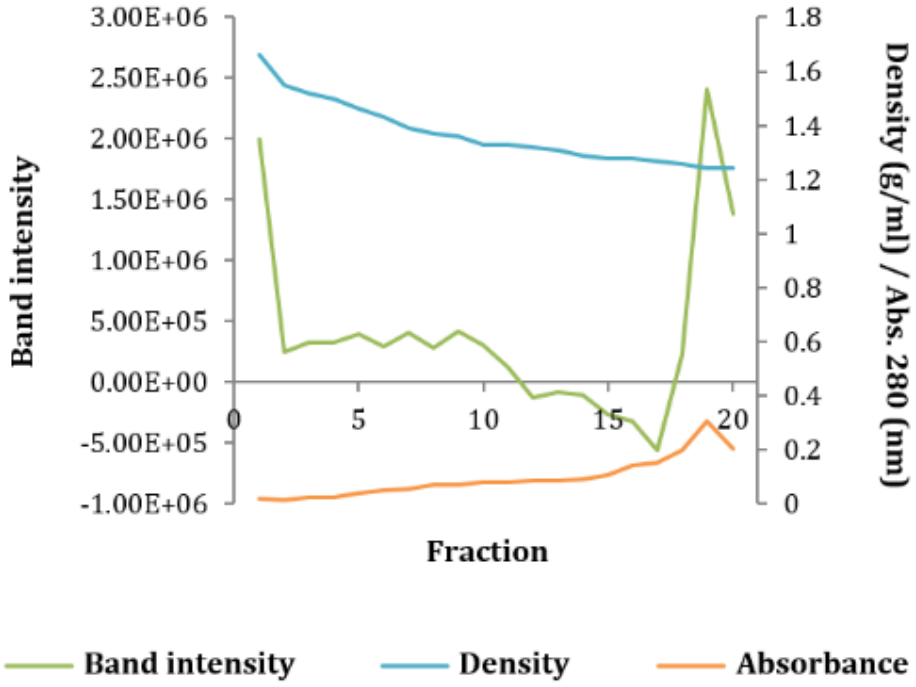

Figure 5.10. Stainings of the *H. mucosa* fractions with Alcian blue.

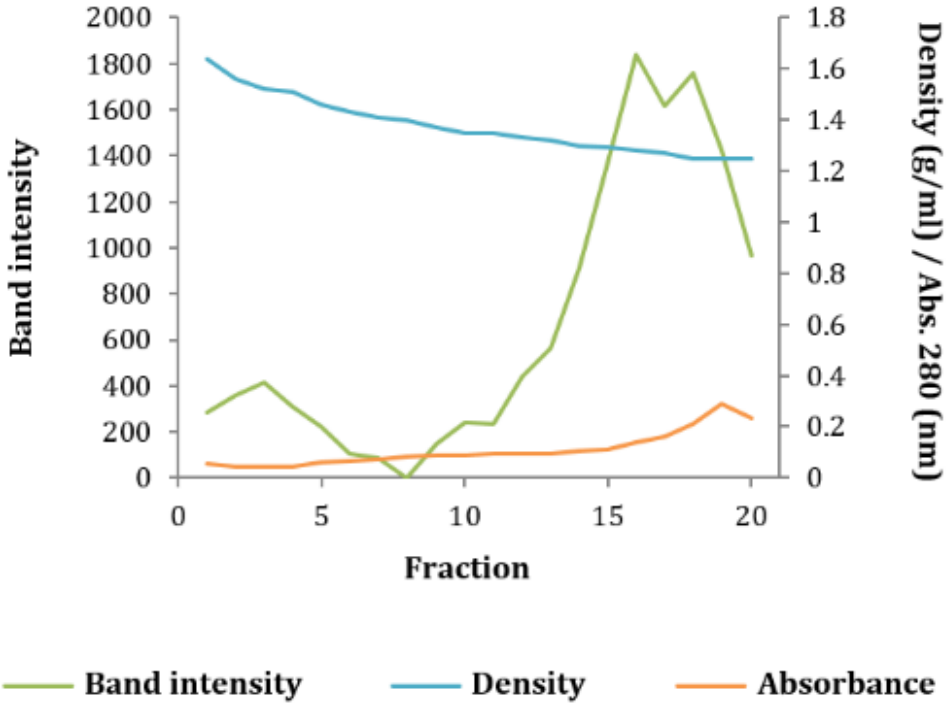

Figure 5.11. Stainings of the *H. mucosa* fractions with Concanavalin A.

Peptides identified for *H. indistincta*

The number of protein families obtained by matching the results of the mass spectrometry analysis against the database of translated sequences is listed in Table 5.1. For the most part (exception being MIIG1321 cell fractions), the number of proteins in the light fractions was always higher than in the heavy fractions, in agreement with the absorbance trends for the same samples. The protein families obtained from the supernatant samples of both individuals are markedly fewer than in the cell samples (i.e. 331 versus 1915 for MIIG1321 and 641 versus 2270 for MIIG1322).

The mass spectrometry analysis detected both mucin and mucin-like sequences in *H. indistincta*. Ten different mucoproteins were detected, some of which were present in more than one fraction (Table 5.2). From a quantitative point of view, the differences in terms of distribution of the mucin/mucin-like sequences among the two *H. indistincta* specimens are not remarkable (Table 5.2): seven different mucin-like sequences were isolated from MIIG1321 and five from MIIG1322, and two were found in both specimens (i.e. sequence 1 and 6). More precisely, for MIIG1321 four sequences were located in the heaviest cell fractions and two in the lightest, while other two mucin-like proteins were found in the fractions 11–20 of the supernatant. For MIIG1322, two mucin-like sequences were isolated from the cell fractions 1–10 and from each of the supernatant fraction pools. In spite of the highest number of protein families yielded, no mucin-like sequences were detected in the pooled fractions 11–20 from the specimen MIIG1322, or the heavy fractions of the supernatant from MIIG1321.

Table 5.1. Protein families isolated from the fractions obtained from the two *H. indistincta* specimens after CsCl/GuHCl isopycnic density gradient ultracentrifugation. In the vast majority of cases the protein families were represented by a single sequence.

	H. indistincta			
	MIIG1321		**MIIG1322**	
Sample	**Density range (g/ml)**	**Number of protein families**	**Density range (g/ml)**	**Number of protein families**
Cell fractions 1-10	1.63 - 1.34	1028	1.68 - 1.36	1081
Cell fractions 11-20	1.33 - 1.24	887	1.34 - 1.24	1189
Supernatant fractions 1-10	1.61 - 1.36	139	1.65 - 1.35	312
Supernatant fractions 11-20	1.34 - 1.24	192	1.35 - 1.23	329

Table 5.2 (next page). List of all the mucin/mucin-like sequences found in *H. indistincta*. In bold the sequences isolated from more than one sample. The number of matches (referred to as the number of PSMs or peptide-spectral-matches) is the number of spectra matching a peptide present in the given protein. The number of significant matches is the number of PSMs that are above the score that Mascot determined as producing a significant hit when looking at the data set and the database I was searching (the more ions from the experimental spectra matching the theoretical spectra, the higher the score and the better a match). The number of sequences is the number of unique peptide sequences identified for this protein. The number of significant sequences is the number of unique peptide sequences identified for this protein that have a higher score for the PSM than the cutoff that Mascot determines (as in the significant matches). The emPAI (exponentially modified Protein Abundance Index or PAI) equals to 10PAI minus one, which is proportional to the protein content in the protein mixture in each dissociated cell and supernatant sample considered (see Ishihama et al., 2005).

Sample	Sequence number	Score	Mass	Number of matches	Number of significant matches	Number of sequences	Number of significant sequences	emPAI
MIIG1321 cell fractions 1-10	**1**	74	16910	3	1	3	1	0.3
	2	63	92950	1	1	1	1	0.05
	3	46	100307	7	1	6	1	0.05
	4	29	13132	3	1	3	1	0.4
MIIG1321 cell fractions 11-20	5	48	100719	12	1	10	1	0.05
	2	46	92950	2	1	1	1	0.05
MIIG1321 supernatant fractions 11-20	**6**	22	29129	4	1	3	1	0.17
	7	15	12638	3	1	2	1	0.42
MIIG1322 cell fractions 1-10	**1**	70	16910	2	1	2	1	0.3
	8	26	90762	7	1	7	1	0.05
MIIG1322 supernatant fractions 1-10	1	59	16910	3	1	2	1	0.3
	9	35	31929	2	1	2	1	0.15
MIIG1322 supernatant fractions 11-20	10	22	43094	5	1	3	1	0.11
	6	20	29129	4	1	4	1	0.17

The Blastp analysis of all the protein families identified by mass spectrometry analysis from *H. indistincta* gave a percentage of similarity always lower than 50% to any database sequence, except for a mucin-like protein isolated from the heavy fractions of the supernatant of MIIG1322 (Table 5.3). This peptide (molecular weight 31929 u) showed a similarity of 53% with a mucin 5B-like protein predicted from the only sponge genome available, i.e. another haplosclerid, *Amphimedon queenslandica*.

The mucoprotein sequence most observed in the two specimens of *H. indistincta* has a mass of 16910 u and was present in the light fractions of the dissociated cells from both sponge samples as well as in the same type of fractions from the supernatant of the sponge MIIG1322. This protein sequence was identified as a mucin-like protein from *A. queenslandica*, but the Blastp search indicated also mucin 4-like protein as possible identification, in any case with low identity (< 40%). Most of the other results of the Blastp search for this sequence were different isoforms of the sushi, nidogen and EGF-like domain-containing protein 1. A peptide sequence with mass of 92950 u and identified as mucin 3A-like protein was isolated from both heavy and light fractions of the specimen MIIG1321. A third mucoprotein (mass of 29129 u) was detected in the light fractions of the supernatant of both sponge specimens and identified as similar to a mucin-like protein isolated from the gastropod *Biomphalaria glabrata*. The Blastp search results with the best scores for this sequence were uncharacterised LOC proteins and tenascin-like proteins, but among the other results there were three mucin-like proteins from molluscs, once again with low similarity percentages.

Table 5.3. Results of the Blastp search for the mucin/mucin-like proteins isolated from *H. indistincta*. In bold the protein sequences for which the Blastp analysis indicated the same GenBank identity as for the analogous sequences in *H. cinerea*.

Sequence number	Description	Max score	Total score	Query cover	E value	Identity	Accession number
1	**PREDICTED: mucin-like protein [*Amphimedon queenslandica*]**	88.6	88.6	83%	1.00E-17	39%	XP_003384557.1
2	**PREDICTED: mucin-3A-like [*Amphimedon queenslandica*]**	286	799	73%	3.00E-77	45%	XP_019851107.1
3	PREDICTED: mucin-4-like [*Amphimedon queenslandica*]	126	126	33%	5.00E-26	34%	XP_019849627.1
4	PREDICTED: mucin-5AC-like, partial [*Amphimedon queenslandica*]	87.4	87.4	76%	9.00E-18	43%	XP_019862133.1
5	PREDICTED: mucin-17-like [*Saccoglossus kowalevskii*]	145	145	20%	4.00E-32	36%	XP_002731731.1
6	PREDICTED: mucin-like protein [*Biomphalaria glabrata*]	47.8	89.7	29%	0.014	40%	XP_013081265.1
7	PREDICTED: mucin-19 [*Drosophila eugracilis*]	37	37	60%	4.30	32%	XP_017064550.1
8	PREDICTED: mucin-like protein [*Amphimedon queenslandica*]	91.7	91.7	27%	2.00E-15	30%	XP_019849626.1
9	PREDICTED: mucin-5B-like [*Amphimedon queenslandica*]	162	162	58%	8.00E-43	53%	XP_011402862.2
10	PREDICTED: mucin-1-like [*Amphimedon queenslandica*]	195	195	77%	3.00E-54	37%	XP_019858873.1

Peptides identified for *H. cinerea*

H. cinerea showed different profiles when compared to *H. indistincta* for the same treatments with the dissociated cells and the supernatant yielding similar numbers of protein families for this sponge species (Table 5.4). This might be due to the fact that for *H. cinerea* only a part of the fractions obtained were processed for mass spectrometry, contrary to *H. indistincta* and *H. mucosa*. In spite of that, 27 mucin/mucin-like sequences were detected in *H. cinerea*, and some of these proteins were present in multiple fractions as already seen for *H. indistincta.* Nineteen different mucoproteins were found in the cell fractions and 20 in the supernatant (Table 5.5). For both the cells and the supernatant of *H. cinerea* the pools of fractions 3-5 yielded the highest number of mucins/mucin-like proteins: 16 different proteins were detected in the supernatant pool and 17 from the cell pool. Three mucins/mucin-like proteins were isolated from the cell fractions 15-17, while four and three proteins were isolated respectively from the pools of supernatant fractions 13-15 and 18-20.

Table 5.4. Protein families isolated from the dissociated cells and supernatant fractions of *H. cinerea* selected based on the values of density, absorbance, PAS and CF subunit staining.

H. cinerea		
Sample	**Density range (g/ml)**	**Number of protein families**
Cell fractions 3-5	1.53 - 1.45	185
Cell fractions 15-17	1.32 - 1.30	236
Supernatant fractions 3-5	1.49 - 1.45	108
Supernatant fractions 13-15	1.33 - 1.30	185
Supernatant fractions 18-20	1.30 - 1.28	222

Table 5.5. List of all the mucin/mucin-like sequences found in *H. cinerea*. Marked in bold the sequences obtained from more than one fraction. For both cells and supernatant, the heavy fractions yielded a number of sequences much higher than the light fractions.

Sample	Sequence number	Score	Mass	Number of matches	Number of significant matches	Number of sequences	Number of significant sequences	emPAI
Supernatant fractions 3-5	1	243	20357	25	15	10	8	3.8
	2	191	173181	24	6	17	6	0.15
	3	111	294376	16	3	14	3	0.04
	4	100	43954	9	3	4	2	0.2
	5	95	15482	1	1	1	1	0.29
	6	90	169532	3	2	2	1	0.05
	7	73	104686	13	2	11	2	0.08
	8	65	28619	2	1	2	1	0.15
	9	57	77881	3	1	3	1	0.05
	10	52	27637	4	1	4	1	0.16
	11	50	267237	17	1	12	1	0.02
	12	41	39528	4	2	3	2	0.23
	13	40	14806	3	1	3	1	0.31
	14	34	14809	2	1	2	1	0.31
	15	33	37550	6	2	4	2	0.24
	16	33	18370	4	1	3	1	0.24

Sample	Sequence number	Score	Mass	Number of matches	Number of significant matches	Number of sequences	Number of significant sequences	emPAI(*)
Supernatant fractions 13-15	17	122	17691	10	4	5	3	0.96
	1	88	20357	15	3	9	3	0.8
	18	25	46463	21	1	14	1	0.09
	19	23	12932	4	1	3	1	0.35
Supernatant fractions 18-20	20	163	100441	12	6	10	6	0.28
	17	82	17691	4	2	3	2	0.57
	1	40	20357	3	1	3	1	0.22
Cell fractions 3-5	15	181	37550	11	6	5	4	0.71
	1	172	20357	29	12	8	7	2.94
	4	165	43954	11	4	4	2	0.2
	3	80	294376	27	3	23	3	0.04
	21	73	19053	3	1	2	1	0.23
	6	66	169532	10	3	8	2	0.08
	10	59	27637	3	1	3	1	0.16
	22	59	164178	8	1	5	1	0.03
	23	54	35159	8	1	4	1	0.12
	11	53	267237	16	1	11	1	0.02
	20	49	100441	5	2	4	2	0.08
	24	33	11323	3	2	2	2	0.98
	13	30	14806	4	1	4	1	0.31
	2	23	173181	25	1	18	1	0.02
	14	20	14809	2	1	2	1	0.31
	12	17	39528	4	1	3	1	0.11
	25	17	36654	11	1	8	1	0.12
Cell fractions 15-17	20	113	100441	7	4	7	4	0.18
	26	32	99374	12	1	9	1	0.04
	27	16	150290	16	1	11	1	0.03

The Blastp analysis of all the mucoproteins identified via mass spectrometry from *H. cinerea* gave a percentage of similarity always lower than 50% except in the case of a mucin–like protein isolated from the supernatant fractions 3–5. This protein (molecular weight 28619 u) was 52% identical to a mucin 4–like sequence predicted from the genome of *A. queenslandica*.

Thirteen mucins/mucin-like proteins in *H. cinerea* were present in different fraction pools. The most distributed protein found in *H. cinerea* has a mass of 20357 u and was identified as an integumentary mucin C.1–like protein predicted from the genome of *A. queenslandica*; it was isolated from all the supernatant pools of fractions and from the heavy cell fractions. From the fractions 3–5 of both dissociated cells and supernatant the mass spectrometry analysis detected a protein sequence of mass 39528 u that was identified as an isoform of an apomucin-like protein from the scorpion *Centruroides sculpturatus* with a percentage similarity of 44%. In the same fractions the mass spectrometry analysis located the sequence of a mucin-like protein with mass of 169532 u; many other types of mucin-like proteins (especially mucin 4–like) are present among the results of the Blastp for this mucoprotein, but also in this case the similarity percentages are always low. A complete list of the Blastp results for all the mucins/mucin-like proteins detected in *H. cinerea* is given in Table 5.6.

Table 5.6. Results of the Blastp search for the mucin/mucin–like proteins detected in *H. cinerea*. Eight out of 27 sequences refer to the same protein sequence from *A. queenslandica* predicted as a mucin 17–like protein. In bold the protein sequences for which the Blastp analysis indicated the same GenBank identity as for the analogous sequences in *H. indistincta*.

Sequence number	Description	Max score	Total score	Query cover	E value	Identity	Accession number
1	PREDICTED: integumentary mucin C.1-like [*Amphimedon queenslandica*]	101	219	88%	6.00E-24	40%	XP_011405517.1
2	PREDICTED: mucin-5AC-like [*Monomorium pharaonis*]	42.7	42.7	9%	9.9	26%	XP_012530444.1
3	PREDICTED: mucin-17-like [*Amphimedon queenslandica*]	313	1269	99%	2.00E-81	25%	XP_011403877.1
5	PREDICTED: mucin-17-like [*Amphimedon queenslandica*]	61.2	138	84%	4.00E-08	34%	
11	PREDICTED: mucin-17-like [*Amphimedon queenslandica*]	283	705	94%	2.00E-72	25%	
13	PREDICTED: mucin-17-like [*Amphimedon queenslandica*]	48.9	48.9	61%	6.00E-04	37%	
14	PREDICTED: mucin-17-like [*Amphimedon queenslandica*]	46.6	83.6	85%	0.004	32%	
21	PREDICTED: mucin-17-like [*Amphimedon queenslandica*]	58.2	183	90%	1.00E-06	34%	
23	PREDICTED: mucin-17-like [*Amphimedon queenslandica*]	80.5	293	98%	6.00E-13	28%	
24	PREDICTED: mucin-17-like [*Amphimedon queenslandica*]	50.4	93.2	98%	6.00E-05	27%	
4	PREDICTED: mucin-3A-like [*Amphimedon queenslandica*]	127	191	92%	3.00E-28	28%	XP_019851150.1
15	PREDICTED: mucin-3A-like [*Amphimedon queenslandica*]	194	194	95%	1.00E-51	40%	
6	**PREDICTED: mucin-like protein [*Amphimedon queenslandica*]**	540	540	66%	1.00E-165	33%	XP_003384557.1

Sequence number	Description	Max score	Total score	Query cover	E value	Identity	Accession number
7	PREDICTED: mucin-3A-like [*Amphimedon queenslandica*]	73.9	118	76%	2.00E-09	23%	XP_019851107.1
9	PREDICTED: mucin-3A-like [*Amphimedon queenslandica*]	65.1	122	60%	6.00E-07	25%	
8	PREDICTED: mucin-4-like [*Amphimedon queenslandica*]	48.5	48.5	18%	0.009	52%	XP_019849592.1
10	LOW QUALITY PROTEIN: mucin-4 [*Lingula anatina*]	49.7	49.7	37%	0.003	36%	XP_013395977.2
12	apomucin-like isoform X1 [*Centruroides sculpturatus*]	47.8	47.8	26%	0.02	44%	XP_023232651.1
16	PREDICTED: mucin-16 isoform X2 [*Chinchilla lanigera*]	39.3	39.3	25%	2.9	42%	XP_013364621.1
18	mucin-2-like [*Acanthaster planci*]	45.1	45.1	20%	0.34	32%	XP_022109176.1
19	mucin-5AC-like [*Stylophora pistillata*]	43.5	43.5	51%	0.019	42%	XP_022785618.1
17	Mucin-19 [*Papilio machaon*]	52.4	93.2	67%	0.00005	32%	KPJ07805.1
20	Mucin-19 [*Nephila clavipes*]	100	326	86%	6.00E-19	29%	PRD33209.1
22	**PREDICTED: mucin-3A-like [*Amphimedon queenslandica*]**	580	971	43%	3.00E-170	47%	XP_019851107.1
25	PREDICTED: mucin-5B-like [*Saccoglossus kowalevskii*]	50.4	50.4	39%	0.003	31%	XP_006812161.1
26	PREDICTED: mucin-5AC-like [*Amphimedon queenslandica*]	150	150	57%	2.00E-33	26%	XP_019859564.1
27	mucin-like protein [*Orbicella faveolata*]	222	222	31%	2.00E-56	36%	XP_020608532.1

Peptides identified for *H. mucosa*

A large difference in terms of number of protein families observed in the 'mucus' collected from *H. mucosa* was evident between fractions 1-10 and 11-20, with the light fractions yielding almost four times the protein families present in the heavy fractions when the mass spectrometry data for *H. mucosa* were matched against the database of translated sequences from *H. indistincta* and twice when compared to the data from *H. cinerea* (Table 5.7). No mucin/mucin-like sequences were detected when the mass spectrometry data were compared against the translated sequences from *H. indistincta*, while the comparison with the *H. cinerea* proteins highlighted the presence of two mucoproteins in the pool of fractions 11-20 (Table 5.8). The two mucoproteins detected in the 'mucus' of *H. mucosa* were identified as a mucin 5AC-like protein and an isoform of a mucin 17 respectively predicted from the genome of *A. queenslandica* and the sea urchin *Strongylocentrotus purpuratus* (Table 5.9). While these two glycoproteins were not isolated from either cell or supernatant fractions of *H. cinerea*, a sequence identified as the same mucin 5AC–like protein from *A. queenslandica* was found in the heavy cell fractions of the *H. indistincta* sample MIIG1321.

Table 5.7. Numbers of protein families isolated from the mucus of *H. mucosa*. The sequences obtained from the mass spectrometry analysis of the fractions from this sponge species were matched against the database of translated sequences from the other two *Haliclona* species.

	H. mucosa		
Sample	**Density range (g/ml)**	***H. indistincta* transcriptome**	***H. cinerea* transcriptome**
Mucus fractions 1-10	1.66 - 1.33	36	70
Mucus fractions 11-20	1.33 - 1.24	122	148

Table 5.8. Results of mass spectrometry data for *H. mucosa* searched against the transcriptome of *H. cinerea*.

Sample	Sequence number	Score	Mass	Number of matches	Number of significant matches	Number of sequences	Number of significant sequences	emPAI(*)
Fractions 11-20	1	30	62201	30	1	17	1	0.07
	2	29	51337	5	1	2	1	0.08

Table 5.9. Blastp results for *H. mucosa*.

Sequence number	Description	Max score	Total score	Query cover	E value	Identity	Accession number
1	PREDICTED: mucin-5AC-like [*Amphimedon queenslandica*]	122	122	24%	9.00E-26	42%	XP_019862133.1
2	PREDICTED: mucin-17 isoform X2 [*Strongylocentrotus purpuratus*]	85.9	85.9	80%	6.00E-14	27%	XP_011671719.1

Analysis of the mucins/mucin–like proteins across the three *Haliclona* species

The two types of mucins/mucin-like proteins located in *H. mucosa*, i.e. mucin 5AC-like protein and mucin 17, were found also in *H. indistincta* and *H. cinerea*, even though the accession numbers for all of them are different. One of the mucin 5AC–like proteins from *H. cinerea* and the one from *H. indistincta* were determined to be similar to sequences predicted from the genome of *A. queenslandica*; the other two mucin 5AC–like proteins from *H. cinerea* were considered similar to a protein predicted from the genome of an ant (*Monomorium pharaonis*) and a mucin 5AC–like protein isolated from the coral *Stylophora pistillata*, in this case with a percentage of similarity of 42%. All the eight protein sequences from *H. cinerea* identified as mucin 17–like protein were identified with percentages of similarity between 25% and 37% with the same mucin 17–like protein predicted from the genome of *A. queenslandica*. The mucin 17-like protein detected in *H. indistincta*, however had 36% identity to an analogous protein isolated from the acorn worm *Saccoglossus kowalevskii*. Other types of mucins/mucin-like proteins observed both in *H. cinerea* and *H. indistincta* were the mucin 3A-like, mucin 4-like, mucin 5B-like proteins and the mucin 19.

No specific protein sequences returned from mass spectrometry were shared among the three *Haliclona* species, but in two cases the Blastp search for the mucoprotein sequences from *H. indistincta* and *H. cinerea* indicated the same GenBank sequence as identity. One case is represented by two mucoproteins identified as a predicted mucin 3A-like protein from *A. queenslandica* with a similarity percentage of 45% for *H. indistincta* and 47% for *H. cinerea*. The query cover is 73% for the protein sequence from *H. indistincta* and 43% for the one from *H. cinerea*, but both sequences earned the highest Blastp max score for each sponge species (see Table 5.3 and Table 5.6). In the second case a protein sequence from *H. cinerea* and a sequence from *H. indistincta* were identified as the same mucin-like protein from *A. queenslandica* (GenBank code: XP_003384557.1) respectively with a similarity

percentage of 33% and 39% for a query cover of the 83% for *H. indistincta* and the 66% for *H. cinerea*.

Discussion

This work represents the first report focused on identifying the diversity of mucins in Porifera through a proteomic approach. The presence of mucin-like proteins in sponges was already highlighted in previous studies. The first was an analysis of the extracellular matrix of *Geodia cydonium*, in which Schutze et al. (2001) isolated a protein rich in threonine that acted as ligand for the receptor tyrosine kinase from this sponge, a similar role played by mucin 4 in higher organisms (Carraway et al., 2003), and in fact the authors defined this ligand as mucus-like protein. Meyer et al. (2006) found via histochemistry methods that the exopinacoderm of the Mediterranean sponge C*hondrilla nucula* is covered by a compact cover of mucus made of O-linked high molecular weight glycoproteins containing sialic acids, a typical component of mucins (Baos et al., 2012). Wang et al. (2013) and Wang and Müller (2015) characterised a mucin 4-like protein found in *Suberites domuncula* for which they proposed a role in the process of water extrusion/suctioning from biosilica after enzymatic synthesis during spicule formation via a mechanism of 'polymerization-induced phase separation'. The work I carried on shows the presence of multiple mucin-like proteins in Poriferans for the first time.

Jatkar et al. (2010) reported that for corals the CsCl/GdmCl isopycnic density gradient ultracentrifugation concentrates the mucins in the density range of 1.4–1.5 g/ml, as previously seen for vertebrates. This correlation does not appear to be respected in the sponges I analysed. Moreover, the density range for the fractions obtained from *H. cinerea* was lower than for *H. indistincta* and *H. mucosa*, for which the density ranges were similar. Dissimilarities in the mucin density have been correlated with different physical properties of these glycoproteins (Pillai et al., 2017), and this could be considered also for the three target

Haliclona species, given the noticeably different appearance of the mucus in *H. cinerea* compared to the other two species.

Most of the proteins isolated in this study identified as mucin–like proteins showed some similarity with mucins described from the human gastrointestinal tract (Tailford et al., 2015). For instance, the only two types of mucin–like proteins found in all the three sponge species were mucin 5AC–like and mucin 17–like. Mucin 5AC, as well as mucins 5B, 19 and 2, are gel-forming mucins secreted by goblet cells respectively in stomach surface, salivary glands and intestine of mammals (Chen et al., 2004, Pelaseyed et al., 2014). The mucins 5AC and 5B are also found in the respiratory tract (Ma et al., 2018). In the various organs these mucins play different roles: as regards the digestive tract, for instance, mucin 5AC protects the stomach wall from damage caused mechanically by ingested food or chemically by gastric acid, while mucin 2, expressing different properties between the small and the large intestine, ensures absorption of nutrients in the first and protection from bacteria in the second tract (Ermund et al., 2013). In this study I found proteins similar to mucins 5B and 19 in *H. indistincta* and *H. cinerea*, and to mucin 2 only in *H. cinerea*. Mucin 17 is a cell-surface mucin and represents one of the most common components of the glycocalyx covering the epithelial cells of the human intestine (Schneider et al., 2018). Other mucin-like proteins found both in *H. indistincta* and *H. cinerea* are membrane-bound mucins, i.e. mucin 3A, normally expressed in the epithelia of the gastrointestinal tract, mucins 1 and 4 that, similarly to mucin 16, are produced by different types of epithelial cells (Hattrup and Gendler, 2008). Cell-surface mucins are involved in blocking pathogens adhesion to mucosal epithelial cells (McGuckin et al., 2015) and are also considered to play an important role in cell signalling (Carraway et al., 2003, Jonckheere and Van Seuningen, 2010, Van Putten and Strijbis, 2017).

A further characterisation of the mucin-like proteins in the target *Haliclona* species could allow identifying the cells responsible for their production using these glycoproteins as markers. As described in chapter 4, we have observed in *H. indistincta* and in other two closely

related species (i.e. the sibling species *H. viscosa* and *H. sarai*) a type of cell characterised by inclusions with granular content and other features that are consistent with mucous cells in higher organisms. Donadey (1982) attributed the production of mucus in *H. mucosa* to a different type of cells with inclusions defined as spumeuse cell and characterised by fibrillary content. The TEM analysis of *H. cinerea* did not show the presence of cells with morphology comparable to the cells with inclusions in *H. indistincta* and *H. mucosa*. Only few cell types can be recognised in the tissue of this sponge species and the only cell morphology considered consistent with the production of mucus was a type of cell with inclusions characterised by electron-dense peripheral membrane-enclosed inclusions, showing sometimes a darker core, and the presence of an electron-dense region that occupies the centre of the cell. These cells show the tendency to come in touch with each other assuming the aspect of a pearl necklace and the inner regions of every cell align and connect with each other, as it can be observed at the intercellular space in between two adjacent cells (Figure 5.7). Similar cells were described in detail by Lévi (1967) in *Haliclona elegans* (Bowerbank, 1866), now redefined as *H. cinerea* (http://www.marinespecies.org/porifera/). Lévi (1967) proposed for these cells a role in the production of fibres distinct from spongin and collagen, but still involved in the skeletal production of the sponge. The author stated that assays carried out to search for polysaccharides in these cells gave negative results, but the Alcian blue-PAS staining of semithin sections of *H. cinerea* we performed along with sections of *H. indistincta* (described in Chapter 4) proved that this is true for the core of the segmented cells, but not for the peripheral inclusions, that instead stained intensively pink, suggesting the presence of neutral polysaccharides (Figure 5.7). Donadey and Vacelet (1977) disagreed with Levi's view and suggested that these cells were spumeuse cells, thus possible mucus-producers.

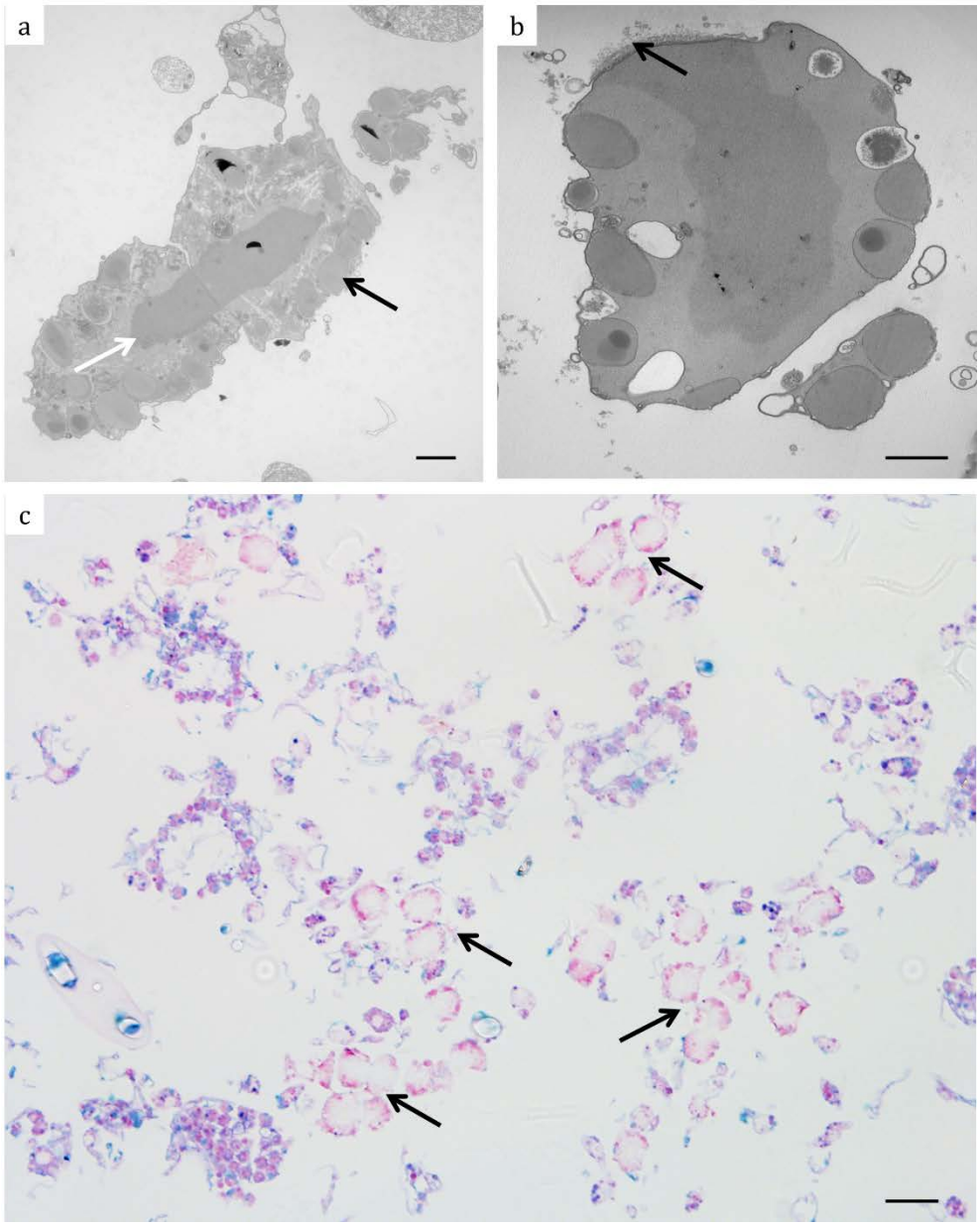

Figure 5.7. Cells with inclusions in *H. cinerea*. **a**. Two adjacent cells with inclusions showing several vesicles with electron dense content and darker core at the cell edge (black arrow), while the dark cores of the two cells are in touch with each other (white arrow). **b**. The arrow points at the fibres visible at the connection point between two adjacent cells; **c**. Inclusions intensively stained with PAS. Scale bars: **a** – **b**: 2 µm; **c**: 20 µm.

However, the exceptional diversity of mucin-like proteins found between the three *Haliclona* species might suggest that more than one cell type can be responsible for the production of different types of these glycoproteins. In fact, in the mucosal surfaces of human body cavities two cell types produce mucins, i.e. the goblet cells secrete gel-forming mucins, while membrane-bounded mucins are produced by both goblet cells and enterocytes (Van Putten and Strijbis, 2017). The latter cells represent the main cell type in the intestine and are characterised by a columnar shape and an apical membrane expanded by microvilli and specialised in the uptake of nutrients and small ions through special transporters. Besides the microvilli, the apical surface of these cells is covered with a thick glycocalyx rich in carbohydrates (Pelaseyed et al., 2014). Similar characteristics are shown in sponges by choanocytes. In fact, in these cells a collar of microvilli surrounds the flagellum and it has been proved that genes associated with the microvilli of vertebrate cells are expressed in sponge choanocytes (Peña et al., 2016). Choanocytes are also characterised by the presence of an organised glycocalyx at the bottom of the collar that was defined as mucus in *Spongilla lacustris* (Fjerdingstad, 1961) and it was proposed by Simpson (1984) to ensure integrity to the collars giving rigidity to the microvilli. Besides creating the water flow that carries the food, the choanocytes are also responsible for the phagocytosis of bacteria and other microorganisms (Leys and Hill, 2012). However, it has been recently shown with *Halisarca caerulea* and other sponge species from different environments that choanocytes also absorb dissolved organic matter (DOM) and based on this discovery it has been proposed the "sponge loop" model for which sponges in coral reefs feed on the dissolved organic matter excreted as mucus by corals and produce particulate organic matter (POM) that is used by other organisms to feed, recirculating the nutrients (De Goeij et al., 2013, Rix et al., 2016). The authors of these studies also found out that the energy derived from the consumption of DOM by sponges is not used to increase their biomass, but to fuel a very rapid turnover of the choanocytes in the chambers and the choanocyte renewal contributes to the POM

production (De Goeij et al., 2009, Alexander et al., 2014). Cell exfoliation is also a characteristic of the intestinal cells and the exfoliated cells contribute to the formation of feces (Albaugh et al., 1992, Williams et al., 2015). Moreover, Kahn and Leys (2016) observed via time-lapse microscopy that the turnover of the choanocytes is not due to mitosis, but to amebocytes inserting into the choanocyte chamber, similarly to what happens to the intestinal epithelial cells (Umar, 2010). The ability of sponges to transform the organic matter from dissolved to particulate has promoted the idea that sponges can be used as models to study the human intestine. In this view, it is of interest the fact that I have found in all the three *Haliclona* sponge species studied proteins that showed similarity to both gel-forming and membrane-bound mucins expressed in this organ and further analyses aimed at determining their distribution could help to understand if they play similar roles to the human counterparts.

One of the main functions played by mucins in the digestive tract of mammals is to act as a barrier against pathogens. In fact, mucins in the gut can trap bacteria through their carbohydrate residues that interact with adhesins on the bacterial cell surface (Cornick et al., 2015). Meyer et al. (2006) already suggested that the glycoconjugates in the exopinacoderm of *C. nucula* act as defence barrier against different microorganisms. In fact these molecules supply ligands for the microbial binding and subsequently the microorganism captured are eliminating with the mucus. However, this antimicrobial function of mucin-like proteins in sponges can be hypothesised not only for the outer layer, but also for the lumen of the canals. In this sense it is of particular interest the fact that we have found in all the three *Haliclona* species peptides identified as mucin 17-like proteins. In fact, this mucin type in the glycocalyx of the human intestine enterocytes has been supposed to bind bacteria and internalise them via transfer to an intracellular vesicle localisation (Pelaseyed et al., 2014).

Meyer et al. (2006) has also suggested an antifouling role for the mucus coating of *C. nucula* by preventing DOM adhesion on the sponge

exopinacoderm via continuous renewal of the mucus layer. In a study focused on the antifouling defence mechanisms of sponges, Müller et al. (2013) demonstrated that the surface of *S. domuncula* is covered by the mucin 4–like protein previously described by the same research group in this sponge, but the authors hypothesised for this protein a different protection mechanism, i.e. by promoting the formation of a coating made of silica. Even though the mechanism is not known, an antifouling activity of the mucus produced by the target sponge species can be also hypothesised, especially for *H. indistincta*. In fact, the population of this species living in Corranroo bay is exposed to a high level of sedimentation due to the geo-hydrodynamic conditions of this area, thus a mechanism of surface cleaning may be necessary for sponge survival.

H. indistincta has been recently determined to produce 3-alkyl pyridinium alkaloids (3-APs) similar to other two species in the subgenus *Rhizoniera*, i.e. *H. viscosa* and *H. sarai* (Firsova, 2017). In particular, the compounds produced by *H. indistincta* seem to behave like a specific group of compounds produced by *H. sarai*, i.e., the 3-alkyl pyridinium salts, as already described in Chapter 1. These compounds are oligomers that bind head-to-tail to each other producing polymers that in water solution have been observed to form supramolecular aggregates with spherical shape (Sepčič et al., 1997b, Sepčič et al., 1999). Moreover, the 3-AP compounds in *H. sarai* are cationic polymers and act like surfactants (Sepčič, personal communication), thus if the similar compounds synthesised by *H. indistincta* are proven to be present in the mucus produced by this sponge, they could contribute to the surface cleaning role attributed to the mucus or even comprise the mucus itself.

All the mucins or mucin-like proteins identified in our study show a low similarity to the proteins in GenBank, in most cases lower than 50%. Lang et al. (2004) stated that methods like BLAST that are based on sequence similarity are not reliable to identify mucin domains due to the poor conservation of their amino acid sequences, therefore additional analyses will be necessary to determine how actually comparable are the mucin-like proteins we found in the three *Haliclona* species with the true

mucins in terms of properties and roles played. However, the method applied can still be considered an efficient way to explore the presence of mucins in organisms for which nothing is known about this matter.

From a methodological point of view, the method we applied seems to be efficient for an initial screening of the mucin/mucin-like proteins even in sponge species from which is not possible to 'milk' the mucus. Unfortunately we had no opportunity to obtain a transcriptome from *H. mucosa*, thus the very low number of protein hits obtained for this species could be explained by the fact that we used for comparison the database of translated sequences from *H. cinerea* and *H. indistincta*, which are not closely related (Longakit et al., unpublished data). However, it cannot be excluded either that the mucus from *H. mucosa* could have been partially lost and/or excessively diluted during collection and some of the mucin components lost, therefore processing the whole sponge tissue would be recommended when possible. Similarly, the higher number of mucin-like proteins found in *H. cinerea* compared to *H. indistincta* is probably due to the larger transcriptome available for that species (100 Mb for *H. cinerea* versus 10 Mb for *H. indistincta*). For both *H. indistincta* and *H. cinerea* we obtained similar results in terms of mucin-like protein diversity between cells and supernatant, and this disproves the original assumption that secreted mucins could be present in the supernatant and not in the cells. The presence of the same proteins in the cells and the supernatant could be explained considering either that the centrifugation speed during the process of cells separation is not enough to restrain all the cells, but also that a certain percentage of cells do not maintain their integrity during the process and release their content in the medium. However, for both *H. indistincta* and *H. cinerea* we did find a mucin-like protein in the supernatant that was not detected in the dissociated cell fractions, therefore it is advisable to process both fractions if possible. The opportunity to repeat the same procedure with sponges belonging to different taxonomical groups and characterised by different body

structures would be interesting in order to assess the efficiency/consistency of the methods applied here.

Concluding remarks

The work described in this chapter represents the first attempt to verify the presence of mucins in Porifera applying a proteomic approach. I analysed three *Haliclona* species producing mucus with different physical properties and I have found in all of them several different types of mucin-like proteins. Unfortunately, the lack of a transcriptome for *H. mucosa* has limited the data obtained from this sponge species, but the retrieval of two mucin–like proteins from its mucus after matching the mass spectrometry data with the transcriptome of *H. cinerea*, and the fact that one of these mucin-like proteins has the same identity as one of the mucin-like proteins isolated from *H. indistincta*, highlights the possible presence of common traits in the evolution of these glycoproteins in the *Haliclona* species studied. Further studies aimed at isolating these mucin-like proteins and localising them in the sponge body would contribute to determine their actual similarity to mucins in higher organisms as well as their gel-forming or cell membrane-bounded nature, which could help clarifying the role these proteins play in sponge biology.

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CHAPTER 6

General discussion

The general aim driving the studies carried out in this project was to investigate factors potentially involved in the production of the bioactive compounds detected in a number of marine sponges currently placed in the genus *Haliclona* Grant, 1841, which is well known for the exceptional variety of natural products synthesised (Tribalat et al., 2016).

The first objective pursued was to investigate the microbial associations of the target species, specifically bacteria, archaea and fungi. In fact, these microorganisms have been shown to be the actual responsible for the production of natural products of interest isolated from different sponge species (Faulkner et al., 1994, Piel, 2006, Simmons et al., 2008). The integrated next generation sequencing (NGS) – transmission electron microscopy (TEM) study of the bacterial and archaeal communities of the five Irish *Haliclona* species considered in this study (i.e. *H. oculata*, *H. cinerea*, *H. simulans*, *H. viscosa* and *H. indistincta*) established that these sponges are all low microbial abundance (LMA; Chapter 2). I considered that the discrepancies noticed between the large bacterial/archaeal diversity highlighted in most of the species analysed by the NGS study and the few microbial morphotypes observed in the tissues sampled via TEM can be explained by factors other than symbiosis, e.g. bacteria used as food by the sponges and/or environmental contamination from the substratum, or possibly by the fact that it is difficult to spot low abundance bacteria in TEM sections of the sponge mesohyl. However, an environmental origin of the bacteria and archaeal sequences detected in LMA sponges was already suggested by Bayer et al. (2014) who also showed via real-time PCR that the numbers of microbial sequences obtained from LMA sponge species are three or more orders of magnitude lower than in high microbial abundance (HMA) sponges. Therefore, I assume that our TEM analysis depicts the abundance of bacteria in the sponges studied realistically.

Even though the relationship between the microbial abundance in sponges and the production of bioactive compounds has not been extensively studied, exception being Hochmuth et al. (2010) and Sacristán-Soriano et al. (2011), I do not expect that the scarce bacteria

observed in the target sponge species could be responsible for the production of their bioactive compounds. However, it will be worthwhile to carry on further studies to prove the accuracy of this conclusion in cases as e.g. *H. cinerea*. In fact, this species has been shown to produce different fatty acids than is usually observed in sponges, especially because these molecules miss branched-chain parts (Joh et al., 1997). While mid-chain-branched fatty acids are common in sponges and their production is attributed to bacterial symbionts (Gillan et al., 1988, Hochmuth et al., 2010), currently I do not have enough information to determine whether the long rod-shape bacterium found within intracellular vacuoles in this sponge and apparently horizontally transmitted (as suggested by its absence in the embryos, Chapter 2) is involved in the production of the fatty acids identified in *H. cinerea*. The NGS study was not sufficiently informative to identify the rod-shaped bacterium, while this would be a crucial step in order to explore the roles it plays in the biology of this sponge species. A similar consideration can be done for *H. simulans*. In fact, this species is known to produce steroids (Viegelmann et al., 2014) which are considered to be typical eukaryotic lipids, but a recent bioinformatic study has revealed that several different bacterial taxa present sterol synthesis genes in their genomes (Wei et al., 2016), therefore an involvement of the bacteria in the bioactivity of this sponge species cannot be excluded based on the available data. The isolation of the symbiotic bacteria from *H. cinerea* and *H. simulans* via e.g. flow cytometry methods and the application of a single cell genomics approach to characterise them would be probably the best way to shed light on the role that these bacteria play in the host sponges and perhaps on their involvement in the production of the bioactive compounds.

As described in Chapter 1, *H. sarai*, *H. viscosa* and *H. indistincta* produce similar compounds, i. e. 3-alkyl pyridinium alkaloids (3–APs). However, the TEM analysis has highlighted totally different bacterial associations for the three species, confirmed for *H. viscosa* and *H. indistincta* by the NGS study. In fact, while the latter species have been shown to be clear

examples of LMA sponges, *H. sarai* represents an intermediate between LMA and HMA sponges because the mesohyl of this species hosts several different bacterial morphotypes that occupy almost all the intercellular space (Chapter 2). The production of the 3–APs in *H. sarai* has already been suggested to involve a contribution of the symbiotic bacteria as source of the precursor nicotinic acid produced from tryptophane and/or aspartate (Tribalat, 2016), therefore the bacteria observed in *H. viscosa* and at least some of the bacteria found in *H. sarai* could play this role in the host sponges. In *H. indistincta*, which does not show associated bacteria either intracellularly or in the mesohyl, the 3–APs precursor molecules of bacterial origin could derive from the ingested bacteria observed in high numbers in the digestive phagosomes.

The investigation of possible fungal associates which could be responsible for the production of the bioactive compounds produced by the *Haliclona* species studied was incentivised by the presence of compartmentalised intracellular vacuoles shown via TEM, especially in the cells of *H. indistincta* and *H. oculata* (Chapter 3). In spite of a cultivation method carefully selected to promote the isolation of specific putative fungal symbionts, from most of the target sponges (including for this survey also the Mediterranean species *H. mediterranea*, *H. fulva*, *H. mucosa* and *H. sarai*) I did not obtain a consistent pattern of fungal biodiversity which I would have expected in case of symbiotic associations (Chapter 3). Therefore, it cannot be excluded that the observed intracellular vacuoles were actually very complex secondary lysosomes as suggested by Prof. Peter Dockery (Anatomy Department, NUI Galway) rather than fungal spores protected by the sponges in the intracellular environment, the latter interpretation endorsed by Dr. Maria Tuohy and Dr. Vijai Gupta (Biochemistry Department, NUI Galway). As already explained in Chapter 3, only through new attempts to isolate the fungal associates from the target *Haliclona* species applying different isolation and cultivation techniques could confirm whether host species-specific fungal communities, which could be implicated in the production of the bioactive compounds detected in these sponges,

actually exist. In any case, my work showed how sponges represent an important target of marine biodiscovery not only for the bioactive compounds produced by themselves or the bacterial communities they host, but also because, acting like a sieve that filters fungal spores from the surrounding seawater, they constitute a rich substratum for the isolation of diverse fungi characterised by chemical properties still only partially explored (Proksch et al., 2010).

The TEM analysis of the *Haliclona* species studied highlighted in some cases the presence of cells with inclusions. As already reported (Introduction and Chapter 4), cells with inclusions have been indicated as the production and/or storage site of bioactive compounds in several distantly related sponge species. In particular I have observed cells with inclusions in the three *Rhizoniera* species (i.e. *H. indistincta*, *H. viscosa* and *H. sarai*; Chapter 4), *H. cinerea* (Chapter 5) and *H. simulans* (not described in the thesis). The abundance and the novel aspect of the cells with inclusions observed in *H. indistincta*, *H. viscosa* and *H. sarai* motivated the decision of selecting *H. indistincta* (the most available of the three) as the first species to perform experiments of cell separation and fractionation aimed at isolating the target cells with inclusions and characterising them from an ultrastructure and chemical point of view, mainly in order to assess the presence of the 3–APs produced by this sponge within the cells with inclusions. The first approach followed was to fractionate the dissociated sponge cells based on their size through the density centrifugation medium Ficoll. In spite of the multiple attempts performed, only in one occasion I was able to separate cell fractions, while in most of the other cases it was not possible to obtain a distinctive separation of cell layers, possibly due to the excess of mucus characterising this sponge species.

The TEM analysis of the cell fractions obtained showed that one of them was mainly made of released inclusions. Therefore, I adopted a different approach and I used flow cytometry to try to isolate the target cells with inclusions. A first analysis of the cell fractions obtained via Ficoll density centrifugation highlighted that the fraction rich in the inclusions emitted

red autofluorescence, which could be associated with the presence of a pink/red pigment, as suggested by Dr Shirley Hanley (Flow Cytometry unit, NUI Galway). The pink pigment could be contained within the inclusions, as already considered by Stephens (2013) who showed via TEM how the larvae of *H. indistincta*, which are pink in colour, contain a large number of the target inclusions. Because of this hypothesis, as well as the knowledge that the bioactive compound dercitamide isolated from *Oceanapia sagittaria* – which is also an alkaloid as the compounds produced by *H. indistincta* – fluoresces at different excitation wavelenghts (Salomon et al., 2001), I attempted to isolate the target cells with inclusions via fluorescent activated cell sorting (FACS). However, also this second approach did not prove to be conclusive to isolate the target cells. In fact, the cell population isolated by FACS was not big enough to be analysed via TEM (the size of the cell pellet is important because a large part of cells are lost during each step of dehydration/embedding), thus I carried out a confocal microscopy analysis of the same cells assisted by Dr Peter Owens (Centre for Microscopy and Imaging, NUI Galway) and I observed that in most cases the fluorescence was concentrated within vacuoles of cells more resembling amebocytes. This fluorescence pattern is consistent with the possible presence within the vacuoles of purple bacteria (Madigan and Jung, 2009) or even red algae chloroplasts highlighted by the NGS study of *H. indistincta* described in Chapter 2, and could mask the autofluorescence of a putative pink pigment stored within the sponge inclusions, making this approach not feasible to isolate the target cells with inclusions.

In order to define a new marker for isolating the cells with inclusions in *H. indistincta*, I decided to pursue the idea introduced in Chapter 4 that the target cells could produce both the bioactive compounds and the mucus characteristic of this sponge. The production of mucosubstances by toxin-producing cells has been observed already in other animals as e.g. sea urchins (O'Connell et al., 1974) and scorpions (Soliman et al., 2013). Even though I was aware of the existence of a fluorescent dye for

localising mucins, i.e. the acridine orange (Shumilov et al., 2014), I followed the suggestion of Prof. David J. Thornton (Wellcome Trust Centre for Cell-Matrix Research, University of Manchester) to apply a proteomic approach to identify the putative mucins present in *H. indistincta*. This in the view of obtaining a more precise marker to localise the target cells with inclusions. A dedicated study of the mucins in Porifera has not been carried out previously, therefore I decided in agreement with my supervisor to include in the survey also *H. cinerea* and *H. mucosa* which produce mucus with different characteristics, as described in Chapter 5. Even though I did not have chance to study the microbial associations of *H. mucosa* via neither TEM nor NGS, this species was reported by Vacelet and Donadey (1977) to host only few rod shaped bacteria between the collagen fibres of the mesohyl, similarly to what I have observed in *H. viscosa*. The study of the mucus in sponges was also consistent with the main aim of this project since the mucus can represent a carrier for the biologically active compounds released by the sponges, as observed with some *Syphonodictyon* species (Sullivan et al., 1983). Actually, the production of mucus has been suggested as indicator of the presence of bioactive molecules in sponges by Muricy et al. (1993) who determined that the mucus-producing species *Polymastia janeirensis*, *Amphimedon viridis*, cf. *Arenosclera* sp. and *Tedania* sp. showed antibacterial or antifungal activity.

Based on the considerations made in Chapter 5, I believe that the antibacterial and/or antifungal function of mucus in sponges is not limited to carrying the bioactive compounds on the sponge surface and surroundings. In fact, the mucus in my opinion plays an important role also in determining the composition of the microbiome hosted within the sponges acting against non-symbiotic bacteria like a selective barrier, as observed e.g. in the gastrointestinal (GI) tract of mammals. Here the mucus first of all entraps the antibacterial compounds produced by the epithelial cells and prevents them from being quickly diluted into the lumen (Birchenough et al., 2015). However, this does not seem to be the case in sponges like *H. indistincta* for which the extracts produced were

proved not to have significant antibacterial activity (Khan, 2015). Moreover, as already seen, mucins in the GI tract also offer multiple binding sites for the adhesion of both commensal and pathogenic microbes via their carbohydrate ligands. The interactions between mucins and microbes can vary largely depending on the type and localisation of the mucins as well as the extent of their O-glycosylation (Thornton and Sheehan, 2004, Derrien et al., 2010). Therefore, mucins play an important role as targets for bacterial adhesion, while the epithelium of the intestinal mucosa can distinguish commensal microbiota from pathogenic microorganisms through cell surface and cytoplasmic pattern recognition receptors (Magalhaes et al., 2007, Kim and Khan, 2013).

It is known that sponges can discriminate between food and symbiotic bacteria (Wilkinson et al., 1984, Wehrl et al., 2007), but the mechanism of this phenomenon has not been determined yet. A key role is considered to be played by bacterial proteins characterised by repeat domains characteristically found in eukaryotes and involved in protein-protein interactions, therefore called eukaryotic-like proteins (Reynolds and Thomas, 2016, Díez-Vives et al., 2017). Müller et al. (1981) proposed that the relationship found between the sponge *Halichondria panicea* and the predominant bacterial species in this sponge, for which Knobloch et al. (2019) have proposed the candidate status '*Candidatus* Halichondribacter symbioticus', is mediated by a lectin. Subsequently, additional lectins were described from both Demosponges and Hexactinellida and supposed to be implicated in both microbial symbioses and defence against pathogens (Garderes et al., 2015). Although the results of my research on the presence of mucins in the target *Haliclona* species are still preliminary, the observation that all the sponges considered have proteins similar to mucins isolated in the digestive tract of mammals (Chapter 5) suggests that these proteins might also mediate the interactions between sponges and their bacterial associates. In this sense, I consider that putative dissimilarities in terms of mucin-like protein composition and/or glycosylation patterns in the

three species *H. indistincta*, *H. viscosa* and *H. sarai*, that all produce a similar gel-like substance as mentioned in Chapter 4, could explain their extremely different microbiomes observed via TEM. The opportunity to apply the same proteomic approach on *H. viscosa* and *H. sarai*, as well as an NGS analysis aimed at obtaining 16S rRNA gene data from *H. sarai* would give useful information for a comparison (even though it has to be taken in account that the geographical origin of *H. sarai* can also play a role in shaping the symbiotic microbial community; Zaballos et al., 2006). Another aspect to consider regarding mucins in the human gastrointestinal tract is that they also act as a nutrient source (represented by the carbohydrate ligands) and a matrix for bacteria proliferation (Derrien et al., 2010, Pelaseyed et al., 2014). I believe that both these functions can be likely also for the mucin-like proteins detected in the target sponge species. The hypothesis that these glycoproteins can be part of the sponge extracellular matrix and/or contribute to the formation of the spongin fibres has been already proposed for other sponge species (e.g. Donadey and Vacelet, 1977, Pomponi, 1976). In this sense, the original interpretation of the cells with inclusions in *H. cinerea* as responsible for the production of different skeletal fibres than collagen and spongin made by Lévi (1967) can be considered consistent with my hypothesis that these cells are the putative site of production of the mucin-like proteins in this species. Finally, still considering the possibility that the host–microbial symbiont interactions in sponges can be mediated by the oligosaccharides O-linked to the mucin-like proteins, the presence of these glycoproteins in association with the spongin could explain the close relationship observed between bacteria and collagen fibres in sponges as *H. simulans*, *H. viscosa* and *H. mucosa*.

The preliminary data I produced about the mucin-like proteins in the target *Haliclona* species represent an important starting point to further characterise structure, distribution in the sponge body and function of these glycoproteins via molecular, histochemical and ultrastructural techniques in order to accomplish the original objective to locate a

marker for the isolation of the putative mucous cells in *H. indistincta*, *H. viscosa* and *H. sarai*. However, a similar development could also provide useful tools to verify the involvement of these glycoproteins in the host-microbe interactions observed in all the species studied, even in species as *H. oculata* and *H. simulans* that apparently do not produce gel forming glycoproteins, but could still present cell membrane mucin-like proteins.

Concluding remarks

Even though I have not accomplished the ultimate aim of proving the producers of the bioactive compounds isolated from the target *Haliclona* species, I expect for them to be the cells with inclusions in species as *H. sarai*, *H. viscosa* and *H. indistincta*. Furthermore, the information collected about the microbial associations and the presence of cell types of interest in the species studied will constitute the basis for multiple future studies more focused on each of the components I have explored during my research project, especially the duplex role as producers of mucus and bioactive compounds of the cells with inclusions in the three *Haliclona* species from clade C and the role of the bacteria observed in *H. cinerea* and *H. simulans*.

Moreover, as anticipated in Chapter 1, the data I produced about bacterial symbionts, cells with inclusions and mucin-like proteins will feed into the revision of the classification of the genus *Haliclona* in the integrative taxonomic effort currently carried on at the Molecular Evolution and Systematics Laboratory (Zoology Department, NUI Galway) as well as significantly contributing to a greater understaning of the biology of these species.

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PUBLICATIONS

Does the Chemical Diversity of the Order Haplosclerida (Phylum Porifera: Class Demospongia) Fit with Current Taxonomic Classification?

Authors Marie-Aude Tribalat[1], Maria V. Marra[2], Grace P. McCormack[2], Olivier P. Thomas[1,3]

Affiliations
[1] Géoazur UMR Université Nice Sophia Antipolis-CNRS-IRD-OCA, Valbonne, France
[2] National University of Ireland Galway, Zoology, School of Natural Sciences, University Road, Galway, Ireland
[3] National University of Ireland Galway, Marine Biodiscovery, School of Chemistry, University Road, Galway, Ireland

Key words
- sponges
- Haplosclerida
- alkaloids
- polyacetylenic
- chemotaxonomy

Abstract

Sponges and their associated microbiota are well known to produce a large diversity of natural products, also called specialized metabolites. In addition to their potential use in the pharmaceutical industry, these rather species-specific compounds may help in the classification of some particular sponge groups. We review herein compounds isolated from haplosclerid sponges (Class Demospongia, Order Haplosclerida) in order to help in the revision of this large group of marine invertebrates. We focus only on 3-alkylpyridine derivatives and polyacetylenic compounds, as these two groups of natural products are characteristic of haplosclerid species and are highly diverse. A close collaboration between chemists and biologists is required in order to fully apply chemotaxonomical approaches, and whenever possible biological data should include morphological and molecular data and some insight into their microbial abundance.

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Correspondence
Prof. Grace P. McCormack
Ryan Institute
School of Natural Sciences
National University of Ireland
University Road
Galway
Ireland
Phone: + 353 91 49 23 21
Fax: + 353 91 49 45 35
grace.mccormack@nuigalway.ie

Correspondence
Prof. Olivier P. Thomas
School of Chemistry
National University of Ireland
University Road
Galway
Ireland
Phone: + 353 91 49 35 63
Fax: + 353 91 49 55 76
olivier.thomas@nuigalway.ie

Introduction

Sponges (Phylum Porifera) are sessile invertebrates distributed in most aquatic ecosystems. In marine areas like the Caribbean Sea, they may represent the largest substrate cover and/or the largest organic biomass of living organisms, thus contributing significantly to several nutrient cycles due to their outstanding filtering capabilities [1,2]. In addition, during their long evolutionary history, most, if not all, of the diverse species of this group (> 8000 described species to date) together with their associated microbiota have developed unique metabolic pathways leading to a huge diversity of natural products, also called specialized metabolites [3]. Taxonomic classification in this particular group of marine invertebrates is still highly challenging due to a paucity of morphological characters and a discrepancy between molecular and morpological data in many cases. For this reason, biochemical information has been recently used as a complementary tool (particularly within the framework of targeted or untargeted metabolomic approaches), leading to the recent concept of integrative systematics [4].

Within the Class Demospongiae, sponges belonging to the Order Haplosclerida are considered among the most prolific sources of bioactive marine natural products, including alkaloids, polyacetylenes, or terpene derivatives. This group is also one of the most diverse of the sponge groups in terms of numbers of species and habitats, and its members also have few distinguishing morphologial characteristics. The current classification of the order as outlined in Systema Porifera, based primarily on morphology, is comprised of three suborders [two marine (Haplosclerina and Petrosina) and one freshwater (Spongillina)]. The marine suborders together comprise six families; Callyspongiidae, Chalinidae, Niphatidae, Petrosiidae, Phloeodictyidae, and Calcifibrospongiidae [5]. Analysis of sterol chemistry had indicated possible difficulties with their classification [6], suggesting patterns of relatedness that did not agree with morphological data, while in contrast, a review of 3-alkylpiperidine alkaloids appeared to agree with the current classification [7]. Subsequent molecular phylogenetic studies reveal an evolutionary history that is not completely compatible with Systema Porifera, indicating that the freshwater sponges belong elsewhere in the Demospongiae, and that while the marine Haplosclerida do form a clade, the suborders and the families (where there is enough data) are polyphyletic [8]. For this reason, a reassessment of the Order Haplosclerida is underway by multiple research groups, as an assessment of chemical

diversity may help in the development of a robust integrative classification of the group and, in return, current systematic studies in this group may also direct the search for related compounds of interest.

This review will focus on two major classes of specialized metabolites found in the Order Haplosclerida (3-alkylpyridine derivatives and polyacetylene derivatives) and will discuss them in view of the currently accepted classification for five of the six families described in Systema Porifera [5], with no compound being reported so far from sponges of the sixth family Calcifibrospongiidae [9]. Sterols and fatty acids have been reported for some members of this group in 1994 [6], and the large chemical diversity produced by this prolific sponge group was last collated in 1996 for 3-alkylpiperidine derivatives [7]. This review is not aimed to be exhaustive but discusses representatives of 3-alkylpyridine derivatives and polyacetylene derivatives. Given that they are both widely distributed amongst haplosclerid species, are largely restricted to this group, and are rather unique in the field of natural products, we consider that focusing our review on these two chemical families provides ample information to help identify discrepancies in the classification, areas for focus in the construction of a revised integrative classification and valuable avenues for future research focus.

Major issues with publishing descriptions of compounds from species that have not been fully identified will become apparent. This issue is relevant not only for the group of marine sponges included here, but for any organism of interest for bioprospecting. Accompanying morphological identification with a DNA sequence is a highly recommended way forward (in addition to deposition of a specimen voucher in a respected institute/museum). We intend this review to encourage better connections between chemists and biologists in an effort to identify novel compounds of interest while also striving to understand the origins and evolution of the same. A solid taxonomic description and classification will help in the rational collection of closely related species that will produce a large diversity of analogues of a targeted natural product family. Greater collaborative efforts will therefore increase avenues for identification of bioactive compounds, increase the likelihood of finding exploitable sources and perhaps even a biotechnological route for production. Knowledge of the complex comparative chemistry will assist in unravelling the controversial systematics specifically within the Order Haplosclerida, but will also lead to fruitful considerations in terms of chemotaxonomy within the phylum itself (Porifera, the sponges).

Distribution of Simple 3-Alkylpyridine Derivatives in the Haplosclerida

Several 3-alkylpyridine derivatives have been isolated from the Haplosclerida, including in some cases the coupling of one or more similar moieties. This group of compounds is differentially distributed across Niphatidae, Callyspongiidae, Chalinidae, and Petrosiidae, but they are not yet reported from confirmed members of the Phloeodictyidae. The presence/absence of these classes of compounds in haplosclerid sponges are summarized in **Table 1**.

Family Niphatidae

Within the family Niphatidae, an outstanding diversity of alkaloids has been isolated from members of the genus *Amphimedon*. Monomers of 3-alkylpyridines named hachijodines E–G as well as glycosylated monomers named amphimedosides A–E were isolated from *Amphimedon* sp. collected off Hachijo-Jima Island (Japan; **Fig. 1**) [10,11]. Additional mono- and dimers, pyrinodemins B–I, were reported from *Amphimedon* sp. found in the same area (Nakijin, Okinawa) along with a high number of dimers bearing a hydroxylamine moiety, starting with the *bis*-3-alkylpyridine derivative pyrinodemin A and nakinadines A–F (with a *β*-aminoacid) [12–16]. All of the compounds mentioned were found to exhibit cytotoxicity. *Niphates* sp. yielded some mono-3-alkylpyridine derivatives named niphatynes A–B, again from the Northwestern Pacific (Fiji; **Fig. 2**) [17], and Kobayashi's group more recently described some very close analogues named niphatesines A–H from a Japanese sponge of the same genus (**Fig. 2**) [18,19]. Pyrinadines A–G have been isolated from *Cribrochalina* sp., also from the Japanese coast (**Fig. 2**) [20,21]. It is not known if the two *Amphimedons* or *Niphates* sp. mentioned here are the same species or two separate species in each case given that the specimens were collected in the same geographical area. Comparison of the specimen vouchers from the various studies will have to be performed to confirm if this is the case and preferably also comparison of DNA sequences.

It would appear that simple mono- and bis-3-alkylpyridine derivatives are restricted to species belonging to the genera *Amphimedon, Cribrochalina*, and *Niphates*, all belonging to the family Niphatidae (**Table 1**). While molecular data confirms that certain *Niphates* and *Amphimedon* species are related to each other, the position of *Cribrochalina* and *Pachychalina* in relation to other members of the Niphatidae family remains unresolved [22]. As more focus is placed on resolving haplosclerid taxonomy, and compounds are isolated from more fully described species, it will be interesting to confirm if this pattern remains.

Also, within the family Niphatidae, a dimeric cyclostellettamine (3 alkylpyridiniums) was isolated from the Northwestern Pacific sponge *Amphimedon compressa* [23]. Complex mixtures of polymeric halitoxins were isolated from several species present in the Northern Caribbean (*Haliclona. rubens, H. viridis* and *H. erina)* that later were classified as *A. compressa, Amphimedon viridis*, and *Amphimedon erina* (**Fig. 3**) [24]. Later, analogous and bioactive polymers named amphitoxins were isolated as major constituents of *A. compressa* from the Caribbean [25,26]. Some trimeric 3-alkylpyridinium salts named niphatoxins A–B were isolated from *Niphates* sp. collected in the Red Sea by Talpir et al. (**Fig. 3**) [27]. No DNA sequence has been generated yet for *A. erina*, and while molecular data does not cluster *A. compressa* and *A. viridis* directly together, they are both placed in the same subgroup (Clade C), and are thus related to each other and with *Niphates* species [8,22]. The chemical content of the Southwestern Atlantic (Brazil) sponge *Pachychalina alcaloidifera* also includes the presence of antimicrobial and antimycobacterial cyclostellettamines A–K (**Fig. 3**) [28,29]. In addition, C-C and C–N bis- and tris-3-alkylpyridinium derivatives named pachychalines A–C were isolated from *Pachychalina* sp. collected in the Northwestern Atlantic (Caribbean; **Fig. 3**) [30]. Relationships of these *Pachychalina* species to each other and to other Haplosclerida have not yet been resolved using morphological and molecular data.

Table 1 Distribution of 3-alkylpyridine derivatives and polyacetylenic compounds within Haplosclerida sponges.

	3-Alkyl-pyridine	3-Alkyl-pyridiniums	Simple C4–C5′ bis-3-alkylpiperidine	Complex C4–C5′ bis-3-alkylpiperidine	Manzamines	Polyacetylenes
Callyspongiidae						
Arenosclera brasiliensis			X			
Callyspongia fistularis						X
Callyspongia pseudoreticulata						X
Callyspongia ridleyi		X				
Callyspongia siphonella						X
Callyspongia truncata						X
Callyspongia sp.		X				X
Siphonochalina sp.						X
Chalinidae						
Haliclona densaspicula				X		
Haliclona fulva						X
Haliclona osiris						X
Haliclona sarai				X		
Haliclona viscosa		X	X			
Haliclona (Haliclona)						X
Haliclona sp.	X	X	X		X	X
Haliclona (Reniera)		X		X		
Niphatidae						
Amphimedon sp.	X	X	X		X	
Cribrochalina dura						X
Cribrochalina vasculum						X
Cribrochalina sp.	X					
Niphates lunisimisis						X
Niphates sp.	X	X				X
Pachychalina alcaloidifera		X	X	X		
Pachychalina sp.		X				
Petrosiidae						
Acanthostrongylophora ingens			X		X	
Acanthostrongylophora sp.					X	
Neopetrosia exigua			X			
Neopetrosia proxima			X			
Neopetrosia seriata			X			
Petrosia corticata						X
Petrosia ficiformis						X
Petrosia solida						X
Petrosia strongylata						X
Petrosia volcano						X
Petrosia (Strongylophora)						X
Petrosia sp.						X
Xestospongia muta						X
Xestospongia testudinaria						X
Xestospongia sp.					X	X
Phloeodictyidae						
Oceanapia triangulata						X
Oceanapia sp.						X

Family Callyspongiidae

Alkylation at the nucleophilic nitrogen N-1 of the pyridine leading to 3-alkylpyridinium salts is observed in some species of this family. A tris-3-alkylpyridine derivative called niphatoxin C was found in *Callyspongia* sp. from the Western Pacific (Northwestern Australia), while Buchanan et al. showed the presence of 3-alkylpyridinium polymeric salts from *Callyspongia (Toxochalina) ridleyi* also collected in the Western Pacific (Papua New Guinea; **Fig. 4**) [31]. These compounds with MW between 5 and 6 kDa cause an irreversible membrane potential depolarization.

In some particular cases, 3-alkylpyridiniums are functionalized through reduction of the aromatic ring leading to highly reactive iminium/enamines intermediates that most commonly lead to the coupling of two units through a C-4/C-5′ bond. This function to connect the two chemical moieties is highly specific and the capability is present only in Haplosclerida and within this group in only a small number of species. The presence of this enzymatic capacity is likely to have phylogenetic significance and thus represents a link between chemistry and taxonomy. Within the family Callyspongiidae, Torres et al. [32] described the arenosclerins A, B, and C but also haliclonacyclamine E, macrocylic bis-3-alkylpiperidine derivatives from the Southwestern Pacific sponge *Arenosclera brasiliensis* (**Fig. 5**) [32]. Inversion of configurations at several asymmetric centers is intriguing and suggests a non-specificity of the enzymes involved. Later, the same authors reported some antibacterial activities of arenosclerins but also some cytotoxic activities against tumoral cell lines [33]. *Arenosclera* as a member of the family Callyspongidae is a genus well

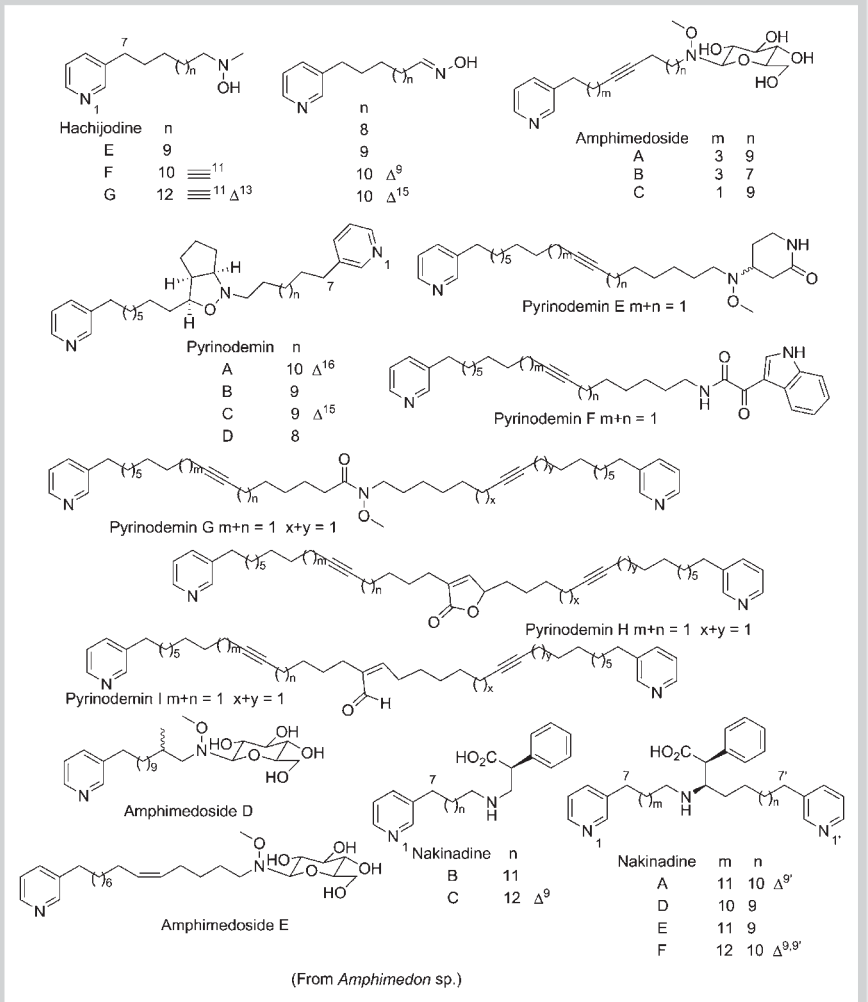

Fig. 1 3-Alkylpyridine derivatives isolated from the species *Amphimedon* sp. (Niphatidae)

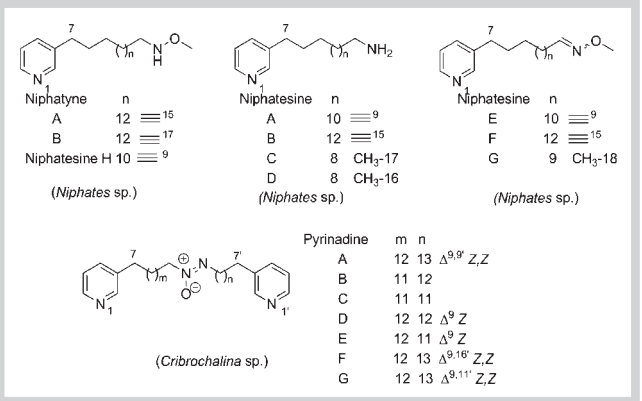

Fig. 2 3-Alkylpyridine derivatives isolated from the species *Cribrochalina* sp. and *Niphates* sp. (Niphatidae).

separated from *Haliclona* (Chalinidae) where bis-3-alkylpiperidines are more commonly found. However, recent molecular studies suggest that members of the Callyspongidae family are closely related to the type species of *Haliclona* including the only *Arenosclera* species so far sequenced (*Arenosclera heroni*) [8, 22, 34].

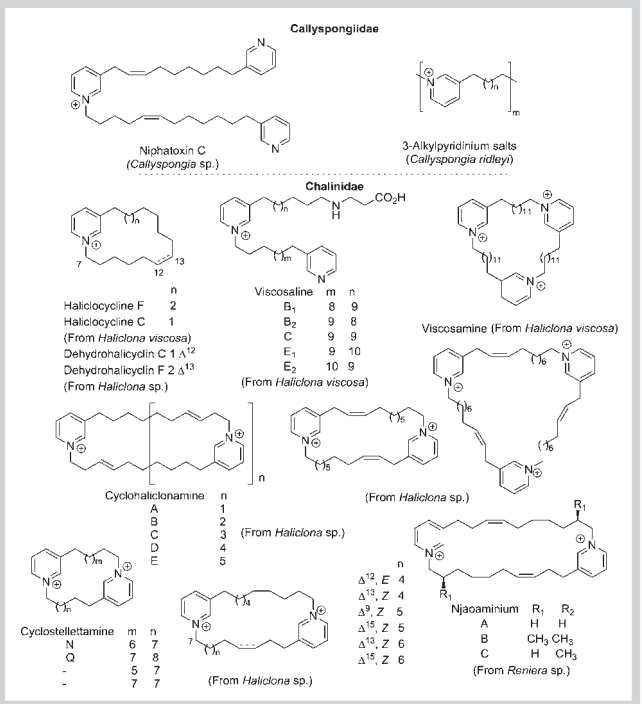

Fig. 3 3-Alkylpyridinium salts isolated from Niphatidae sponges.

Family Chalinidae

Within this family, the genus *Haliclona* is the most represented and diverse, and it has led to an outstanding diversity of natural products, most of them being alkaloids. It is worth underlining, however, that the classification of *Haliclona* urgently needs revisiting. Molecular data indicates that many very distantly related species have been placed in this genus and will eventually be placed in other families of the Haplosclerida [8]. An impressive diversity of 3-alkylpyridinium natural products has been isolated from the Arctic (Svalbard, Norway) sponge *Haliclona (Rhizoniera) viscosa*, including monomers like haliclocycline C and F [35,36], dimers like the viscosalines [37], and trimers like viscosamine [38] (**Fig. 3**). It will be very interesting to determine if related compounds exist in the closely related species *H. indistincta* (currently being investigated by the authors).

Many additional compounds have been isolated from unidentified *Haliclona* species (listed all together as *Haliclona* sp. in **Table 1**). The compounds so far isolated include cyclohaliclonamines A–E, which are cyclic and polymeric 3-alkylpyridinium salts isolated from a Okinawan species [39]; dimeric and trimeric unsaturated cyclized 3-alkylpyridiniums from a Guatemalan species [40]; a large diversity of bis-3-alkylpyridiniums of the cyclostellettamine type from a Korean species [41]; cyclic monomers called dehydrohalicyclins C and F from a Southeastern Pacific (New Zealand) *Haliclona* sp. [42] (**Fig. 4**), and finally methyl-substituted cyclostellettamines called njaoaminiums A–C isolated from *Reniera* sp. (*Reniera* has been reclassified as a subgenus of *Haliclona*) collected in the Western Indian ocean [43] (**Fig. 4**). All of these results strongly illustrate the high propensity of the Haplosclerida (and perhaps a subgroup within it) to produce 3-alkylpyridinium derivatives and therefore to connect at least two 3-alkylpyridines through the nucleophilic nitrogen atom of at least one pyridine.

While the biosynthesis of the huge polyamine family of sponge alkaloids remains unknown due to the lack of experimental data, we can hypothesize a key role of the polyamine pathway in the construction of bis-3-alkylpyridinium intermediates [44]. Indeed some other alkaloids produced by Chalinidae sponges exhibit a key N-C_3-N moiety, e.g., papuamine and haliclonadiamine, epimers isolated from possibly two specimens of the same *Haliclona* sp. from the Western Pacific (Papua New Guinea and Palau; **Fig. 6**) [45,46]. Halitulins are macrocyclic tertiary amines linked to aromatic components. These cytotoxic compounds were identified by Kashman et al. [47] from *Haliclona tulearensis* in the Southwestern Indian Ocean (Madagascar). Later, isomer and macrocyclic amines named haliclorensins were isolated from the same species, suggesting their implication in the biosynthesis of these bioactive alkaloids (**Fig. 6**) [47–50].

The family Chalinidae has led to the largest diversity of bis-3-alkylpiperidine derivatives. Rather simple macrocyclic bis-3-alkylpiperidines named haliclamines A and B, a reduced form of the previous 3-alkylpyridinium cyclostellettamines, were isolated from *Haliclona* sp. growing in the Northwestern Pacific (Japan; **Fig. 5**) [51]. Additional haliclamines C–H were isolated from the Arctic sponge *H. viscosa*, thus linking bis-3-alkylpyridiniums with their reduced forms [52–54]. These simple cyclic dimers have led to more complex products after connection of both piperidine cycles through the C-4/C-5′ carbons. Halicyclamine A is a C-4/C-5′ connected bis-3-alkylpiperidine, isolated from *Haliclona* sp. collected in the Western Pacific (Indonesia; **Fig. 5**) [55]. This compound has mainly displayed antimycobacterial activities [56,57]. Close analogues named haliclonacycl-

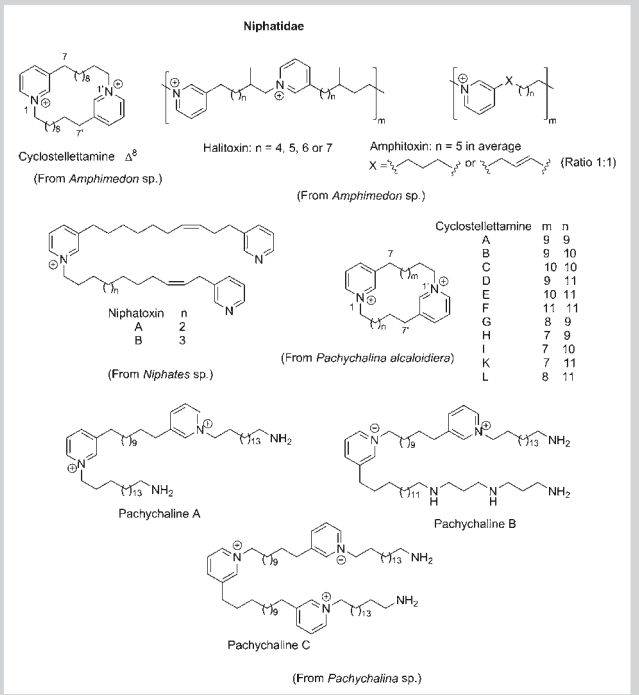

Fig. 4 3-Alkylpyridinium salts isolated from Callyspongiidae and Chalinidae sponges.

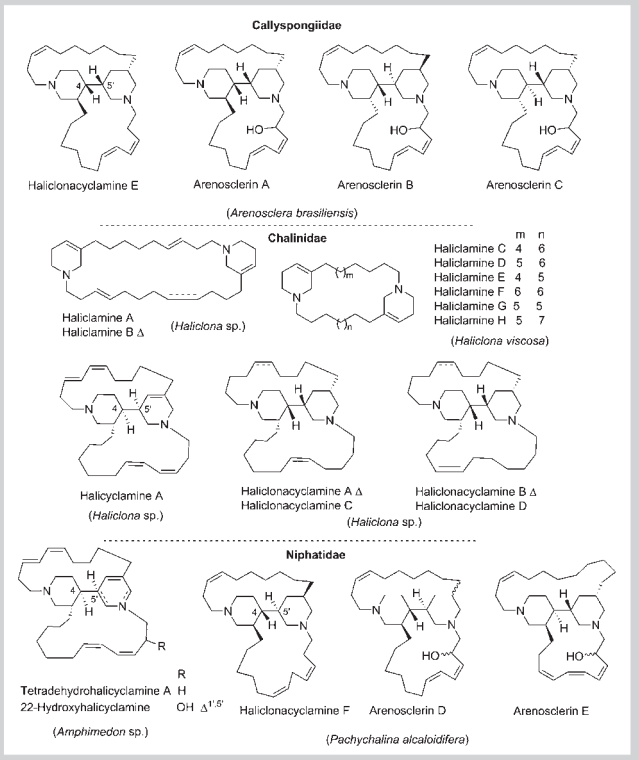

Fig. 5 Simple 3-alkylpiperidines isolated from Callyspongiidae, Chalinidae, and Niphatidae sponges.

amines A–D were isolated from another *Haliclona* sp. in the Western Pacific (Australia; ▶ **Fig. 5**) [58,59]. These compounds were found to be cytotoxic, but also displayed antibacterial and antifungal properties [54].

There are some taxonomic connections between the families Chalinidae and Niphatidae that are currently under investigation, but are relevant to the organization of this review. Very few C-4/C-5′ connected bis-3-alkylpiperidine derivatives have been isolated from the Niphatidae family, but include oxidized analogues of halicyclamines identified in *Amphimedon* sp. from the Northwestern Pacific (Japan; ▶ **Fig. 5**) [60] and some bis-3-alkylpiperidine alkaloids identified from *Pachychalina alcaloidifera*, namely haliclonacyclamine F and arenosclerins D and E (▶ **Fig. 5**) [61]. However, *H. viscosa* mentioned above is more related via molecular data to species of *Niphates* than it is to the type species of *Haliclona* (Stephens, unpublished PhD thesis, National University of Ireland, Galway). Some of the Australian *Haliclona* also cluster with *Niphates* species rather than the type *Haliclona* species, meaning that this group of compounds may end up being more characteristic of Niphatidae than the Chalinidae in time, and make better sense of the distribution of these specific derivatives.

Family Petrosiidae

A large diversity of alkaloids has been described from sponges of the genus *Acanthostrongylophora*, but only a small number of simple bis-3-alkylpiperidines. Garson's group isolated a derivative named acanthocyclamine A from *Acanthostrongylophora ingens* collected from the Western Pacific (Indonesia; ▶ **Fig. 7**) [62]. Nakagawa et al. [63] first isolated Xestospongins A–D from the Northwestern Pacific (Japan) sponge *Xestospongia exigua* that was subsequently renamed *Neopetrosia exigua* (▶ **Fig. 7**). Araguspongines A–H are stereoisomers of these compounds isolated from *Xestospongia* sp. collected in the same area [64]. An additional xestospongin analogue was further isolated from *Xestospongia* sp. collected in the Southwestern Pacific (New Caledonia) [65]. Some macrocyclic bis-3-alkylpiperidine named araguspongines C and D, but also xestospongin D, were also isolated from *N. exigua* collected in the Northeastern Indian Ocean (India; ▶ **Fig. 7**), while Araguspongin M was isolated from this species collected in Palau and a dimethyl analogue of xestospongin C was also isolated from this species from the Northwestern Pacific [66–68]. Wei et al. [69] isolated the C-4/C-5′ connected neopetrosiamine A from a related species from the Caribbean, i.e., *Neopetrosia proxima* (▶ **Fig. 7**). This compound was shown to exhibit cytotoxic bioactivities, and the relatedness of the two *Neopetrosia* species is evident also from molecular data [22]. Later, xestoproxamines were described from the same Caribbean sponge (*N. proxima*) [70]. Petrosins A and B isolated from *Petrosia seriata* collected in the Western Pacific (Indonesia) are unique reports of 3-alkylpiperidine derivatives from this genus (▶ **Fig. 7**) [71,72]. This sponge has been renamed as *Neopetrosia seriata* and two separate pieces of DNA evidence (a short piece of the 28 S ribosomal RNA gene and a region of the mitochondrial cytochrome oxidase 1 gene) place this species with another but unnamed *Petrosia* species [8]. The relationships of both species to other *Petrosia* or *Neopetrosia* or indeed any other Haplosclerida currently remain unresolved.

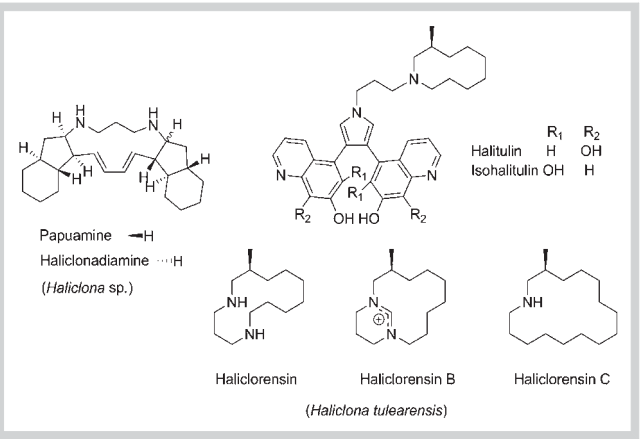

Fig. 6 Polyamines from Chalinidae sponges.

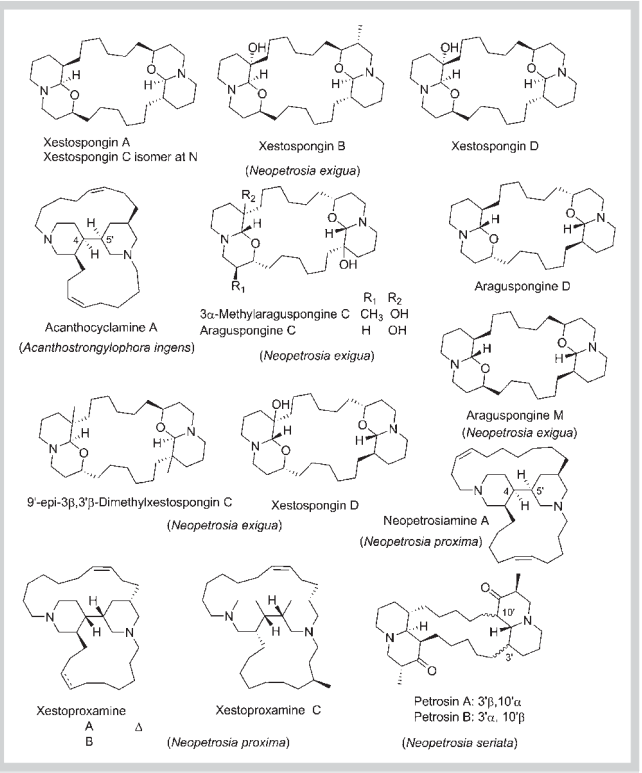

Fig. 7 Simple 3-alkylpiperidines isolated from Petrosiidae sponges.

Distribution of Complex 3-Alkylpiperidine Derivatives in the Haplosclerida

The complexity of 3-alkylpiperidine derivatives culminates with the biosynthesis of outstanding complex molecules, most of them including the C-4/C-5′ but also additional C-C connections and then becoming real cage compounds. Many such compounds are present in members of Chalinidae and Niphatidae families, which, as mentioned above, require reassessment, e.g., some *Haliclona* should be renamed and are more closely related to members of the Niphatidae family. Hence, they are treated here in a separate section. Saraines 1–3 and A–C were isolated from the Northern Mediterranean sponge *Haliclona (Rhizoniera) sarai*

(**Fig. 5**) [73,74]. Both piperidine cycles are also connected through the C-4/C-5′ bond, but in the case of saraines A–C, other connections were established between both cycles. A large array of biological activities have been described for these compounds [75]. Finally a bis-3-alkylpiperidine derivative called haliclonin A was isolated from *Haliclona* sp. in the Northwestern Pacific (Korea) [76]. Just like saraines A–C, this compound exhibits a C-2/C-3′ bond and one of the piperidines has been opened, maybe after oxidation/hydrolysis processes.

An unusual connection with a quinoline aromatic ring on one alkyl chain is characteristic of the njaoamines A–H isolated from the same *Reniera* sp. as the njaoaminiums in the Western Indian Ocean (**Fig. 8**) [77,78]. In these compounds, both piperidine cycles are connected through C-4/C-5′ as before but also through a second C-3/C-2′ connection. Densanins were isolated from *Haliclona densaspicula* in the shallow waters of the Northwestern Pacific (Japan) [79]. They are highly complex pyrrole alkaloids, but their biosynthesis may originate from a common bis-3-alkylpiperidine group through contraction of a piperidine. We still observe the usual C-4/C-5′ connection between both cycles. Complex bis-3-alkylpiperidine alkaloids ingenamine G and madangamine F have been isolated from the Southwestern Atlantic sponge *Pachychalina alcaloidifera,* a species currently placed in the family Niphatidae via morphology (**Fig. 8**) [28,61,80]. Preliminary molecular data suggest that *H. sarai* is actually more closely related to species of *Niphates* than to the *Haliclona* species (*H. oculata*). Much more work relating to morphology, DNA, and chemical evidence is again needed to understand the origin and development of such compounds. Despite the complexity of the chemistry in *P. alcaloidifera*, the authors were not able to isolate more advanced derivatives belonging to the manzamine alkaloids from this sponge.

In some cases, the opening of one piperidine ring can lead to condensation of aromatic rings through Pictet-Spengler condensation. This is the case for the well-known manzamines A and B that maintain the C-4/C-5′ connection and which were first isolated from *Haliclona* sp. in the Northwestern Pacific (Japan; **Fig. 9**) [81,82]. Manzamine C, isolated in the same study, lacks the 3-alkylpiperidine parts but keeps the *β*-carboline. These compounds are among the most promising natural antiparasitic and antitumoral compounds to date [83]. A close analogue called manzamine Y was isolated later from a similar sponge (**Fig. 9**) [84]. At the same time, similar compounds named keramamimes A and B were isolated from *Pellina* sp. [85]. However, its taxonomic status has not been clearly confirmed and it may be the same *Haliclona* species mentioned above. The structure of keramamime B has subsequently been revised and corresponds to manzamine F, a structure much more consistent with biosynthetic hypotheses (**Fig. 9**) [86].

A large diversity of manzamine alkaloids has also been isolated from the family Niphatidae and especially from one *Amphimedon* species. First, 6-hydroxymanzamine and 3,4-dihydromanzamine were isolated from *Amphimedon* sp. in the Northwestern Pacific (Japan) (**Fig. 9**) [87]. Keramaphidin B is a plausible biogenetic precursor of the bis-3-alkylpiperidine parts of manzamine [88], while keramaphidin C and keramamine C [89] are precursors of manzamine C (**Fig. 9**), and ircinols are antipode of the putative biosynthetic precursor of the manzamines [90]. Tetrahydro-*β*-carbolines manzamines H and L were isolated from the same species of *Amphimedon* (**Fig. 9**) [91] and additional manzamines were isolated later: 3,4-dihydromanzamine J, 3,4-dihydro-6-hydroxymanzamine A and manzamine M [92]. Finally, manz-

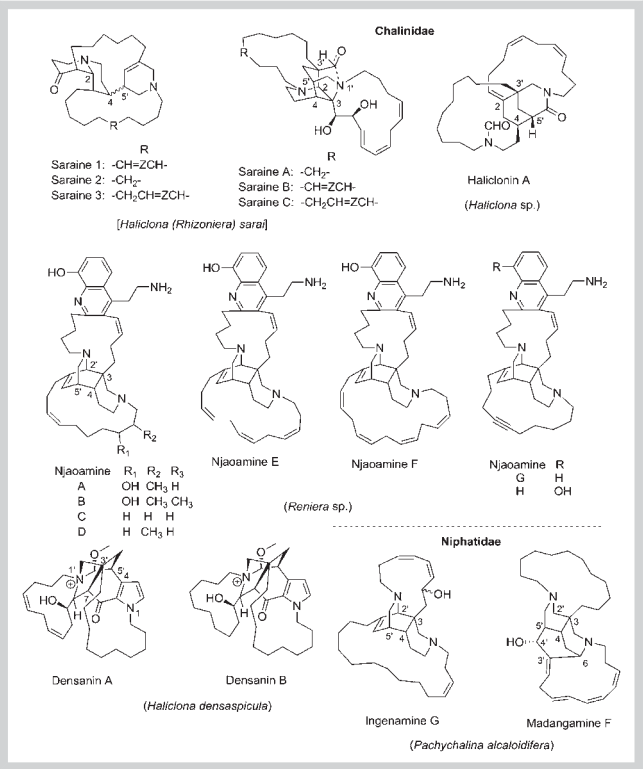

Fig. 8 Complex bis-3-alkylpiperidines from Chalinidae sponges.

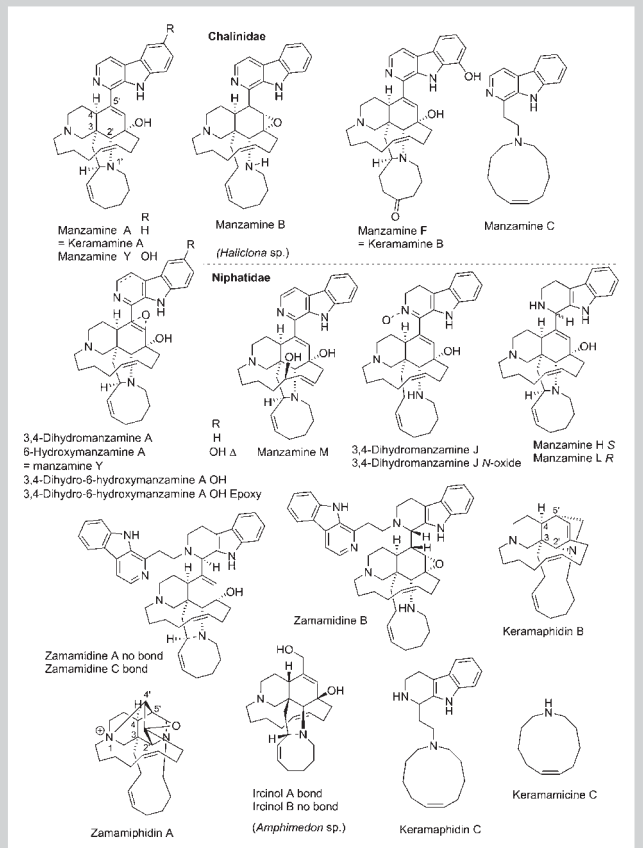

Fig. 9 Manzamine alkaloids isolated from Chalinidae and Niphatidae sponges.

amine derivatives condensed with a second *β*-carboline unit gave a name to the zamamidines, but also 3,4-dihydromanzamine J *N*-oxide and 3,4-dhydro-6-hydroxymanzamine A [93]. Finally a derivative of keramaphidin B named zamamiphidin A was isolated from the same *Amphimedon* sp. (**Fig. 9**) [94].

Isolated from *Acanthostrongylophora* sp., a sponge currently placed in the family Petrosiidae and collected in the Northwestern Pacific, are quite a number of manzamines, i.e., ircinol A, ircinal A, 12,28-oxaircinal A, manzamine A, manzamine A *N*-oxide, 3,4-dihydromanzamine A *N*-oxide, and 8-hydroxymanzamine A as well as manzamine B, 8-hydroxymanzamine B, manzamine E, 12,34-oxamanzamine E and both 6-hydroxy derivatives, 12,28-oxamanzamine E, manzamine F, manzamine J, 8-hydroxymanzamine J, manzamine X, 6-deoxymanzamine X, and acantholactone (**Fig. 10**) [95–97]. It is important to notice here that the extraordinary complex dimer named *neo*-kauluamine was isolated from this sponge but had also been isolated previously from a species belonging to a new genus (and as yet undescribed) within the Petrosiidae family [98]. The huge pharmacological potential of these compounds showing antimicrobial and antialzheimer activities has been demonstrated [91]. Other researchers demonstrated the presence of manzamine A and its 8-hydroxy derivative as well as a highly original series of acanthomanzamines A–E and additional manzamine derivatives like acantholactam and pre-*neo*-kauluamine (**Fig. 11**) from *A. ingens* collected in the same area [99–101]. Two precursors of manzamine alkaloids were isolated from *Xestospongia* sp. collected in the Western Pacific (Papua New Guinea) and the structure of xestocyclamine A was later revised (**Fig. 11**) [102, 103]. Finally, a large chemical diversity of complex bis-3-alkylpiperidines was isolated from *Xestospongia ingens* (Petrosiidae) collected in the Western Pacific (Papua New Guinea). It is worth noting here that the name of this sponge is no longer accepted and instead refers to *A. ingens* already mentioned previously. Ingenamine A was first isolated [104] and then ingamines A and B [105], madangamine A [106], and other analogues (**Fig. 11**) [107]. The unique alkaloid 8-hydroxymanzamine A was isolated from *Pachypellina* sp. (Phloedictyidae) but this species was later assigned to the same unnamed genus from the Petrosiidae family mentioned above [98, 108].

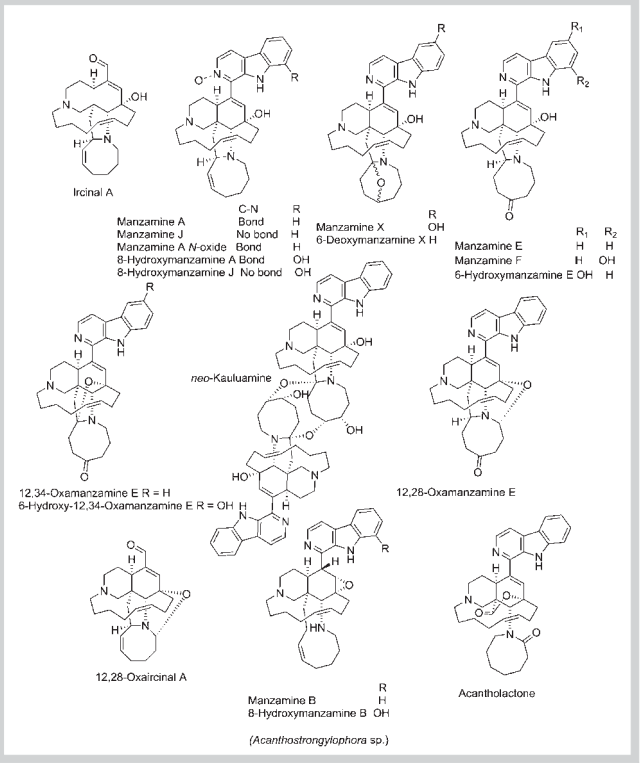

Fig. 10 Manzamine alkaloids from the sponge *Acanthostrongylophora* sp. (Petrosiidae).

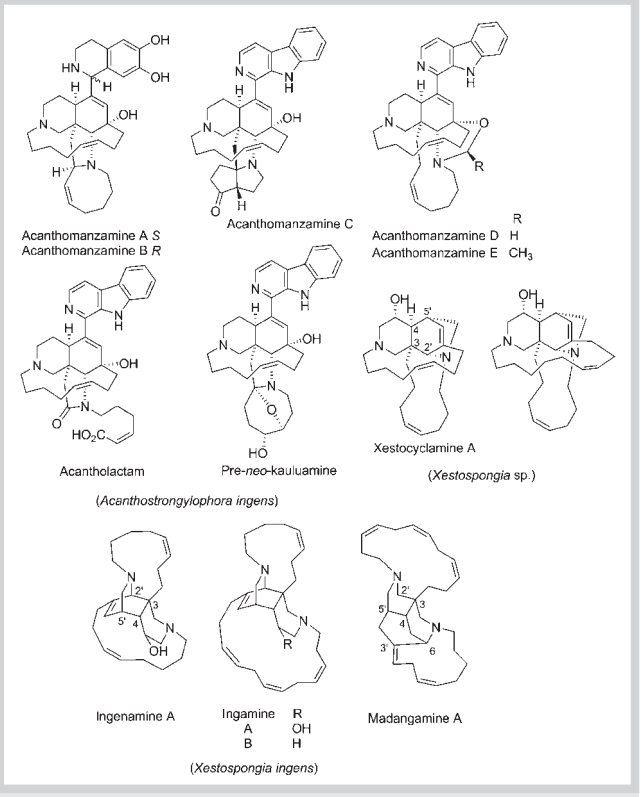

Fig. 11 Manzamine alkaloids from sponges *A. ingens* and *Xestospongia* (Petrosiidae).

Distribution of Polyacetylenic Derivatives across the Haplosclerida

It has been impossible to find a chemical and logical classification of the polyacetylenic compounds produced by members of the Haplosclerida and the chemical structures do not give valuable information for identification of key enzymes leading to their synthesis. A pattern, however, does emerge linking *either* alkaloid *or* polyacetylene pathways in each species but never both (**Table 1**). Therefore, we report on a selection of compounds from this group in view of the taxonomic classification of the species in question as we did for the alkaloids.

A large diversity of polyacetylenes, mostly from the aikupikanyne group, was isolated from *Callyspongia* species, including *Callyspongia truncata, Callyspongia pseudoreticulata*, and *Callyspongia fistularis* (Callyspongiidae) [109–115]. Previously classified as *Siphonochalina* (also Callyspongiidae), *C. truncata and Callyspongia siphonella* uniquely produce polyacetylenic derivatives [116–118]. The change of genus indicates the difficulty with identifying haploslerid sponges via morphological methods alone.

From Chalinidae sponges, several renierins followed by fulvinol and the polyhydroxylated fulvynes were isolated from the Mediterranean sponge *Haliclona (Halichoclona) fulva* [119–121]. Some polyacetylenes were also isolated from *Haliclona* sp. collected in the Western Pacific (Palau) while polyhydroxylated osirisynes were isolated from *H. (Reniera) osiris* collected in the Northwestern Pacific (China) [122,123]. Other compounds from unidentified *Haliclona* species include Haliclonyne [124], lembehynes (Northwestern Pacific, Japan) [125,126], Halycinones A and B [127] (Western Pacific, Micronesia), and brominated derivative [128]. *Adocia* sp. provided adocyacetylenes A–D, and while this genus is now accepted as *Haliclona (Haliclona)* [129], it is likely that the genus *Adocia* will be re-errected in the near future (McCormack, unpublished data). How many different species are included in the above list and what their actual final classification will be is therefore as yet unknown and needs attention.

Among the family Niphatidae, the genus *Cribrochalina* has provided a large diversity of polyacetylenic compounds. Duryne was first isolated from *C. dura* collected in the Northwestern Atlantic (Bahamas) [130], while the Caribbean sponge *C. vasculum* afforded several simple acetylenic alcohols, including vasculyne [131–134]. Nepheliosyne B was isolated from *Niphates* sp. collected in the Western Pacific (New Caledonia) [135], and chlorinated acetylenic compounds were isolated from *Haliclona lunisimisis*, later renamed *Niphates lunisimisis* de Jesus & Faulkner [136].

The family Petrosiidae has yielded a large diversity of polyacetylenic products, mostly from sponges of the genus *Petrosia,* a genus that, unfortunately, also needs taxonomic revision. The Mediterranean sponge *Petrosia ficiformis* has afforded a large panel of polyacetylenic derivatives, including petroformynes 1–10 [137–144], while petrosolic acid was later isolated from *Petrosia* sp. from the red sea [145]. Aztequynols A and B were isolated from a Caribbean *Petrosia* sp. [146]. Northwestern Pacific species from this genus have generated many compounds, including Petrosynol and petrosynone isolated from *Petrosia* sp., [147], brominated derivatives from *P. (Petrosia) volcano* [148], corticatic acids A–E from *P. (Strongylophora) corticata* [149,150], petrocortynes A–H and petrosiacetylenes A–D and other analogues from *Petrosia* sp. collected in Korea [151–157], strongylodiols from *P. (Strongylophora)* sp. [158,159], polyacetylene carboxylic acids [160], neopetroformynes A–D and later miyakosynes A–F [161, 162], petroacetylene from *P. (Petrosia) solida* [163], and finally, petrosiols A–E from *P. (Strongylophora) strongylata* [164].

Research focused on another petrosid genus led to the isolation of some polyacetylenic compounds from the Caribbean sponge *Xestospongia muta* [165], while several brominated derivatives were isolated from *Xestospongia testudinaria* from the Southwestern Pacific (Australia) [166–168]. Other compounds isolated from *Xestospongia* sp. include unsaturated fatty acids [169], melynes A–C [170], a polyhydroxylated derivative named nepheliosyne A analogous to fulvynes and osisirynes [171], and brominated unsaturated fatty acids [172].

With regard to the family Phloedictyidae, their unique isolated compounds were polyacetylenic derivatives, triangulynes A–H and pellynols A–D from the Western Pacific sponge (New Caledonia) *Oceanapia triangulate* [173,174], and another polyacetylene from *Oceanapia* sp. [175]

Integrating Chemistry and Biology

A large diversity of novel compounds of interest have been isolated from haplosclerid sponges and this group is likely to be a focus of continued efforts in this area for the foreseeable future given the diversity of species and habitats available providing a huge diversity of chemicals in turn. This review provides tantalizing insights into possible patterns of shared structures and biosynthetic pathways between species. However, real insights are hampered by the fact that most species included have not been identified to species and have not been included in any modern phylogenetic study. It is important to convey to chemists about the ever-changing world of taxonomic classification given the new methods being employed, including phylogenetic analysis of DNA sequence data. This is never truer than for haplosclerid sponges. There is an urgent need to revisit the studied sponges to place them in the new phylogenetic classification (including patterns of ancestry and descent between species) being built for demosponges. Only an approach of fully identifying sponges at the species level for analysis of chemistry will provide real additional value to the usual tools of systematics, thus contributing significantly to integrative taxonomy. Marine natural chemists should work tightly with taxonomic specialists (including specifically a phylogenetic approach) of each group in order to publish relevant data. For example, some species groups, i.e., of unidentified *Haliclona* and *Xestospongia* species, produce alkaloids but also polyacetylenic compounds, which prevents us from reaching any clear conclusion regarding patterns of distribution of specific compounds across taxonomic groups. However, once understood, the presence of each family of compounds could help significantly in a proper classification and avoid misinterpretation of species identification/classification.

From **Table 1**, several clues are provided for assisting sponge classification. First of all, the presence of both polyacetylenic and alkaloid compounds have never been reported in a single species. Both biosynthetic genes/pathways cannot, therefore, be present in a species. This observation leads us to suggest a clear separation between species producing alkaloids and species producing polyacetylenes. For example, *C. ridleyi* is the only known species of the genus *Callyspongia* that does not produce polyacetylenic compounds. In the same way, only one species of *Cribrochalina* sp. was found to produce alkaloids. This could lead to a clear revision of the taxonomy of these sponges. Finally, *Neopetrosia* species were found to produce alkaloids, while *Petrosia* species produce only polyacetylenic compounds. However, these two genera are currently placed in the same family, Petrosiidae, indicating that revision is required at different levels of the taxonomic hierarchy.

One undefined species of *Cribrochalina* (Niphatidae) produces only 3-alkylpyridine derivatives. Simple alkylation at the nitrogen atom of the pyridine leads to 3-alkylpyridiniums that can polymerize. Only the species of *Callyspongia* (Callyspongiidae), *Niphates*, and *Pachychalina* (Niphatidae) seem to stop the biosynthesis at this step for this group of alkaloids. Because these genera are distinct, revisiting the voucher samples could help to identify similarities between these species. The subsequent ability of some species to reduce the aromatic rings enabling a C-4/C-5′ connection between two cycles is shared by several species, including *Arenosclera brasiliensis* (Callyspongiidae) and *H. viscosa* (Chalinidae). Contrary to other families, members of Callyspongiidae seem unable to develop more complex alkaloids. In the same way, *Neopetrosia* (Petrosiidae) species only produce simple

bis-3-alkylpiperidine derivatives. *Neopetrosia* could be hypothesized then to be closer to some *Callyspongia* sponges, and recent phylogenetic classification suggests that some *Neopetrosia* are placed within the same major clade as *Callyspongia* (Clade A) [8, 21]. Much more complex alkaloids are produced by few species distributed in several families, which also raises questions regarding the reliability of the classification. *H. densaspicula, H. sarai,* a *Reniera* sp. (Chalinidae), and *Pachychalina alcaloidifera* (Niphatidae) are able to produce complex alkaloids, but no manzamines have been isolated from these species. It appears that clearly this ability should gather some *Haliclona* species. As for the extremely complex alkaloid manzamines, members of the genera *Acanthostrongylophora* (Petrosiidae), *Haliclona* (Chalinidae), and *Amphimedon* (Niphatidae) were found to produce them. These compounds are not characteristic of the Petrosiidae, as most species of this family produce only polyacetylenes.

A diversity of polyacetylenic compounds is found distributed across all of the five families of Haplosclerida included so far and their patterns of occurrence should trigger careful examination of the studied species. For example, four *Callyspongia* species (Callyspongiidae) produce compounds very similar to two *Haliclona* species (Chalinidae), two *Cribrochalina* species, and one *Niphates* species (Niphatidae), but most of the polyacetylenic compounds are really representative of *Petrosia* and *Xestospongia* genera (Petrosiidae). Recent molecular phylogenies suggest that *Callyspongia* and some *Haliclona* fall into one major clade (Clade A) at the base of which appears *Cribochalina* [8, 22]. *Niphates* species fall into a distantly related clade, also with some *Haliclona* (Clade C), while species from *Petrosia* and *Xestospongia* are distributed across multiple clades (but distinct from the aforementioned genera) and are poorly represented on phylogenetic trees drawn from molecular data as yet [8, 22].

The value of a chemosystematics approach for haplosclerid sponges has been questioned in the past due to disagreement between patterns of chemical diversity and morphological classification [176]. With molecular data throwing light on possible evolutionary pathways in sponges, it is evident that the morphological classification is flawed and is likely to change, and this is especially true for the Haplosclerida. An additional concern could be the varying influence of microbes residing in sponge tissue, which may be responsible for some of the compounds isolated. A diversity of microbial sequences has been reported from some *Haliclona* species and *Xestospongia*, amongst others [177–180], even though these studies do not show the presence of microbes in the sponge tissue nor do they confirm the source of compounds of interest as being from sponge or microbial cells. Sponges filter feed and concentrate microbes and microbial constituents from their environment, and these elements will be present in approaches that only isolate DNA sequences from sponge cells (even though they may only represent food for the sponge). Therefore, the presence of many bacterial DNA sequences in sponges does not equate directly to a sponge-bacterial association or to the production of detectable levels of compounds of interest by said bacteria. Ultrastructural studies on Irish and Mediteranean *Haliclona* species (Marra, unpublished data) show extremely low numbers of bacterial cells in four species, while an association between one or very few bacteria is evident in three species and many bacterial species are evident in one. Consideration of such patterns in these and other species will also be required to truly understand how patterns of microbial diversity impact patterns of chemical diversity.

Conclusion

Our main conclusion is that chemical diversity does not fit with the current classification of this major group of marine sponges, but supports a clear revision of all the species of the Order Haplosclerida included in chemical publications taking into account the presence or absence of key enzymes leading to 3-alkylpyridine derivatives or polyacetylenes. Despite apparent discrepancies there are tantalizing insights that patterns of chemical diversity may well agree in good part with an updated classification that incorporates molecular and other data including morphology. Assessment of the possible bacterial origin of sponge-derived compounds should also be considered. Three other limitations to this approach are the existence of mistakes made in the identification of the species under investigation, a discrepancy between a classical taxonomic approach utilizing shared sponge morphological characters and the approach that uses molecular phylogenetic methods, and, thirdly, poor sampling of the huge diversity of sponges and the chemistry they contain. However, we believe that this review should help in the further development of an integrative approach between biology and chemistry for this group of sponges. The issues highlighted here are not restricted to the group of marine sponges included, but are relevant to all organisms that are of interest in the search for novel chemistry for whatever reason and where taxonomy is still unresolved. New advances in 'omics' sciences, and especially metabolomic approaches using mass spectrometry and nuclear magnetic resonance with some characteristic signals, could quickly give important clues for integrative systematics.

Conflict of Interest

The authors declare no conflict of interest.

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Data Note

DATA NOTE

The sponge microbiome project

Lucas Moitinho-Silva[1], Shaun Nielsen[1], Amnon Amir[2], Antonio Gonzalez[2], Gail L. Ackermann[2], Carlo Cerrano[3], Carmen Astudillo-Garcia[4], Cole Easson[5], Detmer Sipkema[6], Fang Liu[7], Georg Steinert[6], Giorgos Kotoulas[8], Grace P. McCormack[9], Guofang Feng[7], James J. Bell[10], Jan Vicente[11], Johannes R. Björk[12], Jose M. Montoya[13], Julie B. Olson[14], Julie Reveillaud[15], Laura Steindler[16], Mari-Carmen Pineda[17], Maria V. Marra[9], Micha Ilan[18], Michael W. Taylor[4], Paraskevi Polymenakou[8], Patrick M. Erwin[19], Peter J. Schupp[20], Rachel L. Simister[21], Rob Knight[2,22], Robert W. Thacker[23], Rodrigo Costa[24], Russell T. Hill[25], Susanna Lopez-Legentil[19], Thanos Dailianis[8], Timothy Ravasi[26], Ute Hentschel[27], Zhiyong Li[7], Nicole S. Webster[17,28] and Torsten Thomas[1,*]

[1]Centre for Marine Bio-Innovation and School of Biological, Earth and Environmental Sciences, The University of New South Wales, Sydney, 2052, Australia, [2]Department of Pediatrics, University of California - San Diego, La Jolla, CA 92093, USA, [3]Department of Life and Environmental Sciences, Polytechnic University of Marche, Ancona, 60131, Italy, [4]School of Biological Sciences, University of Auckland, Auckland, New Zealand, [5]Halmos College of Natural Sciences and Oceanography, Nova Southeastern University, Dania Beach, FL 33004, USA, [6]Wageningen University, Laboratory of Microbiology, Stippeneng 4, 6708 WE Wageningen, The Netherlands, [7]State Key Laboratory of Microbial Metabolism and School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, P.R. China, [8]Hellenic Centre for Marine Research, Institute of Marine Biology, Biotechnology and Aquaculture, Thalassocosmos, 71500 Heraklion, Greece, [9]Zoology, School of Natural Sciences, Ryan Institute, National University of Ireland Galway, University Rd., Galway, Ireland, [10]School of Biological Sciences, Victoria University of Wellington, Wellington, New Zealand, [11]Hawaii Institute of Marine Biology, 46-007 Lilipuna Road, Kaneohe, HI 96744-1346, [12]Galvin Life Science Center, University of Notre Dame, Notre Dame, IN 46556, USA, [13]Ecological Networks and Global Change Group, Theoretical and Experimental Ecology Station, CNRS and Paul Sabatier University, Moulis, France, [14]Department of Biological Sciences, University of Alabama, Tuscaloosa, AL 35487, USA, [15]INRA, UMR1309 CMAEE; Cirad, UMR15 CMAEE, 34398 Montpellier, France, [16]Department of Marine Biology, Leon H. Charney School of Marine Sciences, University of Haifa, Haifa, Israel, [17]Australian Institute of Marine Science (AIMS), Townsville, 4810, Queensland, Australia, [18]Department of Zoology, George S. Wise Faculty of Life Sciences, Tel Aviv University,

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Tel Aviv 69978, Israel, [19]Department of Biology and Marine Biology, University of North Carolina Wilmington, Wilmington NC 28409, USA, [20]Institute for Chemistry and Biology of the Marine Environment (ICBM), Carl-von-Ossietzky and University Oldenburg, Schleusenstr. 1, 26382 Wilhelmshaven, Germany, [21]Department of Microbiology and Immunology, University of British Columbia, Canada, V6T 1Z3, [22]Department of Computer Science and Engineering, and Center for Microbiome Innovation, University of California - San Diego, La Jolla, CA 92093, USA, [23]Department of Ecology and Evolution, Stony Brook University, Stony Brook NY 11794, USA, [24]Institute for Bioengineering and Biosciences (IBB), Department of Bioengineering, IST, Universidade de Lisboa, Lisbon, Portugal, [25]Institute of Marine and Environmental Technology, University of Maryland Center for Environmental Science, 701 East Pratt Street, Baltimore, MD 21202, USA, [26]KAUST Environmental Epigenetic Program (KEEP), Division of Biological and Environmental Sciences & Engineering, King Abdullah University of Science and Technology, Thuwal, Kingdom of Saudi Arabia, [27]RD3 Marine Microbiology, GEOMAR Helmholtz Centre for Ocean Research, Kiel, and Christian-Albrechts-University of Kiel, Germany and [28]Australian Centre for Ecogenomics, School of Chemistry and Molecular Biosciences, University of Queensland, St Lucia, QLD, Australia

*Correspondence address. Torsten Thomas, Centre for Marine Bio-Innovation and School of Biological, Earth and Environmental Sciences, The University of New South Wales, Sydney, 2052, Australia; E-mail: t.thomas@unsw.edu.au

Abstract

Marine sponges (phylum Porifera) are a diverse, phylogenetically deep-branching clade known for forming intimate partnerships with complex communities of microorganisms. To date, 16S rRNA gene sequencing studies have largely utilised different extraction and amplification methodologies to target the microbial communities of a limited number of sponge species, severely limiting comparative analyses of sponge microbial diversity and structure. Here, we provide an extensive and standardised dataset that will facilitate sponge microbiome comparisons across large spatial, temporal, and environmental scales. Samples from marine sponges ($n = 3569$ specimens), seawater ($n = 370$), marine sediments ($n = 65$) and other environments ($n = 29$) were collected from different locations across the globe. This dataset incorporates at least 268 different sponge species, including several yet unidentified taxa. The V4 region of the 16S rRNA gene was amplified and sequenced from extracted DNA using standardised procedures. Raw sequences (total of 1.1 billion sequences) were processed and clustered with (i) a standard protocol using QIIME closed-reference picking resulting in 39 543 operational taxonomic units (OTU) at 97% sequence identity, (ii) a *de novo* clustering using Mothur resulting in 518 246 OTUs, and (iii) a new high-resolution Deblur protocol resulting in 83 908 unique bacterial sequences. Abundance tables, representative sequences, taxonomic classifications, and metadata are provided. This dataset represents a comprehensive resource of sponge-associated microbial communities based on 16S rRNA gene sequences that can be used to address overarching hypotheses regarding host-associated prokaryotes, including host specificity, convergent evolution, environmental drivers of microbiome structure, and the sponge-associated rare biosphere.

Keywords: marine sponges; archaea; bacteria; symbiosis; microbiome; 16S rRNA gene; microbial diversity

Data Description

Purpose of data acquisition

Sponges (phylum Porifera) are an ancient metazoan clade [1], with more than 8500 formally described species [2]. Sponges are benthic organisms that have important ecological functions in aquatic habitats [3, 4]. Marine sponges are often found in symbiotic association with microorganisms, and these microbial communities can be very diverse and complex [5, 6]. Sponge symbionts perform a wide range of functional roles, including vitamin synthesis, production of bioactive compounds, and biochemical transformations of nutrients or waste products [7–9]. The diversity of microorganisms associated with sponges has been the subject of intense study (the search of "sponge microbial diversity" returned 348 publications in the Scopus database) [10]. Most of these studies were performed on individual species from restricted geographic regions [e.g., 11, 12]. A comparative assessment of these studies is often hindered by differences in sample processing and 16S rRNA gene sequencing. However, 2 recent studies incorporating a large number of sponge microbiomes (>30) [5, 13] revealed the potential of large-scale, standardised, high-throughput sequencing for gaining insights into the diversity and structure of sponge-associated microbial communities. The purpose of this global dataset is to provide a comprehensive 16S rRNA gene-based resource for investigating and comparing microbiomes more generally across the phylum Porifera.

Sample collection, processing, and 16S rRNA gene sequencing

Sample collection and processing, species identification, and DNA extractions were conducted as previously described [13]. A total of 3569 sponge specimens were collected, representing at least 268 species, including several yet unidentified taxa (hereafter collectively referred to as species) (Supplementary Table S1). Of all species, 213 were represented by at least 3 specimens. *Carteriospongia foliascens* had the highest replication, comprising

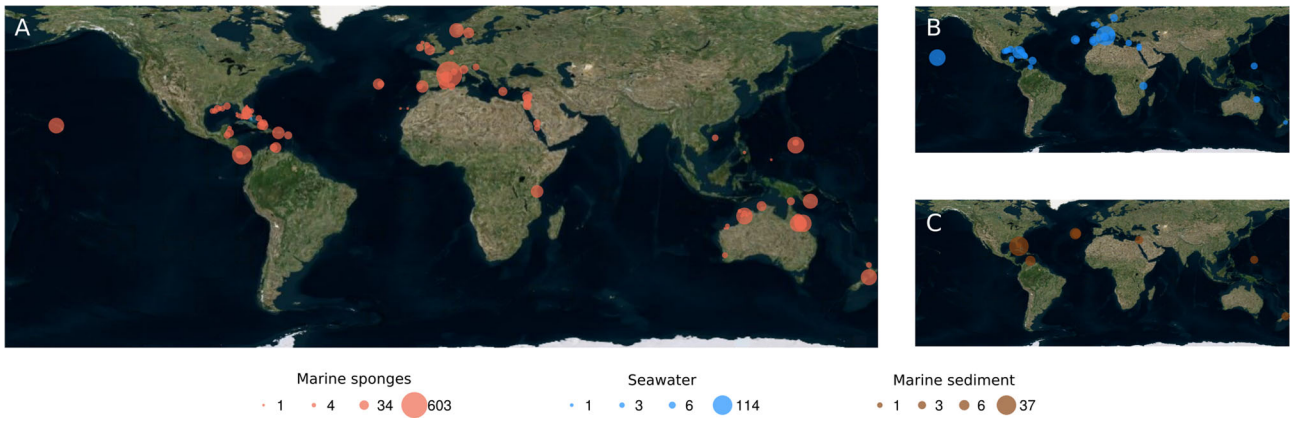

Figure 1: Global sample collection sites. Bubbles indicate collection sites of (**A**) marine sponges, (**B**) seawater, and (**C**) marine sediment samples. Bubble sizes are proportional to number of samples as indicated.

150 individuals. Seawater ($n = 370$), sediment ($n = 65$), algae ($n = 1$), and echinoderm ($n = 1$) samples as well as biofilm swabs ($n = 21$) of rock surfaces were collected in close proximity to the sponges for comparative community analysis. Six negative control samples (sterile water) were processed to identify any potential contaminations. Of the samples included in this current dataset, 973 samples had been analysed previously [13]. Samples were collected from a wide range of geographical locations (Fig. 1; Supplementary Table S1). Total DNA was extracted as previously described [13] and used as templates to amplify and sequence the V4 region of the 16S rRNA gene using the standard procedures of the Earth Microbiome Project (EMP) [14, 15].

Processing of sequencing data

Clustering using the EMP standard protocols in QIIME

Raw sequences were demultiplexed and quality controlled following the recommendations of Bokulich et al. [16]. Quality-filtered, demultiplexed fastq files were processed using the default closed-reference pipeline from QIIME v. 1.9.1 (QIIME, RRID:SCR_008249). Briefly, sequences were matched against the GreenGenes reference database (v. 13_8 clustered at 97% similarity). Sequences that failed to align (e.g., chimeras) were discarded, which resulted in a final number of 300 140 110 sequences. Taxonomy assignments and the phylogenetic tree information were taken from the centroids of the reference sequence clusters contained in the GreenGenes reference database (Greengenes, RRID:SCR_002830). This closed-reference analysis allows for cross-dataset comparisons and direct comparison with the tens of thousands of other samples processed in the EMP and available via the Qiita database [17].

Clustering using Mothur

Quality-filtered, demultiplexed fastq files were also processed using Mothur v. 1.37.6 (Mothur, RRID:SCR_011947) [18] and Python v. 2.7 (Python Programming Language, RRID:SCR_008394) [19] custom scripts with modifications from previously established protocols [13]. Detailed descriptions and command outputs are available at the project notebook (see Availability of supporting data). Briefly, sequences were quality-trimmed to a maximum length of 100 bp. To minimize computational effort, the dataset was reduced to unique sequences, retaining total sequence counts. Sequences were aligned to the V4 region of the 16S rRNA gene sequences from the SILVA v. 123 database (SILVA, RRID:SCR_006423) [20]. Sequences that aligned at the expected positions were kept, and this dataset was again reduced to unique sequences. Further, singletons were removed from the dataset, and the remaining sequences were preclustered if they differed by 1 nucleotide position. Sequences classified as eukaryote, chloroplast, mitochondria, or unknown according to the Greengenes (v. 13_8 clustered at 99% similarity) [21] and SILVA taxonomies [22] were removed. Chimeras were identified with UCHIME (UCHIME, RRID:SCR_008057) [23] and removed. Finally, sequences were *de novo* clustered into operational taxonomic units (OTUs) using the furthest neighbour method at 97% similarity. Representative sequences of OTUs were retrieved based on the mean distance among the clustered sequences. Consensus taxonomies based on the SILVA, Greengenes, and RDP (v. 14_03 2015; Ribosomal Database Project, RRID:SCR_006633) [24] databases were obtained based on the classification of sequences clustered within each OTU. The inclusion of these taxonomies is helpful considering that they have substantial differences, as recently discussed [25]. For example, Greengenes and RDP have the taxon Poribacteria, a prominent sponge-enriched phylum [26], which did not exist in the SILVA version used.

De-noising using Deblur

Recently, sub-OTU methods that allow views of the data at single-nucleotide resolution have become available. One such method is Deblur [27], which is a de-noising algorithm for identification of the actual bacterial sequences present in a sample. Using an upper bound on the polymerase chain reaction and read-error rates, Deblur processes each sample independently and outputs the list of sequences and their frequencies in each sample, enabling single nucleotide resolution. For creating the deblurred biom table, quality-filtered, demultiplexed fasta files were used as input to Deblur using a trim length of 100 and min-reads of 25 (removing sOTUs with <25 reads total in all samples combined). Taxonomy was added to the resulting biom table using QIIME [28], RDP classifier [29], and Greengenes v. 13.8 [21].

Database metadata category enrichment

For enrichment analysis of metadata terms in a set of sequences, each unique metadata value is tested using both a binomial test and a ranksum test. All analysis is performed on a randomly subsampled (5000 reads/sample) table.

Figure 2: Microbial taxonomic profile of marine sponge samples processed with Mothur. (**A**) SILVA, (**B**) Greengenes, and (**C**) RDP taxonomies are shown. OTU sequence counts were grouped according to phylum and class. Taxa with relative abundances ≤0.5% were grouped as "others." Classes with relative abundances >1% are shown in the legend (phylum ";" class). Relative abundances are represented on the x-axes.

The binomial (presence/absence) P-value for enrichment calculated as follows

For a bacterial sequence s and metadata value v, denote N the total number of samples, O(s) the number of samples where s is present, K_v(s) the number of samples with value v where s is present, and T(v) the total number of samples with value v.

$$P\text{-value} = binomial\_cdf\,(\mathrm{T(v)} - \mathrm{K}_v(\mathrm{s}), \mathrm{T(v)}, 1 - P_{\mathrm{Null}}(\mathrm{s}))$$

where $P_{\mathrm{Null}}(\mathrm{s}) = \mathrm{O(s)}/N$

The ranksum (frequency aware) P-value is calculated using the Kruskal-Wallis test (implemented in scipy 0.19) as follows.

For a bacterial sequence s and metadata value v, denote by $F_v(s)$ the vector of relative frequencies of bacteria s in all samples with metadata value v, and denote by $\widehat{F_v(s)}$ the vector of relative frequencies of bacteria s in all samples with metadata other than v. The ranksum P-value is then calculated using the Kruskal-Wallis test for $F_v(s)$ and $\widehat{F_v(s)}$ and shown only if significantly enriched in samples containing v (i.e., rank difference of $F_v(s)$—$\widehat{F_v(s)} > 0$).

We have set up a webserver [30] that performs this enrichment analysis for user-defined sequence submissions. The code for the webserver is also available in Github for a local installation.

Data description

The dataset covers 4033 samples with a total of 1 167 226 701 raw sequence reads. These sequence reads clustered into 39 543 OTUs using QIIME's closed-reference processing, 518 246 OTUs from *de novo* clustering using Mothur (not filtered for OTU abundances), and 83 908 sOTUs using Deblur (with a filtering of at least 25 reads total per sOTU). We recommend that data users consider the differences in sequencing depths per sample and abundance filtering for certain downstream analyses, such as when calculating diversity estimates [16] and comparing OTU abundances across samples [31]. In terms of taxonomic diversity, most Mothur OTUs were assigned to the phylum Proteobacteria, although more than 60 different microbial phyla were recovered from the marine sponge samples according to SILVA ($n = 63$) and Greengenes classifications ($n = 72$) (Fig. 2).

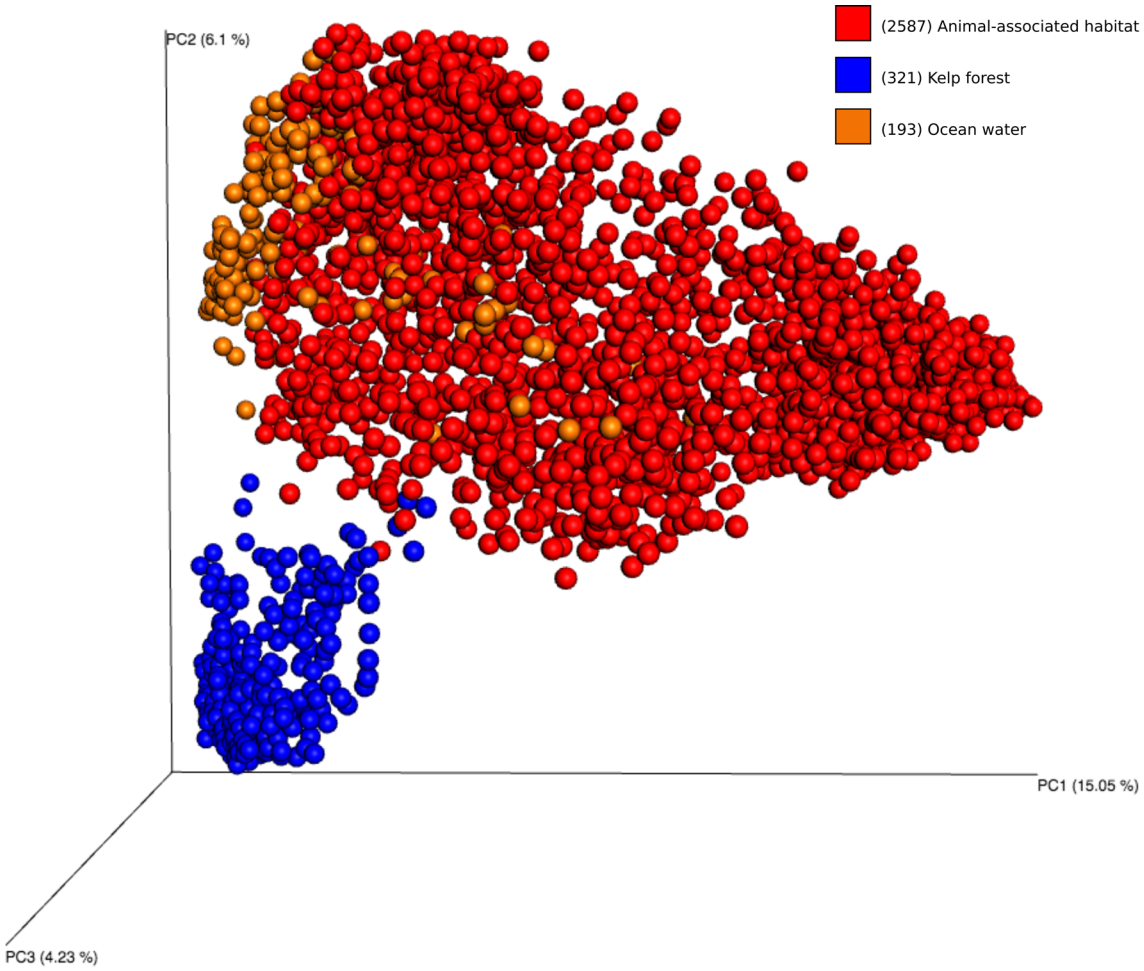

Figure 3: Unweighted UniFrac Principal Coordinates Analysis (PCA) of samples from sponges ("animal-associated habitat"), kelp forest, and ocean water. Samples were rarefying to 10 000 sequences per sample. A movie showing the PCA plot in 3D is provided in the supporting information.

Potential uses

This dataset can be utilised to assess a broad range of ecological questions pertaining to host-associated microbial communities generally or to sponge microbiology specifically. These include: (i) the degree of host specificity, (ii) the existence of biogeographic or environmental patterns, (iii) the relation of microbiomes to host phylogeny, (iv) the variability of microbiomes within or between host species, (v) symbiont co-occurrence patterns, and (vi) assessing the existence of a core sponge microbiome. An example of this type of analysis is shown in Fig. 3, where samples were clustered using unweighted UniFrac data [10] with a Principal Coordinates Analysis and visualization in Emperor [15] based on their origins from sponges, seawater, or kelps [17].

Availability and requirements

Project name: The Sponge Microbiome Project

Project home page: www.spongeemp.com; https://github.com/amnona/SpongeEMP

Operating system(s): Unix

Programming language: Python and R

Other requirements: Python v. 2.7, Biopython v. 1.65, Python 3.5, R v. 3.2.2, Mothur v. 1.37.6, QIIME v. 1.9.1, Deblur

License: MIT

Any restrictions to use by non-academics: none

Availability of supporting data

Raw sequence data were deposited in the European Nucleotide Archive (accession number: ERP020690). Quality-filtered, demultiplexed fastq files, QIIME resulting OTU tables are available at the Qiita database (Study ID: 10 793) [17]. The additional datasets that support the results of this article are available in the *GigaScience* repository, *Giga*DB [32] and include an OTU abundance matrix (the output ".shared" file from Mothur, which is tab delimited), an OTU taxonomic classification table (tab delimited text file), an OTU representative sequence FASTA file, a table of samples' metadata, the biom files from QIIME and Deblur analyses, and the QIIME-generated tree file. The project workflow, Mothur commands, and additional scripts are available as HTML in *Giga*DB [32].

The deblurred dataset has also been uploaded to an online server [19] that supplies both html and REST-API access for querying bacterial sequences and obtaining the observed

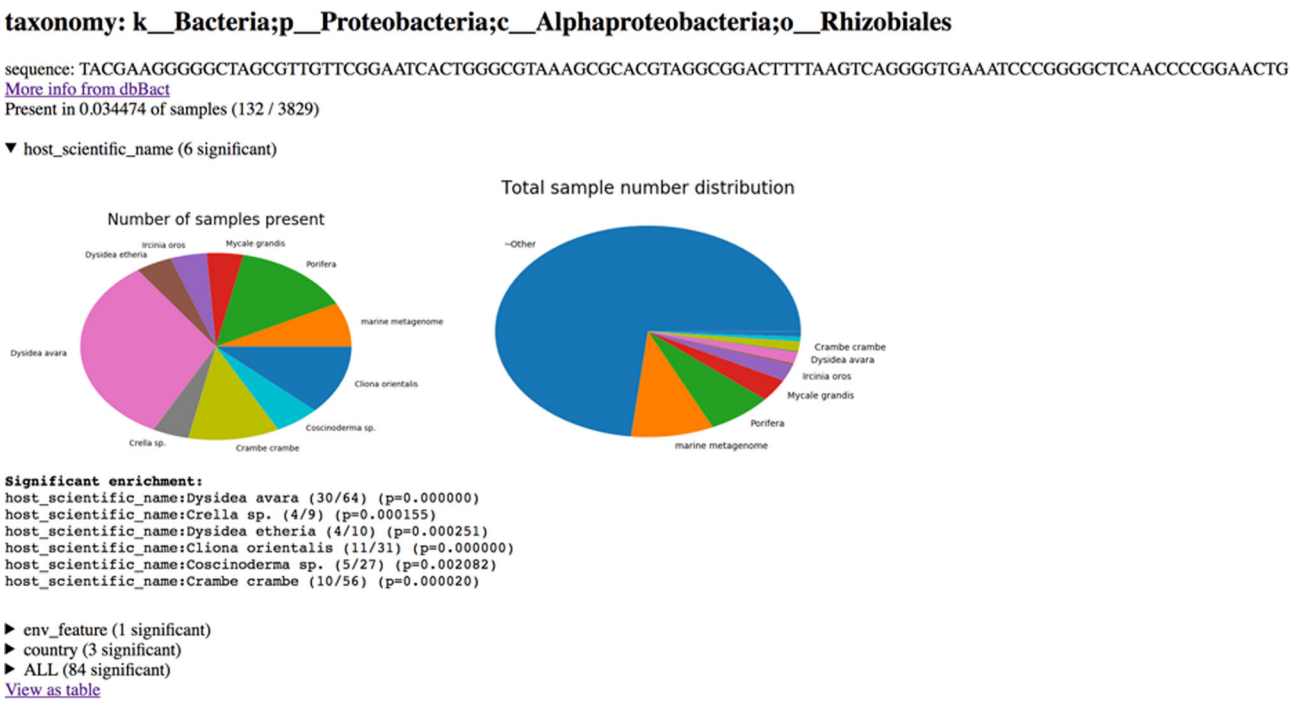

Figure 4: Output of the enrichment analysis through the online server www.spongeemp.com. Top line shows taxonomic assignment for the user-submitted sequence in the second line. Pie charts below show the total number of samples (right) and the number of samples where the submitted sequence is present (left) based on the scientific names of the host, followed by the significantly enriched host names containing the submitted sequence (using either presence/absence binomial test or relative frequency–based ranksum test). At the bottom, fields can be opened to show results of the enrichment analyses for other metadata types (e.g., country).

prevalence and enriched metadata categories where the sequence is observed (Figure 4). This allows an interactive view of which sequences are associated with which specific parameters, such as depth or salinity.

Additional file

sample.metadata

Abbreviations

EMP: Earth microbiome project; bp: base pairs; OTU: operational taxonomic unit; rRNA: ribosomal RNA.

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Competing interests

The authors declare that they have no competing interests.

Author contributions

L.M.-S., N.S.W., and T.T. designed the study. C.A.G., D.S., F.L., G.S., G.K., G.McC., G.-F. F, J.J.B., J.V., J.R.B., J.M.M., J.R., L.S., M.C.P, M.V.M., M.W.T., N.S.W., P.P., P.M.E., P.J.S., R.L.S, R.W.T., R.C., R.T.H., S.L-L., T.D., T.R., U.H., and Z-Y. L. collected samples. C.A.G., D.S., J.V., J.R.B., L.S., M.C.P., M.W.T., N.S.W., P.M.E., R.L.S, R.W.T., S.L-L., and U.H. extracted DNA. G.L.A. and R.K. sequenced DNA. L.M.-S., S.N., A.A., A.G., G.L.A., and T.T. performed data processing and analysis. L.M.-S., N.S.W., and T.T. wrote the manuscript. All authors contributed to the writing of the manuscript.

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