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Elena Bovio

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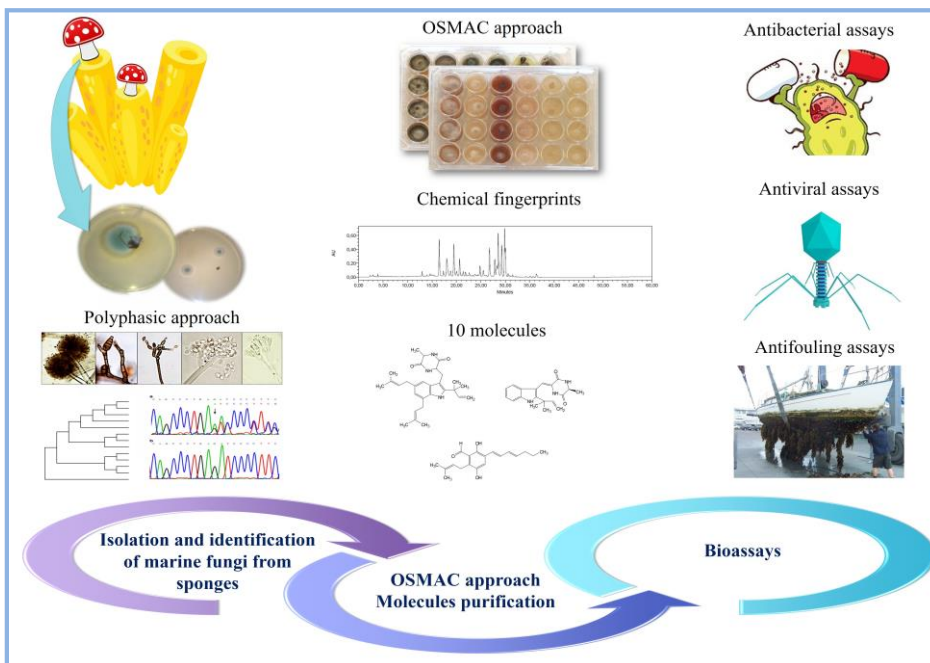
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Ph.D. Program in Biology and Applied Biotechnologies
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Marine fungi from sponges: biodiversity, chemodiversity and biotechnological applications

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**Marine fungi from sponges: biodiversity,
chemodiversity and biotechnological applications**

Elena Bovio

**Tutors: Prof. Giovanna Cristina Varese
Prof. Mohamed Mehiri**

XXXI Cycle: 2015 – 2018

*The sea, once it casts its spell,
holds one in its net of wonder forever*

Jacques Y. Cousteau

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List of Abbreviations

AC	AlgoBank of Caen
ACT	Actin gene
AIC	Akaike Information Criterion
AMR	Antimicrobial resistance
ANOVA	Analysis of variance
ATCC	American type culture collection
BHI	Brain-Heart Infusion
BPP	Bayesian posterior probabilities
CA	Carrot Agar
CAL	Calmodulin gene
CAP	Canonical analysis of principal coordinates
CFU	Colony forming unit
CLSI	Clinical and laboratory standards institute
CMASW	Corn meal agar seawater
cRNP	Ribonucleoprotein
DAAs	Directly acting antivirals
DBIOS	Department of life sciences and system biology
DDD	1,1-dichloro-2,2-bis-(4-chlorophenyl)ethane
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTPs	Deoxynucleotides
EPS	Extracellular polymeric substances
FA	Formic acid
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GA	Gelatin agar
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase gene
GAS	Gelatin agar added with 3% NaCl
GASW	Gelatin agar seawater

GNPS	Global natural products social molecular networking
HAAs	Host-acting antivirals
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HDAC	Histone deacetylase
HIV	Human immunodeficiency virus
HPLC	High Performance Liquid Chromatography
HR-MS/MS	High-resolution tandem mass spectrometry
HSV1	Herpes simplex virus 1
HSV2	Herpes simplex virus 2
IAV	Influenza A virus
IBV	Influenza virus B
IC	Inhibitor concentration
ITS	Internal transcribed spacer regions
LBCM	Laboratoire de Biotechnologie et Chimie Marines
LC-MS	Liquid chromatography - mass spectrometry
LOEC	Low observable effect concentration
LSU	Large subunit
MBC	Minimal bactericidal concentration
MBM	Marine bacterial medium
MCMC	Markov chains monte carlo
MDCK	Madin darby canine kidney cells
MDR	Multi drug resistant
MEA	Malt extract agar
MEASW	Malt extract agar seawater
MeOH	Methanol
MHBCA	Mueller-hinton broth cation adjusted
MHF	Mueller hinton fastidius agar
MIC	Minimal inhibitory concentration
mRNA	Messenger RNA
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MUT	<i>Mycotheca Universitatis Taurinensis</i>
NGS	Next Generations Sequencing
NMR	Nuclear magnetic resonance

nrRNA	Nuclear ribosomal RNA
OD	Optical density
OSMAC	One strain - many compounds
PC	Positive control
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDAS	Potato dextrose agar added with 3% NaCl
PDB	Potato dextrose broth
PERMANOVA	Permutational multivariate analysis of variance
PFU	Plaque forming unit
PRA	Plaque reduction assays
rDNA	Ribosomal DNA
RNA	RiboNucleic Acid
SPE	Solid phase extraction
SW	Seawater
SWA	Seawater agar
TBT	Tributyltin
TSA	Tryptic soy agar
TUB	Beta-tubulin gene
VERO	African green monkey kidney cells (<i>verda reno</i>)
vRNAs	Viral ribonucleoproteins
WH	Wickerham's medium
WHO	World health organisation
WHS	Wickerham's medium added with 3% NaCl

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1. INTRODUCTION

1.1 Marine environment

The Oceans cover almost three quarter of the Earth's surface and host 50% – 80% of life forms (Choudhary *et al.*, 2017). Marine ecosystems are the result of complex interactions between the physiochemical environment and the inhabiting organisms, and have a dramatic impact on climate and human life (Raghukumar, 2017). Marine species are perfectly adapted to harsh conditions, represented by an average salinity of 3.5%, reduced light penetration and high hydrostatic pressure (Nicoletti and Andolfi, 2018; Raghukumar, 2017). On the contrary, others parameters like pH ranging from 7.5 to 8.5 and temperature (-2 to 30 °C, on average) are more stable than in other terrestrial ecosystems, with some exceptions, i.e. the hydrothermal vents (Raghukumar, 2017). These different physical and chemical conditions define the zones in which the marine environment is divided (Figure 1.1).

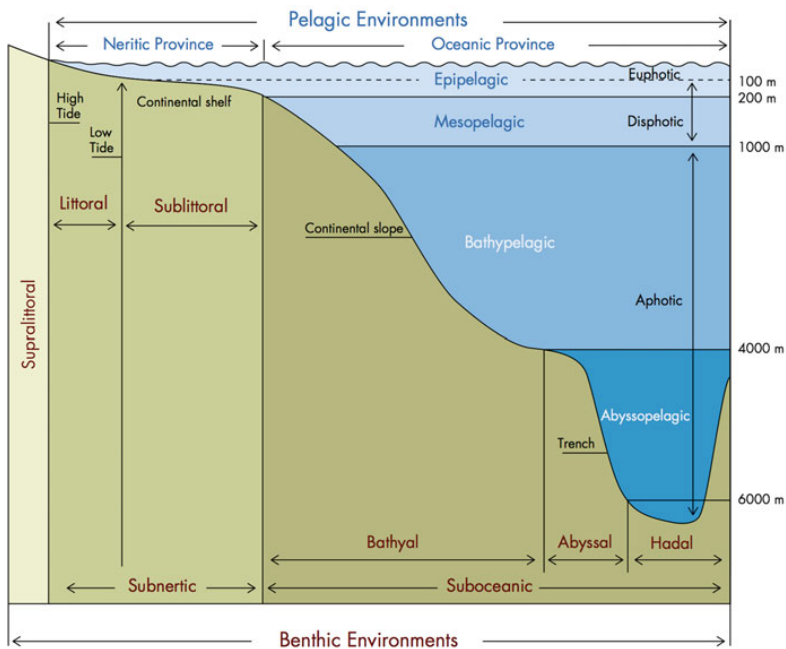


Figure 1.1 Division of the marine environment (Raghukumar, 2017).

Horizontally, the coastal habitat (neritic zone) from the littoral (high tide level) to the edge of the continental shelf (about 200 m depth) is markedly influenced by land. The remaining part, less conditioned by land, is named oceanic habitat (Matthiessen *et al.*, 2018; Raghukumar, 2017). The vertical division highlights the pelagic and the benthic zone, each with several subdivisions. The pelagic zone is the largest habitat on Earth counting for 99.5% of inhabitable space from the sea-surface, to the sea-bottom (Matthiessen *et al.*, 2018). The upper waters are influenced by the atmosphere conditions (i.e. temperature, seasonal variability, organic and inorganic material transported); while, light penetration delimits the euphotic zone, where the photosynthesis occurs. Thus, all the inhabitants of the deeper zones (except chemoautotrophic organisms) depend on oxygen coming from the sea-surface (Matthiessen *et al.*, 2018). As regards the benthic zone, it extends from the shore to the unexplored deep sea floor and represents a sink for the organic matter; here most of invertebrates, algae and plants live (Matthiessen *et al.*, 2018).

The vastness of the oceans makes that the biodiversity and the ecological role of several life forms are still largely unknown even in the more accessible habitats for humans (Nicoletti and Andolfi, 2018). The classified eukaryotes represent about 200,000 species, while the 90% is awaiting to be discovered, among them the majority of marine fungi (Mora *et al.*, 2011).

1.2 Marine fungi

1.2.1 Origin

The first report of a fungus in the marine environment is dated back in 1849 when the Ascomycota *Phaeosphaeria typharum* was isolated from *Thypha* sp.; while, the first record of a yeast in marine environment occurred only in 1922 (Raghukumar, 2017). Today, the origin, distribution and occurrence of marine fungi remain largely unexplored.

Based on a limited number of fossil records, fungi were already present on earth in the Proterozoic, 900-750 millions of years ago (Taylor *et al.*, 2014); however, **whether fungi evolved in the sea or on land remains an open question**. Most of the

life forms evolved in the sea and the Kingdom Fungi and Metazoa (Animalia) both grouped in the supergroup Opisthokonta (Figure 1.2) have a common ancestor (Adl *et al.*, 2012).

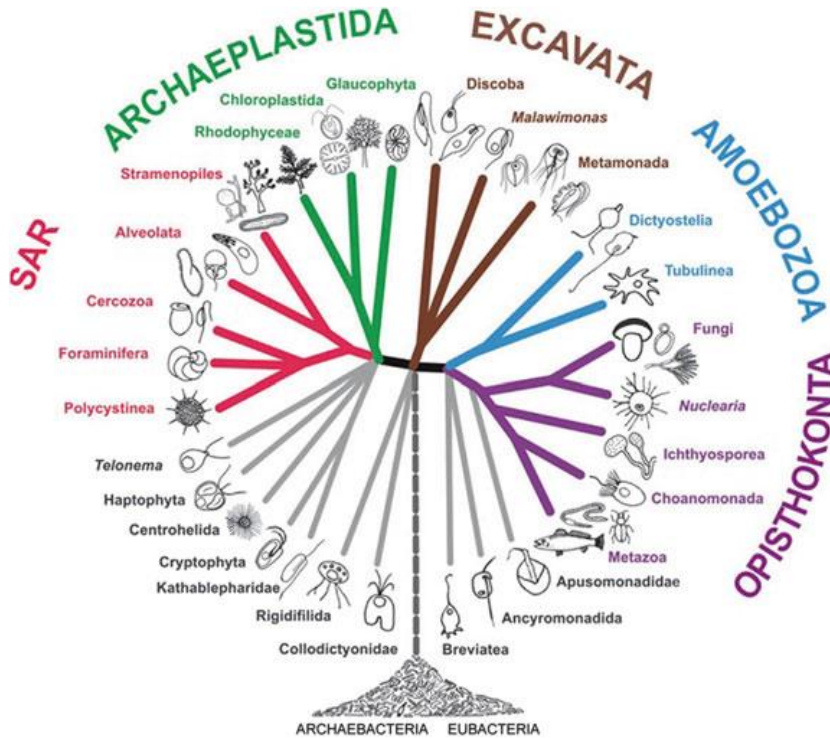


Figure 1.2 General eukaryote phylogenetic tree. The clade Fungi and Metazoa have a common ancestor and are grouped under the Opisthokonta (Adl *et al.*, 2012).

The early divergent branches of the Kingdom Fungi is represented by Microsporidia, Cryptomycota and Aphelida (Figure 1.3) mainly freshwater organisms with some representatives of marine origin, usually parasites of algae and animals (Raghukumar, 2017). In the last years, several sequences of Cryptomycota were retrieved in samples coming from hydrothermal vents and anoxic regions that most probably present environmental conditions closer to the ancestral Earth (Manohar and Raghukumar 2013; Le Calvez *et al.*, 2009). However, despite these findings, the fresh water origin seems to be the most correct (Raghukumar, 2017). Indeed, divergent of

extant fungi probably occurred from ancestral Chytridiomycota that represent the basal clade of Kingdom fungi (Figure 1.3). Chytridiomycota are aquatic organisms, with flagellated spores, that were more likely replaced by the peculiar microscopic structures of “higher fungi”, including terrestrial Ascomycota and Basidiomycota (James *et al.*, 2013).

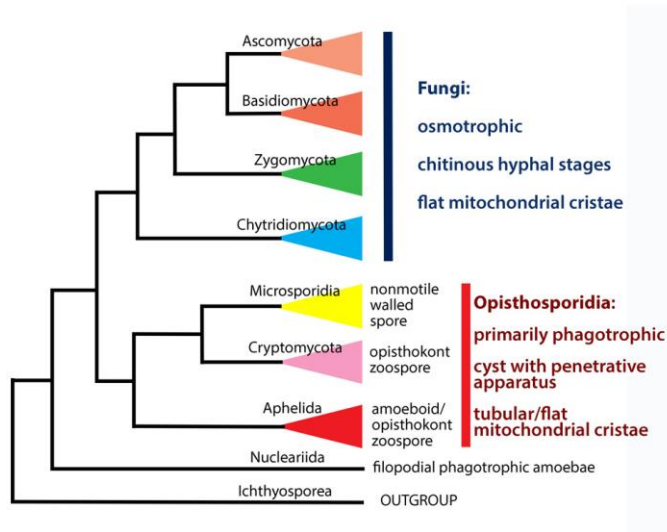


Figure 1.3 Divergent evolution of Kingdom fungi from their ancestors (Microsporidia, Cryptomycota and Aphelida) (Karpov *et al.*, 2014).

Overall, the hypothesis on the terrestrial origin of fungi is both based on the phylogenetic analysis, which sees Chytridiomycota closer to the “higher fungi”, and the recovery of the same mainly on land. To this concern, Chytridiomycota early believed to be terrestrial, are more and more found in the marine environment; surprisingly, several studies point out the dominance of Chytridiomycota sequences above all the others groups of fungi, with putative new lineages (Comeau *et al.*, 2016; Hassett and Gradinger, 2016; Richards *et al.*, 2015).

In conclusion, only new samples and data could be able to clarify the origin of fungi. In this context, SCUBA (Self-Contained Underwater Breathing Apparatus) diving

and ROV (Remotely Operated underwater vehicles) are making Oceans more and more accessible to collect samples in extreme and unexplored environments.

1.2.2 Diversity and occurrence of fungi in the marine environment

Historically, the fungal identification was based on the morphology of the reproductive structures and on the physiology of fungi. However, the morphological variability of some groups and the increasing number of cryptic isolates, indistinguishable for their morpho-physiology, created confusion within the fungal systematic (Wang *et al.*, 2012a). The advent of molecular techniques significantly increased the knowledge on marine fungal diversity; over the last two decades, thanks to the availability of nucleotide sequences and to the discovery of new marine lineages, the systematics of marine fungi has been rewritten. Today, the most updated review of marine fungi by Jones *et al.* (2015) listed 1,112 known species of marine fungi represented by Ascomycota (943 species), Basidiomycota (96 species), Chytridiomycota and related phyla (26 species), Zygomycota (three species), Blastocladiomycota (one species) and asexual morphs of filamentous fungi (43 species). The phylum most represented in terms of species (Ascomycota followed by Basidiomycota) is also the most frequently retrieved, using cultural dependent techniques (Jones *et al.*, 2015). New species and genera are continuously described in the marine environment (Bovio *et al.*, 2018; Devadatha *et al.*, 2018; Abdel-Wahab *et al.*, 2017), up-to-date the website <http://marinefungi.org/> reports 1,254 marine fungal species described. Moreover, well known terrestrial taxa are repeatedly reported for the first time in marine ecosystems (Garzoli *et al.*, 2018; Bovio *et al.*, 2017; Garzoli *et al.*, 2015a). The narrow definition of a marine fungus, proposed by Kohlmeyer and Kohlmeyer (1979): “obligate marine fungi” are those growing and reproducing only in the marine environment; “facultative marine fungi” are terrestrial species actively growing also in the marine environment, was overcome by Jones *et al.* (2015).

Despite the increasing effort of marine mycologist to contribute to the discovery of new species, thus mapping the worldwide biodiversity, marine fungi are still an understudied group compared to others marine microorganisms (Tisthammer *et al.*,

2016). Jones and collaborators (2015) estimated that about 10,000 marine fungi are still waiting to be described. The only seawater gives an idea of the fungal occurrence in the marine environment: few drops of water (one milliliter) contain over 10^3 fungal propagules (Wang *et al.*, 2012a). The distribution of fungi in the water column follow the abundance of the organic matter and its seasonal variability, occurring more frequently in the first meters and close to the coastline, compared to the deep seawaters (Wang *et al.*, 2012a). On the contrary, deep-sea sediments represent a sink for the organic matter creating a habitat where fungi are the dominant eukaryotic microbes (Nicoletti and Andolfi, 2018). Also in the deep-sea hydrothermal vents, 120 fungal species (including new ones) have been retrieved (Xu *et al.*, 2018). Among these fungi, there are pathogens of animals and saprobe that can contribute to nutrient recycling (Nicoletti and Andolfi, 2018; Xu *et al.*, 2018). The challenge for these studies is to demonstrate if the isolated fungi were also active and capable to grow in the environment from which they were recovered. The research of Burgaud and colleagues (2015) highlighted that yeasts frequently isolated from hydrothermal vents tolerate, under laboratory conditions, the pressure at which they were isolated (20 MPa), by studying their vital sign as biomass production, ribosomal and protein activity.

Several others substrates have been investigated for the isolation of marine fungi, however, due to the increasing interest in natural products, the most studied fungal communities are those associated with invertebrates, algae and plants (Raghukumar, 2017). The role of these fungi and the interactions with their hosts will be described in the next sections.

Despite all the efforts made to describe the marine fungal community, the studies available are not enough to cover the vastness of the oceans. Another important factor that influenced the knowledge of marine fungi is our capability to cultivate these organisms under standard laboratory conditions. Nowadays, the implementation of different media and isolation techniques increased the possibility of success in the cultivation of a higher number of fungi. However, the uncultivable fungi still represent the major component of the marine fungal community, hence, to describe the real biodiversity (cultivable and uncultivable species) Next Generation Sequencing (NGS)

techniques started to be largely employed. Up to date, several studies reported the fungal community of marine environment based on NGS analysis, in some case confirming at least the phyla abundance reported in cultural dependent studies. This was the case of deep-sea sediments of a hydrothermal vents system in India, where Ascomycota counted for 96.96% of the fungal community, followed by Basidiomycota (3.00%) and unidentified fungi (Xu *et al.*, 2018). A recent review of the Arctic marine fungi, reported the dominance of Ascomycota in driftwood, sediments, seawater and ice (Rämä *et al.*, 2017). On the contrary, sediments under Arctic land highlighted the dominance of Chytridiomycota; from an ecological point of view, this environment is characterised by abundant phytoplankton biomasses that provide the substrate and the niche for parasitic fungi such as Chytridiomycota (Rämä *et al.*, 2017). Chytridiomycota were also the dominant group in the water samples of Arctic and temperate zones, representing more than three-quarter of fungi in the samples from the Atlantic Ocean, while the others phyla were in the order of abundance Ascomycota, Cryptomycota and Basidiomycota (Comeau *et al.*, 2016).

Certainly, also the microbial community described by the mean of NGS analysis is strongly influenced by environmental factors; moreover, the sampling methods, the DNA extraction procedure and the sequencing techniques can condition the results. To overcome this problem, thanks to the International Census of Marine Microbes (ICoMM) project (Amaral-Zettler *et al.*, 2010), water and sediments samples were collected across the world and standard protocols were used for the identification of marine fungi. In parallel, in correspondence of the sampling sites, the environmental parameters (e.g. temperature, salinity, concentrations of phosphate, nitrate, dissolved oxygen and silicate) were registered. By analysing these data, Tisthammer and colleagues (2016) observed the dominance of Ascomycota and Basidiomycota in the sediments and water column, together with a scarce overlap (15.4%) between water and sediments OTUs. Environmental factors played the major role in shaping the fungal community (73% of the total explainable variance) compared to the geographic location (18%). Depth, dissolved oxygen and nitrate were the three main factors that explained the fungal variability. Other studies underlined the influence of temperature and salinity

in determining the distribution of marine fungi (Shearer *et al.* 2007). Overall, several physicals and chemicals factors modulate marine fungal distribution, this without taking into account the availability of abiotic and biotic substrates, where fungi are abundantly recorded.

In conclusion, although very useful to outline the real biodiversity, NGS usually do not allow low taxonomic rank classification, description of new species and biotechnological exploitation of fungi. Therefore, the improvement of specific isolation techniques and cultural media, mimicking the fungal natural environment, could be the key factor to increase the number of cultivable fungi (Raghukumar, 2017).

1.2.3 Ecology

Marine fungi are widespread in the oceans and colonise different ecological niches; they were found associated with organisms of all trophic levels and can act as saprobes, symbionts and parasites (Raghukumar, 2017). This wide ecological diversity is a consequence of the evolution of fungal cell biology and feeding strategies.

Saprotrophic

Saprotrophic fungi use dead organic matter as a food source by secretion of enzymes; compared to their prokaryotic counterpart (bacteria), they are able to penetrate organic and inorganic materials, thanks to the development of hyphae, rhizoids, or ectoplasmic nets (Raghukumar, 2017). These features make saprobe terrestrial fungi the major contributors to nutrient recycling of organic matter. Several facts indicate that marine fungi too are critical and abundant components of nutrient cycling in the oceans (Tisthammer *et al.*, 2016).

Among the most recalcitrant natural substrates, there are cellulose and chitin, the first and second most abundant biomass in nature (Alamgir, 2017). Lignocellulose materials are extremely abundant in seawater for the presence of plants and due to the wood coming from lands (Raghukumar, 2017). The recovery of fungi on these substrates is influenced by the type of wood and by climate, which determines slow colonisation rate in temperate regions (Panebianco *et al.*, 2002) compared to tropical waters

(Vishwakiran *et al.*, 2001). Moreover, it has been observed that marine fungi isolated from algae and seaweeds produce oxidative enzymes and tannases involved in the degradation of lignocellulose materials (Balabanova *et al.*, 2018). Ascomycota are the main producers of these key enzymes, replacing the role of Basidiomycota in terrestrial environment as degraders of recalcitrant organic matter (Balabanova *et al.*, 2018; Pointing and Hyde, 2000). In this context fungi offer peculiar ecosystems services: by degrading organic matter, new space becomes available for the development of seaweeds such as *P. oceanica* meadows and, by decomposing recalcitrant organic matter, nutrients make the return in the web food chain (Panno *et al.*, 2013).

Likewise the most persistent polymers of plants, the most complex matrixes of animals too are degraded by fungi, while soft tissues remain of bacteria competence: fungi degrade carbonatic shells, chitinous exoskeleton and bones. The retrieval of a fungus on an oyster shell goes back to 1891 when *Ostracoblabe implexa* was first described (Golubic *et al.*, 2005; Bornet, 1891). Shells colonised by fungi present peculiar uniform tunnels, made up by the mineralisation process of developing hyphae (Raghukumar, 2017). Similarly, Bongiorni and colleagues (2005) demonstrated that fungi recorded on the exoskeleton of dead crabs' were able to produce several enzymes, including chitinase (N-acetyl- β -glucosaminidase), involved in the exoskeleton decomposition. Interestingly, prawn wastes increased chitinase production in the marine fungus *Beauveria bassiana* (Suresh and Chandrasekaran, 1998).

Parasites and pathogens

Fungal parasites and pathogens are associated with organisms of all trophic levels and play an essential role in the ecosystem functioning (Raghukumar, 2017). Indeed, they maintain a balance in the ecosystems diversity and only under exceptional conditions, they can devastate a population; hence, the organic matter becomes soon available to saprotrophs to return in the nutrient cycles (Raghukumar, 2017).

Environmental factors, such as the increase of water temperature or oceans acidification have been addressed as one of the causes that determine commensal or mutualistic fungi to become pathogens. This was the case of *Aspergillus sidowii*, the

causal agent of “sea fans diseases”, detected both on healthy and diseased *Gorgonia* spp. (Toledo-Hernandez *et al.*, 2008); experimental studies highlighted that *A. sidowii* grows better as temperature rise, becoming able to extensively parasitize corals (Alker *et al.*, 2001). Generally, climate changes and others stress factors, as livestock overpopulation may induce physiological stresses in organisms, making them susceptible to diseases; therefore, weak parasitic fungi may become highly virulent (Harvell *et al.*, 1999). Indeed, what we know about fungal pathogens of marine animals mainly comes from aquacultures studies (Raghukumar, 2017). Several species of commercial prawns, shrimps, lobsters, crabs and fish have been affected by mycosis. To cite the most virulent diseases, the mortality rate of *Penaeus japonicus* raised up to 100% when injected with a conidial suspension of the pathogen *Acremonium* sp. (Raghukumar, 2017). The same mortality, caused by *Atkinsiella dubia* is observed in the larvae of the Japanese mitten crab *Eriocheir japonicus* (Raghukumar, 2017). As for fish, *Ochroconis humicola* determine the death of several striped jack *Pseudocaranx dentex* in a fish farm in Japan; the same fish was infected by *Exophiala xenobiotica* in another fish farm in Japan (Raghukumar, 2017). Probably, the same pathogens of aquaculture are found in wild animals, with repercussions on the global food chain.

Parasitic fungi can be also among the major contributors in the nutrient cycling; this is the case of Chytridiomycota, (pathogens of phytoplankton, zooplankton, animals, fungi and plants) the main actors of the “mycoloop”, firstly described in fresh water (Kagami *et al.*, 2014). The “mycoloop” concept provides that large inedible phytoplankton species are infected by Chytridiomycota; the nutrients within host cells are consumed by Chytrids, and transferred, thanks to the zoospores, to zooplankton (Kagami *et al.*, 2014). Therefore, Chytridiomycota transfer nutrients that otherwise would be lost by sinking from the euphotic zone (Kagami *et al.*, 2014). Noteworthy, zooplankton is able to control the population of Chytridiomycota with a positive impact for example on the reduction of chytridiomycosis in fresh water amphibians (Kagami *et al.*, 2014).

Despite the “mycoloop” have been mainly studied in fresh water ecosystems, also marine Chytridiomycota are recognised as actors of the “mycoloop” (Reich *et al.*,

2017; Hassett and Gradinger, 2016). Probably several others “mycoloop” exist and are still waiting to be discovered; recently a new one has been reported in the lakes, where Chytridiomycota play a key role transferring the nutrients from the inedible pollens to the zooplankton (Kagami *et al.*, 2017).

Symbionts

The two major mutualistic symbioses known in terrestrial environment are represented by mycorrhizae (plant-fungus association) and lichens (alga/cyanobacterium-fungus association). Lichens count about 700 species in the marine ecosystem, with Ascomycota mainly involved in the symbiosis. Marine lichens are frequently reported in the supralittoral and intertidal zone (Raghukumar, 2017), where they are exposed to high solar irradiance and, with the high tide, they are covered by seawater (Lipnicki, 2015). These extreme conditions induced phenotypic variability (morphologically and anatomically) in lichens and influenced their distribution (Lipnicki, 2015). Interestingly, few lichens have been reported also below the tide level, on algae and shells (Raghukumar, 2017).

As for the other most common symbiosis in the terrestrial environment, mycorrhizae have been reported in marine ecosystems only in the salt-marsh plant (Hyde *et al.*, 1998). However, preliminary data hypothesised mycorrhizal relationship also in the roots of the seagrasses *P. oceanica* (Vohník *et al.*, 2017) and *Enhalus acoroides* (Sakayaroj *et al.*, 2010).

Several other symbiotic interactions have been hypothesised in the marine environment (sponges, corals, echinoderms, arthropods, etc.). For instance, fungi were retrieved in the gut and digestive tract of sea urchins and marine arthropods, probably contributing to break down complex polymers (Raghukumar, 2017). In the three species of the marine sponge *Chondrilla* sp., yeast cells were found to be maternally-transmitted, transferred from the soma through the oocytes to the fertilized eggs (Maldonado *et al.*, 2005). Noteworthy, this symbiosis was found in different geographical locations (Mediterranean area, Caribbean and Australia).

Within corals, fungi may be involved in the nitrogen cycle, making this element available to corals that usually live in low-nitrogen waters; in parallel, fungi may protect their host from UV radiations. All these findings are supported by metabolomic studies of fungi inhabiting corals (Raghukumar, 2017).

However, few studies clearly state the role of fungi within their hosts; because of this, the term symbiosis should refer only to different organisms that live together for a long period of time (Taylor *et al.*, 2007).

1.3 Marine organisms and microorganisms: an unlimited source of novel compounds

Bioactive marine natural products, deriving from both primary and secondary metabolisms, count for 28,609 new molecules with 1,277 new compounds only described in 2016 (Blunt *et al.*, 2018). This research field has important economic implications: the global market of marine-derived natural products is around 5 billion dollars (Thompson *et al.*, 2017).

Up to date, the main problem concerning the use of marine organisms is due to the difficulties to grow them under controlled conditions and the impossibility to collect large amounts of samples from the environment. As an example, a huge amount (1 tonnes - wet weight) of the tunicate *Ecteinascidia turbinata* or of the sponge *Halichondria okadai* would be necessary to obtain few milligrams of two promising anticancer compounds without resorting to the chemical synthesis (Jackson *et al.*, 2015; Proksch *et al.*, 2003).

In order to overcome the supply problem, natural products research has started focusing on marine microorganisms, including fungi that can be easily cultivated and manipulate (Silber *et al.*, 2016). In addition, it is more than likely that bioactive molecules known produced by marine organisms are of bacterial or fungal origin (Jackson *et al.*, 2015; Henriquez *et al.*, 2014).

1.3.1 The Mycotechnology

The term mycotechnology was introduced to indicate the wide range of applications of fungi in biotechnology, from industrial processes to bioremediation and development of new drugs (Nicoletti and Andolfi, 2018). In this context marine fungi, in light also of the putative ecological role that cover in the sea, represent a promising source of new secondary metabolites, extremophilic enzymes and bioremediation agents.

Secondary metabolites

Marine fungi are the candidates to supply the huge amount of pharmaceutical required worldwide, i.e. 100,000 tons per year only for antibiotics (Silber *et al.*, 2016). In addition, their biotechnological potential is incontestable: among the 1,277 new natural products described in 2016, marine fungi account for 36% of these newly described molecules (Blunt *et al.*, 2018). In five years (2010-2015), 285 antibacterial and antifungal compounds were isolated from marine fungi (Nicoletti and Andolfi, 2018). Several molecules isolated from marine fungi proved to be active against the high priority strains of *Staphylococcus aureus* methicillin resistant (MRSA). For instance, two compounds produced by the fungi *Diaporthaceae* sp. PSU-SP2/4 and *Nigrospora* sp. MA75 displayed a Minimal Inhibitory Concentration (MIC) of 2 µg/mL and 8 µg/mL against *S. aureus* MRSA (Xu *et al.*, 2015). As for the most interesting antifungal compounds, Didymellamide A produced by the marine fungus *Stagonosporopsis cucurbitacearum* was active against azole-resistant and -sensitive *Candida albicans*, *Candida glabrata*, and *Candida neoformans* strains with MIC values ranging from 1.6 to 3.1 µg/mL (El-Hossary *et al.*, 2017).

New anticancer with a better pharmaco-toxicological profile are also needed, malignant tumours are responsible for about 8.8 million victims every year (1/6 of deaths) (Calcabrini *et al.*, 2017). Up to date, several compounds produced by marine fungi have been described for the anticancer activity; however, molecules with cytotoxic activity are often wrongly named anticancer compounds. An anticancer should be selective for cancer cells and preferably be active against cancer cells resistant to already

known drugs and with a mechanism different from the induction of apoptosis (Deshmukh *et al.*, 2018; Gomes *et al.*, 2015). There are some promising compounds produced by marine fungi. For instance, Pinophilin A produced by the marine fungus *Penicillium pinophilum* is selective against cancer cells line, with a non-apoptotic mechanism (Gomes *et al.*, 2015; Myobatake *et al.*, 2012). An unidentified species of *Penicillium* sp. produced several gliotoxin analogous with an innovative anticancer mechanism, targeting enzymes commonly involved in the malignant transformation of cells (Gomes *et al.*, 2015; Sun *et al.*, 2011). Ophiobolin O originally isolated from *Aspergillus ustus* and its synthetic analogous present several mechanisms of actions and was found able to kill glioblastoma cells that are apoptosis-resistant (Lv *et al.*, 2015). Another strain of *A. ustus* produced a molecule named phenylahistin, whose synthetic derivate (plinabulin) after 20 years is still in the clinical stage (phase II) despite the promising anti-cancer activity, underlining the long time required before obtain a new drug ready to enter into the market (Mohanlal *et al.*, 2018; Nicoletti and Andolfi, 2018; Kanoh *et al.*, 1997).

As for the antiviral, there are promising compounds produced by marine fungi targeting several viruses with different replication mechanisms (Moghadamtousi *et al.*, 2015). For instance, two fungi, *Aspergillus terreus* and an unidentified deep sea fungus were able to inhibit Influenza strains resistant to the currently available drugs (Abdelmohsen *et al.*, 2017). The fungus *Dichotomomyces cejpilii* F31-1 produced the scequinadoline A, significantly able to inhibit the dengue virus (Wu *et al.*, 2018). Two fungi *Fusarium heterosporum* and *Phoma* sp. produced Equisetin and its enantiomeric homologous, phomasetin, respectively, targeting an important enzyme for the replication of the Human Immunodeficiency Virus (HIV) (Moghadamtousi *et al.*, 2015).

Enzymes

Marine fungi are an appealing source of extremophilic enzymes that preserve their structure and activity at low temperatures, high salt concentrations and pH (Nicoletti and Andolfi, 2018). Several applications can be found for these enzymes. For instance, in the degradation of recalcitrant plants residues as cellulose and inulin, to

obtain ethanol suitable for the biofuel (Nicoletti and Andolfi, 2018; Rawat *et al.*, 2017). Also, the recalcitrant waste of the paper industries can be treated with fungal enzymes or using the fungus alive, as demonstrated by Chen and collaborators (2014) with *Penicillium janthinellum* and *Pestalopsis* sp.. Other environmental applications are based on the use of marine fungal enzymes such as azoreductases, peroxidases and laccases for the degradation of textile effluents (Nicoletti and Andolfi, 2018; Theerachat *et al.*, 2018). Overall, the advantages of using marine fungi lie in their ability to grow at high concentrations of salt or with high osmotic pressure, as it is common in industrial wastewaters (Nicoletti and Andolfi, 2018).

Others recalcitrant polymers as lignin and chitin can be degraded by marine fungi, as already mentioned in the section of saprotrophic fungi with, as result, the valorisation of waste materials.

Bioremediation agents

Bio-based systems to treat crude oil polluted sites, offer interesting alternatives to the classic methods, is an economic and environmentally friendly alternative (Zhang *et al.*, 2011). Due to their robustness and adaptation skill, marine fungi can be directly employed as bioremediation agents and proved to be highly successful in crude oil degradation, facing one of the biggest environmental issues, oil spill. Several fungi can use aliphatic compounds as a sole carbon source, while a lower percentage can also use aromatic compounds. This is the case of several *Aspergillus* spp. capable of degrading benzo[a]pyrene (Nicoletti and Andolfi, 2018; Passarini *et al.*, 2011). The main limit to the degradation process is the availability of the compounds to microorganisms, in this context biosurfactants and emulsificants can play a key role. This is even better if they are produced by marine fungi, for instance, hydrophobins are the most powerful surface-proteins known so far (Artini *et al.*, 2017; Cicatiello *et al.*, 2017; Piscitelli *et al.*, 2017; Cicatiello *et al.*, 2016).

Another environmental issue is represented by heavy metals, such as Cd(II), Hg(II), and Pb(II) that are abundant in the environment due to industrialisation and are responsible for several human pathologies (Mahmoud *et al.*, 2017). Scientific studies

underlined a correlation between halophilism and tolerance to heavy metals, making of marine fungi the best candidates in bioremediation of heavy metals (Nicoletti and Andolfi, 2018). For instance, several deep-sea fungi accumulate heavy metals in different cells districts, including cell wall, vacuoles and cytoplasm (Nicoletti and Andolfi, 2018). Certainly, the selective pressure played by the accumulation of heavy metals in the ocean floor might have been the driving force for the outliving of these highly efficient fungi. Finally, even dead fungal biomass is extremely useful for the biosorption of heavy metals, as in the case of *A. ustus* (Mahmoud *et al.*, 2017).

1.4 The sponge holobiont

Marine sponges (phylum Porifera) are the oldest metazoans on Earth; over their 660–635 M years evolution formed a close association with a wide variety of microorganisms including bacteria, archaea, fungi, and algae (Zumberge *et al.*, 2018; Taylor *et al.*, 2007). This close association was described for the first time by Vacelet and Donadey (1977) who observed bacteria within the sponges' tissues. Today, it is well recognised that microorganisms represent up to 40–60% of the sponge biomass (Yarden 2014). Therefore, the term “sponge holobiont” is more appropriate when sponges and the associated microbial communities are considered as a whole (Webster and Thomas 2016; He *et al.*, 2014). Holobionts are complex systems whose function is determined by both the processes carried out by the single members and those resulting from their interactions (Pita *et al.*, 2018). The stability and health of the holobiont are the results of a dynamic equilibrium characterised by the resistance and the resilience to external pressure (Pita *et al.*, 2018). Therefore, improving our knowledge of the sponge holobiont, will help us to understand their ability to survive to climate changes, maintaining their key role in the ecosystems.

1.4.1 Sponges

1.4.1.1 Origins, anatomy and physiology

Porifera counts more than 8,600 described species and about 15,000 estimated, not yet discovered (Webster and Thomas 2016). Sponges are globally distributed (Figure

1.4) with the main hotspots of biodiversity across the coasts of Europe (Mediterranean Sea and Atlantic Ocean); among the benthic community, they are the most abundant organisms in terms of coverage, biomass and volume (Jackson *et al.*, 2015).

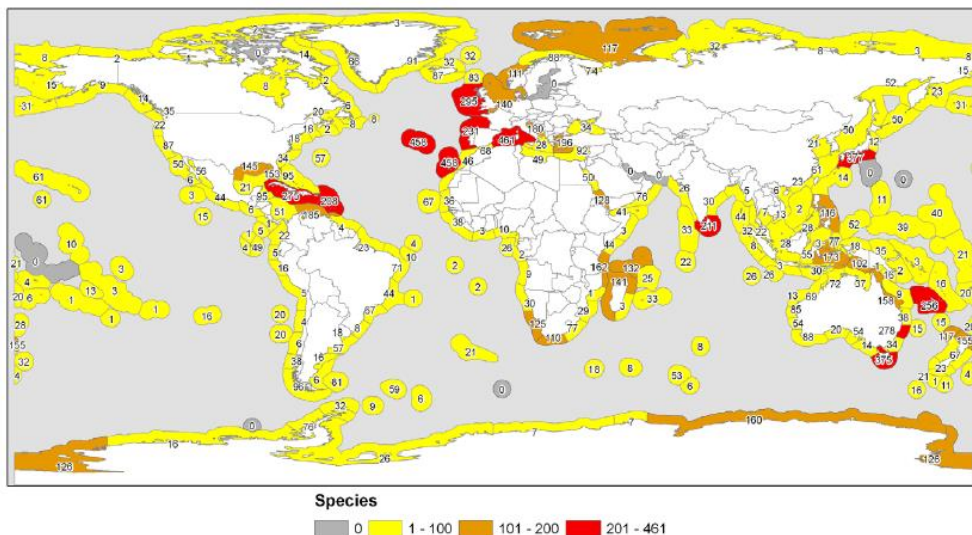


Figure 1.4 Global distribution of marine sponges (Van Soest *et al.*, 2012).

Sponge architecture and morphology greatly affect sponge biology, including interactions with microorganisms (Taylor *et al.*, 2007). Their aspects vary from branching, barrel and cup-shape organisms, to encrusting species closely associated with the substrate. Their “skeleton” composition can be incredibly different and is one of the major morphological features used for classification. The Classes Calcarea and Hexactinellida have calcareous and siliceous spicules, respectively. Demospongia and Homoscleromorpha can be made by a combination of siliceous spicules and spongin (a collagenous protein) or be aspiculate with a sponging “skeleton” (Jackson *et al.*, 2015). The sponge body presents an outer epithelial layer (pinacoderm) made by pinacocytes interspersed with porocytes cell, forming an aquiferous system that connects the extern environment with the inner part. Inside the sponges, flagellated cells (choanocytes) pump and filter water through the ostia (Figure 1.5). Microorganisms present in the water are then phagocytate by archaeocytes in the mesohyl (Jackson *et al.*, 2015). These

morphological features make marine sponges able to process up to 24,000 L/kg of seawater per day and to detain over 80% of its particles (Taylor *et al.*, 2007). The only exception to the general sponges-morphology is made by few deep-sea carnivorous species (about 120) that capture their preys on the outer surface; then, specialised cells migrate and phagocytise the prey prior to digest it (Jackson *et al.*, 2015).

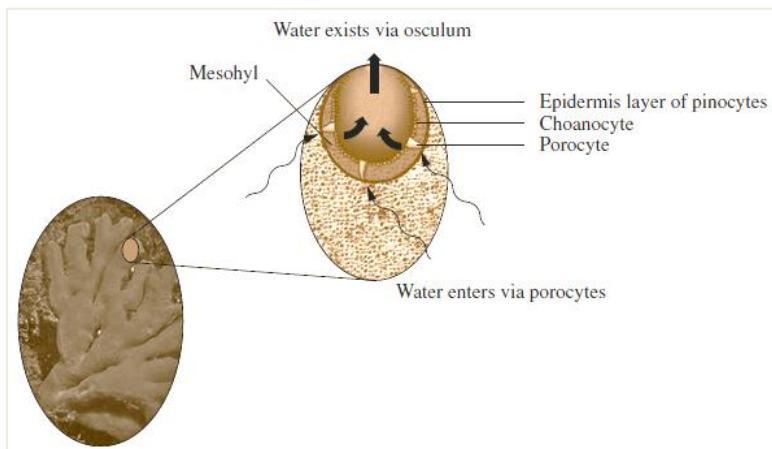


Figure 1.5 General anatomy of a marine sponge (Jackson *et al.*, 2015).

1.4.1.2 Sponges, as producers of secondary metabolites

In 1951 the chemistry of natural products began with the isolation from the sponge *Cryptotethya crypta* of two nucleosides: spongouridin and spongothymidin, used as the base structure for new antiviral drugs as Ara-A (Anjum *et al.*, 2016). Up to date, more novel bioactive compounds were obtained from sponges than from any other marine organism (Mehbub *et al.*, 2014; Taylor *et al.*, 2007). Indeed, marine sponges are the richest source of natural products with about 200 new molecules described every year for a total of 5,300 compounds, counting for 30% of marine natural products discovery (Anjum *et al.*, 2016; Mehbub *et al.*, 2014). The ecological meaning of the production of secondary metabolites by marine sponges must be sought in their lifestyle: attached to the substrate, they are unable to escape from predators and any organisms could grow on them (Anjum *et al.*, 2016). Therefore, sponges have evolved both chemical defence mechanisms and physiological responses (Anjum *et al.*, 2016). As

other sessile organisms, they harbour toxic compounds or produce chemicals against predators, pathogens or fouling organisms (Anjum *et al.*, 2016). Concerning the physiological responses, it has been demonstrated that sponges produce collagen to confined bacterial and fungal pathogens and survive to the infections (Maldonado *et al.*, 2010).

The same molecules produced by sponges with interesting ecological implications on the other side have important pharmaceutical applications, as anti-cancer, anti-viral and antibiotics (Anjum *et al.*, 2016).

1.4.2 Fungi associated with sponges

Marine sponges are one of the richest sources of fungal diversity; however, when compared to bacteria, very little is known about fungi and their ecological role within sponges (Suryanarayanan, 2012). In the next months and years, the “Global Sponge Microbiome Project” that aims to study the microorganisms associated with sponges and that already provided data on bacteria, will focus also on fungi (Pita *et al.*, 2018). As a consequence, more data on the distribution, abundance and specificity of fungi associated with sponges will emerge.

Up to date, fungi have been detected inside sponges using both cultural dependent and independent methods (NGS analysis), underlining a dynamic fungal community (Yarden, 2014).

The culturomic approach requires the isolation of marine fungi from sponges. These animals can be extremely fragile and difficult to manipulate, therefore strong sterilisation process should be avoided to preserve tissues integrity (Suryanarayanan, 2012; Höller *et al.*, 2000). In order to remove external contaminants and sediments, sponges can be surface sterilised with ethanol and then washed several times with seawater (Suryanarayanan, 2012). To isolate fungi, the sponges can be: i) directly plated on agar media, ii) squeezed to extract the water contained in the inner body onto plates with media, iii) homogenised and spread on the plates. Innovative methods report also the isolation of fungi from single sponge cells (Rozas *et al.*, 2011). As for media, those salt-based are widely used to promote the development of halophytic fungi; the use of

antibiotics is recommended to reduce bacterial contaminations (Diep *et al.*, 2016; Sayed *et al.*, 2016; Henríquez *et al.*, 2014; Passarini *et al.*, 2013; Suryanarayanan, 2012; Ding *et al.*, 2011; Wiese *et al.*, 2011; Paz *et al.*, 2010; Li and Wang 2009; Proksch *et al.*, 2008; Wang *et al.*, 2008; Höller *et al.*, 2000).

Overall, the culturomic approach highlights Ascomycota as the phylum most frequently reported in marine sponges and usually includes several representatives of the orders Capnodiales, Eurotiales, Hypocreales and Pleosporales (Suryanarayanan, 2012). As for the others phyla, studies based on cultural dependent approach, reported from three (Paz *et al.*, 2010) to one (Ding *et al.*, 2011; Li and Wang, 2009), or none (Passarini *et al.*, 2013; Thirunavukkarasu *et al.*, 2012; Proksch *et al.*, 2008; Wang *et al.*, 2008; Pivking *et al.*, 2006; Höller *et al.*, 2000) Basidiomycota. Even less frequent are the reports of Mucoromycota, with only few individuals recorded in a limited number of sponges (Passarini *et al.*, 2013; Thirunavukkarasu *et al.*, 2012; Morrison-Gardiner, 2002; Höller *et al.*, 2000).

As for the fungal species retrieved within sponges, several are of terrestrial origins, probably due to the filter feeding nature of sponges, able to process incredible volume of water and to retain suspended particles also coming from land (Raghukumar, 2017). However, even fungi of terrestrial origins have been reported to have peculiar growth (in presence of salts) and to produce metabolites never detected before in their terrestrial counterpart (Suryanarayanan, 2012).

Li and Wang (2009) to distinguish between transient mycobiota, abundant in the water column and true sponge associated fungi proposed a more useful classification. In detail, “sponge specialist” are those genera exclusive of one sponge species, “sponge associates” is used for genera present in more than one species of sponge and “sponge generalist” for fungal genera present on all the species of sponges analysed.

Overall, until now the data presented were referred to the culture dependent method to study marine fungi associated with sponges, aiming also to the biotechnological exploitation of fungi. However, there are also studies covering the real biodiversity of fungi inhabiting sponges, using cultural independent approaches. When the data obtained from NGS studies are compared with those of the fungi effectively

isolated from sponges, confirm on one side the dominance of Ascomycota (De Mares *et al.*, 2017; Passarini *et al.*, 2015; He *et al.*, 2014; Jin *et al.*, 2014), on the other side, shown an almost equal distribution of Ascomycota and Basidiomycota. For instance, in *Suberites zeteki* and *Mycale armata* (Hawaii) 7 and 4 orders of fungi belonging to Ascomycota and Basidiomycota, respectively (Gao *et al.*, 2008). Overall, a comparison of the performance of the culture-dependent and culture-independent methods would be possible only focusing on the same sponge species. Indeed, with the available data, it is possible to state that fungal diversity within sponges vary with the geographical location (Bolaños *et al.*, 2015) and could be influenced from several others factors as the sponge structure (Pivkin *et al.*, 2006). Moreover, bias in our capability to properly isolate and cultivate marine fungi exists (Ding *et al.*, 2011).

A long-standing question to which microbiologists addressed their studies concerned **how sponges discriminate between food and symbionts** (Jackson *et al.*, 2015). To date a univocal answer does not exist; however, looking for cell recognition, adhesion, and signaling mechanisms, there are proofs that sponges are able to recognise microorganisms. For instance, the discovery of sponge mitochondrial introns of fungal origin and of (1-3)- β -d-glucan-binding proteins on the sponge surface for fungus recognition are the strongest proof supporting the ability of sponges to identify and select fungi (Suryanarayanan, 2012).

However, once recognised, **which is the role of fungi within sponges?** Different degrees of complexity characterise the interactions among sponge holobiont components, including mutualism, commensalism and parasitism (Rodríguez-Marconi 2015). Non-pathogenic microorganisms can positively contribute to sponge metabolism, by increasing the uptake of nitrogen, sulphur and carbon, producing hydrolytic enzymes able to convert complex organic matter in easily accessible nutrients for sponges (Pita *et al.*, 2018; Debbab *et al.*, 2012; Taylor *et al.*, 2007). This concept is behind the “sponge loop”, up to date defined only for bacteria (Figure 1.6). The prokaryotic component of the sponge holobiont can be responsible for of up to 90% of the holobiont’s total heterotrophic carbon uptake, the dissolved organic matter is then transformed in the

particulate organic matter, easily accessible to the inhabiting of the reef (Pita *et al.*, 2018).

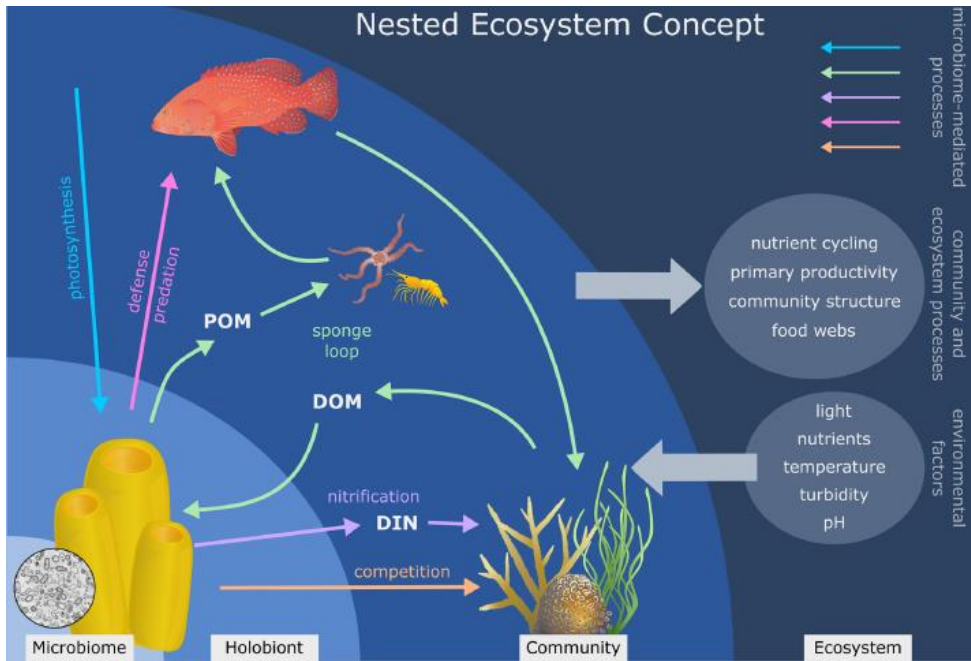


Figure 1.6 The coloured arrows report the key functions carried out by the microbiome that directly influences the sponge holobiont and indirectly play ecosystems services influencing nutrient cycles and the web food chain (Pita *et al.*, 2018).

The most fascinating theory is that marine fungi produce a wide range of bioactive molecules acting as a chemical defence against sponges’ predators, pathogens and fouling organisms (Debbab *et al.*, 2012). This statement is supported by the high yield of bioactive molecules isolated from sponge’s derived fungi (Debbab *et al.*, 2012). So far, metabolites knew to be produced by sponges could be synthesised by fungi, as partially proved for bacteria (Figure 1.6) producing molecules structurally similar to those isolated from sponges (Henríquez *et al.*, 2014; Imhoff and Stöhr 2003).

1.4.3 Secondary metabolites from marine fungi associated with sponges

Sponge's derived fungi are one of the most prolific sources of new molecules (Imhoff, 2016; Indraningrat *et al.*, 2016; Suryanarayanan, 2012). They count for the highest number of total compounds (33%) and novel metabolites (28%) isolated from marine fungi (Imhoff, 2016). However, these high values could be explained by the biochemical interest for sponges and the high number of fungal isolates usually recorded from these filter-feeding organisms (Imhoff, 2016).

The first discovery of a molecule from a sponge derived fungus is dated back to 1993 when the Trichoharzin was isolated from a strain of *Trichoderma harzianum* associated with the sponge *Mycale cecilia* (Kobayashi *et al.*, 1993). Up to date several sponge derived fungi yielded new molecules with antimicrobial activity (antibacterial, antiviral, antifungal, antiprotozoal), especially if compared with bacteria (Figure 1.7A). Figure 1.7B report the most prolific genera, where *Aspergillus* and *Penicillium* play the major role (Blunt *et al.*, 2018; Nicoletti and Andolfi, 2018; Imhoff, 2016; Indraningrat *et al.*, 2016). As for the antibacterial activity, where most of the molecules are active only on Gram-positive strains, an *Aspergillus* sp. showed the lowest MIC value against *Escherichia coli* among all sponges associated microbes (Indraningrat *et al.*, 2016). While, a strain of *Trichoderma* sp. produce the compound trichoderin A with interesting activity on different *Mycobacterium* spp. responsible for tuberculosis, by inhibiting the adenosine triphosphate (ATP) synthesis (Indraningrat *et al.*, 2016).

The massive use of antibiotics has on one side increased the cases of antibiotic resistance, on the other side, together with the immunosuppressive agents used after transplantation, have drastically raised the cases of fungal infections (Indraningrat *et al.*, 2016). Particular promising for the broad spectrum of antifungal activity is a sponge derived fungus *Phoma* sp. that produce a new lactone compound capable to contrast several human pathogens, from *C. albicans* to *Aspergillus fumigatus* (Indraningrat *et al.*, 2016).

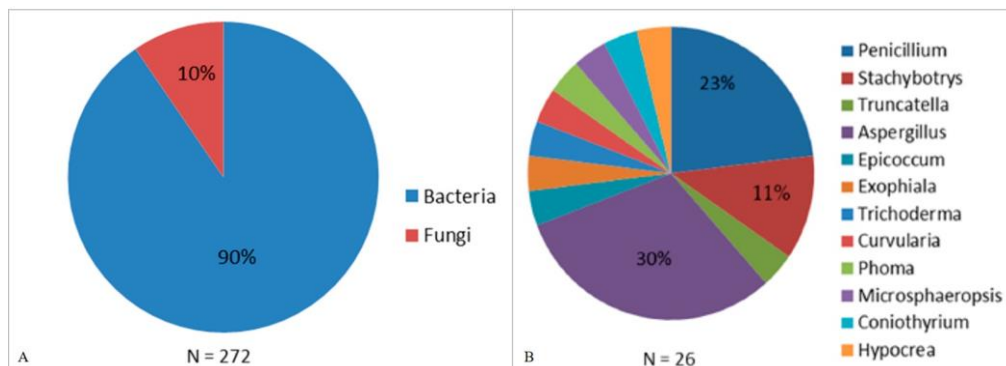


Figure 1.7 Percentage of microorganisms associated with sponges that produce antimicrobial molecules (antibacterial, antiviral, antifungal, antiprotozoal). **B.** Sponge derived fungal genera from which new molecules with antimicrobial activity were isolated (Indraningrat *et al.*, 2016).

Despite being usually neglected to evaluate the biological activity of new molecules, the viruses are a worldwide threat. To this regards, several sponges derived fungi have been found to inhibit at different stages of the HIV viral cycle, they include *Penicillium chrysogenum* and *Stachybotrys chartarum* (Abdelmohsen *et al.*, 2017; Indraningrat *et al.*, 2016), typical terrestrial fungi with unique metabolites when recovered in the marine environment. Noteworthy, the metabolites produced by the sponge derived fungus *Truncatella angustata* are able to inhibit the Influenza A virus, with truncateol M active six-fold lower than an anti-Influenza drug (oseltamivir) (Indraningrat *et al.*, 2016).

Several metabolites produced by fungi isolated from sponges can have also an implication in the agriculture field. For instance, the sponge derived fungus *Penicillium adametzioides* AS-53 produce the peniciadametizine A, selective active against the plant pathogenic fungus *Alternaria brassicae* (MIC 4.0 µg/mL), that damage important crops such as broccoli, cabbage and oilseed rape (El-Hossary *et al.*, 2017). Other two strains identified as plant pathogens, the yeasts *Cryptococcus neoformans* and *Saccharomyces cerevisiae* were positively contrasted (Inhibitor concentration - IC₅₀ 1.56 µg/mL) by a molecule (YM-202204) produced by *Phoma* sp. Q60596, isolated from the marine sponge *Halichondria japonica* (El-Hossary *et al.*, 2017). Remaining in the same sector, fungi isolated from sponges proved to be bioremediation agents, for instance by

degrading the pesticide DDD (1,1-dichloro-2,2-bis-(4-chlorophenyl)ethane), nowadays banned but still persistent in the environment (Nicoletti and Andolfi, 2018)

Fungi isolated from sponges produce also several enzymes, including chitinase and several lignocellulolytic enzymes with an important environmental implication in the management of waste, as above described (Nicoletti and Andolfi, 2018; Batista-Garcia *et al.*, 2017). Further environmental applications of the molecules produced by sponges-derived fungi, such as in the formulation of new eco-friendly antifouling will be later described.

1.5 Classical and epigenetic approach to diversify the production of secondary metabolites in marine fungi

The production of secondary metabolites by fungi is strictly related to the growth conditions. Indeed, one of the driving forces for the production of new metabolites, compared to the constitutive one, usually produced in any conditions, is the simulation of marine fungi-native environment, including the interactions with other microorganisms as usually occur in nature (Vallet *et al.*, 2017; Takahashi *et al.*, 2013). As consequence, growing fungi under standard conditions do not always promote the production of interesting secondary metabolites or of at least the native ones (Reich and Labes, 2017). In 2002, Bode and collaborators highlighted that the modification of easily and accessible growing parameters can activate silent genes that determine the synthesis of new secondary metabolites; this method was called OSMAC approach (One Strain - Many Compounds, Bode *et al.*, 2002). Today, the manipulation of the fermentation conditions and the use of epigenetic modifiers are widely recognised as an effective tool to stimulate the production of secondary metabolites and find new bioactive molecules in fungi (Romano *et al.*, 2018; Adpressa and Loesgen, 2016; González-Menéndez *et al.*, 2016; Yue *et al.*, 2015; VanderMolen *et al.*, 2013; Bills *et al.*, 2008).

The main parameters manipulated to diversify the metabolome of marine fungi are here summarised.

Cultural media and abiotic factors

Fungi are heterotrophic organisms and present different nutrient requirements, therefore, the use of several media increase the possibility of successful isolate new metabolites. Bills and colleagues (2008) in a large screening program aimed to assess the antifungal activity of fungal crude extracts highlighted that, the positive results increase an average of 89% using twelve media compared to three. Bode and colleagues (2002) in the work describing the OSMAC approach highlighted that a strain of *Aspergillus ochraceus* known to synthesize the only aspinonene, when growth in different media, produced up to 15 new molecules.

Carbohydrates are the main nutrients driving the fungal metabolisms, by modulating their concentration and the type of supply is possible to increase the production of the desired molecules or to obtain new ones (Takahashi *et al.*, 2013). For instance, to increase the yield of the target molecule Calcaride A, the medium of the marine fungus *Calcarisporium* sp. KF525 was supplemented with different carbon sources (glucose, sucrose, fructose, maltose, lactose, malt extract or starch). Sucrose and fructose significantly increased the production of Calcaride A compared to the others carbon supplies (Tamminen *et al.*, 2015). Likewise, the modulation of the nitrogen source (yeast extract or peptone) demonstrated to be fundamental to differentiate the metabolic production of the marine fungus *A. sydowii* (Da Silva Lima *et al.*, 2018).

The introduction of salts in the media is another frequently used condition to diversify the production of secondary metabolites, with contrasting results (Takahashi *et al.*, 2013). A study involving 47 marine fungal isolates underlined that most of them (91.5%) grew better with salts, but only few fungi (14.9%) increased their antimicrobial activity (Huang *et al.*, 2011). However, the only evaluation of the antimicrobial activity could be limiting, considering the wide range of target that fungal extracts have. Zheng and colleagues (2013) demonstrated, by analysing the chemical fingerprint of the marine strain *Aspergillus flocculosus* PT05-1, that more and different metabolites were produced with 10% of salts compared to 3%. Interestingly, salts and in general osmotic stress, diversify the metabolome also of terrestrial fungi (Overy *et al.*, 2017; Wijesekera *et al.*, 2017).

Another interesting parameter directly involved in enzymatic activation is pH; for instance, its modification can change the fatty acids and sterols composition in the marine fungus *Epicoccum nigrum* (Ahumada-Rudolph *et al.*, 2014). The control of pH becomes crucial for the successful scale-up of fungal culture in bioreactors (Silber *et al.*, 2016).

The creation of fungal mutants has become another commonly employed technique for the activation of silent genes. High concentrations of antibiotics can generate resistance in the fungal strains. For instance, the marine derived fungus *Penicillium purpurogenum* G59 after exposure to neomycin produced the new molecule Chromosulfine thanks to the activation of a biosynthetic pathway silent in the wild type strain (Yi *et al.*, 2016). Another successful way to create mutants is based on the exposure of fungi to different UV radiations. For instance, Kramer and colleagues (2014) after UV exposure of *Scopulariopsis brevicaulis* LF580 obtained mutants able to increase up to tenfold the production of the target metabolite scopularide A.

Overall, there are plenty of possibilities to modify the culture conditions to obtain new molecules; the challenge is to find the one successful for the target fungal strain.

Biotic factors (co-culture)

Microorganisms in nature produce secondary metabolites to interact with the surrounding living communities. In order to mimic these interactions, the co-culture (solid media) or mixed fermentation (liquid media) with others organisms is a widely applied strategy (Romano *et al.*, 2018). The interactions can be modulated to mimic the natural environment for chemical-ecological study, including symbiosis studies; while, specific organisms can be introduced to promote the synthesis of antibiotics (Romano *et al.*, 2018; Bertrand *et al.*, 2014). The same technique can stimulate the production of molecules modulating the quorum sensing, with an application as antibiotics (Bertrand *et al.*, 2014).

The production of new molecules has been demonstrated to be related to the activation of silent genes, however, how this happens usually remain unknown (Bertrand

et al., 2014). Probably microorganisms are able to produce molecules that act as epigenetic modifiers; this was demonstrated for a strain of *A. fumigatus* that in co-culture with a bacterium activates the same silent pathway as with the addition of an epigenetic modifier (König *et al.*, 2013). Unfortunately, few studies focus on the reasons behind the synthesis of new molecules or compare the results obtained by the co-culture with the pure culture. In this regard, Özkaya and collaborators (2017) worked on the extracts of 70 marine fungi isolated from sponges and tested their bioactivity against bacterial pathogens of aquaculture. Overall, 23% of the extracts showed antibacterial activity. Five of the most promising fungi were subsequently grown in co-culture with the bacteria tested. Two fungi (*Aspergillus carneus* and *Aspergillus iizukae*) increased their range of activity inhibiting the bacterial pathogens not inhibited in pure culture. While the range of activity of the extracts of the three remaining fungi decreased when cultivated in co-culture. Undoubtedly, bacteria may produce effector molecules capable to alter the fungal growth or toxins that at high concentrations may inhibit the fungal development and the production of target antibiotic molecules (Özkaya *et al.*, 2017).

Overall, the co-culture can enhance or decrease the production of secondary metabolites, as also previously reported for other culture conditions. To this concern, the co-culture presents also a drawback in light of the industrial exploitations: the fungus usually needs the presence of the bacteria to activate specific metabolic pathway, while, the bacterial metabolites alone do not induce any modification in the fungus metabolome (Bertrand *et al.*, 2014).

Epigenetic modifiers

Epigenetic modifiers are small molecules that can interfere with the biosynthetic mechanisms of the secondary metabolisms of fungi, by promoting the expression of silent genes (González-Menéndez *et al.*, 2016). The two main group of epigenetic modifiers are methyltransferase (DNMT) and histone deacetylase (HDAC); DNMT inhibitors, as 5-azacitidine can reduce DNA-methylation promoting the expression of silent genes, as successfully reported in several fungi (González-Menéndez *et al.*, 2016).

In parallel, HDAC promotes the removal of acetylation, related to gene silencing (Takahashi *et al.*, 2013).

Up to date, it is not common to use epigenetic modifiers to modulate the production of secondary metabolites in marine fungi as the above-mentioned strategies (the variation of media composition and the co-culture). However, few studies report the success of the epigenetic modification in the synthesis of new metabolites. For instance, Vervoort and collaborators (2011) treated 24 marine fungal strains with the suberoylanilide hydroxamic acid (belonging to the HDAC group), observing a significant variation in the chemical fingerprints compared to the controls. Among the tested fungi, *Microascus* sp. was further studied and in presence of the epigenetic modifier produced a new cyclodepsipeptide (EGM-556) (Vervoort *et al.*, 2011). In the presence of the epigenetic molecule butyrate (HDAC inhibitor) *Leucostoma persoonii*, another marine derived fungus, produced the new compound Cytosporone R; as for other three known molecules, their production was increased from three to 8 fold compared to the untreated control (Beau *et al.*, 2012).

2. AIM OF THE WORK

Due to the lack of knowledge on marine fungi and their incredible biotechnological potential, this Ph.D. thesis focuses on a highly promising group of fungi: those associated with marine sponges. These fungi are both characterised by high biodiversity and chemodiversity, being the most successful producers of new bioactive molecules (Imhoff, 2016; Indraningrat *et al.*, 2016; Suryanarayanan, 2012). On these premises, the main goal of the research was to cover the firsts and fundamentals aspects of the natural products discovery pipeline: from the isolation and identification of fungi from sponges to the isolation of molecules and the evaluation of their biological activity. This resulted in a multidisciplinary Ph.D. project that enclosed mycology, chemistry, biochemistry and biotechnology.

In a “funnel-like” perspective, using multidisciplinary experimental approaches three main parts were developed:

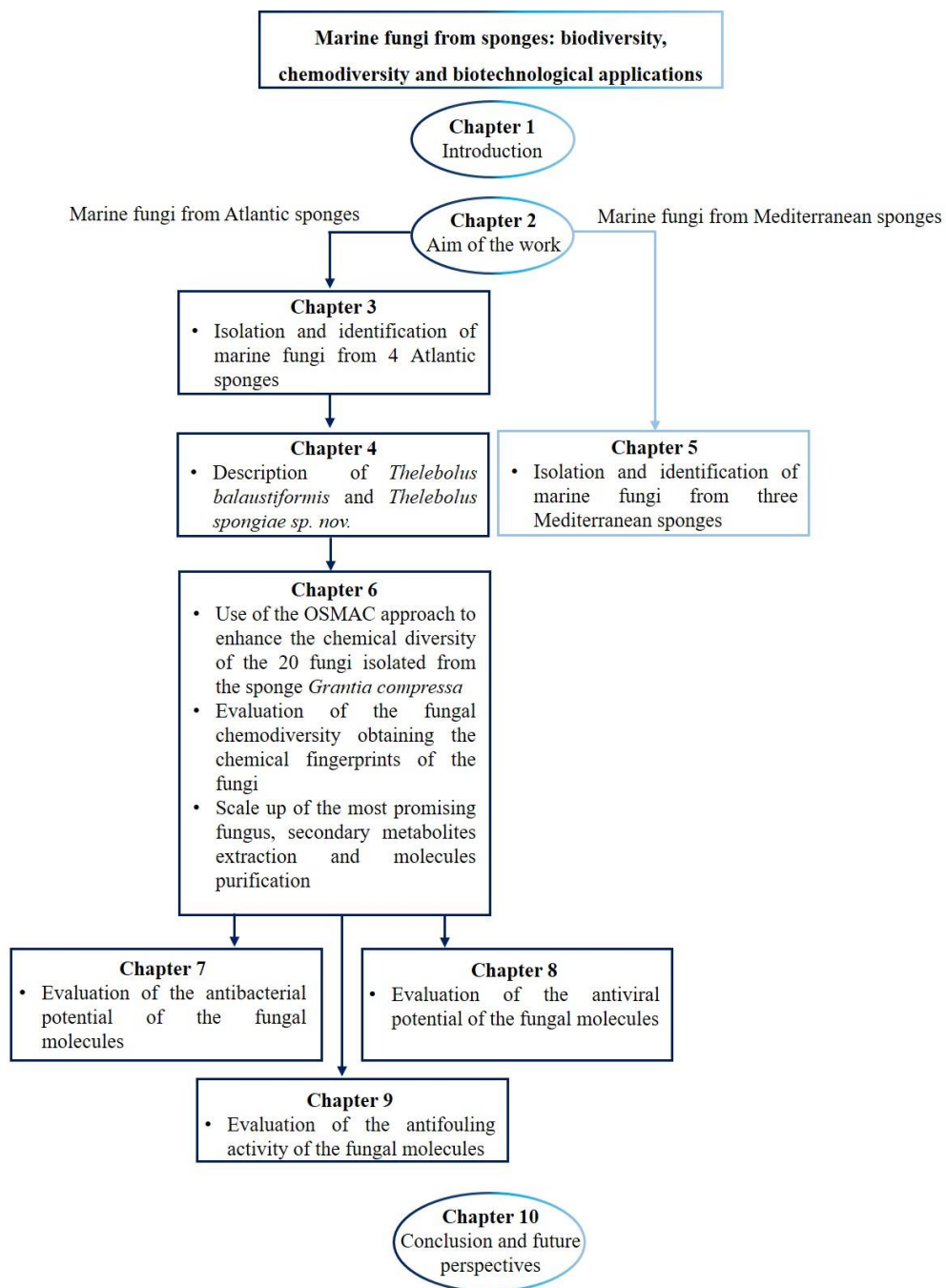
- The first aim was to isolate the fungal communities associated with sponges using several isolation techniques to increase the number of cultivable fungi. Four sponges were collected in the Atlantic Ocean, thanks to the collaboration with the National University of Ireland Galway (Galway, Ireland) and three in the Mediterranean Sea with the support of the University Nice Côte d’Azur (Nice, France). In order to make ecological hypotheses about the capability of the sponges to recruit their mycobiota to exploit these organisms for the production of secondary metabolites and to handle them in accordance with the latest biosafety regulations, fungi were identified at the lowest rank possible. This allowed highlighting two new species that were subsequently described.
- The second goal was to enhance the chemical diversity of all the fungal community associated with one sponge using the OSMAC approach and compare the metabolic profile of each fungus growth in different conditions. The most promising candidate was selected for the purification of high added value molecules. This second part of the project has been achieved thanks to the collaboration with the Marine Natural Products team of the Insitute of Chemistry

of the University Nice Côte d'Azur (Nice, France), which has resulted in a cotutelle agreement for this Ph.D.

- The third aim of the project was to define the bioactivity of the molecules produced by the selected marine fungus. Two main research fields, pharmaceutical and environmental, were chosen as potential targets of the molecules. Thanks to the collaboration with the Clinical Microbiology and Virology Laboratory of the Department of Medicine of the University Milano-Bicocca (Monza, Italy) the antibacterial properties of the molecules have been evaluated. Likewise, the antiviral activity was performed in the Laboratory of Microbiology and Virology of the Department of Life Sciences and System Biology (DBIOS) of the University of Turin (Turin, Italy).

As for the environmental field, the antifouling potential of the fungal compounds was evaluated. The collaboration with the Laboratoire des sciences de l'environnement marin of the University of Western Brittany (Brest, France), aims to assess the molecules for the inhibition of the bacterial/microalgal adhesion and growth. In addition, with the purpose to contrast the byssus production in mussels (common fouling organisms), the inhibition activity of tyrosinase, a model enzyme, was also evaluated.

2.1 Project outline



3. The culturable mycobiota associated with 4 Atlantic sponges

Fungi are a suitable biotechnological resource, but require specific expertise for the isolation and the correct identification. Many taxa already known for their bioactive secondary metabolites lack a precise identification and a correct preservation in culture collections hampering their possible future exploitation.

In the present chapter, relevance will be done to the isolation and low rank identification of marine fungi from 4 Atlantic sponges *Dysidea fragilis* (Demospongiae), *Grantia compressa* (Calcarea), *Pachymatisma johnstonia* (Demospongiae) and *Sycon ciliatum* (Calcarea), Figure 3.1 (A-D). The mycobiota inhabiting the 4 mentioned sponges is reported for the first time, while the bacterial community has been previously (scarcely) studied for *D. fragilis* (De Rosa *et al.*, 2000), *G. compressa* (Macintyre *et al.*, 2014) and *P. johnstonia* (Wichels *et al.*, 2004).

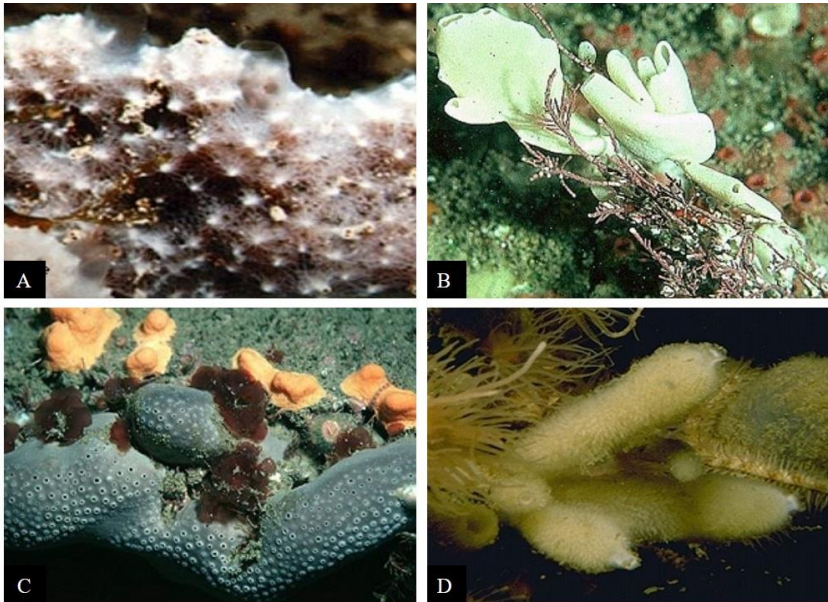


Figure 3.1 A. *D. fragilis* (Ph. B. Picton). B. *G. compressa* (Ph. B. Picton). C. *P. johnstonia* (Ph. B. Picton). D. *S. ciliatum* (Ph. M. De Kluijver).

Interestingly, the two *Demospongiae* have been extensively examined for the production of secondary metabolites. The metabolome of *D. fragilis* was characterised by Yu and colleagues (2006), although the biological activity was demonstrated only for a single compound, which acted as fish feeding deterrent (Marin *et al.*, 1998). *P. johnstonia* is also well known for its production of secondary metabolites, whose anticancer (Ferreira *et al.*, 2011; Zidane *et al.*, 1996) and antibacterial (Warabi *et al.*, 2004) activity has been demonstrated. On the contrary, the metabolome of *G. compressa* and *S. ciliatum* have never been studied.

3.1 MATERIAL AND METHODS

3.1.1 Sampling sites and axenic isolation

The sponges *D. fragilis* and *P. johnstonia* (three specimens each) were collected by scuba divers in Gurraig Sound (Co. Galway, Ireland; N 53° 18' 944'', W 09° 40' 140''). The sampling site was at 15 m depth, characterised by a fairly strong current and suspended sediments. Three specimens of *S. ciliatum* and of *G. compressa* exposed to a fast water flow due to the tide going out were collected in Coranroo rapids (Co. Clare, Ireland; N 53° 09' 100'', W 09° 00' 550''). The sampling sites are reported in Figure 3.2.

Specimens were surface sterilised with ethanol 70% (for 30 s) to prevent contaminants and serially washed (three times) in artificial sterile Seawater (SW) to get rid of unrefined sediments and to washout propagules in order to leave only fungi actively growing on the surface or into the sponge tissues.

Working in sterile conditions, the sponge samples were divided into three parts to be used for two different fungal isolation techniques and for a taxonomic voucher of the sponge. For the first isolation method, one third of the sponge was further cut in 20 pieces of about 0.5 cm³ by means of sterile tools and directly plated in Petri dishes (6 cm Ø) containing two different media: Seawater Agar – SWA (Sea Salts 30 g, Agar 15 g - Sigma-Aldrich, Saint Louis, USA - up to 1 L dH₂O) and Corn Meal Agar Seawater - CMASW (Corn Meal 2 g, Agar 15 g, Sea Salts 30 g - Sigma-Aldrich, Saint Louis, USA

- up to 1 L dH₂O). Five replicates for each medium and incubation temperatures (15 °C and 25 °C) were performed.

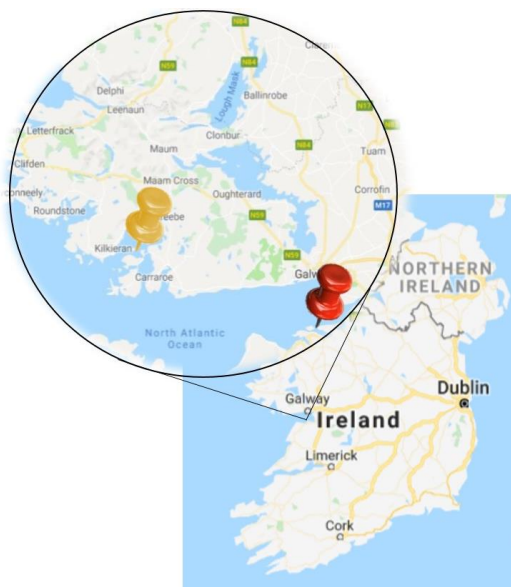


Figure 3.2 Sampling site of *D. fragilis* and *P. johnstonia* marked with the yellow pin; *G. compressa* and *S. ciliatum* sampling site is shown by the red pin.

Approximately 5 g of each sample were also homogenized (homogenizer blade Sterilmixer II - PBI International) and diluted 1:10 w/v in SW. One mL of suspension was included in Petri dishes (9 cm Ø) containing CMASW or Gelatin Agar Seawater–GASW (Gelatin 20 g, Sea Salts 30 g, Agar 15 g - Sigma-Aldrich, Saint Louis, USA - up to 1 L dH₂O), rich in collagen and mimicking sponge tissue composition. Only for the sponge *G. compressa*, due to the few fungal isolates obtained, the Malt Extract Agar Seawater – MEASW (Malt 20 g, Glucose 20 g, Peptone 2 g, Agar 15 g, Sea Salts 30 g - Sigma-Aldrich, Saint Louis, USA - up to 1L dH₂O) was also used as isolation medium.

Five replicates for each medium and incubation temperatures (15 °C and 25 °C) were performed. All media were supplemented with an antibiotic mix (Gentamicin Sulphate 40 mg/L, Piperacillin plus Tazobactam 11 mg/L) to prevent bacterial growth. Plates were incubated in the dark and periodically checked for 30 d to isolate slow growing fungi.

3.1.2 Fungal identification

Fungal morphotypes were isolated in pure culture and identified by means of a polyphasic approach combining morpho-physiological and molecular features. After determination of genera via macroscopic and microscopic features (Kiffer and Morelet, 1997; Von Arx, 1981; Domsch *et al.*, 1980), fungi were transferred to the genus specific media (Samson and Frisvad, 2004; Braun *et al.*, 2003; Klich, 2002).

In parallel, for molecular analyses, fungi were pre-grown on MEA (without SW) at 25 °C for 1 week, for fast growing fungi, and 2–4 weeks for slow growing fungi. DNA was extracted using the NucleoSpin kit (Macherey Nagel GmbH, Duren, DE, USA), according to the manufacturer instructions. Based on the taxonomic assignment attributed to each fungus by morphological observations, specific primers were used for the PCR as detailed in Table 3.1. Briefly, PCR reactions were performed in 50 µL final volumes and consisted of 0.5 µL Taq DNA Polymerase (Qiagen 5 U/µL), 10 µL PCR Buffer (10x), 2.5 µL dNTPs Mixture (dATP, dCTP, dGTP, dTTP; 10 mM), 2 µL MgCl₂ (25 mM), 2.5 µL of each primer (10 µM), 1 µL genomic DNA extract (80 ng/mL) and 34 µL distilled-deionized water. PCR products were visualized under UV light (BIO-RAD Universal Hood II) on 1.5% agarose electrophoresis gels stained with ethidium bromide. Macrogen, Inc. (Seoul, South Korea) Europe Lab carried out the purification and sequencing of PCR products.

Taxonomic assignments were based both on high percentage homologies with sequences available in public databases (GenBank - NCBI database and CBS-KNAW Collection, Westerdijk Fungal Biodiversity Institute) and the consistency of morphological features with available literature descriptions. The taxonomic position of doubtful strains (low homologies with sequences available in public databases) or sterile mycelia (i.e. not showing morphological features useful to confirm taxonomical assignments) were inferred via molecular phylogenetic analyses based on DNA sequences from the large ribosomal subunit LSU (Vilgalys and Hester, 1990). Separate alignments were created for the orders *Pleosporales*, *Capnodiales* and *Chaetothyriales* and two for the classes *Leotiomycetes* and *Sordariomycetes*. Alignments were generated using MEGA v. 7.0 and manually refined. The appropriate evolutionary model under the

Akaike Information Criterion (AIC) was determined with jModelTest 2 (Darriba *et al.*, 2012). Bayesian Inference was performed with MrBayes 3.2.2 (Ronquist *et al.*, 2012) under GTR + I + G evolutionary model (best model). The alignment was run for 10 million generations with two independent runs each containing 4 Markov Chains Monte Carlo (MCMC) and sampling every 1000 iterations. The first 2,500 trees were discarded as “burn-in” (25%). Using the Sumt function of MrBayes a consensus tree was generated and Bayesian posterior probabilities (BPP) were estimated.

For the genus *Emericellopsis*, due to the low number of LSU sequences available, a dataset based on internal transcribed spacer regions (ITS) and partial beta-tubulin gene (*TUB*) was created. Alignments of each gene were generated as above mentioned; then, they were concatenated into a single data matrix with SequenceMatrix v. 1.8 (Vaidya *et al.*, 2011). The appropriate evolutionary model and the alignment were performed as above mentioned

Representative strains of each species isolated in pure culture during this work are preserved at *Mycotheca Universitatis Taurinensis* (MUT- www.mut.unito.it) of the Department of Life Sciences and Systems Biology, University of Turin (Italy). The Accession numbers of the sequences deposited in GenBank are available in Annexe 1.

Table 3.1 Gene loci sequenced, primers for molecular analysis and PCR programs.

Fungi	Gene loci and DNA regions sequenced*	Primers (Forward and Reverse)	PCR amplification Conditions	References for primers
<i>Alternaria</i>	<i>GAPDH</i>	GPD1 and GPD2	96 °C: 2 min, (96 °C: 1 min, 50 °C: 1 min, 72 °C: 50 sec) × 35 cycles; 72 °C: 5 min	[1]
<i>Aspergillus</i>	<i>CAL</i>	CL1 and CL2a	95 °C: 10 min, (95 °C: 50 sec, 55 °C: 50 sec, 72 °C: 1 min) × 35 cycles; 72 °C: 7 min	[2]
<i>Aspergillus, Emericellopsis, Penicillium, Thelebolus</i>	<i>TUB</i>	BT-2a and BT-2b	94 °C: 4 min, (94 °C: 35 sec, 58 °C: 35 sec, 72 °C: 50 sec) × 35 cycles; 72 °C: 5 min	[3]
<i>Cladosporium</i>	<i>ACT</i>	ACT-512F and ACT-783R	94 °C: 8 min, (94 °C: 15 sec, 61 °C: 20 sec, 72 °C: 40 sec) × 35 cycles; 72 °C: 10 min	[4]

Yeast like fungi (<i>Holtermanniella</i> , <i>Metschnikowia</i> , <i>Pseudozyma</i> , Sporidiobolales)	D1-D2	NL1 and NL2	94 °C: 4 min, (94 °C: 1 min, 52 °C: 35 sec, 72 °C: 1.5 min) × 35 cycles; 72 °C: 5 min	[5]
<i>Alternaria</i> , <i>Thelebolus</i> , sterile mycelia and taxa for whom no specific primers are required	ITS	ITS1 and ITS4	95 °C: 5 min, (95 °C:40 s, 55 °C: 50 s, 72 °C: 50 sec) × 35 cycles; 72 °C: 8 min	[6]
Sterile mycelia	LSU	LROR and LR7	95 °C: 5 min, (95 °C: 1 min, 50 °C: 1 min, 72 °C: 2 min) × 35 cycles; 72 °C: 10 min	[7]

**GAPDH*: partial glyceraldehyde-3-phosphate dehydrogenase gene; *CAL*: partial calmodulin gene; *TUB*: partial beta-tubulin gene; *ACT*: partial actin gene; D1-D2: D1-D2 region of the nuclear rDNA large subunit; ITS: internal transcribed spacer regions and intervening 5.8S nrRNA gene; LSU: partial nuclear ribosomal DNA large subunit.

[1] Berbee *et al.*, 1999, [2] O'Donnell *et al.*, 2000, [3] Glass and Donaldson, 1995, [4] Carbone and Kohn, 1999, [5] De Barros Lopes *et al.*, 1998, [6] White *et al.*, 1990, [7] Vilgalys and Hester, 1990.

3.1.3 Statistical analyses

Statistical analyses on the fungal community associated with sponges were performed using PRIMER v. 7.0 (Plymouth Routines In Multivariate Ecological Research; Clarke and Warwick, 2001). The Permutational Multivariate Analysis of Variance (PERMANOVA; pseudo-F index; $p < 0.05$) underlined the differences between the sponge mycobiotas to be assessed. The Canonical Analysis of Principal Coordinates (CAP) visualized data.

3.2 RESULTS AND DISCUSSION

3.2.1 Influence of isolation techniques on the fungal community

The use of different isolation techniques or culture conditions resulted in an increase in the number of fungal isolates. As reported in Figure 3.3A, the majority of the isolates were obtained exclusively by homogenisation of sponge tissues (from 56% in *S. ciliatum* to 86% in *G. compressa*), while the remaining by directly plating the sponge

tissue; less than 18% were recovered with both techniques. The different performance of the isolation techniques could be due to the specific requirements of marine fungi and the technique itself. Direct plating resulted in the isolation of only one fungus for each piece of sponge plated; on the contrary, the homogenization best suits the isolation of more marine fungi. These results are in agreement with other comparative studies (Sayed *et al.*, 2016; Paz *et al.*, 2010). Noteworthy, the direct plating, even if performing less well, allowed the isolation of fungi that otherwise would have not been recorded.

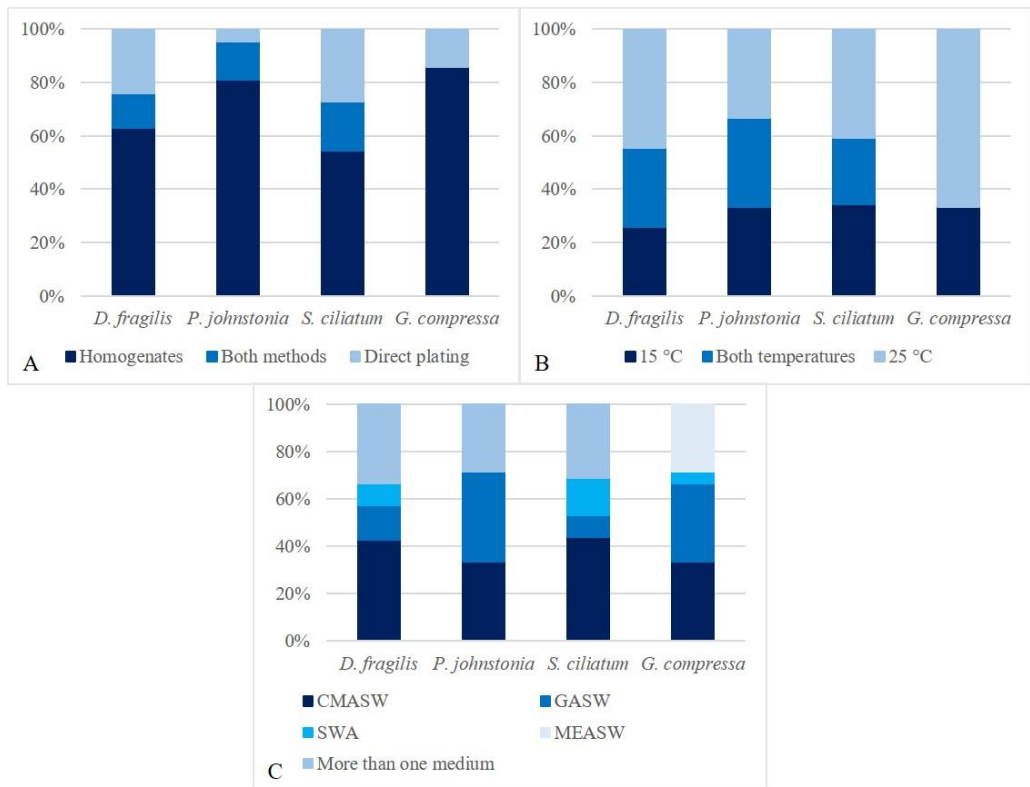


Figure 3.3 Influence of **A.** isolation methods; **B.** incubation temperatures; **C.** growth media, on the fungal community associated with *D. fragilis*, *G. compressa*, *P. johnstonia* and *S. ciliatum*.

We also considered the possible influence of the temperature in the isolation of marine fungi from sponges. To mimic marine conditions as much as possible, two different temperatures were set: 25 °C commonly used to culture fungi and 15 °C closer to the environmental conditions of the sponge sampling sites. Interestingly, the majority

of the taxa, from 67% (*P. johnstonia*) to the totality (*G. compressa*), grew exclusively at one temperature condition (Figure 3.3B). Despite, the temperature is one of the parameter driving fungal diversity in the oceans (Jones, 2000) this is the first time that it has been taken into account to isolate fungi associated with sponges.

As for the isolation techniques, the use of three different media, also mimicking marine environment and sponge composition resulted in an increase in the number of cultivable fungi. The best performing condition for both *D. fragilis* and *S. ciliatum* was CMASW, where almost half of the taxa grew exclusively on it (Figure 3.3C). This medium is not extremely rich in nutrients but usually well-promote the fungal growth; sea salts provide a condition as much as possible similar to the marine environment. About one quarter of fungal taxa from *D. fragilis* and *S. ciliatum* were isolated only on oligotrophic media, mimicking sponges' composition (GASW) or marine water (SWA), Figure 3.3C. The majority of the fungi associated with *P. johnstonia* were only isolated on GASW (38%) or on CMASW (33%); no exclusive taxa were reported on SWA (Figure 3.3C). Interestingly, the medium that yielded the higher number of isolates in *P. johnstonia* was the gelatine-based medium, specifically developed in this research to mimic the host organism. Usually, media rich in nutrients allow the isolation of a high number of fungi, but this not necessary means high biodiversity (Caballero-George *et al.*, 2013).

Concerning *G. compressa*, due to the low number of taxa recorded with the three mentioned media, also an extremely rich substrate (MEASW) was used to isolate the associated fungi. The retrieved taxa were almost equally distributed and exclusively isolated on CMASW GASW and MEASW, while only one fungus was found on SWA (Figure 3.3C).

Interestingly, 66%, 62%, 56% and 38% of taxa from *S. ciliatum*, *G. compressa*, *D. fragilis* and *P. johnstonia*, respectively not only were recovered in one condition but also were isolated only from one plate. Annexe 1 reports all the fungi isolated in the different culture conditions.

Currently, several works on sponge-associated fungi employed different isolation techniques (Diep *et al.*, 2016; Henríquez *et al.*, 2014; Passarini *et al.*, 2013;

Ding *et al.*, 2011; Wiese *et al.*, 2011; Li and Wang, 2009; Proksch *et al.*, 2008; Wang *et al.*, 2008; Höller *et al.*, 2000). It would be extremely important to share these results to point out if some methods are more promising than other ones. In the attempt to increase the number of cultivable fungi, more efforts should be focused on the development of innovative isolation techniques. For example, Rozas *et al.* (2011) succeeded in the isolation of fungi from single sponge cells. In parallel, the micro-Petri dishes, as well as the iChip, could be promising tools for the isolation of “uncultivable” (marine) microorganisms (Nichols *et al.*, 2010; Ingham *et al.*, 2007).

3.2.2 Fungal diversity

A total of 97 taxa were isolated: 54 taxa from *D. fragilis*, 17 from *G. compressa*, 32 from *S. ciliatum* and 21 from *P. johnstonia*; 83.5% of the taxa were recognised at species level, 11.4% at genus level, 4.1% at family level and 1% at order level (Table 3.2). Literature reports that the identification at species level of marine fungi isolated from different substrates ranges from 11.2% to 80.5% (Nicoletti and Andolfi, 2018), placing this work above the average, with a higher percentage of fungi identified at species level.

About one-third of taxa were sterile despite the attempt to stimulate the production of reproductive structures using different culture media. The high abundance of sterile mycelia is not unusual in the marine environment and was reported in several studies (Raghukumar, 2017; Panno *et al.*, 2013; Pivkin *et al.*, 2006; Höller *et al.*, 2000). In order to better define the systematic position of sterile mycelia showing the same similarity percentages with different species and/or low homology with sequences deposited in public databases, different phylogenetic analyses were performed. In detail, Capnodiales (Figure 3.4) were represented by one fungal isolate (MUT 2352) identified at the genus level as *Pseudocercospora* sp. within the Mycosphaerellaceae. The sequences available did not allow a further classification. In the future, the creation of a multi-marker dataset will define if *Pseudocercospora* sp. MUT 2352 represents a new species.

Table 3.2 Fungal taxa isolated from *D. fragilis* (DF), *G. compressa* (GC), *P. johnstonia* (PJ) and *S. ciliatum* (SC) and their relative abundance in percentage (RA%). The species already found in the marine environment (MA) and associated with sponges (SP) are reported, as well as the first record (FR).

	RA%					
	DF	GC	PJ	SC	MA	SP
Ascomycota						
<i>Acremonium breve</i>	0.6				[20]	FR
<i>Acremonium implicatum</i>	0.6				[1, 2, 3, 4, 5, 19]	[3]
<i>Acremonium persicinum</i>	1.3				[7]	[33]
<i>Acremonium potronii</i> *	5.8			1.0	[7, 34]	FR
<i>Acremonium tubakii</i>	5.8				[1, 34]	FR
<i>Acremonium zonatum</i>				2.9	FR	FR
<i>Alternaria molesta</i> *			1.4		[30]	FR
<i>Alternaria</i> sp.*				1.0	-	-
<i>Aspergillus creber</i>	4.5		2.7		[23]	FR
<i>Aspergillus flavipes</i>			5.4		[22]	[35]
<i>Aspergillus fumigatus</i>	2.6			1.0	[1, 4, 5, 7, 19, 42]	[6, 18, 27]
<i>Aspergillus jensenii</i>	0.6		2.7		FR	FR
<i>Aspergillus puulaauensis</i>	3.8				FR	FR
<i>Aureobasidium pullulans</i>	1.3		2.7		[2, 7, 19, 22, 34, 42, 47, 54]	[9, 36, 54]
<i>Beauveria bassiana</i>	1.9			2.9	[7, 34, 47]	[17, 37]
<i>Bimuria novae-zelandiae</i> *	0.6				[31]	FR
<i>Boeremia exigua</i> *	0.6				[32, 34]	FR
<i>Botrytis cinerea</i>	0.6				[5, 24, 34]	FR

<i>Cadophora luteo-olivacea</i>	0.6			4.9	[11]	FR
<i>Cladosporium aggregatocicatricatum</i>	0.6				FR	FR
<i>Cladosporium allicinum</i>	2.6	13.0	1.4	3.9	[2, 42]	FR
<i>Cladosporium cladosporioides</i>	2.6	4.3	6.8	1.0	[1, 2, 5, 7, 19, 21, 22, 34, 42, 47]	[9, 10, 12, 14, 15, 18, 27]
<i>Cladosporium halotolerans</i>			5.4	10.8	[5, 11, 22, 42]	FR
<i>Cladosporium perangustum</i>	0.6				[25]	FR
<i>Cladosporium pseudocladosporioides</i>	2.6	8.7	4.1		[5, 42]	FR
<i>Cladosporium psychrotolerans</i>	1.3				[22]	FR
<i>Cladosporium subtilissimum</i>	0.6				[22, 42]	FR
<i>Cladosporium subuliforme</i>			2.7		FR	FR
<i>Cladosporium xylophilum</i>	0.6				[42]	FR
<i>Coniothyrium obiones*</i>				1.0	[7]	FR
<i>Cyphellophora</i> sp.*				1.0	-	-
<i>Emericellopsis alkalina</i> (anamorph)	1.3				FR	FR
<i>Emericellopsis maritima</i> (anamorph)			1.4		[7, 47]	FR
<i>Emericellopsis pallida</i> (anamorph)	1.3	8.7			[7]	FR
<i>Epicoccum nigrum</i>				4.9	[5, 34]	[40]
<i>Eurotium chevalieri</i>		4.3			[7, 19, 22, 28, 43]	[27]
<i>Euthypella scoparia*</i>		4.3			[44]	[41]
<i>Fusarium pseudograminearum</i>	0.6				FR	FR
<i>Fusarium solani</i>		4.3		1.0	[26, 47]	[3, 41]
<i>Gremmenia infestans*</i>				1.0	FR	FR
<i>Hypocreaceae</i> sp.*	0.6				-	-
<i>Metschnikowia bicuspidata</i>				5.9	[7, 22, 47]	[38]

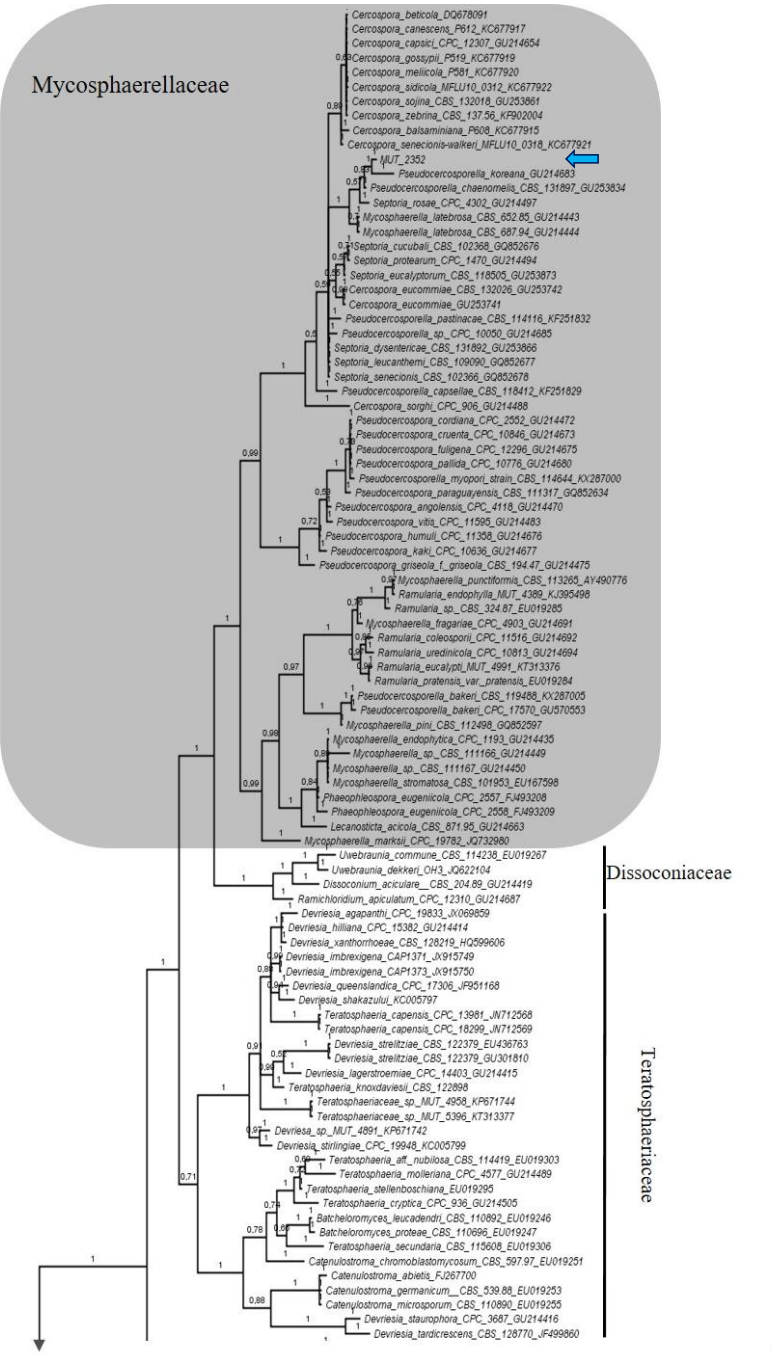
<i>Microascaceae</i> sp.*	0.6			-	-
<i>Mollisia</i> sp.*				1.0	-
<i>Myrothecium cinctum</i> *	1.3				[39]
<i>Neocamarosporium betae</i>				1.0	FR
<i>Neocamarosporium calvescens</i>	0.6				FR
<i>Paraphaeosphaeria neglecta</i> (anamorph)	0.6			1.0	FR
<i>Penicillium antarcticum</i>	10.9		37.8	18.6	[2, 5, 42]
<i>Penicillium brevicompactum</i>	3.2				[1, 2, 3, 4, 5, 7, 19, 22, 34, 42]
<i>Penicillium canescens</i>			1.4		[5, 21, 34]
<i>Penicillium chrysogenum</i>	12.2	4.3	13.5		[5, 7, 22, 34, 47]
<i>Penicillium citreonigrum</i>				1.0	[5, 7, 21]
<i>Penicillium inflatum</i>	0.6				FR
<i>Penicillium janczewskii</i>	5.8				[7, 34]
<i>Penicillium oxalicum</i>		4.3			[7, 22, 45, 46, 47, 49]
<i>Penicillium paneum</i>		8.7			[7, 50]
<i>Penicillium roqueforti</i>				1.0	[40]
<i>Penicillium spinulosum</i>			2.7		[1, 7, 34, 42]
<i>Penicillium thomii</i>			1.4		[7, 34]
<i>Penicillium waksmanii</i>	1.3				[7, 34]
<i>Periconia minutissima</i>				1.0	[7]
<i>Periconia</i> sp.*			1.4		-
<i>Phaeosphaeria olivacea</i> *				1.0	[7]
<i>Phaeosphaeria oryzae</i> *				1.0	FR
<i>Phaeosphaeriopsis</i> sp.*	0.6			4.9	-
Pleosporales sp.*				1.0	-

<i>Pleosporaceae</i> sp.*	0.6				-	-
<i>Pochonia suchlasporia</i>	0.6				[34]	FR
<i>Preussia</i> sp.*	0.6				-	-
<i>Pseudeurotium bakeri</i>				1.0	FR	FR
<i>Pseudocercospora</i> sp.*			1.4		-	-
<i>Pyrenochaetopsis microspora</i> *	1.9				[42]	FR
<i>Roussoellaceae</i> sp.*	0.6				-	-
<i>Sarocladium strictum</i>				14.7	[2, 7, 34]	FR
<i>Scopulariopsis brevicaulis</i>				1.0	[5]	[6, 13]
<i>Tetracladium</i> sp.*		4.3			-	-
<i>Thelebolus balaustiformis</i>	0.6				FR	FR
<i>Thelebolus spongiae</i>	0.6				FR	FR
<i>Thyronectria</i> sp.*				1.0	-	-
<i>Tilachlidium brachiatum</i>	0.6				[28]	FR
<i>Tolypocladium album</i>	1.9				FR	FR
<i>Tolypocladium cylindrosporum</i>	2.6	8.7	1.4	4.9	[29, 34, 47]	FR
<i>Volutella ciliata</i>	0.6				[4]	[40]
<i>Xanthothecium peruvianum</i>	0.6				FR	FR
Basidiomycota						
<i>Lopharia spadicea</i> *	0.6				FR	FR
<i>Coprinellus</i> sp.1*		4.3			-	-
<i>Coprinellus</i> sp.2*		4.3			-	-
<i>Ceriporia lacerata</i> *		4.3			[51, 52]	FR
<i>Holtermanniella festucosa</i>				1.0	[54]	[54]
<i>Irpex lacteus</i> *	0.6				[52]	FR

<i>Psathyrella candolleana</i> *	4.3			FR	FR
<i>Pseudozyma aphidis</i> *		1.4		[47]	FR
<i>Rhodotorula mucilaginosa</i>	4.3			[7, 53, 54]	FR
<i>Rhodotorula graminis</i>		1.4		[7, 47]	FR
<i>Trametes gibbosa</i> *	1.3			FR	FR
Mucoromycota					
<i>Absidia glauca</i>	0.6			[19]	FR
Tot. taxa	54	17	21	32	
Tot. exclusive taxa	38	10	11	20	

*Sterile mycelia

[1] Panno *et al.*, 2013, [2] Gnani *et al.*, 2017, [3] Paz *et al.*, 2010, [4] Costello *et al.*, 2001, [5] Bovio *et al.*, 2017, [6] Ding *et al.*, 2011, [7] Jones *et al.*, 2015, [8] Thirunavukkarasu *et al.*, 2012, [9] Henríquez *et al.*, 2014, [10] Passarini *et al.*, 2013, [11] Garzoli *et al.*, 2015a, [12] Rozas *et al.*, 2011, [13] Yu *et al.*, 2008, [14] Manriquez *et al.*, 2009, [15] San-Martin *et al.*, 2005, [16] Park *et al.*, 2014, [17] Yamazaki *et al.*, 2012, [18] Sayed *et al.*, 2016, [19] Oren and Gunde-Cimerman, 2012, [20] Kis-Papo *et al.*, 2001, [21] Raghukumar and Ravindran, 2012, [22] Zajc *et al.*, 2012, [23] Jurjevic *et al.*, 2012, [24] Suryanarayanan, 2012, [25] Liu *et al.*, 2017, [26] Hatai, 2012, [27] Pivkin *et al.*, 2006, [28] Gomes *et al.*, 2008, [29] Rämä *et al.*, 2014, [30] Tóth *et al.*, 2011, [31] Suetrong *et al.*, 2009, [32] Di Piazza *et al.*, 2017, [33] Fraser *et al.*, 2013, [34] Rämä *et al.*, 2017, [35] Ratnaweera *et al.*, 2016, [36] Shigemori *et al.*, 1998, [37] Zhang *et al.*, 2017, [38] Baker *et al.*, 2009, [39] Wang *et al.*, 2008, [40] Wiese *et al.*, 2011, [41] Bolaños *et al.*, 2015, [42] Garzoli *et al.*, 2018, [43] Butinar *et al.*, 2005, [44] Ciavatta *et al.*, 2008, [45] Sun *et al.*, 2013, [46] Yang *et al.*, 2017, [47] Raghukumar, 2017, [48] Gao *et al.*, 2008, [49] Alwakeel *et al.*, 2013, [50] Li *et al.*, 2013, [51] Zhao *et al.*, 2013, [52] Zhang *et al.*, 2016, [53] Takami, 1999, [54] Kachalkin, 2014, [55] El-Gendy *et al.*, 2018.



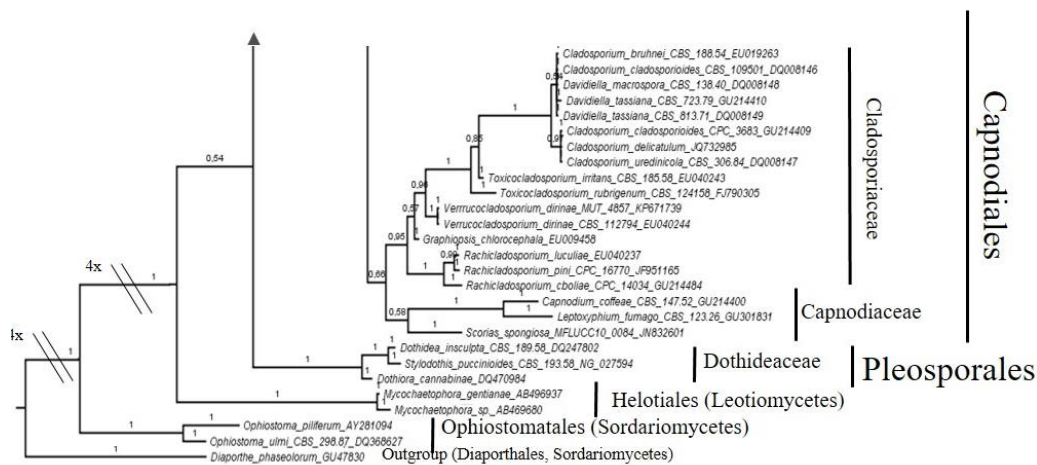


Figure 3.4 Bayesian phylogram of *Capnodiales* (Dothideomycetes) based on rDNA large subunit (LSU). One fungal isolate (MUT 2352) is included and identified as *Pseudocercospora* sp.. Branch numbers indicate BPP values.

Within the *Chaetothyriales* (Figure 3.5), MUT 2862 belongs to the genus *Cyphellophora*, which includes widespread species recorded on both animals and plants, but never described before in marine environment. This genus is in constant revision and Gao *et al.* (2015) have recently described three new species; for this reason, further analysis might reveal that MUT 2862 is a new species.

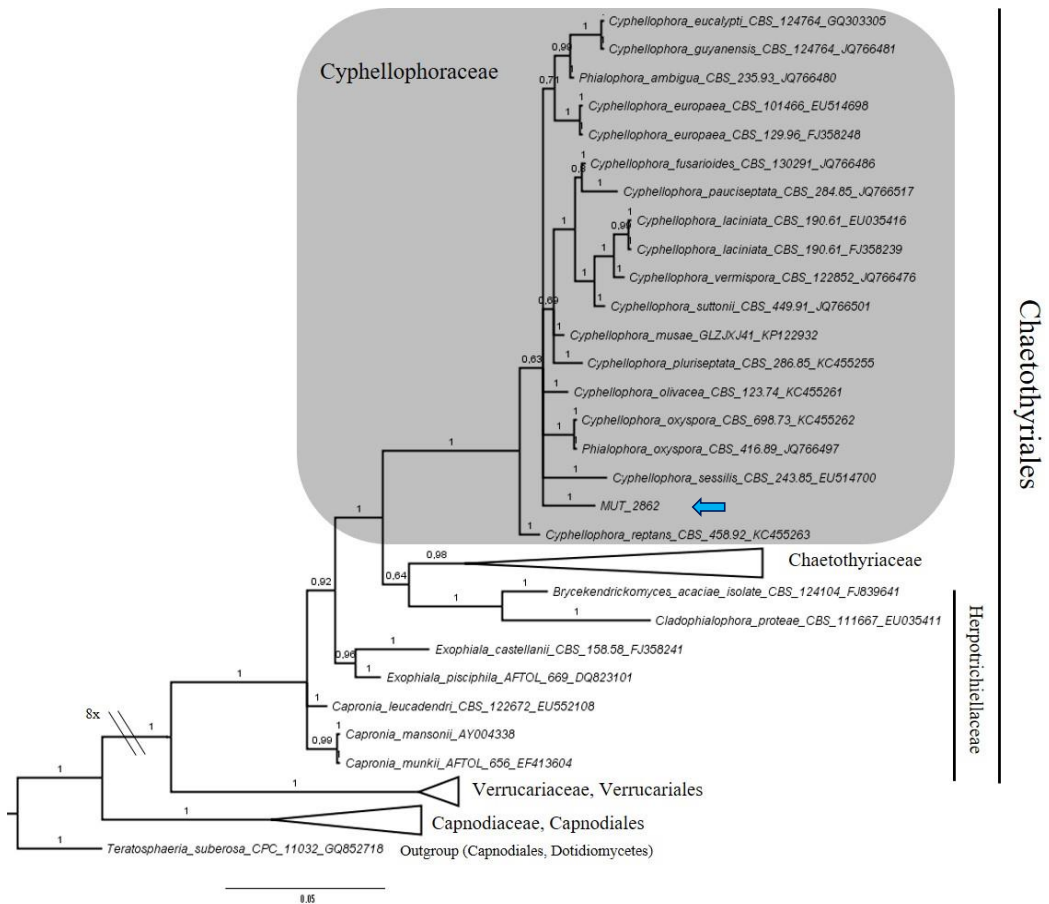


Figure 3.5 Bayesian phylogram of *Chaetothyriales* (*Eurotiomycetes*) based on rDNA large subunit (LSU). MUT 2862 is included and clusters within the genus *Cyphellophora*. Branch numbers indicate BPP values.

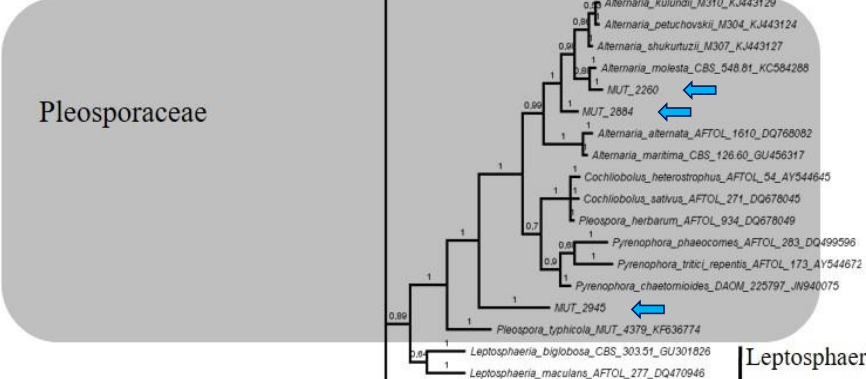
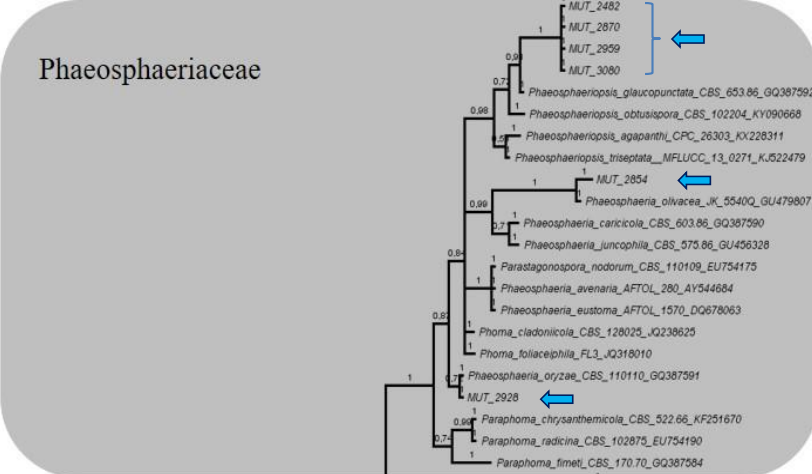
Pleosporales represent the largest group of *Dothideomycetes* and one of the most represented entities in this study with 17 strains (Figure 3.6). Four strains from *S. ciliatum* (MUT 2949, MUT 2870, MUT 3080) and *D. fragilis* (MUT 2482) clustered together and were identified as *Phaeosphaeriopsis* sp.; this genus was already found in the marine environment, including the extreme conditions of deep sea sediments (Nagano *et al.*, 2016; Zhang *et al.*, 2013). Its presence in two different sponges, not sampled in close proximity, might shade a new fungal species leaving in association with

these animals. In the same group of Phaeosphaeriaceae, MUT 2854 and MUT 2928 were identified as *Phaeosphaeria olivacea* and *Phaeosphaeria oryzae*, respectively.

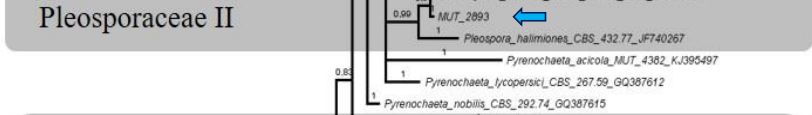
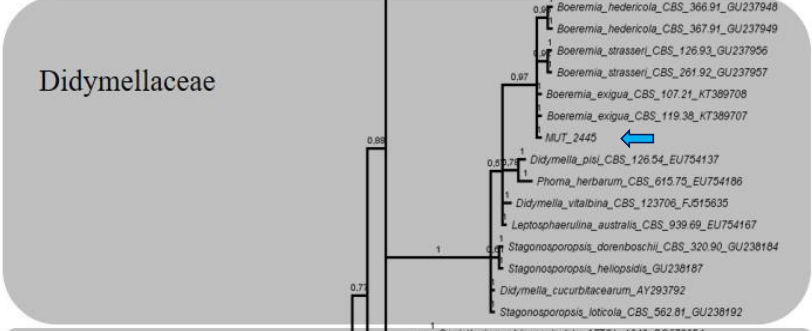
Among the Pleosporaceae, MUT 2260 and MUT 2884 belong to *A. molesta* and *Alternaria* sp., respectively, a genus commonly reported in the marine environment (Raghukumar, 2017).

More cryptic the position of other Pleosporaceae: MUT 2945 (*Pleosporaceae* sp.), MUT 2489 (*Pleosporales* sp.) and MUT 2452 (*Roussoellaceae* sp.) for which further studies, are necessary. In detail, as part of a bigger project aiming to define the systematic position of sterile mycelia in the MUT culture collection, the multi marker phylogenetic analysis identified MUT 2452 as a new species within the genus *Parathyridaria*. The remaining fungi were classified at species level: MUT 2445 (*B. exigua*), MUT 2893 (*C. obiones*), MUT 2374 (*P. microspora*) and MUT 2425 (*B. novae-zealandiae*) or at genera level, MUT 2263 (*Periconia* sp.), and MUT 2390 (*Preussia* sp.), which have been already reported in the sea (Raghukumar, 2017).

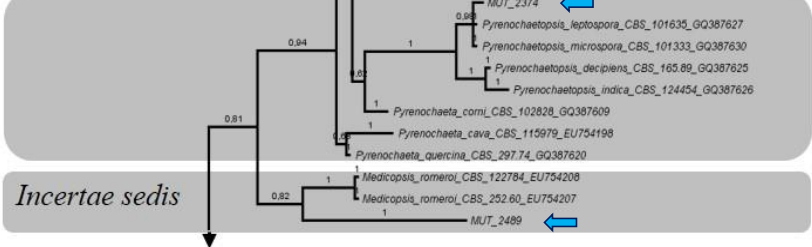
Within *Leotiomyces* (Figure 3.7), MUT 2783 was identified as *Gremmenia infestans*, while more doubtful was the position of MUT 2878, well supported in the genus *Mollisia* but within a clade containing different unidentified species. This genus was already retrieved in the marine environment (Costello *et al.*, 2001); further analysis will be necessary to clarify whether MUT 2878 might be or not a new species.



Leptosphaeriaceae



Cucurbitariaceae



Pleosporales I

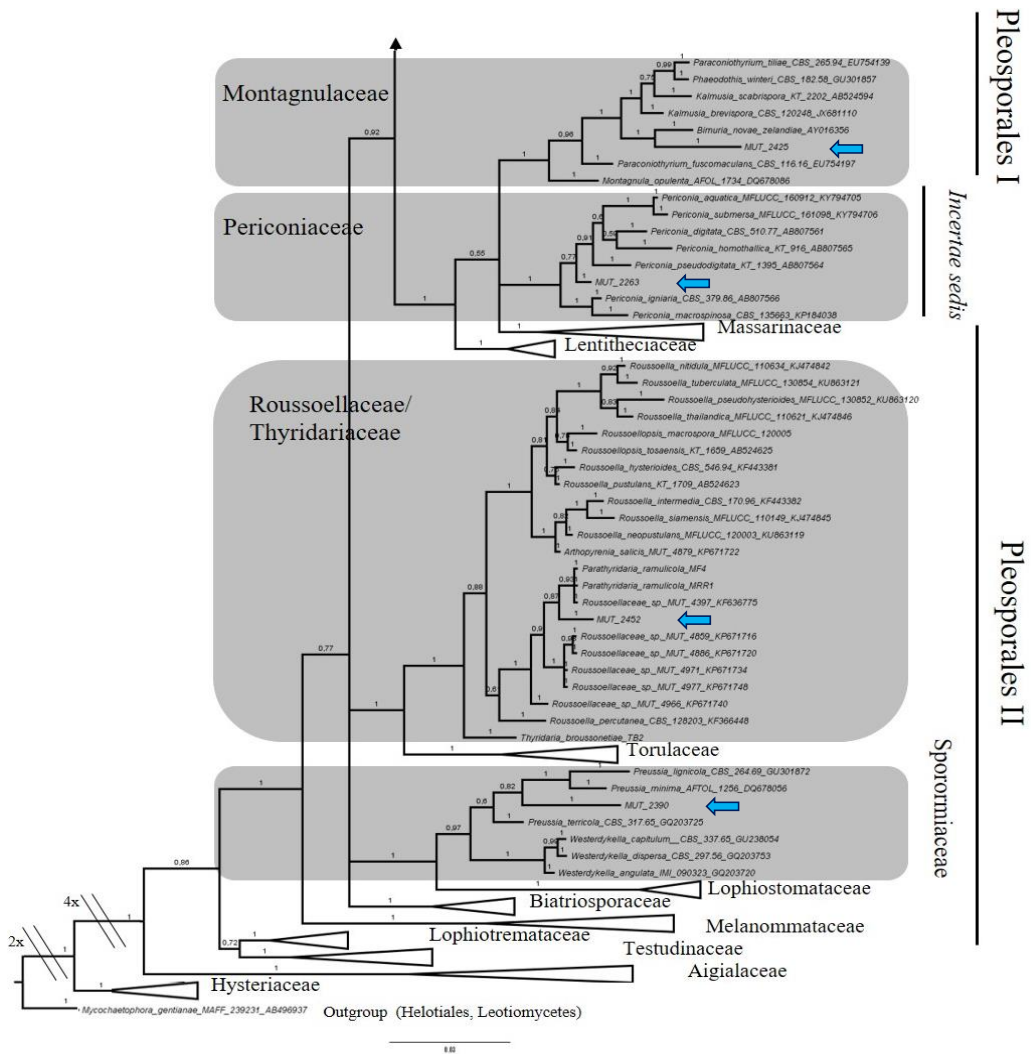
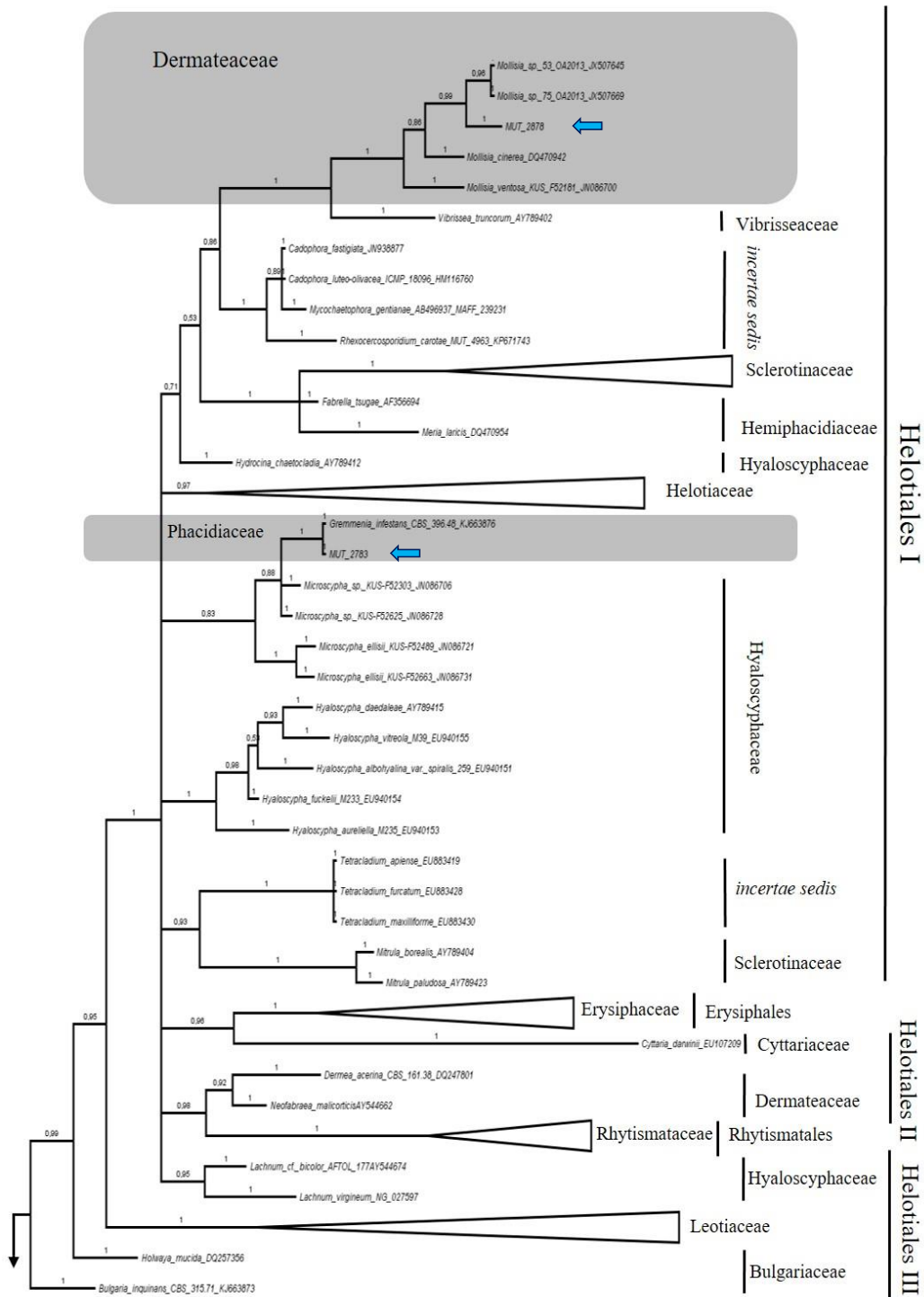


Figure 3.6 Bayesian phylogram of Pleosporales (Dothideomycetes) based on rDNA large subunit (LSU). Six and four fungal isolates clustered within the Phaeosphaeriaceae and the Pleosporaceae, respectively. Six fungal taxa clustered individually within the Didymellaceae, Cucurbitariaceae, Montagnulaceae, Periconiaceae, Sporormiaceae and Roussoellaceae/Thyridariaceae. One fungus was included in the Pleosporales order. Branch numbers indicate BPP values.



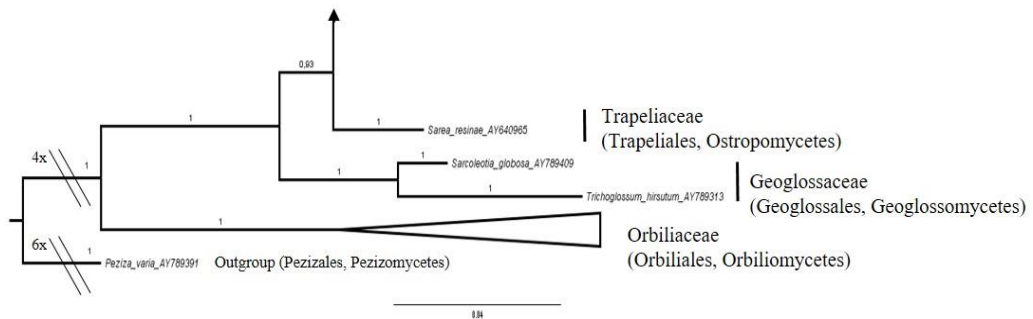


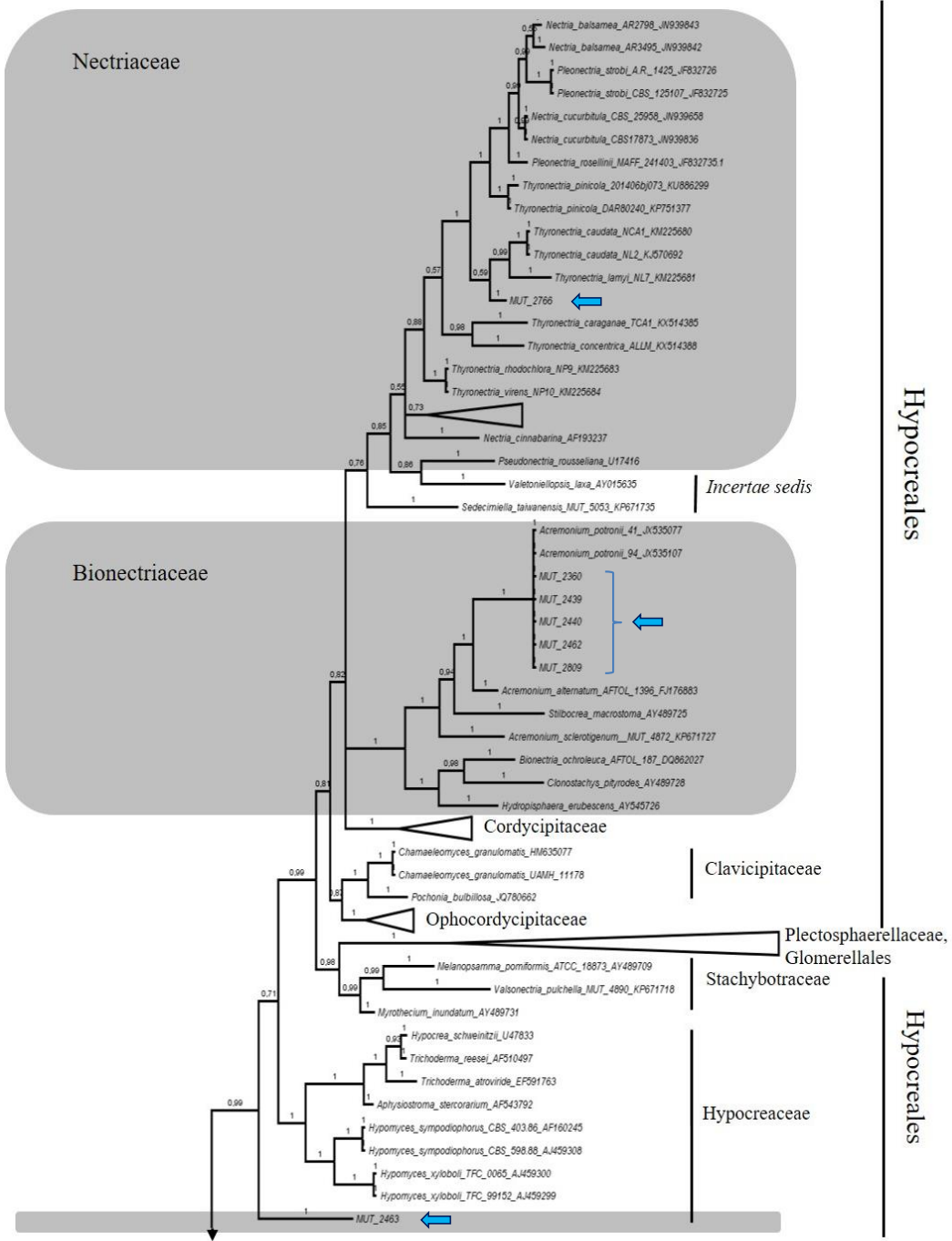
Figure 3.7 Bayesian phylogram of *Leotiomyces* based on rDNA large subunit (LSU). Two fungal isolates were identified as *Mollisia* sp. and *Gremmenia infestans* within the Dermateaceae and Phacidiaceae, respectively. Branch numbers indicate BPP values.

The *Sordariomycetes* were represented by 8 strains (Figure 3.8), MUT 2766 belong to the genus *Thyronectria*, whose presence was described in the Antarctic environment by Seeler *et al.* (1940). More doubtful is the systematic classification of MUT 2463 and MUT 2377 that clustered within *Hypocreaceae* and *Microascaceae*, respectively; they both represent families already recorded in the marine environment (Jones *et al.*, 2015). Several taxa from *D. fragilis* (MUT 2360, MUT 2439, MUT 2462, MUT 2440) and *S. ciliatum* (MUT 2809) were all identified as *A. potronii*.

Finally, the last phylogenetic analysis was carried out for two species within the *Emericellopsis* genus; due to the low number of LSU sequences available a two-markers dataset based on ITS and beta-tubulin partial sequences was used. MUT 2273 and MUT 2274 were identified as *Emericellopsis pallida* (Figure 3.9).

Phylogenetic analyses were undertaken also to clarify the position of the 11 Basidiomycota in separate research by Poli and colleagues (2018). All the taxa were classified at species level (Table 3.2), with the exception of two *Coprinellus* spp. for whom it would be risky to claim a novel species, since the lack of the sequences in GenBank amplifying the translation elongation factor of *Coprinellus* spp., is an issue that must be taken into account (Poli *et al.*, 2018).

The description of the two new species *Thelebolus balaustiformis* and *Thelebolus spongiae* is provided in the next chapter.



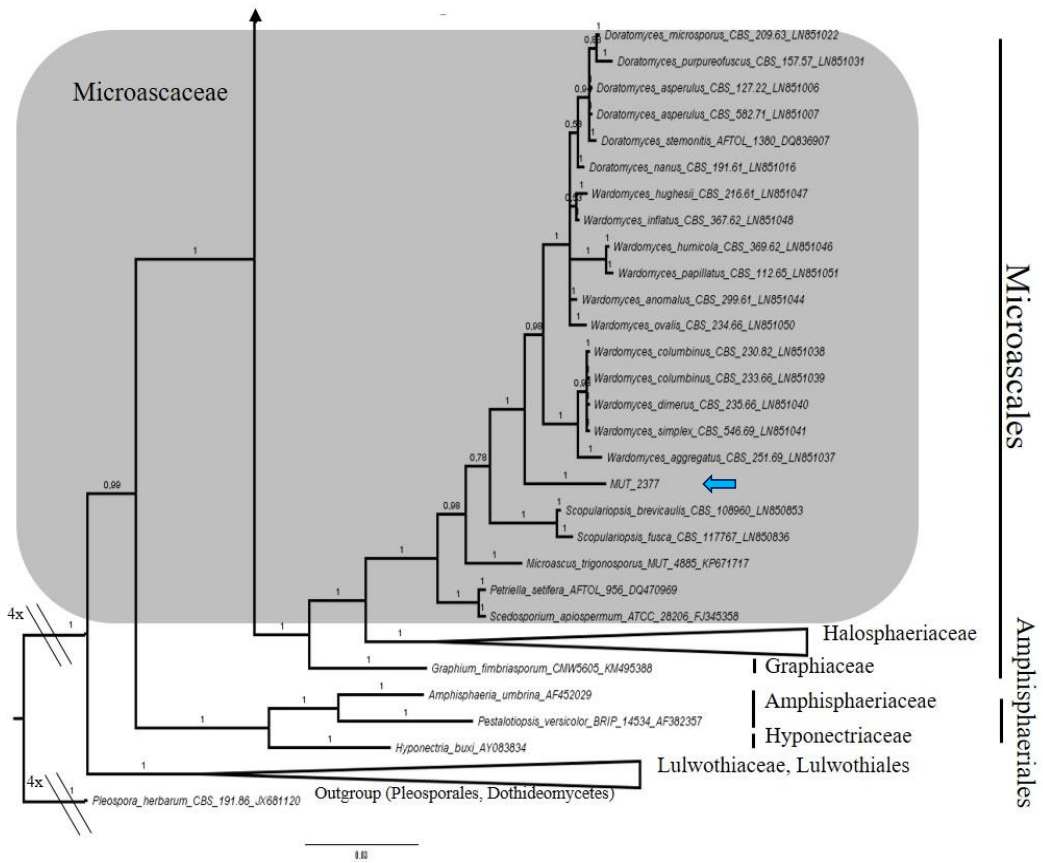


Figure 3.8 Bayesian phylogram of *Sordariomycetes* based on rDNA large subunit (LSU). Three fungal taxa clustered individually within the Nectriaceae, Hypocreaceae and Microasceae. Five fungal isolates clustered within the Bionectriaceae. Branch numbers indicate BPP values.

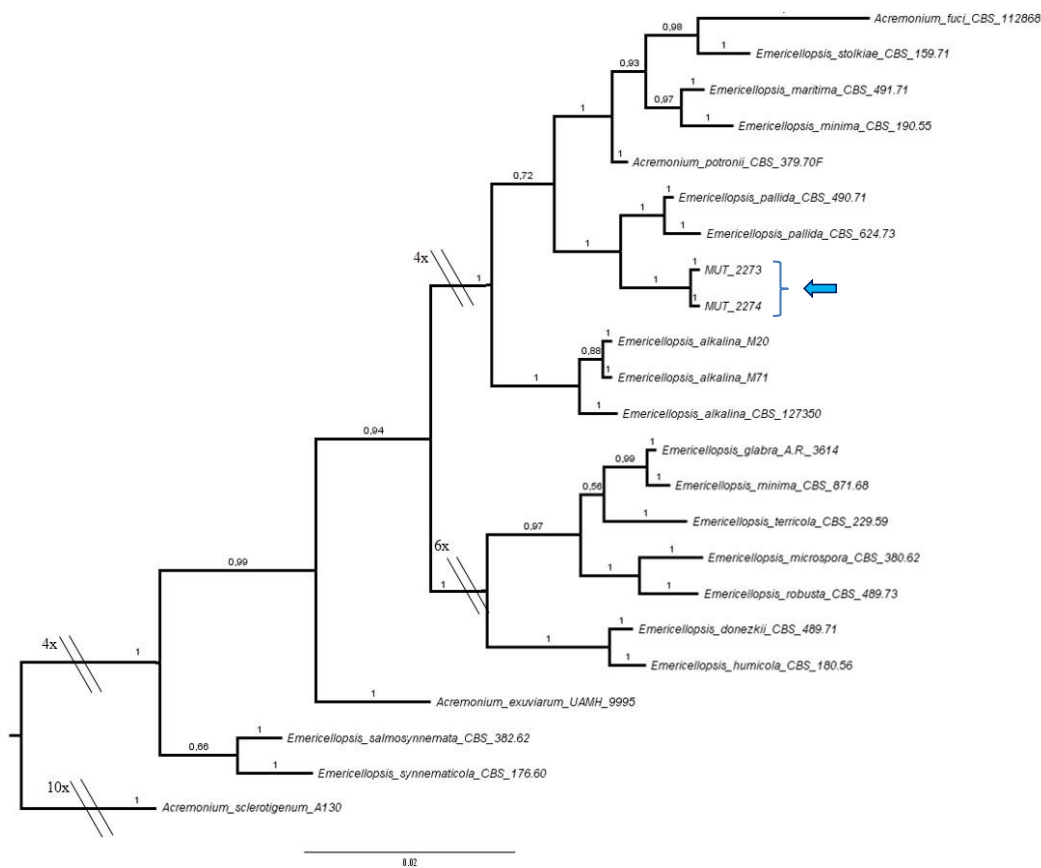


Figure 3.9 Bayesian phylogram of the genus *Emericellopsis* based on a combined dataset of ITS and beta-tubulin partial sequences. MUT 2273 and MUT 2274 were identified as *Emericellopsis pallida*. Branch numbers indicate BPP values.

Overall, the mycobiota of the 4 Atlantic sponges were mainly composed by Ascomycota (88%), with few representatives of *Basidiomycota* (11%) and *Mucoromycota* (1%). The dominance of Ascomycota is confirmed in all marine environments (Rakukumar *et al.*, 2017; Jones *et al.*, 2015) and in studies dealing with sponges' mycobiota (Suryanarayanan, 2012). *Basidiomycota* represented a small percentage of isolates, although their presence in *G. compressa* is surprising, counting for 29% of taxa. Indeed, several studies indicate the occurrence of *Basidiomycota* in sponges as lower than 10% (Ding *et al.*, 2011; Paz *et al.*, 2010; Li and Wang, 2009) or null (El-Gendy *et al.*, 2018; Passarini *et al.*, 2013; Thirunavukkarasu *et al.*, 2012;

Proksch *et al.*, 2008; Wang *et al.*, 2008; Pivkin *et al.*, 2006; Höller *et al.*, 2000). These results do not relate with the systematic classification of sponges; the occurrence of Basidiomycota could be species specific or subordinate to the sponge structure that can be more or less permissive to the fungal entrance (Pivkin *et al.*, 2006). However, their ecological role should not be underestimated: members of *Agaricales*, also detected in the present study, have already been acknowledged for their predominant role the mineralization of the organic matter in the marine environment (Hyde *et al.*, 1998).

Only one fungus (*A. glauca*) belonging to the phylum *Mucoromycota* was detected in association with *D. fragilis*. This species has already proven to withstand high salinities, as it was isolated also from the Dead Sea. Members of *Mucoromycota* were also recorded in a small percentage in few sponges (Passarini *et al.*, 2013; Thirunavukkarasu *et al.*, 2012; Höller *et al.*, 2000).

According to Raghukumar (2017), sponges generally yield from zero to 21 genera of culturable fungi: while *P. johnstonia* (11 genera) and *G. compressa* (12) hosted an average biodiverse community, *S. ciliatum* (25) and *D. fragilis* (32) host fungal communities above the mean values reported from other sponges worldwide. The most represented genera in terms of species were *Penicillium* (13) and *Cladosporium* (11), followed by *Acremonium* (6) and *Aspergillus* (5). The presence of these genera and their abundance is not a surprise: they are among the most common within sponges and the most investigated for new secondary metabolites (Imhoff, 2016).

Interestingly, despite the presence of genera commonly retrieved in the marine environment, the fungal community of the 4 Atlantic sponges highlighted 57 and 21 species first recorded here as being associated with sponges and the marine environment, respectively (Table 3.2).

3.2.3 Fungal diversity among the sponges

The 4 sponges host specific fungal communities: *D. fragilis* mycobiota was represented by 70% exclusive taxa, followed by *S. ciliatum* (63%), *G. compressa* (59%) and *P. johnstonia* (52%). The specificity of the sponge-mycobiota association was highlighted also by the Permanova analysis that reported a significant difference

($p=0.007$) among sponges. The CAP was evaluated as the best analysis to represent the data because shows the axes that best discriminate among the mycobiota of the 4 sponges (Figure 3.10).

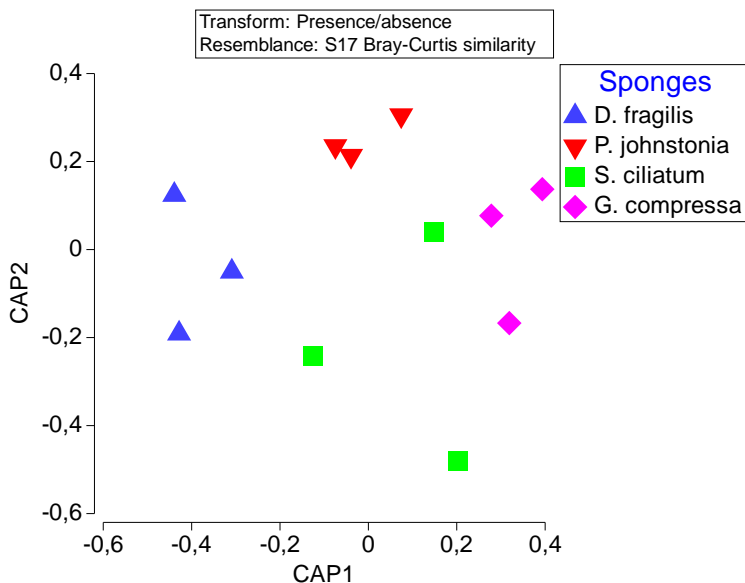


Figure 3.10 CAP of the fungal communities of the 4 Atlantic sponges *D. fragilis* (DF), *G. compressa* (GC), *P. johnstonia* (PJ) and *S. ciliatum* (SC).

Li and Wang (2009), worked on the mycobiota of three marine sponges and in the attempt to discriminate between fungi not strictly associated with sponges from those closely associated, proposed to classify in: “sponge specialist”, “sponge associates” and “sponge generalist”, as already presented in the introduction. By applying this classification to the species of the present study, it was clear that the 4 sponges species host a specific mycobiota, as supported also by statistical analysis. For confirmation of how restricted the fungal strains are to specific sponges versus specific habitat, additional sponge species from each location should be assessed and compared to the diversity reported here.

In detail, “sponge specialist” fungi, represented more than half of the fungal community of each sponge species. Eighteen species were “sponge-associated” and common to at least two sponges (Table 3.2); *D. fragilis* and *S. ciliatum* with 6 of these

resulted the most similar sponges in terms of cultivable mycobiota. Among the “sponge-associated” strains, three species (*A. jensenii*, *A. puulaauensis* and *P. neglecta*) were reported for the first time in the marine environment. The other 8 species have never been retrieved in sponge samples but have already been sampled in seawater, marine plants and algae (references in Table 3.2). Two species were widespread, reported both in the marine environment and associated with sponges: *B. bassiana* and *P. chrysogenum* (references in Table 3.2).

In the present study, the “sponge generalist” fungi were represented by *C. allicinum*, *C. cladosporioides* and *T. cylindrosporium*; all of them have been previously recorded in the marine environment and can be considered as widespread species. *C. cladosporioides* has been reported in several marine environments, from the coral reef (Raghukumar and Ravindran 2012) to the extreme conditions of the salterns (Oren and Gunde-Cimerman 2012; Zajc *et al.*, 2012) or of the crude oil contaminated environments (Bovio *et al.*, 2017). *C. cladosporioides* was also reported in association with marine algae (Gnavi *et al.*, 2017), plants (Panno *et al.*, 2013) and wood (Garzoli *et al.*, 2015a). Not least the presence on the sponges *Amphilectus digitata* (Pivkin *et al.*, 2006), *Haliclona melana* (Rozas *et al.*, 2011), *Cliona* sp. (San-Martin *et al.*, 2005) and on 4 Red Sea sponges (Sayed *et al.*, 2016). *C. allicinum* and *T. cylindrosporium* were recorded only once in the marine environment, on algae (Gnavi *et al.*, 2017) and wood substrates (Rämä *et al.*, 2014), respectively; while, here we documented the first report in association with marine sponges.

Considering the fact that some species, common to more than one sponge, have never been retrieved in the marine ecosystems, it is hard to say if the classification proposed by Li and Wang (2009) is suitable to distinguish between transient mycobiota, abundant in the water column and true sponge-associated mycobiota. This is probably due to our still scant knowledge on fungi inhabiting sea sponges and marine environment.

The specificity of the fungal community of each sponge could be related to several factors. Pivkin *et al.* (2006) highlighted that the number of fungi associated with sponges can be influenced by the sponge structure: the harder the structure, the lower

the number of fungi. Interestingly, this hypothesis is well supported in our study. *D. fragilis* has a soft structure and hosted the highest number of fungal taxa (54). Actually, sponge name is due to its fragility outside water (Marine species identification portal <http://species-identification.org/index.php>). *S. ciliatum* was the second sponge in terms of the number of taxa (32) and also in a scale of body rigidity since it presents calcareous spicules although the choanocyte chambers are free from each other, giving a "loose" consistency (Marine species identification portal <http://species-identification.org/index.php>). *G. compressa* and *P. johnstonia*, which hosted the lowest number of taxa 17 and 21, respectively, are characterized by the hardest structure. *G. compressa* has strong and plastic consistency and can be bent without breaking; while the strong cortex of up to one mm thickness and the presence of both macro (megascleres) and micro (microscleres) spicules guarantee the resistance of *P. johnstonia* (Marine species identification portal <http://species-identification.org/index.php>).

Several other factors could be involved in sponge recruitment of specific fungi, not least the sponge bioactivity (Pivkin *et al.*, 2006); *D. fragilis* and *P. johnstonia* are known for the production of bioactive metabolites, although their antifungal activity has never been demonstrated. As for *G. compressa* that has never been studied for the production of secondary metabolites, the reduced number of fungi isolated in this study (17), found correspondence also with the few bacterial strains (12) recorded by Macintyre and collaborators (2014).

However, the strongest proof supporting the hypothesis of the ability of the sponge to recognise and select fungi is the discovery of sponge mitochondrial introns of fungal origin and of (1→3)-β-d-glucan-binding proteins on the sponge surface for fungus recognition (Suryanarayanan, 2012).

Overall, the mycobiota of the 4 sponges examined in this study resulted one of the most diverse compared to other sponges of the same environment. Baker *et al.* (2009) identified 19 fungal genotypes from the sponge *Haliclona simulans* isolated in the same study area (Gurraig Sound, Co. Galway) and interestingly 85% of the identified fungal orders were also recorded in our research. In the deep-sea sponge *Stelletta normani*,

sampled at 751 m in the North Atlantic Ocean (Irish waters), 14 strains were isolated, with some genus as *Emericellopsis* spp. and *Cadophora* spp. common to the four Atlantic sponges here presented (Batista-Garcia *et al.*, 2017).

The highest overlap (7 species) among the fungal communities of the 4 sponges here described was with the Mediterranean sponge *Tethya aurantium* (from the Adriatic Sea) studied by Wiese *et al.* (2011). In detail, from one to three species were shared with each of the Atlantic sponges (Table 3.2). However, for the fungi associated with *T. aurantium*, the ITS1-5.8S-rRNA-ITS2 region was amplified, that usually it is not able to properly discriminate at intraspecific level; therefore, some grade of uncertainty remain.

Other 5 species recorded in our sponges were also found by Pivkin and collaborators (2006) that sampled different sponges close to Sakhalin Island (Russia). These species have been already retrieved in several other sponges and in seawater (Table 3.2), with the exception of *Aspergillus chevalieri* firstly retrieved in *Halichondria panacea* by Pivkin *et al.* (2006) and also present in *G. compressa* as teleomorph (*E. chevalieri*).

Four species were common to at least one of the sponge of the present study and the Mediterranean sponge *Psammocinia* sp., (Table 3.2) with *A. implicatum* only recorded in *D. fragilis* and *Psammocinia* sp. (Paz *et al.*, 2010). Finally, an overlap of two species was observed among the Atlantic sponges here described and other 7 sponges sampled worldwide (Table 3.2).

Overall, the similarities of the fungal communities among the sponges is strongly influenced by the techniques used for the identification; indeed, only few fungi and usually the most common (i.e. *Penicillium* spp. and *Cladosporium* spp.) are identified at species level. Thus preventing a proper comparison with our results.

Finally, few species reported in the present study and isolated from healthy sponges, have been previously reported as pathogenic on marine plants and animals. *A. molesta* was found on skin lesion of *Phocoena phocoena*, a marine mammal (Tóth *et al.*, 2011), and was first recorded in association with a sponge in the present study. *F. solani* and *M. bicuspidata* are a threat to shrimp and prawn aquaculture (Hatai *et al.*, 2012;

Baker *et al.*, 2009); both species have already been reported in apparently healthy sponges (Bolaños *et al.*, 2015; Paz *et al.*, 2010; Baker *et al.*, 2009). Concerning plants, *C. perangustum* (isolated from marine water) showed pathogenic activity against mangrove leaves under laboratory conditions (Liu *et al.*, 2017). These fungi are probably opportunistic pathogens, not properly able to affect healthy organisms, like the sponges of the present studies; however, further studies will be necessary to better understand their ecological role.

3.3 CONCLUSION

This chapter highlighted the great mosaic of largely unknown marine microbial diversity. The use of several isolation methods improved the yield of cultivable fungi that with few techniques and growth media, would have been impossible to isolate.

The sponges proved to be able to recruit a specific mycobiota that was possible to highlight thanks to a low rank classification. For the first time, several species were reported in the marine environment and in association with sponges. This might lead to think that the species already known in the terrestrial environment were not strictly associated with the sponges. The proximity of the sampling sites with the coast might have determined contamination from land, also considering the nature of sponges as filter feeding organisms. However, the presence of a terrestrial fungus in the sea does not mean that it has no ecological meaning (Poli *et al.*, 2018). Moreover, it has to be noted that all fungi were isolated on media containing seawater, proving that they are able to grow in a salty environment.

Finally, more than 200 sequences were deposited in GenBank, contributing with new molecular data to the knowledge of marine fungi.

The results showed in this chapter have been published in:

Fungal Systematics and Evolution. Bovio E., Garzoli L., Poli A., Prigione V., Firsova D., McCormack G. P., Varese G. C. (2018). The culturable mycobiota associated with three Atlantic sponges, including two new species: *Thelebolus balaustiformis* and *T. spongiae*, 1: 141–167.

Marine Drugs. Bovio E., Garzoli L., Poli A., Luganini A., Villa P., Musumeci R., McCormack G. P., Cocuzza C. E., Gribaudo G., Mehiri M., Varese G. C. (2019). Marine fungi from the sponge *Grantia compressa*: biodiversity, chemodiversity and biotechnological potential.

4. *Thelebolus balaustiformis* and *Thelebolus spongiae*: two new fungal species from the Atlantic sponge *D. fragilis*

4.1 MATERIAL AND METHODS

4.1.1 *Thelebolus* spp. growth conditions and molecular study

Thelebolus spp. MUT 2357 and MUT 2359 were pre-grown on Potato Dextrose Agar - PDA (Potato extract 4 g, dextrose 20 g, agar 15 g - Sigma-Aldrich, Saint Louis, USA - up to 1 L dH₂O) at 25 °C and then inoculated in triplicate onto Petri dishes (9 cm Ø) containing MEA, PDA and Carrot Agar - CA (grated carrot 20 g boiled and filtered, agar 20 g - Sigma-Aldrich, Saint Louis, USA - up to 1 L dH₂O) alone and with different concentrations of NaCl (2.5%, 5%, 10%, 15%) and incubated at 4 °C, 15 °C and 25 °C. The fungal growth, the macroscopic and microscopic features were evaluated at three, seven, 10, 14, 17, 21 d after the inoculum. Mature reproductive structures were observed and photographed with an optical microscope (LEICA DM4500 B) equipped with a camera (LEICA DFC320). Morphological data (microscopic and macroscopic) were compared with the available description of *Thelebolus* species. DNA was extracted and the ITS and beta-tubulin regions were amplified (as mentioned in chapter 3) following the recommendations of previous studies (Crous *et al.*, 2015; de Hoog *et al.*, 2005). A two-marker dataset was built for a phylogenetic analysis, which was performed as described in chapter 3.

4.2 RESULTS AND DISCUSSION

4.2.1 *Thelebolus balaustiformis* and *Thelebolus spongiae* sp. nov.

The sponge *D. fragilis* presented the most diverse mycobiota, including two fungi MUT 2357 and MUT 2359, attributed to the genus *Thelebolus* both by molecular and morphological analyses. No matches in morphological features were observed among our strains and the 16 species and two varieties of *Thelebolus* known (CBS-KNAW Collection, Westerdijk Fungal Biodiversity Institute, MycoBank). The phylogenetic tree (Figure 4.1) based on two markers (ITS and beta-tubulin) confirmed the uniqueness of *Thelebolus* MUT 2357 and *Thelebolus* MUT 2359.

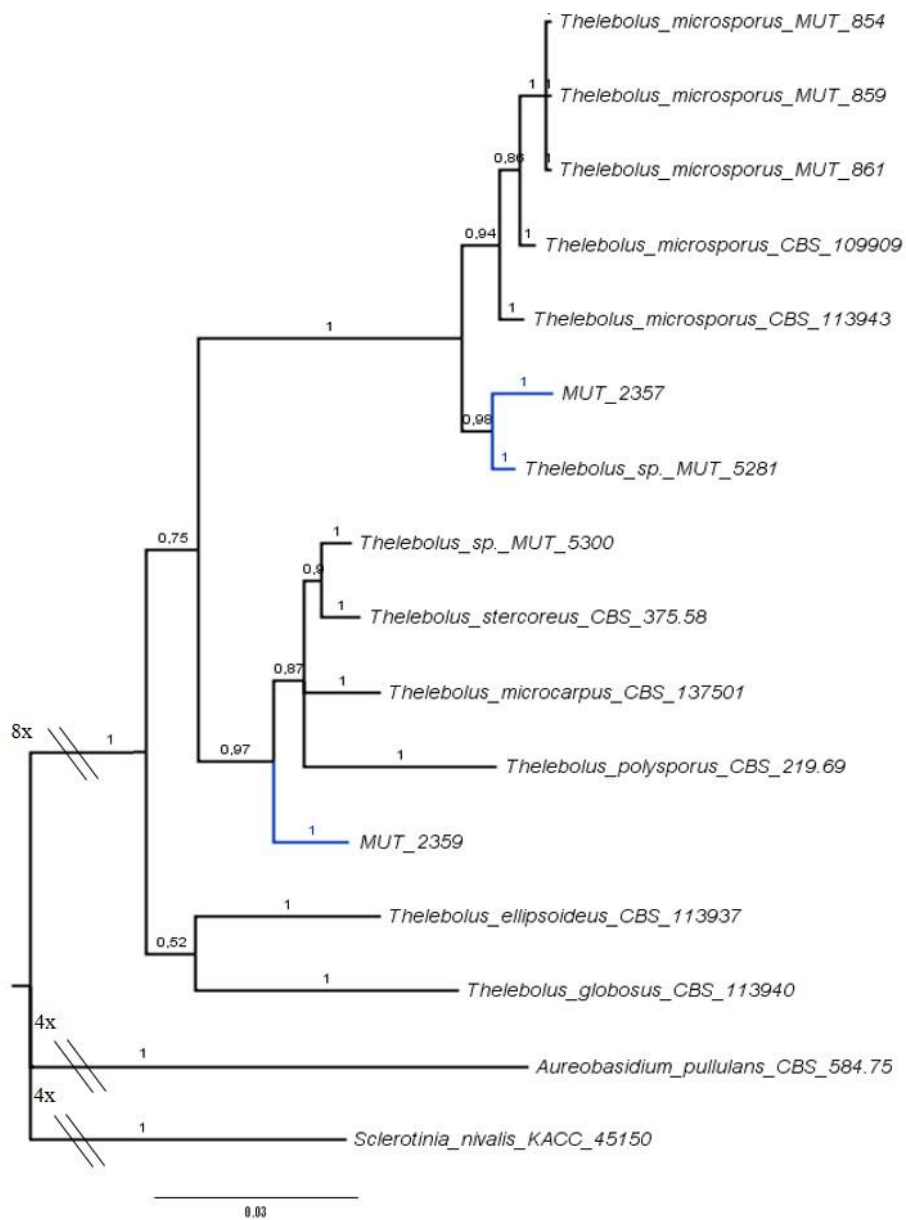


Figure 4.1 Bayesian phylogram of the genus *Thelebolus* based on a combined dataset of ITS and beta-tubulin partial sequences. MUT 2357 and MUT 2359 were identified as new species, *T. balaustiformis* and *T. spongiae*, respectively. Branch numbers indicate BPP values.

4.2.2 Taxonomy

4.2.2.1 Description of *Thelebolus balaustiformis*

Classification: Thelebolaceae, Thelebolales, Leotiomyces.

Thelebolus balaustiformis E. Bovio, L. Garzoli, A. Poli, V. Prigione, G. C. Varese, *sp. nov.* MycoBank MB824102.

Etymology: The specific epithet *balaustiformis* is derived from the similarity of ascomata, either whole or in section, with the pomegranate (*Punica granatum*) fruit, which, in botanical terms, is called *balaustum*.

Ascomata were produced only on MEA at 4 °C, after 3 weeks of incubation (Figure 4.2). *Mycelium* hyaline to pale yellow consisting of irregularly swollen, septate hyphae 1.5–5 µm wide. *Ascomata* hyaline or pale yellow, partially immersed in the colony, (87–)100–120 × 100 µm, at first subglobose cleistohymenial then opening by rupturing of the cortical excipulum in the upper part and becoming semiglobular, appearing “apothecoid” at maturity. *Hymenium* with a palisade of asci. *Cortical excipulum* ca. 6–10 µm thick, consisting of several layers of flattened cells (*textura epidermoidea*). *Asci* 20–30 per ascoma, broadly clavate, rather thick-walled (1–1.5 µm), 48–64-spored, 11–20 × 43–57 µm. *Ascospores* irregularly disposed, ellipsoid with rounded ends (length/width ratio 1.4–1.6), 4–4.7 × 2.8–3 µm, hyaline with a homogenous content, smooth-walled, without mucilaginous substance. *Spores* are forcefully discharged as a single projectile through the subapical part of the ascus. *Paraphyses* absent. *Asexual morph* not observed.

Colony description and physiological features: Colonies on CA attaining 12–17 mm diam in 21 d at 25 °C, plane, thin, mycelium mainly submerged, margins irregular (also at 5% NaCl), becoming regular in presence of 2.5% NaCl; at 15 °C and 4 °C colonies very similar with regular margins, reaching 56–59 mm and 36–37 mm diam in 21 d, respectively.

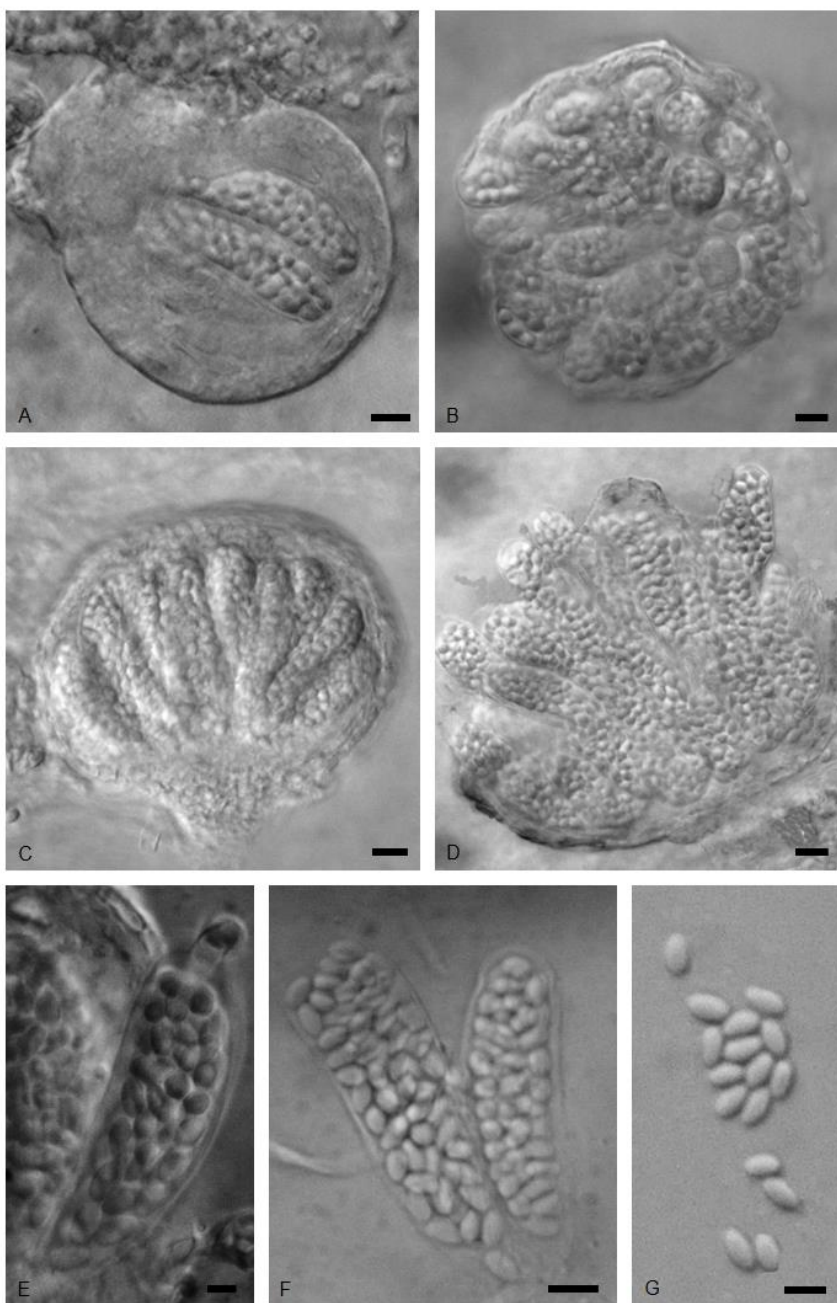


Figure 4.2 *T. balaustiformis* MUT 2357. **A, B.** Closed subglobose ascoma in the first stage of development. **C.** Ascoma becoming apothecial with mature asci. **D.** Apothecial ascoma with cortical excipulum dehiscent; **E, F.** Mature asci with 48–64 ascospores. **G.** Ascospores. Scale bars: **A–D, F** = 10 μ m; **E, G** = 5 μ m.

Colonies on PDA attaining 8–9 mm diam in 21 d at 25 °C, developing in height, pink to orange, the reverse of the same colour of the surface. At 15 °C colonies reaching 65–73 mm diam in 21 d, plane, pink–orange, margins regular (slightly irregular at 5% NaCl), slimy; reverse of the same colour of the surface. At 4 °C colonies very similar, reaching 42–48 mm diam in 21 d. Colonies on MEA not growing at 25 °C in 21 d; in presence of 2.5% and 5% NaCl, mycelium developing in height, pale orange, attaining 11–13 mm (2.5% NaCl) and 8–11 mm (5% NaCl) diam in 21 d. At 15 °C and 4 °C colonies plane, pink, reverse as the surface, reaching 46–48 mm and 25–27 mm diam in 21 d, respectively.

The sizes of the colonies with different media, salt concentrations and temperature are shown in Figure 4.3 (A–I); the morphologies in Figure 4.4 – 4.5 – 4.6.

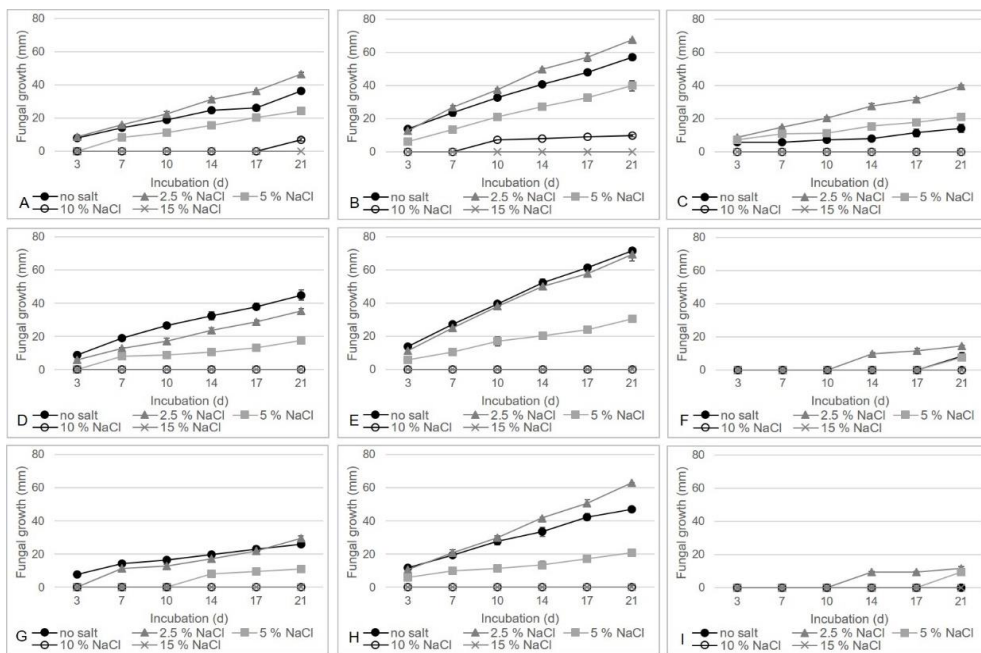


Figure 4.3 *T. balaustriformis* MUT 2357 growth curve with no and different NaCl concentrations on CA at **A.** 4 °C; **B.** 15 °C; **C.** 25 °C; on PDA at **D.** 4 °C; **E.** 15 °C; **F.** 25 °C; on MEA at **G.** 4 °C; **H.** 15 °C; **I.** 25 °C.

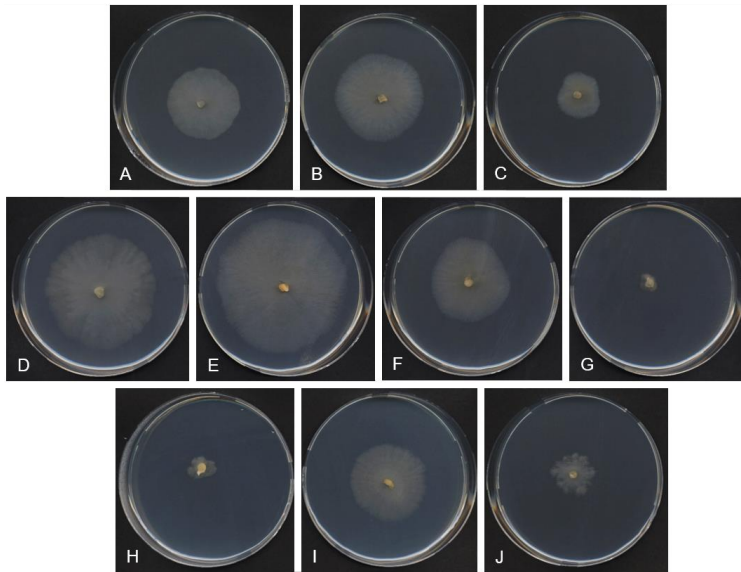


Figure 4.4 *T. balaustiformis* MUT 2357: 21-d-old colonies on CA at 4 °C with **A.** 0% NaCl; **B.** 2.5% NaCl; **C.** 5% NaCl; at 15 °C with **D.** 0% NaCl; **E.** 2.5% NaCl; **F.** 5% NaCl; **G.** 10% NaCl; at 25 °C with **H.** 0% NaCl; **I.** 2.5% NaCl; **J.** 5% NaCl.

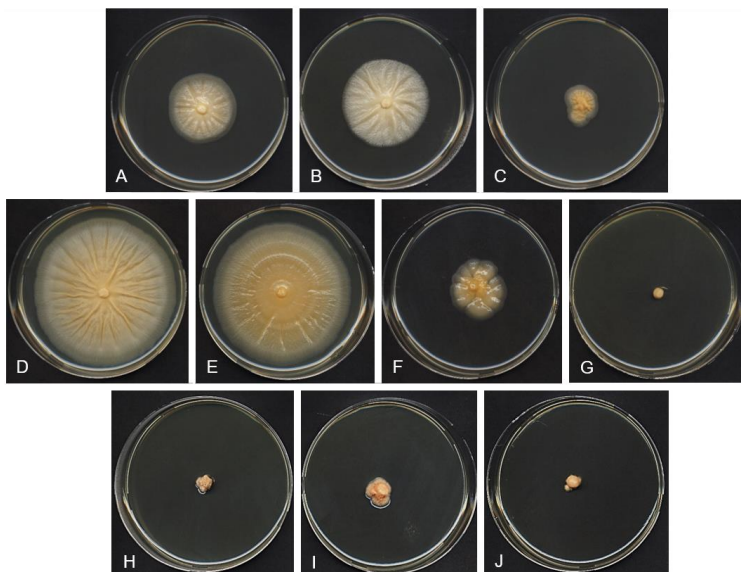


Figure 4.5 *T. balaustiformis* MUT 2357: 21-d-old colonies on PDA at 4 °C with **A.** 0% NaCl; **B.** 2.5% NaCl; **C.** 5% NaCl; at 15 °C with **D.** 0% NaCl; **E.** 2.5% NaCl; **F.** 5% NaCl; **G.** 10% NaCl; at 25 °C with **H.** 0% NaCl; **I.** 2.5% NaCl; **J.** 5% NaCl.

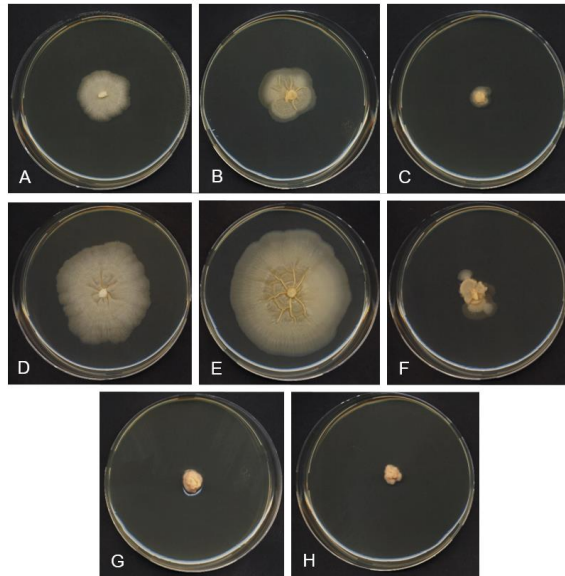


Figure 4.6 *T. balaustiformis* MUT 2357: 21-d-old colonies on MEA at 4 °C with **A.** 0% NaCl; **B.** 2.5% NaCl; **C.** 5% NaCl; at 15 °C with **D.** 0% NaCl; **E.** 2.5% NaCl; **F.** 5% NaCl; at 25 °C with **G.** 2.5% NaCl; **H.** 5% NaCl.

T. balaustiformis reached the optimal growth at 15 °C, regardless of media and/or salt concentrations utilised (Figure 4.3); 25 °C was the most inhibiting temperature (Figure 4.3). Regarding the salt concentration, the fungus grew up to 10% NaCl only on CA at 4 °C (Figure 4.3A) and 15 °C (Figure 4.3B). On CA at 4 °C and 15 °C the faster growth was reached at 2.5% NaCl, followed by 0%, 5% and 10% NaCl (Figure 4.3A, B). At 25 °C the media with NaCl (2.5% and 5%) better supported the fungal growth (Figure 4.3). On PDA at 4 °C the growth was faster with the decreasing of salt (until 0% NaCl). At 15 °C the conditions with 0% and 2.5% NaCl were comparable, while the slower growth was observed at 5% NaCl (Figure 4.3E). At 25 °C the fungus displayed a similar behaviour compared to CA at the same temperature, the growth was slow and better supported by salt (Figure 4.3F). The growth of *T. balaustiformis* on MEA at 4 °C (Figure 4.3G), 15 °C (Figure 4.3) and 25 °C (Figure 4.3) was similar to CA (in the same conditions), but there was a less evident difference

between 0% and 2.5% NaCl at 4 °C, that became more evident at 15 °C, with a faster growth.

Specimen examined: Ireland, Galway, Gurraig Sound, Co. Galway, N 53°, 18.944; W 09°, 40.140, on the sponge *D. fragilis*, 4 Jun. 2015, *G. P. McCormack* and *D. Firsova*. Holotype preserved as metabolically inactive culture MUT 2357.

Note: T. balaustiformis MUT 2357 was isolated by homogenisation of sponge tissues on CMASW, incubated at 15 °C.

4.2.2.2 Description of *Thelebolus spongiae*

Thelebolus spongiae E. Bovio, L. Garzoli, A. Poli, V. Prigione, G. C. Varese, *sp. nov.* MycoBank MB824103.

Etymology: The specific epithet *spongiae* is derived from the isolation of the fungus from a marine sponge and its strict association with it, due to the isolation by direct plating of the sponge.

Ascomata were produced only on PDA at 4 °C, after 3 weeks of incubation (Figure 4.7). *Mycelium* hyaline consisting of septate hyphae 3.2–4.7 µm wide, sometimes organised into bundles. *Ascomata* hyaline, superficial, scattered to grouped, from 50 × 40 µm for uni-ascal to 250 × 200 µm diam for multi-ascal, globose to subglobose cleistohymenial not becoming “apothecoid” with age. *Cortical excipulum* clearly differentiated, pale, 6–7 µm thick of 1–2 layers of flattened cells (*textura epidermoidea*). *Asci* 1–6 per ascoma, from globular to sacciform, rather thick-walled (1.5–3 µm), containing hundreds of spores, 37–57 × 50–70 µm. *Ascospores* irregularly disposed, ellipsoid with rounded ends (length/width ratio 2.2–2.4), 7–9.5 × 3.2–4 µm, hyaline with a homogenous content, smooth-walled, without mucilaginous substance. *Paraphyses* absent. *Asexual morph* not observed.

Colony description and physiological features: Colonies on CA attaining 47–51 mm diam in 21 d at 15 °C, smooth, mycelium sparse, pale pink, margins irregular also in presence of NaCl, reverse of the same colour of the surface.

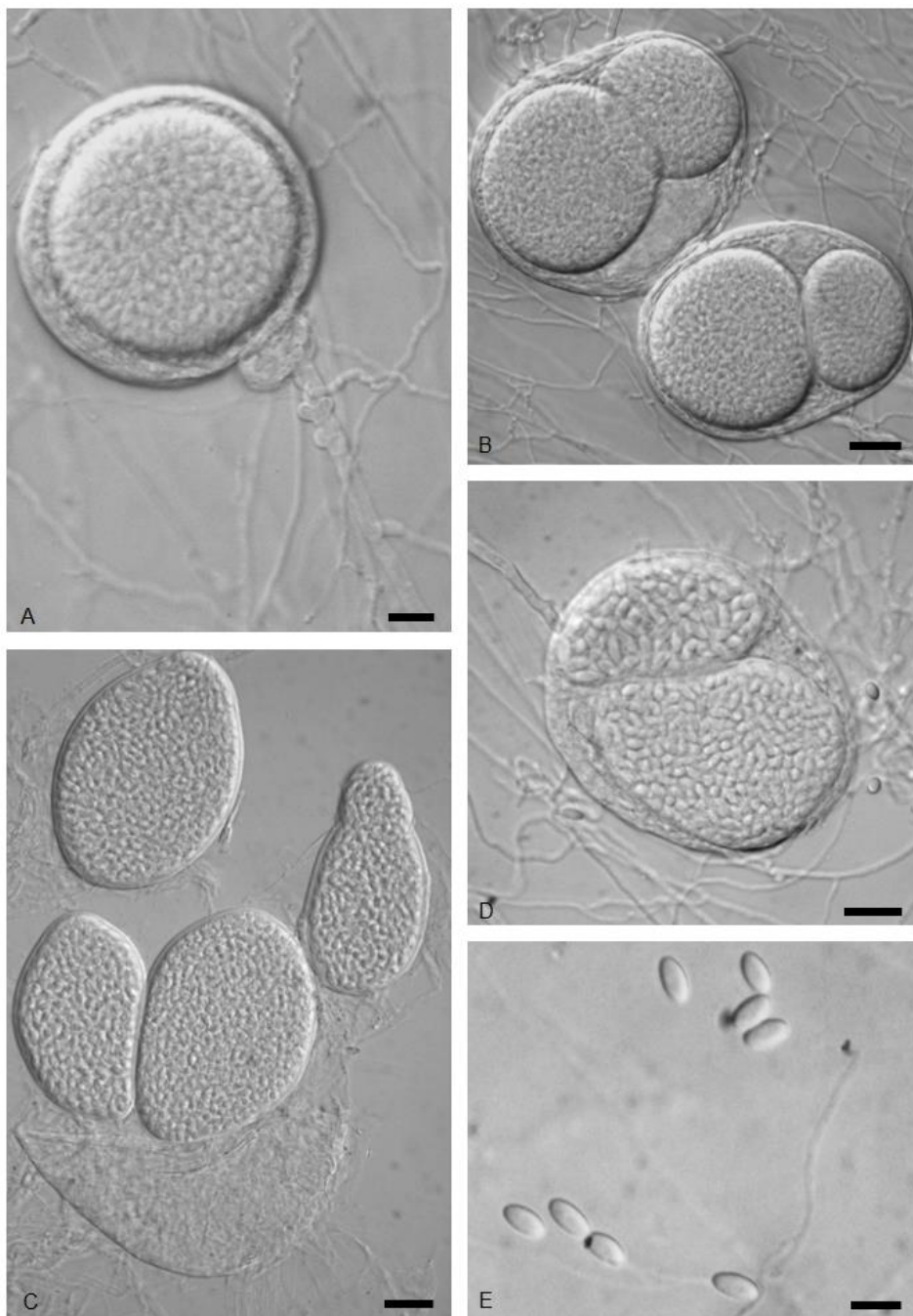


Figure 4.7 *T. spongiae* MUT 2359: **A.** Initial ascoma; **B.** Ascomata with two globular asci; **C.** Mature ascoma opening with four asci; **D.** Ascoma with two sacciform asci; **E.** Ascospores. — Scale bars: **A, E** = 10 μm; **B–D** = 30 μm.

At 15 °C colonies similar but with more regular margins, reaching 49–50 mm diam in 21 d. Colonies with regular margins at 4 °C, 28–30 mm diam in 21 d. Colonies on PDA attaining 70–74 mm diam in 21 d at 25 °C, smooth, pale pink, radially sulcate (also in presence of 2.5% NaCl, not with 5% NaCl), margins mainly submerged; reverse of the same colour of the surface. At 15 °C and 4 °C colonies very similar, reaching 60–64 mm and 35–38 mm diam in 21 d, respectively. Colonies not radially sulcate, mucoid at 4 °C. Colonies on MEA 30–32 mm diam in 21 d at 25 °C, smooth, mycelium partially submerged, pale pink, margins irregular (regular in presence of 2.5% and 5% NaCl); reverse of the same colour of the surface. At 15 °C and 4 °C colonies very similar with margins only slightly irregular, reaching 28–29 mm and 16 mm diam in 21 d, respectively.

The sizes of the colonies with different media, salt concentrations and temperature are shown in Figure 4.8 (A–I); the morphologies in Figure 4.9 – 4.10 – 4.11.

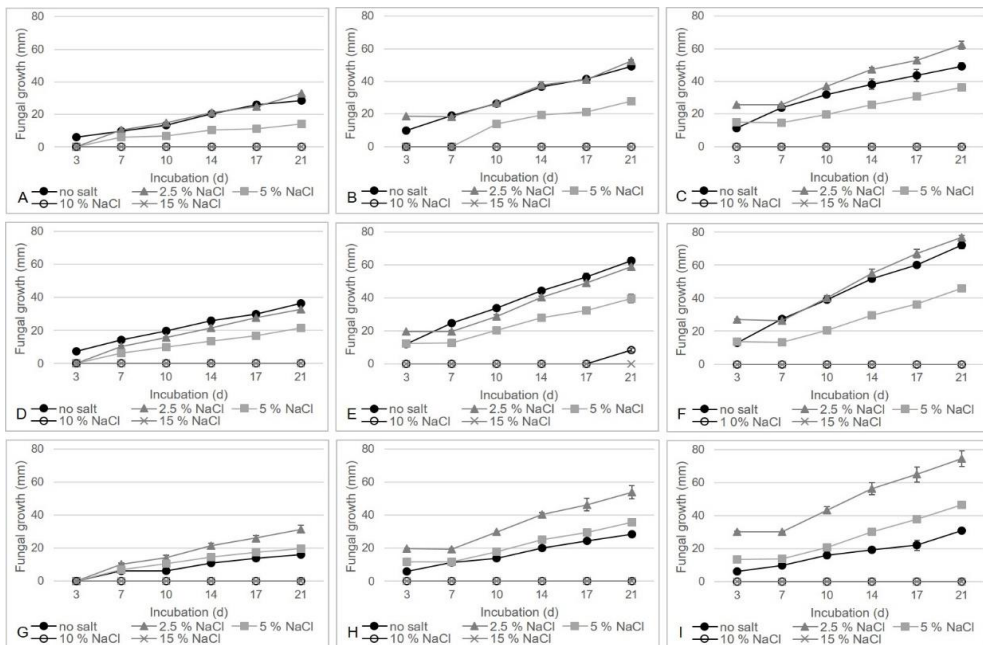


Figure 4.8 *T. spongiae* MUT 2359 growth curve with no and different NaCl concentrations on CA at **A.** 4 °C; **B.** 15 °C; **C.** 25 °C; on PDA at **D.** 4 °C; **E.** 15 °C; **F.** 25 °C; on MEA at **G.** 4 °C; **H.** 15 °C; **I.** 25 °C.

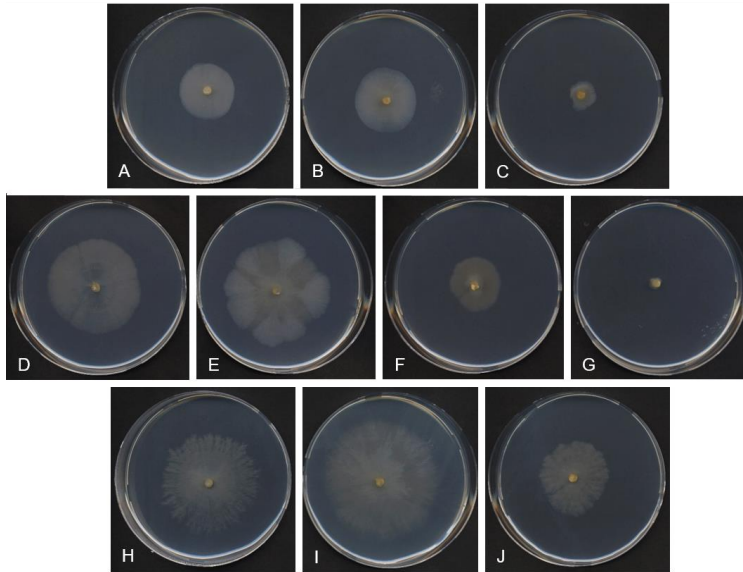


Figure 4.9 *T. spongiae* MUT 2359: 21-d-old colonies on CA at 4 °C with **A.** 0% NaCl; **B.** 2.5% NaCl; **C.** 5% NaCl; at 15 °C with **D.** 0% NaCl; **E.** 2.5% NaCl; **F.** 5% NaCl; **G.** 10% NaCl; at 25 °C with **H.** 0% NaCl; **I.** 2.5% NaCl; **J.** 5% NaCl.

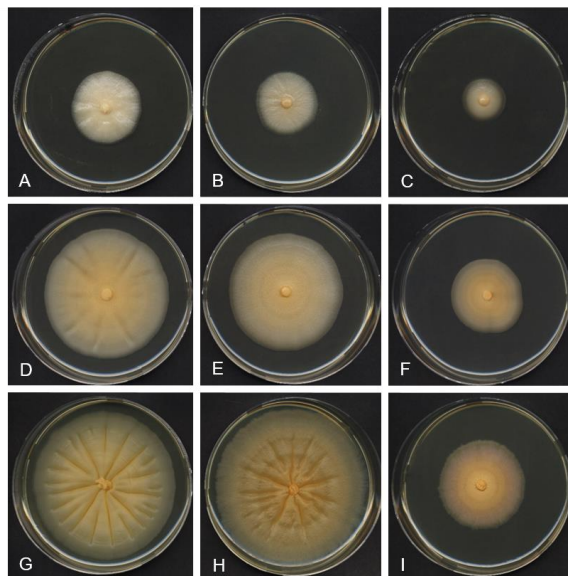


Figure 4.10 *T. spongiae* MUT 2359: 21-d-old colonies on PDA at 4 °C with **A.** 0% NaCl; **B.** 2.5% NaCl; **C.** 5% NaCl; at 15 °C with **D.** 0% NaCl; **E.** 2.5% NaCl; **F.** 5% NaCl; at 25 °C with **G.** 0% NaCl; **H.** 2.5% NaCl; **I.** 5% NaCl.

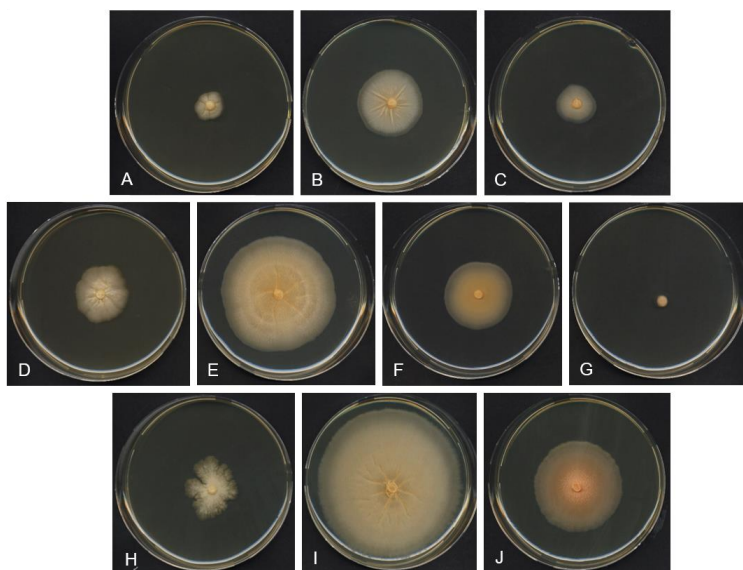


Figure 4.11 *T. spongiae* MUT 2359: 21-d-old colonies on MEA at 4 °C with **A.** 0% NaCl; **B.** 2.5% NaCl; **C.** 5% NaCl; at 15 °C with **D.** 0% NaCl; **E.** 2.5% NaCl; **F.** 5% NaCl; **G.** 10% NaCl; at 25 °C with **H.** 0% NaCl; **I.** 2.5% NaCl; **J.** 5% NaCl.

T. spongiae, as reported in Figure 4.8, grew without NaCl and at 2.5% and 5% of NaCl; while at 10% NaCl exhibited no growth, with the exception of PDA at 15 °C where the growth started 17 d after the inoculum and reached 7–9 mm diam in 21 d (Figure 4.8E). The fungus grew better with the increasing of the incubation temperature, from 4°C to 25 °C. On PDA and CA at all temperatures, the growth of *T. spongiae* without and with 2.5% NaCl was comparable (Figure 4.8A–F); only at 25 °C on CA the difference was more pronounced: after 10 days the fungus started to grow faster with 2.5% NaCl (Figure 4.8C). The presence of 5% NaCl made slower *T. spongiae* growth. *T. spongiae* grew faster on MEA in the presence of NaCl (2.5–5%) compared to its absence; this difference was evident since the first stage of development at 15 °C and 25 °C (Figure 4.8H, I), while at 4 °C it took 10 d to take shape (Figure 4.8G).

Specimen examined: **Ireland**, Galway, Gurraig Sound, Co. Galway, N 53°, 18.944; W 09°, 40.140, on the sponge *D. fragilis*, 4 Jun. 2015, *G. P. McCormack* and *D. Firsova*. Holotype preserved as metabolically inactive culture MUT 2359.

Note: T. spongiae MUT 2359 was isolated by direct plating of the sponge on SWA plate and incubated at 15 °C.

4.2.2.3 Ecology and peculiar features of *Thelebolus* spp.

The genus *Thelebolus* has been isolated from Tropical to Arctic regions, often on animal dung and from freshwater and saline lakes (de Hoog *et al.*, 2005). In the marine environment, members of *Thelebolus* were recorded also associated with *Padina pavonica*, a Mediterranean brown algae (Garzoli *et al.*, 2018) and from an Antarctic marine sponge (Henríquez *et al.*, 2014). In both cases, isolates were reported as *Thelebolus* sp. and the identification was based on molecular data.

Morphological characters useful to classify this genus have been long debated and, since the 70's, the number of spores per ascus represents the main character for species definition (de Hoog *et al.*, 2005). At present, the genus *Thelebolus* includes 16 species and two varietas, most of which described at the end of the 19th or in the first half of the 20th century. For this reason, many of the described species, are lacking: i) original exhaustive descriptions (i.e. microscopic characters poorly described); ii) DNA barcode sequences available in public databases; iii) type strains preserved in culture collections.

Since the two *Thelebolus* species isolated in this study presented unique morphological and molecular features, we performed a deep bibliographic search to define the main characters for each described *Thelebolus* species (Table 4.1) and for those available in culture collections, we obtained comparable sequences, which are now available to the scientific community. The two new marine species can be easily distinguished because they form well-defined lineage within the genus (Figure 4.1). Interestingly, the isolates MUT 2357 clustered with a marine strain (*Thelebolus* sp. MUT 5281), already present in MUT culture collection and isolated from a Mediterranean brown alga; this indicates the strong affinity of this species with the marine environment. From a morphological point of view, all the dichotomous keys of the genus point out as a first statement the presence of 8-spored or multispored asci (de Hoog *et al.*, 2005; Doveri, 2004). Therefore, considering only the multi-spored species (not included in the

tree because there were no available sequences) we can first exclude the similarity of *T. balaustiformis* with *T. monoascus* and *T. pilosus*, in fact, the last two mentioned species present only one ascus per ascomata and a higher number of spores compared to MUT 2357. *T. balaustiformis* differ also from the two varieties of *T. dubius*, by presenting a higher number of asci (20–30) and a lower number (48–64) of smaller spores.

T. spongiae MUT 2359, is characterised by a variable number of asci (from one to six), while in *T. monoascus* and *T. pilosus* is strictly limited to one; the latest mentioned species differs from *T. spongiae* MUT 2359 also for the shape and size of ascospores. *T. dubius* var. *lagopi* and *T. dubius* var. *dubius* present a variable number of asci, starting from three; the shape of asci, as well as those of ascospores, differ from *T. spongiae*. In fact, MUT 2359 presents globular to sacciform asci and peculiar ascospores with a different ratio (2.2–2.4) from *T. dubius* var. *lagopi* (1.5) and *T. dubius* var. *dubius* (1.7).

Interestingly, *T. balaustiformis* was isolated by homogenisation of sponges tissues on CMASW at 15 °C, while *T. spongiae* was isolated by direct plating of sponge on SWA at 15 °C, highlighting one more time the importance of using different isolation techniques and culture conditions.

In conclusion, with the present work, we highlighted the great and still unexplored fungal diversity that characterises the marine environment. The use of several isolation methods improved the yield of cultivable fungi that with few techniques and growth media, would have been impossible to isolate. The sponges proved to host a specific mycobiota and several fungi identified with the contribution of morphological, molecular and phylogenetic approach, were first reported in the marine environment, while *T. balaustiformis* and *T. spongiae* were here described as new. The present study again highlights the great mosaic of largely unknown marine microbial diversity

Table 4.1 *Thelebolus* species and main morphological features (ascomata, asci and ascospores).

Species	Ascomata	Number of asci per ascoma	Asci	Number of ascospores	Ascospores	References
<i>T. coemansii</i>	-	Numerous	85–110 × 20–25 µm, cylindric-clavate	8	-	[13]
<i>T. delicatus*</i>	Subglobosus	-	-	-	-	[5]
<i>T. dubius var. dubius</i>	-	3–5	40–45 × 24.4 µm, broadly ovate or oblong-ovate	128 (?)	6 × 4 µm, ellipsoid, rather pointed at the ends	[4]
<i>T. dubius var. lagopi</i>	80–150 µm diam subglobosus	10–16	87–100 × 25–33 µm, cylindric-clavate	more than 200	6.2–7.6 × 3.6–4.3 µm, ellipsoid to ovoid	[4]
<i>T. ellipsoideus</i>	17–46 µm diam, subglobosus or ovoid to ellipsoid	1–8 (rarely up to 25)	22 × 11–16 µm, shortly ellipsoid to subglobose	8	5–9.2 × 4–5.3 µm, shortly-ellipsoid	[6]
<i>T. globosus</i>	300–520 µm diam, subglobosus or ovoid to ellipsoid	1–4	12–15 × 9–12 µm, irregular shortly ellipsoid to subglobose	8	5–7.5 × 4.1–5.1 µm, broadly ellipsoid	[6]
<i>T. hirsutus*</i>	-	-	-	-	-	[2]
<i>T. lignicola</i>	-	-	-	60-100	3.4 x 4– 4.5 µm	[8]
<i>T. microcarpus</i>	18–70 µm diam, globose to subglobose	1–5	12–17 × 10–15 µm, subglobose to broadly ellipsoid	8	5–9 × 3–4 µm, ellipsoid	[1]

<i>T. microsporus</i>	45–500 µm diam, subglobose, hemispheric or subcylindric	5–100	80–125 × 20–26 µm, cylindric to cylindric-cvaviform	8	6–10 × 3–5	[4, 6, 7]
<i>T. minutissimus*</i>	-	-	-	-	-	[3]
<i>T. monoascus</i>	150–200 µm diam, hemispheric	1	150–170 µm	500	5–6.5 × 4–4.5 µm, ovate	[9]
<i>T. pilosus</i>	-	1	300 × 250 µm	about 100	9–11 × 7–8 µm	[11]
<i>T. polysporus</i>	60–200 µm diam	2–5	50–160 × 18–90 µm, subellipsoid to ovoid or sacciform	256	5–7.5 × 3–4 µm, ovoid to oblong-ellipsoidal	[4, 7, 13]
<i>T. stercoreus</i>	135–400 µm diam, ellipsoid to ovoid or subglobose	1, rarely 2–3	165–262 × 120–205 µm, ellipsoid to ovoid or subglobose	up to 3000 spores	5–7.7 × 2.3–4.5 µm, spores smooth, broadly elliptic, ellipsoid or oblong	[4, 7, 13]
<i>T. striatus</i>	-	-	124–162 × 9–10.8 µm, cylindric elongate	8	11.3–13.5 × 6–6.7 µm, narrow ellipsoid	[12]
<i>T. terrestris</i>	-	-	-	-	18.4–25.6 × 8–9.6 µm	[10]

*The original description does not present any information about the microscopic structures

[1] Crous *et al.*, 2015, [2] De Lamarck and De Candolle, 1815, [3] De Schweinitz, 1834, [4] Doveri, 2004, [5] Fries, 1823, [6] de Hoog *et al.*, 2005, [7] Kimbrough, 1981, [8] Lloyd, 1918, [9] Mouton, 1886, [10] Pfister, 1993, [11] Schroeter, 1908, [12] Thind *et al.*, 1959, [13] Van Brummelen 1998.

The results showed in this chapter have been published in:

Fungal Systematics and Evolution. Bovio E., Garzoli L., Poli A., Prigione V., Firsova D., McCormack G. P., Varese G. C. (2018). The culturable mycobiota associated with three Atlantic sponges, including two new species: *Thelebolus balaustiformis* and *T. spongiae*, 1: 141-167.

5. The culturable fungal communities inhabiting the Mediterranean sponges *Aplysina cavernicola*, *Crambe crambe* and *Phorbas tenacior*

In the present chapter, the culturable fungal communities associated with three Mediterranean Demospongiae, *Aplysina cavernicola*, *Crambe crambe* and *Phorbas tenacior* (Figure 5.1A–C), is presented.



Figure 5.1 A. *A. cavernicola* (Ph. J. de Vaugelas). B. *C. crambe* (Ph P. Amade). C. *P. tenacior* (Ph. J. de Vaugelas).

All the above mentioned sponges are known for the production of bioactive secondary metabolites. Indeed, *A. cavernicola* produces bromotyrosine-derived metabolites with anti-feeding, antibacterial and cytotoxic properties (Reverter *et al.*, 2016). Interestingly, Reverter and colleagues (2016) observed a seasonal variation in the production of *A. cavernicola* metabolites, mainly influenced by water temperature.

C. crambe has been widely studied since the early '90; in 1996, Uriz *et al.* pointed out that the outer layer of this animal was more toxic than the inner one. This toxicity is probably due to polycyclic guanidine alkaloids, whose anticancer activity has been demonstrated (Ternon *et al.*, 2016). Likewise, *A. cavernicola*, also for *C. crambe* the production of secondary metabolites shows a seasonal fluctuation (Becerro *et al.*, 1997). Compared to the other two sponges our knowledge on the metabolome of *P. tenacior* (syn. *Anchinoe tenacior*) is still scarce: Casapullo and collaborators (1993, 1994a, b) recorded few pseudopeptides alkaloids, whose biological activity has not yet been demonstrated.

Having regard to the importance of the low rank classification mentioned in this thesis and to better understand the ecological role of marine fungi, the mycobiotas associated with three Mediterranean sponges, *A. cavernicola*, *C. crambe* and *P. tenacior*, are presented for the first time.

5.1 MATERIAL AND METHODS

5.1.1 Sampling sites and fungal isolation

The Mediterranean sponges *A. cavernicola*, *C. crambe* and *P. tenacior* (three specimens each) were collected by scuba divers in the Mediterranean Sea at Villefranche sur Mer, France (N 43° 41' 31.48707839999", E 7° 19' 12.185658623999"), along the coast, at about 25 m depth (Figure 5.2). *A. cavernicola* and *P. tenacior* were sampled on the same rock, whereas *C. crambe* was collected on a separate rock few meters apart.

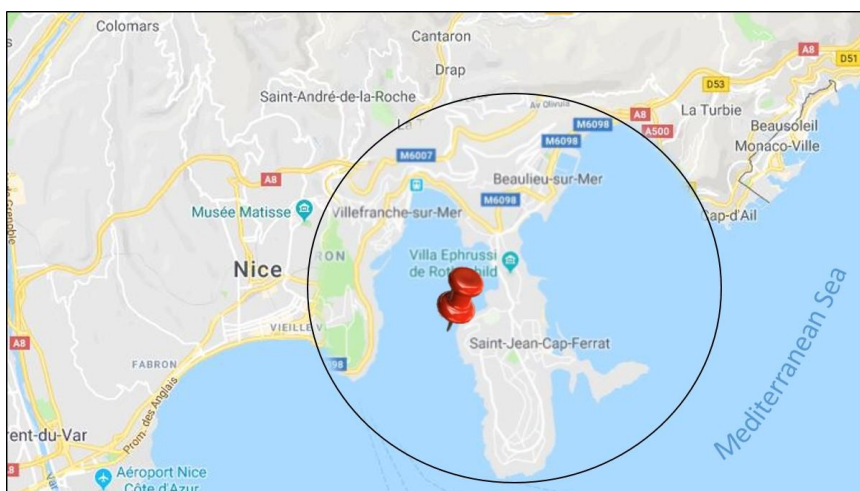


Figure 5.2 Sampling site of *A. cavernicola*, *C. crambe* and *P. tenacior* along the coast of Villefranche sur Mer, France.

Specimens were surface sterilised and homogenate as described for the Atlantic sponges in chapter 3. Differently, to what already described in the chapter, the diluted homogenate was spread in each Petri dish avoiding the inclusion technique that requires to find a balance between the medium that has to be warm and liquid to be plated, but

not too hot to avoid fungal damage. Three different growth media were used: SWA, CMASW and GASW. Three replicates for each medium and incubation temperature (15 °C and 25 °C) were performed. To prevent bacterial growth, all media were supplemented with an antibiotic mix (Gentamicin Sulfate 40 mg/L, Piperacillin and Tazobactam 11 mg/L). Plates were incubated in the dark and monitored for 30 days. Fungal strains that developed were transferred to axenic cultures and taxonomically identified.

In the case of *A. cavernicola*, due to the abundance of samples, also the direct plating was applied as already described in chapter 3. The media, the incubation temperatures and the number of replicates were the same as above mentioned.

5.1.2 Fungal identification

Fungi were identified with the polyphasic approach mentioned in chapter 3, using the same media, primers and PCR programs, accordingly to the requirement of each fungal genus. Sterile mycelia, whose identification was not unveiled with the above-mentioned procedure, underwent phylogenetic analysis. Following amplification of the LSU, using primer pair LROR/LR7 (Vilgalys and Hester, 1990), three datasets were created: two for the orders Pleosporales and Capnodiales and one for the class Sordariomycetes.

Alignments and the Bayesian Inference were performed as described in chapter 3.

Isolates of the same species underwent mini-satellite screening, using as the universal primer M-13 (Poli *et al.* 2016), in order to recognize identical strains. Briefly, PCR reactions were performed in 25 µL final volumes and consisted of 0.3 µL Taq DNA Polymerase (Qiagen 5 U/µL), 2.5 µL PCR Buffer (10x), 1.0 µL dNTPs Mixture (dATP, dCTP, dGTP, dTTP; 10 mM), 2 µL MgCl₂ (25 mM), 1.5 µL of each primer (10 µM), 5 µL genomic DNA extract (5 ng/mL) and 12.7 µL distilled-deionized water. Amplicons were separated on 1.5% agarose gel stained with ethidium bromide and a GelPilot 1 kb plus DNA Ladder was used; images were acquired under UV light (BIO-RAD Universal Hood II) and fingerprints were analysed using Bionumerics 7.1 software.

Strains are preserved at the MUT. Sequences were deposited in GenBank (Annexe 2).

5.1.3 Statistical analyses

Statistical analyses to compare the fungal community among the Atlantic and Mediterranean sponges were performed using PRIMER v. 7.0 (Plymouth Routines In Multivariate Ecological Research; Clarke and Warwick 2001). PERMANOVA (pseudo-F index; $p < 0.05$) allowed the differences between the mycobiotas of the Atlantic and Mediterranean sponge. Principal Coordinate Analysis (PCO) visualized data.

5.2 RESULTS AND DISCUSSION

5.2.1 Influence of isolation techniques on the fungal community

The homogenization of samples was preferred and applied to all the sponges, due to the higher success in the isolation of fungi, compared to the direct plating, as reported in chapter 3 and in other publications (Sayed *et al.*, 2016; Paz *et al.*, 2010)

The use of different incubation temperatures increased the possibility of fungal isolation: even if the sponges were sampled in the same place and at the same depth, the cultivable mycobiota of each organism showed a preference for different incubation temperatures. Overall, 80.0% and 66.7% of taxa were exclusively isolated at 25 °C from *P. tenacior* and *C. crambe*, respectively (Figure 5.3A). As for *A. cavernicola*, most of the taxa (53.8%) were exclusively isolated at 15 °C, 23.1% were recovered at 25 °C and the remaining 23.1% were isolated at both temperatures. Whether an ecological explanation of this phenomenon exists is an issue that requires further studies. In our case, the main difference between *A. cavernicola* and the others two sponges (where a higher number of fungi were recorded at 25 °C) is the ecological niche: *A. cavernicola* usually grows under rocks and, being less exposed to the sun could prefer lower temperatures.

The use of different growth media proved to be important to increase the number of isolated taxa. Fungi from *A. cavernicola* and *P. tenacior* were isolated on all the media at different percentages (Figure 5.3B). Fungi from *C. crambe* grew exclusively on GASW and SWA, media specifically selected to mimic the isolation substrate and the

environment. These two media were particularly effective for the isolation of Basidiomycota. For instance, *Irpex lacteus*, reported for the first time in association with an Atlantic sponge (Chapter 3) using GSWA medium, was here isolated on SWA. *Psathyrella candolleana* and *Schizophyllum commune* were both isolated on GSWA. Thus, Basidiomycota were reported on unconventional poor media, re-enforcing the hypothesis that marine Basidiomycota require specific growth conditions (Ding *et al.*, 2011).

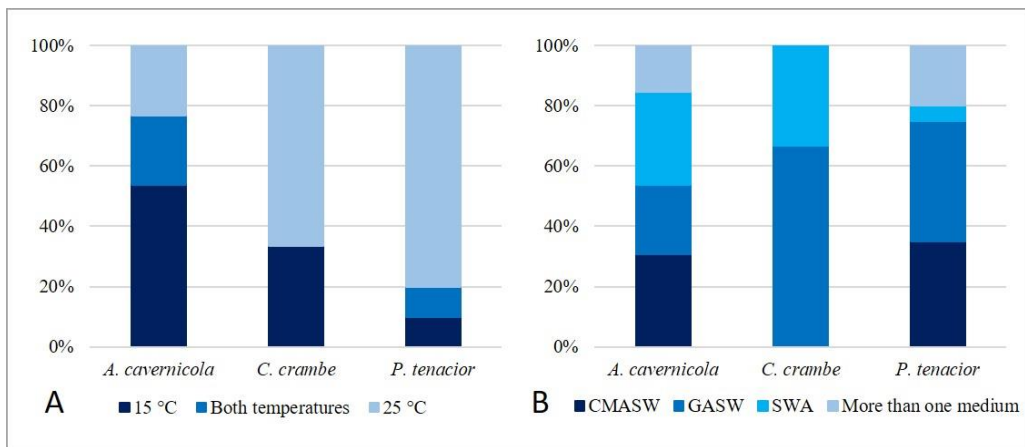


Figure 5.3 A. Influence of incubation temperatures and B. growth media on the fungal community isolated from *A. cavernicola*, *C. crambe* and *P. tenacior*.

The use of media that mimic the environmental conditions seems to be one of the leading factors that increase the number of cultivable fungi. Noteworthy, all *Penicillium* spp. were isolated on CMASW and GASW only, indicating that SWA can be considered as a selective medium that limits the growth of highly sporulating fungi. Annexe 2 reports all the fungi isolated in the different culture conditions.

5.2.2 Fungal diversity

Overall, 29 taxa were isolated from the three sponges: *P. tenacior* hosted 19 taxa (26 isolates), followed by *A. cavernicola* (13 species and 25 isolates), and *C. crambe* (3 species-isolates), as shown in Table 5.1.

Table 5.1 Fungal taxa isolated from *A. cavernicola* (AC), *C. crambe* (CC) and *P. tenacior* (PT) and their relative abundance in percentage (RA%). First record (FR) and species already found in the marine environment (MA) and associated with sponges (SP) are reported.

	RA (%)				SP
	AC	CC	PP	MA	
Ascomycota					
<i>Arthrinium arundinis</i> *			3.7	[2, 4, 5]	[26, 31]
<i>Aspergillus protuberus</i>			3.7	[6, 7, 11]	FR
<i>Beauveria brongniartii</i>			3.7	[7]	FR
<i>Cladosporium cladosporioides</i>	25.0		14.8	[1, 2, 5, 7, 19, 21, 22, 25, 32]	[8, 9, 12, 13, 14, 15, 20]
<i>Cladosporium delicatulum</i>	4.2			FR	FR
<i>Cladosporium perangustum</i>	4.2		3.7	[25]	[9]
<i>Cladosporium pseudocladosporioides</i>	20.8	33.3	11.1	[5, 32]	[9]
<i>Cladosporium ramotenellum</i>			3.7	[22]	FR
<i>Epicoccum nigrum</i>			7.4	[5]	[9, 17]
<i>Eutypella scoparia</i>	4.2			[23]	[16]
Hypocreales sp.*			3.7	-	-
<i>Kernia geniculotricha</i>		33.3		FR	FR
<i>Lecanicillium antillanum</i>			7.4	FR	FR
<i>Neosetophoma samararum</i> *	4.2		3.7	[7]	FR
<i>Penicillium brevicompactum</i>	8.3		3.7	[1, 2, 3, 5, 7, 19, 22]	[3, 6, 9, 10, 15, 31, 55]
<i>Penicillium catenatum</i>			3.7	FR	FR
<i>Penicillium citrinum</i>	4.2	33.3		[4, 5, 7, 11, 18, 21, 22, 24, 30]	[3]
<i>Penicillium corylophilum</i>	8.3			[19, 28]	[15]

<i>Penicillium glabrum</i>		3.7	[7, 18, 19, 28]	[3, 17]
<i>Penicillium murcianum</i>	4.2		FR	FR
<i>Penicillium cinereoatrum</i>		3.7	-	-
<i>Penicillium steckii</i>		3.7	[4, 18, 22]	[3, 6]
<i>Torula herbarum</i> *		3.7	[1, 27]	FR
<i>Uwebraunia dekkeri</i> *		3.7	FR	FR
<i>Virgaria nigra</i>	4.2		FR	FR
<i>Xylaria badia</i> *		3.7	FR	FR
Basidiomycota				
<i>Irpex lacteus</i> *	4.2		[9]	[9]
<i>Psathyrella candolleana</i> *		3.7	[9]	[9]
<i>Schizophyllum commune</i> *	4.2		[2, 7]	[6]
Tot. Taxa	11	3	19	
Tot. exclusive taxa of each sponge	8	1	17	

*Sterile mycelia

[1] Panno *et al.*, 2013, [2] Gnani *et al.*, 2017, [3] Paz *et al.*, 2010, [4] Raghukumar, 2017, [5] Bovio *et al.*, 2017, [6] Gao *et al.*, 2008, [7] Jones *et al.*, 2015, [8] Henríquez *et al.*, 2014, [9] Bovio *et al.*, 2018, [10] Passarini *et al.*, 2013, [11] Garzoli *et al.*, 2015a, [12] San-Martin *et al.*, 2005, [13] Manriquez *et al.*, 2009, [14] Rozas *et al.*, 2011, [15] Pivkin *et al.*, 2006, [16] Bolaños *et al.*, 2015, [17] Wiese *et al.*, 2011, [18] Rämä *et al.*, 2017, [19] Oren and Gunde-Cimerman, 2012, [20] Sayed *et al.*, 2016, [21] Raghukumar and Ravindran, 2012, [22] Zajc *et al.*, 2012, [23] Ciavatta *et al.*, 2008, [24] Suryanarayanan, 2012, [25] Liu *et al.*, 2017, [26] Wang *et al.*, 2015, [27] Blunt *et al.*, 2014, [28] Gomes *et al.*, 2008, [29] Rämä *et al.*, 2014, [30] Debbab *et al.*, 2012, [31] El-Gendy *et al.*, 2018, [32] Garzoli *et al.*, 2015b.

By means of morphological, molecular and phylogenetic approaches, all taxa were identified at the species level, with the exception of one strain (*Hypocreales* sp.) that was not able to grow in axenic conditions. A remarkable number of fungi (31%), belonging to Capnodiales, Pleosporales and Sordariomycetes, remained sterile in pure culture (Table 5.1) and their taxonomic placement was inferred with phylogenetic analyses. In detail, for the Capnodiales tree (figure 5.5), MUT 3036 clustered within the Mycosphaerellaceae and was identified as *U. dekkeri*.

Among the Pleosporaceae (Figure 5.5) all taxa were identified at the species level, MUT 3045 and MUT 3046 clustered within the Phaeosphaeriaceae and were both identified as *N. samararum*. Within the Torulaceae Sporormiaceae, MUT 3038 was identified as *T. herbarum*.

Thanks to the phylogenetic analysis of Sordariomycetes, the taxa were identified at the species level; in detail, MUT 3468, MUT 3034 and MUT 3035 were assigned to *L. antillanum* (Cordycipitaceae), *X. badia* (Xylariaceae) and *A. arundinis* (Apiosporaceae), respectively.

The three sterile Basidiomycota, they were further analyzed by Poli *et al.* (2018) and their identification is reported in Table 5.1.

The fungal diversity associated with the three Mediterranean sponges was mainly ascribable to Ascomycota (89.7%), whereas Basidiomycota represent the 10.3% of the taxa; as already mentioned in chapter 3, this is not surprising both in the marine environment and in association with sponges. A possible explanation could be that marine Ascomycota are involved in processes that in terrestrial environments are normally carried out by Basidiomycota, such as the degradation of lignocellulosic materials (Balabanova *et al.*, 2018; Raghukumar, 2017; Panno *et al.*, 2013) or hydrocarbons (Bovio *et al.*, 2017; Garzoli *et al.*, 2015a). However, it must be considered that Basidiomycota could be poorly retrieved in the marine environment (Jones *et al.*, 2015) for issues in the isolation techniques (Ding *et al.*, 2011).

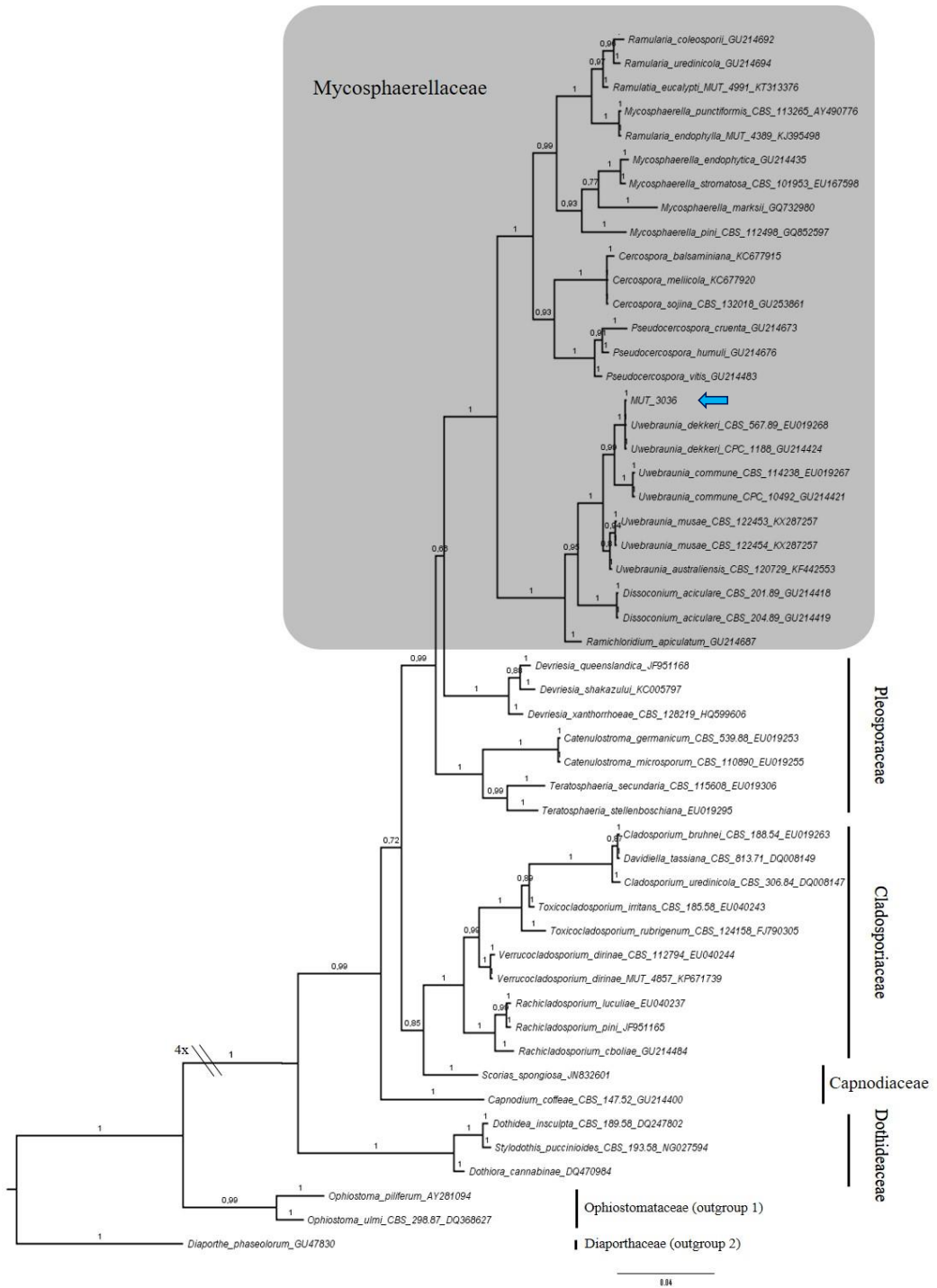
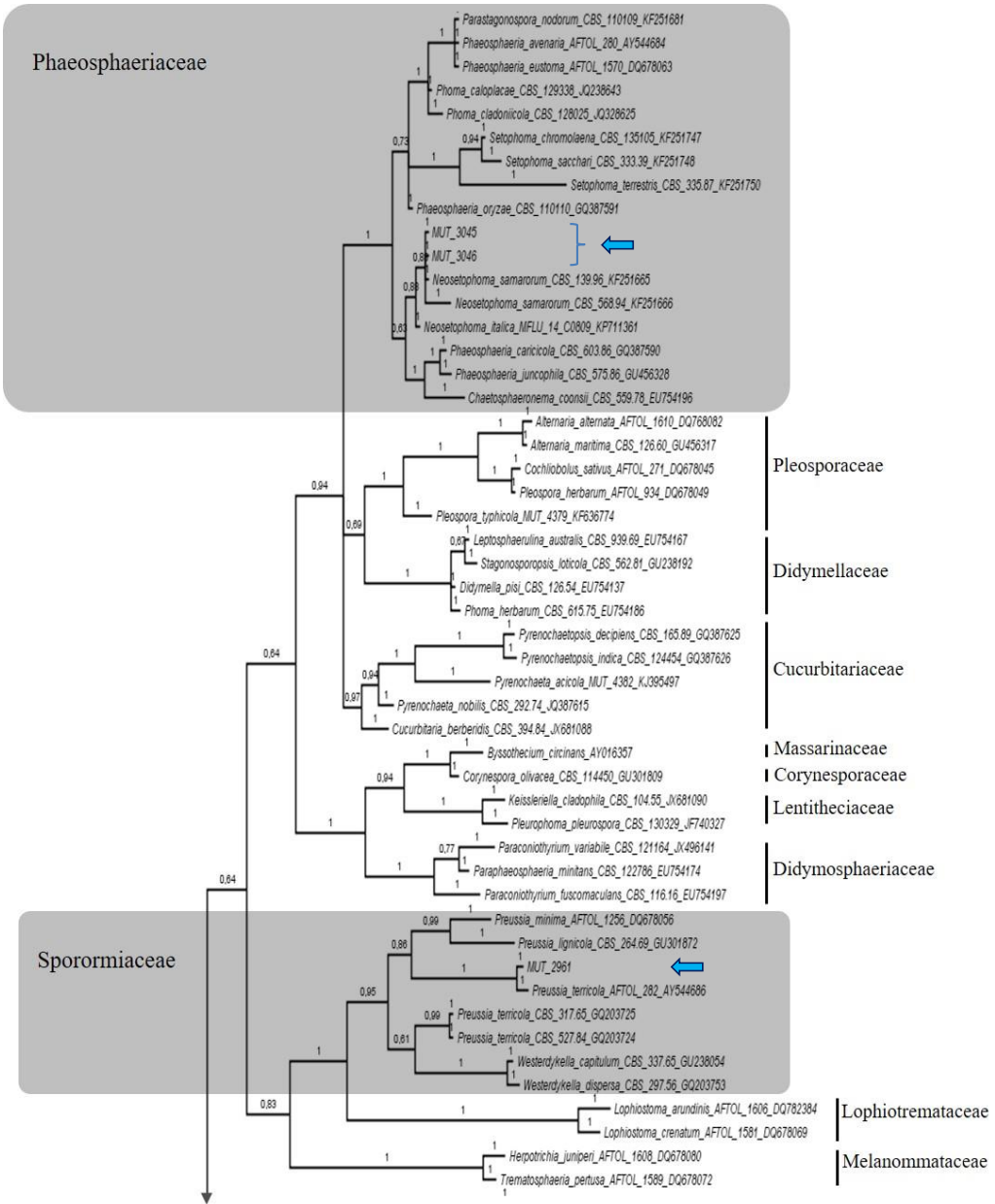


Figure 5.4 Bayesian phylogram of Capnodiales (Dothideomycetes) based on rDNA large subunit (LSU). MUT 3036 is identified as *U. dekkeri*. Branch numbers indicate BPP values.



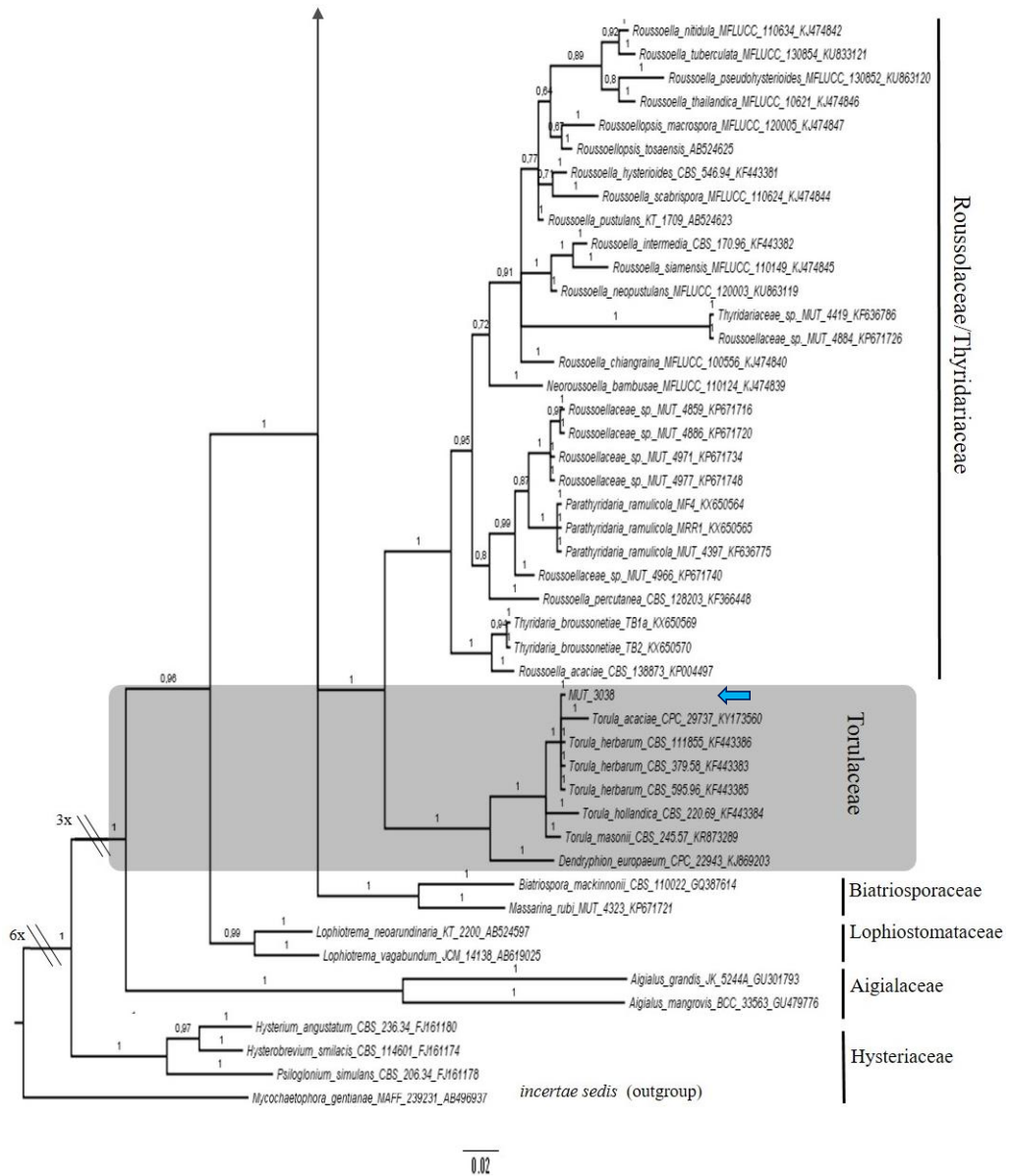
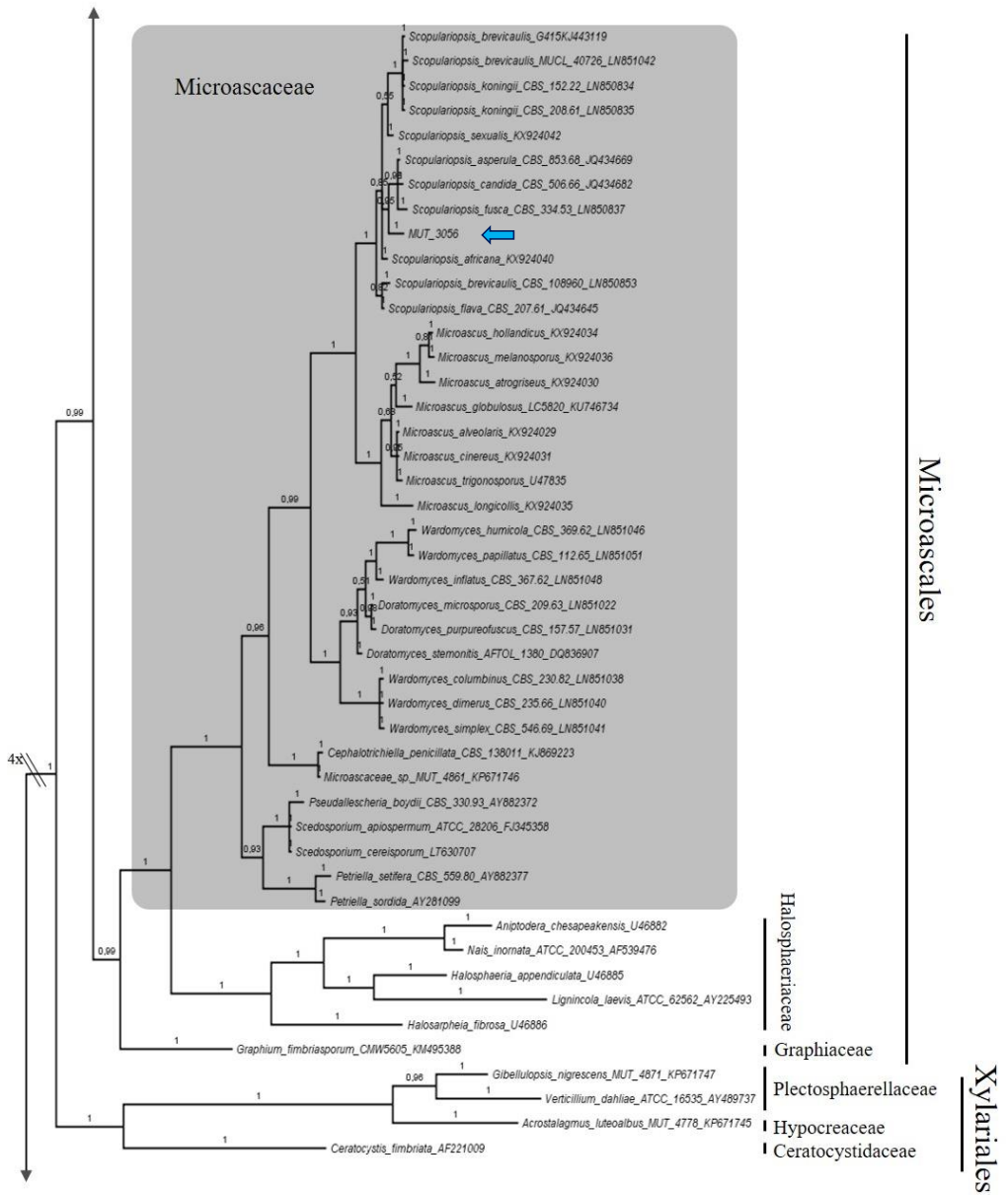


Figure 5.5 Bayesian phylogram of Pleosporales (Dothideomycetes) based on rDNA large subunit (LSU). Two taxa clustered within the Phaeosphaeriaceae. Of the two remaining taxa, one belongs to the Sporormiaceae and one to the Torulaceae. Numbers indicate BPP values.





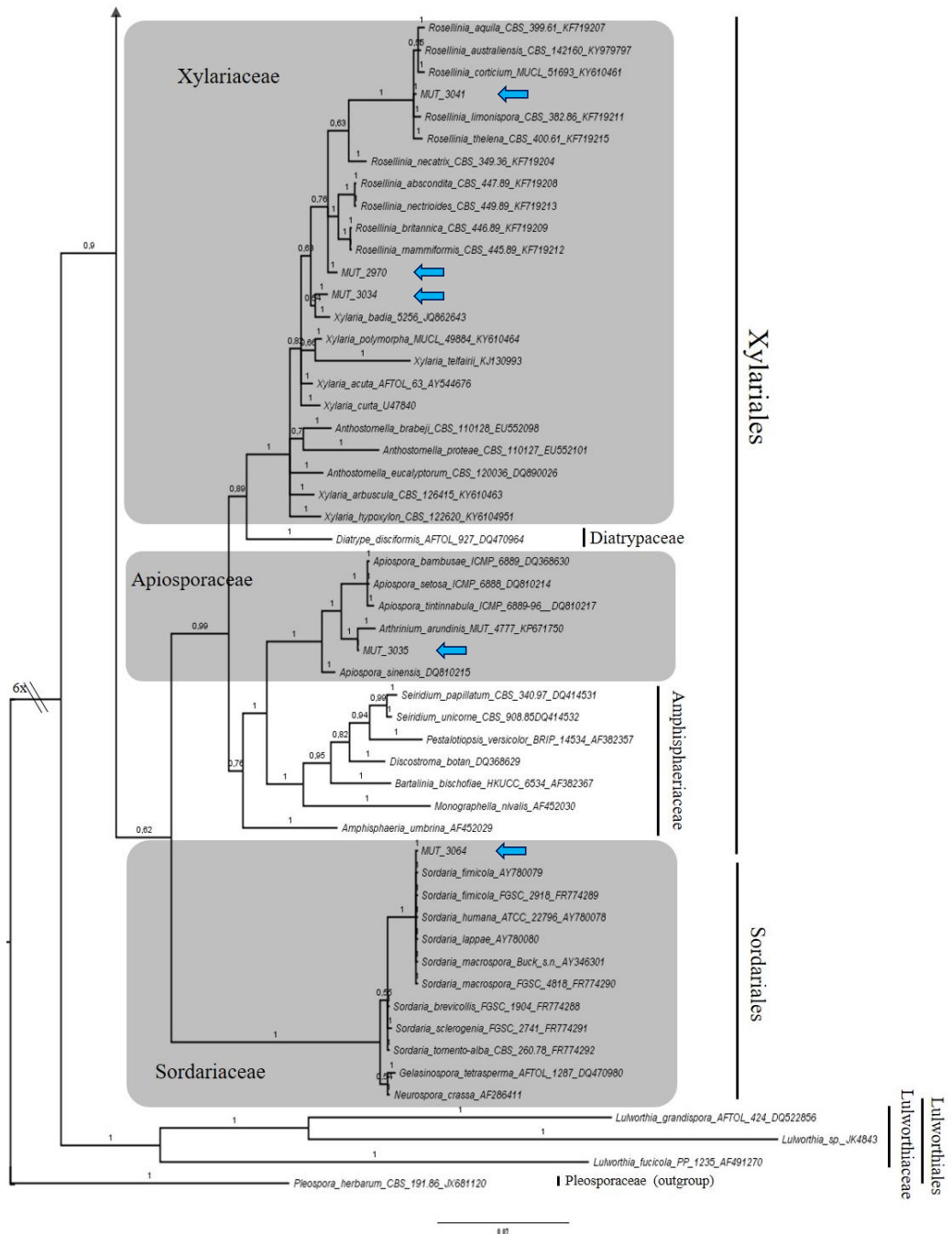


Figure 5.6 Bayesian phylogram of Sordariomycetes based on rDNA large subunit (LSU). Of 6 taxa, each one individually cluster in the families Cordycipitaceae, Microascaceae, Sporormiaceae, Apiosporaceae and Sordariaceae; while one is in the *incertae sedis* group. Three taxa were within the Xylariaceae. Branch numbers indicate BPP values.

The most represented genera in terms of species were *Penicillium* (8 species) and *Cladosporium* (5 species), two genera commonly retrieved in association with sponges and among the most studied for the isolation of new natural products (Imhoff, 2016).

Thanks to the low rank classification, it was possible to compare the fungal species retrieved in the present study with those already reported in marine ecosystems: 28.6% and 42.9% of the species were detected for the first time in the marine environment and in association with sponges, respectively (Table 5.1).

5.2.3 Fungal culturable diversity among the sponges

Considering the mycobiota of each sponge, two out of three of the studied animals hosted a specific fungal community (Figure 5.7), with 85.0% and 61.5% of the species “sponge specialist” of *P. tenacior* and *A. cavernicola*, respectively. On the contrary, only one species out of three was exclusive of the mycobiota of *C. crambe*. This species, *K. geniculotricha*, reported here for the first time from both a sponge and in the marine environment, is a coprophilous fungus on land ecosystems (Saxena and Mukerji, 1970; Seth, 1968). The occurrence of *K. geniculotricha* in a sponge might be related to the close proximity of the sampling site to the coast, therefore influenced by human impact and wastewater. However, there might be also another explanation; an unidentified species of *Kernia* sp. was able to produce an antifungal molecule, active against the fungus *C.albicans*, (Iwamoto *et al.*, 1990). This might shade a potential ecological meaning on the presence of *K. geniculotricha* in *C. crambe*, related to its ability to protect the host and avoid fungal colonisation.

As for the species in common (Figure 5.7), *C. pseudocladosporioides* was shared among the three sponges and can be defined “sponge generalist”. The same species was recorded in the Mediterranean Sea in an oil-spilled site (Bovio *et al.*, 2017) and, in association with Atlantic sponges (chapter 3). *P. citrinum* was shared by *A. cavernicola* and *C. crambe*, and was previously found in the Mediterranean sponge *Psammocinia* sp. (Paz *et al.*, 2010). The retrieval of this species is not surprising since it

is ubiquitous in Mediterranean and Oceanic ecosystems (including three out of 4 of the Atlantic sponges described in chapter 3).

A. cavernicola and *P. tenacior* shared additional four species (“sponge-associated”); *C. cladosporioides* and *P. brevicompactum* already reported in association with sponges and *N. samararum* and *C. perangustum* firstly isolated from a sponge (see Table 5.1 for references).

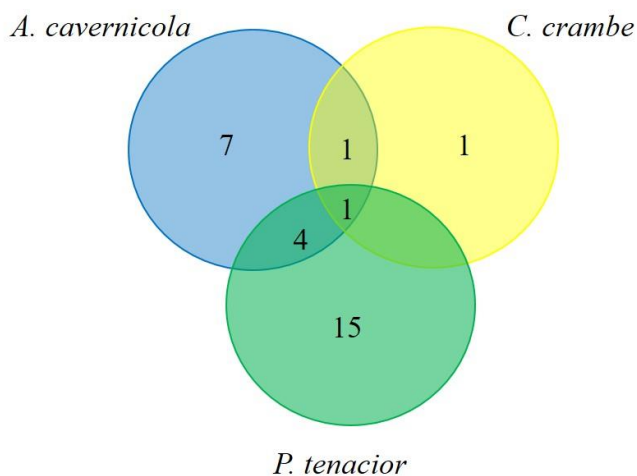


Figure 5.7 Number of exclusive and common fungal taxa in the three Mediterranean sponges’ *A. cavernicola*, *C. crambe* and *P. tenacior*.

The mini-satellite analysis highlighted that in most cases isolates of the same species were different strains. There were two exceptions: *C. pseudocladosporioides* (MUT 3559 and MUT 3580) and *C. cladosporioides* (MUT 3540 and MUT 3571) found both in *A. cavernicola* and *P. tenacior*, respectively. The overlap of only two strains between *A. cavernicola* and *P. tenacior*, collected on the same rock, underline the species-specificity of sponges’ fungal communities. However, due to the high variability within the sample and the low number of fungal isolates, no significant difference can be observed between the mycobiota of the sponges.

The fungal-sponge specificity is still debated. Metagenomics studies showed the overlapping of sponges-fungal OTUs with those of water samples (Naim *et al.*, 2017);

on the contrary, De Mares *et al.* (2017) and Jin *et al.* (2014) demonstrated the specific association between fungi and sponges.

Up to date, only few studies are available on the cultivable fungal community of the Mediterranean sponges, reporting only an apparent higher microbial diversity compared to the present work; indeed, few fungi were identified at species level, thus preventing a proper comparison with our results. For instance, among the 81 fungal strains, belonging to 20 genera recorded in *Suberites domuncula*, only 4 were identified, none common to our sponges (Proksch *et al.*, 2008); while, *Cladosporium* spp. and *Penicillium* spp. were the most represented fungi both in *S. domuncula* (Proksch *et al.*, 2008) and in the sponges here presented.

In the Mediterranean sponge *T. aurantium* (from the Adriatic Sea) 81 fungi were isolated, the ITS1-5.8S-rRNA-ITS2 region was amplified and the results compared with the public databases. Thus, two fungi *P. glabrum* and *E. nigrum* were found both in *T. aurantium* and in *P. tenacior* (Table 5.1). However, *P. glabrum* present only 99% similarity with the sequences reported in the databases and usually, the beta-tubulin region has to be amplified to better discriminate within the genus *Penicillium*.

Last, 85 taxa were isolated from *Psammocinia* sp. by Paz *et al.* (2010) in the Mediterranean Sea. Only 30% of taxa were identified at the lowest rank possible; five of them were also present in at least one of the three Mediterranean sponges here presented (Table 5.1).

Therefore, due to the few studies reporting the identification at species level, it is not surprising that the highest similarity (8 common species) in the fungal communities was found with the Atlantic sponges reported in the chapter. Indeed, we provided a low rank classification that reaches the 83.3% and 100% of fungal species associated with the Atlantic and the Mediterranean sponges, respectively.

Looking at the global picture of the fungal communities inhabiting sponges, several sponges worldwide, hosted a low fungal diversity. For instance, 22 fungal species were found in seven sponges in the Red Sea (Egypt) (Sayed *et al.*, 2016), 24 fungal genotypes were isolated from ten Antarctic sponges (Henríquez *et al.*, 2014) and 29 fungal strains were obtained from 9 Indonesian sponges (Sibero *et al.*, 2017).

Analogously to what we found in *C. crambe*, the Pacific sponge *Myxilla incrustans* hosted only two fungal taxa; the authors explained this phenomenon as a consequence of the habitat where the sponge was collected since the same species displayed a higher number of fungi when sampled in others places (Pivkin *et al.*, 2006). Considering that the fungal community of *C. crambe* is reported for the first time in the present study, it is not possible to achieve a similar conclusion; further studies on different specimens would be necessary to better understand the stability of the mycobiota inhabiting this sponge. This has been done for bacteria: *C. crambe* was collected in clean and polluted sites in Spain (Mediterranean Sea), and the bacterial community appeared to be stable and dominated by Betaproteobacteria (Gantt *et al.*, 2017). Interestingly, the same community was observed on specimens of *C. crambe* sampled at about 150 km far away (Gantt *et al.*, 2017; Croué *et al.*, 2013).

The diversity of fungi inhabiting sponges might be influenced by several factors, therefore more studies on the same sponge species collected at different sites would be necessary.

5.2.4 Mycobiotas vs metabolome

Several factors can shape the fungal community associated with the sponges, such as the sponge structure, more or less accessible to fungi, as hypothesised by Pivkin and collaborators (2006). In the case of the three Mediterranean sponges here presented, the metabolome might play a major role.

C. crambe hosts the lowest biodiversity, with only three species. Actually, this sponge is well known for the production of toxic compounds, which are concentrated in the outer layer (Uriz *et al.*, 1996), making this organism scarcely colonisable by bacteria (Becerro *et al.*, 1997). In fact, *C. crambe* belongs to the “low-microbial-abundance sponges” characterized by an average amount of bacteria per g of sponge-wet weight lower than that of seawater (Sipkema *et al.*, 2015). In addition, in comparison to seawater, also the bacterial diversity within the sponge is lower; different studies reported that Betaproteobacteria dominates the bacterial community of *C. crambe*, that on average are the 85% of the population (Gantt *et al.*, 2017; Croué *et al.*, 2013). By

using a culturomic approach, Öztürk and collaborators (2013) obtained only 107 bacterial isolates from *C. crambe*, despite the use of 16 isolation media and several sponge specimens. Indeed, the toxic compounds produced by this sponge could strongly select the fungal community able to grow on the sponge and/or inhibit the fungal growth in the isolation plates. From an ecological point of view, these compounds, once released, cause teratogen effects on ascidians embryos, supporting the hypothesis that encrusting sponges like *C. crambe*, use toxic metabolites to mediate the colonization of new habitats (Ternon *et al.*, 2016).

The production of bromotyrosine-derived compounds reported in *A. cavernicola* (Reverter *et al.*, 2016) could contribute to the recruitment of the fungal community. Indeed, bromotyrosine-derivatives were isolated with the aim of finding new antibiotics of marine origin and they proved to be active against several Gram-positive and Gram-negative bacteria, as well as against fungi (Peng *et al.*, 2005). Besides, several bacteria isolated from *A. cavernicola* produced antimicrobial compounds (Hentschel *et al.*, 2001) arising once more the question of whether sponges or the associated microorganisms are the real producers of antimicrobial compounds.

Interestingly, the production of chemicals from the host, have been previously related with the scarce colonisation from microorganisms. For instance, red algae produce halogenated compounds capable to strongly limit and select the associated fungal community (Garzoli *et al.*, 2015b).

P. tenacior displayed the highest fungal diversity and the highest percentage of exclusive taxa (85%). However, due to the lack of information about its metabolome, no hypothesis on the ability of this sponge to select its mycobiota can be drawn. As for bacteria, the low biodiversity reported in association *P. tenacior* might be the result of not always sufficient isolation techniques (Dupont *et al.*, 2013).

5.2.5 Fungi from *A. cavernicola* isolated by direct plating

For the isolation of fungi from *A. cavernicola* a second isolation method, the direct plating was applied. This was not possible for *C. crambe* and *P. tenacior* since,

being encrusting sponges and living in a close association with the rocky substrate, their collection was particularly hard.

Overall, 43% of the fungal community of *A. cavernicola* was isolated by direct plating; no overlap was observed between the taxa isolated with the two methods. Interestingly, as for the fungi recorded by homogenization of sponges' tissues, more taxa (60%) were isolated at 15 °C than at 25 °C (40%). The use of different media proved fundamental to increase the number of cultivable fungi: each fungal species was isolated only on one medium (Table 5.2).

Table 5.2 Fungal taxa isolated from *A. cavernicola* by direct plating and their relative abundance in percentage (RA%). First record (FR) and species already found in the marine environment (MA) and associated with sponges (SP) are reported.

	RA%	MA	SP
Ascomycota			
<i>Chaetomium globosum</i>	10.0	[2, 4, 5, 7, 13, 14, 18, 19]	[15]
<i>Cladosporium halotolerans</i>	10.0	[5, 11, 17]	[9]
<i>Preussia terricola</i> *	10.0	[12]	FR
<i>Rosellinia limonispota</i>	10.0	FR	FR
<i>Rosellinia</i> sp.*	10.0	-	-
<i>Sarocladium glaucum</i> *	10.0	FR	FR
<i>Scopulariopsis</i> sp.*	10.0	-	-
<i>Sordaria fimicola</i> *	10.0	[4]	FR
<i>Stachybotrys chartarum</i>	10.0	[5, 7, 8, 11, 20]	[1, 6]
<i>Trichoderma atroviride</i>	10.0	[7, 11, 18]	[3, 10, 16]
Tot taxa	10		

*Sterile mycelia

[1] Ma *et al.*, 2015, [2] Gnani *et al.*, 2017, [3] Paz *et al.*, 2010, [4] Raghukumar, 2017, [5] Bovio *et al.*, 2017, [6] Li *et al.*, 2014, [7] Jones *et al.*, 2015, [8] Kis-Papo *et al.*, 2001, [9] Bovio *et al.*, 2018, [10] Passarini *et al.*, 2013, [11] Garzoli *et al.*, 2015a, [12] Costello *et al.*, 2001, [13] Suryanarayanan, 2012, [14] Gomes *et al.*, 2008, [15] Pivkin *et al.*, 2006, [16] Bolaños *et al.*, 2015, [17] Zajc *et al.*, 2012, [18] Rämä *et al.*, 2017, [19] Oren and Gunde-Cimerman, 2012, [20] Raghukumar and Ravindran, 2012.

Noteworthy, half of the fungal community isolated by direct plating was represented by sterile mycelia (Table 5.2). The phylogenetic analysis performed together with the taxa previously isolated from the homogenization of the sponge's tissues, allowed to identify MUT 2961 as *P. terricola* (Sporomiaceae), within the Pleosporales (Figure 5.5). As for the 5 Sordariomycetes (Figure 5.6), MUT 3643 was identified as *S. glaucum*. MUT 3056 remained identified at the genus level (*Scopulariopsis* sp.; Microascaceae). Within the Xylariaceae MUT 3041, MUT 2970 were identified as *R. limonispora* and *Rosellinia* sp., respectively. MUT 3064 was identified as *S. fimicola* (Sordariaceae).

The mycobiota of *A. cavernicola* was exclusively represented by Ascomycota; the genera reported as the most frequent using the homogenization, are here missing (*Penicillium* spp.) or poorly represented, with only one species for the genus *Cladosporium*. This underlines once more that the isolation techniques strongly select the fungal community; the direct plating seems to favour actively growing fungi present inside the sponge, probably reducing the fungi only present as propagules.

The most frequent genus was *Rosellinia* (two species), reported for the first time within sponges but already documented in marine ecosystems (mangroves plants and/or their surrounding soil) (Chareprasert *et al.*, 2012).

Of the 10 taxa (Table 5.2) isolated by the means of the direct plating; four species (*S. fimicola*, *P. terricola*, *R. limonispora* and *S. glaucum*) were reported for the first time from marine sponges and for *S. glaucum* this was the first record from the marine environment.

Thanks to the collaboration with the University Côte d'Azur (Nice, France), Dr. Estelle Sfecci further studied the fungi isolated from the three Mediterranean sponges for the production of secondary metabolites (Sfecci, 2018). Among all the assessed fungi, *S. chartarum* resulted the most interesting for the production of secondary metabolites. Five already known molecules were obtained: Satatroxine h (**Sc1**), 2',3'-dihydrosatratroxiene H (**Sc2**), 2 α -acetoxystachybotrylactam acetate (**Sc3**), stachybotrylactame acetate (**Sc4**) and the stachybotrylactame (**Sc5**). Moreover, one new

molecule never detected before was named 2',3'-dihydrosatratoxine H (**Sc6**). Interestingly, (**Sc4**) and (**Sc5**) were active against three strains of *S. aureus* at concentrations ranging from 50 µM to 100 µM. As for (**Sc3**) and (**Sc6**) they were slightly active only against two and one *S. aureus* strains, respectively. None of the tested molecules was active against the Gram-negative *E. coli*. These findings support the capability of marine fungi to produce bioactive metabolites that might have ecological implications; moreover, a well-known fungus for the production of different metabolites (Sfecci, 2018) still yielded a new molecule, underlining that we are far to completely explore the chemodiversity of fungi and in particular of those derived from marine environment.

Box 1

Mediterranean vs Atlantic sponges

The isolation of fungi from both Mediterranean and Atlantic sponges in this PhD thesis allowed to retrieve 129 fungal taxa, including two new species here described (*T. balaustiformis* and *T. spongiae*). The Permanova analysis reported a significant difference ($p=0.0005$) when the origin of the sponge is compared, based on the cultivable fungal community. About 25% of the multivariate variability of fungi can be explained by the sponges' origins via two-dimensional PCO (Figure 5.8). In this regard, it would be interestingly to compare the mycobiota of the same sponge species, when possible, in two different environments. Indeed, as already mentioned, the sponge species and its metabolome could play the major role in shaping the fungal community. However, several other environmental factors as depth, dissolved oxygen and nitrates, that have been found to shape the fungal community in the oceans, more than the location (Tisthammer *et al.*, 2016), might influence also the mycobiota associated with the sponges.

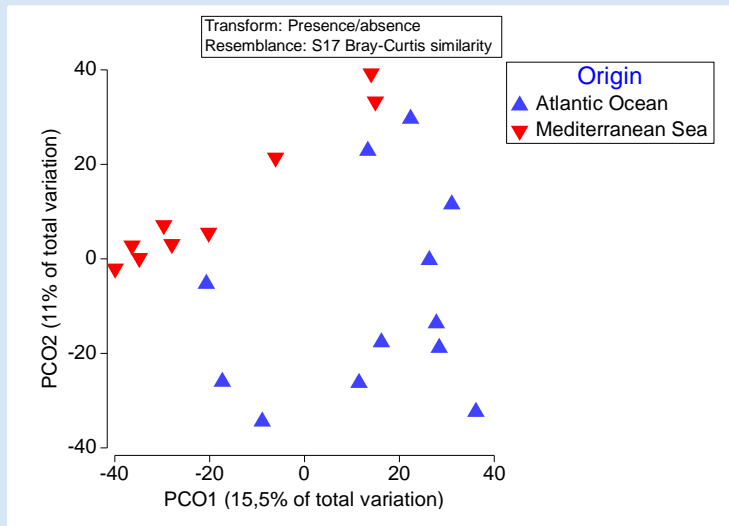


Figure 5.8 PCO on the fungal communities of the Atlantic and Mediterranean sponges.

Overall, only 9 species (*C. cladosporioides*, *C. halotolerans*, *C. perangustum*, *C. pseudocladosporioides*, *E. nigrum*, *E. scoparia*, *I. lacteus*, *P. brevicompactum* and *P. candolleana*) were isolated in at least one Mediterranean and one Atlantic sponge. Interestingly, most of these species were already mentioned (in this chapter and in the chapter 3) as ubiquity, probably not strictly associated with the sponges. Indeed, *C. cladosporioides* was reported as a "sponge generalist" species in the Atlantic sponge, with *C. pseudocladosporioides* and *C. halotolerans* being "sponge associates". As for the Mediterranean sponges, *C. pseudocladosporioides* was a "sponge generalist" to which are added *C. cladosporioides*, *C. perangustum* and *P. brevicompactum* as "sponge associates". Surprisingly, two Basidiomycota (*I. lacteus* and *P. candolleana*) were found in both Atlantic and Mediterranean sponges, leading to think that their ecological role might be underestimated in the marine environment.

In the future, it would be interesting to investigate whether or not the sponges of the same species sampled in the different environments are able to preserve a stable fungal composition and to which extent they are influenced from the external conditions.

5.3 CONCLUSION

The Mediterranean sponges *A. cavernicola*, *C. crambe* and *P. tenacior* hosted specific mycobiotas with only one species shared. The mini-satellite analysis highlighted the uniqueness of the fungal community of three sponge species, living in close proximity.

The different fungal communities observed might be the result of the strong selection operated by the sponges. This was particularly evident for *C. crambe*, a sponge that produces several toxic metabolites, with one of the lowest fungal diversity ever reported.

Overall, 9 and 15 species were reported for the first time in the marine environment and in association with sponges, respectively. This was due on one hand to the different isolation techniques employed that once again proved to be of fundamental importance. On the other hand, to the low rank classification applied; this laid the foundation for the chemical studies on the fungal metabolites performed (Sfecci, 2018).

Finally, more than 80 newly generated sequences were deposited in GenBank, increasing the molecular data relative to marine fungi.

The results showed in this chapter have been submitted to:

Mycological Progress. Bovio E., Sfecci E., Gnavi G., Poli A., Prigione V., Lacour T., Mohamed M., Varese G. C. (2018). The culturable mycobiota associated with the Mediterranean sponges *Aplysina cavernicola*, *Crambe crambe* and *Phorbas tenacior*.

6. Chemical diversity of the fungal community associated with the Atlantic sponge *G. compressa*

Considering the fungal community associated with the four Atlantic sponges, *G. compressa* was the one with the smallest number of fungal taxa. In parallel, as already mentioned, Macintyre and colleagues (2014) found also a reduced number of bacteria from the same sponge species. According to these findings, it can be hypothesised that the sponge is able to select its own fungal community. As consequence, it might be extremely interesting to investigate the role of the associated fungi for the production of secondary metabolites probably involved in sponge defence.

6.1 MATERIALS AND METHODS

6.1.1 Small scale fermentation of the fungal community and OSMAC approach

6.1.1.1 Multi-well culture and co-culture conditions

All the fungal strains (20) ascribable to 17 taxa from the sponge *G. compressa*, (described in chapter 3), were screened for the production of secondary metabolites by applying the OSMAC approach, with the exception of *Tetracladium* sp. that no longer grew in axenic culture. Fungi were pre-grown in Petri dishes (9 cm Ø) containing Wickerham's medium – WH (Yeast extract 3 g, Malt extract 3 g, Peptone 5 g, Glucose 10 g, Agar 15 g - Sigma-Aldrich, Saint Louis, USA - up to 1L dH₂O, pH adjusted to 7.2–7.4, Wickerham, 1951, modified) and incubated in the dark at 24 °C for 7 days.

Afterward, 24 multiwell plates were prepared with 1.5 mL/well of the following solid media:

- WH
- WHS (WH added with 3% NaCl)
- PDA
- PDAS (PDA added with 3% NaCl)
- Gelatin Agar - GA (Gelatin 20 g, Agar 15 g, up to 1L dH₂O)
- GAS (GA added with 3% NaCl)

Four replicates for each condition were prepared.

Two cultural lines were set:

- Fungal pure cultures of the tested strains were inoculated into multiwell plates by placing 4 mm Ø mycelium disks (from the margin of actively growing colonies) in the centre of each well.
- Co-culture lines were prepared by inoculating a smear of *Streptomyces* sp. MUT 2498 on the opposite side of fungal pure cultures disks. This bacterial strain, previously isolated from the Atlantic sponge *D. fragilis*, was chosen according to its ability to grow in all the used media.

Four replicates for each culture condition were set-up. Inoculated multiwell plates were incubated in the dark at 24 °C for two weeks. The production of exudates (*e*), soluble pigments (*s*), and the high production of conidia (*h*) were recorded. Fungal growth was classified as follows: no growth (**class 0**), development of the fungal mycelium only on the inoculum disk (**class 1**), mycelium covering half the area of the well (**class 2**), fungal mycelium covering the entire surface of the well (**class 3**).

Interactions with *Streptomyces* sp. MUT 2498 were determined as contrast (*c*) the growth of the fungus stopped at a distance from the bacterium colony, predominance (*p*) the fungus grow on the bacterium, and no growth of the bacterium (*n*) (Skidmore and Dickinson 1976, modified).

6.1.1.2 Extraction procedure and chemical analysis

The fungal mycelium and the medium contained in each well were freeze-dried. Dried samples were homogenated with an Ultra Turrax device and extracted three times by sonication with 50 mL of CH₂Cl₂:MeOH (1:1, v/v) and filtration of the suspension. The filtrates were evaporated under vacuum.

In order to obtain the chemical fingerprint of each fungus (tested in different conditions), the crude extracts were re-suspended in a mixture of CH₂Cl₂:MeOH (1:1, v/v) to reach the final concentration of 10 mg/mL. High-Performance Liquid Chromatography (HPLC) analyses were performed with a Waters Alliance 2695 HPLC system (Waters Corporation, Milford, MA) coupled with a Waters 996 photodiode array

detector, using a Macherey-Nagel NUCLEODUR® Sphinx column (250 x 4.6 mm, 5 µm). The mobile phase was composed with H₂O (plus 0.1% formic acid - FA) and acetonitrile (ACN + 0.1% FA), with the following gradient: H₂O:ACN 90:10 to 0:100 in 30 min, 0:100 for 5 min, 0:100 to 90:10 in 15 min.

Changing in the chemical diversity was evaluated on the basis of the presence of absorbance peaks at a selected wavelength (280 nm) between 5 and 35 minutes of retention time. The following classes were attributed: 0-3 peaks (*class 1*), 4-7 peaks (*class 2*), 8-11 peaks (*class 3*), and ≥ 12 peaks (*class 4*).

6.1.2 Scale-up of *E. chevalieri* MUT 2316 and molecules purification

The test performed in multiwell plates recognised the fungus *E. chevalieri* MUT 2316 on PDAS as the richest in secondary metabolites. Therefore, this strain was selected for further studies; both solid and liquid culture conditions were assessed.

6.1.2.1 Solid media culture condition and secondary metabolites extraction and purification from *E. chevalieri*

E. chevalieri MUT 2316 was pre-grown on PDA and mycelium disks (4 mm Ø) were taken from the margin of actively growing colonies to inoculate 200 Petri dishes (6 cm Ø) with PDAS. Plates were incubated for two weeks at 24 °C. Petri dishes were then lyophilized. The obtained sample was processed as summarised in Figure 6.1, by homogenization with the Ultra Turrax, and the secondary metabolites were extracted firstly 10 times with 2.5 L of EtOAc:CH₂Cl₂ (1:1, v/v) and filtered under vacuum. The second extraction was processed (12 times) with 3 L of CH₂Cl₂:MeOH (1:1, v/v) and the sample was filtered under vacuum. The EtOAc:CH₂Cl₂ fraction was further processed by adding RP C18 silica to the re-solubilized crude extract. The obtained mixture was then evaporated under reduced pressure and fractionated by Solid Phase Extraction (SPE) over RP C18 silica gel with a gradient of H₂O, MeOH and CH₂Cl₂.

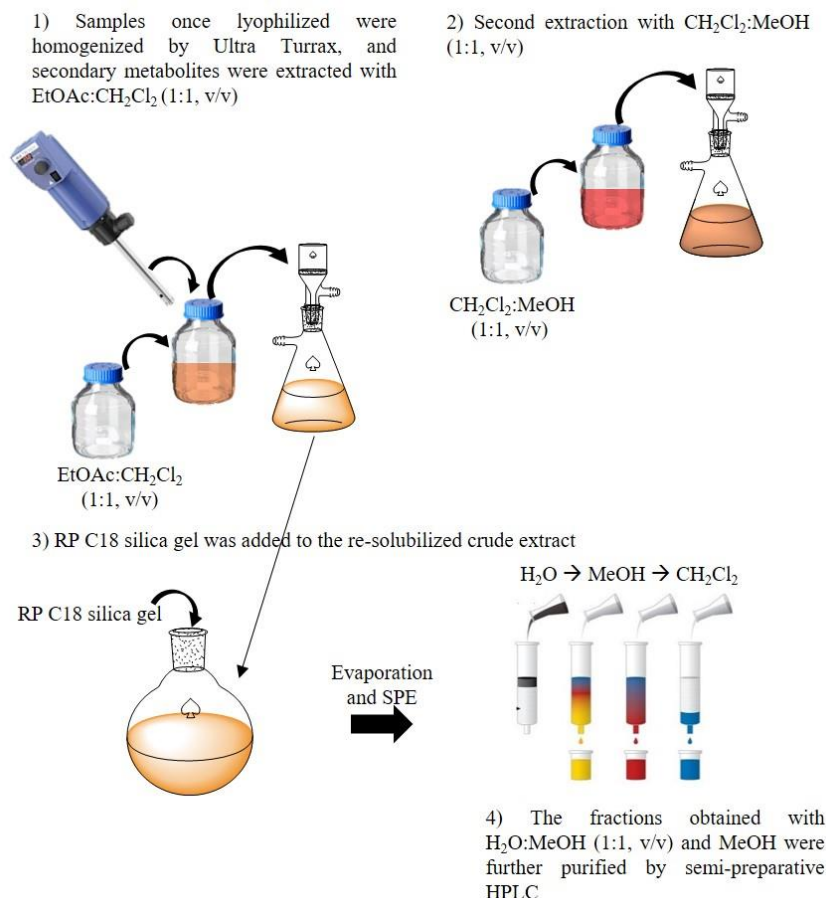


Figure 6.1 Secondary metabolites extraction from *E. chevalieri* MUT 2316 in solid culture condition.

The H₂O:MeOH fraction (from the SPE) was further processed by semi-preparative HPLC (Macherey-Nagel NUCLEODUR® Sphinx RP, 250 x 10 mm, 5 μm) with a flow rate of 3 mL/min. Samples were eluted with different percentage of chemicals (H₂O and ACN plus 0.1% FA) and running time, to improve the purification in the attempt to obtain pure molecules. In addition, also the first MeOH fraction (from the SPE) was further purified by semi-preparative normal phase HPLC (Lichrosorb Diol, 250 x 10 mm, 5 μm). Elution was carried out with Cyclohexane (Cy) and EtOAc with the following gradient: Cy:EtOAc 90:10 to 0:100 in 30 min, 0:100 for 5 min, 0:100 to 90:10 in 10 min, with a flow rate of 3 mL/min. However, in order to increase the yield

of the fractions/molecules obtained, the original MeOH fraction was subsequently processed on Silica Diol gel open column with a gradient from 100% Cyclohexane to 100% EtOAc, to mimic the HPLC conditions above experimented. The obtained fractions were purified by semi-preparative HPLC (Macherey-Nagel NUCLEODUR® Sphinx RP, 250 x 10 mm, 5 µm) with a flow rate of 3 mL/min. Samples were eluted with H₂O and ACN using different percentage of chemicals and running time, in order to improve the purification of each single fraction.

After the purification step, fractions were checked for purity by HPLC and LC-MS with the above-mentioned conditions. Structures of isolated compounds were elucidated through extensive NMR spectroscopy (1D and 2D) analyses and mass spectrometry.

6.1.2.2 Liquid media culture condition and secondary metabolites extraction from *E. chevalieri*

Ten agar plugs (obtained from the margin of an actively growing colony) of *E. chevalieri* MUT 2316 were inoculated in 20 - 250 mL Erlenmeyer flasks containing 180 mL of Potato Dextrose Broth – PDB - (Potato extract 4 g, Dextrose 20 g, Agar 15 g, - Sigma-Aldrich, Saint Louis, USA - up to 1L dH₂O) supplemented with 3% NaCl. Flasks were incubated for 14 days in the dark at 24°C and 120 rpm. Cultures were filtered to separate the fungal biomass from the broth.

The biomass was freeze-dried and extracted as above mentioned for the solid extraction of *E. chevalieri*. Briefly, 1 L of EtOAc:CH₂Cl₂ (1:1, v/v) and 1.5 L of MeOH:CH₂Cl₂ (1:1, v/v) were used to obtain the crude extracts.

The broth was extracted as shown in Figure 6.2, with 6 L of EtOAc, the resulting EtOAc extract was evaporated under reduced pressure. The remaining water fraction was processed over RP C18 silica gel with MeOH. The EtOAc extract was purified by semi-prep HPLC (Macherey-Nagel NUCLEODUR® Sphinx RP, 250 x 10 mm, 5 µm); as mobile phase, different percentage of chemicals (H₂O and ACN + 0.1% FA) and running time were used to improve the separation, with a flow rate of 3 mL/min. The obtained fractions were checked for purity, as mentioned in section 6.2.2.1.

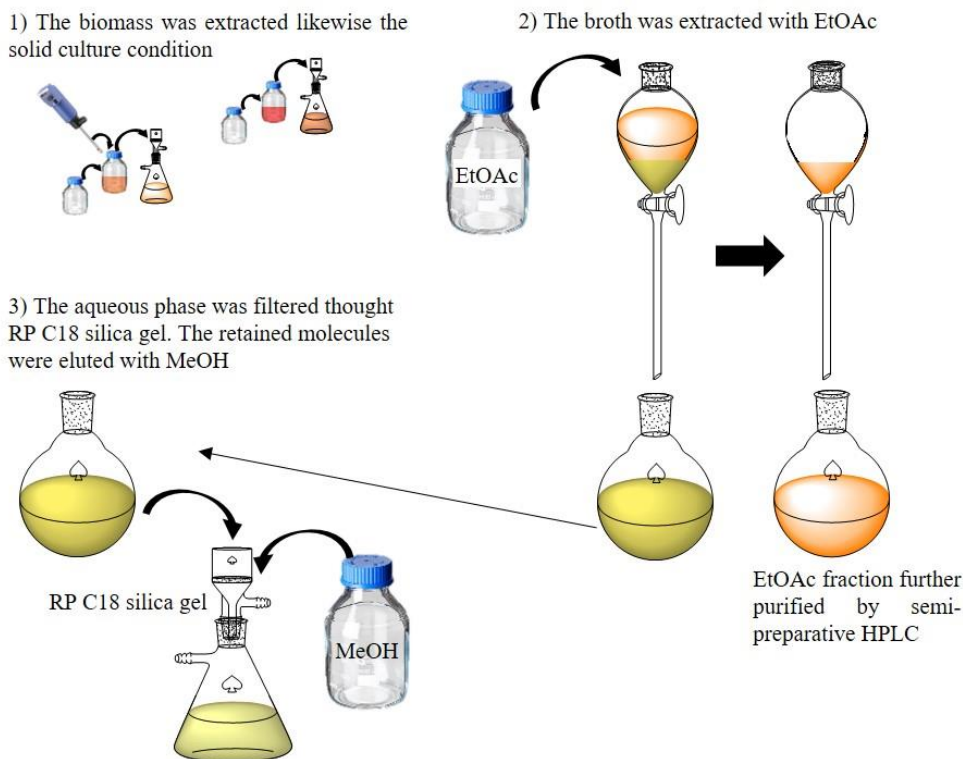


Figure 6.2 Secondary metabolites extraction from *E. chevalieri* MUT 2316 in liquid culture condition.

6.1.2.3 High Resolution Mass Spectrometry analysis and molecular networking

High-resolution tandem mass spectrometry (HR-MS/MS) data were carried out using a Finnigan LTQ Orbitrap coupled to a Surveyor Plus HPLC pump and autosampler (Thermo Fisher, Bremen, Germany) in positive ionization modes using a MS range of m/z 100–2000, a MS2 range of m/z 200–1500, a MSn range of m/z 0–1000 and 30,000 resolution. LC-MS data were acquired using Xcalibur version 2.2. HR-MS/MS raw data files were converted from .RAW to .mzXML format using the Trans-Proteomic pipeline, Institute for Systems biology – Seattle (Deutsch *et al.*, 2010), and clustered with MS-Cluster using Global Natural Products Social Molecular Networking (GNPS - <https://gnps.ucsd.edu>) (Wang *et al.*, 2016a). A molecular network was created using the

online workflow of the GNPS. The following settings were used for generation of the network: minimum pairs cos 0.6; parent mass tolerance, 1.0 Da; ion tolerance, 0.2; network topK, 10; minimum matched peaks, 6; minimum cluster size, 2. Data were visualized and analysed using Cytoscape 3.6.0.

6.2 RESULTS AND DISCUSSION

6.2.1 OSMAC approach

Multiwell assays to test different growth conditions are considered a valuable method for the screening of marine fungi as producers of interesting metabolites (Romano *et al.*, 2018; Linde *et al.*, 2014). Indeed, in comparison to 9 cm Petri dishes or liquid cultures, this method increases the yield of metabolites and decreases the time necessary for their production (Bertrand *et al.*, 2014; Linde *et al.*, 2014). Moreover, several fungi and media can be tested with a miniaturized screening. Different fungal morpho-physiological parameters have been taken into account to register the effects of the OSMAC approach (an exemplification in Figure 6.3); these data were compared with the chemical fingerprints obtained for each culture condition.

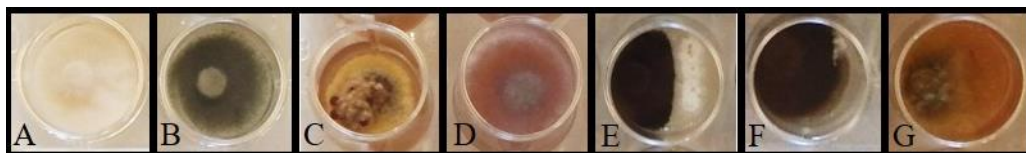


Figure 6.3 **A.** Absence of sporulation on WH and **B.** High sporulation (*h*) on PDAS for *P. paneum* MUT 2327. **C.** Production of exudates (*e*) on WH by *E. chevalieri* MUT 2316. **D.** Production of soluble pigments (*s*) on PDA for *F. solani* MUT 2317. **E.** Contrast (*c*) with the bacterium and the fungus *C. allicinum* MUT 2313 on PDA. **F.** Predominance (*p*) of *C. cladosporioides* MUT 2314 on the bacterium on PDAS. **G.** *E. chevalieri* MUT 2316 on PDAS completely inhibit the bacterial growth (*n*).

6.2.1.1 Effect of media on fungal development and chemical fingerprint

The optimal growth of fungi was the first parameter that was taken into account; indeed, in light of industrial exploitation of the fungus for secondary metabolites production, a fast growing fungus must be preferred (Table 6.1).

Table 6.1 Effect of different growth conditions on the development of the fungus (*class 0, 1, 2, 3*); production of exudates (*e*), soluble pigments (*s*) and high sporulation (*h*); fungal-bacteria interaction as contrast (*c*), predominance of the fungus (*p*) or complete inhibition of the bacterial growth (*n*) are reported in red. The changing in colours from dark to light blue and white, underlined the increasing of growth classes.

MUT code	Taxa	Pure culture						Co-culture					
		WH	WHS	PDA	PDAS	GA	GAS	WH	WHS	PDA	PDAS	GA	GAS
2288	<i>C. lacerata</i>	3 (<i>e, s</i>)	2	3	3	3	3	3 (<i>e, s, p</i>)	3	3 (<i>p</i>)	3 (<i>n</i>)	3	2
2307	<i>C. allacinum</i>	3 (<i>h</i>)	3 (<i>h</i>)	3 (<i>h</i>)	3 (<i>h</i>)	2 (<i>h</i>)	2	2 (<i>h, c</i>)	2 (<i>h, c</i>)	2 (<i>h, c</i>)	3 (<i>h, p</i>)	1 (<i>h</i>)	1
2313	<i>C. allacinum</i>	3 (<i>h</i>)	3 (<i>h</i>)	3 (<i>h</i>)	3 (<i>h</i>)	2 (<i>h</i>)	1	3 (<i>h, c</i>)	3 (<i>h, c</i>)	3 (<i>h, c</i>)	3 (<i>h, c</i>)	2 (<i>h</i>)	1
2314	<i>C. cladosporioides</i>	3 (<i>h</i>)	3 (<i>h</i>)	3 (<i>h</i>)	3 (<i>h</i>)	3 (<i>h</i>)	2	3 (<i>h, c</i>)	3 (<i>h, c</i>)	3 (<i>h, c</i>)	3 (<i>h, p</i>)	2 (<i>h</i>)	2
2315	<i>C. pseudocladosporioides</i>	3 (<i>h</i>)	3 (<i>h</i>)	3 (<i>h</i>)	3 (<i>h</i>)	2 (<i>h</i>)	1	3 (<i>h, c</i>)	3 (<i>h, c</i>)	3 (<i>h</i>)	3 (<i>h, n</i>)	1 (<i>h</i>)	1
2282	<i>Coprinellus</i> sp. 1	3	3	3	3	3	1	3 (<i>p</i>)	2	3 (<i>p</i>)	2	2	1
2332	<i>Coprinellus</i> sp. 2	3	2	3	3	3	0	2 (<i>p</i>)	1	3 (<i>p</i>)	2	1	1
2273	<i>E. pallida</i>	3	3	3	3	2	3	3 (<i>c</i>)	3	3 (<i>c</i>)	3	2	2
2274	<i>E. pallida</i>	3	3	3	3	3	3	3 (<i>c</i>)	3 (<i>p</i>)	3 (<i>c</i>)	3 (<i>p</i>)	2	2
2316	<i>E. chevalieri</i>	3 (<i>e, h, s</i>)	3 (<i>h, s</i>)	3 (<i>h, s</i>)	3 (<i>h, s</i>)	1	2	2 (<i>e, h, s</i>)	3 (<i>h, s, c</i>)	2 (<i>h, s</i>)	3 (<i>h, s, n</i>)	1	2
2334	<i>E. scoparia</i>	3	3	3	3	2	2	3	3	3	2	2	0
2317	<i>F. solani</i>	3 (<i>s</i>)	3	3(<i>s</i>)	3	3	3	3(<i>s</i>)	3 (<i>p</i>)	3(<i>s, p</i>)	3 (<i>p</i>)	3	3
2321	<i>P. chrysogenum</i>	3 (<i>h, s</i>)	3 (<i>h, s</i>)	3 (<i>h</i>)	3 (<i>h</i>)	3 (<i>h, s</i>)	3 (<i>h</i>)	3 (<i>h, s, c</i>)	3 (<i>h, s, c</i>)	3 (<i>h</i>)	3 (<i>h, n</i>)	2 (<i>h, s</i>)	2 (<i>h</i>)
2328	<i>P. oxalicum</i>	3 (<i>h</i>)	3 (<i>h</i>)	3 (<i>h</i>)	3 (<i>h</i>)	2	2	3 (<i>h, p</i>)	3 (<i>h, p</i>)	3 (<i>h, p</i>)	3 (<i>h, p</i>)	3	3
2322	<i>P. paneum</i>	3	3	3 (<i>h</i>)	3 (<i>h</i>)	2	1	3 (<i>c</i>)	3 (<i>c</i>)	3 (<i>h, p</i>)	3 (<i>h, p</i>)	3	1
2326	<i>P. paneum</i>	3	3	3 (<i>h</i>)	3 (<i>h</i>)	3	2	3 (<i>c</i>)	3 (<i>c</i>)	3 (<i>h</i>)	3 (<i>h, n</i>)	3	1
2331	<i>P. candolleana</i>	3	1	3	0	3	0	2 (<i>c</i>)	1	3 (<i>c</i>)	0	2	0
2415	<i>R. mucillaginosa</i>	3	3	3	3	1	1	3	3	3	3	0	1
2413	<i>T. cylindrosporium</i>	3	3	3	3	3	3	3 (<i>c</i>)	3 (<i>c</i>)	3	3	2	2
2410	<i>T. cylindrosporium</i>	3	3	2	2	2	1	2	2	2	2	1	1

Among the culture media tested, WH medium well promoted the fungal growth of all the strains: after 14 days all fungi occupied the entire medium surface (Figure 6.4A). PDA also promoted the fungal growth and 95% of the fungi cover the entire medium surface (Figure 6.4A). These results are in line with a previous work that demonstrated that two weeks are necessary also for slow-growing species to cover the entire surface of a 24-well (Bertrand *et al.*, 2014). From a chemical point of view, rich-nutrients media better induced the metabolic diversity (Figure 6.4B); indeed, the class 3 and 4 (high metabolic production), counted for the 20% of the tested strains both on WH and PDA. On the contrary, the low nutrient gelatin based medium did not promote the proper hyphal development: only 50% of fungi occupy the entire well on GA after two weeks (Figure 6.4A). This affected the metabolic diversity that was strongly reduced and ascribable only to classes 1 and 2 (Figure 6.4B).

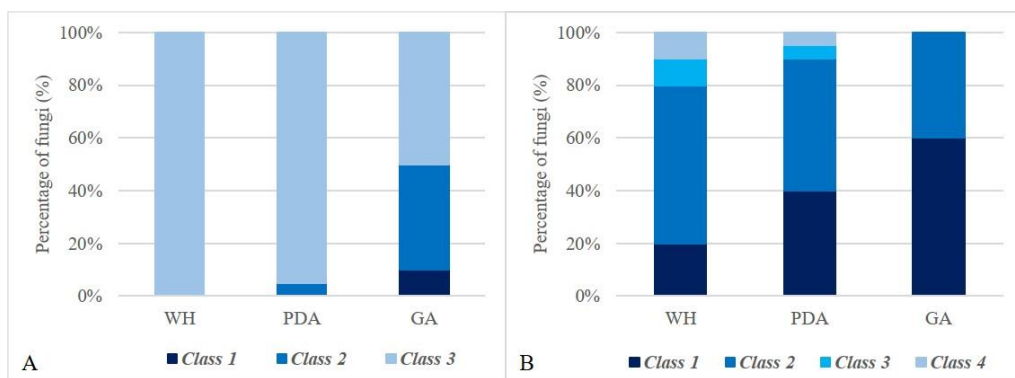


Figure 6.4 A. Fungal growth on three media, classified as development of the fungus on the inoculum (*class 1*), occupation of half the area of the well (*class 2*), complete growth of the fungus on all the surface of the well (*class 3*); **B.** Changing in the chemical diversity of fungal extracts due to the different conditions assessed. Classification based on the number of HPLC-UV 280 nm peaks: 0-3 peaks (*class 1*), 4-7 peaks (*class 2*), 8-11 peaks (*class 3*), ≥ 12 peaks (*class 4*).

The OSMAC approach proved a powerful method, indeed, each of tested fungi showed different metabolic classes in at least one of the three media tested, thus underlining the best conditions for the stimulation of secondary metabolites (Table 6.2).

The only exceptions were represented by *C. allicinum* MUT 2307 and *C. cladosporioides* MUT 2315 where the same metabolic class (**class 2**) was reported in all three conditions, although the occurrence of exclusive peaks characterized both WH and PDA (Table 6.2).

Overall, these results highlighted that low nutrient media as gelatin can be highly successful for the isolation of slow growing fungi (as demonstrated in chapter 3), but are not able to promote the growth and the metabolic diversification of the marine fungi tested in the present study. Similarly, Da Silva Lima *et al.* (2018) reported that the marine fungus *A. sydowii* was able to produce different metabolites in rich nutrients media, containing 150 g/L of glucose, and that the metabolisms was influenced by the different nitrogen and carbon sources. In addition, the marine fungus *Cladosporium* sp. was tested for the production of the compound calcaride A using all rich nutrient media (40 g/L of different carbon sources, plus standard nutrients) (Tamminen *et al.*, 2015). Interestingly, Wang *et al.* (2014) pointed out that although *Ascotricha* sp. in the eutrophic medium yielded more and different metabolites, only using the oligotrophic Czapek medium with NaNO₃ as the sole inorganic nitrogen source, new metabolites were produced.

Table 6.2 Effect of different growth conditions on the HPLC-UV 280 nm chemical fingerprint of fungi, expressed as (*class 1, 2, 3, 4*) at the increasing of the metabolic diversity. Exclusive peaks of each condition indicated in parenthesis. The changing in colours from dark to light blue and white indicated the increasing of the metabolic diversity.

MUT code	Taxa	Pure culture						Co-culture						
		WH	WHS	PDA	PDAS	GA	GAS	WH	WHS	PDA	PDAS	GA	GAS	
2288	<i>C. lacerata</i>	2	1	1	1	1	1	1 (1)	1	1	1	1	1	1
2307	<i>C. allicinum</i>	2 (2)	2	2 (2)	2	2	1	1	1	2 (1)	2	2	2	1
2313	<i>C. allicinum</i>	2 (2)	2 (1)	2	2 (1)	2	2	2 (2)	2	1	1	2	2	2
2314	<i>C. cladosporioides</i>	3	2	2	2	1	1	2	1 (1)	2	2	1 (3)	1	1
2315	<i>C. pseudocladosporioides</i>	2 (1)	1	2 (1)	1 (2)	2	2	1	2	2 (1)	2	2 (2)	1	1
2282	<i>Coprinellus</i> sp. 1	2 (1)	1 (1)	1	1 (1)	1	1	1	1	1	1	1	1	1
2332	<i>Coprinellus</i> sp. 2	2	1 (1)	1	1	1	1	1	1 (1)	1 (1)	2	1	1	1
2273	<i>E. pallida</i>	2	2	4 (1)	3	2	1	1 (2)	1 (1)	3 (3)	3	1	1	1
2274	<i>E. pallida</i>	1 (1)	1 (1)	2	1	1	1	1	1 (1)	2	1	1 (2)	1	1
2316	<i>E. chevalieri</i>	4	4 (1)	4 (1)	4 (4)	1	1	4	4 (2)	4 (3)	4	1	1	1
2334	<i>E. scoparia</i>	2 (2)	1 (1)	1	1	1	1 (1)	1 (3)	1	1	1 (1)	1	1	1
2317	<i>F. solani</i>	1	1	4	2	2	1	1	1	3	2	2	1	1
2321	<i>P. chrysogenum</i>	1	2	2	4	1 (1)	1	3	2	4	3	1	1	1
2328	<i>P. oxalicum</i>	4	3 (1)	3	3	2	2	3 (1)	4 (2)	4	3 (1)	1	1	1
2322	<i>P. paneum</i>	2 (1)	2	2	3	1	1	2	2	3	3	2	1	1
2326	<i>P. paneum</i>	2	2	2 (2)	2	1	1	2	2	2	2	1	1	1
2331	<i>P. candolleana</i>	2 (2)	1	1	1	1	1	2	2	3	1	1 (1)	1	1
2415	<i>R. mucillaginosa</i>	2	2	1 (1)	1 (1)	1	1	1	2	1	1	1	1	1
2413	<i>T. cylindrosporum</i>	1 (1)	2 (1)	2	2	2	2	2	2 (1)	2	2 (1)	1	1	1
2410	<i>T. cylindrosporum</i>	3 (1)	2 (2)	2 (2)	1 (1)	2	2	3	2	1	2	2 (1)	1	1

6.2.1.2 Effect of salts on the fungal development and the chemical fingerprint

The presence of salts differently influences the number of secondary metabolites detected for each fungus when the same medium with and without salts is compared (Figure 6.5).

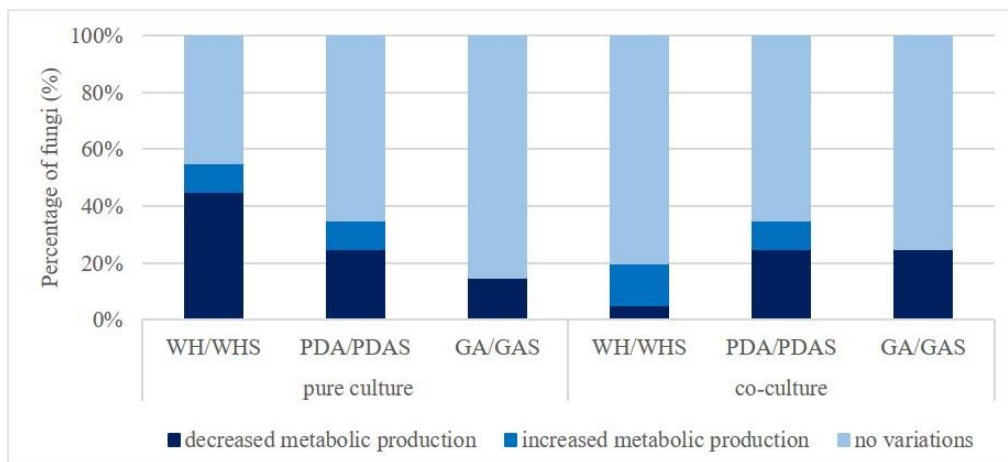


Figure 6.5 Percentage of fungi in which the presence of salts increased, decreased or did not influence the metabolic diversity (same metabolic classes), related to the different conditions tested (three media in pure and co-culture).

Most fungi, from 45% (WH/WHS pure culture) to 85% (GA/GAS pure culture), are not influenced by salts in the number of secondary metabolites produced (Figure 6.5). Only on WHS (pure culture), the number of fungi that decrease the metabolic production in presence of salts is equally abundant (45%). Of them, three are Basidiomycota, namely *C. lacerata* MUT 2288, *Coprinellus* sp. MUT 2332, *P. candolleana* MUT 2331 (Table 6.2) that reported also a decreased growth rate (Table 6.1). Despite none morphological change was observed, the metabolic production increased with salts (on WHS, pure culture) for *P. chrysogenum* MUT 2321 and *T. cylindrosporium* MUT 2413 (Tables 6.2). Of note, *P. chrysogenum* MUT 2321 maintain the same trend also in co-culture with the bacterium (Tables 6.2). In addition, other fungi *C. pseudocladosporioides* MUT 2315, *P. oxalicum* MUT 2328 and *R. mucillaginosa*

MUT 2415 produce more metabolites in presence of salts in co-culture (Table 6.2), none relevant morphological changes were observed (Table 6.1).

On PDA (pure culture), salts decreased the metabolic production for 25% of fungi (Figure 6.5), this did not collimate with any changes of the morpho-physiological parameters evaluated (Table 6.1), with the exception of *F. solani* MUT 2317, where the intense red soluble pigment observed on PDA (metabolic diversity: **class 4**) was not observed on PDAS (metabolic diversity: **class 2**). Of note, *P. chrysogenum* MUT 2321 produce more metabolites in presence of salts also on PDAS (Table 6.2), besides on WHS. To this has to be added also *P. paneum* MUT 2322 (Tables 6.2).

The co-culture (PDA/PDAS) leave unvaried the proportion of fungi influenced by salts in the production of secondary metabolites (Figure 6.5), however, few fungi, *Coprinellus* sp. 2 MUT 2332 and *T. cylindrosporium* MUT 2410 seems stimulated by the combination of salts and bacteria, determining a higher number of secondary metabolites (Table 6.2). Probably, the higher metabolic production of *Coprinellus* sp. 2 MUT 2332 is due to the incapability to prevail on the bacterium, showed on PDAS (Table 6.1), determining a greater investment of metabolites to compete for space and nutrients.

The consequences of the salts on the metabolome were more pronounced on the gelatin based medium both in pure and co-culture, where none of the fungi produced more metabolites in presence of salts. Of note, also the growth and usually the sporulation was reduced on GAS (pure and co-culture) (Table 6.1), with the only exception represented by *E. chevalieri* MUT 2316 that grew better on salts (Table 6.1). Do not surprise that this fungus was repeatedly sampled in the salters with salinity above 17% NaCl (Butinar *et al.*, 2005).

Overall, the different abilities of fungi to tolerate salt could be related to their position within sponges. Fungal endophytes, not exposed to the osmotic pressure have already been detected within sponges (Debbab *et al.*, 2012); as consequence, some of the isolated fungi founded within sponge's tissues could be negatively affected by the presence of salt. However, it has to be considered that the ecology and the environment play the major role in shaping the fungal metabolisms (Reich and Labes, 2017). Despite the salts and the co-culture with a marine bacterium have been used in the attempt to

partially mimic the original habitat, the controlled conditions within a Petri dish are far from the natural environment of a marine fungus, where its metabolites are expected to characterise its lifestyle and mediate different interactions (Reich and Labes, 2017).

Certainly, salt can act as a stress factor for endophytes or mimic environmental conditions for sponge-associated fungi more exposed to seawater, modulating their metabolic production as already demonstrated (Jančič *et al.*, 2016; Kalinia *et al.*, 2017; Wang *et al.*, 2011).

6.2.1.3 Effect of co-culture on the fungal development and the chemical fingerprint

The co-culture differently influenced the fungal growth on the tested media (Table 6.1). These results can be due to space and nutrient competition that generally occur in dual culture (Shehata *et al.*, 2016). On PDAS, 25% of fungi completely inhibited the bacterium (Figure 6.6); this was probably due to the bacterium slow growth compared to the other media. Overall, on WH, WHS, PDA and PDAS, 75%, 60%, 60% and 25% of fungi, respectively, interacted with the bacterium by contrasting its growth or over-growing it (Figure 6.6). These morphological features relate to an increased metabolic production in 15% and 16% of the cases where a contrast (**c**) or dominance of the fungus (**p**) was observed (Table 6.1 and Table 6.2). Noteworthy, even when the number of metabolites was constant or decreased, exclusive peaks interested one-third of the fungi: this was the case of *E. pallida* MUT 2273, that clearly contrasted the bacterium on WH and PDA and produced exclusive peaks not detected in others conditions (Table 6.1 and Table 6.2). The presence of exclusive peaks in co-culture represent direct evidence of the interaction on a specific medium between the two organisms tested; however, whether the fungus or the bacterium is responsible for the molecules synthesis can not be determined.

Overall, even if the co-cultivation have not been considered when the principles of the OSMAC approach were defined, today is a valuable technique to promote the production of new metabolites (Romano *et al.*, 2018). For instance, despite the metabolome of (the marine) *A. fumigatus* is well characterised, the co-cultivation with

two strains of *Streptomyces leeuwenhoekii* allowed to isolate new compounds (Wakefield *et al.*, 2017). More significant of all are the results reported by the work of Özkaya and collaborators (2017), where only the co-cultivation allowed *Aspergillus carneus* and *Aspergillus iizukae* to produce molecules contrasting the target bacterial pathogens. Even most surprising (not from the marine environment), is the research published by Scherlach and co-workers (2012), the fungus *Rhizopus microspores* and the endosymbiotic bacterium *Burkholderia rhizoxinica* both contribute to the synthesis of the rhizoxin. Overall, all these results underline how far we are from completely explore the metabolic diversity of microorganisms and underline that the interactions are key elements to successful stimulate the production of new metabolites.

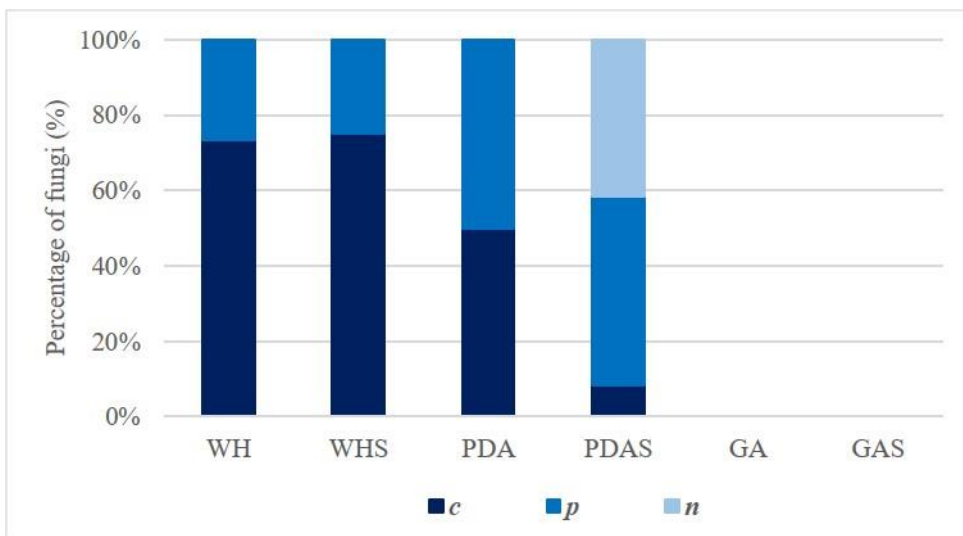


Figure 6.6 Percentage of fungi interacting in different ways with the bacterium. Interactions are classified as contrast (*c*), predominance (*p*) and total inhibition of the bacterial growth (*n*), on the six tested media.

6.2.1.4 General remarks on the OSMAC approach

Considering the 12 conditions tested, it was clear that some fungi presented a reduced metabolic diversity, which was less influenced by the growth conditions. This was the case of *C. lacerata* MUT 2288, *Coprinellus* sp. 1 MUT 2282 and *E. scoparia* MUT 2334, with a metabolic diversity of class 1 for all the conditions, with the only

exception of WH were the production of metabolites slightly increased (*class 2*), Table 6.2. On the contrary, *E. chevalieri* MUT 2316 produced more metabolites than any other tested fungus, in particular on all rich-nutrient media (Table 6.2).

Moreover, the production of secondary metabolites appeared specific for each strain (Table 6.2), this was evident for *T. cylindrosporum* (MUT 2410 and MUT 2413) and *E. pallida* (MUT 2273 and MUT 2274), while it was less marked for *C. allicinum* (MUT 2307 and MUT 2313), *P. paneum* (MUT 2322 and MUT 2326) and *Coprinellus* sp. (MUT 2282 and MUT 2332).

Overall, the OSMAC approach was a valuable method for the induction of different secondary metabolites, still poorly investigated for marine microbes (Romano *et al.*, 2018). Several fungi showed an enhanced metabolic production/diversity together with a clear morphological variation, making these observations useful for the selection of the most diverse cultures. Indeed, as already reported but poorly considered, morphology is one of the main parameters influencing the biosynthesis of metabolites (Silber *et al.*, 2016). In a scale-up fermentation system, the formation of aggregates can make oxygen and nutrients different accessible to the fungal colony (Silber *et al.*, 2016).

The use of several media increases the possibility to isolate new metabolites: in a large screening program performed to assess the antifungal activity of fungal crude extracts, the positive results increase of about 89% by using twelve media instead of three (Bills *et al.*, 2008). However, six to eight media are a good compromise, since the discovery rate of new metabolites tends to decrease (Bills *et al.*, 2008).

Although all the conditions investigated (different media, presence/absence of salts, co-culture) were separately discussed, a synergic action on the gene expression can not be excluded, since the goal of the OSMAC approach is the change of chromatin status in order to activate silent genes (Takahaschi *et al.*, 2013).

6.2.2 Scale up of *Eurotium chevalieri* MUT 2316

E. chevalieri MUT 2316 produced more and different metabolites in comparison to the other fungi tested. For this reason, it was selected for the scale-up and the molecules purification. The condition that better promoted the chemical diversity

(Figure 6.7) and the growth of the fungus was PDAS, compared to all the other conditions assessed. Both solid and liquid culture conditions were tested for the scale-up of *E. chevalieri* MUT 2316.

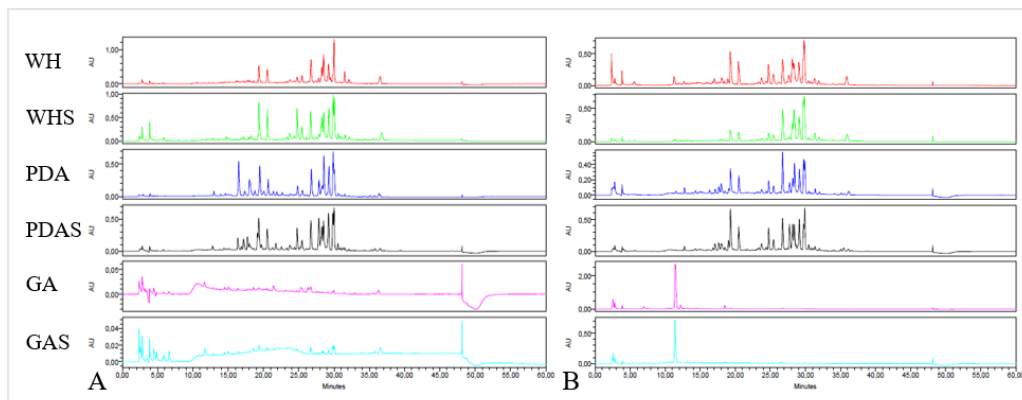


Figure 6.7 Chemical fingerprints ($\lambda = 280$ nm) of the crude organic extract of *E. chevalieri* MUT 2316 on six different media in **A.** pure culture and **B.** Co-culture.

6.2.2.1 *E. chevalieri* MUT 2316 metabolites from solid culture condition

The 200 plates of *E. chevalieri* MUT 2316 yielded 88 g of freeze-dried material; the extraction scheme, the solvents used and the amount of extracts are reported in Figure 6.8.

The first two fractions obtained with EtOAc:CH₂Cl₂ (1:1, v/v) and CH₂Cl₂:MeOH (1:1, v/v) (chemical fingerprints in Figure 6.9A, B), were both represented by an orange-red powder. Due to the high amount of secondary metabolites, the EtOAc:CH₂Cl₂ extract was further processed and fractionate on RP C18 silica gel to obtain six fractions (Figure 6.8). Of these, F3 (chemical fingerprint in Figure 6.9C), eluted with H₂O:MeOH (1:1, v/v), was further purified by semi-preparative HPLC. Four out of the eight compounds obtained were pure but unfortunately, did not give any signals in the NMR analysis to allow structure identification.

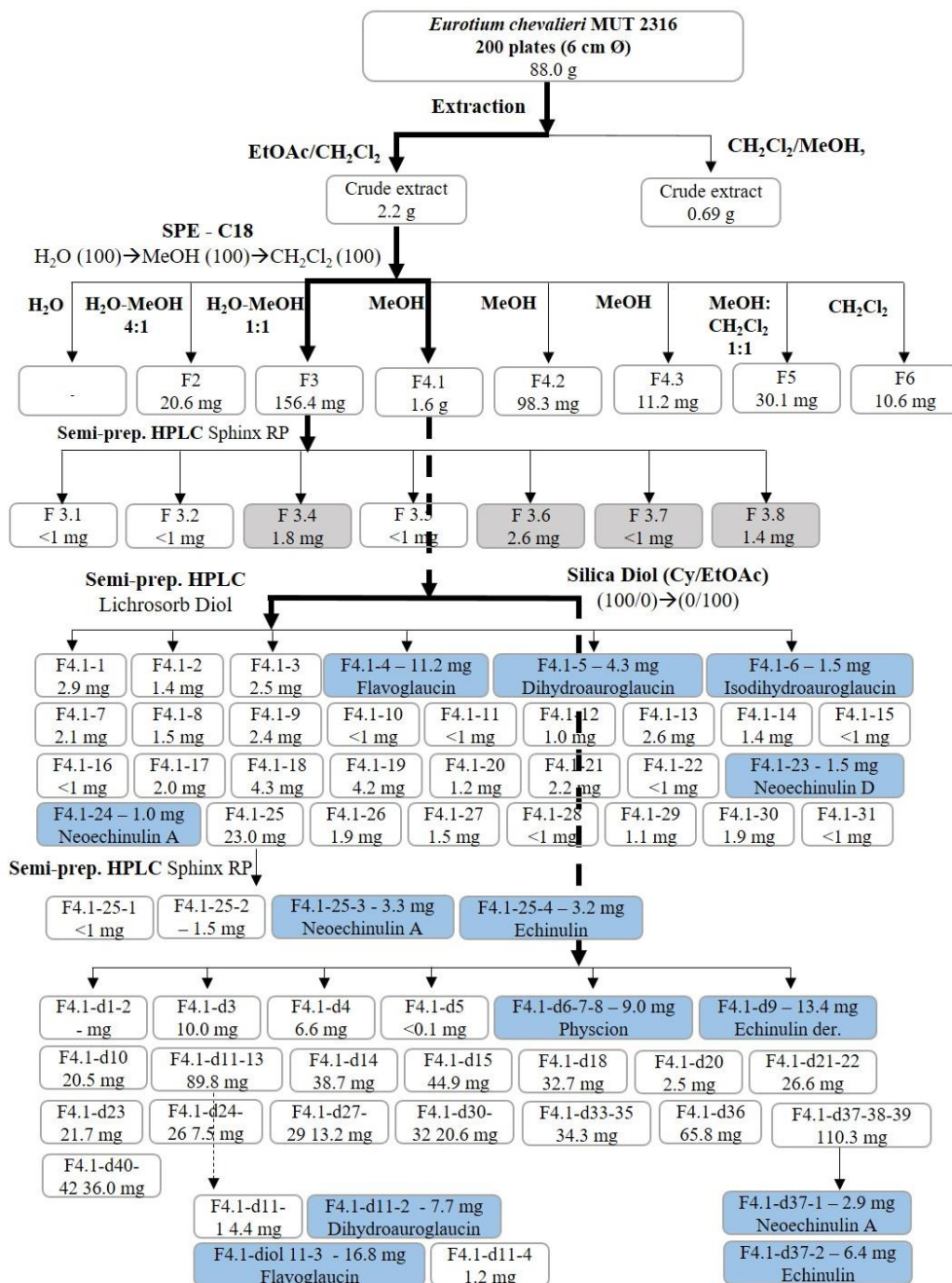


Figure 6.8 Extraction HPLC scheme of *E. chevalieri* MUT 2316 in solid culture condition. The amount of the extracts, the solvents and the techniques used are reported. In light blue the pure molecules obtained and their names, in grey the molecules for which no signals were recorded by extensive NMR analysis.

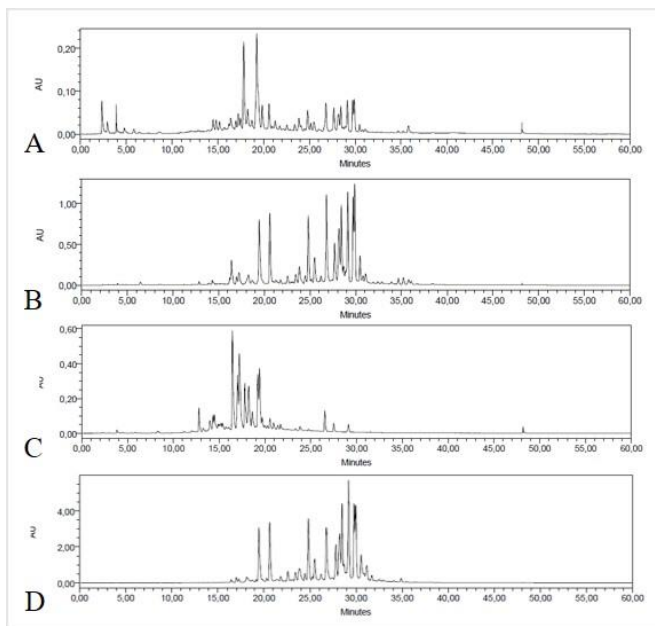


Figure 6.9 Chemical fingerprints (Macherey-Nagel NUCLEODUR® Sphinx column - 250 x 4.6 mm, 5 μ m. H₂O:ACN + 0.1% FA - 90:10 to 0:100 in 30 min, 0:100 for 5 min, 0:100 to 90:10 in 15 min. Flow rate 1 mL/min. λ = 280 nm). **A.** CH₂Cl₂:MeOH crude organic extract. **B.** EtOAc:CH₂Cl₂ crude organic extract. **C.** F3 eluted with H₂O:MeOH. **D.** F4 eluted with MeOH.

More interesting results were obtained by the purification of F4.1 (eluted with MeOH; chemical fingerprint in Figure 6.9D). A first purification step was carried out by semi-preparative normal phase HPLC; but due to the high amount of metabolites and their reduced solubility, the injection volume and, as consequence, the yield of the fractions was low. However, of the 31 fractions collected, and after a further purification of the fraction F4.1-25, 6 pure molecules were obtained and their structure was defined by analyses of NMR spectra coupled with mass spectrometry data; furthermore, molecules spectra were compared with those reported in the literature. The molecules were identified as follow:

Echinulin (Figure 6.10 (**1**)) was isolated as a yellowish powder (3.2 mg). Mass spectrometry analysis gave a pseudomolecular ion $[M + H]^+$ at m/z 462.20. The molecular formula was determined as C₂₉H₃₉N₃O₂.

Neoechinulin A (Figure 6.10 (2)) obtained as a white powder (4.3 mg). Mass spectrometry analysis gave a pseudomolecular ion $[M + H]^+$ at m/z 324.13. The molecular formula was determined as $C_{19}H_{21}N_3O_2$.

Neoechinulin D (Figure 6.10 (3)) isolated as a greyish powder (1.5 mg). Mass spectrometry analysis gave a pseudomolecular ion $[M + H]^+$ at m/z 392.20. The molecular formula was determined as $C_{24}H_{29}N_3O_2$.

Dihydroauroglaucin (Figure 6.10 (4)) was isolated as a yellowish powder (4.3 mg). Mass spectrometry analysis gave a pseudomolecular ion $[M + H]^+$ at m/z 301.13. The molecular formula was determined as $C_{19}H_{24}O_3$.

Flavoglaucin (Figure 6.10 (5)) obtained as a yellowish powder (11.2 mg). Mass spectrometry analysis gave a pseudomolecular ion $[M + H]^+$ at 305.13. The molecular formula was determined as $C_{19}H_{28}O_3$.

Isodihydroauroglaucin (Figure 6.10 (6)) isolated as a yellowish powder (1.5 mg). It presented the same molecular ion pick and molecular formula of its isomer, the compound (4).

In order to make faster the purification process, the remaining F4 fraction was processed on Silica Diol open column mimicking the normal phase HPLC conditions above experimented. Forty-two fractions were obtained (Figure 6.9), the F4.1-d9 was pure but not stable and was no further investigated, while three consequential fractions were pure and identified as:

Pyscion (Figure 6.10 (7)) was isolated as an orange powder (9.0 mg). Unfortunately, mass spectrometry analysis did not highlight pseudomolecular ion pick, however, thanks

to the NMR analysis the molecule was identified with the following molecular formula $C_{16}H_{12}O_5$.

When the amount of fractions and the chemical profile allowed further purifications, the process was carried out by semi-preparative HPLC (Figure 6.9). Echinulin (**1**) 6.4 mg, Neoechinulin A (**2**) 2.9 mg, Dihydroauroglaucin (**4**) 7.7 mg and Flavoglaucin (**5**) 16.8 mg were again obtained and merged with the previous batch for the biological assays.

The structure of the molecules isolated from *E. chevalieri* MUT 2316 in solid culture condition are reported in Figure 6.10.

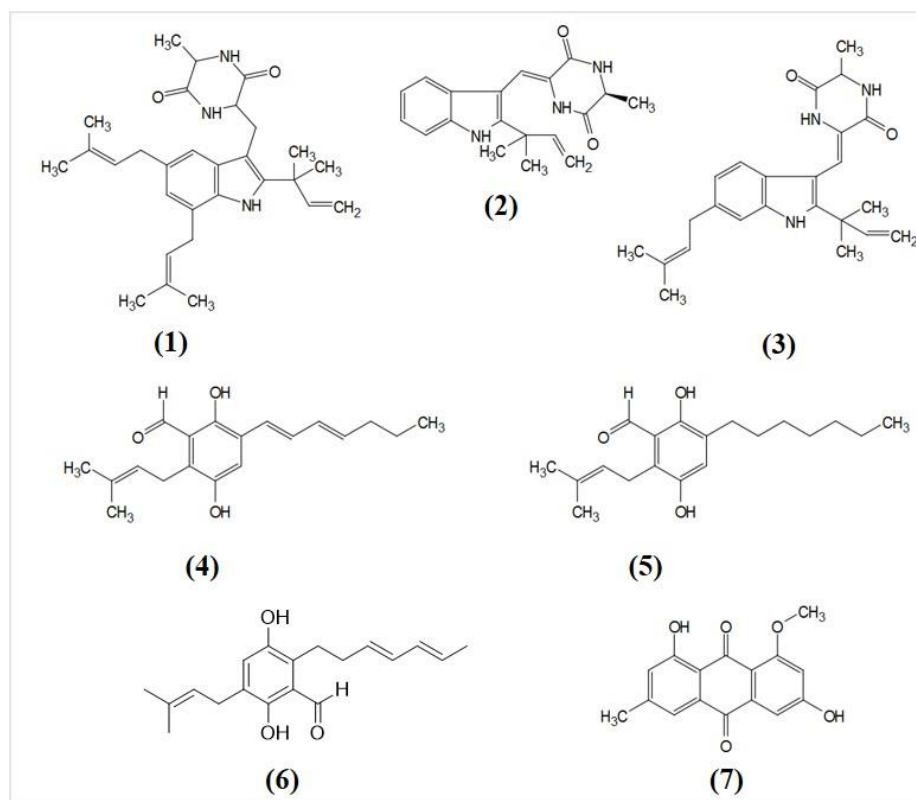


Figure 6.10 Echinulin (**1**), Neoechinulin A (**2**), Neoechinulin D (**3**), Dihydroauroglaucin (**4**), Flavoglaucin (**5**), Isodihydroauroglaucin (**6**) and Physcion (**7**) isolated from *E. chevalieri* MUT 2316 in solid culture condition.

6.2.2.2 *E. chevalieri* MUT 2316 metabolites from liquid culture condition

The separation process of the broth and the mycelium of *E. chevalieri* MUT 2316 in liquid culture condition yielded 3.5 L and 4.2 g (dry weight), respectively. The extraction scheme, the solvents used and the amount of extracts are reported in Figure 6.11.

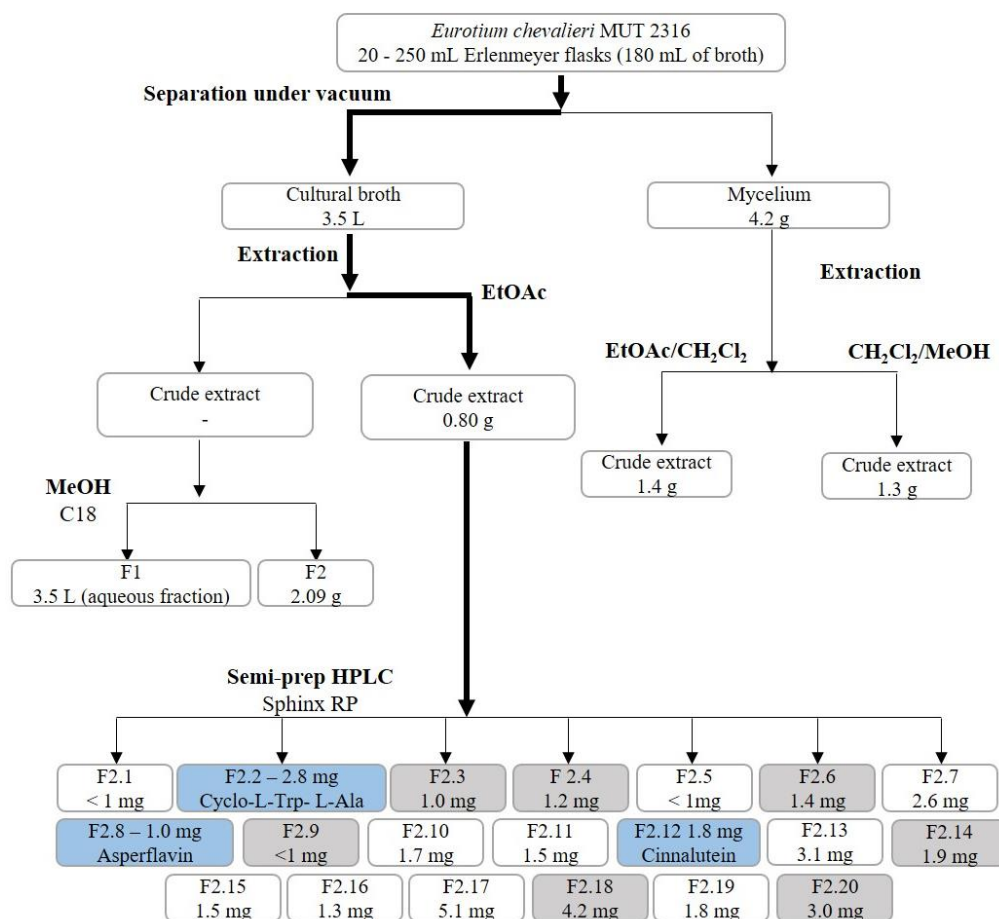


Figure 6.11 Extraction scheme of *E. chevalieri* MUT 2316 in liquid culture condition. The amount of the extracts, the solvents and the techniques used are reported. In light blue the pure molecules obtained and their names, in grey the molecules for which no signals were recorded by extensive NMR analysis.

The chemical fingerprints of the organic extracts associated with the broth and the biomass highlighted the presence of more polar compounds in the medium compared to the mycelium (Figure 6.12). This not surprises to find the polar compounds mainly in the water-based medium, however, the separation of the metabolites between the fungus and its growing medium could make easier the following purification processes also in light of industrial exploitation.

In order to avoid re-extracting the molecules already obtained, the chemical fingerprints of the crude extracts from both solid and liquid culture conditions were compared (Figure 6.9 A, B and Figure 6.12 A–D). The chemical profile with the lowest overlap with the already screened crude extract was selected. This was the case of the EtOAc crude organic extract from the cultural broth (Figure 6.12A), from which, after fractionation, 20 fractions were obtained (Figure 6.11).

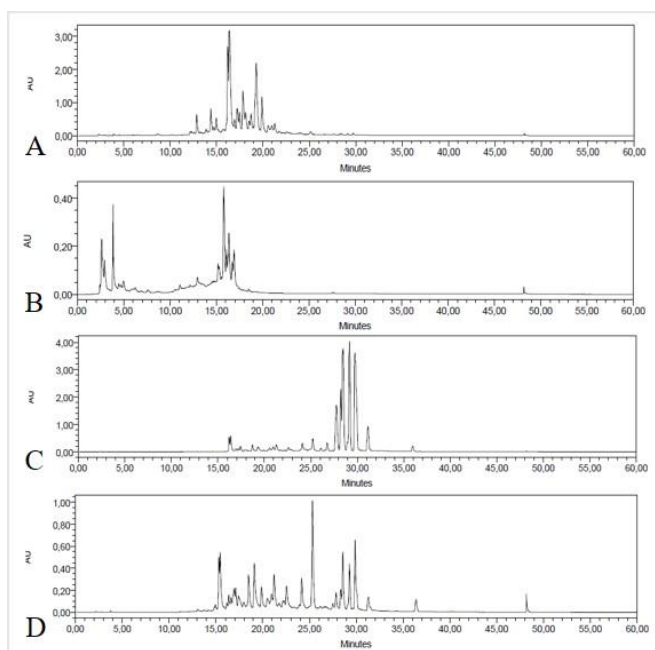


Figure 6.12 Chemical fingerprints (Macherey-Nagel NUCLEODUR® Sphinx column - 250 x 4.6 mm, 5 μ m. H₂O:ACN + 0.1% FA - 90:10 to 0:100 in 30 min, 0:100 for 5 min, 0:100 to 90:10 in 15 min. Flow rate 1 mL/min. λ = 280 nm). **A.** EtOAc crude organic extract from broth. **B.** F2 MeOH fraction from broth **C.** EtOAc:CH₂Cl₂ crude organic extract from the mycelium. **D.** MeOH:CH₂Cl₂ crude organic extract from the mycelium.

The purification process yielded 10 pure molecules (according to the HPLC and LC-MS data), 7 of them did not give any workable NMR data, probably due to the reduced amount of the compounds (Figure 6.9). While, three other molecules were pure and identified by extensive NMR analysis, coupled with mass spectrometry data. The results were also compared with those reported in the literature and the molecules were identified as:

Asperflavin (Figure 6.13 **(8)**) was isolated as a greenish powder (1.0 mg). Mass spectrometry analysis gave a pseudomolecular ion $[M + H]^+$ at m/z 289.07. The molecular formula of the compound was determined as $C_{16}H_{16}O_5$.

Cinnalutein (Figure 6.13 **(9)**) was obtained as a red powder (1.8 mg) with a pseudomolecular ion signal $[M - H]^-$ at m/z 327.05. The molecular formula determined after extensive analysis is $C_{17}H_{12}O_7$.

Cyclo-L-Trp- L-Ala (Figure 6.13 **(10)**) was isolated as a blank-grey powder (2.8 mg). Mass spectrometry analysis gave a pseudomolecular ion $[M - H]^-$ at m/z 256.36. The molecular formula of the compound was determined as $C_{14}H_{15}N_3O_2$.

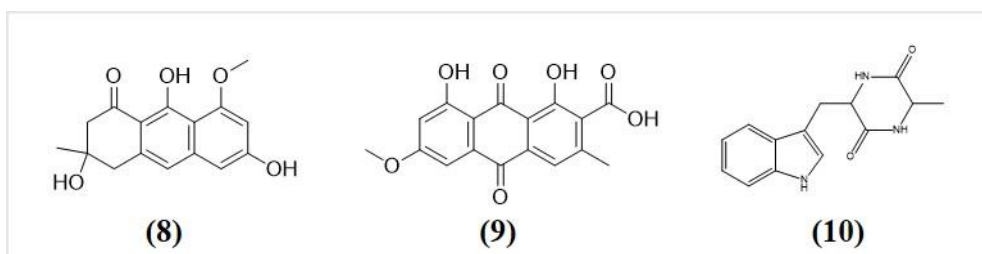


Figure 6.13 Asperflavin (**(8)**), Cinnalutein (**(9)**) and Cyclo-L-Trp- L-Ala (**(10)**), isolated from *E. chevalieri* MUT 2316 in liquid culture condition.

Overall, the 10 molecules obtained by the purification of the extracts of *E. chevalieri* MUT 2316 have already been described in the literature. Eight of them (**(1, 2, 4-8, 10)**) from *E. chevalieri* (Zin *et al.*, 2017; Micheluz *et al.*, 2016; Kanokmedhakul *et*

al., 2011; Fraga *et al.*, 2008; Wu *et al.*, 2014; Ishikawa *et al.*, 1984) or its anamorph *Aspergillus chevalieri* (Tawfik *et al.*, 2017; Hamasaki *et al.*, 1981) isolated from soil, air or plants. To the best of our knowledge, all the molecules are here first reported from a marine-derived *E. chevalieri*. However, these molecules are not new to the marine ecosystem, 8 of them have been previously detected in several marine-derived fungi (Chen *et al.*, 2018; Du *et al.*, 2017; Kwon *et al.*, 2017; Yang *et al.*, 2017; Kamauchi *et al.*, 2016; Du *et al.*, 2014; Kim *et al.*, 2014; Sun *et al.*, 2014; Wijesekara *et al.*, 2014; Sohn *et al.*, 2013; Wijesekara *et al.*, 2013; Wang *et al.*, 2012b; Smetanina *et al.*, 2007; Wang *et al.*, 2007; Li *et al.*, 2006). While Neochinulin D (**3**) and Cinnalutein (**9**) are here firstly recorded in a marine derived fungus and in *E. chevalieri*. Interestingly, some molecules are ubiquitous, for instance (**1**) and (**7**) have been previously isolated from different plants and are known in the Chinese medicine (Pang *et al.*, 2016; Pereira *et al.*, 2016; Wang *et al.*, 2017a; Agarwal *et al.*, 2000). While, (**6**) was isolated also from actinomycetes (Tian *et al.*, 2013).

6.2.2.3 Molecular networking of *E. chevalieri* MUT 2316 in solid and liquid culture condition

The previous sections reported the high metabolic diversity of *E. chevalieri* MUT 2316 in both liquid and solid culture. However, it is incredibly difficult to highlight, only from the chromatograms, which molecules were exclusive of one culture condition. The graphical representation obtained with GNPS and its visualisation with Cytoscape, make easier this process. Indeed, a molecular network is a representation of molecular relatedness (chemical similarity) of any given set of compounds. It is based on the fact that structurally similar molecules share similar MS/MS fragmentation patterns (Yang *et al.*, 2013). Therefore, families tend to cluster together within a network due to the similarity of the structure and the fragmentation pattern (Yang *et al.*, 2013). In the network, a node represents one consensus MS/MS spectrum, a mathematical merging of MS/MS spectra with nearly identical precursor mass and peak patterns, and it is labeled with the precursor mass; edges connect nodes with related consensus MS/MS (Yang *et al.*, 2013).

Figure 6.14 report the molecular network of the crude extracts obtained by solid and liquid media; green, azure and fuchsia nods indicate molecules founded in solid media, liquid media and in both conditions, respectively.

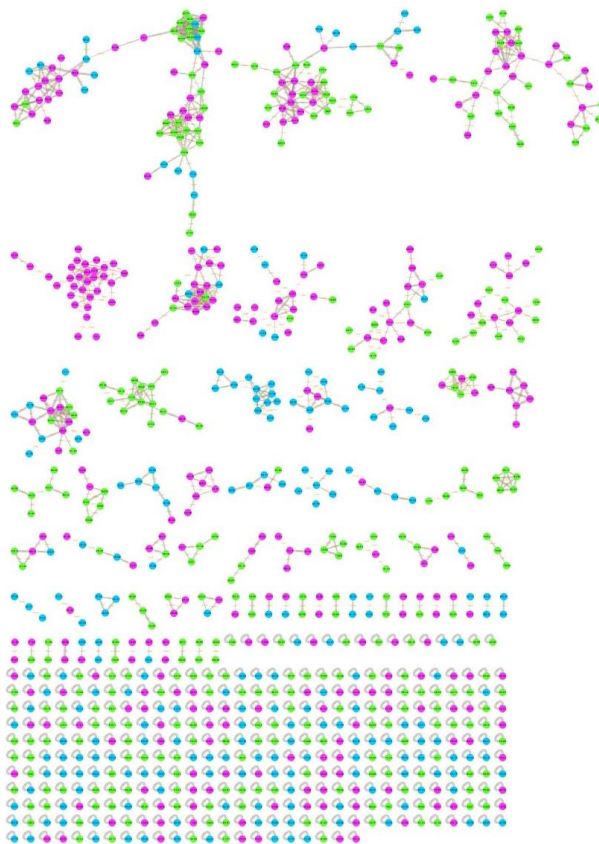


Figure 6.14 Molecular network of the crude extract of *E. chevalieri* MUT 2316 in solid (green), liquid (azure) and both culture conditions (fuchsia).

Overall, *E. chevalieri* MUT 2316 yielded 628 and 536 parent ions in solid and liquid media, respectively. To the best of our knowledge, this is the first report underlining the different molecules produced in the same solid and liquid medium, where the only difference between the two is the absence of the agar. Indeed, the available researches compare solid and liquid culture conditions using different media both for terrestrial (Abdelwahab *et al.*, 2018; Hewage *et al.*, 2018; VanderMolen *et al.*, 2013) and marine fungi (Adpressa *et al.*, 2016; Guo *et al.*, 2015; Liu *et al.*, 2015; Peng

et al., 2014). Therefore, it is not clear if the changes in the metabolic production were due to the growth on a solid or liquid substrate or to the chemical composition of the media.

Overall, we can state that *E. chevalieri* MUT 2316 produce more metabolites in solid culture. Of note, each culture condition contains a high percentage of unique parent ions: 50% were exclusive of solid culture and 42% were only retrieved in the liquid condition. These results support the findings of the OSMAC approach: little modification of the growth parameters strongly enhanced the metabolic diversity (Bode *et al.*, 2002).

Despite the differences between liquid and solid culture, the molecular network indicates that the distribution of the ion mass is similar between the two conditions, with almost half of the molecules having an m/z values higher than 501. The mass values between m/z 301 and 500 were represented by 41% and 34% parent ions in solid and liquid condition, respectively; from this range of molecules 7 out of 10 of the pure compounds were isolated (**1-6, 9**). Small molecules ($m/z < 300$) were poorly represented in both solid (9%) and liquid (11%) cultures, however, the remaining three metabolites (**7, 8, 10**) isolated in the present study are part of the small molecules produced by *E. chevalieri* MUT 2316.

Interestingly, thanks to the graphical representation obtained in Cytoscape, it was possible to highlight also where the pure molecules detected in this study were produced (solid/liquid) (Figure 6.15). Overall, 9 pure compounds were produced both in solid and liquid culture condition, while (**3**) was produced only in the solid medium. Moreover, based on the principle that structurally similar molecules share similar MS/MS fragmentation patterns and tend to cluster together (Yang *et al.*, 2013), it was possible to highlight two families based on the molecules founded in this study (Figure 6.13). This was the case of (**4, 6**) and (**5**), that cluster together due to the close similarity in the fragmentation pattern and in the structure; indeed, (**4, 6**) present two double bonds in position one and three that miss in (**5**). Unfortunately, it is not possible to differentiate the two isomers (**4, 6**).

The second family of compounds that clustered together was represented by **(1)** and **(3)** as shown in Figure 6.15, with **(1)** presenting an additional 3-methylbut-2-enyl chain.

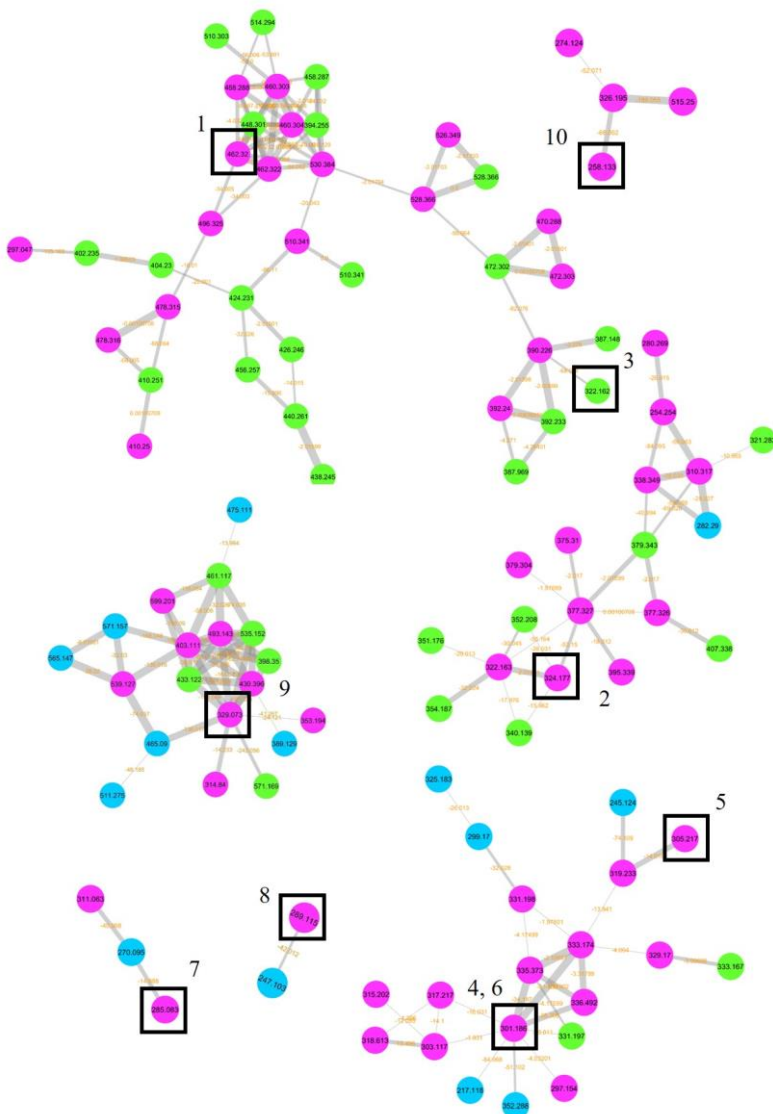


Figure 6.15 Molecular clusters of Echinulin **(1)**, Neoechinulin A **(2)**, Neoechinulin D **(3)**, Dihydroauroglaucin **(4)**, Flavoglaucin **(5)**, Isodihydroauroglaucin **(6)**, Phycion **(7)**, Asperflavin **(8)**, Cinnalutein **(9)** and Cyclo-L-Trp- L-Ala **(10)**. Colours highlight the molecules only detected in solid culture condition (green), in liquid culture condition (azure) and on both (fuchsia).

6.3 CONCLUSION

To the best of our knowledge, this is the first report showing the metabolic diversity of all the fungal community associated with a sponge, using different nutrients media, the co-cultivation with a marine bacterium and evaluating the influence of salts. Moreover, up to date, there are no reports using such high number of OSMAC conditions (12) for marine fungi (Romano *et al.*, 2018).

It is clear, from the scientific literature, that the OSMAC approach has not been extensively applied to marine microorganisms, including fungi (Romano *et al.*, 2018). This appears surprising, since marine microorganisms represent probably the most promising source of new natural products (Blunt *et al.*, 2018; Romano *et al.*, 2018; Silber *et al.*, 2016). However, the OSMAC approach requires extensive experiments to find the best condition for each fungus (Romano *et al.*, 2018), that might be extremely different even for strains of the same species, as here demonstrated. Among the fungi tested, *E. chevalieri* MUT 2316 showed an incredible chemical diversity and, was in parallel capable to grow in axenic culture and produce several metabolites in only two weeks. The use of an innovative approach, based on the creation of a molecular network, allowed to easily underline the molecules common or exclusive to the solid/liquid culture condition, as well as to create molecular families, based on the fragmentation properties. The 10 pure molecules obtained after several purifications steps, although already know are reported for the first time in a marine derived *E. chevalieri* and were tested, for the first time, in several bioassays in the following chapters.

The results showed in this chapter have been published in:

Marine Drugs. Bovio E., Garzoli L., Poli A., Luginini A., Villa P., Musumeci R., McCormack G. P., Cocuzza C. E., Gribaudo G., Mehiri M., Varese G. C. (2019). Marine fungi from the sponge *Grantia compressa*: biodiversity, chemodiversity and biotechnological potential.

7. The antibacterial activity of the molecules produced by *E. chevalieri* MUT 2316

7.1 INTRODUCTION

7.1.1 The Golden Age of antibiotics, the antibiotic crisis and the need of new medicines

For almost three-quarters of a century, the management of bacterial infections has been based on the use of several economic and safe antibiotics (Brown and Wright, 2016). The discovery of the penicillin in 1929 by Flemming, allowed controlling the infections caused by Gram-positive pathogens (mainly *Staphylococcus* spp. and *Streptococcus* spp.). A few years later, the newly discovered streptomycin (1943) positively contrasted the *Mycobacterium tuberculosis* responsible of tuberculosis, definitively opening the Golden Age of antibiotics (Brown and Wright, 2016; Rolain *et al.*, 2016). In the next following years, several new antibiotics were discovered, allowing managing more and more infections and leading the way to organ transplants, immunotherapy and cancer chemotherapy (Wright, 2017). Today, bacterial resistance is going to seriously threaten the quality of life and life expectancy (Brown and Wright, 2016). Ventola (2015) summarised, as shown in Figure 7.1, on one side the discovery of new antibiotics and on the other side the developing of bacterial resistance to previously formulated antibiotics. The results are tragic, bacterial strains resistant to all the known antibiotics have been reported, although with different occurrence and distribution (Ventola, 2015).

Certainly, the bacterial resistance is becoming widespread and it is already representing a worldwide problem: recent estimates report that the number of deaths attributed to antimicrobial resistance (AMR) may rise from the current 700,000 per year to 10 million per year in 2050 (Brogan and Mossialos, 2016; O'Neill, 2016). Several critiques have been moved to these approximations published by a respected economist (O'Neill, 2016), which, however, lack of precise references to background data (de Kraker *et al.*, 2016). The same O'Neill showed disappointment for the absence of institutions worldwide responsible for infection surveillance, or, admitted that the data

are broad estimates and that Academia can guarantee a more precise work (O'Neill, 2016).

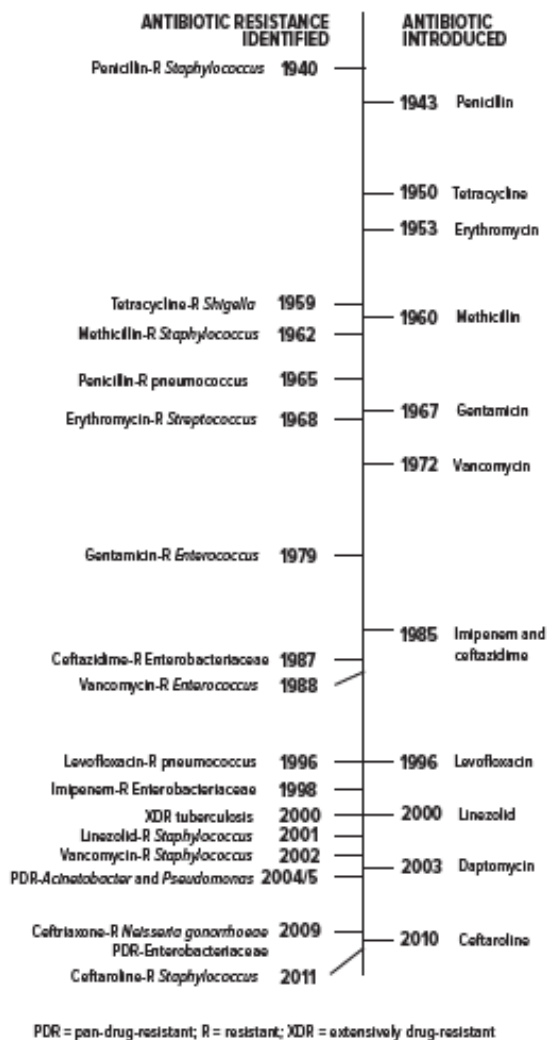


Figure 7.1 Timeline of the developing of new antibiotics and the occurrence of antimicrobial resistance (Ventola, 2015).

Despite this, in 2014, the World Health Organisation (WHO) warned that the antibiotic resistance crisis is becoming real and desperate (Dodds, 2017; Ventola, 2015) and man is the main responsible (Rather *et al.*, 2017). On one side, the self-medication,

the inappropriate prescription of antibiotics and the extensive use in livestock are dramatically increasing the cases of antibiotic resistance (Dodds, 2017; Rather *et al.*, 2017); for instance the 80% of the antibiotic sales are dedicated to animal husbandry (Dodds, 2017). On the other side, the development of new antibiotics could be no more a convenient investment for pharmaceutical industries (Dodds, 2017; Rather *et al.*, 2017). Indeed, before entering the global market of drugs, a new molecule has to follow complex, expensive and long clinical trials. However, it is disconcerting that among the molecules approved by the Food and Drug Administration (FDA) and coming from the marine environment, defined as a promising source of new medicines, there are mainly anti-cancer compounds and none antibacterial (Table 7.1). The same occur looking to the other 22 molecules currently in different stages of the clinical trials (Table 7.1) with cancer, Alzheimer’s disease and pain, as main targets. Therefore, none compound with antibacterial potential is in the chemical trial; in order to remedy to the role that should be played by the pharmaceutical companies, the European Union started financing several drugs discovery projects and new potential antibiotics are entering in the pre-clinical pipelines (Littmann *et al.*, 2015). Choudhary and colleagues (2017) listed about 170 marines derived molecules with antibacterial activity also against the “high priority pathogens” defined by the WHO, including *S. aureus* MRSA and *Enterococcus faecium* vancomycin-resistant.

Table 7.1 Marine pharmaceutical in clinical pipelines (<http://marinepharmacology.midwestern.edu/clinPipeline.htm> accessed on 25th May 2018).

Clinical Status	Compound (name)	Trademark (FDA Approved Year)	Marine organism	Disease
FDA-Approved	Trabectedin (ET-743)	Yondelis® (2015)	Tunicate	Cancer
	Brentuximab vedotin (SGN-35)	Adcetris® (2011)	Mollusk/cyanobacterium	Cancer
	Eribulin Mesylate (E7389)	Halaven® (2010)	Sponge	Cancer
	Omega-3-acid ethyl esters	Lovaza® (2014)	Fish	Hypertriglyceridemia
	Ziconotide	Prialt® (2014)	Cone snail	Pain

	Vidarabine (Ara-A)		Vira-A® (1976)	Sponge	Antiviral
	Cytarabine (Ara-C)		Cytosar-U® (1969)	Sponge	Cancer
Phase III	Plinabulin (NPI-2358)		NA	Fungus	Cancer
	Plitidepsin		Aplidin®	Tunicate	Cancer
	Tetrodotoxin		Tectin®	Pufferfish	Pain
	Lurbinectedin (PM01183)		NA	Tunicate	Cancer
	Depatuxizumab mafodotin (ABT-414)	mafodotin	NA	Mollusk/cyanobacterium	Cancer
	Polatuzumab vedotin (DCDS-4501A)		NA	Mollusk/cyanobacterium	Cancer
	GTS-21 (DMXBA)		NA	Worm	Schizophrenia, Alzheimer's Disease
Phase II	Denintuzumab (SGN-CD19A)	mafodotin	NA	Mollusk/cyanobacterium	Cancer
	AGS-16C3F		NA	Mollusk/cyanobacterium	Cancer
	Plocabulin (PM184)		NA	Sponge	Cancer
	Tisotumab Vedotin		HuMax®-TF-ADC	Mollusk/cyanobacterium	Cancer
	Enfortumab Vedotin (ASG-22ME)		NA	Mollusk/cyanobacterium	Cancer
	Glembatumumab (CDX-011)	Vedotin	NA	Mollusk/cyanobacterium	Cancer
	GSK2857916		NA	Mollusk/cyanobacterium	Cancer
	Ladiratumumab vedotin (SGN-LIV1A)		NA	Mollusk/cyanobacterium	Cancer
	Bryostatatin		NA	Bryozoan	Alzheimer's Disease
	Phase I	ABBV-085		NA	Mollusk/cyanobacterium
Telisotuzumab vedotin (ABBV-399)			NA	Mollusk/cyanobacterium	Cancer
ABBV-221			NA	Mollusk/cyanobacterium	Cancer

ASG-67E	NA	Mollusk/ cyanobacterium	Cancer
ASG-15ME	NA	Mollusk/ cyanobacterium	Cancer
Marizomib (Salinosporamide A; NPI-0052)	NA	Bacterium	Cancer

Therefore, mainly the Academia is focusing on the screening of the molecules produced by microorganisms, usually the product of evolution in nutrient/resource competition and perfectly shaped to enter bacterial cells (Wright, 2017). In parallel to the discovery of new antibiotics, several studies are focusing on molecules enhancing the activity of already known antibiotics as well as on compounds inhibiting biofilm formation and toxins production (Wright, 2017).

In conclusion, as the Golden Age of antibiotics was led by natural products, there are good reasons to think that also today this research field could be able to bring new antimicrobial drugs (Wright, 2017). This hypothesis is supported by the fact that over 60% of new drugs are naturally derived (Choudhary *et al.*, 2017).

7.1.2 Developing of AMR

The AMR is defined as the ability of microbes to grow in the presence of a drug that would normally kill them or limit their growth (Allcock *et al.*, 2017). **What determines AMR?** The leading factor responsible for the AMR is the exposure to antimicrobials that generate a selective pressure on the microorganisms (Allcock *et al.*, 2017; Rolain *et al.*, 2016). In these conditions, a genetic mutation that confers resistance to a bacterium, under selective pressure, can allow the mutant to survive, while the rest of the population die. This bacterium can spread and become dominant (Allcock *et al.*, 2017; Rolain *et al.*, 2016).

The resistance to antibiotics can be acquired by vertical and the horizontal evolution. The first one (Figure 7.2A) is related to the acquisition of DNA mutations that enhance the evaluative success of the bacterium and are transmitted to the progeny (MacGowan and Macnaughton, 2017; Sommer *et al.*, 2017). The horizontal transmission presumes the presence of bacteria (also of different species) already containing antibiotic

resistant genes that through the conjugation, transduction or transformation, are subsequently transmitted to another bacterium (MacGowan and Macnaughton, 2017; Sommer *et al.*, 2017). These three different processes are summarised in Figure 7.2B. The conjugation is based on direct contact between the cells, the DNA transmission is carried out by plasmids, circular molecules of double-stranded DNA independent of the chromosome (MacGowan and Macnaughton, 2017; Sommer *et al.*, 2017). The transduction is mediated by a virus that can incorporate a piece of bacterial DNA during the replication of the viral DNA performed by the bacterial cell (MacGowan and Macnaughton, 2017; Sommer *et al.*, 2017). Last, during the transformation, a bacterium incorporated “naked DNA” from cells that went through the lytic cycle and released their content (MacGowan and Macnaughton, 2017; Sommer *et al.*, 2017).

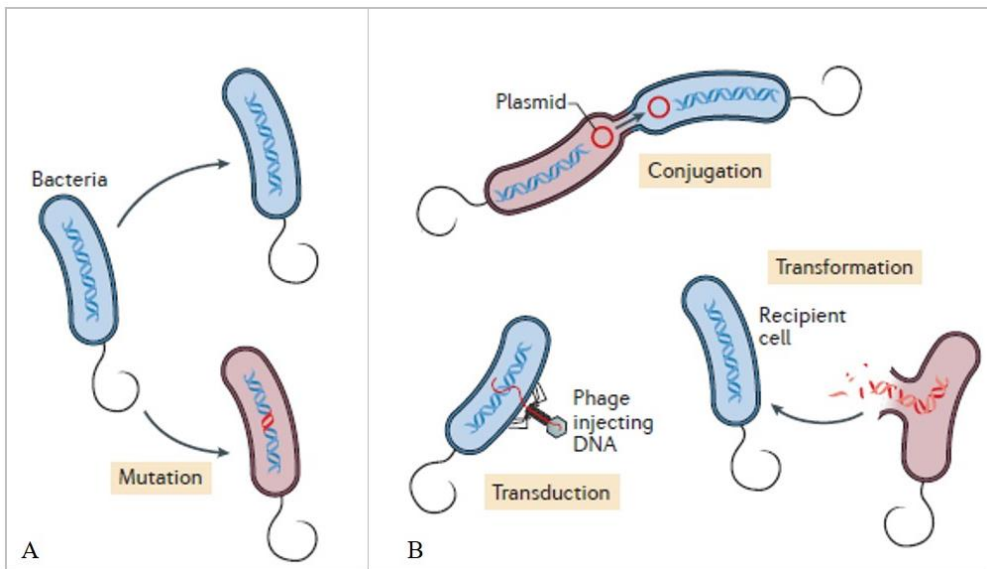


Figure 7.2 Evolution of resistance through **A.** vertical evolution and **B.** horizontal evolution with phage conjugation, transduction or natural transformation. Blue cells are susceptible to bacteria, red cells are resistant bacteria (Sommer *et al.*, 2017).

The acquisition of AMR might determine a reduced fitness, growth and virulence in the bacteria, because antibiotics can target different vital functions, i.e. cell wall synthesis, transcription and translation (Sommer *et al.*, 2017; Rolain *et al.*, 2016).

For instance the bacterium *Bordetella* spp., responsible of pertussis and respiratory infections, have always been treated with erythromycin, with few cases of resistance reported; on the contrary, *S. aureus* and *Streptococcus pneumoniae* treated with the same antibiotic developed alarming resistances (Dewan *et al.*, 2018). In vitro and in vivo studies demonstrated that *Bordetella* spp. is easily able to develop the resistance to erythromycin but this condition determines in bacteria a defective expression of virulence-associated antigens, where all the strains were unable to colonise the respiratory tracts of their hosts (Dewan *et al.*, 2018). Similarly, *Acinetobacter baumannii* resistant to colistin, present a reduced fitness (Geisinger and Isberg, 2017). Depending on the kind of gene resistance acquisition, *S. aureus* MRSA can be slightly less virulent and present a reduced fitness; the same has been observed for *S. aureus* vancomycin-resistant (Geisinger and Isberg, 2017). The growth rate of drug-resistant microbes compared to susceptible strains in absence of antibiotics seems to be a parameter able to successfully predict the evolutionary success of antibiotic-resistant bacteria (Sommer *et al.*, 2017).

Fortunately, it has been showed that “old antibiotics” such as chloramphenicol, clindamycin, clofazimine, colistin, cotrimoxazole, fosfomycin and minocycline, when combined, can successfully treat infections caused by multi-drug resistant (MDR) bacteria, compared to “over-consumed” antibiotics (Rolain *et al.*, 2016). The main problem concerns the availability of such low-cost antibiotics because their production is not any more convenient (Rolain *et al.*, 2016).

7.1.3 What determines a bacterium to become MDR?

Literature usually reports MDR as bacteria resistant to three or more antimicrobial classes’ based on *in vitro* assays (Rolain *et al.*, 2016; Magiorakos *et al.*, 2012). However, a standardised terminology concerning AMR did not exist, even if fundamental to collect and compare epidemiological data worldwide (Magiorakos *et al.*, 2012). In this regard, thanks to an initiative of the European Centre for Disease Prevention and Control and of the American Centres for Disease Control and Prevention, a standardized international terminology have been created for *S. aureus*, *Enterococcus*

spp., *Enterobacteriaceae* (other than *Salmonella* and *Shigella*), *P. aeruginosa* and *Acinetobacter* spp., because of their importance and priority in the healthcare system (Magiorakos *et al.*, 2012).

Focusing on the strains used in the present work, *S. aureus* isolates are defined MDR when they are non-susceptible to at least one agent in three antimicrobial categories (Magiorakos *et al.*, 2012). Sadly, MRSA kills more Americans each year than several other causes of death when considered together, including HIV, Parkinson's disease, emphysema, and homicide (Ventola, 2015). This is the reason why the WHO put MRSA in the least of high priority bacteria with the aim to raise and drive the Academia and industries attention.

Another bacterium responsible for severe diseases is *S. pneumoniae*. It causes pneumonia, meningitis and minor infections (bloodstream, ear, and sinus) responsible of about 7,000 death each year (Ventola, 2015). This species mainly developed resistance to penicillin class and macrolides, however, the development of new vaccines against different strains of *S. pneumoniae* protect on one side from high virulent pathogens and on the other side decrease the spread of resistant strains (Ventola, 2015).

In order to assess the antibacterial potential of the molecules produced by *E. chevalieri* MUT 2316 reference Gram positive and Gram negative strains coming from the American Type Culture Collection (ATCC) strains were used. Based on the obtained results a selection of bacteria resistant to different antibiotics was added to the panel, looking for highly promising molecules to face the current antibiotic crisis.

7.2 MATERIAL AND METHODS

7.2.1 Bacterial growth conditions and inoculum preparation

Representatives of Gram-positive and Gram-negative bacteria were selected for the antimicrobial susceptibility tests, including standard strains and clinical isolates kindly provided and characterized for their resistance by the Hospital of Monza (Italy), Table 7.2.

Table 7.2 Bacterial strains used for the antibacterial tests with their code, resistance and classification (Gram +/-).

Species	Code	Gram +/-	Resistance
<i>Enterococcus faecalis</i>	ATCC 29212	+	-
<i>E. coli</i>	ATCC 25922	-	-
<i>P. aeruginosa</i>	ATCC 27853	-	-
<i>S. aureus</i>	ATCC 29213	+	-
<i>S. aureus</i>	Monza-PFI	+	MRSA
<i>S. aureus</i>	Monza-FD1	+	Fluoroquinolone-resistant
<i>S. pneumoniae</i>	ATCC 49619	+	-
<i>S. pneumoniae</i>	Monza-82	+	Macrolide-resistant

The strains, stored at $-80\text{ }^{\circ}\text{C}$, were inoculated in fresh Mueller-Hinton Broth Cation Adjusted – MHBCA (Acid Digest of Casein 17.5 g, Soluble Starch 1.5 g, Beef Extract 2 g - Sigma-Aldrich, Saint Louis, USA - up to 1 L dH₂O) and incubated overnight at $37\text{ }^{\circ}\text{C}$. *S. pneumoniae* Monza-82, due to the specific growth requirements, was inoculated in fresh Brain-Heart Infusion broth – BHI (Beef Heart infusion 5 g, Calf Brains 12.5 g, Disodium Hydrogen phosphate 2.5 g, D(+)-glucose, 2 g, Peptone 10 g, NaCl 5 g - Sigma-Aldrich, Saint Louis, USA - up to 1 L dH₂O) with the addition of 5% defibrinated sheep blood. The bacterial inoculum was incubated overnight at $37\text{ }^{\circ}\text{C}$ in 5% CO₂ atmosphere for optimal growth (Figure 7.3).

Following the overnight incubation, the bacteria were inoculated in new sterile media (the same used above) and incubated for six hours at $37\text{ }^{\circ}\text{C}$ (in 5% CO₂ for *S. pneumoniae* Monza-82) in order to reach the mid-exponential phase (Figure 7.3). Then, the bacterial inoculum was standardised at 0.5 McFarland, which means about 1.5×10^8 CFU/mL (Colony Forming Unit per mL of suspension).

7.2.2 Stock solutions of the molecules isolated from *E. chevalieri* MUT 2316

The ten pure molecules obtained from the purification of solid and liquid extracts of *E. chevalieri* MUT 2316 were dissolved in 100% dimethyl sulfoxide (DMSO) and named stock 1 (Table 7.3). The concentration of the molecules in the stock 1 was 10

mg/mL, with the exception of Physcion (7), maintained at 1 mg/mL, due to a lower solubility in DMSO. The Stock 1 was preserved at -20 °C in different aliquots, ready to use.

Table 7.3 Molecules isolated from *E. chevalieri* MUT 2316, their weights and concentrations in 100% DMSO (stock 1) and further diluted in the bacterial media for the bioassays (stock 2).

Molecules	Weight (mg)	Stock 1 (mg/mL)	Stock 2 (µg/mL)
Echinulin (1)	9.6	10	256
Neoechinulin A (2)	7.2	10	256
Neoechinulin D (3)	1.5	10	256
Dihydroauroglaucin (4)	12.0	10	256
Flavoglaucin (5)	28.0	10	64
Isodihydroauroglaucin (6)	1.5	10	256
Physcion (7)	9.0	1	64
Asperflavin (8)	1.0	10	256
Cinnalutein (9)	1.8	10	256
Cyclo-L-Trp- L-Ala (10)	2.8	10	256

For the antibacterial assays, the Stock 1 was diluted with the media MHBCA or MHBCA + 5% defibrinated sheep blood (for the test with *S. pneumoniae*) to reach the desired concentration of 256 µg/mL for eight out of ten molecules, and named Stock 2 (Table 7.3). As for the two remaining molecules, the concentration of the stock 2 was 64 µg/mL; this because (5) was not well soluble in the aqueous medium of the bacteria, while for (7), the lower concentration is made to avoid the DMSO toxicity.

7.2.3 Determination of the MIC

The MIC was determined following the Clinical and Laboratory Standards Institute (CLSI) guidelines for broth microdilution method (CLSI guidelines, 2015), summarised in Figure 7.3. Briefly, two-fold serial dilutions of the stock 2 were prepared in MHBCA or MHBCA + 5% defibrinated sheep blood (for *S. pneumoniae*) in 96-multiwell; the bacterial inoculum adjusted to 1×10^6 CFU/mL was equally added to the wells to reach the concentration of 5×10^5 CFU/mL in the final volume of 100 µL.

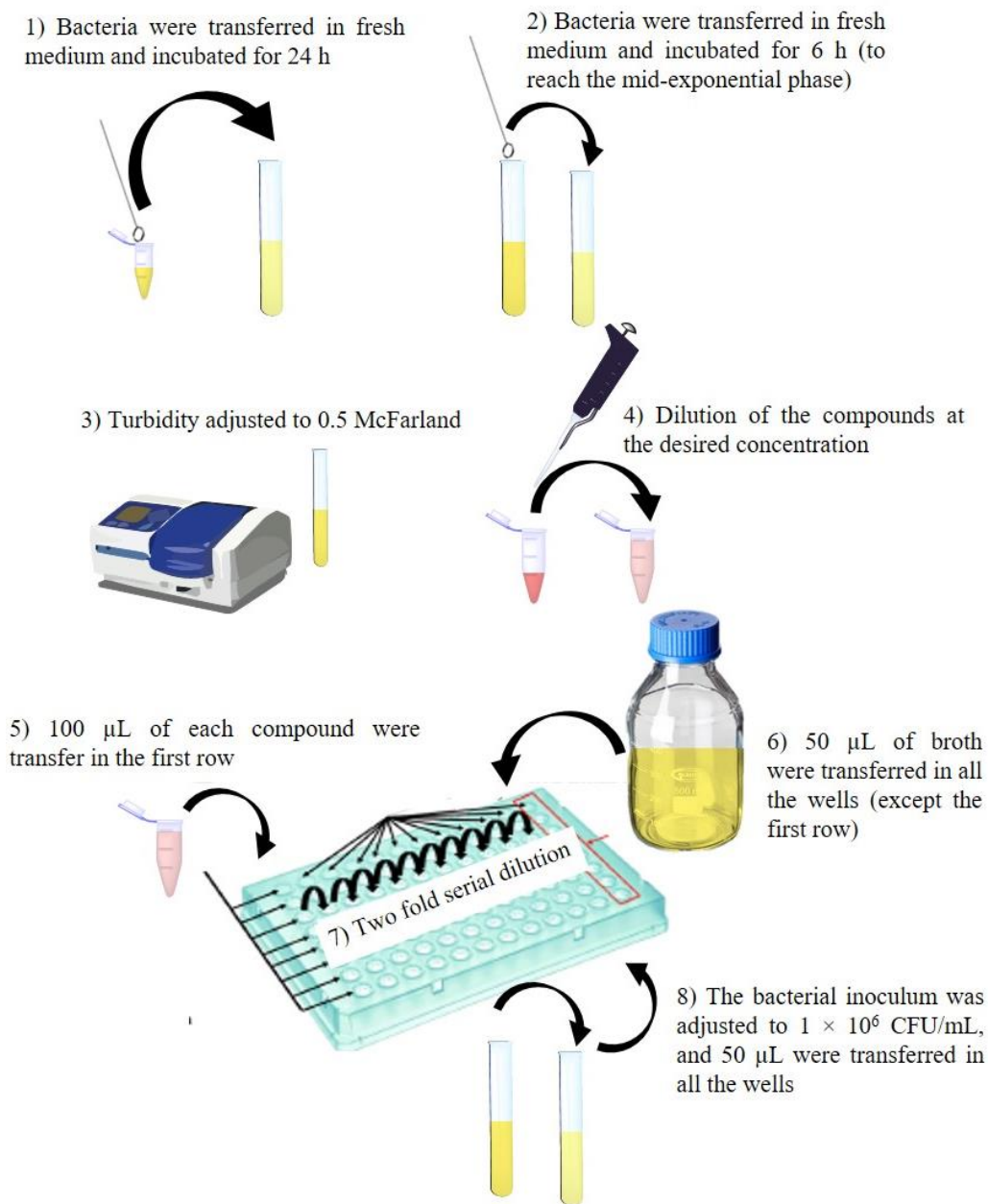


Figure 7.3 Broth microdilution for antibacterial assay as recommended by the CLSI guidelines.

The antibiotics gentamicin, ciprofloxacin, tetracycline, erythromycin and fusidic acid were chosen as controls, according to the sensibility of the bacterial strains tested; the assay was performed in the same way as for the fungal molecules. Controls

were performed also with DMSO, despite appropriate dilutions were set up to test the molecules without the interference of the solvent. Finally, the bacterial strains and the non-inoculated media represented two further controls.

The plates were incubated at 37 °C for 18-24 h. The MIC, defined as the lowest concentration of antimicrobial agent causing visible inhibition of bacterial growth, was evaluated by visualising the absence of bacterial pellets and the clearness of the solution at the lowest molecules/antibiotic concentration.

7.2.4 Determination of the Minimal Bactericidal Concentration (MBC)

The MBC was determined after the MIC assays. The content of the wells representing the MIC values and the next two higher concentration (2xMIC and 4xMIC) were plated on Tryptic Soy Agar - TSA (Pancreatic Digest of Casein 15 g, Peptic Digest of Soybean Meal 5 g, NaCl 5 g, Agar 15 g - Sigma-Aldrich, Saint Louis, USA - up to 1L dH₂O) or Mueller Hinton Fastidious Agar - MHF (Meat Extract 2.0 g, Acid Hydrolysate of Casein 17.5 g, Starch 1.5 g, Agar 17.0 g, Horse Blood mechanically defibrinated 50 g, β-NAD 0.02 g - Sigma-Aldrich, Saint Louis, USA - up to 1L dH₂O) for *S. pneumoniae* strains. Plates were incubated for 24 h at 37 °C (in 5% CO₂ for *S. pneumoniae* Monza-82). Afterward, the MBC, defined as the lowest concentration of antimicrobial agent needed to kill bacteria, was evaluated by determining the absence of bacterial growth in the agar plates.

7.3 RESULTS AND DISCUSSION

7.3.1 *E. chevalieri* MUT 2316 derived compounds show antibacterial activity

The antibacterial activity was evaluated on standard strains of Gram-positive and Gram-negative bacteria, as well as on selected clinical isolates of resistant bacteria. The results of the MIC and MBC are reported in Table 7.4.

Table 7.4 Minimum inhibitory concentration (MIC $\mu\text{g/mL}$) and minimum bactericidal concentration (MBC $\mu\text{g/mL}$) values of compounds (1–10) and reference antibiotics against reference strains and multidrug-resistant isolates. Positive values are highlighted in light blue.

Molecules	Max. conc. ($\mu\text{g/mL}$)	Gram +												Gram -			
		<i>S. aureus</i> ATCC 29213		<i>S. aureus</i> PFI		<i>S. aureus</i> FD1		<i>E. faecalis</i> ATCC 29212		<i>S. pneumoniae</i> ATCC 49619		<i>S. pneumoniae</i> Monza 82		<i>P. aeruginosa</i> ATCC 27853		<i>E. coli</i> ATCC 25922	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Echinulin (1)	128	>128	N/A	>128	N/A	>128	N/A	>128	N/A	>128	N/A	>128	N/A	>128	N/A	>128	N/A
Neoechinulin A (2)	128	>128	N/A	>128	N/A	>128	N/A	>128	N/A	>128	N/A	>128	N/A	>128	N/A	>128	N/A
Neoechinulin D (3)	128	>128	N/A	>128	N/A	>128	N/A	>128	N/A	>128	N/A	64	>128	>128	N/A	>128	N/A
Dihydroauroglaucin (4)	128	128	>128	128	>128	128	>128	64	>128	>128	N/A	8	32	>128	N/A	>128	N/A
Flavoglaucin (5)	32	>32	N/A	>32	N/A	>32	N/A	>32	N/A	>32	N/A	>32	N/A	>32	N/A	>32	N/A
Isodihydroauroglaucin (6)	128	64	128	64	128	32	64	64	>128	>128	N/A	4	16	>128	N/A	>128	N/A
Physson (7)	32	>32	N/A	>32	N/A	>32	N/A	>32	N/A	>32	N/A	16	>32	>32	N/A	>32	N/A
Asperflavin (8)	128	64	>128	128	>128	64	>128	>128	N/A	>128	N/A	32	128	>128	N/A	>128	N/A
Cinnalutein (9)	128	\geq 128	N/A	>128	N/A	128	>128	>128	N/A	>128	N/A	32	>128	>128	N/A	>128	N/A
Cyclo-L-Trp- L-Ala (10)	128	>128	N/A	>128	N/A	>128	N/A	>128	N/A	>128	N/A	>128	N/A	>128	N/A	>128	N/A
Gentamicin	128	0.5	N/D	>128	N/D	1	N/D	>128	N/D	N/D	N/D	N/D	N/D	1	N/D	0.5	N/D
Ciprofloxacin	128	0.25	N/D	64	N/D	8	N/D	0.25	N/D	0.5	N/D	1	N/D	0.12	N/D	\leq 0.06	N/D
Erythromycin	128	0.5	N/D	8	N/D	0.5	N/D	2	N/D	0.12	N/D	16	N/D	N/D	N/D	N/D	N/D
Tetracycline	128	0.12	N/D	1	N/D	1	N/D	8	N/D	0.12	N/D	\leq 0.06	N/D	8	N/D	1	N/D
Fusidic acid	128	0.12	N/D	N/D	N/D	N/D	N/D	4	N/D	4	N/D	N/D	N/D	N/D	N/D	N/D	N/D

N/D Not defined; N/A Not applicable

Compound **(6)** was the most promising and showed activity against almost all Gram-positive bacteria tested (except *S. pneumonia* ATCC 49619), with MIC values ranging from 4 $\mu\text{g/mL}$ to 64 $\mu\text{g/mL}$ (Figure 7.4); the lowest value was observed against the macrolide-resistant *S. pneumoniae* Monza-82. **(6)** was recently assessed for the antibacterial activity and showed activity against the Gram-positive *Bacillus cereus* and the Gram-negative *Salmonella typhimurium* with the same MIC value of 3.7 $\mu\text{g/mL}$ (Liang *et al.*, 2018). The same author (Liang *et al.*, 2018) reported no antibacterial activity against *S. aureus*, *P. aeruginosa* and *E. coli*; however, the method used to determine the MIC and the concentrations assessed were not mentioned, preventing further comparison with our results, that to the best of our knowledge extensively study the antibacterial activity of **(6)**, also against MDR bacteria for the first time.

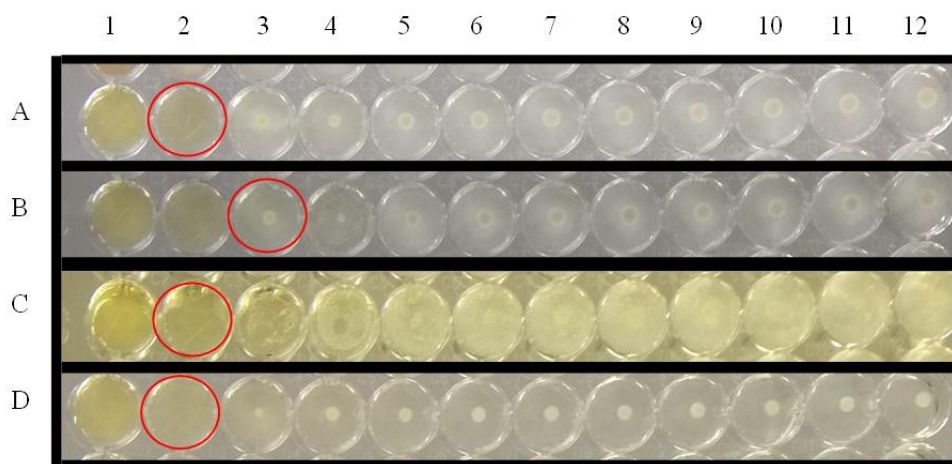


Figure 7.4 Red circles indicate the MIC values for **(6)**, numbers are referred to the different concentrations tested, from one (128 $\mu\text{g/mL}$) to 12 (0.06 $\mu\text{g/mL}$), making two-fold serial dilution. **A.** *S. aureus* ATCC 29213 (MIC 64 $\mu\text{g/mL}$). **B.** *S. aureus* Monza-FD1 (MIC 32 $\mu\text{g/mL}$). **C.** *S. aureus* Monza-PFI (MIC 64 $\mu\text{g/mL}$). **D.** *E. faecalis* ATCC 29212 (MIC 64 $\mu\text{g/mL}$). *S. pneumoniae* Monza-82 is not reported because the colour of the medium did not allow to obtain significant pictures.

Similar to **(6)**, **(4)** was active on almost all Gram-positive isolates (with the exception of *S. pneumonia* ATCC 49619), with a MIC values of 8 $\mu\text{g/mL}$, 64 $\mu\text{g/mL}$ and 128 $\mu\text{g/mL}$ on *S. pneumoniae* Monza-82, *E. faecalis* ATCC 29212 and *S. aureus* strains,

respectively. Interestingly, this molecule was previously classified as not active against the standard and methicillin-resistant *S. aureus* strains due to the low concentration assessed, 20 µg/ml (Gao *et al.*, 2012). Hence, we reported for the first time the antibacterial activity of (**4**).

The compound (**8**) was active against all *S. aureus* strains (MIC 64 µg/mL – 128 µg/mL) and *S. pneumoniae* Monza-82 (MIC 32 µg/mL). Ganihigama *et al.* (2015) reported the antibacterial activity of (**8**) against *M. tuberculosis* (MIC 25 µg/mL). Zhao *et al.* (2018) did not observe activity against *B. cereus* and *Proteus vulgaris* at the highest concentration tested (7.2 µg/mL), in this regard, highest concentrations, comparable to those used by Ganihigama *et al.* (2015) and in the present study should be assessed to clarify the antibacterial activity of (**8**).

The molecule (**9**) was firstly assessed for the antibacterial activity and showed positive results against the fluoroquinolone-resistant *S. aureus* Monza-FD1 (MIC 128 µg/mL) and macrolide-resistant *S. pneumoniae* Monza-82 (MIC 32 µg/mL).

Of note, *S. pneumoniae* Monza-82 showed a particular phenotype of sensitivity to all the molecules mentioned above, including also the compounds (**3**) and (**7**) that were active only against this bacterium with a MIC value of 64 µg/mL and 16 µg/mL, respectively. The compound (**7**) was previously tested against *Micrococcus luteus* (MIC 200 µg/mL) and the Gram-negative *Klebsiella pneumoniae* (MIC 250 µg/mL) and *P. aeruginosa* (MIC 200 µg/mL) (Basu *et al.*, 2005). However, in that previous study, the MIC values were based on the inhibition zone of the agar diffusion assays, a non-optimal method for determining the MIC since it is not possible to quantify the amount of antibiotic that spreads in the agar. A weak antibacterial activity was observed also by Liang *et al.* (2018). On the contrary, Zin *et al.* (2017) observed no antibacterial activity for (**7**). This highlights that the use of different bacterial strains can highly influence the results, therefore reference strains coming from international culture collections should always be preferred.

Overall, in our test, all the above mentioned molecules, were only active against Gram-positive bacteria, that, compared to Gram-negative are usually neglected in the

research programs aiming to find new antibiotics; however, Gram-positive are responsible for severe diseases and sepsis (Dodds, 2017).

Interestingly, the compounds (**3**, **4**, **6-9**) were active against the macrolide-resistant strain of *S. pneumoniae* Monza-82 and not against the susceptible strain *S. pneumoniae* ATCC 49619. This might reveal that the macrolide-resistant bacteria (*S. pneumoniae* Monza-82) has a reduced fitness compared to a susceptible strain, as already demonstrated for several bacteria (Dewan *et al.*, 2018; Geisinger and Isberg, 2017; Sommer *et al.*, 2017; Rolain *et al.*, 2016).

The rest of the tested compounds (Table 7.4) did not show antibacterial activity at the concentration tested, confirming previously reports for (**10**) (Tian *et al.*, 2013) and (**1**) (Zin *et al.*, 2017; Pereira *et al.*, 2016; Meng *et al.*, 2015; Gao *et al.*, 2012), with the only exception of the results obtained by Liang *et al.* (2018) that reported a weak antibacterial activity for (**1**), (IC₅₀ 23.08 µg/mL). Compound (**5**) did not show antibacterial activity in our study, on the contrary, it was previously reported active against two *S. aureus* strains (IC₅₀ 14,3 µg/mL) by Gao *et al.* 2012 and different bacterial strains (IC₅₀ 15.22 µg/mL) by Liang *et al.* (2018). These data are only apparently in contrast with our study since we did not evaluate the IC₅₀; indeed, (**5**) can partially inhibit bacterial growth but not to extinction. This consideration can also be applied to (**2**) which was inactive in our assay at the concentration tested, but shown an IC₅₀ value of 16.17 µg/mL against different bacteria (Liang *et al.*, 2018). Moreover, (**2**) was active against *M. tuberculosis* (MIC 100 µg/mL), *B. cereus* (MIC 2.02 µg/mL) and *P. vulgaris* (MIC 8.08 µg/mL), underlining that this compound might have different target that up to date we did not consider (Zhao *et al.*, 2018; Ganihigama *et al.*, 2015).

Interestingly, the compounds (**4**, **8**, **6**) showed a MIC value lower than that reported for gentamicin against *S. aureus* Monza-PFI; in addition, on the same bacterial strain, (**6**) reported also the same MIC of ciprofloxacin. Similarly, the MIC of (**4**, **6**) was lower than that of erythromycin, while the same MIC value of the antibiotic was reported by (**7**) against *S. pneumoniae* Monza-82. Therefore, in some cases, the molecules tested in this study revealed to be more efficient than the antibiotics (in vitro assays) currently used.

As concern the bactericidal activity, it was reported for the compound **(6)** against all the bacteria where a MIC was reported, whereas molecules **(8)** and **(4)** shown this activity only against the *S. pneumoniae* Monza-82 strain, as reported in Table 7.4; this activity was not observed for the others molecules at the concentrations tested. To the best of our knowledge, **(1-10)** have been firstly assessed for the bactericidal activity.

7.3.2 Structure-activity relationship

Little modifications in the structure of the molecules can determine huge variations in the biological activity. For instance, **(6)** is active against almost all bacterial strains (except *S. pneumoniae* ATCC 49619) with also bactericidal activity; its isomer **(4)** was active against the same targets but at highest concentrations and did not show bactericidal activity. Unfortunately, the use of different concentrations did not allow further comparison among the two molecules above mentioned and **(5)**, that differ only for the presence of two double bonds in position one and three.

The compounds **(1, 2, 3 and 10)**, all inactive in the antibacterial assay, present the same core indole structure. However, at which extent the core structure instead of the different functional groups characterizing each molecule can be responsible for the bioactivity of the compounds, it is hard to predict.

7.4 CONCLUSION AND FUTURE PERSPECTIVES

How can AMR be overcome? The recent campaigns to combat antimicrobial resistance are raising public awareness; the use of antibiotics should be drastically reduced, avoiding self-medication and the inappropriate prescription for humans (Dodds, 2017; Rather *et al.*, 2017). On the other side, both the preventive and the massive use of antibiotics should be avoided in livestock (Dodds, 2017; Rather *et al.*, 2017). However, it is still not clear which is the extent of the reversion of resistant bacteria in susceptible strains after the reduction of the selective pressure generated by the antibiotics (Rolain *et al.*, 2016). In light of this, new molecules able to contrast resistant bacteria should be found. In this study, the marine fungus *E. chevalieri* MUT 2316 demonstrated to be able to produce high added values molecules. More than half of the

compounds (**3**, **4**, **6-9**), were active against at least one bacterium, with (**6**) as the most promising molecules that showed also a broad bactericidal activity. Several molecules were more efficient than already known antibiotics, opening the possibility to future test aiming to assess the mechanisms behind the antibacterial activity and the selectivity of the tested compounds for a specific target.

Certainly, the work already performed, with the isolation of fungi, the purification of the molecules and the biological assays, require different expertise, that, normally the Academia is able to provide. However, future test on this molecules and in general on the high values compounds with promising antibacterial activity should be carried out by the pharmaceutical industry, since the public financing are not able to cover the costs to bring a new drug on the market.

The results showed in this chapter have been published in:

Marine Drugs. Bovio E., Garzoli L., Poli A., Luganini A., Villa P., Musumeci R., McCormack G. P., Cocuzza C. E., Gribaudo G., Mehiri M., Varese G. C. (2019). Marine fungi from the sponge *Grantia compressa*: biodiversity, chemodiversity and biotechnological potential.

8. The antiviral activity of the molecules produced by *E. chevalieri* MUT 2316

8.1 INTRODUCTION

8.1.1 Viruses and antivirals

Viruses are among the major causes of human morbidity and mortality (Szczubiałka *et al.*, 2016; Martinez *et al.*, 2015). For instance, hepatitis B virus (HBV), hepatitis C virus (HCV) and HIV are approximately responsible for about 3.1 million deaths per year (Martinez *et al.*, 2015). Without considering a series of emerging viruses (including dengue and ebola viruses) with high epidemic potential (Martinez *et al.*, 2015). Viruses have also dramatic socio-economic costs; for instance, the impact of zika virus in Latin America and in the Caribbean area in two years has been estimated between 7 and 18 billion dollars (Zitzmann and Kaderali, 2018). The symptomatic dengue with about 60 million cases estimated in 2013 reached 9 billion dollars (Zitzmann and Kaderali, 2018). Unfortunately, these numbers will rise due to several factors, including climate changes and the increase of people traveling around the world (Zitzmann and Kaderali, 2018; Wang *et al.*, 2017a).

Viruses can be defined as intracellular parasites and their life cycles are completely dependent on cellular factors and pathways (Zitzmann and Kaderali, 2018). They can be classified according to their genetic material (DNA or RNA) either double or single-stranded and the orientation of the encoded genes, that can be packaged together in a virion, or be represented by separate virus particles (Simmonds and Aiewsakun, 2018). The genome can be of different size, coding for a minimum of two genes to over 2,500 genes, reflecting the complexity of the replication strategies, the interaction with the host cells and the viral structure (Simmonds and Aiewsakun, 2018). This last mentioned factor is another element useful for the classification of the viruses that have the most diverse nucleocapsids, from icosahedral to rectangular and filamentous (Simmonds and Aiewsakun, 2018).

Nowadays, there are vaccines protecting from several viruses and, analysing the period between 1987 and 2017, every year 2.8 new antiviral are approved by the FDA,

with almost a linear progression (Chaudhuri *et al.*, 2017). The main target, with about half of the total drugs approved in the last 30 years is the HIV, followed by HCV, HBV, cytomegalovirus and herpes simplex virus, as shown in Figure 8.1 (Chaudhuri *et al.*, 2017).

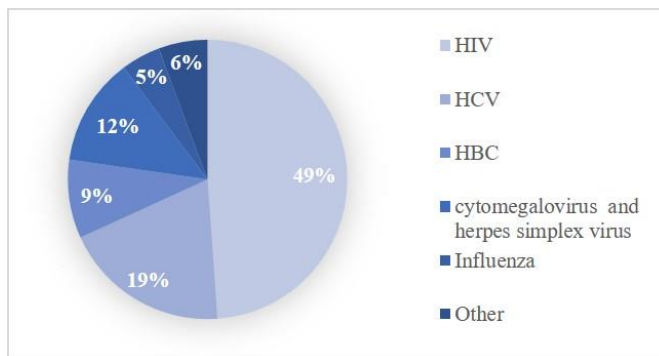


Figure 8.1 Antiviral approved by the FDA in the period 1987-2017 (Chaudhuri *et al.*, 2017, modified).

The antivirals can be divided according to their way of action; directly acting antivirals (DAAs), affect the viral genomes or more commonly the proteins i.e. polymerase, protease and integrase. Host-acting antivirals (HAAs) target the host by modification of factors indirectly affecting the virus life cycle or the immune response components, i.e. antibodies, interferons and vaccines (Chaudhuri *et al.*, 2017; Martinez *et al.*, 2015). The antivirals of the last 30 years mainly belong to the DAAs, counting for 85% of the approved drugs, while the remaining are HAAs (Chaudhuri *et al.*, 2017). Another classification is based on the spectrum of action of the antivirals, with compounds able to target only a specific virus with no or little activity against other viruses, and broad-spectrum antivirals (Martinez *et al.*, 2015). The last mentioned can cover several viruses reducing the possibility of resistances also to DAAs and they could treat acute viral infections (Martinez *et al.*, 2015). Due to their way of action, targeting hosts factors essential on one hand for the viral replication and, on the other hand for the cells, may determine high toxicity (Martinez *et al.*, 2015).

Overall, new drugs are necessary to contrast both well-established viruses, becoming resistant to commercial medicines, and emerging virus. In this contest, two

viruses have been chosen as model, the Influenza A virus (IAV), and the Herpes Simplex virus 1 (HSV-1)

8.1.2 Influenza A virus (IAV)

Influenza is a respiratory infection caused in humans by IAV and Influenza B virus (IBV) (Ferhadian *et al.*, 2018). It is among the viruses with the highest incidence, affecting 10% and 30% of adults and children worldwide, respectively, with about 500,000 deaths (Krammer *et al.*, 2018; Zitzmann and Kaderali, 2018; Martinez *et al.*, 2015).

The influenza virus belongs to the family of orthomyxoviridae and the genome consists of 8 negative-sense single-strand viral RNA (for IAV and IBV), coding for at least 11 proteins (Ferhadian *et al.*, 2018; Krammer *et al.*, 2018; Patil *et al.*, 2018; Zitzmann and Kaderali, 2018), whose replication cycle is reported in Figure 8.2.

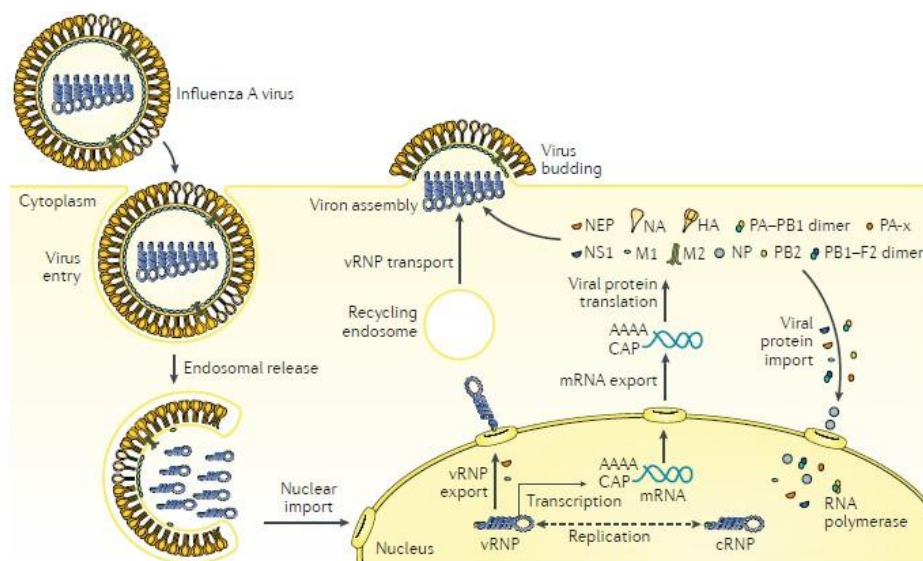


Figure 8.2 Influenza viral cycle. The influenza virus enters into the cell thanks to endosomal uptake; once inside it releases the genetic material as viral ribonucleoproteins (vRNAs) that enter in the nucleus for the transcription of messenger RNA (mRNA) and the replication thanks to the intermediation of a positive-sense complementary ribonucleoprotein (cRNP). In the cytoplasm, the mRNA is translated into the viral proteins that will constitute new virions once assembled together with the vRNPs (Krammer *et al.*, 2018).

Influenza is characterised by the exponential growth of the viral load from the infection to up to two days; afterward, the viral load decrease and at 6-8 days post infection completely disappear (Zitzmann and Kaderali, 2018). Today, to contrast this virus there are both vaccines and drugs. Influenza vaccines have as main drawback a limited time coverage against the virus. They are re-formulated every year according to WHO recommendations (Krammer *et al.*, 2018).

As regards the antivirals, they are fundamental to prevent infections before the vaccines are available and to treat patients that have been already exposed to the virus (Krammer *et al.*, 2018). Of the two classes of drugs approved, adamantanes (amantadine and rimantadine) that act as an M2 ion channel blocker (required for viral entry and exit), are not anymore recommended due to the high resistance developed by IAV (Krammer *et al.*, 2018; Zitzmann and Kaderali, 2018). The other class is represented by neuraminidase inhibitors and includes three drugs (Oseltamivir, Peramivir and Zanamivir) worldwide approved. These compounds avoid the release of viral particles from cells, preventing the spread of the virus (Luganini *et al.*, 2018).

Overall, despite the availability of vaccines and antiviral agents, influenza continues to be the most important cause of viral respiratory disease associated with human hospitalizations and deaths (Krammer *et al.*, 2018). Exactly one century after the Influenza pandemic in 1918 we are still not completely able to prevent this viral infection (Krammer *et al.*, 2018). Certainly, vaccines more durable and with higher protection are necessities, as well as new and more efficient drugs due to the increasing resistance of the compounds currently on the market (Ferhadian *et al.*, 2018; Krammer *et al.*, 2018).

8.1.3 Herpes Simplex virus 1 (HSV-1)

Herpes simplex belongs to the family of Herpesviridae. It is a double-stranded DNA virus encapsulated in a lipid and glycoprotein envelope. Herpes simplex can be further divided into type I (HSV-1) and type II (HSV-2), identifiable by specific glycoproteins I and II, respectively (Lee and Nair, 2017). Recently it has been estimated that from 60% to 80% of individuals worldwide are seropositive for HSV-1 (Benassi-Zanqueta *et al.*, 2018).

The DNA of HSV-1 consists of 152 kilobase pair that codes for over 80 proteins; the lytic cycle is summarised in Figure 8.3.

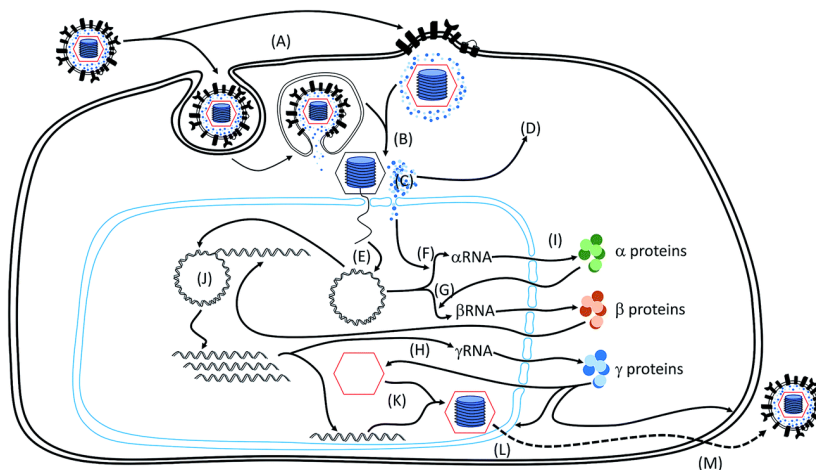


Figure 8.3 HSV-1 cycle. The glycoproteins present on the surface of HSV-1 are fundamental for the virus-receptor interaction; the virus can enter into the cell by membrane fusion or endocytosis. The genetic material is then transferred in the nucleus. Three genes groups mediate the transcription of HSV-1 and the expression of the proteins: α coding for proteins mainly responsible of the first steps related with the infection, β with proteins involved in the viral replication and, γ responsible for the synthesis of structural proteins. These three groups are subsequently activated.

For the replication of the HSV-1 DNA, 7 viral proteins are involved, including the DNA polymerase; first, the replication proceeds *via* the theta form to change in the rolling cycle replication that after the further cut, represent the final genome ready for encapsidation (Szczubiałka *et al.*, 2016).

The HSV-1 has humans as the only host; the way of transmission of the virus is by direct contact with the infected person, with his lesions or his blood (Szczubiałka *et al.*, 2016). Up to date, the infection is incurable, the HSV-1 remain in a latent state in the neurons (Szczubiałka *et al.*, 2016). After the exposure to several factors (i.e. ultraviolet light, immunosuppression or emotional stress), the virus starts moving to the skin to replicate and the first symptoms become visible (Szczubiałka *et al.*, 2016). In addition to the skin damage, there are several other side-effects still understudied, like

neurological disorders, encephalitis, blindness, and probably some relation with neurodegenerative diseases, including the Alzheimer's disease (Szczubiałka *et al.*, 2016; Terlizzi *et al.*, 2016).

The existing antiviral mainly targets the viral DNA polymerase (i.e. Acyclovir, Famcyclovir and Valacyclovir) (de Mello *et al.*, 2016; Terlizzi *et al.*, 2016), followed by molecules contrasting the absorption, entry and intracellular transport (i.e. Docosanol). At the moment, there are no drugs on the market acting on the HSV-1 during its latency, blocking the reactivation (de Mello *et al.*, 2016; Terlizzi *et al.*, 2016). The most desired drugs should be able to block virus attachment or entry (Terlizzi *et al.*, 2016). The progress in the development of a new drug is limited, while the medicines already available on the market are generating more and more resistant HSV-1 strains, due to their limited action (de Mello *et al.*, 2016; Szczubiałka *et al.*, 2016). Likewise, for the vaccines, they should protect from both HSV-1 and HSV-2, due to changing epidemiology; indeed, there are increasing reports of cases of genital herpes caused by HSV-1 and oral infections due to HSV-2 (de Mello *et al.*, 2016; Terlizzi *et al.*, 2016).

Therefore, considering the need for new antiviral and the incredible biotechnological potential of the molecules produced by marine fungi, the 10 compounds isolated from *E. chevalieri* MUT 2316 were tested against IAV and HSV-1.

8.2 MATERIAL AND METHODS

In order to assess the antiviral activity of the molecules produced by *E. chevalieri* MUT 2316, the IAV and the HSV-1 were selected as models, according to the expertise of the Laboratory of Microbiology and Virology of the DBIOS (University of Turin, Italy), with which the antivirals test were performed.

8.2.1 Cell culture conditions and viruses titre determination

The African green monkey kidney cells (VERO, ATCC CCL-81), permissive for the Acyclovir-sensitive HSV-1 and the Madin Darby Canine Kidney cells (MDCK, ATCC CRL-2936™) permissive for the IAV (A/Puerto Rico/8/34) were used in this test. VERO cells were propagated in Dulbecco's Modified Eagle Medium – DMEM

(Euroclone) supplemented with 10% fetal bovine serum – FBS (Euroclone), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin, and 100 µg/mL streptomycin sulphate. The HSV-1, a clinical isolate kindly provided by V. Ghisetti, Amedeo di Savoia Hospital (Turin, Italy) was propagated and titrated by standard plaque assay on VERO cells (Luganini *et al.*, 2011). The IAV, provided by Arianna Loregian (University of Padua, Italy) was propagated and titrated by plaque assay on MDCK cells. Briefly, the infections with IAV were performed in the presence of 2 µg/mL of TPCK-treated trypsin from bovine pancreas (Sigma-Aldrich) and 0.14% of Bovine Serum Albumin (Sigma-Aldrich) in serum-free complete medium (Luganini *et al.*, 2018).

8.2.2 Cytotoxic assays

The VERO and MDCK cells obtained from the culture above described were re-suspended, counted and seeded (in the same medium), in 96-multiwell at the final concentration of 6×10^4 cells per well (final volume 100 µL). Plates were incubated at 37 °C. The next day, cultures were exposed to increasing concentrations of the compounds (diluted from the same stock 1 in DMSO of the previous chapter), at 12.5 µg/mL, 25.0 µg/mL and 50.0 µg/mL. As for (7), to avoid DMSO toxicity, lower concentrations (6.25 µg/mL, 12.5 µg/mL, 25.0 µg/mL) were used. The plates were incubated for 48 h at 37 °C, and then 100 µl of CellTiter-Glo reagent (Promega) was added to lyse the cells and to determine the cell viability following the instruction of the manufacturer. The test was performed in duplicate.

The concentration of the molecules that determined at least 70% of cell viability was assessed for the antiviral activity.

8.2.3 Antiviral assay

For plaque reduction assays (PRA) with IAV and HSV-1 viruses, MDCK and VERO cells were seeded in 24-multiwell plates at a density of 3×10^5 or 6×10^4 cells per well, respectively. The next day, cultures were treated with the selected molecules at different concentrations 1 h prior to infection, and then infected with 35 Plaque Forming Unit (PFU)/well of either HSV-1 or IAV in the presence of the compounds.

Following virus adsorption (1 h at 37 °C for IAV or 2 h at 37 °C for HSV-1), cultures were maintained in medium containing the corresponding compounds supplemented with 0.14% BSA, 2 µg/mL trypsin, and 0.7% Avicel for IAV, or with 3% FBS, and 0.9% Avicel for HSV-1. All these steps are summarised in Figure 8.4.

1) MDCK and VERO cells were seeded in 24-multiwell plates at the desired density



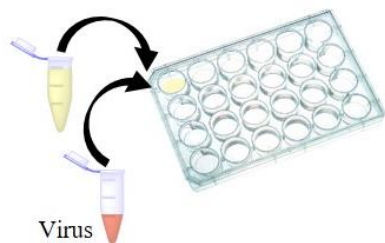
2) Compounds were diluted at the desired concentration



3) The next day, cultures were treated with the selected molecules at different concentrations, 1 h prior to infection with the virus



4) The medium was removed prior to add the molecules at the desired concentration with the virus



5) Following virus adsorption, cultures were maintained in medium containing the corresponding compounds + BSA, trypsin, and Avicel for IAV; + FBS and Avicel for HSV-1.



Figure 8.4 Schema of the treatment of the cells with the molecules for the PRA, before, during and after the infection.

All compounds concentrations were tested in duplicate in two independent experiments. Control wells with mock-infected cells and untreated virus-infected cells were included in each plate. At 48 h post-infection cell monolayers were fixed with 4% formaldehyde 1 h at room temperature, stained with an aqueous solution of 1% crystal violet for 40 min, and viral plaques were microscopically counted. The mean plaque

counts for each compound was expressed as percentage of inhibition of the viral replication compared to the positive control (PC). The analysis of variance (ANOVA) was performed to compare the antiviral activity of the compounds with the control ($p < 0.05$) for each tested virus, using IBM SPSS Statistics software.

8.3 RESULTS AND DISCUSSION

8.3.1 Cytotoxic activity of *E. chevalieri* MUT 2316 compounds

Prior to the assessment of the antiviral activity by PRA, cell viability assays were performed, with three different concentration of the same compound, in order to exclude non-specific cell toxicity against MDCK and VERO cells. The concentration of the molecules that guaranteed at least 70% cell viability after 48 h of treatment was considered not toxic (Table 8.1) and was tested against IAV and HSV-1.

Table 8.1 Concentration of the molecules (1–10) that guaranteed at least 70% of viability of MDCK and VERO cells.

Molecules	Concentration that determines at least 70% of MDCK cells viability (µg/mL)	Concentration that determines at least 70% of VERO cells viability (µg/mL)
Echinulin (1)	50.0	50.0
Neoechinulin A (2)	25.0	50.0
Neoechinulin D (3)	50.0	50.0
Dihydroauroglaucin (4)	25.0	25.0
Flavoglaucin (5)	-	-
Isodihydroauroglaucin (6)	-	12.5
Physcion (7)	25.0	25.0
Asperflavin (8)	12.5	50.0
Cinnalutein (9)	12.5	12.5
Cyclo-L-Trp- L-Ala (10)	25.0	50.0

Unfortunately, due to the high toxicity, (5) was not tested in the antiviral assays against both IAV and HSV-1; for the same reason (10) was not assessed against HSV-1. Overall, as shown in Table 8.1, in most cases the VERO cells demonstrated to be less

sensitive to the tested compounds, for instance, **(2, 6)** and **(8)** were not toxic at 2 and four times the concentration reported against the MDCK cells, respectively.

8.3.2 *E. chevalieri* MUT 2316-derived compounds show antiviral activity against HSV-1 and IAV

The antiviral assays revealed that all the compounds, although differently, were significantly able to inhibit the viral replication of IAV and HSV-1. Figure 8.5A report a viral plaque of HSV-1 seen at the microscope; while Figure 8.5B show the plaques generated by the IAV that are visible without magnification and can be compared to a control (Figure 8.5C) without plaques.

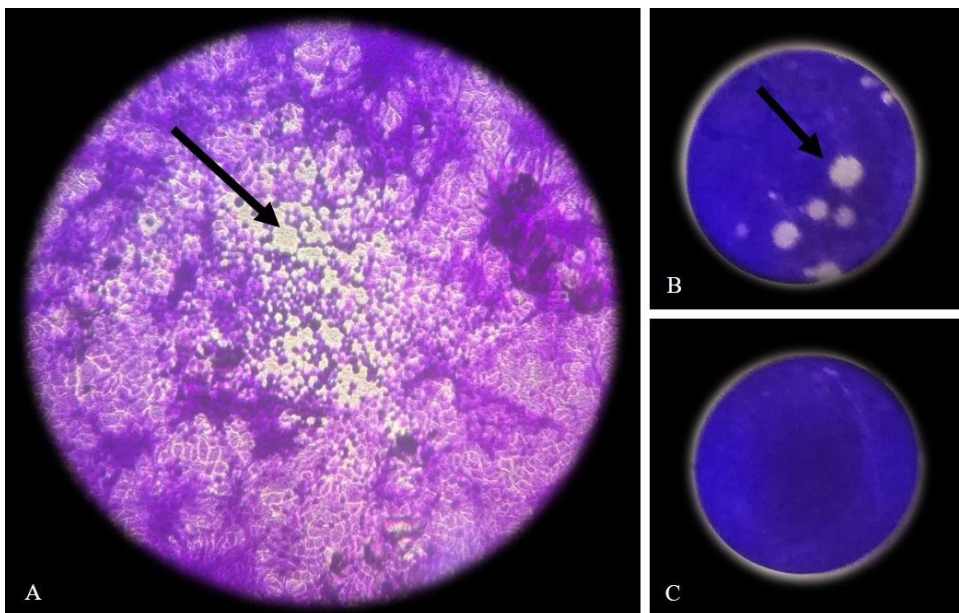


Figure 8.5 **A.** the arrow indicates the viral plaque generated by HSV-1, seen at the microscope; **B.** the arrow indicates the plaques produced by IAV, visible without magnification in the multiwell plates; **C.** control without plaques.

Among the compounds tested, Dihydroauroglucin **(4)** and Physcion **(7)** at 25 $\mu\text{g/mL}$ inhibited the replication of IAV by 100% and 82%, respectively (Figure 8.6A). Neoechinulin D **(3)** exhibited an inhibition rate of 66% at 50 $\mu\text{g/mL}$. The inhibition rate

of the remaining compounds was lower than 50% (Figure 8.6A), although significant if compared to the control.

Interestingly, 5 out of 8 compounds were here first reported for the antiviral activity against IAV. Echinulin (**1**), Neoechinulin A (**2**) and Cyclo-L-Trp- L-Ala (**10**) have already been tested against another strain of Influenza (A/WSN/33) by Chen and collaborators (2015). (**1**) showed an inhibition rate of 13% at 46 $\mu\text{g/mL}$, while in our study inhibited 47% of IAV at 50 $\mu\text{g/mL}$. The different results can be due to the way on which Echinulin works, probably not allowing the attachment of the virus to the cells; contrary to what performed by Chen *et al.*, (2015), we treated the cells with the compounds prior to the viral infection, in order to cover all the viral cycle. Similarly, Cyclo-L-Trp- L-Ala (**10**) might act in the same way, it inhibited the IAV of 36% (at 25 $\mu\text{g/mL}$), while gave almost no inhibition (2% at 26 $\mu\text{g/mL}$) in the work of Chem and collaborators (2015).

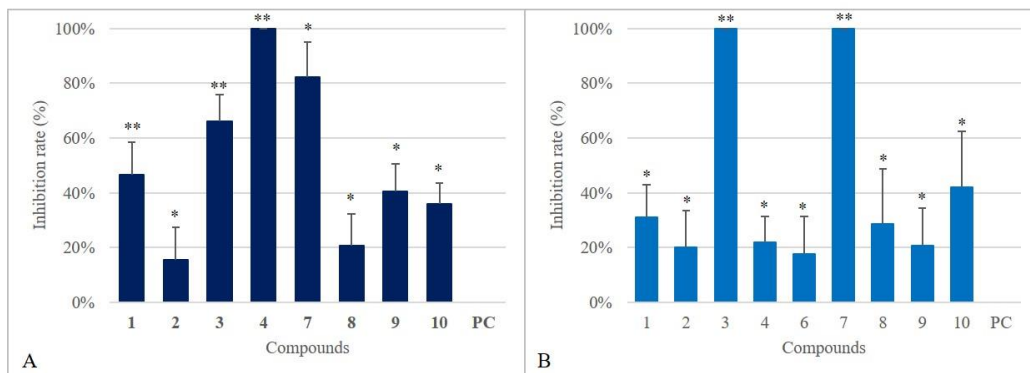


Figure 8.6 Effect of the molecules derived from *E. chevalieri* on **A.** IAV and **B.** HSV-1 replication. The mean plaque counts for each molecule was expressed as percent of inhibition of viral replication compared to the positive control (PC). The data shown are the means \pm standard deviations from two independent experiments performed in duplicate. * $p < 0.05$, ** $p < 0.01$.

Neoechinulin A (**2**) in the paper of Chen *et al.* (2015) inhibited 36% of Influenza virus at 32 $\mu\text{g/mL}$, while we reported 16% of inhibition at 25 $\mu\text{g/mL}$. In this case, the different concentrations could have played a role in determining different results.

Regarding HSV-1, (3) and (7) completely inhibited HSV-1 at 50 µg/mL and 25 µg/mL, respectively (Figure 8.6B). Interestingly, the inhibition of the replication of HSV-1 was here reported for the first time for 8 out of 9 molecules. A previous work of Liang *et al.* (2018) evaluated the antiviral activity of (5) and (6) against HSV-1 reporting as IC₅₀ values 3.90 µg/mL and 2.91 µg/mL, respectively. The same authors underlined that the molecules showed moderate toxicity against the Vero cells. In our case, (5) was not assessed due to the high cytotoxicity, while (6) was tested at the lowest concentration (12.5 µg/mL) that guaranteed at least 70% of cells viability, and was able to inhibit 17.74% of the replication of the HSV-1 virus. The different results obtained might be due to the strains of the virus, in our case a clinical isolate, while Liang and collaborators (2018) did not mention the origin of the HSV-1 strain.

Overall, these results suggest a significant antiviral activity of different *E. chevalieri* MUT 2316 derived bioactive molecules, with (7) probably representing a broad-spectrum antiviral, highly efficient with both virus and (4) and (3), mainly active against IAV and HSV-1, respectively.

8.3.3 Structure-activity relationship

The influence of the structure of the molecule in the bioactivity is particularly evident between the two isomers (4, 6) and also compared to the molecule (5) that differ for the presence of two double bonds in position one and three, as already mentioned in the previous chapter. In detail, these variations in the molecular structure are sufficient to determine high cytotoxicity in (5) and (6), preventing their use in the antiviral activity test against IAV, whereas (4) completely inhibited the IAV replication, without being cytotoxic at the tested concentration. The compounds (1, 2, 3 and 10) although they present the same core indole structure, the highly different functional groups guaranteed unique biological properties.

8.4 CONCLUSION AND FUTURE PERSPECTIVES

The antiviral activity, usually neglected in the evaluation of the biological properties of the molecules revealed promising results. The molecules produced by *E.*

chevalieri MUT 2316 have been tested for the first time against HSV-1. Of note, its replication was completely inhibited by (3, 7).

Five molecules were firstly assessed against IAV; as for the remaining three, thanks to a procedure able to cover all the phase of the lytic cycle of IAV, we highlighted the uncovered potential of two of them compared to a previous study (Chen *et al.*, 2015). Interestingly, (4) was selectively able to completely inhibit the replication of IAV; (7) besides the total inhibition of HSV-1, it demonstrated an inhibition of about 80% against IAV, being probably a broad spectrum antiviral, active with both representatives of a DNA virus (HSV-1) and a negative-sense single-strand RNA virus (IAV).

In the next months, the compounds that completely inhibited the replication of the virus will be further studied using different concentrations in order to define a dose-response against the selected virus. Moreover, their mechanisms of action will be investigated, probably revealing a specific target for (3) and (4), which selectively inhibited a DNA and RNA virus, respectively. While (7) might target a mechanism common to both IAV and HSV-1.

The comparison between the antibacterial and the antiviral activity was not deliberately done, because it would be misleading, due to the different techniques and concentrations used. To this concern, the data have been presented separately and only further studies may confirm the specificity and the mechanisms of action of the 10 molecules produced by *E. chevalieri* MUT 2316.

The results showed in this chapter have been published in:

Marine Drugs. Bovio E., Garzoli L., Poli A., Luganini A., Villa P., Musumeci R., McCormack G. P., Cocuzza C. E., Gribaudo G., Mehiri M., Varese G. C. (2019). Marine fungi from the sponge *Grantia compressa*: biodiversity, chemodiversity and biotechnological potential.

9. The antifouling activity of the molecules produced by *E. chevalieri* MUT 2316

9.1 INTRODUCTION

9.1.1 The biofouling: definition and impact

The marine biological fouling, usually abbreviated as marine biofouling, is defined as the accumulation of microorganisms, algae, and aquatic animals on surfaces biotic and abiotic, including made by man and immersed in seawater (Amara *et al.*, 2018).

The biofouling can seriously affect the worldwide economy and environmental integrity. For instance, its presence on ship's hulls can increase the fuel consumption up to 40%, mainly due to the water resistance and the weight gain of the boats (Amara *et al.*, 2018). Moreover, the biofouling increases boat propulsion up 70% (Trepas *et al.*, 2014a) with a rise of carbon dioxide and sulphur dioxide emissions estimated to be 384 and 3.6 million tonnes per year, respectively (Martins *et al.*, 2018). The transport delays, the hull repairs and the biocorrosion would cost an additional 150 billion dollars per year (Hellio *et al.*, 2015). While the impact of invasive species that might be transported by ships, threatening indigenous aquatic life forms is invaluable (Martins *et al.*, 2018).

Besides the ships, biofouling can also block the aquacultures nets reducing the water, oxygen and nutrients exchange, and increasing the risk of diseases in fish stock (Amara *et al.*, 2018). Biofouling can also negatively affect the materials properties, weight, shape and efficiency of several marine plants, including those for renewable energy (Trepas *et al.*, 2014 b).

9.1.2 Biofouling formation

The biofouling is the assemblage of several life forms, made up of thousands of marine organisms that follow an ecological succession, defined as the process of change in the species structure of an ecological community over time. Amara and collaborators (2018) summarised in four steps the biofouling formation on a surface (Figure 9.1),

represented by both the “microfouling” in the first three stages and as for last, by the “macrofouling”:

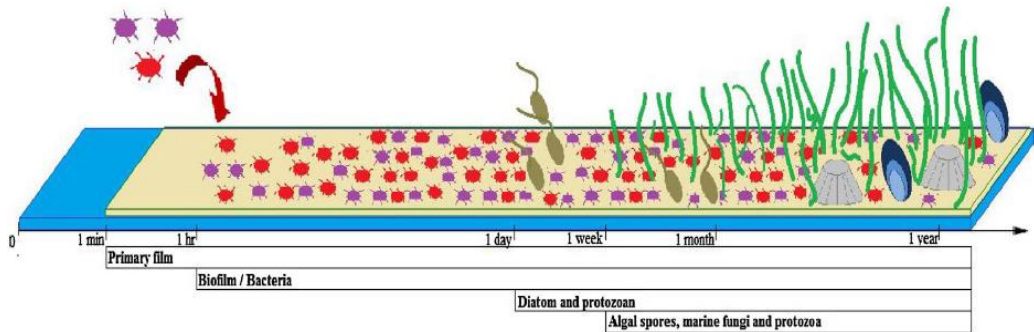


Figure 9.1 Fouling formation on a surface over time (Amara *et al.*, 2018).

a) Primary film formation

Few minutes after the immersion of an object into the water, the biofouling starts, the organic molecules already present in the environment cover the object surface. The outcome is a modification of the physicochemical properties of the surface that promote the bacterial adhesion, providing also a higher concentration of nutrients than seawater.

b) Biofilm formation

The formation of the biofilm is a process taking place in several sub stages. The first colonisers, bacteria, weakly interact with the substrate, adhering reversibly. Along the time, bacteria produce extracellular polymeric substances (EPS) that allow them to colonise irreversibly the surface, also creating irregularity that helps to trap more particles and organisms, including algae.

c) Eukaryotes colonisation

After several days, in some case weeks (from the immersion of an object in seawater), the eukaryotes, mainly represented by diatoms and protozoan, start to stick on the surface thanks to the production of polysaccharides or protein glues.

d) Settlement of invertebrate larvae and algal spores

The last stage of the biofouling formation involved the colonisation of marine invertebrates (i.e. mussels, barnacles, ascidians) and the growth of

macroalgae. In addition, the irregular surface created by the organisms further helps to trap more nutrients and organisms, including algal spores, marine fungi and protozoa.

9.1.3 Antifouling methods

The biofouling has been a problem since man started to sail the seas. The first attempt to control the biofouling encrustation and erosion of wooden ships are credited to Phoenicians and Carthaginians that, probably using pitch and copper were able to prevent the encrustation and erosion of micro and macroorganisms to their boats (Amara *et al.*, 2018; Martins *et al.*, 2018). Today, several methods have been applied as antifouling and several others are understudy; they can be mainly divided into three categories.

Physical methods

The physical methods mainly involve the surface modification of the objects immersed in the water. Up to date, most of the researches focused on microtextured surfaces. However, the main limit is that a specific scale of microtexture can strongly inhibit the settlement of restricted groups of organisms, based on the dimension of the texture itself (Trepas *et al.*, 2014b). The development of multiscale texture, with layers made by different motifs, can amplify their range of action against several fouling organisms (Trepas *et al.*, 2014b). In the attempt to find new solutions and new motifs to contrast the biofouling, researchers were inspired by the capability of several marine organisms to keep their body free from biofouling, i.e. sharks and species without physical defences (molluscs, sponges, algae, seaweeds) (Chapman *et al.*, 2014). This research field based on the development of natural inspired surfaces is known as “biomimicry” (Chapman *et al.*, 2014). Although, one of the main concern about these surfaces is their durability along the time; indeed, a tiny biofilm may compromise the micro-texture and shade the original surface, allowing the biofilm to grow (Trepas *et al.*, 2014b). In this regard, an integrated approach based on “bioinspired” microtextures coupled with chemicals, may represent a solution (Chapman *et al.*, 2014). For instance, 40% of the biofilm formation was repressed coupling algae inspired materials with

brominated furanone compounds, which act on the quorum sensing of microorganisms (Chapman *et al.*, 2014). Similarly, a bioinspired shark-skin-surface treated with TiO₂ can reduce the attachment of *E. coli* and *S. aureus* over 95% and 80%, respectively, compared to a smooth surface (Dundar Arisoy *et al.*, 2018). Indeed, TiO₂ absorbing UV light generates reactive hydroxyl radicals capable to damage the membrane of bacteria causing their death (Dundar Arisoy *et al.*, 2018). However, these results were obtained after one hour of exposure to UV light that on one hand underline the fast colonisation rate of bacteria, on the other hand, point out the necessity of further studies along the time.

Chemical methods

The antifouling paints incorporating biocides are the chemical answer to the fouling issue; the biocides are constantly released in the environment to guarantee a threshold concentration of the compounds in the water that inhibits the development of the biofouling (Amara *et al.*, 2018).

The most powerful antifouling ever existed was composed by organotin compounds, substances with at least one tin-carbon bond (Amara *et al.*, 2018). Among this family of molecules, tributyltin (TBT), and its related agents have been the most largely employed antifouling; they are fungicides active at an extremely low concentration (Amara *et al.*, 2018). However, TBT due to its lyphophyl nature has a side effect: the capability to penetrate the biological membranes, with outcome on the growth, development, survival and reproduction of target and non-target organisms, from bacteria to mammals (Amara *et al.*, 2018; Martins *et al.*, 2018; Satheesh *et al.*, 2016). Therefore, since the early 1990s TBT have been limited, and definitively banned in 2008, by the International Maritime Organisation (Amara *et al.*, 2018; Le Norcy *et al.*, 2017; Satheesh *et al.*, 2016).

Up to date, the main alternative to TBT, are paints based on copper compounds and organic molecules named booster biocides (Amara *et al.*, 2018; Le Norcy *et al.*, 2017). However, these formulations are not environmental satisfactory; for instance, a copper concentration that exceeds three ppb affects various life stages of marine organisms (Le Norcy *et al.*, 2017). As for other commercial formulations, the Sea-Nine,

a fungicide, is bioaccumulated in non-target benthic communities, with effects on all the web chain (Le Norcy *et al.*, 2017). The Diuron and the Irgarol 1051 are pesticides and herbicides that negatively affect the marine wildlife and the environment, besides their function as antifouling (Le Norcy *et al.*, 2017). As consequence, due to the side effects of booster biocides, several of them have been completely banned by different regulations, including the EU Biocidal Product Regulation (EU 528/2012).

The latest novelty in terms of antifouling concerns the foul-release polymeric nanocomposite coatings that can avoid the attachment of fouling species by reducing their adhesion (Selim *et al.*, 2017). They are represented by hydrophilic polymers i.e. polyethylene glycol, hydrogel, zwitterionic and different silicon and titanium oxides (Selim *et al.*, 2017). These nanocomposite coatings have great potential, however, their efficiency, long terms impact and cost must be improved (Selim *et al.*, 2017).

Biological methods

Marine natural products can represent an environmentally friendly alternative to biocides and, an economically advantageous solution to foul-release polymers (Trepos *et al.*, 2014b). Natural products can have reduced or broad spectrum targets: compounds displaying activity against all the set of tested organisms might be general biocides, while those active against a limited group of organisms deserve more attention (Trepos *et al.*, 2014a). This is because the compounds acting in a selective way, may have a reduced impact on non-target organisms, but require several tests to explore their potential (Trepos *et al.*, 2014a, b).

The general mode of action of natural products as antifouling can be summarised as follow:

- a) Protein expression regulators: inhibit the settlement of fouling organisms by altering their protein expression. For instance, meleagrín, an alkaloid isolated from *Penicillium meleagrínium* inhibit the settlement of *Balanus amphitrite* (a cyprid model organism for antifouling studies) determining an up-regulation of a protein with consequent endocrine disruption and prevention of the larval molting cycle (Qian *et al.*, 2013).

b) Blockers of neurotransmission: affect signals transduction during settlement stopping the attachment and metamorphosis of fouling organisms. To this regard, the oleamide produced by the marine mussels *Mytilus edulis* inhibited the adhesion and germination of algal spores (Kang *et al.*, 2016).

c) Oxidative Stress Inducers: avoid the attachment of fouling organisms to the surface, through oxidative damage. For instance, the crude extract of the red alga *Chondrus crispus*, contains hexose oxidase that catalyzes the generation of hydrogen peroxide (Chen and Qian, 2017).

d) Surface Modifiers and biofilm inhibitors: block the attachment site of the bacteria and algae avoiding the formation of biofilm. This can be obtained by physical modification of the substrate or by incorporating natural product in the paints. For instance, the zosteric acid produced by the seagrass *Zostera marina* block the surface attachment sites of bacteria (Qian *et al.*, 2013).

e) Adhesive Production/Release Inhibitors: inhibit the production/release of adhesive compounds in the fouling species. This effect has been experimented also using “live-paints”, immobilising marine bacteria known for the production of antifouling compounds (Chen and Qian, 2017).

f) Toxic killing: natural product can belong to this category represented by biocides. For instance, the 3,30-diindolylmethane, isolated from the bacterium *Pseudovibrio denitrificans* demonstrated to contrast the macrofouling, with an efficiency comparable to the commercial formulation Sea-Nine. However, the stability of this natural product in the environment and its activity against non-target organisms made of it a common biocide and not a candidate to contrast fouling species (Chen and Qian, 2017).

9.1.4 Why sponges and their associated microorganisms as candidates for the production of new antifouling?

The observation that many sponges are free from overgrowing organisms has stimulated the search for naturally antifouling from marine sponges (Bayer *et al.*, 2011). Indeed, these sessile organisms lack a specialised immune system and only developed

physical and chemical defence can protect them from fouling species (Hanssen *et al.*, 2014). Among the most successful antifouling isolated from marine sponges there are bromotyrosine-derived compounds (Hanssen *et al.*, 2014); for instance, barretin was isolated from the sponge *Geodia barrette* and was able to inhibit fouling adhesion in field studies (Hedner *et al.*, 2008). While synthetic derivatives of bastadin produced by the sponge *Ianthella basta* inhibit micro and macrofouling adhesion (Le Norcy *et al.*, 2017).

However, as mentioned in chapter 1, there are facts suggesting that microorganisms inhabiting sponges could be the real producer of molecules previously ascribable to their host (Hanssen *et al.*, 2014). The use of the microbial metabolites as antifouling present several advantages, avoiding the supply problem of several animals from the environment, when the cultivation in aquaria or the chemical synthesis is not feasible (Wang *et al.*, 2017b; Hanssen *et al.*, 2014). The first paper describing the production of antifouling compounds from microorganisms was published in 1995 (Wang *et al.*, 2017b); today several microorganisms have been described as a promising source for the formulation of new antifouling paints. Among fungi, *Aspergillus* spp. (anamorph of *Eurotium* spp.) are the most fruitful producers of antifouling compounds (Wang *et al.*, 2017b).

In light of the above mentioned consideration on the urgent need to find new and environmentally friendly antifouling, and the potential of fungi associated with sponges, the metabolites obtained from *E. chevalieri* MUT 2316 were tested against representative bacterial and algal fouling species and an enzymatic assay was performed to evaluate the inhibition of the byssus formation.

9.2 MATERIAL AND METHODS

In order to assess the antifouling activity of the molecules produced by *E. chevalieri* MUT 2316, 5 marine bacterial species and 5 marine algal species were selected as model, according also to the expertise of the Laboratoire des sciences de l'environnement marin of the University of Western Brittany (Brest, France), with which the antifouling tests were performed.

9.2.1 Marine Antibacterial Assays

The molecules were tested against 5 bacterial species (Table 9.1) representative of fouling species in the estuarine and marine environment (Chambers *et al.*, 2011) and obtained from the ATCC. Based on the availability of bacterial strains in the laboratory, only Gram-negative bacteria were tested.

Table 9.1 Marine bacteria used for the antifouling test.

Species	ATCC code
<i>Halomonas aquamarina</i>	14400
<i>Polaribacter irgensii</i>	700398
<i>Roseobacter litoralis</i>	49566
<i>Vibrio aestuarianus</i>	35048
<i>Pseudoalteromonas citrea</i>	29720

Bacteria were pre-grown in sterile Marine Bacterial Medium - MBM (Peptone 5 g - Sigma-Aldrich, Saint Louis, USA - up to 1 L seawater) at room temperature.

The compounds isolated from *E. chevalieri* MUT 2316 were solubilised in DMSO at the same concentration mentioned in chapter 7 for the antibacterial and antiviral assays (Table 9.2, Stock 1): 10 mg/mL for (**1, 2, 4, 5, 9, 10**) and 1 mg/mL for (**7**). The scarce volume of the remaining molecules (**3, 6, 8**) did not allow further tests.

Table 9.2 Molecules isolated from *E. chevalieri* MUT 2316 tested for the antifouling activity, their concentrations in 100% DMSO (stock 1) and further diluted for the bioassays (stock 2).

Molecules	Stock 1 (mg/mL)	Stock 2 (µg/mL)
Echinulin (1)	10	1000
Neoechinulin A (2)	10	1000
Dihydroauroglaucin (4)	10	1000
Flavoglaucin (5)	10	1000
Physcion (7)	1	1000
Cinnalutein (9)	10	1000
Cyclo-L-Trp- L-Ala (10)	10	1000

9.2.1.1 Growth inhibition

In order to evaluate the bacterial growth inhibition, the compounds were diluted in MBM (Table 9.2, Stock 2) and transferred to clear 96-multiwell plates, to reach the final concentrations of 0.001, 0.01, 0.1, 1, 10 and 100 $\mu\text{g/mL}$ (Figure 9.2). (7) was not tested at the highest concentration (100 $\mu\text{g/mL}$) to avoid DMSO toxicity.

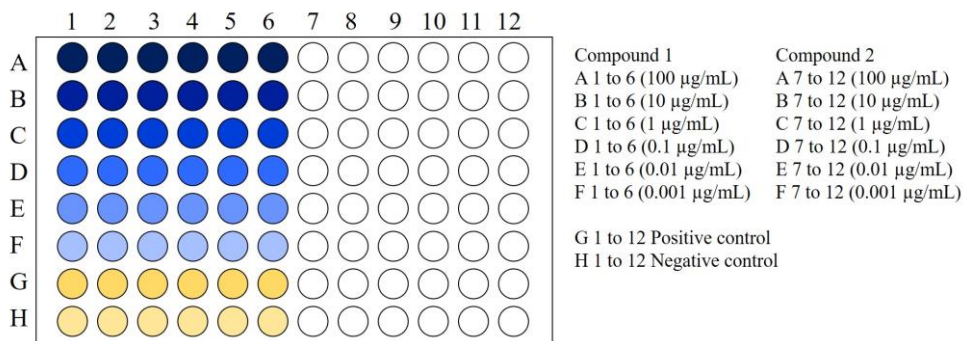


Figure 9.2 Scheme of a multiwell plate prepared with different concentrations of the tested compounds and the controls.

The optical density (OD) of the pre-growth bacteria was measured at 630 nm with the spectrophotometer (Tecan Infinite M200) and the bacterial inoculum was adjusted to 2×10^8 CFU/mL according to the Amsterdam method reported in Table 9.3 (Hellio *et al.*, 2015).

The bacterial inoculum was equally added to the wells, containing the tested compounds, to reach the final volume of 100 μL . The plates were incubated for 72 h at 20 $^{\circ}\text{C}$ (Hellio *et al.*, 2015). Controls were performed with DMSO, making the same dilutions set up to test the molecules, with 1% as the maximum concentration of the solvent; the bacterial strains and the non-inoculated media represented 2 further controls (Figure 9.2). The test was replicated six times.

The Low Observable Effect Concentration (LOEC), defined as the lowest test concentration with an average response that is significantly different from the control (Amara *et al.*, 2018) was calculated, based on the OD values at 630 nm. Therefore, the ANOVA was performed to compare the bacterial growth inhibition of the compounds

with the control ($p < 0.05$) using IBM SPSS Statistics software. Before the statistical analysis, the controls with the DMSO, that unlikely inhibited the bacterial growth, were subtracted to the results produced by the molecules. Moreover, the potential interference due to the coloured molecules was taken into account by measuring the OD at 630 nm immediately after the inoculum.

Table 9.3 Amsterdam table indicating the volume of bacterial suspension to add to the medium (final volume 10 mL) to obtain an inoculum concentration of 2×10^8 CFU/mL, based on the OD value at 630 nm.

OD at 630 nm	Volume (μ L)
0.20	50
0.25	40
0.30	33
0.35	29
0.40	25
0.45	22
0.50	20
0.55	18
0.60	16
0.65	15

9.2.1.2 Adhesion inhibition

The plates used to evaluate the bacterial growth inhibition were emptied and rinsed with 100 μ L of seawater to remove the non-attached cells. Once dry, the bacterial biofilm was stained with 0.3% aqueous crystal violet, then after 30 min the plates were rinsed with water and let dry overnight. The wells were filled with 100 μ L of 80% aqueous ethanol and the OD was measured at 595 nm to evaluate the LOEC, performing the above-mentioned statistical analysis and taking into account the possible influence of the DMSO (Hellio *et al.*, 2015).

9.2.2 Microalgal assay

The molecules were tested against 5 algal species (Table 9.4) known to be involved in surface colonization, coming from the Algobank of Caen - AC (France) and the Laboratoire de Biotechnologie et Chimie Marines – LBCM (University of South Brittany, France).

Table 9.4 Marine algae used for the antifouling test.

Species	Code
<i>Cylindrotheca closterium</i>	AC 170
<i>Exanthemachrysis gayraliae</i>	AC 15
<i>Halamphora coffeaeformis</i>	AC 713
<i>Porphyridium purpureum</i>	AC 122
<i>Phaeodactylum tricornutum</i>	AC 171

The algae were pre-grown in sterile F/2 medium (20 mL of 50× stock solution purchased from Sigma-Aldrich and up to 1 L of sterile seawater) for 7 days at room temperature, exposed to natural daylight irradiance.

9.2.2.1 Growth inhibition

In order to evaluate the algal growth inhibition, the compounds (Table 9.2, Stock 1) were diluted in the F/2 medium (Table 9.2, Stock 2) and transferred to black 96-multiwell plates to reach the same final concentrations tested for the antibacterial activity and as shown in Figure 9.2.

The chlorophyll *a* content of each pre-grown alga solution was assessed accordingly to Hellio and co-authors (2015). Briefly, 5 mL of algae suspension was filtered under vacuum on glass microfiber filters GF/F (Whatman); filters were immediately transferred in vials with 100% MeOH and stored in the dark, at 4 °C for 30 min. The fluorescence of the extracted chlorophyll *a* was measured (Tecan Infinite M200, excitation 485 nm, emission 645 nm) and its concentration was determined based on a calibration curve made with spinach chlorophyll *a*. The algal suspension was then diluted to obtain for each alga a stock solution of 0.1 mg/L of chlorophyll *a*, which was

equally added to the wells (with the test and control line) to reach the final volume of 100 μL . The plates were stored for two weeks at room temperature, exposed to the daylight irradiance. The same controls of the antibacterial test were carried out (with DMSO, with the algae and the medium alone).

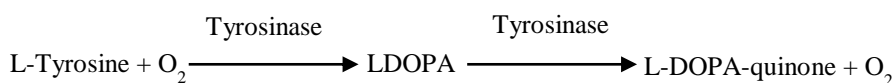
After the incubation period, the content of each well was gently transferred in new black polystyrene 96-multiwell plates; the algae were let settlement prior to removing the supernatant. Finally, 100 μL of 100% MeOH were added to extract and then the concentration of chlorophyll *a* was measured as above mentioned. The LOEC was then calculated ($p < 0.05$); the possible influence of the coloured molecules and of the DMSO was taken into account as above mentioned.

9.2.2.2 Adhesion inhibition

The plates used for the test and emptied in the previous section were rinsed with 100 μL of seawater to remove the non-attached cells. Once dry, 100 μL of 100% MeOH were added to extract and then measure the concentration of chlorophyll *a* as previously mentioned (Hellio *et al.*, 2015). The LOEC was then calculated ($p < 0.05$) after the evaluation of the possible influence of the coloured molecules and the DMSO.

9.2.3 Inhibition of blue mussel *M. edulis* settlement – tyrosinase assay

The inhibition of the mussels' settlement is evaluated through an enzymatic assay based on tyrosinase that plays an essential role in mussel byssus production. Mushroom tyrosinase (EC 1.14.18.1 - Sigma-Aldrich) was used for this test and the following reaction was monitored:



The compounds (Table 9.2, Stock 1) were diluted in 0.5 mM potassium phosphate buffer pH 6.5 (Table 9.2, Stock 2) and 13 μL were transferred to clear polystyrene 96-multiwell plates (Fisher Scientific), to reach the same final

concentrations tested for the antibacterial and antimicrobial assays. Then, a mix with 5 mL of potassium phosphate buffer (0.5 mM, pH 6.5), 5 mL of L-tyrosine (1 mM) and 4.5 mL of dH₂O was prepared; 180 µL of the mix were added to each well and the plates were equilibrated at 25 °C. Afterward, 7 µL of the enzyme tyrosinase (1000 U/mL) was added to each well kept at 25 °C. The change in the absorbance was measured after 800 s, at 410 nm using the spectrophotometer (Tecan Infinite M200), Duckworth and Coleman (1970). The results were expressed as inhibition of tyrosinase activity compared to the control without the enzyme. A positive control was carried out with the kojic acid using the same concentrations of the tested molecules. The test was performed in triplicate and the LOEC was then calculated ($p < 0.05$).

9.3 RESULTS AND DISCUSSION

9.3.1 The molecules produced by *E. chevalieri* MUT 2316 display antifouling activity

In the present chapter the activity of 7 molecules, produced by the marine fungus *E. chevalieri* MUT 2316, was assessed against pioneer bacteria and microalgal species, that subsequently promote the surfaces colonisation by macrofouling (Hanssen *et al.*, 2014). The inhibition of the adhesion and growth of marine bacteria and marine algae, as well as the inhibition of tyrosinase (a key enzyme for mussels settlement), was evaluated for the first time for **(1, 2, 4, 5, 7, 9, 10)**.

In order to remain within a concentration of relevance for industrial exploitation of new antifouling formulations, defined by the U.S. Office of Naval Research <25 µg/mL (Trepas *et al.*, 2014a), more emphasis will be done to the compounds with the lowest significant LOEC values.

All the tested molecules, although in a different way, showed antifouling activity, as summarised in Figure 9.3.

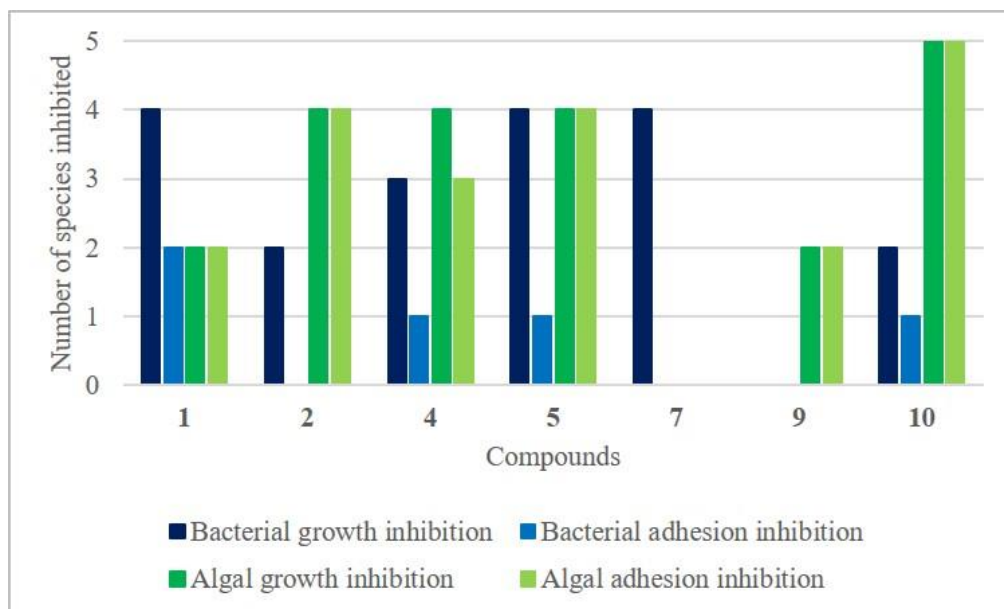


Figure 9.3 Number of bacterial and algal species inhibited in the growth and adhesion by the seven tested (1, 2, 4, 5, 7, 9, 10) molecules produced by *E. chevalieri* MUT 2316.

Among the most promising compounds of the present study, (10) displayed activity in 61.5% of cases at the lowest concentration tested (0.001 $\mu\text{g}/\text{mL}$) as reported in Table 9.5. Of note, (10) inhibited the growth and adhesion of all the algal species, including the two diatoms *C. closterium* and *H. coffeaeformis*, that are key microfouling species, since they form permanent slimy layers on marine surfaces (Trepas *et al.*, 2014a).

In addition, (10) could potentially contrast the macrofouling, limiting the byssus formation in mussels at a concentration lower (0.01 $\mu\text{g}/\text{mL}$) than the PC kojic acid (Table 9.6). The antibacterial activity, although reported only against two and one species, for the growth and adhesion inhibition, respectively (Table 9.5), was never reported before in terrestrial bacteria (Tian *et al.*, 2013), including those assessed in the chapter 7 (even if a different and not easily comparable method was used).

Table 9.5 Low Observable Effect Concentration (LOEC µg/mL) values of compounds (**1, 2, 4, 5, 7, 9, 10**) against the growth (Gr) and adhesion (Ad) of marine bacteria and algae, representatives of fouling organisms. Positive values are highlighted in light blue.

Molecules	Echinulin (1)		Neoechinulin A (2)		Dihydroauroglau cin (4)		Flavoglau cin (5)		Physcion (7)		Cinnalutein (9)		Cyclo-L-Trp-L-Ala (10)		Sea-Nine*	
	Gr	Ad	Gr	Ad	Gr	Ad	Gr	Ad	Gr	Ad	Gr	Ad	Gr	Ad	Gr	Ad
Marine Bacteria																
<i>H. aquamarina</i> ATCC 14400	100	>100	>100	>100	>100	>100	>100	>100	0.01	>100	>100	>100	0.001	>100	0.1	<0.01
<i>P. irgensii</i> ATCC 700398	1	>100	100	>100	0.01	>100	1	>100	1	>100	>100	>100	>100	0.001	1	0.1
<i>P. citrea</i> ATCC 29720	0.01	0.001	>100	>100	0.01	>100	1	>100	0.01	>100	>100	>100	>100	>100	N/A	N/A
<i>R. litoralis</i> ATCC 49566	>100	0.1	>100	>100	>100	0.001	0.01	0.1	>100	>100	>100	>100	>100	>100	1	<0.01
<i>V. aestuarianus</i> ATCC 35048	100	>100	100	>100	0.001	>100	0.001	>100	10	>100	>100	>100	0.001	>100	<0.01	1
Marine microalga																
<i>C. closteriumb</i> AC 170	>100	>100	0.001	1	>100	>100	>100	>100	>100	>100	>100	0.001	0.001	0.001	<0.01	<0.01
<i>E. gayraliae</i> AC 15	>100	0.01	>100	0.01	1	1	0.01	0.01	>100	>100	>100	>100	0.001	0.001	<0.01	<0.01
<i>H. coffeaeformis</i> AC 713	0.1	>100	0.01	1	10	1	1	1	>100	>100	0.01	1	0.001	1	<0.01	<0.01
<i>P. tricor nutum</i> AC 171	>100	0.01	1	0.001	1	>100	10	0.001	>100	>100	1	>100	100	1	N/A	N/A
<i>P. purpureum</i> AC 122	0.001	>100	100	>100	1	0.1	0.1	0.001	>100	>100	>100	>100	10	100	<0.01	<0.01

N/A not applicable; *Data from Trepos *et al.* 2015

Table 9.6 Low Observable Effect Concentration (LOEC $\mu\text{g/mL}$) values of compounds (**1**, **2**, **4**, **5**, **7**, **9**, **10**) in the tyrosinase assay. Positive values are highlighted in light blue.

Molecules	Tyrosinase assays
Echinulin (1)	>100
Neoechinulin A (2)	>100
Dihydroauroglaucin (4)	100
Flavoglaucin (5)	>100
Physcion (7)	>100
Cinnalutein (9)	>100
Cyclo-L-Trp- L-Ala (10)	0.01
Kojic acid	1

The molecule (**5**) showed also a strong and broad antifouling activity, inhibiting all the algae tested (with the exception of *C. closterium*); interestingly, (**5**) was significantly able to inhibit the adhesion of *P. purpureum* and *P. tricornutum* at two and four orders magnitude lower, compared to the growth inhibition of the same algae. This makes of (**5**) a highly desired antifouling for algae, with limited biocide activity and high adhesion inhibition. (**5**) inhibited the growth and the adhesion of 4 and one bacterial species, respectively, and it was the sole compound able to contrast the growth of *R. litoralis* (Table 9.5). The antibacterial activity of this compound was not reported in chapter 7, both due the different methods used and the fact that only the complete absence of growth of the bacteria was evaluated. Instead, Gao *et al.* (2012) by evaluating the IC_{50} reported the activity of (**5**) against *S. aureus* strains.

The compound (**4**) for which the similarity of the chemical structure with (**5**) was already described in chapter 7, showed almost the same antifouling activity of (**5**) (only the adhesion inhibition against *R. litoralis* and *P. tricornutum* was not reported). However, (**4**) was significantly active against bacteria and algae at concentrations usually higher than that reported for (**5**) (Table 9.5). Overall, the antibacterial activity of (**4**) was here first reported against terrestrial and human pathogens (chapter 7) and marine species. (**4**) was able to inhibit the tyrosinase (Table 9.6) even if a too high concentration (100 $\mu\text{g/mL}$) to be considered useful for future applications.

The compound (**2**) revealed to be more active against microalgae than bacteria (Table 9.5); indeed, it was able to inhibit the growth and adhesion of four out of five microalgae, including the two diatoms, relevant for the biofouling formation, as above

mentioned. Regarding bacteria, only two were inhibited at the highest concentration tested (100 µg/mL); this might be due to the specific mechanisms of action of this compound, that was not active with the selected strains of chapter 7; while showed different activity against other bacterial strains (Liang *et al.*, 2018; Zhao *et al.*, 2018; Ganihigama *et al.*, 2015). Of note, (2) was previously reported to inhibit the larval settlement of *B. amphitrite* (Chen *et al.*, 2018; Sun *et al.*, 2014). Although it did not display toxicity against the barnacle larva ($LC_{50} > 50$ µg/mL), showed teratogen effects against the embryos of the zebra fish *Danio rerio* (Chen *et al.*, 2018). In this regards, the further use of (2) as antifouling should be carefully evaluated.

The molecule (1) contrasted the growth of four out of five bacteria, and the adhesion of two bacteria, being the only compound significantly limiting the adhesion of *P. citrea* (Table 9.5). This is the first report of the antibacterial activity of (1) against marine bacteria, while it was inactive against several terrestrial bacteria (Zin *et al.*, 2017; Pereira *et al.*, 2016; Meng *et al.*, 2015; Gao *et al.*, 2012), including those tested in the chapter 7; only Liang and collaborators (2018) reported (1) as active against different bacteria. Of note, (1) limited the growth of *R. litoralis*, *E. gayraliae* and *P. tricornutum* without interfering with the growth of the selected bacteria and algae. Probably, among all the possible mechanisms of action of (1) there is the ability to alter the expression of adhesive proteins in the target species or more generally, it avoids the release of adhesive compounds in selected bacterial and algal species. Certainly, (1) deserve more attention also in light of its capability to inhibit the settlement of *B. amphitrite*, without being toxic against bot the barnacle species and a nontarget zebra fish species *D. rerio* (Chen *et al.*, 2018).

The last two molecules tested, (7) and (9) showed a specific activity only against one of the two target groups (bacteria or algae) assessed in the present chapter. (7) was only able to contrast bacteria (four out of five species) and in particular their growth, not affecting the ability of the selected species to form a biofilm, making of (7) not the best candidate as antifouling. The antibacterial activity of this compound was confirmed also against some human pathogens, as reported in chapter 7 and in the bibliography (Linag *et al.*, 2018; Basu *et al.*, 2005).

As regard **(9)**, it was only able to contrast algae, by inhibiting the growth and/or the adhesion of three species. By contrast, **(9)** showed antibacterial activity against two MDR bacteria, but not on standard strains (chapter 7).

Overall, all the compounds, with the exception of **(9)** displayed activity against the Gram-negative bacteria tested, that are more difficult to access compared to the Gram-positive (Trepas *et al.*, 2015). Only apparently contrasting are the results obtained in chapter 7, where none of the tested molecules was active against Gram-negative bacteria. Indeed, different strains were used and the technique itself did not allow easy comparison since only the extinction of the bacterial growth have been considered in chapter 7.

Noteworthy, all the compounds were active against *V. aestuarianus*; this species has been retrieved worldwide as a pathogen of oysters, with high mortality on both adult and juvenile (Travers *et al.*, 2017). Therefore, the fungal molecules tested in the present study could be suitable for both antifouling and the protection of aquaculture devices, contrasting a pathogenic strain.

Moreover, to put these results in an industrial and applicative context, the minimum inhibitory concentration (comparable to the LOEC) of the biocide TBT oxide and copper sulphate, against the growth of a range of bacteria, was observed to be 6 µg/mL (Hellio *et al.*, 2001). In 73.7% of cases, the molecules here assessed were already able to contrast the bacterial growth at concentrations lower than 1 µg/mL.

By comparing the obtained results with those of the commercial antifouling Sea-Nine (Table 9.5, data from Trepas *et al.* 2015), available for four out of five bacterial and algal species here tested, several *E. chevalieri* MUT 2316 derived molecules can be considered highly promising. For instance, **(1, 2, 5, 10)** were active at 0.001 µg/mL against at least one algal species (Table 9.5), being therefore comparable and probably even more active than the Sea-Nine (MIC <0.01 µg/mL) reported by Trepas and colleagues (2015). As for the antibacterial assays, **(4, 5, 7, 10)**, when active, showed LOEC values lower or comparable to those reported for the Sea-Nine (Table 9.5).

Finally, looking at the antifouling activity of other natural products (from fungi or sponges), tested against the same strains of the present study, it emerges that molecules produced by the sponge derived fungus *Aspergillus* sp. were active at one of the lowest value reported in literature (0.001 µg/mL), as in our case (Zhou *et al.*, 2014). Two

bromotyrosine derivatives (ianthelline and baretin) isolated from the marine sponge *Stryphnus fortis* showed MIC values between 0.1–10 µg/mL (Hanssen *et al.*, 2014). Unfortunately, lower concentrations were not test, not allowing a comparison with the most active molecules reported in the present chapter.

In the future, it would be interesting to better understand the mechanisms behind the inhibition of the bacterial and algal growth by the compounds produced by *E. chevalieri* MUT 2316. For instance, the extracts or the pure molecules of several marine fungi were able to inhibit the quorum sensing of the bacterium *Chromobacterium violaceum* (Martín-Rodríguez *et al.*, 2014; Dobretsov *et al.*, 2011).

Other high added value molecules are represented by the hydrophobins, defined as small amphiphilic proteins self-assembling at hydrophobic/hydrophilic interfaces, able to modify the wettability of different substrates (Artini *et al.*, 2017; Cicatiello *et al.*, 2017; Piscitelli *et al.*, 2017). They are produced by fungi, including several marine fungi (Cicatiello *et al.*, 2016), that are highly promising for the inhibition of the biofilm formation. For instance, the marine fungus *Acremonium sclerotigenum* MUT 4872 produced the Pac3 hydrophobins that were successfully able to contrast the biofilm formation of *Staphylococcus epidermidis* (Artini *et al.*, 2017). The mechanism of action is still under study, but instead involving bacterial proteins and polysaccharides might act on the extracellular DNA that is required for the initial attachment of *S. epidermidis* to the substrate (Artini *et al.*, 2017).

9.4 CONCLUSION AND FUTURE PERSPECTIVES

The molecules produced by *E. chevalieri* MUT 2316 revealed to be promising antifouling. This was particularly evident for **(10)** that was not among the most interesting compounds of the previous chapter (reporting the antibacterial and antiviral activity) but founded in the antifouling tests its target, showing antibacterial, antimicrobial activity and the inhibition of the tyronisase, at comparable or lower concentrations than commercial antifouling formulations. Other compounds, presented a broad-spectrum activity, this was the case of **(4, 5)**. However, it is unrealistic to think that only a molecule would be able to contrast all the fouling species, without being a common biocide, active also against non-target species. Based on this study, a formulation composed by the molecules **(1, 4, 5, 10)**

would be able to contrast the growth and adhesion of all tested bacteria (except the adhesion of *V. aestuarianus*) at concentrations $<0.01 \mu\text{g/mL}$. Similarly, **(2, 5, 10)** would be able to inhibit the growth and adhesion of all tested microalgae concentrations $<1 \mu\text{g/mL}$. In this regard, the requirements of a new antifouling formulation with a concentration $<25 \mu\text{g/mL}$ would be satisfied.

In the following months, thanks to the collaboration with the Laboratoire des sciences de l'environnement marin of the University of Western Brittany (Brest, France), and the financing of the Galileo project by the Italo-French University, this work will continue. In detail, the synergic effects of the molecules will be assessed using marine bacteria and algae; moreover, barnacles and macroalgae, representative of the macrofouling, will be introduced in the screening. The mechanism of action of the molecules, as well as their toxicity, will be assessed. Moreover, field studies will be set up with the most promising compounds.

10. GENERAL CONCLUSION AND FUTURE PERSPECTIVES

Marine environment represents an untapped source of fungal diversity, where it has been estimated that about 10% of fungi have been explored until now (Jones *et al.*, 2015). These fungi are also becoming an appealing source of natural products. Indeed, from the discovery of a novel compound in the marine environment, in 1951 (Anjum *et al.*, 2016), several researchers pointed out the hidden treasure of new molecules in the oceans. Suddenly it has been realised that to obtain a suitable amount of new drugs, it was necessary to take a huge amount of the organisms that produce the molecules from the environment. Therefore, the only solutions were represented by the cultivation of the organisms, usually difficult, or the chemical synthesis when feasible and economically convenient. It is precisely for these circumstances that marine fungi come up, being usually easier to cultivate and manipulate with reduced costs, compared to macroorganisms. Based on recent researches marine fungal metabolites will find application towards human needs: pharmaceuticals, cosmeceuticals, nutraceuticals, etc. Additionally, they will help to understand the functioning of our oceans, regarding the rules that drive the interactions among marine microorganisms and macroorganisms, the role of fungi in the biogeochemical cycles and the impact of global change on marine ecosystems. Therefore, the implications related to the biodiversity, chemodiversity and the biotechnological potential of marine fungi are countless, and explore several research fields, from biology to chemistry, as carried out during this Ph.D. project.

Marine fungi were successfully isolated from both Atlantic and Mediterranean sponges; overall, 129 taxa were obtained and are now preserved at MUT. Certainly, the preservation in a culture collection will allow to further use the fungal strains for several purposes, for instance to characterised taxa not identified at species level that might result in new species, or for the biotechnological exploitation of the strains. In addition, these fungi are also publicly available as their almost newly 300 generated sequences that have been deposited in GenBank database.

For the further exploitation of marine fungi, in particular for the discovery of new metabolites, and to handle them following the biosafety regulations, a low rank classification is fundamental. Thanks to a polyphasic approach based on morphological,

molecular and phylogenetic techniques, 84.5% of the taxa were identified at the species level and even at strain level, providing a value above the average for marine fungi and probably one of the highest available in the literature for such high number of fungal taxa. The low rank classification guarantee a series of trickle-down effects:

- It allows comparing the fungal communities among the sponges. In this case, the statistical analysis highlighted a significant difference in the mycobiotas of the 4 Atlantic sponges. As for the Mediterranean sponges, the mini-satellite analysis underlined such a low number of fungal strains common to the three sponges. Moreover, the fungal communities of the Mediterranean and the Atlantic sponges were significantly different, with only 9 species common, mainly represented by ubiquitous fungi.
- It makes possible a detailed comparison with the literature, and in this case, 72 and 30 fungal species were reported for the first time within sponges and in the marine environment, respectively.
- It allows comparing the metabolites produced by fungi with the available data, with the aim to avoid re-extracting already known compounds.

However, there are several reasons for which the identification at species level results not feasible: i) the abundance of sterile mycelia in the marine environment (about one third of the total taxa in our study), not allow morphological considerations; ii) the reduced number of fungal sequences (with few markers) in public databases, that does not allow the construction of multi-marker dataset for the phylogenetic analysis; iii) the presence of new species. In the last mentioned circumstance, whether sterile, the description of a new species might be complex. This was not the case of *T. balaustiformis* and *T. spongiae* capable to produce reproductive structures in axenic conditions and here first described, updating the knowledge on marine fungal diversity.

The investigation of the chemical diversity of marine fungi associated with sponges covered the second part of this Ph.D. project. For the first time, all the fungal community associated with a sponge, *G. compressa*, was studied using the OSMAC approach, still scarcely applied for studies involving marine fungi (Romano *et al.*, 2018). Not surprisingly, it has been difficult to define a condition that promotes both the development of the mycelium and the secondary metabolites production for all fungi; generally, rich nutrients

media are the best candidates to achieve the above mentioned results. Although the morphology of fungi in some case can easily suggest a different metabolic production, the use of innovative techniques can provide high accuracy results. This was the case of the molecular network created using the online workflow of the GNPS website, that highlighted how the same fungus *E. chevalieri* MUT 2316 produce about half of the total molecules either in liquid or in solid media.

Overall, 10 pure molecules were isolated from *E. chevalieri* MUT 2316, that although already known, have been little investigated for their bioactivity, highlighting the second drawback in the discovery of new metabolites (after the lack of precise systematic identification). Indeed, once a pure molecule is obtained, as much as possible bioassays should be performed, in order to discover the real potential of the tested compound. This requires on one side different facilities and expertise, on the other side the availability of the molecule in the amount necessary to perform several tests.

In this Ph.D. thesis, thanks to national and international collaborations, I had the opportunity to explore the antibacterial, antiviral and antifouling potential of the purified and identified molecules, aiming to face three different worldwide emergencies. First, the increase of antimicrobial resistance and the need to find new antibiotics, drive this Ph.D. project to assess the antibacterial activity of the molecules produced by *E. chevalieri* MUT 2316 against standard and MDR bacteria. The preliminary tests pointed out at least one molecule, (**6**) has been able to inhibit, at already low concentrations, all the Gram-positive (except one), including clinically isolated of MDR bacteria, with bactericidal activity. This lays the foundations for a further study aiming to evaluate a dose-response and the toxicity of the molecule. After these further assessments of the biological activity of the molecules, fully carried out by the Academia, the participation of the pharmaceutical industries, totally absent in the developing of new antibiotics in the last years, would be highly desired. Indeed, several promising compounds obtained from marine fungi and in general from marine organisms will never enter in the pre-clinical pipelines without the external economic support and qualified skills.

Despite the urgent need of new antivirals, the assessment of the antiviral activity is usually neglected for the evaluation of the biological activity of a new molecule. This is

demonstrated by the fact that of the compounds isolated in this project, only few were previously assessed against a virus. Among the molecules produced by *E. chevalieri* MUT 2316, **(3, 4)** were selective able to inhibit the replication of either the IAV or HSV-1, while **(7)** might be defined a broad spectrum antiviral. Ongoing studies aim to assess the mechanism behind the antiviral activity.

Finally, the last series of bioassays were carried out aiming to face the urgent need of environmentally friendly antifouling, and highlighted several molecules already active at extremely low concentrations, inhibiting the adhesion and growth of both bacteria and microalgae. As result, a mix of few compounds produced by *E. chevalieri* MUT 2316 would inhibit all the bacteria and microalgae tested (except the adhesion of *V. aestuarianus*). In the future, the effectiveness of this mix will be assessed and the toxicity of the compounds will be evaluated prior to field test.

Overall, *E. chevalieri* MUT 2316 proved to be a promising candidate for the development of new drugs and antifouling formulations. While, from an ecological point of view, if *E. chevalieri* MUT 2316 would be able to produce the same molecules within the sponge, it might be said that it actively contribute in the defence of its host from the pathogens and the fouling organisms. But, unfortunately, up to now, this has still to be studied.

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Annexe 1 Fungal taxa isolated from *D. fragilis* (DF), *G. compressa* (GC), *P. johnstonia* (PJ) and *S. ciliatum* (SC) and their isolation conditions, including direct plating (DP), homogenization (HO), different media and incubation temperatures. MUT accession numbers (available on: <http://www.mut.unito.it/en/Database>) and GenBank accession numbers are provided for representative strains of each fungal taxa.

MUT CODE	ID DEF	Isolation methods		Isolation media				Incubation temperatures		Spon ges	GenBank accession numbers							
		D P	H O	CMA SW	GAS W	ME A	SW A	15 °C	25 °C		ITS	LSU	TUB	ACT	CAL	GAPDH	D1-D2	
2436	<i>Absidia glauca</i>	x		x					x	DF	MG813 192							
2360	<i>Acremonium potronii</i>	x					x	x		DF	MG813 196	MG816 494						
2462	<i>Acremonium potronii</i>		x	x				x		DF	MG813 198	MG816 496						
2440	<i>Acremonium potronii</i>	x		x				x		DF	MG813 199	MG816 497						
2439	<i>Acremonium potronii</i>	x					x		x	DF	MG813 197	MG816 495						
-	<i>Acremonium potronii</i>	x					x	x		DF								
-	<i>Acremonium potronii</i>		x	x				x		DF								
-	<i>Acremonium potronii</i>	x		x				x		DF								
-	<i>Acremonium potronii</i>		x	x				x		DF								
2809	<i>Acremonium potronii</i>		x	x					x	SC	MG813 210	MG816 501						
-	<i>Acremonium potronii</i>	x					x		x	SC								
2491	<i>Acremonium breve</i>		x	x				x		DF	MG813 193							
2365	<i>Acremonium implicatum</i>		x	x				x		DF	MG813 194							
2367	<i>Acremonium persicinum</i>	x					x	x		DF	MG813 195							
-	<i>Acremonium persicinum</i>	x					x		x	DF								
2355	<i>Acremonium tubakii</i>	x		x				x		DF	MG813 200							
-	<i>Acremonium tubakii</i>	x		x					x	DF								

-	<i>Acremonium tubakii</i>	x	x		x	DF			
-	<i>Acremonium tubakii</i>	x	x		x	DF			
-	<i>Acremonium tubakii</i>	x		x	x	DF			
-	<i>Acremonium tubakii</i>	x		x	x	DF			
2378	<i>Acremonium tubakii</i>	x		x	x	DF	MG813 201		
-	<i>Acremonium zonatum</i>	x	x		x	SC			
-	<i>Acremonium zonatum</i>	x		x	x	SC			
2818	<i>Acremonium zonatum</i>	x		x	x	SC	MG813 213		
-	<i>Acremonium zonatum</i>	x		x	x	SC			
2260	<i>Alternaria molesta</i>	x	x		x	PJ	MG813 166	MG816 482	MG832 209
2884	<i>Alternaria sp.</i>	x	x		x	SC	MG813 214	MG816 502	MG832 211
2346	<i>Aspergillus creber</i>	x	x		x	PJ			MG832 143
-	<i>Aspergillus creber</i>	x		x	x	PJ			
-	<i>Aspergillus creber</i>	x	x		x	DF			
-	<i>Aspergillus creber</i>	x	x		x	DF			
2513	<i>Aspergillus creber</i>	x	x		x	DF			MG832 144
-	<i>Aspergillus creber</i>	x	x		x	DF			
-	<i>Aspergillus creber</i>	x		x	x	DF			
-	<i>Aspergillus creber</i>	x		x	x	DF			
-	<i>Aspergillus flavipes</i>	x	x		x	PJ			
-	<i>Aspergillus flavipes</i>	x	x		x	PJ			
2226	<i>Aspergillus flavipes</i>	x	x		x	PJ			MG832 141
-	<i>Aspergillus flavipes</i>	x		x	x	PJ			
2908	<i>Aspergillus fumigatus</i>	x	x		x	SC		MG832 204	

2518	<i>Aspergillus fumigatus</i>	x	x		x	DF			MG832 191
-	<i>Aspergillus fumigatus</i>	x	x		x	DF			
2347	<i>Aspergillus jensenii</i>	x	x		x	PJ			
2237	<i>Aspergillus jensenii</i>	x	x		x	PJ			MG832 142
2520	<i>Aspergillus jensenii</i>	x	x		x	DF			MG832 145
-	<i>Aspergillus puulaauensis</i>	x	x		x	DF			
2522	<i>Aspergillus puulaauensis</i>	x		x	x	DF			MG832 146
-	<i>Aspergillus puulaauensis</i>	x		x	x	DF			
-	<i>Aspergillus puulaauensis</i>	x		x	x	DF			
-	<i>Aspergillus puulaauensis</i>					DF			
-	<i>Aspergillus puulaauensis</i>	x		x	x	DF			
-	<i>Aureobasidium pullulans</i>	x	x		x	PJ			
2348	<i>Aureobasidium pullulans</i>	x		x	x	PJ		MG813 169	
2441	<i>Aureobasidium pullulans</i>	x	x		x	DF		MG813 203	
-	<i>Beauveria bassiana</i>	x	x		x	SC			
2805	<i>Beauveria bassiana</i>	x		x	x	SC		MG813 215	
2882	<i>Beauveria bassiana</i>	x	x		x	SC		MG813 216	
2523	<i>Beauveria bassiana</i>	x		x	x	DF		MG813 172	
-	<i>Beauveria bassiana</i>	x	x		x	DF			
-	<i>Beauveria bassiana</i>	x		x	x	DF			
2425	<i>Bimuria novae-zelandiae</i>	x		x	x	DF		MG813 173	MG816 486
2445	<i>Boeremia exigua</i>	x		x	x	DF		MG813 174	MG816 487
-	<i>Botrytis cinerea</i>	x	x		x	DF			
-	<i>Cadophora luteo olivacea</i>	x	x		x	SC			

-	<i>Cadophora luteo olivacea</i>	x	x		x	SC		
2817	<i>Cadophora luteo olivacea</i>	x		x	x	SC	MG813 217	
-	<i>Cadophora luteo olivacea</i>	x		x		SC		
2895	<i>Cadophora luteo olivacea</i>	x			x	SC	MG813 218	
2485	<i>Cadophora luteo-olivacea</i>	x		x	x	DF	MG813 204	
2288	<i>Ceriporia lacerata</i>	x		x	x	GC	MF1252 92	MF125 289
2524	<i>Cladosporium aggregatocicatricatum</i>	x	x		x	DF	MG813 175	MG832 112
2241	<i>Cladosporium allicinum</i>	x		x	x	PJ		MG832 106
-	<i>Cladosporium allicinum</i>	x	x		x	SC		MG832 124
2842	<i>Cladosporium allicinum</i>	x		x	x	SC	MG813 219	MG832 122
-	<i>Cladosporium allicinum</i>	x		x	x	SC		
-	<i>Cladosporium allicinum</i>	x		x	x	SC		
-	<i>Cladosporium allicinum</i>	x	x		x	DF		
2528	<i>Cladosporium allicinum</i>	x		x	x	DF		MG832 114
2525	<i>Cladosporium allicinum</i>	x	x		x	DF		MG832 113
-	<i>Cladosporium allicinum</i>	x		x	x	DF		
2307	<i>Cladosporium allicinum</i>	x		x	x	GC		-
2313	<i>Cladosporium allicinum</i>	x	x		x	GC		MH383 514
2243	<i>Cladosporium cladosporioides</i>	x	x		x	PJ		MG832 107
-	<i>Cladosporium cladosporioides</i>	x		x	x	PJ		
-	<i>Cladosporium cladosporioides</i>	x		x	x	PJ		
-	<i>Cladosporium cladosporioides</i>	x	x		x	PJ		
2244	<i>Cladosporium cladosporioides</i>	x		x	x	PJ		

-	<i>Cladosporium cladosporioides</i>	x	x	x	PJ	
2532	<i>Cladosporium cladosporioides</i>	x	x		DF	MG832 116
2529	<i>Cladosporium cladosporioides</i>	x	x	x	DF	MG832 115
2314	<i>Cladosporium cladosporioides</i>	x		x	GC	MH383 515
-	<i>Cladosporium cladosporioides</i>	x	x		DF	
2245	<i>Cladosporium halotolerans</i>	x	x	x	PJ	MG832 108
-	<i>Cladosporium halotolerans</i>	x	x	x	PJ	
2246	<i>Cladosporium halotolerans</i>	x	x	x	PJ	MG813 163
-	<i>Cladosporium halotolerans</i>	x	x	x	PJ	MG832 109
2933	<i>Cladosporium halotolerans</i>	x	x		SC	
-	<i>Cladosporium halotolerans</i>	x	x	x	SC	
-	<i>Cladosporium halotolerans</i>				SC	
-	<i>Cladosporium halotolerans</i>				SC	
-	<i>Cladosporium halotolerans</i>	x	x		SC	
2940	<i>Cladosporium halotolerans</i>	x	x		SC	MG832 125
-	<i>Cladosporium halotolerans</i>	x	x		SC	
-	<i>Cladosporium halotolerans</i>	x	x		SC	
-	<i>Cladosporium halotolerans</i>	x		x	SC	
-	<i>Cladosporium halotolerans</i>	x	x		SC	
-	<i>Cladosporium halotolerans</i>	x	x		SC	
2533	<i>Cladosporium perangustum</i>	x	x		DF	MG832 117
2247	<i>Cladosporium pseudocladosporioides</i>	x	x		PJ	
2248	<i>Cladosporium pseudocladosporioides</i>	x	x		PJ	MG832 110
-	<i>Cladosporium pseudocladosporioides</i>	x	x		PJ	

2932	<i>Cladosporium pseudocladosporioides</i>	x	x	x	SC			MG832 126
-	<i>Cladosporium pseudocladosporioides</i>	x	x	x	DF			
-	<i>Cladosporium pseudocladosporioides</i>	x	x	x	DF			
-	<i>Cladosporium pseudocladosporioides</i>	x	x	x	DF			
2537	<i>Cladosporium pseudocladosporioides</i>	x	x	x	DF			MG832 119
2535	<i>Cladosporium pseudocladosporioides</i>	x	x	x	DF			MG832 118
2315	<i>Cladosporium pseudocladosporioides</i>	x	x	x	GC			MH383 516
2579	<i>Cladosporium psychrotolerans</i>	x	x	x	DF			MG832 120
2580	<i>Cladosporium psychrotolerans</i>	x	x	x	DF			
2583	<i>Cladosporium subtilissimum</i>	x	x	x	DF			MG832 121
2249	<i>Cladosporium subuliforme</i>	x	x	x	PJ			MG832 111
2589	<i>Cladosporium xylophilum</i>	x	x	x	DF			MG832 122
2893	<i>Coniothyrium obiones</i>	x		x	SC	MG813 220	MG816 503	
2282	<i>Coprinellus</i> sp. 1	x		x	GC	MF1404 67	MF140 459	
2332	<i>Coprinellus</i> sp. 2	x	x	x	GC	MF1404 69	MF140 461	
2862	<i>Cyphellophora</i> sp.	x	x	x	SC	MG813 221	MG816 504	
2459	<i>Emericellopsis alkalina</i> (anamorph)	x	x	x	DF	MG813 205		
-	<i>Emericellopsis alkalina</i> (anamorph)	x	x	x	DF			
2351	<i>Emericellopsis maritima</i>	x	x	x	PJ	MG813 170	MG816 484	
2273	<i>Emericellopsis pallida</i>	x	x	x	GC	MH399 734	MG845 233	
2274	<i>Emericellopsis pallida</i>	x	x	x	GC	MH399 735	MG845 234	
-	<i>Emericellopsis pallida</i> (anamorph)	x	x	x	DF			
2458	<i>Emericellopsis pallida</i> (anamorph)	x	x	x	DF	MG813 206		

-	<i>Epicoccum nigrum</i>	x	x		x	SC		
-	<i>Epicoccum nigrum</i>	x	x		x	SC		
-	<i>Epicoccum nigrum</i>	x	x		x	SC		
-	<i>Epicoccum nigrum</i>	x	x		x	SC		
2874	<i>Epicoccum nigrum</i>	x		x	x	SC	MG813 222	
2316	<i>Eurotium chevalieri</i>	x		x	x	GC	MH399 736	MH383 517
2334	<i>Euthypella scoparia</i>	x		x	x	GC	MH399 739	MG845 235
2594	<i>Fusarium pseudograminearum</i>	x		x	x	DF	MG813 176	
2850	<i>Fusarium solani</i>	x	x		x	SC	MG813 223	
2317	<i>Fusarium solani</i>	x		x	x	GC	MH399 737	
2783	<i>Gremmenia infestans</i>	x			x	SC	MG813 224	MG816 505
2943	<i>Holtermanniella festucosa</i>	x	x		x	SC		MF196 244
2463	<i>Hypocreaceae</i> sp.	x	x		x	DF	MG813 207	MG816 499
2370	<i>Irpex lacteus</i>	x		x	x	DF	MF0986 96 ^a	
-	<i>Metschnikowia bicuspidata</i>	x	x		x	SC		
-	<i>Metschnikowia bicuspidata</i>	x	x		x	SC		
-	<i>Metschnikowia bicuspidata</i>	x	x		x	SC		
-	<i>Metschnikowia bicuspidata</i>	x	x		x	SC		
2941	<i>Metschnikowia bicuspidata</i>	x	x		x	SC		MG845 236
-	<i>Metschnikowia bicuspidata</i>	x	x		x	SC		
2377	<i>Microascaceae</i> sp.	x	x		x	DF	MG813 208	MG816 500
2264	<i>Moesziomyces bullatus</i>	x		x	x	PJ		MF521 974

2878	<i>Mollisia</i> sp.	x	x		x	SC	MG813 225	MG816 506
-	<i>Myrothecium cinctum</i>	x	x		x	DF		
-	<i>Myrothecium cinctum</i>	x			x	DF		
2599	<i>Myrothecium cinctum</i>	x			x	DF	MG813 177	
2956	<i>Neocamarosporium betae</i>	x			x	SC	MG813 233	
2404	<i>Neocamarosporium calvescens</i>	x			x	DF	MG813 179	
2806	<i>Paraphaeosphaeria neglecta</i>	x			x	SC	MG813 226	
2453	<i>Paraphaeosphaeria neglecta</i> (anamorph)	x	x		x	DF	MG813 209	
-	<i>Penicillium antarcticum</i>	x	x		x	PJ		
2250	<i>Penicillium antarcticum</i>	x	x		x	PJ		MG832 184
-	<i>Penicillium antarcticum</i>	x	x		x	PJ		
-	<i>Penicillium antarcticum</i>	x	x		x	PJ		
-	<i>Penicillium antarcticum</i>	x	x		x	PJ		
-	<i>Penicillium antarcticum</i>	x	x		x	PJ		
-	<i>Penicillium antarcticum</i>	x	x		x	PJ		
-	<i>Penicillium antarcticum</i>	x	x		x	PJ		
-	<i>Penicillium antarcticum</i>	x	x		x	PJ		
-	<i>Penicillium antarcticum</i>	x	x		x	PJ		
-	<i>Penicillium antarcticum</i>	x	x		x	PJ		
-	<i>Penicillium antarcticum</i>	x	x		x	PJ		
-	<i>Penicillium antarcticum</i>	x	x		x	PJ		
-	<i>Penicillium antarcticum</i>	x	x		x	PJ		
-	<i>Penicillium antarcticum</i>	x	x		x	PJ		
-	<i>Penicillium antarcticum</i>	x	x		x	PJ		

-	<i>Penicillium antarcticum</i>	x	x		x	PJ	
-	<i>Penicillium antarcticum</i>	x		x	x	PJ	
-	<i>Penicillium antarcticum</i>	x		x	x	PJ	
-	<i>Penicillium antarcticum</i>	x		x	x	PJ	
-	<i>Penicillium antarcticum</i>	x		x	x	PJ	
-	<i>Penicillium antarcticum</i>	x		x	x	PJ	
-	<i>Penicillium antarcticum</i>	x			x	x	PJ
2251	<i>Penicillium antarcticum</i>	x		x	x	PJ	MG832 185
-	<i>Penicillium antarcticum</i>	x			x	x	PJ
-	<i>Penicillium antarcticum</i>	x			x	x	PJ
-	<i>Penicillium antarcticum</i>	x			x	x	PJ
-	<i>Penicillium antarcticum</i>	x			x	x	PJ
-	<i>Penicillium antarcticum</i>	x			x	x	PJ
-	<i>Penicillium antarcticum</i>	x			x	x	PJ
-	<i>Penicillium antarcticum</i>	x			x	x	PJ
-	<i>Penicillium antarcticum</i>	x			x	x	PJ
-	<i>Penicillium antarcticum</i>	x	x			x	SC
-	<i>Penicillium antarcticum</i>	x	x			x	SC
-	<i>Penicillium antarcticum</i>	x	x			x	SC
-	<i>Penicillium antarcticum</i>	x	x			x	SC
-	<i>Penicillium antarcticum</i>	x	x			x	SC
-	<i>Penicillium antarcticum</i>	x	x			x	SC
-	<i>Penicillium antarcticum</i>	x	x			x	SC
-	<i>Penicillium antarcticum</i>	x	x			x	SC

-	<i>Penicillium antarcticum</i>	x	x		x		SC	
-	<i>Penicillium antarcticum</i>	x	x			x	SC	
-	<i>Penicillium antarcticum</i>	x	x			x	SC	
-	<i>Penicillium antarcticum</i>	x	x			x	SC	
-	<i>Penicillium antarcticum</i>						SC	
-	<i>Penicillium antarcticum</i>	x	x			x	SC	
-	<i>Penicillium antarcticum</i>	x		x		x	SC	
-	<i>Penicillium antarcticum</i>	x		x		x	SC	
-	<i>Penicillium antarcticum</i>	x		x		x	SC	
-	<i>Penicillium antarcticum</i>	x		x		x	SC	
-	<i>Penicillium antarcticum</i>	x		x		x	SC	
-	<i>Penicillium antarcticum</i>	x		x		x	SC	
-	<i>Penicillium antarcticum</i>	x		x		x	SC	
2926	<i>Penicillium antarcticum</i>	x		x		x	SC	MG832 205
-	<i>Penicillium antarcticum</i>	x	x			x	DF	
-	<i>Penicillium antarcticum</i>	x	x			x	DF	
-	<i>Penicillium antarcticum</i>	x	x			x	DF	
-	<i>Penicillium antarcticum</i>	x	x			x	DF	
-	<i>Penicillium antarcticum</i>	x	x			x	DF	
-	<i>Penicillium antarcticum</i>	x		x		x	DF	
2609	<i>Penicillium antarcticum</i>	x		x		x	DF	MG832 193
-	<i>Penicillium antarcticum</i>	x		x		x	DF	
-	<i>Penicillium antarcticum</i>	x		x		x	DF	

-	<i>Penicillium antarcticum</i>	x		x	x	DF	
-	<i>Penicillium antarcticum</i>	x		x	x	DF	
-	<i>Penicillium antarcticum</i>	x	x		x	DF	
-	<i>Penicillium antarcticum</i>	x	x		x	DF	
-	<i>Penicillium antarcticum</i>	x	x		x	DF	
-	<i>Penicillium antarcticum</i>	x	x		x	DF	
2735	<i>Penicillium antarcticum</i>	x	x		x	DF	MG832 192
2634	<i>Penicillium antarcticum</i>	x		x	x	DF	
-	<i>Penicillium antarcticum</i>	x		x	x	DF	
-	<i>Penicillium brevicompactum</i>	x	x		x	DF	
2665	<i>Penicillium brevicompactum</i>	x	x		x	DF	MG832 195
-	<i>Penicillium brevicompactum</i>	x		x	x	DF	
-	<i>Penicillium brevicompactum</i>	x		x	x	DF	
2664	<i>Penicillium brevicompactum</i>	x	x		x	DF	MG832 194
2252	<i>Penicillium canescens</i>	x	x		x	PJ	MG832 186
2253	<i>Penicillium chrysogenum</i>	x	x		x	PJ	MG832 187
-	<i>Penicillium chrysogenum</i>	x	x		x	PJ	
-	<i>Penicillium chrysogenum</i>	x	x		x	PJ	
-	<i>Penicillium chrysogenum</i>	x	x		x	PJ	
-	<i>Penicillium chrysogenum</i>	x		x	x	PJ	
2254	<i>Penicillium chrysogenum</i>	x		x	x	PJ	MG832 188
2255	<i>Penicillium chrysogenum</i>	x	x		x	PJ	MG832 189
-	<i>Penicillium chrysogenum</i>	x		x	x	PJ	

-	<i>Penicillium chrysogenum</i>	x		x	x	PJ	
2704	<i>Penicillium chrysogenum</i>	x	x		x	DF	MG832 197
-	<i>Penicillium chrysogenum</i>	x		x	x	DF	
-	<i>Penicillium chrysogenum</i>	x		x	x	DF	
-	<i>Penicillium chrysogenum</i>	x		x	x	DF	
-	<i>Penicillium chrysogenum</i>	x		x	x	DF	
-	<i>Penicillium chrysogenum</i>	x		x		DF	
-	<i>Penicillium chrysogenum</i>	x		x		DF	
-	<i>Penicillium chrysogenum</i>	x		x		DF	
-	<i>Penicillium chrysogenum</i>	x		x		DF	
-	<i>Penicillium chrysogenum</i>	x		x		DF	
-	<i>Penicillium chrysogenum</i>	x		x		DF	
-	<i>Penicillium chrysogenum</i>	x		x		DF	
-	<i>Penicillium chrysogenum</i>	x		x		DF	
-	<i>Penicillium chrysogenum</i>	x		x		DF	
-	<i>Penicillium chrysogenum</i>	x		x		DF	
-	<i>Penicillium chrysogenum</i>	x		x		DF	
-	<i>Penicillium chrysogenum</i>	x		x		DF	
-	<i>Penicillium chrysogenum</i>	x		x		DF	
-	<i>Penicillium chrysogenum</i>	x		x		DF	
-	<i>Penicillium chrysogenum</i>	x		x		DF	
-	<i>Penicillium chrysogenum</i>	x		x		DF	
-	<i>Penicillium chrysogenum</i>	x		x		DF	
-	<i>Penicillium chrysogenum</i>	x		x		DF	
2666	<i>Penicillium chrysogenum</i>	x		x	x	DF	MG832 196
2321	<i>Penicillium chrysogenum</i>	x	x		x	GC	MH383 518
2903	<i>Penicillium citreonigrum</i>	x		x		SC	MG832 206
2705	<i>Penicillium inflatum</i>	x	x		x	DF	MG832 198
-	<i>Penicillium janczewskii</i>	x		x		DF	

-	<i>Penicillium janczewskii</i>	x			x	x		DF			
-	<i>Penicillium janczewskii</i>	x			x	x		DF			
2713	<i>Penicillium janczewskii</i>	x			x		x	DF			MG832 200
2710	<i>Penicillium janczewskii</i>	x			x		x	DF			MG832 199
-	<i>Penicillium janczewskii</i>	x	x				x	DF			
-	<i>Penicillium janczewskii</i>	x	x					DF			
-	<i>Penicillium janczewskii</i>	x		x				DF			
2328	<i>Penicillium oxalicum</i>	x		x			x	GC	MH399 738		
2322	<i>Penicillium paneum</i>	x		x				GC			MH383 519
2326	<i>Penicillium paneum</i>	x			x			GC			MH383 520
2906	<i>Penicillium roqueforti</i>	x		x			x	SC			MG832 207
2256	<i>Penicillium spinulosum</i>	x	x					PJ	MG813 164	MG816 481	MG832 190
-	<i>Penicillium spinulosum</i>	x	x					PJ			
2257	<i>Penicillium thomii</i>	x	x				x	PJ	MG813 165		
2734	<i>Penicillium waksmanii</i>	x	x					DF			MG832 201
-	<i>Penicillium waksmanii</i>	x	x					DF			
-	<i>Periconia minutissima</i>	x	x					SC			
2887	<i>Periconia minutissima</i>	x	x					SC	MG813 227		
2263	<i>Periconia sp.</i>	x		x				PJ	MG813 167	MG816 483	
2854	<i>Phaeosphaeria olivacea</i>	x	x				x	SC	MG813 228	MG816 507	
2928	<i>Phaeosphaeria oryzae</i>	x				x	x	SC	MG813 229	MG816 508	
2482	<i>Phaeosphaeriopsis sp.</i>	x				x		DF	MG813 178	MG816 488	
3080	<i>Phaeosphaeriopsis sp.</i>	x	x				x	SC	MG813 232	MG816 510	
2959	<i>Phaeosphaeriopsis sp.</i>	x	x					SC	MG813 230	MG816 509	

2870	<i>Phaeosphaeriopsis</i> sp.	x	x	x	SC	MG813 231	MG816 513
-	<i>Phaeosphaeriopsis</i> sp.	x	x	x	SC		
-	<i>Phaeosphaeriopsis</i> sp.	x	x	x	SC		
2945	Pleosporaceae sp.	x	x	x	SC	MG813 234	MG816 511
2489	Pleosporales sp.	x	x	x	DF	MG813 180	MG816 489
2386	<i>Pochonia suchlasporia</i>	x	x	x	DF	MG813 181	
2492	<i>Porostereum spadiceum</i>	x	x	x	DF	MF1404 68	
2390	<i>Preussia</i> sp.	x	x	x	DF	MG813 182	MG816 490
2331	<i>Psathyrella candolleana</i>	x	x	x	GC	MF1252 93	MF125 290
2812	<i>Pseudeurotium bakeri</i>	x	x	x	SC	MG813 235	
2352	<i>Pseudocercospora</i> sp.	x	x	x	PJ	MG813 171	MG816 485
2374	<i>Pyrenochaetopsis microspora</i>	x	x	x	DF	MG813 202	MG816 498
	<i>Pyrenochaetopsis microspora</i>	x	x	x	DF		
	<i>Pyrenochaetopsis microspora</i>	x	x	x	DF		
2266	<i>Rhodotorula graminis</i>	x	x	x	PJ		MF112 036
2415	<i>Rhodotorula mucilaginosa</i>	x	x	x	GC	MF4237 18	
2452	Roussoellaceae sp.	x	x	x	DF	MG813 183	MG816 491
-	<i>Sarocladium strictum</i>	x	x	x	SC		
-	<i>Sarocladium strictum</i>	x	x	x	SC		
-	<i>Sarocladium strictum</i>	x	x	x	SC		
-	<i>Sarocladium strictum</i>	x	x	x	SC		
-	<i>Sarocladium strictum</i>	x	x	x	SC		
-	<i>Sarocladium strictum</i>	x	x	x	SC		
2830	<i>Sarocladium strictum</i>	x	x	x	SC	MG813 211	

-	<i>Sarocladium strictum</i>	x	x	x	SC			
-	<i>Sarocladium strictum</i>	x	x	x	SC			
-	<i>Sarocladium strictum</i>	x		x	x	SC		
-	<i>Sarocladium strictum</i>	x	x		x	SC		
-	<i>Sarocladium strictum</i>	x	x			SC		
2892	<i>Sarocladium strictum</i>	x		x	x	SC	MG813 212	
-	<i>Sarocladium strictum</i>	x		x	x	SC		
-	<i>Sarocladium strictum</i>	x		x	x	SC		
2849	<i>Scopulariopsis brevicaulis</i>	x		x	x	SC	MG813 236	
-	<i>Tetracladium sp.</i>	x	x		x	GC		
2357	<i>Thelebolus balaustiformis</i>	x	x		x	DF	MG813 184	MG816 492
2359	<i>Thelebolus spongiae</i>	x		x	x	DF	MG813 185	MG816 493
2766	<i>Thyronectria sp.</i>	x	x		x	SC	MG813 237	MG816 512
2364	<i>Tilachlidium brachiatum</i>	x	x		x	DF	MG813 186	
2484	<i>Tolypocladium album</i>	x	x		x	DF	MG813 187	
-	<i>Tolypocladium album</i>	x	x		x	DF		
-	<i>Tolypocladium album</i>	x	x		x	DF		
2267	<i>Tolypocladium cylindrosporum</i>	x	x		x	PJ	MG813 168	
-	<i>Tolypocladium cylindrosporum</i>	x	x		x	SC		
-	<i>Tolypocladium cylindrosporum</i>	x	x		x	SC		
-	<i>Tolypocladium cylindrosporum</i>	x	x		x	SC		
2869	<i>Tolypocladium cylindrosporum</i>	x	x		x	SC	MG813 238	
2875	<i>Tolypocladium cylindrosporum</i>	x		x	x	SC	MG813 239	
-	<i>Tolypocladium cylindrosporum</i>	x	x		x	DF		

-	<i>Tolypocladium cylindrosporum</i>	x		x		x	DF	
2447	<i>Tolypocladium cylindrosporum</i>	x		x		x	DF	MG813 189
2406	<i>Tolypocladium cylindrosporum</i>	x	x			x	DF	MG813 188
2410	<i>Tolypocladium cylindrosporum</i>	x			x	x	GC	MH399 740
2413	<i>Tolypocladium cylindrosporum</i>	x	x			x	GC	MH399 741
2444	<i>Trametes gibbosa</i>	x		x		x	DF	MF0986 90
-	<i>Trametes gibbosa</i>	x		x		x	DF	MF0986 91
2397	<i>Volutella ciliata</i>	x		x		x	DF	MG813 190
2496	<i>Xanthothecium peruvianum</i>	x		x		x	DF	MG813 191

Annexe 2 Fungal taxa isolated from *A. cavernicola* (AC), *C. crambe* (CC) and *P. tenacior* (PT) and their isolation conditions, including direct plating (DP), homogenization (HO), different media and incubation temperatures. MUT accession numbers (available on: <http://www.mut.unito.it/en/Database>) and GenBank accession numbers are provided for each fungal taxa.

MUT CODE	ID DEF	Isolation methods		Isolation media			Incubation temperatures		Sponges	GenBank accession numbers			
		DP	HO	CMASW	GASW	SWA	15 °C	25 °C		ITS	LSU	TUB	ACT
3035	<i>Arthrimum arundinis</i>		x	x			x		PT	MG980573	MG980403		
3393	<i>Aspergillus protuberus</i>		x		x			x	PT				MH047310
3048	<i>Beauveria brongniartii</i>		x		x			x	PT	MG980574			
3236	<i>Chaetomium globosum</i>	x			x		x		AC	MG980575			
3040	<i>Cladosporium cladosporioides</i>		x			x	x		AC				MH047331
3540	<i>Cladosporium cladosporioides</i>		x			x	x		AC				MH047322
3542	<i>Cladosporium cladosporioides</i>		x			x	x		AC				MH047323
3549	<i>Cladosporium cladosporioides</i>		x		x			x	AC				MH047324
3551	<i>Cladosporium cladosporioides</i>		x			x		x	AC				MH047325
3050	<i>Cladosporium cladosporioides</i>		x		x			x	PT				MH047329
3562	<i>Cladosporium cladosporioides</i>		x			x	x		PT				MH047326
3563	<i>Cladosporium cladosporioides</i>		x		x			x	PT				MH047327
3564	<i>Cladosporium cladosporioides</i>		x		x		x		PT				MH047328
3571	<i>Cladosporium cladosporioides</i>		x	x				x	PT				MH047330
3250	<i>Cladosporium delicatulum</i>		x			x	x		AC				MH047332
3271	<i>Cladosporium halotolerans</i>	x				x	x		AC				MH047333
3292	<i>Cladosporium perangustum</i>		x			x	x		AC				MH047334
3397	<i>Cladosporium perangustum</i>		x	x				x	PT				MH047335

3553	<i>Cladosporium pseudocladosporioides</i>	x		x		x	AC		MH047336
3554	<i>Cladosporium pseudocladosporioides</i>	x	x			x	AC		MH047337
3555	<i>Cladosporium pseudocladosporioides</i>	x	x			x	AC		MH047338
3556	<i>Cladosporium pseudocladosporioides</i>	x			x	x	AC		MH047339
3557	<i>Cladosporium pseudocladosporioides</i>	x		x		x	AC		MH047340
3558	<i>Cladosporium pseudocladosporioides</i>	x		x		x	AC		MH047341
3559	<i>Cladosporium pseudocladosporioides</i>	x	x			x	AC		MH047342
3580	<i>Cladosporium pseudocladosporioides</i>	x			x	x	PT		MH047343
3587	<i>Cladosporium pseudocladosporioides</i>	x	x			x	PT		MH047344
3499	<i>Cladosporium pseudocladosporioides</i>	x		x		x	CC		MH047345
3459	<i>Cladosporium ramotenellum</i>	x		x		x	PT		MH047346
3460	<i>Epicoccum nigrum</i>	x	x		x	x	PT	MG980576	
3463	<i>Epicoccum nigrum</i>	x					PT	MG980577	
2975	<i>Eutypella scoparia</i>	x	x			x	AC	MG980578	
-	Hypocreales sp.	x		x		x	PT		
2966	<i>Irpex lacteus</i>	x			x	x	AC	MF098695	MF115837
3058	<i>Kernia geniculotricha</i>	x			x	x	CC	MG980579	
3468	<i>Lecanicillium antillanum</i>	x			x	x	PT	MG980580	MG980404
3045	<i>Neosetophoma samararum</i>	x			x	x	AC	MG980581	MG980405
3046	<i>Neosetophoma samararum</i>	x	x			x	PT	MG980582	MG980406
3471	<i>Penicillium brevicompactum</i>	x	x			x	PT		MH047313
3501	<i>Penicillium brevicompactum</i>	x	x			x	AC		MH047311
3503	<i>Penicillium brevicompactum</i>	x	x			x	AC		MH047312
3498	<i>Penicillium cACenACum</i>	x	x			x	PT		MH047314

3486	<i>Penicillium cinereoatrum</i>	x	x		x	PT			MH047315
3297	<i>Penicillium citrinum</i>	x	x		x	AC			MH047316
3500	<i>Penicillium citrinum</i>	x		x	x	CC			MH047317
3560	<i>Penicillium corylophilum</i>	x		x	x	AC	MG980583		
3561	<i>Penicillium corylophilum</i>	x		x	x	AC			MH047318
3485	<i>Penicillium glabrum</i>	x		x	x	PT			MH047319
3299	<i>Penicillium murcianum</i>	x	x		x	AC			MH047320
3495	<i>Penicillium steckii</i>	x	x		x	PT			MH047321
2961	<i>Preussia terricola</i>	x		x	x	AC	MG980584	MG980407	
3033	<i>Psathyrella candolleana</i>		x	x	x	PT	MG980585	MG980408	
3041	<i>Rosellinia limonispora</i>	x		x	x	AC	MG980586	MG980409	
2970	<i>Rosellinia</i> sp.	x		x	x	AC	MG980587	MG980410	
3643	<i>Sarocladium glaucum</i>	x			x	x	AC	MG980588	MG980411
3019	<i>Schizophyllum commune</i>		x	x	x	AC	MF098694	MF115836	
3056	<i>Scopulariopsis</i> sp.	x			x	x	AC	MG980589	MG980412
3064	<i>Sordaria fimicola</i>	x		x		x	AC	MG980590	MG980413
3308	<i>Stachybotrys chartarum</i>	x		x		x	AC	MG980591	
3038	<i>Torula herbarum</i>		x		x	x	PT	MG980592	MG980414
3313	<i>Trichoderma atroviride</i>	x			x	x	AC	MG980593	
3036	<i>Uwebraunia dekeri</i>	x		x		x	PT	MG980594	MG980415
2971	<i>Virgaria nigra</i>	x		x		x	AC	MG980595	
3034	<i>Xylaria badia</i>	x		x		x	PT	MG980596	MG980416

Ph.D. activities (2015-2018)

Internships

- Institut de Chimie de Nice, University Nice Côte d'Azur (Nice, France), 2016–2018 (10 months)
Supervisor: Prof. Mohamed Mehiri
Evaluation of the chemodiversity of marine fungi associated with the sponge *G. compressa*. Isolation of molecules from *E. chevalieri* MUT 2316.
- Laboratoire des sciences de l'environnement marin, University of Western Brittany, 27/05/2018–15/06/2018.
Supervisors: Prof. Claire Hellio, Dr. Marilyne Fauchon
Evaluation of the antifouling activity of the molecules produced by *E. chevalieri* MUT 2316.
- Laboratory of Microbiology and Virology, University of Turin (Turin, Italy), 09/04/2018–18/04/2018.
Supervisors: Prof. Giorgio Gribaudo, Dr. Anna Luganini
Evaluation of the antiviral activity of the molecules produced by *E. chevalieri* MUT 2316.
- Laboratory of Clinical Microbiology and Virology, University of Milano-Bicocca (Monza, Italy), 06/03/2018–09/03/2018.
Supervisors: Prof. Clementina E. Cocuzza, Prof. Rosario Musumeci
Evaluation of the antibacterial activity of the molecules produced by marine fungi *E. chevalieri* MUT 2316.

During my Ph.D., I attended a total of 29 seminars and 9 courses/workshops, chosen according to my interests, exploring different fields of life sciences, chemistry and giving me the opportunity to improve two foreign languages, English and French.

Grants and Awards

- FEMS travel grant for participation at the 7th Congress of European Microbiologists (FEMS) held in Valencia (Spain), 9–13 July 2017

- MaFNaP travel grant for the participation at the 2nd International Conference of Marine Fungal Natural Product held in Kiel (Germany), 27–29 June 2017.
- Best poster presentation, 4th Mediterranean Young Researchers Days, Société Chimique de France held in Nice (France), 12–13 October 2016.

- Galileo Program from the Italo-French University to develop scientific collaborations between Italian and French institutions and research laboratories on joint projects and foster the mobility of groups of young scientists coming from Italian or French institutions/research centers (2017–2019).
- Vinci Program from the Italo-French University to promote the mobility of Ph.D. students with a cotutelle agreement between Italy and France (2016–2018)

PUBLICATIONS

Full Research Papers

Bovio E., Sfecci E., Gnavi G., Poli A., Prigione V., Lacour T., Mohamed M., Varese G. C. (2019). The culturable mycobiota associated with the Mediterranean sponges *Aplysina cavernicola*, *Crambe crambe* and *Phorbas tenacior*. Submitted to *Mycological Progress*.

Bovio E., Poli A., Garzoli L., Luganini A., Gribaudo G., Villa P., Musumeci R., McCormack G. P., Cocuzza C. E., Mehiri M., Varese G. C. (2019). Marine fungi from the sponge *Grantia compressa*: biodiversity, chemodiversity and antimicrobial potential. *Marine Drugs*.

Bovio E., Garzoli L., Poli A., Prigione V., Firsova D., McCormack G. P., Varese G. C. (2018). The culturable mycobiota associated with three Atlantic sponges, including two new species: *Thelebolus balaustiformis* and *T. spongiae*. *Fungal Systematics and Evolution*, 1: 141–167.

Bovio E., Gnavi G., Prigione V., Spina F., Denaro R., Yakimov M., Calogero R., Crisafi F., Varese G. C. (2017). The culturable mycobiota of a Mediterranean marine site after an oil spill: Isolation, identification and potential application in bioremediation. *Science of The Total Environment*, 576: 310–318.

Bona F., Franchino M., **Bovio E.**, Badino G., Maiorana G. (2016). Microalgae cultivation for biofuel production: optimization of environmental conditions for the intensive culture of *Neochloris oleoabundans* (Chlorophyta). *Biologia Ambientale*, 30: 1–7.

Congresses and Abstract in Peer-Reviewed International Journal or Proceeding Books

Bovio E., Poli A., Garzoli L., Luganini A., Villa P., Musumeci R., Cocuzza C. E., Gribaudo G., Mehiri M., Varese G. C. (2018). The biotechnological potential of marine fungi. 8th International Forum on Industrial Biotechnology and Bioeconomy (IFIB). Turin (Italy) – Poster presentation.

Bovio E., Cocuzza C. E., Garzoli L., Gribaudo G., Luganini A., Musumeci R., Poli A., Villa P., Mehiri M., Varese G. C. (2018). Marine fungi within sponges: biodiversity, chemodiversity and biotechnological potential. XXXVII Annual meeting of the European Culture Collections' Organisation (ECCO). Moscow (Russia) – Poster presentation.

Bovio E., Poli A., Garzoli L., Luganini A., Villa P., Musumeci R., Cocuzza C.E., Gribaudo G., Hellio C., Mehiri M., Varese G.C. (2018). The biodiversity and biotechnological potential of sponges derived fungi. XXII Convegno Nazionale di Micologia. Siena (Italy) – Oral presentation.

Bovio E., Garzoli L., Gnavi G., Poli A., Prigione V., Mehiri M., Varese G. C. (2018). The biotechnological potential of sponges-derived fungi: an ecological perspective. 17th Congress of the International Society for Microbial Ecology (ISME). Leipzig (Germany) – Poster presentation.

Bovio E., Garzoli L., Gnavi G., Marchese P., Poli A., Varese G. C. (2018). The Mediterranean Sea: an untapped source of fungal diversity. 11th International Mycological Congress. San Juan, Puerto Rico (USA) – Poster presentation.

Varese G.C., **Bovio E.**, Garzoli L., Gnavi G., Poli A., Prigione V. (2018). Marine Fungi: the missing tile in the Ocean Biodiversity mosaic. The World Conference on Marine Biodiversity. Montréal (Canada). Published in PeerJ.

Bovio E., Gnavi G., Prigione V., Spina F., Varese G. C. (2017). Oil and plastic in the sea: could marine fungi solve environmental problems? 4^{ème} Journée de Biologie Marine Université de Nice Sophia Antipolis. Nice (France) – Oral presentation.

Bovio E., Garzoli L., Firsova D., Mc Cormack G. P., Mehiri M., Varese G. C. (2017). The unexplored fungal community of the sponge *Grantia compressa*: identification and metabolic fingerprint. 7^o Congress of European Microbiologists. Valencia (Spain) – Poster presentation.

Bovio E., Garzoli L., Mc Cormack G. P., Mehiri M., Varese G. C. (2017). Biodiversity and chemodiversity of the fungal community associated with the Atlantic sponge *Grantia*

compressa. International Conference of Marine Fungal Natural Products. Kiel (Germany)
– Oral presentation.

Bovio E., Garzoli L., Gnavi G., Firsova D., Mc Cormack G. P., Mehiri M., Varese G. C. (2016). Marine fungi isolated from the sponge *Grantia compressa*: biological and chemical diversity. 4th Mediterranean Researchers Days. Nice (France) – Poster presentation.

Bovio E., Garzoli L., Firsova D., McCormack G. P., Varese G. C. (2016). Marine fungi isolated from the sponge *Pachymatisma johnstonia* in the Atlantic Ocean. Congress of the Italian Society of Marine Biology, Turin (Italy) – Oral presentation. Published in *Biologia Marina Mediterranea*, 23: 302–303.

Bovio E., Gnavi G., Spina F., Prigione V., Varese G. C. (2016). Marine fungi from a crude-oil polluted site in the Mediterranean Sea: diversity and selection of potential bioremediation agents. Congress of the Italian Society of Marine Biology, Turin (Italy) – Oral presentation. Published in *Biologia Marina Mediterranea*, 23: 317–318.

Varese G.C., **Bovio E.**, Garzoli L., Gnavi G., Perugini I., Poli A. Prigione V., Reale L., Spina F., Tigini V. (2016). Diversity of marine fungi and their biotechnology potential. ICOMID Microbial Diversity resource potential, Moscow (Russia).

Gnavi G., Prigione V., **Bovio E.**, Varese G.C. (2014). The mycobiota of a marine site contaminated by crude oil: isolation, characterization and strain selection for further application in bioremediation. MedRem-2014, Microbial resource management for polluted marine environments and bioremediation, Hammamet (Tunisia). Proceeding of the Ulixes International conference.

Carnevale G., Barberis M., Bosio N., **Bovio E.**, Delfino M., Girodo A., Labartino M., Novo S., Pavia M., Tamagnone E., Tessore V., Trincas E., Visentin S., Pavia G. (2014). Evidence of possible protective pigmentation in the Messinian cyprinodontid *Aphanius crassicaudus* (Agassiz, 1832). Giornate di Paleotologia XII edizione-volume dei riassunti, Catania (Italy)
– Poster presentation.

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