DEVELOPMENT AND VALIDATION OF A NOVEL APPROACH FOR DELINEATING THE ROLE OF SPONGE-BACTERIA INTERACTIONS: *APLYSILLA ROSEA* AS A MODEL SPONGE

A thesis submitted for the award

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DECLARATION

I certify that this thesis does not contain material which has been accepted for award of any degree or diploma; and to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text of this thesis or in the notes.

Mohammad Ferdous Mehbub

DEDICATION

I would like to take this opportunity to glorify this thesis with the name of Allah, the beneficent, the merciful.

I would like to dedicate my thesis to my family especially my father Professor Md Mamtazur Rahman, my mother Firoza Shameem, my wife Deena and my daughters Ithica and Erana for their endless love and support.

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ABBREVIATIONS

$^{\circ}C$	degree Celsius
µg	microgram
µl	microliter
AAM	Antibiotic Agar Medium
bp	base pairs
bTEFAP	Bacterial Tag–Encoded FLX Amplicon Pyrosequencing
CFU	Colony forming units
$CHCl_3$	chloroform
CTAB	Cetryltrimethylammonium bromide
DNA	Deoxyribonucleic acid
dNTPs	Dinucleotide triphosphates
EDTA	Ethylenediamine tetraacetic acid
ERPA	Enzyme resolving power analysis
ESI-MS	Electrospray ionization mass spectrometry
EtOH	ethanol
f	forward
g	gram
GC	Gas chromatograph
GC-MS	Gas Chromatography Mass Spectrometry
HPDA	Half-strength Potato Dextrose Agar
HPLC	High-performance liquid chromatography
hr	Hour(s)
IPCA	Individual principal component analysis
L	Liter
LB	Luria broth
LC-MS	Liquid Chromatography-Mass Spectrometry
m/z	Mass to charge ratio
MeOH	methanol
mg	milligram
MiCA3	Microbial Community Analysis III
min	Minute(s)
ml	millilitre

MS	Mannitol soy agar
NCBI	National Centre for Biotechnology Information
NRPS	Non-ribosomal peptide synthase
PCA	Principal component analysis
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
PKS	Polyketide synthase
PLS-DA	Partial least squares discriminant analysis
ppt	Parts per thousand
PSPA	Primer sequence prevalence analysis
PVP	Polyvinylpyrrolidone
r	reverse
RDP	Ribosomal Database Project
RFLP	Restriction Fragment Length Polymorphism
sp.	species
spp.	species (plural)
TLC	Thin Layer Chromatography
TREND	Transects for Environmental monitoring and Decision making
TRF	Terminal restriction fragment
TRFLP	Terminal restriction fragment length polymorphism
TSA	Tryptone soya agar
TSB	Tryptone soya broth
UV	Ultraviolet

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ABSTRACT

Sponge-bacteria interactions are hypothesized to play a critical role in the biosynthesis of sponge-derived natural products. However, this hypothesis is experimentally difficult to test as sponges interact with a wide range of bacteria that live in or associate with them, as well as those in the external environment. To experimentally test this hypothesis this study proposed a novel approach to understanding the role of the interactions between bacteria and sponges using a controlled experimental aquarium system whereby a bacterium of choice was exposed to sponge explants at a specific density to initiate the sponge-bacteria interaction. To develop and validate this experimental system, the role of the interactions between a marine sponge *Aplysilla rosea* and two types of bacteria: *Vibrio natriegens* as a representative external bacterium from seawater and *Streptomyces* sp. isolated from the host sponge as a representative sponge-associated bacterium were studied by characterizing the impact on the sponge-associated microbial diversity, its metabolite profile and representative antibacterial bioactivities.

To develop this approach, the first task was to design and establish the experimental system and protocols. The key challenges were to keep the sponges alive with little disruption during collection, transport and maintenance in a controlled aquarium, and to minimize the biological variations. To establish the experimental system, a total of 12 different sponge species from six different orders were collected from South Australian marine waters to establish the protocols for their long-term maintenance to be used in this study. *Aplysilla rosea* was chosen for this study based on its availability, chemical profile and adaptability and its ability to survive up to 40 days under aquarium conditions.

To understand the impact of sponge-bacteria interactions on the sponge-associated microbial community, a method for characterizing these changes was established and validated. The sponge-associated microbial community was investigated using T-RFLP analysis and/or pyrosequencing. TRFLP analysis was successfully optimized to generate results better aligned with pyrosequencing.

To characterize the metabolite changes in sponges, a metabolite profiling approach was established with separate protocols for analyzing apolar metabolites by GC-MS and polar metabolites by TLC, HPLC and ESI-MS. To detect the changes in biological activities produced by the sponge as a result of the challenge by the microorganisms, an antibacterial assay against two common human pathogenic bacteria *Staphylococcus aureus* and *Escherichia coli* as well as a nonpathogenic bacteria *Vibrio natriegens* were used.

While any sponge-associated bacterium can potentially be chosen for a sponge-bacteria interaction study, for this study only sponge-associated actinobacteria were targeted for isolation due to their potential role in sponge ecology and defence. The main aim of the isolation study was to obtain culturable sponge-associated actinobacteria to be used to achieve an understanding of the role of interaction between sponge and sponge-associated bacteria. A *Streptomyces* ACT-52A isolated from *Aplysilla rosea* was selected based on good sporulation and high antimicrobial activity for the validation study.

The first validation study was performed by exposing the sponges to a non-pathogenic external representative bacterium *V. natriegens* from a marine environment not associated with this sponge. The sponges in a series of controlled aquaria were exposed to *V. natriegens* at $5x10^6$ cfu/ml and monitored for 48 hours. Significant shifts in the microbial communities associated with the sponges were found after 24-48 hours, as revealed by TRFLP and pyro-sequencing. The addition of *Vibrio* caused significant changes in the sponge metabolite profile. Some new bioactive compounds in sponges treated with *Vibrio* were found, but not identified. The antibacterial activity against *Staphylococcus aureus* was greater in the extracts of treated sponges compared to the control group.

The second validation experiment was conducted using a sponge-associated actinobacterium ACT-52A, with an improved experimental system and design. The main improvement in the experimental system was to include adsorbent resin XAD-7 added into the aquarium by using a bag during experiments to capture the metabolites released. The changes in microbial community and metabolite profiles of sponge were different when the sponge interacted with *Streptomyces* ACT-52A compared to the *Vibrio* sp. The investigation of metabolites released into the seawater captured by XAD-7 resin during treatment indicated a distinct metabolite profile when compared to the sponge tissue and indicated many new compounds were secreted into the water with some of them demonstrating higher antibacterial activities.

As it is not possible to observe and test the interaction between sponge and microbes under the sea, this controlled experimental aquarium system could be a very valuable experimental tool to simulate a range of sponge-bacteria interaction conditions where experimental data can be obtained and analyzed to test many hypotheses on the roles of the sponge-bacteria interactions. This study has shown for the first time that the exposure of the sponge *A. rosea* to its associated bacterium, *Streptomyces* ACT-52A, in a controlled aquaria system had significantly altered its bacterial community, metabolite profiles and antibacterial activity against *S. aureus*. Another significant finding is that a diverse range of metabolites was secreted into the seawater by the sponges during the exposure experiments. The application of bacteria could be a trigger or an elicitor for the production of new biologically active compounds. This finding opens up a new avenue for sponge-derived natural product discovery for secreted metabolites that is still to be investigated. The experimental approach established in our laboratory could be extended for future studies of the roles of sponge-bacteria interactions, which call for more systematic design focusing on different types of interactions, rather than different types of bacteria.

CHAPTER 1

INTRODUCTION

As the most ancient animals in the world sponges (phylum Porifera) are evolutionarily important multicellular organism (Nichols & Wörheide 2005; Wang et al. 2010). As both human and sponges are holobionts (Webster & Taylor 2012) and host great amounts of bacteria within their bodies, understanding the ecological, evolutionary and biotechnological aspects of sponges are important for human welfare. As the second largest benthic biomass in tropical oceans, sponges are sessile and filter-feeders and their remarkable filter-feeding ability makes them important players in the overall ecosystem by contributing to the benthic communities (Hentschel et al. 2012; Simister 2012). Sponges have attracted great attention due to their capacity to produce a huge diversity of bioactive compounds that are of pharmaceutical and biotechnological interest (Taylor et al. 2007). The complex and as yet not well-understood role of the interaction between sponge and their associated bacteria is hypothesized to be the key regulator of the biosynthetic production of these bioactive compounds. This study aimed to develop and validate an experimental system by which we can study and understand the role of this interaction in biosynthesis regulation.

1.1 Function of sponge-associated bacteria

The sponge body is mainly composed of sponge tissue and bacteria, archaea and eukaryote (Taylor et al. 2007). Of the three domains in life (Archaea, Bacteria and Eukaryota), sponge-associated bacteria play important roles in sponge biology and ecology, such as by producing secondary metabolites (Stierle et al. 1988; Unson & Faulkner 1993; Unson et al. 1994; Salomon et al. 2004; Schmidt, EW 2005; Grozdanov & Hentschel 2007). Sponge body mass can contain up to 40% of bacteria (Taylor et al. 2007). The general body plans of sponges are Asconoid, Syconoid and Leuconoid (Ruppert & Barnes 2004). The outer layers contain cyanobacteria and eukaryotic algae (Wilkinson, CR 1992a), the middle (mesohyl) layer contains heterotrophic and autotrophic bacteria extracellularly (Hentschel et al. 2006; Simister 2012) and a few bacteria can be located intracellularly as endosymbionts (Vacelet & Gallissian 1978; Friedrich et al. 1999; Fuerst et al. 1999; Lee et al. 2001).

Many studies have been conducted to understand the different functions of sponge-associated bacteria. Based on those studies we can categorize the functions of sponge-associated bacteria as follows:

- Carbon metabolism, photosynthesis and nutrition (Wilkinson, CR 1978, 1979b; Wilkinson, CR 1983; Cheshire et al. 1997; Steindler et al. 2002; Yahel et al. 2003; Erwin & Thacker 2007; Yahel et al. 2007; Erwin & Thacker 2008)

- Nitrogen cycling (Wilkinson, CR 1979a; Shieh & Lin 1994; Hentschel et al. 2002; Diaz et al. 2004; Bayer et al. 2008; Mohamed et al. 2008a; Hoffmann et al. 2009; Mohamed et al. 2010; Off et al. 2010).
- Sulphur cycling (Imhoff & Trüper 1976; Schumannkindel et al. 1997; Manz et al. 2000; Webster et al. 2001; Hoffmann et al. 2005; Hoffmann et al. 2006; Taylor et al. 2007; Hoffmann et al. 2008; Meyer & Kuever 2008)
- Methane oxidation (Vacelet et al. 1995; Vacelet et al. 1996)
- UV protection (Sara 1971; Bandaranayake et al. 1996; Shick & Dunlap 2002)
- Removal of toxic metabolites (Brusca & Brusca 1990; Bayer et al. 2008)
- Stabilizing sponge skeleton (Wilkinson, CR et al. 1981; Rutzler 1985)
- Bioactive secondary metabolites production (Bakus et al. 1986; Unson et al. 1994; Bewley et al. 1996; Hentschel et al. 2001; Osinga et al. 2001; Kubanek et al. 2002)

1.2 Bioactive compounds from marine sponges and sponge-associated bacteria

Although there is a debate about the real producers of sponge-derived bioactive metabolites, over 30% of all the marine natural compounds are directly derived from whole sponges, which are considered as the greatest producers of bioactive metabolites of all the marine organisms investigated (Mehbub et al. 2014; Blunt et al. 2015). These metabolites exhibited different types of bioactivity such as anti-viral (Müller et al. 1987; Ford et al. 1999; Qureshi & Faulkner 1999; Cutignano et al. 2000; Kelve et al. 2003), anti-microbial (Blunt et al. 2006), anti-fungal (Schmidt et al. 2000), anti-inflammatory (Abad et al. 2008), anti-fouling (Roper et al. 2009), cytotoxicity (Blunt et al. 2006) and anticancer activity (Hart et al. 2000; Piel et al. 2004; Simmons et al. 2005; Essack et al. 2011; Valeriote et al. 2012).

It is a common notion that new species of sponges are likely providing new compounds. However, the debate started when taxonomically different sponge species showed some structurally similar compounds (Unson & Faulkner 1993; Bewley et al. 1996). It has been already been established that at the species level all sponges have particular microbial associations which are termed as sponge-specific and sponge-species-specific microbial communities (Lee et al. 2011). A number of studies confirmed this theory (Friedrich et al. 2001; Hentschel et al. 2002; Taylor et al. 2004; Webster & Blackall 2009). More than 25 bacterial phyla including Proteobacteria (Alphaproteobacteria, Gammaproteobacteria, Deltaproteobacteria and Epsilonproteobacteria), Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Gemmatimonadetes and Nitrospira, Poribacteria, Planctomycetes, Verrucomicrobia and Chlamydiae (Taylor, Michael William et al. 2007; Webster & Taylor 2012) and Tectomicrobia (Wilson et al. 2014) have been discovered from sponges. Some of these phyla are common in many sponge species and this could be one of the reasons for the similar type of compounds if they are produced by sponge-

associated common bacteria (Hentschel et al. 2003; Lejon et al. 2011). Similarly the different new compounds produced by taxonomically different sponge species may be related to those unique sponge species-specific bacteria.

1.3 Interaction between sponge and sponge-associated bacteria

Interaction between sponge and their associated bacteria particularly their symbionts is very important for sponge survival and maintenance of their ecology and biology, which was identified as a focal point for future research (Taylor et al. 2007). The process of this interaction could occur in many different forms (Taylor et al. 2007). Some of the examples include food sources (Reiswig 1971, 1975; Pile et al. 1996), pathogens/parasites (Kinne, O 1980; Hummel et al. 1988a; Bavestrello et al. 2000; Webster et al. 2002), or mutualistic symbionts (Wilkinson, CR 1983, 1992a). Perhaps the phylogenetic diversity of bacteria controls the types of interaction that occur within host sponges (Taylor et al. 2007). These associated bacteria inhabit sponges in both an intracellular and extracellular state (Vacelet & Donadey 1977). Studies on sponge-bacteria interactions mainly focus on the symbiotic bacteria as a form of symbiosis which is indeed one of the most important aspects. However, the other forms of interaction (Fig. 1) could also be important, as symbiosis is not the only way sponges maintain their microbial community (Selvin et al. 2010), and interact with their environments. In this thesis the other forms of interaction described by Selvin were also considered for a model sponge *Aplysilla rosea* (Fig. 1).

1.4 Types of interaction between sponge and sponge-associated bacteria

Colonization of sponge-associated bacteria (e.g. symbiotic bacteria) is one of the key mechanisms of sponge-bacteria interactions, which may be mediated by receptor ligand interactions by highly specific immunological cross-reactions (Selvin et al. 2010). Simply, the host allows those particular bacteria to grow by recognizing them by an internal mechanism (McFall-Ngai 1994); antibiosis could be one of the mechanisms. Alphaproteobacteria and Gammaproteobacteria play roles to produce N-acyl homoserine lactone (AHLs) which are classified as quorum sensing signalling molecules and are indicated as possible regulators of bioactive secondary metabolite production (Mohamed 2007). Chemical defence is one of the powerful tools used by sponges to protect themselves from predation (Chanas & Pawlik 1995; Burns et al. 2003). Sponges use their endosymbionts as defence against pathogens by inhibiting the colonization of potential pathogens (Kitano & Oda 2006). This inhibiting process could be accomplished in many different ways such as by producing bacteriocins or by altering the host physiology or by priming the immune system (Daw & Falkiner 1996; Falk et al. 1998; Hooper et al. 1998; Hooper et al. 2001; Hooper et al. 2003). This interaction could produce defensive enzymes by the sponge-associated bacteria such as phospholipase A2 (PLA2) that acted against fouling pressure (Selvin et al. 2010). The most

discussed and established mechanism of interaction is symbiosis between host and microbial symbionts for several purposes such as nutrient acquisition (Wilkinson, CR 1992b; Vacelet et al. 1995), stabilization of the sponge skeleton (Rutzler 1985), processing of metabolic waste (Wilkinson, CR 1978; Beer & Ilan 1998), and production of secondary metabolites (Schmidt et al. 2000).

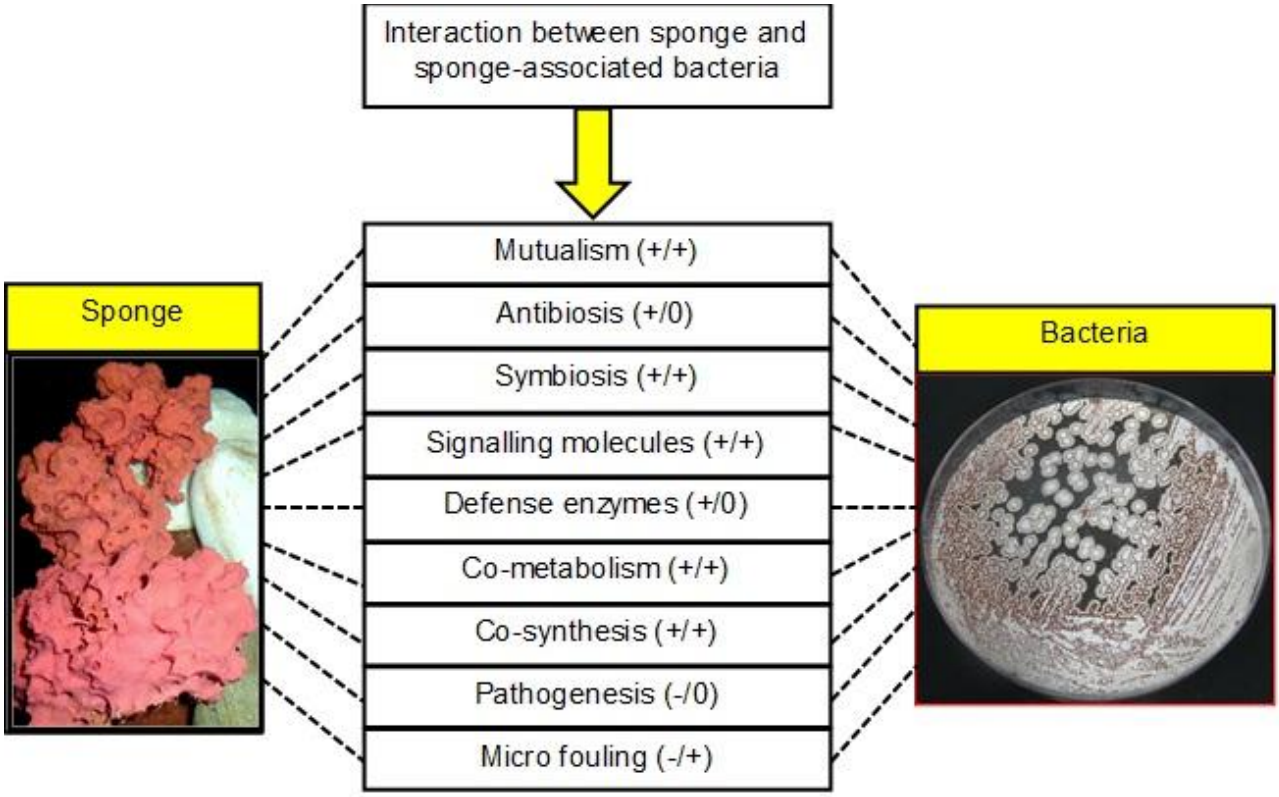

Fig 1. The hypothetical mechanisms of interaction between sponge and sponge-associated bacteria (adapted from Selvin et al. 2010)

1.5 Approach to study the roles of sponge-bacteria interaction

As it is not possible to observe and test the interaction between sponges and microbes in the sea, the controlled experimental aquarium system proposed in this thesis would be very useful to simulate a range of sponge-bacteria interaction conditions where experimental data could be obtained and analysed to test many hypotheses on the roles of sponge-bacteria interactions. A hypothetical model was developed at the beginning of this thesis to achieve the study aims (Fig. 2). Different analytical tools (genomics, transcriptomics, metabolomics and functional bioassays) can be used to gain fundamental insights into the scientifically intriguing questions of sponge-bacteria interactions and their roles in the biosynthetic metabolism of a myriad of marine natural products as well as their ecological functions and evolutionary development. As the first step to validate this experimental system and approach, only molecular analysis of microbial community, instrumental

analysis of metabolite profiles, and antibacterial activity as a demonstration of bioactivity were investigated.

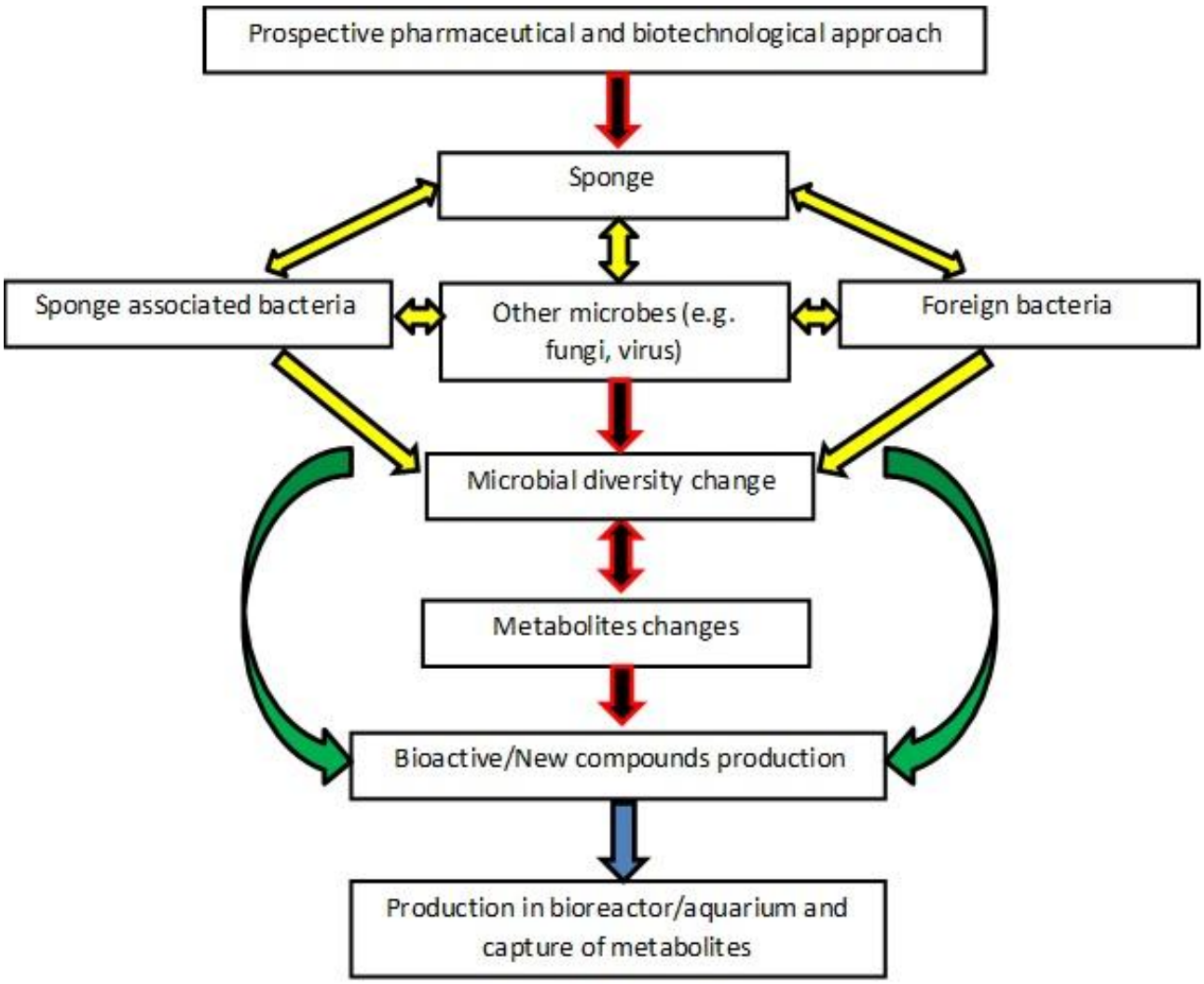

Fig 2. Conceptual study design of the roles of sponge-bacteria interaction.

1.6 Past research on culturing sponges in laboratory settings

Sponge mariculture started with the aim of producing sponge-derived bioactive compounds particularly secondary metabolites (Kim & Dewapriya 2012) as the chemical synthesis of many of these compounds of interest was difficult (Thakur & Müller 2004). Research has also been conducted to achieve optimum conditions for sponge growth and to produce metabolites by *ex situ* cultivation (Sipkema et al. 2005). Manipulation in *ex situ* culture (in tanks) proved more advantageous than mariculture due to the benefit of being able to control many environmental parameters such as salinity, temperature, light, oxygen and food (Thornton & Kerr 2002; Sipkema et al. 2005; Yi et al. 2005; Osinga et al. 1999b; Osinga et al. 2003). It has been arguably concluded that cultivation of sponge explants under controlled aquarium conditions would contribute to

ensuring the supply of pharmaceutical compounds from sponges (Yi et al. 2005), however is still lack of scale-up success.

Sponge explants from different orders have been successfully cultivated in laboratory-based aquaria for metabolite production purposes (Osinga et al. 1999b). Sponge explants were better for cultivation in mariculture due to their ability to grow rapidly compared to whole sponge transplants (Kinne, O 1977; Yi et al. 2005). Explant preparation has been described in the literature, with particular emphasis on rapid collection, conditioning, submerged cutting and bigger intact size pieces (Simpson 1963; Belarbi et al. 2003).

Explant culture has been reported for many different sponges such as *Chondrosia reniformis* (Nickel & Brümmer 2003), *Geodia barretti* (Hoffmann et al. 2003), *Geodia cydonium* (Muller et al. 1999), *Pseudosuberites andrewsi* (Osinga et al. 1999a; Osinga et al. 1999b; Osinga et al. 2003), *Chondrosia reniformis* (Nickel & Brümmer 2003), *Ephydatia fluviatilis* (Francis et al. 1990).The marine sponge *Ircinia strobilina* (order Dictyoceratida: family Irciniidae) was studied by maintaining them in a recirculating aquaculture system (Mohamed et al. 2008b). For feeding purposes, the microalga *Nanochloropsis* sp. was used at a concentration of $4x10^6$ cells/ml every 2 to 3 days and sponge health was inspected visually (Mohamed et al. 2008b). It has been reported that reduced water flow rate promotes bacterial growth on sponges (Hummel et al. 1988b) which has an impact on sponge health. Although biomass production in controlled environments of aquaria have been successful in ensuring consistent yields, many features of aquarium cultivation remain unknown for most sponges (Belarbi et al. 2003).

1.7 Ecological role of natural products in predator defence for sponges

Various ecological studies reported that secondary metabolites generated by sponges often act for protective purposes against predator attacks, microbial infections, biofouling, and overgrowth by other sessile organisms (Paul et al. 2004; Paul et al. 2006). Threatened sponges utilize their stored secondary metabolites that have cytotoxic, antibiotic and feeding deterrent properties (Proksch 1994). A study that used purified sponge secondary metabolites confirmed the presence of antifeedant properties in sponge derived natural products (Waddell & Pawlik 2000). Sponges maintain their ecology in a marine environment against pathogenic bacteria, viruses, parasites, fungus and other predators by applying their natural products, which include both chemical defence mechanisms and physiological responses (Mehbub et al. 2014). Chemical defence mechanisms are used as a safeguard against certain deleterious bacteria (Paul et al. 2001; Mahon et al. 2003; Paul et al. 2006). Perhaps most of the novel natural products derived from sponges against viral, fungal and parasitic diseases evolved in this way (Unson et al. 1994).

1.8 Research aim and overview of the thesis objectives

The hypothesis of this study was that the biosynthesis of sponge-derived bioactive metabolites is regulated by their symbiotic bacteria and influenced by the interactions between sponges and their associated bacteria and/or foreign bacteria. The aim of this study was to establish a novel experimental approach to understanding the role of the interaction between bacteria and sponges using a controlled experimental aquarium system where a bacterium of choice is exposed to sponge explants at a specific density to initiate the sponge-bacteria interaction. Marine sponge *Aplysilla rosea* was chosen as a model sponge for this study. According to the World Porifera Database this sponge belongs to the order Dendroceratida, family Darwinellidae (Van Soest et al. 2014), available under surfaces of stones on the shore, on rocks and stones below low water; depth distribution: littoral to 640 m (Bergquist 1980).

The three main objectives of this study were:

1. To understand the microbial ecology of the sponge *Aplysilla rosea* during a bacterial challenge;
2. To understand the impact of bacterial challenge on metabolic profile and bioactivity;
3. To utilize this knowledge to understand the biosynthesis regulation for improved production of bioactive/new compounds.

1.9 Thesis Outline

As this PhD thesis is a combination of published/submitted papers and written chapters are not in the format of a usual thesis, the relationships between different chapters are explained below for readers to follow the logical arrangements.

Chapter 1 provides a brief introduction and documents the organisation of the thesis.

Chapter 2 comprises a literature review in the form of two papers and one book chapter: two literature reviews on sponge-derived marine natural products (Mehbub et al. 2014; Mehbub et al. 2016) as well as a book chapter about microbes-derived marine natural products associated with sponges (Mehbub et al. 2015). The objective of this chapter is to present a comprehensive understanding of sponge-derived marine natural products from both sponges and their associated microbes. Recommendations of areas for future study and exploration, and suggestions of some possible strategies to mitigate the supply problem of obtaining new compounds as possible drug sources for the future were drawn from the two papers and one book chapter.

Chapter 3 describes the experimental set up of the controlled aquarium system for the study of sponge-bacteria interaction. Information about sponge collection and the conduct of the experiments is included.

Chapter 4 explains the key analytical techniques used for the study of microbial diversity. The focus of this study was the use of TRFLP after validating it with a next generation sequencing technology (454-pyrosequencing) in order to minimize the study costs and rapid profiling.

Chapter 5 describes the isolation of culturable actinobacteria associated with the marine sponges chosen for the present study at the early stage of this PhD project (to be submitted).

Chapter 6 describes the first validation study with the purpose to establish and validate a novel experimental approach to the study of the role of the sponge-bacteria interaction using a model sponge *Aplysilla rosea* and a foreign bacterium *Vibrio natriegens* in a controlled aquarium system (submitted to Applied and Environmental Microbiology, under review).

Chapter 7 describes the second validation study which had an improved experimental design to study the role of sponge-bacteria interaction using sponge *Aplysilla rosea* and a sponge-associated bacterium *Streptomyces* ACT-52A in a controlled aquarium system (submitted to *Applied Microbiology and Biotechnology*, under review). The major improvement was to include resin adsorbents in the seawater of the aquarium to capture the sponge-secreted metabolites.

Chapter 8 presented the major findings of this research, recommendations for future directions and the conclusions drawn from this PhD project.

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CHAPTER-2

LITERATURE REVIEW

2.1 Outline of the literature review

Chapter 2 comprises comprehensive literature review on natural products derived from sponges and sponge associated microbes, summarized in two review papers and one book chapter (Appendices 1, 2, and 3). The two review papers cover on the trend of discovery of marine natural products from sponge from 2001-2010 (Mehbub et al. 2014) and a detailed review on the sponge order that contributes to the highest number of marine natural products discovered, respectively (Mehbub et al. 2016). The book chapter covers the literature review on sponge associated microbes-derived marine natural products from 2000-2012 (Mehbub et al. 2015). The objective of this chapter is to present a comprehensive understanding of sponge-derived marine natural products from both sponges and their associated microbes to understand the trend of discovery, and the relative contribution to novel bioactive marine natural products from both sponges and their associated microbes. Recommendations on areas for future study and exploration, and suggestions on possible strategies to mitigate the supply problem of obtaining new compounds as possible drug sources for the future were drawn from these reviews. Brief outlines of these two review papers and one book chapter follow.

The first review paper entitled 'Marine sponge derived natural products between 2001 and 2010: trends and opportunities for discovery of bioactives' was published in the journal *Marine Drugs* (Mehbub M, Lei J, Franco C, Zhang W. Mar Drugs 2014; 12: 4539-4577; doi:10.3390/md12084539). Marine sponges belonging to the phylum Porifera (Metazoa), evolutionarily the oldest animals are the single best source of marine natural products. This review presents a comprehensive overview of the source, taxonomy, country of origin or geographical position, chemical class, and biological activity of sponge-derived new natural products discovered between 2001 and 2010. The data has been analyzed with a view to gaining an outlook on the future trends and opportunities in the search for new compounds and their sources from marine sponges. The published full paper is attached as Appendix 1.

The second review paper entitled 'New marine natural products isolated from sponges (Porifera) of the order Dictyoceratida (2001 to 2012): a promising source for drug discovery, exploration and future prospects' was published in the journal *Biotechnology Advances* (Mehbub M, Perkins M, Zhang W, Franco C *Biotechnology Advances* 2016; 10.1016/j.biotechadv.2015.12.008). The discovery of new drugs can no longer primarily rely on terrestrial resources, as they have been heavily exploited for over a century. During the last few decades marine sources, particularly sponges, have proven to be a most promising source of new natural products for drug discovery. This review considers the order Dictyoceratida in the phylum Porifera which has produced the

largest number of new marine natural products over the period 2001-2012. This paper examines all the sponges from the order Dictyoceratida that have provided new compounds during the time period in a comprehensive manner. The distinctive physical characteristics and the geographical distribution of the different families are presented. The wide structural diversity of the compounds produced and the variety of biological activities they exhibited are highlighted. As a representative of sponges, insights into this order and avenues for future effective natural product discovery are presented. The research institutions associated with the various studies are also listed with the aim of facilitating collaborative relationships, as well as to acknowledge the major international contributors to the discovery of novel sponge metabolites. The order Dictyoceratida is a valuable source of novel chemical structures which will continue to contribute to a new era of drug discovery. The full paper is attached as Appendix 2.

The book chapter entitled 'Secondary metabolites from microorganisms isolated from marine sponges from 2000 to 2012', Mehbub, M, Franco, C & Zhang, W, 2015, appears in Dharumadurai Dhanasekaran, Nooruddin Thajuddin & A Panneerselvam (eds), *Antimicrobials: Synthetic and Natural Compounds*, CRC Press, pp. 279-316) The continuously developing resistance of pathogenic bacteria, the re-emergence of viral diseases, and cancers that are still incurable make us redouble our efforts to find cures to alleviate human vulnerabilities. The introduction of new drugs is crucial, as older antibiotics and drugs begin to lose their efficacy. Rationally designed drugs have made inroads into the pharmaceutical industry but natural products continue to introduce the chemical diversity required to maintain a contribution of around 60% of the drugs, directly or after chemical modification, that are available in the market (Newman, D.J. & Cragg 2007). Microbial natural products contribute more than 40% of new chemical entities reported between 1981 and 2010 (Newman et al. 2003; Baltz et al. 2005; Koehn & Carter 2005; Fisher 2014). Marine sponges (phylum Porifera) are of particular interest because they are remarkable filter feeders; some can filter $24 m^3.kg^{-1}$ sponge.day^{-1} (Vogel 1977). During the filtration process, they concentrate bacterial cells that are otherwise diluted in seawater. They harbour dense and diverse microbial consortia, which comprise as much as 40% of sponge tissue volume and span all three domains of life (Taylor et al. 2007). This makes sponges excellent models for the study of marine host-associated bacteria as they represent a substantial reservoir of novel microbial diversity (Taylor et al. 2004).Therefore, in recent years the search has intensified for microorganisms from sponges with the expectation that novel compounds will result from their screening. Likewise, the often stated question whether sponge-derived natural products are biosynthesized by sponges or by associated microorganisms remains largely unanswered (Faulkner et al. 1993; Piel et al. 2004). Insights into this issue could have a significant impact on marine pharmacology. For most compounds, drug development is currently not possible due to limited access to the biological material. If the actual source is a bacterium, supply could be ensured by cultivating the producer or by isolating the biosynthetic genes and expressing the

pathway in culturable bacteria (Piel, J 2006; Schmitt et al. 2008). The advantage of the latter approach is that it should be generally applicable to a wide range of compounds independent of cultivation. Although the genetic tools to express bacterial pathways are in principle available (Fujii 2009), the application of this strategy to sponge symbionts is currently highly challenging for several reasons. This review focuses on microorganisms from sponges that have been reported to produce secondary metabolites or bioactive compounds from 2000 to 2012. The microorganisms reported here are the bacteria, with actinobacteria looked at separately due to their recognised ability to produce a wide range of secondary metabolites, and fungi, including yeast. Data has been compiled from the published literature and data reviewed by Faulkner and Blunt (Faulkner, DJ 2002) and (Blunt et al. 2003, 2004, 2005, 2006; Blunt et al. 2007, 2008; Blunt et al. 2009; Blunt et al. 2010, 2011; Blunt et al. 2012; Blunt et al. 2013; Blunt et al. 2014) from Natural Product Reports of 2000–2012. The book chapter can be found in Appendix 3.

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CHAPTER 3

EXPERIMENTAL SETUP OF CONTROLLED AQUARIUM SYSTEM FOR THE STUDY ON THE ROLES OF SPONGE BACTERIA INTERACTION

3.1 Introduction

This project requires live marine sponges in laboratory aquarium conditions. Therefore, efforts were made to study and understand the requirements of experimental sponges to remain healthy in aquarium conditions. This was the first step, but one of the most challenging parts of the entire PhD project. A significant amount of time was devoted to setting up the laboratory aquarium system as well as to studying the requirements of live sponges to make sure they keep alive and healthy in aquarium conditions. As sponges are habituated to living in the wild environment, not all sponges are necessarily suitable for aquarium conditions. It is, in fact, possible to cultivate sponges from cuttings taken from a parent in the controlled environments of aquaria (Belarbi et al. 2003). Although in nature sponge growth varies with the seasons as the food quality and quantity fluctuate, in aquarium conditions most of the parameters can be controlled for consistent maintenance of sponges (Le Pennec et al. 2003). The aquaculture of sponges is a growing field, which has been reviewed previously (Osinga et al. 1999). There are many factors that could influence the growth and survival of the sponges such as food, supply of silicon (for some sponges), temperature, oxygen, light, water quality, toxins, contaminants and diseases. The aim of this chapter is to set up the experimental aquarium system and establish key operating parameters to ensure the survival and consistent health of sponges in aquarium in order to conduct the sponge-bacteria interaction study described in Chapters 6 and 7.

3.2 Materials and Methods

3.2.1 Design of the laboratory aquarium for sponge maintenance

At the beginning of the study big buckets (~200 liters) with seawater (Fig 1 A and B) were used to keep the sponges alive. Later it became evident that sponges cannot survive for long without water exchange, food supply and aeration in the big buckets. Therefore an aquarium was designed and constructed with a recirculating water exchange capacity, aeration facility and bio filtering ability, as shown in Fig 2 A and B.

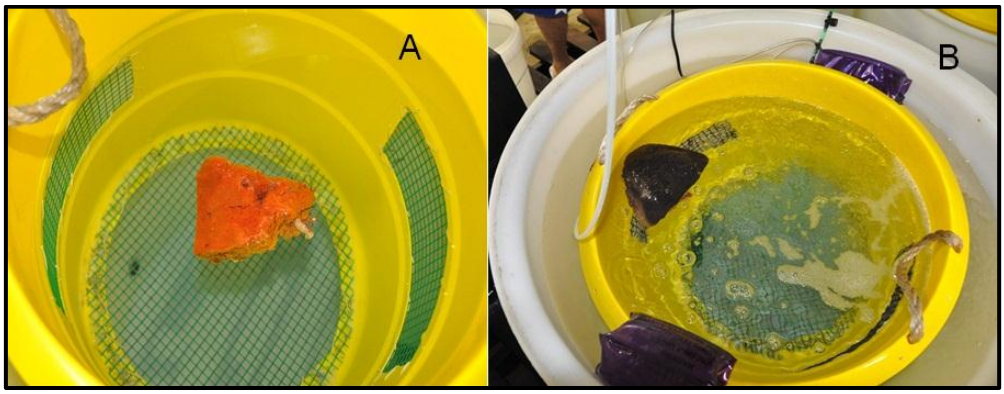

Fig 1. (A) Maintenance of sponge *Tedania* cf. *anhelans* without aeration in buckets and (B) Maintenance of sponge *Psammocinia* sp. with aeration in buckets with ~200 liters water.

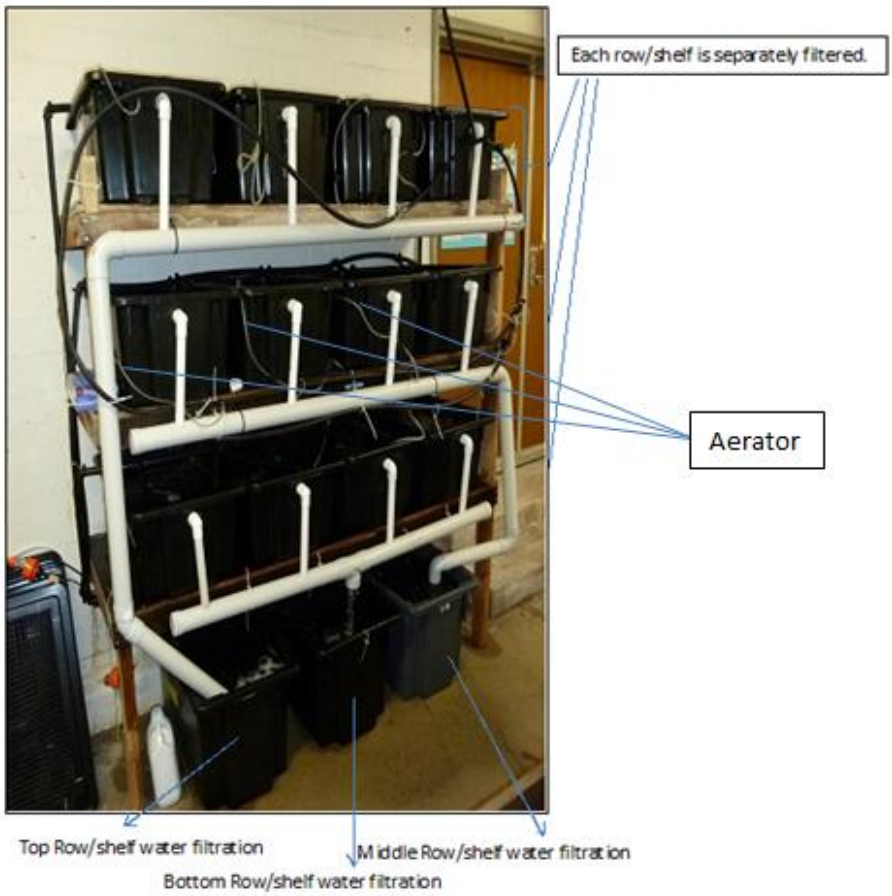

Fig 2. A well-designed aquarium for maintenance of different types of sponges in different aquarium tanks.

These aquaria were reasonably large with a capacity of ~100 liters each and were capable of maintaining 30-40 sponge explants with a dimension of 6 cm each in different tanks. Although these aquaria were found suitable for general maintenance, it was not possible to conduct the sponge-bacteria interaction using this aquaria system due to the continuous flow of seawater from one tank to another with the same filtering system.

3.2.2 Design and set up of the laboratory aquarium for sponge-bacteria interaction study

A controlled closed aquarium system was therefore designed for the conduct of the sponge-bacteria interaction study to fulfil the requirement of an experimental design, as shown in Fig 3. The capacity of this particular aquarium was 10 liters (Fig 4); however, a few 30 liters aquaria were also designed for the experiments requiring higher amount of metabolites for downstream analysis using bigger size (10-15 cm) sponges.

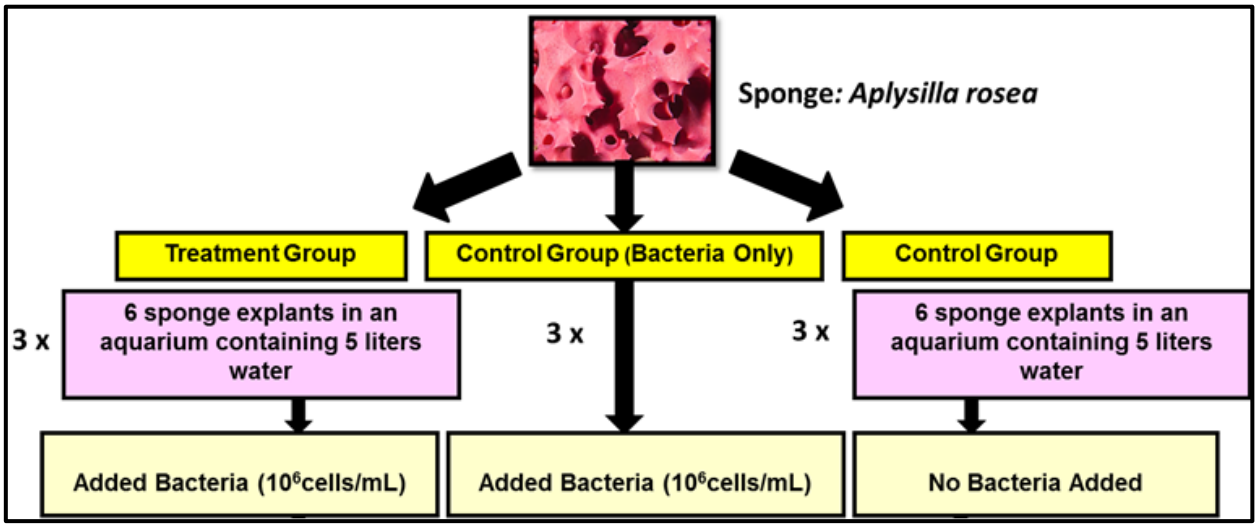

Fig 3. Experimental design for conducting the sponge-bacteria interaction study in a closed aquarium system.

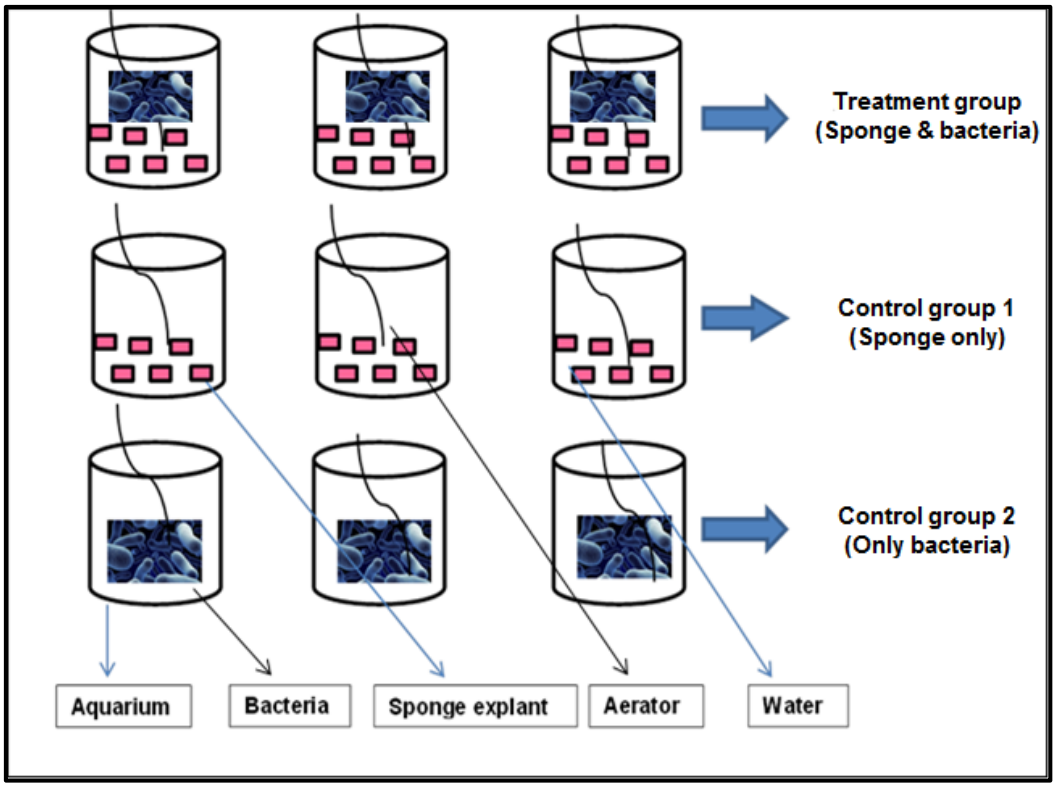

Fig 4. A schematic diagram of control closed aquaria system for conducting sponge-bacteria interaction experiments.

3.2.3 Checklist before commencing field trips

One of the essential activities in this study was the field trip for collecting the sponges from different locations in South Australia. A safe operating procedure was developed and followed in the conduct of the field trips, as shown in Appendix 4.

3.2.4 Collection of different sponges from jetty pylons

Sponges were carefully removed from jetty pylons by scuba diver with a scraper, so as not to damage them or minimize the damages to the sponges. The samples were kept immersed in seawater in plastic bags and immediately transferred to buckets of seawater in the boat on the surface without exposure to air, as shown in Fig 5. Approximately 50 liters capacity big buckets were used to transport the sponges.

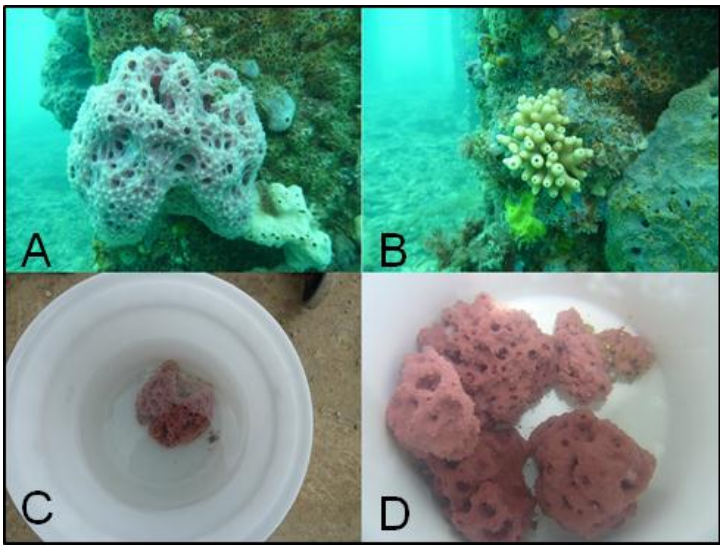

Fig 5. A and B (*Dysidea* sp.) and B (*Euryspongia* sp.) showing sponges attached to jetty pylons in Rapid Bay and C and D showing immersion of *Aplysilla rosea* under seawater in a bucket after collection from jetty pylons.

Once sponges were put in the buckets, the boat came to the jetty and sponges were collected from the jetty stairs, as shown in Fig 6.

Fig 6. Transfer of sponges from boat to the jetty stairs.

3.2.5 Transportation of sponge samples from field to the laboratory aquarium

After receiving the sponges at the jetty, the bucket water was replaced with fresh seawater several times. This procedure was carefully conducted to avoid exposure to the air. Sponges were transported to the aquaria from different locations in South Australia such as Rapid Bay, Second Valley, Outer Harbour, Ardrossan, Klein Point, Stenhouse Bay, and Large Bay. The average time taken to bring the sponges from each location to the aquaria was about 2-3 hours. An air-conditioned car was always used to transport the sponges.

3.2.6 Conditioning of sponges after transportation

Once the buckets with sponges reached the laboratory, conditioning of sponges started by providing fresh seawater stored in a reservoir in the marine aquaria by using a hose pipe. This process was conducted slowly to adapt the sponges to their habitat water to minimize the shock. Five to six water changes were conducted at 20 minute intervals to remove any toxins released as a result of transport stress.

3.2.7 Transfer of sponges from bucket to aquaria

After finishing the conditioning of the sponges, they were transferred to transparent glass or plastic aquaria, as shown in Fig 7. All the good and healthy sponges were sorted accordingly and each was transferred to a big black aquarium (100 liters capacity of water) with some rocks underneath, as shown in Fig 8. The transfers were conducted with a big plastic bag with each individual sponge piece in a submerged condition. Under no circumstances were sponges exposed to air.

Fig 7. Transfer of sponges from buckets to aquaria for sorting purposes

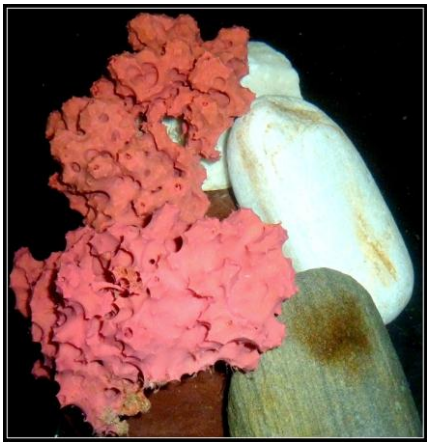

Fig 8. Transfer of sponges from plastic/glass aquarium to a 100 liters capacity aquarium with rocks underneath the sponges.

3.2.8 Cutting sponges under water

The techniques for sponge explant preparation have been previously described (Simpson 1963). For this study, explants were initially prepared from 12 morphologically different types of sponges from six different orders (Table 1). Later on three sponges named *Aplysilla rosea*, *Aplysina* sp. and *Euryspongia* cf. *arenaria* were found to be more suitable in aquarium conditions. However, only *Aplysilla rosea* was used extensively for this project. During the explant preparation healthy sponges were used in a submerged condition in relatively cold water (15°C) and free from any encrusting growths of other organisms (Belarbi et al. 2003). The explant preparation was preferably conducted after conditioning sponges in a big plastic or glass aquarium with a range of 20 to 100 liters capacity depending on the size and number of sponges. A sharp sterile scalpel (Swann-Morton®) was used for cutting explants after putting the sponges onto a cutting board under water. The use of a cutting board avoided squeezing, which is one of the main causes of sponge tissue damage (Osinga et al. 1999).

3.2.9 Food supply and feeding of sponges

Supply of adequate food is one of the key factors of keeping the sponges alive and healthy in aquarium conditions. Two different kinds of food, cyanobacteria Spirulina and microalgae *Nanochloropsis* sp., were mainly provided as foods for the sponges. Sponges were fed with either Spirulina or microalga *Nanochloropsis* sp. (10^6 cells/ml) every 2 to 3 days. During the feeding, the recirculating pump and aerator were turn off for 1-2 hours. After the feeding the pump was turned on again for recirculation and biofiltration.

3.2.10 Control of the temperature

The air-conditioning system in the marine aquarium was set to 15°C as default. This temperature was maintained all the year round. The water temperature was also frequently monitored by thermometer.

3.2.11 Control of oxygen supply

Supply of oxygen was ensured by providing an aerator in each aquarium by bubbling in a confined space. Oxygen was measured by Orion Star™ A329 Dissolved Oxygen Portable Multiparameter (Thermo Scientific).

3.2.12 Control of light

All aquaria were black in colour to control the exposure from too much light. Twelve-hour light cycles were maintained in the aquaria throughout the year.

3.2.13 Control of waste removal and maintenance of water quality

In order to control the waste, recirculating water from the biofiltarion tank was regularly monitored and the water exchanged on alternate days. To monitor the water quality, ammonia, nitrate, nitrite and pH were observed twice a week. Ammonia, nitrite and nitrate were measured by using commercial kit (Aquaone) and pH was measured by pH paper (MN 921 11) with a range of 0 - 14 PPT.

3.2.14 Maintaining salinity

Salinity is a very important factor for sponge survival. Therefore salinity was measured once a week by a portable refractometer (Thermo Scientific) and top up with seawater when required to maintain the salinity.

3.3 Results and Discussion

In this project, 12 morphologically different types of sponges from six different orders (Table 1) were tested in a newly designed aquaria system and a range of different operating conditions. The advantage of maintaining sponge explants in aquaria has been described in literature (Le Pennec

et al. 2003). Only a few sponges, particularly *Euryspongia* cf. *arenaria*, *Aplysina* sp. and *Aplysilla rosea* showed better adaptability in aquarium conditions. Other sponges failed to adapt, perhaps due to their experience of significant changes in environment, or nutrient requirements. Larger explants showed better longevity than smaller explants due to their high proportion of intact skin (Duckworth et al. 1997). All the different types of sponges were evaluated for their survivorship for a maximum 4-8 weeks. As an example shown in Fig 9, although *Aplysilla rosea* and *Aplysina* sp. can easily survive for four weeks in aquaria conditions, *Psammoncinia* sp. and *Holopsamma laminaefavosa* died quickly, in less than four weeks. Based on the initial survival study, only three sponges (*Euryspongia* cf. *arenaria*, *Aplysina* sp. and *Aplysilla rosea)* were further maintained for their survivorship study until they died. *Euryspongia* cf. *arenaria* was able to survive for eight months, *Aplysina* sp. for 6 months and *Aplysilla rosea* almost for 2 months.

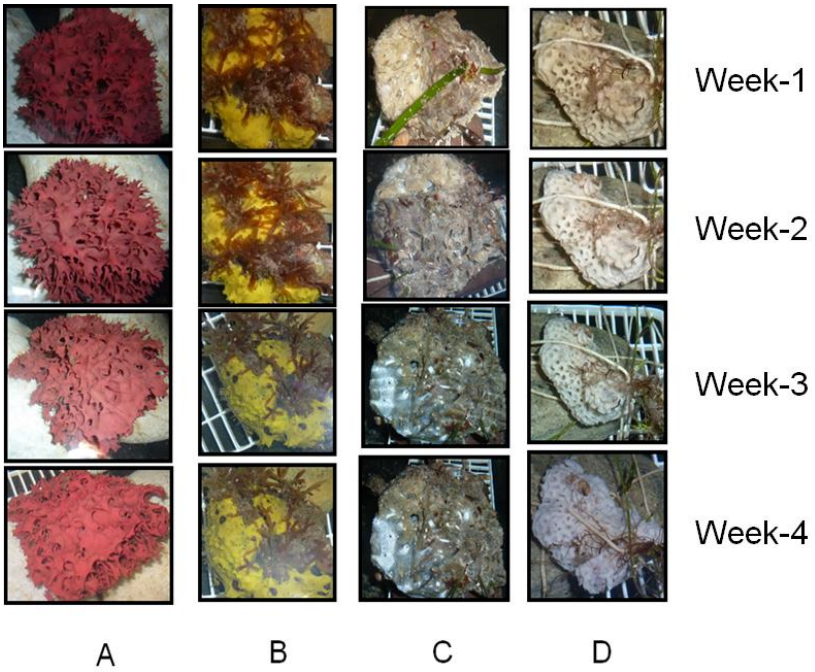

Fig 9. A, B, C and D showing four different sponges *Aplysilla rosea*, *Aplysina* sp., *Psammocinia* sp. and *Holopsamma laminaefavosa* respectively in aquaria condition for four weeks.

Table 1: Name and order of sponges used for the maintenance study in the aquaria conditions

Name of sponge	**Order**
Aplysilla rosea	Dendroceratida
Aplysina sp.	Verongida
Suberites domuncula	Hadromerida
Dendrilla cactos	Dendroceratida
Dysidea avara	Dictyoceratida
Psammocinia sp.	Dictyoceratida
Clathria sp.	Poecilosclerida
Holopsamma laminaefavosa	Poecilosclerida
Euryspongia sp.	Dictyoceratida

Euryspongia cf. *arenaria*	Dictyoceratida
Tedania (Tedania) cf. *anhelans*	Poecilosclerida
Ecionemia robusta	Astrophorida

The sponge *Aplysilla rosea* was primarily chosen for this study due to their high abundance and availability in South Australia. Besides, *A. rosea* possesses a unique chemical profile revealed by GC MS and showed high antimicrobial activity. Although the other two sponges were in the study plan due to time limitation it was not possible to use them.

It has been observed that food supply is one of the most important factors for sponge survival in aquarium conditions. This is due to the use of filtered seawater, which has less food available for the sponges. Both Spirulina and *Nanochloropsis* worked well for the sponges as a food supply. It was important in this study to dilute the food properly. Concentrations of 10^6 to 10^8 cells/ml of either Spirulina or *Nanochloropsis* were found feasible and tolerable for sponges. However, highly concentrated food (~10^{10} cells/ml) was found causing spoilage into the filtration system and the sponges failed to tolerate that high concentration in their confined space. It was also found that *Nanochloropsis* has better palatability for *Aplysilla rosea* compared to Spirulina as these sponges were found healthier by observing surface tissue. This observation is in agreement with the finding that sponge food choice could vary from species to species (Osinga et al. 2003).

The salinity of seawater (35ppt) was maintained in the aquaria. However, it was found that when the salinity accidentally went up more than 40 ppt all *Aplysilla* died overnight, although some sponge species have been reported for their high salinity tolerance, such as *Hippospongia lachne* (Osinga et al. 1999).

The temperature was kept stable throughout the study at 15°C, which was found suitable for all the sponge species studied. Influence of light on sponge survival was observed in *Aplysilla rosea* and *Euryspongia* cf. *arenaria*. These two sponges were found healthier at the top of the aquarium shelf where they were exposed to more light. Perhaps, this is due to the abundance of phototrophic symbionts compared to the heterotrophic species associated with these two sponges. It has been reported that photosynthetic endosymbionts require more light for survival (Belarbi et al. 2003). It might possible that as these symbionts serve as nutrients for their host sponge (Sara 1971), the sponges on the top shelf survived longer than the sponges in the darker area (Fig 2).

Throughout the study the pH was kept stable within a range of 7.8 to 8, which has been reported as suitable for sponge growth (Brown et al. 1995). It was found that the water quality depends on the amount of sponges kept in the aquarium and the water exchange rate. Water exchange every alternate day was found suitable when the ammonia, nitrate and nitrite were examined. However, if too many sponges were kept in the aquarium the ammonia content increased rapidly as a range of

0.1 to 1 PPM due to accumulation of inorganic nitrogenous metabolic waste products in the surrounding water (Osinga et al. 1999) and water exchange was required every day.

Any dead sponges or debris were removed from the aquaria from time to time, as it has been observed that dead particles pollute the water (Osinga et al. 1999). In this study it has been found that dead sponges caused the mortality of the other sponges within 2-3 days and more than one type of sponge in the similar aquarium chamber died quickly, perhaps due to intraspecific chemical interactions or by producing toxins (Pawlik 1995; Chanas et al. 1997).

3.4 Conclusion

Cultivation of marine sponges in aquarium conditions is still a big challenge. In this particular study the focus was to keep the sponges alive and healthy rather than getting good growth. A few sponges *Aplysilla rosea*, *Aplysina* sp. and *Euryspongia* cf. *arenaria* were found adaptable to the aquarium conditions after properly maintaining them with feeding at a concentration of 10^6 cells/ml and monitoring the water quality with a low ammonium level (0-0.2) and pH ranging from 7.5-8. As *Aplysilla rosea* was the targeted sponge for this PhD project, most of the effort was put into optimizing suitable conditions for this particular sponge including conditioning, explant preparation, feeding, health monitoring and water exchange interval. The optimization study of different sponges in aquarium conditions helped to keep the sponges alive and healthy in the aquaria for the conduct of the sponge-bacteria interaction study.

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CHAPTER 4

COMPARISON OF TERMINAL RESTRICTION FRAGMENT LENGTH POLYMORPHISM (TRFLP) AND 454 PYROSEQUENCING FOR THE STUDY OF MICROBIAL DIVERSITY FOR MARINE SPONGE *APLYSILLA ROSEA*

4.1 Introduction

A number of culture independent DNA-based methods such as DGGE, TRFLP, pyrosequencing, and illumina sequencing are commonly used to identify or characterize the microbial diversity associated with marine sponges (Webster et al. 2001; Montalvo et al. 2005; Zhang et al. 2006; Lee et al. 2011; Abdelmohsen et al. 2014). These methods are considered to be more exhaustive as more genera and species can be identified, particularly by deep sequencing of 16S ribosomal RNA (rRNA) gene amplicon libraries (Huber et al. 2007; Costello et al. 2009; Turnbaugh et al. 2009; Andersson et al. 2010; Hollister et al. 2010; Teixeira et al. 2010). However, different molecular methods often give different results with the same samples due to the inherent biases of each method (Silva & Russo 2000). TRFLP and pyrosequencing have been the two most common methods reported in the literature. TRFLP analysis has been viewed as a rapid, convenient, sensitive, high-throughput, and highly reproducible method of characterising strains to the genus level in microbial ecology studies (Kitts 2001; Abdo et al. 2006). In addition, this method can successfully relate and identify differences in microbial community composition between different samples (Chin et al. 1999; Dunbar et al. 2000; Covert & Moran 2001; Derakshani et al. 2001). High-throughput sequencing technologies such as pyrosequencing have introduced a level of accuracy in characterising microbial diversity, often to the species level (Legge 2012), although they are still expensive (Langaee & Ronaghi 2005; Silvar et al. 2011). There are usually discrepancies between results obtained from TRFLP analysis and pyrosequencing (Castro-Carrera et al. 2014). Determining the difference and contributing factor(s) is of paramount importance if both or either of them are applied. TRFLP analysis and pyrosequencing of 16S rRNA genes are to be used in this study for characterizing the microbial communities associated with sponges. Therefore, the main aim of this chapter was to compare these two culture-independent molecular methods for characterizing the microbial diversity of the experimental sponge *Aplysilla rosea* in order to qualify these two methods for further studies in Chapters 6 and 7.

4.2 Materials and Methods

T-RFLP and pyrosequencing both require high quality DNA for PCR amplification. Therefore, in this study, a DNA extraction protocol was refined and established to obtain the community DNA from the sponge *Aplysilla rosea*.

4.2.1 DNA extraction and method optimisation

To reveal the microbial diversity associated with sponges by molecular methods, it is critical to extract high quality community DNA from sponges that could cover the utmost bacterial community within the sponge body. A published DNA extraction protocol (Atashpaz et al. 2010) was further optimized to extract high quality DNA from marine sponge *Aplysilla rosea* and their associated bacteria that can be used for microbial diversity study by molecular approaches.

4.2.1.1 Extraction procedure

Briefly, total genomic DNA was extracted from 20 mg of lypophilized sponge tissue following the literature after required modification (Atashpaz et al. 2010). The main modification was in the buffer composition (3% CTAB, 100 mM Tris-HCl, 2 M NaCl, 20 mM Na_2EDTA, 0.2% LiCl, 2% PVP and 1% β-Mercaptaethanol) along with another buffer (lysozyme 80 mg/mL, 4.8% Triton X, 8 mM EDTA and 80 mM Tris-Cl) and a tissue lyser (Qiagen) used for mechanical disruption with 1 mm beads for 30 sec at 15 Hz speed. Two separate extractions were combined for the same sample to maximize the representation of microbes. DNA was purified using a DNeasy Blood and Tissue Kit (Qiagen) and quantified in a ND-8000 UV-Visible Spectrophotometer (Thermoscientific) (please see Appendix 5 for detailed methodology).

4.2.2 Designing primers for TRFLP

Good quality DNA amplification requires appropriate primer set. This step is very important because the rest of the analysis depends on the result of PCR amplification. In order to design an effective universal primer for TRFLP analysis and to amplify as many members of the microbial community as possible in a sponge, new primers were designed in the present study by using the Primer-BLAST program which is available at the NCBI website (Ye et al. 2012).

Several primers were designed for this study based on the K12 *Escherichia coli* genomic DNA sequence retrieved from the NCBI website. As there are regions of differing variability in the 16S rRNA gene map, priority was given to totally conserved and conserved regions described in the literature, as shown in Fig 1 (Baker et al. 2003).

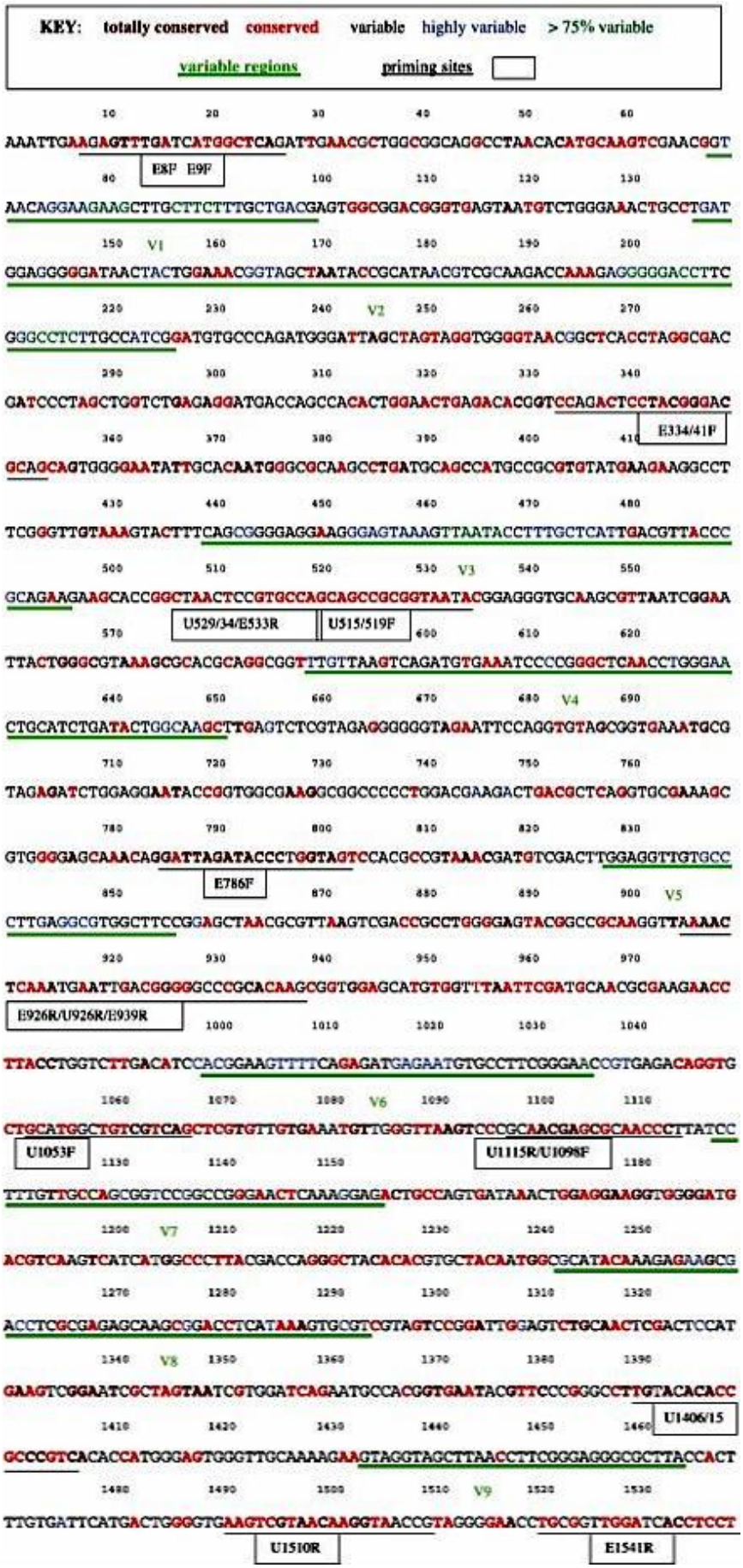


Fig 1. Variable regions V1–V9 of *E. coli* 16S rRNA gene sequence showing ‘‘universal’’ priming

sites. To indicate bacterial sequence variability, colour coding was used based on the variability map for the 16S rRNA gene adapted from the literature (Van de Peer et al. 1996).

The following criteria were considered during primer design:

1. Primer length 18-30 bases;
2. Primer melting temperature (Tm) between 54°C to 72^{0}C;
3. The primer melting temperature difference not more than 5^{0}C;
4. GC concentration 40-60%;
5. A bases not more than 3 or more times in a row;
6. Avoiding 2 G or C bases at their 3′ end within their last 5 bases.

4.2.3 Evaluation of primer sets

For evaluating the primer two different types of tools have been considered (Schutte et al. 2008). Primer sequence prevalence analysis (PSPA) (Shyu et al. 2007) available on the Microbial Community Analysis (MiCA) website (http://mica.ibest.uidaho.edu/) and the Probe Match tool on the Ribosomal Database Project website (http://rdp.cme.msu.edu/) which helps to compare the specificity and selectivity of different primer sets by providing the number of successful amplicons based on the available sequence in the database. In this study PSPA was used as it reports the number of successful amplifications for each primer individually (forward and reverse) and for each primer pair collectively (Shyu et al. 2007).

4.2.4 Template DNA and master mix to minimize PCR inhibition

Two key methods were followed to minimize the contaminants of DNA in the present study. Firstly, a silica-based column was used for purification of DNA at the final step of DNA extraction. Secondly, less DNA template was added to the PCR amplification (Bessetti 2007). Although dilution of the DNA template to minimize inhibitors in the amplification is not recommended due to the possibility of less PCR efficiency (Wintzingerode et al. 1997), in this study concentration of DNA was normalized by diluting to 25 ng/µl before starting to prepare master mix for PCR amplification. Fifty nanogram DNA was used in a total of 50 µl PCR master mix. Finally, $MgCl_2$ was added into the PCR master mix which acted as a co-factor for DNA polymerase (Focher et al. 1989) and made the PCR reaction successful.

4.2.5 Optimization of PCR reactions

A touch down PCR protocol was developed and followed: denaturation at 95°C for 5 min 1 cycle, then denaturation at 95°C for 30 s, annealing at 61°C-51°C with an -0.3°C increment for 45 s, and

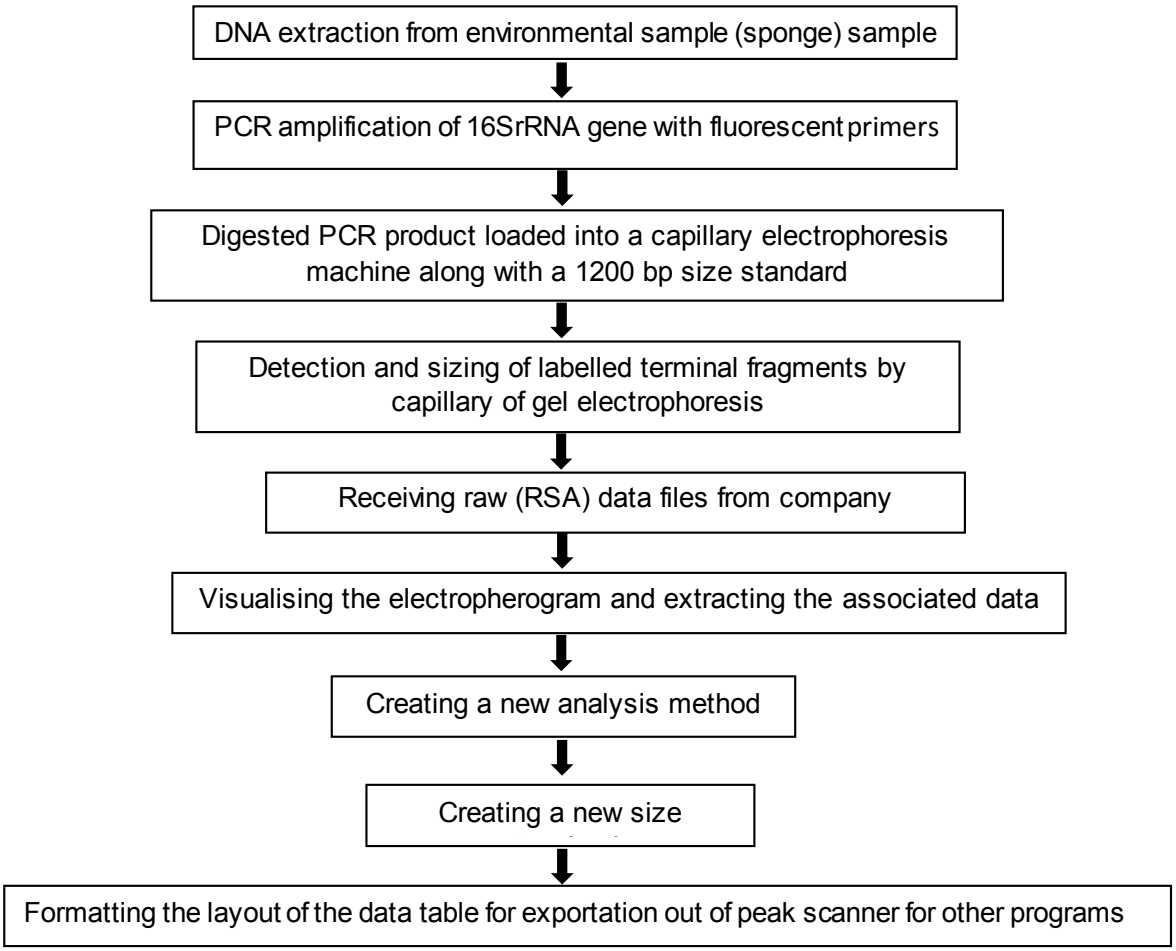
elongation at 72°C for 2 min for 40 cycles, the last cycle being followed by a 10 min elongation at 72°C.

4.2.6 Optimal choice of restriction digestion enzymes

In this study enzyme resolving power analysis (ERPA) (Shyu et al. 2007) was used to select appropriate restriction digestion enzymes, which required a pair of forward and reverse primers in order to produce the amplicons; then the search algorithm iterates through the entire list of restriction enzymes available in MiCA3. Besides, the literature showed that when the distribution of predicted fragments from 16S rRNA genes in a sequence database (Stredwick et al. 2000) were looked at carefully, as reported by Brunk et al. (1996) who used Hha1, Msp1, Rsa1, and a combined digest of both RsaI and HhaI, it was evident that Rsa1 generated restriction fragments were specific for less than four species of the same genus (Dunbar et al. 2001). The ERPA, which was used to explore the specificities of different restriction enzymes *in silico* analysis against the RDP (R10, U27) 16S bacterial rRNA database which contains a large number (1,519,357) of sequences, is a fast search algorithm that can accommodate primers and multiple restriction enzymes.

4.2.7 Analysis of TRFLP data by peak scanner and T-REX

Many steps were followed for TRFLP protocol, as shown in Fig 2.



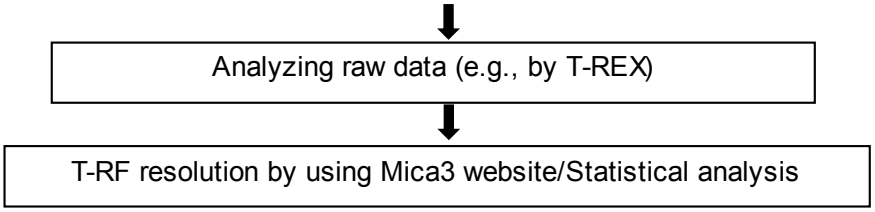


Fig 2. Flow diagram of sample preparation for analysis of TRFLP data

The universal bacterial forward primer 9F, tagged with a 6FAM label, and the reverse primer 928R were used in PCR reactions to amplify 919 bp of the bacterial 16S rRNA gene sequence. The second universal bacterial forward primer EUB8F, tagged with 6 FAM label, and the reverse primer EUB926R, tagged with a VIC label were used in PCR reactions to amplify 918 bp of the bacterial 16S rRNA gene sequence. Approximately 400 ng of purified PCR products were digested with the restriction endonucleases, Hha1, Msp1 and Rsa1 in a total reaction volume of 50 µl following the manufacturer's protocol. Immediately following digestion, samples were inactivated by heating at 65°C for 20 min for Hha1 and Rsa1 digested products. Msp1 was inactivated by adding 2.5 µl of 0.5 M EDTA. Samples were sent to Macrogen, Korea for fragment analysis. The company provided the raw FSA files after fragment analysis. Data were normalized using Dunbar's method (Dunbar et al. 2001). Peak profiles were generated using Peak Scanner Software v 2.0 (Life Technologies). The lengths of individual terminal restriction fragments (TRF) were determined by comparison with internal size standards (GS1200LIZ) using the program Peak Scanner Software™ v 2.0. TRFs beyond the resolution of internal size standards (20 to 1200 bp) or with peak areas of less than 50 fluorescence units removed.

4.2.8 Resolution of terminal fragments

Peak profiles were tabulated using the program Peak Scanner Software™ v 2.0 (Life Technologies Corporation, Carlsbad, CA) and peaks (putative populations) were identified by Microbial Community Analysis III (MiCA3), using PAT and APLAUS+ (Shyu et al. 2007) and Silva reference database (R106).

4.2.9 Analysis of pyrosequencing data

Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) was undertaken by the Research and Testing Laboratory (Lubbock, TX) as described in the literature (Dowd et al. 2008). The 16S rRNA universal eubacterial primers 28F (5′ GAGTTTGATCNTGGCTCAG 3′) and 519R (5′ GTNTTACNGCGGCKGCTG 3′) were used to amplify approximately 500 bp of the variable regions V1 to V3. Bioinformatics was conducted using the Research and Testing Laboratory Standard Pipeline (Crawford et al. 2009) and using Mothur by following the 454 SOP (Schloss et al. 2011).

4.3 Results and Discussion

DNA extraction kits become popular due to their convenience and ease of use. However, they are costly and not optimized for all kinds of DNA samples. Nevertheless, there are some successful kits used for DNA extraction from sponges. These include the Fast DNA spin kit for soil (Q-Biogene) (Hentschel et al. 2002; Thoms et al. 2003; Schmitt et al. 2008; Wilson et al. 2014), the DNeasy® Blood & Tissue kit (Qiagen®) (Erwin et al. 2012) and the Mo Bio soil DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA, USA) (Lee et al. 2011).

Although at the beginning of the study these commercial kits were used to achieve high quality DNA, PCR amplification of DNA was not successful. Careful observation from all of those protocols revealed that the first step of each protocol uses lysis accomplished by either physical disruption, chemical lysis or enzymatic lysis, or, in some cases, a combination of these three; the second step usually follows with the removal of protein and RNA contamination (Amaro et al., 2008, Syn & Swarup, 2000), and in the final step most of the commercial kits use silica-based column purification to obtain high quality DNA. One of the potential problems in the commercial kits is that they lack the power to remove the polysaccharides, glycoprotein and lipopolysaccharides (Atashpaz et al., 2010, Kalia et al., 1999). Perhaps, for this reason when commercial kits were used in this study, PCR amplification of DNA was not successful in several instances. Therefore, an optimized DNA extraction method was established for the marine sponge *Aplysilla rosea* to obtain high quality DNA for further downstream analysis.

Complex microbial communities can be studied by TRFLP based on the variation in the 16S rRNA gene (Osborn et al. 2000). The identification of genus or multiple genera can be achieved by using PCR techniques (Wilcoxson et al. 2005). The first step of PCR is designing primers which target the conserved regions of homologous genes (Polz & Cavanaugh 1998).

This is an important step because enough sequence variability between the primer sites is crucial for distinguishing various bacteria present in the samples (Brunk et al. 1996). Also, a reasonable amplicon length (~900bp) was given priority for PCR amplification, as too short amplicons will result in false patterns and true diversity will be underestimated because short amplicons will not contain a restriction site (Kitts et al. 2001). Therefore, new primers were designed in this study (Table 1).

Inhibition of PCR reaction from community DNA has been reported in the literature for various reasons such as co-extracted contaminants, differential amplification, and formation of artefactual PCR products (Wintzingerode et al. 1997). This study has minimized this inhibition by purifying DNA on a silica-based column as well as diluting the concentrated DNA (Appendix 5).

A common problem in PCR reaction is the generation of multiple undefined and unwanted products which sometimes inhibit amplification of the expected PCR product (Roux 2009). Annealing

temperature is one of the most important factors, which facilitates getting the desired product. A common way to optimize the annealing temperature is gradient PCR but this requires more time and effort, although, at the beginning of the study, gradient PCR was used to confirm optimum annealing temperature (Appendix 5). Finally, Touchdown (TD) PCR was chosen, as this method proved more convenient for PCR optimization (Don et al. 1991). Therefore, in this study TD PCR was used which also adapted well with the suboptimal buffer composition (e.g., Mg^{++} concentration) (Hecker & Roux 1996) that was used in the present study.

TRFLP analysis uses restriction enzymes which resolve putative bacterial population on detecting 16S rRNA gene sequence polymorphisms (Liu et al. 1997). The recognition site for a given restriction enzyme is unique to that particular enzyme. But, finding suitable restriction digestion enzymes depends on how efficiently it can resolve the genera or species. Four base-pair enzymes have been reported for their higher ability of recognition (Schütte et al. 2008). Therefore, in this study the four base-pair enzymes Hha1, Msp1 and Rsa1 were selected based on enzyme resolving power analysis (Fig 3) and previous reports (Erwin et al. 2012; Simister et al. 2012; Olson & Gao 2013).

TRFLP is an inexpensive and widely used technique to study microbial diversity especially for a large number of samples. TRFLP analysis can be used with up to 3 restriction enzymes to characterise microbial populations to the genus level (Conn & Franco 2004). The key to this approach is, firstly, to resolve genera by each individual enzyme by using MiCA website. Secondly, the genera resolved by the three different restriction digestion enzymes are compared and the common genera found which were resolved by the three enzymes or at least two enzymes. In this study this approach was followed in order to resolve all genera present in the sponge sample. Examples of genera which were present in control and treated samples at 48 h are shown in Table 2 and Table 3 and Table 4. This tentative characterisation was further reinforced by the identification of specific TRFs from the pyrosequencing results, which provide a more reliable identification of the microbial diversity up to the genus or species level. The TRFs corresponding to specific genera can then be used to characterise changes in populations of the system under investigation. A total of 15 primers were designed and selected for *in silico* analysis, as shown in Table 1.

Table 1. Different primers set designed for TRFLP study.

S.N	Name of Forward Primer	Forward sequence	Name of Reverse Primer	Reverse sequence	Total Product Size
1	AF	5′ACTCCTACGGGAGGCAGCAGT3′	AR	5′CCGTCAATTCATTTGAGT3′	589
2	BF	5′GAGGATGACCAGCCACACTG3′	BR	5′CCGTCAATTCATTTGAGT3′	628
3	CF	5′GAGAGGATGACCAGCCACACTG3′	CR	5′CCGTCAATTCATTTGAGT3′	630
4	DF	5′GACTCCTACGGGAGGCAGCAGT3′	DR	5′CCGTCAATTCATTTGAGT3′	590
5	EF	5′CAGGCCTAACACATGCAAGTC3′	ER	5′CCGTCAATTCATTTGAGT3′	884
6	FF	5′GTGCCAGCAGCCGCGGTAA3′	FR	5′TAGCTCCGGAAGCCACGCCT3′	350
7	GF	5′CCTACGGGAGGCAGCAG3′	GR	5′CCGTCAATTCATTTGAGT3′	586
8	HF	5′GAGTTTGATCATGGCTCAG3′	HR	5′CCGTCAATTCATTTGAGT3′	918
9	IF	5′GAGAGGATGACCAGCCACACTGG3′	IR	5′CCGTCAATTCATTTGAGT3′	630
10	JF	5′AGAGTTTGATCATGGCTCAG3′	JR	5′CCGTCAATTCATTTGAGT3′	919
11	KF	5′GAGTTTGATCATGGCTCAG′	KR	5′CCCCGTCAATTCATTTGAGT3′	920
12	LF	5′GAGTTTGATCATGGCTCAGA3′	LR	5′CCCCGTCAATTCATTTGAGT3′	920
13	MF	5′GAGTTTGATCATGGCTCAGATT3′	MR	5′CCCCGTCAATTCATTTGAGT3′	920
14	9F	5′GAGTTTGATCATGGCTCAGAT3′	928R	5′CCCCGTCAATTCATTTGAGT3′	919
15	EUB8F	5′AGAGTTTGATCMTGGCTCAG 3′	EUB926R	5′CCGTCAATTCMTTTRAGTTT3′	918

Table 2. Resolving the TRF by three different restriction enzymes Hha1, Msp1 and Rsa1 in control sponges after 48 hours treatment with primer 9F and 928R (experimental details described in Chapter 5).

Name of the Genera	Hha1	Msp1	Rsa1
Achromatium	+	+	+
Achromobacter	-	+	-
Acidithiobacillus	+	+	-
Acidovorax	-	+	-
Acinetobacter	-	+	+
Actinobacillus	-	+	+
Actinomycetales	-	-	+
Aeromonas	-	+	+
Agricultural soil bacterium	+	+	+
Alcaligenes	+	+	-
Alcanivorax	-	+	-
Aliivibrio	-	-	+
Almonella	-	+	-
Alorhosdospira	+	-	-
Alteromonas	-	-	+
Anaplasma	-	-	+
Anthinobacterium	+	-	-
Azoarcus	-	+	-
Azotobacter	-	+	+
Azovibrio	-	+	-
Bacillus	-	+	+
Beggiatoa	-	+	+
Bergeriella	-	+	-
Bermanella	-	-	+

Name of the Genera	Hha1	Msp1	Rsa1
Beta proteobacterium	+	+	-
Bordetella	+	+	-
Buchnera	-	+	-
Burkholderia	+	-	-
Candidatus	+	+	+
Capnocytophaga	+	+	-
Cellvibrio	+	+	+
Chitinimonas	+	-	-
Chromatiaceae	-	-	+
Chromatium	+	+	+
Citrobacter	-	+	-
Clostridia	+	-	-
Collimonas	-	+	-
Comamonadaceae,	-	+	-
Comamonas	+	+	-
Colwellia	+	+	+
Crenothirx	+	-	+
Cupriavidus	+	-	-
Cycloclasticus	+	-	-
Delftia	-	+	-
Deltaproteobacterium	+	-	-
Denitrobacter	+	-	-
Desulfococcus	+	-	-
Desulfovibrio	+	-	-
Diaphorobacter	+	+	-
Dokdonella	+	-	-
Duganella	+	+	-
Eikenella	-	+	-
Eisseria	-	+	-
Elluria	+	-	-
Enhydrobacter	-	-	+
Ensifer	+	-	+
Enterobacter	-	+	-
Ergeriella	-	+	-
Ethylobacter	+	-	+
Ethylocaldum	+	-	-
Ethylococcus	+	+	+
Ethylomicrobium	+	-	+
Ethylomonas	-	-	+
Ethylophilus	-	+	-
Ewingella	-	+	-
Flavobacterium	+	+	-
Francisella	-	+	-
Fusobacteium	+	-	-
Gallibacterium	-	-	+
Gamma proteobacterium	+	+	+
Glaciecola	-	+	+
Haemophilus	-	-	+
Hahella	-	-	+
Halomonas	+	+	+
Halorhodospira	+	-	-
Halothiobacillus	+	-	+
Herbaspirillum	+	-	-
Hodocyclus	+	-	-
Janthinobacterium	-	+	-
Klebsiella	-	-	+
Kocuria	-	+	-
Legionella	+	-	-
Leptothrix	+	-	-

Name of the Genera	Hha1	Msp1	Rsa1
Marinimicrobium	+	+	-
Marinobacterium	-	+	-
Marinomonas	-	-	+
Massilia	+	-	-
Methylobacter	+	-	+
Methylococcaceae	+	-	-
Methylococcus	+	+	-
Methylomicrobium	+	+	+
Methylomonas	+	-	+
Methylosarcina	+	-	-
Methylotenera	-	+	-
Methyloversatilis	-	+	-
Microbulbifer	+	-	+
Mitsuaria	-	+	-
Moraxella	-	-	+
Neisseria	-	+	-
Nitrincola	-	+	+
Nitrosococcus	+	-	-
Nitrosomonas	+	-	-
Nitrospina	+	-	-
Olavius	+	-	-
Ottowia	+	-	-
Oxalobacteriaceae	+	-	-
Pantoea	-	+	-
Paucibacter	-	+	-
Pellagiobacter	+	-	+
Pseudoalteromonas	+	-	+
Photorhabdus	+	-	-
Pigmentiphaga	+	-	-
Polaromonas	+	+	-
Propionibacter	-	+	-
Providencia	-	+	+
Provotella	-	+	-
Pseudidiomarina	-	+	-
Pseudomonas	+	+	+
Psychrobacter	-	-	+
Pusillimonas	+	-	-
Rahnella	-	+	-
Ralstonia	-	+	-
Rhizobium	+	-	-
Rhodoferax	-	+	-
Salinimonas	-	+	-
Salmonella	-	-	-
Samsonia	-	+	-
Serratia	-	+	-
Shewanella	+	+	-
Sideroxydans	-	-	-
Singularimonas	+	-	-
Sodalis	-	+	-
Solimonas	+	-	-
Spongiibacter	-	-	+
Streptomyces	+	+	+
Succinatimona	-	-	+
Tatumella	-	+	-
Teredinibacter	-	+	-
Thioalkalivibrio	-	+	-
Thiobacillus	+	+	-
Thiocapsa	-	-	+
Thiocystis	-	-	+

Name of the Genera	Hha1	Msp1	Rsa1
Thiomonas	+	+	-
Tolumonas	-	+	-
Uncultured	+	+	+
Unidentified	+	+	+
Unknown	+	+	+
Variovorax	+	+	-
Verminephrobacter	+	-	-
Vibrio	+	+	+
Xenorhabdus	-	+	-
Yersinia	-	+	-
Zoogloea	+	+	-

Table 3. Resolving the TRF by three different restriction enzymes Hha1, Msp1 and Rsa1 in treated sponges after 48 hours treatment with primer 9F and 928R (experimental details described in Chapter 5).

Name of the Bacteria	Hha1	Msp1	Rsa1
Achromatium	+	+	+
Acidithiobacillus	+	-	+
Acidovorax	+	-	+
Acinetobacter	+	+	+
Actinobacillus	+	-	+
Achromobacter	-	-	+
Aeromonas	-	+	+
Agarivorans	-	-	+
Agricultural soil bacterium	+	-	+
Alcanivorax	-	+	-
Alcaligenes	+	+	+
Alicycliphilus	+	-	-
Aliivibrio	+	-	+
Alorhosdospira	+	-	-
Alteromonas	+	-	+
Anthinobacterium	+	-	-
Arsenophonus	-	+	-
Arthrobacter	-	-	+
Avibacterium	-	-	+
Azoarcus	-	+	-
Azovibrio	-	+	-
Allochromatium	-	-	+
Azotobacter	-	+	-
Bacillus	-	-	+
Beggiatoa	+	+	+
Bermanella	-	-	-
Beta proteobacterium	+	+	-
Bordetella	+	+	-
Buchnera	+	-	+
Burkholderia	+	+	-
Caldimonas	+	-	-
Candidatus	+	+	+
Capnocytophaga	+	+	+
Castellaniella	+	-	-
Cellvibrio	+	+	+
Chitinimonas	+	-	-
Chloroflexaceae	+	-	-
Chromatium	+	-	-
Chromobacterium	+	-	-

Name of the Bacteria	Hha1	Msp1	Rsa1
Chronobacter	-	-	+
Chromohalobacter	-	-	+
Citrobacter	+	+	+
Clostridia	+	-	-
Clothiorhodospira	+	-	-
Colwellia	+	+	+
Comamonadaceae	+	+	+
Comamonas	+	+	-
Crenothirx	+	+	-
Cupriavidus	+	-	-
Cycloclasticus	+	-	-
Dechloromonas	+	-	-
Delftia	+	-	-
Deltaproteobacterium	+	-	+
Denitrobacter	+	-	-
Desulfococcus	+	-	-
Desulfocurvus	+	-	-
Desulfovibrio	+	-	+
Diaphorobacter	+	-	-
Dickeya	-	+	-
Dokdonella	+	-	-
Duganella	+	-	-
Ectothiorhodospira	+	-	-
Eikenella	-	+	-
Elluria	+	-	-
Ensifer	-	+	-
Enterobacter	-	+	+
Erwinia	-	+	+
Escherichia	+	-	+
Ethylocaldum	-	-	-
Ethylococcus	+	-	-
Ethylomicrobium	+	-	-
Ethylomonas	+	-	-
Ethylophaga	+	-	-
Ewingella	-	+	-
Flavobacterium	+	+	+
Francisella	+	+	-
Fusobacteium	+	-	+
Gallibacterium	-	-	+
Gamma proteobacterium	+	+	+
Glaciecola	+	-	+
Haemophilus	+	-	+
Halomonas	+	+	+
Halorhodospira	+	-	-
Halothiobacillus	+	-	-
Herbaspirillum	+	-	-
Hodocyclus	+	-	-
Idiomarina	+	-	+
Iodobacter	+	-	-
Janthinobacterium	+	-	-
Kangiella	+	-	-
Kerstersia	+	-	-
Klebsiella	-	-	+
Kocuria	-	+	-
Kushneria	-	-	+
Legionella	+	-	+
Leptothrix	+	-	-
Loripes	-	+	-
Marinimicrobium	+	-	-

Name of the Bacteria	Hha1	Msp1	Rsa1
Marinomonas	-	-	+
Marinobacter	-	-	+
Marinobacterium	+	-	-
Massilia	+	-	-
Methylobacter	+	-	+
Methylocaldum	+	-	-
Methylococcaceae	+	-	-
Methylomonas	+	-	-
Methylosarcina	+	-	-
Methyloversatilis	-	+	-
Methylovulum	-	+	-
Methylococcus	+	-	-
Methylomicrobium	-	+	-
Microbulbifer	+	-	+
Moraxella	-	+	-
Moritella	+	-	-
Neisseria	-	+	-
Neptuniibacter	-	-	+
Neptunomonas	+	-	+
Nitrosococcus	+	-	-
Nitrosomonas	-	+	-
Nitrospina	+	-	-
Oceanimonas	-	+	-
Olavius	-	-	+
Olbachia	+	-	-
Ottowia	+	-	-
Oxalobacteriaceae	+	-	-
Pantoea	-	+	+
Pasteurella	+	+	+
Pectobacterium	-	-	+
Paucibacter	-	+	-
Pelagiobacter	+	-	-
Pseudoalteromonas	+	+	+
Photorhabdus	+	-	-
Photobacterium	-	-	+
Pigmentiphaga	+	-	-
Plesiomonas	-	+	-
Propinibacter	-	+	-
Pseudidomarina	-	+	-
Psychrobacter	-	-	+
Providencia	-	+	-
Polaromonas	-	+	+
Pseudomonas	+	+	+
Pusillimonas	+	-	-
Rahnella	-	+	-
Ralstonia	+	+	-
Ranicola	-	-	+
Rhizobacter	+	-	-
Rhizobium	+	-	-
Salmonella	-	-	+
Salinicola	-	-	+
Samsonia	-	+	-
Serratia	-	+	+
Sideroxydans	-	+	-
Singularimonas	+	-	-
Sinobacter	+	-	-
Sodalis	-	+	-
Solimonas	+	-	-
Sphingomonas	-	-	+

Name of the Bacteria	Hha1	Msp1	Rsa1
Shewanella	-	+	+
Tatumella	-	+	+
Teredinibacter	-	+	-
Tetrathiobacter	-	+	-
Thioalkalivibrio	+	-	-
Thiomonas	+	-	-
Tolumonas	-	+	-
Uncultured	+	+	+
Unidentified	+	+	+
Variovorax	+	+	-
Unknown	+	+	+
Verminephrobacter	+	-	-
Vibrio	+	+	+
Wautersia	+	-	-
Xenorhabdus	-	+	-
Yersinia	-	+	-
Zoogloea	+	-	-

Table 4. Comparison of TRF by three different restriction enzymes Hha1, Msp1 and Rsa1 which are common in three enzymes either in control or treated sponges after 48 hours with primer 9F and 928R (experimental details described in Chapter 5).

Name of the Bacteria	Control	Treatment
Achromatium	+	+
Acinetobacter	-	+
Agricultural soil bacterium	+	-
Alcaligenes	-	+
Beggiatoa	-	+
Beta proteobacterium	-	+
Candidatus	+	+
Capnocytophaga	-	+
Cellvibrio	+	+
Citrobacter	+	+
Colwellia	+	+
Comamonadaceae,	-	+
Eikenella	-	+
Ethylococcus	+	-
Flavobacterium	-	+
Gamma proteobacterium	+	+
Halomonas	+	-
Methylomicrobium	+	-
Pasteurella	-	+
Pseudoalteromonas	-	+
Pseudomonas	+	+
Uncultured	-	+
Unidentified	-	+
Unknown	-	+
Vibrio	-	+

A bacterial genus was considered to be present in a sample only if all three corresponding TRFs (from the three separate restriction enzyme digests) within 2-bp range were present in the sample (Tables 2 and 3). In those instances where the TRF for the third restriction enzyme digest (usually Rsa1) did not come up with RDP database, a decision to include the genus was made on

a case-by-case basis after rechecking the T-RFLP electropherograms. A TRF was considered to be validated if it correlated with TRFs from the other two enzyme digests that matched a bacterial genus or species (Conn & Franco 2004)

Bacterial 16S rRNA gene was chosen as a phylogenetic marker. A detailed analysis showed that the primers used in both TRFLP and pyrosequencing techniques must be able to "capture" the same complete compliment of genera/species, even though the primer set is not the same, as different product lengths are required for each analysis. Therefore, the suitable primer set was designed and selected by primer sequence prevalence analysis (PSPA), as shown in Table 5.

Table 5. Several potential combinations of selected forward and reverse primers used for the PSPA analysis. PSPA reports the number of matches for given pairs of forward and reverse primers.

Forward Sequence	Forward Matches	Reverse sequence	Reverse Matches	Primer Matches
ACTCCTACGGGAGGCAGCAGT	1000053	CCGTCAATTCATTTGAGT	112836	96978
GAGGATGACCAGCCACACTG	37057	CCGTCAATTCATTTGAGT	112743	22996
GACTCCTACGGGAGGCAGCAGT	816322	CCGTCAATTCATTTGAGT	112819	87823
GAGAGGATGACCAGCCACACTG	36857	CCGTCAATTCATTTGAGT	112717	22854
CAGGCCTAACACATGCAAGTC	87253	CCGTCAATTCATTTGAGT	112736	39703
CAGGCCTAACACATGCAAGTC	87253	TAGCTCCGGAAGCCACGCCT	16987	10226
GTGCCAGCAGCCGCGGTAA	851277	CCGTCAATTCATTTGAGT	114606	110089
GTGCCAGCAGCCGCGGTAA	843486	TAGCTCCGGAAGCCACGCCT	17414	16844
CCTACGGGAGGCAGCAG	1025996	CCGTCAATTCATTTGAGT	112884	100327
GAGTTTGATCATGGCTCAG	46602	CCGTCAATTCATTTGAGT	112734	10109
AGAGTTTGATCATGGCTCAG	40832	CCGTCAATTCATTTGAGT	112724	8638
GAGAGGATGACCAGCCACACTGG	36817	CCGTCAATTCATTTGAGT	112706	22839
GAGTTTGATCATGGCTCA	47120	CCCCGTCAATTCATTTGAGT	103578	9432
GAGTTTGATCATGGCTCAGA	27765	CCCCGTCAATTCATTTGAGT	103550	9263
GAGTTTGATCATGGCTCAGAT	15859	CCCCGTCAATTCATTTGAGT	103536	8025
GAGTTTGATCATGGCTCAGATT	15792	CCCCGTCAATTCATTTGAGT	103527	8007
AGAGTTTGATCMTGGCTCAG	213397	CCCCGTCAATTCATTTGAGT	103553	20925
AGAGTTTGATCMTGGCTCAG	213397	CCGTCAATTCMTTTRAGTTT	829002	144451

Based on the all above analyses and the literature review two primer sets were finally selected for this study, as shown in Table 6.

Table 6. Name of the primers used for TRFLP study.

Primer for TRFLP	Sequence	Reference
9F	6 FAM 5/ GAGTTTGATCMTGGCTCAGAT 3/	New
928R	5/ CCCCGTCAATTCMTTTGAGT 3/	New
Eub 8F	6 FAM 5/ AGAGTTTGATCMTGGCTCAG 3/	(Jiang et al. 2006)
Eub 926R	VIC 5/ CCGTCAATTCMTTTRAGTTT 3/	(Lane et al. 1985)

A previous study used TRFLP to examine changes or variations in community structure and to recognize those phylotypes that are stable or changing (Marsh 2005). This technique is used in microbial ecology for differentiation of communities (Liu et al. 1997; Moeseneder et al. 1999), but it usually lacks the power to identify specific genera in a diverse microbial population. Even though the method developed by Conn and Franco (Conn & Franco 2004) was followed to identify at the genus level, the result obtained using the first primer set missed the major part of *Cyanobacteria* which was revealed by 454 pyrosequencing. After careful analysis it was found that this primer set was not able to pick up *Synechococcus* which was one of the main genera of *Aplysilla rosea,* as shown in Table 7.

Table 7. Putative genera identified by TRFLP using primer pairs 9F-928R and EUB 8F-EUB 926R with three restriction digestion enzymes Hha1, Msp1 and Rsa1 in virtual digest (ISPaR) and their comparison with pyrosequencing data of sponge sample

Name of bacteria genera/species	Pyrosequencing by primer 28 F and 519R	Primer 9F and 928R	Primer EUB 8F and EUB 926R
Alteromonas sp.	+	+	+
Arcobacter sp.	+	-	-
Clostridium sp.	+	+	+
Colwellia psychrerythraea	+	+	+
Colwellia sp.	+	+	+
Flavobacterium sp.	+	+	+
Glaciecola sp.	+	+	+
Idiomarina sp.	+	+	+
Marinomonas sp.	+	+	+
Moritella sp.	+	+	+
Oleispira sp.	+	+	+
Phaeobacter sp.	+	-	+
Pirellula sp.	+	-	+
Pseudoalteromonas sp.	+	+	+

Name of bacteria genera/species	Pyrosequencing by primer 28 F and 519R	Primer 9F and 928R	Primer EUB 8F and EUB 926R
Pseudomonas sp.	+	+	+
Pseudovibrio sp.	+	-	+
Roseobacter sp.	+	+	+
Ruegeria atlantica	+	-	-
Ruegeria sp.	+	-	+
Salegentibacter sp.	+	-	+
Shewanella sp.	+	+	+
Sinorhizobium sp.	+	-	+
Spongiobacter sp.	+	-	-
Sulfitobacter sp.	+	-	+
Streptomyces sp.	+	+	+
Synechococcus sp.	+	-	+
Tenacibaculum soleae	+	-	-
Tenacibaculum sp.	+	-	+
Vibrio gallaecicus	+	+	-
Vibrio lentus	+	+	+
Vibrio natriegens	+	+	+
Vibrio pomeroyi	+	+	+
Vibrio sp.	+	+	+

Although it is a eubacterial primer set, the data generated by this primer set were not completely comparable to pyrosequencing. This tentative characterisation by TRFLP can be reinforced by confirming the identification of specific TRFs from the results obtained through pyrosequencing, which gives more reliable identification of the microbial diversity up to the genus level. The TRFs corresponding to specific genera can then be used to characterise changes in populations of the system under investigation. This was successfully done in the present study by optimizing the primers used for TRFLP as well as the restriction digestion enzymes to generate results aligned with pyrosequencing (Marsh 2005). Another advantage is that TRFLP can handle the analysis of multiple samples simultaneously, compared to 454-pyrosequencing, and at a much cheaper rate without the laborious computational work of sequence data (Camarinha-Silva et al. 2012). Therefore, the primer sets optimized and designed for TRFLP (Marsh et al. 2000; Shyu et al. 2007; Fortuna et al. 2011) have the ability to detect the genus *Synechococcus* as well as other dominant

genera revealed by pyrosequencing (Table 7). Three restriction digestion enzymes were selected to identify bacterial populations (Liu et al. 1997; Marsh et al. 2000) based on the literature (Fortuna et al. 2011) as well as by doing *in silico* digestion (Shyu et al. 2007), which can characterise microbial populations to the genus level, as shown in Figs 3 and 4.

Restriction Enzyme	Total Hits	Success	5'Unique	5'Average	5'Std. Dev.	3'Unique	3'Average	3'Std. Dev.
AGG^CCT	34225	32614	290	225.130	217.572	336	588.135	161.714
AG^CT	34225	34211	280	124.624	75.938	281	89.268	101.445
A^CGT	34225	32862	500	283.658	215.055	504	382.342	183.611
A^CRYGT	34225	21802	382	699.660	302.535	316	467.261	405.426
A^CTAGT	34225	5286	283	886.176	109.783	241	793.459	288.763
CAN^GT	34225	34157	103	55.516	49.489	504	461.532	303.848
CATG^	34225	34215	80	47.291	25.772	535	433.653	242.597
CAYNN^NNRTG	34225	11253	444	817.535	187.685	389	703.824	325.736
CCTTCNNNNN^N	34225	27769	385	413.633	271.797	407	605.198	194.683
CC^NGG	34225	34224	534	365.135	247.608	56	45.662	9.635
CC^TNAGG	34225	7159	351	777.701	276.471	337	859.465	129.163
CG^CG	34225	34223	304	287.370	124.242	195	168.354	147.055
C^CGG	34225	34202	433	388.496	158.358	300	277.569	109.583
C^TAG	34225	33425	516	237.528	187.529	308	211.031	192.257
C^TNAG	34225	34218	285	244.210	75.603	277	214.600	184.786
C^TTAAG	34225	1467	288	896.882	103.042	270	894.537	111.813
GAGTCNNNNN^	34225	8089	381	846.541	135.439	331	764.888	273.889
GA^TC	34225	34196	303	225.042	63.784	410	397.385	186.106
GCG^C	34225	32580	524	344.991	213.490	449	288.078	217.867
GGATGNN^	34225	31840	482	306.632	204.158	503	495.485	200.334
GG^CC	34225	34222	297	138.845	103.234	288	116.437	166.450
GKGCM^C	34225	9722	448	732.634	310.390	406	777.582	278.796
GT^AC	34225	34160	597	582.904	253.334	90	40.171	44.583
G^AATTC	34225	21227	286	755.903	129.464	224	505.530	321.971
G^ANTC	34225	33895	322	238.654	130.149	422	444.258	202.679
G^ATC	34225	34196	303	224.043	63.793	410	398.384	186.104
G^CGC	34225	32580	521	343.087	213.747	451	289.981	217.591
G^GATCC	34225	1674	264	905.469	69.666	237	880.177	164.111
G^GNCC	34225	34194	289	221.963	65.265	345	381.891	209.716
G^GWCC	34225	28989	465	343.697	274.513	446	606.843	211.752
TARCCANNNNNNNNN^NN	34225	1972	335	877.952	156.390	316	898.882	79.976
TCN^GA	34225	34207	366	182.704	91.988	346	300.437	95.918
TC^NNGA	34225	28897	630	410.811	274.150	512	447.303	287.330
TG^CA	34225	34217	109	51.463	34.863	457	291.100	133.385
T^CGA	34225	33164	228	91.616	165.789	515	514.838	347.611
T^TAA	34225	34195	427	355.158	194.201	178	67.202	107.470
^AATT	34225	34105	524	448.014	136.325	278	239.091	67.895
^GATC	34225	34196	303	223.044	63.801	409	399.383	186.102

Fig 3. Selection of suitable enzymes by enzyme resolving power analysis with primer 9F and 928R

All criteria were fulfilled by the second primer set EUB 8F and 926R (Table 5) used in this study that identified the main genera in the sample as well as other important genera revealed by pyrosequencing. Although Hha1 and Msp1 (Engebretson & Moyer 2003; Harder et al. 2004; Erwin et al. 2011) provided better resolution by generating more TRF, Rsa1 failed to show similar efficacy. Thus, in future HaeIII will be considered as a third restriction digestion enzymes (Erwin, Patrick M et al. 2012).

The results of this study validate the approach taken for aligning the TRFLP data with pyrosequencing by optimizing primer and restriction digestion enzymes.

Restriction Enzyme	Total Hits	Success	5'Unique	5'Average	5'Std. Dev.	3'Unique	3'Average	3'Std. Dev.
AGG^CCT	144451	62937	744	622.097	345.868	616	758.054	199.146
AG^CT	144451	141662	782	191.215	163.018	748	241.467	239.551
A^CGT	144451	137199	863	313.477	235.987	797	345.642	240.494
A^CRYGT	144451	127455	662	282.964	313.771	652	627.950	294.900
A^CTAGT	144451	8956	622	890.853	94.703	507	864.543	177.744
CAN^GT	144451	144274	388	55.943	41.377	827	419.820	259.647
CATG^	144451	144296	376	51.251	35.533	866	502.079	262.139
CAYNN^NNRTG	144451	34343	921	808.968	228.599	748	780.350	270.905
CCTTCNNNNN^N	144451	84283	807	566.796	340.313	648	645.333	292.097
CC^NGG	144451	144234	906	423.886	248.575	272	47.050	46.091
CC^TNAGG	144451	20875	813	826.225	213.494	658	856.676	144.827
CG^CG	144451	144431	530	201.000	123.813	454	264.998	146.438
C^CGG	144451	144285	729	270.798	161.279	564	246.411	125.955
C^TAG	144451	141726	911	289.803	243.422	727	240.151	244.566
C^TNAG	144451	144426	495	224.004	91.989	513	209.329	150.198
C^TTAAG	144451	11930	710	869.710	140.990	575	868.339	143.704
GAGTCNNNNN^	144451	25944	875	835.029	182.106	746	813.243	221.771
GA^TC	144451	134399	919	313.463	203.907	867	474.223	233.248
GCG^C	144451	131744	929	328.022	260.086	852	380.026	272.244
GGATGNN^	144451	114932	880	388.690	285.176	812	579.869	238.156
GG^CC	144451	143134	681	216.921	120.301	713	263.029	238.065
GKGCM^C	144451	44493	939	751.084	275.489	830	754.607	278.949
GT^AC	144451	143794	892	440.799	233.489	500	57.399	112.320
G^AATTC	144451	105914	628	715.471	132.621	434	428.308	291.035
G^ANTC	144451	130793	834	326.503	220.852	832	507.823	220.922
G^ATC	144451	134399	920	312.533	204.110	866	475.153	233.118
G^CGC	144451	131744	932	326.198	260.476	850	381.850	271.906
G^GATCC	144451	10241	648	875.581	133.449	536	873.572	139.124
G^GNCC	144451	144223	615	239.212	73.250	694	406.718	196.860
G^GWCC	144451	104669	966	461.812	302.930	869	609.230	249.879
TARCCANNNNNNNNN^NN	144451	18981	803	839.263	190.030	695	854.164	155.452
TCN^GA	144451	137800	857	257.174	195.066	836	351.243	185.810
TC^NNGA	144451	137526	879	385.859	221.897	776	320.989	183.666
TG^CA	144451	144340	459	52.848	38.711	804	326.951	201.258
T^CGA	144451	141463	791	101.353	173.142	812	425.788	325.574
T^TAA	144451	139849	829	298.060	292.849	607	197.072	254.954
^AATT	144451	139481	858	457.000	208.123	702	277.363	148.264
^GATC	144451	134399	922	311.602	204.314	866	476.084	232.989

Fig 4. Selection of suitable enzymes by enzyme resolving power analysis with primer EUB 8F and EUB 926R.

4.4 Conclusion

This study refined the TRFLP analysis enabling it to reveal the microbial community associated with sponge *A. rosea* which was highly matching that of the pyrosequencing data in order to identify putative genera/species. Thus the results of this study indicate that the TRFLP method could be a very effective way of microbial diversity analysis as it is aligned reasonably well with pyrosequencing. In this thesis, each of these two methods and the two in combination will be used to study the microbial diversity analysis of the experimental sponges.

4.5 References

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CHAPTER 5

ISOLATION OF CULTURABLE ACTINOBACTERIA FROM SPONGE *APLYSILLA ROSEA* AND *APLYSINA* SP. FOR USE IN SPONGE-BACTERIA INTERACTION STUDY

5.1 Introduction

Many different types of bacteria that are associated with sponges have been reported in the literature, as summarized in Chapter 1. Among the associated bacteria, actinobacteria have been reported as a major source of antibiotics compared to other microorganisms (Takahashi & Omura 2003; Jose & Jebakumar 2013). Their ecological role in the marine environment (Haefner 2003) might be very important for sponge survival and growth. Besides, as a producer of bioactive compounds, they play a significant role for sponge protection (Das et al. 2006). This phylum of bacteria have proved economically valuable for biotechnological applications (Manivasagan et al. 2013).

A diverse range of biological activities such as antibacterial, antifungal, antiparasitic, antimalarial, immunomodulatory, anti-inflammatory, antioxidant, antiviral and anticancer activities were reported from sponge-associated actinobacteria (Bull & Stach 2007; Olano et al. 2009; Pimentel-Elardo et al. 2010; Abdelmohsen et al. 2012; Blunt et al. 2013). For this study only the antimicrobial activity of these isolates has been tested. The rationale for evaluating the antimicrobial potential of these sponge-associated actinobacteria was to test their potential roles in altering or modifying the bioactivity of sponges by altering metabolites profile and their abundance during the sponge-bacteria interaction study described in Chapter 7. Antimicrobial activity is easy to test and will serve as one of the many representative bioactivities.

While any sponge-associated bacterium can potentially be chosen for sponge-bacteria interaction study, for this study only actinobacteria were targeted for isolation. One of these culturable sponge-associated actinobacteria from *Aplysilla rosea* will be used in the study of the interaction between sponge and sponge-associated bacteria described in Chapter 7. The isolate was selected based on good sporulation and high antimicrobial activity.

Two marine sponge species from two different orders collected from South Australia waters, which can adapt well to aquarium conditions, were initially selected for this study. The main aim of the isolation study was to obtain culturable sponge-associated bacteria to be used for later experiments to develop an understanding of the role of the interaction between sponge and sponge-associated bacteria.

5.2 Material and Methods

5.2.1 Collection of samples

All the sponge samples were collected by scuba diving from different locations in South Australia during this PhD project, which started in 2011, as, described in Chapter 3. For this particular study, sponges were carefully removed from jetty pylons. Once collected, the samples were kept immersed in seawater in plastic bags and the diver returned to the boat and immediately transferred the sponges into the buckets of seawater without exposure to air. When the boat arrived at the jetty, the buckets were collected from the boat and the water replaced with fresh seawater. This procedure was executed carefully to avoid exposure of the sponges to the air. Sponges were transported to the laboratory in an air-conditioned car. To facilitate aeration to the bucket, a few airways were created in the lid while transporting. Once the buckets with the sponges reached the aquarium, they were conditioned through 5-6 seawater changes at 20 minute intervals to remove any toxins released as a result of transport stress. Next, they were transferred into aquaria (~100 liters capacity). This standard procedure was followed whenever there was a new collection of sponges. All the sponges were identified by S. Sorokin, SARDI Aquatic Science, South Australia.

Collection details of *Aplysilla rose*a and *Aplysina* sp. are shown in Table 1 and Fig 1.

Table 1. Names of the two different sponges with their location, collection date, order and family used for the isolation study during the project.

Name of sponge	Location	Date	Order	Family
Aplysilla rosea	Rapid Bay	27.06.2012	Dendroceratida	Darwinellidae
Aplysina sp.	Rapid Bay	27.06.2012	Verongida	Aplysinidae

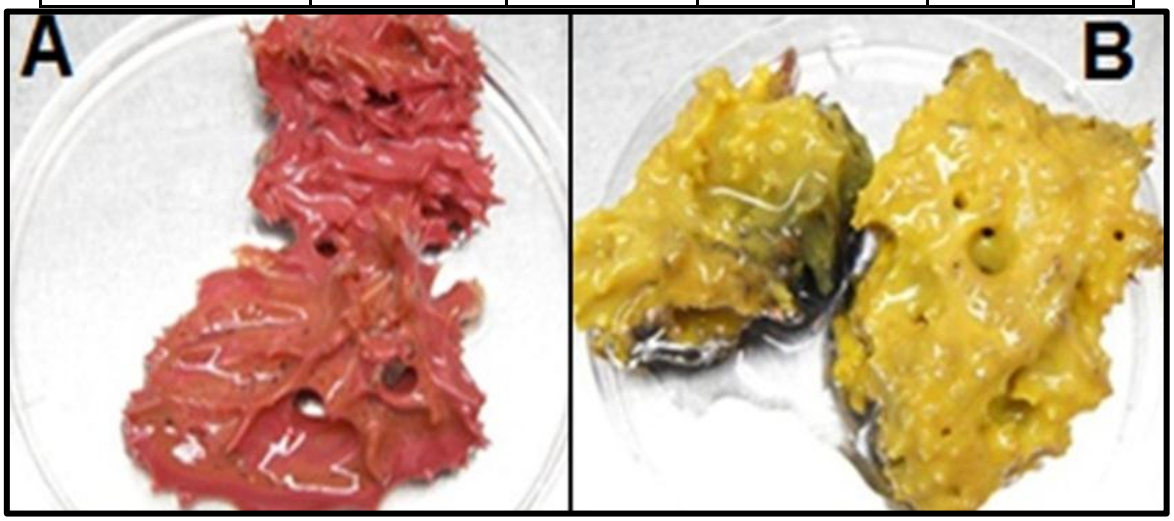


Fig 1. Pictures of the two different sponges *Aplysilla rosea* (A) and *Aplysina* sp. (B) selected for isolation of actinobacteria.

5.2.2 Actinobacteria isolation

Two marine sponges *Aplysilla rosea* and *Aplysina* sp. were collected from Rapid Bay (35°31'15.29"S, 138°11'6.88"E) in South Australia at a depth of 9 m, in June 2012 and processed for the cultivation of actinobacteria while they were fresh by following previously described methods (Jensen et al. 1991; Mincer et al. 2002; Jensen et al. 2005; Mincer et al. 2005). The main characteristics of these methods were pre-treatment, drying in a laminar flow, diluting homogenate in seawater and incubate dilutions at high temperature (40-60°C) before inoculation onto different types of agar media. In addition different antibiotics were incorporated in the media. In this study, after following the pre-treatment according to the above-mentioned literature, the sponge homogenate was prepared by using sterilized phosphate buffered saline (PBS) (Zhang et al. 2008; Abdelmohsen et al. 2010). Briefly, the homogenate was diluted in a series of dilutions up to 10^{-1}, 10^{-2} and 10^{-3} before plating on different agar medium plates in triplicate for each dilution. The isolation plates were incubated at 28°C, generally for 12 weeks. Morphologically diverse strains were selected for further purification.

5.2.3 Preparation of sponge extract for adding into the media

To support the growth of sponge-associated actinobacteria, "aqueous sponge extract" was incorporated into the selected (M5 and M7) medium while the temperature cooled down to 50°C. The "aqueous sponge extract" was prepared according to literature (Abdelmohsen et al. 2010), briefly, by grinding the required amount of sponge biomass in a mortar containing sterile seawater, followed by centrifugation in a 50 ml falcon tube (5000 rpm, 10 min) and sterilization by filtration through a 0.2 µm pore size filter. The freshly prepared supernatant was termed "aqueous sponge extract".

5.2.4 Media used for isolation

Eight different types of isolation media were selected for this study are as follows:

M1 was composed of glycerol 10 ml, casein (Difco-vitamin-free), 0.3 g, KNO_3, 2 g, NaCl, 2 g, K_2HPO_4 2 gm, $MgSO_4.7H_2O$ 0.05 g, $CaCO_3$, 0.02 g, $FeSO_4.7H_2O$, 0.01 g, Bacto agar (Difco), 15 g, milliQ water 1 liter (L), pH 7.2 (Küster & Williams 1964); M2 was composed of humic acid 1.0 g, Na_2HPO_4 0.5 g, KCl 1.71 g, $MgSO_4.7H_2O$ 0.05 g, $FeSO_4.7H_2O$ 0.01 g, $CaCO_3$ 0.02 g, Bactoagar (Difco)18 g, milliQ water 1 L, cycloheximide 100 µg/ml, pH 7.2 (Hayakawa & Nonomura 1987); M3 was composed of soluble starch, 10 g; yeast extract 4 g, peptone 2 g, Bacto agar (Difco) 15 g, natural sea water 1 L, pH 7.4 (Zhang et al. 2006; Zhang, H et al. 2008); M4 was composed of 100% glycerol 6 ml, arginine 1 g, K_2HPO_4 1 g, MgSO4 0.5 g, Bacto agar (Difco) 15 g, natural seawater 1 L, pH 7.4 (Kennedy et al. 2009); M5 was composed of raffinose 10 g, L-Histidine 1 g, $FeSO_4$ 0.01g, K_2HPO_4 1 g, $CaCO_3$ 0.5 g, NaCl 20 g, $MgSO_4$ 0.1 g, peptone 0.1 g, Bacto agar

(Difco) 15 g, natural seawater 1L, sponge extract 10%, pH 7.8 (Gandhimathi et al. 2008); M6 was composed of glucose 4 g, malt extract 10 g, yeast extract 4 g, KNO_3 4 g, Bacto agar (Difco) 15 g, 2% sponge extract, nalidixic acid, 20 µg/ml, nystatin 20 µg/ml and beonmyl 50 µg/ml, natural seawater (50%)1 L, pH 7.2 (Montalvo et al. 2005); M7 was composed of soluble starch 20 g, NaCl 3 g, KNO_3 1 g, $FeSO_4.7H_2O$ 0.014 g, K_2HPO_4, $MgSO_4$ 0.5 g, Bacto agar (Difco) 15 g and milliQ water 1 L. benomyl 50 µg/ml, nalidixic acid, 20 µg/ml, nystatin 20 µg/ml, 2% sponge extract, pH 7.2 (Maldonado, L. A. et al. 2005) and, finally, M8 was composed of fucose 0.1 g, raffinose 5 g, collagen 1 g, asparagine 0.1 g, $FeSO4.7H_2O$ 1 mg, K_2HPO_4 1 g, sodium propionate 4 g, calcium carbonate 0.02 g $MgSO_4$ 0.1 g, $MnCl_2$ 0.1 g, potassium dichromate 50µg/ml + nalidixic acid 15 µg /ml, pH, 1 ml vitamin solutions, Bacto agar (Difco) 15 g, 1 L natural seawater (50%), pH 7.2.

Of the eight media, M2 was modified and M8 was a new media which was used only in this study.

5.2.5 Composition of culture maintenance media

After the cultures were purified, they were maintained in the following media: soluble starch 20 g, NaCl 3 g, KNO_3 1 g, $FeSO_4.7H_2O$ 0.014 g, K_2HPO_4, $MgSO_4$ 0.5 g, Bacto agar (Difco) 15 g and milliQ water 1 L. benomyl 50 µg/ml, nalidixic acid, 20 µg/ml, nystatin 20 µg/ml and pH 7.2.

5.2.6 Antimicrobial bioassay

5.2.6.1 Antibacterial activity assay

Antibacterial activity assay was conducted according to the literature (Vilches et al. 1990; Kaewkla 2009; Wiese et al. 2009). Briefly, the test organisms were grown in tryptone soy broth (TSB: Oxoid) at 37°C by shaking at 150 rpm for 18 hr. The growth of bacteria was assessed by measuring the optical density (OD) using a spectrophotometer at 600 nm and the OD was adjusted to 0.2. The test microorganisms were added to antibiotic assay medium (AAM:Oxoid) at 45°C at the ratio of 1 ml per 25 ml of media and about 20 ml was eventually poured into a 9 cm Petri dish.

The AAM agar, which was seeded with the test organisms, was cut using a 6 mm cork borer to make wells (ten wells per plate). Fifty microliters of methanol extracts from agar media were put into each well. Each sample was repeated in duplicate. Methanol was used as a negative control for all the samples. Vancomycin (200 µg/ml) and Colistin (500 µg/ml) were used against gram-positive and gram-negative bacteria, respectively, as positive controls. The test plates were incubated at 37°C for 18-24 hr and the antibacterial activity was assessed by measuring the zone of inhibition.

5.2.6.2 Antifungal activity assay

Antifungal activity assay was conducted according to the literature (Kaewkla 2009). Briefly, two test fungi were grown on Potato Dextrose agar (PDA, Oxoid) at pH 6.0 for 7 days. A plug was cut at the

edge of the radial growth of each fungus by using a 6 mm cork borer and placed at the centre of a new PDA plate at pH 6.0 and incubated at 27°C for 4 days or until the diameter of the fungi was about 2.5 cm. Then six wells were made by using a 6 mm cork borer with the centre of the wells approximately 1.5 cm away from the edge of the PDA plate. Fifty microliters of extract was prepared in the same way as for the antibacterial test and was put in the wells. Each sample was repeated in duplicate. Methanol was used as a negative control. Cycloheximide (220 µg/ml) and amphotericin B (100 units) were used as positive controls. The growth of fungi was measured toward the direction of each well in 4 and 9 days. Results were recorded as follows: strong inhibition (+++): when the growth of fungi away from the well was more than 14 mm; moderate inhibition (++): when the growth of fungi was away from the well more than or equal to 10 mm; inhibition mm (+): when the growth of fungi was away from the well more than or equal to 5 mm; negative (-): no difference in growth when comparing with the negative control plates without the wells or less than 5 mm.

5.2.7 Morphological comparison

Morphological characterisation to identify duplicate strains was conducted using three different types of media as follows: International *Streptomyces* Project (ISP) 2 media, Half-strength Potato Dextrose Agar (HPDA) and Mannitol Soya agar. The media compositions are shown in Table 2:

Table 2. Composition of media for morphological characterisation of actinobacteria strain

Name of Media	Composition
ISP-2	Malt extract 10 g, Yeast extract 4 g, Glucose 4 g, Bacto agar (Difco) 15 g, and 1 L milliQ, pH 7.3
HPDA	PDA 19.5 g and 7.5 g Bacto agar (Difco) and 1 L milliQ water, pH 7.3,
MS	Mannitol 20g, Soya flour 20 g, Bacto agar (Difco) 15 g and 1 L MilliQ water, pH 7.3

Based on the morphology, isolates were tentatively grouped by observing the characteristics of the colonies on the plates, the presence of aerial mycelia and substrate mycelia, spore mass colour, distinctive reverse colony colour, and diffusible pigment.

5.2.8 Genotypical identification

DNA extraction: The selected isolates were grown on ISP-2 agar media and HPDA media overlaid with cellophane after sterilization and allowed to grow for 7-10 days according to the literature (Kaewkla 2009). The media which showed better growth (ISP-2) was taken for DNA extraction. A reasonable amount of cells (two loops) were resuspended in 900 µl pre-warmed buffer A (2% CTAB, 100 mM Tris HCl, 1.4 M NaCl, 1% PVP and 20 mM EDTA, 0.2% Lici), pH 8 and 100 µl buffer B (80 mM Tris-Cl, 8 mM EDTA, 4.8% Triton X-100 and 80 mg/ml lysozyme) and incubated at 70°C for 15 min. Then the samples were incubated again at 65°C for 2 hours and invert mixed every 10 minutes. Samples were centrifuged at 10000 rpm for 5 minutes and the supernatant was transferred to a new sterile tube. The cells were extracted again by adding equal volumes of chloroform, isoamyl alcohol (24:1) and invert mixed for 5 minutes, then centrifuged at 12000 rpm for 8 min. The supernatant was transferred to a new sterile tube without touching the white layer. This step was repeated twice. Before the last extraction 20 µl of 10 mg/ml of RNAse was added and the samples were incubated at 37° for 30 minutes. In order to extract the DNA, 300 µl 3 M Sodium Acetate and 500 µl filter sterilized isopropanol were added in the tube and invert mixed for 5 minutes. The tube was kept at -20°C for one hour. Then the samples were centrifuged at 8000 rpm to precipitate the DNA.

The supernatant was discarded and the pellet was washed with 600 µl 95% ethanol, 70% ethanol and again 95% ethanol respectively. To confirm the evaporation of ethanol the tube was then dried by placing it in a 55°C heating block for 10 min or until it was dried. The pellet was resuspended by adding 50 µl, sterile Tris (10mM). The DNA was stored in -20°C for further use. The presence of DNA was confirmed by running the extracted DNA on a 0.8% agarose gel, which was then stained with GelRed™ (Biotium, California, USA) for visualisation.

5.2.8.1 Quantification of DNA

Quantification of DNA was completed by using Genequant pro (RNA/DNA calculator, Biochrom) and Nano Drop 8000 spectrophotometer (Thermo scientific). The A_{260}/A_{280} absorbance ratio was used to determine undesired contaminations. To see the band, quality, molecular weight and intactness of the extracted DNA, 0.8 % and 1% agarose gel electrophoresis was used. Gel red was used for staining instead of ethidium bromide in order to avoid hazardous chemicals. For gel image, Fujifilm LAS-4000 imager (GE Healthcare) was used.

5.2.8.2 PCR reaction, 16S rRNA gene sequencing and digestion of PCR product

PCR reaction was performed for 16S rRNA gene sequencing. DNA was firstly amplified by the primers 27f and 765r (5′ CTGTTTGCTCCCCACGCTTTC 3′) (Damiani et al. 1996), and, based on this result, selected strains were further amplified by 704f (5′GTAGCGGTGAAATGCGTAGA3′) (Lane 1991) and 1492r (5′ TACGGYTACCTTGTTACGACTT3′). Finally, a few selected strains were amplified by 27f and 1492 r at both ends by the forward and reverse primer to get a complete sequence to confirm the identity of the strain.

The PCR was carried out in 50-µl reaction volumes with the following reagents: 2 µl of 27f (5µM), 2 µl of 1492r (5 µM), 5 µl Thermopol buffer, 37 µl of injection water, 1 µl dNTPs (dATP, dCTP, dGTP, dTTP - 10 mM each with the water for injection) 1 µl of Taq polymerase (1 U Thermopol), and 2 µl of template DNA (25ng/µl). The reactions were subjected to the following temperature cycling profile: 95°C for 5 min, followed by 40 cycles of 94°C for 1 min, 52°C for 1 min, followed by 72°C for 1 min, and finally 72°C for 10 min. The PCR products were purified by using an ultra-clean PCR purification kit (Mobio). The purified PCR products were digested with Hha1 and run in 1.5% agarose gel to see the different banding patterns to distinguish the strains from each other.

5.2.8.3 Chimera checking and 16S rRNA gene sequence identification

Software Chromas Lite (2.2.1) was used to discard the weak signal from partial sequence and this edited sequence was used for identification by using blastn program in NCBI. To achieve a complete sequence, a commercial software sequencher (5.2.4) was used and contig was prepared for individual strain. These contig sequences were submitted as BLAST queries to GenBank (NCBI). The sequences were also deposited to Genbank for accession numbers as shown in Table 6.

5.2.9 Phylogenetic analysis

The CLUSTAL W program (Mega6) was used to multiple align 16S rRNA sequences of each novel strain with the 16S rRNA gene sequences of each novel strain with the 16S rRNA gene sequences of representatives of the most closely related genera of species, available from GenBank/EMBL. Phylogenetic trees were constructed by the neighbour-joining (Saitou & Nei 1987) and maximum-likelihood (Kimura 1980) tree-making methods using the software package MEGA version 6 (Tamura K 2013). Pairwise distances for the neighbour-joining algorithm were calculated according to the Kimura two-parameter model (Kimura 1980) and close-neighbour-interchange (search level = 2, random addition = 100) and Kimura two-parameter model (Kimura 1980) were applied in maximum-likelihood analysis. The topology of the tree was evaluated by performing a bootstrap analysis (Felsenstein 1985) based on 1000 replications.

5.2.10 Extraction of metabolites from actinobacteria

To conduct the metabolite profiling, isolates were grown in ISP-2 media. Once good growth was observed (7-10days), a 3 cm*3cm agar block with spores from the petri plate was cut using a sterile scalpel in aseptic conditions. The piece of agar was chopped into smaller pieces and placed into falcon tubes and methanol was added at a ratio of 1:1.5 (~6 ml agar + 9 ml methanol) and the tubes were closed tightly with lids. The tubes were set in a Ratek orbital shaker for 4 hours to extract the metabolites from the agar and centrifuged at 5000 rpm for 30 minutes.

5.2.11 Thin Layer Chromatography (TLC)

The crude extract was filtered through a 13 mm 0.2 µm PVDF acrodisc syringe filter and added to 2 ml tubes with glass inserts. Twenty microliter were loaded onto aluminium TLC silica gel 60 F_{254} plates (Merck), using the following solvent system: Ethyl acetate: methanol [9:1]. The migration of the extract in the TLC plate was detected by UV light at 254 nm and 365 nm.

5.2.12 Comet analysis

Metabolites from the actinobacteria were prepared in the above-mentioned way. Then 2 mL extracts were dried by using centrifugal evaporator in a special Eppendorf tube (B147671L/2423, Eppendorf AG, Germany). In the next step samples were kept in -80°C overnight and then freeze-dried for one day. The dried samples were packed in a special box and sent to Dr. Ernest Lacey, Microbial Screening Technologies, Building A, 28-54 Percival Rd, Smithfield in New South Wales for Comet analysis and databatch search against different compounds extracted from actinobacteria.

5.2.13 High Performance Liquid Chromatography (HPLC)

The crude extract was filtered through a 13 mm 0.2 µm PVDF acrodisc syringe filter and added to 2 ml tubes with glass inserts. Then, 50 µl of this filtered solution was injected into the HPLC system described below. The peaks were detected at across the 200 to 800 nm wavelength range.

HPLC analyses were performed with an Agilent 1100 system, including Alliance separation module 2695, column heater, and 2998 photodiode array detector, and run using Empower Chromatography Data Software (Waters). The HPLC conditions consisted of two eluents (eluent A [0.1% aqueous trifluoroacetic acid] and eluent B [100% acetonitrile]) and an elution profile based on a linear gradient from 10% eluent B to 100 % eluent B within 25 min and then holding at 100% eluent B for an additional 20 min. Flow rate was kept constant at 1 ml min^{-1}. Chromatographic separation was performed on an Atlantis T3 C18 column (Agilent, 100 mm x 3 mm ID and 3 µm particle size) in reversed phase with a fixed temperature of 25°C.

5.3 Results and Discussion

5.3.1 Isolation of the actinobacteria from different media

Bacterial colonies started to develop in different media after 7 days but after three weeks a reasonable amount of colonies formed and the numbers were documented. The counting continued until the 12th week, when fewer colonies were found in different media and no consistency was observed. Actinobacteria colonies were recognized by their usual characteristics such as aerial and substrate mycelium of which the colours range from white to yellowish, brownish, red, pink, orange, green or black; by observing spore formation with cottony, velvety or powdery appearance; by their often leathery compactness and conical appearance with a dry surface; and by their 'earthy' odour and tendency to dig into the agar media (Waksman 1967; Starr 1981; Ventura et al. 2007). Although more than 400 different colonies were observed in eight different media, only 118 actinobacteria were isolated from the sponges *Aplysilla rosea* and *Aplysina* sp. after subsequent purification. The numbers of isolated strains were fewer due to the abundance of the same colony in multiple times in different media. Although colonies were revealed in all eight media, the efficacy of the medium was different in relation to colony numbers (Fig 2 and Fig 3). In this study, the use of sponge extracts and the addition of salt or seawater were found effective in growing more and diverse colonies which might indicate the salt requirement of obligate marine actinomycetes (Maldonado et al. 2005). However, once purified, 70% strains were able to grow without seawater or salt. This result indicated that culturable actinobacteria isolated from the marine sponge do not always show an absolute requirement for seawater for their growth (Mincer et al. 2002; Maldonado et al. 2005; Zhang et al. 2006). Among all the media, M7 and M6 showed better efficiency compared to other media in relation to colony number and diversity. However, M8 also provided some unusual colonies. It has been observed that media M6 and M7 contained sponge extract which indicates that mimicking the sponge environment could be one of the supportive ways to obtain more and novel actinobacteria with high diversity (Webster & Hill 2001).

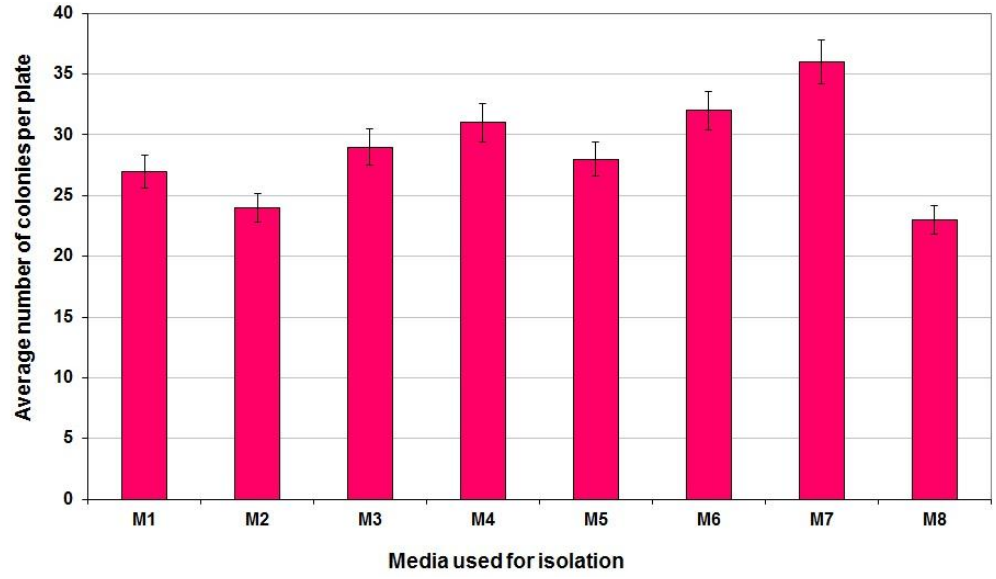


Fig 2. The distribution of sponge-associated actinobacteria colonies from *Aplysilla rosea* in different media during twelve weeks.

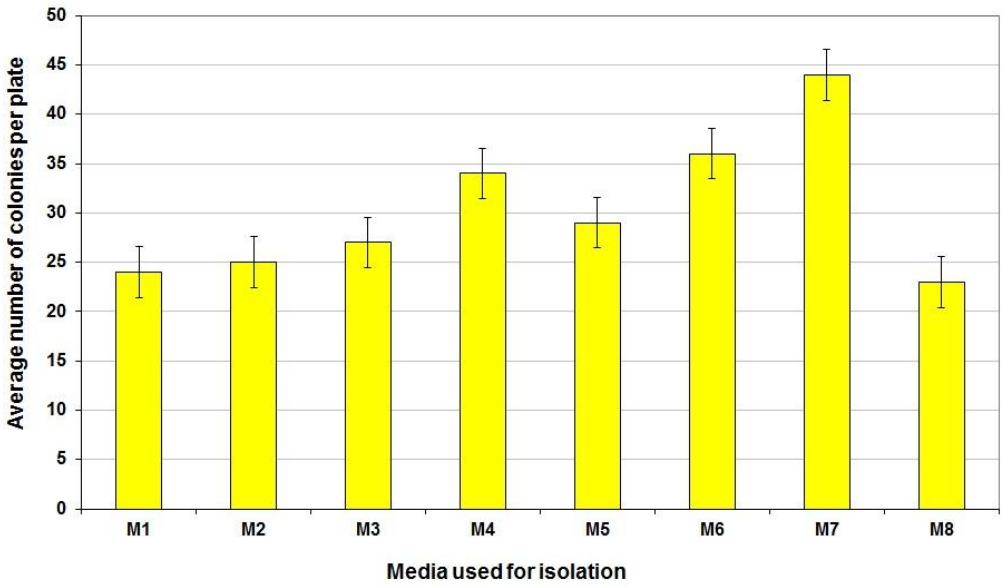


Fig 3. The distribution of sponge-associated actinobacteria colonies from *Aplysina* sp. in different media during twelve weeks.

5.3.2 Antimicrobial analysis

Antimicrobial activity was evaluated for all 118 strains isolated from the two sponges used for this study but only 78 strains showed either antifungal or antibacterial or both types of activity. A total of 22 isolates from *Aplysilla rosea* showed activity against three different types of *S. aureus* (Table 3). Of these, ACT-52A showed the highest activity. On the other hand, only eight strains from *Aplysina* sp. showed activity against the three strains of *S. aureus* used in this study. ACT-39B showed the highest activity among all the isolates in *Aplysina* sp. Among all the isolates from *A. rosea* and

Aplysina sp. only ACT-74 showed activity against *Escherichia coli* with a zone of inhibition 12 mm (Table 3). A total of 14 isolates from *Aplysilla rosea* showed moderate activity (++) against *Rhizoctonia solani*. On the other hand, a total of 10 isolates from *Aplysina* sp. showed moderate to high activity (++/+++) against *Rhizoctonia solani*. The best activity (+++) was observed in two isolates ACT-98 and ET-11. A total of six isolates from *Aplysilla rosea* showed moderate activity (++) against *Fusarium oxysporum* and only two isolates from *Aplysina* sp. showed moderate activity against *F. oxysporum* (Table 3).

Finding new antibiotics against *Staphylococcus aureus* has often been closely followed by the rise of resistant strains. *S. aureus* isolates have been reported for their resistance to beta-lactams which has a significant impact on the community. They have even developed resistance to beta-lactamase-resistant penicillins. Multidrug-resistant *S. aureus* is a big threat for humans, as obtaining effective antibiotics against this strain is becoming more difficult (Schito 2006; Hiramatsu et al. 2014). There are different mechanisms by which bacteria develop resistance. Efflux systems are one mechanism which contributes to antibiotic resistance in bacteria such as *Staphylococcus aureus* (Nor A) (Kaatz & Seo 1995).

This is why *Staphylococcus aureus*, as well as antibiotic resistant *Staphylococcus aureus* (Nor A) (SAK3765) and *Staphylococcus aureus* (2703) efflux-pump gene knocked out were used to evaluate antibacterial activity. These bacteria were used as gram positive bacteria and *Escherichia coli* were used as gram negative bacteria for the antibacterial activity test.

For the antifungal activity test two fungal pathogens *Rhizoctonia solani* and *Fusarium oxysporum* were used. Both are familiar as causative agents for plant diseases.

Table 3. Antimicrobial activity of methanolic extract from actinobacteria isolated from *Aplysilla rosea* and *Aplysina* sp. with the tentative compound identification (NM = no obvious metabolite, ND = Not detected).

Host sponge	Isolate code	*Rhizoctonia solani*	*Fusarium oxysporum*	*S. aureus*	*S. aureus* (SAK 3756)	*S. aureus* SAK 2703	*E. coli*	Putative identification
Aplysilla rosea	ACT-4	++	++	12	10	10	0	New
Aplysilla rosea	ACT-9	+	+	9	8	8	0	Ikarugamycin like
Aplysilla rosea	ACT-14A	++	+	14	12	10	0	Tetranoic acid
Aplysilla rosea	ACT-15	+	+	7	0	0	0	Tetranoic acid
Aplysilla rosea	ACT-16	+	+	18	0	7.5	0	Tetranoic acid
Aplysilla	ACT-17	++	+	12	12	13	0	New

Host sponge	Isolate code	*Rhizoctonia solani*	*Fusarium oxysporum*	*S. aureus*	*S. aureus* (SAK 3756)	*S. aureus* SAK 2703	*E. coli*	Putative identification
rosea								
Aplysilla rosea	ACT-18	+	+	13	10	11	0	NM
Aplysilla rosea	ACT-19A	+	+	13	0	0	0	Tetranoic acid
Aplysilla rosea	ACT-19B	-	+	14	12	12	0	Tetranoic acid
Aplysilla rosea	ACT-20	+	+	12	11	13	0	Tetranoic acid
Aplysilla rosea	ACT-21	-	-	12	10	10	0	Tetranoic acid
Aplysilla rosea	ACT-22A	+	+	12	9	11	0	Ikarugamycin like
Aplysilla rosea	ACT-22B	++	+	12	12	13	0	Tetranoic acid
Aplysilla rosea	ACT-22C	-	+	11	11.5	13	0	Tetranoic acid
Aplysilla rosea	ACT-23	-	+	0	0	0	0	Ikarugamycin like
Aplysilla rosea	ACT-26	+	+	12	9	11	0	Tetranoic acid
Aplysilla rosea	ACT-32	+	+	0	0	0	0	NM
Aplysilla rosea	ACT-33	++	+	33	16	15	0	Actinomycin
Aplysilla rosea	ACT-40	+	+	0	0	0	0	New
Aplysilla rosea	ACT-47	-	-	8	0	0	0	Alteramide like
Aplysilla rosea	ACT-49	-	-	8	0	0	0	NM
Aplysilla rosea	ACT-52	++	++	33	18	17	0	Actinomycin
Aplysilla rosea	ACT-52A	++	+	37	22	20	0	Actinomycin
Aplysilla rosea	ACT-52B	++	-	6	0	0	0	Peptide
Aplysilla rosea	ACT-53	+	+	7	6	6	0	New
Aplysilla rosea	ACT-58	-	+	8	0	0	0	New
Aplysilla rosea	ACT-63	+	+	14	6	6	0	Napthoquinone
Aplysilla rosea	ACT-70	++	++	7	0	0	0	ND
Aplysilla	ACT-70D	++	++	14	11	9	0	New

Host sponge	Isolate code	*Rhizoctonia solani*	*Fusarium oxysporum*	*S. aureus*	*S. aureus* (SAK 3756)	*S. aureus* SAK 2703	*E. coli*	Putative identification
rosea								
Aplysilla rosea	ACT-71	++	++	13	10	9	0	ND
Aplysilla rosea	ACT-74	++	-	13	10	10	12	NM
Aplysilla rosea	ACT-76	+	+	0	0	0	0	Alteramide like
Aplysilla rosea	ACT-77	++	+	13	12	12	0	ND
Aplysilla rosea	ET-8	+	+	8	0	0	0	ND
Aplysilla rosea	ACT-115	++	++	0	0	0	0	ND
Aplysilla rosea	ACT-117	+	+	8	10	10	0	Adenosine type
Aplysilla rosea	ET-22	+	+	9	0	0	0	New
Aplysina sp	ACT-31	-	+	0	0	0	0	NM
Aplysina sp	ET-25	+	+	0	0	0	0	ND
Aplysina sp.	ACT-34	+	+	10	0	0	0	NM
Aplysina sp.	ACT-34A	++	+	10	0	0	0	ND
Aplysina sp.	ACT-34B	-	-	11	0	0	0	ND
Aplysina sp.	ACT-36	+	-	0	0	0	0	Tetranoic acid
Aplysina sp.	ACT-39	++	+	16	18	16	0	Indolmycin
Aplysina sp.	ACT-39B	+	+	22	20	18	0	NM
Aplysina sp.	ACT-43	++	-	7	7	8	0	Alteramide like
Aplysina sp.	ACT-44	++	+	12	0	0	0	ND
Aplysina sp.	ACT-46	+	+	7	7	0	0	Actinomycin
Aplysina sp.	ACT-48	-	-	8	0	0	0	ND
Aplysina sp.	ACT-61	+	-	7	6	6	0	Alteramide like
Aplysina sp.	ACT-67	+	+	21	0	0	0	Indolmycin
Aplysina sp.	ACT-69	+	+	12	10	10	0	New
Aplysina sp.	ACT-69A	+	+	0	0	0	0	ND
Aplysina sp.	ACT-69B	+	+	0	0	0	0	ND
Aplysina sp.	ACT-69C	+	+	0	0	0	0	Ikarugamycin like
Aplysina sp.	ACT-69D	+	+	0	0	0	0	Ikarugamycin like
Aplysina sp.	ACT-69F	-	-	6	0	0	0	ND
Aplysina sp.	ACT-69G	++	+	6	0	0	0	NM
Aplysina sp.	ACT-72	++	++	8	0	0	0	Heronamide
Aplysina sp.	ACT-73	+	+	7	0	0	0	Heronamide
Aplysina sp.	ACT-79	+	+	10	7	7	0	Heronamide
Aplysina sp.	ACT-80	+	+	10	10	10.5	0	Tetranoic acid
Aplysina sp.	ACT-84	+	-	0	0	0	0	NM
Aplysina sp.	ACT-86	+	+	0	0	0	0	NM
Aplysina sp.	ACT-87 A	+	+	0	0	0	0	Tetranoic acid

Host sponge	Isolate code	*Rhizoctonia solani*	*Fusarium oxysporum*	*S. aureus*	*S. aureus* (SAK 3756)	*S. aureus* SAK 2703	*E. coli*	Putative identification
Aplysina sp.	ACT-90	+	+	7	7	7	0	Unknown
Aplysina sp.	ACT-94	+	+	7	7	7	0	ND
Aplysina sp.	ACT-98	+++	++	12	0	0	0	NM
Aplysina sp.	ACT-99	++	+	0	0	0	0	NM
Aplysina sp.	ACT-100	++	+	0	0	0	0	NM
Aplysina sp.	ET-1	+	+	8	0	0	0	NM
Aplysina sp.	ET-2	+	+	8	0	10	0	NM
Aplysina sp.	ET-3	+	+	0	0	0	0	NM
Aplysina sp.	ET-5	+	+	8	0	0	0	Heronamide
Aplysina sp.	ET-7	+	-	5	0	0	0	NM
Aplysina sp.	ET-11	+++	+	10	0	0	0	New (Polyene)
Aplysina sp.	ET-15	+	-	10	0	0	0	ND
Aplysina sp.	ET-28	+	-	10	0	0	0	ND

5.3.3 Metabolite Profiles

To further understand the metabolite profiles of these active strains, firstly, thin layer chromatography (TLC) was applied to visualize the compounds produced by different actinobacteria (Figs 4 and 5). Different banding patterns were observed when the TLC technique was applied for different cultures (Figs 4 and 5). A few cultures showed very unique profiles compared to other cultures. These cultures are ACT-4, ACT-17, ACT-40, ACT-53, ACT-58, ACT-70D, ET-11 and ET-28. They showed unique band pattern in TLC under UV.

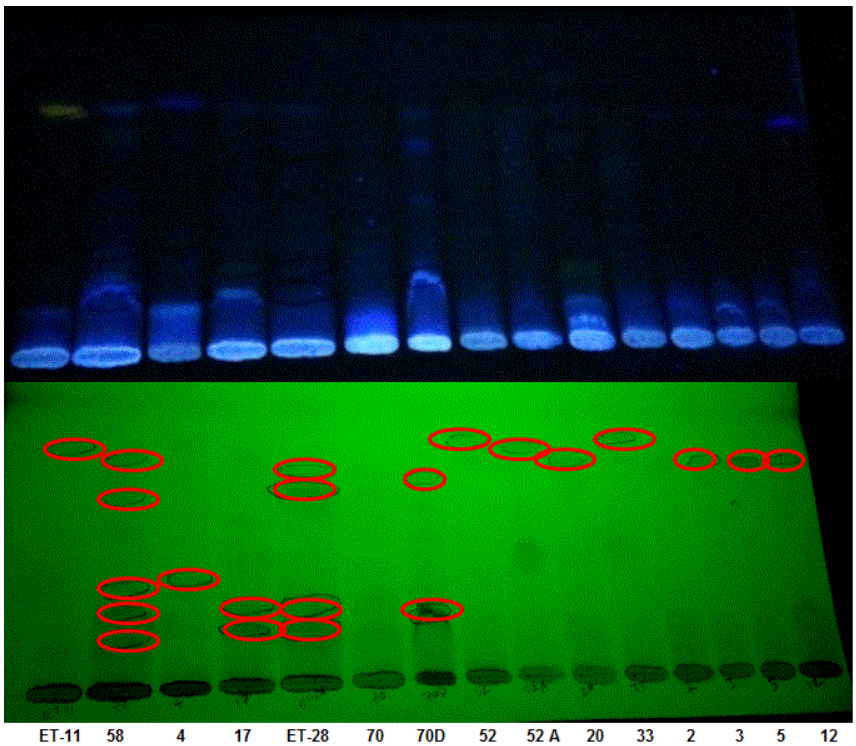


Fig 4. Secondary metabolites extracted by methanol from selected actinobacteria run in TLC plate in ethyl acetate methanol [9:1] solvent system showing different compounds profiles at 254 and 365 nm under UV.

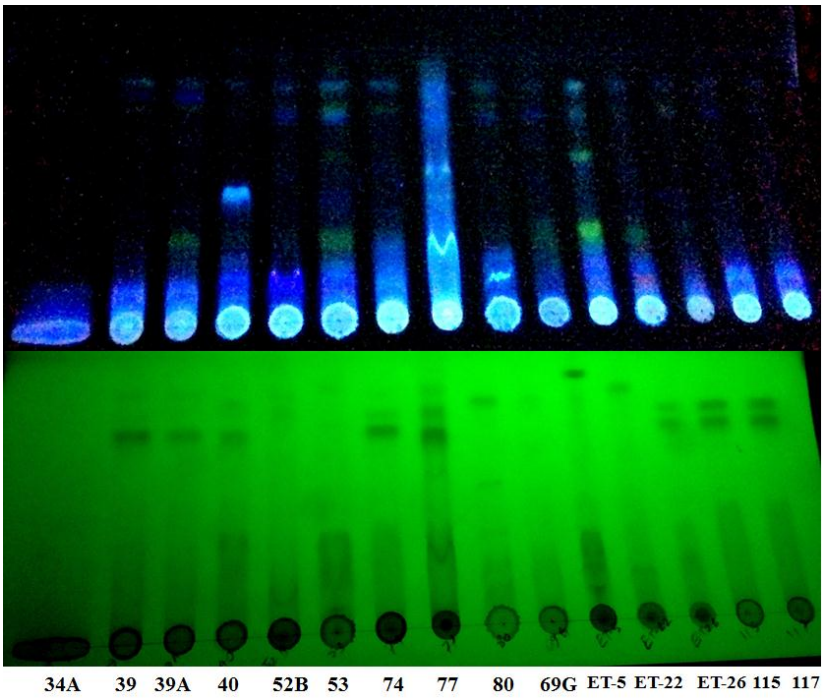


Fig 5. Secondary metabolites extracted by methanol from selected actinobacteria run in TLC plate in ethyl acetate methanol [9:1] solvent system showing different compounds profiles at 254 and 365 nm under UV.

After conducting antimicrobial activity and TLC, most of the cultures were sent to a collaborator company in Sydney for further analysis by HPLC technique (Appendix 6). Different metabolites were identified from the methanolic extract of the actinobacteria strain grown in ISP-2 media. Those compounds were compared with previously recorded compounds produced by many different actinobacteria from many different sources by HPLC technique using a specialized computer database developed by the company for actinobacteria. It was also confirmed by the collaborator company in Sydney (conducted by HPLC) that these extracts showed tentatively new compounds (Appendix 6). Further study is required for purification and downstream analysis for the identification of the new compounds, which was not the intention of this study.

HPLC analyses were conducted in our laboratory with the targeted strain for sponge bacteria interaction study to confirm the results from the Sydney company, as shown in Fig 6. The Sydney result reported that ACT-52A is an actinomycin producing strain, which was confirmed by their UV spectrum shown in Fig 6.

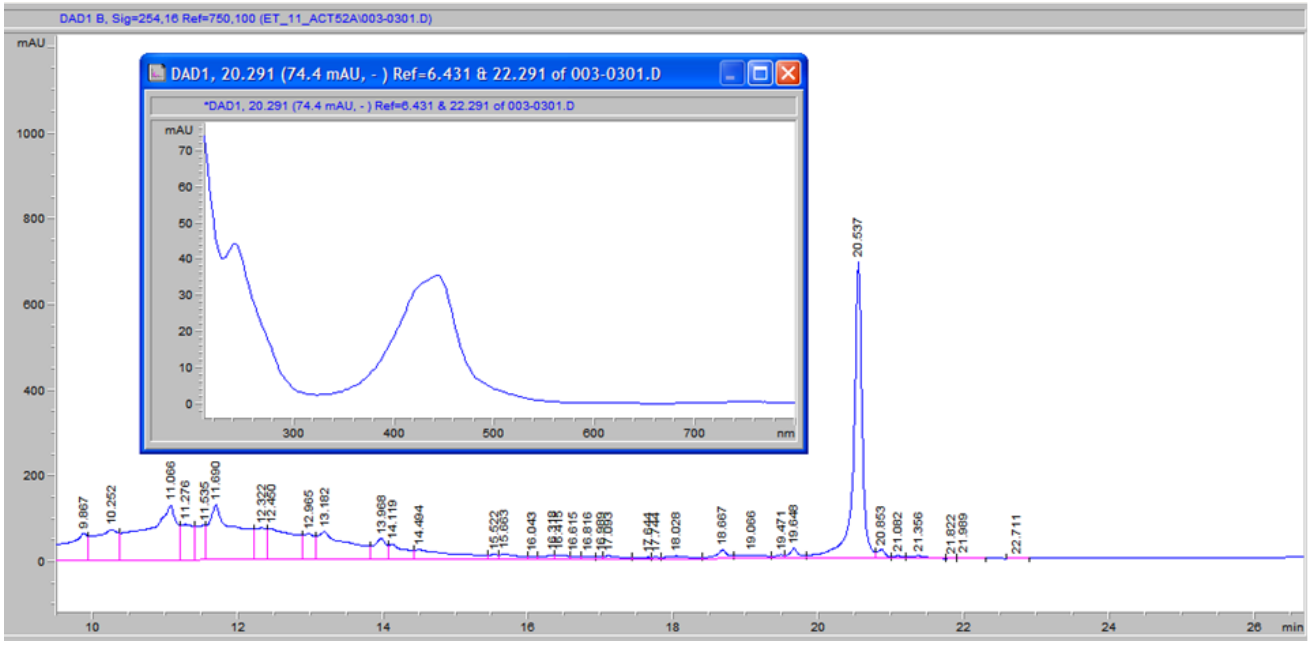


Fig 6. HPLC profile of extracts from ACT-52A, grown in ISP-2 media, showing actinomycin isolated from *Aplysilla rosea*

The roles of secondary metabolites by sponge-associated actinobacteria are not well understood. However, it was hypothesized that sponge-associated bacteria play dominant roles during the interaction between sponge and associated bacteria, as discussed in Chapter 7. This study has isolated sponge-associated actinobacteria for the sponge-bacteria interaction experiment discussed in Chapter 7. The role of actinobacteria in changing the interaction with their host marine sponges has not been explored in the past. The results from the culture dependent study confirmed that *Streptomyces* sp. were common in two sponges selected for this study. The culture independent study of *Aplysilla rosea* also revealed that *Streptomyces* sp. is the abundant actinobacteria in *Aplysilla rosea*. Therefore, *Streptomyces* ACT-52A, of which the nearest sequence was revealed as *Streptomyces costaricanus,* was selected for experiments to study the role of interaction between sponge *Aplysilla rosea* and the sponge-associated bacteria by exposing the sponges with this bacterial spore, as described in Chapter 7.

5.3.4 Morphological evaluation and grouping

Based on the antimicrobial activity, 42 isolates were further grouped according to colour morphology (Appendix 6). Different cultural characteristics of selected actinobacteria were obtained when compared based on the colour dimension in three different media according to the literature (Kornerup & Wanscher 1978). It was found that when comparing colony morphology using ISP-2, MS and HPDA it is possible to distinguish the actinobacteria for further evaluation. Based on the morphology all the isolates were categorized into 14 groups. Representative isolates from different groups were selected for further research.

5.3.5 Genotypical Evaluation

To confirm the accurateness of morphological grouping, one representative isolate from each group was further analysed by RFLP technique. Different banding patterns were observed except 33/61 and 43/46, which were similar from all those 14 isolates (Fig 7). In the next step to identify the actinobacteria, representative isolates from each group were partially sequenced (Table 4). Finally, 5 selected isolates from *Aplysilla rosea* and 5 isolates from *Aplysina* sp. were fully sequenced (Table 5) and revealed as *Streptomyces* sp.

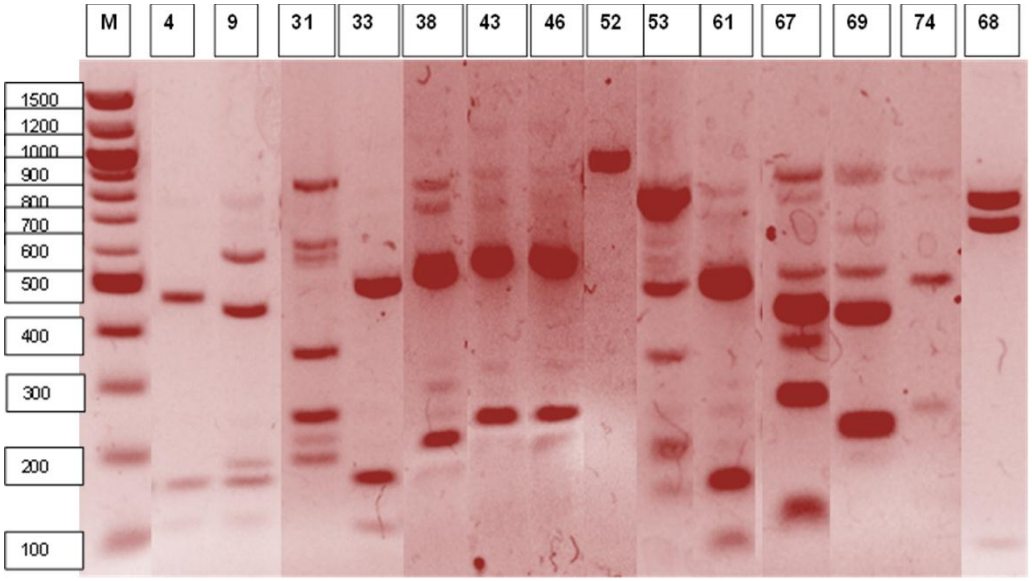


Fig 7. RFLP banding pattern of selected actinobacteria digested by Hha1

Table 4. Partial (P) and full (F) sequence information for selected isolates

Strain ID	Partial/Full sequence	Source	Identification (closes type strain)	Accession no
ACT-4	P (654)	*Aplysilla rosea*	*Streptomyces olivaceus*	-
ACT-9	F	*A. rosea*	*Streptomyces carpaticus*	-
ACT14A	F	*A. rosea*	*Streptomyces Pectum*	KP739981
ACT-17	P (734)	*A. rosea*	*Streptomyces griseobrunneus*	-
ACT-19	P (653)	*A. rosea*	*Streptomyces fimicarius*	-
ACT-22A	F	*A. rosea*	*Streptomyces carpaticus*	KP739982
ACT-25	F	*A. rosea*	*Streptomyces tricolor*	KP739983
ACT-33	P(658)	*A. rosea*	*Streptomyces griseoaurenticaus*	-

ACT-34	F	*Aplysina* sp.	*Streptomyces carpaticus*	KP739984
ACT-36	P (647)	*Aplysina* sp.	*Streptomyces* sp.	-
ACT-38	P (654)	*Aplysina* sp.	*Streptomyces albidoflavus*	-
ACT-39B	F	*Aplysina* sp.	*Streptomyces bambergiensis*	KT630861
ACT-40	F	*A. rosea*	*Streptomyces anulatus*	KP739985
ACT-43	P(650)	*Aplysina* sp.	*Streptomyces fimicarius*	-
ACT-46	P(643)	*Aplysina* sp.	*Streptomyces* sp.	-
ACT-52A	F	*A. rosea*	*Streptomyces costaricanus*	KT253928
ACT-53	P(690)	*A. rosea*	*Streptomyces griseoflavus*	-
ACT-55	P(643)	*Aplysina* sp.	*Streptomyces collinus*	-
ACT-61	P (661)	*Aplysina* sp.	*Streptomyces olivaceus*	-
ACT-67	P (656)	*Aplysina* sp.	*Streptomyces cacaoi*	-
ACT-68	P (655)	*A. rosea*	*Streptomyces drozdowiczii*	-
ACT-69D	F	*Aplysina* sp.	*Streptomyces carpaticus*	KT253930
ET-11	F	*Aplysina* sp.	*Streptomyces halophytocola*	KP739988
ET-25	F	*Aplysina* sp.	*Streptomyces coelescens*	KP739990
ET-22	P (670)	*A. rosea*	*Streptomyces qinglanensis*	-

The closest type strains of those isolates are provided in Table 5.

Table 5. Closest type strain of selected isolates when compared with 16SrRNA gene sequence

Code	**Name**	**Strain**	**Accession**	**Pairwise Similarity (%)**	**Diff/Total nt**	**Complete-ness (%)**
ACT-14A	*Streptomyces pactum*	NBRC 13433(T)	AB184398	99.93	1/1335	99.59
ACT-22A	*Streptomyces carpaticus*	NBRC 15390(T)	AB184641	99.55	6/1348	99.93
ACT-25	*Streptomyces carpaticus*	NBRC 15390(T)	AB184641	99.77	3/1329	99.93
ACT-40	*Streptomyces griseoplanus*	AS 4.1868(T)	AY999894	99.85	2/1348	96.75

Code	Name	Strain	Accession	Pairwise Similarity (%)	Diff/Total nt	Complete-ness (%)
ACT-52A	*Streptomyces costaricanus*	NBRC 100773(T)	AB249939	99.57	6/1396	100
ACT-34	*Streptomyces carpaticus*	NBRC 15390(T)	AB184641	99.7	4/1327	99.93
ACT-39B	*Streptomyces bambergiensis*	NBRC 13479(T)	AB184869	99.3	10/1428	99.59
ACT-69D	*Streptomyces carpaticus*	NBRC 15390(T)	AB184641	99.56	6/1368	99.93
ET-11	*Streptomyces halophytocola*	KLBMP 1284(T)	JQ819259	99.71	4/1368	99.38
ET-25	*Streptomyces tricolor*	NBRC 15461(T)	AB184687	99.4	8/1339	98.98

The full sequence information of these selected isolates are as follows:

ACT-14A (1336 bp), Accession no: K39981

1 TTCGGTGGGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTG
61 GGACAAGCCCTGGAAACGGGGTCTAATACCGGATATTGACCTTCACGGGCATCTGTGAGG
121 TTCGAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAAT
181 GGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGCTGA
241 GACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGC
301 CTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCCGGGTTGTAAACCTCTTTCAGCA
361 GGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAG
421 CCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAG
481 GCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACG
541 GGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGA
601 TATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGC
661 GAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCACGCCGTAAACGGTGGGCA
721 CTAGGTGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCC
781 TGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGC
841 GGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCG
901 GAAACGGCCAGAGATGGTCGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGT
961 CAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGT
1021 TGCCAGCAAGCCCCTTCGGGGGTGTTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGG
1081 AGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCT
1141 ACAATGGCCGGTACAATGAGCTGCGATACCGCAAGGTGGAGCGAATCTCAAAAAGCCGGT
1201 CTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCA
1261 GATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCAC
1321 GAAAGTCGGTAACACC

ACT-22A (1351 bp): Accession no: KP739982

1 GCCGGGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGA
61 TAAGCCCGGGAAACTGGGTCTAATACCGGATACGACACTCCGAGGCATCTTGGGGTGTGG
121 AAAGTTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCC
181 TACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGAC
241 ACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTG
301 ATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGA
361 AGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGC
421 GGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGG
481 TTTGTCGCGTCGATTGTGAAAGCCCGGGGCTTAACCCTGGGTCTGCAGTCGATACGGGCA
541 GGCTAGAGTTCGGCAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATC
601 AGGAGGAACACCGGTGGCGAAGGCGGGGTCTCTGGGCCGATACTGACGCTGAGGAGCGAAA
661 GCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTA
721 GGTGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGG
781 GGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGA
841 GCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACATCGGAA
901 ACGGCCAGAGATGGTCGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAG
961 CTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTGTGTTGC
1021 CAGCGGAGCCTTCGGGCGGCCGGGGACTCACGGGAGACTGCCGGGGTCAACTCGGAGGAA
1081 GGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAAT
1141 GGCCGGTACAATGAGCTGCGATGCCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAG
1201 TTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCA
1261 GCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCATGAAAG
1321 TCGGTAACACCCGAAGCCGGTGGCCTAACCC

ACT-25 (1333 bp): Accession No: KP739983

1 GGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGATAAGCCCGGGAAA
61 CTGGGTCTAATACCGGATACGACACTCCGAGGCATCTTGGGGTGTGGAAAGTTCCGGCGG
121 TGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACG
181 ACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTC
241 CTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCC
301 GCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTG
361 ACGGTACCTGCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG
421 GTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCGA
481 TTGTGAAAGCCCGGGGCTTAACCCTGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGG
541 CAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCG
601 GTGGCGAAGGCGGGTCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGA
661 ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAACA
721 TTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGC
781 AAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAA
841 TTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACATCGGAAACGGCCAGAGATG
901 GTCGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAG
961 ATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTGTGTTGCCAGCGGAGCCTTC
1021 GGGCTGCCGGGGACTCACGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACG
1081 TCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATG
1141 AGCTGCGATGCCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGT
1201 CTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGT
1261 GAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCATGAAAGTCGGTAACACCCG
1321 AAGCCGGTGGCCT

ACT-34 (1331): Accession no: KP739984

1 GGCCGGGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGG
61 ATAAGCCCGGGAAACTGGGTCTAATACCGGATACGACACTCCGAGGCATCTTGGGGTGTG
121 GAAAGTTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGC
181 CTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGA
241 CACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCT
301 GATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGG
361 AAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCG
421 CGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCG
481 GTTTGTCGCGTCGATTGTGAAAGCCCGGGGCTTAACCCTGGGTCTGCAGTCGATACGGGC
541 AGGCTAGAGTTCGGCAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATAT
601 CAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCCGATACTGACGCTGAGGAGCGAA
661 AGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACT
721 AGGTGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTG
781 GGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGG
841 AGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACATCGGA
901 AACGGCCAGAGATGGTCGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCA
961 GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTGTGTTG
1021 CCAGCGGAGCCTTCGGGCTGCCGGGGACTCACGGGAGACTGCCGGGGTCAACTCGGAGGA
1081 AGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAA
1141 TGGCCGGTACAATGAGCTGCGATGCCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCA
1201 GTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATC
1261 AGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCATGAAA
1321 GTCGGTAACAC

ACT-39B (1453): Accession no: KT630861

1 TTTTGATCAGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATG
61 AACCACTTCGGTGGGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCCTC
121 ACTTCGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATGATACCTCCACTCGCATGGG
181 TGGAGGTTGAAAGCTCCGGCGGTGAGGGATGAGCCCGCGGCCTATCAGCTAGTTGGTGAG
241 GTAACGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGG
301 GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGC
361 GCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTT
421 CAGCAGGGAAGAAGCGCAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCC
481 AGCAGCCGCGGTAATACGTAGGGCGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCT
541 CGTAGGCGGCCTGTCGCGTCAATTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCG
601 ATACGGGCAGGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGC
661 GCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGA
721 GGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGG
781 TGGGCACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCCGCAGCTAACGCATTAAGTGC
841 CCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACA
901 AGCAGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACAT
961 ACACCGGAAACGTCTGGAGACAGGCGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGC
1021 TGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTC
1081 CCGTGTTGCCAGCAAGCCCTTCGGGGTGTTGGGGACTCACGGGAGACCGCCGGGGTCAAC
1141 TCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACG
1201 TGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGC
1261 CGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTTGCTAGTAAT
1321 CGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACG
1381 TCACGAAAAGCCGGAACACCCGAAGGCCGGGGGTCCAACCCCCTTGCGGGGGAAGGAGCC
1441 GGTCTAAGGGGGG

ACT-40 (1346): Accession no: KP739985

1 CGGGGTGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTTCACTCTGGG
61 ACAAGCCCTGGAAACGGGGTCTAATACCGGATAACACTCTGTCCCGCATGGGACGGGGTT
121 AAAAGCTCCGGCGGTGAAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGC
181 CTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGA
241 CACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCT
301 GATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGG
361 AAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCG
421 CGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCG
481 GCTTGTCACGTCGGATGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGGGC
541 TAGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATAT
601 CAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAA
661 AGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACT
721 AGGTGTTGGCGACATTCCACGTCGTCGGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTG
781 GGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGG
841 AGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATATACCGGA
901 AGCATCAGAGATGGTGCCCCCCTTGTGGTCGGTATACAGGTGGTGCATGGCTGTCGTCA
961 GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTG
1021 CCAGCATGCCCTTCGGGGTGATGGGGACTCACAGGAGACTGCCGGGGTCAACTCGGAGGA
1081 AGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAA
1141 TGGCCGGTACAATGAGCTGCGATGCCGCGAGGCGGAGCGAATCTCAAAAAGCCGGTCTCA
1201 GTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTTGCTAGTAATCGCAGATC
1261 AGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAA
1321 GTCGGTAACACCTGAAGCCGGTGGCC

ACT-52A (1397): Accession no: KT253928

1 CATGCAAGTCGTACGATGATAGCCCTTACAGGGGTGGATTAGTGGCGAACGGGTGAGTAA
61 CACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGA
121 TATGACCATCTTGGGCATCCTTGATGGTGTAAAGCTCCGGCGGTGCAGGATGAGCCCGCG
181 GCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGA
241 GAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT
301 GGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGC
361 CTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGA
421 AGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGG
481 AATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGATTGTGAAAGCTCGGGGC
541 TTAACCCCGAGTCTGCAGTCGATACGGGCTAGCTAGAGTGTGGTAGGGGAGATCGGAATT
601 CCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGGCGAAGGCGGATC
661 TCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCC
721 TGGTAGTCCACGCCGTAAACGGTGGGAACTAGGTGTTGGCGACATTTCCACGTCGTCGGT
781 GCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAA
841 AGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGA
901 AGAACCTTACCAAGGCTTGACATACACCGGAAAGCATTAGAGATAGTGCCCCCCTTGTGG
961 TCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTC
1021 CCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTTGTGGTGCTGGGGACTC
1081 ACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCC
1141 CTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGTGA
1201 GGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCA
1261 TGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGC
1321 CTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGNCCAAC
1381 CCCTTGTGGGAGGGAGC

ACT-69D (1371): Accession no: KT253930

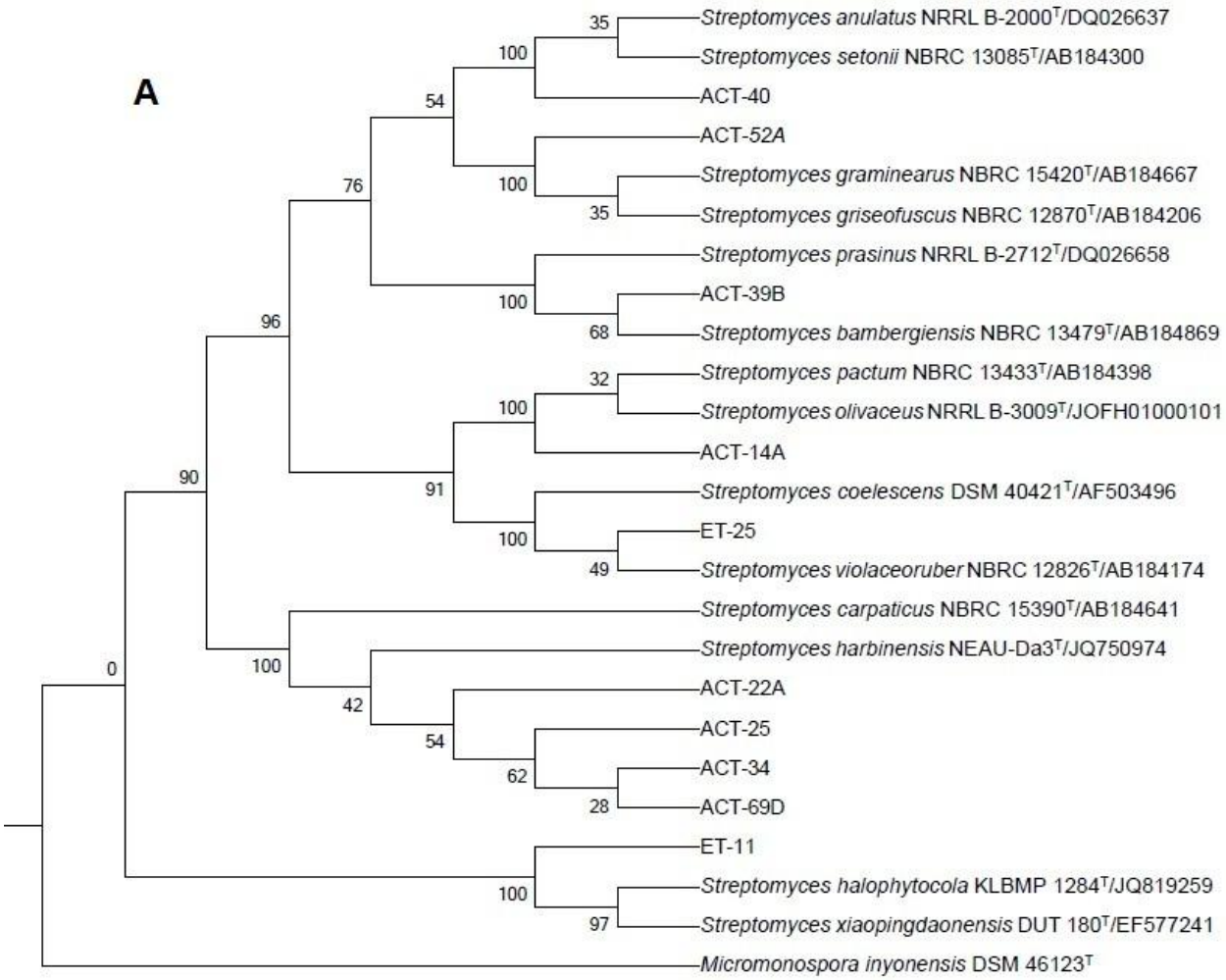
1 CATGCAAGTCGAACGATGAACCGGTTTCGGCCGGGGATTAGTGGCGAACGGGTGAGTAAC
61 ACGTGGGCAATCTGCCCTGCACTCTGGGATAAGCCCGGGAAACTGGGTCTAATACCGGAT
121 ACGACACTCCGAGGCATCTTGGGGTGTGGAAAGTTCCGGCGGTGCAGGATGAGCCCGCGG
181 CCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAG
241 AGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTG
301 GGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCC
361 TTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAA
421 GCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGA
481 ATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCGATTGTGAAAGCCCGGGGCT
541 TAACCCTGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGCAGGGGAGACTGGAATTC
601 CTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTC
661 TGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTG
721 GTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTTGTCCGTGCC
781 GCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGG
841 AATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGA
901 ACCTTACCAAGGCTTGACATACATCGGAAACGGCCAGAGATGGTCGCCCCCTTGTGGTCG
961 GTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG
1021 CAACGAGCGCAACCCTTGTCCTGTGTTGCCAGCGGAGTCTTCGGGCTGCCGGGGACTCAC
1081 GGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCT
1141 TATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATGCCGTGAGG
1201 TGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATG
1261 AAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCT
1321 TGTACACACCGCCCGTCACGTCATGAAAGTCGGTAACACCCGAAGCCGGTG

ET-11 (1370 bp) Accession no: KP739988

1 GTGGTGGATTAGTTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGTACTTTCGG
61 GATAAGCCTTGGAAACGGGGTCTAATACCGGATGGGACTGCCGGCCGCATGGTCTGGTGG
121 TGGAAAGCTCCGGCGGTACAGGATGAGCTCGCGGCCTATCAGCTTGTTGGTGGGGTGATG
181 GCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGA
241 GACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGC
301 CTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAG
361 GGAAGAAGCCTTAAAAAGTGACGGTACCTGCAGAAGAAGCACCGGCTAACTACGTGCCAG
421 CAGCCGCGGTAATACGTAGGGTGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCG
481 TAGGCGGCGTGTCGCGTCGGATGTGAAAGCCCGGGGCTTAACCCCGGGTTGGCATTCGAT
541 ACGGGCAGGCTAGAGTTCGGTAGGGGAGATTGGAATTCCTGGTGTAGCGGTGAAATGCGC
601 AGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGG
661 AGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTG
721 GGCACTAGGTGTGGGCGGCATTCCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTGCCC
781 CGCCTGGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAA
841 GCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATA
901 CACCGGAATCGGCCAGAGATGGTCGCGCCTTTTGGGCTGGTGTACAGGTGGTGCATGGCT
961 GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTC
1021 TGTGTTGCCAGCACGCCCTTCGGGGTGGTGGGGACTCACAGGAGACTGCCGGGGTCAACT
1081 CGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGT
1141 GCTACAATGGCCGGTACAATGAGAGGCGAGGCCGTGAGGTGGAGCGAATCTCAAAAAGCC
1201 GGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATC
1261 GCAGATCAGCAGTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGT
1321 CACGAAAGTCGGTAACACCCGAAGCCGGTGGCCTAACCCCCTTGTGGGGA

ET-25 (1341 bp): Accession no: KP739990

1 GCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAAC
61 GGGGTCTAATACCGGATACTGACCCTCGCAGGCATCTGCGAGGTTCGAAAGCTCCGGCGG
121 TGAAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACG
181 ACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTC
241 CTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCC
301 GCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTG
361 ACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG
421 GCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGG
481 TTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGG
541 TAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCG
601 GTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGA
661 ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAACA
721 TTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGC
781 AAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAA
841 TTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAGCATCAGAGATG
901 GTGCCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAG
961 ATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAAGCCCTTC
1021 GGGGTGTTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACG
1081 TCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATG
1141 AGCTGCGATACCGCAAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGT
1201 CTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATAGGTGCGGT
1261 GAATACGTTCCCGGGCCTTGTACACACCGCCCGATTCCCAAGGACGAAAGTCGGTAACAC
1321 CCGAAGCCGGTGGCCCAACCC



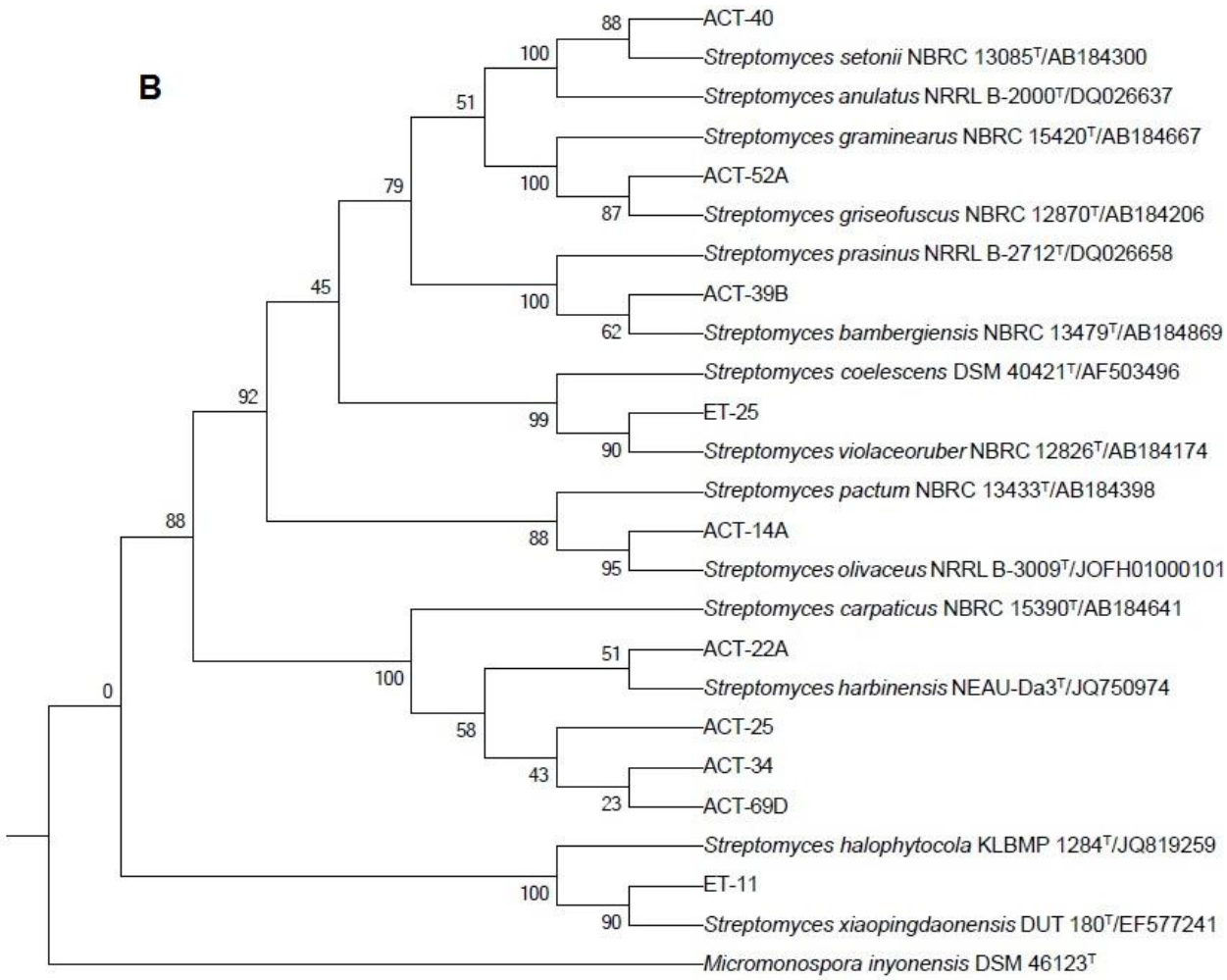


Fig 8. Figure A (neighbour-joining method) and B (maximum-likelihood method) showing phylogenetic tree of selected strains 16S ribosomal gene sequence with their closest type strains.

A fair number of strains from sponge *Aplysilla rosea* and *Aplysina* sp. showed antimicrobial activity. This result confirms that protection against different infectious agents or predators was accomplished in sponges perhaps by their associated bacteria by producing antimicrobial activities (Xi et al. 2012) such as antibacterial and antifungal activity. The noticeable activity against fungi by sponge-associated bacteria might indicate that to reserve their nutrients they need these sponge-associated bacteria which are active against fungi. No literature has reported actinobacteria as sponge pathogens (Schneemann et al. 2010). Rather it may be possible that sponge-associated actinobacteria play important roles in stabilizing sponge ecology by producing antimicrobial compounds (Bose 2014).

5.4 Conclusion

In over 400 colonies only 118 culturable actinobacteria were isolated from *Aplysilla rosea* and *Aplysina* sp. by using eight different media. Antimicrobial activity were observed in 78 strains isolated from *Aplysilla rosea* and *Aplysina* sp. sponges. A range of secondary metabolites including some potential new compounds were tentatively identified by TLC and HPLC techniques from

culturable actinobacteria. These actinobacteria from this study can be used to elucidate the role of interaction between sponge and sponge-associated bacteria for further experiments such as ACT-52A of which closest type strain was revealed as *Stretomyces costaricanus.* ACT-52A is the first sponge-associated actinobacteria which used for sponge-bacteria interaction study discussed in Chapter 7.

5.5 References

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CHAPTER 6

VALIDATION OF A NOVEL APPROACH TO STUDY THE ROLE OF SPONGE-BACTERIA INTERACTION USING SPONGE *APLYSILLA ROSEA* AND *VIBRIO NATRIEGENS* IN A CONTROLLED AQUARIUM SYSTEM

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Validation of a novel approach to study the role of sponge-bacteria interaction using sponge *Aplysilla rosea* and *Vibrio natriegens* in a controlled aquarium system

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Running Head: *Aplysilla rosea* and *Vibrio natriegens* interactions

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6.1 Abstract

Sponge-bacteria interactions are hypothesised to play a critical role in the biosynthesis of sponge derived novel marine natural products; however, this is experimentally difficult to test as sponge interact with a wide range of bacteria that live in or associate with them, as well as those in the external environment. To understand the roles of the interaction between sponge and associated bacteria in relation to their microbial diversity, metabolic profile and bioactivity, we proposed a new approach and designed a controlled experimental aquarium system that allows the study of sponge-bacteria interactions in a well-defined manner. To validate this approach, this aquarium system was used to study the interaction between the sponge *Aplysilla rosea* and a marine bacterium *Vibrio natriegens*. Sponges were exposed to *V. natriegens* at $5x10^6$ cfu/mL in a series of controlled aquaria and monitored for 48 hours. TRFLP and pyro-sequencing revealed significant shifts in microbial communities associated with the sponges after 24-48 hours. Both the control (without added bacteria) and *Vibrio*-exposed sponges (treatment) showed a distinct shift in bacterial diversity and abundance with time. As a result, *Vibrio* addition caused significant changes in the sponge metabolite profile analysed by chemical methods such as TLC, HPLC, GC-MS and ESI-MS. Changes in bioactivity were demonstrated via antibacterial activity which was higher with extracts of treated sponges. These results experimentally support the notion of dynamic and concerted responses by the sponge when interacting with a bacterium, and the feasibility of this novel approach to study the role of sponge bacteria interactions.

6.2 Introduction

Sponges host a diverse and extensive microbial community of symbiotic bacteria, archaea and unicellular eukaryotes in their tissues, but also have the ability to protect themselves from harmful microorganisms (1, 2). Any microorganisms that can resist the digestive processes and immune response of a sponge, can successfully inhabit it (3). The composition and structure of the associated microbial communities are controlled mainly by the host's ecological and biological features (4). It has been reported that this association could be species-specific (5, 6). However, the interactions between marine sponges and their associated bacteria are complicated and not easily understood (7, 8).

These bacterial assemblages are used by sponges for several purposes, such as nutrition (9) by either intracellular digestion or translocation of metabolites, nitrogen fixation (10-12), nitrification (13, 14), photosynthesis or carbon fixation (10, 15, 16), and anaerobic metabolism (17).

Microorganisms also play a crucial role in stabilizing the sponge skeleton, and contribute to protecting the host against predation and biofouling by enhancing its chemical defence system (18).

Sponges contribute over 30% of marine natural products discovered so far, and possess great potential for drug development (19). Sponge-derived natural products can be either biosynthesized directly by the sponge cells, or by their associated microorganisms. In most cases, the producers remain largely unidentified (20, 21). While some sponge-derived natural products seem to have a sponge origin since they are located within sponge cells (22, 23), it has also been observed that many compounds are associated with microbial symbionts, suggesting that microbes are the true producers (24-29). For most sponge-derived bioactive compounds, drug development is currently not possible due to limited access to sponge material and the low concentration of active compounds. Compounds that are produced by associated microorganisms, do not have similar limitations as there is a good potential to culture these microorganisms in bioreactors (30). Sponges produce and utilize diverse chemical classes to protect themselves and utilize symbiotic organisms for additional protection (31), including antifouling and anti-biofilm properties, such as terpenoids (32, 33) and the pyrrole-imidazoles (34-37). Therefore, it is hypothesized that sponges will defend themselves by producing antimicrobial compounds by altering their bacterial populations when challenged with foreign bacteria. Antimicrobial activity typically is monitored using the challenge microorganism, in this case *V. natriegens,* and a representative Gram-stain positive bacterium, such as *Staphylococcus aureus* or *Bacillus subtilis*, and a representative Gram-stain negative bacterium, either *Escherichia coli* or *Pseudomonas aeruginosa*.

To develop a reliable and renewable source of these sponge-derived compounds, it is important to identify the real producer and the biosynthetic pathway (19). Therefore, studying the interactions between sponges and their associated bacteria is important for understanding their role in producing the compounds of interest.

We hypothesize that the interactions between a sponge and its surrounding environment, including the microbes present in that environment, could be the main driving force in its chemical diversity. Secondary metabolites play an important role in many ecological processes, and these processes provide an evolutionary driver for the development of a wide variety of chemical compounds (38-40).

Different approaches have been used to understand the sponge and its association with microbes, such as using microscopy to examine the uptake of microbes (41). Novel molecular techniques (e.g.,16S-rRNA clone library) were firstly used by Althoff and co-workers in 1998 to analyse the microbial community (28). Sponge-derived bacterial sequence data were successfully used by Hentschel and co-workers in 2002 (42), and re-examined by Taylor and colleagues in 2007 (2), to facilitate the understanding of sponge-microbe associations. Recently, deep sequencing technology has added a new dimension to the study of the diversity of sponge associated bacteria, and will be a powerful tool for understanding sponge bacteria interactions in the near future (5, 43, 44). Interactions of sponges with bacteria are classified as competition, predation or symbiotic associations (45). Symbiotic associations can also differ, ranging from mutually beneficial to commensal (46). It is important to understand the role of sponge-bacteria interactions because of their ecological and biological significance (26, 28, 30). Moreover, uncovering the interaction between sponge-bacteria is critical to understand the biosynthetic origin of sponge derived novel metabolites (47).

In this study, we propose a novel approach to study the role of the sponge-bacteria interaction using a controlled aquarium system in which sponge explants are exposed to a selected bacterium at a specific density to initiate the sponge-bacteria interaction. While any sponge and sponge-associated bacterium can potentially be chosen for sponge-bacteria interaction study, for this study to validate this experimental system, a model sponge *Aplysilla rosea* and model bacterium *Vibrio natriegens* were chosen to demonstrate how the interaction between the two influences on the sponge-associated microbial diversity, the metabolic profile and the bioactivity.

Aquatic environments are a rich source of *Vibrio* spp., particularly estuaries, marine coastal waters, sediments and aquaculture settings worldwide (48-55), and the presence of high densities of *Vibrio* species in sponges has been reported (56, 57). *Vibrio natriegens* was originally isolated from salt marsh mud (58). We selected this bacterium because it grows in the marine environment, has a very short generation time of less than 10 min under optimal growth conditions (59) and has not been reported in association with *Aplysilla rosea*.

Three parameters have been used to demonstrate the impacts of sponge-bacteria interactions, which include microbial community, metabolic profiles and potential bioactivity. Terminal restriction fragment length polymorphism (TRFLP) and pyrosequencing were employed to investigate the diversity of the bacterial assemblages of *A. rosea,* and GC-MS for profiling the changes of non-targeted apolar metabolites. The changes in polar metabolic profile were investigated using TLC, HPLC and MS techniques. As a simple demonstration of potential

changes in bioactivity, due to changes in microbial community and metabolite profiles, antibacterial assays were performed with *V. natriegens, S. aureus* and *E. coli* as test organisms.

6.3 Materials and Methods

6.3.1 Sponge collection, transport and laboratory maintenance.

Twelve large pieces (>10 cm) of the shallow water sponge *A. rosea* were collected from Rapid Bay, Gulf St Vincent (35°31'15.29"S, 138°11'6.88"E) in South Australia at a depth of 9 m, in October 2011. All sponges collected were identified as *A. rosea* by S. Sorokin, (SARDI Aquatic Science, South Australia). Sponges were carefully removed from jetty pylons with a scraper, so as not to damage them. The samples were kept immersed in seawater in plastic bags and immediately transferred to buckets of seawater on the surface without exposure to air. Sponges were transported to the laboratory with aeration and held in the transport buckets through 5-6 water changes at 20-minute intervals to remove any toxins released as a result of transport stress. Next, they were transferred into aquaria (~100 liter capacity) for 1-2 weeks with regular water exchange on alternate days. Sponges were fed with 40 ml of the microalga *Nanochloropsis* sp. at 10^6 cfu/ml every 2 to 3 days.

6.3.2 Media preparation, culture of *Vibrio natriegens* and cell count.

Luria agar (LA) and broth (LB), (60) were prepared to grow *V. natriegens* with slight modification: tryptone, 10 g; yeast extract, 5 g; NaCl, 22.5 g; MilliQ water, 1,000 ml; (pH 7.0) with and without 15 g agar. A liquid culture of *V. natriegens* was prepared by inoculating cells from a single colony grown on LA medium into 250 ml LB medium and incubated at 37°C for 12 h on a rotary shaker at 200 rpm.

For inoculating into the tanks, *V. natriegens* was grown in 100 ml LB. Cultures were centrifuged to remove the growth medium, re-suspended in sea water and added to tanks to a final concentration of 5×10^6 cfu/mL at the start of the experiment (Time 0 h).

Cell counts of *V. natriegens* from sea water was conducted by dilution series and plating onto LA media using Miles and Misra method (61) (Supplementary File A). *V. natriegens* was identified by comparison of growth rate and colony morphology with the pure culture.

6.3.3 Experimental design.

Several pilot scale experiment was conducted repeatedly with the following aquarium set up to confirm the consistency of the result at the beginning of the experiment. Finally as shown in Fig 1 a small aquarium with 10 L capacity was used with 5 L of sea water for the experiment. Three replicate aquaria were used for the control and each treatment. Fine bubble aeration was provided to maintain dissolved oxygen levels (6-10 ppm). Sponge explants (n=30) were cut with a sterilized sharp knife under sea water, and averaged 6.02 ±1.11g wet weight and ca. 6 cm in size. Five randomly selected sponge explants were added to each aquarium 4-5 days prior to conducting the experiment to allow acclimatization.

Three tanks without sponges were for bacteria only control. For sampling, one sponge explant was collected at 0, 6, 12, 24 and 48 hours from each tank, placed in a sterile 50 ml falcon tube, frozen immediately with liquid nitrogen, and then freeze-dried, ground with a sterile mortar and pestle into a fine powder and stored at -80°C for microbial diversity analysis, metabolic profiling, bioactivity test and other required analyses. Sea water in each tank was monitored for cell count, pH, ammonia, nitrate, nitrite, and absorbance at 0, 3, 6, 12, 24 and 48 hours.

Samples were labelled with the following format: *Vibrio* treatment (T) or Control (C) followed by replicate tank number then time of sampling in hours. For example, C1-0 is Control tank 1 at 0 hours; T3-48 is treatment (added *V. natriegens*) tank 3 at 48 hours.

6.3.4 Genomic DNA extraction and PCR.

Briefly, total genomic DNA was extracted from 20 mg of lypophilized sponge tissue using the modified DNA extraction method of Atashpaz et al. (62). The main modification was in the buffer composition (3% CTAB, 100 mM Tris-HCl, 2 M NaCl, 20 mM Na_2EDTA, 0.2% LiCl, 2% PVP and 1% β-Mercaptaethanol) along with another buffer (lysozyme 80mg/mL, 4.8% Triton X, 8mM EDTA and 80mM Tris-Cl) and used a tissue lyser (Qiagen) for mechanical disruption with 1 mm beads for 30 sec at 15 Hz speed. Two separate extractions were combined for the same sample to maximize the representation of microbes. DNA was purified using a DNeasy Blood and Tissue Kit (Qiagen) and quantified in a ND-8000 UV-Visible Spectrophotometer (Thermoscientific). DNA quality was checked by running the gDNA on a 0.8% agarose gel at 100V for 40 minutes. The 16S rRNA genes were amplified from 50 ng of DNA using corresponding primers as shown in Table 1 for TRFLP and Table 2 for pyrosequencing (63, 64). Two different sets of primers were used for TRFLP and one set of primer was used for

pyrosequencing. The PCR reactions for TRFLP were performed in an Axygen Maxygene Thermal Cycler (MaxyGene Gradient).

Table 1. Name of the primers used for TRFLP study.

Name of Primer for TRFLP	Sequence	Reference
9F	6 FAM 5′ GAGTTTGATCMTGGCTCAGAT 3′	New
928R	5′ CCCCGTCAATTCMTTTGAGT 3′	New
Eub 8F	6 FAM 5′ AGAGTTTGATCMTGGCTCAG 3′	(63)
Eub 926R	VIC 5′ CCGTCAATTCMTTTRAGTTT 3′	(63)

Table 2. Primer used for Pyrosequencing.

Name of Primer for Pyrosequencing	Sequence	Reference
pyro28F	5′ GAGTTTGATCNTGGCTCAG 3′	(64)
pyro519R	5′ GTNTTACNGCGGCKGCTG 3′	(64)

The PCR was carried out in 50 µl reaction volumes with the following reagents: 2 µl of forward labelled primer (5 µM), 2 µl of reverse labelled primer (5 µM), 5 µl Thermopol buffer, 37 µl of injection water, 1 µl dNTPs (dATP, dCTP, dGTP, dTTP - 10 mM each with the water for injection), 1 µl of Taq polymerase (1 U Thermopol), and 2 µl of template DNA (25 ng/µl). The following touchdown PCR program was used: initial denaturation at 95°C for 5 min 1 cycle, then denaturation at 95°C for 30 s, annealing at 61°C-51°C with the -0.3°C increment for 45 s, and elongation at 72°C for 2 min for 40 cycles, the last cycle being followed by a 10 min elongation at 72°C.

PCR products were separated by electrophoresis in 1% (w/v) agarose gel to confirm amplicon size. To obtain sufficient PCR products for TRFLP analysis, four separate (50 µl each) PCR reactions were combined and purified using the UltraClean® PCR Clean-Up Kit (Mo Bio), then quantified using a Nano Drop 8000 Spectrophotometer (Thermo Scientific).

6.3.5 TRFLP analyses.

Microbial community analysis by TRFLP was carried out on sponge DNA samples at 0, 24 and 48 hours. Approximately 400 ng of purified PCR products were digested with the restriction endonucleases, Hha1, Msp1 and Rsa1 in a total reaction volume of 50 µl following the manufacturer's protocol. Immediately following digestion, samples were inactivated by heating at 65°C for 20 min for Hha1 and Rsa1 digested products. Msp1 was inactivated by adding 2.5 µl of 0.5 M EDTA. Samples were sent to Macrogen, Korea for fragment analysis. The company provided the raw FSA files after fragment analysis. Data were normalized using Dunbar's method (65). Peak profiles were generated using Peak Scanner Software v 2.0 (Life Technologies), and peaks (putative populations) were identified using Microbial Community Analysis III (MiCA3) (66) together with the Ribosomal Database Project (RDP) database.

6.3.6 Statistical analysis and 16S rRNA gene 454 pyrosequencing.

Bacterial tag-encoded titanium amplicon pyrosequencing (bTEFAP) was undertaken by the Research and Testing Laboratory (Lubbock, TX) as described by Dowd et al. (67). The 16S rRNA universal eubacterial primers 28F and 519R (Table 2) were used to amplify approximately 500 bp of the variable regions V1 to V3.

Bioinformatics was conducted using the Research and Testing Laboratory Standard Pipeline (68) and using Mothur (69). The percentage of each microbial species was used for hierarchical cluster analysis, and ANOVA and PERMANOVA were used to determine differences in microbial composition between treatments and sampling times. Treatments compared were without *V. natriegens* at t=0 (2 replicates) and with *V. natriegens* at t=24 and 48 hrs (3 replicates). Species of *Endozoicomonas*, *Ruegeria*, *Tenacibaculum* and *Vibrio* (except for *V. natriegens*) were combined to the individual genus, as the percentage of individual species was low (generally <1%). The microbial community in each sample was determined by Shannon's Diversity Index. The percentage of each microbial taxon (genus or species) was compared between treatments using a two-way ANOVA design, compared with and without *V. natriegens* addition at 24 and 48 hrs. Pairwise tests were conducted as needed using the LSD method. Permutational multivariate analysis of variance (PERMANOVA) (70) was used to reveal the effects of experimental treatments on the composition of the entire microbial community. PERMANOVA was conducted using 4999 permutations of residuals under a reduced model.

6.3.7 Metabolic profiling of non-polar metabolites from sponges by GC-MS.

All chemicals used were of analytical grade suitable for mass spectrometry. Briefly, 0.1 g of freeze-dried ground sponge tissue was extracted three times with $CHCl_3$/MeOH (2:1 v/v). Prior to the first extraction, 4 µl of octadecane ($C_{18}H_{38}$) was added as an internal standard. The powder and solvent mixtures were vortexed, sonicated for 15 minutes in an ultrasonic bath and incubated on a shaker for 60 minutes at room temperature. Samples were centrifuged for 5 minutes at 13793 ×*g* (Eppendorf-5415C) and the supernatant was transferred to a new safe lock tube (B147671L/2423, Eppendorf AG, Germany) which is stable to use with 100% chloroform and methanol. Sponge tissue was re-extracted twice more, using 0.75 mL of the same solvent system and omitting the 60 minute incubation. Extracts were combined, 0.25 mL water was added and samples were mixed and left to stand for 10 minutes to allow phase separation. Samples were centrifuged again for 5 minutes at 13793 ×*g* and the top aqueous phase containing the polar metabolites was carefully discarded and the bottom phase was collected. Finally, extracts were dried under a stream of nitrogen and dissolved with chloroform and transferred to a glass vial (see Supplementary File B for flow chart of procedure).

GC-MS analysis was performed using an Agilent 7890A Gas Chromatograph (GC) and Agilent 5975C inert XL EI/CI MSD (mass selective detector). Chromatographic separation was achieved on a 30 m Rxi® 5Sil MS with 10 m Integra-Guard® (low polarity Crossbond® silarylene crossbond; similar to 5% phenyl/95% dimethyl polysiloxane, RESTEK) column with dimensions: 0.25 mm ID and 0.25 µm phase thickness. Mass spectra were acquired at 70 eV from m/z 25-650 using MSD Chemstation software version E.02.00.493 (Agilent Technologies). Helium was the carrier gas at a flow rate of 1.2 mL/minute. Inlet and interface temperatures were 300°C and 250°C, respectively. The ion source temperature was set at 230°C. The oven temperature program was as follows: 70°C for 3 minutes, then a ramp of 8°C/ minute to 330°C where the temperature was held for 10 minutes before re-equilibration to 70°C. One µL sample in chloroform was injected in splitless mode.

6.3.8 Analysis and clustering of Cluster Mass Function (CMF) data.

GC-MS data files were converted to netCDF format using the embedded file conversion function included in MSD ChemStation. Raw data files were imported into MetAlign (version 4.11011) and processed to give aligned ion and retention time (ion-RT) tables (71). MSClust was used to perform unsupervised clustering. All detected features belonging to the same putative compounds were grouped together to allow data reduction for further analysis. Data extraction was optimized for several of the program settings including maximum amplitude, peak threshold, and average peak width at half height. All ion-wise alignments were clustered into putative

mass-spectra with MSClust and reduced data frames were subsequently used for multivariate statistical analysis. Multivariate statistical methods were applied to reduce dimensionality for visualization and subsequent interpretation of the chemo-diversity of the sponges.

All statistical data analysis was done in the R statistical environment (R Development Core Team 2012). Apolar metabolite profile was estimated by comparison of the reduced CMF data through hierarchical clustering using the complete Ward method with the Chemometrics and mixOmics packages for R (72, 73).

6.3.9 Thin Layer Chromatography (TLC).

Samples were prepared in a similar way as HPLC (see below) and finally 20 µl were loaded onto aluminium TLC silica gel 60 F_{254} plates (Merck), using the following solvent systems: Ethyl acetate: methanol [7:3] and Butanol: Acetic acid: water [4:1:1].

Preparative scale layer chromatography (PLC) was carried out using PLC glass silica gel (Kieselgel 60 F_{254}, size 5x10 cm) with the active extracts against *S. aureus* to obtain purified compounds for further analysis. Antibacterial activity was evaluated by students' t-test with two independent groups using R version 3.1.2

6.3.10 High Performance Liquid Chromatography (HPLC).

HPLC analyses were performed with an Agilent 1100 system, including Alliance separation module 2695, column heater, and 2998 photodiode array detector, and run using Empower Chromatography Data Software (Waters). The HPLC conditions consisted of two eluents (eluent A [0.1% aqueous trifluoroacetic acid] and eluent B [100% acetonitrile]) and an elution profile based on a linear gradient from 10% eluent B to 100 % eluent B within 25 min and then holding at 100% eluent B for an additional 20 min. Flow rate was kept constant at 1 ml min^{-1}. Chromatographic separation was performed on an Atlantis T3 C18 column (Agilent, 100 mm x 3 mm ID and 3 µm particle size) in reversed phase with a fixed temperature of 25°C. For the profiling of the polar metabolites, 100 mg of freeze-dried sponge tissues were extracted three times according to Turon et al. 2009 (74). Briefly, powdered sponge tissue was dissolved with 1 ml of MeOH in an ultrasonic tank for 5 min, centrifuged and retaining the pellet after transferring the supernatant. The pellet was extracted twice more with 10 and 15 min sonication. The combined crude extract was filtered through a 13 mm 0.2µm PVDF acrodisc Syringe Filter and added to a 2 ml tube with glass insert. Then, 50 µl of this filtered solution was injected into the

HPLC system described above. The peaks were detected at 215, 250, 310, 365 nm from the data collected across the 200- to 800-nm wavelength range.

6.3.11 Matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization Mass Spectrometry (ESI-MS).

Four samples were selected for further analysis by ESI-MS: C1-24, and C2-48 and T1-48 and T2-48 based on their TLC and HPLC profiles.

The ESI mass spectra were obtained with a Waters Synapt HDMS (Waters, Manchester, UK). Mass spectra were obtained in the positive ion mode with a capillary voltage of 3.0 kV and a sampling cone voltage of 100 V. The other conditions were as follows: extraction cone voltage, 4.0 V; ion source temperature, 80 °C; desolvation temperature, 350°C; desolvation gas flow rate, 500 L/h. Data acquisition was carried out using Waters MassLynx (V4.1, Waters Corporation, Milford, CT, USA). Positive ion mass spectra were acquired in the V resolution mode over a mass range of 0–2000 *m/z* using continuum mode acquisition. MS/MS spectra were obtained by mass selection of the ion of interest using the quadrupole, fragmentation in the trap cell where argon was used as collision gas. Typical collision energy (Trap) was 50.0 V.

6.3.12 Antibacterial activity and bioautogram.

S. aureus and *E.coli* were grown on Tryptone soy agar (TSA) (Tryptone soy 30 g, agar 15 g and RO water 1 L) (75) and added to Antibiotic agar medium (AAM) (Sigma-Eldrich) at 1% (v/v) after adjusting the bacterial concentration to an OD of 0.2 at 600 nm. Sponge extracts (50 ul) were added to wells in the agar. Plates were incubated at 37°C for 16 to 24 hours, and zones of inhibition were measured. *Vibrio natriegens* was grown in *Vibrio* agar (Brain heart infusion agar 38g, NaCl, 20 g and MilliQ water, 1L) and the antibacterial test was conducted in the same manner as described above except *Vibrio* agar was used instead of AAM.

6.3.13 Ammonia, Nitrite, Nitrate measurement.

Ammonia, nitrite and nitrate were measured using a commercial kit (Aquaone) and salinity by a portable refractometer (Thermo Fisher Scientific).

6.3.14 Sequence accession numbers.

All sequence data were deposited, with MIMARKS-compliant metadata, in the NCBI Sequence Read Archive under BioProject number PRJNA276919, with accession numbers from SRX950227 to SRX950240 (Supplementary File-C).

6.4 Results

6.4.1 Maintenance of the sponge *Aplysilla rosea*.

Large pieces (>10cm) of *A. rosea* were successfully maintained in large recirculating aquarium systems (~100 liters) for up to 40 days with feeding and regular seawater exchange. In the small experimental tanks (10 liters), 5-6 small pieces (≤6 cm) could survive for a maximum of 10 days without feeding, and with added *Vibrio* at 10^6 cfu/ml, they could survive for a maximum of 54 hours in a reliably healthy condition. Therefore, the experiments were restricted to 48 hours. Sponge health status was observed and assessed visually by colour, tissue compactness and the appearance of necrotic spots.

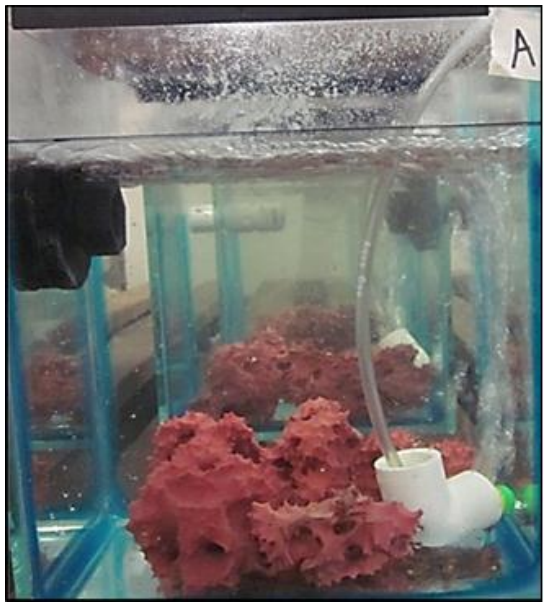

Fig 1. Experimental aquarium containing *Aplysilla rosea* sponges at the start of the experiment with no *Vibrio natriegens* addition.

6.4.2 Enumeration of total *Vibrio natriegens* cell count.

V. natriegens was added to the tanks at an final density of $5x10^6$ cfu/mL. In control tanks without sponges, the *Vibrio* culture grew rapidly within 10 minutes and increased to $3x10^8$cfu/mL at 24 hours and maintained this level until 48 hours (Fig 2). In treatment tanks that contained sponges,

the density of *V. natriegens* declined to $2x10^3$cfu/mL at 12 hours, followed by an increase to $9x10^6$ cfu/mL at 24 hours (Fig 2). No *Vibrio* colonies were detected in tanks containing only sponges.

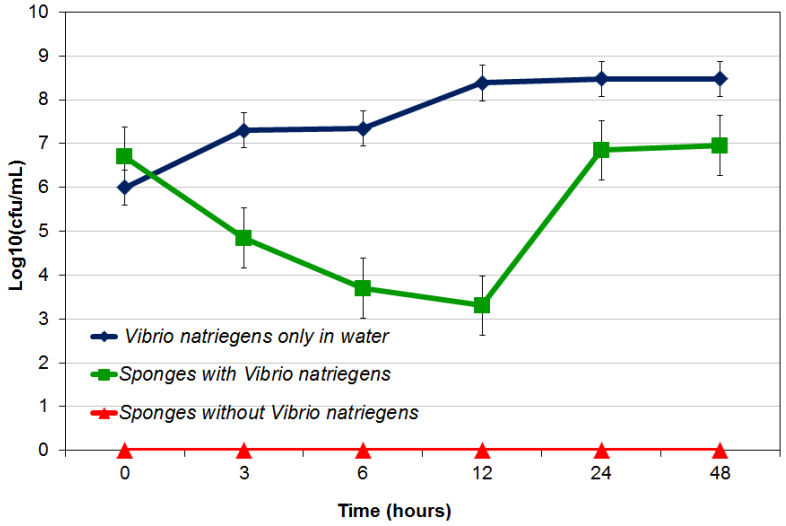

Fig 2. CFU counts of *Vibrio natriegens* in tank water at 0, 3, 6, 12, 24 and 48 hours after *V. natriegens* addition to tanks with no sponge and with sponges. Error bars indicate SEM, n=3.

6.4.3 Characterization of microbial community by TRFLP.

Using the primer pairs 9F and 928R, a total of 116 different TRFs were obtained from the sponges, with 12 to 25 from each sample for each enzyme; the numbers were generally higher when digested with Hha1 and Msp1. At the start of the experiment (t=0 hr), a total of 32 bacterial genera were identified (Table 3). After 24 hours, this increased to 60 genera in the treated sponges, but remained relatively stable at 28 genera in the control sponges. After 48 hours, only 19 genera were found in the *Vibrio*-treated sponges and 11 genera in the control sponges.

When the same samples were analysed with the primer pair EUB8F and EUB926R, 18 genera were found at 0 hour. After 24 hours 26 genera were found (Table 3), along with unidentified bacteria for the treated sponges. In contrast, in the control sponges only 10 genera were detected at 24 hours (Table 3). After 48 hours, 45 genera were identified concurrently by three restriction digestion enzymes (Table 3) in the treated sponges compared to only 11 in the control sponges.

Table 3. Putative genera identified by TRFLP using primer pairs 9F-928R and EUB 8F-EUB 926R.

Sample and Time	Primer 9F and 928R	Primer EUB 8F and EUB 926R
Treatment+ Control (T=0h, C=0h)	*Acinetobacter, Burkholderia,* ***Colwellia**, **Pseudoalteromonas**, **Pseudomonas**, Ralstonia, *Vibrio, Janthinobacterium, **Microbulbifer**, Pusillimonas,* **Unknown**	*Bacillus, Bacteroidetes*, *Bradyrhizobium*, ***Candidatus***, *Clostridium*, *Comamonas*, *Mesorhizobium*, *Rhizobiales*, ***Rhizobium***, *Streptomyces*, ***Synechococcus***, *Streptococcus*, **Vibrio.* Unidentified and unknown
Treatment (T=24h)	***Achromatium***, *Achromobacter*, ***Acidithiobacillus***, ***Acidovorax***, *Aeromonas*, ***Alcaligenes***, *Alicycliphilus*, ***Aliivibrio***, *Arthrobacter*, ***Bacillus***, *Beggiatoa*, ***Buchnera***, ***Candidatus***, ***Chromatium***, **Comamonadaceae**, ***Comamonas***, ***Delftia***, ***Diaphorobacter***, *Enterobacter*, *Escherichia*, *Gallibacterium*, ***Haemophilus***, *Malikia*, ***Neptunomonas***, *Nitrosococcus*, *Olavius*, ***Pasteurella***, *Polaromonas*, *Serratia*, ***Shewanella***, *Shigella*, ***Streptomyces***, *Thioalkalivibrio*, *Uchneara*, ***Variovorax***	***Achromatium***, *Acidiphylum*, *Acidithiobacillus*, *Acidovorax*, *Actinobacillus*, *Acinetobacter*, ***Actinomyces***, *Aliivibrio*, **Alteromonas, *Archobacter*, ***Azorhizobium***, *Bacillus*, *Buchnera*, *Candidatus*, ***Colwellia***, *Clostridium*, *Flavobacteria*, **Mesorhizobium* , *Pseudomonas*, *Pseudoalteromonas*, ***Roseobacter***, ***Ruegeria***, **Sinorhizobium*, *Vibrio*, **Synechococcus*, ***Streptomyces***, *Shewanella*, unidentified and unknown
Control (C=24h)	*Capnocytophaga*, ***Duganella*,** *Ethylomicrobium*, *Ethylophaga*, ***Ethylophilus***, *Massillia*, ***Psychrobacter*** and *Zoogloea*	Actinobacteria, *Arthrobacter*, *Bacillus*, *Candidatus*, *Clostridium*, ***Pseudomonas***, ***Synechococcus***, *Streptomyces* and ***Rhizobium***

Sample and Time	Primer 9F and 928R	Primer EUB 8F and EUB 926R
Treatment (T=48h)	***Acinetobacter***, *Beggitoa*, , *Capnocytophaga*, *Citrobacter*, Comamonadaceae, *Eikenella*, ***Flavobacterium***, *Pasteurella,* Uncultured, **Unidentified**	*Achromatium*, *Achromobacter*, *Acidithiobacillus*, ***Acinetobacter***, *Actinobacillus*, *Actinomyces,* **Alteromonas*, *Arthrobacter*, *Azorhizobium*, *Azospirillum*, *Bacillus*, *Beggiatoa*, *Bordetella*, *Burkholderia*, *Candidatus*, *Capnocyophaga*, *Cellvibrio*, *Clostridium*, ****Colwellia***, *Corynebacterium*, *Deinococcus*, *Desulfovibrio*, ***Flavobacteria,*** *Halobacillus*, *Lactobacillus*, ***Mesorhizobium***, *Methylobacter*, ***Phaeobacter***, *Pseudoalteromonas*, *Pseudomonas*, *Rhizobium*, *Rhodococcus*, ****Roseobacter***, ****Ruegeria***, *Salnimicrobium*, *Shewanella*, **Sinorhizobium*, *Sphingomonas*, *Streptomyces*, **Synechococcus*, **Vibrio,* Unknown
Control (C=48h)	*Achromatium*, ***Candidatus***, *Chromatium* and *Ethylococcus*	*Bacillus*, ***Candidatus***, *Clostridium*, **Flavobacteria*, *Pseudoalteromonas*, *Pseudomonas*, *Rhizobium*, ****Sinorhizobium***, *Streptomyces*, **Synechococcus*.

* indicating genera matching with pyrosequencing result. **Bold** indicates most abundant genera

6.4.4 Characterization of microbial diversity by 454 Pyrosequencing.

Pyrosequencing revealed a significant change in the sponge associated microbial communities over time and with the addition of *Vibrio* (Table 4). Analysis of microbial diversity using Shannon's Diversity Index indicated that diversity changed significantly (P=0.001) with time between T=0 and 24 hours (H=0.38, 1.52 and 1.24 for T=0, 24 and 48 hours respectively,

maximum LSD at 0.05=0.47) and was significantly (*P*=0.001) more diverse in the *Vibrio*-treated sponges (*H*=2.07) compared to the control sponges (*H*=0.80). The bacterial community structure of treated and untreated sponge is shown in Fig 3. In the control tanks, sponges were initially dominated by *Synechococcus,* which decreased with time and became dominated by *Sinorhizobium.*

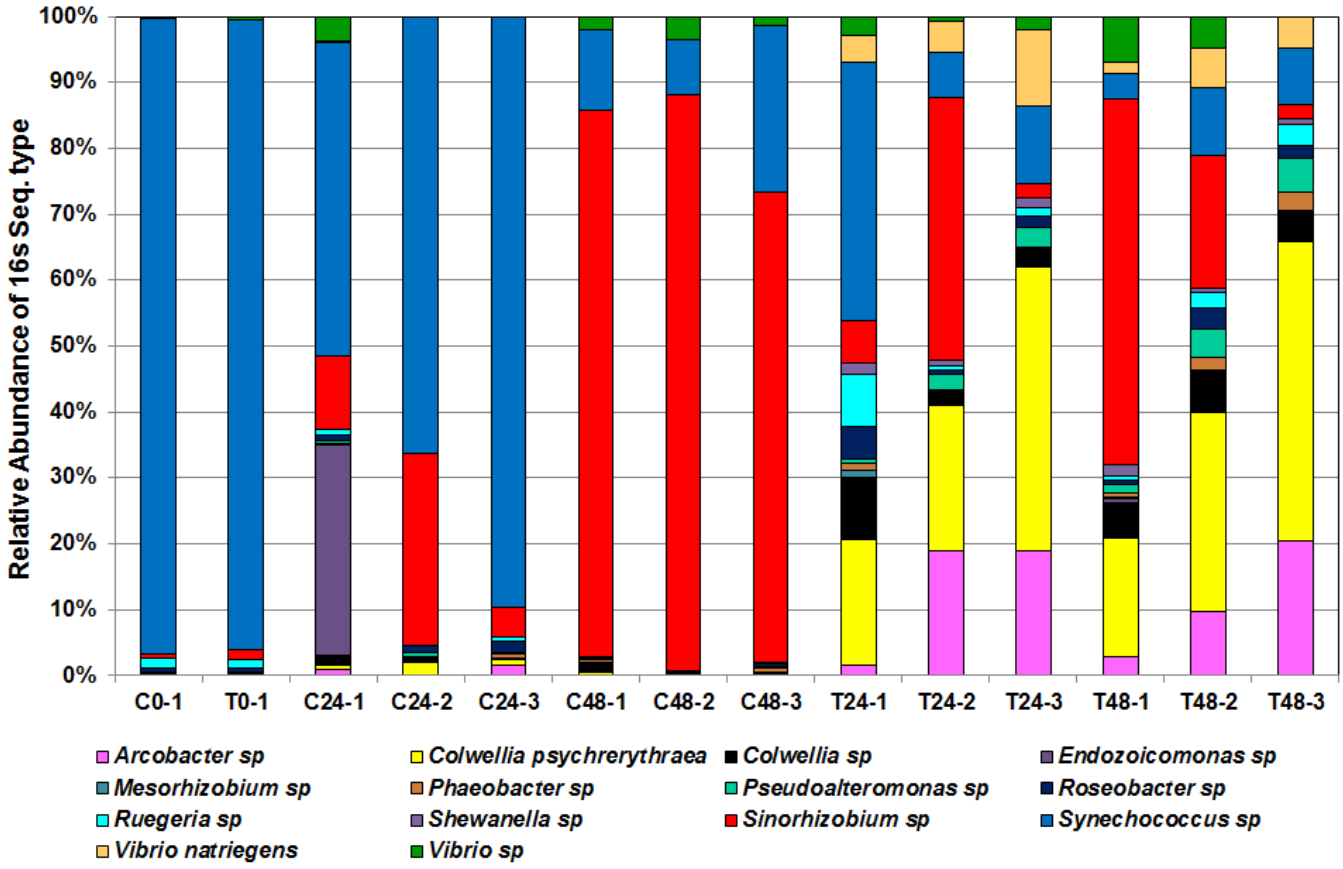

Fig 3. Major bacterial genera including *Vibrio natriegnes* inferred from 16S rRNA relative sequence abundances derived from 454 pyrosequencing of sponges from control tanks (C, no added bacteria) and treated tanks (T, added *V. natriegens*) at 0, 24 and 48 h for each replicate tank.

In the treated tanks, Principal coordinates analysis ordination (PCO) (Fig 4) showed a clear separation of *Vibrio*- treated and untreated samples and indicated four distinct groupings.

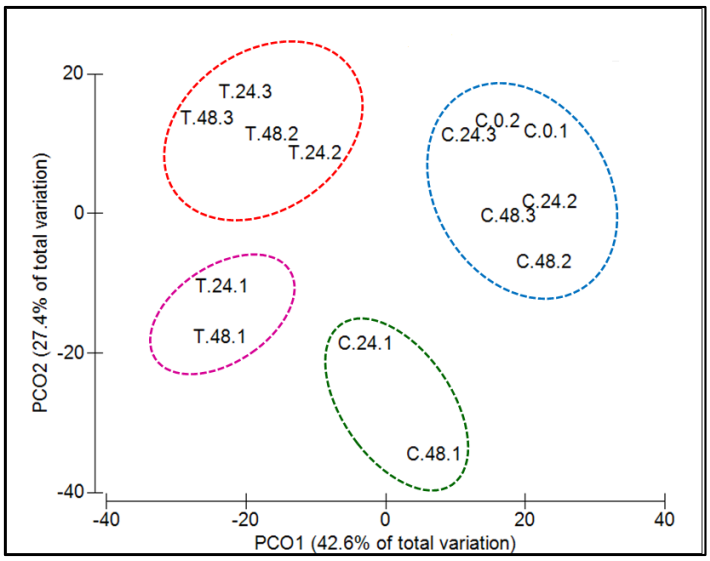

Fig 4. Principle Coordinate Ordination (PCO) presenting the bacterial communities associated with treated and control *Aplysilla rosea* sponges based on Bray Curtis similarity using 454 pyrosequencing of 16SrRNA.

Table 4. Results from the multivariate permutational analysis (PERMANOVA) for the effect of time (Ti) and treatment (Tr) on bacterial assemblages in the sponge *Aplysilla rosea*.

Source	df	SS	Pseudo-F	P(perm)
Time	2	2499	3.6283	0.0052
Treatment	1	3749.8	5.4881	0.0002
Ta (Tr)	4	2902.5	2.1071	0.0908
Ti x Tr	1	349.46	0.4314	4986
Res	5	1721.9		

PERMANOVA indicated these changes were significant *(P*<0.01, Table 4). C1-24 and C1-48 were distinguished by a high abundance of *Sinorhizobium* sp. whereas C2-24, C2-48 and C3-48 had high abundances of *Synechococcus* sp., *Oleispira* sp., and *Pirellula* sp. On the other hand T1-48 was very different from the others because it was dominated by *Mesorhizobium* spp., *Paracoccus* spp., *Oceanisphaera* spp., *Amphritea balenae* and *Colwellia* spp. T1-24 was

dominated by *Colwellia* spp., *Ruegeria* spp., *Phaeobacter* spp., *Roseobacter* spp., *Shewanella* spp., *Flavobacterium* spp., *Tenacibaculum* spp, *Arcobacter* spp., and *Pseudovibrio* spp.

The largest differences between treatments at different times were in *Synechococcus* spp., which made up 92% of sequences in sponge tissue at the start of the experiment, with significant interaction (ANOVA; F (1,8) =6.12 P=0.038) between time and *Vibrio* addition. In the control sponges, *Synechococcus* spp. were reduced to 60% and 15% of their original abundance after 24 and 48 hr respectively, and in the *V. natriegens*-treated sponges to 15% and 6%.

There was also a significant interaction between time and *Vibrio* treatment for the percentage of *Sinorhizobium* spp., increasing from 13% at 24 hr for treated and untreated samples to 21% in *Vibrio* treated samples and 80% in untreated samples. *Vibrio* addition also resulted in a number of other taxa increasing as a percentage of the community (Table 5). Finally, treatment and time had a main effect for *Phaeobacter* spp., which increased from 0.26 to 0.9% (F (1,8) =5.62, P=0.045) of the community from 24 to 48 hrs.

Table 5. Main effect of *Vibrio* addition on relative abundance of microbial 16SrRNA sequences in sponges for microbial groups with a significant difference between control (sponge only) and *Vibrio* treatment. Results from 2 way ANOVA. n=6.

Microbial group	% of sequences		$F(df_{time}, df_{error})$ = F-value, P = p-value
	Control	+*Vibrio*	
Alteromonas sp.	0.08	2.21	F(1,8) = 8.77, P = 0.018
Arcobacter sp.	0.41	9.09	F(1,8) = 9.26, P = 0.016
Colwellia psychrerythraea	0.66	22.25	F(1,8) = 40.70, P = <0.001
Colwellia sp.	0.58	3.96	F(1,8) = 12.45, P = 0.008

Microbial group	% of sequences	$F(df_{time}, df_{error})$ = F-value, P = p-value	
	Control	+*Vibrio*	
Marinomonas sp.	0.01	0.41	F(1,8) = 7.50, P = 0.026
Moritella abyssi	0	1.02	F(1,8) = 15.18, P = 0.005
Pseudoalteromonas sp.	0.23	2.07	F(1,8) = 14.32, P = 0.005
Shewanella sp.	0.01	0.98	F(1,8) = 31.67, P = <0.001
Vibrio natriegens	0.03	4.08	F(1,8) = 49.30, P = 0.002
Vibrio sp.	0.57	7.65	F(1,8) = 14.32, P = 0.005

6.4.5 Changes in non-polar metabolite profile of sponges.

Both control and treated sponges had similar non-polar metabolite profiles at the start of the experiment, with the exception of one divergent replicate from each (C2-0 and T3-0; Fig 5). After 24 hours, this divergence had disappeared, and both groups of sponges experienced a similar shift in metabolite profiles. After 48 hours, however, there were distinct differences between control and treated sponges due to increased abundance of a several metabolites in the treated sponges and reduced metabolites in control sponges and vice versa. Moreover, several unknown new metabolites were observed in the treated sponges, which were not revealed in control sponges.

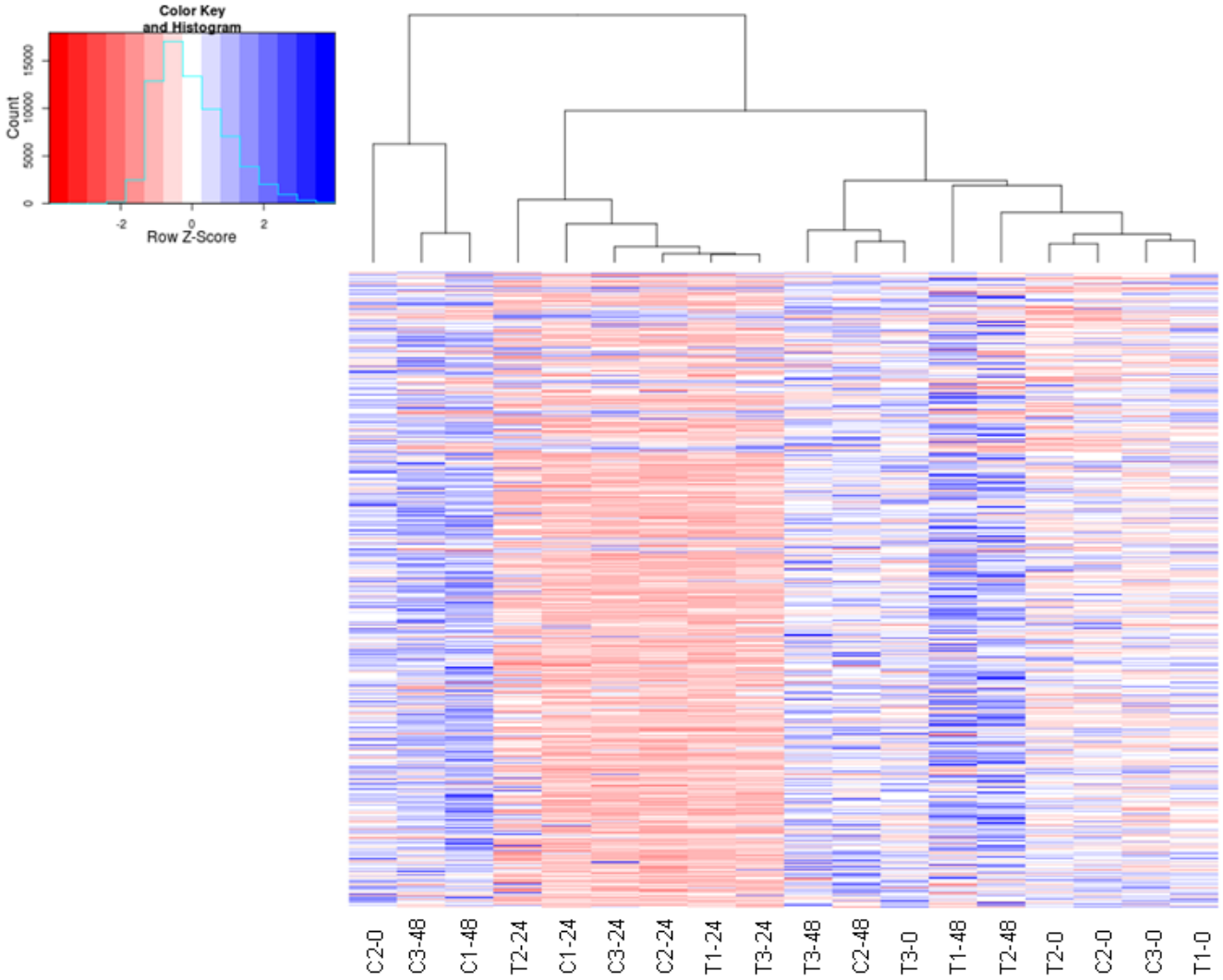

Fig 5. Cluster analysis and distance metrics of non-polar extract of control and treated sponges during 48 hours clearly showing different metabolic profile patterns between control and treated sponges.

6.4.6 Changes in polar metabolite profiles of sponges.

HPLC analysis of polar metabolites revealed different peak profiles between control and treated sponges after 24 and 48 hours. Most of the peaks were detected at 215 nm wavelength. Principal component analysis (PCA) of selected samples with triplicate HPLC profile from three different extraction at this particular wavelength showed distinct clustering patterns after 24 and 48 hours between control and treated sponge extract (Supplementary File D).

ESI-MS profiling was applied to four samples: C1-24; C2-48, T1-24 and T2-48 (Fig 6) for further evaluation.

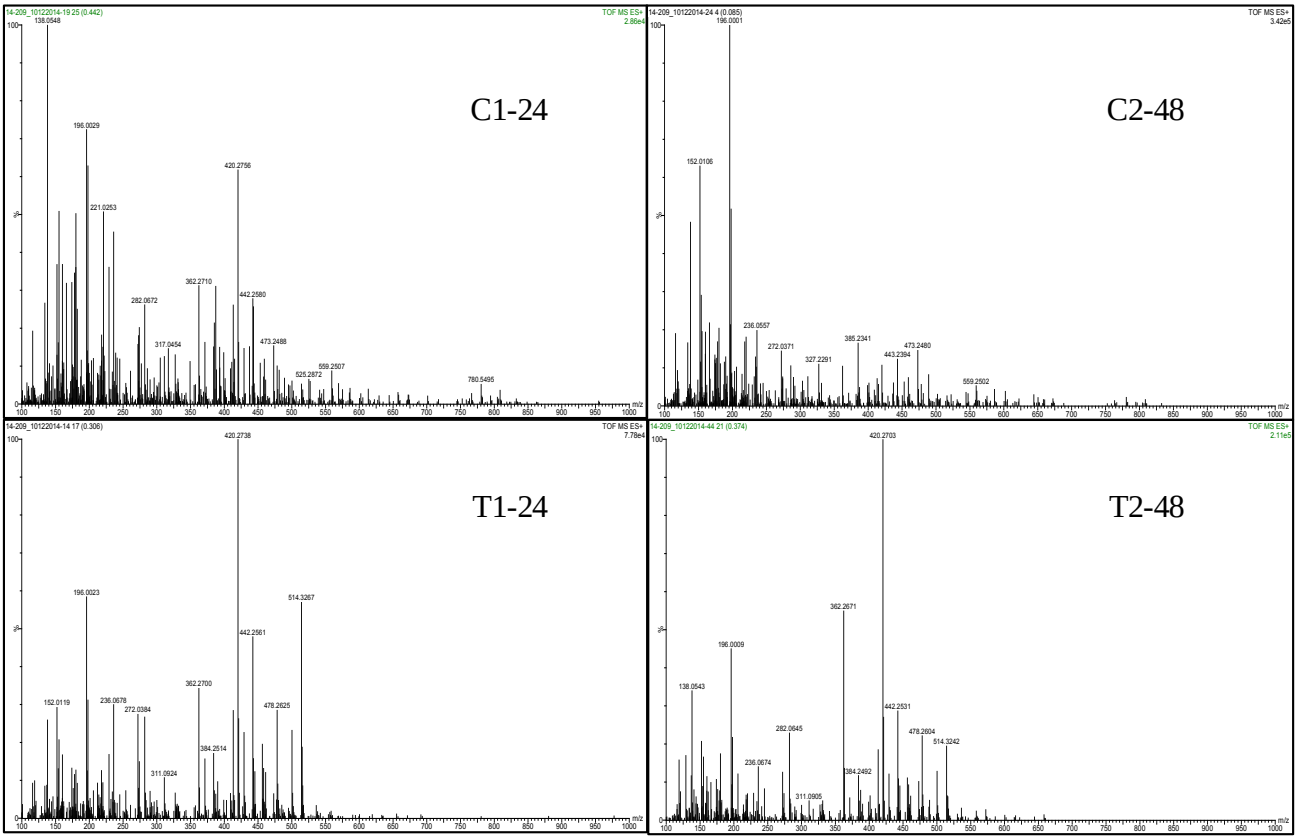

Fig 6. Comparison of ESI-MS chromatogram between control and treated sponges during 24 and 48 hours.

The main differences observed were a shift of lower molecular weight compounds in control and higher molecular weight compounds in treated samples. The four most abundant peaks were observed in the control sponges at *m/z* 420, 138, 152 and 196; in contrast, the four most abundant peaks were detected in the treated sponges at *m/z* 196, 362, 514 and 420. The ESI-MS profiles of the sponges between treated and control sponges were substantially different (Supplementary File E).

6.4.7 Water quality.

Throughout the experiment, the pH was stable at 7.8. The ammonia content in the control tanks ranged between 0 and 0.5 ppm and in the treatment tank between 0 and 5 ppm. Nitrite in the control tank was undetectable throughout the experiment but in the treatment tank after 48 hours it reached 1 ppm. In the tanks which only contained *Vibrio* without any sponge, ammonia content ranged between 0 and 5 ppm and nitrite was undetectable.

6.4.8 Metabolite profile changes in sponges demonstrated by antibacterial activity and bioautogram.

To test if changes in bioactivity correlated with changes in metabolite profiles extracts of sponges from both treatment and control tanks samples were tested against the 3 test bacteria. The 0 hour samples showed similar inhibition zones (9mm) vs *S aureus* only, but after 24 hours the sponge extracts in the treated tank showed greater inhibition vs *S aureus* than that of the control tank. This trend remained even after 48 hours (Supplementary File F).

No antibacterial activity was observed a when the sponge tissue extracts were evaluated against *Escherichia coli* and *Vibrio natriegens*.

Further evaluation of different spots from the preparative TLC plate against *S. aureus*, showed that both T1-24 and T2-48 contained five active spots and the highest activity (9 mm) was found in T2-48. The student t-test illustrated that T1-24 and T2-48 (P<0.0001) possessed significantly higher bioactivity compared to C1-24 and C2-48.

TLC plate showed good separation of compounds in the solvent system Ethyl acetate: methanol [7:3] and Butanol: Acetic acid: water [4:1:1] (Supplementary File F). When we compared the retention factors between the treatments (T1-24 and T2-48) and the control (C1-24 and C2-48), many spots were found in the treated sponges but not in the control sponges, including the bioactive spots (Supplementary File G), confirmed by bioautogram.

6.5 Discussion

In the marine environment, marine sponges constantly interact with the bacteria within and surrounding the sponges (76, 77). To our knowledge few experimental studies have been conducted to understand the role of this interaction (78), therefore a new experimental approach was developed to study this role by which any sponge of interest could be exposed to selected bacteria either associated or non-associated with sponges in a controlled aquarium system. The advantage of this approach is that the roles of the selected sponge-bacteria interactions could be studied experimentally in a variety of controlled conditions. As the first validation study, a model sponge *A. rosea* was challenged by a fast growing non-associated marine bacterium *V. natriegens* to develop and optimize the experimental aquarium system and methodology to understand the role of their interaction. The impact of this interaction was characterized by the changes in sponge-associated microbial community, the sponge metabolic profiles in terms of

both polar and non-polar metabolites, as well as their bioactivities (antibacterial activity as demonstration only).

To successfully develop this experimental system, it is important to ensure that the sponge of interest is handled without stress during collection, transport and maintenance in the laboratory and aquarium before the exposure experiments. Using *A. rosea* as a model, this study developed a complete set of protocols that could successfully maintain the live sponge for an extended period of time (40 days). In particular, a specific feeding regime was established to acclimatise the sponges in the aquaria. This acclimatisation is essential to standardise the sponges for the exposure experiments in order to minimise the biological variability of each sponge explants, though it could not be completely eliminated as demonstrated in the experimental results (Fig 5). While the specific conditions developed for *A. rosea* may not be generally applicable to other sponge species, it is important to optimise or fine-tune the basic protocols established in this study for different types of sponges.

There are several limitations in this study. The small volume aquarium used for this study may not be ideal for sponges, as the confined space caused the microbial diversity change in the control tanks. Secondly, feeding during the experiment, particularly for long term experiments, should be continued to avoid starvation stress and by adding a few more tanks with regular feeding and without feeding as the comparison controls. Thirdly, to minimize the biological variability, a large number of samples may be used and the statistical test can be used to obtain more reliable data though the study cost may be prohibitive. Finally, the current experiment did not consider capturing the metabolites released into the sea water (79) by the sponge. Here the system can be improved by introducing bags of absorbents such as XAD7 or XAD4 into the tanks. In this case, a continuous flow-through system is desirable rather than using only an aerator. As shown in Figs 5 and 6, many compounds were produced during the exposure treatment. Further studies can be directed to elucidate these metabolites by purification (80) and using LC-MS and NMR (81) which would reveal the metabolite structures.

The change in sponge-associated bacteria diversity is of utmost interest, as this change could trigger the changes in sponge metabolism and metabolite composition (82). It is not known how rapidly the sponge-associated microbial community could change in response to the bacterial exposure. TRFLP and pyrosequencing demonstrated that the change in microbial community could occur within 24 hours (Table 3, Fig 3) (6, 83, 84). Sponges use their chaonocytes to capture and take up the trapped ‘food’. This includes viruses (85), bacteria and cyanobacteria (41, 86). While the sponge-associated microbial community also changed in the control tank

without exposure to *V. natriegens*, there was a vastly different community composition in the sponge treated with *V. natriegens* (Fig 3). The sponge in the control tank was dominated by *Synechococcus sp* (97%) at the start of the experiment. After 24 hours the control sponges were dominated by *Cyanobacteria* (41%), mainly *Synechococcus* sp. and after 48 hours *Alphaproteobacteria* (79%), mainly *Sinorhizobium* sp. Under normal conditions *Synechococcus* sp. contributes to the functional role in sponges, for instance photosynthetic carbon assimilation and the transfer of surplus carbon stores to their hosts (87). On the other hand, *Sinorhizobium* sp. can degrade carbohydrates and this group of bacteria is well suited to nutrient poor conditions (88) which is likely to be the case in this experiment. Moreover, this bacterium utilises nutrient acquisition strategies that reduce the cells' requirement for organic substances and allows replication under low nutrient conditions (88). Further study is necessary regarding sponge nutrition and how nutrition can affect growth and metabolite production, as proposed by Wilson (89).

A previous study found that sponges alter their bacterial population for survival in different environmental situations and to maintain their ecology (90). In the treatment tank, sponge-associated microbial community demonstrated a significant change at both 24 and 48 hours, with a highly diverse microbial structure. The exposure to *V. natriegens* activated many minor groups of bacteria with many different genera detected in the treated sponges at 24 and 48 hours which were not evident in the control (90). The altered bacterial community in the treated sponges were dominated by *Gammaproteobacteria* with similar percentages of 49.5% and 48.6% after 24 and 48 hours, respectively, but with a different bacterial composition. After 24 hours, *Colwellia* spp., *Vibrio* spp., *Ruegeria* spp. and *Roseobacter* spp. were the main genera and after 48 hours this changed to *Colwellia* spp., *Vibrio* spp., *Pseudoalteromonas* spp. At both time points *Colwellia* was one of the major genera found in the treated sponges. From the literature it is evident that *Colwellia psychrotropica* (*Gammaproteobacteria*) is resistant to vibriostatic agent (91). Furthermore, the other *Colwellia* genera have their own characteristics and functions in relation to their role in interacting with sponges. For example, *Colwellia psychrerythraea* produces polyhdroxyalkanoate (PHA) compounds, which serve as intracellular carbon and energy reserves linked to pressure adaption (92). *Ruegeria* and *Roseobacter* spp. respond to bio-fouling (93). Moreover, *Colwellia*, *Ruegeria* and *Roseobacteria* spp. are known for the production of bioactive compounds (94-96) that may serve as defence response to *Vibrio* exposure.

It is noted that *V. natriegens* grew rapidly in the seawater (Fig 2), and reached its maximum density within 12 hours in the experimental conditions of this study. When the *V. natriegens* was added to the treatment tank, the number decreased rapidly over the first 12 hours due to the rate of filter feeding activity of sponges (41, 97-100) which exceeded the growth rate of *V. natriegens*. After 12 hours *V. natriegens* started to grow again and reached a maximum density at 24 hours (Fig 2). Fu *et al*. (101) have reported the similar observation of *Vibrio* sp. in their study of sponge *Hymeniacidon perleve* where overgrowth of *Vibrio* sp. could cause significant changes and lead to death of sponges. It is also likely that the unhealthy condition of treated sponges was due to the stress caused by *V. natriegens, and* could be responsible for the significant shift in their microbial community structure. It was observed that sponges that were treated with *V. natriegens* could only maintain a healthy status up to 56 hours.

It is now well known that bacteria can effectively communicate among themselves (102) using quorum sensing (QS) to synchronize the activities of a group of bacteria in a colony or biofilm (103). Acyl-homoserine lactones (AHLs) are important molecules for QS (56). Marine sponges harbour a high amount of bacteria which can produce AHLs (104, 105) although there is no evidence of the QS activity of *Vibrio natriegens.*

TRFLP analysis along with pyrosequencing of 16S rRNA genes can be used to characterize the microbial communities of sponges. TRFLP analysis used 3 restriction enzymes to characterise microbial populations to the genus level (106). This tentative characterisation has been further reinforced by the identification of specific TRFs from the pyrosequencing results, which provides a more reliable identification of the microbial diversity up to the genus or species level. The TRFs corresponding to specific genera were used to characterise changes in populations of the system under investigation. TRFLP was chosen to examine changes or variations in community structure and to recognize those phylotypes that are stable or changing (107). This technique is used in microbial ecology for differentiation of communities (83, 108), but it usually lacks the power to identify specific genera in a diverse microbial population. Even though we followed the method of Conn and Franco (106) to identify at the genus level, the result obtained using the first primer set missed the major part of *Cyanobacteria* which was revealed by 454 pyrosequencing. After careful analysis we found that this primer set was not able to detect *Synechococcus*. However, TRFLP can handle the analysis of multiple samples simultaneously, compared to 454-pyrosequencing, at a much cheaper rate without the laborious computational work of sequence data (109). Therefore, the primer sets were optimized (66, 110, 111) to pick up the genus *Synechococcus* as well as other dominant genera revealed by pyrosequencing.

Although Hha1 and Msp1 (112-114) provided better resolution by generating more TRFs, Rsa1 failed to show similar efficacy. Thus, we recommend the use of HaeIII as a third restriction digestion enzymes for future studies (115).

The second significant impact of sponge-bacteria interaction is the change in the metabolite profiles (Fig 5 and 6). Parallel to the change in sponge-associated microbial community, metabolite profiles changed over the 48 hours treatment. In terms of apolar metabolites, significant changes were observed for 24 and 48 hours, but were not significant for up to 12 hours (data not shown). Direct comparisons between the control and the treatment indicated a very different pattern and composition of metabolites that also changed with time (Fig 5).

As a result of the changes in the metabolite profiles caused by sponge-bacteria interaction, it is reasonable to expect that the biological activities of sponge-derived extracts would also change. Overall, this study developed and validated an experimental aquarium system that could be used to simulate sponge-bacteria interactions and to experimentally investigate the roles of such interactions in manipulating the sponge-associated bacteria community, metabolite profiles as well as the resultant biological activities. The results demonstrated significant impacts on all three levels of sponges upon the exposure of *Vibrio natriegens*.

As it is not possible to observe and test the interaction between sponge and microbes under the sea, this controlled experimental aquarium system would be very useful tool to simulate a range of sponge-bacteria interaction conditions where experimental data can be obtained and analysed to test many hypotheses on the roles of the sponge-bacteria interactions. As this was a validation study of the system under development, future studies can improve the experimental system design, and also apply a range of advanced analytical tools including omics tools (genomics, transcriptomics, metabolomics and functional bioassays) to gain fundamental insights into the scientifically intriguing questions of sponge-bacteria interactions and their roles in biosynthetic metabolism of a myriad of marine natural products, their ecological functions and evolutionary development over 680 million years.

For the development of sponge derived marine drugs, the insufficient supply of sponge biomass is always a critical issue. The results presented here on the role of sponge-bacteria interaction in enhancing the production and diversity of bioactive compounds are very valuable for the development of innovative production processes. Application of bacteria as a trigger or an elicitor can improve the production of biologically active compounds (116, 117) and even produce new compounds.

6.6 Conclusions

This study successfully developed and validated a novel experimental system to study the role of sponge-bacteria interaction using a sponge *A. rosea* and a bacterium *V. natriegens*. The findings on the dynamic and concerted changes in microbial diversity, metabolite profile and bioactivity of the sponge *A. rosea* exposed to the bacterium *V. natriegens* are significant using this controlled aquaria system. The rapid responses within 24 h at all three levels demonstrated that the sponge is highly responsive and adaptive to bacteria interaction. The plasticity of sponges when interacting with bacteria of high diversity in their body as associated bacteria and surrounding seawater make it an impressive mechanism for maintaining the sponge metabolism. The results indicated that the high diversity of sponge derived marine natural products is likely contributed by this diverse sponge-bacteria interaction. The significance of this first baseline study of sponge-bacteria interaction using this novel experimental approach shows that it can be applied for a range of studies of sponge-bacteria interactions. Further studies should focus on the improvement of the experimental system design; the conduct of systematic studies using different host sponges and their associated bacteria to fully elucidate the underlying mechanisms of different sponge-microbe interactions and their adaptation to ecological challenges.

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The authors declare no conflict of interest.

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114. **Erwin PM, Olson JB, Thacker RW.** 2011. Phylogenetic diversity, host-specificity and community profiling of sponge-associated bacteria in the northern Gulf of Mexico. PLoS One **6:**e26806.

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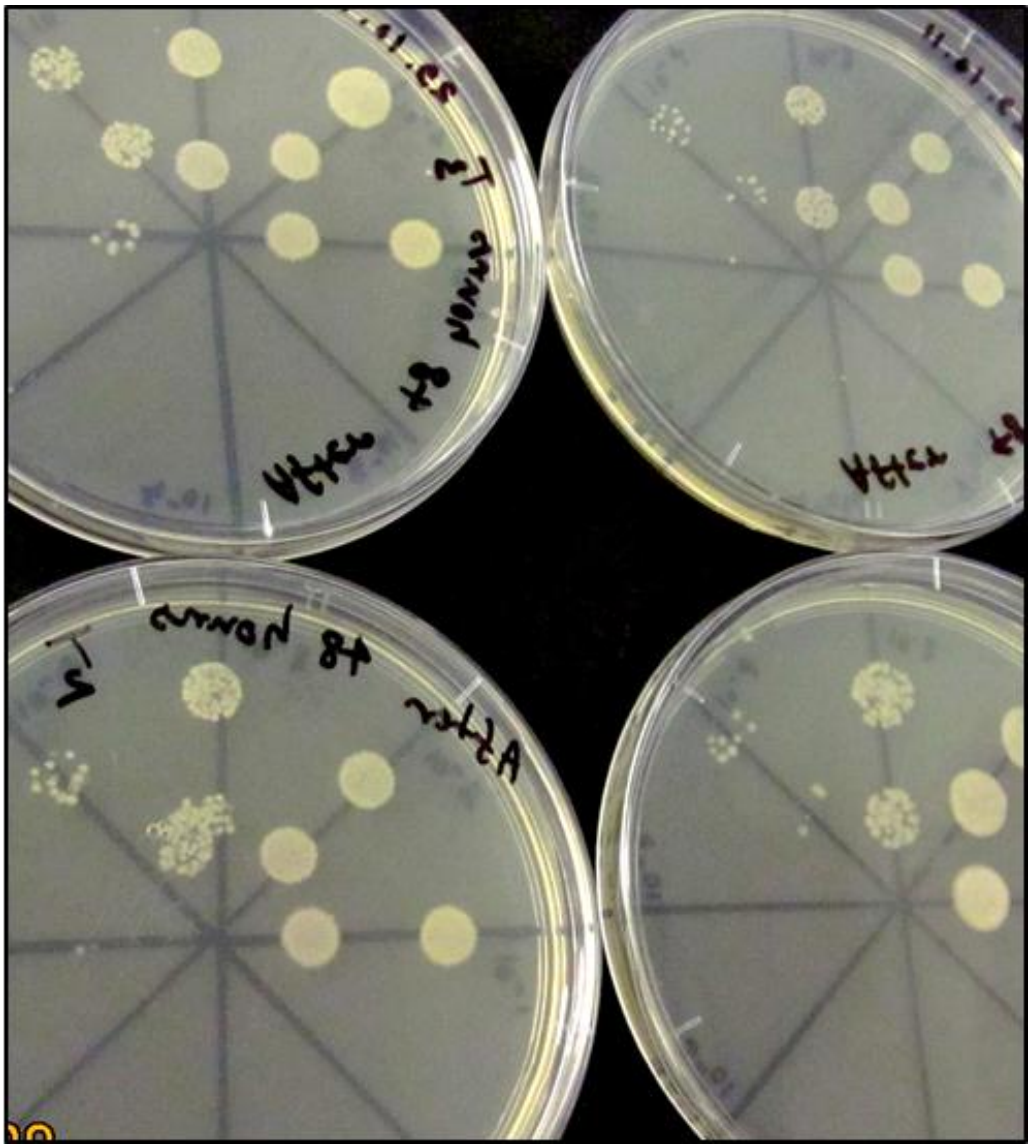
117. **Piel J.** 2006. Bacterial symbionts: prospects for the sustainable production of invertebrate-derived pharmaceuticals. Curr Med Chem **13:**39–50.

6.9 Supplementary Information

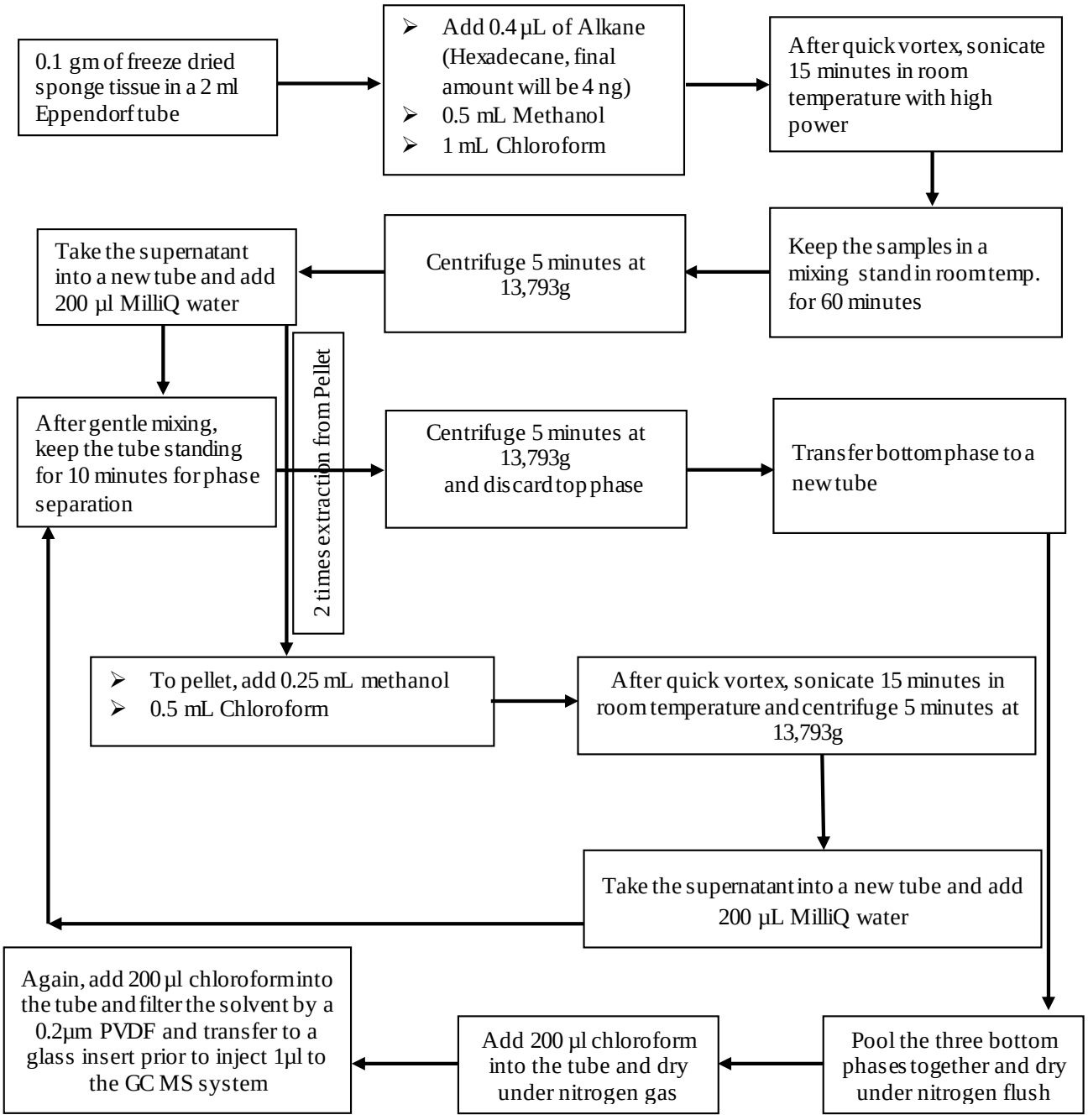
Supplementary File A

CFU count of *Vibrio natriegens* from seawater from different tanks in Luria agar medium during 48 hours.



Supplementary File B

Flow diagram of the extraction of non-polar metabolites from sponge tissue



Supplementary File C

Accession numbers of 454 pyrosequencing samples with download link.

RNA-seq of Sponge: bacteria: Sample Treatment.48.3

1 LS454 (454 GS FLX Titanium) run: 4,253 spots, 2.3M bases, 4.8Mb downloads

Accession: SRX950240

RNA-seq of Sponge: bacteria: Sample Treatment.48.2

1 LS454 (454 GS FLX Titanium) run: 4,659 spots, 2.5M bases, 5.2Mb downloads

Accession: SRX950239

RNA-seq of Sponge: bacteria: Sample Control.48.3

1 LS454 (454 GS FLX Titanium) run: 11,766 spots, 6.2M bases, 13.1Mb downloads

Accession: SRX950238

RNA-seq of Sponge: bacteria: Sample Control.48.2

1 LS454 (454 GS FLX Titanium) run: 10,220 spots, 5.4M bases, 11.4Mb downloads

Accession: SRX950237

RNA-seq of Sponge: bacteria: Sample Treatment.24.3

1 LS454 (454 GS FLX Titanium) run: 7,872 spots, 4.2M bases, 8.9Mb downloads

Accession: SRX950236

RNA-seq of Sponge: bacteria: Sample Treatment.24.2

1 LS454 (454 GS FLX Titanium) run: 21,798 spots, 11.7M bases, 24.5Mb downloads

Accession: SRX950235

RNA-seq of Sponge: bacteria: Sample Control.24.2

1 LS454 (454 GS FLX Titanium) run: 2,340 spots, 1.2M bases, 2.6Mb downloads

Accession: SRX950234

RNA-seq of Sponge: bacteria: Sample Control.R.0

1 LS454 (454 GS FLX Titanium) run: 6,300 spots, 3.3M bases, 6.9Mb downloads

Accession: SRX950233

RNA-seq of Sponge: bacteria: Sample Control.0

1 LS454 (454 GS FLX Titanium) run: 10,079 spots, 5.4M bases, 11.4Mb downloads

Accession: SRX950232

RNA-seq of Sponge: bacteria: Sample Control.24.3

1 LS454 (454 GS FLX Titanium) run: 10,301 spots, 5.4M bases, 11.4Mb downloads

Accession: SRX950231

RNA-seq of Sponge: bacteria: Sample Control.48.1

1 LS454 (454 GS FLX Titanium) run: 4,300 spots, 2.3M bases, 5.2Mb downloads

Accession: SRX950230

RNA-seq of Sponge: bacteria: Sample Treatment.48.1

1 LS454 (454 GS FLX Titanium) run: 15,858 spots, 8.7M bases, 19Mb downloads

Accession: SRX950229

RNA-seq of Sponge: bacteria: Sample Control.24.1

1 LS454 (454 GS FLX Titanium) run: 4,538 spots, 2.4M bases, 5.3Mb downloads

Accession: SRX950228

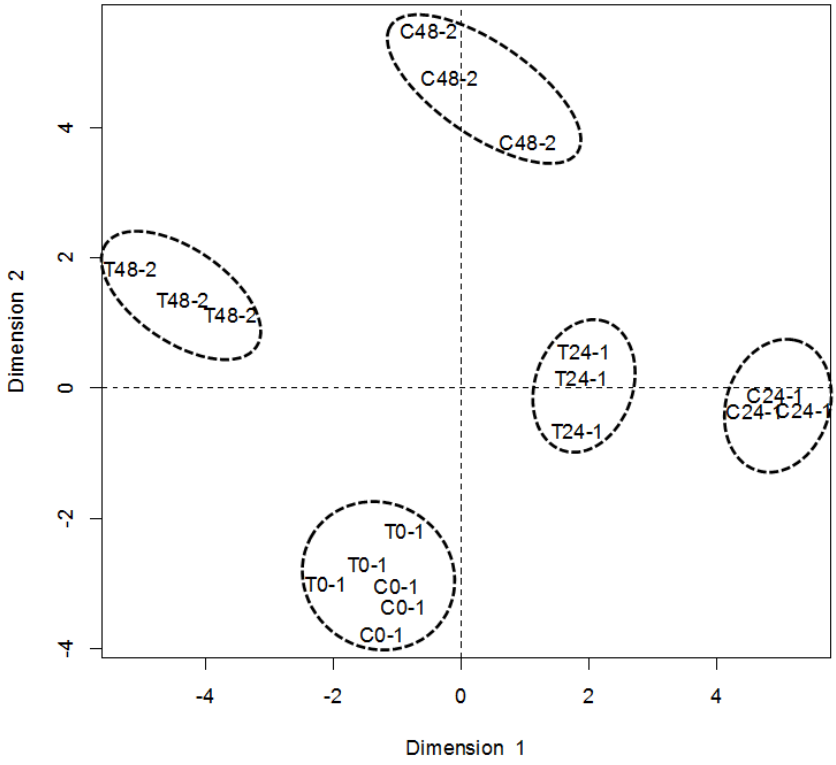
RNA-seq of Sponge: bacteria: Sample Treatment.24.1

1 LS454 (454 GS FLX Titanium) run: 5,529 spots, 2.9M bases, 6.4Mb downloads

Accession: SRX950227

Supplementary File D

Principle Component Analysis (PCA) of selected polar metabolites of sponge *Aplysilla rosea* analysed by HPLC at 215 nm wavelength between control (labelled as C-1, C24-1 and C48-2) and treatment tanks (labelled as T-1, T24-1 and T48-2) at 0, 24 and 48 h of experiments (n=3) clearly showing different clustering patterns.



Supplementary File E

Comparison of ions at different *m/z* by ESI-MS between treated and control sponges with the high abundance (bold) including all common and exceptional ions.

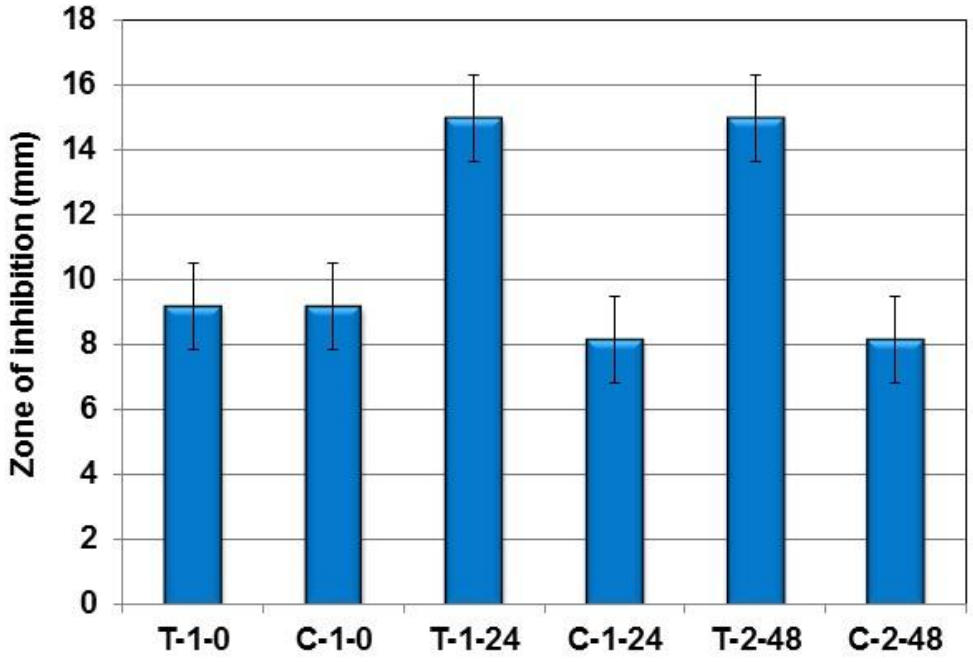
m/z		m/z	
C1-24	T1-241	C2-48	T2-48
116	116	**116**	116
120	120	120	120
-	-	-	**130**
135	135	**135**	135
138	**138**	**138**	**138**
152	**152**	**152**	152
155	155	**155**	**155**
160	160	160	160
166	166	166	166
175	175	**175**	175
180	180	**180**	180
196	**196**	**196**	**196**
198	198	-	-
205	205	205	-
-	-	-	210
215	215	215	215
220	220	220	220
236	**236**	**236**	236
245	245	245	245
272	**272**	272	272
282	282	-	**282**
285	285	285	285
301	301	-	-
311	311	-	-

m/z		m/z	
C1-24	T1-241	C2-48	T2-48
317	-	-	-
327	327	-	-
362	**362**	362	**362**
370	370	-	-
-	384	-	384
385	385	**385**	385
400	400	400	400
	413	-	413
415	415	415	415
420	420	420	**420**
429	-	429	-
-	430	-	430
-	**442**	-	**442**
443	443	443	443
-	**457**	-	457
460	460	460	460
473	473	473	473
478	**478**	478	**478**
489	489	489	489
-	501	-	501
-	**514**	-	**514**
545	-	545	-
559	559	559	559
587	-	587	587
645	645	645	645

Bold indicates the most abundant peaks revealed by ESI-MS chromatogram

Supplementary File F

Activity of sponge extract against *Staphylococcus aureus* during 48 hours clearly showed that treated sponges' bioactivity has increased due to the addition of *Vibrio* and interaction effect.

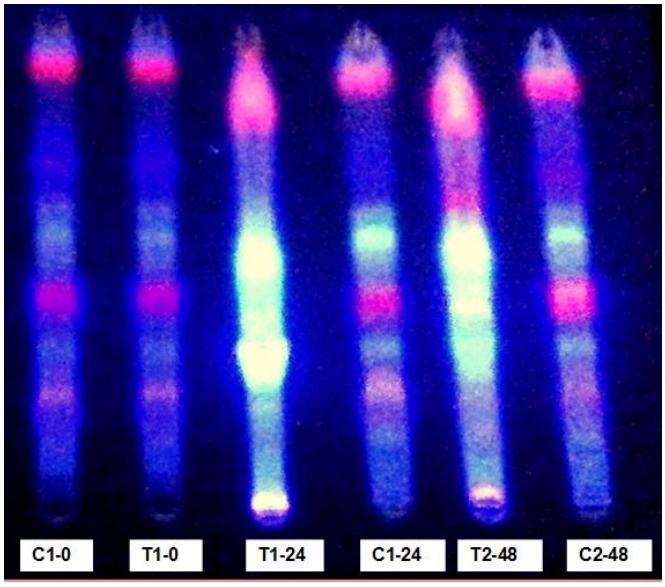


Supplementary File G

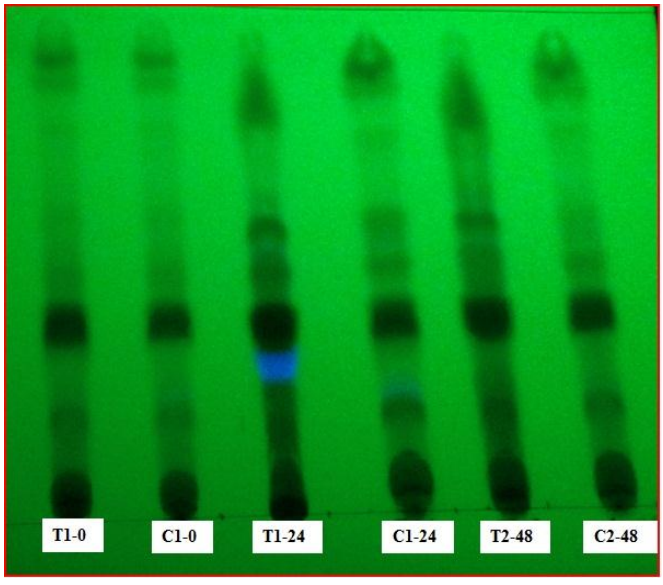
Retention factors of sponge extract with Ethylacetate 7: Methanol 3 and Butanol 4; Acetic acid 1: Water 1, solvent system and their TLC profile at 365 nm and at 254 nm respectively.

Name of Sample	Spot	Retention Factor	
		E:M=7:3 (365 nm)	B:A:W=4:1:1 (254 nm)
T1-0 and C1-0	1	0.13	0.15
	2	0.62	0.4
	3	0.75	0.53
	4	-	0.66
	5	-	0.85
	6	-	0.88
T124	1	0.09	**0.28**
	2	**0.19**	**0.37**
	3	**0.38**	0.41
	4	**0.53**	**0.48**
	5	**0.60**	0.56
	6	0.69	**0.80**
	7	**0.74**	-
	8	0.82	-
C1-24	1	0.09	0.23
	2	0.17	0.31
	3	0.34	0.41
	4	0.69	0.52
	5	0.75	0.56
	6	0.82	0.83
T2-48	1	**0.09**	**0.30**

Name of Sample	Spot	Retention Factor	
		E:M=7:3 (365 nm)	B:A:W=4:1:1 (254 nm)
	2	0.19	**0.38**
	3	**0.22**	0.41
	4	**0.39**	**0.47**
	5	**0.51**	**0.52**
	6	**0.67**	**0.60**
	7	**0.72**	**0.78**
	8	**0.75**	-
C2-48	1	0.11	0.23
	2	0.19	0.32
	3	0.35	0.41
	4	0.71	0.50
	5	0.76	0.53
	6	0.85	0.65
	7	-	0.83
	8	-	0.88



Ethylacetate 7: Methanol 3 at 365 nm



Butanol 4; Acetic acid 1: Water 1 at 254 nm

CHAPTER 7

THE ROLE OF SPONGE-BACTERIA INTERACTIONS: THE SPONGE *APLYSILLA ROSEA* CHALLENGED BY ITS ASSOCIATED BACTERIUM *STREPTOMYCES* ACT-52A IN A CONTROLLED AQUARIUM SYSTEM

This chapter is a paper which was submitted to *Applied Microbiology and Biotechnology* and is under review.

The role of sponge-bacteria interactions: the sponge *Aplysilla rosea* challenged by its associated bacterium *Streptomyces* ACT-52A in a controlled aquarium system

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Key words: *Aplysilla rosea*, *Streptomyces* ACT- 52A, role of interaction, sponge (Porifera), *Actinobacteria*, controlled aquarium, metabolites, bioactivity.

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7.1 Abstract

Sponge-associated bacteria play a critical role in sponge biology, metabolism and ecology, but how they interact with their host sponges and the role of these interactions are poorly understood. This study investigated the role of the interaction between the sponge *Aplysilla rosea* and its associated actinobacterium, *Streptomyces* ACT-52A in modifying sponge microbial diversity, metabolite profile and bioactivity. A novel experimental approach previously established by exposing sponges to bacteria of interest in a controlled aquarium system was improved by including the capture and analysis of secreted metabolites by absorbent in the seawater. In a series of controlled aquaria, *A. rosea* was exposed to *Streptomyces* ACT-52A at 10^6 cfu/ml and monitored for up to 360 hours. Shifts in microbial communities associated with the sponges occurred within 24 to 48 hours after bacterial exposure, and continued for at least 360 hours, as revealed by TRFLP. The metabolite profiles of sponge tissues also changed substantially as the microbial community shifted. Control sponges (without added bacteria) and *Streptomyces* ACT-52A-exposed sponges released different metabolites into the seawater. The antibacterial activity of compounds collected from the seawater increased at 96 and 360 hours of exposure for the treated sponges compared to the control group, due to new compounds being produced and released. Increased antibacterial activity of metabolites from treated sponge tissue was observed only at 360 hours, whereas that of control sponge tissue remained unchanged. The results demonstrate that the interaction between sponges and their associated bacteria plays an important role in regulating secondary metabolite production.

7.2 Introduction

Marine sponges have received considerable attention in recent decades due to their extraordinary ability to produce a diverse array of novel chemical compounds (Blunt et al. 2015; Mehbub et al. 2014) that show a wide range of biological activity, including anti-viral (Cutignano et al. 2000; Ford et al. 1999; Kelve et al. 2003; Müller et al. 1987; Qureshi and Faulkner 1999), anti-microbial (Blunt et al. 2006), anti-fungal (Bakus et al. 1986; Schmidt et al. 2000), anti-cancer (Hart et al. 2000; Piel et al. 2004; Simmons et al. 2005), anti-inflammatory (Abad et al. 2008), and anti-fouling (Roper et al. 2009). In most cases, drug development has been hindered by the limited availability of sponge sourced materials. The body mass of sponges can contain up to 40% bacteria (Taylor et al. 2007), and many reports have demonstrated that these bacteria play a key role, and are sometimes the true producers of bioactive compounds of

interest (Bultel-Poncé et al. 1997; Hentschel et al. 2006; Jensen and Fenical 1994). Examples of compounds that are produced by sponge-associated bacteria include polyketides and nonribosomal peptide compounds (Grozdanov and Hentschel 2007; Salomon et al. 2004; Schmidt 2005; Stierle et al. 1988; Unson and Faulkner 1993; Unson et al. 1994), 2-undecyl-4-quinolone (Bultel-Poncé et al. 1999), manzamine A (Ang et al. 2000), organohalogens (2,4,4'-trichloro-2'-hydroxydiphenylether) (Bultel-Poncé et al. 1998), N-Hexadecanoic- acid (Selvin 2009), pyrone I (Ang et al. 2000), and Rifamycin B and Rifamycin SV (Kim et al. 2006). Consequently, sponge-associated bacteria may offer a solution to the metabolite supply problem due to their potential for large-scale production through industrial fermentation. However, due to the limited success in the isolation of bacteria responsible for specific bioactive metabolites, the problem remains (Amann et al. 1991; Schmidt et al. 1991). The limited understanding of the sponge-bacteria interaction (Steindler et al. 2005; Wilkinson 1992b) also makes it difficult to identify which bacteria to focus on. Interaction between sponges and their associated bacteria particularly their symbionts is very important for sponge survival and maintenance of their ecology and biology, which was identified as a focal point for future research (Taylor et al. 2007). The process of this interaction could occur in many different forms (Taylor et al. 2007). Some of the examples include food sources (Pile et al. 1996; Reiswig 1971; Reiswig 1975), pathogens/parasites (Bavestrello et al. 2000; Hummel et al. 1988; Kinne 1980; Webster et al. 2002), or mutualistic symbionts (Wilkinson 1983; Wilkinson 1992a). Perhaps the phylogenetic diversity of bacteria controls the types of interaction that occur within host sponges (Taylor et al. 2007). Environmental factors like temperature and nutrients also are considered as influential parameters that change the composition and structure of microbial symbionts in sponges (Mohamed et al. 2008b; Turque et al. 2010; Webster et al. 2008; Webster et al. 2011). Innovative ideas and novel approaches are necessary to understand the role of the interaction between sponges and their associated bacteria in producing bioactive metabolites, and how this knowledge can be used to develop advanced solutions to the supply problem.

We have been developing a novel experimental approach to test the hypothesis that the sponge-bacteria interaction plays an important role in regulation of sponge-derived metabolite biosynthesis. *Aplysilla rosea* is the model sponge used in our study, as it is abundant in South Australian marine waters, and has an encrusting morphology that makes it easy to handle in an aquarium system. It belongs to the order *Dendroceratida,* which is known to produce a diverse range of diterpene compounds (Bobzin and Faulkner 1989; Bobzin and Faulkner 1992), and the family *Darwinellidae,* reported for their high antimicrobial properties such as *Dendrilla nigra*

which is a rich source of cultivable actinomycetes. It was found that *Micromonospora-Saccharomonospora-Streptomyces* group was the major cultivable actinobacteria found in this particular sponge (Selvin et al. 2009). In our laboratory, we proposed a novel approach to experimentally study the role of the sponge-bacteria interactions using a controlled experimental aquarium system where sponge explants were exposed to a bacterium of choice at a specific density. We have previously used this experimental system to show how the interaction between the fast growing non-pathogenic, foreign (non-sponge-associated) marine bacterium *Vibrio natriegens* and the sponge *A. rosea* influenced the sponge-associated microbial diversity, its metabolic profile and anti-bacterial bioactivity (Mehbub et al. 2015). We found that this system could be valuable in the study of the role of sponge-bacteria interactions, and could provide insights into the manipulation of sponge-derived metabolites and biosynthesis.

Here, the role of the sponge-bacteria interactions was further studied by examining the changes of *A. rosea* in relation to microbial diversity, metabolite production and bioactivity when exposed to an associated actinobacterium *Streptomyces* ACT-52A, using an improved experimental system and design. Firstly, it was ensured that sponges remain healthy during the experiment and this was confirmed before conducting the final experiment by adding *Streptomyces* ACT-52A in the experimental aquaria. The main improvement in the current experimental system was the introduction of Amberlite resin XAD-7 to capture the metabolites released from the sponges. XAD-7 is widely used for adsorbtion of antibiotics from fermentation broths (Sigma-Aldrich 1998). In addition, the treatment time was extended from 48 h up to 360 h, with larger aquaria and larger sponge explants in order to have sufficient material for further characterization. Increasing evidence suggests that the high diversity of secondary metabolites in sponges is produced by the sponges in response to symbiotic and foreign bacteria (Oclarit et al. 1994; Schmidt et al. 2000; Stierle and Stierle 1992). There are several reasons for choosing a member of the phylum *Actinobacteria* for this experiment: they are important, ubiquitous components of sponge-associated microbial communities (Hentschel et al. 2002; Zhang et al. 2006), including those associated with *A. rosea* and they are prolific sources of natural products, and pharmacologically relevant secondary metabolites (Santos-Gandelman et al. 2014) that are frequently targeted in biodiscovery programs (Franco and Coutinho 1991; Schmidt et al. 2000; Stierle and Stierle 1992).

At the beginning of this study, pyrosequencing revealed that the *Aplysilla rosea* selected for this study was mainly dominated by 0.7% of actinobacteria and only one genus *Streptomyces* was

identified by pyrosequencing. Therefore, *Streptomyces* was chosen for this study. Although it was reported in the literature that similar type of sponge *Dendrilla nigra from Darwinellidae family dominated with culturable Micromonospora–Saccharomonospora–Streptomyces* group (Selvin et al. 2009), in this study only *Streptomyces* was found from *A. rosea* in both culture dependent and culture independent study.

7.3 Materials and Methods

7.3.1 Sample collection, transport and laboratory maintenance

Twenty five large individuals (>10 cm) of the shallow water sponge *A. rosea* were collected from Ardrossan, Gulf St Vincent (34°25'29.3"S, 137°54'20.5"E), in South Australia at a depth of 6-8 m, in March 2013. All sponges were identified as *A. rosea* by S. Sorokin (SARDI Aquatic Science, South Australia). Sponges were carefully removed from jetty pylons with a scraper and kept immersed in seawater in plastic bags and immediately transferred to buckets of seawater on the surface without exposure to air. Sponges were transported to the laboratory with aeration and held in the transport buckets through 5-6 water changes at 20 minute intervals to remove any toxins released as a result of transport stress. They were then transferred into aquaria (~100 L capacity) for 2 weeks with water exchange on alternate days. Sponges were fed with 40 ml of the microalga *Nannochloropsis* sp. (10^6 cells/ml) every 2 to 3 days.

7.3.2 Production and enumeration of spores from *Streptomyces* ACT-52A

A special medium (20 g mannitol, 20 g soy, 20 g oatmeal, 1 L seawater, MSOS) was prepared to grow the desired amount of actinobacterial spores from *Streptomyces* ACT-52A (Figure S1) for this experiment. The strain was cultured on MSOS at 27°C for 7 days. Spores were harvested in 2 ml of 0.9% sterile saline water per plate and collected by cut tips using a 1 ml micropipette. Two replicates of a 1 in 10 serial dilution series of 10^{-1} to 10^{-12} were prepared in 900 µl of 0.9% sterile saline. Absorbance was measured at 530 nm for each dilution using a spectrophotometer, and spore density in each dilution was measured by plating 10 µl onto the same medium and counting using the Miles and Misra method (Miles et al. 1938), and a standard curve prepared to relate absorbance to cell number.

7.3.3 Experimental design and aquarium setup

To assess the effect of bacterial addition on sponge metabolite production, five small sponge explants (for 96 hour experiments) and three large sponge explants (for 360 hour experiments) were placed in each aquarium one hour before the experiment commenced, with three replicate treatment (sponges + added bacteria), sponge control (sponges only) and bacterial control (bacteria only) aquaria in each experiment. Shorter experiments (96 hours) were conducted using replicate 10 L glass aquaria with 5 L of water, while 360 hour experiments used 30 L aquaria with 15 L of water. Experiment was started (Time 0 h) by adding 10^6 cfu/ml *Streptomyces* ACT-52A as a final concentration to the treatment tank and bacterial control. Fine bubble aeration was provided to maintain dissolved oxygen levels above 6 ppm. Sponge explants were removed from intact parent colonies with a sterilized sharp knife under water. For the 96 hour experiments, explants were ca. 4 cm in size (n=36), and averaged 4.02 ± 0.15 g wet weight. For the 360 hour experiments, explants were ca. 12 cm (n=6), and averaged 12.34 ± 0.13 g wet weight. All sponges were conditioned for two weeks after collection before explants were taken, and a further 4-5 days after cutting prior to experimental use, in large recirculating aquaria with water changes on alternate days. Following commencement of the experiment, one explant from each replicate aquarium was collected at 0, 24, 48, 72 and 96 h in the 96 hour experiment, and all explants were collected at 360 hours in the longer experiment. Upon collection, the explants were placed into sterile 50 ml falcon tubes and immediately frozen with liquid nitrogen, and freeze-dried, ground with a sterile mortar and pestle into fine powder and stored at −80°C for microbial diversity analysis, metabolic profiling, bioactivity testing and further downstream analysis. Water in each tank was monitored for absorbance, pH, ammonia, nitrate, and nitrite at 5, 10, 15, 20, 24, 40, 48, 72, 96 and 360 hours.

For abbreviated labeling of samples we used the following format; *Streptomyces* Treatment (T) or Control (C) or Bacterial Control (BC) followed by replicate tank number then time of sampling in hours. For example C.1.0 is Control tank 1 at time 0 hours, T.1.48 is Treatment (added *Streptomyces*) tank 1 at 48 hours.

7.3.4 Set up of Amberlite absorbent in the aquaria

Amberlite XAD-7 (100 g) was packed into cheesecloth bags, tightened and put into each of the 360 hour experimental aquaria prior to starting the experiment. In a similar way, for the 96 hour

experiment, 60 g of XAD-7 in bags was put into all aquaria with the exception of BC.2. XAD-7 resins were collected at the end of each experiment.

7.3.5 Genomic DNA extraction

DNA was extracted by following a bacterial DNA extraction method (Atashpaz et al. 2010) after making necessary modifications to make it suitable for sponges. Lyophilized sponge tissue (20 mg), 900 µl buffer (3% CTAB, 100 mM Tris-HCl, 2 M NaCl, 20 mM Na_2EDTA, 0.2% LiCl, 2% PVP and 0.1% β-Mercaptoethanol) and 10 mg of lysozyme were added to a 2 ml tube, mixed and incubated at 65°C for 2 hours. Then 100 µl acid-washed sterilized glass beads (1 mm) in the same buffer were added and cells lysed in a Qiagen tissue lyser for 30 sec at 15 Hz. Samples were then centrifuged for 30 min at 13,400 × g, the supernatant was transferred to a new tube and 20 µl proteinase K added prior to incubating at 37°C for 30 minutes, mixing every 15 minutes. An equivalent amount of chloroform:isoamyl alcohol (24:1) was then added, mixed for 5 minutes by inverting the tube by hand, and the supernatant collected by centrifugation. This step was repeated twice. Next, 5 µl RNAase (10 mg/ml) was added to the tube, incubated at 37°C for 30 minutes and again extracted with chloroform: isoamyl alcohol (24:1). DNA was precipitated by adding one volume of 3 M sodium acetate and three volumes of ice-cold isopropanol (by slow dripping) and freezing at −20°C for 1 hour. The DNA pellet was collected after centrifugation at 13,400 × g for 10 minutes. DNA was cleaned first with 95%, then 70%, and finally 95% ethanol, and the residual ethanol evaporated. The DNA was then dissolved in 50 µl 10 mM Tris (Mobio). Two replicate 50 µl extractions were pooled and used for final DNA purification. The Qiagen blood and tissue kit was used to clean up the DNA. Buffer AL (200 µl Qiagen) was added to the 100 µl DNA and mixed by inversion, before being incubated at 56°C for 10 minutes. Then, 200 µl of 100% ethanol was added and mixed thoroughly. This solution was passed through a silica column and drained. DNA was washed with manufacturer provided buffer and finally 50 µL buffer AE (Qiagen) was used to elute the DNA with two consecutive elutions to obtain a higher yield. DNA was quantified using an ND-8000 UV-Visible Spectrophotometer (Thermoscientific), and DNA quality was checked on a 0.8% agarose gel.

7.3.6 PCR and TRFLP for microbial diversity analysis

For TRFLP, the 16S rRNA genes were amplified from 50 ng of genomic DNA using the forward primer EUB 8F (6 FAM 5′ AGAGTTTGATCMTGGCTCAG 3′) (Jiang et al. 2006) and reverse primer (VIC 5′ CCGTCAATTCMTTTRAGTTT 3′) (Lane et al. 1985). The PCR was carried out in

50 µl reaction volumes with the following reagents: 2 µl of forward primer (5 µM), 2 µl of reverse primer (5 µM), 5 µl Thermopol buffer, 5 µl $MgCl_2$ (10 mM), 32 µl of injection water, 1 µl dNTPs (dATP, dCTP, dGTP, dTTP - 10 mM each with the water for injection), 1 µl of Taq polymerase (1 U Thermopol), and 2 µl of template DNA (25 ng/µl). The following touchdown PCR program was used: initial denaturation at 95°C for 5 min 1 cycle, then denaturation at 95°C for 30 s, annealing at 61°C-51°C with a −0.3°C increment for 45 s, and elongation at 72°C for 2 min for 40 cycles to generate sufficient amplicons from the template DNA, the last cycle being followed by a 10 min elongation at 72°C. The PCR were performed in an Axygen Maxygene Thermal Cycler (Maxygene Gradient). PCR products were separated by electrophoresis in 1% (w/v) agarose gel to confirm amplicon size. To get sufficient PCR products for TRFLP analysis, four separate (50 µl each) PCR reactions were combined and purified using the UltraClean® PCR Clean-Up Kit (Mo Bio), then quantified using a Nano Drop 8000 spectrophotometer (Thermo scientific).

For microbial diversity analysis, approximately 200 ng of purified PCR products were digested with the Hha1 restriction endonuclease, in a total reaction volume of 50 µl, following the manufacturer's protocol (New England Biolabs Inc.). All products were digested at 37°C for 5 hours. Immediately following digestion, samples were inactivated by heating at 65°C for 20 minutes. Samples were sent to Macrogen, Korea for fragment analysis. The company provided the raw FSA files after fragment analysis.

7.3.7 Data conversion and statistical analyses

Peak profiles were tabulated using the program Peak Scanner Software™ v 2.0 (Life Technologies Corporation, Carlsbad, CA) and peaks (putative populations) were identified by Microbial Community Analysis III (MiCA3), using APLAUS+ (Shyu et al. 2007) and the Silva reference database (R106). The lengths of individual terminal restriction fragments (T-RF) were determined by comparison with internal size standards (GS1200LIZ). T-RFs beyond the resolution of internal size standards (20 to 1200 bp) or with peak areas of less than 50 fluorescence units were removed, and peak profiles were imported into the program T-REX (Culman et al. 2009). Prior to T-RF alignment in T-REX, the objective filtering algorithm was followed (Abdo et al. 2006) based on peak area and a cut off value of 3 standard deviations (SD) was applied to denoise the data set by eliminating background peaks. Following noise reduction, T-RFs were aligned across samples using a 0.5 bp clustering threshold, and peak profiles were

standardized using relative abundance (percentage total fluorescence). As two labelled primers were used for TRFLP, 6 FAM (blue) and VIC (green), two sets of data were obtained. Relative abundances were used to calculate the distance matrix and clustering pattern of different samples by Partial Least Squares Discriminant Analysis (PLS-DA) using the mixOmics package (Dejean et al. 2011) in the R statistical environment (R Development Core Team 2012).

To compare bacterial community profiles between treatments, permutational multivariate analysis of variance (PERMANOVA) (Anderson 2001) was used separately for the blue and green channels, followed by non-metric multidimensional scaling to visualise patterns, with Bray-Curtis similarities and 9999 permutations under a reduced model. Treatment and Time were fixed factors, while Tank was random and nested within Treatment. All analyses were performed using Primer v6 and PERMANOVA+ (Plymouth Marine Laboratory, United Kingdom).

7.3.8 Characterization of microbial diversity by 454 Pyrosequencing

To assess the microbial diversity further, a few selected samples were analysed by 454 pyrosequencing for validation purposes. As a baseline, the 0 hour control was selected to determine the initial microbial diversity in the sample. We also selected the treatment at 96 hours to visualize any major changes along with TRFLP to validate the result.
The extracted gDNA was amplified using primers targeting the V1 to V3 hypervariable regions of the bacterial 16S rRNA gene (V1-27F: 5'-*X*-AC-GAGTTTGATCMTGGCTCAG-3' (Chun et al. 2010) and V3-518R: 5'-X-AC- WTTACCGCGGCTGCTGG-3' (Ibarbalz et al. 2014) where *X* denotes a 10 nucleotide long barcode uniquely designed for each sample followed by a AC common linker. These primers amplify approximately 500 bp of the variable regions V1 to V3. PCR reactions were carried out in a thermocycler under the following conditions: initial denaturation at 94°C for 3 min; followed by 25 to 35 cycles of denaturation at 94°C for 15 sec, annealing at 55-65°C for 30 sec, and elongation at 72°C for 8 min. Both PCR and DNA sequencing was performed by Macrogen Incorporation (Seoul, Korea) using the standard shotgun sequencing reagents and a 454 GS FLX Titanium Sequencing System (Roche).

7.3.9 Processing of raw sequence data

All raw sequences were processed by Macrogen Inc (Korea) using their pipeline. Briefly, all sequences were filtered and denoised using the MOTHUR (Schloss et al. 2009) implementation

of AMPLICONNOISE (Quince et al. 2011) to segregate the reads from each sample by matching the initial and final bases of the reads to the known tag sequences used in the preparation of the libraries. BLAST was used with E-value=0.01 and the Silva rRNA database (http://www.arb-silva.de/) to find regions of local similarity between sequences. The top-five E-values from the BLAST results (candidate hits) were aligned using Needle, which uses the Needleman-Wunsch global alignment algorithm to find the optimum alignment (including gaps) of two sequences along their entire length. A global alignment of the selected candidate hits was performed and the best alignment used for taxonomic classification.

7.3.10 Metabolic profiling of non-polar extract from sponges

Briefly, 0.1 g of freeze-dried ground sponge tissue was extracted three times with Chloroform ($CHCl_3$)/Methanol (MeOH) (2:1 v/v). Prior to the first extraction, 4 µl of octadecane ($C_{18}H_{38}$) was added as an internal standard. The powder and solvent mixture were vortexed, sonicated for 15 minutes in an ultrasonic bath and incubated on a shaker for 60 minutes at room temperature. Samples were centrifuged for 5 minutes at 13793 *g* (Eppendorf-5415C) and the supernatant was transferred to a new safe lock tube (B147671L/2423, Eppendorf AG, Germany) which is stable to use with 100% $CHCl_3$ or MeOH. Again 0.20 ml water was added and the sample mixed and left to stand for 10 minutes to allow phase separation. The sample was centrifuged again for 5 minutes at 13793 *g* and the top aqueous phase was carefully discarded and the bottom phase was collected. Sponge tissue was re-extracted twice more, using 0.75 ml of the same solvent system and omitting the 60 minute incubation. Finally, the three bottom phases were pooled together to be dried under a stream of nitrogen and dissolved with 200 µl $CHCl_3$ and filtered by 0.2 µm PVDF and finally, transferred to a glass insert for gas chromatography-mass spectrometry (GC-MS) analysis. All chemicals used were of analytical grade suitable for mass spectrometry (MS).

7.3.11 GC-MS analysis of non-polar extracts

GC-MS analysis was performed using an Agilent 7890A gas chromatograph (GC) and Agilent 5975C inert XL EI/CI MSD (mass selective detector). Chromatographic separation was achieved on a 30 m Rxi® 5Sil MS with 10 m Integra-Guard® (low polarity Crossbond® silarylene crossbond; similar to 5% phenyl/95% dimethyl polysiloxane, RESTEK) column with dimensions: 0.25 mm ID and 0.25 µm phase thickness. Mass spectra were acquired at 70 eV from m/z 25-650 using MSD Chemstation software version E.02.00.493 (Agilent Technologies).

Helium was the carrier gas at a flow rate of 1.2 ml/minute. Inlet and interface temperatures were 300°C and 250°C, respectively. The ion source temperature was set at 230°C. The oven temperature program was as follows: 70°C for 3 minutes, then a ramp of 8°C/minute to 330°C where the temperature was held for 10 minutes before re-equilibration to 70°C. One µL of sample in chloroform was injected in splitless mode.

7.3.12 Analysis and clustering of Cluster Mass Function (CMF) data

GC-MS data files were converted to netCDF format using the embedded file conversion function included in MSD ChemStation. Raw data files were imported into MetAlign (version 4.11011) and processed to give aligned ion and retention time (ion-RT) tables (Lommen 2009). MSClust was used to perform unsupervised clustering. All detected features belonging to the same putative compounds were grouped together to allow data reduction for further analysis. Data extraction was optimized for several of the program settings including maximum amplitude, peak threshold, and average peak width at half height. All ion-wise alignments were clustered into putative mass-spectra with MSClust, and reduced data frames were subsequently used for multivariate statistical analysis to reduce dimensionality for visualization and subsequent interpretation of the chemo-diversity of the sponges.

GC MS was used to profile non-polar metabolites from sponges. Principal component analysis (PCA), individual Principal component analysis (IPCA) and partial least squares discriminant analysis (PLS-DA) were employed to compare the metabolite profiles from control and treated sponges at different time points. All statistical data analysis was done in the R statistical environment (R Development Core Team 2012). Apolar metabolite diversity was estimated by comparison of the reduced CMF data through hierarchical clustering using the complete Ward method with the Chemometrics and mixOmics packages for R (Dejean et al. 2011; Wehrens 2011).

7.3.13 Metabolite profiling of polar extracts (Methanolic extract)

7.3.13.1 Extraction of metabolites from sponge tissue

For the profiling of metabolites by TLC and HPLC, 100 mg of freeze-dried sponge tissue was extracted three times according to the literature (Turon et al. 2009). Briefly, sponge tissue powder was transferred to a new safe lock tube (B147671L/2423, Eppendorf AG, Germany) and

dissolved with 1 ml of methanol (MeOH). Mixing was accomplished using an ultrasonic tank for 5, 10 and 15 min respectively, retaining the pellet after transferring the supernatant each time. The crude extract was concentrated by using a centrifugal evaporator and filtered through a 13 mm 0.2 µm PVDF acrodisc Syringe Filter and transferred to a 2 ml tube with glass insert.

7.3.13.2 Elution of metabolites from Amberlite XAD-7 resin

The XAD resin (60-100g) was added to a glass column and washed with MilliQ water. The compounds were then eluted with 250 ml methanol, collected in 15 ml fractions, followed with 200 ml acetone and collected in the similar way.

7.3.14 Thin Layer Chromatography (TLC)

Twenty microlitres of the sponge tissue extract or XAD-7 eluate was loaded onto an aluminium TLC silica gel 60 F_{254} plate (Merck), and developed using the following solvent systems: (1) Butanol: Acetic acid: distilled water (dH_2O) [60:15:15], (2) Ethyl acetate (EtOAC): MeOH [9:1], and (3) EtOAc:MeOH [7:3].

7.3.15 High Performance Liquid Chromatography (HPLC)

HPLC analyses were performed with an Agilent 1100 system, including Alliance separation module 2695, column heater, and 2998 photodiode array detector, and run using Empower Chromatography Data software (Waters). The HPLC conditions consisted of two eluents (eluent A [0.1% aqueous trifluoroacetic acid] and eluent B [100% acetonitrile]) and an elution profile based on a linear gradient from 10% eluent B to 100 % eluent B within 25 min and then holding at 100% eluent B for an additional 20 min. Flow rate was kept constant at 1 ml min^{-1} and 50 µl sample was injected. Chromatographic separation was performed on an Atlantis T3 C18 column (Agilent, 100 mm x 3 mm ID and 3 µm particle size) in reversed phase with a fixed temperature of 24°C. Fifty microliter extract was injected into the HPLC system. Peaks were monitored across the 200-800 nm wavelength range.

7.3.16 Antibacterial activity and bioautogram

The crude extracts of sponges, and eluates collected from the Amberlite XAD-7 resin from all the tanks were tested for antibacterial activity against the pathogenic bacteria *S. aureus*, *E. coli* and the non-pathogenic bacterium *V. natriegens*. Activity against *S. aureus* was also tested

using a bioautogram. *S. aureus* and *E.coli* were grown on Tryptone soy agar (TSA) (Tryptone soy 30 g, agar 15 g and RO water 1 L) (Martin 1950) and finally on Antibiotic agar medium (AAM) (27g Antibiotic agar medium Sigma-Aldrich in 1 L MilliQ water). AAM was used to test for antibacterial activity after adjusting the *S. aureus* and *E. coli* concentration at 1% (v/v) with the OD measurement of 0.2 at 600 nm. Sponge extracts (50 µl) from treatment and control tanks were inserted into the well, dried for 30 minutes to 1 hour and incubated at 37°C for 16 to 24 hours, and zones of inhibition were measured. *Vibrio natriegens* were grown in *Vibrio* agar (Brain heart infusion agar 38g, NaCl, 20 g and MillQ water, 1L) and antibacterial tests were conducted in the same way except that *Vibrio* medium was used instead of AAM medium.

TLC plates in EtOAC:MeOH [9:1] were found best for bioautogram. For the bioautogram, the AAM-*S. aureus* medium was prepared and poured into 19 cm glass petri dishes. TLC plates were placed on the medium for 30 minutes after detecting the 'spots' UV at 364 and 254 nm. These spots were recorded on the underside of the petri dish and the TLC plates were removed and the medium incubated at 37°C for 16 hours. The active spots were identified by a zone of inhibition in the plate. Preparative scale layer chromatography (PLC) was carried out using PLC glass silica gel (Kieselgel 60 F_{254}, size 5×10 cm) with the active extracts against *S. aureus* to obtain purified compounds for further analysis.

7.3.17 Sequence accession numbers

The 16S rRNA gene sequence of *Streptomyces* ACT-52A (GenBank Accession number KT253928) showed a 99.57% pairwise similarity to *S. Streptomyces costaricanus* (completeness 100%), *S. graminearus* (completeness 99.93%), *S. griseofuscus* (completeness 99.93%) and *S. murinus* (completeness 99.93%). However, according to its position in the phylogenetic tree it falls in the same clade as *S. griseofuscus,* followed by *S. costaricanus* and *S. murinus* (Figure S2). All 454 pyrosequencing data were deposited, with MIMARKS-compliant metadata, in the NCBI Sequence Read Archive under the accession numbers from SRS1063494- SRS1063499 (details in Table S1).

7.4 Results

7.4.1 Health, maintenance and growth of *Aplysilla rosea*

Large pieces (>10cm) of *A. rosea* were successfully maintained in the large recirculating aquarium systems (~100 liters) for up to 40 days with feeding and regular water exchange. In the small experimental tanks (10 liters), 5-6 small pieces (≤6 cm) could survive in a reliably healthy condition for a maximum of 6-7 days without feeding and with added *Streptomyces* ACT-52A at 10^6 cfu/ml, while in the big tanks (30 liters) they could survive 16-17 days in healthy condition. Consequently, all sponges appeared to still be healthy at the conclusion of each experiment. The sponges were found to adapt well in the large aquarium system (~100L) when small rocks were provided in the aquarium as a substrate for attachment (Fig 1).

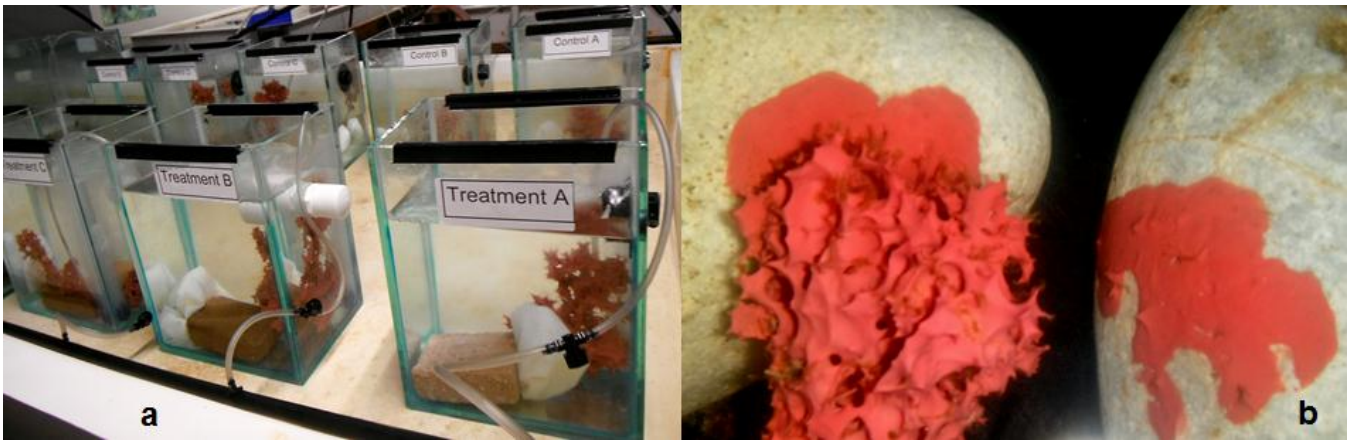

Fig 1. **(a)** Experimental aquarium set up to study the role of the interaction between the sponge *Aplysilla rosea* and sponge associated bacteria *Streptomyces* ACT-52A and **(b)** Adaptation of *A. rosea* sponges in the aquarium up to 40 days and encrusting behaviour of *A. rosea* on rocks.

7.4.2 *Streptomyces* ACT-52A density

The addition of *Streptomyces* ACT-52A to aquaria containing sponges did not result in increased bacterial cell densities compared to those found in aquaria with sponges but no bacteria added. In the absence of sponges, bacterial cell density did not respond for 10 hours, but then increased substantially for a short period, prior to declining to original levels.

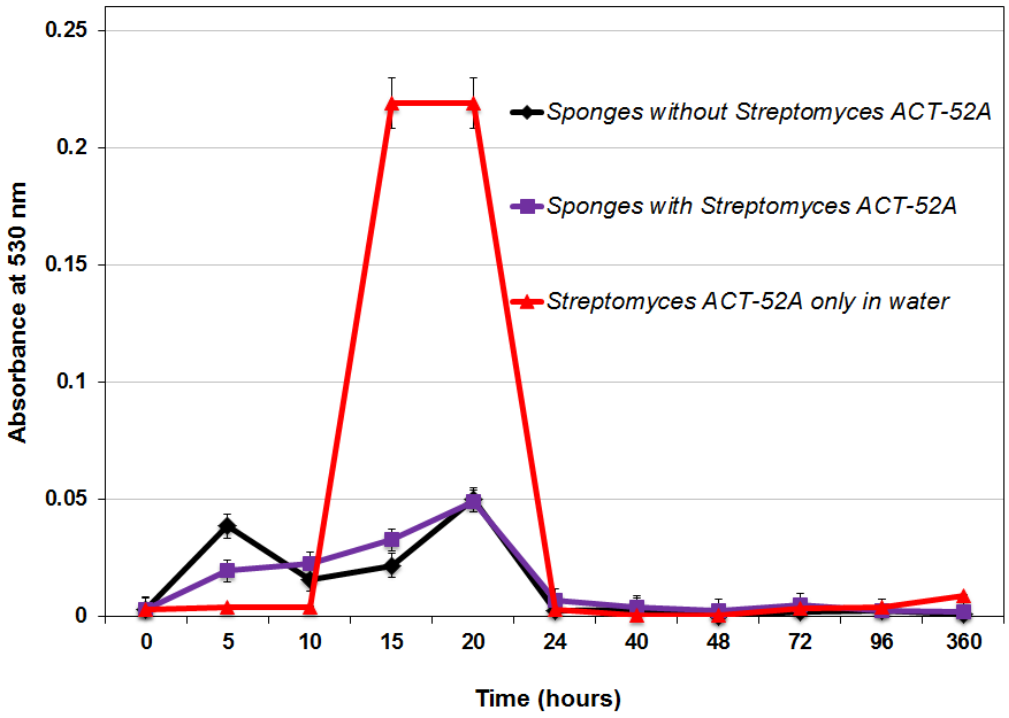

Fig 2. Absorbance at 530 nm, indicating microbial density, in experimental aquaria with 10^6 CFU/mL of *Streptomyces* ACT-52A added at time 0 with and without the sponge *Aplysilla rosea,* and with *A. rosea* only, over 360 hours. Error bars indicate SEM, n=3.

7.4.3 Characterization of sponge microbial diversity by TRFLP

A total of 467 valid TRFs were identified. The highest diversity (97 TRFs) was found in the control sponges at 48 hours. Overall, six major fragment patterns were observed at 0 hour. Different fragment patterns and abundances were observed between the control and treated sponges at 24 hours. At 48 hours, control and treated sponges showed similar fragment patterns and abundances. However, the abundance then diverged, with a few new fragment patterns in treated sponges compared to controls at 96 hours and at 360 hours. Putative identification of TRFs is presented in Table 1.

Table 1. Putative taxonomic assignments of terminal-restriction fragments (TRFs) were done by using MiCA3 server application using APLAUS+ algorithm with SILVA (R106) reference database.

Fragment Size		**Identification**
Forward	Reverse	Class/Genus/Species
54	-	New
207	347	*Alteromonas* sp.
370	227	*Arthrobacter* sp., *Gordonia alkanivorans, Gordonia bronchialis, Gordonia defluvii, Gordonia namibiensis, Gordonia* sp., *Gordonia sputi, Leucobacter aridicollis, Mycobacterium barrassiae, Mycobacterium elephantis, Mycobacterium goodii, Mycobacterium kuopiense*, *Mycobacterium smegmatis, Salinibacterium* sp., *Streptomyces thermovulgaris, Yonghaparkia alkaliphila*
207	342	*Burkholderia pseudomallei*
93	342	*Cytophaga* sp.
93	347	*Denitrovibrio acetiphilus , alpha proteobacterium*
93	346	*Desulfovibrio* sp.
60	346	*Halanaerobacter salinarius, Phaeobacter gallaeciensis, Lactobacillus antri, Bacillus* sp., *Halobacillus dabanensis, Halobacillus profundi, Halobacillus* sp., *Halobacillus trueperi, Lactococcus lactis, Halobacillus litoralis, Methylopila capsulata, Microbulbifer maritimus, Sporosarcina ureae, Paracoccus methylutens, Rhodovulum* sp., *Roseobacter denitrificans, Sporolactobacillus inulinus, Shewanella frigidimarina*
60	227	*Jeotgalicoccus* sp., *Macrococcus brunensis,*
370	342	*Klebsiella* sp.
75	45	*Lachnospiraceae bacterium,*
461	45	*Lactobacillus vitulinus*
370	45	*Leucobacter* sp.
370	346	*Marinomonas* sp., *Sideroxydans lithotrophicus, Erwinia amylovora, Marinomonas blandensis, Citrobacter freundii, Erwinia pyrifoliae, Methylobacillus flagellatus, Pantoea* sp., *Erwinia amylovora, Enterobacter* sp., *Erwinia amylovora, Erwinia rhapontici, Klebsiella milletis, Klebsiella oxytoca, Klebsiella pneumoniae, Klebsiella* sp., *proteobacterium*
60	64	*Mesorhizobium albiziae*

Fragment Size		Identification
Forward	Reverse	Class/Genus/Species
77	811	*Microscilla marina, Bacteroidetes, Flavobacterium columnare, Subsaxibacter* sp.*, Polaribacter irgensii, Bizionia* sp.*, Cellulophaga* sp.*, Polaribacter* sp.*,*
201	227	*Mycobacterium tuberculosis, Mycobacterium bovis, Thermomicrobium roseum,* Unidentified, *Acidimicrobium ferrooxidans*
207	346	*Nitrosomonas europaea, Burkholderia* sp.*, Candidatus, Nitrosomonas* sp.*,* Unidentified, *Thauera aromatica, Cycloclasticus pugetii, Thauera aromatic, Chlorochromatium aggregatum, Dechloromonas aromatica, Burkholderia cepacia, Burkholderia hospita, Burkholderia* sp.*, Burkholderia xenovorans, gamma proteobacterium*
60	347	*Phaeobacter gallaeciensis, Silicibacter lacuscaerulensis, Citreicella* sp.*, Phaeobacter gallaeciensis, Ruegeria* sp.*, Roseobacter denitrificans, Sagittula stellata, Rhodobacteraceae, Octadecabacter antarcticus, Roseobacter litoralis, Citreicella* sp.*, Roseovarius nubinhibens, Ruegeria pomeroyi, Phaeobacter gallaeciensis, Jannaschia* sp.*, Sagittula stellata, alpha proteobacterium, Jannaschia rubra, Leisingera methylohalidivorans, Microbulbifer variabilis, Paracoccus halophilus, Paracoccus pantotrophus, Paracoccus* sp.*, Pelagibaca* sp.*, Paracoccus denitrificans, Roseobacter* sp.*, Roseovarius* sp.*, Rubrimonas* sp.*, Sagittula* sp.*, Salipiger* sp.*, Silicibacter* sp.*, Silicibacter pomeroyi, Thalassobacter* sp.*, Thalassobacter stenotrophicus,* Unidentified*, Wenxinia marina*
201	342	*Polaromonas hydrogenivorans*
207	64	*Pseudomonas* sp.
339	347	*Roseovarius* sp.*, Parvibaculum* sp.*, Rhodobacter sphaeroides, Roseobacter* sp.*, alpha proteobacterium, Maritimibacter* sp.
370	227	*Ruminococcus albus,* Unidentified, *actinobacterium, Clavibacter michiganensis, Klugiella xanthotipulae, Leifsonia ginsengi, Leifsonia poae, Leifsonia* sp.
60	227	*Salinicoccus alkaliphilus, Salinicoccus* sp.*, Streptomyces* sp.
339	346	*Roseivivax halotolerans*
339	227	*Streptomyces* sp.
201	64	*Thermomicrobium roseum*
560	342	*Thermotogales*
336	346	Unidentified
207	342	*Variovorax paradoxus*
959	961	Uncultured
657	-	New

There was a clear interaction between Treatment and Time in the TRFLP data for the TRFs labelled with 6 FAM (PERMANOVA: $F_{4,16}$=52, P<0.0001). At the beginning of the experiment, there was no difference between treatments, but at 24 hours they differed greatly (Fig 3a). While the difference decreased at 48 and 96 hours, there was a slight increase again at 360 hours. All the samples were clustered into three distinct groups: 0 hour treatment and controls; 48 hours treatment and control and 24 hours treatment; and the remaining samples separated into a third cluster (Fig 3b).

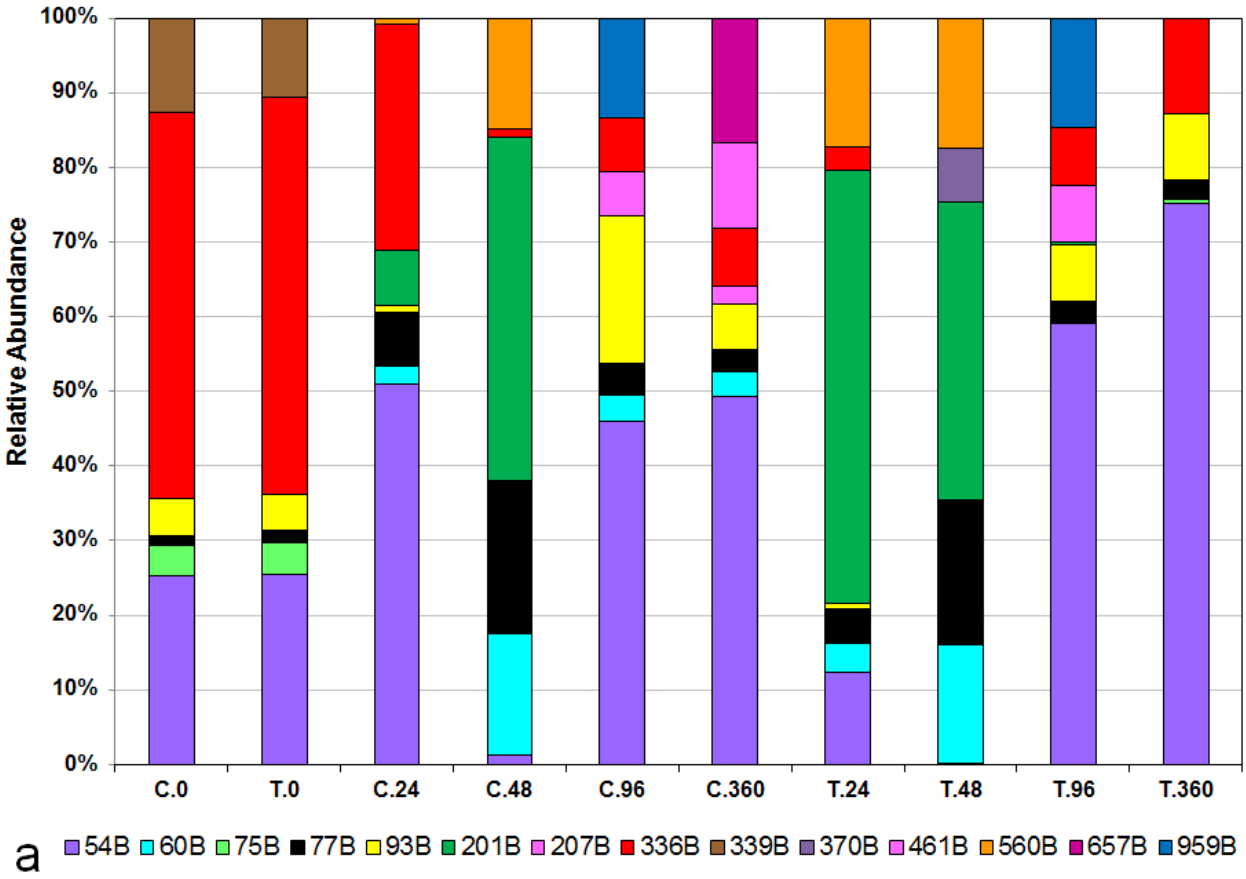

Fig 3. (a) Mean relative abundance of major bacterial TRF (≥ 1%) derived from TRFLP from control tanks (C, no added bacteria) and treatment tanks (T, added *Streptomyces* ACT-52A) at 0, 24, 48, 96 and 360 h (n=3).

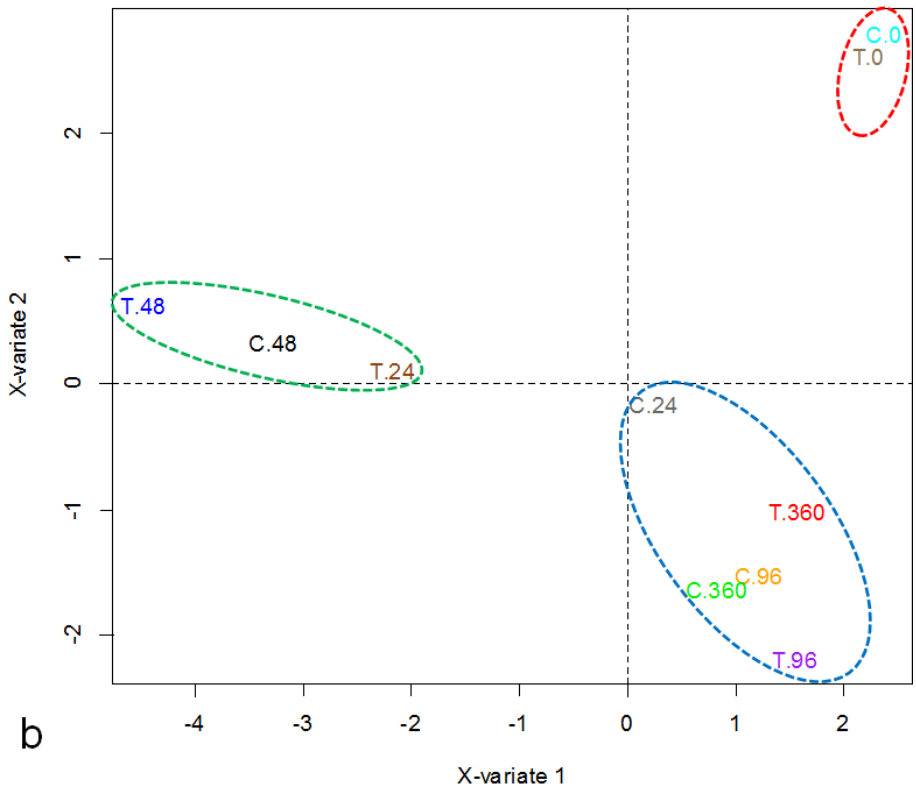

Fig 3. (b) Partial Least Squares-Discriminant Analysis of mean TRF (n=3) of *Aplysilla rosea* showing differences between control (C) and treatment (T) tanks at 0, 24,48,72, 96 and 360 h.

7.4.4 Characterization of microbial diversity by 454 pyrosequencing

Data for the TRF fragments labelled with 6 FAM and VIC provided very similar patterns (Fig 4) when visualized by nMDS. Different TRFs correlated strongly with different sample groups. This trend was observed at 0 hour until 360 hours.

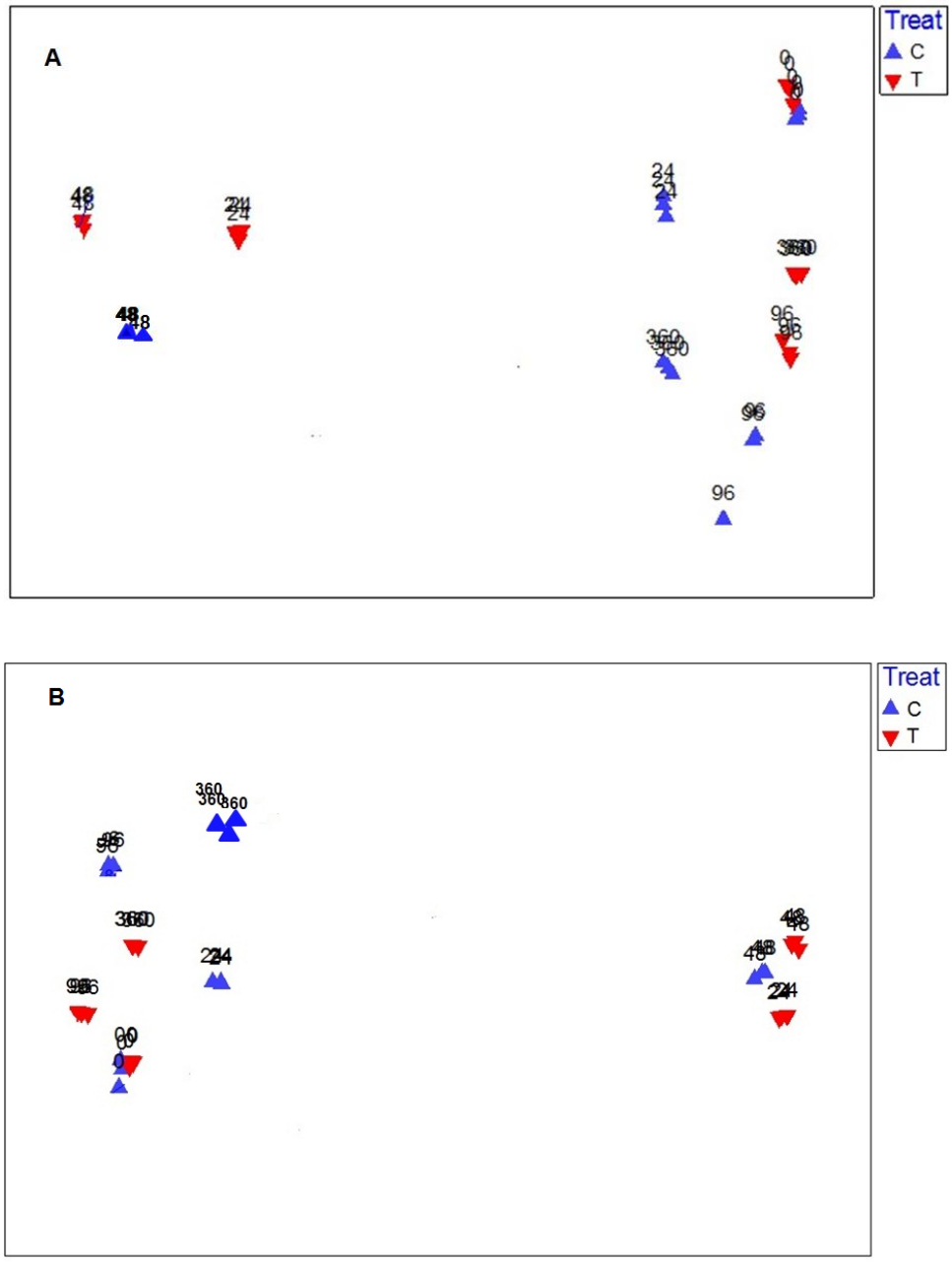

Fig 4. Bray-Curtis based nMDS plot of bacterial diversity (TRFs) changes as a result of the interaction between *Aplysilla rosea* and *Streptomyces* ACT-52A where **A** is based on the 6 FAM forward primer and **B** on the VIC reverse primer. Fig legends C=Control and T=Treatment and 0, 24, 48, 96 and 360 signifies time (hours).

On average, 91% of sequence reads were revealed as bacteria when 0 hour samples were analysed by 454 pyrosequencing, with 21 different phyla observed in *A. rosea*. *Proteobacteria*, *Cyanobacteria*, *Bacteriodetes*, *Planctomycetes*, *Firmicutes*, *Nitrospirae*, *Acidobacteria* and

Actinobacteria were the most prominent phyla (Figure S3) and other phyla includes *Verrucomicrobia*, *Elusimicrobia*, *Chlorobi*, *Deferribacteres*, *Tenericutes*, *Lentisphaerae*, *Deinococcus-Thermus*, *Gemmatimonadetes*, *Spirochaetes*, *Chloroflexi*, *Fusobacteria*, *Fibrobacteria*, *Thermomicrobia* and unknown. Dominant classes were *Alphaproteobacteria* (53.56%), *Gammaproteobacteria* (7.40%), *Flavobacteria* (6.10%), *Deltaproteobacteria* (2.27%), *Betaproteobacteria* (1.30%), *Sphingobacteria* (1.20%), *Phycisphaerae* (0.70%) and *Planctomycetacia* (0.52%), with 24.25% unknown. At the genus level, this sponge was dominated by *Pseudovibrio* (8.07%), *Prochlorococcus* (7.79%), *Ruegeria* (6.99%), *Synechococcus* (1.46%), *Algibacter* (1.12%), *Pelagibius* (1.11%), *Nitrosomonas* (1.05%), Nitrospira (0.87%), *Nitrospina* (0.69%), *Roseobacter* (0.83%), *Flagellimonas* (0.54), with 56.76% unknown. At 96 hours *Prochlorococcus* increased to 22.56% and *Streptomyces* to 1.35 % in treated sponges, indicating an increased prevalence of *Cyanobacteria* and *Actinobacteria* compared to 0 hour (Fig 5).

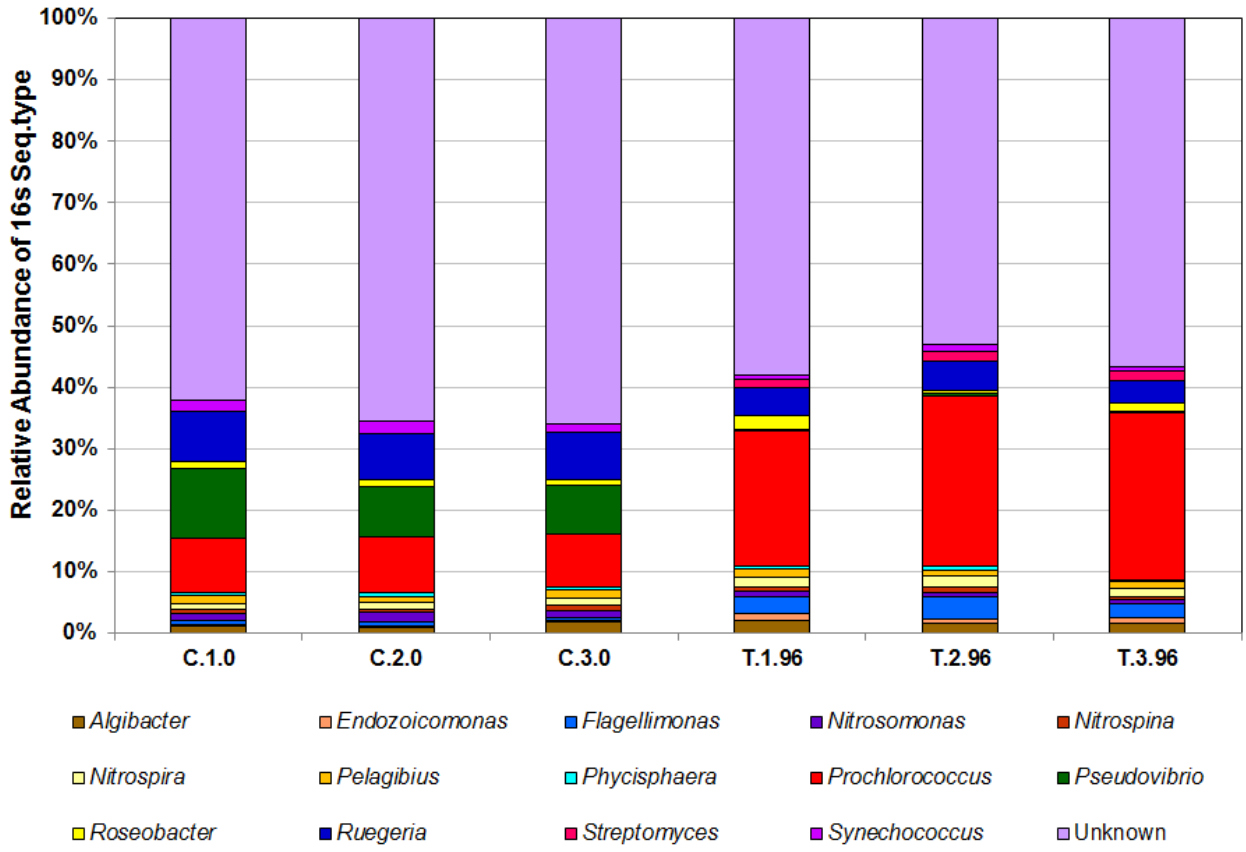

Fig 5. Major bacterial genera inferred from 16S rRNA relative sequence abundances derived from 454 pyrosequencing of sponges from the start of the experiment (0 hour control) and from the treated tanks (T.96) after 96 hours.

7.4.5 Non-polar metabolite profiles of sponges

At the start of the experiment, both control and treated sponges had similar non-polar metabolite profiles as expected (Fig 6), and while profiles changed throughout the course of the experiment, the differences between the two groups remained relatively small. Interestingly, at 360 hours, the metabolite profiles converged towards those expressed at 0 hours (Fig 6).

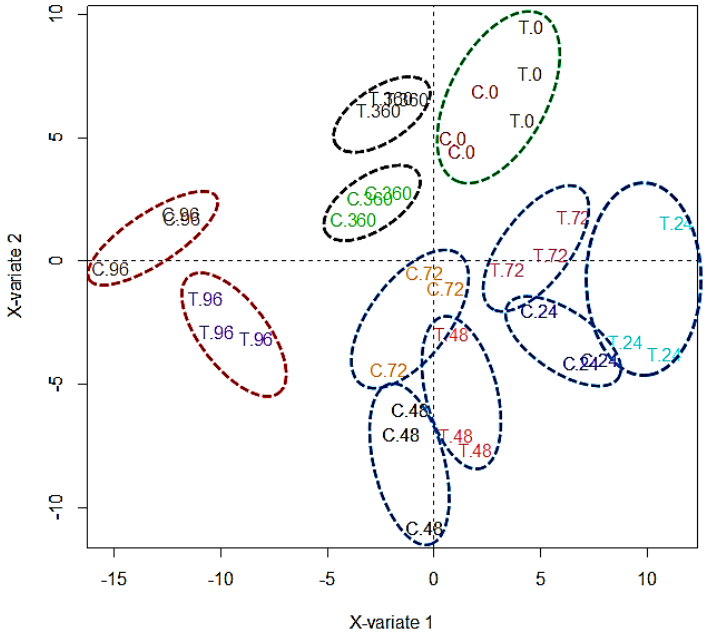

Fig 6. Partial Least Squares-Discriminant Analysis (PLS-DA) of non-polar metabolites of *Aplysilla rosea* analysed by GC-MS between control (C) and treatment (T) tanks at 0, 24,48,72, 96 and 360 hours for each replicate tank (n=3). Ellipses enclose all replicates from the same time and treatment.

7.4.6 Changes in bioactivity and polar metabolite profiles of sponge and seawater

Metabolites eluted from Amberlite XAD-7 resin collected from treated and control tanks at 96 and 360 hours showed bioactivity. A total of 5 and 6 bioactive fractions were collected from treatment tanks, and 3 and 5 from the control tank at 96 and 360 hours, respectively. Metabolites from the treated tanks showed higher activity than those from the control tanks, particularly fraction 5, which was the most active (Table 2 and Figure S4). PLC active bands were further analysed by HPLC using gradient CH_3CN/H_2O as solvents. The spectra showed that band 5 had two compounds and the main compound matched with the fraction 5 major peak (Figure S5, e). Therefore, we assume this peak could be a new compound resulting from the sponge-bacteria interaction, as its presence is not evident in the control. In order to

determine the novelty of this compound further LC MS analysis was conducted but was not possible to identify due to lack of sufficient material.

When PCA, IPCA and PLS-DA (Fig 7) were employed to compare the polar metabolite profiles from the control and treated sponges at different time points, different clustering patterns were observed.

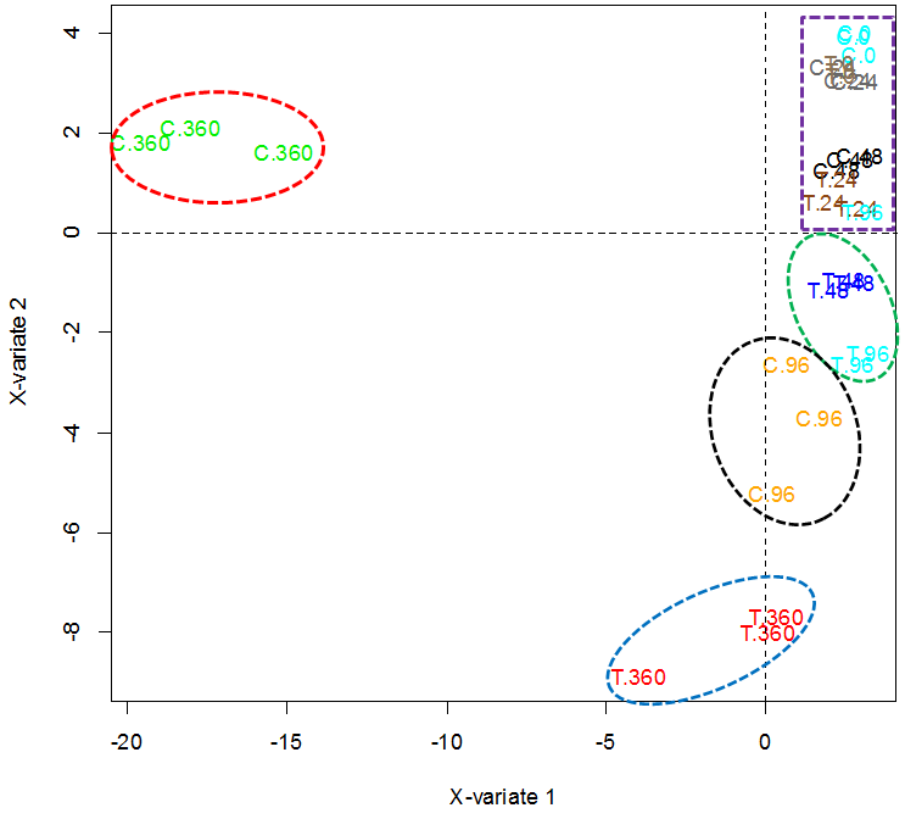

Fig 7. Partial Least Squares-Discriminant Analysis of polar metabolites of *Aplysilla rosea* analysed by HPLC between control (C) and treatment (T) tanks at 0, 24,48,72, 96 and 360 hours for each replicate tank (n=3) at 215 nm wavelength.

We show PLS-DA for our analysis because it results in a classical PLS regression where the response variable is a categorical one expressing the class membership of the statistical units. As PLS-DA does not allow for other response variables than the one used for defining the groups of individuals, all measured variables play the same role with respect to the class assignment (Boulesteix and Strimmer 2007). Methanolic extracts profiled by HPLC showed higher peaks firstly at 215 nm and then 254 nm, therefore, PLS-DA was conducted only for 215 (Fig 7) and 254 nm wavelengths (data not shown). At 215 nm PLS-DA analysis showed that

there were five clusters of metabolites. At both wavelengths, C-360 and T-360 showed substantial differences to each other (chromatogram shown in Figure S6).

7.4.7 TLC profiles of polar metabolites of sponge and seawater for control and treatment sponges

Four different solvent systems showed good separation of compounds on TLC plates; they are $CHCl_3$: MeOH: H_2O [65:35:10], Butanol: Acetic acid: H_2O [4:1:1], EtOAC:MeOH [9:1] and EtOAC:MeOH [7:3]. Different compound profiles were obtained from the sponge tissue extract while compared with XAD-7 eluent. A bioautogram was conducted using EtOAC:MeOH [9:1] TLC plates. XAD-7 eluent TLC plates revealed that the bacteria only controls showed limited active profiles, while the treatment and sponge control tanks showed many active profiles against *S. aureus*. More active bands were collected from the treatment tanks compared to the control tanks (Figure S7).

Based on the antibacterial activity of XAD-7 fractions against *S. aureus*, we chose three for TLC analysis; fractions 4 and 5 from the treatment tank and fraction 3 from the control tank at 360 hours. It needs to be noted that these fractions showed no activity against *E. coli* or *V. natriegens,* but they did show activity against *S. aureus* which also confirmed. A total of nine bands were found from fraction 5 (Table 3), five bands from fraction 4 and 2 bands from fraction 3 (Figure S4). Based on the antibacterial activity, band 5 from fraction 5 was run again using the same solvent system, with six spots observed (Figure S8). When we compared the sponge tissue extracts from control and treated sponges at different time points with the active excreted metabolites captured using XAD-7, there were substantial differences (Figure S9).

TLC confirmed that the main difference in sponge tissue extracts between control and treatment was prominent at 360 hours. Further investigation revealed some new active spots in the TLC plates (Figure S10).

7.4.8 Antibacterial activity and bioautogram of sponge tissue extract and Amberlite XAD - 7 fractions

All the sponge extracts from control and treatment tanks showed antibacterial activity against *S. aureus* at all sampling times. However, theses samples displayed no activity against *E. coli* or *V. natriegens*. Higher antibacterial activity was observed at 360 hours in treated sponge tissue

compared to the control. To find out more details, large-scale extraction (from 5 g tissue) was conducted, and treated sponge tissue showed higher antibacterial activity against *S. aureus* (16 mm) compared to the control (11 mm). Fractions eluted from the PLC plate confirmed there were more active bands in treated sponge tissues compared to the controls. A total of 4 active bands were revealed from treated sponge tissues, while only one active spot (6) was revealed in control sponge tissue, at 360 hours (Figure S11 and Table S2). When the metabolites from XAD-7 were evaluated against *S. aureus*, the highest activity (28 mm inhibition zone against *S. aureus*) was revealed from treatment tanks at 360 hours whereas the control showed a 21 mm zone of inhibition against *S. aureus* (Table 2a and 2b).

Table 2a. Activity of Amberlite XAD-7 fractions collected after 360 hours from treatment tanks against *Staphylococcus aureus*

Name of sample	Fraction No	Zone of inhibition against *S. aureus* (mm)
XAD-7 fraction from treatment tank	1	Not tested (Water wash)
	2	None (Water wash)
	3	None (Water wash)
	4	22 (eluted with Methanol)
	5	28 (eluted with Methanol)
	6	27 (eluted with Methanol)
	7	25 (eluted with Methanol)
	8	20 (eluted with Methanol)
	9	15 (eluted with Methanol)
	10	None (eluted with Acetone)
	11	None (eluted with Acetone)

Table 2b. Activity of Amberlite XAD-7 fractions collected after 360 hours from control tank against *Staphylococcus aureus*

Name of sample	No of Fraction	Zone of inhibition against *S. aureus* (mm)

XAD-7 fraction from control tank	1	None (Water wash)
	2	None (Water wash)
	3	21 (eluted with Methanol)
	4	19 (eluted with Methanol)
	5	20 (eluted with Methanol)
	6	15 (eluted with Methanol)
	7	11 (eluted with Methanol)
	8	None (eluted with Acetone)
	9	None (eluted with Acetone)
	10	None (eluted with Acetone)
	11	None (eluted with Acetone)

Table 3. Antibacterial activity of preparative thin layer chromatography (PLC) purified metabolites from fraction five, eluted from Amberlite XAD-7 from the treatment tank at 360 hours

Number of the band from fraction 5	**Zone of inhibition (mm)**
Band-1	7
Band-2	8
Band-3	6
Band-4	**12**
Band-5	**20**
Band-6	0
Band-7	0

Number of the band from fraction 5	Zone of inhibition (mm)
Band-8	0
Band-9	6

7.5 Discussion

Sponge metabolism is highly influenced by their associated bacteria and external bacteria in the environment, which can regulate metabolite production and promote the release of metabolites as a form of chemical defence (Taylor et al. 2007). A newly developed experimental approach along with improved design facilitated the present study to understand the role of the interaction between a sponge (*Aplysilla rosea*) and its associated bacteria. In this study, a sponge-associated bacterium *Streptomyces* ACT-52A, which was isolated from the host sponge, was used to initiate the interaction. The idea of capturing metabolites released into the sea water (Mendez and Salas 2001) by the sponge added a new dimension in this study as it gave a more complete profile of bioactive compounds produced by sponges. In addition, we believe it removes toxic compounds from the circulating water thus allowing the sponge to survive for much longer in a healthy state.

Changing microbial diversity is a key mechanism influencing sponge survival, growth, and metabolite production (Hentschel et al. 2003). This study revealed that, in response to sponge-associated bacterial exposure, the microbial diversity, profiles of metabolites and the bioactivities of sponges changed. Previous studies have also reported that sponges change their microbial diversity when there is competition of space, food and interaction with other animals or microbes (Januar et al. 2015; Mohamed 2007). Sponges have the capacity to be colonized by a diverse range of bacteria (Ducklow 1990; Rohwer et al. 2002). To cope with the challenge of ubiquitous microorganisms in the marine environment, benthic invertebrates like sponges need to regulate the bacteria they encounter and to resist microbial colonization and the invasion of potential pathogens (Kelman et al. 2009). One of the key methods of combating microbial attack is through chemical defence.

The bacterium (*Streptomyces* ACT-52A) used in this experiment for exposure to *A. rosea* was an endosymbiont from the host sponge, which showed high antibacterial activity. Previous studies have shown that *Streptomyces* sp. isolated from similar type of sponges also showed high antibacterial activity and have reported for their host defence (Kim 2015; Selvin 2009). The endosymbionts are important to marine sponges due to their intimate association with their host (Selvin et al. 2010). One of the main roles of sponge symbionts is the production of bioactive secondary metabolites (Bakus et al. 1986; Bewley et al. 1996; Hentschel et al. 2001; Kubanek et al. 2002; Osinga et al. 2001; Unson et al. 1994). In our study, we hypothesized that the as yet not well-understood interaction between sponge and associated bacteria regulates the production of bioactive compounds. Being filter feeders, any challenge with bacteria will occur immediately and impact the whole microbial population.

To document changes in microbial diversity, both TRFLP and Pyrosequencing were used. Pyrosequencing revealed that at 0 hour, both control and treatment sponges had a high prevalence of *Prochlorococcus*, *Pseudovibrio* and *Ruegeria* (Fig 5). *Prochlorococcus* is a photosynthetic *Cyanobacteria*, which is the most abundant photosynthetic organism on Earth, and has previously been reported in *A. rosea* (Alex and Antunes 2015). Once the sponge *A. rosea* was exposed to the *Streptomyces* ACT-52A, they responded within 24 hours. The microbial population, as revealed by TRFLP, changed from *Alphaproteobacteria*, *Gammaproteobacteria*, *Flavobacteria* to *Betaproteobacteria*, *Actinobacteria*, *Chlorofexi* and gram negative bacteria *Thermotogae*. From the literature, it is evident that sponges respond quickly when they face any external stimuli or adverse condition or competition with other organisms (Allison and Martiny 2008; Morrow et al. 2013; Nogales et al. 2011). For instance, a sponge-associated Alphaproteobacterium symbiont of *Rhopaloeides odorabile* declined dramatically within 24 hours when the sponge was exposed to elevated temperature (Webster et al. 2008). Similarly, in our previous study of *A. rosea*, we found that the sponge explants responded to the *V. natriegens* challenge within 24 hours (Mehbub et al. 2015). It has been stated that depletion of nutrients is a powerful driving force for sponges to host microbes (Santos-Gandelman et al. 2014). The increasing abundance of cyanobacteria (*Prochlorococcus*) during this time period (48 hours) in both control and treated sponges in the current study supports the role of cyanobacteria in carbon metabolism, photosynthesis and nutrition (Cheshire et al. 1997; Erwin and Thacker 2007; Erwin and Thacker 2008; Steindler et al. 2002; Wilkinson 1978; Wilkinson 1979; Wilkinson 1983; Yahel et al. 2003; Yahel et al. 2007). From the

pyrosequencing data it is evident that at 96 hours, the treated sponges were colonized by more *Streptomyces* sp. compared to 0 hour.

Understanding the complex sponge microbiota requires a basic knowledge of how these communities change over time (Erwin et al. 2011). Although the literature shows evidence that under normal living conditions the sponge microbial community is very stable (Friedrich et al. 2001; Lemoine et al. 2007; Mohamed et al. 2008a; Pita et al. 2013; Simister et al. 2013; Thoms et al. 2003; Webster et al. 2008; White et al. 2012), this study provided experimental evidence that external stimuli such as exposure to even sponge-associated bacteria can facilitate sponges to alter their microbial assemblage. It is also evident that the microbial assemblage in the present study was not as divergent as the microbial assemblage in the earlier study when challenged with an external, fast growing bacterium *V. natriegens* (Mehbub et al. 2015). The results may be related to the nature of these two types of bacteria, one being closely associated with the host sponge, while the other is an external foreign bacterium. The question that this new experimental system can answer is whether different types of compounds are produced due to challenges with different microorganisms.

Metabolomics approaches can provide insight into ecophysiology and ecotoxicology by characterising the physiological responses of an organism (Bundy et al. 2009; Prince and Pohnert 2010; Viant 2009). One of the significant influences of the sponge-bacteria interaction is the change in the metabolite profiles (Fig 6 and Fig 7). PLS-DA analysis of non-polar metabolites revealed that the main changes occurred at 96 hours. This delay in response is possibly due to the slow rate of biosynthesis by the sponge, or the slow growth of *Actinobacteria* spores. The non-polar metabolites are mainly produced to defend against bacteria outside the body of the sponges. As *Streptomyces* ACT-52A is an endosymbiont it is unlikely to be harmful to *A. rosea*, nevertheless a chemical response was noted with substantial changes at 96 hours, but at 360 hours the profiles of non-polar metabolites return to their original state. This reflects that the sponge does have the ability to adapt to changes in the environment. In contrast, the methanolic (polar) extracts showed different patterns compared to non-polar metabolites, particularly at 360 hours. It is evident that polar metabolites produced by sponges are one of the major tools for the prevention of epibiosis and predation (Davis and Bremner 1999). In this study, the interaction between sponges and associated bacteria enhanced the production of polar compounds in both sponge tissues and those captured from the seawater.

As a representative bioactivity, antibacterial activity of extracts and fractions of sponge tissues and metabolites excreted into the seawater against *S. aureus* was investigated. The results clearly demonstrated that bioactivity could change due to changes in the microbial assemblage and metabolite profiles of the sponges when exposed to sponge-associated *Actinobacteria*. It has been reported that sponges regulate the antagonizing growth of different microbes by utilizing their associated bacteria to produce antimicrobial or other secondary metabolites (Burgess et al. 1999). The TLC result of polar compounds both from sponge tissue (Figure S10) and XAD-7 (Figure S7) revealed that different types of active compounds have been generated in treated sponges compared to the control particularly noted at 360 hours. Perhaps in this study sponges produced antimicrobials with the help of their associated bacteria to facilitate a group of bacteria to maintain their ecology.

Until now most sponge-derived natural products were discovered from sponge tissue extract, and, to our knowledge, metabolites released from sponges into the seawater have not been studied. Our results demonstrate that the metabolites in the sponge tissue are very different from those excreted into the seawater. These unexplored released metabolites from different sponges could open a new avenue of biodiscovery of sponge-derived natural products. In our study, as we only collected the released metabolites at 96 and 360 hours, the time for optimum production of bioactive metabolites was not determined. This could be achieved by adding and removing resin bags on a daily basis and evaluating the level and composition of the metabolites. Once the maximum production time was known the process of challenging the sponges could be repeated many times with the chosen bacterium and the absorbent harvested after each challenge. This could significantly overcome the 'sponge metabolite supply' problem.

Our novel approach and controlled aquarium system are therefore very valuable for exploring these extracellular released metabolites from sponges under a variety of challenging conditions such as different bacteria, fungi, temperature, dissolved oxygen and nutrition. Further studies will be required to demonstrate the potential of this field for bio-discovery.

It is still unknown how sponges respond to different types of bacteria. From our limited studies of two different types of bacteria (one external environmental bacterium *Vibrio natriegens* (Ferdous et al 2016) and one sponge-associated *Actinobacteria Streptomyces* ACT-52A), concerted changes in sponge-associated bacterial diversity, metabolite profiles and bioactivity were common responses when exposed to bacteria challenges. While the changes at all three

levels are very different for the exposures to these two different types of bacteria, the speed of responses and the degree of changes are also different. Further studies on the roles of sponge-bacteria interactions should be designed in such a way that different types of sponge-bacteria interactions, including mutualism, antibiosis, symbiosis, co-metabolism, co-synthesis, pathogenesis, micro fouling etc (Selvin et al, 2010) can be differentiated and tested experimentally.

This study has shown for the first time that the exposure of the sponge *A. rosea* to its associated bacterium, *Streptomyces* ACT-52A, can significantly alter its bacterial community, metabolite profile, and antibacterial activity against *S. aureus*. A diverse range of metabolites was also secreted into the seawater by the sponges during the exposure experiments. This finding could open a new avenue for sponge-derived natural product discovery for extracellular released metabolites that are yet to be explored. The experimental approach established in our laboratory could be extended for future studies of the roles of sponge-bacteria interactions, which call for a more systematic design focusing on different types of interactions, rather than different types of bacteria.

7.6 Ethical Statement

This study does not contain any studies with human participants or animals performed by any of the authors.

7.7 Conflict of interest

The authors declare that they have no competing interests.

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7.10 Supplementary Files

Applied Microbiology and Biotechnology

The role of sponge-bacteria interactions: the sponge *Aplysilla rosea* challenged by its associated bacterium *Streptomyces* ACT-52A in a controlled aquarium system

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Supplementary materials

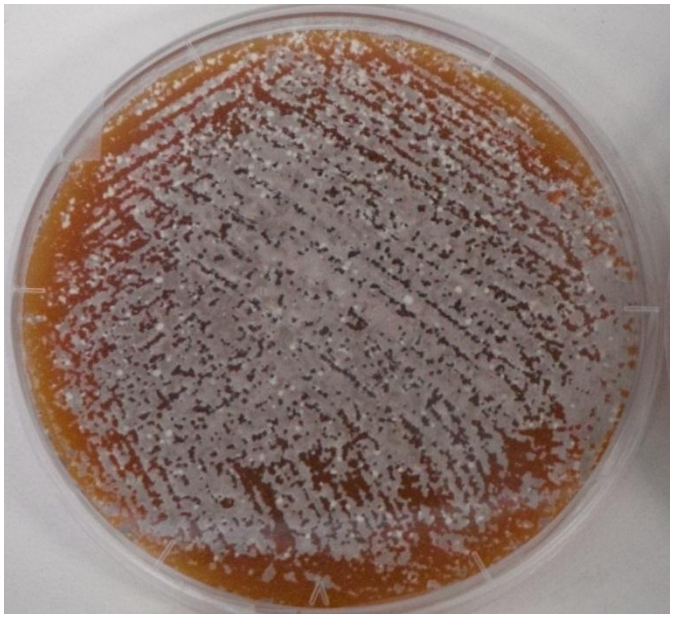


Figure S1 Growth of *Streptomyces* ACT-52A in spore production media

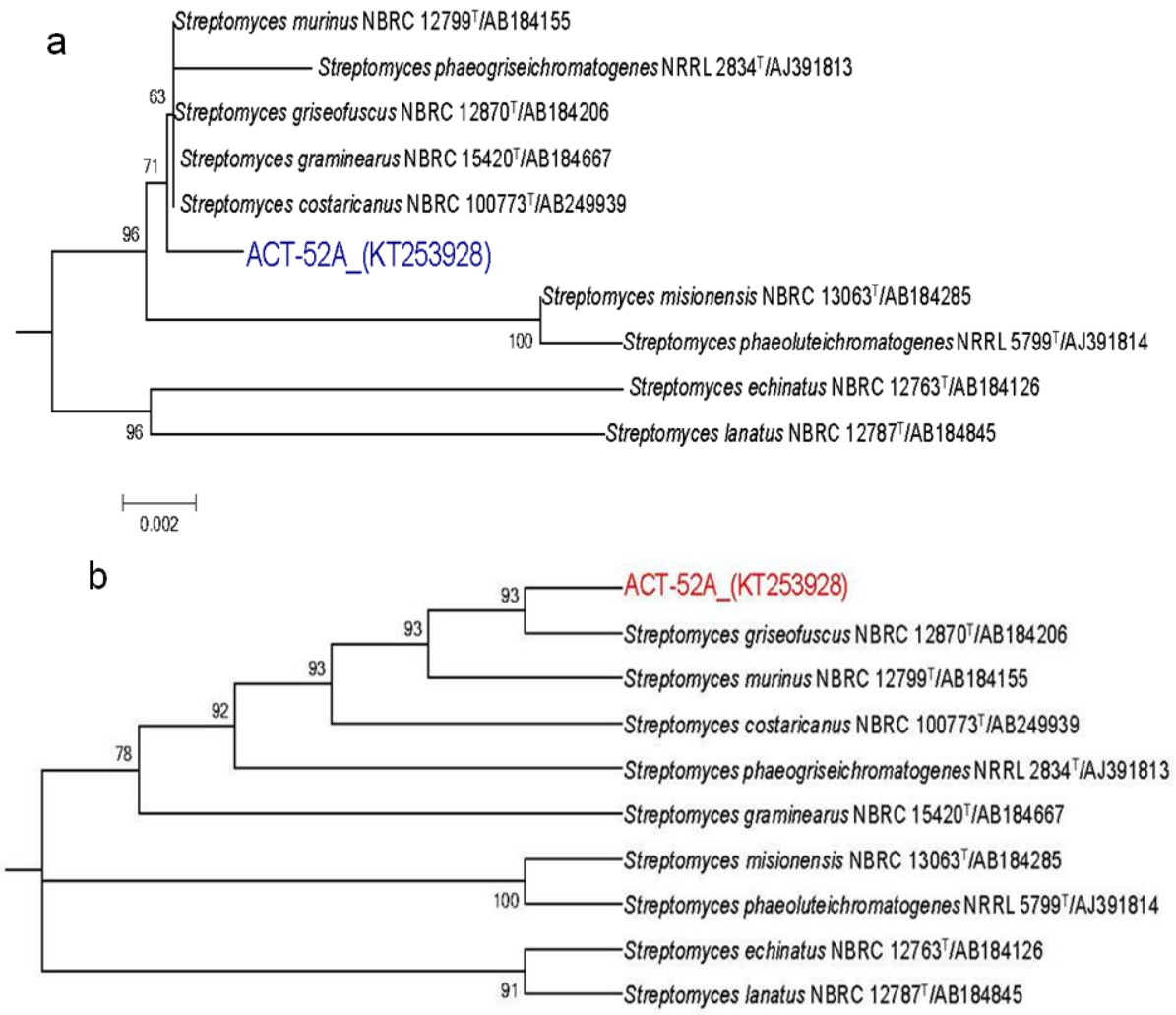


Figure S2 Phylogenetic tree of *Streptomyces* ACT-52A 16S ribosomal gene (GenBank Accession No. KT253928 and DSMZ open collection as DSM 101673) **(a)** the evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length = 0.05086998 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) and are in the units of the number of base substitutions per site. The analysis involved 10 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1309 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura K 2013). **(b)** The evolutionary history was inferred by using the Maximum

Likelihood method based on the Kimura 2-parameter model (Kimura 1980). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein 1985) is taken to represent the evolutionary history of the taxa analyzed (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Initial tree(s) for the heuristic search were obtained automatically by applying the Maximum Parsimony method. The analysis involved 10 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1309 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura K 2013).

Table S1 SRA accession numbers for pyrosequencing data with sample code and web link.

Sample Name	Accession numbers	Web Link
C.0.1	SRS1063499	http://www.ncbi.nlm.nih.gov/biosample/4038424
C.0.2	SRS1063498	http://www.ncbi.nlm.nih.gov/biosample/4038425
C.0.3	SRS1063497	http://www.ncbi.nlm.nih.gov/biosample/4038426
T.96.1	SRS1063496	http://www.ncbi.nlm.nih.gov/biosample/4038427
T.96.2	SRS1063495	http://www.ncbi.nlm.nih.gov/biosample/4038428
T.96.3	SRS1063494	http://www.ncbi.nlm.nih.gov/biosample/4038429

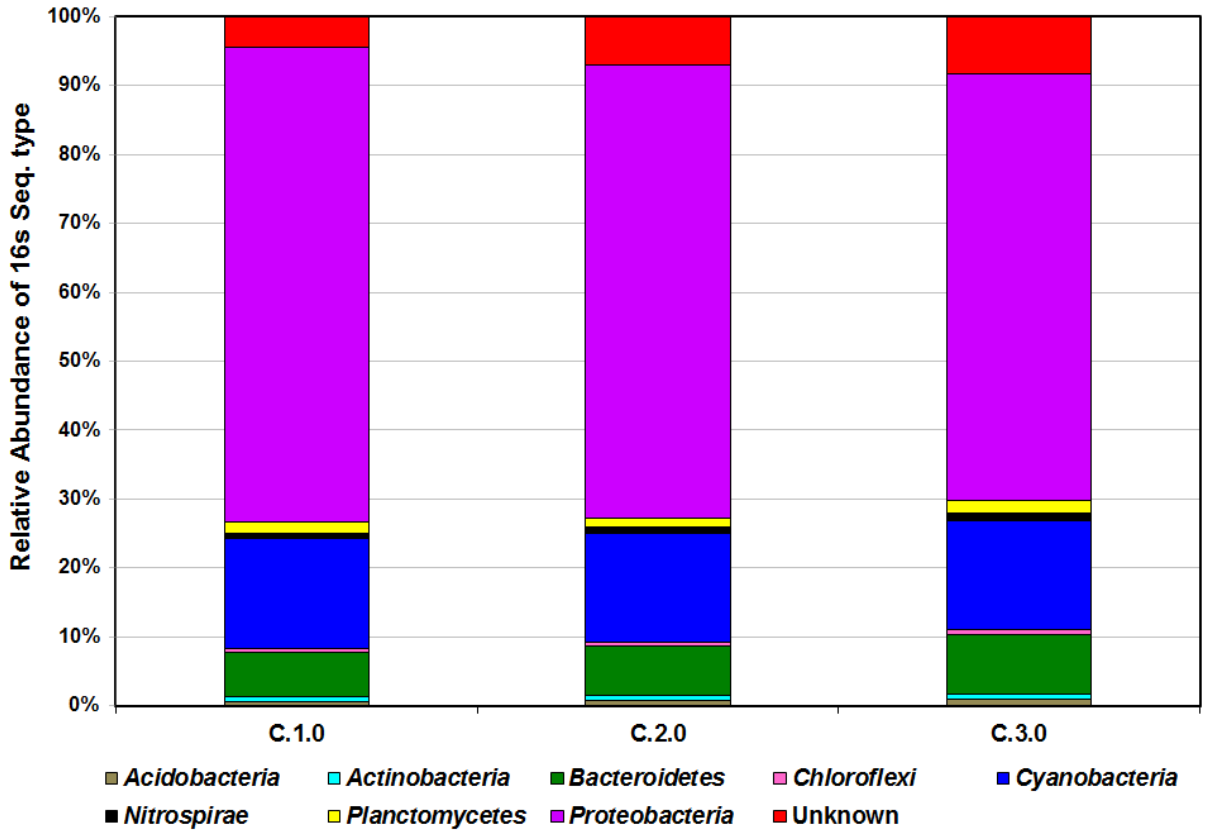


Figure S3 Major bacterial phyla inferred from 16S rRNA relative sequence abundances derived from 454 pyrosequencing of sponges from the start of the experiment (0 hour control).

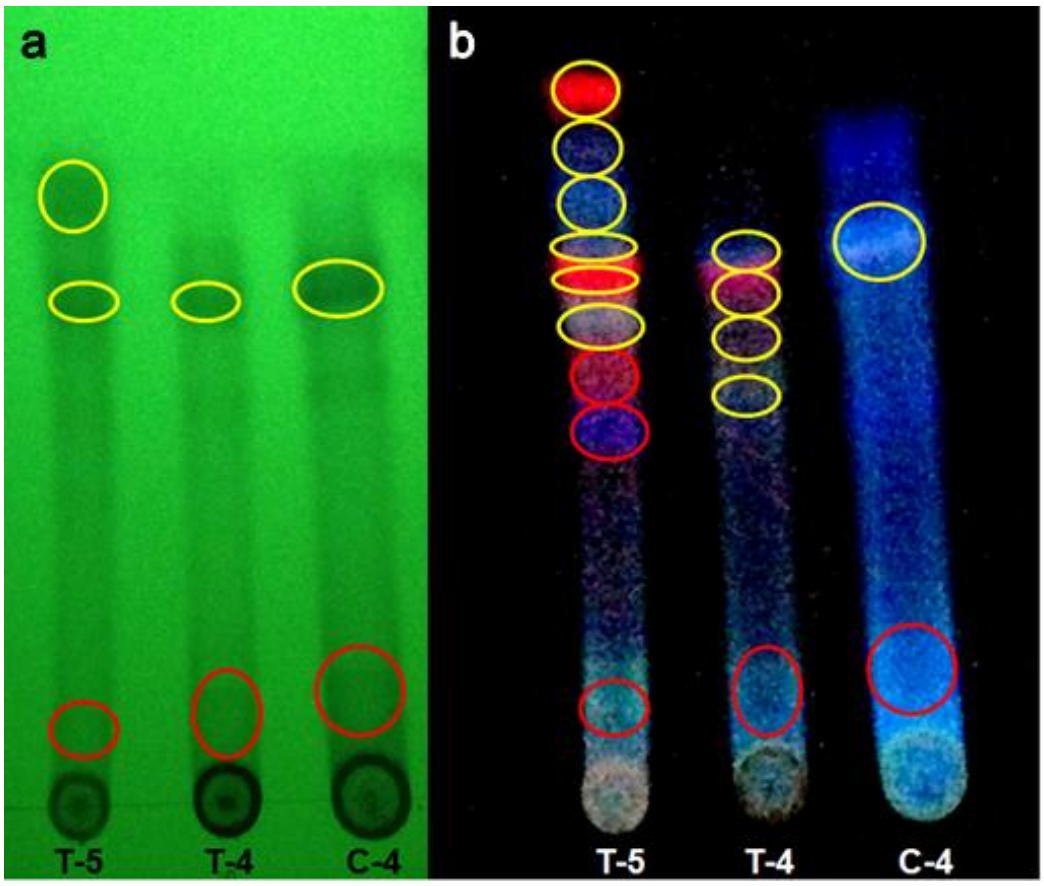
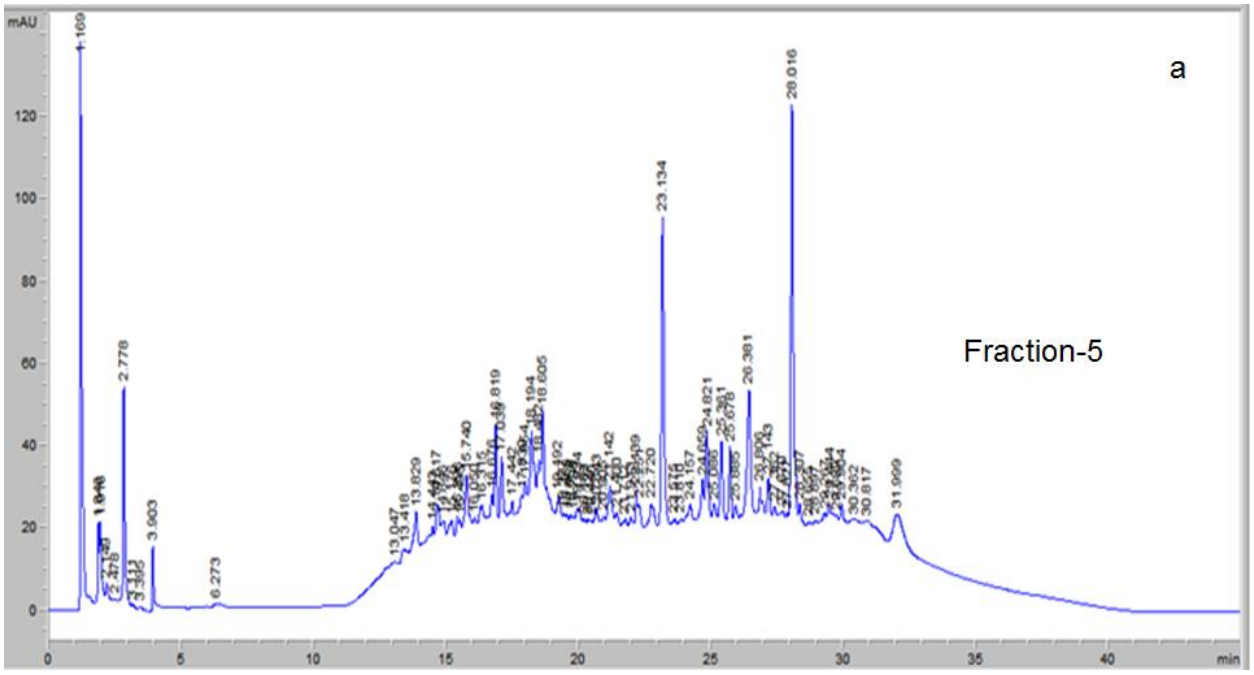


Figure S4 TLC result of polar metabolites of few key fractions collected from Amberlite XAD-7 after 360 hours and their antibacterial activity against *Staphylococcus aureus* when run as a bioautogram. (a) Two active XAD-7 fractions from treatment tank (T-5,T-4) and one active XAD-7 fractions from control tank (C-4) when run in EtOAc:MeOH [9:1].and seen under UV at 254 nm. (b) The same fractions under UV absorbance at 365nm. Red circles indicate active spots against *Staphylococcus aureus* whereas yellow circles show only spots without activity against *S. aureus.* T-5=Fraction 5 from treatment tank, T-4=Fraction 4 from treatment tank and C-4= Fraction 4 from control tank.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Fraction-5
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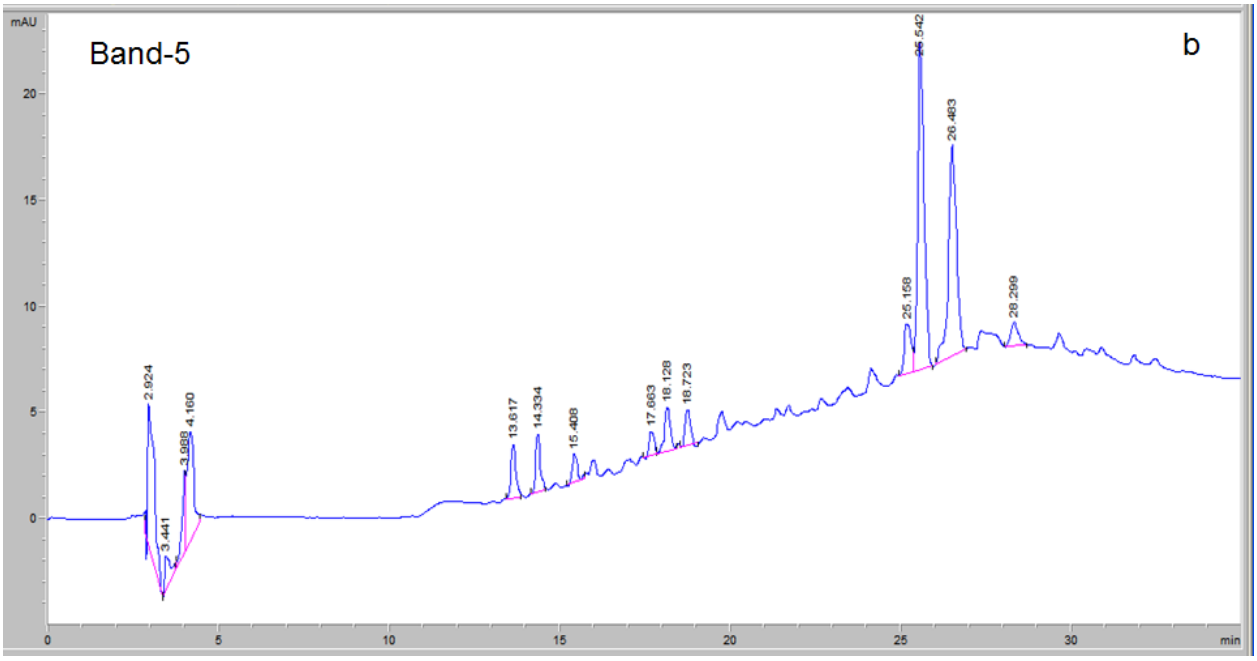
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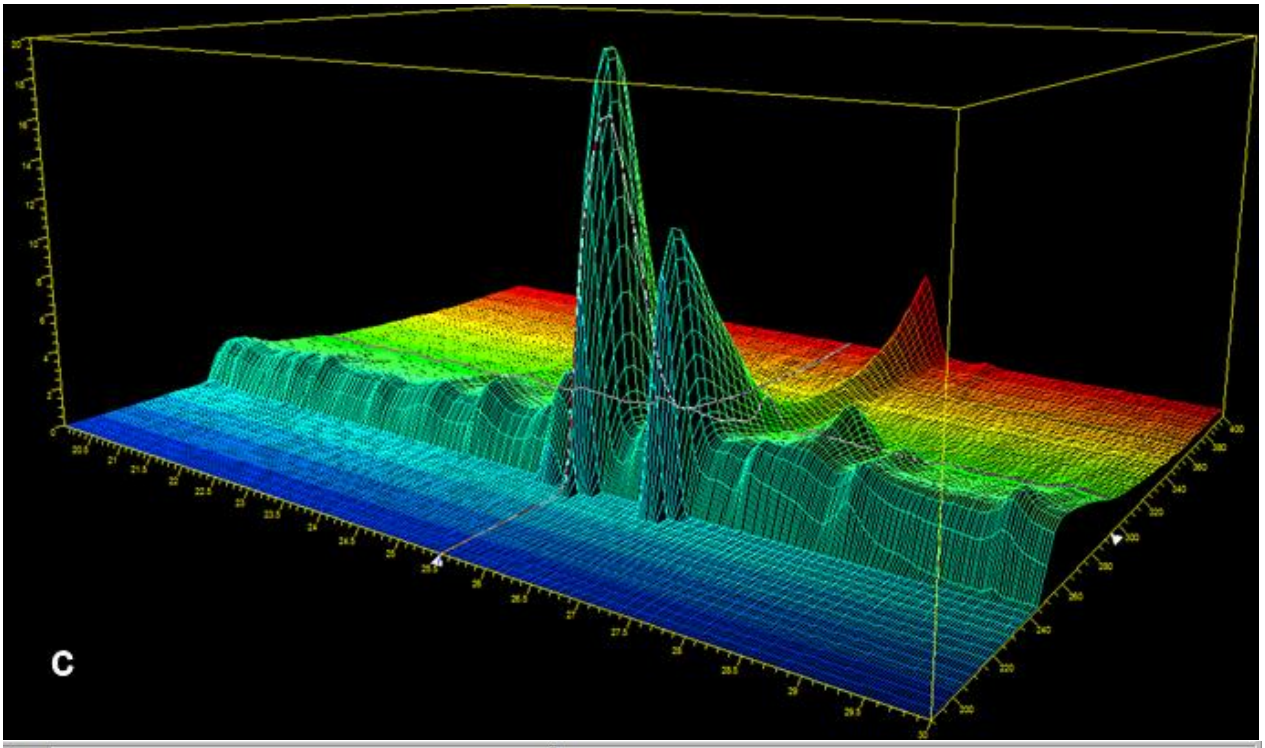
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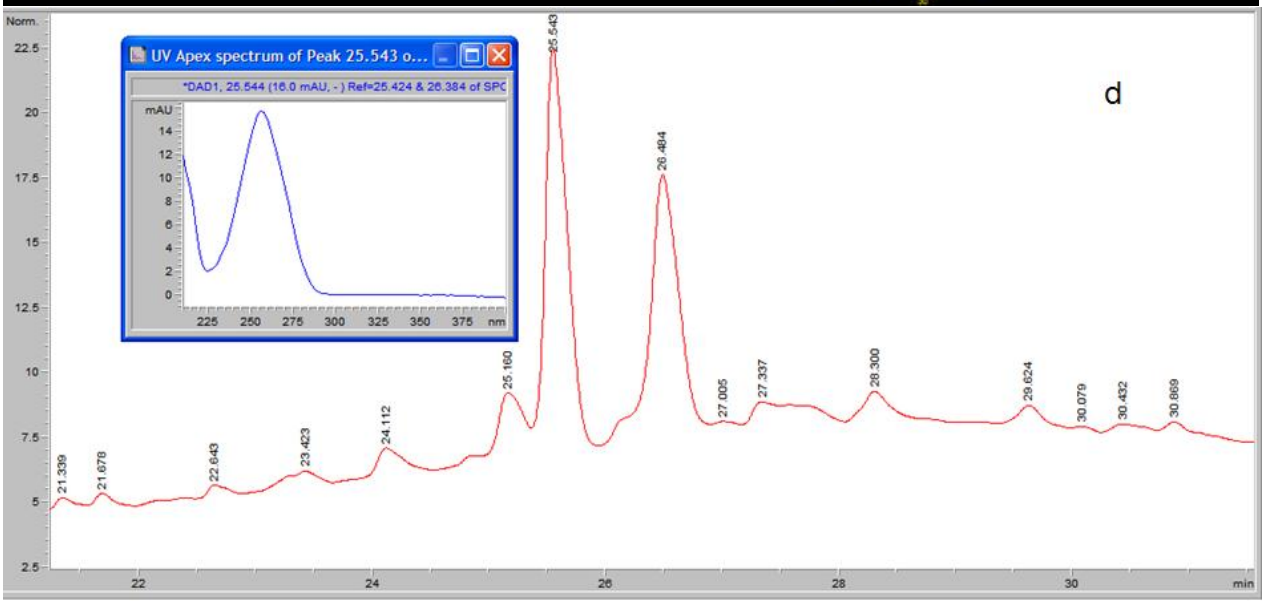
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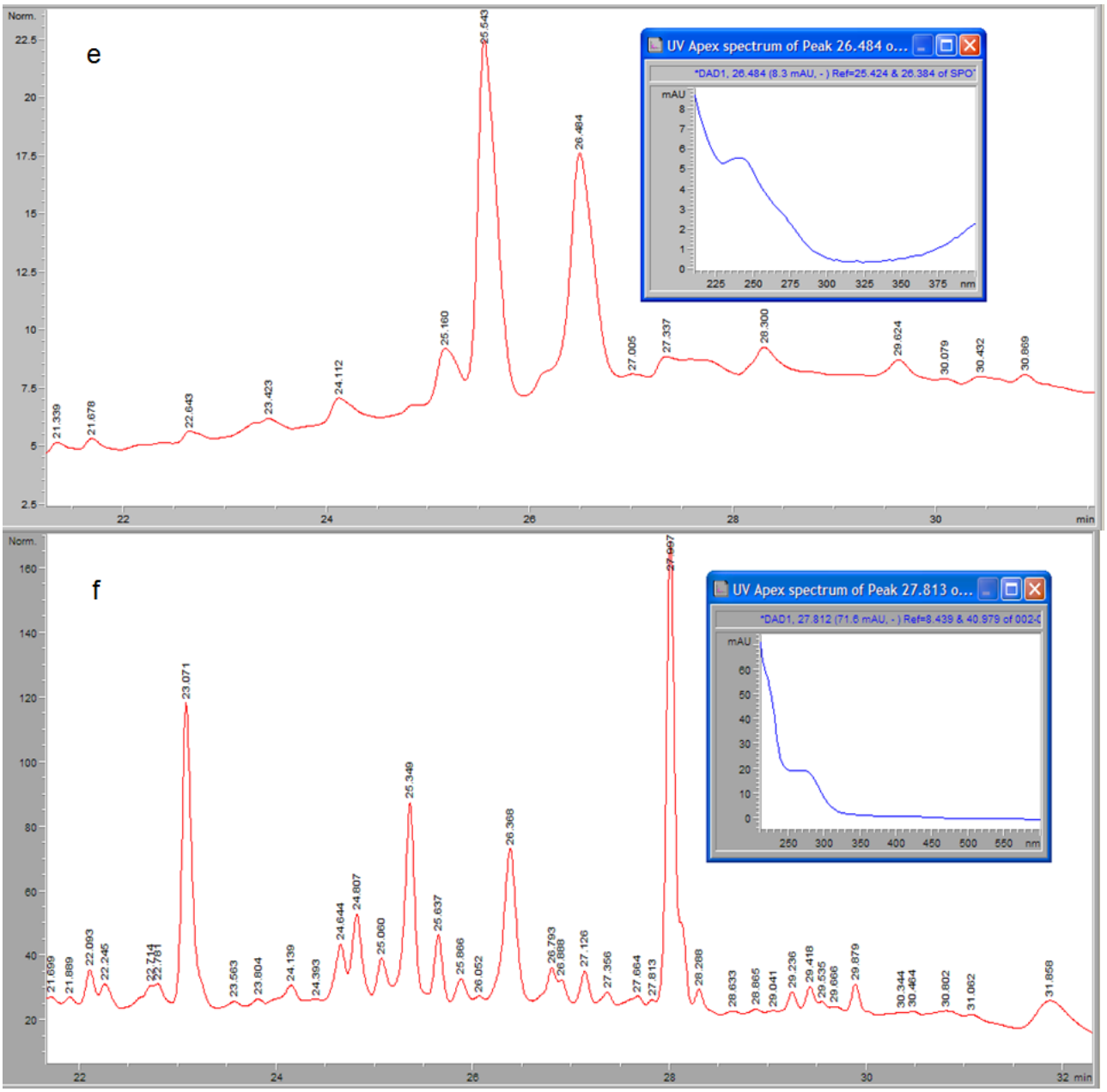
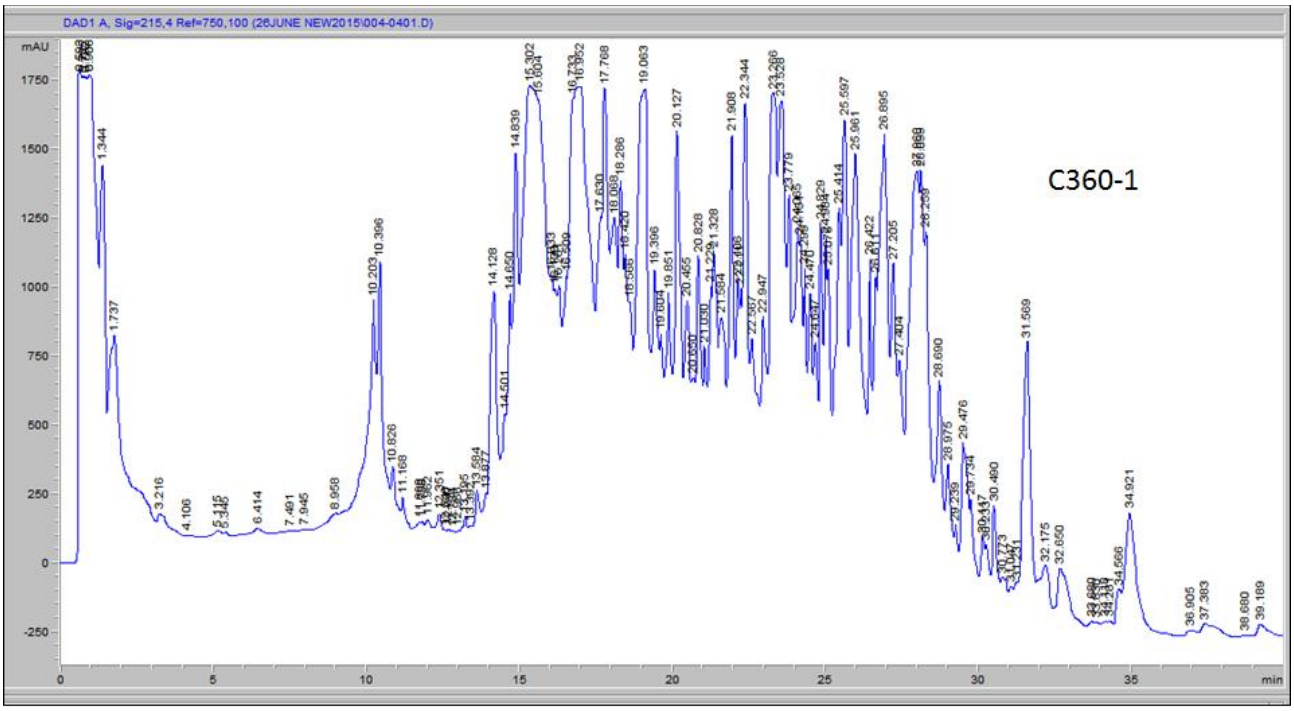


Figure S5 HPLC analysis of metabolites. (a) HPLC analysis revealed three main peaks from fraction 5 collected from Amberlite XAD-7 from treatment tank at 360 hours when observed at 254 nm with retention times of 23.134, 26.381 and 28.016 min. (b) Further analysis of band 5 revealed two main peaks, their retention times being 25.542 and 26.483 min. (c) A three dimensional picture of band 5. (d) The major peaks for band 5 at 25.542 min. (e) Fraction 5 at 28.016 min had the same UV spectrum at 260 nm, which is possibly the reason for increased bioactivity.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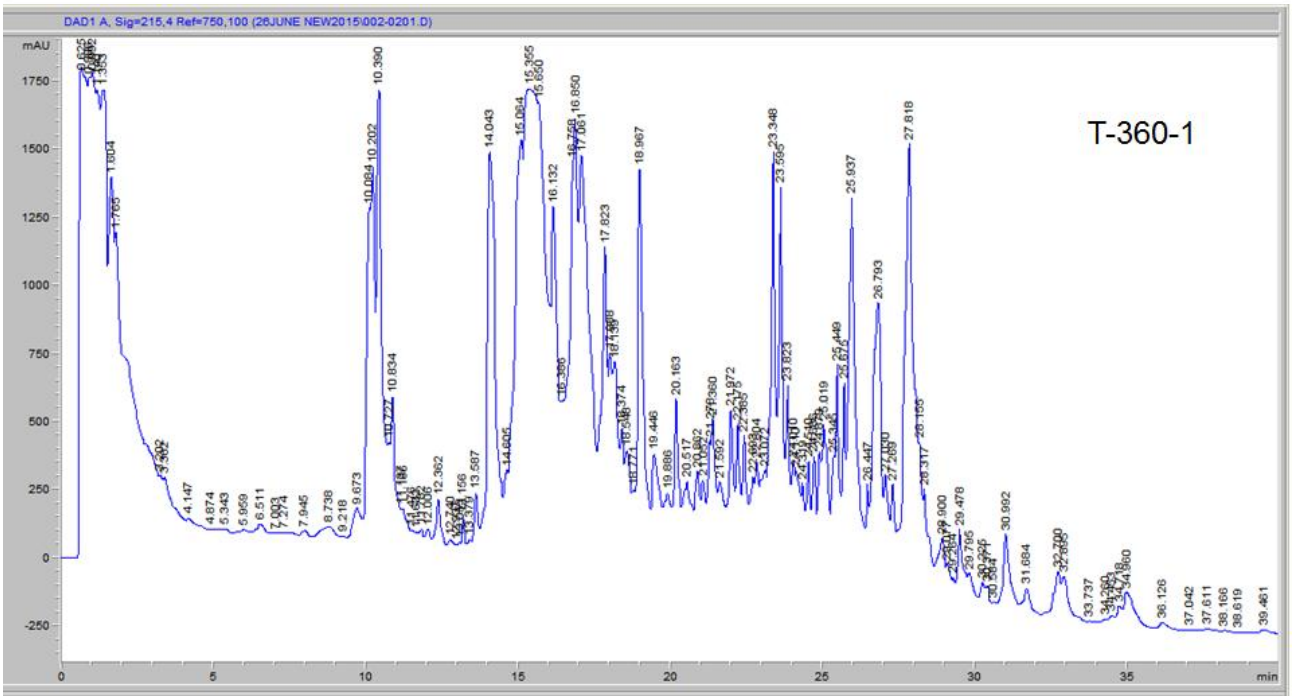
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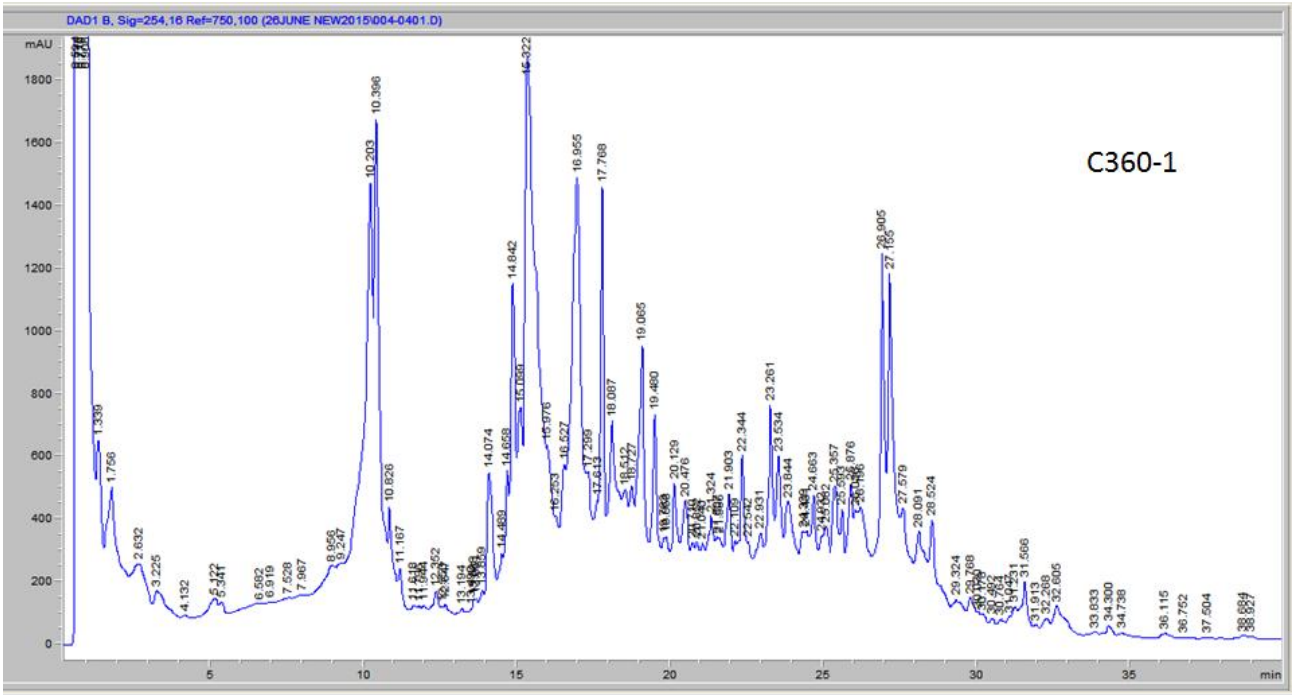
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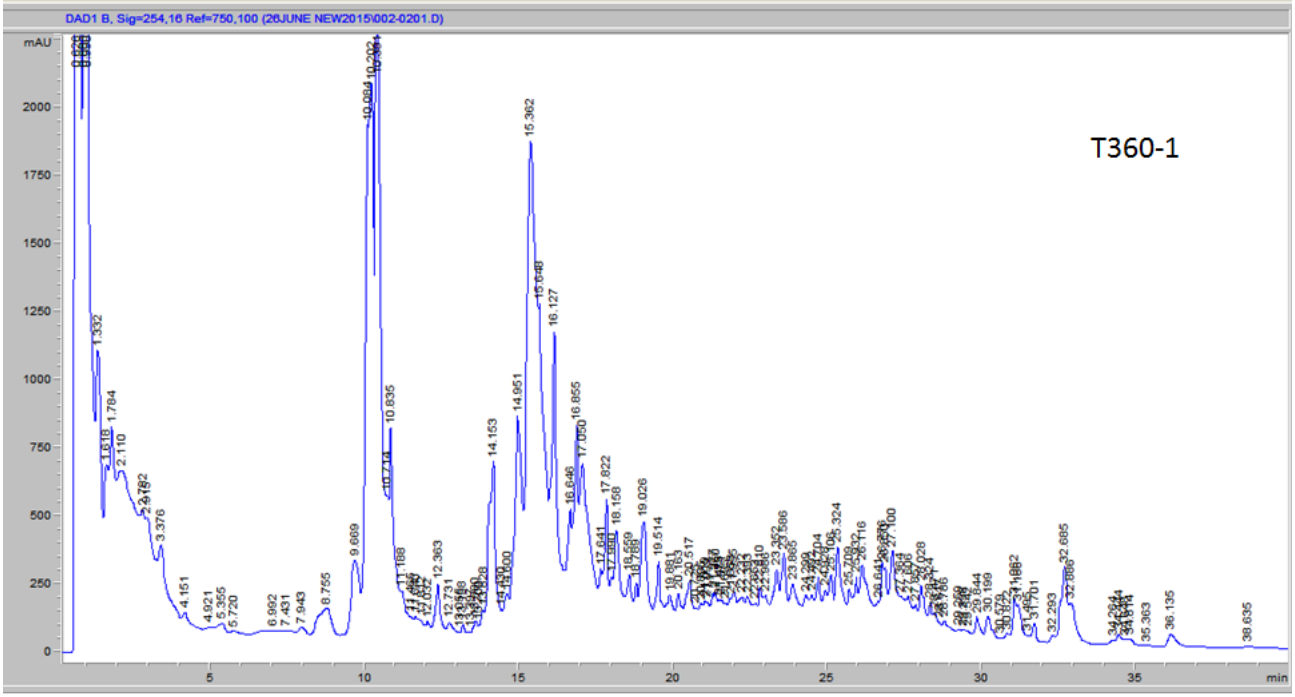
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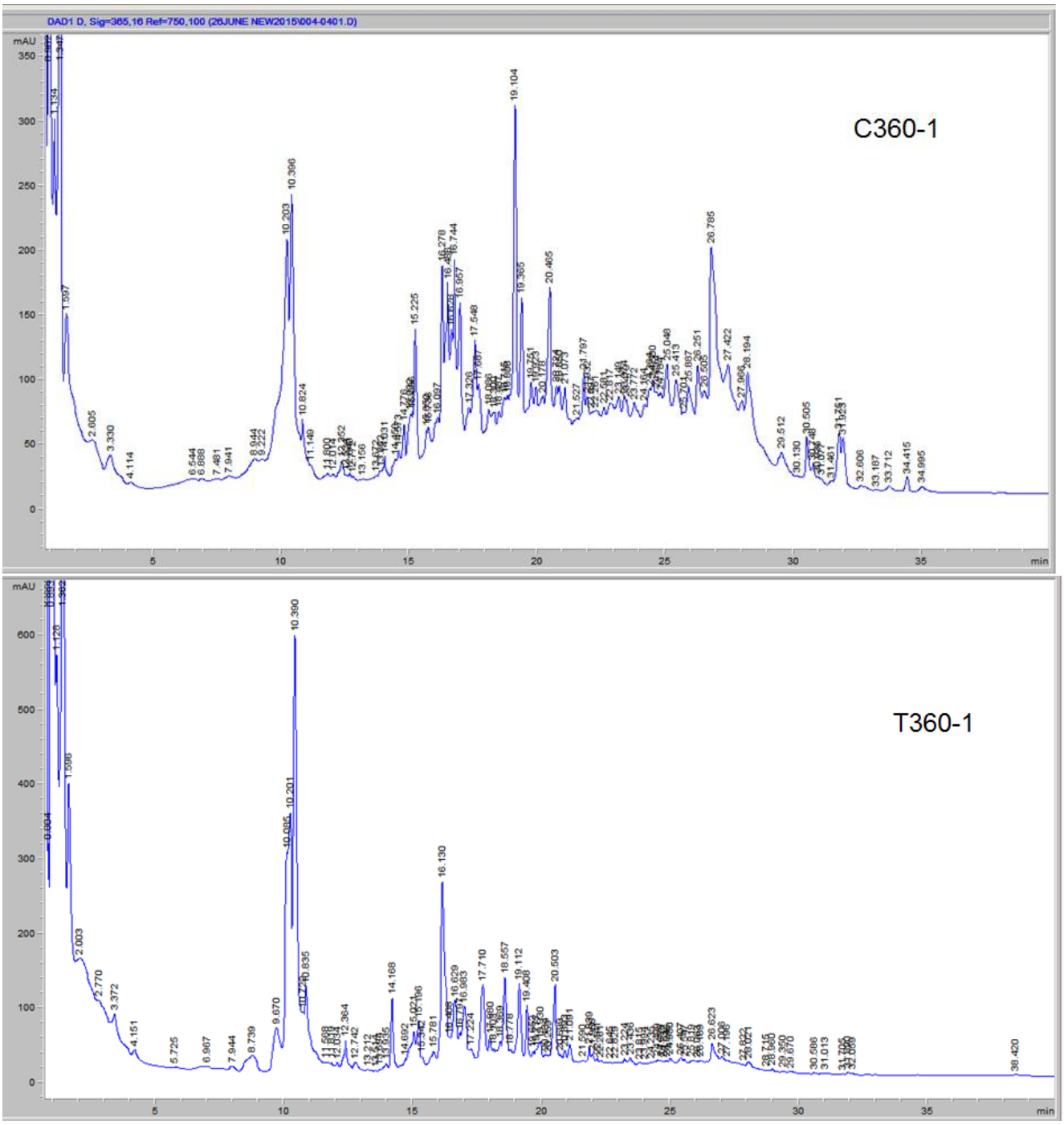

Figure S6 Representative HPLC chromatogram of methanolic extracts from treatment and control sponges showing distinct peak profile patterns at different wavelengths at 360 hours.

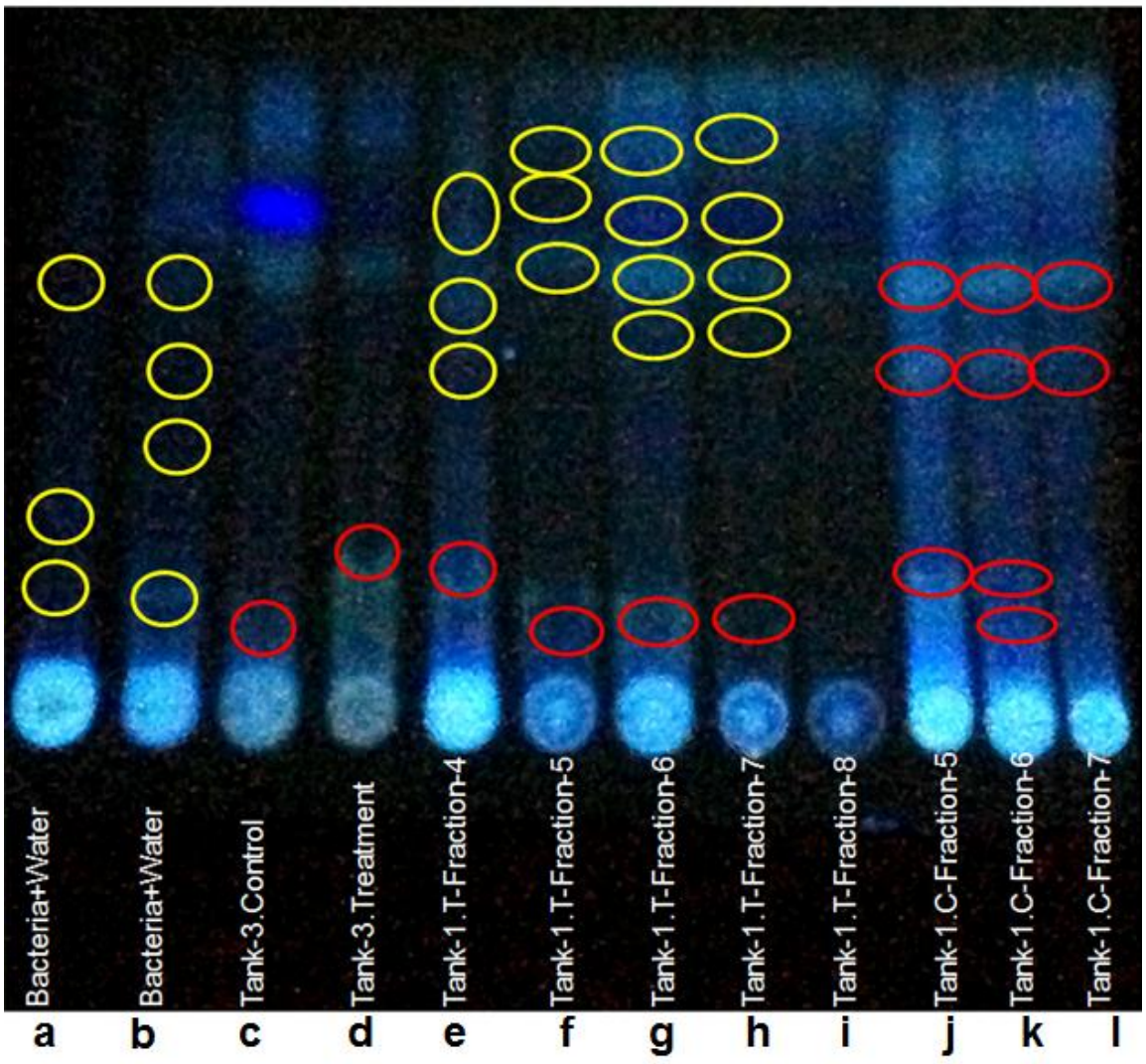

Figure S7 TLC result of different XAD-7 fractions run on Ethlacetate:Methanol (9:1). XAD-7 collected at 96 hours. Yellow circles show moderately active spots, whereas red circles show very active spots. Legends are explain below:

a= BW (bacteria +water), Tank-1. Fraction-5

b= BW, Tank-1, Fraction 6

c=Control tank-3, Fraction 5

d=Treatment tank-3, Fraction 7

e= Treatment tank-1, Fraction 4

f= Treatment tank-1, Fraction 5

g= Treatment tank-1, Fraction 6

h= Treatment tank-1, Fraction 7

i = Treatment tank-1, Fraction 8

j= Control tank-1, Fraction 5

k=Control tank-1, Fraction 6

l= Control tank-1, Fraction 7

l= Control tank-1, Fraction 7

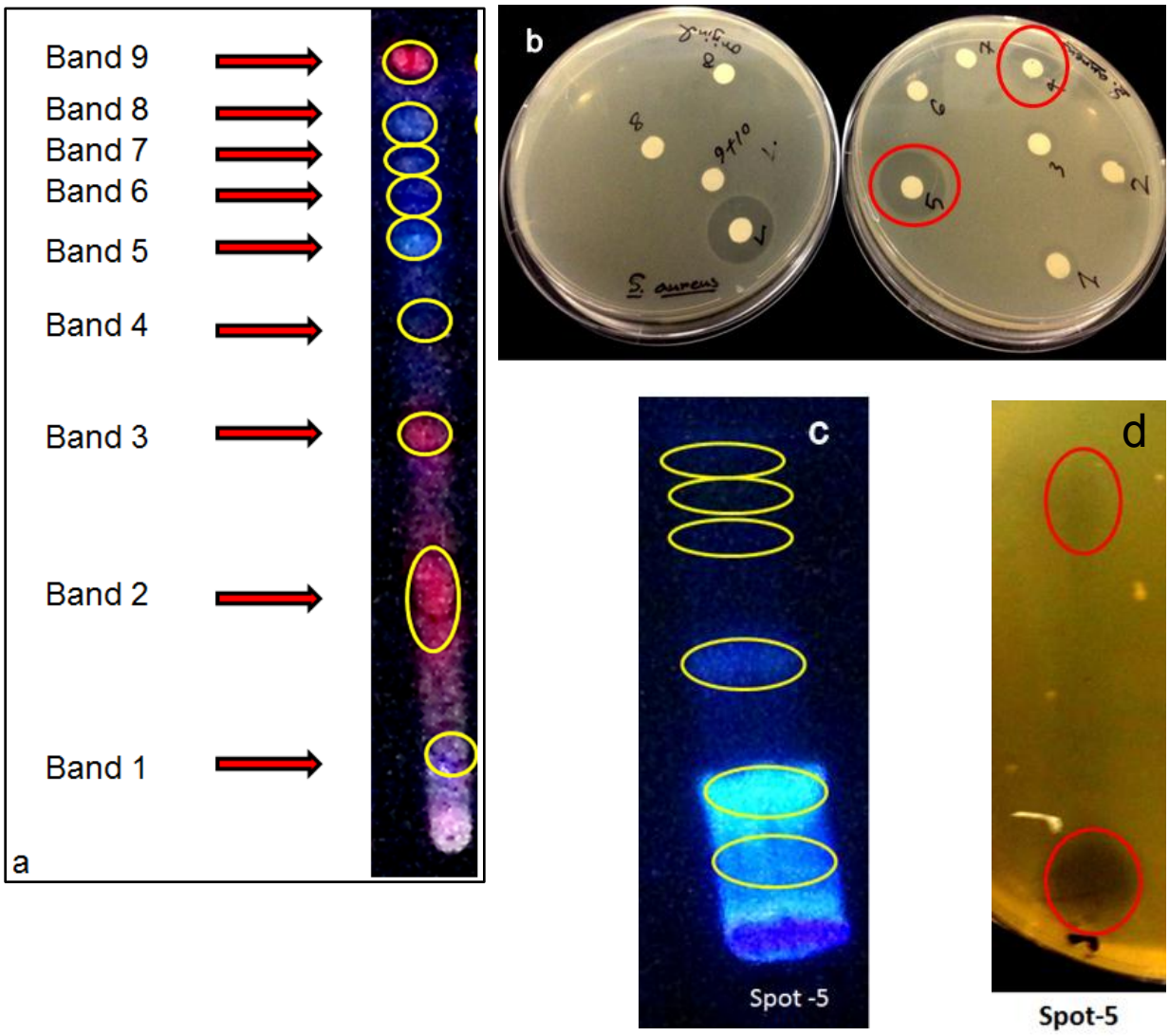

Figure S8 Antibacterial activity of fraction five eluted from Amberlite XAD-7 in the treatment tank after 360 hours. (a) Nine spots revealed by thin layer chromatography (TLC) in Ethylacetate: Methanol [9:1]. (b) The zone of inhibition (red circles) against *Staphylococcus aureus*. (c) Further TLC of band 5 in the same system. (d) The zone of inhibition (red circles) of band 5 against *S. aureus* during the bioautogram.

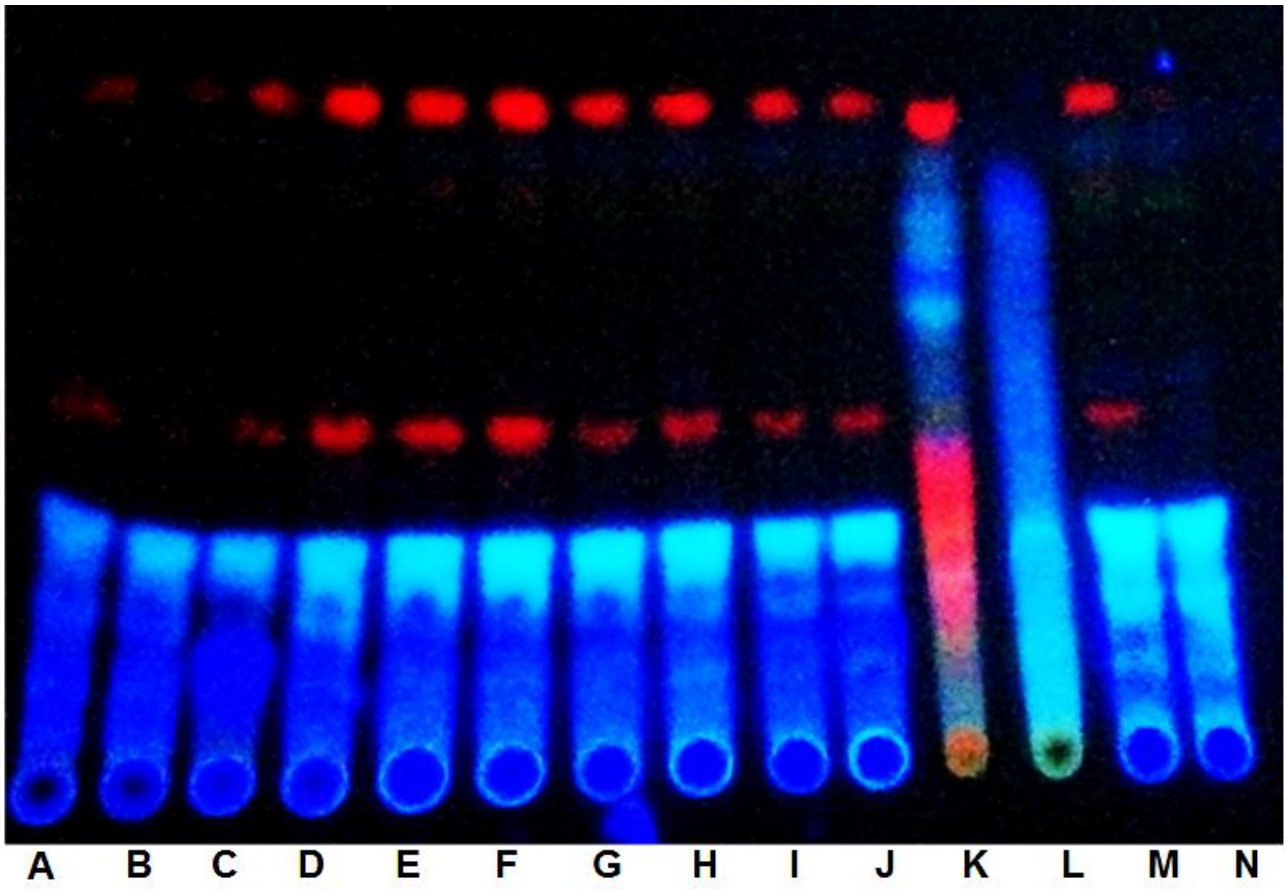

Figure S9 TLC of sponge tissue extract (A-J and M, N) and extract collected from XAD-7 (K, L) run in 9:1 Ethylacetate Methanol.

Legend: A= T-0-1, B=C-0-1, C=T-24-1, D=C-24-1, E=T-48-1, F= C-48-1, G=M-48, H=T-72-1, I=C-72-1, J=M-72, K=T-360-XAD7-Fraction 5, L=T-360-XAD7-Fraction 3, M=C-96-1, N=T-96-1. (Treatment labelled as T and Control labelled as C. M signifies sample from holding aquarium (sponge maintenance) which were not used in the interaction experiment).

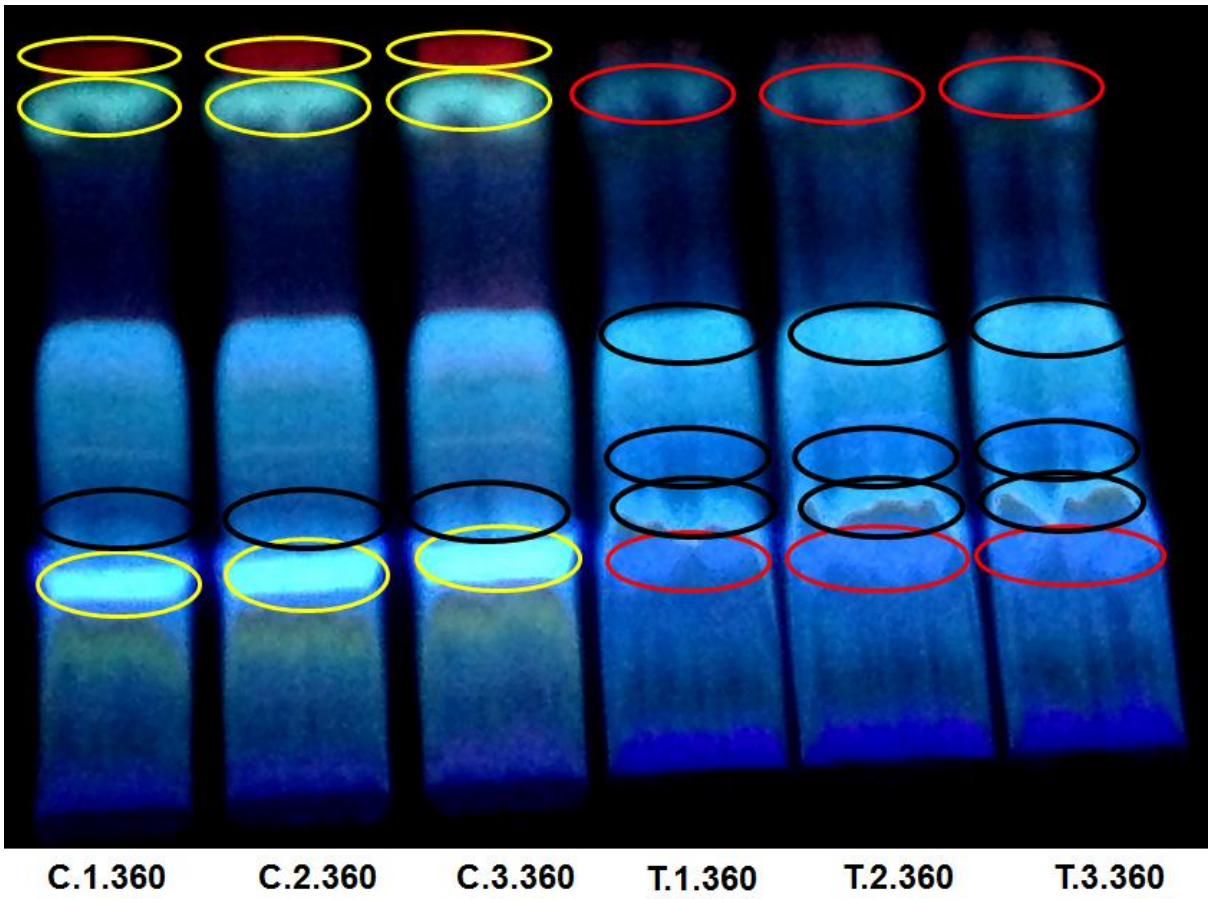

Figure S10 Distinct TLC profiles between extracts from control and treated sponges after 360 hours when run in Ethylacetate: Methanol [7:3]. Black circles indicate active spots against *Staphylococcus aureus*. Three spots were clearly visible under UV from control sponge (yellow circles) extracts and 2 from treatment sponges (red circles) when measured at 365 nm.

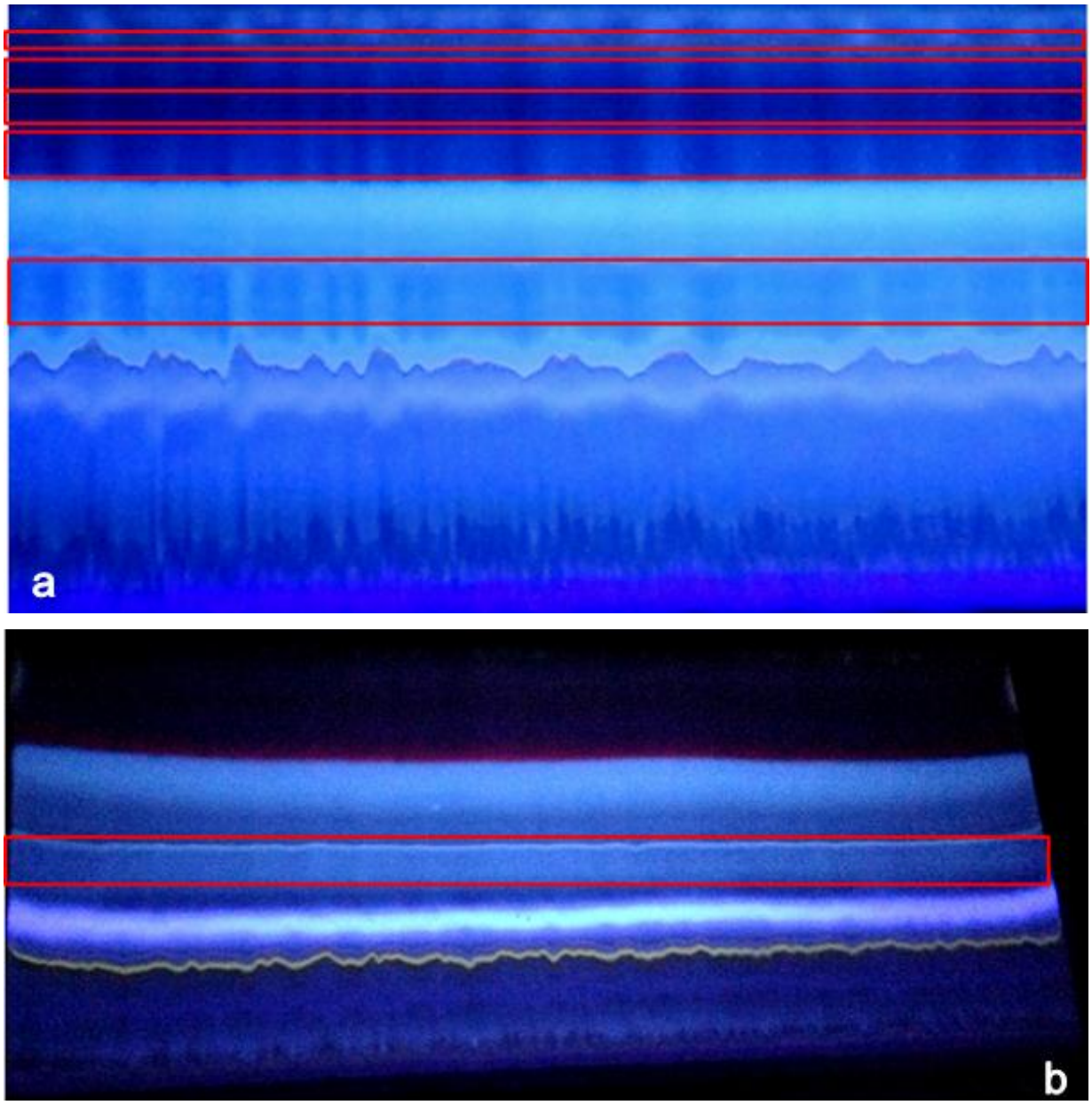

Figure S11 PLC showing metabolites under UV at 365 nm from treated and control sponge extract at 360 hours when run in Ethylacetate:Methanol [7:3]. (a) Treated tank sponge tissue metabolites. (b) Control tank sponge tissue metabolites. Red rectangles indicate active spots against *Staphylococcus aureus.* (c) The antibacterial activity of sponge tissue against *S. aureus* from treatment (T-360) and control (C-360) sponges at 360 hours when run as a bioautogram.

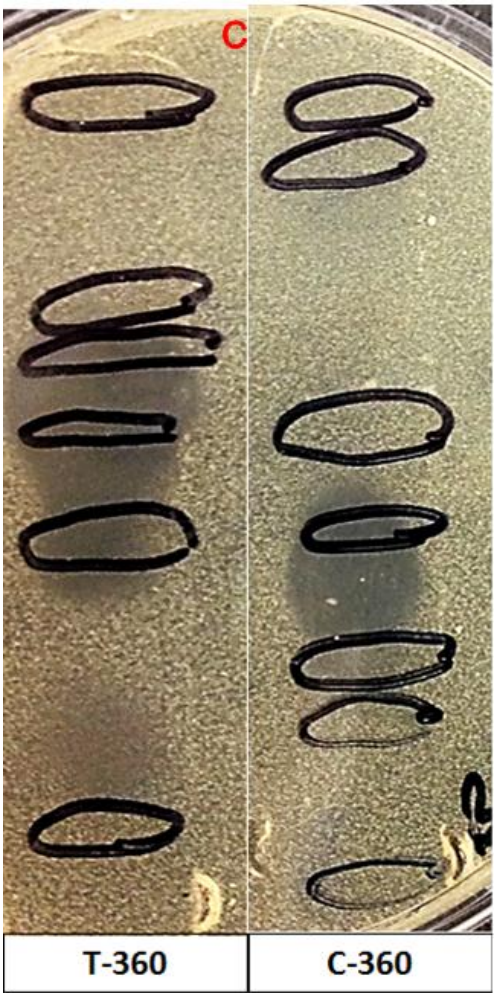

Table S2 The zone of inhibition of different bands against *S. aureus* from treatment (T-360) and control (C-360) sponge tissue extracts at 360 hours.

T-360h	Zone of inhibition (mm)	**C-360h**	Zone of inhibition (mm)
Band-6	12	Band-6	10
Band-9	10		
Band-10	11		
Band-11	15		
Band-12	6		

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CHAPTER 8

CONCLUSION

8.1 Summary of the Research

Marine biotechnology is anticipated to produce a multitude of scientific and industrial outputs from the biodiversity of marine ecosystems and the genetic uniqueness of marine organisms in fostering the development of functional products and bioproducts that have medical, agricultural and industrial applications. Marine sponges have received significant attention as important source of bioactive compounds and the highest producers of novel metabolites, which have potential to contribute to the production of novel therapeutics for the prevention and cure of an array of human diseases (Ireland et al. 1993; Blunt et al. 2005; Laport et al. 2009). In the decade from 2001–2012 marine sponges continued to be the most promising source of marine natural products, contributing nearly 30% of all marine natural products discovered during this decade (Mehbub et al. 2014). Sponges protect themselves from predators or any potential threat by using their secondary metabolites. They are multicellular animals (Nichols & Wörheide 2005; Wang et al. 2010) and their bodies are composed of a good amount of microbes with up to 40%–60% of microbial biomass (Schmitt et al. 2007). Among those microbes, bacteria were found to be the most dominant and to play a vital role in the maintenance of the sponge ecology and biology. In the coming decade sponge-associated microorganisms promise to be outstanding sources of new marine natural products (Mehbub et al. 2015). Although there are more than 18 orders of sponges, the following 5 orders were found to produce more than 70% of new metabolites – Astrophorida, Dictyoceratida, Halichondrida, Haplosclerida and Poecilosclerida (Mehbub et al. 2014). The order Dictyoceratida was found to be the most prolific producer of new compounds among all the sponge orders studied during 2001-2012 (Mehbub et al. 2016). Within this order the most promising genera were found to be *Dysidea* sp. from Dysideidae family; *Sarcotragus* sp. and *Ircinia* sp. from Irciniidae family; *Spongia* from Spongiidae family and *Hyrtios* sp. and *Phyllospongia* sp. for their contribution of large numbers of new bioactive compounds. Further research could be fruitful if other species from these genera can be collected. Regardless of the order, the examination of the contribution from an individual species revealed that each species contributed on average 3–5 compounds. Therefore, unstudied species from any sponge order need to be examined to explore more new compounds.

The numbers of new compounds reported from microorganisms associated with sponges may seem less significant at around 35 compounds per year in the decade of 2001-2012 (Mehbub et al. 2015). The success rate in terms of sponge-derived therapeutic drug development is still not satisfactory. Part of the reason is that in many laboratories the chemists who isolate, purify and determine the structures of the compounds do not have the capacity to produce these compounds

in sufficient quantity and test their compounds in a large range of biological assays. However, the current insurmountable problem of securing a continuous supply of a sponge metabolite for drug development if chemical synthesis is not economic will lead to a more intensive search for microbial sources as fermentation production is feasible. In addition efforts have also been made to culture sponge cells with the capability to produce these desired compounds on a large scale. With regard to the true producers of sponge-derived metabolites, it is a hot topic to understand the roles of sponge cells and bacteria as well as their interactions in relation to the biosynthesis of the largest number of marine natural products and their potential commercial development for specific applications.

Understanding of the ecological and environmental impacts of collection sites on sponge biodiversity and chemical diversity could be gained by making collaboration between natural product chemists and marine biologists. Partnerships between natural product chemists and biologists could be advantageous for the discovery of multiple valuable activities if a wide range of biological assays could be tested.

Based on the literature review and previous studies it was hypothesized that the biosynthesis of sponge-derived bioactive metabolites is regulated by their symbiotic bacteria and influenced by the interactions between sponges and their associated bacteria and/or foreign bacteria. The aim of this study was to establish a novel experimental approach to achieving an improved understanding of the role of the interaction between bacteria and sponges using a controlled experimental aquarium system where a bacterium of choice is exposed to sponge explants at a specific density in order to initiate the sponge-bacteria interaction.

To conduct the interaction study, cultivation of marine sponges in aquarium conditions was necessary, which is still a big challenge. However, based on their availability, chemical profile and adaptability in aquaria two sponges, *Aplysilla rosea* and *Aplysina* sp., from two different orders, Dendroceratida and Verongida, respectively, were targeted for this PhD project and most of the effort was put into optimizing suitable conditions for these particular sponges.

Although sponges interact with a lot of different types of bacteria to maintain their ecology and biology, in this study one foreign bacterium and one sponge-associated bacterium were mainly chosen as a validation for the interaction study. Of the different types of sponge-associated bacteria, actinobacterium, *Streptomyces* ACT-52A, was chosen due to high bioactivity and possible role in sponge defence.

This interaction between sponge and sponge-associated bacteria is a continuous process, as sponges are filter feeders and during this filtration process they accumulate microbes and microscopic particles into their body as a food source. It was hypothesized that to protect themselves from unwanted microbes they used their internal microbes as a defence system. This

study revealed that changes in the diversity of sponge-associated bacteria, metabolite profiles and bioactivity are the key to maintaining their ecology and biology. A novel approach was applied in order to test the hypothesis that some of the sponge-derived bioactive compounds may be the result of different interactions between sponge and sponge-associated bacteria, which are regulated by the foreign bacterium or an added sponge-associated bacterium. To conduct this experiment an aquarium experimental system was developed and the methodology was validated. This approach and methods could be used in a diverse range of different sponge-bacteria interaction studies.

This is the first study which revealed that capture of metabolites is possible in an aquarium system. The excreted metabolites from the sponge tissue in the seawater inside the tank captured by Amberlite XAD-7 were discovered to be mostly different from the sponge tissue extract. This finding would be valuable for future exploration of new metabolites from sponges as no literature has reported these findings in the past. The main advantage of this approach and system is it does not require extraction of the sponge tissue to obtain the metabolites.

8.2 Major Findings of the Project

The novel experimental approach developed in this study could be used to understand the roles of sponge-bacteria interaction.

The first interaction study successfully developed and validated a novel experimental system to study the role of sponge-bacteria interaction using a sponge *A. rosea* and an external bacterium *V. natriegens*. The findings on the dynamic and concerted changes in the microbial diversity, metabolite profile and bioactivity of the sponge *A. rosea* exposed to the bacterium *V. natriegens* are significant using this controlled aquaria system. The rapid responses within 24 h at all three levels demonstrated that the sponge is highly responsive and adaptive to bacteria interaction. The results indicated that the sponge-bacterium interaction lead to the production of a new set of natural products. Some of these metabolites showed high bioactivity which was not evident in the control sponge tissue. The significance of this first baseline study of sponge-bacteria interaction using this novel experimental approach shows that it can be used to evaluate the effect of a range of added bacterium.

In the first study, the treated sponges seem to be unhealthy after 48 hours which would end up with dying. This is perhaps due to the secreted metabolites as a result of sponge-bacteria interaction. In this study the released metabolites accumulated in the confined system was not captured by any absorbent which was a shortcoming for the first experiment.

In the second study the sponge-bacterium interaction was conducted with one sponge-associated actinobacterium, *Streptomyces* ACT-52A, once again concerted changes in the diversity of sponge-associated bacteria, metabolite profiles and bioactivity were found.

The novel experimental approach was improved in this study by including the capture and analysis of secreted metabolites by absorbent in the seawater. The results clearly demonstrated that bioactivity could change due to changes in microbial population and metabolite profiles of the sponges when exposed to sponge-associated actinobacteria. Until now most of the sponge-derived natural products were discovered from sponge tissue extract but to our knowledge metabolites released from sponges into the seawater have not been studied. These results demonstrate that the metabolite profiles from the sponge tissue are very different between from those excreted into the seawater. These unexplored released metabolites from different sponges could open a new avenue of biodiscovery of sponge-derived natural products.

While the changes at all three levels (microbial diversity, metabolites and bioactivity) were very different for the exposures to these two different types of bacteria, the speed of responses and the degree of changes in microbial diversity, metabolites and bioactivity were also different. However, more systematic studies are required to confirm these findings.

This study has shown for the first time that the exposure of the sponge *A. rosea* to its associated bacterium, *Streptomyces* ACT-52A, and an external bacterium, *V. natriegens,* in a controlled aquarium system significantly altered its bacterial community, metabolite profiles and antibacterial activity against *S. aureus*. Another significant finding is that a diverse range of metabolites was secreted into the seawater by the sponges during the exposure experiments. This finding could open a new avenue for the discovery of sponge-derived natural products of extracellular released metabolites that are yet to be explored. The experimental approach established in our laboratory could be extended for future studies of the roles of sponge-bacteria interactions, which would require a more systematic design focusing on different types of bacteria such as Proteobacteria, Cyanobacteria, Poribacteria; different types of bacterial density; different duration; and different types of interactions.

8.3 Application of Analytical Techniques

One of the essential parts of this study was to conduct a microbial diversity analysis. T-RFLP was chosen to conduct this microbial diversity analysis after aligning this method with a next generation sequencing technology such as 454 pyrosequencing. The results from this part of the study indicate that the T-RFLP method could be a very effective and inexpensive way of microbial diversity analysis as it is aligned reasonably well with pyrosequencing.

The novel approach and model system used in this study can use for other sponges and adding different types of bacterium. Metabolites released in the seawater can be captured multiple times by adding and removing resin bags based on the optimum production time and evaluating the level and composition of the metabolites. By knowing the maximum production time, the process of challenging the sponges could be repeated many times with the chosen bacterium and the absorbent harvested after each challenge. This could significantly overcome the 'sponge metabolite supply' problem.

8.4 Past Research and Novelty of Present Study

Firstly, studies have been conducted on sponge growth *in vitro* to produce metabolites, and the importance of different parameters on sponge growth was discussed (Turon et al. 1998; Muller et al. 1999; Sipkema et al. 2005;; Garcia et al. 2006; Hausmann et al. 2006; Duckworth 2009; Koopmans et al. 2009; Sacristán-Soriano et al. 2016). Most studies mentioned that sponge explant cultures can be promising as they produce metabolites that are similar to the types of metabolites often extracted from the wild sponges; good growth has also been achieved during sponge explant cultivation. Secondly, the importance of sponge-associated microbes in the metabolite production has been discussed in the literature (Faulkner et al. 1993; Faulkner et al. 1994; Unson et al. 1994; Bultel-Poncé et al. 1998; Piel 2004, 2009; Quévrain et al. 2009; Devi et al. 2010; Thomas et al. 2010; Croué et al. 2013; Santos-Gandelman et al. 2014). These studies have revealed that sponge-associated bacteria can be a good source of these secondary metabolites, and in some cases indeed the real producers of these sponge-derived metabolites. Moreover, sponge-bacteria interaction was recognised to play a significant role in sponge ecology (Taylor et al. 2007), and hypothesized to play a key role in synthesizing these metabolites, however with limited studies. Many studies have been devoted to understand the sponge-bacteria interaction (Reiswig 1971, 1975; Vacelet & Donadey 1977; Kinne 1980; Wilkinson 1983; Hummel et al. 1988; Wilkinson 1992; Pile et al. 1996; Bavestrello et al. 2000; Webster et al. 2002; Webster et al. 2011).

The present study was the first one to investigate the roles of sponge-bacteria interaction using a controlled aquarium system where the sponge explants can be exposed to foreign and symbiotic bacteria. The new findings of the present study demonstrated that sponge-bacteria interactions change the sponge microbial communities, therefore the metabolites profiles, and the biological activities upon exposure to foreign and symbiotic bacteria. In addition, the discovery that many of such metabolites including new metabolites are produced and released into the seawater during sponge-bacteria interaction is novel and would be valuable for sustainable production of sponge metabolites for pharmaceutical and industrial uses. The methodology developed and validated in this present study will definitely promote systematic understanding of the roles of sponge-bacteria interactions in the future.

8.5 Future Directions

A better designed aquarium is necessary for the conduct of a long-term challenge experiment. As nutrition is one of the major catalysts of sponge ecology, feeding the sponges should be considered also as a positive control. The approach in further studies on the roles of sponge-bacteria interactions should be designed in such a way that different types of sponge-bacteria interactions, including mutualism, antibiosis, symbiosis, co-metabolism, co-synthesis, pathogenesis, and micro fouling etc. (Selvin et al. 2010) could be differentiated and tested experimentally.

The novel approach used in this study and the controlled aquarium system are therefore very valuable for exploring these extracellular released metabolites from sponges under a variety of challenging conditions such as exposure to different bacteria, fungi, temperature, dissolved oxygen and nutrition. It might be possible that different types of interaction could produce different types of compounds. In this PhD project the particular sponges were exposed to only a single type of bacterium to initiate the sponge-bacteria interaction. It would be interesting to challenge different kinds of sponges with multiple bacteria, multiple fungi, bacteria and fungi together and even use cancer cells to see the sponge response. The extra cellular metabolites released by the sponges and captured by the Amberlite resin required further purification and structure elucidation. A modified aquarium system with larger capacity would be able to produce enough metabolites for clinical or preclinical trial in the future.

As sponges change their microbial diversity during interaction, it is also important to quantify the genetic changes with their microbial diversity change. For example the PKS or NRPS transcripts need to be quantified. As sponges use quorum sensing molecules, the type of quorum sensing molecules also needs to be elucidated. Further, the biosynthetic pathway responsible for metabolite production also needs to be discovered. Further studies will be required to demonstrate the potential of this field of bio-discovery.

Sponges and microbes associated with sponges are both very important for the discovery of new compounds, the production of new antibiotics and many biotechnological applications. The novel approach and interaction model used in this study between sponge and microbe is just the beginning. Further study in this field could lead us to a new era where the supply of new drugs could be a reality rather a dream.

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Appendix 1

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Review

Marine Sponge Derived Natural Products between 2001 and 2010: Trends and Opportunities for Discovery of Bioactives

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Abstract: Marine sponges belonging to the phylum Porifera (Metazoa), evolutionarily the oldest animals are the single best source of marine natural products. The present review presents a comprehensive overview of the source, taxonomy, country of origin or geographical position, chemical class, and biological activity of sponge-derived new natural products discovered between 2001 and 2010. The data has been analyzed with a view to gaining an outlook on the future trends and opportunities in the search for new compounds and their sources from marine sponges.

Keywords: marine sponges; porifera; marine natural products; anticancer; sponge-associated bacteria; drug; bioactive compounds

1. Introduction

Discovery of marine derived natural products is a promising, but comparatively new field, which started with the discovery of unusual nucleoside derivatives in the sponge *Tethya crypta* in the 1950s by Bergmann and Feeney [1,2]. In the early 1960s, research on marine natural products was driven by

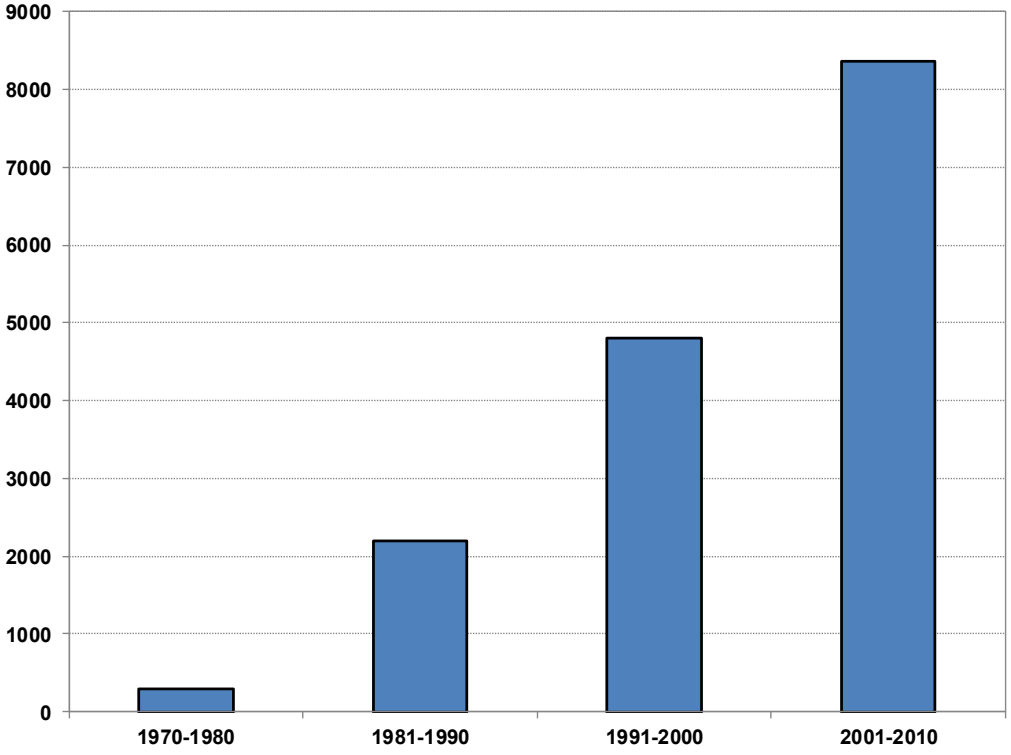
chemical studies and few compounds were tested for any relevant bioactivity [3] such as production of a pyrrole antibiotic by a marine bacterium *Pseudomonas bromoutilis* [4]. However, utilization of marine organisms as sources of bioactive metabolites started seriously at the end of 1960s [5] with the isolation of prostaglandin derivatives from the Caribbean Gorgonian *Plexaura homomalla* [6]. In the 1980s effective collaborations were established between marine chemists and pharmacologists and the investigations were focused on central nervous system membrane active toxins, ion channel effectors, anticancer and anti-viral agents, tumor promoters and anti-inflammatory agents [7]. In the 1990s pharmaceutical and biotechnological industries focused their screens on chemical libraries of both natural products, as well as synthetic compounds produced by combinatorial methods [8]. Invertebrates, mainly sponges, tunicates, bryozoans or molluscs provided the majority of the marine natural products involved in clinical or preclinical trials [9].

The discovery of marine natural products has accelerated over the last two decades with the number of new compounds discovered annually increasing from 20 to more than 200 [10]. It has been estimated that by 2010 more than 15,000 new marine natural products (NMNP) had been discovered [11–13] with 8368 new compounds recorded for the decade between 2001 and 2010. This constitutes over half of all the compounds discovered since 1951.

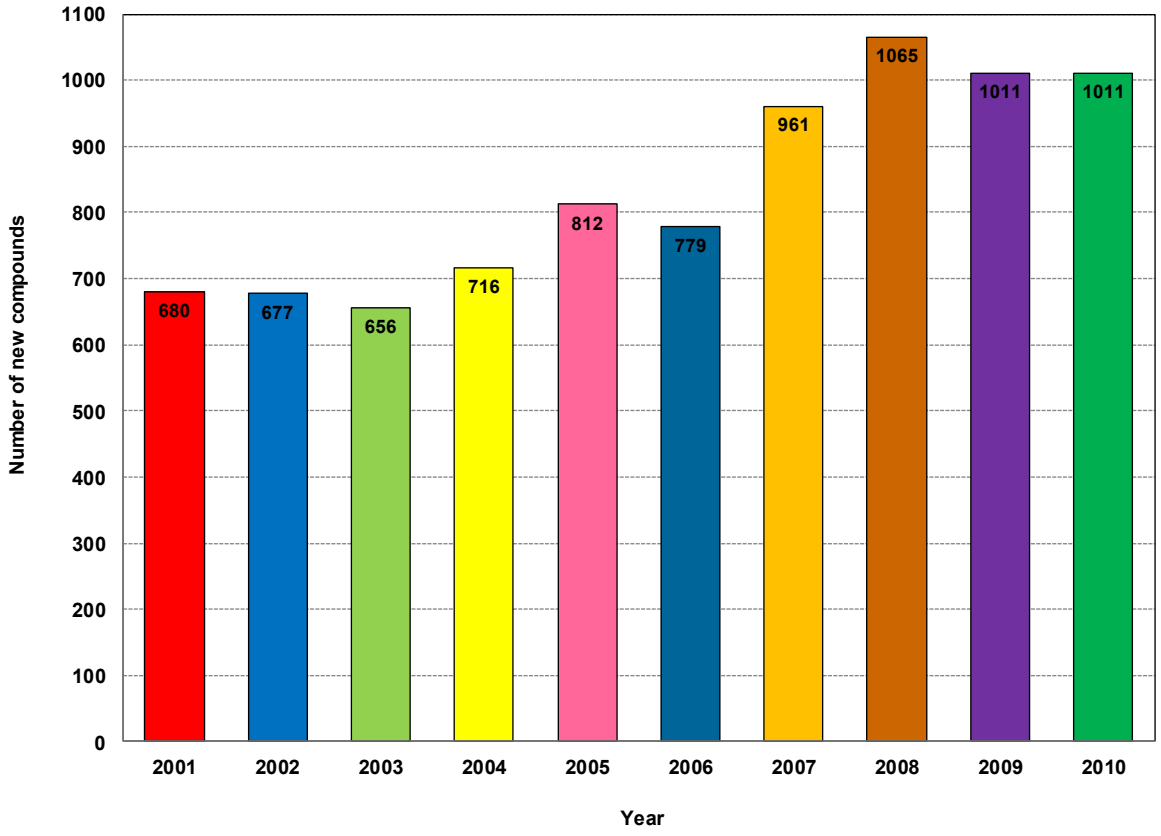
Among all the marine organisms investigated, marine sponges (Porifera) are recognized as the richest sources of NMNP, with about 4851 compounds to date, contributing to nearly 30% of all marine natural products discovered so far. It should be noted that of these, 1499 new compounds were isolated in the five years from 2008 to 2012 [14–18]. This makes sponges the most prolific marine producers of compounds with more than 200 new compounds reported each year for the last decade [19]. With this myriad of NMNP available, numerous studies have revealed a broad spectrum of biological activities for these compounds, including anticancer, antiviral, antibacterial, antifungal, antiprotozoal, anthelmintic, anti-inflammatory, immunosuppressive, neurosuppressive, neuroprotective, antifouling and a range of other bioactivities [20]. In addition, as infectious microorganisms evolve and develop resistance to existing pharmaceuticals, marine sponges provide novel leads against bacterial, fungal and viral diseases [19,21]. Figure 1a shows the almost linear growth of new compounds over the last three decades. It is predicted that if this rate can be sustained, the discovery of marine natural products from sponges, in particular, and as well as other major marine organisms will bring about new and effective therapies against human diseases [22–24].

Figure 1b shows the trends of novel marine natural products discovered from different phyla of marine organisms during 2001–2010. The annual discovery of marine natural products remained at a constant level of about 500 products in the late 1990s [10] but this number has increased from 600 to over 1000 compounds from 2008 to 2010, a significant increase which was partly driven by new developments in modern analytical technology and instruments, especially the development of the high resolution nuclear magnetic resonance spectrometer (NMR) and mass spectrometry (MS) coupled with high-performance LC and GC [10].

Figure 1. (**a**) Number of new compounds isolated from marine organisms per decade from 1970 to 2010; (**b**) Total number of new compounds isolated from different marine organisms from 2001 to 2010.



(**a**)



(**b**)

Although sponges have shown the highest potential for natural product discovery, no comprehensive reviews have been published that focus only on compounds from sponges in terms of their source areas, modes of action, chemical class and taxonomy. Two review papers written by Hu *et al*. 2011 and Leal *et al.* 2012 [10,25] described the overall trends in marine natural products, including those from Porifera, during the last two decades. Therefore, we prepared this manuscript based on sponge-derived new natural products from 2001 to 2010 and all the graphs and tables generated for this paper are based on the data reviewed by Blunt *et al.* from the *Natural Product Reports* of 2003–2012 [13–16,20,26–30]. Information was collected from each individual published paper during this time period and data was generated and analyzed accordingly.

Sponges are exclusively aquatic animals that dominate in many benthic habitats. They are sessile and do not have tissues or sensory organs but have different types of cells which conduct all forms of bodily function. They consume food and excrete waste products within cells without a body cavity [31].

Numerous ecological studies have shown that secondary metabolites produced by sponges often serve defensive purposes to protect them from threats such as predator attacks, microbial infections, biofouling, and overgrowth by other sessile organisms [32,33]. For this reason Porifera are attractive subjects for natural product chemists due to the sheer number of metabolites produced, the novelty of structure encountered, and the therapeutic potential of these compounds in the treatment of human diseases [7]. There is evidence that some compounds originally found in sponge cells are synthesized by microorganisms associated with sponges, since the mesohyl of sponges is often inhabited by microbes and many poriferan natural products resemble metabolites produced by marine microbes [34].

However, these natural products have interesting biomedical potential, pharmaceutical relevance and diverse biotechnological applications [5,35–39]. Moreover, sponge-derived antifouling molecules have been found to be less toxic, environmentally friendly biocides that are often very effective [40].

It is of both scientific and industrial interest as to why and how marine sponges possess such a high diversity of novel marine natural products. As the oldest metazoan, sponges have survived in the ocean for over 600 million years [41] throughout the vast changes experienced by the ocean. The fact that sponges still exist in all waters from fresh to saline, from intertidal to deep-sea, from tropical to frozen waters indicates the tremendous ability of sponges to respond and adapt to the varied environmental conditions over this period. In addition, sponges are one of the most efficient sessile filter feeders: they can filter up to 24 $m^3 \cdot kg^{-1}$ day^{-1} [42]. Bacterial numbers in sponge tissue often exceed those of the surrounding seawater by two to three orders of magnitude as the sponge mesohyl provides a unique ecological niche for particular bacterial species. In many cases, sponge mesohyl harbours the bacterial symbionts (30%–60%) [43]. Bacteria provide their hosts with products of their metabolism, thereby granting the sponge access to bacteria-specific traits such as autotrophy, nitrogen fixation and nitrification [44]. Other examples show that sponge-associated bacteria can process metabolic waste compounds, stabilize the sponge skeleton and provide protection against UV radiation [35,45,46]. The most prominent example of sponge bacterial symbiosis, however, is the involvement of bacteria in the production of bioactive metabolites [47] that have a role in defense [48].

These highly intensive, constant interactions with the environment have given sponges a unique biochemistry to produce the high diversity of metabolites that can either help them survive or prompt them to evolve. Being attached to a solid surface, a sponge is unable to escape when confronted with a predator, and so, when threatened they release stored secondary metabolites that have cytotoxic,

antibiotic and feeding deterrent properties [48]. Some chemicals prevent settlement of fouling organisms on the sponge surface and restrict competition for space with neighbors. The sponge bacterial associations and interactions have been widely studied, with evidence that the sponge-associated bacteria can help the sponges to produce secondary metabolites to protect them against their predators [49]. In an ecological context, sponges have developed special mechanisms to protect themselves from pathogenic bacteria, viruses, parasites, fungus and other predators that include both chemical defense mechanisms and physiological responses. Chemical defense mechanisms help to protect sponges against certain deleterious bacteria [33,50,51]. In this way, sponges provide novel leads against viral, fungal and parasitic diseases [52]. By producing different types of toxins or malevolent tastes and odors, sponges protect themselves against predators or inhibit coral overgrowth that could threaten the sponge osculum or other systems. As a physical defense they have spicules and collagen. Sponges may also succumb to microbial and fungal infections which could result in the disintegration of the sponge fibers/tissue and ultimately lead to sponge death [53]. The fact that sponges are susceptible to microbial infection suggests that they should also possess mechanisms to prevent these types of diseases [54]. Maldonado and co-workers [55] showed how sponges recover from a bacterial infection: their ultrastructural study revealed that the sponges secrete successive collagen barriers at the diseased area and abandon decaying body parts external to the barrier.

Recently, the ubiquitous defense enzyme, phospholipase A2 (PLA2) detected in a sponge associated bacterium envisaged the possible functional role in the ecological succession of the host sponge against predatory/fouling pressure in the habitat [56]. In response to predators and pathogens, sponges have engineered complex secondary metabolites from a diverse set of biological precursors. Secondary metabolites are organic compounds that are not directly involved in the normal growth, development or reproduction of organisms. These metabolites produced by sponges and their associated microflora can be classified chemically as alkaloids, terpenoids, glycosides, phenols, phenazines, polyketides, fatty acid products and peptides, amino acid analogues, nucleosides, porphyrins, aliphatic cyclic peroxides and sterols [57,58]. Many of these compounds are very potent because the diluting effect of the ocean drives the construction of molecules that are highly active and stable in saline conditions [59].

Given the significance of sponges in marine natural product discovery, the aim of this review is to present a comprehensive overview of sponge-derived natural product discovery during the recent decade from 2001 to 2010, in order to understand the defining trends and provide insights into avenues for further compound discovery. The temporal trends of the discovery of sponge-derived marine natural products and their biological activities, the sources of discovery in terms of sponge taxonomy, the chemical classes of these natural products, and the countries of collection have been categorized. Our analysis also includes a short description of the relative distribution and contribution of these discoveries with reference to governmental funding, policies and known national priorities given to marine natural products. Finally, the opportunities and challenges have been identified for future R and D in this fast growing field.

The new compounds isolated during the last decade were classified into 18 chemical classes including acid, alkaloid, ester, fatty acid, glycoside, ketone, lipid, macrolide, alcohol, peptide, peroxide, polyketide, quinone, steroid, sterol, terpene, terpenoid and unclassified, based on the data reviewed by Blunt *et al.* from *Natural Product Reports* of 2003 to 2012 [13–16,20,26–30]. The World Porifera Database [60] was used for the taxonomic classification of the sponges mentioned in the Natural Product

Reports [13–16,20,26–30]. The World Register of Marine Species (WoRMS) database [60] was also used to cross check detailed taxonomical information (order and family) for each surveyed species and to validate and/or update their scientific names [61].

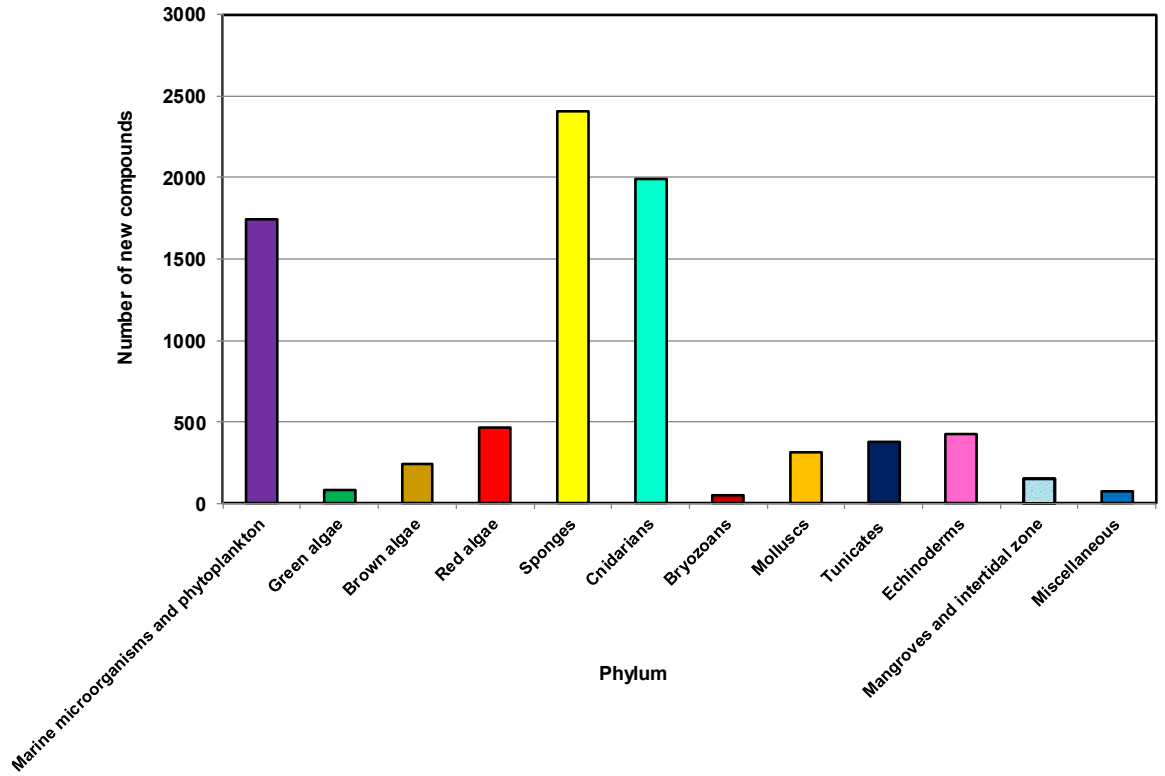
During the recent decade the sponges collected were from 19 known orders as well as 12 sponges of unknown identity which provided new compounds. These included Agelasida, Astrophorida, Axinellida, Chondrosida, Choristida, Clathrinida, Dendroceratida, Dictyoceratida, Hadromerida, Halichondrida, Haplosclerida, Homosclerophorida, Leucosolenida, Lithistida, Lyssacinosida, Ocilosclerida, Poecilosclerida, Spirophorida and Verongida. Sixty two countries (with Antarctica labeled as a country for reporting purposes) were the sources for the sponge samples studied. The bioactivities were mainly classified as anti-Alzheimer's, antibacterial, antituberculosis, anticancer, antifungal, anti-inflammatory, antimalarial, antiviral and anti-HIV.

2. New Compounds and Their Distribution 2001–2010

2.1. Yearly Distribution of Phyla that Produce Natural Products Discovered from 2001 to 2010

To investigate the distribution of sources of NMNP, 12 different categories including a separate group comprising marine microorganisms and phytoplankton were used for this review (Figure 2). Invertebrates comprise approximately 60% of all marine animal diversity [62]. Most belong to the phyla Porifera (sponges), Annelida, Arthropoda, Bryozoa, Cnidaria, Echinodermata, Mollusca and Chordata. Several studies addressing marine invertebrates also include these groups of organisms [32,33,63].

Figure 2. Total number of new compounds isolated from different types of marine sources, 2001–2010.



As highlighted in other reviews [10,64], the phyla Porifera and Cnidaria have been the two main sources of NMNP. During the last 10 years from 2001 to 2010, more than 2400 new natural products have been discovered from 542 genera and 671 species of sponges. These sponges belong to 19 known orders and 74 families, contributing about 29% of the marine natural products discovered during this decade, making it the largest source among all marine organisms [48,65].

2.2. Sponges (Porifera) as a Source of New Natural Products and Drugs for the Future

A review in 2003 collected the most important marine natural compounds which were undergoing preclinical and clinical trials (I, II, III) for anticancer activity. Among those, compounds from sponges were the following: Discodermolide, Hemiasterlins A & B, modified Halichondrin B, KRN-7000, Alipkinidine (alkaloid), Fascaphysins (alkaloid), Isohomohalichondrin B, Halichondrin B, Laulimalide/Fijianolide, 5-Methoxyamphimedine (alkaloid) and Variolin (alkaloid) [66]. A review paper was published by Sipkema *et al.* 2004 about the drugs from marine sponges [67].

Marine natural sources as potential anticancer agents were reviewed in 2011 which mentioned 39 marine-derived potential anticancer agents and among them 18 compounds from sponges with different mechanisms of action [68]. Interestingly, from the 16 marine natural products that are currently under preclinical trials as new drug candidates, most are derived from invertebrates. Of these, Porifera remain the most important phylum, with six of the 16 compounds [69–71]. A review paper published in 2013 classified anticancer molecules according to their current status in the clinical phase trials (approved/phase IV/phase III/phase II/phase I) and updated the data to April 2012 [72]. A very recent review, published in 2014, showed the compounds derived from marine sources currently in clinical trials against cancer with more updated information on clinical and late preclinical developments [73]. This paper also mentioned that although many compounds showed potential against cancer and entered clinical trials in cancer, to date, only Cytarabine, Yondelis® (ET743), Eribulin (a synthetic derivative based on the structure of Halichondrin B), and the Dolastatin 10 derivative, monomethylauristatin E (MMAE or vedotin) as a warhead, have been approved for use in humans (Adcetris®) [73].

Although a number of compounds from sponges showed promising activity to be potential drug candidates over the last few decades they are generally not ready for further development due to the challenge of obtaining continuous and larger supplies of the compounds, unless they can be chemically synthesized. Considerable quantities of a drug candidate are vital for clinical trials, but only a few milligrams of most natural products can be isolated from marine samples [74]. One solution is farming sponges to source bioactive metabolites [75]. On the other hand, sponge compounds that are produced by sponge-associated microorganisms can be scaled up as the microorganisms are able to flourish independently of the sponge. Another way to ensure supply is using sponge cell culture, although this is still a growing area. Muller *et al.* in 2000 described the production of bioactive compounds by sponge cell culture [76] and Zhang *et al.* in 2003 and Cao *et al.* in 2007 showed improved cell proliferation and spiculogenesis from primmorphs of sponges and dynamics of spicule production in *in vitro* sponge cell culture systems [77]. The sustainable production of bioactive compounds from sponges was reviewed in 2004 [78] and again in 2009 where the advantage and disadvantage of sponge cell culture was discussed, with the conclusion that the understanding of the metabolic pathways is one of the potential advantages of sponge cell culture systems [79]. However, the most promising solution was

answered by Wilson *et al.* in 2014; his findings illuminated two promising approaches for addressing the supply problem [80]: firstly, large-scale cultivation of the microorganisms that produce interesting metabolites, and secondly, expressing the biosynthetic pathway of interest in an easily cultivable surrogate host. The discovery of Wilson and Piel and their colleagues identified *Entotheonella* and members of the newly proposed phylum Tectomicrobia as a "biochemically talented" phylum on a par with the actinomycetes [80–82]. Thus, these results could facilitate a new era of drug discovery.

2.3. The Distribution of New Marine Natural Products from Sponges

To date, about 11,000 species of sponges have been formally described of which approximately 8500 are considered valid, but as many as twice those numbers are thought to exist [83]. Well known sponge fauna in the Caribbean, Mediterranean, and the British Isles each contain 500–800 species, whereas less well characterized sponge fauna in Australia, Papua New Guinea and Indonesia possess a high biodiversity [84]. Sponges are currently divided into four distinct classes, 25 orders, 128 families and 680 genera [59,60].

The sponges reported to produce new compounds in the last ten years were from 19 known orders although a number of sponges were not identified. A careful analysis of the trends of the discovery of new bioactive compounds from different orders of sponges is presented here to guide scientists in future discoveries.

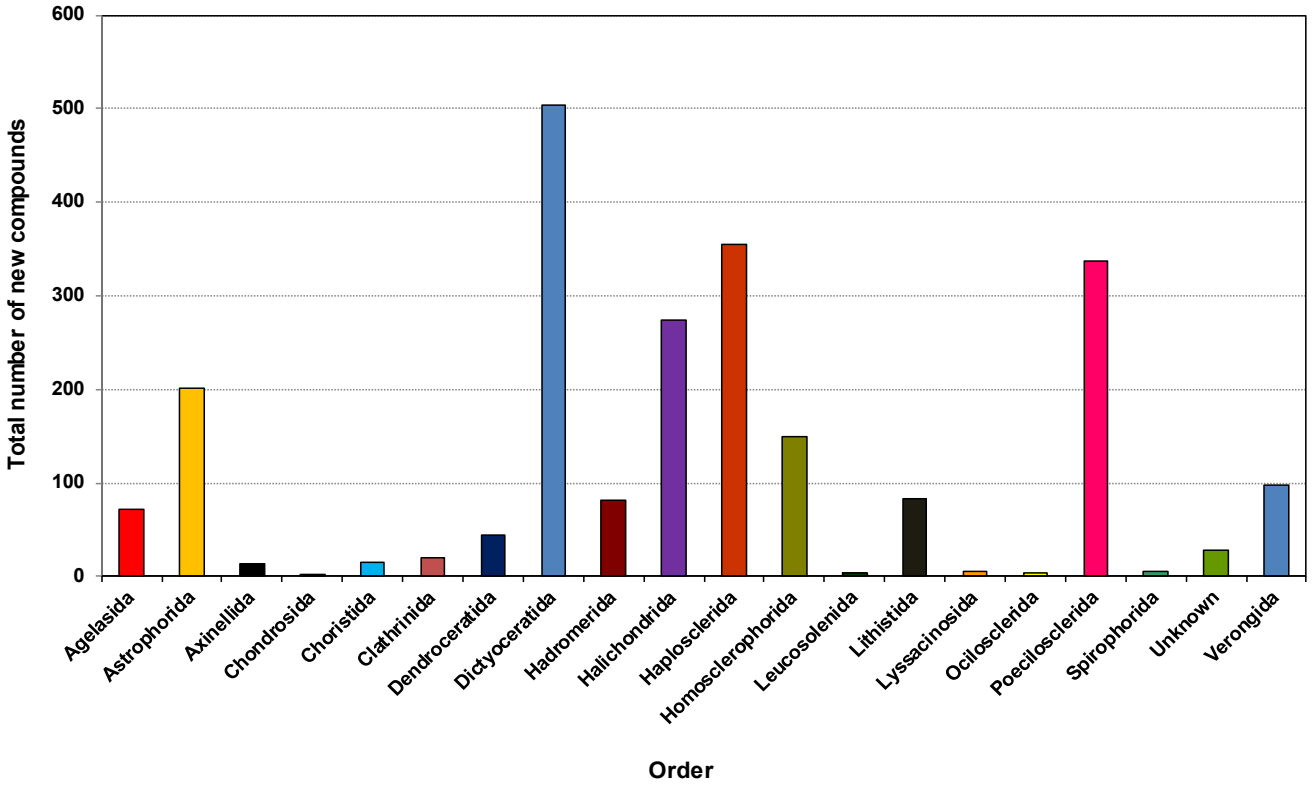
As shown in Figure 3a, 504, 355, 337, 274 and 201 new compounds were found from the five orders Dictyoceratida, Haplosclerida, Poecilosclerida, Halichondrida and Astrophorida, respectively. Notably, these five orders contributed more than 70% of the new compounds. The highest numbers of new compounds were found from Dictyoceratida with 72 found in 2008, 66 in 2004, 63 in 2009, 58 in 2001 and 51 in 2005.

Table 1 shows that some orders have been found to be productive sources of NMNP, many with a large chemical diversity. Astrophorida, Dictyoceratida, Halichondrida, Haplosclerida and Poecilosclerida are the orders from which more than 50 species were studied [83].

Dictyoceratida contributed at least 16% of new compounds each year except in 2010 (11.9%), reaching a peak of 28.5% in 2005 (Figure 3b). Haplosclerida contributed at least 13.58% of new compounds each year except 2004 and 2008 with values of 9.4% and 7.7% respectively. This order yielded the highest number of compounds in 2001 at 24.6%. In the case of Poecilosclerida, a gradual increase was observed until 2004 and reached a peak in 2009 at 22.1%. From 2004 to 2008 Halichondrida contributed at least 11.4% new compounds each year.

This corresponds with a recent review on clinically active compounds from sponges which had Astrophorida, Chondrosida, Dendroceratida, Dictyoceratida, Hadromerida, Halichondrida, Haplosclerida, Lithistida, Poecilosclerida, Spirophorida and Verongida as the main orders from which clinically active compounds were found [85]. Leal *et al.* 2012, covering 1990–2009, found that NMNP were recorded in 17 orders of Demospongiae, and about 89% of the natural products were derived from only eight of those orders, namely Astrophorida, Dictyoceratida, Halichondrida, Haplosclerida, Homosclerophorida, Lithistida, Poecilosclerida and Verongida [25].

Figure 3. (**a**) Total number of new compounds isolated from different orders of marine sponges 2001–2010; (**b**) Distribution of new compounds isolated from different orders of marine sponges as a percentage found within the year, 2001–2010.



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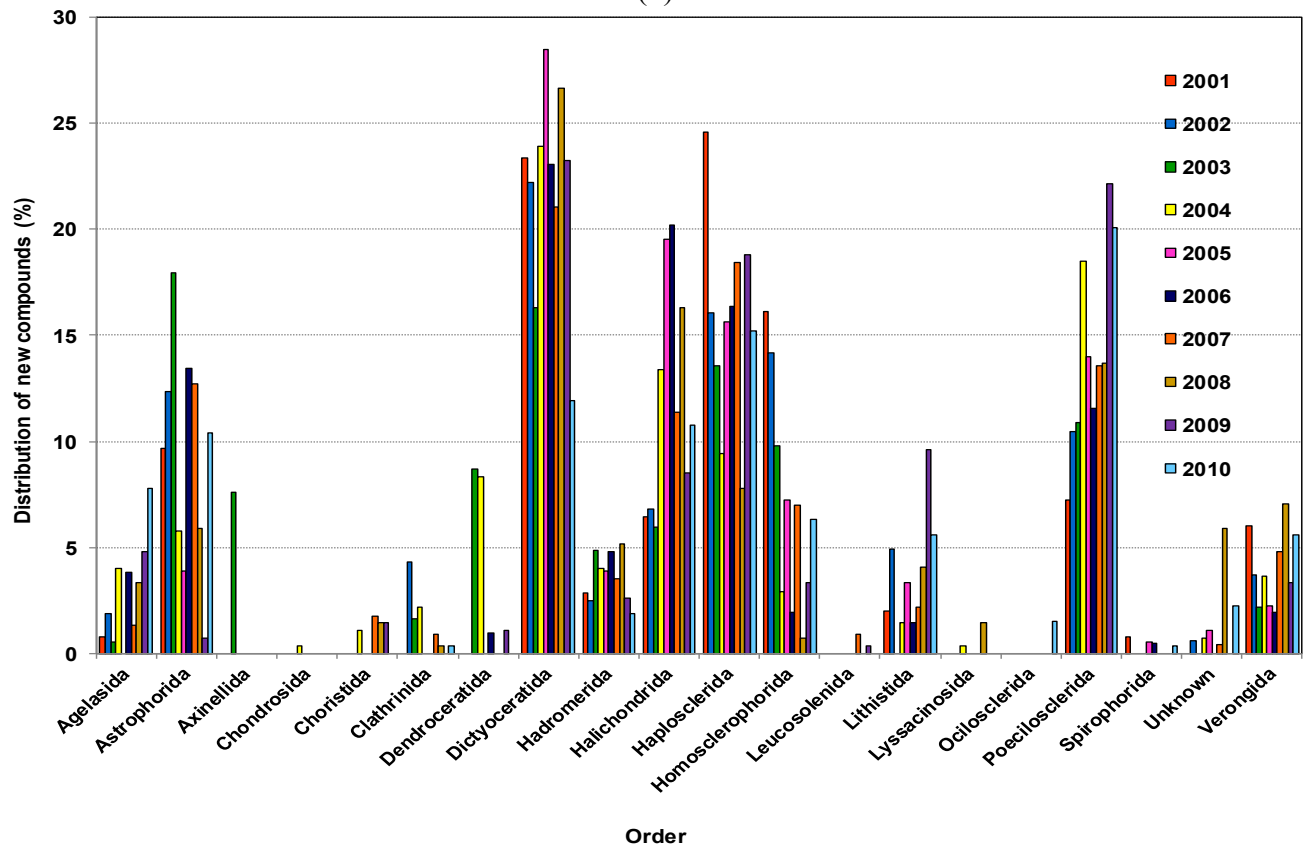


(**b**)

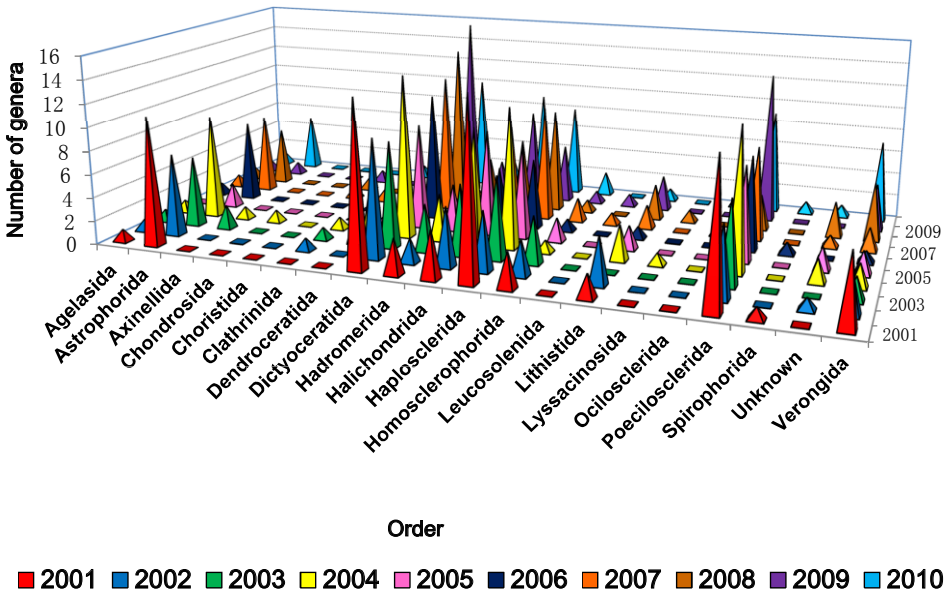
Table 1. Total number of new compounds isolated from different orders of marine sponges with number of families, genera, species and number of references published from 2001 to 2010.

Order	Number of Families	Number of Genera	Number of Species	Number of References
Agelasida	9	9	21	24
Astrophorida	26	58	63	62
Axinellida	2	2	2	2
Chondrosida	1	1	1	1
Choristida	3	3	3	3
Clathrinida	5	5	7	7
Dendroceratida	7	10	11	13
Dictyoceratida	40	117	145	161
Hadromerida	24	31	32	33
Halichondrida	31	69	86	84
Haplosclerida	52	80	100	120
Homosclerophorida	10	20	39	50
Leucosolenida	1	1	1	1
Lithistida	14	20	23	32
Lyssacinosida	2	2	2	2
Ocilosclerida	1	1	1	2
Poecilosclerida	67	68	81	83
Spirophorida	4	4	4	5
Unknown	8	12	12	10
Verongida	27	29	37	46
Total	334	542	671	741

All NMNP discovered since 1990 were recorded in 64 families belonging to the phylum Porifera. However, about 51% of these products were derived from only nine families: Spongiidae, Dysideidae and Thorectidae in the order Dictyoceratida; Chalinidae and Petrosiidae in the order Haplosclerida; Halichondriidae in the order Halichondrida; Ancorinidae belonging to the order Astrophorida; Plakinidae belonging to the order Homosclerophorida and Theonellidae of the order Lithistida. Here, the highest increase in the number of NMNP annually discovered was recorded for the families Chalinidae and Spongiidae. The family Theonellidae has yielded a number of unique compounds [86] with a broad spectrum of biological activities, including antifungal [87] and cancer cell growth inhibitors [88,89].

Figure 4 shows that between 9 and 16 genera were found to produce new molecules each year from the order Dictyoceratida which revealed a high availability of different genera from this particular order, with the highest number found in 2009. This order will be examined in greater detail in a subsequent review covering 2001–2010. Genera belonging to the orders Haplosclerida, Halichondrida, Poecilosclerida, and Astrophorida also showed more than five productive genera for most of the last decade. The number of new compounds correlated with the high diversity of sponges because the higher the diversity the higher the possibility of getting more novel compounds. Another possible reason that makes Dictyoceratida the most prolific producers of NMNP as well as Astrophorida, Haplosclerida, Homosclerophorida and Halichondrida is because these orders harbor high densities of microorganisms [25,85].

Figure 4. Number of different types of genera used for isolation of new compounds from different orders of marine sponges from 2001 to 2010.



2.4. Distribution of New Compounds per Species from Different Orders

During the last decade each individual species from different sponges contributed on an average four new compounds under different orders (Figure S1, Supplementary Information). Although very few species have been studied from the orders Axinellida and Choristida, on an average they also produce almost the same number of compounds. This finding permits the inference that each individual species has the potential for contributing similar numbers of new compounds regardless of the order.

2.5. Symbiotic Relationships: Sponge Associated Microorganisms

Hentchel *et al.* 2002 reported that although it is generally believed that symbiotic interactions exist between sponges and specific microorganisms, consideration of alternative explanations such as the selective enrichment of ubiquitous seawater bacteria is also important [42]. Moreover, they stated that very specific type of selective pressures, perhaps the hostility to digestion, must exist to establish the uniform composition arrangements of the microbial communities existing in sponges that have otherwise few commonalities [42].

However, it was consequently observed that marine sponges host abundant and diverse communities of symbiotic microorganisms [90]. Webster *et al.* 2012 mentioned that microbial symbionts are undoubtedly important to sponge health, and therefore it is likely that interruptions to symbiosis as a result of climate change/environmental stress will influence sponge health, growth rates or their ability to defend themselves from predation, fouling and disease [91]. Symbiotic interactions between sponges and microorganisms could contribute to sponge nutrition as well [92]. Important roles of the symbionts include photosynthetic carbon fixation [93], nitrification [94,95], nitrogen fixation [44,96,97], and anaerobic metabolism [98]. Another important role of sponge-associated bacteria is the production of potential secondary metabolites, such as antibiotics, antifungal compounds and anti-predation or

antifouling compounds [39]. Whilst the microbes associated with the sponges produce the secondary metabolites, possibly all of these sponges have particular microbial associations [99]. More research is necessary to explore the relationship between microorganisms and phytoplankton associated with sponges because it has been suggested that (at least) some of the bioactive secondary metabolites isolated from sponges are produced by functional enzyme clusters which originated from these microorganisms. The role of these microorganisms in sponge biology varies from source of nutrition to mutualistic symbiosis with the sponge [100].

It has been recognized that metabolites synthesized by marine microorganisms associated with sponges could become a major source for the discovery of new drugs, not only because the biological diversity in marine ecosystems like coral reefs or deep sea floors is probably higher than in the rainforest, but because marine microorganisms offer a renewable resource for the scale-up and development of potentially new drugs [101,102].

A number of examples of the functions of the sponge-associated microorganisms are provided to signify the diversity of functions. Dudler and Eberl 2006 conducted a study on interactions between bacteria and eukaryotes which showed increasing evidence to support the hypothesis that secondary metabolites produced by symbiotic bacteria are a result of bacterial cell-to-cell signaling [103]. In a similar vein, Schmidt *et al.* 2008 looked at how organisms cooperate in the synthesis of natural products. They found that partners may exchange and modify the natural products produced by each other and also explained how these secondary metabolites are utilized by the host organisms [104].

Many sponge-derived metabolites resemble bacterial natural products or belong to substance classes typical for these microorganisms [82]. In a recent paper, the Piel group demonstrated beyond doubt that almost all bioactive polyketides and peptides known from the marine sponge *Theonella swinhoei* were attributed to a single phylotype, *Entotheonella* spp. and are extensively distributed in sponges [80].

The diversity in the locations (Okinawa, the Philippines, Indonesia, the Red Sea, Italy, South Africa, and Papua New Guinea) and genera of sponges (*Amphimedon* sp. and *Acanthostrongylophora*) responsible for the production of manzamine alkaloids are widely believed to be a result of a symbiotic relationship between these sponges with common or closely related microorganism(s), which may account for the generation of manzamine enantiomers [105].

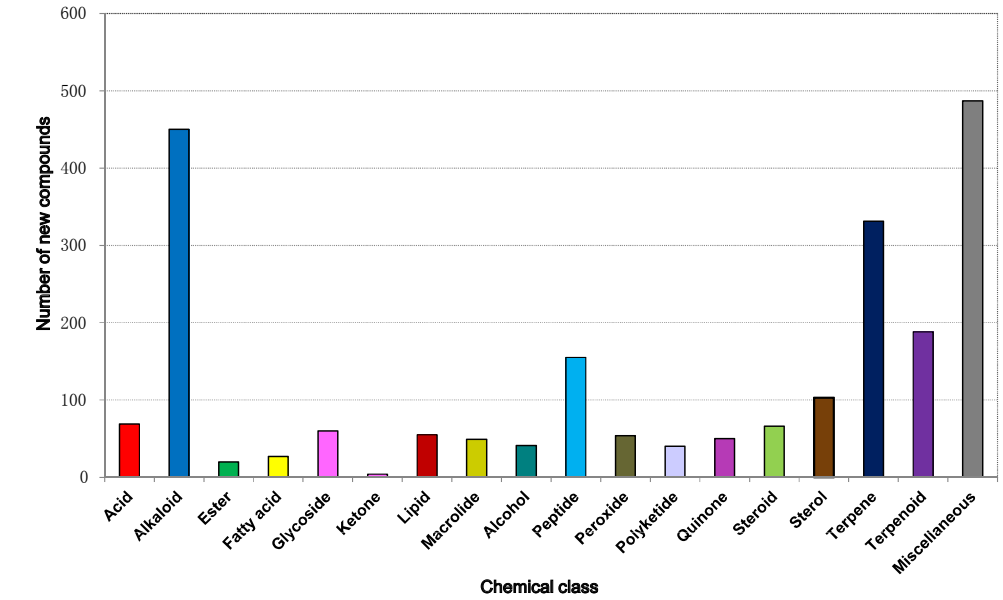
Crambe crambe (Schmidt, 1862) (Poecilosclerida) is a red incrusting marine sponge present in the Mediterranean Sea and reported to produce diverse PGAs, namely crambescidins 800, 816, 830, 844, as well as isocrambescidin [106,107]. Using 16S rRNA gene pyrosequencing it has been found that the associated bacterial community of *C. crambe* is dominated by a single bacterial species affiliated to the Betaproteobacteria [108].

2.6. The Distribution of Chemical Classes

Although acid, ester, ketone, peroxide do not directly fall into chemical classes, we considered these as chemical classes just to show the distribution as such described in the original paper as well as mentioned by Blunt *et al.* [13–16,20,26–30] in their annual review of marine natural products that were used in preparing this manuscript. In fact, classes of natural products should be classified based on their biosynthesis. However, it is important to remember that a macrolide is often a polyketide and a sterol a steroid and fatty acid belongs to lipid.

A wide range of chemical and functional diversity has been observed among new compounds during the last decade. The analyzed data (Figure 5a) has shown that in the last ten years 450, 331, 188 and 155 new compounds were classified as alkaloids, terpenes, terpenoids and peptides, respectively, and these four classes make up approximately 50% of compounds discovered. While alkaloids contributed 20% of the new molecules the terpene/terpenoid classification which are often not readily distinguishable, together made up 23% of the total within the decade.

Figure 5. (**a**) Chemical classes of new compounds isolated from marine sponges from 2001 to 2010; (**b**) The distribution of different chemical classes of new compounds isolated from marine sponges from 2001 to 2010.



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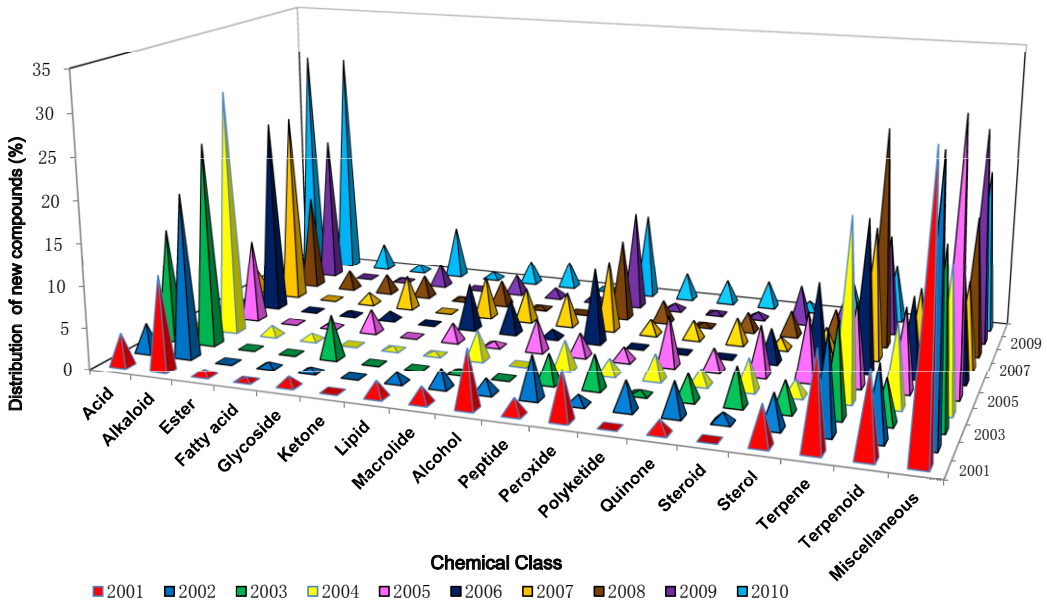


(**b**)

The chemical diversity of bioactive compounds reportedly produced by sponge-microbe associations showed that certain chemical classes such as quinones, steroids, fatty acids, diketopiperazines,

alkaloids, terpenes, terpenoids, trichoverroids and prodigiosin derivatives, diglucosyl-glycerol, polyketides, cyclopeptides, glycoglycerolipid, benzoic acid derivatives are responsible for anticancer or antitumor activity; quinolone derivatives for anti HIV activity; fatty acid esters and fatty acids for anti-inflammatory activity; alkaloids and quinolone derivatives for antimalarial activity; polyketides glycopeptides, α-pyrone derivatives, peptides, proteins, antimycin, lipopeptides, polybrominated biphenyl ether, cyclic depsipetide, terpenes, pentaketides, furan carboxylic acid, alkaloid, diketopiperazine, anthraquinone, chromones, steroid, lactone, quinolone derivative, trisindole derivative, macrolactam, ethers, phenol derivative for antimicrobial activity; and dihydropyridine for neuroprotective activity [85]. In this review it was also evident that anticancer, antimicrobial, anti-HIV, antiflammatory, antimalarial, and neuroprotective disease and antituberculosis were the main classes activity exhibited by most of the new compounds during the recent decade. Terpenoid quinones and hydroquinones, the chemical classes found mainly from Dictyoceratid sponges [11,12,20], have been continuously reviewed and updated. Sesquiterpenoid quinones and hydroquinones showed versatile activities and even a hydro-quinone displays multiple activities [109].

The reason that most sponges produce alkaloids could be for protection from predators such as fish as alkaloids act as good toxicants against predators. Assman *et al.* 2000 presented data suggesting that bromopyrrole alkaloids fulfill multiple ecological functions in the defense mechanisms of the common and diverse genus *Agelas* [110]. A striking example of the significance of marine alkaloids for chemical defense against fish is provided by the red coloured sponge *Latrunculia magnificea* (order Poecilosclerida) from the Red Sea. Even though *L. magnificea* growth is exposed, it is apparently avoided by fish, whereas other sponges from the same habitat that are cryptic are readily consumed by fish when artificially exposed [111].

Further information on the different classes of secondary metabolites of marine sponges and their bioactivities are the subject of previous reviews [14,93,112,113].

2.7. The Distribution of Bioactive Compounds

Table 2 shows that during the decade until 2010, 332, 229, 227, 173 and 149 new bioactive compounds were found from Dictyoceratida, Haplosclerida, Poecilosclerida, Halichondrida and Astrophorida, respectively, the highest number of new bioactive compounds isolated from different orders of sponges in this period. Dictyoceratida alone contributed 20.6% of all bioactives, Haplosclerida and Poecilosclerida both contributed 14%, whereas Halichondrida and Astrophorida contributed 10.8% and 9.3% respectively. The detail of yearly distribution is presented in Figure 6. Therefore, these five orders contributed 69% of bioactive compounds among all the different orders of sponges.

In sponges the role of the chemical constituents is clouded by the complexity of the sponge-symbiont relationship [114]. The current body of evidence is too limited to make broad generalizations, but it suggests complex chemical and biological interactions that have not yet been resolved [115]. This knowledge will feed into strategies to relieve a major bottleneck for sponge metabolite production, namely: understanding metabolites production in the sponge [79].

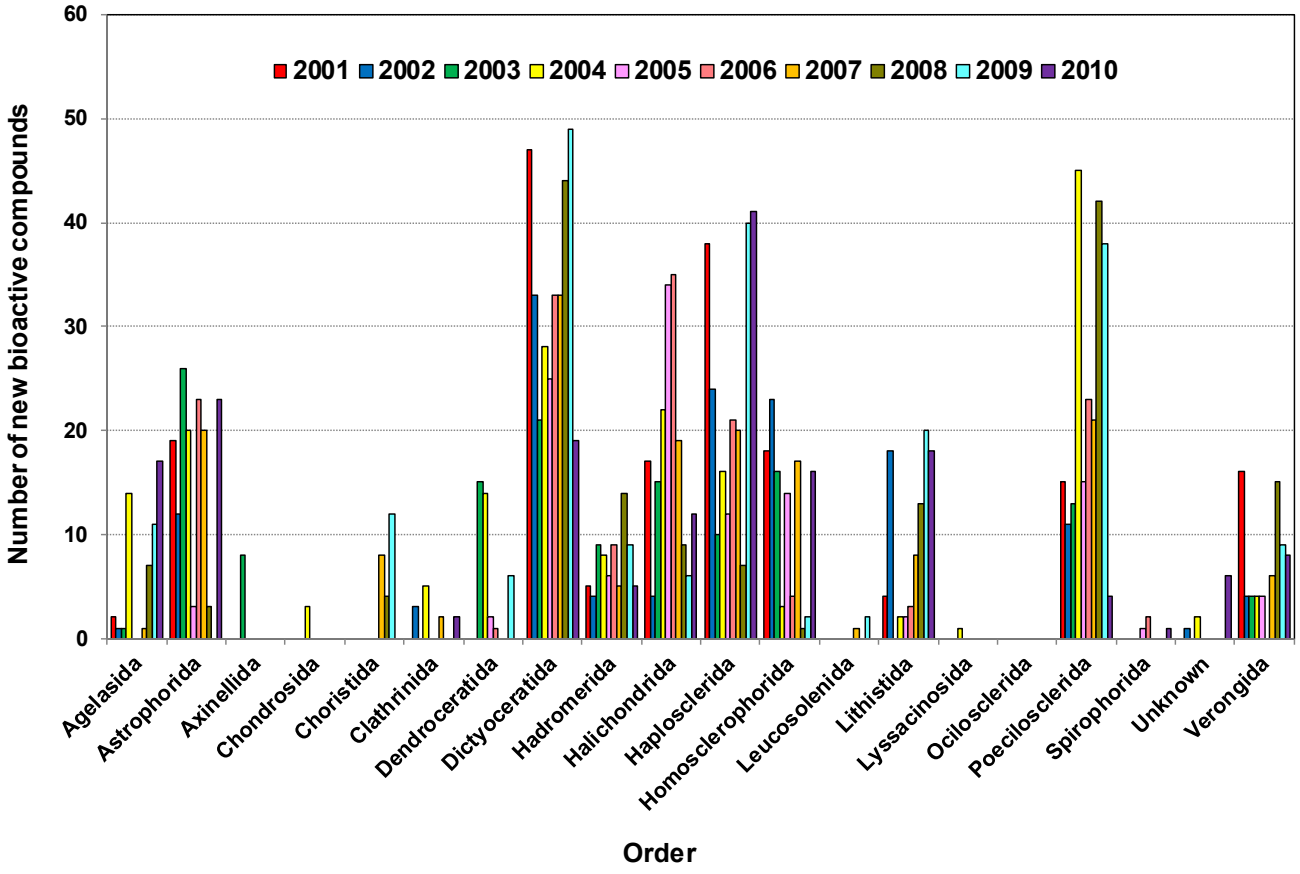
Table 2. Total number of new compounds isolated from different orders of marine sponges with different bioactivities from 2001 to 2010.

Orders of Sponges	Anti-Alzheimer's	Antibacterial	Antituberculosis	Anticancer/ Cytotoxicity	Antifungal	Anti-inflammatory	Antimalarial	Anti-HIV	Antiviral	Miscellaneous	Total
Agelasida	0	17	0	11	6	0	6	0	0	14	54
Astrophorida	0	8	6	97	7	0	1	5	3	22	149
Axinellida	0	0	0	0	0	0	0	0	0	8	8
Chondrosida	0	0	0	3	3	0	0	0	0	0	6
Choristida	0	0	0	12	0	0	0	0	0	12	24
Clathrinida	0	4	3	3	2	0	0	0	0	0	12
Dendroceratida	0	4	0	14	3	3	0	0	0	14	38
Dictyoceratida	0	38	3	182	11	2	1	5	0	90	332
Hadromerida	0	2	3	45	1	0	0	5	0	18	74
Halichondrida	1	18	4	99	16	1	2	1	0	31	173
Haplosclerida	8	15	2	100	20	0	7	4	0	73	229
Homosclerophorida	0	2	3	55	10	0	9	1	0	34	114
Leucosolenida	0	1	0	2	0	0	0	0	0	0	3
Lithistida	0	5	2	38	9	2	0	16	0	16	88
Lyssacinosida	0	0	0	1	0	0	0	0	0	0	1
Ocilosclerida	0	0	0	0	0	0	0	0	0	0	0
Poecilosclerida	0	17	5	143	21	1	1	4	1	34	227
Spirophorida	0	0	0	4	0	0	0	0	0	0	4
Unknown	0	0	0	2	0	0	0	0	0	7	9
Verongida	0	14	0	17	5	0	0	0	0	34	70
Total	9	145	31	828	114	9	27	41	4	407	1615

The origin and role of a number of compounds such as bioactive peptides within the sponges have yet to be clarified, as many of these compounds have potent activities not always clearly related to their *in situ* role [116]. However, it was subsequently found that antimicrobial peptides (AMPs) are components of innate immunity, forming the first-line of defense used by any organisms against the invading pathogens [117]. Pasupuleti *et al.* 2012 termed AMPs as the key component of the innate immune system [118]. Two good examples of AMPs produced by Porifera are Stylisin and Discodermin A [119,120], although many AMPs have been produced since the first production of Discodermin from sponges [121]. In 2013 a review paper was published on antimicrobial peptides with versatile biological activities which included a few sponges producing AMPs [122].

There is a sign that the quick evolution of molecules related to cell adhesion and pathogen killing (AMP precursors) has been acute in the successful alteration of sponges [123]. In their 2010 review Otero-González *et al.* discussed the new frontier for microbial infection control by antimicrobial peptides including Porifera [121]. A review published by Brogden *et al.* 2005 commented that translocated peptides can modify cytoplasmic membrane septum creation, hinder cell-wall synthesis, constrain nucleic-acid synthesis, inhibit protein synthesis, or obstruct enzymatic activity [124].

Figure 6. Distribution of bioactive compounds isolated from various marine sponge orders 2001–2010.



Some external activities of these peptides are as antitumorals, antivirals, immunosuppressive and antimicrobial agents, as well as neurotoxins, hepatotoxin, and cardiac stimulants. The various functional roles of some terpenoids are considered as hormones (gibberellins), photosynthetic pigments (phytol,

carotenoids), electron carriers (ubiquinone, plastoquinone), and mediators of polysaccharide assembly, as well as communication and defense mechanisms [125]. Terpenes may also act as safeguard for a variety of organisms in the marine world, including algae, sponges, corals, mollusks and fish [33].

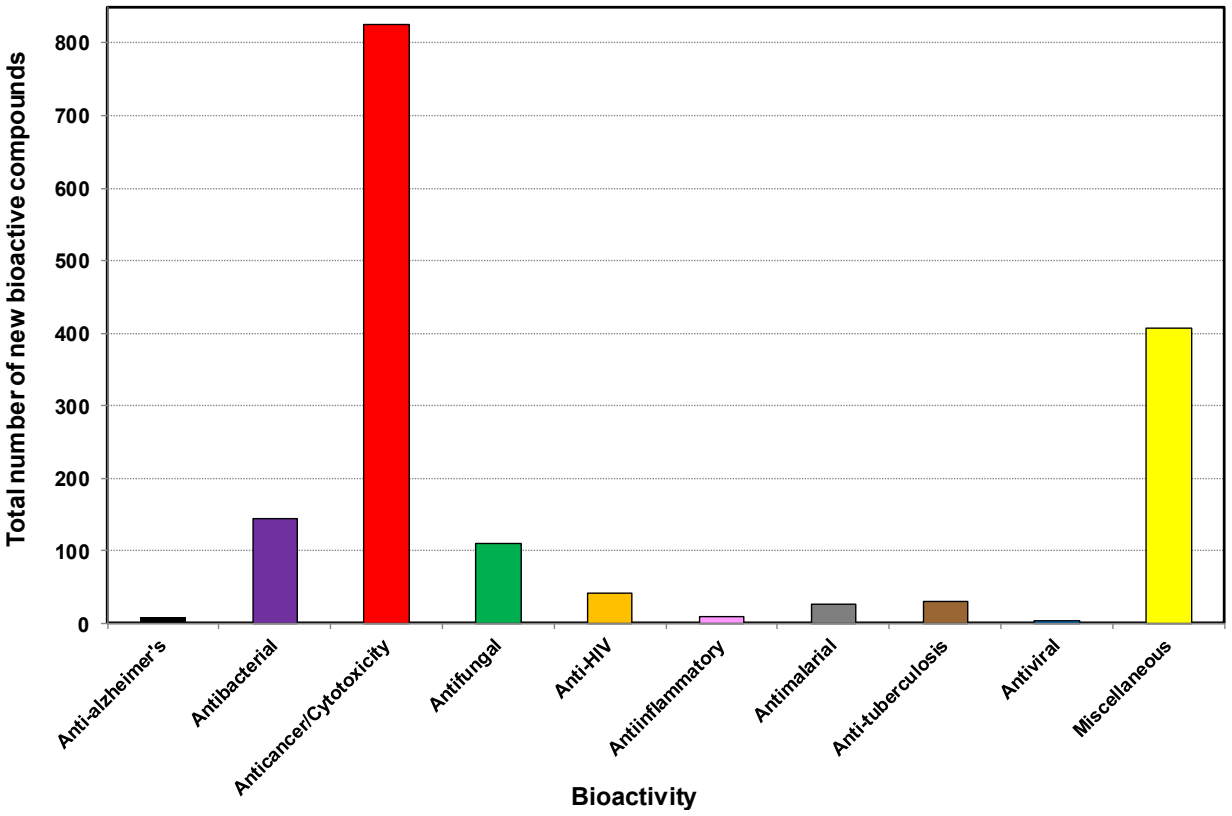
It is important to note that most of the orders showed cytotoxicity or anticancer activity although only a few have been tested for *in vivo* antitumor activity. In the five year period from 1986 to 1991, more than 400 novel marine natural products with cytotoxic activity were reported in the literature [126] with the majority of compounds only tested for cytotoxicity in cell culture assays. Although cytotoxic activity is regarded as the first indicator in identifying anticancer drugs [127], we have considered these compounds to be cytotoxic unless further experimentation indicates their potential role as an anticancer drug. Increasing evidence has shown that cell death can be induced via three different mechanisms: apoptosis, autophagy and oncosis [128]. Most of these sponge-derived novel compounds have been screened for cytotoxic activity but not for apoptosis [129–131], although analysis should focus on a number of cancer relevant targets associated with the cell cycle, signal transduction, angiogenesis or apoptosis [132–135]. A recent overview (2011) retrieved scientific papers identifying 39 compounds from marine sponges with apoptosis-inducing anticancer properties [136]. In another example that distinguishes cytotoxicity from antitumor activity, a recent study used a novel *in vitro* assay to screen 2036 extracts from 683 individual sponges that led to the identification of bioactive compounds (which were prepared in pure form and in sufficient quantities) that could treat solid tumors [137].

Figure 7a,b show that the number of compounds with reported inhibition of cancer cell lines (or cytotoxicity) was highest with the number of 825, antibacterial activity at 145, and antifungal and anti-HIV activities at 111 and 41, respectively. Thus, cytotoxicity or anticancer activity contributed at least half of the reported activity. Progress towards marine anticancer drugs dominates, with the prime source phylum being sponges, followed by microorganisms, tunicates and mollusks [22]. Antiviral and anti-HIV activities have been observed from samples of Astrophorida*,* Lithistida and Poecilosclerida. The most promising antiviral substances from sponges appear to be 4-methylaaptamine, manzamines [19], besides Papuamides C and D [138], haplosamates A and B [139] and avarol [140] which are examples of HIV-inhibiting compounds from different sponges.

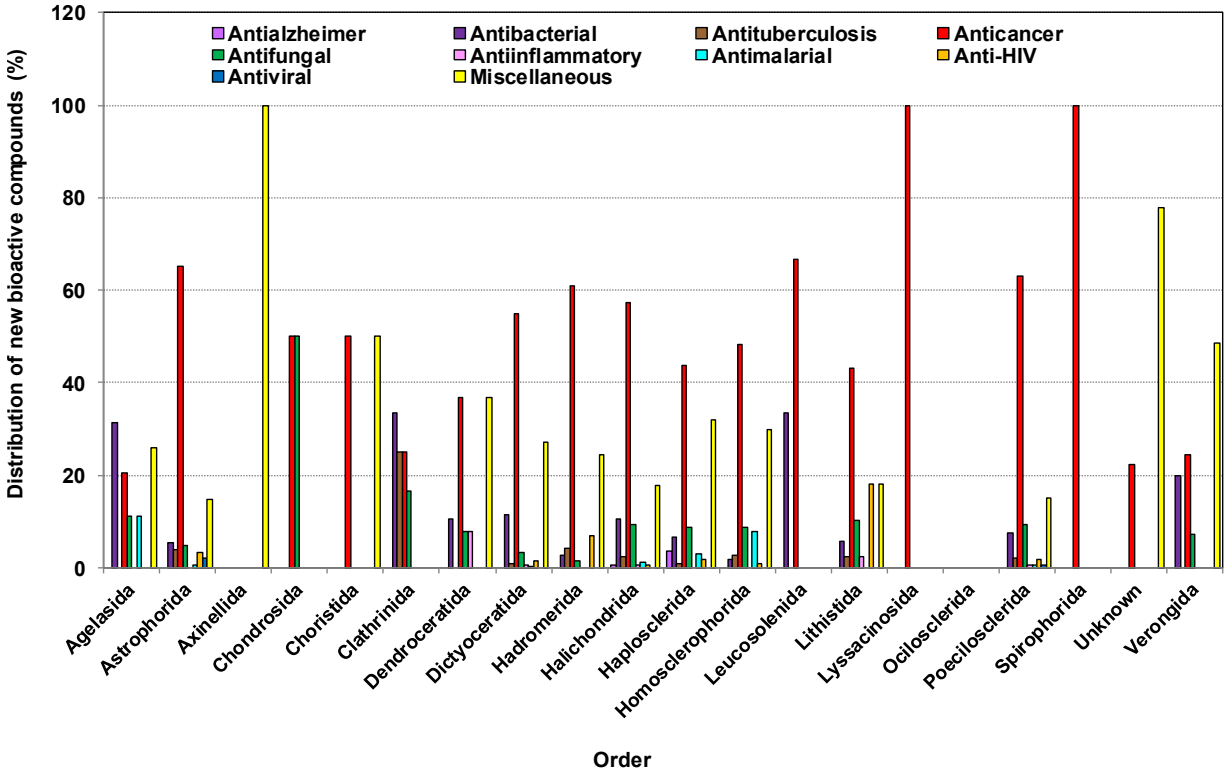
In a recent overview of 132 natural products from marine sources obtained during the period 2002–2011, which exhibited anti-HIV activity, it was reported that sponges contributed more than half of all anti-HIV natural products from marine organisms. These were mainly alkaloids and cyclic depsipeptides [141], such as Cortistatin A (CA), a recently discovered natural steroidal alkaloid isolated from the marine sponge *Corticium simplex* [142]. It has been reported to display anti-proliferative properties towards human umbilical vein endothelial cells (HUVECs) with an average half-maximal inhibitory concentration (IC_{50}) of 0.35 μM [142,143]. A recent study showed that Cortistatin A potently suppresses Tat-dependent HIV transcription [144]. Further reading on the structural characteristics of sponge derived cyclodepsipeptides can be found in a recent review [145]. It is important to note that Halichondrida and Haplosclerida are the only orders from which anti-Alzheimer's activity was reported during the last decade.

There have been many interesting compounds with unusual structures with potential activity which have been observed during the last decade. Some of the compounds are illustrated in Table 3.

Figure 7. (**a**) Total number of new compounds isolated from different marine sponges with various bioactivities from 2001 to 2010; (**b**) The distribution of new compounds isolated from different orders of marine sponges with various bioactivities from 2001 to 2010.



(**a**)



(**b**)

Table 3. Selected compounds with unusual structure and significant activity from sponges.

Organism	Order	Compound Name	Chemical Class	Special Feature/Activity	Source, Country, Year,/Depth	Reference
Sarcotragus sp.	Dictyoceratida	Sarcotragin A, & B	Trisnorsesterterpenoid lactam	Showed moderate cytotoxicity (LC_{50} 207 μg/mL) toward the leukemia cell-line K562	Seoguipo, Jaeju Island, Korea, 2001	[146]
Polymastia tenax	Hadromerida	5α,6α-epoxy-24*R**-ethylcholest-8(14)-en-3β,7α-diol and 5α,6α-epoxy-24*R**-ethylcholest-8-en-3β, 7α-diol	Sterol	Exhibited significant cytotoxic activity *vs.* human lung carcinoma (A-549), human colon carcinomas (HT-29 and H-116), and human prostate carcinoma (PC-3) cell lines with the LC_{50} (μg/mL) value of 5–10, 1–5, 1–5, 0.5–1 and 1–5	Punta de Betín, Bahía de Santa Marta, in the Colombian Caribbean, Colombia, 2002	[147]
Crella spinulata	Poecilosclerida	Benzylthiocrellidone	Bis-dimedone thioether	First report of a natural product containing a dimedone moiety. No activity reported	Davies and Bowden Reefs Australia, 2002	[148]
Ectyoplasia ferox	Poecilosclerida	Ectyoceramide	Galactofuranosylceramide (GSL)	The first example of a monohexofuranosylceramide and the first natural GSL with its first sugar in the furanose form. No activity reported	Island of Rum Cay, Bahamas, 2000	[149]
Cribrochalina olemda	Haplosclerida	Kapakahine E	Peptide (cyclic)	Kapakahine E showed moderate cytotoxicity against P388 murine leukemia cells at IC_{50} of 5.0 μg/mL	Pohnpei, Micronesia, 2003	[150]
Haliclona Viscosa	Haplosclerida	Viscosamine	Trimeric 3-alkyl pyridinium alkaloid	First trimeric 3-alkyl pyridinium compound from a marine environment. No activity reported	Coast of Blomstrandhalvøya, near Hansneset, Kongsfjorden, Arctic Ocean, 2003	[151]
Phakellia fusca	Axinellida	Compound **1**, **2**, **3**	5-Fluorouracil alkaloid	First report of fluorine containing natural products from a marine source. No activity reported	Yongxiong Island of the Xisha Islands, South China Sea, China 2003	[152]

Table 3. *Cont.*

Agelas clathrodes	Agelasida	Clarhamnoside	Rhamnosylated *R*-Galactosylceramide	The first Rhamnosylated *R*-Galactosylceramide, a glycolipid containing an unusual L-rhamnose unit. No activity reported	Grand Bahamas Island (Sweetings Cay), Bahamas, 2004	[153]
Psammocinia sp.	Dictyoceratida	Psymberin	Cytotoxin (distantly related to the Pederin family)	Several melanoma, breast, and colon cancer cell lines demonstrated high sensitivity ($LC_{50} < 2.5 \times 10^{-9}$ M) to psymberin, and all six leukemia cell lines proved comparably insensitive	Papua New Guinea, 2004	[154]
Callyspongia abnormis	Haploscerida	Callynormine A	Cyclic Peptide	Represents a new class of heterodetic cyclic peptides (designated endiamino peptides). This compound possessing an α-amido-β-aminoacrylamide cyclization functionality	Shimoni reef, Kenya, 2004	[155]
Axinella infundibula	Halichondrida	Axinelloside A	Lipopolysaccharide (Sulfated)	Axinelloside A, a complex polysulfated glycolipid, which strongly inhibited the activity of human telomerase with an IC_{50} value of 0.4 μM	Shikine-jima Island, the Izu Islands, Japan, 2005	[156]
Theonella swinhoei	Lithistida	Plytheonamide A, B	Polypeptide	Showed cytotoxicity against P388 murine leukemia cells with IC_{50} values of 78 and 68 pg/mL, respectively. Linear polypeptides with unprecedented structural features	Hachijo-jima Island, Japan, 2005	[157]
Neopetrosia sp.	Haplosclerida	Neopetrosiamide A, B	Peptide (diastereomeric tricyclic)	Active in inhibiting the amoeboid invasion by human tumor cells	Near Milne Bay, Papua New Guinea, 2005	[158]
Prianos osiros	Haplosclerida	(3*R*,3′*R*,5*S*)-3,3′,5,19′-tetrahydroxy-7′,8′-didehydro-γ,ε-carotene-8-one	Acetylenic carotenoid	Contains an unusual cytotoxic carotenoid	Pohnpei, Micronesia, 2005	[159]

Table 3. *Cont.*

Ircinia sp.	Dictyoceratida	Irciniasulfonic acid B	Fatty acid derivative (taurine conjugated)	Reversed the multi-drug resistance to vincristine in KB/VJ300 cells at the concentration of 100 μM	Tsuzumi Island, Fukuoka Prefecture, Japan, 2006	[160]
Suberites japonicus	Hadromerida	Seragamide A–F	Depsipeptide (actin targeting)	Caused multinuclei formation in cells at 0.01–0.02 μg/mL	Seragaki, Okinawa, Japan, 2006	[161]
Theonella swinhoei	Lithistida	Hurghadolide A	Macrolide	Caused disruption of the actin cytoskeleton at concentrations of 7.3 nM. Active against *Candida albicans* (MIC 31.3 μg/mL)	Red Sea, Egypt, 2006	[89]
Theonella swinhoei	Lithistida	Swinholide I	Macrolide	as above	Red Sea, Egypt, 2006	[89]
Coelocarteria cfr. *singaporensis*	Poecilosclerida	Coelodiol and Coelic acid	Diterpene (ent-isocopalane)	Inhibit the *in vitro* growth of MKN-45 cell line (human gastric adenocarcinoma) at 20 and 40 μg/mL respectively	Bunaken, Marine Park (North Sulawesi), Indonesia, 2006	[162]
Lendenfeldia sp.	Dictyoceratida	(*S*)-2,2′-Dimethoxy-1, 1′-binaphthyl-5,5′,6,6′-tetraol	Naphthalene dimer	Significantly inhibited both hypoxia-induced (IC_{50} values 4.3 μM) and iron chelator (1, 10-phenanthroline)-induced HIF-1 activation in T47D breast tumor cells. This compound inhibited HIF-1 activation at concentrations that were significantly lower than those that suppressed tumor cell viability	Collected at 2 m depth on May 22, 1993 (sample C011337), from a sea grass bed, Indonesia, 2007	[163]
Erylus formosus	Astrophorida	Eryloside F1–F4	Triterpene glycoside	At a concentration of 100 μg/mL were found to activate Ca2 influx into mouse spleenocytes. biosides having aglycons related to penasterol with additional oxidation patterns in their side chains	Puerto Morelos (the Caribbean Sea), Mexico 2007	[164]

Table 3. *Cont.*

Erylus formosus	Astrophorida	Eryloside M–Q	Triterpene glycoside	As above, contain new variants of carbohydrate chains with three, four and six sugar units. Contain 14-carboxy-24-methylenelanost-8(9)-en-3β-ol	Puerto Morelos (the Caribbean Sea), Mexico, 2007	[164]
Cacospongia mycofijiensis	Dictyoceratida	CTP-431	Thiopyrone	Showed only mild cytotoxicity (IC_{50}: 18 μM) against human colon carcinoma HCT-116. This compound has no previous precedent in natural products chemistry. Its structure including absolute configuration as 8*R*,9*R*,10*S*,13*S*	Beqa Lagoon, Fiji, 2008	[165]
Homophymia sp.	Lithistida	Homophymine A	Cyclodepsipeptide	Exhibited cytoprotective activity against HIV-1 infection with a IC_{50} of 75 nM	Coast of New Caledonia, 2008	[166]
Ianthella sp.	Verongida	Petrosterol-3,6-dione and 5α,6α-epoxy-petrosterol	C29 sterol	Showed growth-inhibitory effects with IC_{50} values of 8.4, 19.9, 17.8, 16.2 and 22.1 μM against lung (A549), colon (HT-29), breast (MCF-7), ovary (SK-OV-3), and two types of leukemia (HL-60 and U937) human cancer cell lines	Namyet Island, Khanh Hoa province, Vietnam, 2009	[167]
Topsentia sp.	Halichondrida	Geodisterol-3-*O*-sulfite and 29-demethylgeodisterol-3-*O*-sulfite	Sterol (sulphated)	Reverses efflux pump mediated fluconazole resistance. Also enhances fluconazole activity in a *Saccharomyces cerevisiae* strain overexpressing the *Candida albicans* efflux pump MDR1, as well as in a fluconazole-resistant *Candida albicans* clinical isolate known to overexpress MDR1	Chuuk, Micronesia, 2009	[168]

Table 3. *Cont.*

Spongia (*Heterofibria*) sp.	Dictyoceratida	Heterofibrin A1–A3 and B1–B3	Fatty acid	Possess a diyne-ene moiety, while the monolactyl and dilactyl moiety featured in selected heterofibrins is unprecedented in the natural products literature. Inhibited lipid droplet formation in A431 fibroblast cells (up to 60% at 10 μM)	Great Australian Bight, Australia, 2010	[169]
Xestospongia sp.	Haplosclerida	Xestosaprol F–M	Xestosaprol (pentacyclic compound)	Showed moderate inhibition of the aspartic protease BACE1 (memapsin-2), which has a central role in the etiology of Alzheimer's disease with the IC_{50} value of 135 ± 11 μM. First examples of a monooxygenated A-ring	Coral reef at Sangalaki, Indonesia, 2010	[170]
Theonella swinhoei	Lithistida	Paltolides A–C	Peptides (Anabaenopeptin like)	Closely related to a group of anabaenopeptins that are submicromolar inhibitors of carboxypeptidase U with greater than 50 fold selectivity over other carboxypeptidases	Uchelbeluu Reef, Palau, 2010	[171]
Neopetrosia proxima	Haplosclerida	Neopetrosiamine A	Alkaloid (tetracyclic bis-piperidine)	Exhibited strong inhibitory activity against MALME-3M melanoma cancer, CCRF-CEM leukemia, and MCF7 breast cancer with IC_{50} values of 1.5, 2.0, and 3.5 μM, respectively. *In vitro* activity *vs.* pathogenic strain of *Mycobacterium tuberculosis* (H37Rv) and *Plasmodium falciparum*	Mona Island, Puerto Rico, 2010	[172]
Iotrochota baculifera	Poecilosclerida	Baculiferins A–O	*O*-sulfated pyrrole alkaloids	Baculiferins C, E–H, and K–N (4, 6–9, 12–15) are potent inhibitors of HIV-1 IIIB virus in both MT4 and MAGI cells. Additionally could bind to the HIV-1 target proteins Vif, APOBEC3G, and gp41	Inner coral reef, Hainan Island, China, 2010	[173]

2.8. Distribution of New Compounds Based on Country/Geographical Area

One important aspect of sponges is their geographical location. A high percentage of bioactive sponge species were reported from different geographical regions [174–176].

Figure 8 shows that the new compounds were mainly isolated from sponges collected from 61 countries. Of these, sponges from Japan had the highest number of compounds (332) followed by Indonesia (235), Korea (211). Sponge samples from Australia and China contributed 187 and 146 compounds, respectively, during the last decade. The Bahamas, Mexico, Palau, Papua New Guinea, Philippines and Vanuatu are the other countries which were the source of more than 50 new compounds found in the last ten years. The obvious question is why do some regions show a high diversity of compounds as well as an abundance of sponges? Studies showed that the highest concentration of toxic or antioxidant sponge metabolites are found in habitats such as coral reefs that are characterized by intense competition and feeding pressure from carnivorous fish [177]. The adaptive significance of the chemical defenses of sponges are that they are highly effective against most species of fish and a group of shell-less gastropods, the nudibranchs that feed on sponges and sequester their chemical armory [177]. An excellent paper on the global diversity of sponges includes global sponge diversity information which was collected from different regional projects and resources and also reviewed was information on invasive sponges that might well have some influence on distribution patterns in the future [83].

Figure 8. Total number of new compounds isolated from different marine sponges and their source locations from 2001 to 2010.

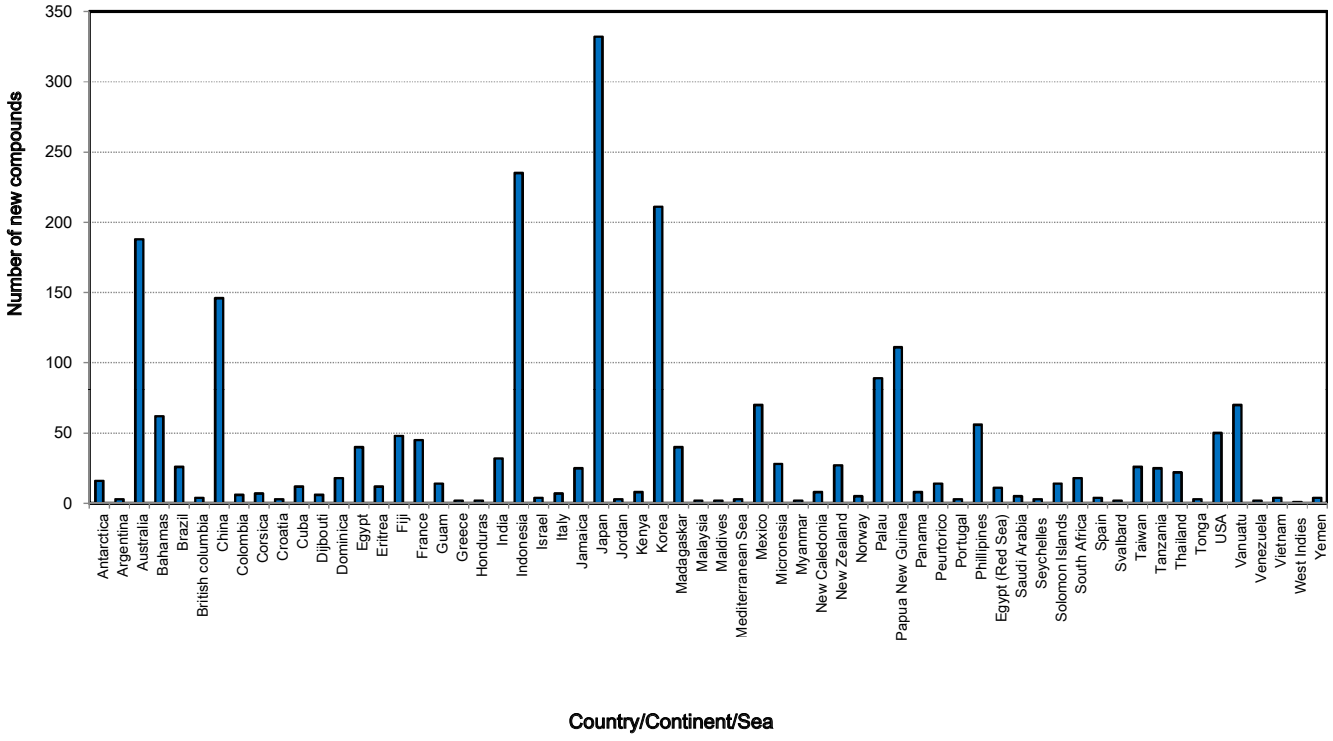
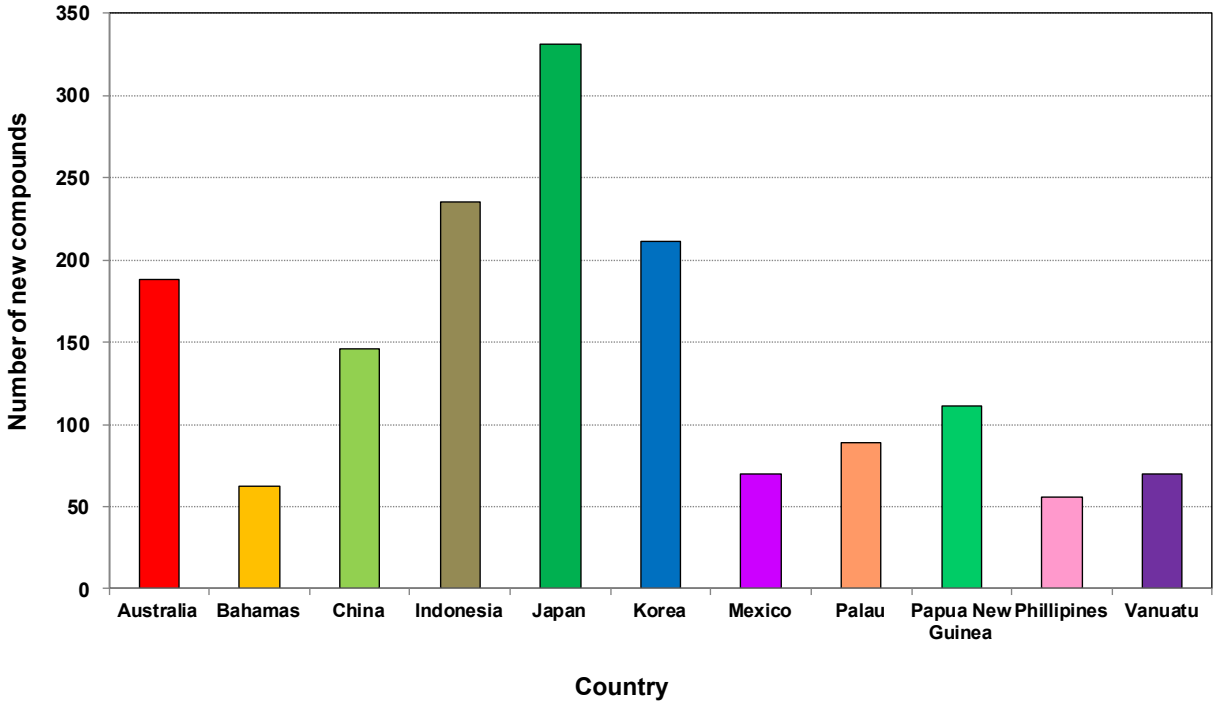


Figure 9 shows the total number of new compounds isolated from the top ten countries. It has been found that each year from 2001 to 2010 at least 20 new compounds were isolated from Japanese sponge samples, with the highest output occurring in 2004 and 2005 with 46 new compounds (Figure S2, Supplementary Information). From 2006 to 2009 at least 20 compounds were found each year in Indonesia. Marine invertebrates, which are plentiful in the Indo-Pacific regions including Indonesia, are rich in secondary metabolites and are becoming targets for the continuing search for bioactive compounds [178].

Figure 9. Total number of new compounds isolated from marine sponges from the top 10 source countries from 2001 to 2010.



Although in 2001 the highest contributors of new compounds derived from sponges were from Japan, Korea and Australia at 22.9%, 18.9% and 14.3%, respectively, the scenario changed during 2010 where the highest contributions of new compounds came from China, Australia and Indonesia at 23.9%, 21.3%, and 21%, respectively [179]. China started its marine high-tech program since 1996, and after 15 years it emerged as one of the highest contributors to marine sponge natural products discovery in 2010. This achievement demonstrates how significant the government science and technology funding policy can impact the development of marine biotechnology. Australia is one of only 17 recognised megabiodiverse countries primarily based on its highly biodiverse and endemic terrestrial flora and fauna [180,181]. While the full extent of Australian marine biodiversity remains relatively unexplored [182], several marine biodiversity hotspots including centers of endemicity have been recognized, especially in coral reefs [183,184], the temperate coastline [185] and the Great Australian Bight off the coast of South Australia [186]. There have been reports on the high species diversity of sponges in the north west [187–190], in the deep sea off the south west [191,192] of the Bight, and the Great Barrier Reef [189]. Therefore, there is urgent need to explore those potential locations in order to obtain new compounds and drugs for the future.

The Australian Institute of Marine Science (AIMS) conducted a study based on the Australian marine habitat and identified biogeographic bioactivity hotspots that correlated with biodiversity hotspots. AIMS found that high-level phylogeny, and therefore the metabolic machinery available to an organism, is a major basis of bioactivity, while habitat diversity and ecological circumstance are possible drivers in the stimulation of this machinery and bioactive secondary metabolism [193]. Therefore, in near future, knowledge of metabolomics coupled with genomics tool and bioinformatics could be a high level device for the exploration of target specific bioactive compounds from sponges.

In addition, microbes associated with marine sponges could vary with the geographical area, so, if these associated bacteria are responsible for producing compounds, then it is possible that the same sponge species in different geographical locations could produce different secondary metabolites [194,195]. However, a recent study showed the stability of bacterial communities in two temperate sponges exposed to environmental variation, which is consistent with previous research on other temperate sponges. This study used next generation sequencing and revealed how different components of bacterial communities associated with *Ecionemia alata* and *Tethya bergquistae* responded to environmental variation *in situ* [196]. The similarity observed in bacterial communities among specimens occupying different habitats suggests that environmental variation occurring in those habitats does not affect the stability of the community, and hence, most likely does not radically alter the metabolism of these sponges. The study recommends further study to improve the understanding of the role of microbial symbiont communities which may affect the physiology and ecology of sponges on temperate rocky reefs [196].

A recent pyrosequencing analysis of 32 sponge species from eight locations around the world identified few bacterial species that are common to more than a handful of sponge species [177]. They stated that different sponges were found to contain different bacterial species (species-specific community) but share only very few bacterial species (core community) [197]. However, the bacterial species in different sponges are still more closely related to each other than, for example, to seawater bacteria (indicated by Plus-OTUs and sponge-specific clusters), consistent with previous studies suggesting at least partially overlapping communities among different sponges. Sponges therefore contain a uniform, sponge-specific bacterial community although each sponge species contains different bacterial species [197]. Perhaps, this is one of the main reasons for getting new compounds in species level regardless of the genera and order. Although we commented on this, based on our study of sponges of the last decade, further study to prove this hypothesis would be worthwhile.

3. Conclusions

In the decade 2001–2010 marine sponges continued to be the most promising source of marine natural products. Marine microorganisms and phytoplankton grew rapidly to become the third largest source by increasing their contribution from 9% to 39% during the decade. Sponges are a reservoir of marine microorganisms with up to 40%–60% as microbial biomass. In the coming decade sponge associated microorganisms promise to be an outstanding source of the NMNP.

From the current trend of discovery it could be predicted that China, Australia and Indonesia will be the source of more new compounds in the future competing with Japan and Korea. However, although Indonesia is an excellent source of sponges, most of their studies were conducted by scientists in other

countries. Certainly, exploration of new compounds from marine sponges is dependent on government funding and policy, industrial interest and investment, research facilities, the expertise of scientists, infrastructure and laboratory facilities, equipment, machinery and institutional support.

Astrophorida, Dictyoceratida, Halichondrida, Haplosclerida and Poecilosclerida were the key orders of sponges studied during the first decade of the 21st century. The examination of the contribution from an individual species revealed that regardless of the order each species contributed on average 3–5 compounds. The high number of new compounds was the result of the high diversity of species from these particular orders. Alkaloids (20%), terpenes (14.7%) or terpenoids (8.3%) and peptides (6.8%) represented the three main chemical classes of compounds discovered from sponges in this period, and together with the other chemical classes showed a range of biological activities. Of all the biological activities investigated cytotoxicity or anticancer activity against different cancer cell lines was most frequently reported at 53.6%. Antibacterial and antifungal activity were two other areas where new compounds showed potential activity at 9.4% and 7.2%, respectively. Anti-Alzheimer's, antibacterial, antituberculosis, anticancer, antifungal, anti-inflammatory, antimalarial, antiviral, anti-HIV were the other activities exhibited by the new compounds from sponges. Because sponge extracts showed potent cytotoxic activity, which is often reported as anticancer activity, it is very important to study the mode of action of these extracts by isolating pure compounds rather than only testing cytotoxicity.

The order Dictyoceratida was found to be the most prolific producer of new compounds among all the sponge orders studied. *Dysidea* sp. and *Ircinia* sp. were found to be the most promising genera because of their capacity for producing new bioactive compounds.

In any event, the discovery of marine natural products from sponges relies particularly on finding new genera and species from the most prolific to the least abundant orders, which is still achievable. In order to overcome the problem of production of sponge-derived compounds, synthesis of bioactive, low molecular weight compounds by cloning biosynthetic gene clusters using recombinant techniques could be applied. Most importantly, sponge derived compounds should be utilized with a combination of innovative technologies which could develop new fields of application that will impact significantly on biotechnology.

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Author Contribution

Mohammad Ferdous Mehbub did the coordination for collecting all the information to prepare a comprehensive database for writing this manuscript, proof read the database, wrote a major part of the manuscript and generates the graphs and tables, proof reading the manuscript as well and made necessary correction.

Jie Lei helped to collect the information to develop the database for preparing this manuscript.

Christopher Franco wrote a significant part of the manuscript, decided on the format and figures to be included and did the final proofreading and corrections.

Wei Zhang has the original idea, structure and plan for this manuscript, and planned and refined the graphs and tables with Mohammad Ferdous Mehbub, and wrote the abstract, and wrote a significant portion for the introduction.

Conflicts of Interest

The authors declare no conflict of interest.

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Appendix 2

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Research review paper

New marine natural products from sponges (Porifera) of the order Dictyoceratida (2001 to 2012); a promising source for drug discovery, exploration and future prospects

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ABSTRACT

The discovery of new drugs can no longer rely primarily on terrestrial resources, as they have been heavily exploited for over a century. During the last few decades marine sources, particularly sponges, have proven to be a most promising source of new natural products for drug discovery. This review considers the order Dictyoceratida in the Phylum Porifera from which the largest number of new marine natural products have been reported over the period 2001–2012. This paper examines all the sponges from the order Dictyoceratida that were reported as new compounds during the time period in a comprehensive manner. The distinctive physical characteristics and the geographical distribution of the different families are presented. The wide structural diversity of the compounds produced and the variety of biological activities they exhibited is highlighted. As a representative of sponges, insights into this order and avenues for future effective natural product discovery are presented. The research institutions associated with the various studies are also highlighted with the aim of facilitating collaborative relationships, as well as to acknowledge the major international contributors to the discovery of novel sponge metabolites. The order Dictyoceratida is a valuable source of novel chemical structures which will continue to contribute to a new era of drug discovery.

Contents

1. Introduction

Sponges (Porifera) and their associated microorganisms are the largest contributors (Blunt et al., 2015) of marine natural products. During the period from 2001 to 2012 (full details of discovery profiles are presented in Table S1) (Mehbub et al., 2014), the order of Dictyoceratida (Phylum Porifera, Class Demospongiae, Order Dictyoceratida) contributed over 20% of all sponge-derived new compounds discovered, making it the highest among all the sponge orders. In contrast, the orders of Haplosclerida, Poecilosclerida, Halichondrida, and Astrophorida contributed respectively 14.2%, 14%, 10.7% and 9.2%,

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respectively. The order of Lithistida, as one of the most prolific contributors reported in the past, only contributed 5.5%. Importantly, the compounds discovered from Dictyoceratida demonstrated a diverse range of biological activities, including antibacterial, anticancer, antifungal, anti-HIV, anti-inflammatory, and antimalarial. To advance the future discovery of sponge-derived natural products, a systematic review this particular order, on the collection sites, the types of compounds and their biological activities will generate important insights into the bioprospecting of other sponge orders for potential new drug discovery.

Dictyoceratida sponges are identified by anastomosing sponge fibers and the absence of a mineral skeleton. They are commonly present on coral reefs, often outnumbering all other sponge groups (Duckworth and Wolff, 2007). Dictyoceratida consists of five families which can easily be distinguished by the fine collagenous filaments in the irciniids, the homogeneous skeletal fibres of spongiids, the eurypylous choanocyte chambers in dysideids and the opposite of these characteristics in the thorectids (such as diplodal choanocyte chambers, pithed and laminated fibres and an absence of fine filaments)(Cook, 2007). There are thirty-six described genera, which are defined in terms of their skeletal architecture, mucus production, and whether or not they are armored. Skeletal characters cover primary, secondary or tertiary fibers, fascicular fibers, foreign coring, fiber diameter, skeletal density, collagen deposition, and general skeletal morphology and distribution. Several additional genera are known but have not yet been described.

Dictyoceratida sponges are tough and flexible, but can be hard or soft. Infiltration of debris into the matrix and condensing of skeletal mesh can make them hard, but those with a higher amount of soft tissue volume compared to the density of the fiber and skeleton are soft (Cook and Bergquist, 2002c). A conulose surface is common but a few species of *Psammocinia* are densely armored. Fibers are fused together in a vein-like network but two genera (*Ircinia* and *Psammocinia*) have a hierarchical arrangement with respect to diameter and surface orientation with primary, secondary and sometimes tertiary elements (Cook and Bergquist, 2002c).

In this review, the original data was collected from the *Natural Product Reports* published by Blunt et al., 2003 to 2014 (Blunt et al., 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2011, 2012, 2014) as well as all the literature published during the time frame from 2001 to 2012 regarding novel compounds from the order Dictyoceratida. Bacteria and fungi associated with the order Dictyoceratida are known to synthesize a range of compounds with a variety of biological activities (Amagata et al., 2003; Bringmann et al., 2003, 2007; Elyakov et al., 1991; Hentschel et al., 2001; Müller et al., 2004; Oclarit et al., 1994; Thakur et al., 2005). The microoganisms isolated from Dictyoceritida species that are reported as producers of new compounds were isolated from *Dysidea ampha*, *D. avara*, *D fragilis*, *Hyrtios proteus*, *Ircinia facultata* and *Psammocinia* sp. (Mehbub et al., 2015). During the review period of 2001 to 2012, 559 new compounds were reported from the order Dictyoceratida with an average of 47 compounds per year (Fig. 1)(Mehbub et al., 2014).

2. Taxonomical origins of new compounds from Dictyoceratida

According to the World Porifera Database (van Soest et al., 2014), Dictyoceratida has 5 direct families: Dysideidae, Irciniidae, Spongiidae, Thorectidae, and Verticilliitidae. The last family has been reclassified recently into this order and contains only one genus. New compounds were reported only from the four families: Dysideidae, Irciniidae, Spongiidae and Thorectidae during 2001 to 2012 (Table 1).

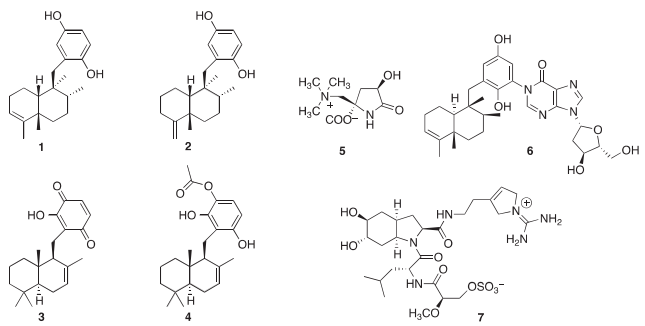
Over 42% of the studies conducted during the period 2001–to 2012 (in terms of the number of publications) were on the family Thorectidae and around 38% of the new compounds were reported from this family (15 genera and 27 species, Table 1). The Thorectidae family consists of two subfamilies (Thorectinae, Phyllospongiinae), which have 23 valid genera and 129 species (Hooper and van Soest, 2002). Therefore only 20% of these named species from this family have been studied for new compounds discovery. Thorectids range from low and encrusting to massive in growth form. These sponges have been collected from throughout tropical and temperate oceans but not from polar waters. This family can be distinguished by their diplodal choanocyte chambers, strongly laminated skeletal fibres, and the absence of fine skeletal filaments (Cook and Bergquist, 2002b).

Spongiidae family composed of 6 valid genera and 91 nominal species (Cook and Bergquist, 2002a). During the review period only 4 genera and 11 species were studied (Table 1), accounting for only 12% of the named species. 86 new compounds were reported, with the genera *Spongia* being most productive with 49 new compounds reported (Fig. 4). The genera within the family were distinguished by the characteristics of surface armouring, skeletal morphology and the development of sub-dermal or internal lacunae (Cook and Bergquist, 2002a). They have collagen in their matrix that is often varied and the choanocyte chambers are diplodal. The species of these genera have a dense structure, dominated by the secondary fiber reticulum and their surface can be deeply armored with a structured dermal crust of sand, foreign spicules and detritus (Cook and Bergquist, 2002a).

The family Dysideidae (4 genera and 17 species, Table 1) and Irciniidae (3 genera and 10 species, Table 1) provided 118 and 103 new compounds respectively. The genus *Dysidea* from the Dysideidae family and genus *Sarcotragus* and *Ircinia* from the Irciniidae family were the most productive genera, giving 91, 47 and 46 new compounds, respectively (Fig. 4). They both warrant further study as 10 or fewer species from each genus were examined, whereas *Dysidea* contains 58 species and *Ircinia* has 76 species. The Irciniidae in particular could be a good model taxon for further study because of their high microbial abundance, rich chemical profile, high metabolic activity and diversified life strategy within marine sponges (Hardoim and Costa, 2014).

Dysideidae are uniquely characterized within the order Dictyoceratida by the presence of eurypylous choanocyte chambers. Members of the Dysideidae can be distinguished from other members of the order as they possess a tangential network of cellular tracts which produce a lacy pattern of the surface and have only light collagen deposition within the mesohyl (Mau et al., 1996). Marine sponges belonging to the genus *Dysidea* (Phylum: Porifera, Class: Demons*pongiae*, Order: Dictyoceratida, Family: Dysideidae) have been investigated for over 40 years as a source of diverse natural products (Blunt et al., 2009). *Dysidea* is a large genus that is widely distributed in tropical and subtropical waters around the world (Yang et al., 2010b). Among the sponges, the genus *Dysidea* is well documented as a rich source for structurally unique and biologically active compounds, including polybrominated phenyl ethers, chlorinated amino acid derivatives, furano-sesquiterpenes, polyhydroxy steroids and sesquiterpenes.

Notable metabolites that have been isolated include isoarenarol 1 and arenarol 2, the anti-inflammatory sesquiterpene-quinones 3 and 4 (McNamara et al., 2005; Yoo et al., 2003) the neurotoxic amino acid dysibetain 5 (Sakai et al., 1999), the meroterpenenucleoside conjugate avinosol 6 (Diaz-Marrero et al., 2006), and a guanidine-bearing peptide 7 (Carroll et al., 2002).



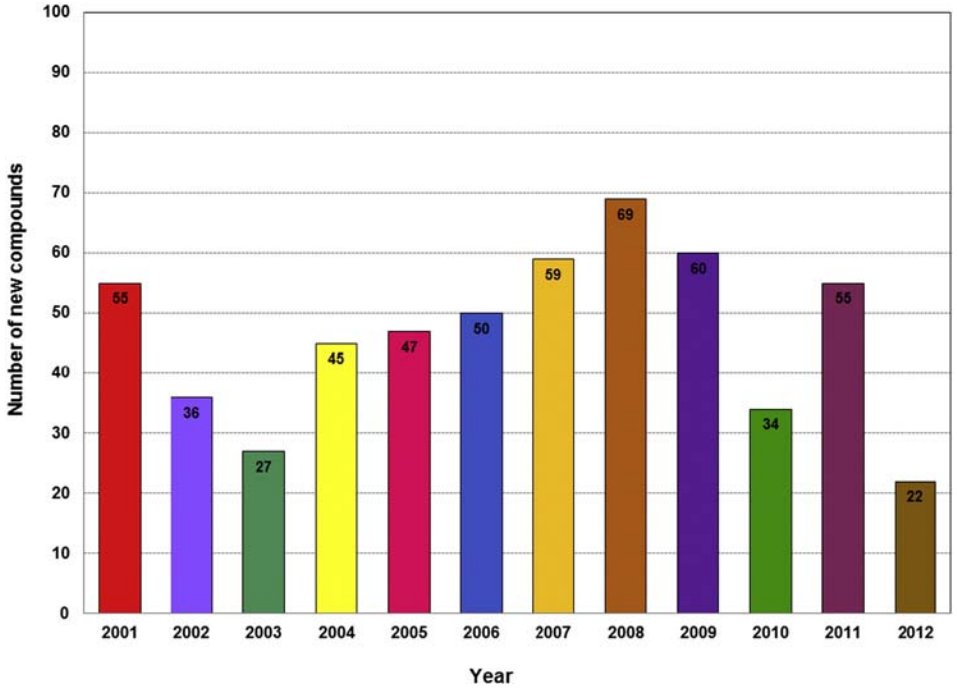


Fig. 1. Total number of new compounds reported yearly from the order Dictyoceratida from 2001 to 2012.

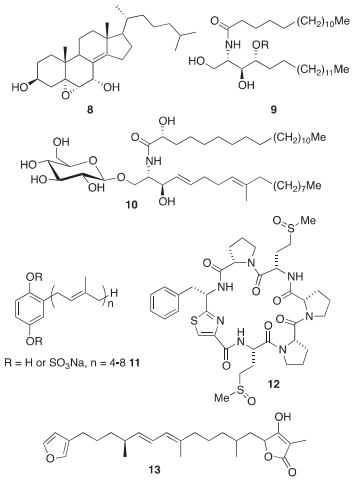
Several of the metabolites first reported from *Dysidea* spp. have subsequently been ascribed to microbial symbionts, such as cyanobacteria (Lemloh et al., 2009). A large number of natural products from *Dysidea* spp. are likely to be derived from marine microorganisms, which are known to be the source of notable bioactive secondary metabolites (Blunt et al., 2009). The close association with symbionts that *Dysidea* sponges have evolved helps to make them a unique source of bioactive and structurally diverse secondary metabolites (Williams et al., 2009b).

"The family Irciniidae Gray, 1867 has autapomorphic features distinguishing this taxon from other demosponge families: its taxa possess fine collagen filaments in the mesohyl, which gives the sponges a rubber-like texture" (Pöppe et al., 2010). The Irciniidae currently consists of three genera with 111 described species (van Soest et al., 2014) with an assumed worldwide distribution. The genus *Ircinia* currently comprises 76 described species (van Soest et al., 2014) and differs from the genus *Sarcotragus* Schmidt, 1862 (11 known species) by the nature of the primary fibres. The genus *Psammocinia* Lendenfeld, 1889 for which 23 species are currently described, is distinguished by a dermis armored with a thick crustose layer of foreign debris. However, the classification of the species is more difficult, increasing the probability of the existence of cryptic species among the known specimens (fibers Cook and Bergquist, 1998; Hooper and van Soest, 2002) and their characterisation purely by means of morphology appears unlikely.

The species of the genus *Ircinia* are easily recognized by the presence of fine filaments in the mesohyl, strong primary fiber fascicles, the foreign coring in primary fibers and an unarmoured cortex. Sponges of this genus have been reported to produce and exude low-molecular-mass volatile compounds such as dimethyl sulfide, methyl isocyanide and methyl isothiocyanate that give them an unpleasant garlic odor (Pawlik et al., 2002). Steroid **8** (Venkateswarlu et al., 1996), sphingolipid (Zhang et al., 2005) **9** and **10** hydroquinone, (Venkateswarlu and Reddy, 1994) **11** and cyclic hexapeptide (Mau et al., 1996) **12** derivatives have been reported from this genus, in addition to furanosesterterpenes (e.g., compound **13**) which are considered as one of the major constituents (Capon and Macleod, 1987; Liu et al., 2006b).

Table 1
Summary of the total number of new compounds reported from the order Dictyoceratida with the number of families, genera, species and number of references published from 2001 to 2012.

Family	Genera	Species	Number of new compounds	References
Dysideidae	4	17	118	42
Irciniidae	3	10	103	30
Spongiidae	4	11	114	35
Thorectidae	15	27	215	78
Unknown	1	1	9	3



In recent studies, a variety of chemical classes of compounds have been reported from this genus including compounds such as sterols **14–19** (Xu et al., 2008), the pyran derivatised ester **20** (Granato et al., 2005), irciformonins E–K **21-28** (Shen et al., 2009) irciformonins A–D **29-33** (Shen et al., 2006), furano sesquiterpenes **33–34** (Issa et al., 2003), ircinolin A **35** (Su et al., 2011), irciniastatin A **36** (Pettit et al., 2004), the macrolide tedanolide C **37** (Chevallier et al., 2006; Rashid

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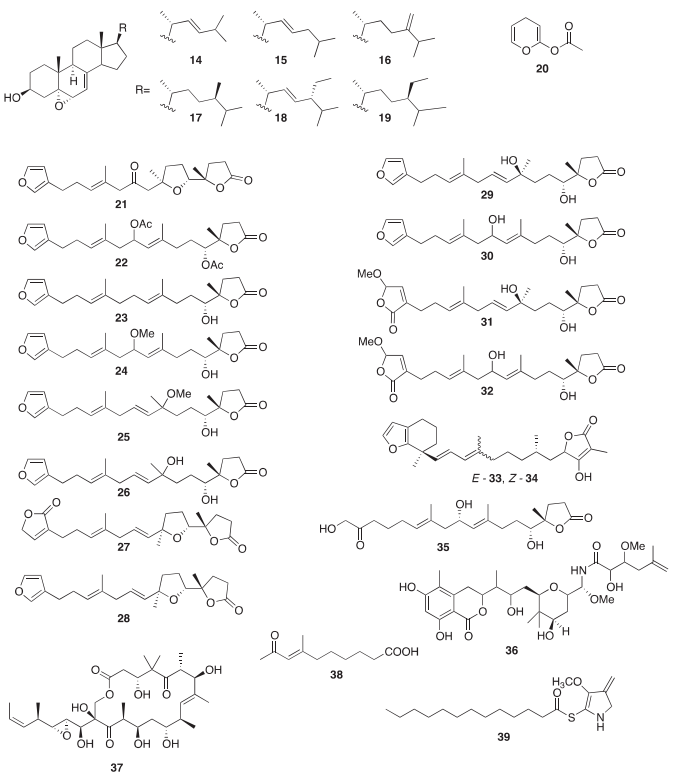
et al., 2001), ketone **38** (Tatli et al., 2008) and the novel nitrogen containing ircinamine **39** (Kuramoto et al., 2002).



Ircinia spp. are mainly dominated by Alpha- and Gammaproteobacteria (Muscholl-Silberhorn et al., 2008). Besides, Acidobacteria, Chloroflexi and Poribacteria also showed high abundance with this particular genus and most of the cases of associated bacterial communities were species-specific (Schmitt et al., 2012; Webster et al., 2010). The main roles of these associated bacterial species are to maintain the physiological and metabolic activity which ultimately benefits the sponge (Hardoim and Costa, 2014). Moreover, they can control the growth of their own cells by producing bioactive compounds which were mainly a result of the quorum sensing (QS) mechanism (Bandara et al., 2012).

The sponges belonging to the Irciniidae family are potentially valuable because of their ability to host bacterial species; however these are not easy to grow by culture dependent techniques (Hardoim and Costa, 2014). The dynamics of this particular family are mainly controlled by two types of quorum-sensing molecules of which the salient features are microbe-microbe and host-microbe signaling (Hardoim and Costa, 2014).

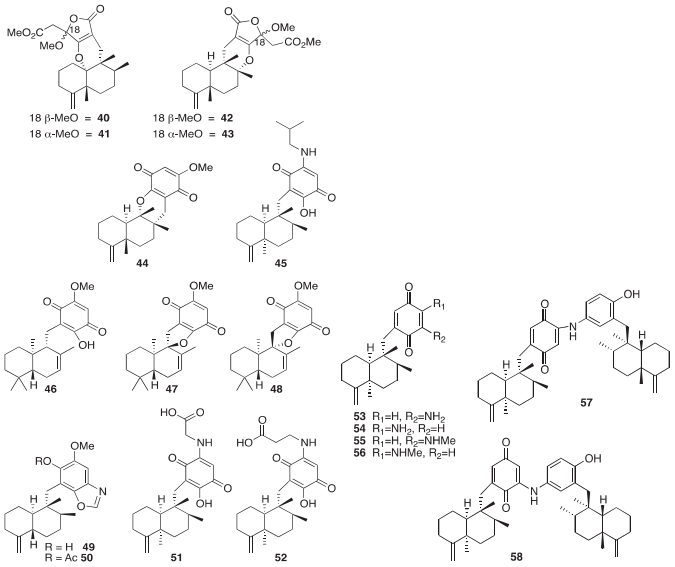
3. Geographical distribution and source countries for Dictyoceratida

Dictyoceratida is a cosmopolitan sponge, but identification of the collection sites will help to identify the areas where this order is abundant and also inform the marine ecological and environmental impact on chemical diversity if these data are available. Dictyoceratida sponges were reported from the waters of 31 'source' countries and of these, the 6 major contributors are Korea, Japan, Australia, China, Papua New Guinea and Indonesia (Fig. 2).

In the search for new natural products the past geographical trends of discovery can be used as a guide to identify areas for future sponge collection of the sponges. The reports from the order Dictyoceratida (25 genera and 52 species) have been analyzed based on data from the top six source countries (Fig. 3). Of the 25 genera, 12 were collected from Indonesia and analyzed for new compounds and their bioactivity evaluated. Of these, only one genus comprised three different species. On the other hand, of the total of 5 genera collected from China, there were 14 individual species. Eight genera were collected from Japan, containing 9 individual species. A total of 13 genera of sponges were collected from Australia with 19 individual species, while 13 individual species were collected from Papua New Guinea from 10 genera. Lastly, five individual sponge species were collected from Korea, which were from five different genera. Of all the genera, *Dysidea* and *Ircinia* possess the highest diversity of chemical classes. The highest number of different types of *Dysidea* and *Ircinia* sponges was found in China followed by Australia and then Japan.

It is clear that sponge species from this order has been studied sporadically even from these top six source countries (Table 2). While this type of chemistry research often depends on random collection of the source materials, this analysis together with the biodiversity studies of sponge distribution in different source countries will definitely lead to rational selection of sites and sponge species for a more productive discovery. During 2001–2012 new compounds were reported from a total of 27 known genera (66 different species) of the order Dictyoceratida. Collection sites from the six most prolific countries contributed 25 known genera and 52 species.

It has been shown to be of value to extract the same species of sponge from different locations with the later extractions giving new compounds that are often but not always structurally related to the previous isolates. For example an examination (Mitome et al., 2002; 2003) of the Okinawan sponge *Dactylospongia elegans* was shown to contain the previously reported sesquiterpenoid quinones dactyloquinones A–E **40–43** and the new sesquiterpenoid quinone, neodactyloquinone **44**. In a later study (Aoki et al., 2004) 5-epi-smenospongorine **45**, together with nine known sesquiterpenequinone/phenols were reported and in a 2008 report (Yong et al., 2008b), three new sesquiterpene quinones isohyatellaquinone **46**, 7,8-dehydrocyclospongiaquinone-2 **47** and 9-epi-7,8-dehydrocyclospongiaquinone-2 **48** together with the known quinones, dictyoceratidaquinone, mamanuthaquinone, ilimaquinone, hyatellaquinone and the sesterterpene furospinosulin were reported. Finally in 2011 a new sesquiterpene benzoxazole, nakijinol B **49**, its acetylated derivative, nakijinol B diacetate **50**, and two new sesquiterpene quinones, smenospongines B **51** and C **52**, were also reported (Ovenden et al., 2011b).



But structurally related compounds can also be found in extracts of sponges from different genera and species. For example, the four

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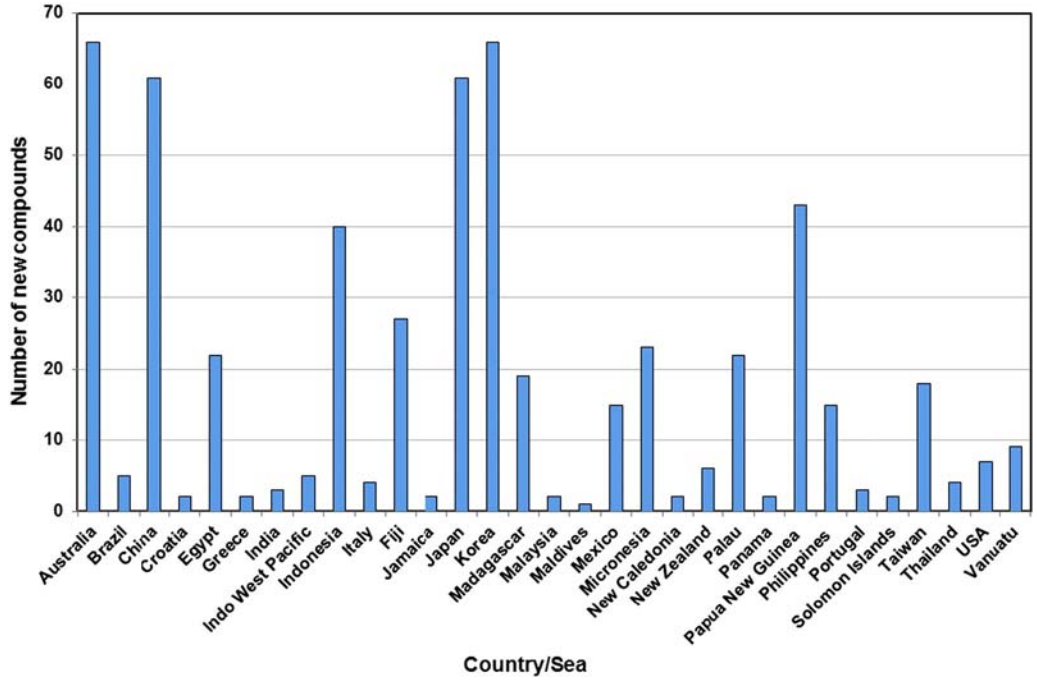

Fig. 2. Total number of new compounds reported from marine sponges belonging to the order Dictyoceratida and their source countries from 2001 to 2012.

new structurally related derivatives, 18-aminoarenarone **53**, 19-aminoarenarone **54**, 18-methylaminoarenarone **55**, and 19-methylaminoarenarone **56**, and the new dimeric popolohuanone F **57**, and were reported (Utkina et al., 2010) from the Australian marine sponge *Dysidea* sp. together with the known compounds arenarol **2** and popolohuanone A **58**.

Dysidea, *Hyrtios*, *Ircinia* and *Spongia* were the four main genera producing new compounds which were reported in many countries during the recent decade, including Australia, Brazil, China, Egypt, Fiji, India, Indonesia, Japan, Korea, Maldives, Mexico, Micronesia, New Caledonia, New Zealand, Papua New Guinea, Philippines, Red Sea, Thailand, Taiwan and Vanuatu. However, China is the only country where all four genera were studied (South China Sea) (Hooper et al., 2000) (Table S1).

Sarcotragus was one of the best yielding genera providing 47 new compounds reported during the 2001 to 2012 time period but interestingly it was only reported from Korean waters (He et al., 2012; Liu et al., 2001, 2002a, 2002b, 2003, 2006a, 2011; Shin et al., 2001; Wang et al., 2008). The variety of compounds included a large range of sesquiterpenoid natural products such as sarcotragins A **59** and B **60** (Shin et al., 2001), furanosesterterpene tetronic acids **61–67** (Liu et al., 2001), new norsesterterpenoids **68** and **69** (Liu et al., 2003),

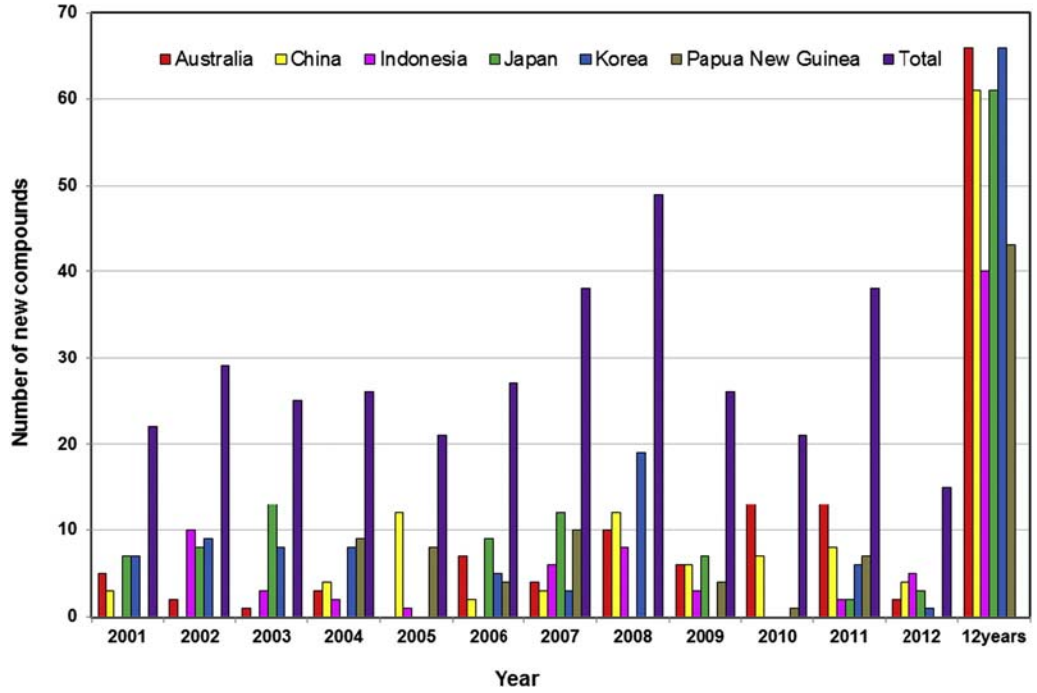

Fig. 3. Total number of new compounds reported from the order Dictyoceratida reported from the top 6 countries from 2001 to 2012.

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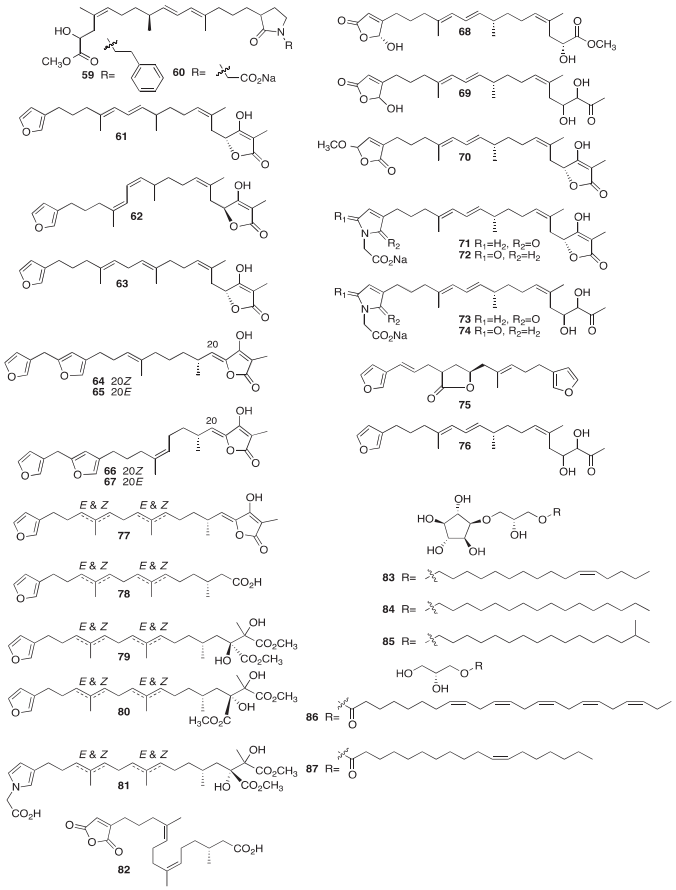
Table 2
The sponges from the order Dictyoceratida reported from Indonesia, Japan, Korea, Australia and China and Papua New Guinea during the period 2001–2012 (+ collected, − not collected).

Name of sponges	Country of collection					
	Japan	Indonesia	Korea	Australia	China	Papua New Guinea
Cacospongia mycofijiensis	−	−	−	−	−	+
Cacospongia sp.	−	−	−	−	−	+
Carteriospongia foliascens	−	+	−	−	−	−
Candidaspongia sp.	−	+	−	−	−	+
Coscinoderma mathewsi	−	−	−	+	−	−
Citronia astra	−	−	−	+	−	−
Dactylospongia elegans	+	+	−	+	−	−
Dactylospongia n. sp.	−	−	−	+	−	−
Dysidea arenaria	−	−	−	−	+	−
Dysidea cf. *arenaria*	+	−	−	−	−	−
Dysidea avara	−	−	−	−	+	−
Dysidea chlorea	+	−	−	−	−	−
Dysidea dendyi	−	−	−	+	−	−
Dysidea fragilis	−	−	−	−	+	−
Dysidea herbacea	−	−	−	+	−	+
Dysidea septosa	−	−	−	−	+	−
Dysidea sp.	+	+	−	+	+	+
Dysidea villosa	−	−	−	+	+	−
Fascaplysinopsis reticulata	−	+	−	−	−	−
Fasciospongia sp.	−	−	−	+	−	−
Hippospongia cf. *metachrome*	+	−	−	−	−	−
Hippospongia lachne	−	−	−	−	+	−
Hippospongia sp.	−	+	−	−	−	−
Hyattella sp.	−	−	+	+	−	−
Hyattella intestinalis	−	−	−	+	−	−
Hyrtios erectus	+	+	−	+	+	−
Hyrtios reticulatus	−	+	−	−	−	+
Hyrtios sp.	+	+	−	−	−	−
Ircinia aruensis	−	−	−	−	+	−
Ircinia fasciculata	−	−	−	−	+	−
Ircinia ramosa	−	−	−	+	−	−
Ircinia selaginea	−	−	−	−	+	−
Ircinia sp.	+	−	−	+	−	+
Lamellodysidea chlorea	−	−	−	+	−	−
Lamellodysidea herbacea	−	+	−	−	−	−
Lendenfeldia sp.	−	+	−	−	−	−
Luffariella sp.	+	−	−	−	−	−
Luffariella variabilis	−	−	−	+	−	−
Phyllospongia foliascens	−	−	−	−	+	−
Phyllospongia papyracea	−	−	−	−	+	+
Phyllospongia sp.	−	−	−	−	+	−
Psammoclema sp.	−	−	−	+	−	−
Psammocinia aff. *Bulbosa*	−	−	−	−	−	+
Psammocinia sp.	−	+	+	−	−	+
Sarcotragus sp.	+	−	+	−	−	−
Smenospongia sp.	−	+	+	−	−	+
Spongia irregularis	−	−	−	−	−	+
Spongia sp.	+	−	+	+	+	−
Spongia zimocca	−	−	−	−	+	−
Strepsichordaia lendenfeldi	−	+	−	−	−	−
Thorecta reticulata	−	−	−	+	−	−
Thorectandra sp.	−	−	−	−	−	+
Unknown	−	−	−	+	−	−

sesterterpenoid **70** (Liu et al., 2003), pyrroloterpenoids **71–74** (Liu et al., 2002a), a stereoisomer of kurospongin **75** (Liu et al., 2003) and norsesterterpenoid **76** (Takahashi et al., 2007a). In 2008 a large range of related structures **77–82** were published (Wang et al., 2008) with a number of double bond positions and geometries. A number of lipids made up the other main chemical class reported. New cyclitol derivatives **83–84** were reported in 2002 (Liu et al., 2002b) and then **85** in 2011(Liu et al., 2011) also two new simple glyceroplipids **86–87** reported in 2006 (Liu et al., 2006a).



This sponge has a rich association with microbes and further study is required to determine the real producer of the sesterterpenoid natural products, as it is possible that microbes associated with this sponge produce this particular type of chemical class of compounds (Takahashi et al., 2007a).

It was found that 6 genera in particular produced the majority of the compounds (56%) as shown in Fig. 4. Although the genus *Dysidea* was the highest producer with 91 new compounds, there are 5 other genera (*Spongia*, *Sarcotragus*, *Hyrtios*, *Ircinia*, and *Phyllospongia*), which also produced more than 30 new compounds during the period 2001–2012. During the 12-year period a total of 27 genera of the 41 known genera in the order were reported to produce novel compounds. Some genera were only reported in a single country such as *Citronia*, *Psammoclema* and *Thorecta* in Australia; *Sarcotragus* in Korea; *Scalarispongia* in the Red Sea (Egypt) and *Strepsichordala* in Indonesia. The three genera, which were only found in Australia, indicated the unique reservoir of sponges in Australia. Exploration in Australian waters is thus likely to be more productive for the isolation of new compounds. One prime example is the Ningaloo Reef which is identified as a marine biodiversity hotspot and is considered to be one of the 18 richest multi-taxon centers of endemism vulnerable to extinction worldwide (Roberts et al., 2002). Finding novel sponges from such areas could provide unique new metabolites.

Marine sponges of the genus *Hyrtios* (Thorectidae, Dictyoceratida) occur throughout tropical and temperate oceans. They are a rich source of bioactive secondary metabolites. During the last two decades mainly three chemical classes of compounds were reported from *Hyrtios* sponges: terpenoids (mainly sesterterpenes and sesquiterpene quinones) (Nasu et al., 1995; Ryu et al., 1996; Salmoun et al., 2000;

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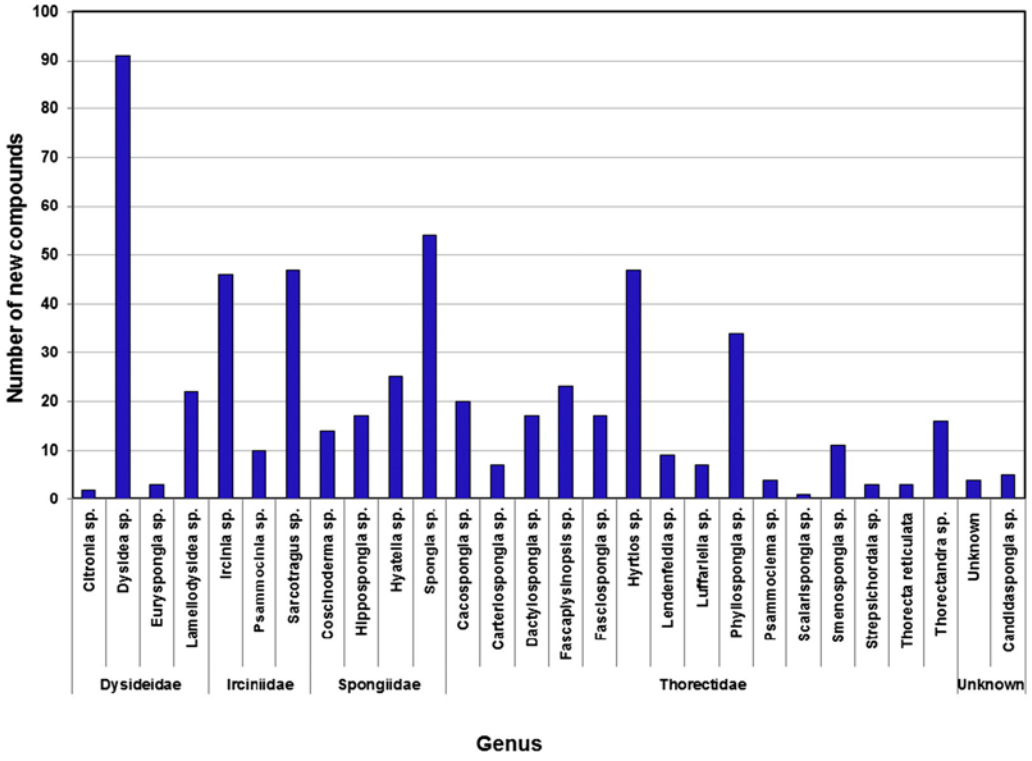
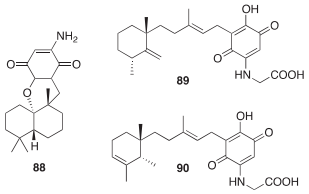


Fig. 4. Total number of new compounds reported from different genera of the order Dictyoceratida during 2001 to 2012.

Williams et al., 1999; Youssef et al., 2002), tryptamine-derived alkaloids (Bourguet-Kondracki et al., 1996; Kobayashi et al., 1990; Salmoun et al., 2002; Sato et al., 1998) and, less frequently, macrolides (Kobayashi et al., 1993, 1994; Pettit, 1996; Pettit et al., 1993a, 1993b). Sponges of the genus *Spongia* are known to be a rich source of terpenoids (such as cyclosmenospongine **88** (Utkina et al., 2003) and Metachromins L **89** & M **90** (Takahashi et al., 2007a) and polyketides (Pettit et al., 1994).



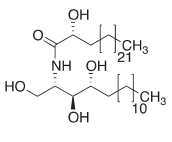
4. Chemical classes of new compounds reported from Dictyoceratida

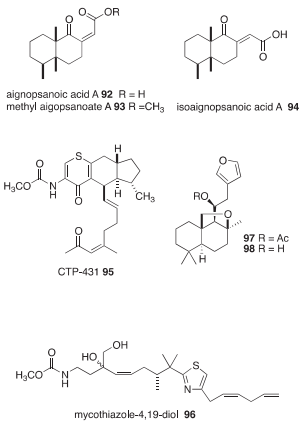
Terpenes, terpenoids, alkaloids and quinones were the main chemical classes which were reported from Dictyoceratida (Fig. 5), making up over 65% of all compounds.

Notably the same genera, when collected in different countries, often produce compounds that belong to different chemical classes. This may reflect that these sponges or member(s) of their associated microbial communities actually produce different chemical structures, specific to the collection site. The genus *Fasciospongia*, yielded terpenes as the main metabolites when collected from Croatia, India, Palau and Portugal, with the one exception being the isolation of ceramide **91** from an Indian collection (Ramesh et al., 2001).

However, care must be taken when making comparisons at the genus level, as the organism may be different species when that level of classification is applied.

The compounds produced by the same species *Cacospongia mycofijiensis* sampled from 3 countries (Papua New Guinea, Fiji, Vanuatu) are varied. The terpenes; aignopsanoic acid A **92**, methyl aignopsanoate A **93**, and isoaignopsanoic acid A **94** were isolated from a Papua New Guinea collection (Johnson et al., 2009) but the thiopyrone (CTP-431 **95**) was isolated from a Fijian collection (Johnson et al., 2008) and the thiazole derivative (mycothiazole-4,19-diol **96**) was isolated from a Vanuatu collection (Sonnenschein et al., 2006). Notably the Fijian sponge metabolite CTP-431 (a novel thiopyrone) (Johnson et al., 2008) shares some structural analogy to latrunculin A (Kakou et al., 1987) but the thiopyrone fragment is unprecedented in natural product chemistry. Yet another pair of compounds cacofuran A **97** & B **98** was isolated from a Japanese collection of a *Cacospongia* sp. but the





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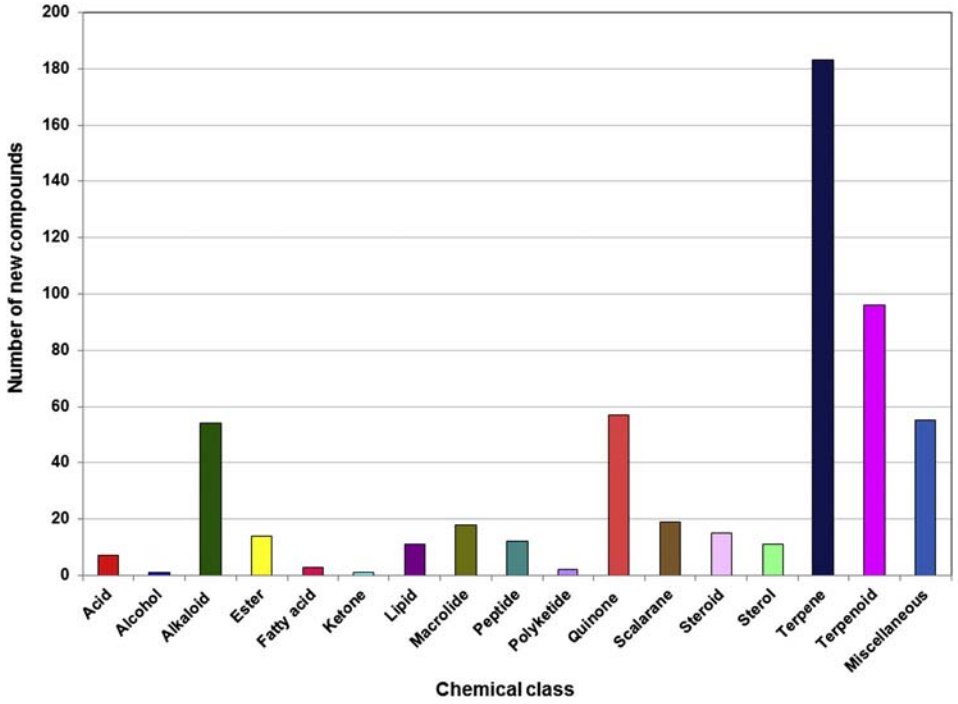
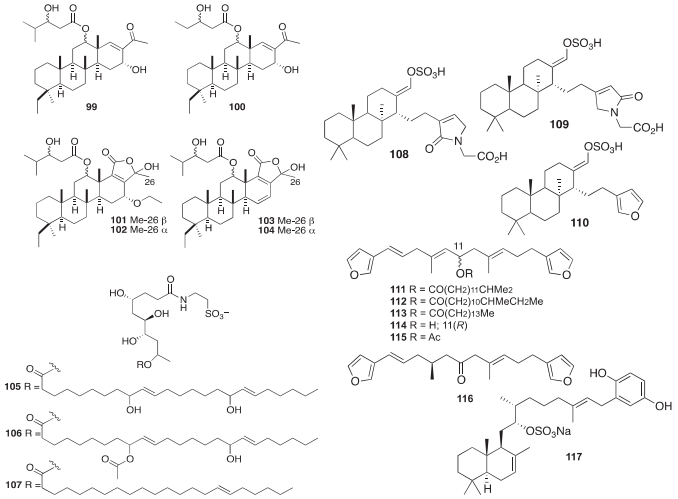


Fig. 5. Total number of new compounds belonging to specific chemical classes reported from marine sponges from the order Dictyoceratida from 2001 to 2012.

particular species of this sponge was not identified (Tanaka et al., 2001a).

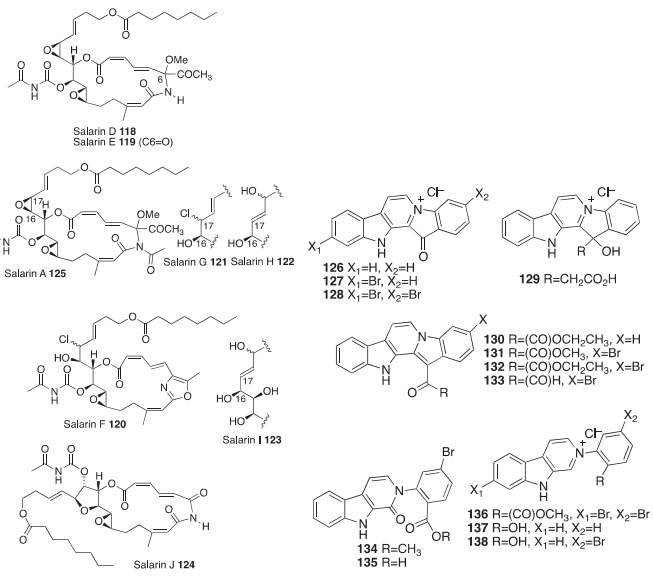
Some other examples of the same genus producing different chemical classes when found in samples from a different location are *Carteriospongia* spp. from Indonesia and Philippines, which produce a terpene and sulfonic acids (McCulloch et al., 2009; Williams et al., 2009a). The two new 20, 24-bishomo-25-norscalaranes **99** and **100** and four 20, 24-bishomoscalaranes **101–104** were isolated from the Indonesian marine sponge *Carteriospongia foliascens* (Williams et al., 2009a). But the three novel natural products, carteriosulfonic acids A



105, B **106**, and C **107**, were identified from a Philippine collection of a *Carteriospongia* sp. (McCulloch et al., 2009).

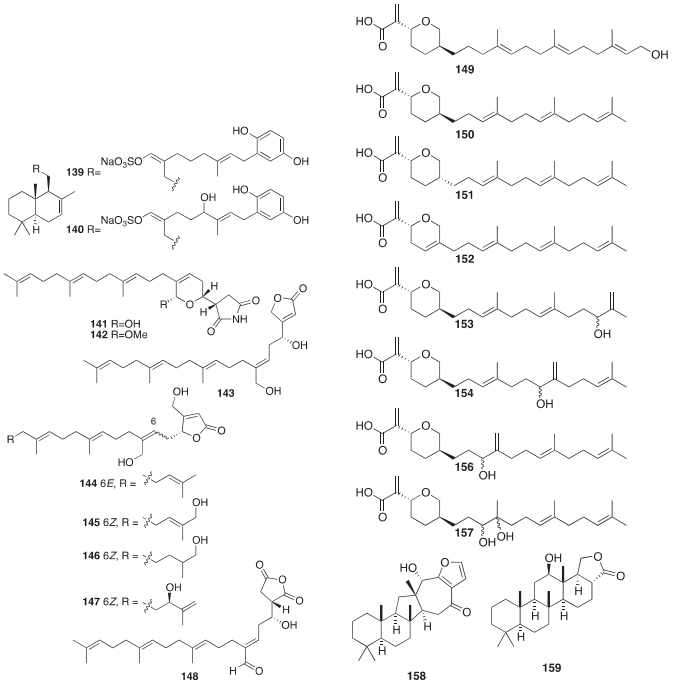
Coscinoderma spp. were studied from Australia, Solomon Islands, Japan and New Caledonia (De Marino et al., 2009; Loukaci et al., 2001; Tsukamoto et al., 2001; Yong et al., 2008a, 2008b). Extraction of a *Coscinoderma mathewsi* from the Solomon Islands led to the isolation of two novel nitrogen containing cheilanthane sesterterpenoids, coscinolactams A **108** and B **109**, together with known suvanine **110** (De Marino et al., 2009). But extraction of *Coscinoderma mathewsi* collected near Mooloolaba, S.E. Queensland, led to the isolation of the furanoterpene esters **111–113** together with the known furanoterpenes **114–116**. The CDC25A inhibitor coscinosulfate **117**, a sesquiterpene sulfate was identified from the New Caledonian sponge *Coscinoderma matthewsi* (Yong et al., 2008a).

Fascaplysinopsis spp. were reported from Fiji and Madagascar and they produced alkaloids and macrolides (Bishara et al., 2010; Segraves et al., 2004). Seven new nitrogenous macrolides, designated salarins D–J **118–124**, closely related to salarin A **125**, were reported from the Madagascar *Fascaplysinopsis sp*. sponge (Bishara et al., 2010). The Fijian collection (Segraves et al., 2004) on the other hand yielded



the known fascaplysin **126** and 12 new structurally related alkaloids **127–138**.

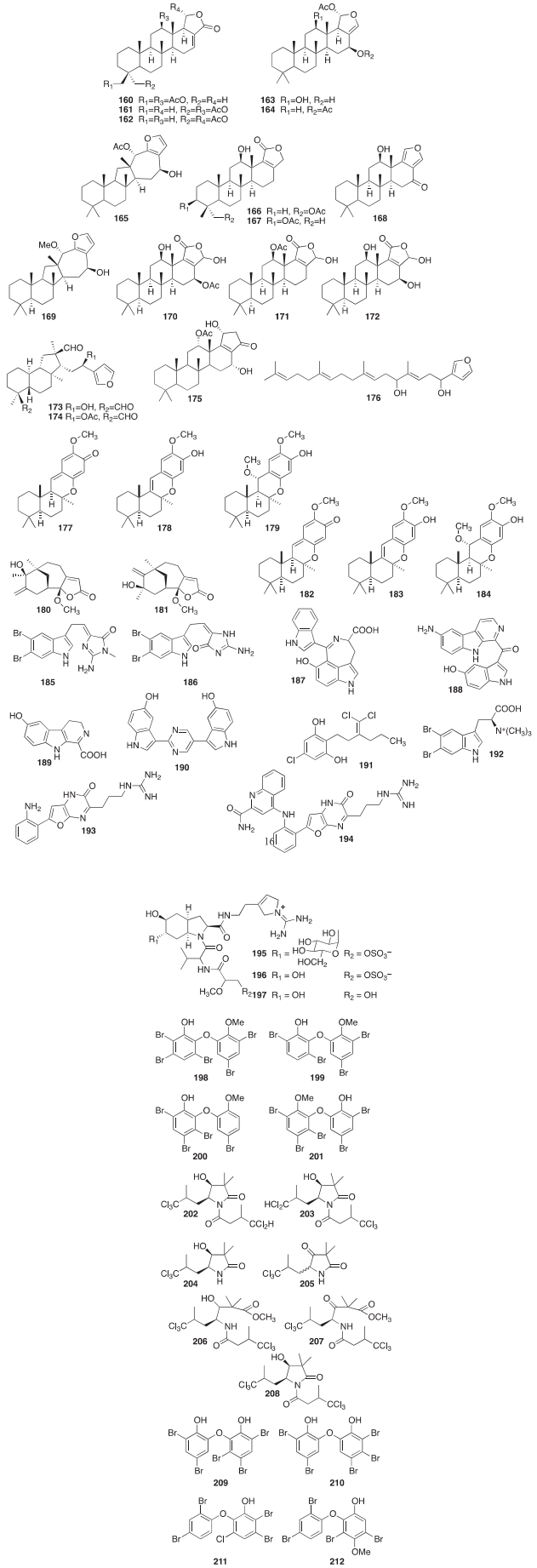
Hippospongia spp. have been reported from Japan, Taiwan, Madagascar and Indonesia and a variety of chemical classes have been reported, including terpene, hydroquinone and α-oxoamide (Chang et al., 2012; Craig et al., 2002; Musman et al., 2001; Piao et al., 2011). Two new sesterterpene sulfates, hipposulfates A **139** and B **140** were isolated from an Okinawan sponge, *Hippospongia* cf. *metachromia* (Musman et al., 2001). While eight new acyclic manoalide-related sesterterpenes, hippolides A–H **141-148** were isolated from the South China Sea sponge *Hippospongia lachne* (Piao et al., 2011). A *Hippospongia* sp. collected in Indonesia gave barangcadoic acid A **149** and rhopaloic acids D **150** to G **157** which exhibited RCE protease inhibitory activity (Craig et al., 2002). A *Hippospongia* sp. collected from Taiwan was reported to give the pentacyclic sesterterpene,



hippospongide A **158**, and one new scalarane sesterterpenoid, hippospongide B **159** (Chang et al., 2012).

Hyrtios spp. have been reported from a wide range of countries including Maldives, Egypt, China, Japan, Indonesia, Papua New Guinea, and Micronesia and Thailand. A large range of terpenoid and alkaloid natural products (**160–194**) has been reported from this genus (Aoki et al., 2001; Endo et al., 2007; Inman et al., 2010; Kamel et al., 2009; Lee et al., 2009; Longeon et al., 2011; Mahidol et al., 2009; Piña et al., 2003; Qiu et al., 2004; Robinson et al., 2009; Salmoun et al., 2002; Sata et al., 2005; Sauleau et al., 2006; Takahashi et al., 2011; Yamanokuchi et al., 2012; Youssef, 2005a; Youssef et al., 2002, 2004).

Lamellodysidea spp. produce chemical classes which vary with country. An Australian collection (Carroll et al., 2004) yielded dysinosins B–D **195–197,** from the sponge *Lamellodysidea chlorea*, and the Indonesian Sponge *Lamellodysidea herbacea* gave polybrominated diphenyl ethers **198–201** (Hanif et al., 2007). But the same sponge *Lamellodysidea herbacea* collected from the Red Sea (Sauleau et al., 2005) gave new polychlorinated pyrrolidinones **202–208** and a Fiji collection (Calcul et al., 2009) gave the new polyhalogenated diphenyl ethers **209–212** along with a number of known compounds (Calcul et al., 2009; Carroll et al., 2004; Faulkner et al., 1994; Handayani et al., 1997; Hanif et al., 2007; Hattori et al., 2001;



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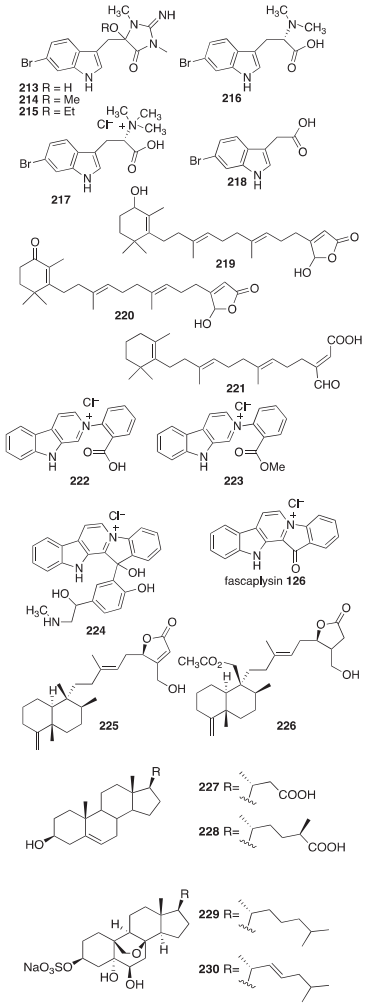
Kuniyoshi et al., 1985; Liu et al., 2004; Salvá and Faulkner, 1990; Sauleau et al., 2005; Sharma and Vig, 1972; Sionov et al., 2005; Unson and Faulkner, 1993; Unson et al., 1994).

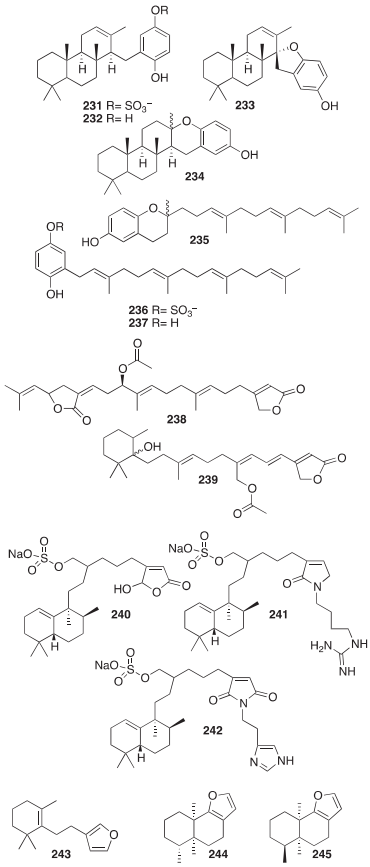
Collection of *Thorectandra* spp. from Papua New Guinea gave the brominated tryptophan derivatives **213–218** (Segraves and Crews, 2005) and three new sesterterpenoids, 16-oxoluffariellolide **219**, 16-hydroxyluffariellolide **220** and trienoic acid **221** (Cao et al., 2005). But collections of the same genus from Palau gave alkaloids **222** and **223**(Charan et al., 2004) and **224** (Charan et al., 2002b) along with known compound fascaplysin **126**. Another collection from Palau gave the terpenes thorectandrol A **225** and B **226** (Charan et al., 2001).

In contrast, other genera were found to produce the same chemical class when sampled from different locations. For example *Euryspongia* spp. from Vanuatu (Mandeau et al., 2005) gave the new steroids **227** and **228** and samples from Palau gave different compounds (eurysterols



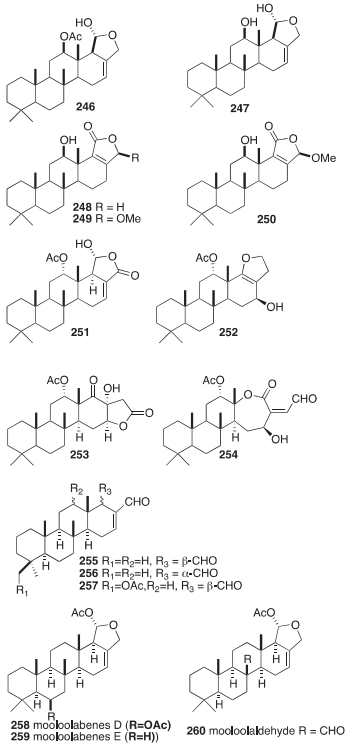
A **229** and B **230**) of the same chemical class (Boonlarppradab and Faulkner, 2007; Mandeau et al., 2005).

Fasciospongia spp. have been reported to produce a wide range of terpenoids from locations in Australia **231–237** (Zhang et al., 2011),



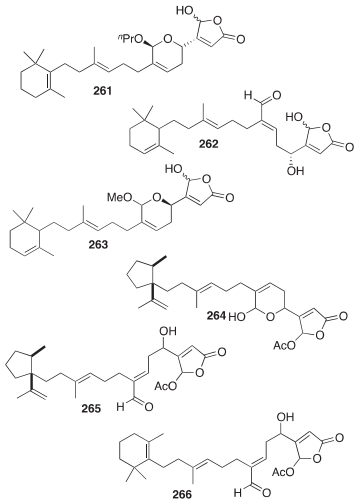
Croatia **238–239** (De Rosa and Carbonelli, 2006), Palau **240–242** (Yao et al., 2009) and Portugal **243–245** (Gaspar et al., 2008).

Hyatella cribriformis from India (Kumar et al., 2008) gave the scalarane sesterterpenes **246–249** and *Hyatella intestinalis* from the Gulf of California, Mexico (Hernández-Guerrero et al., 2006) contains a variety of new scalarane-related sesterterpenes including **251–254** and the same species from Australia (Somerville

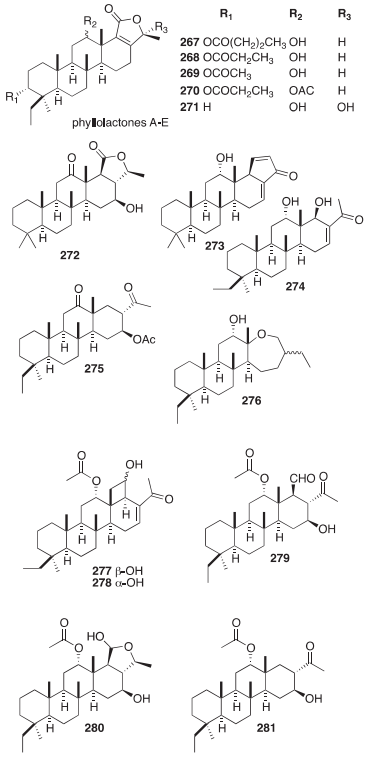


et al., 2006) gave a variety of norsesterterpenes, mooloolabenes **255–259** and mooloolaldehyde **260**.

Similarly, the same broad chemical class was produced by *Luffariella* spp. collected from a variety of areas. A collection from Micronesia (Zhou and Molinski, 2006) gave a derivative of manoalide **261**, a collection from Japan (Tsuda et al., 2002) gave luffariolides H **262** and J **263**, and an Australian (Ettinger-Epstein et al., 2007) collection of *Luffariella variabilis* gave structurally related compounds **264–266**.

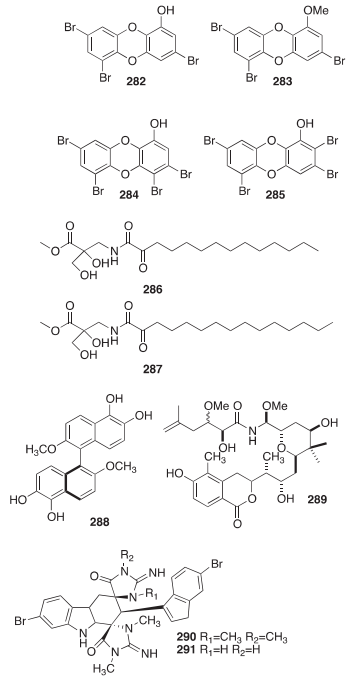


A rich source of scalarane sesterterpenes has been the genus *Phyllospongia*. With *Phyllospongia lamelosa* from the Indo West Pacific (Chang et al., 2001) giving bishomoscalarane sesterterpenes **267–271**, *Phyllospongia foliascens* from China (Zhang et al., 2009) providing phyllofolactone L **272**, phyllofenone D **273** and phyllofenone **274**, and *Phyllospongia madagascarensis* from Madagascar, (Ponomarenko et al., 2004) providing compounds **275** and **276**. A Papua New Guinea (Li et al., 2007) sample of *Phyllospongia papyracea* gave six new bishomoscalarane sesterterpenes including two rare scalaranes **277** and **278** with a cyclobutane ring in the molecule and an



Indonesian (Roy et al., 2002) sample gave eight new scalarane class sesterterpenes including compounds **279–281**.

A number of comparatively uncommon chemical classes were reported from *Sarcotragus spp.* These classes of compounds included the glycerolipids **86** and **87** (Liu et al., 2006a) the furanoterpene **76** (an isomer of kurospongin) (Liu et al., 2003), the cyclitol derivatives **82** and **83** (Liu et al., 2002b) and the pyrroloterpenoids **72–75** (Liu et al., 2003). Unusual brominated compounds were also reported, such as the bromo-tryptophans **213–218** from *Thorectandra* spp. (Segraves and Crews, 2005), the tribromodibenzo-*p*-dioxins **282** and **283** (Utkina et al., 2002) and the tetrabrominated dibenzo-*p*-dioxins **284** and **285** (Utkina et al., 2001) from *Dysidea dendyi*. Further examples of structurally interesting compounds include the alpha-oxoamides salaramide A **286** and salaramide B **287** from *Hippospongia* sp. (Bensemhoun et al., 2010), a pyran derivative **20** from *Ircinia felix* (Granato et al., 2005), pyrrolidiones **202–208** from *Lamellodysidea herbacea* (Sauleau et al., 2005), the naphthalene dimer **288** from *Lendenfeldia* sp. (Dai et al., 2007), psymberin which is the same compound as Irciniastatin A (Pettit et al., 2004) **289** from *Psammocinia* sp. (Cichewicz et al., 2004b)



and the bisspiroimidazolidinone derivatives dictazolines A **290** and B **291** from *Smenospongia cerebriformis* (Dai et al., 2008).

The structural diversity produced by these sponges is further indicated by the isolation of the thiazole derivative **96** (Sonnenschein et al., 2006), the thiopyrone **95** (Johnson et al., 2008), the latrunculin analogs **292–293** (Amagata et al., 2008), the betaines **294–296** (Sakai et al., 2004), the ceramides **9** (Guan and Zeng, 2010; Ibrahim et al., 2008; Ramesh et al., 2001; Zhang et al., 2005), polychlorinated compounds **191** (Harrigan et al., 2001; Sata et al., 2005; Sauleau et al., 2005), the puupehenone-related dimer **307** (Utkina et al., 2011), the indole derivatives **185–189** (Longeon et al., 2011), bromophenols **308–311** (Hattori et al., 2001), cyclitol derivatives **83–85** (Liu et al., 2002b),

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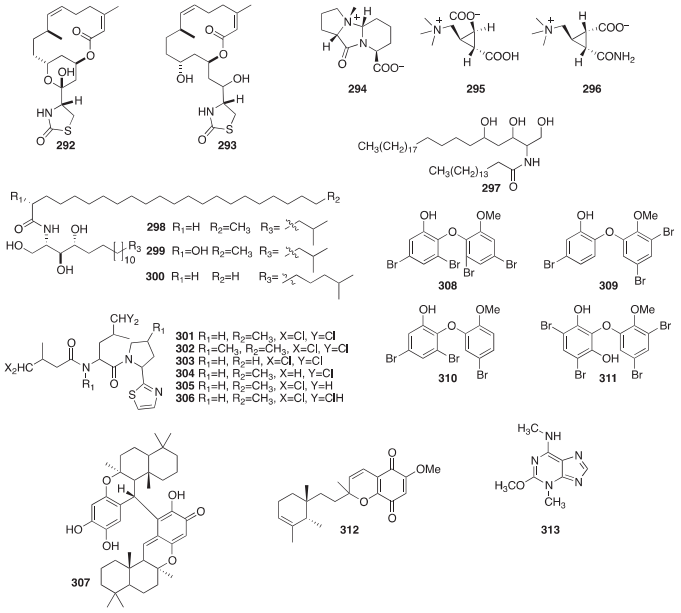


metachromin J **312** (Takahashi et al., 2006) and the dimethyl purine **313** (Suciati et al., 2011).

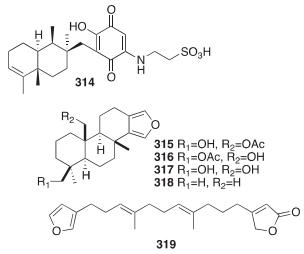
5. The biological activity and bioactive compounds reported from different genera of Dictyoceratida

As shown in Table 3, the compounds reported from the order Dictyoceratida exhibit a diverse range of bioactivities, including antibacterial, anticancer, antifungal, anti-HIV, anti-inflammatory, cytotoxicity, Gsk-3β inhibitor, inhibitor of phosphatase (Cdc25B), inhibitor of PLA2, inhibitor of protein kinase, and other miscellaneous activities. The compounds extracted from the six genera- *Dysidea*, *Ircinia*, *Sarcotragus*, *Spongia*, *Hyrtios* and *Hyatella*- comprised 55% of the total reported bioactivities.

The biological activities reported showed a high proportion of compounds with cytotoxic (43%) and anticancer activity (10%). The compounds were generally tested with various cancer cell lines and at certain concentrations showed toxicity. In this review the definition of an anticancer compound is one which has a known mode of action such as apoptosis or defined cell cycle arrest, or one which is effective in animal models. However, further studies are required to obtain development candidates with anticancer potential such as solid tumor selectivity, followed by trials in tumor-bearing mice (Valeriote et al., 2011).

The antimicrobial activity is relatively easy to test and makes up just over 10% of all reported activities. Other specific bioactivities were reported for approximately one third of compounds but as is often the case with negative results there is little information in the reports as to whether compounds were tested but showed no activity in particular assays.

Compounds within the terpenoid chemical class were reported to have a wide range of activities such as antibacterial, anticancer, cytotoxicity, anti-HIV, antituberculosis, Trh receptor 2 binding activity, PLA2 activity, brine shrimp toxicity and inhibition of fertilized sea urchin eggs. Terpenes isolated from different genera were specifically reported to have different types of activity. For example, the terpenes **267–271** produced by a *Phyllospongia* sp. showed anti-HIV activity (Chang et al., 2001); but some derivatives of compounds from *Hyrtios* sp. showed antibacterial activity (Kamel et al., 2009); The terpene dysidine **314** produced by a *Dysidea* sp. showed PLA2 activity (Giannini et al., 2001) while steroids **315–318** produced by *Spongia* sp. showed Trh receptor 2 binding activity (Carroll et al., 2008). The bromophenols **308–311** showed antimacro and microalgal activity (Hattori et al., 2001); irciniastatin A **36** showed anticancer (Pettit et al., 2004) activity, while acids **105–107** were reported as a Gsk-3β inhibitors (McCulloch et al., 2009). Although anti-inflammatory activity was reported for different chemical classes of compounds, this activity was reported only with compounds from three genera, namely *Coscinoderma*, *Scalarispongia* and *Dysidea*. Lipids are common in marine sponges and the genus *Lendenfeldia* produced furanolipid **319** which showed activity against breast tumor cells (Liu et al., 2008).



6. Potential solution for the supply of bioactive compounds derived from Dictyoceratida

A major issue with compounds produced by sponges is that if the compound is required for clinical development and cannot be produced by other means, vast amounts of the sponge will be required (Lipton and Sunith, 2009). Therefore, consideration must be given for the sustainable aquaculture of Dictyoceratida sponges. *In situ* aquaculture methods for *Dysidea avara* have been successfully tried in the Northwestern Mediterranean and the results indicated that the rope method was successful (de Caralt et al., 2010). The best method will depend on the sponge species and the environmental characteristics of the culture location. This implies previous knowledge of the biology and physiology (e.g., elasticity, recovery capability, growth) of the target species and the environmental conditions of the selected zone (e.g., water flow, temperature, etc.) (de Caralt et al., 2010).

Therefore, the features of the environment in those particular areas could be instructive for the sustainable aquaculture of sponges belonging to Dictyoceratida. One study strongly supports hydrodynamic features (wave exposure and current) as determinants of the distribution of several species (Bannister et al., 2007). As hydrodynamic features are related to rates and types of sediment (Larcombe et al., 2001) as well as to food availability (Riisgard, 1988), the specific effects of the hydrodynamic features are clearly relevant for the determination of suitable sites for the aquaculture of sponges. Duckworth et al., 2008, found that the spatial variability of Dictyoceratida sponges in Torres Strait is influenced by a combination of environmental, biological and stochastic processes (Duckworth et al., 2008). The distribution patterns of sponges are also influenced by environmental and biological factors, including water flow and depth (Wilkinson and Evans, 1989), predation (Dunlap and Pawlik, 1996), light intensity (Wilkinson and Trott, 1985) and substrate and habitat type (Adjeroud, 1997; Schubauer et al., 1990). In addition to environmental and biological factors, the spatial variation of sponges may be influenced by chance or stochastic events (Alcolado, 1994; Zea et al., 1994).

Dictyoceratida sponges are well known for their symbiotic bacteria and some sponge-derived compounds have been reported to be produced by sponge-associated microorganisms. Although bioactive compounds like terpenoids and polyketides had been explored from Irciniidae sponges, their original producer has not been figured out (Hardoim and Costa, 2014). If the real producer belongs to a sponge-associated bacterium, it may provide an alternative option with easy scale-up fermentation for its production. Secondly, expressing the biosynthetic pathway of interest in an easily cultivable surrogate host are the two most promising approaches to meet the supply problem to date (Wilson et al., 2014; Wenzel et al., 2015). Another way to

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Table 3
Total number of new compounds isolated from different genera of marine sponges with reported bioactivities from 2001 to 2012 (T = Thorectidae, S = Spongiidae, D = Dysideidae, I = Irciniidae and U = Unknown) (Family).

Name of Genera	Family	Antibacterial	Anticancer	Antifungal	Anti-HIV	Anti-inflammatory	Cytotoxicity	Gsk-3β inhibitor	Inhibitor of phosphatase (Cdc25B)	Inhibitor of PLA2	Inhibitor of protein kinase	Inhibitor of RCE protease	Miscellaneous	Total
Citronia sp.	D	–	–	1	–	–	–	–	–	–	–	–	–	1
Dysidea sp.	D	–	2	–	–	1	23	–	1	3	–	–	21	51
Euryspongia sp.	D	–	–	2	–	–	2	–	–	–	–	–	1	5
Lamellodysidea sp.	D	4	–	–	–	–	2	–	–	–	–	–	4	10
Ircinia sp.	I	–	6	–	–	–	15	–	–	–	3	–	5	29
Psammocinia sp.	I	–	1	–	–	–	3	–	–	–	–	–	4	8
Sarcotragus sp.	I	5	1	–	–	–	29	–	–	–	–	–	9	44
Coscinoderma sp.	S	–	–	–	–	2	8	–	1	–	–	–	10	21
Hippospongia sp.	S	–	–	–	–	2	3	–	–	–	–	2	2	9
Hyatella sp.	S	5	6	–	–	–	11	–	–	–	–	–	7	29
Spongia sp.	S	8	3	1	–	–	14	–	–	–	–	–	19	45
Cacospongia sp.	T	1	6	–	–	–	2	–	–	–	1	–	2	12
Carteriospongia sp.	T	–	–	–	–	–	2	3	–	–	–	1	-	6
Dactylospongia sp.	T	1	1	–	–	–	9	–	–	–	–	–	-	11
Fascaplysinopsis sp.	T	–	3	–	–	–	6	–	–	–	–	–	2	11
Fasciospongia sp.	T	7	–	–	–	–	1	–	–	–	–	–	-	8
Hyrtios sp.	T	6	3	2	–	1	15	–	–	–	–	–	9	36
Lendenfeldia sp.	T	–	2	–	–	–	2	–	–	–	–	–	1	5
Luffariella sp.	T	2	–	1	–	–	3	–	–	–	–	–	-	6
Phyllospongia sp.	T	–	1	–	5	–	13	–	–	–	–	–	6	25
Psammoclema sp.	T	–	4	–	–	–	–	–	–	–	–	–	–	4
Scalarispongia sp.	T	–	–	–	–	2	–	–	–	–	–	–	–	2
Smenospongia sp.	T	2	–	–	–	–	5	–	–	–	–	–	3	10
Strepsichordaia sp.	T	–	–	–	–	–	3	–	–	–	–	–	–	3
Thorecta reticulata	T	–	–	–	–	–	3	–	–	–	–	–	–	3
Thorectandra sp.	T	4	3	–	–	–	5	–	3	–	–	–	1	16
Candidaspongia sp.	U	–	–	–	–	–	5	–	–	–	–	–	–	5
Unknown	U	–	3	–	–	–	–	–	–	–	–	–	–	3
Total		45	45	7	5	8	185	3	5	3	4	3	110	423

Table 4
Organizations reporting on compounds from Dictyoceratida sponges during 2001 to 2012.

Organization	Location/city	Country
University of Queensland	Brisbane	Australia
Eskitis Institute for Drug Discovery, Griffith University	Brisbane	Australia
The Western Australian Museum	Fremantle	Australia
University of Newcastle	Newcastle	Australia
Calvary Mater Newcastle Hospital	Newcastle	Australia
Children's Medical Research Institute	Sydney	Australia
Australian Institute of Marine Science (AIMS)	Townsville	Australia
University of São Paulo	São Paulo	Brazil
University of British Columbia	Vancouver	Canada
Genome Sciences Centre	Vancouver	Canada
Chinese Academy of Sciences	Beijing	China
Peking University	Beijing	China
Beijing University of Chinese Medicine	Beijing	China
Changchun University of Chinese Medicine (CUCM)	Changchun	China
Guangzhou First Municipal People's Hospital	Guangzhou	China
Sun Yat-Sen University	Guangzhou	China
Jinan University	Guangzhou	China
South China Sea Institute of Oceanology (SCSIO)	Guangzhou	China
Shanghai Institutes for Biological Sciences (SIBS)	Shanghai	China
Second Military Medical University (SMMU)	Shanghai	China
Donghua University	Shanghai	China
Shenyang Pharmaceutical University (SPU)	Shenyang	China
Xiamen University (XMU)	Xiamen	China
Fourth Military Medical University (FMMU)	Xi'an	China
Suez Canal University	Ismailia	Egypt
Assiut University	Assiut	Egypt
The University of the South Pacific (USP)	Suva	Fiji
Pierre Fabre Research Institute	Ramonville	France
National Museum of Natural History	Paris	France
Heinrich Heine University (HHU)	Dusseldorf	Germany
Institute for Pharmaceutical Biology, University of Bonn	Bonn	Germany
University of Athens	Athens	Greece
Indian Institute of Chemical Technology	Hyderabad	India
Andhra University	Visakhapatnam	India
University of Bandar Lampung	Bandar Lampung	Indonesia
Research and Development Centre for Oceanology	Jakarta	Indonesia
Research Center of Marine and Fisheries Product Processing and Biotechnology	Jakarta	Indonesia
Hasanuddin University	Makassar	Indonesia
Diponegoro University	Semarang	Indonesia
Tel Aviv University	Tel Aviv	Israel
University of Naples	Naples	Italy
University Study Naples	Naples	Italy
Institute of Biomolecular Chemistry of the National Research Council	Naples	Italy
University of Salerno	Salerno	Italy
Kyushu University	Fukuoka	Japan
Kanazawa University	Kanazawa	Japan
Ehime University	Matsuyama	Japan
Nagoya University	Nagoya	Japan
The University of the Ryukyus	Okinawa	Japan
Hokkaido University	Sapporo	Japan
Osaka University	Suita	Japan
Kirin Brewery Company	Tokyo	Japan
The Korean Ocean Research and Development Institute (KORDI)	Ansan	Korea
Pusan National University	Busan	Korea
Dong-eui University	Busan	Korea
Korea Maritime and Ocean University	Busan	Korea
Korea Basic Science Institute (KBSI)	Daejeon	Korea
Hannam University	Daejeon	Korea
Korean Research Institute of Chemical Technology (KRICT)	Daejeon	Korea
Inje University	Gimhae	Korea
Kunsan National University	Gunsan	Korea
National Institute of Biological Resources(NIBR)	Incheon	Korea
Kyung Hee University	Seoul	Korea
Korea Institute of Science and Technology	Seoul	Korea
The University of Amsterdam	Amsterdam	Netherlands
University of Otago	Dunedin	New Zealand
National Institute of Engineering, Technology and Innovation (INETI)	Lisbon	Portugal
Institute of Bioorganic Chemistry of the Russian Academy of Sciences	Vladivostok	Russia
Pacific Institute Of Bio-Organic Chemistry	Vladivostok	Russia
University of Cadiz	Cadiz	Spain
Zapateira University of Coruña	Coruña	Spain
National Sun. Yat-Sen University	Kaohsiung	Taiwan
National Taiwan University	Taipei	Taiwan
Ramkhamhaeng University	Bangkok	Thailand
Ubon Ratchathani Rajabhat University	Muang	Thailand
Henry Ford Hospital	Detroit	USA
The University of Wisconsin	Madison	USA
The University of Hawaii	Manoa	USA
The University of Oklahoma	Norman	USA
The University of California	San Diego Santa Cruz	USA
Texas Biomedical Research Institute	San Antonio	USA

ensure supply is using sponge cell culture, which is still a growing field (de Caralt et al., 2007; Koopmans et al., 2009; Pomponi, 2006).

7. Sponge source country vs country of study

Although the sponges have been collected from all over the world only a limited number of countries have carried out the research (Table S1). Japanese research groups have studied 21 sponge genera of which 11 were collected in Japan while the rest were collected from Micronesia, Indonesia, Egypt and Palau. Of the 8 sponge genera studied by Chinese research groups only *Luffariella* sp. was collected from Micronesia, *Sarcotragus* sp. was collected from Korea and the rest were collected from China. Egyptian researchers studied two genera collected from Egyptian waters and one collected from Indonesia. French researchers studied 6 different genera and the sponges were collected from New Caledonia, Vanuatu, Madagascar, Egypt, Fiji and India. Israeli research groups studied 2 genera, all collected from Madagascar. Italian groups studied 5 genera which were collected from the Solomon Islands, Vanuatu, Croatia and Italy. Among the 11 sponge genera studied by researchers based in Australia, the majority were collected from Australia except *Dysidea arenaria* which was from New Caledonia. Korean researchers studied a total of 8 sponge genera of which only *Hyrtios*, *Coscinoderma* and *Ircinia* were collected from Micronesia while the rest belonged to their own collection sites in Korea. Russian researchers studied 6 sponge genera which were collected from Australia, New Zealand, Madagascar and USA. Spanish groups studied 2 sponge genera collected from Mexico and Indonesia. Indian and Taiwanese researchers each studied two sponge genera and Thai and Portuguese groups studied one sponge genus each, all collected from their own waters. Researchers in the USA studied the 16 sponge genera which were collected from many different countries with only two *Hyrtios* spp. and one *Lendenfeldia* sp., collected from USA waters. All Papua New Guinean sponges were collected by USA or Canadian-based research groups.

The USA, Japan, Australia, China and Korea stand out as the leading countries with ample scientific expertise and resources for marine sponge natural products discovery. Countries like Indonesia and Papua New Guinea, though having significant sponge resources, require more international collaboration to build their capacity. All US NIH funded researchers have to adhere to the NCI's Letter of Collection (first used in 1989, prior to the CBD). This was made a requirement in the middle 1990s and has always been the case in any collections made by the NCI. Even, only in very few cases has information on

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collection permits (which is essential according to the Nagoya Protocol (Protocol, 2010)) been reported in the literature.

In most instances there has been no information in the published literature about the permit process, benefit sharing and conservation of biodiversity as required under the UN Convention on Biodiversity and the Nagoya Protocol (Anonymous, 1992; Protocol, 2010) and this is thus an area for improvement for any future studies.

8. Institutions researching Dictyoceratida

The institutions, universities, hospitals or research organizations working on Dictyoceratida sponges over the 2001–2012 are presented in Table 4.

9. Conclusions

The order Dictyoceratida was found to be the most prolific producer of new compounds among all the sponge orders studied during 2001–2012. Within this order the most promising genera were found to be *Dysidea* sp. from Dysideidae family; *Sarcotragus* sp. and *Ircinia* sp. from Irciniidae family; *Spongia* from Spongiidae family and *Hyrtios* sp. and *Phyllospongia* sp. for their contribution of large numbers of new bioactive compounds. Further research can be fruitful if other species from these genera can be collected. And the understanding of the ecological and environmental impacts of collection sites on sponge biodiversity and chemical diversity can be established. This will call form partnership between natural product chemistry and marine biologist.

The success rate in terms of sponge-derived therapeutic drug development is still not satisfactory. Part of the reason is that in many laboratories the chemists who isolate, purify and determine the structure of the compounds do not have the capacity to test their compounds in a large range of biological assays. Similarly, partnerships between natural product chemists and biologist can be beneficial in discovering multiple valuable activities if a wide range of biological assays could be tested.

This review highlights the importance of complying with the UN Convention on Biological Diversity and following the Nagoya Protocol for future studies to ensure the R&D outcomes and commercial benefits of sharing between the countries where samples are collected and the research is carried out.

The wide range of chemical structural diversity and bioactivities produced by the Dictyoceratida sponges make this order a potential source of new therapeutic molecules. Future studies could focus on the large number of unexplored species in this order, expecting many more new compounds to be discovered.

The information in the Supplementary Table (Table S1) contains all the new compounds from marine sponges of the order Dictyoceratida from 2001 to 2012, therefore, in addition to the chemical structures and descriptions already discussed the table contains compounds from the following sponge species: *Cacospongia mycofijiensis* (Johnson et al., 2011), *Cacospongia scalaris* (Tsoukatou et al., 2003), other *Cacospongia* spp. (Tanaka et al., 2001b; Rubio et al., 2007), *Candidaspongia* spp. (Trianto et al., 2011; Whitson et al., 2011), *Carteriospongia flabellifera* (Diyabalanage et al., 2012), *Coscinoderma* sp. (Bae et al., 2011), *Dactylospongia* spp. (Jankam et al., 2007; Cutignano et al., 2001), *Dysidea arenaria* (Piggott and Karuso, 2005; Qiu and Wang, 2008), *Dysidea avara* (Jiao et al., 2011); *Dysidea* cf. *arenaria* (Agena et al., 2009), *Dysidea chlorea* (Ueda et al., 2006), *Dysidea fragilis* (Yu et al., 2006; Yu et al., 2009), *Dysidea herbacea* (Deschamps et al., 2007; Sakai et al., 2001; Utkina and Denisenko, 2006), *Dysidea septosa* (Huang et al., 2008), *Dysidea* spp. (Katavic et al., 2012; Suna et al., 2009; Goetz et al., 2001; Govindam et al., 2012; Huang et al., 2005; Pérez-García et al., 2005; Ren et al., 2010; Sadar et al., 2008; Shao et al., 2006; Zhang et al., 2008; Ardá et al., 2005), *Fasciospongia* sp. (Yao and Chang, 2007), *Hippospongia metachromia* (Shen et al., 2001), *Hippospongia metachromia* (Skepper and Molinski, 2008), *Hyattella* spp. (Jeon et al. 2011, Yang et al., 2010a), *Hyrtios erectus (erecta)* (Yu et al., 2005), *Ircinia selaginea* (Lamark) (Yan et al., 2001); *Ircinia* spp. (Buchanan et al., 2001; Emura et al., 2006; Lee et al., 2012, Li et al., 2009; Murakami et al., 2002), *Lendenfeldia chondrodes* (Sakai and Kamiya, 2006), *Lendenfeldia* sp. (Chill et al., 2004; Chaturvedula et al., 2004); *Luffariella* cf. *variabilis* (Gauvin-Bialecki et al., 2008), *Phyllospongia foliascens* (Zhang et al., 2010), *Phyllospongia papyracea* (Lan and Li, 2007), *Psammocinia* aff. *bulbosa* (Robinson et al., 2007), *Psammocinia* sp. (Cichewicz et al., 2004a), *Psammoclema* sp. (Holland et al., 2009), *Scalarispongia aqabensis* (Youssef et al., 2010), *Smenospongia aurea* (Hu et al., 2002), *Smenospongia* sp. (Rho et al., 2004), *Spongia (Heterofibria)* sp. (Salim et al., 2010; Ponomarenko et al., 2007); *Spongia officinalis* (Manzo et al., 2011); *Spongia* spp. (Choi et al., 2004; Grassia et al., 2001; Gross et al., 2009; Mori et al., 2007; Nam et al., 2006; Nam et al., 2007; Takahashi et al., 2007b; Tokue et al., 2006; Tsukamoto et al., 2003; Cao et al., 2004), Carroll et al., 2009), *Spongia zimocca* (Zeng et al., 2001), *Spongia irregularis* (Carr et al., 2007), *Thorecta reticulata* (Ovenden et al., 2011a) and *Thorectandra* sp. (Charan et al., 2002a).

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Table S1. New compounds isolated from marine sponges of the order Dictyoceratida from 2001 to 2012; including genus/species, family, compound name, chemical class, activity, source (depth), country of collection, country of study and reference. (NR=None Reported).

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Cacospongia mycofijiensis	Thorectidae	Aignopsanoic acid A	Sesquiterpene	Antiparasitic	Kimbe Bay	Papua New Guinea	USA	(Johnson et al., 2009)
Cacospongia mycofijiensis	Thorectidae	Methyl aignopsanoic acid A	Sesquiterpene	Antiparasitic	Kimbe Bay	Papua New Guinea	USA	(Johnson et al., 2009)
Cacospongia mycofijiensis	Thorectidae	Isoaignopsanoic acid A	Sesquiterpene	NR	Kimbe Bay	Papua New Guinea	USA	(Johnson et al., 2009)
Cacospongia mycofijiensis	Thorectidae	Aignopsanoic acid B	Sesquiterpenoid (bicyclic)	NR	Northern coastlines of New Britain	Papua New Guinea	USA	(Johnson et al., 2011)
Cacospongia mycofijiensis	Thorectidae	Apo-latrunculin T	NR	Antiprotozoal	Northern coastlines of New Britain	Papua New Guinea	USA	(Johnson et al., 2011)
Cacospongia mycofijiensis	Thorectidae	20-methoxy-fijianolide A	NR	Modest microtubule-stabilizing effects	Northern coastlines of New Britain	Papua New Guinea	USA	(Johnson et al., 2011)
Cacospongia mycofijiensis	Thorectidae	Aignopsane ketal	Sesquiterpenoid (bicyclic)	NR	Northern coastlines of New Britain	Papua New Guinea	USA	(Johnson et al., 2011)
Cacospongia mycofijiensis	Thorectidae	Ctp-431	Thiopyrone	Inhibitor of DNA - dependent protein kinase	Beqa Lagoon (15-20 m)	Fiji	USA	(Johnson et al., 2008)
Cacospongia mycofijiensis	Thorectidae	Latrunculol A	Latrunculin analogue	Anticancer	Beqa Lagoon (15-20 m)	Fiji	USA	(Amagata et al., 2008)
Cacospongia mycofijiensis	Thorectidae	Latrunculol B	Latrunculin analogue	Anticancer	Beqa Lagoon (15-20 m)	Fiji	USA	(Amagata et al., 2008)
Cacospongia mycofijiensis	Thorectidae	18-epi-latrunculol A	Latrunculin analogue	Anticancer	Beqa Lagoon (15-20 m)	Fiji	USA	(Amagata et al., 2008)
Cacospongia mycofijiensis	Thorectidae	Latrunculol C	Latrunculin analogue	Anticancer	Beqa Lagoon (15-20 m)	Fiji	USA	(Amagata et al., 2008)
Cacospongia mycofijiensis	Thorectidae	Latrunculone A	Latrunculin analogue	Anticancer	Beqa Lagoon (15-20 m)	Fiji	USA	(Amagata et al., 2008)
Cacospongia mycofijiensis	Thorectidae	Latrunculone B	Latrunculin analogue	Anticancer	Beqa Lagoon (15-20 m)	Fiji	USA	(Amagata et al., 2008)
Cacospongia mycofijiensis	Thorectidae	Mycothiazole-4,19-diol	Thiazole derivative	NR	Mele Bay (15-20 m)	Vanuatu	USA	(Sonnenschein et al., 2006)
Cacospongia scalaris	Thorectidae	16-acetoxy-dihydrodeoxoscalarin	Sesterterpene	NR	Astakos Gulf, Etoloakarnania (30 m)	Greece	Greece	(Tsoukatou et al., 2003)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Cacospongia scalaris	Thorectidae	Astakolactin	Sesterterpene	NR	Astakos Gulf, Etoloakarnania (30 m)	Greece	Greece	(Tsoukatou et al., 2003)
Cacospongia sp.	Thorectidae	(+)-Isojaspic acid	Meroditerpene	Antibacterial	Milne Bay (9.1-18.3m)	Papua New Guinea	USA	(Rubio et al., 2007)
Cacospongia sp.	Thorectidae	Cacofuran A	Diterpene (furano)	Cytotoxicity	Yonaguni Island, Okinawa (20 m)	Japan	Japan	(Tanaka et al., 2001b)
Cacospongia sp.	Thorectidae	Cacofuran B	Diterpene (furano)	Cytotoxicity	Yonaguni Island, Okinawa (20 m)	Japan	Japan	(Tanaka et al., 2001b)
Candidaspongia sp.	Unknown	Precandidaspongiolide A	Macrolide	Cytotoxicity	NR	Papua New Guinea	USA	(Whitson et al., 2011)
Candidaspongia sp.	Unknown	Precandidaspongiolide B	Macrolide	Cytotoxicity	NR	Papua New Guinea	USA	(Whitson et al., 2011)
Candidaspongia sp.	Unknown	Spongiolide A	Macrolide	Cytotoxicity	NR	Papua New Guinea	USA	(Whitson et al., 2011)
Candidaspongia sp.	Unknown	Compound 2 ($C_{52}H_{88}NaO_{14}$)	Macrolide	Cytotoxicity	Kupang, West Timor, East Nusa Tenggara (15–25 m)	Indonesia	Japan	(Trianto et al., 2011)
Candidaspongia sp.	Unknown	Compound 3($C_{32}H_{50}NaO_{13}$)	Macrolide	Cytotoxicity	Kupang, West Timor, East Nusa Tenggara (15–25 m)	Indonesia	Japan	(Trianto et al., 2011)
Carteriospongia flabellifera	Thorectidae	Flabelliferin A	Sesterterpenoid	Cytotoxicity	Tutuba Island (200m)	Vanuatu	USA	(Diyabalanage et al., 2012)
Carteriospongia flabellifera	Thorectidae	Flabelliferin B	Sesterterpenoid	Cytotoxicity	Tutuba Island (200m)	Vanuatu	USA	(Diyabalanage et al., 2012)
Carteriospongia foliascens	Thorectidae	Bishomonorscalarane 1	C-22 Sesterterpenoid	Inhibited RCE-protease activity	Palau Barang Lompo, Makassar, Sulawesi (10 m)	Indonesia	Canada	(Williams et al., 2009a)
Carteriospongia foliascens	Thorectidae	Bishomonorscalarane 2	Sesterterpenoid	NR	Palau Barang Lompo, Makassar, Sulawesi (10 m)	Indonesia	Canada	(Williams et al., 2009a)
Carteriospongia sp.	Thorectidae	Carteriosulfonic acid A	Acid (glycyl conjugates of the ω-brominated lipid (e)-14,14-dibromotetradeca-2, 13-dienoic acid)	Gsk-3β inhibitor	San Miguel Island, Sorsogon (10 m)	Philippines	USA	(McCulloch et al., 2009)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Carteriospongia sp.	Thorectidae	Carteriosulfonic acid B	Acid (glycyl conjugates of the ω-brominated lipid (e)-14,14-dibromotetradeca-2, 13-dienoic acid)	Gsk-3β inhibitor	San Miguel Island, Sorsogon (10 m)	Philippines	USA	(McCulloch et al., 2009)
Carteriospongia sp.	Thorectidae	Carteriosulfonic acid C	Acid (glycyl conjugates of the ω-brominated lipid (e)-14,14-dibromotetradeca-2, 13-dienoic acid)	Gsk-3β inhibitor	San Miguel Island, Sorsogon (10 m)	Philippines	USA	(McCulloch et al., 2009)
Citronia astra	Dysideidae	Citronamide A	Peptide (tetra)	Antifungal	Day Reef, North Queensland	Australia	Australia	(Carroll et al., 2009)
Citronia astra	Dysideidae	Citronamide B	Peptide (tetra)	NR	Day Reef, North Queensland	Australia	Australia	(Carroll et al., 2009)
Coscinoderma mathewsi	Spongiidae	Coscinolactam A	C22- sesterterpenoid	Anti-inflammatory and Inhibited pge2 and no production	Barrier Reef, Vangunu Island	Solomon Islands	Italy	(De Marino et al., 2009)
Coscinoderma mathewsi	Spongiidae	Coscinolactam B	C22- sesterterpenoid	Anti-inflammatory and Inhibited pge2 and no production	Barrier Reef, Vangunu Island	Solomon Islands	Italy	(De Marino et al., 2009)
Coscinoderma mathewsi	Spongiidae	Tetradehydrofurospongin-1 fatty acid ester derivative 1A	Furanoterpene fatty acid ester	NR	Inner Gneerings, off Mooloolaba (10-15 m)	Australia	Australia	(Yong et al., 2008a)
Coscinoderma mathewsi	Spongiidae	Tetradehydrofurospongin-1 fatty acid ester derivative 1B	Furanoterpene fatty acid ester	NR	Inner Gneerings, off Mooloolaba (10-15 m)	Australia	Australia	(Yong et al., 2008a)
Coscinoderma mathewsi	Spongiidae	Tetradehydrofurospongin-1 fatty acid ester derivative 1C	Furanoterpene fatty acid ester	NR	Inner Gneerings, off Mooloolaba (10-15 m)	Australia	Australia	(Yong et al., 2008a)
Coscinoderma mathewsi	Spongiidae	Coscinosulfate	Sesquiterpene sulfate	CDC25 phosphatase inhibitor	Eastern coast	New Caledonia	France	(Loukaci et al., 2001)
Coscinoderma sp.	Spongiidae	Compound 2 ($C_{31}H_{47}O_2$)	Sesterterpene	Cytotoxicity and Miscellaneous	The shore of Weno Island, Chuuk state	Micronesia	Korea	(Bae et al., 2011)
Coscinoderma sp.	Spongiidae	Compound 5 ($C_{31}H_{47}O_6SNa_2$)	Sesterterpene	Cytotoxicity and Miscellaneous	The shore of Weno Island, Chuuk state	Micronesia	Korea	(Bae et al., 2011)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Coscinoderma sp.	Spongiidae	Compound 10 ($C_{25}H_{37}O_6SNa_2$)	Sesterterpene	Cytotoxicity and Miscellaneous	The shore of Weno Island, Chuuk state	Micronesia	Korea	(Bae et al., 2011)
Coscinoderma sp.	Spongiidae	Compound 11 ($C_{26}H_{40}O_7SNa$)	Sesterterpene	Cytotoxicity and Miscellaneous	The shore of Weno Island, Chuuk state	Micronesia	Korea	(Bae et al., 2011)
Coscinoderma sp.	Spongiidae	Compound 12 ($C_{26}H_{39}O_7SNa_2$)	Sesterterpene	Cytotoxicity and Miscellaneous	The shore of Weno Island, Chuuk state	Micronesia	Korea	(Bae et al., 2011)
Coscinoderma sp.	Spongiidae	Compound 13 ($C_{27}H_{43}O_7SNa_2$)	Sesterterpene	Cytotoxicity and Miscellaneous	The shore of Weno Island, Chuuk state	Micronesia	Korea	(Bae et al., 2011)
Coscinoderma sp.	Spongiidae	Compound 14 ($C_{27}H_{45}O_9SNa_2$)	Sesterterpene	Cytotoxicity and Miscellaneous	The shore of Weno Island, Chuuk state	Micronesia	Korea	(Bae et al., 2011)
Coscinoderma sp.	Spongiidae	Compound 15 ($C_{25}H_{38}NO_5SNa_2$)	Sesterterpene	Cytotoxicity and Miscellaneous	The shore of Weno Island, Chuuk state	Micronesia	Korea	(Bae et al., 2011)
Dactylospongia elegans	Thorectidae	Isohyatellaquinone	Sesquiterpene	Cytotoxicity	Inner Gneerings reef, near Mooloolaba (10–15 m)	Australia	Australia	(Yong et al., 2008c)
Dactylospongia elegans	Thorectidae	7,8-dehydrocyclo*Spongia*quinone-2	Sesquiterpene	Cytotoxicity	Inner Gneerings reef, near Mooloolaba (10–15 m)	Australia	Australia	(Yong et al., 2008c)
Dactylospongia elegans	Thorectidae	9-epi-7,8-dehydrocyclo*Spongia*quinone	Sesquiterpene	Cytotoxic	Inner Gneerings reef, near Mooloolaba (10–15 m)	Australia	Australia	(Yong et al., 2008c)
Dactylospongia elegans	Thorectidae	5-epi-smenospongorine	Sesquiterpene aminoquinone	Anticancer	West Flores	Indonesia	Japan	(Aoki et al., 2004)
Dactylospongia elegans	Thorectidae	Nakijinol B	Sesquiterpene benzoxazole	Cytotoxicity	Pugh Shoal, northeast of Truant Island, NT	Australia	Australia	(Ovenden et al., 2011b)
Dactylospongia elegans	Thorectidae	Smenospongine B	Sesquiterpene quinone	Cytotoxicity	Pugh Shoal, northeast of Truant Island, NT	Australia	Australia	(Ovenden et al., 2011b)
Dactylospongia elegans	Thorectidae	Smenospongine C	Sesquiterpene quinone	Cytotoxicity	Pugh Shoal, northeast of Truant Island, NT	Australia	Australia	(Ovenden et al., 2011b)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Dactylospongia elegans	Thorectidae	Neodactyloquinone	Sesquiterpenoid quinone	Cytotoxicity	Coral reef off Ishigaki Island, Okinawa (5 m)	Japan	Japan	(Mitome et al., 2003)
Dactylospongia elegans	Thorectidae	Dactylolactone A	Sesquiterpenoid quinone	NR	Coral reef off Ishigaki Island, Okinawa (5 m)	Japan	Japan	(Mitome et al., 2003)
Dactylospongia elegans	Thorectidae	Dactylolactone B	Sesquiterpenoid quinone	NR	Coral reef off Ishigaki Island, Okinawa (5 m)	Japan	Japan	(Mitome et al., 2003)
Dactylospongia elegans	Thorectidae	Dactylolactone C	Sesquiterpenoid quinone	NR	Coral reef off Ishigaki Island, Okinawa (5 m)	Japan	Japan	(Mitome et al., 2003)
Dactylospongia elegans	Thorectidae	Dactylolactone D	Sesquiterpenoid quinone	NR	Coral reef off Ishigaki Island, Okinawa (5 m)	Japan	Japan	(Mitome et al., 2003)
Dactylospongia elegans	Thorectidae	Dactyloquinone C	Sesquiterpenoid quinone	NR	Coral reef off Ishigaki Island, Okinawa (5 m)	Japan	Japan	(Mitome et al., 2002)
Dactylospongia elegans	Thorectidae	Dactyloquinone D	Sesquiterpenoid quinone	NR	Coral reef off Ishigaki Island, Okinawa (5 m)	Japan	Japan	(Mitome et al., 2002)
Dactylospongia elegans	Thorectidae	Dactyloquinone E	Sesquiterpenoid quinone	NR	Coral reef off Ishigaki Island, Okinawa (5 m)	Japan	Japan	(Mitome et al., 2002)
Dactylospongia n. sp.	Thorectidae	Dactylospongiaquinone	Meroterpenoid (sesquiterpene quinone)	Cytotoxicity, antibacterial	Inner Gneerings, near Mooloolaba (10–15 m)	Australia	Australia	(Jankam et al., 2007)
Dactylospongia sp.	Thorectidae	Dactylolide	Macrolide	Cytotoxicity	Vanuatu islands	Vanuatu	Italy	(Cutignano et al., 2001)
Dysidea arenaria	Dysideidae	19-hydroxypolyfibrospongol B	Sesquiterpenoid hydroquinone	NR	Coral reefs near Hainan Island, South China Sea	China	China	(Qiu and Wang, 2008)
Dysidea arenaria	Dysideidae	9-hydroxyfurodysinin-O-ethyl lactone	Sesquiterpene	NR	NR	New Caledonia	Australia	(Piggott and Karuso, 2005)
Dysidea avara	Dysideidae	Dysidavarone A	Sesquiterpene quinone	Cytotoxicity and (PTP1B) inhibitory activity	Coast of Yongxing Island in Xiasha	China	China	(Jiao et al., 2011)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Dysidea avara	Dysideidae	Dysidavarone B	Sesquiterpene quinone	NR	Coast of Yongxing Island in Xiasha	China	China	(Jiao et al., 2011)
Dysidea avara	Dysideidae	Dysidavarone C	Sesquiterpene quinone	NR	Coast of Yongxing Island in Xiasha	China	China	(Jiao et al., 2011)
Dysidea avara	Dysideidae	Dysidavarone D	Sesquiterpene quinone	Cytotoxicity and (PTP1B) inhibitory activity	Coast of Yongxing Island in Xiasha	China	China	(Jiao et al., 2011)
Dysidea cf. *arenaria*	Dysideidae	7a,11a-diacetoxyisoagatholactone or 7,11-di-epi-dorisenone	Diterpene (*Spongia*n)	Cytotoxicity	Manza in Okinawa Island (35 m)	Japan	Japan	(Agena et al., 2009)
Dysidea cf. *arenaria*	Dysideidae	$C_{24}H_{34}O_7$	Diterpene (*Spongia*n)	Cytotoxicity	Manza in Okinawa Island (35 m)	Japan	Japan	(Agena et al., 2009)
Dysidea cf. *arenaria*	Dysideidae	$C_{24}H_{34}O_6$	Diterpene (*Spongia*n)	Cytotoxicity	Manza in Okinawa Island (35 m)	Japan	Japan	(Agena et al., 2009)
Dysidea cf. *arenaria*	Dysideidae	$C_{22}H_{32}O_4$	Diterpene (*Spongia*n)	Cytotoxicity	Manza in Okinawa Island (35 m)	Japan	Japan	(Agena et al., 2009)
Dysidea cf. *arenaria*	Dysideidae	$C_{22}H_{32}O_4$	Diterpene (*Spongia*n)	Cytotoxicity	Manza in Okinawa Island (35 m)	Japan	Japan	(Agena et al., 2009)
Dysidea cf. *arenaria*	Dysideidae	$C_{27}H_{42}O_6$	Diterpene (*Spongia*n)	Cytotoxicity	Manza in Okinawa Island (35 m)	Japan	Japan	(Agena et al., 2009)
Dysidea cf. *arenaria*	Dysideidae	$C_{27}H_{42}O_7$	Diterpene (*Spongia*n)	Cytotoxicity	Manza in Okinawa Island (35 m)	Japan	Japan	(Agena et al., 2009)
Dysidea cf. *cristagalli*	Dysideidae	21-hydroxy-ent-isozonarone	Sesquiterpene quinone	Anti-inflammatory	A reef flat in Spirits Bay, Northland (33 m)	New Zealand	New Zealand	(McNamara et al., 2005)
Dysidea chlorea	Dysideidae	Haterumadysin A	Sesquiterpene	Inhibition of the first cleavage of fertilized sea urchin eggs	Hateruma Island, Okinawa	Japan	Japan	(Ueda et al., 2006)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Dysidea chlorea	Dysideidae	Haterumadysin B	Sesquiterpene	Inhibition of the first cleavage of fertilized sea urchin eggs	Hateruma Island, Okinawa	Japan	Japan	(Ueda et al., 2006)
Dysidea chlorea	Dysideidae	Haterumadysin C	Sesquiterpene	Inhibition of the first cleavage of fertilized sea urchin eggs	Hateruma Island, Okinawa	Japan	Japan	(Ueda et al., 2006)
Dysidea chlorea	Dysideidae	Haterumadysin D	Sesquiterpene	Inhibition of the first cleavage of fertilized sea urchin eggs	Hateruma Island, Okinawa	Japan	Japan	(Ueda et al., 2006)
Dysidea dendyi	Dysideidae	Spongiadioxin C	Tribromodibenzo-p-dioxin	Inhibiton of fertilized sea urchin eggs	Scott Reef, Northwest Australia (3 m)	Australia	Russia	(Utkina et al., 2002)
Dysidea dendyi	Dysideidae	Methyl ether of Spongiadioxin C	Tribromodibenzo-p-dioxin	Inhibiton of fertilized sea urchin eggs	Scott Reef, Northwest Australia (3 m)	Australia	Russia	(Utkina et al., 2002)
Dysidea dendyi	Dysideidae	Spongiadioxin A	Polybrominated dibenzo-p-dioxin	Cytotoxicity	Scott Reef, Northwest Australia (3 m)	Australia	Russia	(Utkina et al., 2001)
Dysidea dendyi	Dysideidae	Spongiadioxin B	Polybrominated dibenzo-p-dioxin	Cytotoxicity	Scott Reef, Northwest Australia (3 m)	Australia	Russia	(Utkina et al., 2001)
Dysidea fragilis	Dysideidae	Dysifragilisin A	Sesquiterpene aminoquinone	Inhibitory activity against HPTP1b	Sanya, Hainan province (10 m)	China	China	(Yu et al., 2009)
Dysidea fragilis	Dysideidae	Dysifragilisin B	Sesquiterpene quinone	Inhibitory activity against hPTP1B	Sanya, Hainan province (10 m)	China	China	(Yu et al., 2009)
Dysidea fragilis	Dysideidae	(4z,15z)-(s)-1	Lipid (long-chain 2h-azirine)	Cytotoxicity	Arrow Wall, Pohnpei	Micronesia	USA	(Skepper and Molinski, 2008)
Dysidea fragilis	Dysideidae	(4e,15z)-(s)-2	Lipid (long-chain 2h-azirine)	Cytotoxicity	Arrow Wall, Pohnpei	Micronesia	USA	(Skepper and Molinski, 2008)
Dysidea fragilis	Dysideidae	(4e)-(r)-3	Lipid (long-chain 2h-azirine)	Cytotoxicity	Arrow Wall, Pohnpei	Micronesia	USA	(Skepper and Molinski, 2008)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Dysidea fragilis	Dysideidae	Spirofragilin	Sesquiterpene isocyanide	NR	South China Sea	China	China	(Yu et al., 2006)
Dysidea herbacea	Dysideidae	Neodysidenin	Trichloroleucine peptide	Platelet-type 12-human lipoxygenase selective inhibitor	Duchess Island (30 feet)	Papua New Guinea	USA	(Deschamps et al., 2007)
Dysidea herbacea	Dysideidae	2-(3/,5/-dibromo-2/-hydroxyphenoxy)-3,5-dibromophenol	Macrolide	NR	Large Barrier Reef	Australia	Australia	(Utkina and Denisenko, 2006)
Dysidea herbacea	Dysideidae	Dysibetaine PP	Betaine	NR	Yap State	Micronesia	Japan	(Sakai et al., 2004)
Dysidea herbacea	Dysideidae	Dysibetaine CPa	Betaine	NR	Yap State	Micronesia	Japan	(Sakai et al., 2004)
Dysidea herbacea	Dysideidae	Dysibetaine CPb	Betaine	NR	Yap State	Micronesia	Japan	(Sakai et al., 2004)
Dysidea herbacea	Dysideidae	Neodysiherbaine A	Amino acid (excitatory)	NR	Yap State	Micronesia	Japan	(Sakai et al., 2001)
Dysidea robusta	Dysideidae	Isopyrodysinoic acid	Sesquiterpene	NR	São Salvador da Bahia de Todos os Santos	Brazil	Brazil	(Williams et al., 2009b)
Dysidea robusta	Dysideidae	13-hydroxyisopyrodysinoic acid	Sesquiterpene	NR	São Salvador da Bahia de Todos os Santos	Brazil	Brazil	(Williams et al., 2009b)
Dysidea robusta	Dysideidae	Pyrodysinoic acid B	Sesquiterpene	NR	São Salvador da Bahia de Todos os Santos	Brazil	Brazil	(Williams et al., 2009b)
Dysidea septosa	Dysideidae	Lingshuiolide A	Sesquiterpene	NR	Lingshui Bay, Hainan Province	China	China	(Huang et al, 2008)
Dysidea septosa	Dysideidae	Lingshuiolide B	Sesquiterpene	NR	Lingshui Bay, Hainan Province	China	China	(Huang et al, 2008)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Dysidea septosa	Dysideidae	Lingshuiperoxide	Sesquiterpene	NR	Lingshui Bay, Hainan Province	China	China	(Huang et al., 2008)
Dysidea septosa	Dysideidae	Isodysetherin	Sesquiterpene	NR	Lingshui Bay, Hainan Province	China	China	(Huang et al., 2008)
Dysidea septosa	Dysideidae	Spirolingshuiolide	Sesquiterpene	NR	Lingshui Bay, Hainan Province	China	China	(Huang et al., 2008)
Dysidea sp.	Dysideidae	18-aminoarenarone	Sesquiterpenoid quinone	NR	Scott Reef, Northwest Australia (15 m)	Australia	Russia	(Utkina et al., 2010)
Dysidea sp.	Dysideidae	18-methylaminoarenarone	Sesquiterpenoid quinone	NR	Scott Reef, Northwest Australia (15 m)	Australia	Russia	(Utkina et al., 2010)
Dysidea sp.	Dysideidae	19-aminoarenarone	Sesquiterpenoid quinone	NR	Scott Reef, Northwest Australia (15 m)	Australia	Russia	(Utkina et al., 2010)
Dysidea sp.	Dysideidae	19-methylaminoarenarone	Sesquiterpenoid quinone	NR	Scott Reef, Northwest Australia (15 m)	Australia	Russia	(Utkina et al., 2010)
Dysidea sp.	Dysideidae	Popolohuanone F	Sesquiterpenoid quinone	NR	Scott Reef, Northwest Australia (15 m)	Australia	Russia	(Utkina et al., 2010)
Dysidea sp.	Dysideidae	Chromodorolide D	Diterpene	NR	Inner Gneerings Reef, South East Queensland	Australia	Australia	(Katavic et al., 2012)
Dysidea sp.	Dysideidae	Chromodorolide E	Diterpene	NR	Inner Gneerings Reef, South East Queensland	Australia	Australia	(Katavic et al., 2012)
Dysidea sp.	Dysideidae	Dysideanin A	Alkaloid	NR	Lingshui, Hainan Island	China	China	(Ren et al., 2010)
Dysidea sp.	Dysideidae	Dysideanin B	Alkaloid	Antibacterial	Lingshui, Hainan Island	China	China	(Ren et al., 2010)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Dysidea sp.	Dysideidae	Scalarester	Sesterterpene	NR	Sanya, Hainan Island	China	China	(Yang et al., 2010b)
Dysidea sp.	Dysideidae	Dysideamine	Sesquiterpene aminoquinone	Radical scavenging and antineuronal activity	NR	Indonesia	Japan	(Suna et al., 2009)
Dysidea sp.	Dysideidae	3,4,5-tribromo-6-methoxy-2-(2′,4′-dibromophenoxy)phenol	Polybrominated diphenyl ether	Antibacterial	NR (1 m)	Micronesia	USA	(Zhang et al., 2008)
Dysidea sp.	Dysideidae	Sintokamide A	Peptide (Chlorinated)	Anticancer	Palau Sintok, Karimunjawa archipelago (15 m)	Indonesia	Canada	(Sadar et al., 2008)
Dysidea sp.	Dysideidae	Sintokamide B	Peptide (Chlorinated)	Novel antagonist	Palau Sintok, Karimunjawa archipelago (15 m)	Indonesia	Canada	(Sadar et al., 2008)
Dysidea sp.	Dysideidae	Sintokamide C	Peptide (Chlorinated)	Miscellaneous	Palau Sintok, Karimunjawa archipelago (15 m)	Indonesia	Canada	(Sadar et al., 2008)
Dysidea sp.	Dysideidae	Sintokamide D	Peptide (Chlorinated)	Miscellaneous	Palau Sintok, Karimunjawa archipelago (15 m)	Indonesia	Canada	(Sadar et al., 2008)
Dysidea sp.	Dysideidae	Sintokamide E	Peptide (Chlorinated)	Miscellaneous	Palau Sintok, Karimunjawa archipelago (15 m)	Indonesia	Canada	(Sadar et al., 2008)
Dysidea sp.	Dysideidae	Dysideasterol F	Polyoxygeneted Steroid	Cytotoxicity	Ishigaki Island, Okinawa	Japan	Japan	(Govindamet al., 2012)
Dysidea sp.	Dysideidae	Dysideasterol G	Polyoxygeneted Steroid	Cytotoxicity	Ishigaki Island, Okinawa	Japan	Japan	(Govindamet al., 2012)
Dysidea sp.	Dysideidae	Dysideasterol H	Polyoxygeneted Steroid	Cytotoxicity	Ishigaki Island, Okinawa	Japan	Japan	(Govindamet al., 2012)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Dysidea sp.	Dysideidae	Avinosol	Meroterpenoid	Anticancer	NR	Papua New Guinea	Canada	(Diaz-Marrero et al., 2006)
Dysidea sp.	Dysideidae	3′-aminoavarone	Meroterpenoid	Cytotoxicity	NR	Papua New Guinea	Canada	(Diaz-Marrero et al., 2006)
Dysidea sp.	Dysideidae	3′-phenethylaminoavarone analogue	Meroterpenoid	NR	NR	Papua New Guinea	Canada	(Diaz-Marrero et al., 2006)
Dysidea sp.	Dysideidae	O-methyl nakafuran-8 lactone	Sesquiterpenoid	Inhibitory bioactivity against phospho-tyrosine protein phosphatase (PTP1B)	Hainan Island	China	China	(Shao et al., 2006)
Dysidea sp.	Dysideidae	Dysithiazolamide	Tetrachloro amino acid	NR	Mayo Island near Sulawesi Island (40 feet)	Indonesia	Spain	(Ardá et al., 2005)
Dysidea sp.	Dysideidae	20-O-acetyl-21-hydroxy-ent-isozonarol	Merosesquiterpene	Cytotoxicity	Topolobampo Bay, Pacific Ocean	Mexico	Spain	(Pérez-García et al., 2005)
Dysidea sp.	Dysideidae	20-O-acetylneoavarol	Merosesquiterpene	Cytotoxicity	Topolobampo Bay, Pacific Ocean	Mexico	Spain	(Pérez-García et al., 2005)
Dysidea sp.	Dysideidae	Ent-yahazunol	Merosesquiterpene	Cytotoxicity	Topolobampo Bay, Pacific Ocean	Mexico	Spain	(Pérez-García et al., 2005)
Dysidea sp.	Dysideidae	Dysienone	Merosesquiterpene	Cytotoxicity	Topolobampo Bay, Pacific Ocean	Mexico	Spain	(Pérez-García et al., 2005)
Dysidea sp.	Dysideidae	Dysideasterol A	Steroid (polyhydroxylated)	Cytotoxicity	Hainan Island (10 m)	China	China	(Huang et al, 2005)
Dysidea sp.	Dysideidae	Dysideasterol B	Steroid (polyhydroxylated)	NR	Hainan Island (10 m)	China	China	(Huang et al, 2005)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Dysidea sp.	Dysideidae	Dysideasterol C	Steroid (polyhydroxylated)	NR	Hainan Island (10 m)	China	China	(Huang et al, 2005)
Dysidea sp.	Dysideidae	Dysideasterol D	Steroid (polyhydroxylated)	NR	Hainan Island (10 m)	China	China	(Huang et al, 2005)
Dysidea sp.	Dysideidae	Dysideasterol E	Steroid (polyhydroxylated)	NR	Hainan Island (10 m)	China	China	(Huang et al, 2005)
Dysidea sp.	Dysideidae	Furodysin lactone	Sesquiterpenoid	NR	Siquijor	Philippines	USA	(Goetz et al., 2001)
Dysidea sp.	Dysideidae	Pyrodysinoic acid	Sesquiterpenoid	NR	Siquijor	Philippines	USA	(Goetz et al., 2001)
Dysidea sp.	Dysideidae	Dysidenone A	Sesquiterpene cyclopentenone	Inhibition of human synovial phospholipase A2 (PLA2)	Lahdu	Vanuatu	Italy	(Giannini et al., 2001)
Dysidea sp.	Dysideidae	Dysidenone B	Sesquiterpene cyclopentenone	Inhibition of human synovial phospholipase A2 (PLA2)	Lahdu	Vanuatu	Italy	(Giannini et al., 2001)
Dysidea sp.	Dysideidae	Dysidine 4	Sesquiterpene aminoquinone	Inhibition of human synovial phospholipase A2 (PLA2)	Lahdu	Vanuatu	Italy	(Giannini et al., 2001)
Dysidea sp.	Dysideidae	Dysideaproline A	Polychlorinated compound	NR	Bararin Island (20-40 feet)	Philippines	USA	(Harrigan et al., 2001)
Dysidea sp.	Dysideidae	Dysideaproline B	Polychlorinated compound	NR	Bararin Island (20-40 feet)	Philippines	USA	(Harrigan et al., 2001)
Dysidea sp.	Dysideidae	Dysideaproline C	Polychlorinated compound	NR	Bararin Island (20-40 feet)	Philippines	USA	(Harrigan et al., 2001)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Dysidea sp.	Dysideidae	Dysideaproline D	Polychlorinated compound	NR	Bararin Island (20-40 feet)	Philippines	USA	(Harrigan et al., 2001)
Dysidea sp.	Dysideidae	Dysideaproline E	Polychlorinated compound	NR	Bararin Island (20-40 feet)	Philippines	USA	(Harrigan et al., 2001)
Dysidea sp.	Dysideidae	Dysideaproline F	Polychlorinated compound	NR	Bararin Island (20-40 feet)	Philippines	USA	(Harrigan et al., 2001)
Dysidea sp.	Dysideidae	Barbaleucamide A	Polychlorinated compound	NR	Bararin Island (20-40 feet)	Philippines	USA	(Harrigan et al., 2001)
Dysidea sp.	Dysideidae	Barbaleucamide B	Polychlorinated compound	NR	Bararin Island (20-40 feet)	Philippines	USA	(Harrigan et al., 2001)
Dysidea sp.	Dysideidae	Diplopuupehenone	Puupehenone-related dimer	Antioxidant	Tutuila Island, American Samoa (10 m)	USA	Russia	(Utkina et al., 2011)
Dysidea villosa	Dysideidae	21-dehydroxybolinaquinone	Alkaloid (bromotyrosine)	PTP1B inhibitory activity and Cytotoxic	Hainan	China	China	(Li et al., 2009)
Euryspongia n. sp.	Dysideidae	3β-hydroxy-26-norcampest-5-en-25-oic acid	Steroid	Inhibition of 6-keto-prostaglandinfl (6KPGF1) production	Epi Lamen Bay	Vanuatu	France	(Mandeau et al., 2005)
Euryspongia sp.	Dysideidae	Eurysterol A	Steroid sulfate	Cytotoxicity, antifungal	Light House Reef, Koror (37 m)	Palau	USA	(Boonlarppradab and Faulkner, 2007)
Euryspongia sp.	Dysideidae	Eurysterol B	Steroid sulfate	Cytotoxicity, antifungal	Light House Reef, Koror (37 m)	Palau	USA	(Boonlarppradab and Faulkner, 2007)
Fascaplysinopsis reticulata	Thorectidae	10-bromofascaplysin	Alkaloid (fascaplysin)	NR	Thanggalai Island, Side Streets and 7 Sisters	Fiji	USA	(Segraves et al., 2004)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Fascaplysinopsis reticulata	Thorectidae	3,10-dibromofascaplysin	Alkaloid (fascaplysin)	NR	Thanggalai Island, Side Streets and 7 Sisters	Fiji	USA	(Segraves et al., 2004)
Fascaplysinopsis reticulata	Thorectidae	Homofascaplysate A	Alkaloid (fascaplysin)	NR	Thanggalai Island, Side Streets and 7 Sisters	Fiji	USA	(Segraves et al., 2004)
Fascaplysinopsis reticulata	Thorectidae	Homofascaplysin B-1	Alkaloid (fascaplysin)	NR	Thanggalai Island, Side Streets and 7 Sisters	Fiji	USA	(Segraves et al., 2004)
Fascaplysinopsis reticulata	Thorectidae	3-bromohomofascaplysin B	Alkaloid (fascaplysin)	NR	Thanggalai Island, Side Streets and 7 Sisters	Fiji	USA	(Segraves et al., 2004)
Fascaplysinopsis reticulata	Thorectidae	3-bromohomofascaplysinB-1	Alkaloid (fascaplysin)	NR	Thanggalai Island, Side Streets and 7 Sisters	Fiji	USA	(Segraves et al., 2004)
Fascaplysinopsis reticulata	Thorectidae	3-bromohomofascaplysin C	Alkaloid (fascaplysin)	NR	Thanggalai Island, Side Streets and 7 Sisters	Fiji	USA	(Segraves et al., 2004)
Fascaplysinopsis reticulata	Thorectidae	7,14-dibromoreticulatine	Alkaloid (fascaplysin)	NR	Thanggalai Island, Side Streets and 7 Sisters	Fiji	USA	(Segraves et al., 2004)
Fascaplysinopsis reticulata	Thorectidae	Reticulatol	Alkaloid (fascaplysin)	NR	Thanggalai Island, Side Streets and 7 Sisters	Fiji	USA	(Segraves et al., 2004)
Fascaplysinopsis reticulata	Thorectidae	14-bromoreticulatol	Alkaloid (fascaplysin)	NR	Thanggalai Island, Side Streets and 7 Sisters	Fiji	USA	(Segraves et al., 2004)
Fascaplysinopsis reticulata	Thorectidae	3-bromosecofascaplysin A	Alkaloid (fascaplysin)	NR	Thanggalai Island, Side Streets and 7 Sisters	Fiji	USA	(Segraves et al., 2004)
Fascaplysinopsis reticulata	Thorectidae	3-bromosecofascaplysin B	Alkaloid (fascaplysin)	NR	Thanggalai Island, Side Streets and 7 Sisters	Fiji	USA	(Segraves et al., 2004)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Fascaplysinopsis sp.	Thorectidae	Salarin D	Macrolide (nitrogenous)	Cytotoxicity	Salary Bay, West coast (25–35 m)	Madagascar	Israel	(Bishara et al., 2010)
Fascaplysinopsis sp.	Thorectidae	Salarin E	Macrolide (nitrogenous)	Cytotoxicity	Salary Bay, West coast (25–35 m)	Madagascar	Israel	(Bishara et al., 2010)
Fascaplysinopsis sp.	Thorectidae	Salarin F	Macrolide (nitrogenous)	Cytotoxicity	Salary Bay, West coast (25–35 m)	Madagascar	Israel	(Bishara et al., 2010)
Fascaplysinopsis sp.	Thorectidae	Salarin G	Macrolide (nitrogenous)	Cytotoxicity	Salary Bay, West coast (25–35 m)	Madagascar	Israel	(Bishara et al., 2010)
Fascaplysinopsis sp.	Thorectidae	Salarin H	Macrolide (nitrogenous)	Cytotoxicity	Salary Bay, West coast (25–35 m)	Madagascar	Israel	(Bishara et al., 2010)
Fascaplysinopsis sp.	Thorectidae	Salarin I	Macrolide (nitrogenous)	NR	Salary Bay, West coast (25–35 m)	Madagascar	Israel	(Bishara et al., 2010)
Fascaplysinopsis sp.	Thorectidae	Salarin J	Macrolide (nitrogenous)	Cytotoxicity	Salary Bay, West coast (25–35 m)	Madagascar	Israel	(Bishara et al., 2010)
Fascaplysinopsis sp.	Thorectidae	Tauslarin C	Cyclodepsipeptide	Anticancer	NR (25-35 m)	Madagascar	Israel	(Bishara et al., 2010)
Fascaplysinopsis sp.	Thorectidae	Taumycin A	Lipodepsipeptide	Toxic to brineshrimp, anticancer	NR	Madagascar	Israel	(Bishara et al., 2010)
Fascaplysinopsis sp.	Thorectidae	Taumycin B	Lipodepsipeptide	Toxic to brineshrimp	NR	Madagascar	Israel	(Bishara et al., 2010)
Fascaplysinopsis sp.	Thorectidae	Salarin C	Macrolide (nitrogenous)	Anticancer	Salary Bay, ca. 100 km north of Tulear	Madagascar	Israel	(Bishara et al., 2010)
Fasciospongia cavernosa	Thorectidae	Compound 1a ($C_{27}H_{36}O_6$)	Sesterterpenoid	NR	Rovinj (25 m)	Croatia	Italy	(De Rosa and Carbonelli, 2006)
Fasciospongia cavernosa	Thorectidae	Compound 2 ($C_{27}H_{40}O_5$)	Sesterterpenoid	NR	Rovinj (25 m)	Croatia	Italy	(De Rosa and Carbonelli, 2006)
Fasciospongia cavernosa	Thorectidae	Ceramide 1	Ceramide	NR	Mandapam coast, Gulf of Myanomar	India	India	(Ramesh et al., 2001)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Fasciospongia sp.	Thorectidae	19-oxofasciospongine A	Sesterterpene (scalarane)	Cytotoxicity	NR (1 m)	Palau	USA	(Yao et al., 2009)
Fasciospongia sp.	Thorectidae	Fascioquinol A	Meroterpene	Antibacterial	West of Cape Leeuwin, Western Australia (100 m)	Australia	Australia	(Zhang et al., 2011)
Fasciospongia sp.	Thorectidae	Fascioquinol B	Meroterpene	Antibacterial	West of Cape Leeuwin, Western Australia (100 m)	Australia	Australia	(Zhang et al., 2011)
Fasciospongia sp.	Thorectidae	Fascioquinol C	Meroterpene	NR	West of Cape Leeuwin, Western Australia (100 m)	Australia	Australia	(Zhang et al., 2011)
Fasciospongia sp.	Thorectidae	Fascioquinol D	Meroterpene	NR	West of Cape Leeuwin, Western Australia (100 m)	Australia	Australia	(Zhang et al., 2011)
Fasciospongia sp.	Thorectidae	Fascioquinol E	Meroterpene	NR	West of Cape Leeuwin, Western Australia (100 m)	Australia	Australia	(Zhang et al., 2011)
Fasciospongia sp.	Thorectidae	Fascioquinol F	Meroterpene	NR	West of Cape Leeuwin, Western Australia (100 m)	Australia	Australia	(Zhang et al., 2011)
Fasciospongia sp.	Thorectidae	Fasciospongine C	Sesterterpene	Antibacterial	NR (1 m)	Palau	USA	(Yao et al., 2009)
Fasciospongia sp.	Thorectidae	25-hydroxyhalisulfate 9	Sesterterpene	Antibacterial	NR (1 m)	Palau	USA	(Yao et al., 2009)
Fasciospongia sp.	Thorectidae	Isomicrocionin-3	Furanosesquiterpene (Isomeric)	NR	Berlengas Islands, Western Portugal (4 m)	Portugal	Portugal	(Gaspar et al., 2008)
Fasciospongia sp.	Thorectidae	(−)-Microcionin-1	Furanosesquiterpene (Isomeric)	Antibacterial	Berlengas Islands, Western Portugal (4 m)	Portugal	Portugal	(Gaspar et al., 2008)
Fasciospongia sp.	Thorectidae	(−)-Isomicrocionin-1	Furanosesquiterpene (Isomeric)	NR	Berlengas Islands, Western Portugal (4 m)	Portugal	Portugal	(Gaspar et al., 2008)
	Thorectidae	Fasciospongine A	Sulfated sesterterpene alkaloid	Antibacterial	NR	Palau	USA	(Yao and Chang, 2007)
Fasciospongia sp.*Fasciospongia* sp.	Thorectidae	Fasciospongine B	Sulfated sesterterpene alkaloid	Antibacterial	NR	Palau	USA	(Yao and Chang, 2007)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Hippospongia cf. *metachromia*	Spongiidae	Hipposulfate A	Sesterterpene sulfate	Cytotoxicity	Hedo, Okinawa	Japan	Japan	(Musman et al., 2001)
Hippospongia cf. *metachromia*	Spongiidae	Hipposulfate B	Sesterterpene sulfate	NR	Hedo, Okinawa	Japan	Japan	(Musman et al., 2001)
Hippospongia lachne	Spongiidae	Hippolide A	Sesterterpene	Cytotoxicity, PTP1B inhibitory activity, antiinflammatory	Yongxing Island and Seven Connected Islets in the South China Sea	China	China	(Piao et al., 2011)
Hippospongia lachne	Spongiidae	Hippolide B	Sesterterpene	Cytotoxicity, PTP1B inhibitory	Yongxing Island and Seven Connected Islets in the South China Sea	China	China	(Piao et al., 2011)
Hippospongia lachne	Spongiidae	Hippolide C	Sesterterpene	NR	Yongxing Island and Seven Connected Islets in the South China Sea	China	China	(Piao et al., 2011)
Hippospongia lachne	Spongiidae	Hippolide D	Sesterterpene	NR	Yongxing Island and Seven Connected Islets in the South China Sea	China	China	(Piao et al., 2011)
Hippospongia lachne	Spongiidae	Hippolide E	Sesterterpene	Antiinflammatory	Yongxing Island and Seven Connected Islets in the South China Sea	China	China	(Piao et al., 2011)
Hippospongia lachne	Spongiidae	Hippolide F	Sesterterpene	NR	Yongxing Island and Seven Connected Islets in the South China Sea	China	China	(Piao et al., 2011)
Hippospongia lachne	Spongiidae	Hippolide G	Sesterterpene	NR	Yongxing Island and Seven Connected Islets in the South China Sea	China	China	(Piao et al., 2011)
Hippospongia lachne	Spongiidae	Hippolide H	Sesterterpene	NR	Yongxing Island and Seven Connected Islets in the South China Sea	China	China	(Piao et al., 2011)
Hippospongia metachromia	Spongiidae	Hippochromin A	Sesquiterpene hydroquinone	NR	Nan-Wan	Taiwan	Taiwan	(Shen et al., 2001)
Hippospongia metachromia	Spongiidae	Hippochromin B	Sesquiterpene hydroquinone	NR	Nan-Wan	Taiwan	Taiwan	(Shen et al., 2001)
Hippospongia sp.	Spongiidae	Barangcadoic acid A	Terpenoid	RCE protease inhibitory activity	Barangcadi Island, Ujung Pandang, Sulawesi	Indonesia	Canada	(Craig et al., 2002)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Hippospongia sp.	Spongiidae	Rhopaloic acid D	Terpenoid	RCE protease inhibitory activity	Barangcadi Island, Ujung Pandang, Sulawesi	Indonesia	Canada	(Craig et al., 2002)
Hippospongia sp.	Spongiidae	Hippospongide A	Sesterterpene	NR	Coast of Tai-tung (20 m)	Taiwan	Taiwan	(Chang et al., 2012)
Hippospongia sp.	Spongiidae	Hippospongide B	Sesterterpenoid	NR	Coast of Tai-tung (20 m)	Taiwan	Taiwan	(Chang et al., 2012)
Hippospongia sp.	Spongiidae	Salaramide A	Alpha-oxoamide	NR	Salary Bay	Madagaskar	France	(Bensemhoun et al., 2010)
Hippospongia sp.	Spongiidae	Salaramide B	Alpha-oxoamide	NR	Salary Bay	Madagaskar	France	(Bensemhoun et al., 2010)
Hyattella cribriformis	Spongiidae	24-β-methoxyscalarolide	Sesterterpene (scalarane)	NR	Kanyakumari	India	India	(Kumar et al., 2008)
Hyattella intestinalis	Spongiidae	Hyatelone A	Sesterterpene (scalarane-related)	Anticancer	Gulf of California	Mexico	Spain	(Hernández-Guerrero et al., 2006)
Hyattella intestinalis	Spongiidae	Hyatelone B	Sesterterpene (scalarane-related)	Anticancer	Gulf of California	Mexico	Spain	(Hernández-Guerrero et al., 2006)
Hyattella intestinalis	Spongiidae	Hyatelone C	Sesterterpene (scalarane-related)	NR	Gulf of California	Mexico	Spain	(Hernández-Guerrero et al., 2006)
Hyattella intestinalis	Spongiidae	Hyatolide A	Sesterterpene (scalarane-related)	Anticancer	Gulf of California	Mexico	Spain	(Hernández-Guerrero et al., 2006)
Hyattella intestinalis	Spongiidae	Hyatolide B	Sesterterpene (scalarane-related)	NR	Gulf of California	Mexico	Spain	(Hernández-Guerrero et al., 2006)
Hyattella intestinalis	Spongiidae	Hyatolide C	Scalarane	Anticancer	Gulf of California	Mexico	Spain	(Hernández-Guerrero et al., 2006)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Hyattella intestinalis	Spongiidae	Hyatolide D	Scalarane	NR	Gulf of California	Mexico	Spain	(Hernández-Guerrero et al., 2006)
Hyattella intestinalis	Spongiidae	Hyatolide E	Scalarane	NR	Gulf of California	Mexico	Spain	(Hernández-Guerrero et al., 2006)
Hyattella intestinalis	Spongiidae	Hyatelactam	Scalarane	Anticancer	Gulf of California	Mexico	Spain	(Hernández-Guerrero et al., 2006)
Hyattella intestinalis	Spongiidae	12-O-deacetyl-19-epi-scalarin	Scalarane	NR	Gulf of California	Mexico	Spain	(Hernández-Guerrero et al., 2006)
Hyattella intestinalis	Spongiidae	12-O-deacetylnorscalaral B	Norscalarane	Anticancer	Gulf of California	Mexico	Spain	(Hernández-Guerrero et al., 2006)
Hyattella intestinalis	Spongiidae	Mooloolabene A	Norsesterterpene	Cytotoxicity	Inner Gneerings, off Mooloolaba (10-15 m)	Australia	Australia	(Somerville et al., 2006)
Hyattella intestinalis	Spongiidae	Mooloolabene B	Norsesterterpene	Cytotoxicity	Inner Gneerings, off Mooloolaba (10-15 m)	Australia	Australia	(Somerville et al., 2006)
Hyattella intestinalis	Spongiidae	Mooloolabene C	Norsesterterpene	Cytotoxicity	Inner Gneerings, off Mooloolaba (10-15 m)	Australia	Australia	(Somerville et al., 2006)
Hyattella intestinalis	Spongiidae	Mooloolabene D	Norsesterterpene	Cytotoxicity	Inner Gneerings, off Mooloolaba (10-15 m)	Australia	Australia	(Somerville et al., 2006)
Hyattella intestinalis	Spongiidae	Mooloolabene E	Norsesterterpene	Cytotoxicity	Inner Gneerings, off Mooloolaba (10-15 m)	Australia	Australia	(Somerville et al., 2006)
Hyattella intestinalis	Spongiidae	Mooloolaldehyde	Sesterterpene	Cytotoxicity	Inner Gneerings, off Mooloolaba (10-15 m)	Australia	Australia	(Somerville et al., 2006)
Hyattella sp.	Spongiidae	Psammaplysin G	Alkaloid bromotyrosine	Antimalarial	Hervey Bay, Little Woody, Sponge Garden, Queensland, (19 m)	Australia	Australia	(Yang et al., 2010a)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Hyattella sp.	Spongiidae	Compound 7 ($C_{29}H_{44}O_6Na$)	Sesterterpene	Cytotoxicity, antibacterial activity, inhibitory activity against isocitrate lyase	Shore of Soheuksan-do, West Sea (20 m)	Korea	Korea	(Jeon et al., 2011)
Hyattella sp.	Spongiidae	Compound 8 ($C_{27}H_{42}O_5Na$)	Sesterterpene	Cytotoxicity, antibacterial activity, inhibitory activity against isocitrate	Shore of Soheuksan-do, West Sea (20 m)	Korea	Korea	(Jeon et al., 2011)
Hyattella sp.	Spongiidae	Compound 9 ($C_{28}H_{44}O_4Na$)	Sesterterpene	Cytotoxicity, antibacterial activity, inhibitory activity against isocitrate lyase	Shore of Soheuksan-do, West Sea (20 m)	Korea	Korea	(Jeon et al., 2011)
Hyattella sp.	Spongiidae	Compound 10 ($C_{28}H_{42}O_6Na$)	Sesterterpene	Cytotoxicity, antibacterial activity, inhibitory activity against isocitrate	Shore of Soheuksan-do, West Sea (20 m)	Korea	Korea	(Jeon et al., 2011)
Hyattella sp.	Spongiidae	Compound 11 ($C_{31}H_{46}NO_7Na_2$)	Sesterterpene	Cytotoxicity, antibacterial activity, inhibitory activity against isocitrate	Shore of Soheuksan-do, West Sea (20 m)	Korea	Korea	(Jeon et al., 2011)
Hyrtios erectus (erecta)	Thorectidae	Hyrtiosin A	Sesterterpene (scalarane)	NR	Lingshui Bay, Hainan Province (10 m)	China	China	(Yu et al., 2005)
Hyrtios erectus (erecta)	Thorectidae	Hyrtiosin B	Sesterterpene (scalarane)	NR	Lingshui Bay, Hainan Province (10 m)	China	China	(Yu et al., 2005)
Hyrtios erectus (erecta)	Thorectidae	Hyrtiosin C	Sesterterpene (scalarane)	NR	Lingshui Bay, Hainan Province (10 m)	China	China	(Yu et al., 2005)
Hyrtios erectus (erecta)	Thorectidae	Hyrtiosin D	Sesterterpene (scalarane)	NR	Lingshui Bay, Hainan Province (10 m)	China	China	(Yu et al., 2005)
Hyrtios erectus (erecta)	Thorectidae	Hyrtiosin E	Sesterterpene (scalarane)	NR	Lingshui Bay, Hainan Province (10 m)	China	China	(Yu et al., 2005)
Hyrtios erectus	Thorectidae	Salmahyrtisol A	Sesterterpene	Cytotoxicity	Hurghada, Red Sea	Egypt	Egypt	(Youssefet al., 2002)
Hyrtios erectus	Thorectidae	5,6-dibromo-29-demethylaplysinopsin (Z)	Alkaloid (aplysinopsin-type indole)	Neuronal nitric oxide synthase (nNOS) selective inhibitor	Iriomote-Island, Okinawa Prefecture	Japan	Japan	(Aoki et al., 2001)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Hyrtios erectus	Thorectidae	5,6-dibromo-29-demethylaplysinopsin (E)	Alkaloid (aplysinopsin-type indole)	Neuronal nitric oxide synthase (nNOS) selective inhibitor	Iriomote-Island, Okinawa Prefecture	Japan	Japan	(Aoki et al., 2001)
Hyrtios erectus	Thorectidae	6,7-dibromo-4-hydroxy-2-quinolone	Alkaloid (aplysinopsin-type indole)	Neuronal nitric oxide synthase (nNOS) selective inhibitor	Iriomote-Island, Okinawa Prefecture	Japan	Japan	(Aoki et al., 2001)
Hyrtios erectus	Thorectidae	Hyrtiazepine	Alkaloid (manzamine-type)	NR	Safaga, Red Sea	Egypt	France	(Sauleau et al., 2006)
Hyrtios erectus	Thorectidae	5-hydroxy-1h-indole-3-carboxylic acid methyl ester	Alkaloid (azepino-indole-type)	NR	Safaga, Red Sea	Egypt	France	(Sauleau et al., 2006)
Hyrtios erectus	Thorectidae	(+)-20-formylhyrtiosal	Sesterterpene	NR	Hainan Island	China	China	(Qiu et al., 2004)
Hyrtios erectus	Thorectidae	(+)-16-O-acetyl-20-formylhyrtiosal	Sesterterpene	NR	Hainan Island	China	China	(Qiu et al., 2004)
Hyrtios erectus	Thorectidae	12-α- O -acetylhyrtiolide	Sesterterpene	NR	Hainan Island	China	China	(Qiu et al., 2004)
Hyrtios erectus	Thorectidae	5,10-dihydroxyfurospinulosine-1	Sesterterpene	NR	Hainan Island	China	China	(Qiu et al., 2004)
Hyrtios erectus and *H. reticulatus*	Thorectidae	Hyrtiosulawesine	Alkaloid (β-carboline)	NR	Lankai Island (11 m) and west side of Bone Lola Reef (13 m), off Makassar, Sulawesi	Indonesia	Belgium	(Salmoun et al., 2002)
Hyrtios erectus	Thorectidae	Hyrtioerectine A	Alkaloid	Cytotoxicity	Hurghada, Red Sea (10-25 m)	Egypt	Egypt	(Youssef, 2005b)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Hyrtios erectus	Thorectidae	Hyrtioerectine B	Alkaloid	Cytotoxicity	Hurghada, Red Sea (10-25 m)	Egypt	Egypt	(Youssef, 2005b)
Hyrtios erectus	Thorectidae	Hyrtioerectine C	Alkaloid	Cytotoxicity	Hurghada, Red Sea (10-25 m)	Egypt	Egypt	(Youssef, 2005b)
Hyrtios gumminae	Thorectidae	Similan A	Sesterterpenoid	Cytotoxicity	Similan Island, Andaman Sea (30-40 feet)	Thailand	Thailand	(Mahidol et al., 2009)
Hyrtios gumminae	Thorectidae	12beta,20-dihydroxy-16 beta-acetoxy-17-scalaren-19,20-olide	Sesterterpenoid (cheilanthane)	Cytotoxicity	Similan Island, Andaman Sea (30-40 feet)	Thailand	Thailand	(Mahidol et al., 2009)
Hyrtios gumminae	Thorectidae	12beta-acetoxy-20-hydroxy-17-scalaren-19,20-olide	Sesterterpenoid (cheilanthane)	Cytotoxicity	Similan Island, Andaman Sea (30-40 feet)	Thailand	Thailand	(Mahidol et al., 2009)
Hyrtios gumminae	Thorectidae	12beta,16alpha,20-trihydroxy-17-scalaren-19,20-olide	Sesterterpenoid	Cytotoxicity	Similan Island, Andaman Sea (30-40 feet)	Thailand	Thailand	(Mahidol et al., 2009)
Hyrtios reticulatus	Thorectidae	Hyrtiocarboline	Alkaloid (1-imidazoyl-3-carboxy-6-hydroxy—carboline)	Anticancer	Bismarck Sea (13-20 m)	Papua New Guinea	USA	(Inman et al., 2010)
Hyrtios reticulatus	Thorectidae	Hyrtioreticulin A	Alkaloid (tetrahydro-β-carboline)	inhibited ubiquitin-activating enzyme, Cytotoxicity	North Sulawesi (10 m)	Indonesia	Japan	(Yamanokuchi et al., 2012)
Hyrtios reticulatus	Thorectidae	Hyrtioreticulin B	Alkaloid (tetrahydro-β-carboline)	inhibited ubiquitin-activating enzyme, Cytotoxicity	North Sulawesi (10 m)	Indonesia	Japan	(Yamanokuchi et al., 2012)
Hyrtios reticulatus	Thorectidae	Hyrtioreticulin C	Alkaloid (tetrahydro-β-carboline)	Cytotoxicity	North Sulawesi (10 m)	Indonesia	Japan	(Yamanokuchi et al., 2012)
Hyrtios reticulatus	Thorectidae	Hyrtioreticulin D	Alkaloid (azepinoindole-type)	Cytotoxicity	North Sulawesi (10 m)	Indonesia	Japan	(Yamanokuchi et al., 2012)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Hyrtios reticulatus	Thorectidae	Hyrtioreticulin E	Alkaloid (tetrahydro-b-carboline)	Cytotoxicity	North Sulawesi (10 m)	Indonesia	Japan	(Yamanokuchi et al., 2012)
Hyrtios sp.	Thorectidae	1-carboxy-6-hydroxy-3,4-dihydro-β-carboline	Alkaloid (Manzamine)	NR	NR	Micronesia	Korea	(Lee et al., 2009)
Hyrtios sp.	Thorectidae	20 -epi-hydroxyhaterumadienone	Unknown	NR	NR	Papua New Guinea	USA	(Robinson et al., 2009)
Hyrtios sp.	Thorectidae	16- O -methylsesterstatin 4	Sesterterpenoid	Antibacterial	American Samoa	USA	USA	(Kamel et al., 2009)
Hyrtios sp.	Thorectidae	17, 24-dihydroheteronemin	Sesterterpenoid	Cytotoxicity, antibacterial	American Samoa	USA	USA	(Kamel et al., 2009)
Hyrtios sp.	Thorectidae	16, 25-deacetoxy-17, 24-dihydroheteronemin	Sesterterpene (scalarane)	Antibacterial	American Samoa	USA	USA	(Kamel et al., 2009)
Hyrtios sp.	Thorectidae	16-deacetoxy-25-methoxy-17, 24-dihydroheteronemin	Sesterterpene (scalarane)	Antibacterial	American Samoa	USA	USA	(Kamel et al., 2009)
Hyrtios sp.	Thorectidae	Hyrtinadine A	Alkaloid (bis-indole)	Cytotoxicity	Unten-Port, Okinawa	Japan	Japan	(Endo et al., 2007)
Hyrtios sp.	Thorectidae	Poipuol	Trichlorinated metabolite (polyketide-derived)	NR	Brenneke's Ledge, Kauai Island, Hawaii (18 m)	USA	USA	(Sata et al., 2005)
Hyrtios sp.	Thorectidae	Hyrtiosenolide A	Sesquiterpene γ-methoxybutenolide	Antibacterial	Hurghada, Red Sea (8-10 m)	Egypt	Japan	(Youssefet al., 2004)
Hyrtios sp.	Thorectidae	Hyrtiosenolide B	Sesquiterpene γ-methoxybutenolide	Antibacterial	Hurghada, Red Sea (8-10 m)	Egypt	Japan	(Youssefet al., 2004)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Hyrtios sp.	Thorectidae	(+)-(5s,8s,9r,10s)-20-methoxy puupehenone	Merosesquiterpene (puupehenone congener)	NR	Togian Island, Tomini Bay North Sulawesi	Indonesia	USA	(Piña et al., 2003)
Hyrtios sp.	Thorectidae	(+)-(5s,8s,10s)-20-methoxy-9,15-ene-puupehenol	Merosesquiterpene (puupehenone congener)	NR	Togian Island, Tomini Bay North Sulawesi	Indonesia	USA	(Piña et al., 2003)
Hyrtios sp.	Thorectidae	(+)-(5s,8s,9r,10s)-15,20-dimethoxypuupehenol	Merosesquiterpene (puupehenone congener)	NR	Togian Island, Tomini Bay North Sulawesi	Indonesia	USA	(Piña et al., 2003)
Hyrtios sp.	Thorectidae	5,6-dibromo-L-hypaphorine	Indole derivative	Anti-inflammatory and antioxidant	NR	Fiji	France	(Longeon et al., 2011)
Hyrtios sp.	Thorectidae	Hyrtioseragamine A	Alkaloid	Antimicrobial	Seragaki, Okinawa	Japan	Japan	(Takahashi et al., 2011)
Hyrtios sp.	Thorectidae	Hyrtioseragamine B	Alkaloid	Antimicrobial	Seragaki, Okinawa	Japan	Japan	(Takahashi et al., 2011)
Ircinia aruensis	Irciniidae	5 alpha,6 alpha-epoxy-26,27-dinorergosta-7,22-en-3 beta-ol	Sterol	Cytotoxicity	Zhanjiang, Naozhou Island, South China Sea (15-20 m)	China	China	(Xu et al., 2008)
Ircinia aruensis	Irciniidae	5 alpha,6 alpha-epoxycholesta-7,22-en-3 beta-ol	Sterol	Cytotoxicity	Zhanjiang in Naozhou Island, South China Sea (15-20 m)	China	China	(Xu et al., 2008)
Ircinia aruensis	Irciniidae	5 alpha,6 alpha-epoxyergosta-7,24(28)-en-3 beta-ol	Sterol	Cytotoxicity	Zhanjiang in Naozhou Island, South China Sea (15-20 m)	China	China	(Xu et al., 2008)
Ircinia aruensis	Irciniidae	5 alpha,6 alpha-epoxyergosta-7-en-3 beta-ol	Sterol	NR	Zhanjiang in Naozhou Island, South China Sea (15-20 m)	China	China	(Xu et al., 2008)
Ircinia aruensis	Irciniidae	5 alpha,6 alpha-epoxystigmasta-7,22-en-3 beta-ol	Sterol	NR	Zhanjiang in Naozhou Island, South China Sea (15-20 m)	China	China	(Xu et al., 2008)
Ircinia aruensis	Irciniidae	5 alpha,6 alpha-epoxystigmasta-7-en-3 beta-ol	Sterol	NR	Zhanjiang in Naozhou Island, South China Sea (15-20 m)	China	China	(Xu et al., 2008)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Ircinia fasciculata	Irciniidae	Ircisulfamide	Ceramide (4-sulfated)	NR	Wei-Zhou Island, Beihai	China	China	(Zhang et al., 2005)
Ircinia fasciculata	Irciniidae	Ircicerebroside	Glycosphingolipid	NR	Wei-Zhou Island, Beihai	China	China	(Zhang et al., 2005)
Ircinia felix	Irciniidae	4H-pyran-2ol acetate	Pyran derivative	NR	NR	Brazil	Brazil	(Granato et al., 2005)
Ircinia felix	Irciniidae	4H-pyran-2ol acetate	Ester	NR	NR	Brazil	Brazil	(Granato et al., 2005)
Ircinia formosana	Irciniidae	Irciformonin E	C22-sesterterpenoid	NR	Eastern Taiwan (20 m)	Taiwan	Taiwan	(Shen et al., 2009)
Ircinia formosana	Irciniidae	Irciformonin F	C22-sesterterpenoid	NR	Eastern Taiwan (20 m)	Taiwan	Taiwan	(Shen et al., 2009)
Ircinia formosana	Irciniidae	Irciformonin G	C22-sesterterpenoid	NR	Eastern Taiwan (20 m)	Taiwan	Taiwan	(Shen et al., 2009)
Ircinia formosana	Irciniidae	Irciformonin H	C22-sesterterpenoid	NR	NR (20 m)	Taiwan	Taiwan	(Shen et al., 2009)
Ircinia formosana	Irciniidae	Irciformonin I	C22-sesterterpenoid)	Inhibition of peripheral blood mononuclear cell proliferation	NR (20 m)	Taiwan	Taiwan	(Shen et al., 2009)
Ircinia formosana	Irciniidae	Irciformonin J	C22- sesterterpenoid	Inhibition of peripheral blood mononuclear cell proliferation	NR (20 m)	Taiwan	Taiwan	(Shen et al., 2009)
Ircinia formosana	Irciniidae	Irciformonin K	C22- sesterterpenoid	Inhibition of peripheral blood mononuclear cell proliferation	NR (20 m)	Taiwan	Taiwan	(Shen et al., 2009)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Ircinia formosana	Irciniidae	Irciformonin A	C22-sesterterpene	Cytotoxicity	East coast	Taiwan	Taiwan	(Shen et al., 2006)
Ircinia formosana	Irciniidae	Irciformonin B	C22-sesterterpene	NR	East coast	Taiwan	Taiwan	(Shen et al., 2006)
Ircinia formosana	Irciniidae	Irciformonin C	C22-sesterterpene	Cytotoxicity	East coast	Taiwan	Taiwan	(Shen et al., 2006)
Ircinia formosana	Irciniidae	Irciformonin D	C22-sesterterpene	Cytotoxicity	East coast	Taiwan	Taiwan	(Shen et al., 2006)
Ircinia ramosa	Irciniidae	Irciniastatin A	Irciniastatin	Anticancer	Barrier reef near Semporna, Sabah (Borneo), (20-36 m)	Malaysia	USA	(Pettit et al., 2004)
Ircinia ramosa	Irciniidae	Irciniastatin B	Irciniastatin	Anticancer	Barrier reef near Semporna, Sabah (Borneo), (20-36 m)	Malaysia	USA	(Pettit et al., 2004)
Ircinia ramosa	Irciniidae	73-deoxychondropsin A	Macrolide lactam (Polyketide-derived)	Cytotoxicity	NR	Australia	USA	(Rashid et al., 2001)
Ircinia ramosa	Irciniidae	Chondropsin C	Macrolide lactam (Polyketide-derived)	Cytotoxicity	NR	Philippines	USA	(Rashid et al., 2001)
Ircinia selaginea (Lamark)	Irciniidae	Irciniketene	Diterpene ketene (Conjugated)	Anticancer	Beihai, Guangxi Province	China	China	(Yan et al., 2001)
Ircinia sp.	Irciniidae	7-methyl-9-oxo-dec-7-eneoic acid	Ketone	NR	NR	Egypt	USA	(Tatli et al., 2008)
Ircinia sp.	Irciniidae	Irciniasulfonic acid B	Fatty acid	Anticancer	Tsuzumi Island, Fukuoka Prefecture (10 m)	Japan	Japan	(Emura et al., 2006)
Ircinia sp.	Irciniidae	Tedanolide C	Macrolide	Anticancer	Milne Bay, Papua New Guinea	Papua New Guinea	USA	(Chevallier et al., 2006)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Ircinia sp.	Irciniidae	Terpenoid 1 ($C_{25}H_{32}O_4$)	Sesterterpene (furano)	Cytotoxicity	Iriomote Island, Okinawa	Japan	Japan	(Issa et al., 2003)
Ircinia sp.	Irciniidae	Terpenoid 3 ($C_{25}H_{32}O_4$)	Sesterterpene (furano)	Cytotoxicity	Iriomote Island, Okinawa	Japan	Japan	(Issa et al., 2003)
Ircinia sp.	Irciniidae	Terpenoid 5 ($C_{24}H_{36}O_3$)	Sesterterpene (furano)	Cytotoxicity	Iriomote Island, Okinawa	Japan	Japan	(Issa et al., 2003)
Ircinia sp.	Irciniidae	Terpenoid 6 ($C_{24}H_{35}ClO_3$)	Sesterterpene (furano)	Cytotoxicity	Iriomote Island, Okinawa	Japan	Japan	(Issa et al., 2003)
Ircinia sp.	Irciniidae	Terpenoid 7 ($C_{24}H_{35}ClO_3$)	Sesterterpene (furano)	Cytotoxicity	Iriomote Island, Okinawa	Japan	Japan	(Issa et al., 2003)
Ircinia sp.	Irciniidae	Cyclotheonamide E4	Peptide	Tryptase inhibitor	Miyako Island, Okinawa	Japan	Japan	(Murakami et al., 2002)
Ircinia sp.	Irciniidae	Cyclotheonamide E5	Peptide	Tryptase inhibitor	Miyako Island, Okinawa	Japan	Japan	(Murakami et al., 2002)
Ircinia sp.	Irciniidae	Ircinamine	Alkaloid	Anticancer	NR	Japan	Japan	(Kuramoto et al., 2002)
Ircinia sp.	Irciniidae	25-hydroxy-13(24),15,17-cheilanthatrien-19,25-olide	Sesterterpenoid (cheilanthane)	Protein kinase inhibitor	Porpoise Cay, Wreck Reef (21 m)	Australia	Australia	(Buchanan et al., 2001)
Ircinia sp.	Irciniidae	25-hydroxy-13(24),17-cheilanthadien-16,19-olide	Sesterterpenoid (cheilanthane)	Protein kinase inhibitor	Porpoise Cay, Wreck Reef (21 m)	Australia	Australia	(Buchanan et al., 2001)
Ircinia sp.	Irciniidae	16,25-dihydroxy-13(24),17-cheilanthadien-19,25-olide	Sesterterpenoid (cheilanthane)	Protein kinase inhibitor	Porpoise Cay, Wreck Reef (21 m)	Australia	Australia	(Buchanan et al., 2001)
Ircinia sp.	Irciniidae	Polyprenyl chromene 1	Polyprenyl Hydroquinone Derivative	NR	Weno Is., Chuuk State	Micronesia	Korea	(Lee et al., 2012)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Ircinia sp.	Irciniidae	Polyprenyl chromene 2	Polyprenyl Hydroquinone Derivative	NR	Weno Is., Chuuk State	Micronesia	Korea	(Lee et al., 2012)
Ircinia sp.	Irciniidae	Polyprenyl chromene 3	Polyprenyl Hydroquinone Derivative	NR	Weno Is., Chuuk State	Micronesia	Korea	(Lee et al., 2012)
Ircinia sp.	Irciniidae	Ircinolin A	Terpenoid	NR	Orchid Island (20 m)	Taiwan	China	(Su et al., 2011)
Ircinia sp.	Irciniidae	15-acetylirciformonin B	Terpenoid	Cytotoxicity	Orchid Island (20 m)	Taiwan	China	(Su et al., 2011)
Ircinia sp.	Irciniidae	10-acetylirciformonin B	Terpenoid	Cytotoxicity	Orchid Island (20 m)	Taiwan	China	(Su et al., 2011)
Lamellodysidea chlorea	Dysideidae	Dysinosin B	Peptide	NR	Southeast tip of Wooded Islet, Low Isles, Queensland (16 m)	Australia	Australia	(Carroll et al., 2004)
Lamellodysidea chlorea	Dysideidae	Dysinosin C	Peptide	NR	Southeast tip of Wooded Islet, Low Isles, Queensland (16 m)	Australia	Australia	(Carroll et al., 2004)
Lamellodysidea chlorea	Dysideidae	Dysinosin D	Peptide	NR	Southeast tip of Wooded Islet, Low Isles, Queensland (16 m)	Australia	Australia	(Carroll et al., 2004)
Lamellodysidea herbacea	Dysideidae	2,3,5- tribromo-6-(3′,5′-dibromo-2′-methoxyphenoxy)phenol	Ether (Polybrominated diphenyl)	Antibacterial	Sangiang Island, West Java	Indonesia	Japan	(Hanifet al., 2007)
Lamellodysidea herbacea	Dysideidae	2,5- dibromo-6-(3′,5′-dibromo-2′-hydroxyphenoxy)phenol	Ether (Polybrominated diphenyl)	Antibacterial, Cytotoxicity	Sangiang Island, West Java	Indonesia	Japan	(Hanifet al., 2007)
Lamellodysidea herbacea	Dysideidae	2,4,5-tribromo-6- (5′-bromo-2′-hydroxyphenoxy)phenol	Ether (Polybrominated diphenyl)	Antibacterial, Cytotoxicity	Sangiang Island, West Java	Indonesia	Japan	(Hanifet al., 2007)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Lamellodysidea herbacea	Dysideidae	2,4,5-tribromo-6-(3′,5′- dibromo-2′-hydroxyphenoxy)anisole	Ether (Polybrominated diphenyl)	Antibacterial	Sangiang Island, West Java	Indonesia	Japan	(Hanifet al., 2007)
Lamellodysidea herbacea	Dysideidae	Dysidamide D	Pyrrolidinone (polychlorinated)	NR	Red Sea (20 m)	Egypt	France	(Sauleau et al., 2005)
Lamellodysidea herbacea	Dysideidae	Dysidamide E	Pyrrolidinone (polychlorinated)	NR	Red Sea (20 m)	Egypt	France	(Sauleau et al., 2005)
Lamellodysidea herbacea	Dysideidae	7,7,7-trichloro-3-hydroxy-2,2,6-trimethyl-4-(4,4,4-trichloro-3-methyl-1-oxobutylamino)-heptanoic acid methyl ester	Pyrrolidinone (polychlorinated)	NR	Red Sea (20 m)	Egypt	France	(Sauleau et al., 2005)
Lamellodysidea herbacea	Dysideidae	7,7,7-trichloro-2,2,6-trimethyl-3-oxo-4-(4,4,4-trichloro-3-methyl-1-oxobutylamino)- heptanoic acid methyl ester	Pyrrolidinone (polychlorinated)	NR	Red Sea (20 m)	Egypt	France	(Sauleau et al., 2005)
Lamellodysidea herbacea	Dysideidae	Dysidamide F	Pyrrolidinone (polychlorinated)	NR	Red Sea (20 m)	Egypt	France	(Sauleau et al., 2005)
Lamellodysidea herbacea	Dysideidae	Dysidamide G with 5-epi-dysidamide G	Pyrrolidinone (polychlorinated)	NR	Red Sea (20 m)	Egypt	France	(Sauleau et al., 2005)
Lamellodysidea herbacea	Dysideidae	Dysidamide H	Pyrrolidinone (polychlorinated)	NR	Red Sea (20 m)	Egypt	France	(Sauleau et al., 2005)
Lamellodysidea herbacea	Dysideidae	Cholesta-8-en-3 ,5 ,6 ,25-tetrol	Steroid (polyhydroxy)	NR	Red Sea (20 m)	Egypt	France	(Sauleau et al., 2005)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Lamellodysidea herbacea	Dysideidae	Cholesta-8(14)-en-3 ,5 ,6 ,25-tetrol	Steroid (polyhydroxy)	NR	Red Sea (20 m)	Egypt	France	(Sauleau et al., 2005)
Lamellodysidea herbacea	Dysideidae	Cholesta-8,24-dien-3 ,5 ,6 -triol	Steroid (polyhydroxy)	Antifungal	Red Sea (20 m)	Egypt	France	(Sauleau et al., 2005)
Lamellodysidea herbacea	Dysideidae	Cholesta-8(14),24-dien-3 ,5 ,6 -triol	Steroid (polyhydroxy)	Antifungal	Red Sea (20 m)	Egypt	France	(Sauleau et al., 2005)
Lamellodysidea herbacea	Dysideidae	2-(3′,5′-dibromo-2′-hydroxyphenoxy)-3,4,6-tribromophenol	Polyhalogenated diphenyl ether	Bcl-2 activity	Vim Levu	Fiji	USA	(Calcul et al., 2009)
Lamellodysidea herbacea	Dysideidae	2-(3′,5′-dibromo-2′-hydroxyphenoxy)-4,5,6-tribromophenol	Polyhalogenated diphenyl ether	Bcl-2 activity	Vim Levu NR (9.1-18.3 m)	Fiji	USA	(Calcul et al., 2009)
Lamellodysidea herbacea	Dysideidae	2-(2′,4′-dibromophenoxy)-5,6-dibromo-3-chlorophenol	Polyhalogenated diphenyl ether	Bcl-2 activity	Vim Levu	Fiji	USA	(Calcul et al., 2009)
Lamellodysidea herbacea	Dysideidae	2-(2′,4′-dibromophenoxy)-3,5-dibromo-4-methoxyphenol	Polyhalogenated diphenyl ether	Bcl-2 activity	Vim Levu	Fiji	USA	(Calcul et al., 2009)
Lendenfeldia chondrodes	Thorectidae	1-deoxynojirimycin-6-phosphate	Amino acid derivative	NR	Yap (6-8m)	Micronesia	Japan	(Sakai and Kamiya, 2006)
Lendenfeldia chondrodes	Thorectidae	N-methyl-1-deoxynojirimycin-6-phosphate	Amino acid derivative	NR	Yap (6-8m)	Micronesia	Japan	(Sakai and Kamiya, 2006)
Lendenfeldia sp.	Thorectidae	Furospongolide	Furanolipid (terpene derived)	Inhibited breast tumor cells	Saipan, Commonwealth of the Northern Mariana Islands	USA	USA	(Liu et al., 2008)
Lendenfeldia sp.	Thorectidae	(S)-2,2′-dimethoxy-1,1′-binaphthyl-5,5′,6,6′-tetraol	Naphthalene dimer	Inhibited hypoxia induced hif-1, anticancer	NR	Indonesia	USA	(Dai et al., 2007)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Lendenfeldia sp.	Thorectidae	3-[(3e,7e)-4,8-dimethylpentadeca-3,7- dienyl	Furanolipid	NR	NR	Indonesia	USA	(Dai et al., 2007)
Lendenfeldia sp.	Thorectidae	16β-hydroxy-24-methyl-12,24-dioxoscalaran-25-al	C22 furanoterpenoid	Ctyotoxicity	Barren Island, South of Nosy Lava	Madagascar	Israel	(Chill et al., 2004)
Lendenfeldia sp.	Thorectidae	2β,16β,22-trihydroxy-24-methyl-24-oxoscalaran-25-oxo methyl ester	Sesterterpene	Ctyotoxicity	Barren Island, South of Nosy Lava	Madagascar	Israel	(Chill et al., 2004)
Lendenfeldia sp.	Thorectidae	12β,16β,22-trihydroxy-24-methyl-24-oxoscalaran-25-oic acid	Sesterterpene	NR	Barren Island, South of Nosy Lava	Madagascar	Israel	(Chill et al., 2004)
Lendenfeldia sp.	Thorectidae	12b,16b-dihydroxy-22-acetoxy-24-methyl-24-oxoscalaran- 25-oxo methyl ester	Sesterterpene	NR	Barren Island, South of Nosy Lava	Madagascar	Israel	(Chill et al., 2004)
Luffariella cf. *variabilis*	Thorectidae	24-O-ethylmanoalide	Sesterterpene	NR	Mayotte Island, Indian Ocean	India	France	(Gauvin-Bialecki et al., 2008)
Luffariella variabilis	Thorectidae	25 acetoxyluffariellin A	Sesterpene (acetylated)	NR	Orpheus Island (10 m)	Australia	Australia	(Ettinger-Epstein et al, 2007)
Luffariella variabilis	Thorectidae	25 acetoxyluffariellin B	Acetylated sesterpene	NR	Orpheus Island (10 m)	Australia	Australia	(Ettinger-Epstein et al, 2007)
Luffariella variabilis	Thorectidae	25-acetoxyseco-manoalide	Acetylated sesterpene	NR	Orpheus Island (10 m)	Australia	Australia	(Ettinger-Epstein et al, 2007)
Luffariella sp.	Thorectidae	24-n-propyl-O-manoalide	Sesquiterpenoid	Cytotoxicity, antifungal	Pohnpei	Micronesia	China	(Zhou and Molinski, 2006)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Luffariella sp.	Thorectidae	Luffariolide H	Sesterterpenoid	Cyotoxicity, antibacterial	Nakijin, Okinawa	Japan	Japan	(Tsuda et al., 2002)
Luffariella sp.	Thorectidae	Luffariolide J	Sesterterpenoid	Cyotoxicity, antibacterial	Nakijin, Okinawa	Japan	Japan	(Tsuda et al., 2002)
Phyllospongia lamellosa	Thorectidae	Phyllolactone A	Sesterterpene (bishomoscalarane)	Anti-HIV, Cytotoxicity	NR	Indo West Pacific	USA	(Chee Chang et al., 2001)
Phyllospongia lamellosa	Thorectidae	Phyllolactone B	Sesterterpene (bishomoscalarane)	Anti-HIV, Cytotoxicity	NR	Indo West Pacific	USA	(Chee Chang et al., 2001)
Phyllospongia lamellosa	Thorectidae	Phyllolactone C	Sesterterpene (bishomoscalarane)	Anti-HIV, Cytotoxicity	NR	Indo West Pacific	USA	(Chee Chang et al., 2001)
Phyllospongia lamellosa	Thorectidae	Phyllolactone D	Sesterterpene (bishomoscalarane)	Anti-HIV, Cytotoxicity	NR	Indo West Pacific	USA	(Chee Chang et al., 2001)
Phyllospongia lamellosa	Thorectidae	Phyllolactone E	Sesterterpene (bishomoscalarane)	Anti-HIV, Cytotoxicity	NR	Indo West Pacific	USA	(Chee Chang et al., 2001)
Phyllospongia dendyi	Thorectidae	$C_{13}H_6Br_6O_3$	Polybrominated diphenyl ether	NR	NR	Palau	Japan	(Liu et al., 2004)
Phyllospongia dendyi	Thorectidae	$C_{13}H_7Br_5O_3$	Polybrominated diphenyl ether	NR	NR	Palau	Japan	(Liu et al., 2004)
Phyllospongia dendyi	Thorectidae	3,5-dibromo-2-(4,6-dibromo-2-hydroxyphenoxy) anisole	Bromophenol	Antimacro and microalgal activity	NR	Palau	Japan	(Hattori et al., 2001)
Phyllospongia dendyi	Thorectidae	4,6-dibromo-2-(4-bromo-2-hydroxyphenoxy) anisole	Bromophenol	Antimacro and microalgal activity	NR	Palau	Japan	(Hattori et al., 2001)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Phyllospongia dendyi	Thorectidae	4-bromo-2-(4,6-dibromo-2-hydroxyphenoxy) anisole	Bromophenol	Antimacro and microalgal activity	NR	Palau	Japan	(Hattori et al., 2001)
Phyllospongia dendyi	Thorectidae	4,6-dibromo-2-(3,5-dibromo-6-hydroxy-2- methoxyphenoxy) anisole	Bromophenol	Antimacro and microalgal activity	NR	Palau	Japan	(Hattori et al., 2001)
Phyllospongia foliascens	Thorectidae	Phyllofolactone M	Sesterterpene (scalarane)	NR	Yongxing Island, South China Sea	China	China	(Zhang et al., 2010)
Phyllospongia foliascens	Thorectidae	(24e)-5α,6α-epoxystigmasta-7,24(28)-dien-3β-ol	Sterol	NR	Yongxing Islan, South China Sea	China	China	(Zhang et al., 2010)
Phyllospongia foliascens	Thorectidae	Phyllofolactone L	Sesterterpene	NR	Yongxing Island, South China Sea	China	China	(Zhang et al., 2009)
Phyllospongia foliascens	Thorectidae	Phyllofolactone D	Sesterterpene	Cytotoxicity	Yongxing Island. South China Sea	China	China	(Zhang et al., 2009)
Phyllospongia foliascens	Thorectidae	Phyllofolactone E	Sesterterpene	NR	Souch China Sea	China	China	(Zhang et al., 2009)
Phyllospongia madagascarensis	Thorectidae	16β-acetoxy-20,24-dimethyl-12,24-dioxo-25-norscalarane	Sesterterpene (scalarane-based)	NR	Near northwest coast	Madagascar	Russia	(Ponomarenko et al., 2004)
Phyllospongia madagascarensis	Thorectidae	12β-hydroxy-20,24-dimethyl-13,18-oxa-25-norscalarane	Sesterterpene (scalarane-based)	NR	Near northwest coast	Madagascar	Russia	(Ponomarenko et al., 2004)
Phyllospongia papyracea	Thorectidae	12α-acetoxy-13β,18β-cyclobutane-20,24-dimethyl-24-oxoscalar- 16-en-25β-ol	Sesterterpene (bishomoscalarane)	NR	NR (18.3 m)	Papua New Guinea	USA	(Li et al., 2007)
Phyllospongia papyracea	Thorectidae	12α-acetoxy-13β,18β-cyclobutane-20,24-dimethyl-24-oxoscalar- 16-en-25α-ol	Sesterterpene (bishomoscalarane)	NR	NR (18.3 m)	Papua New Guinea	USA	(Li et al., 2007)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Phyllospongia papyracea	Thorectidae	12α-acetoxy-13β,18β-cyclobutane-20,24-dimethyl-24-oxoscalar- 16-en-25β-ol	Sesterterpene (bishomoscalarane)	NR	NR (18.3 m)	Papua New Guinea	USA	(Li et al., 2007)
Phyllospongia papyracea	Thorectidae	12α-acetoxy-16α-(3/-ydroxypentanoyloxy)-20,24-dimethyl-24-oxoscalaran-25β-oic acid	Sesterterpene (bishomoscalarane)	NR	NR (18.3 m)	Papua New Guinea	USA	(Li et al., 2007)
Phyllospongia papyracea	Thorectidae	25-nor-12α-acetoxy-20,24-dimethyl-24-oxoscalar-16-en-18β-ol	Sesterterpene (bishomoscalarane)	NR	NR (18.3 m)	Papua New Guinea	USA	(Li et al., 2007)
Phyllospongia papyracea	Thorectidae	12α-acetoxy-20,24-dimethyl-16,24-dioxoscalara-14,17-dien-24-ol- 25,24-olide	Sesterterpene (bishomoscalarane)	NR	NR (18.3 m)	Papua New Guinea	USA	(Li et al., 2007)
Phyllospongia papyracea	Thorectidae	(12α,16β)-12-acetoxy-16-hydroxy-20,24-dimethyl-25-norscalar-17-en-24-one	Sesterterpenoid (scalarane-type)	Anticancer	Hainan Island (10-15 m)	China	China	(Lan and Li, 2007)
Phyllospongia papyracea	Thorectidae	(12α,24r)-	Sesterterpenoid (scalarane-type)	NR	Hainan Island (10-15 m)	China	China	(Lan and Li, 2007)
Phyllospongia papyracea	Thorectidae	(12α,24s)-12-[(3-hydroxypentanoyl)oxy]-20,24-dimethyl-25-oxoscalar-15,17-dien-25,24-olide	Sesterterpenoid (scalarane-type)	NR	Hainan Island (10-15 m)	China	China	(Lan and Li, 2007)
Phyllospongia sp.	Thorectidae	Compound 1 ($C_{28}H_{44}O_5$)	Sesterterpene	Cytotoxicity	Makassar (10-15 m)	Indonesia	Japan	(Roy et al., 2002)
Phyllospongia sp.	Thorectidae	Compound 1a ($C_{28}H_{44}O_5$)	Sesterterpene	Cytotoxicity	Makassar (10-15 m)	Indonesia	Japan	(Roy et al., 2002)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Phyllospongia sp.	Thorectidae	Compound 3 ($C_{28}H_{44}O_4$)	Sesterterpene	Cytotoxicity	Makassar (10-15 m)	Indonesia	Japan	(Roy et al., 2002)
Phyllospongia sp.	Thorectidae	Compound 4 ($C_{29}H_{46}O_4$)	Sesterterpene	Cytotoxicity	Makassar (10-15 m)	Indonesia	Japan	(Roy et al., 2002)
Phyllospongia sp.	Thorectidae	Compound 5 ($C_{30}H_{49}O_6$)	Sesterterpene	Cytotoxicity	Makassar (10-15 m)	Indonesia	Japan	(Roy et al., 2002)
Phyllospongia sp.	Thorectidae	Compound 6 ($C_{27}H_{42}O_3$)	Sesterterpene	Cytotoxicity	Makassar (10-15 m)	Indonesia	Japan	(Roy et al., 2002)
Phyllospongia sp.	Thorectidae	Compound 7 ($C_{27}H_{42}O_4$)	Sesterterpene	Cytotoxicity	Makassar (10-15 m)	Indonesia	Japan	(Roy et al., 2002)
Psammocinia aff. *bulbosa*	Irciniidae	Psymbamide A	Peptide (brominated cyclic)	NR	Milne Bay	Papua New Guinea	USA	(Robinson et al., 2007)
Psammocinia sp.	Irciniidae	Psammocinin A1	Furanosesterterpene	Cytotoxicity	Ulleung Island (20 m)	Korea	Korea	(Choi et al., 2004)
Psammocinia sp.	Irciniidae	Psammocinin A2	Furanosesterterpene	Cytotoxicity	Ulleung Island (20 m)	Korea	Korea	(Choi et al., 2004)
Psammocinia sp.	Irciniidae	Psammocinin B	Furanosesterterpene	Cytotoxicity	Ulleung Island (20 m)	Korea	Korea	(Choi et al., 2004)
Psammocinia sp.	Irciniidae	Psymberin	Pederin	Anticancer	NR	Papua New Guinea	USA	(Cichewicz et al., 2004b)
Psammocinia sp.	Irciniidae	Chromarol A	Meroditerpene	Exhibiting selective (>25-166-fold) inhibition against 15- hLO versus 12- hLO	Madang region	Papua New Guinea	USA	(Cichewicz et al., 2004a)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Psammocinia sp.	Irciniidae	Chromarol B	Meroditerpene	Exhibiting selective (>25-166-fold) inhibition against 15- hLO versus 12- hLO	Madang region	Papua New Guinea	USA	(Cichewicz et al., 2004a)
Psammocinia sp.	Irciniidae	Chromarol C	Meroditerpene	Exhibiting selective (>25-166-fold) inhibition against 15-hLO versus 12- hLO	Madang region	Papua New Guinea	USA	(Cichewicz et al., 2004a)
Psammocinia sp.	Irciniidae	Chromarol D	Meroditerpene	Exhibiting selective (>25-166-fold) inhibition against 15-hLO versus 12-hLO	Madang region	Papua New Guinea	USA	(Cichewicz et al., 2004a)
Psammocinia sp.	Irciniidae	Chromarol E	Meroditerpene	Inhibition both 15-hLOand 12-hLO	Madang region	Papua New Guinea	USA	(Cichewicz et al., 2004a)
Psammoclema sp.	Thorectidae	3α,12α,16α-Trihydroxy-24ξ -ethylcholest-25-ene	Sterol (trihydroxy)	Anticancer	Nelson Bay, New South Wales (8 m)	Australia	Australia	(Holland et al., 2009)
Psammoclema sp.	Thorectidae	3α,12α,16α-Trihydroxy-24R-methylcholest-22e-ene	Sterol (trihydroxy)	Anticancer	Nelson Bay, New South Wales (8 m)	Australia	Australia	(Holland et al., 2009)
Psammoclema sp.	Thorectidae	3α,12α,16α-Trihydroxy-24-methylcholest-24(28)-ene	Sterol (trihydroxy)	Anticancer	In Nelson Bay, New South Wales (8 m)	Australia	Australia	(Holland et al., 2009)
Psammoclema sp.	Thorectidae	3α,12α,16α-Trihydroxycholestane	Sterol (trihydroxy)	Anticancer	In Nelson Bay, New South Wales (8 m)	Australia	Australia	(Holland et al., 2009)
Sarcotragus sp.	Irciniidae	Compound 1	Sesterterpenoid	Antibacterial	Jeju Island (25 m)	Korea	Korea	(Wang et al., 2008)
Sarcotragus sp.	Irciniidae	Compound 2	Sesterterpenoid	Antibacterial	Jeju Island (25 m)	Korea	Korea	(Wang et al., 2008)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Sarcotragus sp.	Irciniidae	Compound 3	Sesterterpenoid	Antibacterial, Cytotoxicity	Jeju Island (25 m)	Korea	Korea	(Wang et al., 2008)
Sarcotragus sp.	Irciniidae	Compound 4	Sesterterpenoid	NR	Jeju Island (25 m)	Korea	Korea	(Wang et al., 2008)
Sarcotragus sp.	Irciniidae	Compound 5	Sesterterpenoid	NR	Jeju Island (25 m)	Korea	Korea	(Wang et al., 2008)
Sarcotragus sp.	Irciniidae	Compound 6	Sesterterpenoid	Inibitory against isocitrate lyase	Jeju Island (25 m)	Korea	Korea	(Wang et al., 2008)
Sarcotragus sp.	Irciniidae	Compound 7	Sesterterpenoid	NR	Jeju Island (25 m)	Korea	Korea	(Wang et al., 2008)
Sarcotragus sp.	Irciniidae	Compound 8	Sesterterpenoid	NR	Jeju Island (25 m)	Korea	Korea	(Wang et al., 2008)
Sarcotragus sp.	Irciniidae	Compound 9	Sesterterpenoid	NR	Jeju Island (25 m)	Korea	Korea	(Wang et al., 2008)
Sarcotragus sp.	Irciniidae	Compound 10	Sesterterpenoid	NR	Jeju Island (25 m)	Korea	Korea	(Wang et al., 2008)
Sarcotragus sp.	Irciniidae	Compound 11	Sesterterpenoid	Cytotoxicity	Jeju Island (25 m)	Korea	Korea	(Wang et al., 2008)
Sarcotragus sp.	Irciniidae	Compound 12	Sesterterpenoid	Cytotoxicity	Jeju Island (25 m)	Korea	Korea	(Wang et al., 2008)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Sarcotragus sp.	Irciniidae	Compound 13	Sesterterpenoid	Inhibitory against isocritate clees	Jeju Island (25 m)	Korea	Korea	(Wang et al., 2008)
Sarcotragus sp.	Irciniidae	Compound 14	Sesterterpenoid	NR	Jeju Island (25 m)	Korea	Korea	(Wang et al., 2008)
Sarcotragus sp.	Irciniidae	Compound 15	Sesterterpenoid	NR	Jeju Island (25 m)	Korea	Korea	(Wang et al., 2008)
Sarcotragus sp.	Irciniidae	Compound 16	Sesterterpenoid	NR	Jeju Island (25 m)	Korea	Korea	(Wang et al., 2008)
Sarcotragus sp.	Irciniidae	Compound 17	Sesterterpenoid	Antibacterial	Jeju Island (25 m)	Korea	Korea	(Wang et al., 2008)
Sarcotragus sp.	Irciniidae	Compound 18	Sesterterpenoid	Antibacterial	Jeju Island (25 m)	Korea	Korea	(Wang et al., 2008)
Sarcotragus sp.	Irciniidae	Compound 19	Sesterterpenoid	NR	Jeju Island (25 m)	Korea	Korea	(Wang et al., 2008)
Sarcotragus sp.	Irciniidae	Melophlin ($C_{26}H_{44}O_4$)	Glycerolipid	Cytotoxicity	Cheju Island (15-25 m)	Korea	China	(Liu et al., 2006a)
Sarcotragus sp.	Irciniidae	Compound 3 ($C_{21}H_{40}O_4$)	Glycerolipid	Cytotoxicity	Cheju Island (15-25 m)	Korea	China	(Liu et al., 2006a)
Sarcotragus sp.	Irciniidae	Sarcotin N	Norsesterterpenoid	Cytotoxicity	Jeju Island (15- 25 m)	Korea	Korea	(Liu et al., 2003)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Sarcotragus sp.	Irciniidae	Sarcotin O	Norsesterterpenoid	Cytotoxicity	Jeju Island (15- 25 m)	Korea	Korea	(Liu et al., 2003)
Sarcotragus sp.	Irciniidae	ent-kurospongin	Kurospongin	Cytotoxicity	Jeju Island (15- 25 m)	Korea	Korea	(Liu et al., 2003)
Sarcotragus sp.	Irciniidae	epi-sarcotin F	Sesterterpenoid	Cytotoxicity	Jeju Island (15- 25 m)	Korea	Korea	(Liu et al., 2003)
Sarcotragus sp.	Irciniidae	Sarcotrine E	Pyrroloterpenoid	Cytotoxicity	Jeju Island (15- 25 m)	Korea	Korea	(Liu et al., 2003)
Sarcotragus sp.	Irciniidae	Isosarcotrine E	Pyrroloterpenoid	Cytotoxicity	Jeju Island (15- 25 m)	Korea	Korea	(Liu et al., 2003)
Sarcotragus sp.	Irciniidae	Sarcotrine F	Pyrroloterpenoid	Cytotoxicity	Jeju Island (15- 25 m)	Korea	Korea	(Liu et al., 2003)
Sarcotragus sp.	Irciniidae	Isosarcotrine F	Pyrroloterpenoid	Cytotoxicity	Jeju Island (15- 25 m)	Korea	Korea	(Liu et al., 2003)
Sarcotragus sp.	Irciniidae	Sarcotride B	Cyclitol derivative	Cytotoxicity	Cheju Island (15-25 m)	Korea	Korea	(Liu et al., 2003)
Sarcotragus sp.	Irciniidae	Sarcotride C	Cyclitol derivative	Cytotoxicity	Cheju Island (15-25 m)	Korea	Korea	(Liu et al., 2003)
Sarcotragus sp.	Irciniidae	Sarcotrine A	Pyrrolosesterterpene	Anticancer	Cheju Island (15-25 m)	Korea	Korea	(Liu et al., 2002a)
Sarcotragus sp.	Irciniidae	epi-sarcotrine A	Pyrrolosesterterpene	Cytotoxicity and toxic to brine shrimp larvae	Cheju Island (15-25 m)	Korea	Korea	(Liu et al., 2003)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Sarcotragus sp.	Irciniidae	Sarcotrine B	Pyrrolosesterterpene	Cytotoxicity and toxic to brine shrimp larvae	Cheju Island (15-25 m)	Korea	Korea	(Liu et al., 2003)
Saeeercotragus sp.	Irciniidae	epi-sarcotrine B	Pyrrolosesterterpene	Cytotoxicity and toxic to brine shrimp larvae	Cheju Island (15-25 m)	Korea	Korea	(Liu et al., 2003)
Saercotragus sp.	Irciniidae	Sarcotrine C	Pyrrolosesterterpene	Cytotoxicity and toxic to brine shrimp larvae	Cheju Island (15-25 m)	Korea	Korea	(Liu et al., 2003)
Seeaercotragus sp.	Irciniidae	epi-sarcotrine C	Pyrrolosesterterpene	Cytotoxicity and toxic to brine shrimp larvae	Cheju Island (15-25 m)	Korea	Korea	(Liu et al., 2003)
Sarcotragus sp.	Irciniidae	Sarcotrine D	Pyrrolosesterterpene	Cytotoxicity and toxic to brine shrimp larvae	Cheju Island (15-25 m)	Korea	Korea	(Liu et al., 2003)
Sarcotragus sp.	Irciniidae	Sarcotin A	Sesterterpene	Cytotoxicity	Cheju Island (15-25 m)	Korea	Korea	(Liu et al., 2001)
Sarcotragus sp.	Irciniidae	Sarcotin B	Sesterterpene	Cytotoxicity	Cheju Island (15-25 m)	Korea	Korea	(Liu et al., 2001)
Sarcotragus sp.	Irciniidae	Sarcotin C	Sesterterpene	Cytotoxicity	Cheju Island (15-25 m)	Korea	Korea	(Liu et al., 2001)
Sarcotragus sp.	Irciniidae	Sarcotin D	Sesterterpene	Cytotoxicity	Cheju Island (15-25 m)	Korea	Korea	(Liu et al., 2001)
Searcotragus sp.	Irciniidae	Sarcotin E	Sesterterpene	Cytotoxicity	Cheju Island (15-25 m)	Korea	Korea	(Liu et al., 2001)
Searcotragus sp.	Irciniidae	Sarcotragin A	Sesterterpenoid alkaloid	Cytotoxicity	Seoguipo, Jaeju Island (25 m)	Korea	Korea	(Shin et al., 2001)
Sarcotragus sp.	Irciniidae	Sarcotragin B	Sesterterpenoid alkaloid	Cytotoxicity	Seoguipo, Jaeju Island (25 m)	Korea	Korea	(Shin et al., 2001)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Sarcotragus sp.	Irciniidae	Sarcotride D	Glycolipid (Cyclitol derivative)	Cytotoxicity	Cheju Island (15-25m)	Korea	China	(Liu et al., 2011)
Sarcotragus sp.	Irciniidae	Sarcotin P	Norsesterterpenoid	Cytotoxicity and toxicity to brine shrimp	Cheju Island (15–25 m)	Korea	Korea	(He et al., 2012)
Scalarispongia aqabaensis	Thorectidae	Scalaristerol (5α,8α-dihydroxycholest-6-en-3β-ol)	Sterol	Antiinflammatory	Red Sea	Egypt	Egypt	(Youssef et al. 2010)
Smenospongia aurea	Thorectidae	N-3/-ethylaplysinopsin	Indole alkaloid	Antiinfective and human 5-ht2 receptor binding	Discovery Bay (16-30 m)	Jamaica	USA	(Hu et al., 2002)
Smenospongia aurea	Thorectidae	Makaluvamine O	Pyrroloiminoquinone	Antiinfective and human 5-ht2 receptor binding	Discovery Bay (16-30 m)	Jamaica	USA	(Hu et al., 2002)
Smenospongia cerebriformis	Thorectidae	Dictazoline A	Bisspiroimidazolidinone derivative	NR	Hospital Point on Solarte Isle, Boca del Toro, northwest coast (2-3 m)	Panama	USA	(Dai et al., 2008)
Smenospongia cerebriformis	Thorectidae	Dictazoline B	Bisspiroimidazolidinone derivative	NR	Hospital Point on Solarte Isle, Boca del Toro, northwest coast (2-3 m)	Panama	USA	(Dai et al., 2008)
Smenospongia sp.	Thorectidae	12-deacetoxy-23-acetoxy-19-O-acetylscalarin	Sesterterpene	Cytotoxic	Gagu-Do (Island), southwestern Korea (15-20 m)	Korea	Korea	(Rho et al., 2004)
Smenospongia sp.	Thorectidae	12-deacetoxy-23-acetoxyscalarin	Sesterterpene	Cytotoxicity	Gagu-Do (Island), southwestern Korea (15-20 m)	Korea	Korea	(Rho et al., 2004)
Smenospongia sp.	Thorectidae	12-deacetoxy-23-hydroxyheteronemin	Sesterterpene	Cytotoxicity	Gagu-Do (Island), southwestern Korea (15-20 m)	Korea	Korea	(Rho et al., 2004)
Smenospongia sp.	Thorectidae	4-hydroxy-9-deoxoidiadione	Sesterterpene	Cytotoxicity	Gagu-Do (Island), southwestern Korea (15-20 m)	Korea	Korea	(Rho et al., 2004)
Smenospongia sp.	Thorectidae	4-acetoxy-9-deoxoidiadione	Sesterterpene	Cytotoxicity	Gagu-Do (Island), southwestern Korea (15-20 m)	Korea	Korea	(Rho et al., 2004)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Smenospongia sp.	Thorectidae	Smenospongine	Sesquiterpene aminoquinone	Induces erythroid differentiation in k562 cells	NR	Indonesia	Japan	(Aoki et al., 2004)
Smenospongia sp.	Thorectidae	6-bromo-1h-indole-3-carboxylic acid methyl ester	Brominated tryptophan derivative	Antibacterial	Southwest tip of Misima Island in the Louisiade Achipelago (6-30.5 m)	Papua New Guinea	USA	(Segraves and Crews, 2005)
Spongia (Heterofibria) sp.	Spongiidae	19-acetoxy*Spongia*-13(16),14-dien-3-one	Diterpenoid	NR	Near Suwarrow atoll (Northern Cook Islands) (5 m)	New Zealand	Russia	(Ponomarenko et al., 2007)
Spongia (Heterofibria) sp.	Spongiidae	3α,19-diacetoxy*Spongia*-13(16),14-diene	Diterpenoid	Lysosomal activation	Near Suwarrow atoll (Northern Cook Islands) (5 m)	New Zealand	Russia	(Ponomarenko et al., 2007)
Spongia (Heterofibria) sp.	Spongiidae	3α-acetoxy*Spongia*-13(16),14-diene	Diterpenoid	NR	Near Suwarrow atoll (Northern Cook Islands) (5 m)	New Zealand	Russia	(Ponomarenko et al., 2007)
Spongia (Heterofibria) sp.	Spongiidae	3r-acetoxy*Spongia*-13(16),14-diene	Diterpenoid	NR	Near Suwarrow atoll (Northern Cook Islands) (5 m)	New Zealand	Russia	(Ponomarenko et al., 2007)
Spongia (Heterofibria) sp.	Spongiidae	3,18-methylene-2r-acetoxy*Spongia*-13(16),14-diene	Diterpenoid	NR	Near Suwarrow atoll (Northern Cook Islands) (5 m)	New Zealand	Russia	(Ponomarenko et al., 2007)
*Spongia (Heterofibria)*sp.	Spongiidae	Heterofibrin A1	Fatty acid	Antibacterial	Great Australian Bight (125 m)	Australia	Australia	(Salimet al., 2010)
Spongia (Heterofibria) sp.	Spongiidae	Heterofibrin A2	Ester	Antibacterial	Great Australian Bight (125 m)	Australia	Australia	(Salimet al., 2010)
Spongia (Heterofibria) sp.	Spongiidae	Heterofibrin A3	Ester	Antibacterial	Great Australian Bight (125 m)	Australia	Australia	(Salimet al., 2010)
Spongia (Heterofibria) sp.	Spongiidae	Heterofibrin B2	Ester	Antibacterial	Great Australian Bight (125 m)	Australia	Australia	(Salimet al., 2010)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Spongia (Heterofibria) sp.	Spongiidae	Heterofibrin B3	Ester	Antibacterial	Great Australian Bight (125 m)	Australia	Australia	(Salimet al., 2010)
Spongia (Heterofibria) sp.	Spongiidae	Heterofibrins B1	Fatty acid	Antibacterial	Great Australian Bight (125 m)	Australia	Australia	(Salimet al., 2010)
Spongia irregularis	Spongiidae	Irregularasulfate	Sesterpenoid	Inhibited calcineurin	Near Keviang	Papua New Guinea	Canada	(Carr et al., 2007)
Spongia officinalis	Spongiidae	7,8-Epoxyfurospongin-1 ($C_{21}H_{30}O_4$)	Terpene	NR	Mazara del Vallo along the Sicily coast (80 m)	Italy	Italy	(Manzo et al., 2011)
Spongia officinalis	Spongiidae	Officinoic acid A	Terpene	NR	Mazara del Vallo along the Sicily coast (80 m)	Italy	Italy	(Zhang et al., 2011)
Spongia officinalis	Spongiidae	Officinoic acid B	Terpene	NR	Mazara del Vallo along the Sicily coast (80 m)	Italy	Italy	(Zhang et al., 2011)
Spongia officinalis	Spongiidae	Isofurospongin-4 ($C_{26}H_{36}O_5$)	Terpene	NR	Mazara del Vallo along the Sicily coast (80 m)	Italy	Italy	(Zhang et al., 2011)
Spongia sp.	Spongiidae	N-palmitoyl-heptacosane-1,3,5-triol	Ceramide (sphingolipid)	Antibacterial, anticancer	Sanya, Hainan Island	China	China	(Guan and Zeng, 2010)
Spongia sp.	Spongiidae	N-palmitoyl-heptacosane-1,3,5-triol	Ceramide (sphingolipid)	Antibacterial, anticancer	Sanya, Hainan Island	China	China	(Guan and Zeng, 2010)
Spongia sp.	Spongiidae	Furanoditerpenoid 1	Diterpene	NR	NR	Fiji	Germany	(Gross et al., 2009)
Spongia sp.	Spongiidae	Furanoditerpenoid 2	Diterpene	NR	NR	Fiji	Germany	(Gross et al., 2009)
Spongia sp.	Spongiidae	Furanoditerpenoid 3	Diterpene	NR	NR	Fiji	Germany	(Gross et al., 2009)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Spongia sp.	Spongiidae	20-acetoxy-19-hydroxy*Spongia*-13(16),14-diene	Diterpene	Trh receptorr2 binding activity	West Islet, Wreck Reef, Coral Sea (20 m)	Australia	Australia	(Carroll et al., 2008)
Spongia sp.	Spongiidae	19-acetoxy-20-hydroxy*Spongia*-13(16),14-diene	Diterpene	Trh receptorr2 binding activity	West Islet, Wreck Reef, Coral Sea (20 m)	Australia	Australia	(Carroll et al., 2008)
Spongia sp.	Spongiidae	19,20-diacetoxy*Spongia*-13(16),14-diene	Diterpene	Trh receptorr2 binding activity	West Islet, Wreck Reef, Coral Sea (20 m)	Australia	Australia	(Carroll et al., 2008)
Spongia sp.	Spongiidae	19,20-dihydroxy*Spongia*-13(16),14-diene	Diterpene	Trh receptorr2 binding activity	West Islet, Wreck Reef, Coral Sea (20m)	Australia	Australia	(Carroll et al., 2008)
Spongia sp.	Spongiidae	Metachromin L	Sesquiterpenoid quinone	Cytotoxicity	Gesashi, Okinawa	Japan	Japan	(Takahashi et al., 2007a)
Spongia sp.	Spongiidae	Metachromin M	Sesquiterpenoid quinone	Cytotoxicity	Gesashi, Okinawa	Japan	Japan	(Takahashi et al., 2007a)
Spongia sp.	Spongiidae	Metachromin N	Sesquiterpenoid quinone	NR	Gesashi, Okinawa	Japan	Japan	(Takahashi et al., 2007a)
Spongia sp.	Spongiidae	Metachromin O	Sesquiterpenoid quinone	NR	Gesashi, Okinawa	Japan	Japan	(Takahashi et al., 2007a)
Spongia sp.	Spongiidae	Metachromin P	Sesquiterpenoid quinone	NR	Gesashi, Okinawa	Japan	Japan	(Takahashi et al., 2007a)
Spongia sp.	Spongiidae	Metachromin Q	Sesquiterpenoid quinone	NR	Gesashi, Okinawa	Japan	Japan	(Takahashi et al., 2007a)
Spongia sp.	Spongiidae	Metachromin R	Sesquiterpenoid	NR	Gesashi, Okinawa	Japan	Japan	(Takahashi et al., 2007b)
Spongia sp.	Spongiidae	Metachromin S	Sesquiterpenoid	Cytotoxicity	Gesashi, Okinawa	Japan	Japan	(Takahashi et al., 2007b)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Spongia sp.	Spongiidae	Metachromin T	Sesquiterpenoid	Cytotoxicity	Gesashi, Okinawa	Japan	Japan	(Takahashi et al., 2007b)
Spongia sp.	Spongiidae	Spongolactam A	Diterpenoid (Nitrogenous)	Ftase inhibition and Cytotoxicity	Akajima Island in Okinawa (20 m)	Japan	Japan	(Mori et al., 2007)
Spongia sp.	Spongiidae	Spongolactam B	Diterpenoid (Nitrogenous)	Ftase inhibition and Cytotoxicity	Akajima Island in Okinawa (20 m)	Japan	Japan	(Mori et al., 2007)
Spongia sp.	Spongiidae	Spongolactam C	Diterpenoid (Nitrogenous)	Ftase inhibition and Cytotoxicity	Akajima Island in Okinawa (20 m)	Japan	Japan	(Mori et al., 2007)
Spongia sp.	Spongiidae	12-O-deacetyl-12-epi-19-deoxy-21-hydroxyscalarin	Sesterpene	Inhibition against the farnesoid x-activated receptor (fxr)	Near Tong-Yong City (10 m)	Korea	Korea	(Nam et al., 2007)
Spongia sp.	Spongiidae	12-O-deacetyl-12-epi-19-deoxy-22-hydroxyscalarin	Sesterpene	Inhibition against the farnesoid x-activated receptor (fxr)	Near Tong-Yong City (10 m)	Korea	Korea	(Nam et al., 2007)
Spongia sp.	Spongiidae	12-O-deacetyl-12-epi-19-O-methylscalarin	Sesterpene	NR	Near Tong-Yong City (10 m)	Korea	Korea	(Nam et al., 2007)
Spongia sp.	Spongiidae	Metachromin J	Sesquiterpenoid	NR	Okinawa	Japan	Japan	(Takahashi et al., 2006)
Spongia sp.	Spongiidae	Metachromin K	Sesquiterpenoid	NR	Okinawa	Japan	Japan	(Takahashi et al., 2006)
Spongia sp.	Spongiidae	Deacetoxyscalarin	Sesterterpene	Neurotrophic activity	Toyama Bay	Japan	Japan	(Tokue et al., 2006)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Spongia sp.	Spongiidae	Compound 1 ($C_{29}H_{44}O_6$)	Alcohol (nitrobenzyl)	Inhibition of transactivation for the nuclear hormone receptor, fxr (farnesoid x-activated receptor antagonists) , Cytotoxicity	Tong-Yong City in the South Sea	Korea	Korea	(Nam et al., 2006)
Spongia sp.	Spongiidae	12-O-deacetoxyl-24-hydroxyldeoxoscalarin	Sesterterpene	Inhibition of transactivation for the nuclear hormone receptor, fxr (farnesoid x-activated receptor) , Cytotoxicity	Tong-Yong City in the South Sea	Korea	Korea	(Nam et al., 2006)
Spongia sp.	Spongiidae	12-O-deacetoxyl-19-omethydeoxoscalarin	Sesterterpene	Inhibition of transactivation for the nuclear hormone receptor, fxr (farnesoid x-activated receptor) , Cytotoxicity	Tong-Yong City in the South Sea	Korea	Korea	(Nam et al., 2006)
Spongia sp.	Spongiidae	17-O-isoprenyldictyoceratin-C	Sesterterpenoid (scalarane-type)	NR	Negros Oriental (18 m)	Philippines	USA	(Cao et al., 2004)
Spongia sp.	Spongiidae	12-O-deacetylscalarafuran	Sesterterpenoid (scalarane-type)	Cytotoxicity	Toyama Bay in the Japan Sea (5 m)	Japan	Japan	(Tsukamoto et al., 2003)
Spongia sp.	Spongiidae	12-O-deacetyl-12-epi-scalarin	Sesterterpenoid (scalarane-type)	Cytotoxicity	Toyama Bay (5 m)	Japan	Japan	(Tsukamoto et al., 2003)
Spongia sp.	Spongiidae	12-O-acetyl-16-O-methylhyrtiolide	Scalarane-type sesterterpenoid	Cytotoxicity	Toyama Bay (5 m)	Japan	Japan	(Tsukamoto et al., 2003)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Spongia sp.	Spongiidae	Cyclosmenospongine	Sesquiterpenoid aminoquinone	Cytotoxicity, hemolytic activity	NR	Australia	Australia	(Utkina et al., 2003)
Spongia sp.	Spongiidae	Spongidepsin	Macrolide	Anticancer	Vanuatu Island	Vanuatu	Italy	(Grassia et al., 2001)
Spongia zimocca	Spongiidae	Zimoclactone B	Diterpene (*Spongia*n)	NR	Beihai, Guangxi Province	China	China	(Zeng et al., 2001)
Spongia zimocca	Spongiidae	Zimoclactone C	Diterpene (*Spongia*n)	NR	Beihai, Guangxi Province	China	China	(Zeng et al., 2001)
Strepsichordaia lendenfeldi	Thorectidae	Strepsiamide A	Ceramide	Cytotoxicity	NR	Indonesia	Egypt	(Ibrahim et al., 2008)
Strepsichordaia lendenfeldi	Thorectidae	Strepsiamide B	Ceramide	Cytotoxicity	NR	Indonesia	Egypt	(Ibrahim et al., 2008)
Strepsichordaia lendenfeldi	Thorectidae	Strepsiamide C	Ceramide	Cytotoxicity	NR	Indonesia	Egypt	(Ibrahim et al., 2008)
Thorecta reticulata	Thorectidae	Metachromin U	Merosesquiterpenoid	Cytotoxicity	East of Hunter Island, North West Tasmania (6 m)	Australia	Australia	(Ovenden et al., 2011a)
Thorecta reticulata	Thorectidae	Metachromin V	Merosesquiterpenoid	Cytotoxicity	East of Hunter Island, North West Tasmania (6 m)	Australia	Australia	(Ovenden et al., 2011a)
Thorecta reticulata	Thorectidae	Metachromin W	Merosesquiterpenoid	Cytotoxicity	East of Hunter Island, North West Tasmania (6 m)	Australia	Australia	(Ovenden et al., 2011a)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Thorectandra sp.	Thorectidae	16-oxoluffariellolide	Sesterterpenoid	Inhibition of phosphatase (Cdc25B)	Coral Reef Research Foundation (30 m)	Papua New Guinea	USA	(Cao et al., 2005)
Thorectandra sp.	Thorectidae	16-hydroxyluffariellolide	Sesterterpenoid	Inhibition of phosphatase (Cdc25B)	Coral Reef Research Foundation (30 m)	Papua New Guinea	USA	(Cao et al., 2005)
Thorectandra sp.	Thorectidae	(2e,6e,10e)-3-formyl-7,11-dimethyl-13-(2,6,6-trimethylcyclohex-1-enyl)trideca-2,6,10-trienoic acid	Sesterterpenoid	Inhibition of phosphatase (Cdc25B)	Coral Reef Research Foundation (30 m)	Papua New Guinea	USA	(Cao et al., 2005)
Thorectandra sp.	Thorectidae	Compound 1	Alkaloid (β-carboline)	Anticancer	NR	Palau	USA	(Charan et al., 2004)
Thorectandra sp.	Thorectidae	1-deoxysecofascaplysin A	Alkaloid (β-carboline)	Anticancer	NR	Palau	USA	(Charan et al., 2004)
Thorectandra sp.	Thorectidae	Fascaplysin	Alkaloid (β-carboline)	Anticancer	NR	Palau	USA	(Charan et al., 2004)
Thorectandra sp.	Thorectidae	Thorectandramine	Alkaloid (β-carboline)	Cytotoxicity, anti-proliferative activity	NR	Palau	USA	(Charan et al., 2002b)
Thorectandra sp.	Thorectidae	Thorectandrol C	Sesterterpene	Cytotoxicity	Palau (10 m)	Palau	USA	(Charan et al., 2002a)
Thorectandra sp.	Thorectidae	Thorectandrol D	Sesterterpene	Cytotoxicity	NR (10 m)	Palau	USA	(Charan et al., 2002a)
Thorectandra sp.	Thorectidae	Thorectandrol E	Sesterterpene	NR	NR (10 m)	Palau	USA	(Charan et al., 2002a)
Thorectandra sp.	Thorectidae	Thorectandrol A	Sesterterpene	Cytotoxicity	NR (10 m)	Palau	USA	(Charan et al., 2001)

Name of sponge	Family	Compound name	Chemical class	Activity	Source (depth)	Country of collection	Country of study	Ref.
Thorectandra sp.	Thorectidae	Thorectandrol B	Sesterterpene	Cytotoxicity	NR (10 m)	Palau	USA	(Charan et al., 2001)
Thorectandra sp.	Thorectidae	(-)-5-Bromo-N,N-dimethyltryptophan	Brominated tryptophan derivative	Antibacterial	Milne Bay region of Papua New Guinea. Coast Nuakata Island (9.1-15.2 m)	Papua New Guinea	USA	(Segraves and Crews, 2005)
Thorectandra sp.	Thorectidae	(+)-5-Bromohypaphorine	Brominated tryptophan derivative	Antibacterial	Milne Bay region of Papua New Guinea. Coast Nuakata Island (9.1-15.2 m)	Papua new guinea	USA	(Segraves and Crews, 2005)
Thorectandra sp.	Thorectidae	6-Bromo-1'-hydroxy-1', 8-dihydroaplysinopsin	Brominated tryptophan derivative	Antibacterial	Milne Bay region of Papua New Guinea. Coast Nuakata Island (9.1-15.2 m)	Papua New Guinea	USA	(Segraves and Crews, 2005)
Thorectandra sp.	Thorectidae	6-Bromo-1-ethoxy-1-8-dihydroaplysinopsin	Brominated tryptophan derivative	Antibacterial	Milne Bay region of Papua New Guinea. Coast Nuakata Island (9.1-15.2 m)	Papua New Guinea	USA	(Segraves and Crews, 2005)
Unidentified	Unknown	N6-methyl mucronatine ($C_8H_{12}N_5O$)	N,N-Dimethyl Purine	NR	Inner Gneerings shoals, near Mooloolaba in South East Queensland (10-12 m)	Australia	Australia	(Suciati et al., 2011)
Unknown	Unknown	19-acetoxy*Spongia*-13(16),14-diene	Diterpenoid	Inhibition of lyase activity of DNA polymerase β	Island of Rabaul (26 m)	Papua New Guinea	USA	(Chaturvedula et al., 2004)
Unknown	Unknown	Methyl-ent-15,16-dinorisocopal-12-en-13-ol-19-oate	Bis-norditerpenoid	Inhibition of lyase activity of DNA polymerase β	Island of Rabaul (26 m)	Papua New Guinea	USA	(Chaturvedula et al., 2004)
Unknown	Unknown	ent-15,16-Dinorisocopal-12-en-13-ol-19-oic acid	Bis-norditerpenoid	Inhibition of lyase activity of DNA polymerase β	NR (26 m)	Papua New Guinea	USA	(Chaturvedula et al., 2004)

Appendix 3

chapter fifteen

Secondary metabolites from microorganisms isolated from marine sponges from 2000 to 2012

Mohammad F. Mehbub, Christopher M.M. Franco, and Wei Zhang

Contents

15.1 Introduction

The continuously developing resistance of pathogenic bacteria, the reemergence of viral diseases, and cancers that are still incurable make us redouble our efforts to find cures to alleviate human vulnerabilities. The introduction of new drugs is crucial, as older antibiotics and drugs begin to lose their efficacy. Rationally designed drugs have made inroads into the pharmaceutical industry, but natural products continue to introduce the chemical diversity required to maintain a contribution of around 60% of the drugs, directly or after chemical modification, which are available in the market (Newman and Cragg, 2007). Microbial natural products contribute more than 40% of new chemical entities reported between 1981 and 2010 (Newman et al., 2003; Baltz et al., 2005; Koehn and Carter, 2005; Fisher, 2014).

Whereas the majority of microorganisms that produce valuable products have been obtained predominantly from terrestrial sources, there has been a recent trend to collect microorganisms that are associated with other life forms such as endophytes of plants (Govindasamy et al., 2014). There has also been an evolving realization that the oceans, which cover a larger proportion of the earth's surface, have a different microbial diversity,

and here too there are associations with marine life forms such as sponges. It was noted that due to the aqueous milieu, their metabolites have more potent activities and are usually structurally very different from those found from terrestrial-based samples. Recent studies have borne out this hypothesis, and the marine environment is proving to be an eclectic source of novel chemical diversity that is contributing to drug discovery. Many bioactive substances have been isolated from a variety of marine organisms, including phytoplankton, bryozoans, algae, sponges, tunicates, and molluscs (Faulkner, 2002; Proksch et al., 2002; Zhang et al., 2005; Mehbub et al., 2014). Microorganisms associated with these animals have shown the ability to adapt, and this adaptation capacity may be the key reason for their secondary metabolite production capacity (Piel, 2004, 2009; König et al., 2006; Brady et al., 2009; Valliappan et al., 2014).

Marine sponges (phylum Porifera) are of particular interest because they are remarkable filter feeders; some can filter 24 m^3 kg^{-1} sponge day^{-1} (Vogel, 1977). During the filtration process, they concentrate bacterial cells that are otherwise diluted in seawater. They harbor dense and diverse microbial consortia, which comprise as much as 40% of sponge tissue volume and span all three domains of life (Taylor et al., 2007). This makes sponges excellent models for the study of marine host-associated bacteria as they represent a substantial reservoir of novel microbial diversity (Taylor et al., 2004). Therefore, in recent years, the search has intensified for microorganisms from sponges with the expectation that novel compounds will result from their screening.

There are more than 8500 sponge species (Van Soest et al., 2012) that contain very diverse microbial consortia (Taylor et al., 2004); and, it has been reported that individual species from sponges during the past decade contains at least a few different types of bioactive natural products (Mehbub et al., 2014). Therefore, sponges could be termed the "drugstore of the sea" (Blunt et al., 2009). Many of these compounds likely serve as agents of defense that protect the immobile animals from being overgrown or ingested (Pawlik, 1992; Paul and Ritson-Williams, 2008), but for most substances, an ecological function has not been experimentally demonstrated. Likewise, the often-stated question whether sponge-derived natural products are biosynthesized by sponges or by associated microorganisms remains largely unanswered (Faulkner et al., 1993; Piel et al., 2004). Insights into this issue could have a significant impact on marine pharmacology. For most compounds, drug development is currently not possible due to a limited access to the biological material. If the actual source is a bacterium, supply could be ensured by cultivating the producer or by isolating the biosynthetic genes and expressing the pathway in culturable bacteria (Piel, 2006; Schmitt et al., 2008). The advantage of the latter approach is that it should be generally applicable to a wide range of compounds independent of cultivation. Although the genetic tools to express bacterial pathways are in principle available (Fujii, 2009), the application of this strategy to sponge symbionts is currently highly challenging for several reasons.

This review focuses on microorganisms from sponges that have been reported to produce secondary metabolites or bioactive compounds from 2000 to 2012. The microorganisms reported here are the bacteria, with actinobacteria looked at separately due to their recognized ability to produce a wide range of secondary metabolites, and fungi, including yeast. Data have been compiled from the published literature and data reviewed by Faulkner (2002) and Blunt et al. (2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2011, 2012, 2013, 2014) from Natural Product Reports.

Species isolated previously from marine sediments but subsequently reported from sponges, for example, *Salinispora* strains isolated from the Great Barrier Reef sponge *Pseudoceratina clavata* (Kim et al., 2005), will not be included.

15.2 Microbial sources

The microbial populations include archaea and bacteria (Webster et al., 2001; Hentschel et al., 2003), fungi (Höller et al., 2000), cyanobacteria (Thacker and Starnes, 2003), unicellular algae (Vacelet, 1981), dinoflagellates (Garson et al., 1998), and actinobacteria (Maldonado et al., 2005b), which make up at least half the tissue volume in some sponge species (Vacelet and Donadey, 1977; Hentschel et al., 2003).

Members of the phylum Actinobacteria and specifically the order Actinomycetales have been identified as abundant members of sponge-associated microbial communities (Hentschel et al., 2002; Zhang et al., 2006). However, in this survey, it is evident that there has been a shift from actinobacteria to fungi as the main microorganisms. The number of fungi from sponge samples reported as producers of new compounds is now at least three times higher compared to the reports for actinobacteria from the same source.

Over the 2000–2012 period, a total of 269 new compounds were isolated from sponge-associated microbes (Figure 15.1). Of these, 186 new compounds were isolated from 27 genera of fungi including two compounds from one yeast genus, compared to just 56 new compounds from seven actinobacterial genera and 27 new compounds from seven bacterial genera (Table 15.1). Confirming the switch to fungal sources are the 69 publications on fungal metabolites compared to 20 for actinobacteria and 15 for bacteria during this review period. However, our understanding of the sponge-associated fungal, actinobacterial, and bacterial communities and their structures is still inadequate.

15.2.1 Marine fungi

Fungi from marine sponges are now the primary source of novel metabolites from microbial sources that have industrial as well as medicinal values. By 1998, more than 100 metabolites from marine-derived fungi had been described by different researchers (Biabani and Laatsch, 1998). However, the majority of the reports focused on natural product

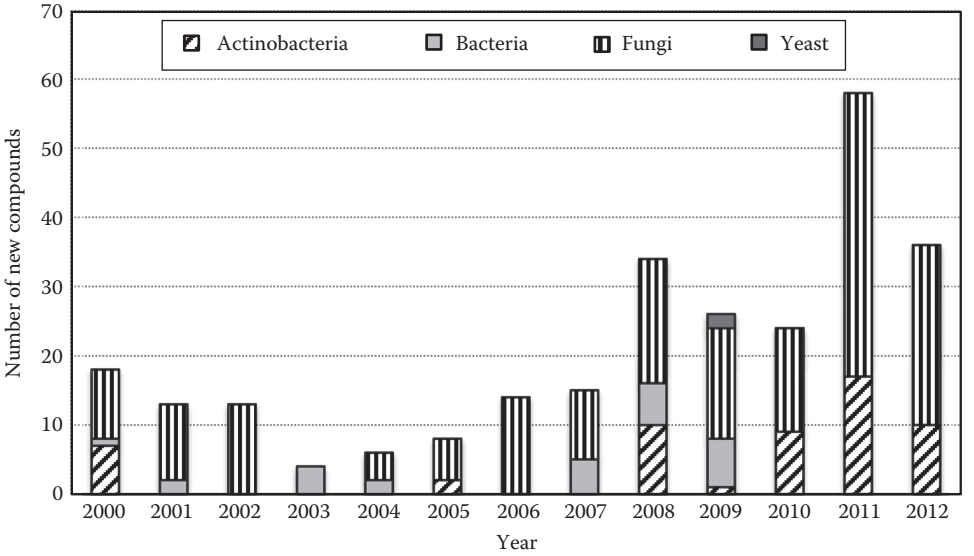

Figure 15.1 The total number of new compounds isolated from sponge-associated microorganisms from 2000 to 2012.

Table 15.1 New compounds isolated from microorganisms from marine sponges from 2000 to 2012: their chemical class, country of collection, and reported activity

Compound name	Chemical class	Country of collection	Name of microorganism	Type of microorganism	Name of sponge	Reported activity	References
GGL.1 1,2-*O*-diacyl-3-[α-glucopyranosyl-(1–6)-α-glucopyranosyl)]glycerol	Glycoglycerolipid	Croatia	*Microbacterium* sp.	Actinobacterium	*Halichondria panicea*	Anticancer	Wicke et al. (2000)
GGL.2 1-*O*-acyl-3-[R-glucopyranosyl-(1–3)-(6-*O*-acyl-R-mannopyranosyl)] glycerol	Glycoglycerolipid	Croatia	*Microbacterium* sp.	Actinobacterium	*Halichondria panicea*	Anticancer	Wicke et al. (2000)
GGL.3 1-*O*-acyl-3-[6-*O*-acetyl-*R*-glucopyranosyl-(1–3)-(6-*O*-acyl-*R*-mannopyranosyl)] glycerol	Glycoglycerolipid	Croatia	*Microbacterium* sp.	Actinobacterium	*Halichondria panicea*	Anticancer	Wicke et al. (2000)
GGL.4 1,2-*O*-diacyl-3-[α-galactofuranosyl)]glycerol	Glycoglycerolipid	Croatia	*Microbacterium* sp.	Actinobacterium	*Halichondria panicea*	Anticancer	Wicke et al. (2000)
DPG tetraacyldiphosphatidyl glycerol	Diphosphatidylglycerol	Croatia	*Microbacterium* sp.	Actinobacterium	*Halichondria panicea*	Anticancer	Wicke et al. (2000)
Compound 1 ($C_{25}H_{31}N_3O_6$)/β-aminopimelic acid	Tripeptide	Bulgaria	*Pseudomonas/ Alteromonas*	Bacterium	*Dysidea fragilis*	Antiviral	De Rosa et al. (2000)
4′-*N*-Methyl-5′-hydroxystaurosporine	Indolocarbazole alkaloid	Spain	*Micromonospora* sp.	Actinobacterium	*Clathrina coriacea*	Cytotoxic	Hernández et al. (2000)
5′-Hydroxystaurosporine	Indolocarbazole alkaloid	Spain	*Micromonospora* sp.	Actinobacterium	*Clathrina coriacea*	Cytotoxic	Hernández et al. (2000)
Iso-cladospolide B	Hexaketide	Indonesia	Not identified	Fungus	Not identified	NR	Smith et al. (2000)
Seco-patulolide C	Hexaketide	Indonesia	Not identified	Fungus	Not identified	NR	Smith et al. (2000)
Pandangolide 1	Polyketide	Indonesia	Not identified	Fungus	Not identified	NR	Smith et al. (2000)
Pandangolide 2	Polyketide	Indonesia	Not identified	Fungus	Not identified	NR	Smith et al. (2000)
Spiciferone A	Spiciferone derivative	Indonesia	*Drechslera hawaiiensis*	Fungus	*Callyspongia aerizusa*	NR	Edrada et al. (2000)
Spiciferone B	Spiciferone derivative	Indonesia	*Drechslera hawaiiensis*	Fungus	*Callyspongia aerizusa*	NR	Edrada et al. (2000)

(*Continued*)

Table 15.1 (Continued) New compounds isolated from microorganisms from marine sponges from 2000 to 2012: their chemical class, country of collection, and reported activity

Compound name	Chemical class	Country of collection	Name of microorganism	Type of microorganism	Name of sponge	Reported activity	References
Spiciferol A	Spiciferone derivative	Indonesia	*Drechslera hawaiiensis*	Fungus	*Callyspongia aerizusa*	NR	Edrada et al. (2000)
Spiciferone A (1)	Spiciferone derivative	Indonesia	*Drechslera hawaiiensis*	Fungus	*Callyspongia aerizusa*	NR	Edrada et al. (2000)
Butoxyl-spiciferin	Spiciferone derivative	Indonesia	*Drechslera hawaiiensis*	Fungus	*Callyspongia aerizusa*	NR	Edrada et al. (2000)
Asperic acid	Pyran derivative	United States	*Aspergillus niger*	Fungus	*Hyrtios proteus*	NR	Varoglu and Crews (2000)
Nafuredin	Lactone	Palau	*Aspergillus niger*	Fungus	Not identified	Cytotoxic	Takano et al. (2001)
2-Acetamido-2-deoxy-D-galacturonic acid	Amino sugar	Russia	*Pseudoalteromonas distincta*	Bacterium	Not identified	NR	Muldoon et al. (2001)
5-Acetamido-3,5,7,9-tetradeoxy-7-formamido-L-glycero-L-manno-nonulosonic acid	Amino sugar	Russia	*Pseudoalteromonas distincta*	Bacterium	Not identified	NR	Muldoon et al. (2001)
Aspergione A	Chromone	Indonesia	*Aspergillus versicolor*	Fungus	*Xestospongia exigua*	NR	Lin et al. (2001a)
Aspergione B	Chromone	Indonesia	*Aspergillus versicolor*	Fungus	*Xestospongia exigua*	NR	Lin et al. (2001a)
Aspergione C	Chromone	Indonesia	*Aspergillus versicolor*	Fungus	*Xestospongia exigua*	NR	Lin et al. (2001a)
Aspergione D	Chromone	Indonesia	*Aspergillus versicolor*	Fungus	*Xestospongia exigua*	NR	Lin et al. (2001a)
Aspergione E	Chromone	Indonesia	*Aspergillus versicolor*	Fungus	*Xestospongia exigua*	NR	Lin et al. (2001a)
Aspergione F	Chromone	Indonesia	*Aspergillus versicolor*	Fungus	*Xestospongia exigua*	NR	Lin et al. (2001a)
Aspergillone	Chromone	Indonesia	*Aspergillus versicolor*	Fungus	*Xestospongia exigua*	NR	Lin et al. (2001b)

(*Continued*)

Table 15.1 (Continued) New compounds isolated from microorganisms from marine sponges from 2000 to 2012: Their chemical class, country of collection, and reported activity

Compound name	Chemical class	Country of collection	Name of microorganism	Type of microorganism	Name of sponge	Reported activity	References
Aspergillodiol	Chromone	Indonesia	*Aspergillus versicolor*	Fungus	*Xestospongia exigua*	NR	Lin et al. (2001b)
Aspergillol	Chromone	Indonesia	*Aspergillus versicolor*	Fungus	*Xestospongia exigua*	NR	Lin et al. (2001b)
12-Acetyl-aspergillol	Chromone	Indonesia	*Aspergillus versicolor*	Fungus	*Xestospongia exigua*	NR	Lin et al. (2001b)
Lunatin	Anthraquinone	Indonesia	*Curvularia lunata*	Fungus	*Niphates olmeda*	Antibacterial	Jadulco et al. (2002)
Herbarin A	α-Pyrone	France	*Cladosporium herbarum*	Fungus	*Aplysina aerophoba*	Miscellaneous	Jadulco et al. (2002)
Herbarin B	α-Pyrone	France	*Cladosporium herbarum*	Fungus	*Aplysina aerophoba*	Miscellaneous	Jadulco et al. (2002)
Herbaric acid	Phthalide	Indonesia	*Cladosporium herbarum*	Fungus	*Callyspongia aerizusa*	NR	Jadulco et al. (2002)
Varitriol	Macrotetrolide	Venezuela	*Emericella variecolor*	Fungus	Not identified	Anticancer	Malmstrøm et al. (2002)
Varioxirane	NR	Venezuela	*Emericella variecolor*	Fungus	Not identified	NR	Malmstrøm et al. (2002)
Dihydroterrein	NR	Venezuela	*Emericella variecolor*	Fungus	Not identified	NR	Malmstrøm et al. (2002)
Varixanthone	NR	Venezuela	*Emericella variecolor*	Fungus	Not identified	Antimicrobial	Malmstrøm et al. (2002)
Xestodecalactone B	Decalactone	Indonesia	*Penicillium* cf. *montanense*	Fungus	*Xestospongia exigua*	Antiyeast	Edrada et al. (2002)
Microsphaerone A	ɣ-Pyrone	France	*Microsphaeropsis* sp.	Fungus	*Aplysina aerophoba*	Anticancer	Wang et al. (2002)
Microsphaerone B	ɣ-Pyrone	France	*Microsphaeropsis* sp.	Fungus	*Aplysina aerophoba*	Anticancer	Wang et al. (2002)
Xestodecalactone A	Decalactone	Indonesia	*Penicillium* cf. *montanense*	Fungus	*Xestospongia exigua*	NR	Edrada et al. (2002)

(*Continued*)

Table 15.1 (Continued) New compounds isolated from microorganisms from marine sponges from 2000 to 2012: Their chemical class, country of collection, and reported activity

Compound name	Chemical class	Country of collection	Name of microorganism	Type of microorganism	Name of sponge	Reported activity	References
Xestodecalactone C	Decalactone	Indonesia	*Penicillium* cf. *montanense*	Fungus	*Xestospongia exigua*	NR	Edrada et al. (2002)
YM-266183	Thiopeptide	Japan	*Bacillus cereus*	Bacterium	*Halichondria japonica*	Antibacterial	Nagai et al. (2003)
YM-266184	Thiopeptide	Japan	*Bacillus cereus*	Bacterium	*Halichondria japonica*	Antibacterial	Nagai et al. (2003)
Pseudoalterobactin A	Siderophore	Palau	*Pseudoalteromonas* sp.	Bacterium	*Cinachyrella australiensis*	NR	Kanoh et al. (2003)
Pseudoalterobactin B	Siderophore	Palau	*Pseudoalteromonas* sp.	Bacterium	*Cinachyrella australiensis*	NR	Kanoh et al. (2003)
Cyclo-(glycyl-L-seryl-L-prolyl-L-glutamyl)	Cyclic peptide	Italy	*Ruegeria* sp.	Bacterium	*Suberites domuncula*	Antibacterial	Mitova et al. (2004)
Cyclo-(glycyl-L-prolyl-L-glutamyl)	Cyclic peptide	Italy	*Ruegeria* sp.	Bacterium	*Suberites domuncula*	Antibacterial	Mitova et al. (2004)
Communesin C	Indole alkaloid (communesin derivative)	Italy	*Penicillium* sp.	Fungus	*Axinella verrucosa*	Anticancer	Jadulco et al. (2004)
Communesin D	Indole alkaloid (communesin derivative)	Italy	*Penicillium* sp.	Fungus	*Axinella verrucosa*	Anticancer	Jadulco et al. (2004)
Petrosifungins A	Cyclic peptide	Italy	*Penicillium brevicompactum*	Fungus	*Petrosia ficiformis*	NR	Bringmann et al. (2004)
Petrosifungins B	Cyclic peptide	Italy	*Penicillium brevicompactum*	Fungus	*Petrosia ficiformis*	NR	Bringmann et al. (2004)
Sorbicillactone A	Sorbicillin alkaloid	Italy	*Penicillium chrysogenum*	Fungus	*Ircinia fasciculata*	Anti-HIV	Bringmann et al. (2005)
Sorbicillactone B	Sorbicillin alkaloid	Italy	*Penicillium chrysogenum*	Fungus	*Ircinia fasciculata*	Anti-HIV	Bringmann et al. (2005)
Sorbivinetone	Sorbicillin alkaloid	Italy	*Penicillium chrysogenum*	Fungus	*Ircinia fasciculata*	NR	Bringmann et al. (2005)

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Table 15.1 (Continued) New compounds isolated from microorganisms from marine sponges from 2000 to 2012: Their chemical class, country of collection, and reported activity

Compound name	Chemical class	Country of collection	Name of microorganism	Type of microorganism	Name of sponge	Reported activity	References
(S)-2,4-Dihydroxy-1-butyl(4-hydroxy)benzoate	Benzoate	China	*Penicillium aurantiogriseum*	Fungus	*Mycale plumose*	Cytotoxic	Xin et al. (2005)
Dehydroxynocardamine	Cyclic peptide	Korea	*Streptomyces* sp.	Actinobacterium	Not identified	Miscellaneous	Lee et al. (2005)
Desmethylenylnocardamine	Cyclic peptide	Korea	*Streptomyces* sp.	Actinobacterium	Not identified	Miscellaneous	Lee et al. (2005)
Clonostachysin A	Cyclic peptide	Japan	*Clonostachys rogersoniana*	Fungus	*Halichondria japonica*	Miscellaneous	Adachi et al. (2005)
Clonostachysin B	Cyclic peptide	Japan	*Clonostachys rogersoniana*	Fungus	*Halichondria japonica*	Miscellaneous	Adachi et al. (2005)
Guangomide A	Cyclic depsipeptide	Papua New Guinea	Not identified fungus	Fungus	*Ianthella* sp.	Antibacterial	Amagata et al. (2006)
Guangomide B	Cyclic depsipeptide	Papua New Guinea	Not identified fungus	Fungus	*Ianthella* sp.	Antibacterial	Amagata et al. (2006)
Homodestcardin	Cyclic depsipeptide	Papua New Guinea	Not identified fungus	Fungus	*Ianthella* sp.	NR	Amagata et al. (2006)
RHM1	Peptide	Papua New Guinea	*Acremonium* sp.	Fungus	*Teichaxinella* sp.	Cytotoxic	Boot et al. (2006)
RHM2	Octapeptide	Papua New Guinea	*Acremonium* sp.	Fungus	*Teichaxinella* sp.	Cytotoxic	Boot et al. (2006)
Tropolactone A	Meroterpene	United States	*Aspergillus* sp.	Fungus	Not identified	Cytotoxic	Cueto et al. (2006)
Tropolactone B	Meroterpene	United States	*Aspergillus* sp.	Fungus	Not identified	Cytotoxic	Cueto et al. (2006)
Tropolactone C	Meroterpene	United States	*Aspergillus* sp.	Fungus	Not identified	Cytotoxic	Cueto et al. (2006)

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Table 15.1 (Continued) New compounds isolated from microorganisms from marine sponges from 2000 to 2012: Their chemical class, country of collection, and reported activity

Compound name	Chemical class	Country of collection	Name of microorganism	Type of microorganism	Name of sponge	Reported activity	References
Tropolactone D	Meroterpene	United States	*Aspergillus* sp.	Fungus	Not identified	Cytotoxic	Cueto et al. (2006)
IB-01212	Cyclic depsipeptide	Japan	*Clonostachys* sp.	Fungus	Not identified	Cytotoxic	Cruz et al. (2006)
Roridin R	Macrocyclic trichothecene	Indonesia	*Myrothecium* sp.	Fungus	Not identified	Cytotoxic	Xu et al. (2006)
12-Hydroxyroridin E	Macrocyclic trichothecene	Indonesia	*Myrothecium* sp.	Fungus	Not identified	Cytotoxic	Xu et al. (2006)
Roridin Q	Macrocyclic trichothecene	Indonesia	*Myrothecium* sp.	Fungus	Not identified	Cytotoxic	Xu et al. (2006)
2′,3′-Deoxyroritoxin D	Macrocyclic trichothecene	Indonesia	*Myrothecium roridum*	Fungus	Not identified	Cytotoxic, antiyeast	Xu et al. (2006)
5-*cis*-3-Oxo-C12-HSL (compound 1)	*N*-Acyl-L-homoserine lactone	Norway	*Mesorhizobium* sp.	Bacterium	*Phakellia ventilabrum*	Antibacterial, cytotoxic	Krick et al. (2007)
5-*cis*-3-Oxo-C12-homoserine lactone	*N*-Acyl-L-homoserine lactone	Norway	*Mesorhizobium* sp.	Bacterium	*Phakellia ventilabrum*	NR	Krick et al. (2007)
Bromoalterochromide A	Brominated chromopeptide	Australia	*Pseudoalteromonas maricaloris*	Bacterium	*Fascaplysinopsis reticulata*	Cytotoxic	Speitling et al. (2007)
Bromoalterochromide A′	Brominated chromopeptide	Australia	*Pseudoalteromonas maricaloris*	Bacterium	*Fascaplysinopsis reticulata*	Cytotoxic	Speitling et al. (2007)
Aurantiomide B	Quinazoline alkaloid	China	*Penicillium aurantiogriseum*	Fungus	*Mycale plumose*	Cytotoxic	Xin et al. (2007)
Aurantiomide C	Quinazoline alkaloid	China	*Penicillium aurantiogriseum*	Fungus	*Mycale plumose*	Cytotoxic	Xin et al. (2007)
Glyco-C_{30}-carotenoic acid	Diapolycopenedioic acid	NR	*Rubritalea squalenifaciens*	Bacterium	*Halichondria okadai*	Miscellaneous	Shindo et al. (2007)
Trichodermanone A	Sorbicillin–polyketide	Dominica	*Trichoderma* sp.	Fungus	*Agelas dispar*	Miscellaneous	Neumann et al. (2007)
Trichodermanone B	Sorbicillin–polyketide	Dominica	*Trichoderma* sp.	Fungus	*Agelas dispar*	Miscellaneous	Neumann et al. (2007)

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Table 15.1 (Continued) New compounds isolated from microorganisms from marine sponges from 2000 to 2012: Their chemical class, country of collection, and reported activity

Compound name	Chemical class	Country of collection	Name of microorganism	Type of microorganism	Name of sponge	Reported activity	References
Trichodermanone C	Sorbicillin–polyketide	Dominica	*Trichoderma* sp.	Fungus	*Agelas dispar*	Miscellaneous	Neumann et al. (2007)
Trichodermanone D	Sorbicillin–polyketide	Dominica	*Trichoderma* sp.	Fungus	*Agelas dispar*	Miscellaneous	Neumann et al. (2007)
Aurantiomide A	Quinazoline alkaloid	China	*Penicillium aurantiogriseum*	Fungus	*Mycale plumose*	NR	Xin et al. (2007)
Aspinotriol A	Pentaketide	Micronesia	*Aspergillus ostianus*	Fungus	Not identified sponge	NR	Kito et al. (2007)
Aspinotriol B	Pentaketide	Micronesia	*Aspergillus ostianus*	Fungus	Not identified sponge	NR	Kito et al. (2007)
Aspinonediol	Pentaketide	Micronesia	*Aspergillus ostianus*	Fungus	Not identified sponge	NR	Kito et al. (2007)
Streptophenazine A	Phenazine	Germany	*Streptomyces* sp.	Actinobacterium	*Halichondria panicea*	Antibacterial	Mitova et al. (2008)
Streptophenazine B	Phenazine	Germany	*Streptomyces* sp.	Actinobacterium	*Halichondria panicea*	Antibacterial	Mitova et al. (2008)
Streptophenazine C	Phenazine	Germany	*Streptomyces* sp.	Actinobacterium	*Halichondria panicea*	Antibacterial	Mitova et al. (2008)
Streptophenazine D	Phenazine	Germany	*Streptomyces* sp.	Actinobacterium	*Halichondria panicea*	Antibacterial	Mitova et al. (2008)
Streptophenazine E	Phenazine	Germany	*Streptomyces* sp.	Actinobacterium	*Halichondria panicea*	Antibacterial	Mitova et al. (2008)
Streptophenazine F	Phenazine	Germany	*Streptomyces* sp.	Actinobacterium	*Halichondria panicea*	Antibacterial	Mitova et al. (2008)
Streptophenazine G	Phenazine	Germany	*Streptomyces* sp.	Actinobacterium	*Halichondria panicea*	Antibacterial	Mitova et al. (2008)
Streptophenazine H	Phenazine	Germany	*Streptomyces* sp.	Actinobacterium	*Halichondria panicea*	Antibacterial	Mitova et al. (2008)
Chlorohydroaspyrone A	Aspyrone derivative (polyketide)	Korea	*Exophiala* sp.	Fungus	*Halichondria panicea*	Antibacterial	Zhang et al. (2008a)

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Table 15.1 (Continued) New compounds isolated from microorganisms from marine sponges from 2000 to 2012: Their chemical class, country of collection, and reported activity

Compound name	Chemical class	Country of collection	Name of microorganism	Type of microorganism	Name of sponge	Reported activity	References
Chlorohydroaspyrone B	Aspyrone derivative (polyketide)	Korea	*Exophiala* sp.	Fungus	*Halichondria panicea*	Antibacterial	Zhang et al. (2008a)
Scopularide A	Cyclic depsipeptide	Croatia	*Scopulariopsis brevicaulis*	Fungus	*Tethya aurantium*	Antibacterial, cytotoxic	Yu et al. (2008)
Scopularide B	Cyclic depsipeptide	Croatia	*Scopulariopsis brevicaulis*	Fungus	*Tethya aurantium*	Antibacterial, cytotoxic	Yu et al. (2008)
Gymnastatin Q	NR	Japan	*Gymnascella dankaliensis*	Fungus	*Halichondria japonica*	Anticancer	Amagata et al. (2008)
Dihydroinfectopyrone	Pyrone	Thailand	Order pleosporales	Fungus	Not identified	Anticancer	Proksch et al. (2008)
Aspergillide A	Polyketide	Micronesia	*Aspergillus ostianus*	Fungus	Not identified	Cytotoxic	Kito et al. (2008)
Aspergillide B	Polyketide	Micronesia	*Aspergillus ostianus*	Fungus	Not identified	Cytotoxic	Kito et al. (2008)
Aspergillide C	Polyketide	Micronesia	*Aspergillus ostianus*	Fungus	Not identified	Cytotoxic	Kito et al. (2008)
Gymnastatin R	NR	Japan	*Gymnascella dankaliensis*	Fungus	*Halichondria japonica*	Cytotoxic	Amagata et al. (2008)
Dankastatin A	NR	Japan	*Gymnascella dankaliensis*	Fungus	*Halichondria japonica*	Cytotoxic	Amagata et al. (2008)
Dankastatin B	NR	Japan	*Gymnascella dankaliensis*	Fungus	*Halichondria japonica*	Cytotoxic	Amagata et al. (2008)
(Z)-6-Benzylidene-3-hydroxymethyl-1,4-dimethyl-3-methylsulfanylpiperazine-2,5-dione	Diketopiperazine	Thailand	Strain CRIF2 (order Pleosporales)	Fungus	Not identified	Cytotoxic	Prachyawarakorn et al. (2008)
(3*S*,3′*R*)-3-(3′-Hydroxybutyl)-7-methoxyphthalide	Diketopiperazine	Thailand	*Strain CRIF2* (order Pleosporales)	Fungus	Not identified	Cytotoxic	Prachyawarakorn et al. (2008)
Diapolycopenedioic acid xylosyl ester A	Glyco-C_{30}-carotenoic acid	Japan	*Rubritalea squalenifaciens*	Bacterium	*Halichondria okadai*	Miscellaneous	Shindo et al. (2008)

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Table 15.1 (Continued) New compounds isolated from microorganisms from marine sponges from 2000 to 2012: Their chemical class, country of collection, and reported activity

Compound name	Chemical class	Country of collection	Name of microorganism	Type of microorganism	Name of sponge	Reported activity	References
Diapolycopenedioic acid xylosyl ester B	Glyco-C_{30}-carotenoic acid	Japan	*Rubritalea squalenifaciens*	Bacterium	*Halichondria okadai*	Miscellaneous	Shindo et al. (2008)
Diapolycopenedioic acid xylosyl ester C	Glyco-C_{30}-carotenoic acid	Japan	*Rubritalea squalenifaciens*	Bacterium	*Halichondria okadai*	Miscellaneous	Shindo et al. (2008)
Compound 3 ($C_{10}H_{11}NO_4$)	Siderophore	Indonesia	*Pseudoalteromonas* sp.	Bacterium	*Halisarca ectofibrosa*	Miscellaneous	You et al. (2008)
Cebulactam A1	Macrolactam	Philippines	*Saccharopolyspora cebuensis*	Actinobacterium	*Haliclona* sp.	NR	Pimentel-Elardo et al. (2008)
Cebulactam B1	Macrolactam	Philippines	*Saccharopolyspora cebuensis*	Actinobacterium	*Haliclona* sp.	NR	Pimentel-Elardo et al. (2008)
Cyclo-[phenylalanyl-leucyl]2	Peptide	Thailand	*Pseudoalteromonas* sp.	Bacterium	*Halisarca ectofibrosa*	NR	Rungprom et al. (2008)
Cyclo-[leucyl-isoleucyl]2	Peptide	Thailand	*Pseudoalteromonas* sp.	Bacterium	*Halisarca ectofibrosa*	NR	Rungprom et al. (2008)
1-(2,8-Dihydroxy-1,2,6-trimethyl-1,2,6,7,8,8ahexahydro-naphthalen-1-yl)-3-methoxy-propan-1-one	Polyketide	NR	*Mycelia sterilia*	Fungus	Not identified	NR	Hao et al. (2008)
4,8-Dihydroxy-7-(2-hydroxy-ethyl)-6-methoxy-3,4-dihydro-2-naphthalen-1-one	Polyketide	NR	*Mycelia sterilia*	Fungus	Not identified	NR	Hao et al. (2008)
1-Methyl-naphthalene-2,6-dicarboxylic acid	Polyketide	NR	*Mycelia sterilia*	Fungus	Not identified	NR	Hao et al. (2008)
Circumdatin J	Alkaloid	Micronesia	*Aspergillus ostianus*	Fungus	Not identified	NR	Ookura et al. (2008)
6-Hydroxymethyl-1-phenazine-carboxamide	Phenazine	Korea	*Brevibacterium* sp. KMD 003	Bacterium	*Callyspongia* sp.	Antibacterial	Choi et al. (2009)
1,6-Phenazinedimethanol	Phenazine	Korea	*Brevibacterium* sp. KMD 003	Bacterium	*Callyspongia* sp.	Antibacterial	Choi et al. (2009)

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Table 15.1 (Continued) New compounds isolated from microorganisms from marine sponges from 2000 to 2012: Their chemical class, country of collection, and reported activity

Compound name	Chemical class	Country of collection	Name of microorganism	Type of microorganism	Name of sponge	Reported activity	References
Angucyclinone (PM070747)	Benz[α]anthraquinone	Tanzania	*Saccharopolyspora taberi (PEM-06-F23–019B)*	Actinobacterium	Unidentified	Anticancer	Pérez et al. (2009)
Hydroxydecylparaben, 4-hydroxybenzoic acid 3-hydroxy-decyl ester	Paraben	France	*Microbulbifer* sp.	Bacterium	*Leuconia nivea*	Antimicrobial	Quévrain et al. (2009)
Methyldecylparaben, 4-hydroxybenzoic acid methyl-decyl ester	Paraben	France	*Microbulbifer* sp.	Bacterium	*Leuconia nivea*	Antimicrobial	Quévrain et al. (2009)
Hydroxymethyldecylparaben, 4-hydroxybenzoic acid 3-hydroxy-methyl-decyl ester	Paraben	France	*Microbulbifer* sp.	Bacterium	*Leuconia nivea*	Antimicrobial	Quévrain et al. (2009)
Dodec-5-enylparaben, 4-hydroxybenzoic acid dodec-5-enyl ester	Paraben	France	*Microbulbifer* sp.	Bacterium	*Leuconia nivea*	Antimicrobial	Quévrain et al. (2009)
Aspergillusol A	Tyrosine	Thailand	*Aspergillus aculeatus*	Fungus	*Xestospongia testudinaria*	Antiyeast, cytotoxic	Ingavat et al. (2009)
Beauversetin	Tetramic acid	Germany	*Beauveria bassiana*	Fungus	*Myxilla incrustans*	Cytotoxic	Neumann et al. (2009)
Epoxyphomalin A	Prenylated polyketide	Dominica	*Phoma* sp.	Fungus	*Ectyplasia perox*	Cytotoxic	Mohamed et al. (2009)
Epoxyphomalin B	Prenylated polyketide	Dominica	*Phoma* sp.	Fungus	*Ectyplasia perox*	Cytotoxic	Mohamed et al. (2009)
2-(1H-Indol-3-yl)ethyl 2-hydroxypropanoate	Indole	Japan	*Pichia membranifaciens*	Yeast	*Halichondria okadai*	Miscellaneous	Sugiyama et al. (2009)
2-(1H-Indol-3-yl)ethyl 5-hydroxypentanoate	Indole	Japan	*Pichia membranifaciens*	Yeast	*Halichondria okadai*	Miscellaneous	Sugiyama et al. (2009)
JBIR-37	Glycosyl benzenediol	Japan	*Acremonium* sp.	Fungus	Not identified	NR	Izumikawa et al. (2009)

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Table 15.1 (Continued) New compounds isolated from microorganisms from marine sponges from 2000 to 2012: Their chemical class, country of collection, and reported activity

Compound name	Chemical class	Country of collection	Name of microorganism	Type of microorganism	Name of sponge	Reported activity	References
JBIR-38	Glycosyl benzenediol	Japan	*Acremonium* sp.	Fungus	Not identified	NR	Izumikawa et al. (2009)
Paecilopyrone A	α-Pyrone	Korea	*Paecilomyces lilacinus*	Fungus	*Petrosia* sp.	NR	Elbandy et al. (2009)
Paecilopyrone B	α-Pyrone	Korea	*Paecilomyces lilacinus*	Fungus	*Petrosia* sp.	NR	Elbandy et al. (2009)
Phomaligol B	Cyclohexenone	Korea	*Paecilomyces lilacinus*	Fungus	*Petrosia* sp.	NR	Elbandy et al. (2009)
Phomaligol C	Cyclohexenone	Korea	*Paecilomyces lilacinus*	Fungus	*Petrosia* sp.	NR	Elbandy et al. (2009)
Neobacillamide A	Alkaloid	China	*Bacillus vallismortis* C89	Bacterium	*Dysidea avara*	NR	Yu et al. (2009)
Chlorocylindrocarpol	Sesquiterpene	Korea	*Acremonium* sp.	Fungus	*Stelletta* sp.	NR	Zhang et al. (2009)
Acremofuranone A	Sesquiterpene	Korea	*Acremonium* sp.	Fungus	*Stelletta* sp.	NR	Zhang et al. (2009)
Acremofuranone B	Sesquiterpene	Korea	*Acremonium* sp.	Fungus	*Stelletta* sp.	NR	Zhang et al. (2009)
Dihydroxybergamotene	Sesquiterpene	Korea	*Acremonium* sp.	Fungus	*Stelletta* sp.	NR	Zhang et al. (2009)
Trichopyrone [6-(4-hydroxy-1-pentenyl)-4-meth oxy-3-methyl-2H-pyran-2-one]	Pyranone	Dominica	*Trichoderma viride*	Fungus	*Agelas dispar*	NR	Abdel-Lateffa et al. (2009)
JBIR-15	Aspochracin derivative	Japan	*Aspergillus sclerotiorum*	Fungus	*Mycale* sp.	NR	Motohashi et al. (2009)
Mayamycin	Polyketide	Germany	*Nocardiopsis* sp.	Actinobacterium	*Halichondria panicea*	Antibacterial, cytotoxic	Schneemann et al. (2010a)
JBIR-58	Salicylamide	Japan	*Streptomyces* sp. SpD081030ME-02	Actinobacterium	Not identified	Cytotoxic	Ueda et al. (2010a)

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Table 15.1 (Continued) New compounds isolated from microorganisms from marine sponges from 2000 to 2012: Their chemical class, country of collection, and reported activity

Compound name	Chemical class	Country of collection	Name of microorganism	Type of microorganism	Name of sponge	Reported activity	References
Fellutamide C	Lipopeptide	Japan	*Aspergillus versicolor*	Fungus	*Petrosia* sp.	Cytotoxic	Lee et al. (2010)
Epoxyphomalin D	Prenylated polyketide	Dominica	*Paraconiothyrium sporulosum*	Fungus	*Ectyplasia perox*	Cytotoxic	Mohamed et al. (2010)
JBIR-97	NR	Japan	*Tritirachium* sp.	Fungus	*Pseudoceratina purpurea*	Cytotoxic	Ueda et al. (2010b)
JBIR-98	NR	Japan	*Tritirachium* sp.	Fungus	*Pseudoceratina purpurea*	Cytotoxic	Ueda et al. (2010b)
JBIR-99	NR	Japan	*Tritirachium* sp.	Fungus	*Pseudoceratina purpurea*	Cytotoxic	Ueda et al. (2010b)
Trichoderin A	Aminolipopeptide	NR	*Trichoderma* sp.	Fungus	Not identified	Antituberculosis	Pruksakorn et al. (2010)
Trichoderin A1	Aminolipopeptide	NR	*Trichoderma* sp.	Fungus	Not identified	Antituberculosis	Pruksakorn et al. (2010)
Trichoderin B	Aminolipopeptide	NR	*Trichoderma* sp.	Fungus	Not identified	Antituberculosis	Pruksakorn et al. (2010)
JBIR-65	Diterpene	Japan	*Actinomadura* sp. SpB081030SC-15	Actinobacterium	Not identified	Miscellaneous	Takagi et al. (2010a)
JBIR-34	Indole-containing peptide	Japan	*Streptomyces* sp. Sp080513GE-23	Actinobacterium	*Haliclona* sp.	Miscellaneous	Motohashi et al. (2010)
JBIR-35	Indole-containing peptide	Japan	*Streptomyces* sp. Sp080513GE-23	Actinobacterium	*Haliclona* sp.	Miscellaneous	Motohashi et al. (2010)
Nocapyrone A	ɣ-Pyrone	Germany	*Nocardiopsis* sp.	Actinobacterium	*Halichondria panicea*	NR	Schneemann et al. (2010b)
Nocapyrone B	ɣ-Pyrone	Germany	*Nocardiopsis* sp.	Actinobacterium	*Halichondria panicea*	NR	Schneemann et al. (2010b)
Nocapyrone C	ɣ-Pyrone	Germany	*Nocardiopsis* sp.	Actinobacterium	*Halichondria panicea*	NR	Schneemann et al. (2010b)
Nocapyrone D	ɣ-Pyrone	Germany	*Nocardiopsis* sp.	Actinobacterium	*Halichondria panicea*	NR	Schneemann et al. (2010b)

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Table 15.1 (Continued) New compounds isolated from microorganisms from marine sponges from 2000 to 2012: Their chemical class, country of collection, and reported activity

Compound name	Chemical class	Country of collection	Name of microorganism	Type of microorganism	Name of sponge	Reported activity	References
JBIR-74	Roquefortine C analog (mycotoxin)	Japan	*Aspergillus* sp.	Fungus	Not identified	NR	Takagi et al. (2010b)
JBIR-75	Roquefortine C analog (mycotoxin)	Japan	*Aspergillus* sp.	Fungus	Not identified	NR	Takagi et al. (2010b)
Epoxyphomalin C	Prenylated polyketide	Dominica	*Paraconiothyrium sporulosum*	Fungus	*Ectyplasia perox*	NR	Mohamed et al. (2010)
Epoxyphomalin E	Prenylated polyketide	Dominica	*Paraconiothyrium sporulosum*	Fungus	*Ectyplasia perox*	NR	Mohamed et al. (2010)
Sorbifuranone A	Sorbicillin	Italy	*Penicillium chrysogenum*	Fungus	*Ircinia fasciculata*	NR	Bringmann et al. (2010)
Sorbifuranone B	Sorbicillin	Italy	*Penicillium chrysogenum*	Fungus	*Ircinia fasciculata*	NR	Bringmann et al. (2010)
Sorbifuranone A	Sorbicillin	Italy	*Penicillium chrysogenum*	Fungus	*Ircinia fasciculata*	NR	Bringmann et al. (2010)
Marilone B	Phthalide (polyketide)	Australia	*Stachylidium* sp.	Fungus	*Callyspongia* sp. cf. *C. flammea*	Antagonistic of serotonin receptor	Almeida et al. (2011)
Butylrolactone-VI	Dibenzylbutyrolactone	Chile	*Aspergillus* sp. (2P-22)	Fungus	*Cliona chilensis*	Antibacterial	San-Martin et al. (2011)
Cillifuranone	Intermediate in sorbifuranone	Croatia	*Penicillium chrysogenum* strain LF066	Fungus	*Tethya aurantium*	Antibiotic	Wiese et al. (2011)
Insuetolide A	Meroterpene	Israel	*Aspergillus aculeatus*	Fungus	*Psammocinia* sp.	Antifungal	Cohen et al. (2011)
Bendigole D	3-Keto sterol	NR	*Actinomadura* sp.	Actinobacterium	*Suberites japonicus*	Anti-inflammatory, cytotoxic	Simmons et al. (2011)
Bendigole F	3-Keto sterol	NR	*Actinomadura* sp.	Actinobacterium	*Suberites japonicus*	Anti-inflammatory	Simmons et al. (2011)

(*Continued*)

Table 15.1 (Continued) New compounds isolated from microorganisms from marine sponges from 2000 to 2012: Their chemical class, country of collection, and reported activity

Compound name	Chemical class	Country of collection	Name of microorganism	Type of microorganism	Name of sponge	Reported activity	References
(3*S*,8*R*)-Methyl 8-hydroxy-3-methoxycarbonyl-2-methylenenonanoate	Hexylitaconic acid	Korea	*Penicillium* sp.	Fungus	*Stelletta* sp.	Anti-inflammatory	Li et al. (2011b)
(3*S*)-Methyl-9-hydroxy-3-methoxycarbonyl-2-methylenenonanoate	Hexylitaconic acid	Korea	*Penicillium* sp.	Fungus	*Stelletta* sp.	Anti-inflammatory	Li et al. (2011b)
Marilone A	Phthalide (polyketide)	Australia	*Stachylidium* sp.	Fungus	*Callyspongia* sp. cf. *C. flammea*	Antimalarial and anticancer	Almeida et al. (2011)
Marilone C	Phthalide (polyketide)	Australia	*Stachylidium* sp.	Fungus	*Callyspongia* sp. cf. *C. flammea*	Antimalarial and Anticancer	Almeida et al. (2011)
Myrocin D	Diterpene	Italy	*Arthrinium* sp.	Fungus	*Geodia cydonium*	Anticancer	Ebada et al. (2011)
22-Deoxythiocoraline	Thiocoraline analog	United States	*Verrucosispora* sp.	Actinobacterium	*Chondrilla caribensis* f. *caribensis*	Cytotoxic	Wyche et al. (2011)
Thiochondrilline C	Thiocoraline analog	United States	*Verrucosispora* sp.	Actinobacterium	*Chondrilla caribensis* f. *caribensis*	Cytotoxic	Wyche et al. (2011)
12-Sulfoxythiocoraline	Thiocoraline analog	United States	*Verrucosispora* sp.	Actinobacterium	*Chondrilla caribensis* f. *caribensis*	Cytotoxic	Wyche et al. (2011)
Acremostrictin	Tricyclic lactone	Korea	*Acremonium strictum*	Fungus	Not identified	Antibacterial, miscellaneous	Julianti et al. (2011)
Insuetolide C	Meroterpene	Israel	*Aspergillus aculeatus*	Fungus	*Psammocinia* sp.	Cytotoxic	Cohen et al. (2011)
(*E*)-6-(4′-Hydroxy-2′-butenoyl)-strobilactone A	Sesquiterpene	Israel	*Aspergillus aculeatus*	Fungus	*Psammocinia* sp.	Cytotoxic	Cohen et al. (2011)

(*Continued*)

Table 15.1 (Continued) New compounds isolated from microorganisms from marine sponges from 2000 to 2012: Their chemical class, country of collection, and reported activity

Compound name	Chemical class	Country of collection	Name of microorganism	Type of microorganism	Name of sponge	Reported activity	References
Fellutamide F	Lipopeptide	Korea	*Aspergillus versicolor*	Fungus	*Petrosia* sp.	Cytotoxic	Lee et al. (2011)
Dihydrotrichodermolide	Polyketide (sorbicillin dimer)	NR (East Pacific)	*Phialocephala* sp.	Fungus	*Stelletta* sp.	Cytotoxic	Li et al. (2011a)
Dihydrodemethylsorbicillin	Polyketide (sorbicillin monomer)	NR (East Pacific)	*Phialocephala* sp.	Fungus	*Stelletta* sp.	Cytotoxic	Li et al. (2011a)
Phialofurone	Benzofuranone	NR (East Pacific)	*Phialocephala* sp.	Fungus	*Stelletta* sp.	Cytotoxic	Li et al. (2011a)
Bendigole E	3-Keto sterol	NR	*Actinomadura* sp.	Actinobacterium	*Suberites japonicus*	NR	Simmons et al. (2011)
JBIR-56	Peptide	Japan	*Streptomyces* sp.	Actinobacterium	Not identified	NR	Motohashi et al. (2011)
JBIR-57	Peptide	Japan	*Streptomyces* sp.	Actinobacterium	Not identified	NR	Motohashi et al. (2011)
Streptomycindole	Indole alkaloid	China	*Streptomyces* sp.	Actinobacterium	*Craniella australiensis*	NR	Huang et al. (2011)
Thiochondrilline A	Thiocoraline analog	United States	*Verrucosispora* sp.	Actinobacterium	*Chondrilla caribensis f. caribensis*	NR	Wyche et al. (2011)
Thiochondrilline B	Thiocoraline analog	United States	*Verrucosispora* sp.	Actinobacterium	*Chondrilla caribensis f. caribensis*	NR	Wyche et al. (2011)
Arthrinin A	Diterpene	Italy	*Arthrinium* sp.	Fungus	*G. cydonium*	NR	Ebada et al. (2011)
Arthrinin B	Diterpene	Italy	*Arthrinium* sp.	Fungus	*G. cydonium*	NR	Ebada et al. (2011)
Arthrinin C	Diterpene	Italy	*Arthrinium* sp.	Fungus	*G. cydonium*	NR	Ebada et al. (2011)
Arthrinin D	Diterpene	Italy	*Arthrinium* sp.	Fungus	*G. cydonium*	NR	Ebada et al. (2011)
Asperaculin A	Sesquiterpene	Thailand	*Aspergillus aculeatus*	Fungus	*Xestospongia testudinaria*	NR	Ingavat et al. (2011)
Pre-aurantiamine	Diketopiperazine	Thailand	*Aspergillus aculeatus*	Fungus	*Stylissa flabelliformis*	NR	Antia et al. (2011)

(*Continued*)

Table 15.1 (Continued) New compounds isolated from microorganisms from marine sponges from 2000 to 2012: Their chemical class, country of collection, and reported activity

Compound name	Chemical class	Country of collection	Name of microorganism	Type of microorganism	Name of sponge	Reported activity	References
(−)-9-Hydroxyhexylitaconic acid	Diketopiperazine	Thailand	*Aspergillus aculeatus*	Fungus	*S. flabelliformis*	NR	Antia et al. (2011)
(−)-9-Hydroxyhexylitaconic acid-4-methyl ester	Diketopiperazine	Thailand	*Aspergillus aculeatus*	Fungus	*S. flabelliformis*	NR	Antia et al. (2011)
Insuetolide B	Meroterpene	Israel	*Aspergillus aculeatus*	Fungus	*Psammocinia* sp.	NR	Cohen et al. (2011)
Austalide M	Meroterpene	Italy	*Aspergillus* sp.	Fungus	*Tethya aurantium*	NR	Zhou et al. (2011)
Austalide N	Meroterpene	Italy	*Aspergillus* sp.	Fungus	*Tethya aurantium*	NR	Zhou et al. (2011)
Austalide O	Meroterpene	Italy	*Aspergillus* sp.	Fungus	*Tethya aurantium*	Cytotoxic	Zhou et al. (2011)
Austalide P	Meroterpene	Italy	*Aspergillus* sp.	Fungus	*Tethya aurantium*	Cytotoxic	Zhou et al. (2011)
Austalide Q	Meroterpene	Italy	*Aspergillus* sp.	Fungus	*Tethya aurantium*	Cytotoxic	Zhou et al. (2011)
(3*S*,8*R*)-8-Hydroxy-3-carboxy-2-methylenenonanoic acid	Hexylitaconic acid	Korea	*Penicillium* sp.	Fungus	*Stelletta* sp.	NR	Li et al. (2011b)
(3*S*)-9-Hydroxy-3-carboxy-2-methylenenonanoic acid	Hexylitaconic acid	Korea	*Penicillium* sp.	Fungus	*Stelletta* sp.	NR	Li et al. (2011b)
Stachyline A	Tyrosine-derived metabolite	Australia	*Stachylidium* sp.	Fungus	*Callyspongia* sp. cf. *C. flammea*	NR	Almeida et al. (2010)
Stachyline B	Tyrosine-derived metabolite	Australia	*Stachylidium* sp.	Fungus	*Callyspongia* sp. cf. *C. flammea*	NR	Almeida et al. (2010)
Stachyline C	Tyrosine-derived metabolite	Australia	*Stachylidium* sp.	Fungus	*Callyspongia* sp. cf. *C. flC. fla*	NR	Almeida et al. (2010)

(*Continued*)

Table 15.1 (Continued) New compounds isolated from microorganisms from marine sponges from 2000 to 2012: Their chemical class, country of collection, and reported activity

Compound name	Chemical class	Country of collection	Name of microorganism	Type of microorganism	Name of sponge	Reported activity	References
Stachyline D	Tyrosine-derived metabolite	Australia	*Stachylidium* sp.	Fungus	*Callyspongia* sp. cf. *C. flammea*	NR	Almeida et al. (2010)
Compound 1, new (5a,6a)-isomer of ophiobolin H	Sesterterpene	NR (Adriatic Sea)	*Aspergillus ustus*	Fungus	*Suberites domuncula*	No significant cytotoxicity found	Liu et al. (2011)
Compound 2, (5a,6a)-5-*O*-methylophiobolin H	Sesterterpene	NR (Adriatic Sea)	*Aspergillus ustus*	Fungus	*Suberites domuncula*	No significant cytotoxicity found	Liu et al. (2011)
Compound 3, 5-*O*-methylophiobolin H	Sesterterpene	NR (Adriatic Sea)	*Aspergillus ustus*	Fungus	*Suberites domuncula*	No significant cytotoxicity found	Liu et al. (2011)
Compound 4, s(6a)-21,21-*O*-dihydroophiobolin G	Sesterterpene	NR (Adriatic Sea)	*Aspergillus ustus*	Fungus	*Suberites domuncula*	No significant cytotoxicity found	Liu et al. (2011)
Compound 5, (6a)-18,19,21,21-Otetrahydro-18,19-dihydroxyophiobolin G	Sesterterpene	NR (Adriatic Sea)	*Aspergillus ustus*	Fungus	*Suberites domuncula*	No significant cytotoxicity found	Liu et al. (2011)
Tetromycin 1	Tetromycin	France	*Streptomyces axinellae*	Actinobacterium	*Axinella polypoides*	Antiparasitic, inhibition of cathepsin L-like proteases	Pimentel-Elardo et al. (2011)
Tetromycin 2	Tetromycin	France	*Streptomyces axinellae*	Actinobacterium	*Axinella polypoides*	Antiparasitic, inhibition of cathepsin L-like proteases	Pimentel-Elardo et al. (2011)
Tetromycin 3	Tetromycin	France	*Streptomyces axinellae*	Actinobacterium	*Axinella polypoides*	Antiparasitic, inhibition of cathepsin L-like proteases	Pimentel-Elardo et al. (2011)

(*Continued*)

Table 15.1 (Continued) New compounds isolated from microorganisms from marine sponges from 2000 to 2012: Their chemical class, country of collection, and reported activity

Compound name	Chemical class	Country of collection	Name of microorganism	Type of microorganism	Name of sponge	Reported activity	References
Tetromycin 4	Tetromycin	France	*Streptomyces axinellae*	Actinobacterium	*Axinella polypoides*	Antiparasitic, inhibition of cathepsin L-like proteases	Pimentel-Elardo et al. (2011)
Lobophorin C	Kijanimicin	China	*Streptomyces microflavus*	Actinobacterium	*Hymeniacidon perlevis*	Cytotxic	Wei et al. (2011)
Lobophorin D	Kijanimicin	China	*Streptomyces microflavus*	Actinobacterium	*Hymeniacidon perlevis*	Anticancer	Wei et al. (2011)
JBIR-109	Trichostatin analog	Japan	*Streptomyces* sp. *strain* RM72	Actinobacterium	Not identified	Anticancer	Hosoya et al. (2012)
JBIR-110	Trichostatin analog	Japan	*Streptomyces* sp. *strain* RM72	Actinobacterium	Not identified	Anticancer	Hosoya et al. (2012)
JBIR-111	Trichostatin analog	Japan	*Streptomyces* sp. *strain* RM72	Actinobacterium	Not identified	Anticancer	Hosoya et al. (2012)
Urdamycinone E	Glycosylated benz[α] anthraquinone	Thailand	*Streptomyces*sp. BCC45596	Actinobacterium	*Xestospongia* sp.	Antimalarial, antituberculosis	Supong et al. (2012)
Urdamycinone G	Glycosylated benz[α] anthraquinone	Thailand	*Streptomyces* sp. BCC45596	Actinobacterium	*Xestospongia* sp.	Antimalarial, antituberculosis	Supong et al. (2012)
Dehydroxyaquayamycin	Glycosylated benz[α] anthraquinone	Thailand	*Streptomyces* sp. BCC45596	Actinobacterium	*Xestospongia* sp.	Antimalarial, antituberculosis	Supong et al. (2012)
Acremolin	Methyl guanine	Korea	*Acremonium strictum*	Fungus	Not identified Choristida sponge	Cytotoxic	Julianti et al. (2012)
Disydonol A	Sesquiterpene	China	*Aspergillus* sp.	Fungus	*Xestospongia testudinaria*	Cytotoxic	Sun et al. (2012)
Disydonol C	Sesquiterpene	China	*Aspergillus* sp.	Fungus	*Xestospongia testudinaria*	Cytotoxic	Sun et al. (2012)
Aspergilusidone A	Depsidone	Thailand	*Aspergillus unguis* CRI282–03	Fungus	Not identified	Cytotoxic	Sureram et al. (2012)
Aspergilusidone B	Depsidone	Thailand	*Aspergillus unguis* CRI282–03	Fungus	Not identified	Cytotoxic	Sureram et al. (2012)

(*Continued*)

Table 15.1 (Continued) New compounds isolated from microorganisms from marine sponges from 2000 to 2012: Their chemical class, country of collection, and reported activity

Compound name	Chemical class	Country of collection	Name of microorganism	Type of microorganism	Name of sponge	Reported activity	References
Aspergilusidone C	Depsidone	Thailand	*Aspergillus unguis* CRI282–03	Fungus	Not identified	Cytotoxic	Sureram et al. (2012)
New compound	Diaryl ether	Thailand	*Aspergillus unguis* CRI282–03	Fungus	Not identified	Cytotoxic	Sureram et al. (2012)
Aspergiterpenoid A	Sesquiterpene	China	*Aspergillus* sp.	Fungus	*Xestospongia testudinaria*	Antibacterial, cytotoxic	Li et al. (2012)
(−)-Sydonol	Sesquiterpene	China	*Aspergillus* sp.	Fungus	*Xestospongia testudinaria*	Antibacterial, cytotoxic	Li et al. (2012)
(−)-Sydonic acid	Sesquiterpene	China	*Aspergillus* sp.	Fungus	*Xestospongia testudinaria*	Antibacterial, cytotoxic	Li et al. (2012)
(−)-5-(hydroxymethyl)-2-(2′,6′,6′-trimethyltetrahydro-2H-pyran-2-yl)phenol	Sesquiterpene	China	*Aspergillus* sp.	Fungus	*Xestospongia testudinaria*	Antibacterial, cytotoxic	Li et al. (2012)
JBIR 124	Sorbicillin	Japan	*Penicillium citrinum* sp. *I080624G1f01*	Fungus	Not identified	Miscellaneous	Kawahara et al. (2012)
Cyclodysidin A	Cyclic lipopeptide	Croatia	*Streptomyces strain* RV15	Actinobacterium	*Dysidea tupha*	NR	Abdelmohsen et al. (2012)
Cyclodysidin B	Cyclic lipopeptide	Croatia	*Streptomyces strain* RV15	Actinobacterium	*Dysidea tupha*	NR	Abdelmohsen et al. (2012)
Cyclodysidin C	Cyclic lipopeptide	Croatia	*Streptomyces strain* RV15	Actinobacterium	*Dysidea tupha*	NR	Abdelmohsen et al. (2012)
Cyclodysidin D	Cyclic lipopeptide	Thailand	*Streptomyces strain* RV15	Actinobacterium	*Dysidea tupha*	NR	Abdelmohsen et al. (2012)
Disydonol B	Sesquiterpene	China	*Aspergillus* sp.	Fungus	*Xestospongia testudinaria*	None	Sun et al. (2012)
1-Hydroxy-10-methoxy-dibenz[b,e]oxepin-6,11-dione	Dibenz[b,e]oxepine	Japan	*Beauveria bassiana TPU942*	Fungus	Not identified	None	Yamazaki et al. (2012)
(5E)-2-methyl-5-[(1′R*, 5′R*)-2-methylidene-7-oxobicyclo[3.2.1]oct-6-ylidene]-4-oxopentanoic acid	Sesquiterpene	Thailand	*Emericellopsis minima*	Fungus	*Hyrtios erecta*	None	Pinheiro et al. (2012)

(*Continued*)

Table 15.1 (Continued) New compounds isolated from microorganisms from marine sponges from 2000 to 2012: Their chemical class, country of collection, and reported activity

Compound name	Chemical class	Country of collection	Name of microorganism	Type of microorganism	Name of sponge	Reported activity	References
Eurocristatine	Diketopiperazine	Thailand	*Eurotium cristatum*	Fungus	Not identified	None	Gomes et al. (2012)
JBIR-113	Depsipeptide	Japan	*Penicillium* sp.	Fungus	Not identified	NR	Kawahara et al. (2012)
JBIR-114	Depsipeptide	Japan	*Penicillium* sp.	Fungus	Not identified	NR	Kawahara et al. (2012)
JBIR-115	Depsipeptide	Japan	*Penicillium* sp.	Fungus	Not identified	NR	Kawahara et al. (2012)
Cyclomarinone	Phthalide (polyketide)	Australia	*Stachylidium* sp.	Fungus	*Callyspongia* sp. cf. *C. flammea*	NR	Almeida et al. (2012)
Maristachone A	Phthalide (polyketide)	Australia	*Stachylidium* sp.	Fungus	*Callyspongia* sp. cf. *C. flammea*	NR	Almeida et al. (2012)
Maristachone B	Phthalide (polyketide)	Australia	*Stachylidium* sp.	Fungus	*Callyspongia* sp. cf. *C. flammea*	NR	Almeida et al. (2012)
Maristachone C	Phthalide (polyketide)	Australia	*Stachylidium* sp.	Fungus	*Callyspongia* sp. cf. *C. flammea*	NR	Almeida et al. (2012)
Maristachone D	Phthalide (polyketide)	Australia	*Stachylidium* sp.	Fungus	*Callyspongia* sp. cf. *C. flammea*	NR	Almeida et al. (2012)
Maristachone E	Phthalide (polyketide)	Australia	*Stachylidium* sp.	Fungus	*Callyspongia* sp. cf. *C. flammea*	NR	Almeida et al. (2012)
Marilactone	Phthalide (polyketide)	Australia	*Stachylidium* sp.	Fungus	*Callyspongia* sp. cf. *C. flammea*	NR	Almeida et al. (2012)

NR, not reported.

chemistry rather than describing the biological aspects of fungi associated with sponges (Höller et al., 2000). Fungal associations with sponges have been well established (Yarden, 2014) though not well characterized (Suryanarayanan, 2012) and require further in-depth study (Webster and Taylor, 2012). In this report, the fungi isolated from sponges between 2000 and 2012 that were reported to produce novel compounds belong to the following genera: *Acremonium*, *Arthrinium*, *Aspergillus*, *Beauveria*, *Cladosporium*, *Clonostachys*, *Curvularia*, *Drechslera*, *Emericella*, *Emericellopsis*, *Eurotium*, *Exophiala*, *Gymnascella*, *Microsphaeropsis*, *Myrothecium*, *Paecilomyces*, *Paraconiothyrium*, *Penicillium*, *Phialocephala*, *Phoma*, *Scopulariopsis*, *Stachylidium*, *Trichoderma,* and the yeast *Pichia* (Table 15.1). *Aspergillus* spp. produced 59 new compounds followed by *Penicillium* spp. that produced 26. Of a total of 272 natural products that were isolated from fungi during the period of 1970–2002, 28% came from sponges (Bugni and Ireland, 2004). During the current review period, 69% of the 269 new compounds were produced by fungi from sponges.

As the ecology of fungi in the marine environment is revealed through molecular ecology methods (Gao et al., 2008), the relationships between fungi and their hosts in the marine environment will help our understanding of the marine ecosystem and could lead to improved collection and isolation methods and the identification of chemically unexplored species (Bugni and Ireland, 2004; Abdelmohsen et al., 2014).

15.2.2 Actinobacteria

Actinobacterial associations have been found in reef and deepwater sponges, and evidence for sponge-specific symbioses exists (Hentschel et al., 2006). Moreover, marine actinobacteria in particular have yielded a higher percentage of novel secondary metabolites as compared to fungi (Lam, 2006) in the past. In this review, we report on only seven genera of actinobacteria isolated from sponges that have been reported to produce 57 new compounds. The genera are *Actinomadura*, *Microbacterium*, *Micromonospora*, *Nocardiopsis*, *Saccharopolyspora,* and *Verrucosispora*, with *Streptomyces* spp. producing the highest number of compounds (32).

In at least one case, actinobacterial symbionts such as a *Micromonospora* sp. have been shown to produce bioactive compounds (manzamines) that have no terrestrial equivalents (Montalvo et al., 2005) and are of considerable interest. Sponges in the South China Sea harbor a large diversity of actinobacteria and show evidence of host specificity (Li et al., 2006).

15.2.3 Bacteria

Bacteria associated with sponges that were reported to produce new metabolites were from four classes and seven genera only—Alphaproteobacteria (*Mesorhizobium*, *Ruegeria*), Firmicutes (*Bacillus*), Gammaproteobacteria (*Microbulbifer*, *Pseudoalteromonas*, *Pseudomonas*), and Verrucomicrobia (e.g., *Rubritalea*). However, no new compound has been reported from sponge-associated cyanobacteria since 2000, which revealed that these photosynthetic microorganisms were given less attention.

15.3 Sponge sources

Over the 13-year period (2000–2012), from a total of 104 reported studies, which would have used at least one sample per study, 35 sponge genera and 30 unidentified sponges were obtained from 23 countries or regions around the world. The sponge genera with the highest number of reported compounds are *Halichondria* with 34 compounds from three species from 12 separate studies. All three groups of microorganisms were isolated, but from different studies. This was a common feature of the studies, where each publication

reported on compounds from only one type of microbe. *Xestospongia* with 25 compounds from fungi and actinobacteria were reported in eight separate studies, *Callyspongia* spp. with 22 compounds from fungi and bacteria in six separate studies, *Stelletta* spp. with 11 compounds from fungi only in four separate studies, and *Suberites* spp. with 10 compounds from all three types of microorganism in three studies. The rest of the sponge samples produced between 1 and 8 compounds were reported in 1–3 studies for each genus.

15.4 Chemical diversity among the microbial metabolites and their activities

A diverse array of compounds has been discovered from sponges such as terpenoids, steroids, phenolic compounds, alkaloids, polysaccharides, peptides, polyketides, and fatty acids, which all showed potential biological activity (Mehbub et al., 2014). Among the 269 new compounds isolated from sponge-associated microbes, only 159 compounds showed bioactivity that includes antibacterial, anticancer/cytotoxicity, antifungal, anti-HIV, anti-inflammatory, antiparasitic, antimalarial, antituberculosis, antiviral, and antiyeast (Figure 15.2). It was noted that the fungi associated with sponges in particular produce similar classes of compounds as sponges. Of particular note are the multiple congeners, with about two-thirds of the compounds produced as a set of three or more congeners, which is common in secondary metabolism. At least 40% of compounds had no activity reported when the publication of the compounds first appeared in the literature. Subsequent testing is likely to have taken place as it is unlikely that these new resources would not be assessed for their bioactive potential. However, it was noted that there were few screening assays reported that were based on receptor-binding activity or mode of action studies.

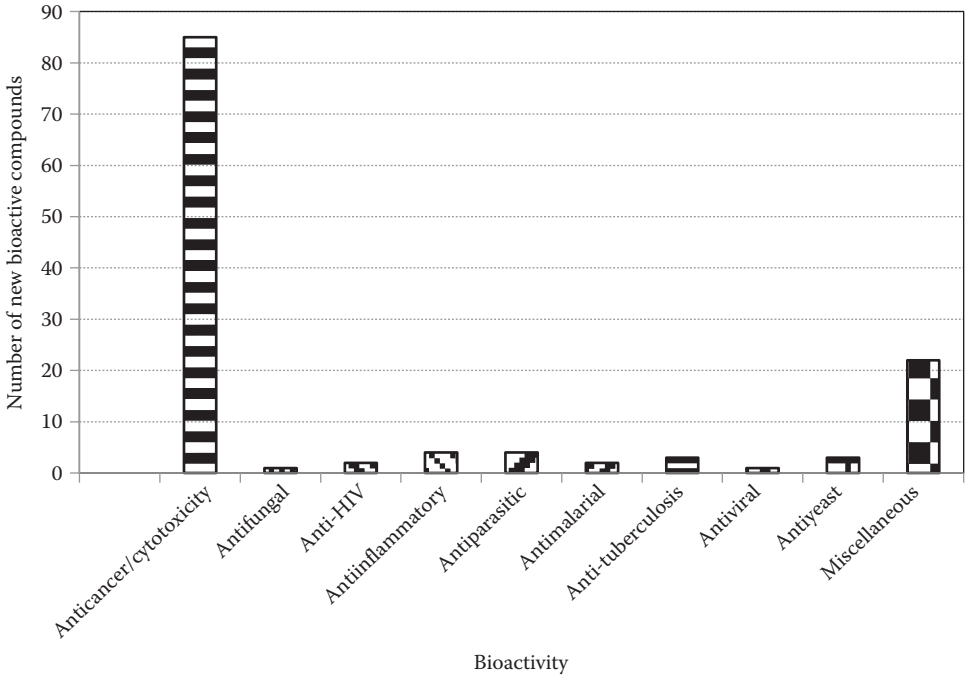

Figure 15.2 The reported bioactivity of compounds isolated from microorganisms associated with marine sponges from 2000 to 2012.

15.5 Identification of microbial diversity

Identification of microorganisms is important because biotechnology relies on defining their activities (Hugenholtz and Pace, 1996). Microbial species identified rapidly by sequencing their signature ribosomal genes; 16S rRNA for bacteria and actinobacteria and 28S rRNA for fungi are the most common. Full characterization is achieved using polyphasic methodology that employs both phenotypic and genotypic characteristics (Vandamme et al., 1996; Achtman and Wagner, 2008). Phenotypic methods include classical taxonomy, colony characteristics, biochemical and physiological studies, numerical taxonomy, cell wall composition, fatty acid analyses, isoprenoid quinones and whole-cell protein analyses, polyamines, cytochromes, and in some cases advanced spectroscopic methods (FTIR, pyrolysis mass spectrometry, UV resonance Raman spectroscopy). The genotypic methods include 16S and 28S rRNA gene sequencing and, increasingly, multilocus gene sequencing, DNA base content, and DNA–DNA hybridization (Sarethy et al., 2014).

15.6 Isolation techniques for bacteria, actinobacteria, and fungi from sponges

The use of improved cultivation approaches for the discovery of marine natural products from marine microorganisms is of paramount importance for the development of new pharmaceuticals (Bull et al., 2005). Conventional isolation methods are used to cultivate the most abundant microorganisms, but more selective methods are required to culture the uncommon and new microbial species and genera that have not been isolated previously (Ferguson et al., 1984; Eilers et al., 2000). It has been shown recently that previously uncultivable microorganisms could be grown in pure culture if provided with the chemical components of their natural environment (Kaeberlein et al., 2002; Rappe et al., 2002).

The main parameters that have influenced the isolation of novel strains include enrichment, pretreatment of samples, choice of nutrients in isolation, application of appropriate antibiotics, use of seawater and salt concentrations, inclusion of sponge extracts, and above all cultivation techniques that mimic nature (Hameş-kocabaş and Uzel, 2012). Moreover, temperature, pH, culture conditions, incubation time, and removal of emerging isolates are also very important (Kaewkla and Franco, 2013).

Using specific enrichment techniques, primarily by varying the media, hundreds of different organisms from the family of Streptosporangiacae were isolated, and many were hypothesized to produce novel antibiotics on the basis of observations of other members of this family (Lazzarini et al., 2001). In a separate example, by changing the media in combination with using selective agents for motile microorganisms, two new antibiotic-producing actinobacterial species were discovered, demonstrating the usefulness of a guided-culturing approach for the isolation of rare strains (Otoguro et al., 2001). Finally, the culturing of rare organisms has been improved by the development of new techniques. This is illustrated by the use of microcapsules, derived from a single encapsulated cell, for high-throughput screening (Zengler et al., 2005). These culturing techniques will undoubtedly improve access to natural products as new microorganisms are isolated in large numbers.

15.6.1 Sponge sampling and isolation of microorganisms

Through the use of culture-independent molecular techniques, new insights into the structure of microbial communities have been gained (Vanwonterghem et al., 2014). Molecular tools have a great potential to assist in developing strategies for identifying previously uncultured

bacteria. Identification of organisms via molecular tools can also help in determining appropriate culture media, most effective for isolation and purification of specific microorganisms. These tools allow us to further investigate or exploit microorganisms (Stewart, 2012).

After deciding on the sponges to be sampled and their location, during the sponge collection, careful attention is required to minimize contamination. Sterile ziplock bags or sterile tubes are used accompanied by the rapid transfer of samples to avoid any possible contamination from runoff (Fenical and Jensen, 2006).

Sterilized seawater is used to remove loosely attached microorganisms, and then the sponge sample is cut into small pieces (~1 cm^3). These parts are then homogenized in a prechilled sterilized mortar and pestle using sterilized seawater or phosphate-buffered saline (Zhang et al., 2008b; Abdelmohsen et al., 2010). The process of isolation of actinobacteria or fungi is to dilute the crushed sponge samples with sterile seawater or one-quarter strength Ringer's solution (Pathom-Aree et al., 2006; Bredholdt et al., 2007). After mixing by either vortexing or shaking, they are transferred to the isolation agar media in petri dishes (Jensen et al., 1991; Pathom-Aree et al., 2006).

Isolation media that were used for actinobacteria and for fungi from sponges are presented in Table 15.2 and Table 15.3, respectively.

Table 15.2 Media used for the successful isolation of actinobacteria from sponges

Medium ingredients	References
GA: 6 mL 100% glycerol, 1 g arginine, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 18 g agar, and 1 L natural seawater.	Mincer et al. (2002)
Noble: 8 g noble (purified) agar, 500 mg mannitol, 100 mg peptone, 1 L natural seawater, rifampicin (5 μg mL^{-1}), and cycloheximide (100 μg mL^{-1}).	Jensen et al. (2005)
Seawater: Eighteen grams of agar, 1 L natural seawater, rifampicin (5 μg mL^{-1}), and cycloheximide (100 μg mL^{-1}).	Jensen et al. (2005)
PA: 2 g peptone, 0.1 g asparagine, 4 g sodium propionate, 0.5 g K_2HPO_4, 0.1 g $MgSO_4$, 0.01 g $FeSO_4 \cdot 7H_2O$, 5 g glycerol, 20 g NaCl, 18 g Difco Bacto agar, 1 l distilled water, $K_2Cr_2O_7$ (50 μg mL^{-1}), and 15 μg nalidixic acid (15 μg mL^{-1}).	Zhang et al. (2008b)
Artificial seawater medium (per liter): 23.0 g NaCl, 0.75 g KCl, 1.47 g $CaCl_2 \cdot 2H_2O$, 5.08 g $MgCl_2 \cdot 6H_2O$, 6.16 g $MgSO_4 \cdot 7H_2O$, 5 g NH_2Cl, 3.5 g yeast extract, 3.5 g peptone, 0.89 g $Na_2HPO_4 \cdot 2H_2O$, and 20 g glucose.	Wicke et al. (2000)
Bennett agar (per liter): 1 g yeast extract, 1 g beef extract, 2 g tryptone, 10 g dextrose, and 15 g agar.	Lee et al. (2005)
Bennett agar (as previous) supplemented with nalidixic acid (0.02%) and cycloheximide (0.02%).	Pérez et al. (2009)
Gause's starch medium: 20 g soluble starch, 1 g KNO_3, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g NaCl, 0.01 g $7H_2O$, and 18 g agar 1 l water containing 50% natural seawater, and 0.1 mg mL^{-1} $K_2Cr_2O_7$. (The pH of the solution was set to 7.2 before sterilization.)	Li et al. (2005)
RH: 10 g raffinose, 1 g L-histidine, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 5H_2O$, 0.01 g $FeSO_4$, and 15 g agar; pH 7.2.	Maldonado et al. (2009)
Starch casein: 10 g soluble starch, 0.3 g casein, 2 g K_2HPO_4, 2 g KNO_3, 2 g NaCl, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.02 g $CaCO_3$, 0.01 g $FeSO_4 \cdot 7H_2O$, 15 g agar, and 1 L distilled water.	Maldonado et al. (2005b)
Salts: solution A (750 mL artificial seawater containing 1 g K_2HPO_4 and 10 g Bacto Agar) and solution B (250 mL artificial seawater containing 1 g KNO_3, 1 g $MgSO_4 \cdot 7H_2O$, 1 g $CaCl_2 \cdot 2H_2O$, 0.2 g $FeCl_3$, 0.1 g $MnSO_4 \cdot 7H_2O$). (Solutions A and B are autoclaved separately and mixed and supplemented with 1 mL trace element solution)	Magarvey et al. (2004)

Table 15.3 Media used for isolation of fungi from sponges

Formula	References
YMP (per liter): 3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g glucose, and 24.4 g sea salt. (The pH of the medium was adjusted to 7.2–7.4 using 0.1 N NaOH or HCl prior to inoculation.)	Jadulco et al. (2004)
Oatmeal agar: supplemented with 100% seawater.	Cruz et al. (2006)
MEA medium (prepared with 75% seawater, obtained locally): 20 g glucose, 20 g malt extract, 20 g agar, 1 g peptone, penicillin (10,000 units mL^{-1}), and streptomycin (5 mg mL^{-1}).	Lee et al. (2010)
GPY agar: based on natural seawater of 30 PSU, containing per liter 1 g glucose, 0.5 g peptone, 01 g yeast extract, and 15 g agar.	Wiese et al. (2011)
PDA: potato dextrose agar (Difco) with 250 mg L^{-1}. (Chloramphenicol was also mended with different fungicides.)	Paz et al. (2010)
Potato carrot agar (KM) per liter: 20 g cooked and mashed potatoes, 20 g cooked and mashed carrots, 20 g agar with and without artificial sea salts, and cyclosporine A (0.5 mg L^{-1}).	Proksch et al. (2008), Höller et al. (2000)
Modified ISP-4	Huang et al. (2012)

15.6.2 *Pretreatment of sponges for selective isolation of actinobacteria*

Pretreatment methods have been found successful to isolate rare or selective actinobacteria. Physical methods such as drying the sponges in laminar airflow and dilution have been described (Jensen et al., 2005; Mehbub and Amin, 2012). In addition, dilution and incubation in water bath at 50°C for 6 min and 40°C for 60 min have been described (Kim et al., 2005; Selvin et al., 2009).

However, many pretreatment methods that have been described for isolation of actinobacteria from marine sediments can also be applied for sponge samples: mechanical disruption of sponge tissue using glass beads (Maldonado et al., 2009); physical treatment such as drying, stamping, and dilution (Mincer et al., 2002; Jensen et al., 2005; Gontang et al., 2007); heat treatment such as using dry heat and incubation in different temperatures (Jensen et al., 1991; Mincer et al., 2002, 2005; Bredholt et al., 2008); freezing (Jensen et al., 2005; Bredholdt et al., 2007); radiation (Bredholdt et al., 2007); heat and radiation (Eccleston et al., 2008); centrifugation (Maldonado et al., 2005b); and chemical treatment such as using 1.5% phenol (Bredholt et al., 2008). It has been established that for obligate marine actinobacteria, sodium or salt is a prerequisite (Maldonado et al., 2005a). And, methods could be used to isolate actinobacteria according to the desired result. Even careful use of antibiotics is important for isolating targeted actinobacteria, such as the *Salinispora* strain for which cycloheximide and rifampicin are commonly used (Mincer et al., 2005). Besides, it has been revealed that UV irradiation is another effective method for getting selective isolates of different actinobacterial genera. For instance, high frequency irradiation helped to isolate *Streptosporangium* and *Rhodococcus*, and extremely high frequency irradiation was favorable for isolating *Nocardiopsis*, *Nocardia*, and *Streptosporangium* spp., and UV radiation was effective for isolating *Nocardiopsis*, *Nocardia*, and *Pseudonocardia* spp. (Bredholdt et al., 2007).

Careful selection of different nutrient sources is very important to isolate novel bacteria or fungi because the microbes residing in the sponges are likely to grow under environmental conditions that mimic their source. Moreover, the use of sponge extracts, sediment extracts, different salts, and natural seawater has been practiced in the past. In addition,

soluble starch, glycerol, glucose, raffinose, and mannitol were popular as carbon sources, while peptone, yeast, casein, nitrate, histidine, and L-asparagine were popular as nitrogen sources (Jensen et al., 2005; Maldonado et al., 2005b, 2009; Mincer et al., 2005; Gontang et al., 2007; Kennedy et al., 2009).

15.7 Concluding remarks

The discovery of new compounds from the oceans is increasing with over 1000 new compounds per year reported from marine sponges alone. In light of this abundance, the numbers of new compounds reported from microorganisms associated with sponges may seem less significant at around 35 compounds per year in recent years. However, the currently insurmountable problem of getting a continuous supply of a sponge metabolite for drug development if chemical synthesis is not economic will lead to a more intensive search for microbial sources, while efforts are made to culture sponge cells with the capability to produce the desired compounds on a large scale.

It is not surprising that fungi have overtaken the prokaryotes in the search for new secondary metabolites due to their ability to synthesize compounds belonging to the same chemical classes as their sponge hosts. To date, there have been no reports of fungal symbionts producing a sponge-derived metabolite. While fungi from sponge continue to be the dominant type of microbial producers of new compounds, it is important that studies on their ecology and interactions be undertaken to improve our understanding of their role in this ecosystem. One of the benefits will be to realize new isolation methods and techniques to reveal a broader diversity than is currently known from sponge samples. It was noted that a high proportion of the new metabolites are reported without any reported bioactivity associated with them; when there are reports, the bioactivity is from a nonspecific evaluation such as antimicrobial activity, rather than a specific mode of action assay based on a new target site that might reveal a new compound early. Most reports of anticancer activity are also based on nonspecific cytotoxic activity against one or more mammalian cell lines without further detailed assessment as to their mechanisms of action.

Further analysis of the reporting of bioactivity also shows that most compounds are reported to be tested in one assay. Therefore, the challenge is not only to find new compounds but also to develop mechanisms to evaluate their activity in a range of assays. This will allow the full potential of these varied molecules to be realized in the search for new and effective therapies for the range of diseases that are still awaiting a cure. Microbial secondary metabolites are produced by organisms that can be readily scaled up so that the supply of the compound can meet future demand.

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Appendix 4

Safe Operating Procedure

Checklist before commencing a field trip

S.O.P NUMBER	RISK ASSESSMENT NUMBER	RISK ASSESSMENT
SOP#337	**RA#337 Field Trips**	**Low**

DATE CREATED	Employees Involved in SOP Creation	REVIEW DATE
10/03/2011	Mohammad Ferdous Mehbub (Etu)	**10/03/2016**

Field Trip Checklist

Checklist has to be completed at least one day before commencing the field trip.

Personal Equipment:

Swimwear
A full length wetsuit (it will be worn to protect from cold and animal bites specially from blue ring octopus)
Towel and other personal equipment such as for sun protection and hydration
Mask and snorkel and fins (of correct fit, recently tested and serviced)
Wet Shoes that can be worn in the water and protect feet when walking over sharp surfaces
Personal floatation devices / life vests for all persons entering the water
Charged mobile phones with reception in areas of operation.

Equipment for sample collection:

Buckets with lids for transport of sponges
Plastic bags for transportation of samples in water
Empty ice container or other floating device that serves to place sponges and cutting equipment in while being in the water
Saltwater ice
Double bags
Catch bags
Nally bins
Trolley to transport buckets
Cutting equipment, such as Knife, Scalpel blade and Handle, Scissors etc., in a suitable storage container with lid.
Booking of University vehicle

Equipment for documentation:

Camera (Underwater camera if available)
Notebook and Pen

Things to be checked before commencing the field trip:

1. Obtained & read the University Field Trip Guidelines
2. Dive gear checked and marked off
3. Dive briefing
4. Collection briefing
5. All participants briefed and issues with the Field Trip Itinerary; the checklists should be read out.

a. The latest weather forecast should be checked and measures should be taken to address inclement weather (heat / cold / wind / rain / hail / frost / UV etc.)

6. Snorkel Registration forms have to be filled out and handed to the University Diving Officer for approval
    a. Only persons who are registered to snorkel, have relevant training and/or a recent dive medical are allowed into the water.

Approvals:

7. Complete Flinders University Dive Plan and submit for approval to the Executive Dean of the School no later than 72 hours before the first dive is scheduled to occur.
    a. Particular attention should be paid to Water Safety, water conditions and shark risk.
    b. It is advised that participants wear high visibility clothing.
8. A copy of the Participant Safety on Field Trips form issues to all Participants (to be signed and returned to Field Trip Supervisor/Leader).
    a. Participants must declare all known medical conditions and medical history
    b. Persons requiring certain medications should ensure that the relevant personal prescription medicines are carried and in a location and dosage known to the other participants. The medicine should carry an appropriate prescription.
9. Completed the Field Trip Risk Assessment, Management and Emergency Contingency Plan. The plan must be lodged with the supervisor and the department.
    a. Contingency plans should include the contact details of the local lifesaving or surf club if necessary.

10. All relevant permits and permissions obtained from National Parks and land owners. Particular attention should be paid to boundaries with private and traditional lands.

Communication:

11. Obtained appropriate and approved communication devices (the hire of a satellite phone if necessary).
    a. If boating activity is involved, the boat must be equipped with a recently serviced and registered Emergency Satellite Beacon (406MHz) (http://beacons.amsa.gov.au/)
    b. If VHF radio is available (such as on a government vehicle fitted with a radio set), the correct emergency frequencies should be programmed and ready to use (http://www.sascan.net.au/?page=infPages/infMarineRescue).
    c. Participants should be briefed on the correct use of these communication devices, the method of setting or changing frequencies, talking into such devices (radio discipline), dialing emergency numbers (satellite phone) and what constitutes misuse.
    d. Only commercially available UHF should be used for short-range non-emergency communications. VHF channels should not be misused.

Safety and medical equipment:

12. Approved First Aid Kit (including animal bite, allergy treatments and sun block), recently checked and serviced (materials should not be out of date or contaminated)

13. Oxygen Resuscitation equipment, with sufficient medical oxygen supplies for the area of work, must be on site. At least two personnel involved must have qualifications in the use of both, and the equipment should be tested before departure.

14. Trained First Aid person attending, regardless of the size of group. The level of First Aid

training should be appropriate to the activity.

15. Volunteers & visitors advised to arrange their own insurance coverage; indemnity forms should be signed for all non-university personnel.

16. Ensure safety equipment is of an approved design and meets the minimal legal requirements & is inspected (eg. personal floatation devices).
    a. Information on Personal Floatation Devices: http://www.sa.gov.au/subject/Transport,+travel+and+motoring/Boating+and+marine/Boat+and+marine+safety/Marine+safety+equipment/Personal+flotation+devices

17. If working alone in remote area, participant/s are advised that regular contact must be made with the field trip supervisor/leader.

Vehicle Conditions:

18. Ensure that vehicles are registered, roadworthy and covered by insurance. It is recommended a university vehicle be used.
    a. Vehicles should not be driven onto beaches unless it is a four-wheel drive suited for such terrain (eg. Correct tyre type and tyre pressure), and at places approved for vehicular traffic. If parking on an unpaved car park is required, the parking position should be above the highest tide mark on level ground.
    b. Speeding and other dangerous driving behaviour is strictly forbidden and it is the duty of all participants to ensure the driver(s) drive according to road rules and laws, and are aware of road signs and hazards.
    c. A 'vehicle commander' seated in the front passenger seat should be awake at all times to accompany the driver to ensure that driver is awake as well.
    d. Vehicle should not be parked on the grass when hot, as fire will occur.
    e. Driver should take a rest every 2 hours or driver must be changed every 2 hours.

19. Advice staff if private vehicle is used; details of the vehicle must be given to the nominated University contact person. The private vehicle must not be defective.

20. No firearms, spears, explosives or other weapons are permitted on field trips.

21. Any other relevant safety items to be considered
    a. Any activity that has the possibility of encountering nightfall should have torchlights and other safety light devices, and sufficient batteries. For example, any boating activity must prepare for the possibility of night fall in the case of engine failure or stranding, and must include equipment approved for water and wet weather use.

Sample handling:

Bucket transportation to the aquarium:
Samples will be placed in the buckets with seawater, and secured well for transport.
The nearest car park (which is car park1 near Biology) will be used.
One bucket will be carried by one person at a time when using the stairs.
Before transferring the samples from car park to aquarium make sure that the door in the marine aquarium is open for access.
Place the buckets onto the floor and fill slowly with seawater to replace the water in the bucket while taking precaution that the sponges remain in the bucket under water.

After replacing the bucket with clean water, sponges will be kept for 2 hours to adjust to the temperature of the aquarium.

Then the sponges will be transferred in the main tank by using plastic jar or plastic bags and sponges remain under water.

Sponges will be kept under water all the time and not exposed in the air in any circumstances because they will be die immediately.

Sponges will be cut after one day and feeding will be started and feeding will stop just one day before the experiment.

Approved by Wei Zhang

Appendix 5

1. Amount of source materials

   20 mg of sponge powder is the best for this method

2. Chemicals/reagents

3. Tris-EDTA: 10 mM Tris HCl, 1 mM EDTA pH 8 (autoclaved)
4. 1 M Tris, pH 8 (F.W. 121.1) 500 ml:
   60.55 g Trizma base
   ~400 ml H2O
   Approximately 21.1 ml concentrated HCl (Use pH meter)
   Bring up to volume with H_2O
   Sterilize by autoclaving (15 minutes)
5. 3 M Sodium acetate (F.W. 136.10) 500 ml
   204.15 g Sodium acetate
   ~200 ml H2O
   90 ml Glacial Acetic Acid (must remember, add acid to water)
   Dissolve, then bring up to volume with H2O
   Filter sterilized
6. 0.5 M EDTA, pH 8.0 (F.W. 336.2) 500 ml
   84.05 g EDTA
   ~250 ml H2O
   EDTA will not dissolve yet. Add 5 M NaOH slowly with stirring until the EDTA dissolves, and then reaches pH 8.0 (takes approx. 71 ml)
   Bring up to volume with H_2O
   Sterilize by autoclaving (15 minutes)
7. PVP (polyvinylypyrrolidone)
8. Water for injection
9. 70% ethanol (ice-cold) (filter sterilized)
10. 95% ethanol (filter sterilized)
11. Iso propenol (filter sterilized)

Recipe 10x TE

for 1 litre of 10x TE stock solution

volume	reagent	final conc.
100 ml	1M Tris-HCl pH 7.5 or 8.0 (see notes)	100 mM
20 ml	0.5M EDTA pH 8.0	10 mM
880 ml	ddH_2O	

Recipe 1x TE

for 1 liter of 1x TE solution

volume	reagent	final conc.
10 ml	1M Tris-HCl pH 7.5 or 8.0 (see notes)	10 mM
2 ml	0.5M EDTA pH 8.0	1 mM
988 ml	ddH_2O	

→ 1x TE is 10 mM Tris-HCl and 1 mM EDTA

Note: For the Tris-HCl use Tris base and adjust to desired pH using HCl

12. Equipment/materials

Cryogloves, fume hood, laminar flow, centrifuge, fridge, and freezer, heat block, crushed ice, polypropylene tube, sterilize pipette tips, sterilize cut pipette tips.

Buffer composition: 3% (W/V) CTAB

100 mM Tris HCl (pH8)

2 M NaCl

20 mM Na_2EDTA (pH8)

0.2% Lici

(Before autoclave adjust the pH 8) Stir the buffer until it becomes clear and if you can make the correct pH, the buffer will be clear but allow sufficient time to dissolve.

Add 2 % PVP and 1% β mercaptoethanol after autoclaving the buffer (Do this under fume hood and mix well by vortex or invert mix)

Note: To get the best activity always calculate how much buffer you need and prepare fresh. Maximum storage 1 month at room temperature)

13. Method

- In a sterile 2 ml polypropylene tube add 800 µl Buffer A (3% CTAB, 100 mM Tris-HCl, 2 M NaCl, 20 mM Na_2EDTA, 0.2% LiCl, 2% PVPP and 1% β-Mercaptaethanol) (but before using the buffer keep the buffer 60^0C for 5 minutes and invert mix until it becomes cloudy), add 20 mg of fine powder (even from 5 mg to 10 mg is fine but better to use 20 mg) to the tube. Then add 100 µl buffer B (1M Tris-Cl (pH 8), 0.5M EDTA, 4.8% Triton X and 80 mg/ml lysozyme. Molecular grade water was used to prepare the buffer.) Use molecular grade water to prepare the buffer.
  [Note: This buffer needs to be adjusted at pH 8 and filter sterilized. Note: To get a sufficient amount of DNA and getting more diversity do the extraction in replicate]

  After adding this buffer, invert mix several times as well.

- Mix the sample by inversion of the tubes several times by using hands, do not vortex. Then incubate the tube at 65°C for 2 hours.
- Gently shake the sample (inversion) every 10 min.
- Centrifuge the tube at 10,000 rpm for 10 minutes
- Transfer the supernatant to a new 2 ml tube (supernatant is not so clear that's ok)
- Add equal volume of chloroform isoamayl alcohol (24: 1) to the tube and gently flipped and invert several times.
- Then centrifuge the sample at 12000 rpm for 8 min
- Transfer the supernatant to a new tube (The most critical step is this one, don't touch the white layer by any means with the pipette tips)
- Repeat the previous step two times which started by adding chloroform isoamayl alch ohol.
- Add 100 µl of 3 M sodium acetate and flip gently.
- Add 500 µl of ice cold isopropanol, add the isopropanol by using cut tips and add by several drops.
- Mix the sample by flipping slowly and then by inversion for 5 to 10 minutes by hand.

- Store the sample in -20^{0}C for one hour. (Longer storage will reduce the extraction efficiency by precipitating other stuff).
- Centrifuge the tube at 13.2 rpm for 10 minutes and remove the supernatant without touching the pellet.
- The pellet should be at the bottom, if you do not see the pellet add 100µl more isopropanol and invert mix several times and centrifuge again.
- Discard the supernatant to a new tube and add 500 µl of 95% cold ethanol and flick mix several times.
- Centrifuge the tube at 12,000 rpm for 15 min and discard the supernatant
- Wash the sample again with cold 70% ethanol at 12000 rpm for 15 minutes. (Note: Repeat both washing steps if you find that the pellet is not clear, otherwise it is not necessary to do it)
- Discard the supernatant without disturbing the pellet
- Dry the pellet at 37^{0}C for 30 minutes and then under fume hood for 5 to 10 minutes and check that there is no ethanol, don't over dry then it will be difficult to dissolve.
- Add 50 µl TE or Tris buffer or injection water to the tube to dissolve the DNA pellet.
  [Note: Injection water is suitable instead of TE, sometimes EDTA could inhibit PCR but for longer time preservation TE is good]
- Pool together the two tubes (replicate), which means 100 µl of injection water with DNA.
  [Note: DNA can be kept at 4°C overnight at this stage if there is shortage of time and the later step can be continued]
- Add 15 µl of proteinase K and 200 µl of Buffer AL (from DNeasy blood & tissue kit-Qiagen), mix well by inverting and incubated at 70°C for 10 min.
- Added 200 µl 100% filter sterilized ethanol and mixed well. Transfer the mixture to silica based Spin-column and centrifuged at 13.2 rpm at room temperature for 1 min.
- Discard the flow through, add 500 µl of Buffer AW1 (Qiagen) and centrifuge at 13.2 rpm for 1 min at 4°C
- Discard the flow through, add 500 µl of Buffer AW2 (Qiagen) and centrifuge at 13.2 rpm for 1 min at 4°C
- After discard the flow through, dry the column by centrifugation at 4 C at 13.2 rpm for 1 min.
- Transfer the spin-column to a new tube and add 100 µl of prewarm Buffer AE (Qiagen) [kept at 60°C for 5 min] and incubate at room temperature for 2 min.
- Elute DNA by centrifuging at 8000 rpm at 4°C.
- Again Add 100 µl of prewarm Buffer AE in the tube and incubate at room temperature for 2 min and elute at 8000 rpm at 4°C.
- Finally, 200 µl DNA will be ready to use for PCR and other downstream application which should be stored at -20°C.

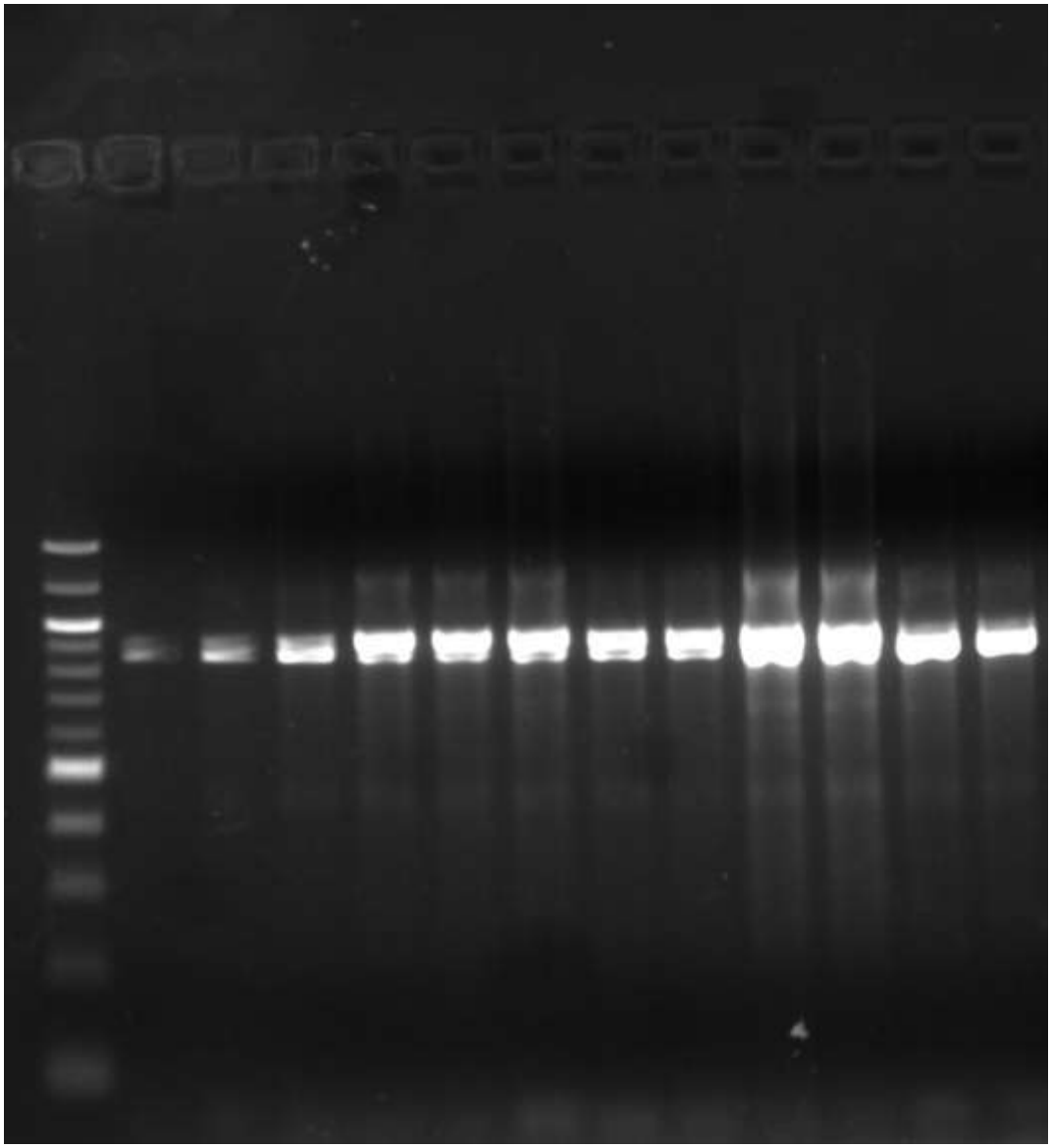


Fig S1. Gradient PCR with sponge sample to reveal the appropriate annealing temperature by using primer 9F and 928R.

Appendix 6

Fig: Act 4

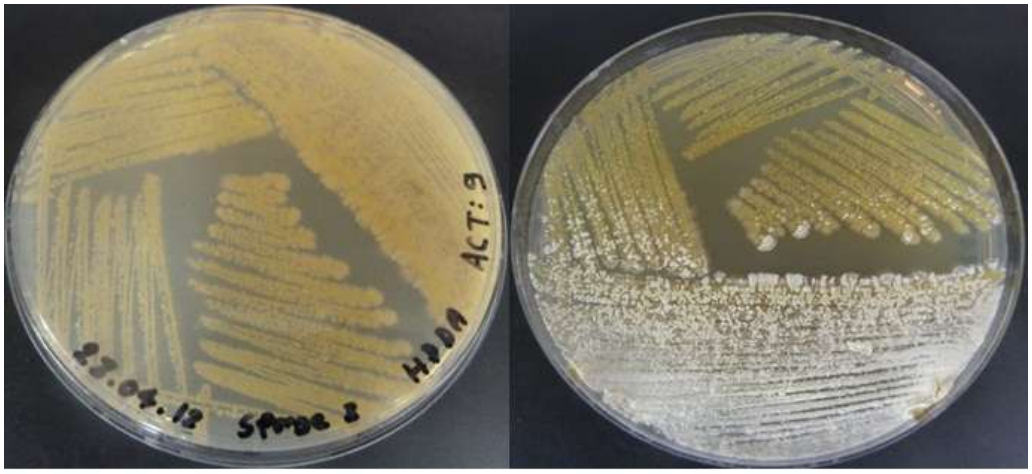


Fig: Act 9

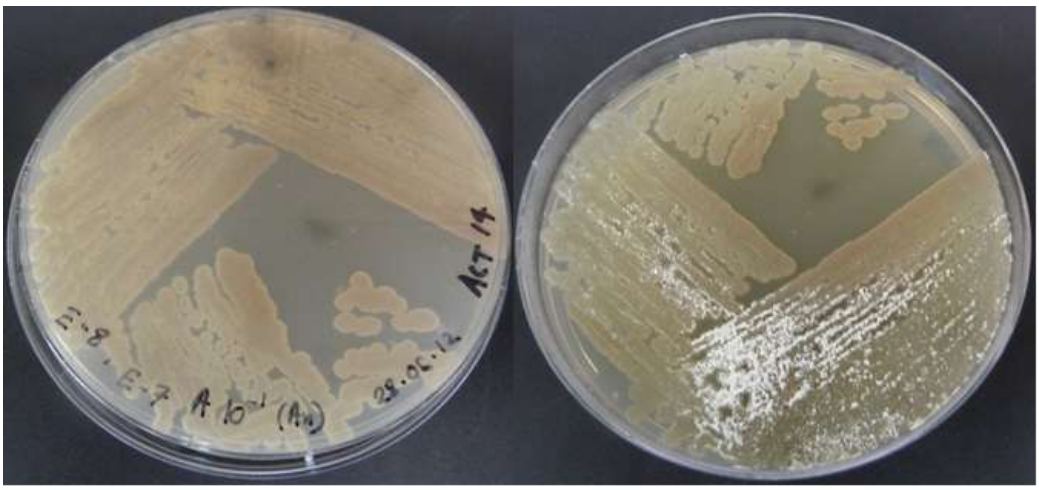
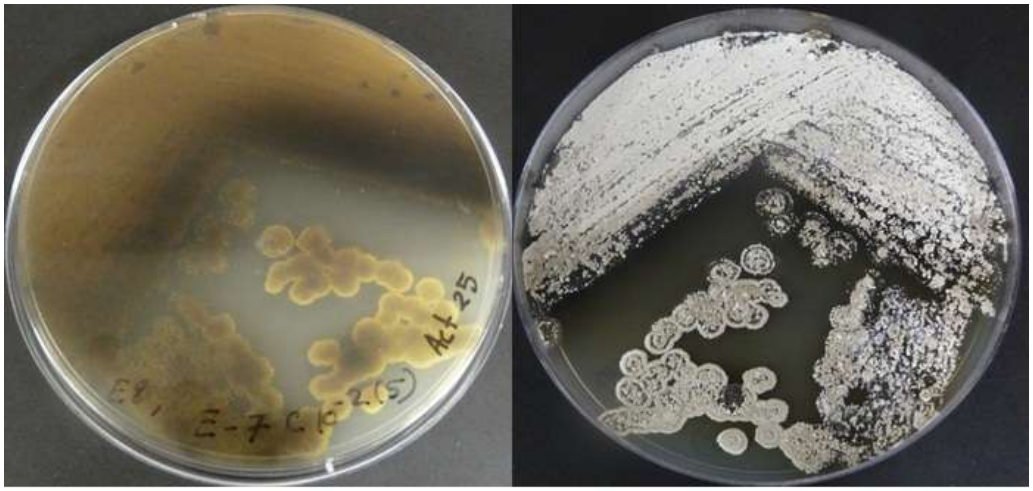


Fig: Act 14

Fig: Act 22



Act: 25

Fig: Act 31

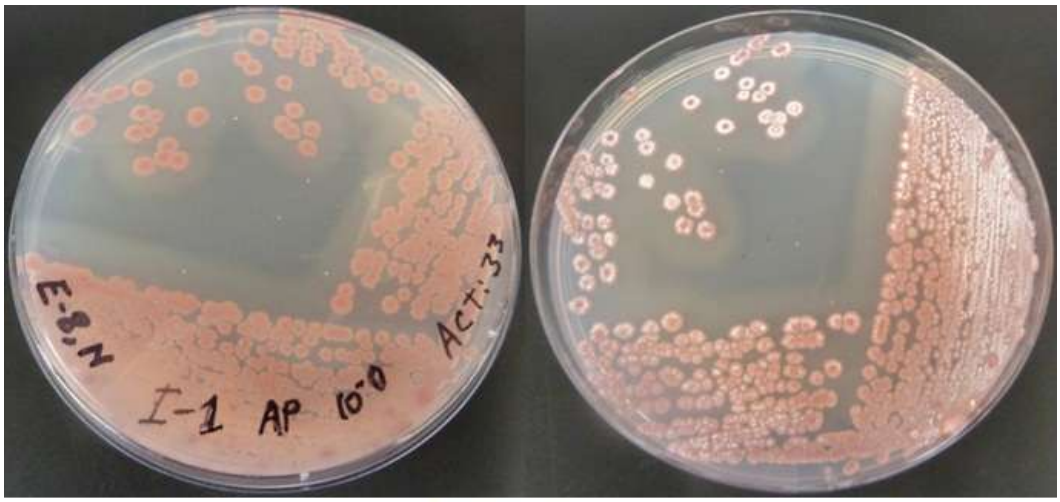


Fig: Act 33

Fig: Act 34

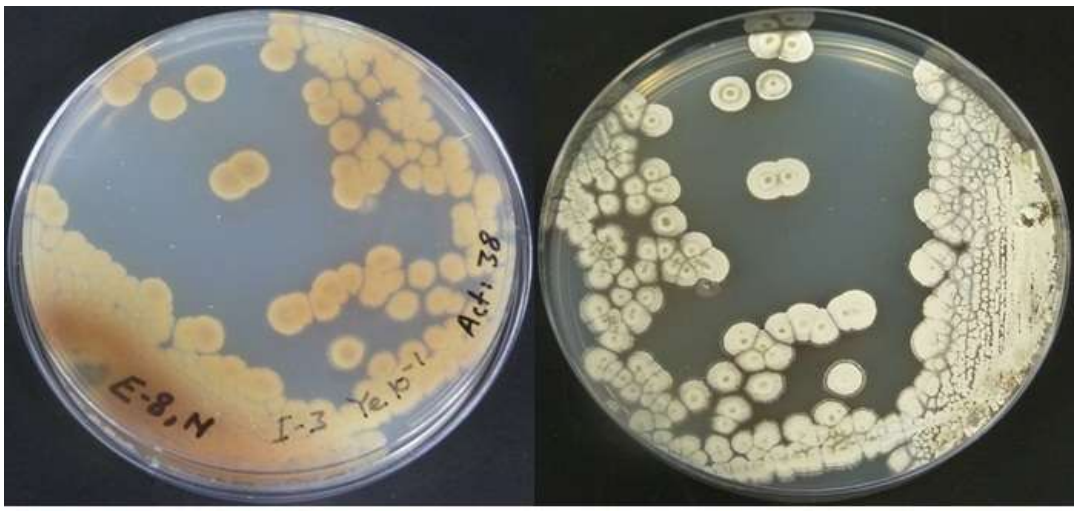


Fig: Act 38

Fig: Act 39

Fig: Act 40

Fig: Act 43

Fig: Act 46

Fig: Act 52

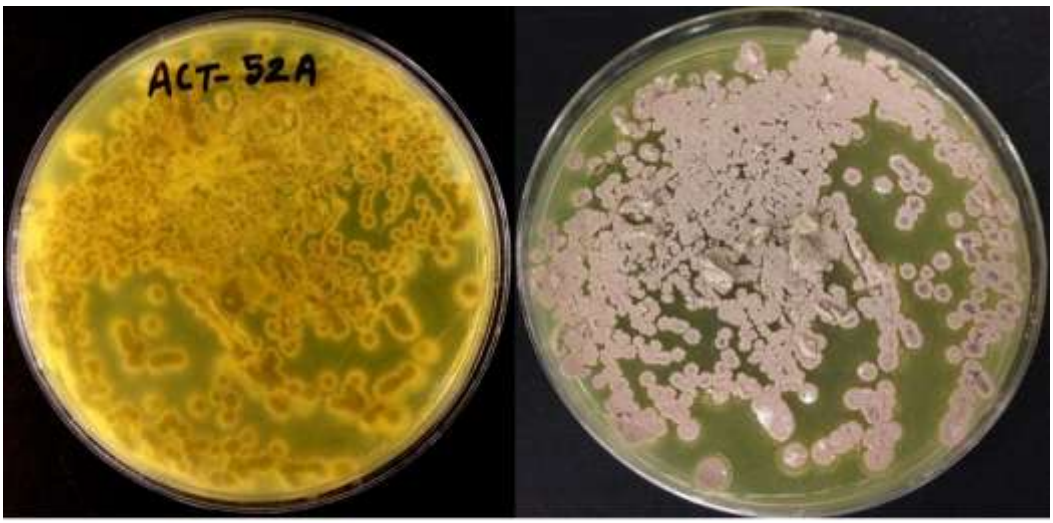


Fig: ACT-52A

Fig: Act 53

Act: 61

Fig: Act 67

Fig: Act 68

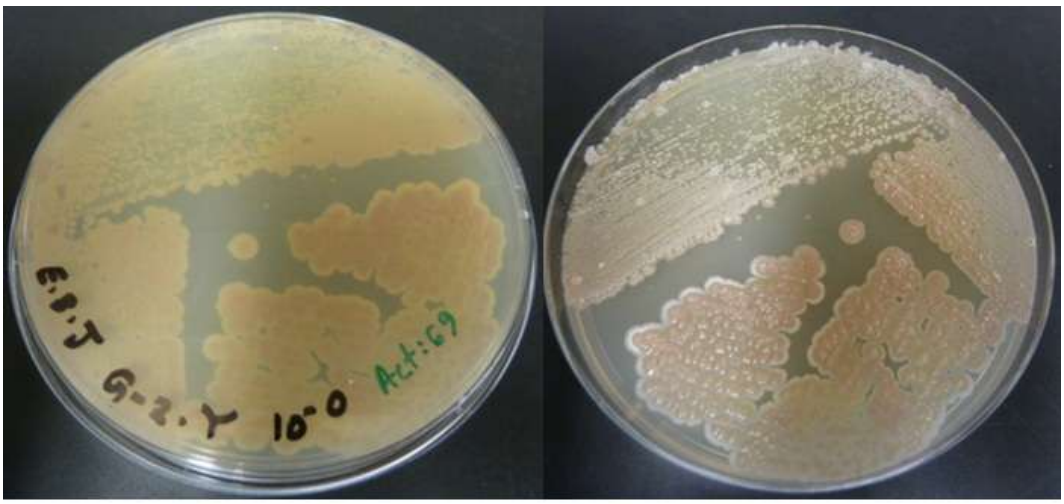


Fig: Act 69

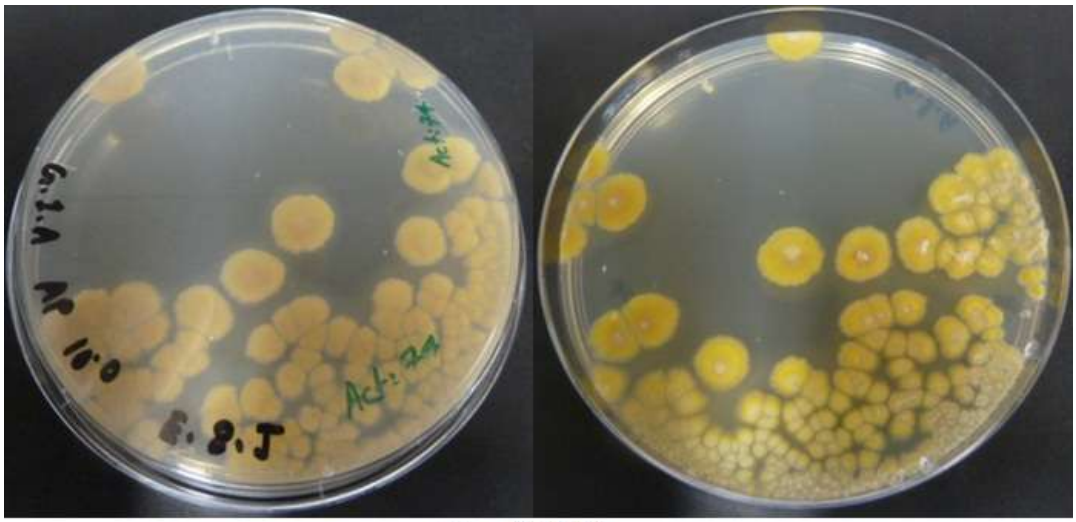


Fig: Act 74

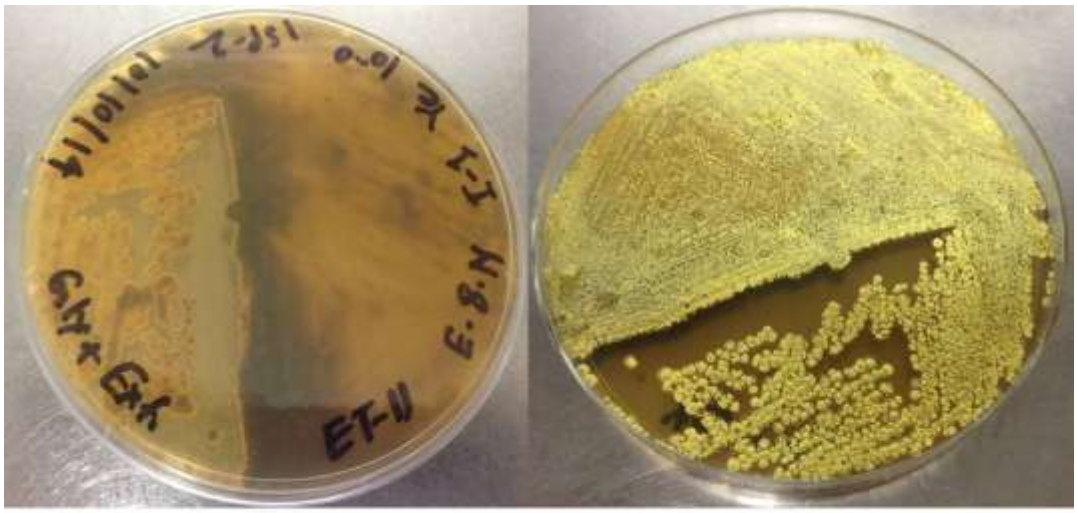
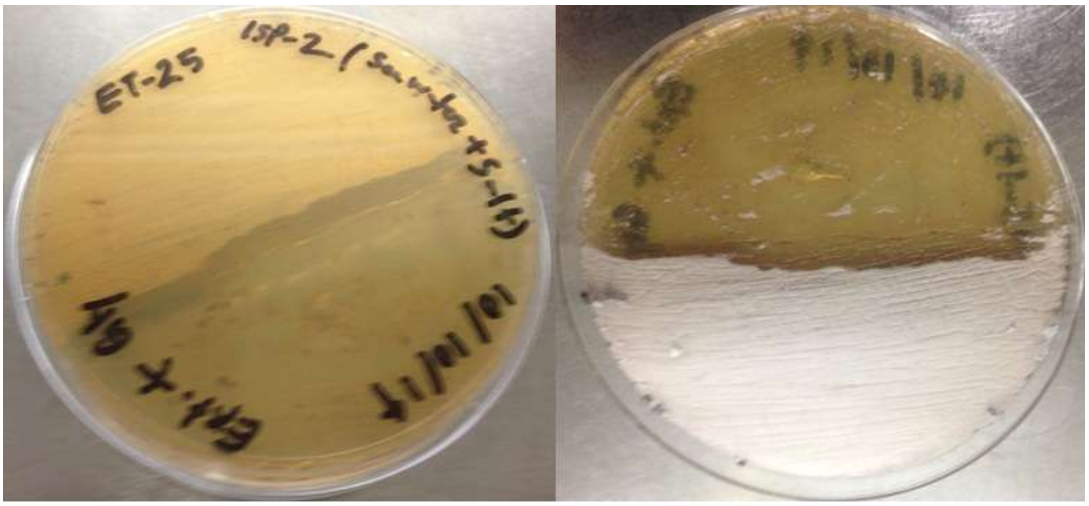


Fig: ET-11



ET-25

Figure: Some representative isolates showing the morphological difference when maintaining in ISP-2 media.

Table: Cultural characteristics of the selected strains nominated for full sequencing. Colour dimension based on Kornerup and Wanscher (1978).

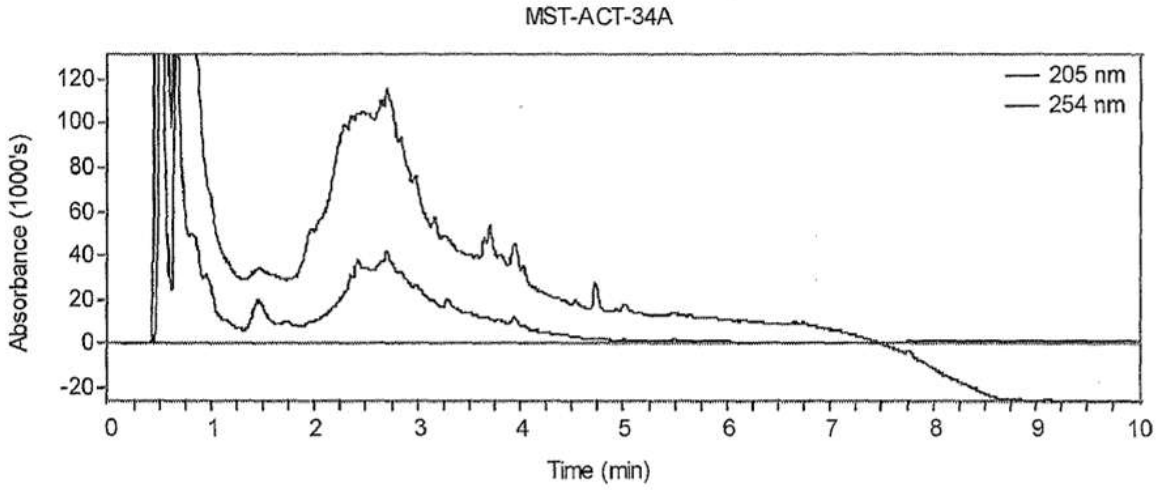
Strain	Medium	Substrate mycelium		Aerial mycelium	
ACT-14A	MS	Good	Grey	Good	White
	ISP-2	Poor	Butter yellow	Poor	White
	HPDA	Poor	Cream	Poor	White
ACT-22A	MS	Good	Burnt amber	Moderate	White
	ISP-2	Moderate	Amber yellow	Poor	White
	HPDA	Poor	Cream	Good	Grey

Strain	Medium	Substrate mycelium		Aerial mycelium	
ACT-25	MS	Good	Grey	Good	White
	ISP-2	Good	Butter yellow	Good	White
	HPDA	Good	Cream	Good	White
ACT-40	MS	Medium	Champagne	Medium	White
	ISP-2	Good	Brown	Good	White
	HPDA	Good	Brown	Medium	White
ACT-52A	MS	Good	Brown	Good	White
	ISP-2	Good	Rust	Good	White
	HPDA	Good	Yellow	Good	White
ACT-34	MS	Medium	Cream	Medium	White
	ISP-2	Medium	Brown	Medium	White
	HPDA	Good	Marine blue	Good	White
ACT-39B	MS	Medium	White	Medium	White
	ISP-2	Medium	Oak brown	Medium	White
	HPDA	Medium	White	Medium	White
ACT-69D	MS	Good	Dark turquoise	Good	White
	ISP-2	Good	Light turquoise	Good	White
	HPDA	Poor	Champagne	Poor	White
ET-11	MS	Good	Grey	Medium	Yellow
	ISP-2	Moderate	Brown	Medium	Yellow
	HPDA	Good	Grey	Medium	Yellow
ET-25	MS	Good	Grey	Good	White
	ISP-2	Moderate	Brown	Medium	White
	HPDA	Poor	Grey	Medium	White

HPLC analyses conducted in Sydney for selected isolates grown in ISP-2 media.

Comet analysis of ACT-34A HPLC Trace under TFA conditions.

Production	Poor
Total Peaks (254 nm)	6
Non-Polar Peaks (254 nm)	3
Non-Polar Peaks (205 nm)	1
Resolved UV Spectra	0
No. of UV Classes	0

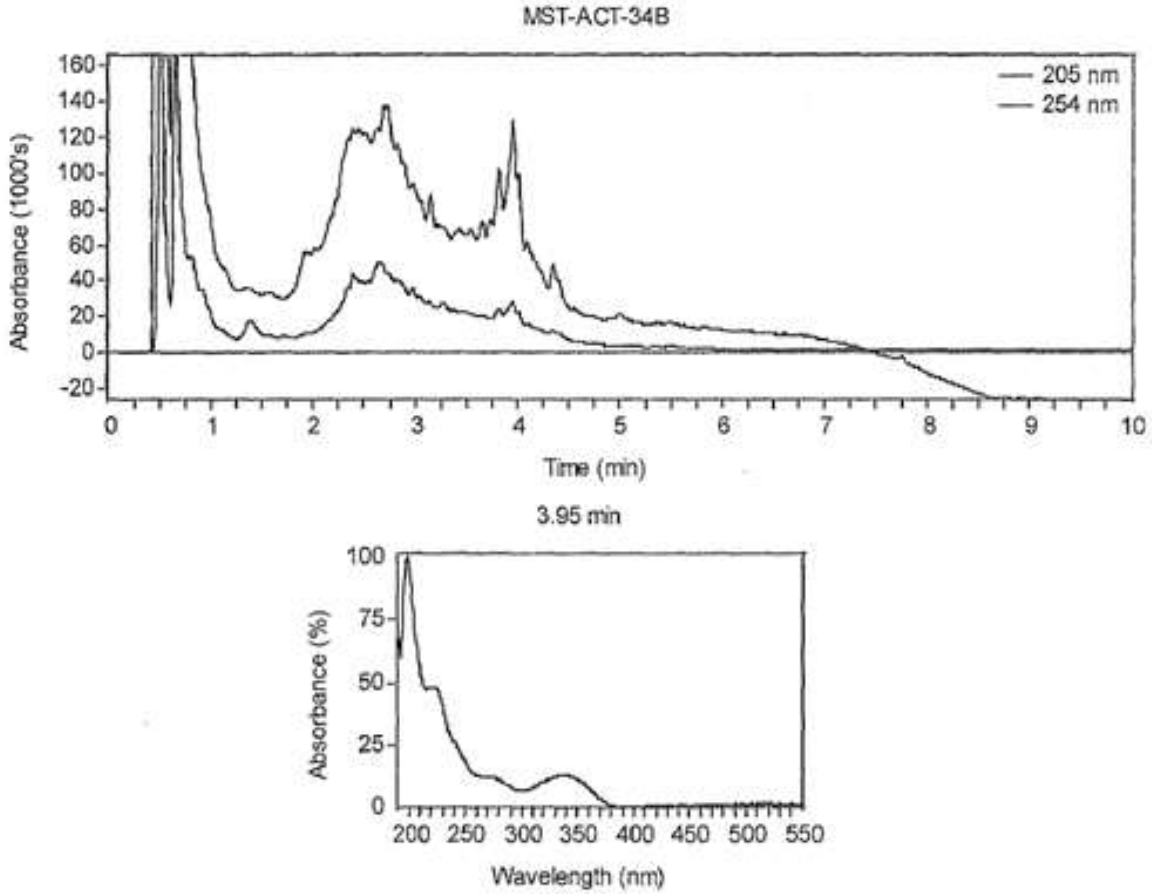


Comet analysis of ACT-34B HPLC Trace under TFA conditions.

Production	Low
Total Peaks (254 nm)	9
Non-Polar Peaks (254 nm)	6
Non-Polar Peaks (205 nm)	4
Resolved UV Spectra	1
No. of UV Classes	0

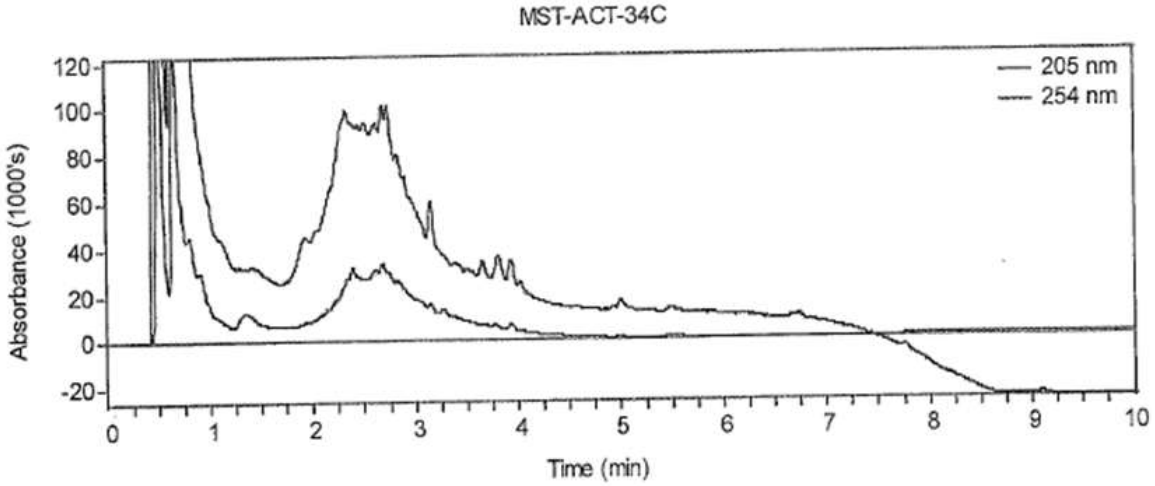
Resolved UV Spectra of ACT-34B HPLC Trace under TFA conditions.

Peak	RT	Height	Class	UV Description
9	3.95	6589		199 (p, 100%)



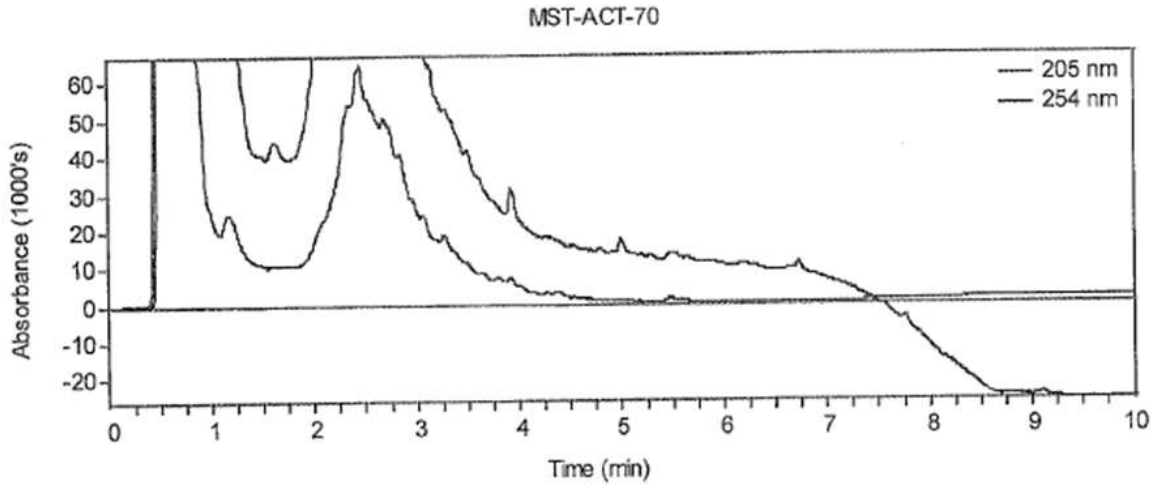
Comet analysis of ACT-34C HPLC Trace under TFA conditions.

Production	Poor
Total Peaks (254 nm)	5
Non-Polar Peaks (254 nm)	2
Non-Polar Peaks (205 nm)	1
Resolved UV Spectra	0
No. of UV Classes	0



Comet analysis of ACT-70 HPLC Trace under TFA conditions.

Production	Low
Total Peaks (254 nm)	4
Non-Polar Peaks (254 nm)	2
Non-Polar Peaks (205 nm)	5
Resolved UV Spectra	0
No. of UV Classes	0

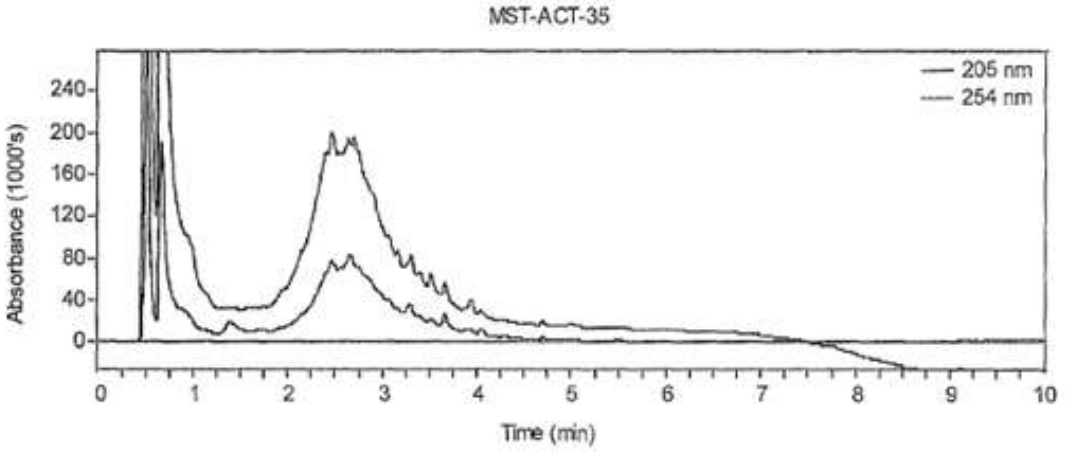


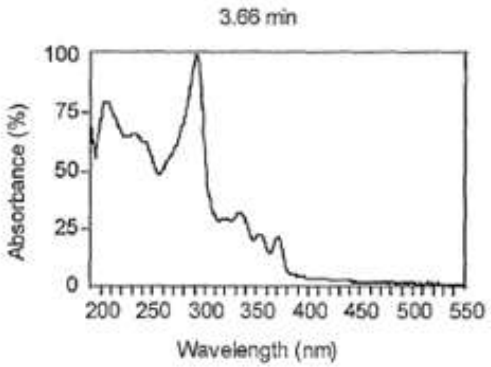
Comet analysis of ACT-35 HPLC Trace under TFA conditions.

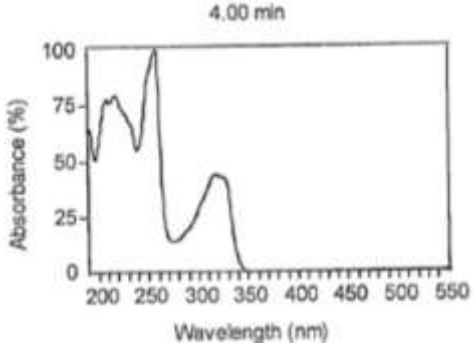
Production	Low
Total Peaks (254 nm)	8
Non-Polar Peaks (254 nm)	6
Non-Polar Peaks (205 nm)	4
Resolved UV Spectra	1
No. of UV Classes	0

Resolved UV Spectra of ACT-35 HPLC Trace under TFA conditions.

Peak	RT	Height	Class	UV Description
7	3.66	12172		233 (p, 66%), 267 (s, 56%), 292 (p, 100%), 319 (p, 29%), 333 (p, 32%), 353 (p, 23%), 371 (p, 21%)





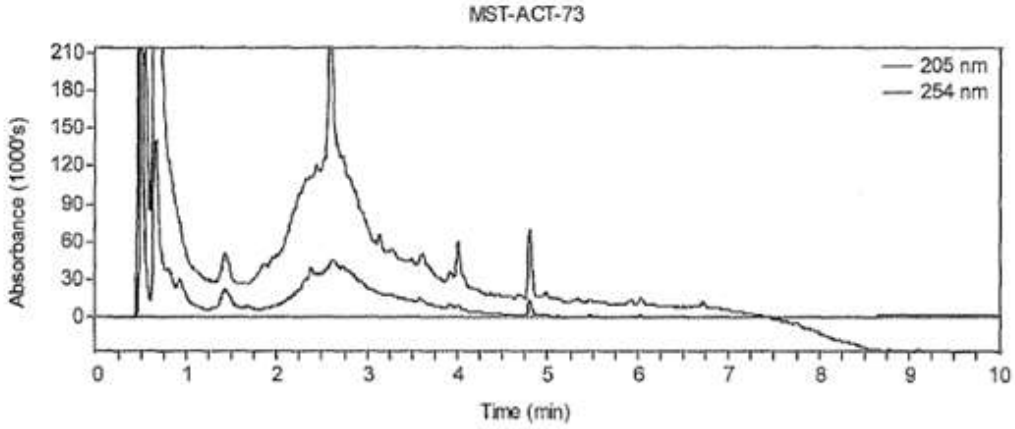


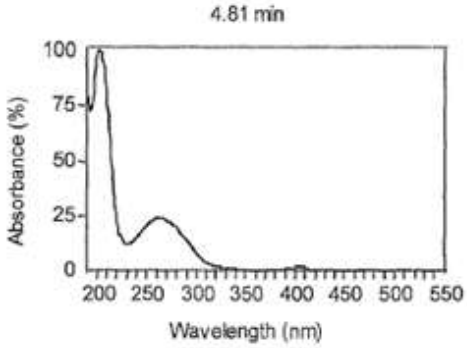
Comet analysis of ACT-73 HPLC Trace under TFA conditions.

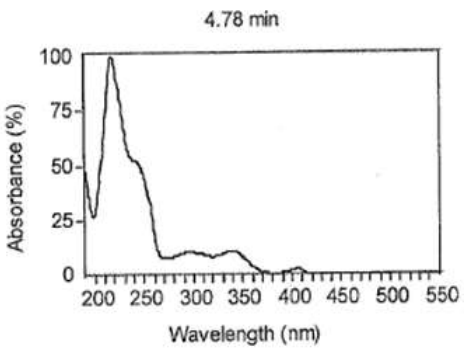
Production	Poor
Total Peaks (254 nm)	7
Non-Polar Peaks (254 nm)	4
Non-Polar Peaks (205 nm)	4
Resolved UV Spectra	1
No. of UV Classes	0

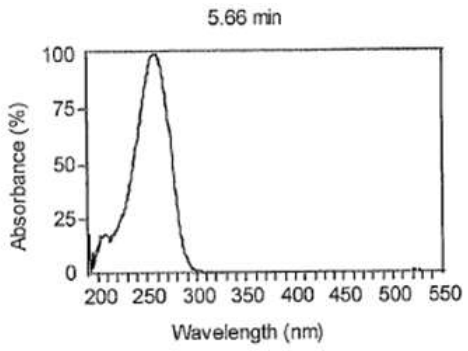
Resolved UV Spectra of ACT-73 HPLC Trace under TFA conditions.

Peak	RT	Height	Class	UV Description
7	4.81	12080		201 (p, 100%), 264 (p, 24%)







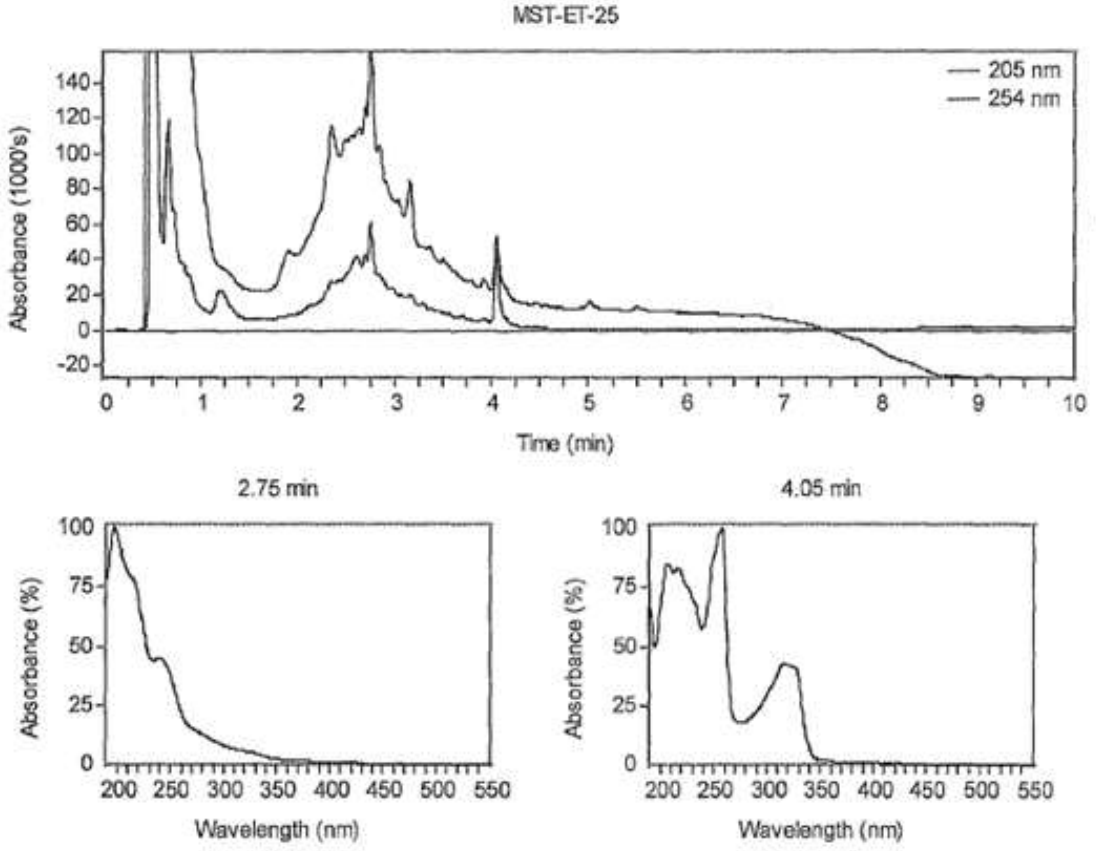


Comet analysis of ET-25 HPLC Trace under TFA conditions.

Production	Low
Total Peaks (254 nm)	7
Non-Polar Peaks (254 nm)	5
Non-Polar Peaks (205 nm)	4
Resolved UV Spectra	2
No. of UV Classes	0

Resolved UV Spectra of ET-25 HPLC Trace under TFA conditions.

Peak	RT	Height	Class	UV Description
6	2.75	23518		197 (p, 100%), 214 (s, 79%), 241 (p, 44%)
7	4.05	42726		207 (p, 84%), 217 (p, 83%), 257 (p, 100%), 317 (p, 43%)

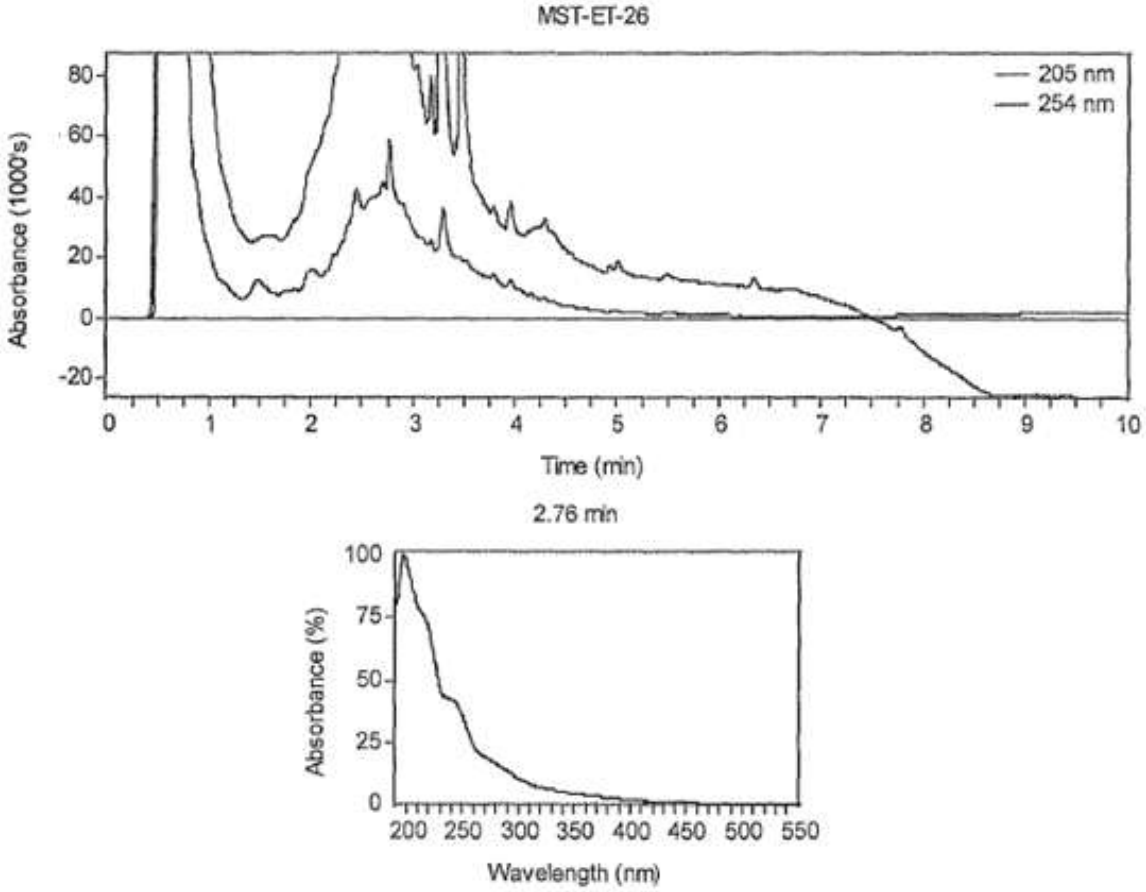


Comet analysis of ET-26 HPLC Trace under TFA conditions.

Production	Low
Total Peaks (254 nm)	7
Non-Polar Peaks (254 nm)	4
Non-Polar Peaks (205 nm)	5
Resolved UV Spectra	1
No. of UV Classes	0

Resolved UV Spectra of ET-26 HPLC Trace under TFA conditions.

Peak	RT	Height	Class	UV Description
6	2.76	17941		198 (p, 100%)

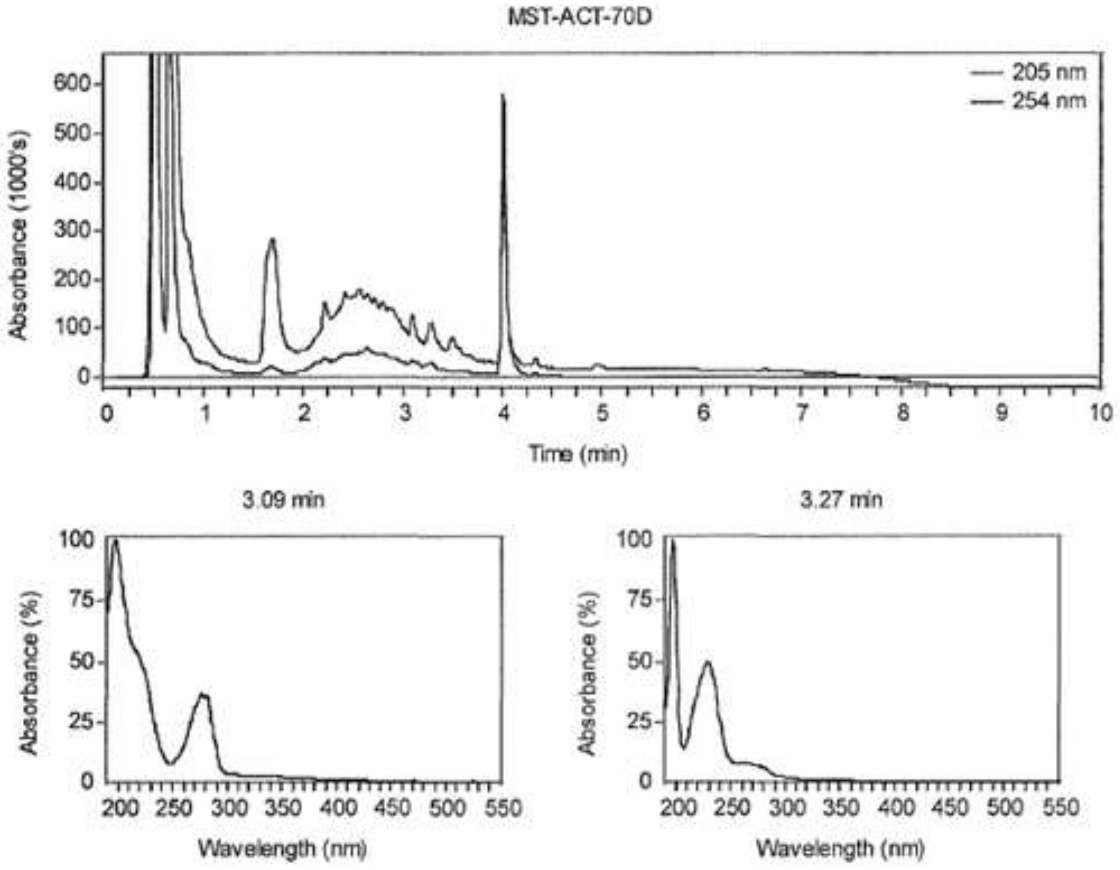


Comet analysis of ACT-70D HPLC Trace under TFA conditions.

Production	Med
Total Peaks (254 nm)	11
Non-Polar Peaks (254 nm)	9
Non-Polar Peaks (205 nm)	12
Resolved UV Spectra	3
No. of UV Classes	0

Resolved UV Spectra of ACT-70D HPLC Trace under TFA conditions.

Peak	RT	Height	Class	UV Description
8	3.09	6420		197 (p, 100%)
9	3.27	8716		197 (p, 100%), 229 (p, 49%), 272 (s, 8%), 280 (s, 6%)
10	4.00	573495		217 (p, 79%), 257 (p, 100%), 303 (s, 31%), 317 (p, 44%), 324 (s, 42%)

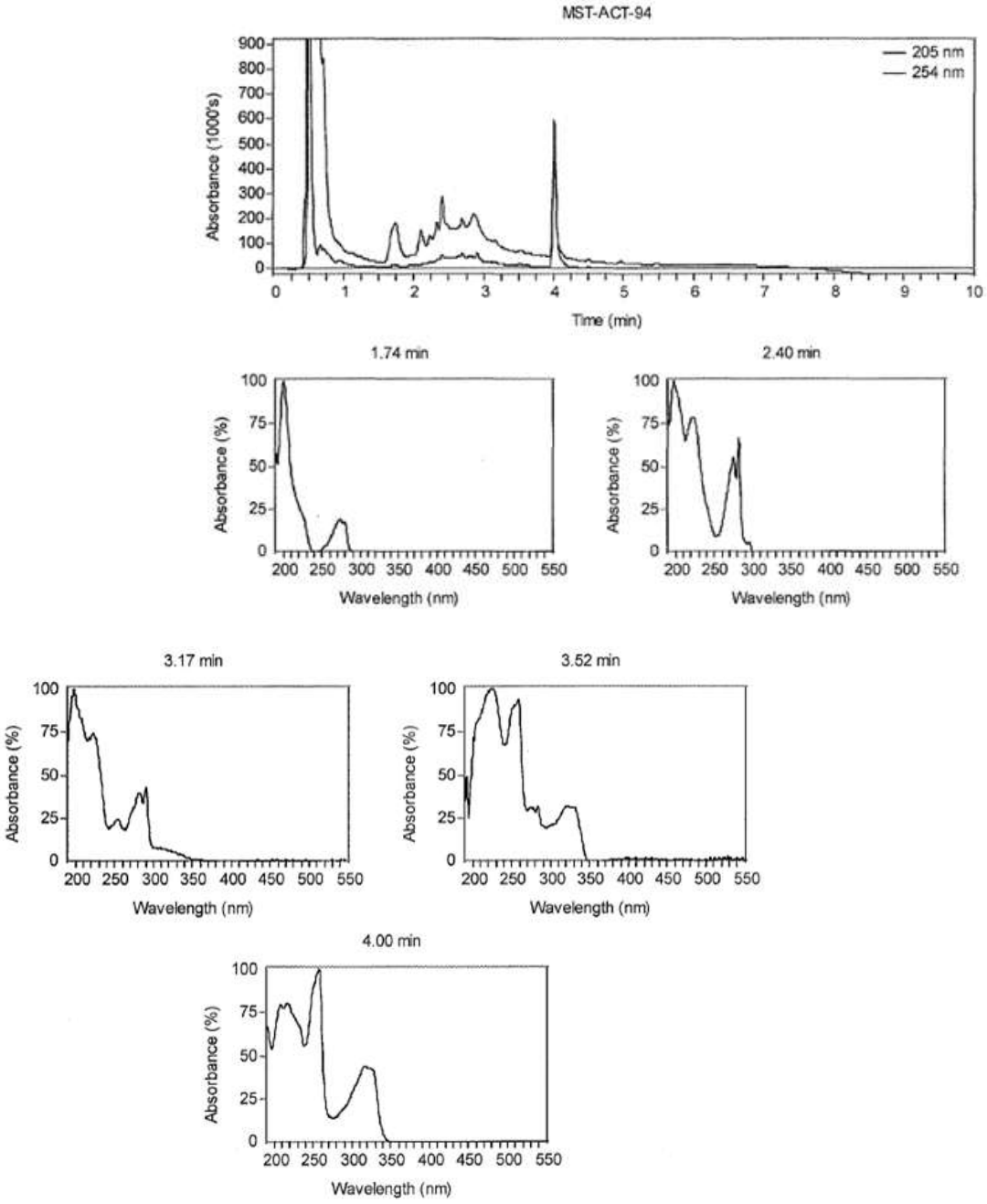


Comet analysis of ACT-94 HPLC Trace under TFA conditions.

Production	Med
Total Peaks (254 nm)	14
Non-Polar Peaks (254 nm)	10
Non-Polar Peaks (205 nm)	9
Resolved UV Spectra	5
No. of UV Classes	0

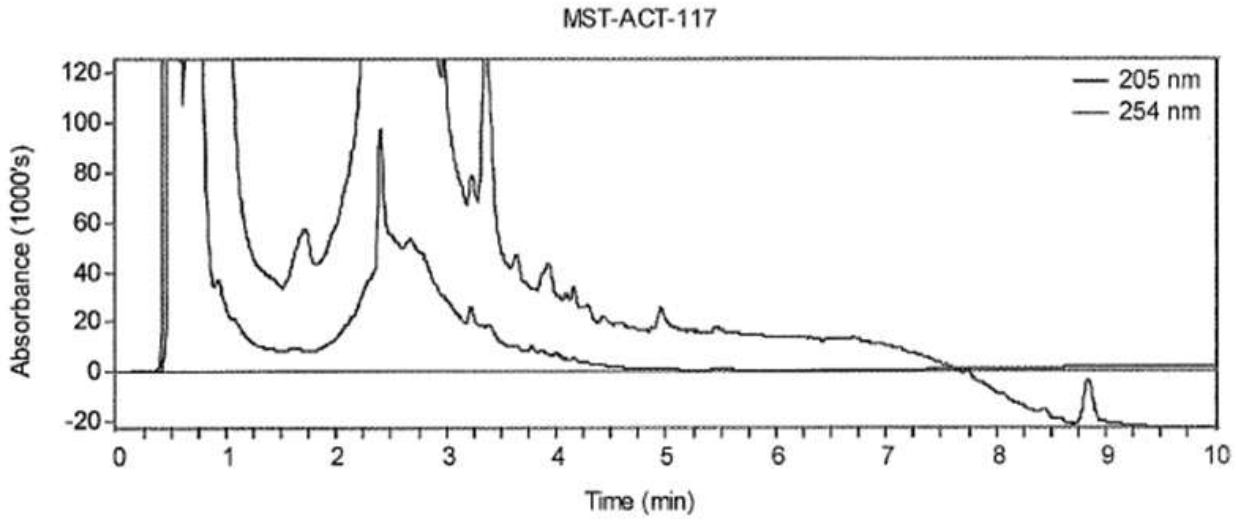
Resolved UV Spectra of ACT-94 HPLC Trace under TFA conditions.

Peak	RT	Height	Class	UV Description
5	1.74	6297		200 (p, 100%)
8	2.40	16989		198 (p, 100%), 222 (p, 79%), 275 (p, 55%)
12	3.17	4533		198 (p, 100%), 223 (p, 74%), 254 (p, 24%), 282 (p, 40%)
13	3.52	9900		258 (p, 93%)
14	4.00	590658		208 (p, 79%), 217 (p, 80%), 232 (s, 67%), 257 (p, 100%), 317 (p, 44%), 324 (s, 42%)



Comet analysis of ACT-117 HPLC Trace under TFA conditions.

Production	Low
Total Peaks (254 nm)	7
Non-Polar Peaks (254 nm)	3
Non-Polar Peaks (205 nm)	8
Resolved UV Spectra	0
No. of UV Classes	0

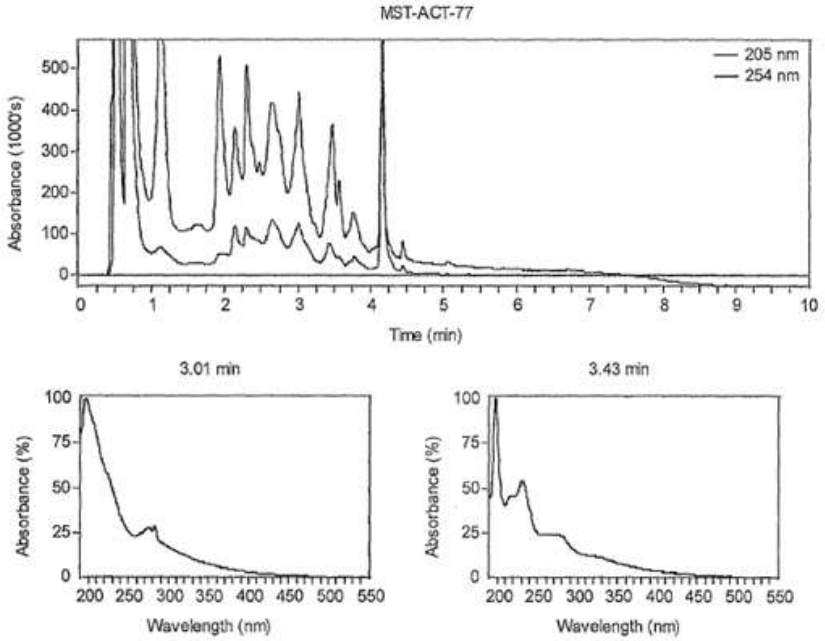


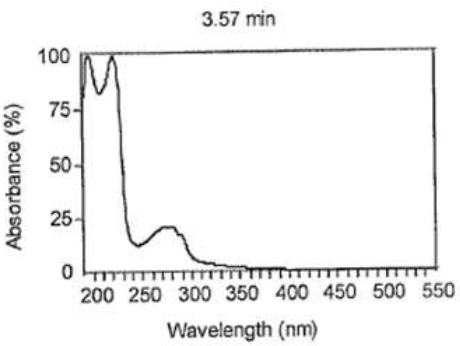
Comet analysis of ACT-77 HPLC Trace under TFA conditions.

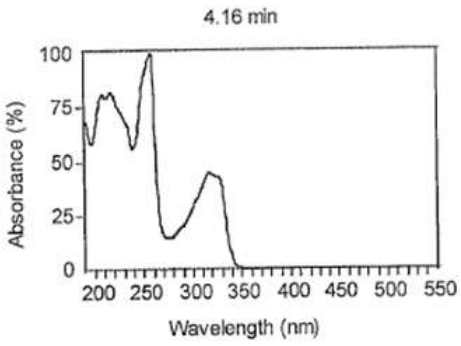
Production	Med
Total Peaks (254 nm)	14
Non-Polar Peaks (254 nm)	11
Non-Polar Peaks (205 nm)	13
Resolved UV Spectra	5
No. of UV Classes	0

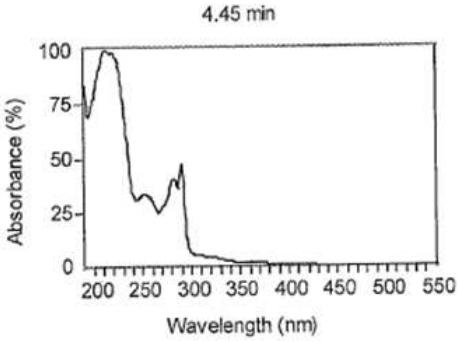
Resolved UV Spectra of ACT-77 HPLC Trace under TFA conditions.

Peak	RT	Height	Class	UV Description
9	3.01	70549		196 (p, 100%), 276 (p, 28%), 296 (s, 18%)
10	3.43	41200		198 (p, 100%), 218 (p, 46%), 231 (p, 54%), 266 (p, 24%)
11	3.57	10007		195 (p, 100%), 221 (p, 99%)
13	4.16	608598		217 (p, 82%), 257 (p, 100%), 317 (p, 44%), 324 (s, 43%)
14	4.45	12510		212 (p, 100%), 252 (p, 33%), 283 (p, 41%)







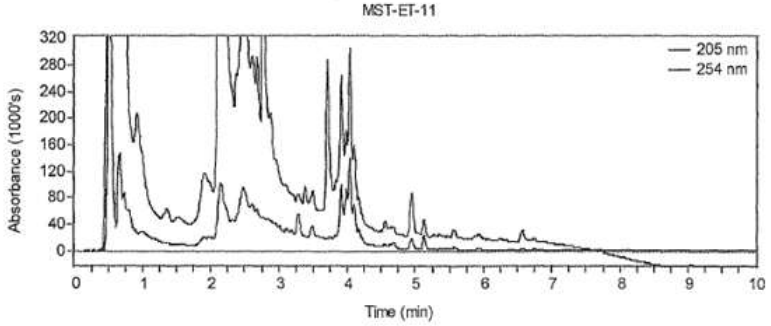


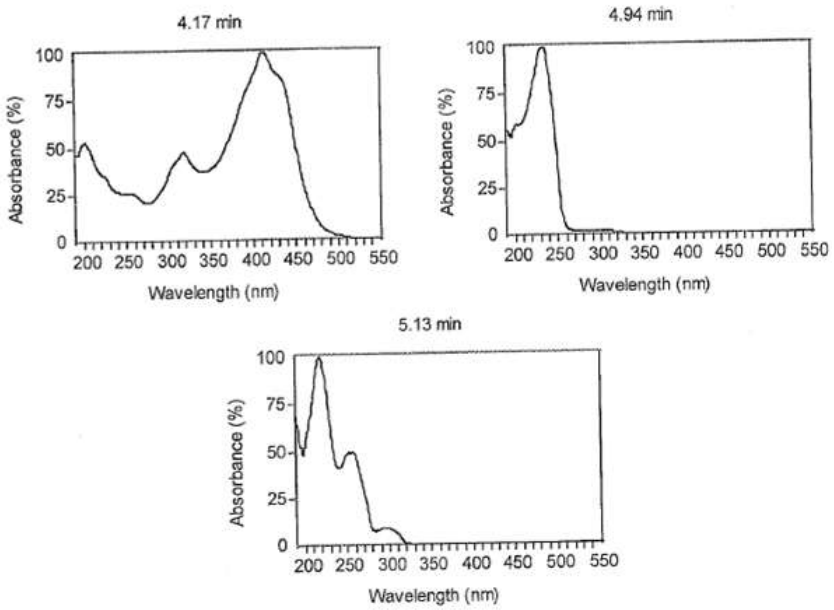
Comet analysis of ET-11 HPLC Trace under TFA conditions.

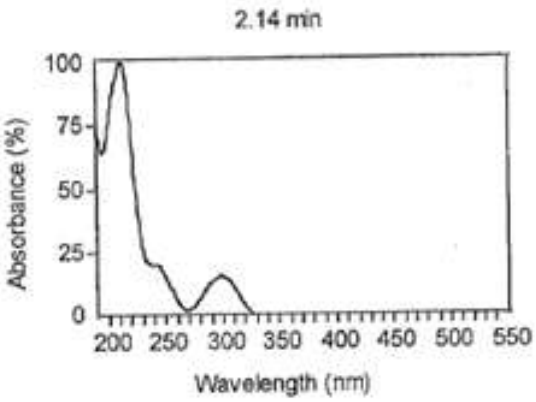
Production	Low
Total Peaks (254 nm)	19
Non-Polar Peaks (254 nm)	15
Non-Polar Peaks (205 nm)	21
Resolved UV Spectra	11
No. of UV Classes	0

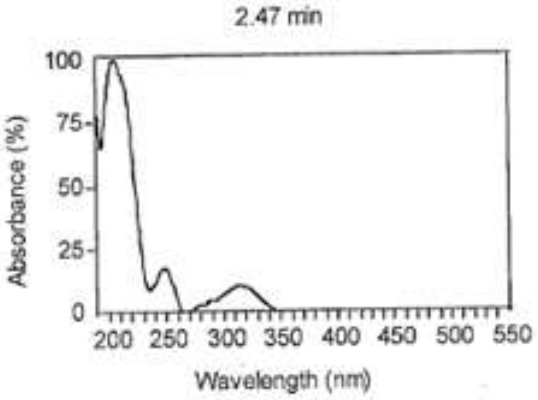
Resolved UV Spectra of ET-11 HPLC Trace under TFA conditions.

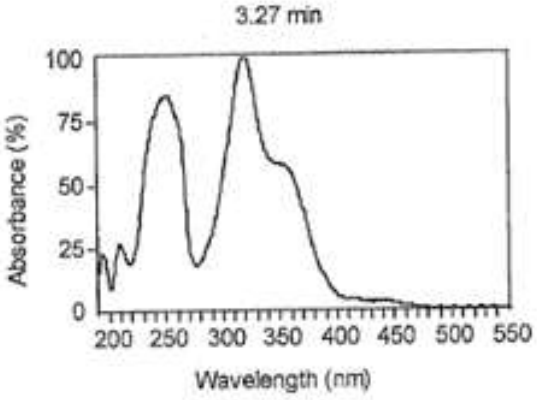
Peak	RT	Height	Class	UV Description
5	2.14	73288		211 (p, 100%), 240 (s, 20%), 299 (p, 15%)
6	2.47	38904		206 (p, 100%), 248 (p, 17%), 283 (s, 3%), 315 (p, 10%)
10	3.27	30582		209 (p, 27%), 252 (p, 85%), 261 (s, 74%), 319 (p, 100%)
11	3.49	15645		201 (p, 100%), 253 (p, 45%)
12	3.91	55307		201 (p, 33%), 310 (p, 10%), 386 (s, 73%), 404 (p, 100%), 424 (p, 95%)
13	3.98	23363		250 (s, 0%), 298 (p, 13%), 384 (p, 75%), 402 (p, 100%), 426 (p, 78%)
14	4.03	71345		204 (p, 33%), 256 (p, 17%), 318 (p, 28%), 414 (p, 100%)
15	4.11	26648		204 (p, 33%), 220 (s, 25%), 255 (p, 17%), 311 (s, 34%), 319 (p, 38%), 415 (p, 100%)
16	4.17	7459		318 (p, 47%), 413 (p, 100%), 430 (s, 88%)
18	4.94	15928		235 (p, 100%)
19	5.13	19676		218 (p, 100%), 269 (s, 31%)




4.17 min
Absorbance (%)
100
75
50
25
0
200 250 300 350 400 450 500 550
Wavelength (nm)
4.94 min
Absorbance (%)
100
75
50
25
0
200 250 300 350 400 450 500 550
Wavelength (nm)
5.13 min
Absorbance (%)
100
75
50
25
0
200 250 300 350 400 450 500 550
Wavelength (nm)


2.14 min
Absorbance (%)
100
75
50
25
0
200 250 300 350 400 450 500 550
Wavelength (nm)


2.47 min
Absorbance (%)
100
75
50
25
0
200 250 300 350 400 450 500 550
Wavelength (nm)


3.27 min
Absorbance (%)
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50
25
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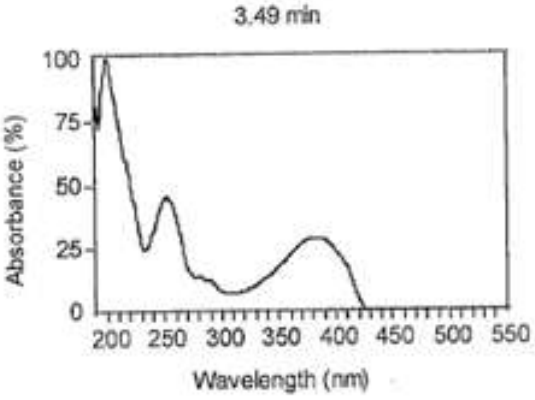
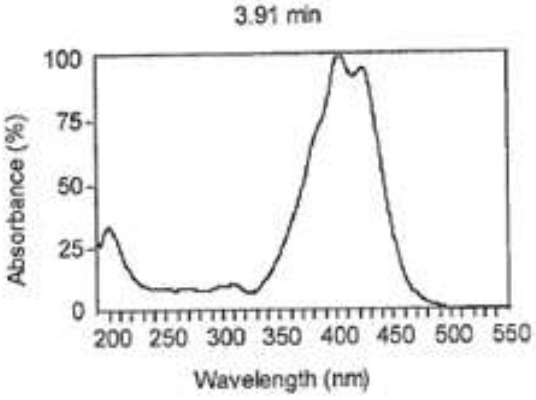
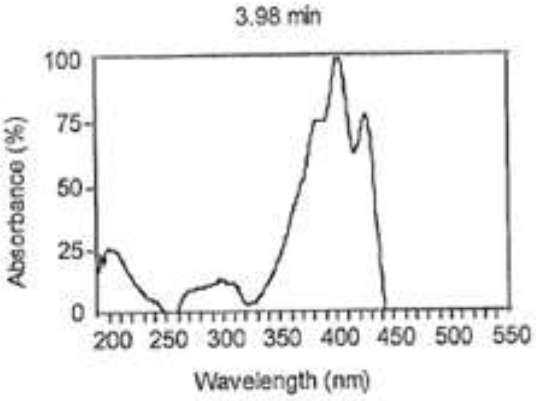
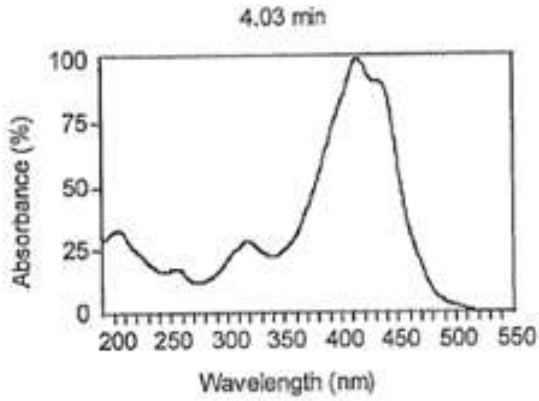
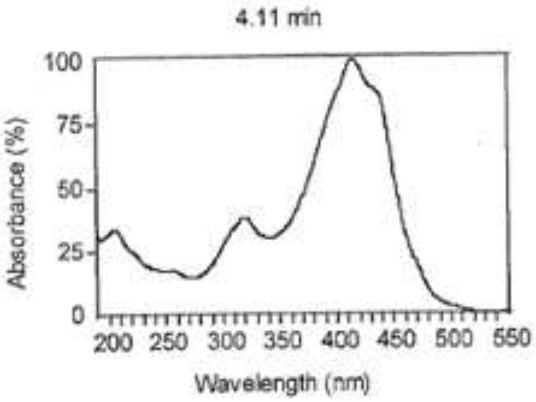
200 250 300 350 400 450 500 550
Wavelength (nm)


3.49 min
Absorbance (%)
100
75
50
25
0
200 250 300 350 400 450 500 550
Wavelength (nm)


3.91 min
Absorbance (%)
100
75
50
25
0
200 250 300 350 400 450 500 550
Wavelength (nm)


3.98 min
Absorbance (%)
100
75
50
25
0
200 250 300 350 400 450 500 550
Wavelength (nm)


4.03 min
Absorbance (%)
100
75
50
25
0
200 250 300 350 400 450 500 550
Wavelength (nm)

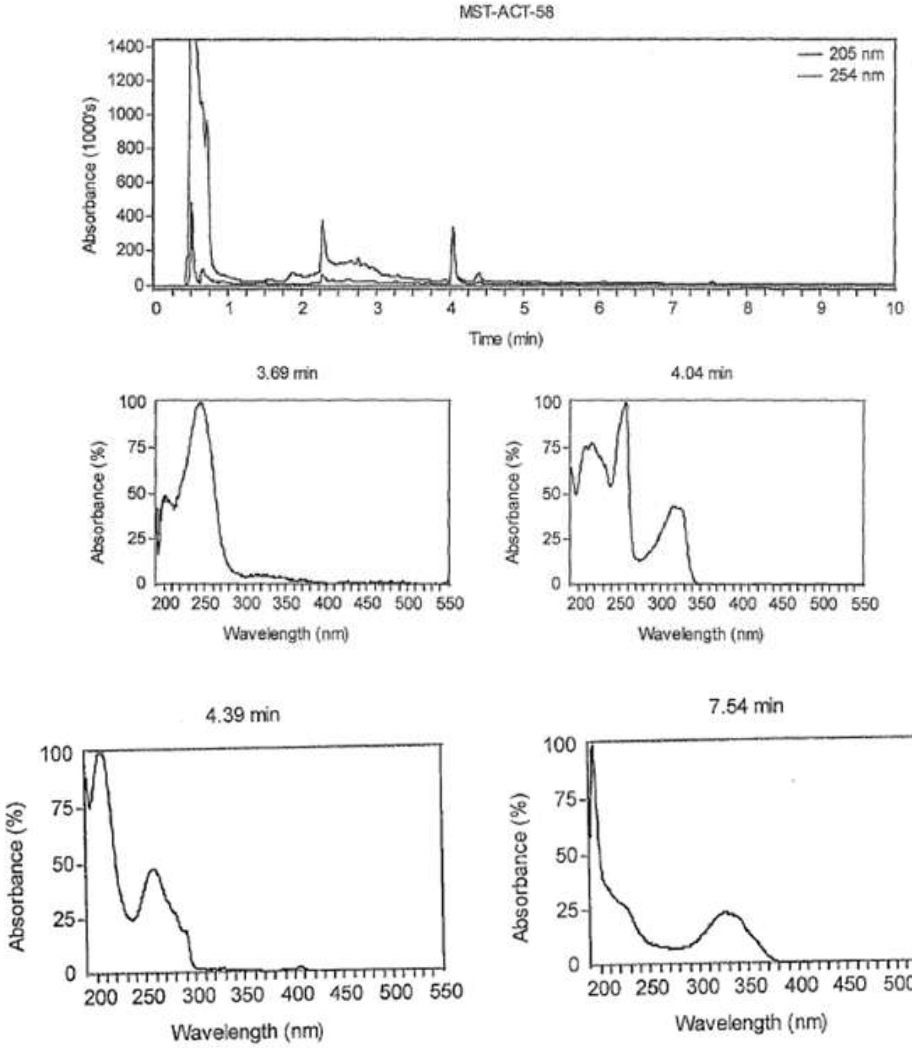

4.11 min
Absorbance (%)
100
75
50
25
0
200 250 300 350 400 450 500 550
Wavelength (nm)

Comet analysis of ACT-58 HPLC Trace under TFA conditions.

Production	Med
Total Peaks (254 nm)	13
Non-Polar Peaks (254 nm)	10
Non-Polar Peaks (205 nm)	10
Resolved UV Spectra	4
No. of UV Classes	0

Resolved UV Spectra of ACT-58 HPLC Trace under TFA conditions.

Peak	RT	Height	Class	UV Description
10	3.69	8383		unresolved
11	4.04	330525		217 (p, 78%), 257 (p, 100%), 317 (p, 43%), 324 (s, 42%)
12	4.39	21022		206 (p, 100%), 259 (p, 47%), 278 (s, 28%)
13	7.54	5861		194 (p, 100%), 326 (p, 23%)

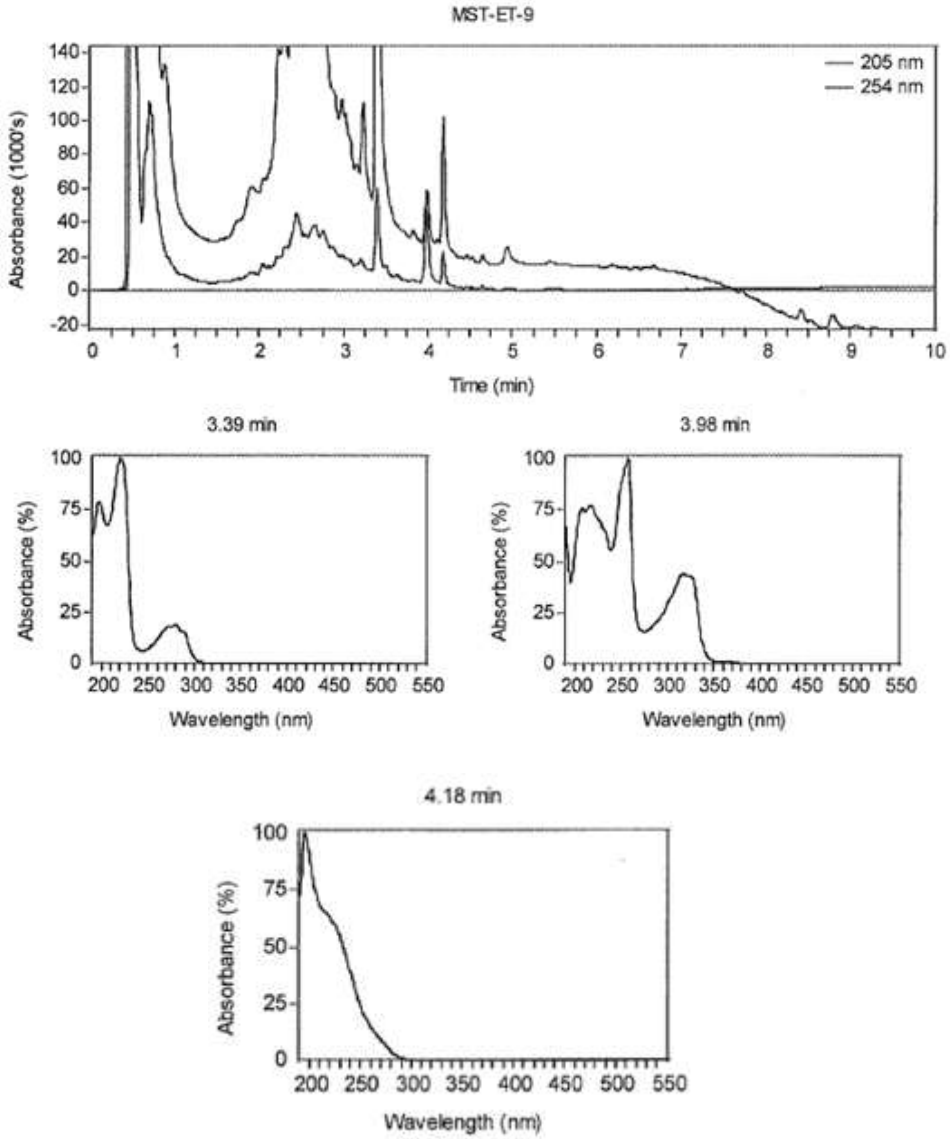


Comet analysis of ET-9 HPLC Trace under TFA conditions.

Production	Low
Total Peaks (254 nm)	8
Non-Polar Peaks (254 nm)	6
Non-Polar Peaks (205 nm)	9
Resolved UV Spectra	3
No. of UV Classes	0

Resolved UV Spectra of ET-9 HPLC Trace under TFA conditions.

Peak	RT	Height	Class	UV Description
6	3.39	47820		197 (p, 79%), 221 (p, 100%), 280 (p, 19%)
7	3.98	41463		207 (p, 76%), 257 (p, 100%), 317 (p, 44%)
8	4.18	19203		195 (p, 100%), 216 (s, 65%)

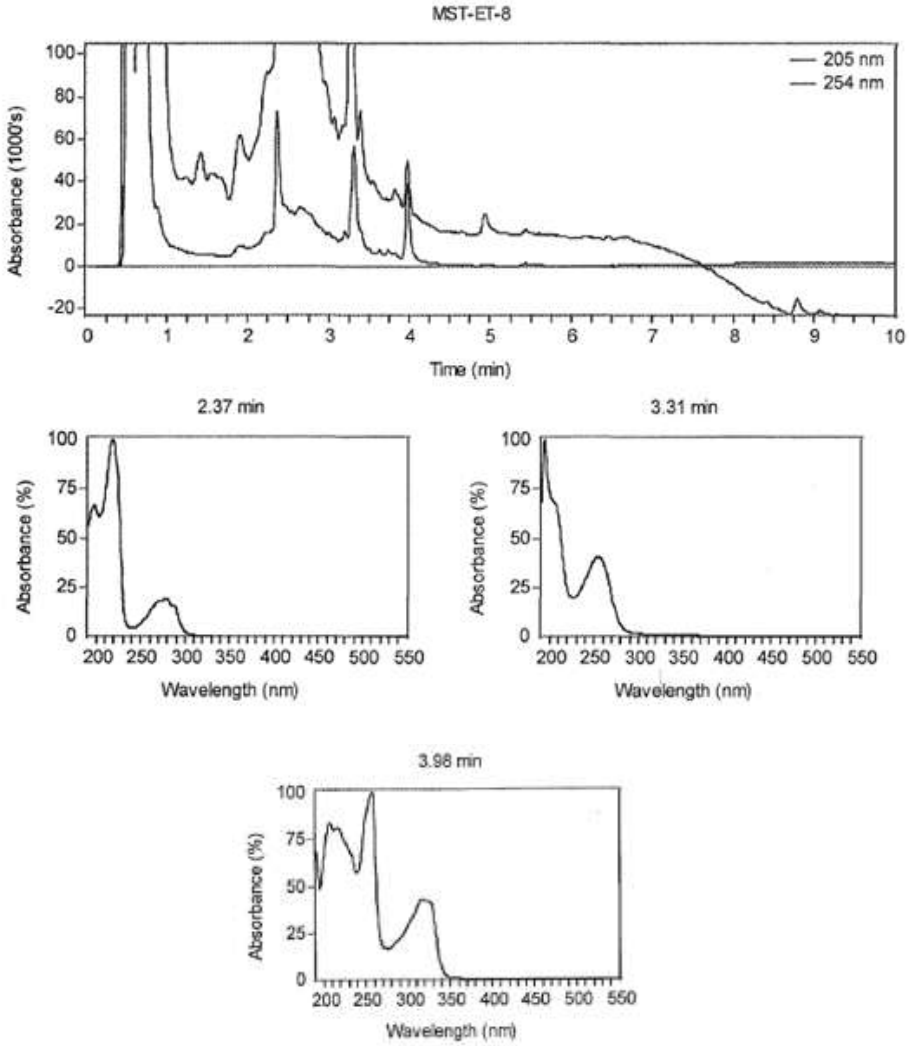


Comet analysis of ET-8 HPLC Trace under TFA conditions.

Production	Low
Total Peaks (254 nm)	6
Non-Polar Peaks (254 nm)	3
Non-Polar Peaks (205 nm)	7
Resolved UV Spectra	3
No. of UV Classes	0

Resolved UV Spectra of ET-8 HPLC Trace under TFA conditions.

Peak	RT	Height	Class	UV Description
4	2.37	50500		197 (p, 67%), 219 (p, 100%), 279 (p, 19%)
5	3.31	45161		194 (p, 100%), 254 (p, 40%)
6	3.98	35322		207 (p, 83%), 257 (p, 100%), 317 (p, 43%), 324 (s, 42%)



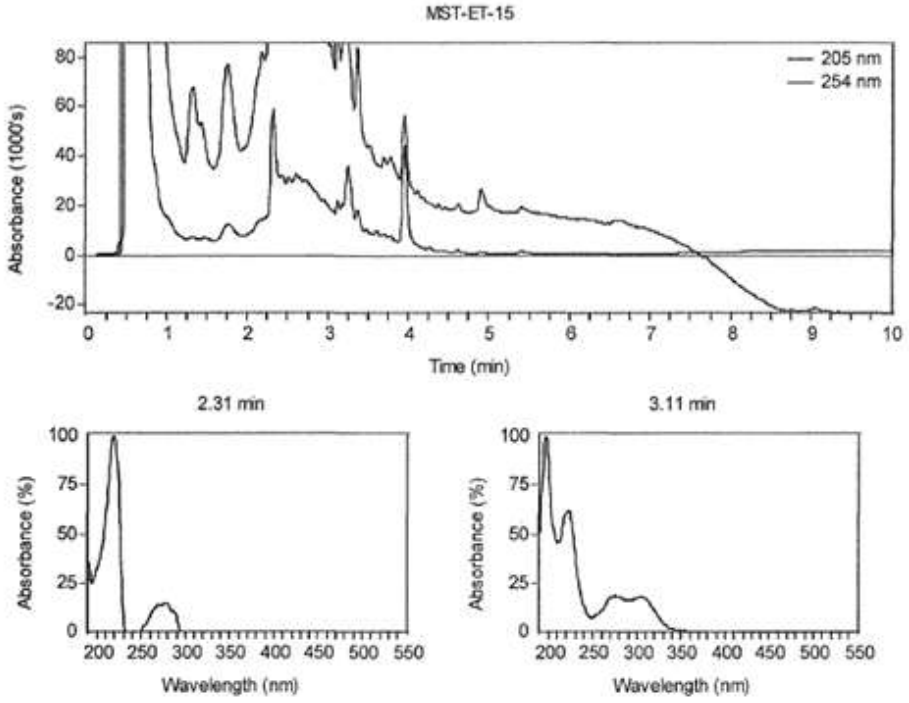
COMET Analysis of ET-15

Comet analysis of ET-15 HPLC Trace under TFA conditions.

Production	Low
Total Peaks (254 nm)	8
Non-Polar Peaks (254 nm)	6
Non-Polar Peaks (205 nm)	8
Resolved UV Spectra	5
No. of UV Classes	0

Resolved UV Spectra of ET-15 HPLC Trace under TFA conditions.

Peak	RT	Height	Class	UV Description
4	2.31	31468		219 (p, 100%), 278 (p, 15%)
5	3.11	4093		197 (p, 100%), 222 (p, 61%)
6	3.25	18233		194 (p, 100%), 205 (s, 67%)
7	3.37	5157		196 (p, 100%), 221 (p, 89%), 268 (p, 23%)
8	3.95	39462		207 (p, 86%), 218 (p, 86%), 232 (s, 70%), 257 (p, 100%), 317 (p, 44%)

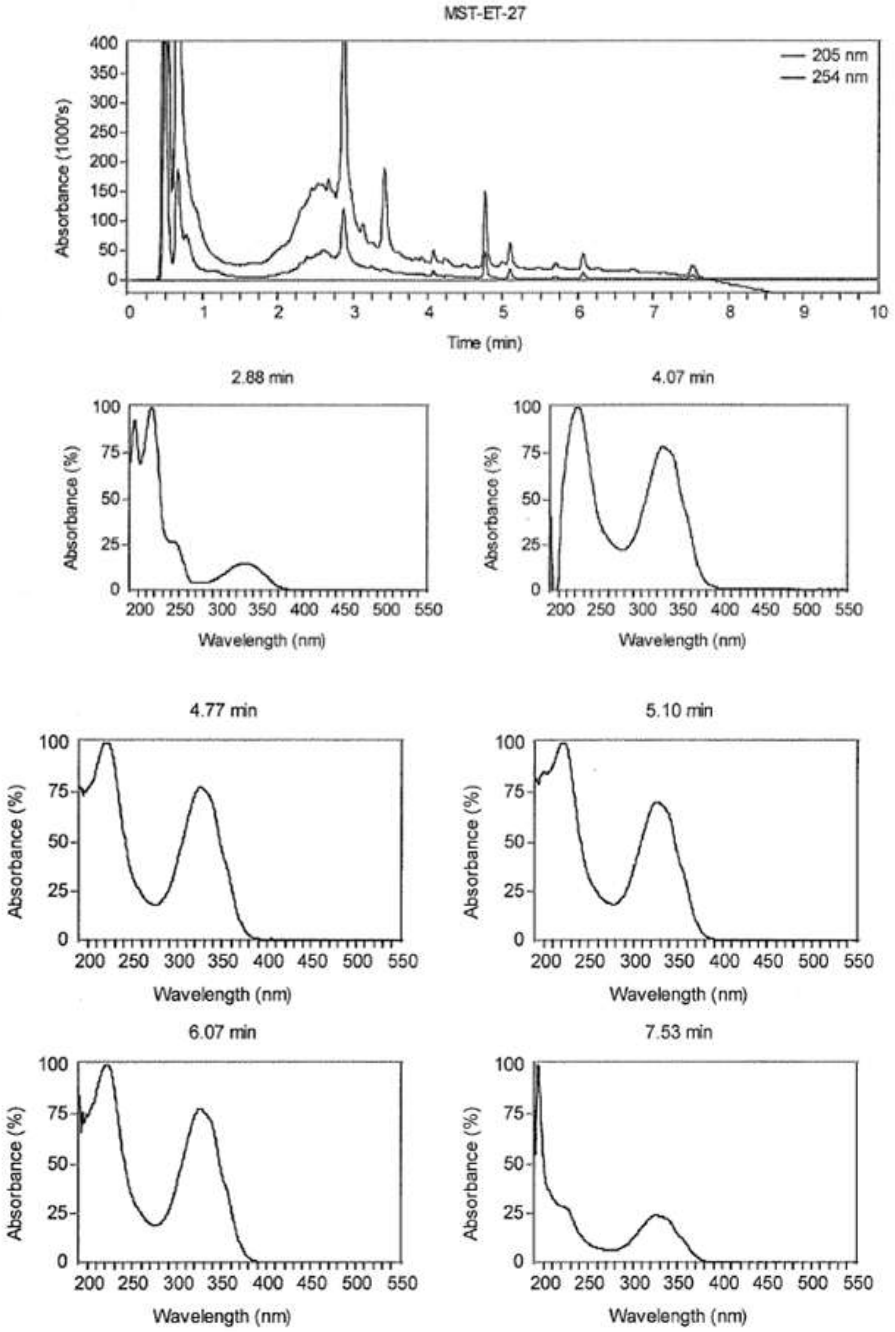


Comet analysis of ET-27 HPLC Trace under TFA conditions.

Production	Low
Total Peaks (254 nm)	12
Non-Polar Peaks (254 nm)	9
Non-Polar Peaks (205 nm)	9
Resolved UV Spectra	6
No. of UV Classes	0

Resolved UV Spectra of ET-27 HPLC Trace under TFA conditions.

Peak	RT	Height	Class	UV Description
6	2.88	88066		196 (p, 93%), 217 (p, 100%), 330 (p, 15%)
8	4.07	7501		224 (p, 100%), 326 (p, 78%)
9	4.77	43496		221 (p, 100%), 325 (p, 77%), 353 (s, 40%)
10	5.10	13877		199 (p, 86%), 221 (p, 100%), 326 (p, 70%), 354 (s, 36%)
11	6.07	9661		222 (p, 100%), 326 (p, 78%)
12	7.53	5833		326 (p, 24%)

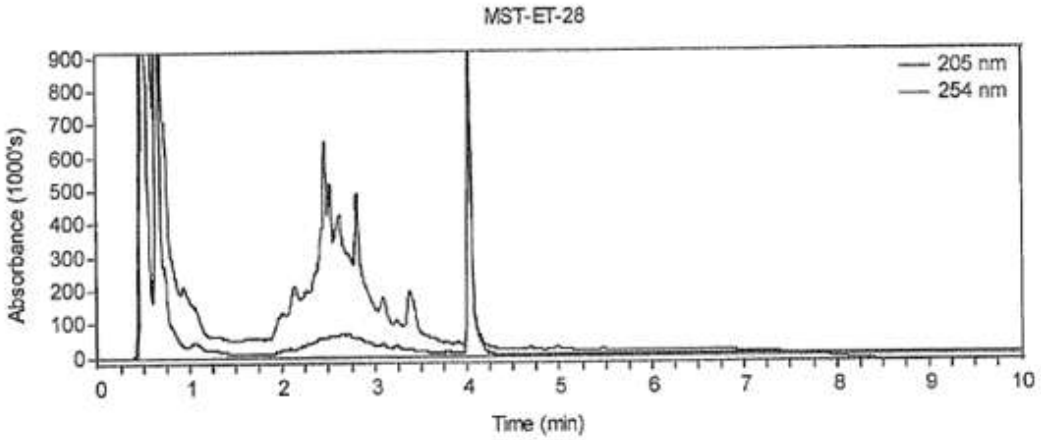


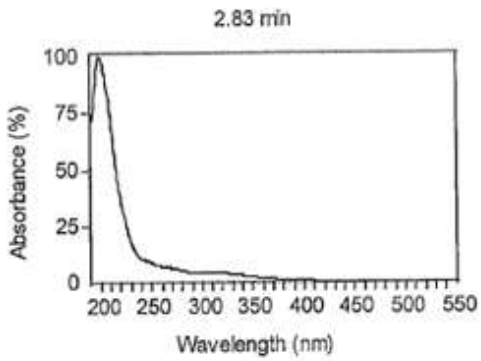
Comet analysis of ET-28 HPLC Trace under TFA conditions.

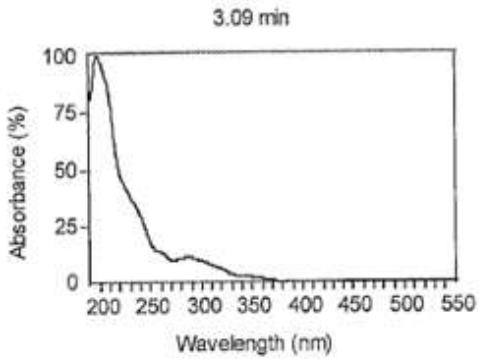
Production	Med
Total Peaks (254 nm)	12
Non-Polar Peaks (254 nm)	10
Non-Polar Peaks (205 nm)	10
Resolved UV Spectra	3
No. of UV Classes	0

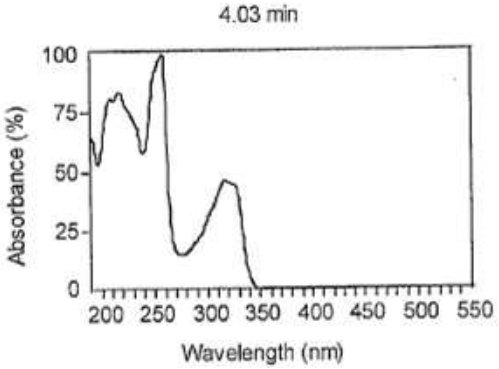
Resolved UV Spectra of ET-28 HPLC Trace under TFA conditions.

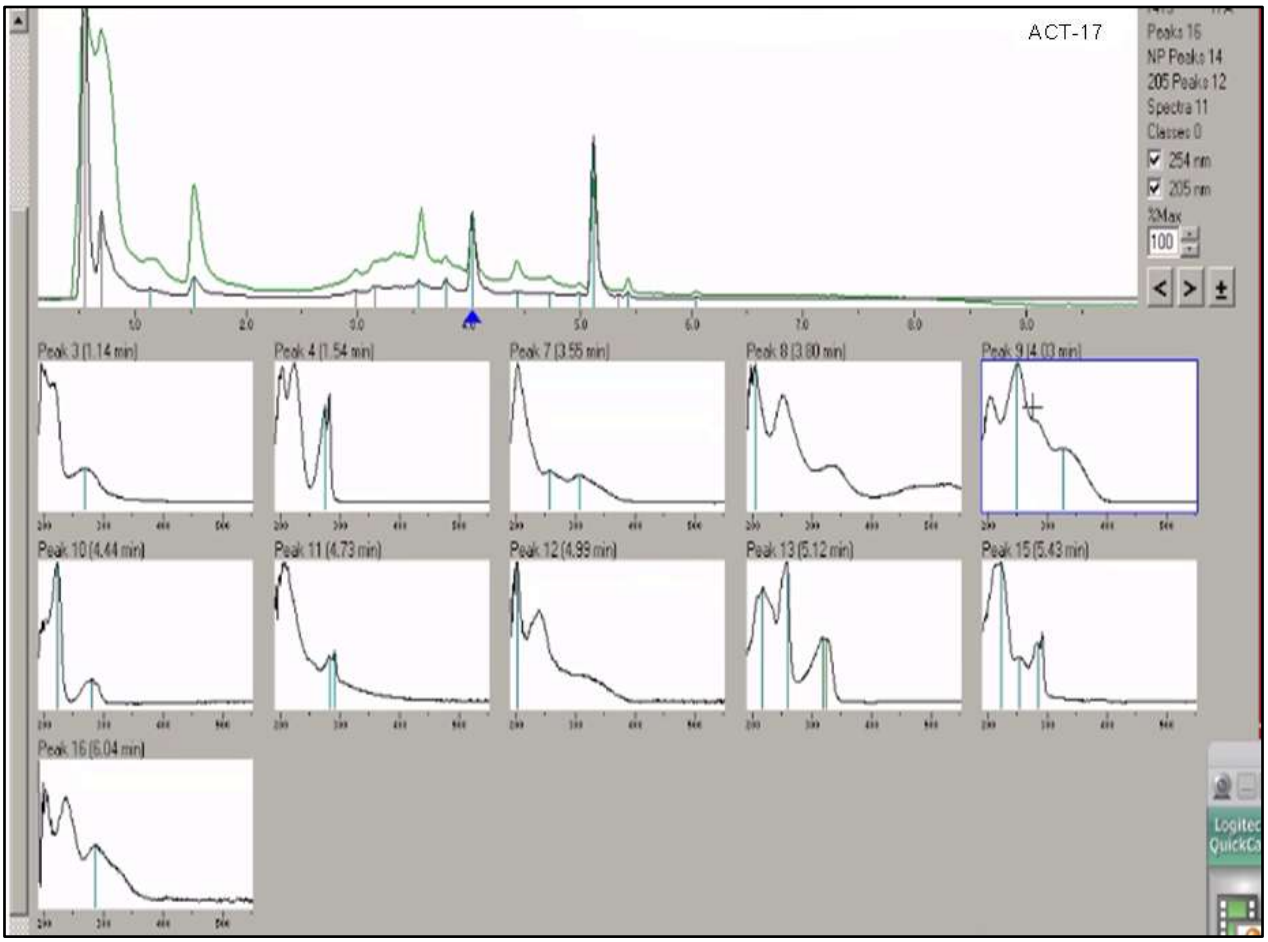
Peak	RT	Height	Class	UV Description
8	2.83	8045		199 (p, 100%), 266 (s, 7%)
9	3.09	6802		197 (p, 100%), 288 (p, 11%)
12	4.03	959909		217 (p, 83%), 257 (p, 100%), 303 (s, 33%), 317 (p, 46%), 324 (s, 45%)

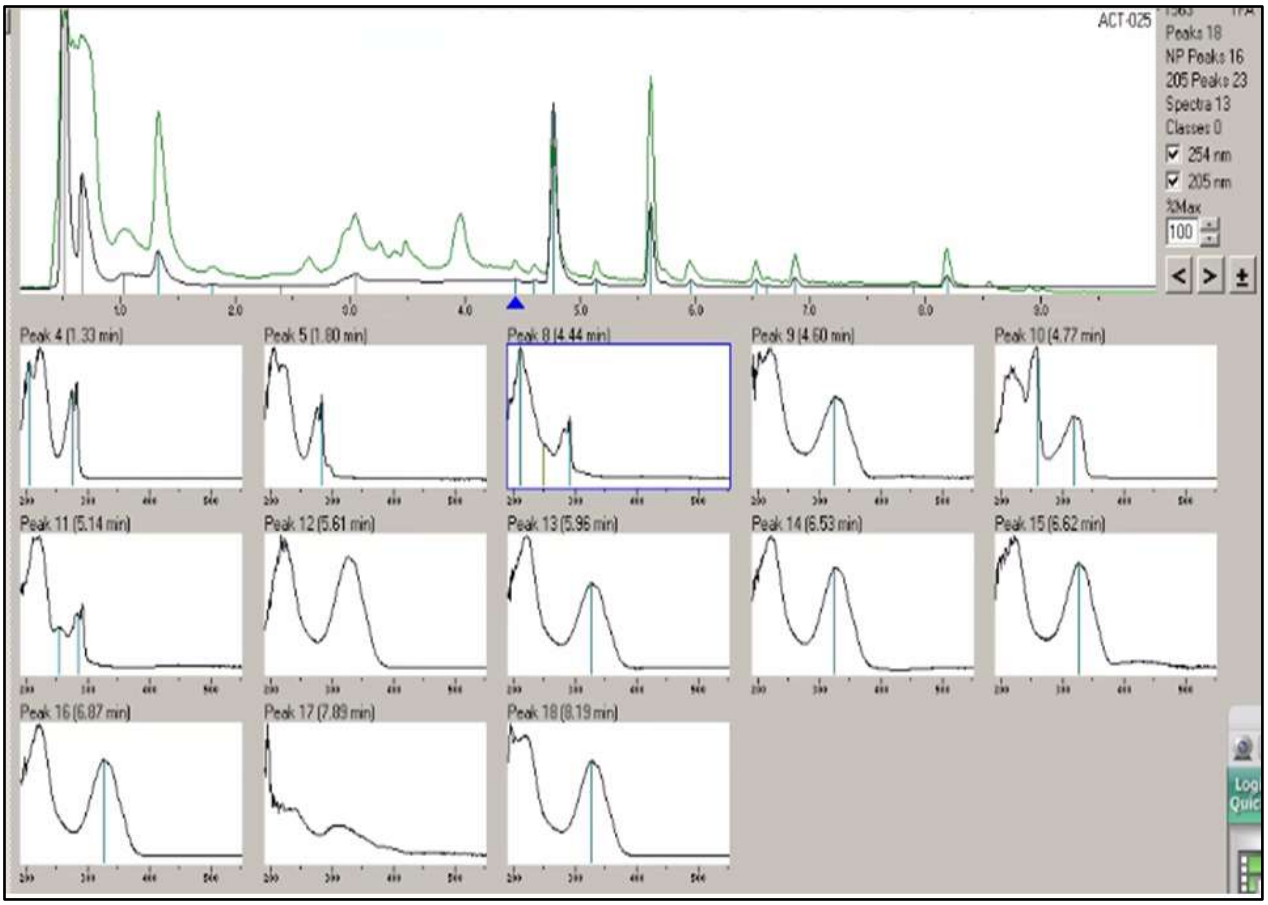


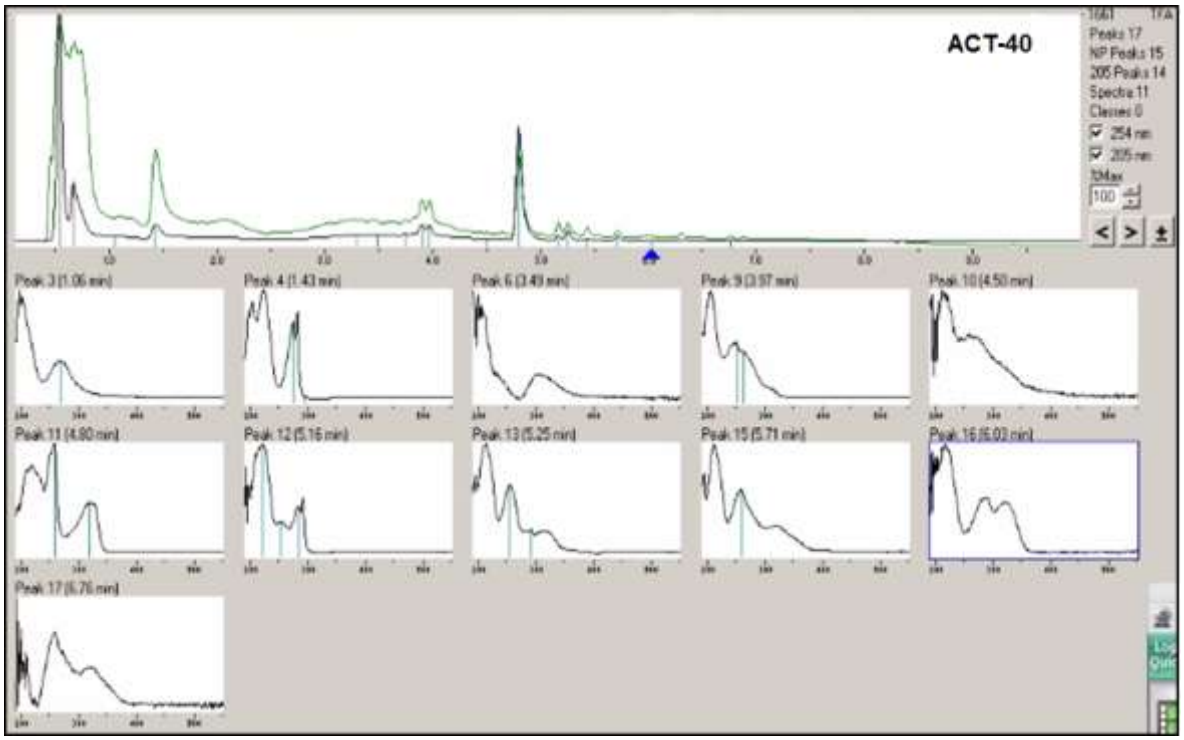


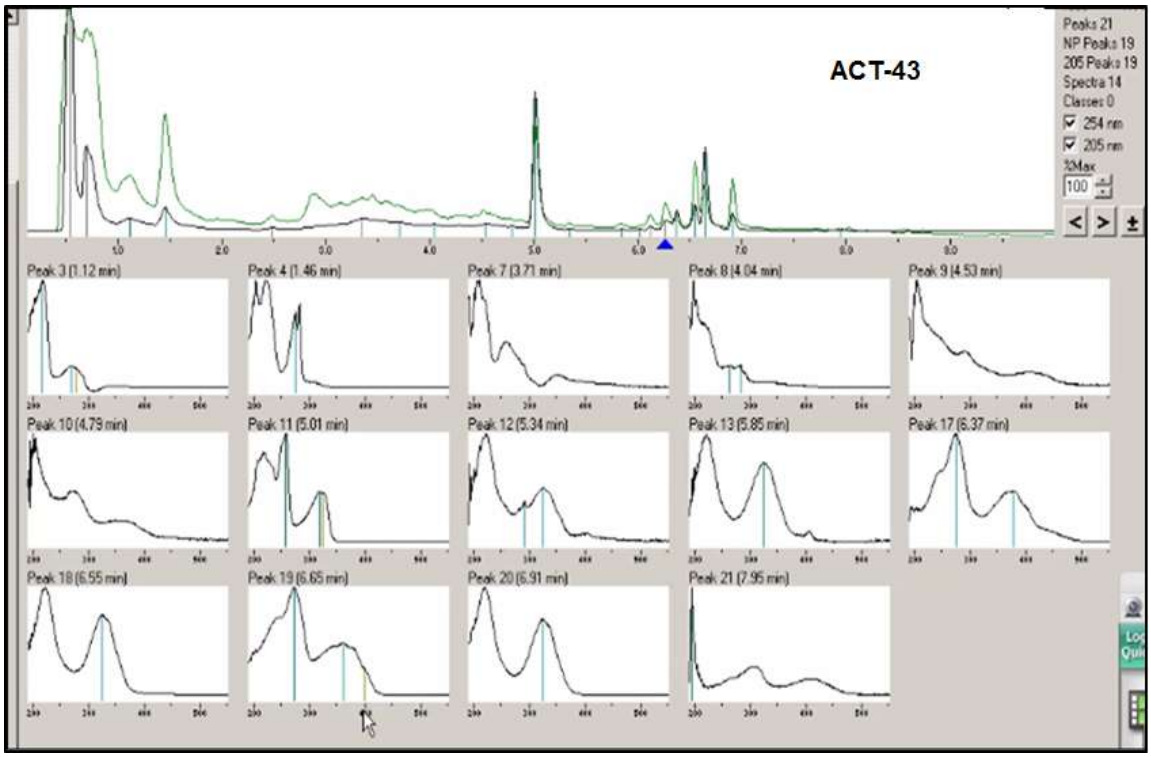


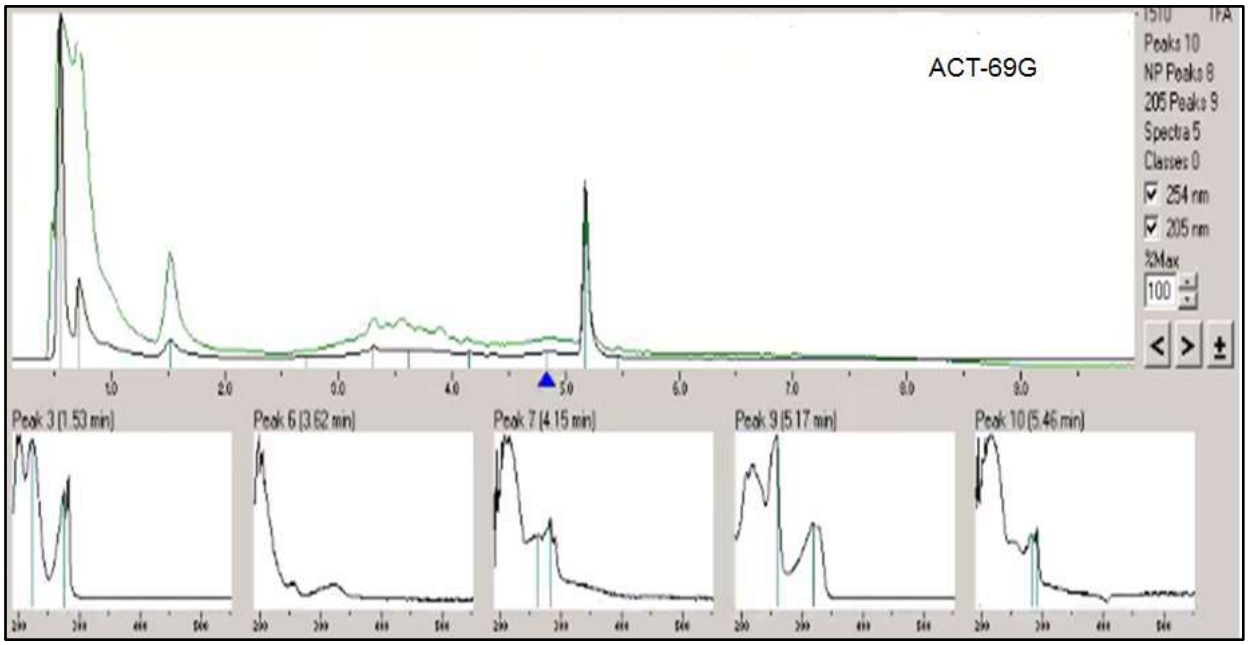


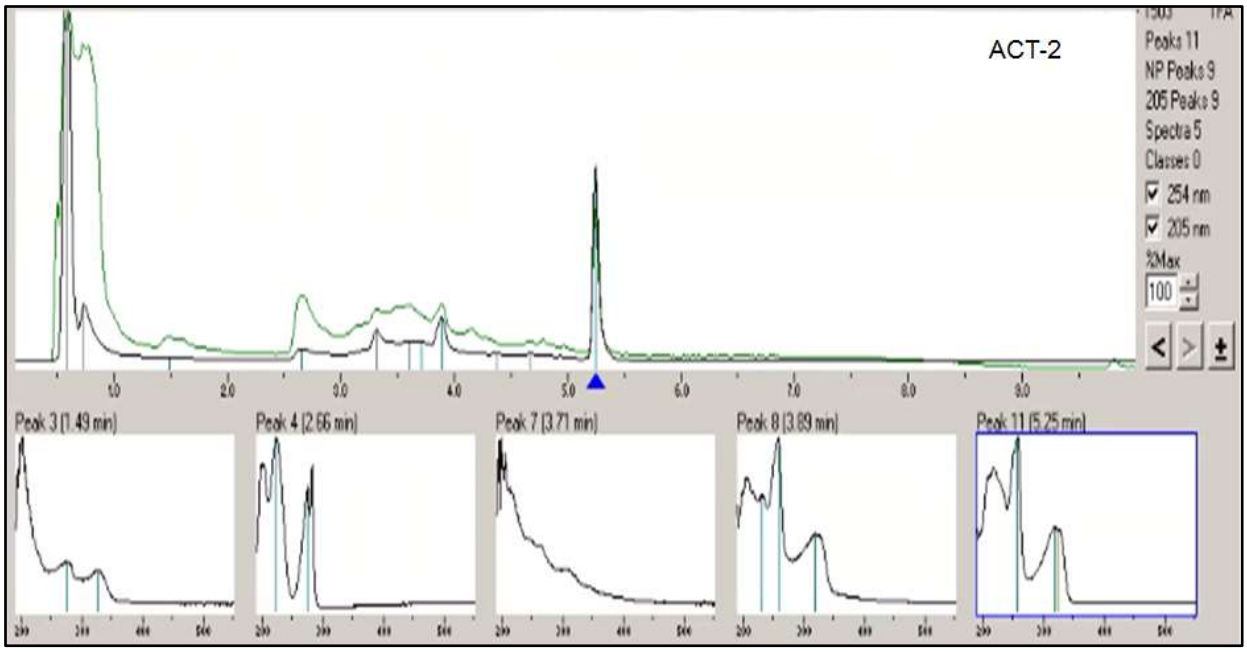

ACT-17
Peaks 16
NP Peaks 14
205 Peaks 12
Spectra 11
Classes 0
254 nm
205 nm
%Max
100
Peak 3 (1.14 min)
Peak 4 (1.54 min)
Peak 7 (3.55 min)
Peak 8 (3.80 min)
Peak 9 (4.03 min)
Peak 10 (4.44 min)
Peak 11 (4.73 min)
Peak 12 (4.99 min)
Peak 13 (5.12 min)
Peak 15 (5.43 min)
Peak 16 (6.04 min)

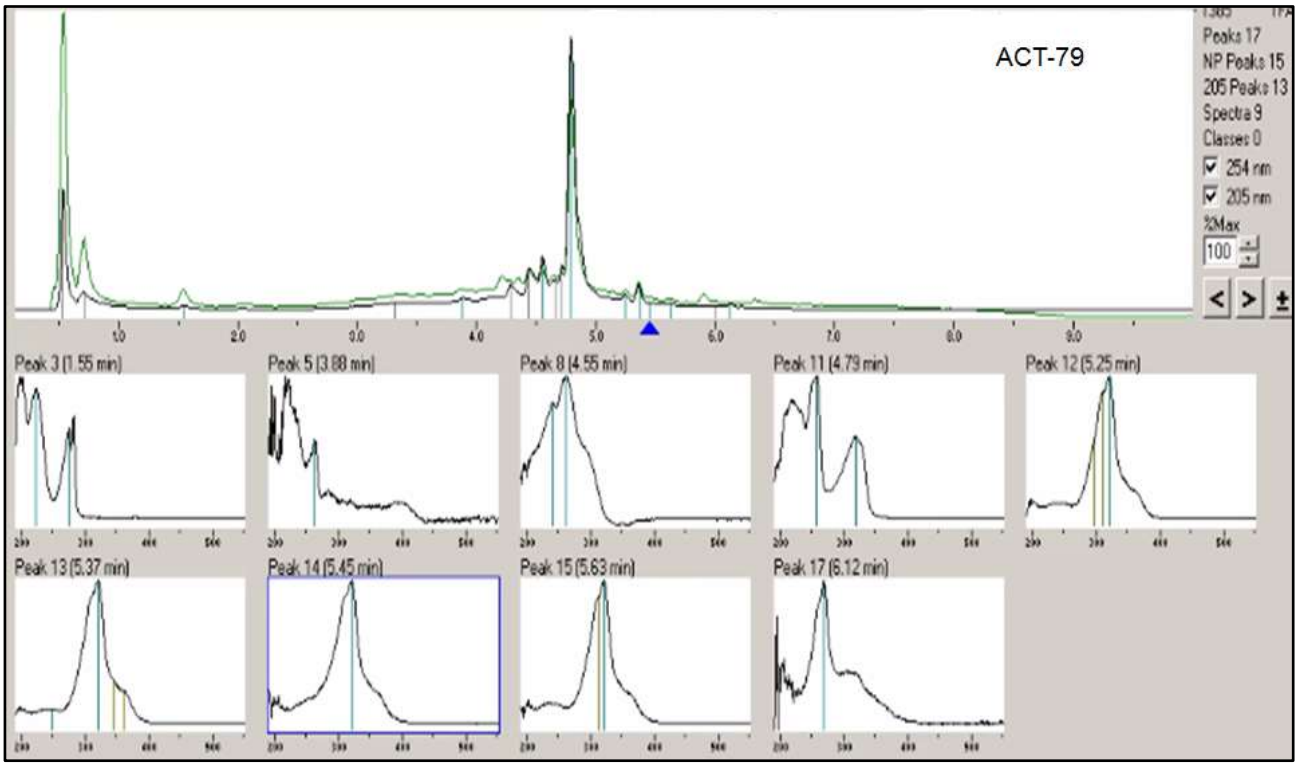

ACT-025
Peaks 18
NP Peaks 16
205 Peaks 23
Spectra 13
Classes 0
254 nm
205 nm
%Max
100
Peak 4 (1.33 min)
Peak 5 (1.80 min)
Peak 8 (4.44 min)
Peak 9 (4.60 min)
Peak 10 (4.77 min)
Peak 11 (5.14 min)
Peak 12 (5.61 min)
Peak 13 (5.96 min)
Peak 14 (6.53 min)
Peak 15 (6.62 min)
Peak 16 (6.87 min)
Peak 17 (7.89 min)
Peak 18 (8.19 min)

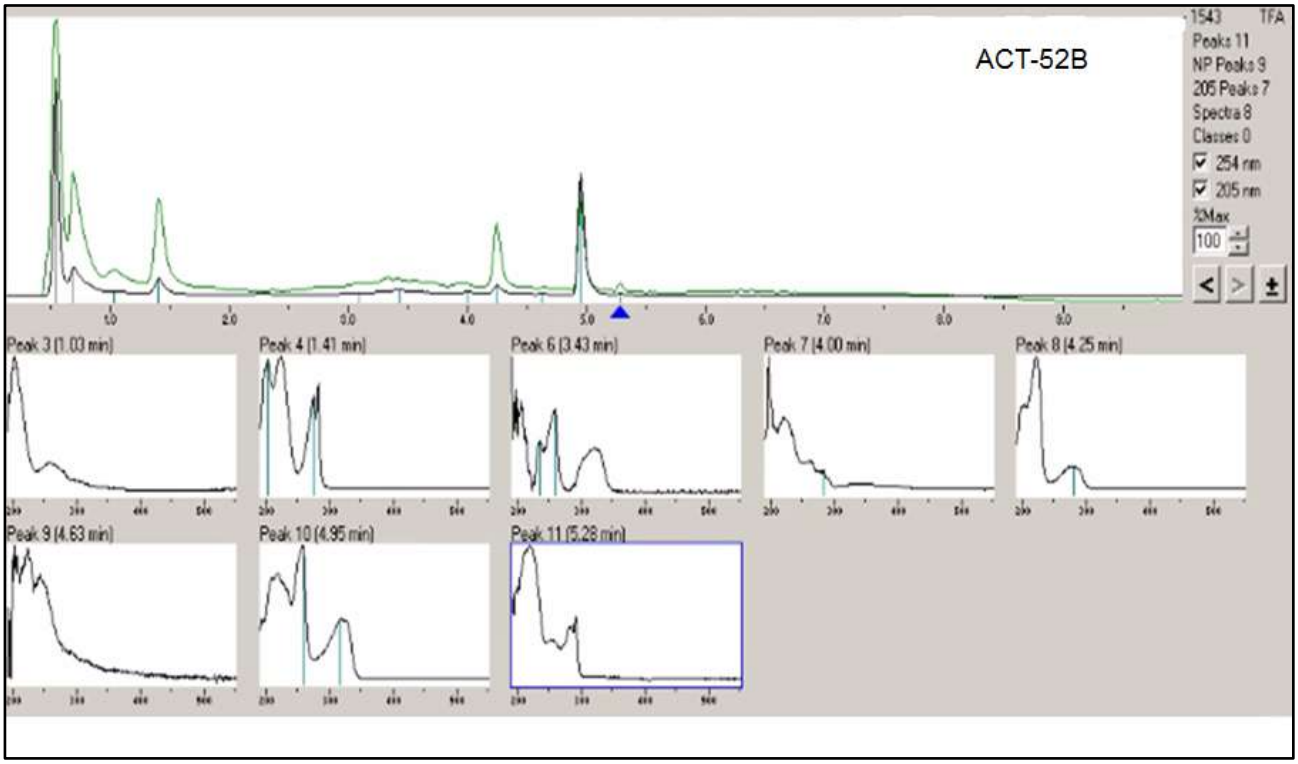

ACT-40
Peaks 17
NP Peaks 15
205 Peaks 14
Spectra 11
Classes 0
254 nm
%Max
100
Peak 3 (1.06 min)
Peak 4 (1.43 min)
Peak 6 (3.49 min)
Peak 9 (3.97 min)
Peak 10 (4.50 min)
Peak 11 (4.80 min)
Peak 12 (5.16 min)
Peak 13 (5.25 min)
Peak 15 (5.71 min)
Peak 16 (6.02 min)
Peak 17 (6.76 min)

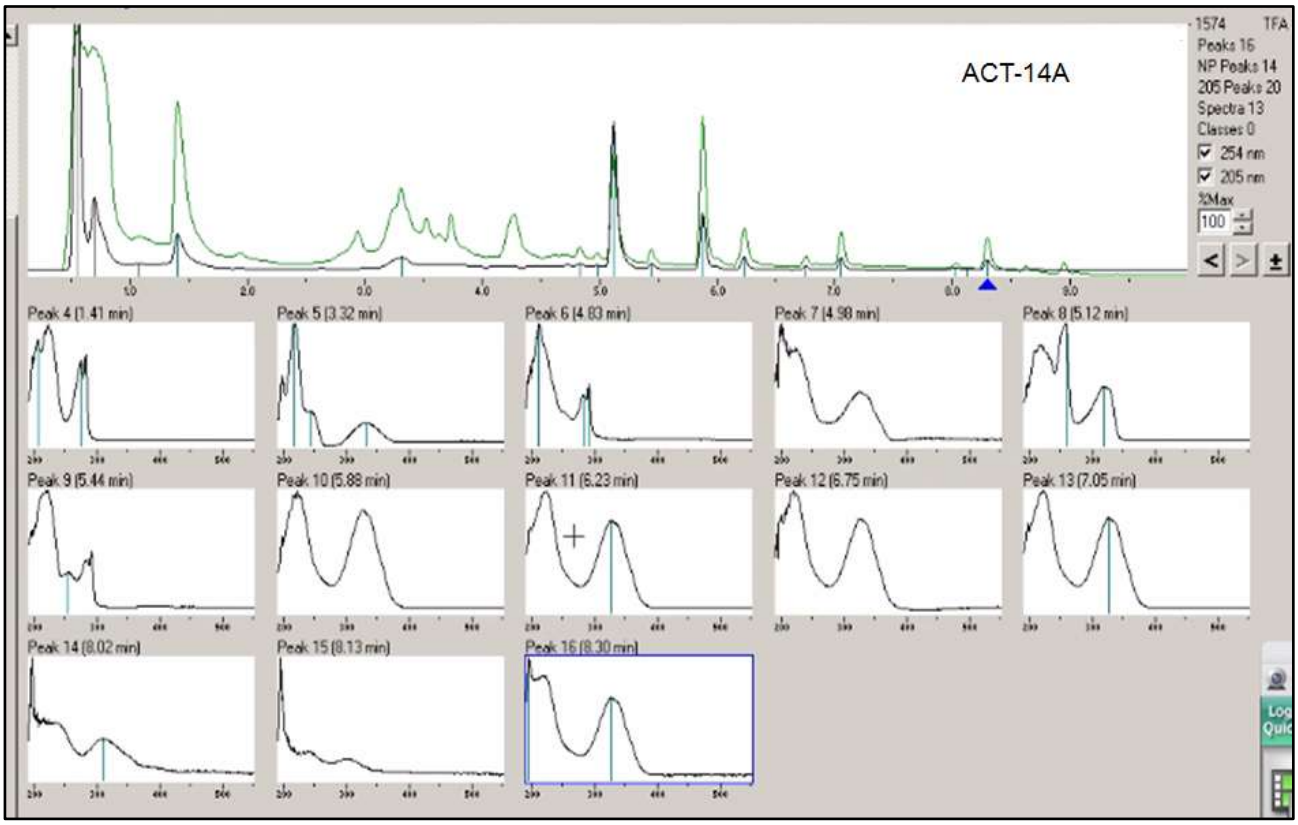

ACT-43
Peaks 21
NP Peaks 19
205 Peaks 19
Spectra 14
Classes 0
254 nm
205 nm
%Max
100
Peak 3 (1.12 min)
Peak 4 (1.46 min)
Peak 7 (3.71 min)
Peak 8 (4.04 min)
Peak 9 (4.53 min)
Peak 10 (4.79 min)
Peak 11 (5.01 min)
Peak 12 (5.34 min)
Peak 13 (5.85 min)
Peak 17 (6.37 min)
Peak 18 (6.55 min)
Peak 19 (6.65 min)
Peak 20 (6.91 min)
Peak 21 (7.95 min)

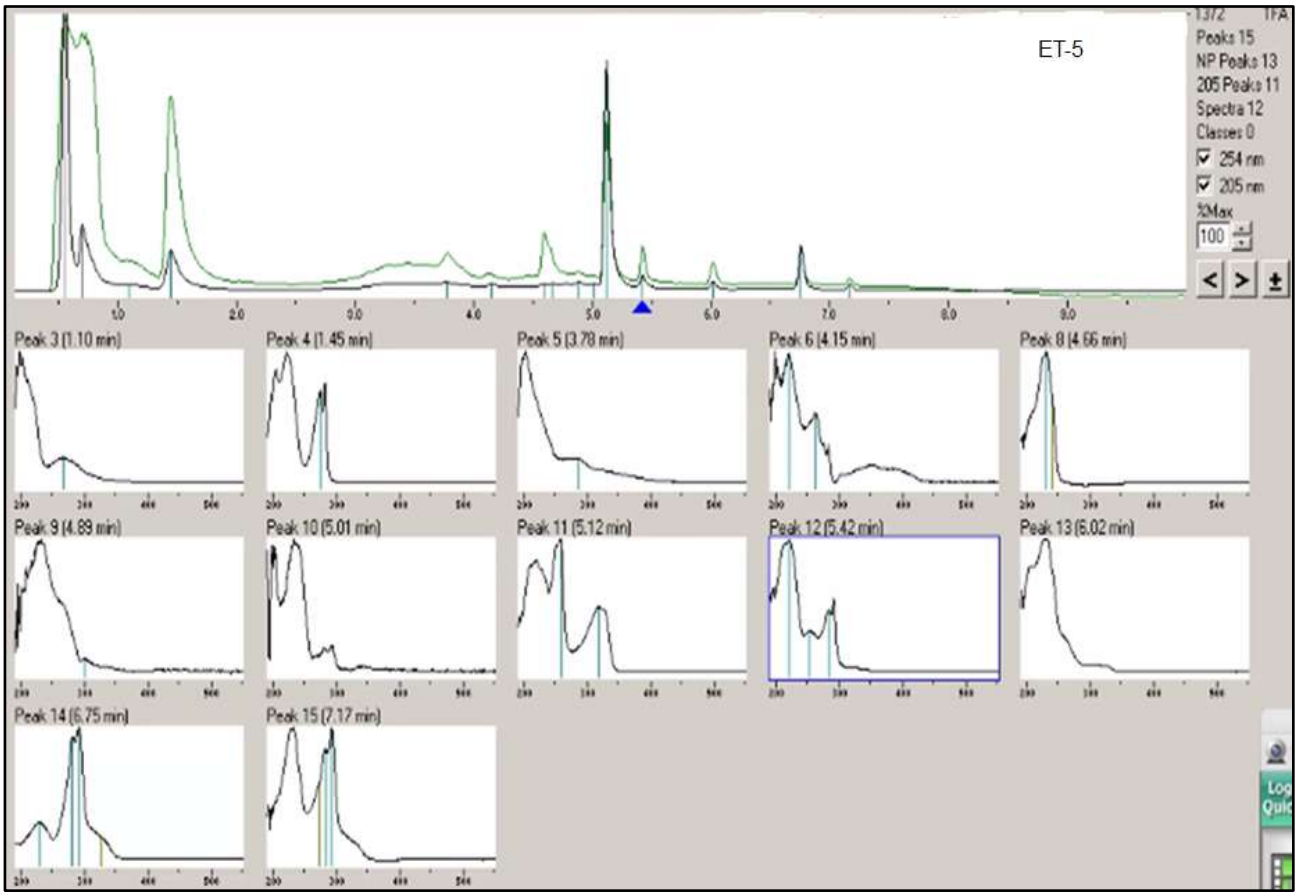

ACT-69G
Peaks 10
NP Peaks 8
205 Peaks 9
Spectra 5
Classes 0
254 nm
205 nm
%Max
100
Peak 3 (1.53 min)
Peak 6 (3.62 min)
Peak 7 (4.15 min)
Peak 9 (5.17 min)
Peak 10 (5.46 min)

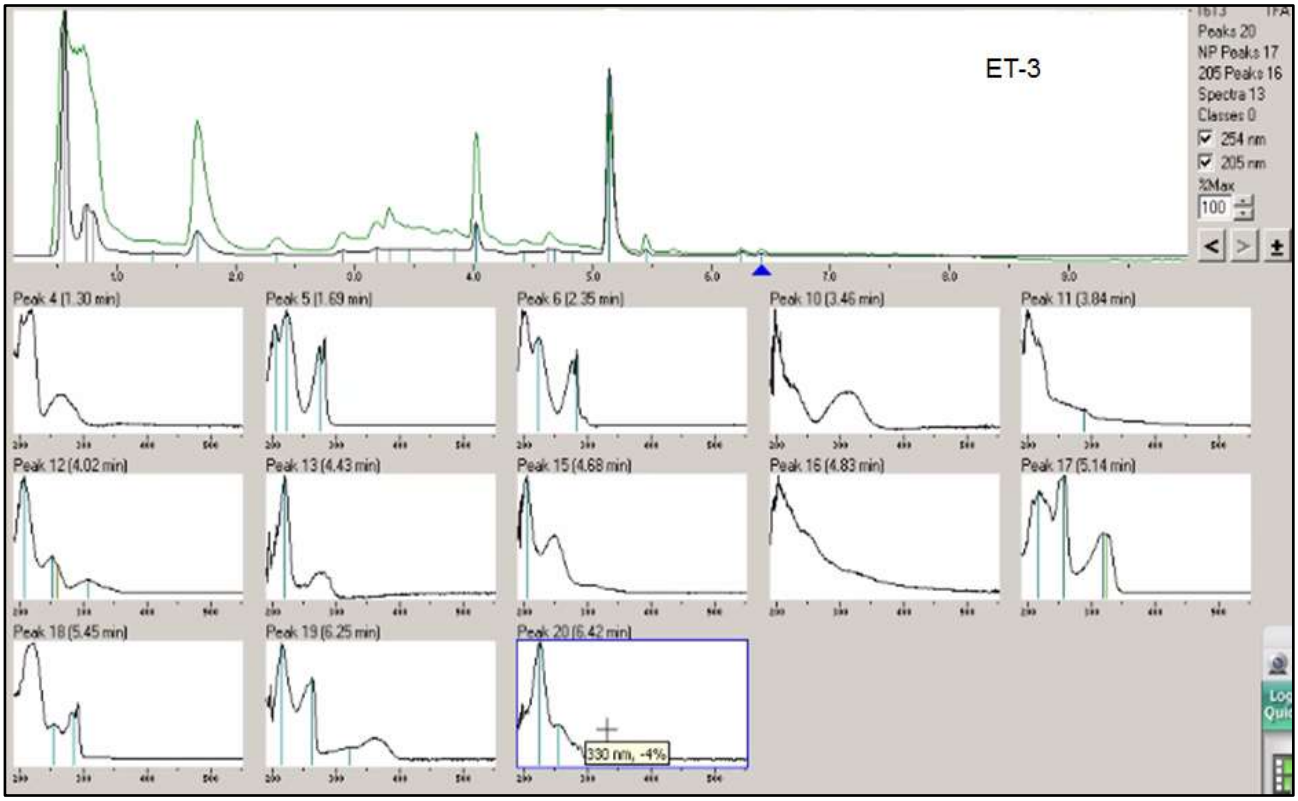

ACT-2
Peaks 11
NP Peaks 9
205 Peaks 9
Spectra 5
Classes 0
254 nm
205 nm
%Max
100
Peak 3 (1.49 min)
Peak 4 (2.66 min)
Peak 7 (3.71 min)
Peak 8 (3.89 min)
Peak 11 (5.25 min)


ACT-79
Peaks 17
NP Peaks 15
205 Peaks 13
Spectra 9
Classes 0
254 nm
205 nm
%Max
100
Peak 3 (1.55 min)
Peak 5 (3.88 min)
Peak 8 (4.55 min)
Peak 11 (4.79 min)
Peak 12 (5.25 min)
Peak 13 (5.37 min)
Peak 14 (5.45 min)
Peak 15 (5.63 min)
Peak 17 (6.12 min)


ACT-52B
1543
TFA
Peaks 11
NP Peaks 9
205 Peaks 7
Spectra 8
Classes 0
254 nm
205 nm
%Max
100
Peak 3 (1.03 min)
Peak 4 (1.41 min)
Peak 6 (3.43 min)
Peak 7 (4.00 min)
Peak 8 (4.25 min)
Peak 9 (4.63 min)
Peak 10 (4.95 min)
Peak 11 (5.28 min)


ACT-14A
1574 TFA
Peaks 16
NP Peaks 14
205 Peaks 20
Spectra 13
Classes 0
254 nm
205 nm
%Max
100
Peak 4 (1.41 min)
Peak 5 (3.32 min)
Peak 6 (4.83 min)
Peak 7 (4.98 min)
Peak 8 (5.12 min)
Peak 9 (5.44 min)
Peak 10 (5.88 min)
Peak 11 (6.23 min)
Peak 12 (6.75 min)
Peak 13 (7.05 min)
Peak 14 (8.02 min)
Peak 15 (8.13 min)
Peak 16 (8.30 min)


ET-5
Peaks 15
NP Peaks 13
205 Peaks 11
Spectra 12
Classes 0
254 nm
205 nm
%Max
100
Peak 3 (1.10 min)
Peak 4 (1.45 min)
Peak 5 (3.78 min)
Peak 6 (4.15 min)
Peak 8 (4.66 min)
Peak 9 (4.89 min)
Peak 10 (5.01 min)
Peak 11 (5.12 min)
Peak 12 (5.42 min)
Peak 13 (6.02 min)
Peak 14 (6.75 min)
Peak 15 (7.17 min)


ET-3
Peaks 20
NP Peaks 17
205 Peaks 16
Spectra 13
Classes 0
254 nm
205 nm
%Max
100
Peak 4 (1.30 min)
Peak 5 (1.69 min)
Peak 6 (2.35 min)
Peak 10 (3.46 min)
Peak 11 (3.84 min)
Peak 12 (4.02 min)
Peak 13 (4.43 min)
Peak 15 (4.68 min)
Peak 16 (4.83 min)
Peak 17 (5.14 min)
Peak 18 (5.45 min)
Peak 19 (6.25 min)
Peak 20 (6.42 min)
330 nm, -4%