Quorum Sensing in Australian Soft Corals

Thesis submitted by

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BSc Chem (Hons.) - Marine Biol., BA – Japanese

For the Degree of Doctor of Philosophy

in the School of Pharmacy and Molecular Sciences,

James Cook University

19th January 2015

Publications arising from this thesis

Objective	Title	Intended Journal	Status
1, 4	Evidence of quorum sensing in Australian soft corals	ISME Journal	In draft
2	The influence of lactone ring size on quorum sensing in *Agrobacterium tumefaciens* and *Chromobacterium violaceum*	Marine Drugs	In draft
3	Quorum sensing without a lactone: A novel sterol from *Nephthea* sp.	Chemistry and Biology	In draft

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Declaration

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institute of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

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Acknowledgments

This thesis, like so many others, would not exist in its present form without the help, support and guidance of many. The following is an incomplete list of those that helped me along this path and to whom I will always be grateful.

First and foremost I must thank my supervisors, A/Prof Bruce Bowden, Dr Lone Hoj and Prof. Rocky de Nys, for taking me on and believing in me. Bruce, thank you for introducing me into the world of chemistry. I would never have had the confidence to take that leap without your guidance and support. The trips to Orpheus Island have been amongst the most rewarding times of my life. Lone, you stepped in and took me on when circumstances had left me stranded, I will be forever grateful for your patience and understanding. Rocky, every time I needed your advice you were always there.

My greatest appreciation for Dr Cherie Motti and Dr Dianne Tapiolas at the Australian Institute of Marine Science chemistry lab, supervisors in all but name. The mentoring I received from you in the lab has been invaluable. Peter Thomas-Hall, thanks for keeping me looking forward. You have always been there with a helpful hand in the lab, a ready cup of tea and a chat. To the members of the 'Futures Project' team, thank you for the opportunity to work alongside you, it has been an honour. To Prof Linda Blackall, this project started with you and despite that you were not able to be here at the end I will be forever grateful to the start you gave me and the lessons I learned from you. Dr Tilmann Harder, thank you for inviting me to Sydney and the Centre of Marine Biofouling and Bio-Innovation, everything I know of quorum sensing stems from there. Dr David Abrego, thank you for everything.

The community of scientists at AIMS is an incredible thing to be a part of. You welcomed me with open arms and made a student into a peer. A special thanks to Dr Libby Evans-Illidge, no one could take better care of the AIMS@JCU crew than you! Thank you for always being there when we need you. My fellow AIMS@JCU students and the beloved Kia Carnival, that 100 km round commute was many things but unsupportive was definitely not one of them. Dr Sebastian Schmidt-Roach, my office mate and amazing friend; your friendship will be forever treasured thanks for being alongside in the trenches.

The financial support of the university in the form of both a scholarship and a tutoring position made this journey possible. To the students of MB2080 and CH2042, thank you for reminding me why I started. Your enthusiasm is contagious and it has been a privilege to be a part of your education.

All PhD students have times when they wonder what on earth they have gotten themselves into. In those moments, I was lucky to be surrounded by friends who would be family, but for the small issue of not being related. Special mentions to: Mrs Janine Sheaves (pot), who does so much for so many; Mr Chris Williams, whose 'outsider perspective' helped me surmount the insurmountable; Dr Vivian Cumbo, for celebrating the good days and understanding the bad ones; Mr Bebe Worsfield, for reminding me of life outside the lab; Mr Max Ellis, for believing and never doubting and of course, Dr Shelley Templeman (kettle), words will never be enough, thank you from the bottom of my heart.

And finally, to my family, for encouraging me and believing in me even when my dreams took me and kept me far from home, I can never repay what you have given although I will spend the rest of my life trying.

Statement of Contribution of Others

Nature of Assistance	Contribution	Names and Affiliations of Co-Contributors
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Intellectual support	Data Analysis	Assoc. Prof. B Bowden (JCU), Dr L Hoj (AIMS)
	Image Production	Kirsty Freckelton
	Editorial assistance	Assoc. Prof. B Bowden (JCU), Dr L Hoj (AIMS)
Financial support	Field research	Assoc. Prof. B Bowden, Dept. of Chemistry, JCU
	Research Support	Futures Project, AIMS
	Stipend	Australian Postgraduate Award
	Write-up Grant	Graduate Research School, JCU
	Travel Grant	Australian Coral Reef Society (ACRS) to the ACRS conference 2010 Coffs Harbour, Australia
		Australian National Network in Marine Science (ANNiMS) to the ANNiMS conference 2011 Perth, Australia
Data collection	Field Work	Assoc. Prof. B Bowden (JCU), Mark Slavin, Louise Forster, Sophie Gordon
	Laboratory Guidance	Assoc. Prof. B Bowden (JCU), Dr Cherie Motti (AIMS), Dr Brett Baille (AIMS), Peter Thomas-Hall (AIMS), Dr Nete Bernbom (AIMS)
	Chemical Modelling	Dr Ken Adam (JCU), Assoc. Prof. B Bowden (JCU)

Abstract

Quorum Sensing (QS) has emerged as a potential pathway of interaction between eukaryotic host organisms and their associated microbial communities. QS is the term that has been applied to describe the indirect regulation of gene expression in many micro-organisms. This is achieved by the use of small diffusible molecules that enable coordination of gene expression across an entire population. The extracellular nature of these compounds also allows their interception, interference and mimicry. Consequently, the molecules that regulate QS, and those produced to interfere with QS, are of increasing interest for biotechnology products. This thesis targeted QS interference in soft coral holobionts, as many soft coral species commonly contain compounds with structural similarities to well-studied bacterial QS molecules (acyl homoserine lactones).

The presence of QS activity within the soft coral holobiont was investigated in order to determine if there were chemical or taxonomic patterns to the QS interference capability. Extracts (across three polarity ranges) of fifteen species of soft coral from four different families were screened against two QS bacterial biosensors, *Agrobacterium tumefaciens* A136 and *Chromobacterium violaceum* CV026, for both induction and inhibition of QS. The QS interference observed in the soft coral species differed between the two biosensor strains and was not restricted to one family, but rather extended across taxonomic bounds. Bioassay-guided fractionation of these extracts revealed chemical patterns that related to the observed activity, particularly in the induction of QS. To investigate the source of the chemical patterns, cembranoid diterpenes from active fractions were purified and tested for QS interference activity to confirm their presence as one source of QS activity in soft coral holobionts. Interestingly, the form of this activity (induction or inhibition) was found to correlate with structural variability of the secondary oxygen ring of these compounds. The potential for non-cembranoid diterpene QS activity

in soft corals was investigated with an extract of *Nephthea chabroli* that had previously demonstrated strong QS inductive activity in *A. tumefaciens* A136 but was not known to contain cembranoid diterpenes. Bioassay guided fractionation identified one QS compound as 17,22-dihydroxy-24-methylene cholesterol. Finally, bacteria within the mucosal layer of the soft coral species *Sinularia flexibilis* and *Lobophytum compactum* were cultured to investigate the potential for the previously observed QS interference of cembranoid diterpenes to have an *in situ* ecological role. Isolobophytolide, which is the dominant cembranoid diterpene of *L. compactum* and has demonstrated QS activity, was used as an optional selection agent in the isolation media for strains of this species. The isolated strains were identified by their 16s rRNA gene and screened for induction and inhibition of QS using their culture medium. The identity and QS activity of the isolates, varied between the host corals and with the presence of isolobophytolide.

These results suggest that QS interference could be a mechanism of bacterial regulation in soft corals and that soft coral holobionts could be a good model system to explore QS interference. The identification of QS interference in cembranoid diterpenes demonstrates their potential for inter-kingdom interference with bacterial QS gene expression. In addition, the structure activity information revealed in these compounds may be transferable to other model systems and for the design of biotechnology products. The selective effect of isolobophytolide on the growth of QS bacterial strains in the mucosal layers of *L. compactum* is further evidence of the possible ecological role these compounds may play in influencing the composition of the associated bacterial community and their QS regulated gene expression.

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Chapter 1

Introduction

1.1 Antibiotics and Antibiotic Resistance

The golden age of antibiotics, which started with the discovery of penicillin, was heralded as the end of infectious disease. It is now less than a hundred years later and the rate of discovery and approval of new antibiotics has been outstripped by the incidence of antibiotic resistance (1). Traditional antibiotics rely on their ability to reduce growth and survival of pathogenic organisms. This approach of directly targeting mechanisms that reduce survival provides pressure for selection of resistance genes. In Gram negative bacteria, this selection pressure is compounded by the frequent location of resistance genes on mobile plasmid units that can spread rapidly through a population (2).

Only two new structural classes of compounds have been approved for use as antibiotics since 1962: an oxazolidinone in 2003 (3) and a cyclic lipopeptide in 2000 (4). Both of these are antibiotics that are primarily active against Gram positive bacteria: no new Gram negative specific antibiotics have been approved in recent times. The main classes of antibiotic drugs were discovered largely by empirical screening of either natural products or synthetic and semi-synthetic libraries. These screening protocols exploited a limited range of both bacterial physiology and chemical space (5, 6). New strategies involving *in silico* technology and structure based drug design are improving the rate of screening and efficacy of traditional style antibiotics (6, 7).

Modern antibiotics are typically administered at concentrations far higher than those found *in situ* in nature (8, 9). There is evidence to suggest that at the lower ecologically relevant concentrations, some small organic antibiotics are actually involved in cell to cell signalling, either within or between species (8, 9). The discovery of the prevalence and extent of cell to cell signalling in bacterial communities has dramatically altered how bacteria and bacterial infections are perceived. New mechanisms for targeting unwanted bacterial infections may be possible from a deeper understanding of how bacteria, bacterial communities and infections are regulated *in situ*.

1.2 Cell to Cell Signalling and Quorum Sensing

Bacteria use chemical cues to gain information either directly or indirectly from the environment (10). Quorum Sensing (QS) is one system of indirectly acquiring or conveying environmental information (10). In QS, bacterial gene expression is regulated in response to small diffusible molecules (11, 12). These molecules may be produced by the bacterium itself, conspecifics or even bacteria from different species (10). Bacteria use QS systems to interact with their physical and biological environment (11), to form biofilms (13), secrete virulence factors (14) and regulate metabolite production (15). Bacterial QS is regulated by the constant release of low levels of QS molecules and can provide a bacterial population with elements of multi-cellularity (15). The QS molecules diffuse into the surrounding environment and as the bacterial population cell density increases, QS molecules then accumulate in the surrounding environment (Figure 1.1). As a consequence, the concentration of QS molecules can act as a proxy measurement of cell density (11). QS regulated genes are differentially expressed as a function of the concentration of these molecules thereby allowing gene expression to be simultaneously triggered across the bacterial population (Figure 1.1) (11). An increasing number of QS molecules and receptors are being discovered (15) and QS is one of the most promising pathways to emerge for potential bacterial regulation (16). QS could be expected to have a reduced selection pressure for resistance when compared to traditional antibiotics because it does not directly target growth and survival of bacteria (17, 18).

1.3 QS in Gram Negative Bacteria

Gram negative bacteria include in their number many important symbionts from mutualists to pathogens. Of particular concern, is the rapidity with which Gram negative bacteria develop antibiotic resistance (2). Gram negative bacteria possess various forms of cell signalling pathways including the QS signalling system known as the Auto-Inducer One or AI-1 system. AI-1 is a two gene system where the genes are homologues of LuxI

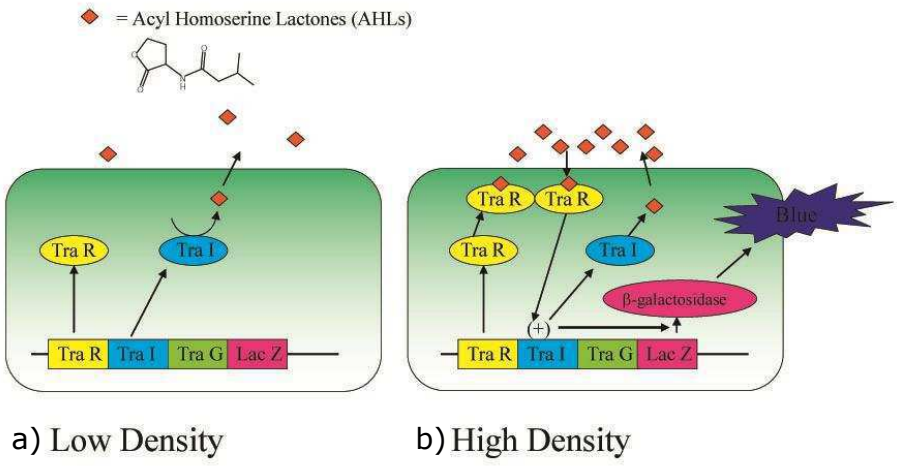

Figure 1.1: Quorum sensing schematic based on the QS biosensor strain *Agrobacterium tumefaciens* A136 used in this study. The genes TraR and TraI found in this biosensor strain are homologues of the LuxR/LuxI genes. At low density (a), signal molecules are secreted and detected but no gene expression occurs. At high density (b), the concentration of signal molecules has reached a critical point, triggering gene expression to occur.

and LuxR. LuxI, or its homologue (eg. TraI), is the induction gene that encodes for the production of the QS signal molecule (11). The signal molecules of AI-1 are of the type known as acyl homoserine lactones (AHLs) (Figure 1.2). These signal molecules can then be detected by the LuxR protein, or its homologue (eg. TraR). The complex formed between the AHL signal molecule and the LuxR type receptor protein triggers expression of QS regulated genes (Figure 1.1). In addition, a positive feedback loop is triggered when the QS molecules are detected by LuxR thereby ensuring their continued release by LuxI (Figure 1.1). Multiple examples of AHL signal molecules have been isolated and the AHL molecules have been found to consist of three functional components: a γ-lactone ring (5 membered), an amide bond (at the C-3 position) and an acyl side chain of variable length and substitution (Figure 1.2). The acyl side chain can vary from 4-12 carbons in length and feature varying degrees of saturation and / or oxygenation (Figure 1.2) (19). The lactone ring moiety allows the signal molecule to bind to the receptor protein providing the signal with its activity (20), whereas the variability of the side chain provides specificity to the signal (19, 21).

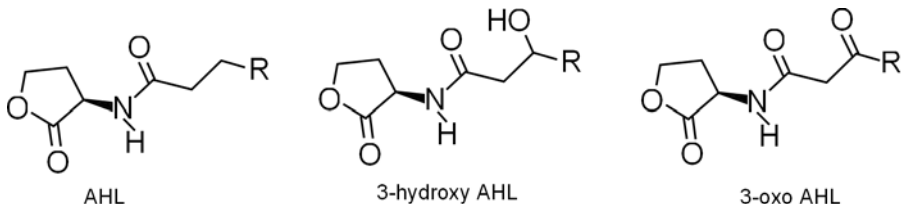

Figure 1.2: Acyl homoserine lactone (AHL) structure showing variability of the acyl side chain. R = C_1 - C_{12}

1.4 AI-1 and Inter-Species Communication

Initially, AI-1 was viewed solely as an intra-species mechanism of communication within Gram negative bacteria. Complementary systems such as AI-2 were considered to fulfil the role of inter-species communication (11). This assumption has been challenged by the discovery of uncoupled or incomplete AI-1 QS systems within Gram negative bacteria, predominantly in those species found in multi-species biofilms (22). Uncoupled receptors allow species to "eavesdrop" on neighbours and competitors and to alter gene expression accordingly (23). The ability to alter gene expression as a result of the presence or activities of other species in close proximity could provide a competitive advantage (22). If this form of AI-1 interaction is prevalent it would act as a mechanism of community regulation.

1.5 Bacterial QS Biosensors

The ability of some bacterial species to detect non-natant QS molecules has allowed the development of bacterial biosensor strains. QS bacterial biosensor strains are usually bacteria that have been genetically modified so that QS can only occur under certain circumstances. Usually this is achieved by a QS controlled promoter gene to a reporter gene (24) and that genetic modification regulates a measurable response. Bacterial biosensors provide a simple but sensitive method to detect and investigate QS behaviours and interactions. The reporter gene of bacterial biosensors typically involves the production of pigments or bioluminescence that are readily quantified and / or detected (24). Bacterial

biosensors designed to detect the presence of QS signals are genetically modified so that they possess only the ability to respond to exogenously added QS signals and cannot produce such signals themselves (24).

A small number of bacterial biosensors have also been designed to specifically detect molecules that can inhibit QS. The design of these strains generally takes one of two strategies (24, 25). The first comprises a gene encoding a lethal protein fused to a QS-controlled promoter with the result that the biosensor is unable to grow in the presence of AHL signal molecules unless a functional nontoxic QS inhibiting compound is present at a sufficiently high concentration (25). An alternative strategy employs an antibiotic resistance gene controlled by a repressor, which is in turn controlled by a QS-regulated promoter (25). In this instance the presence of AHL causes growth inhibition in the presence of the appropriate antibiotic, however, when a QS inhibiting compound is present, down-regulation of the repressor enables growth of the bacterium (25).

Each LuxI / LuxR QS gene homologue pair has slightly different sensitivities to the length and type of acyl side chain of AHL molecules. Consequently, many bacterial biosensors have been developed to detect either short, long or oxo- forms of AHLs (24). Two of the most commonly used AI-1 bacterial biosensor strains are based on the species *Chromobacterium violaceum* and *Agrobacterium tumefaciens*. In *C. violaceum*, QS regulates the production of the secondary metabolite violacein (26). In addition to its antimicrobial properties, violacein is a deep purple pigment that is easily detected (Figure 1.3). Bacterial biosensors based on this strain utilise the LuxI / LuxR homologue genes CviI / CviR to enact QS (26). These QS genes are sensitive to AHLs with $C_4 - C_8$ carbon chains as well as 3-oxo-C_6 and -C_8 carbon chains (26, 24).

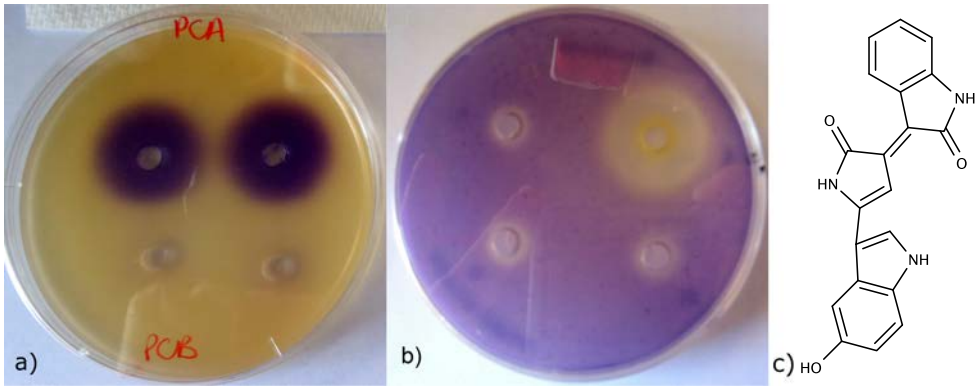

Figure 1.3: Bacterial biosensor assays showing positive results for a) induction of QS and b) inhibition of QS in the bacterial biosensor *Chromobacterium violaceum* CV026. The bacterial biosensor strain is embedded in the agar and the test substance is added to the wells. Purple pigment colouration is caused by the production of violacein (c).

A. tumefaciens is a plant pathogen in which QS induces the formation of galls in their plant host utilising the TraI / TraR QS genes (27, 28, 29). In its unmodified form these genes are located on a large Ti plasmid (27, 28, 29). Frequently, QS biosensors strains based on this species have this pathogenic plasmid removed (27, 28, 29). The plasmid is then replaced with a smaller plasmid bearing the QS genes fused to the gene LacZ and resistance to a certain antibiotic to enable selectivity (27, 28, 29). The LacZ fusion results in the production of an enzyme when the strain detects QS signal molecules (27, 28, 29). If the *A. tumefaciens* strain is cultured in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), this enzyme causes the breakdown of X-gal by hydrolysis of the glycoside linkage at the indole 3-position to form an indigo product (27, 28, 29). This indigo metabolite can then be detected and measured in a similar fashion to the violacein of *C. violaceum* (Figure 1.4). TraR is sensitive to AHLs with $C_4 - C_{14}$ acyl side chains as well their equivalent 3-oxo-acyl side chains and C_6-C_{10} hydroxy acyl side chains (27, 28, 29, 24).

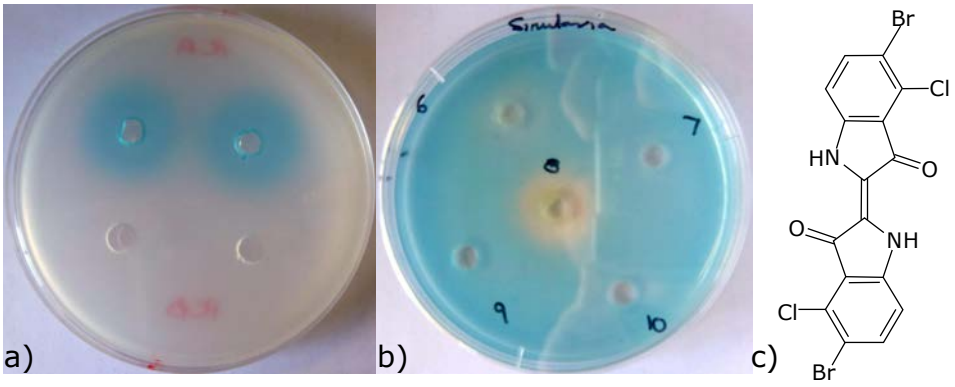

Figure 1.4: Bacterial biosensor assays showing positive results for a) induction of QS and b) inhibition of QS in the bacterial biosensor *Agrobacterium tumefaciens* A136. The bacterial biosensor strain is embedded in the agar and the test substance is added to the wells. Blue pigment colouration is caused by the breakdown of X-gal (c).

1.6 Inter-Kingdom Communication

QS regulated phenotypes are often crucial to successful interactions with eukaryotic host organisms regardless of whether the interaction is beneficial, harmful or benign. It is therefore unsurprising that QS is prevalent in bacteria that frequently associate with eukaryotes (30, 31). Some QS signals have been observed to directly impact eukaryotic cells (32). Furthermore, some bacterial QS genes are directly affected by eukaryotic products (33). This could indicate that QS systems improve the success of interaction with a host (22). Chemical communication such as this could allow participants to coordinate gene expression in order to establish and maintain associations (33). The implication of this is that QS through AI-1 could represent interplay of communication and regulation between host and symbiont.

1.7 Host Interference and QS Mimics

The response and interaction of eukaryotes with bacterial QS systems has been investigated in a number of model systems including humans, squid and algae. These host

organisms have evolved mechanisms to detect and interfere with QS, so they can respond quickly and reliably to the presence of pathogenic or mutualistic bacteria (34). Molecules that act as analogues of AHLs to interfere with QS systems are termed QS mimics. QS mimic compounds have been detected in algae (31, 35, 36), plants (34, 37), animals (38, 39) and fungi (40). Interactions between hosts and symbiont bacteria can be considerably affected by manipulation of QS systems (38).

One of the best studied of these interactions is that between the temperate red algae *Delisea pulchra* and the microbes that associate with the surface of its thallus (35). *D. pulchra* produces compounds known as furanones in pores that open out onto the surface of the thallus (35). Furanones contain similar functionality to AHLs and are capable of binding to QS receptor proteins. Unlike the complex that forms between an AHL and the receptor protein, when furanones bind to these receptors the resulting complex does not trigger gene expression (36). Instead, QS gene expression becomes inhibited as the furanones reduce the binding sites available for AHL molecules and result in an increased turnover of the receptor complex (36). Ecologically, this QS interference reduces the ability of bacteria to form biofilms (36). An active biofilm is a requirement for many fouling organisms to settle and as such these compounds aid the *D. pulchra* in keeping the thallus surface unfouled.

1.8 Biotechnology Applications of QS Mimics

The inhibition of fouling organisms revealed by the furanone compounds opened up the possibility of biotechnological applications of QS inhibitors and mimics (41). Biofilm associated fouling communities can cover ships and any structures placed in aquatic environments (42). The increased drag caused by fouling communities adhered to ships can reduce speed by up to 10% and increase fuel consumption by 30% (43). Consequently, compounds that can inhibit the formation of biofilms are of considerable economic importance. Bacterial biofilms are important not just for shipping concerns but also in the medical field. Bacterial biofilms can protect pathogens from antibiotic compounds as

seen in chronic *Pseudomonas aeruginosa* cystic fibrosis lung infections (44, 18). Promisingly, the inclusion of QS inhibitors in combination with the antibiotics used to treat *P. aeruginosa* infections was demonstrated to markedly increase treatment efficacy (18). The ability to control the formation of such biofilms could have far reaching implications, saving not only money but lives.

The development of QS mimics into biotechnological products is hindered by the complexity of bacterial communities and their interactions. Eukaryotes provide numerous habitats for bacterial biofilm formation (45). Eukaryotic associated bacterial biofilms are typically highly diverse but also differ from the surrounding environment (46, 47). In addition, these complex communities often interact with their host organism. For instance, many bacterial species have also recently been revealed to play essential roles in the health and development of host eukaryotic organisms. Further compounding this complexity is the diversity of phenotypes that can be regulated by QS and therefore potentially affected by the presence of QS mimicking products. Some pathogens such as *Vibrio cholera* use QS systems to leave host organisms, enabling cholera infections to spread quickly through host populations (48), whereas other bacteria such as *Chromobacterium violaceum* are triggered to produce antibiotic compounds to inhibit the growth of competitors (26). Any product designed to interfere with QS would need to take this complexity into account, which would require strong foundational knowledge of the mechanism of action and structural plasticity of QS mimics.

1.9 Structural Understanding of QS Mimic Compounds

QS mimics have been detected in the extracts of a number of plant (49, 50, 37), animal (51) and fungal (52) species. Unfortunately though, in the majority of cases, the structural identity of mimic compounds remains unknown. While bacterial biosensors provide a reliable means of detecting the presence of QS mimic compounds, the low

concentration of those compounds combines with the complexity of the natural extracts in which they are found to produce a considerable isolation and elucidation challenge. For example, over 20 chromatographically resolvable fractions demonstrating QS mimicking activity were isolated from extracts of the common pea, *Pisa sativum* and the structural identities of the mimic compounds were unresolvable (37).

The lack of structural knowledge of QS mimics hampers understanding of their mechanism of interaction with QS receptors, directly impacting the search for new mimics and the design of new pharmaceuticals. The lactone ring of the acyl homoserine lactone (Figure1.5a) has been demonstrated to be required for their successful binding to the QS receptor proteins (20). This requirement is also true of the *D. pulchra* furanones (Figure1.5b): both molecule types lost their ability to interact with this system upon experimentally opening the oxygenated ring (36). Unfortunately, of those QS mimic compounds that have been elucidated the role of an oxygenated ring is not as clear; an oxygenated ring has been observed in penicillic acid and petulin (Figure1.5c,d) of a *Penicillum* species (40) but not in the N,N'-alkylated imidazolium-derivatives (Figure 1.5e) from potato tubers (53). Further, the mechanism by which these other mimic compounds interact with the QS system is often not well established. A greater number of elucidated mimic compounds would significantly improve the understanding of the mechanisms and plasticity of these interactions and provide essential structure function relationships required for any biotechnological application.

1.10 Host – Microbe Interactions

Investigations of QS mimics reveal not only important information for biotechnological design, but also a fundamental understanding of the interactions that occur between host and symbiont (54, 55, 56). Macro-organisms provide a multitude of niches for microbial colonisation and as a result all macro-organisms have an associated community of micro-organisms. The specificity of many of these associations indicates that the relationship can be more than a passive association with host tissues and is likely to be actively

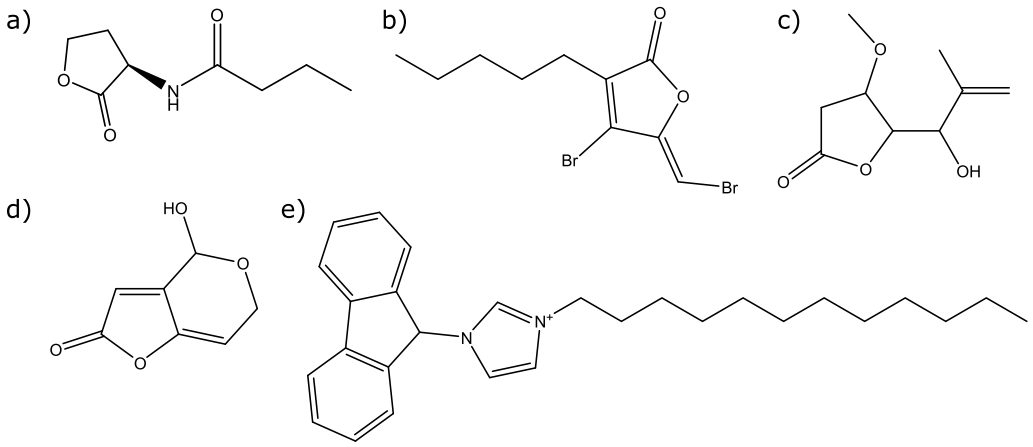

Figure 1.5: Structural variation of QS mimics that have been identified. a) AHL, b) *Delisea pulchra* furanones, c) penicillic acid d) petulin e) N,N'-alkylated imidazolium-derivatives.

maintained (47, 57, 45, 58). Micro-organisms are also important factors in the health and resilience of the host organism (59, 60, 61). Loss or changes of diversity in the associated microbial community can be a first indication of stress and reduced resilience of the host organism (62, 63). To a large extent, however, the specific role and interactions of these important microbial communities remain unclear (62).

1.11 The Coral Holobiont

Holobiont is a term introduced by Mindell (64) and later expanded upon by Rohwer (65) that refers to a host organism and its symbiotic microorganisms (and viruses) (Figure 1.6). The holobiont is an important concept because it encompasses the interdependence of all members of the holobiont for survival. The coral holobiont is comprised of the coral host, algal symbionts and microbial communities (and viruses) (Figure 1.6). The symbiosis between corals and zooxanthellae has long been recognised, however, the role of the microbial communities is only just becoming apparent (Figure 1.6).

A coral represents a number of different niches available for colonisation. The surface mucosal layer (SML) is a niche that is also the first and largest point of interaction between a coral and the environment. This layer houses a complex community of mi-

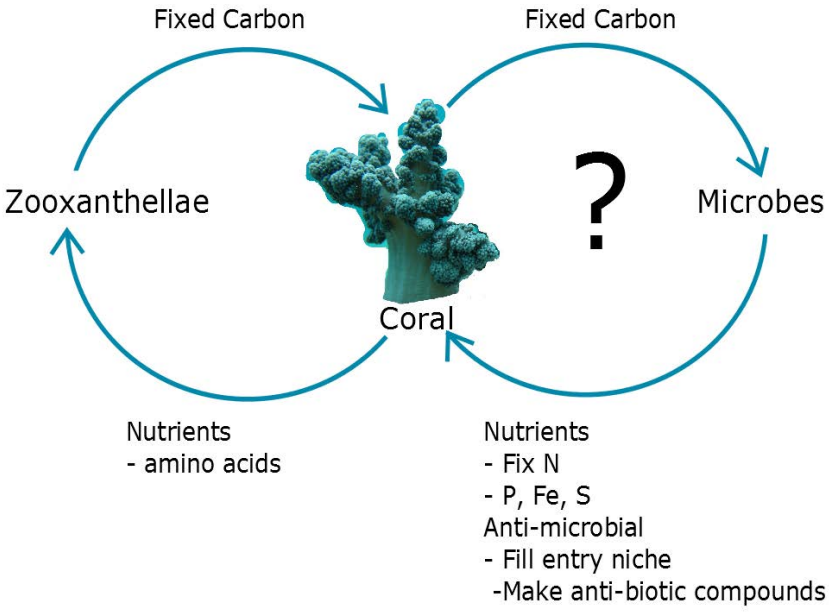

Figure 1.6: The soft coral holobiont showing all symbionts and the interactions that link them. Figure adapted from (65).

croorganisms, which is hypothesised to be an essential component of the health and resilience of the coral host (66, 67, 46, 68). Furthermore, bacteria from this layer have been implicated in both the nitrogen and sulphur cycles (69, 70). At this stage, however, little is understood about the mechanisms regulating this community or its interaction with the coral host (62).

The survival of a holobiont is dependent on the balance between all of the members (57). In corals, microbial communities appear to be integral to resilience of the holobionts and potentially act as a first line of defence against bacterial pathogens (68, 71, 66). Microbial communities associated with corals have been observed to rapidly shift with changes to the local environment (eg. temperature) and the health of the host organism (57, 62, 61). It is therefore important to to understand the interactions that occur between host and microbes to regulate this balance.

Corals can influence their SML bacteria through physiological, physical or chemical means. Physiological measures may involve a direct immune response, whereas, physical mechanisms consist of a sloughing of the mucous layer (72, 73, 74). Chemical metabolites produced to inhibit growth or pathogenesis provide potential for adaptive responses

(75, 76, 77). Selection of bacteria through chemical signals, could involve: toxicity (cell death or growth inhibition), chemotaxis (movement along a chemical gradient), or QS interaction. In addition, microbes can self-regulate their community through QS cross talk, whereby interception or manipulation of QS compounds can alter gene expression in different populations of bacteria rather than their natal population (23, 15).

1.12 Coral Reefs and Associated Microbial Interactions

A fundamental understanding of the mechanisms regulating these microbial communities is becoming increasingly important. Stress, reduced resilience and increased disease susceptibility threaten coral reefs and marine benthos globally (78). Octocoral communities of the Caribbean have already become decimated by microbial disease outbreaks (79, 80). The Indo-Pacific is also facing reports of increased disease outbreaks (81, 82). Although the causative agent of many coral diseases remain elusive, changes in microbial community composition associated with coral disease are now well established. Many coral disease infections are associated with loss of diversity in the associated microbial community following a period of stress such as increased temperature (83, 84). For reasons that remain unclear, shifts in the composition of the associated communities allow microbes that may have been present at low levels in the healthy holobiont dominate in new low diversity communities (85, 86, 57). QS, as a mechanism of both microbial community regulation and microbe-host interaction, could provide a pathway to investigate these observed changes.

1.13 Soft Coral Cembranoid Diterpenes

Soft corals have long been a source of small molecules of biological and pharmaceutical interest (87 and previous in series; 88, 89). These small molecules appear to fulfil

ecological rather than direct survival roles and are frequently referred to as secondary metabolites. While a large number of different types of metabolites have been isolated from soft coral, perhaps the best known are the cembranoid diterpenes (90). Typically, these molecules come in the form of a 14 membered isoprenoid ring (carbocyclic diterpenes), generally with an isopropyl residue at C-1 and three symmetrically dispersed methyl groups at C-4, C-8 and C-12 (Figure 1.7; 91). In addition, the isopropyl residue at C-1 becomes cyclised to form a secondary ring often in the form of a lactone or furan ring (Figure 1.7; 91).

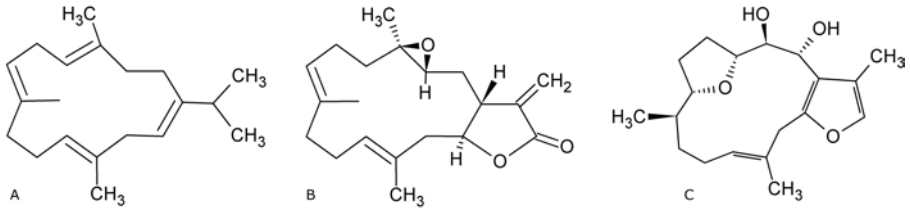

Figure 1.7: Diversity of cembranoid diterpenes of soft coral. a) Isoneocembrene A showing the isopropyl group at C-1 and symmetrically dispersed methyls at C-4, C-8 and C-12. b) Isolobophytolide: a cembranoid diterpene from *Lobophytum compactum*; the isopropyl group has become cyclised in the form of a γ-lactone. c) Pachyclavulariadiol: a cembranoid diterpene from *Pachyclavularia violacea*; the isopropyl group has become cyclised in the form of a furan ring.

Cembranoid diterpenes have a strong taxonomic linkage with the coral species they are isolated from, however, they have also been isolated from organisms other than soft corals (90). Their presence in other organisms highlights the potential for micro-organisms to be the true producers of these compounds (92), although this linkage has yet to be conclusively demonstrated (90). Regardless of the true producers of these secondary metabolites, they are largely attributed as the reason behind the ecological and evolutionary success of soft corals (93, 94). Many cembranoid diterpenes have demonstrated strong bioactivity profiles including ichthyotoxicity, cytotoxicity and antimicrobial properties (94, 89, 90, 95). In addition, a study examining the QS interference potential of extracts of marine invertebrate taxa indicated a high level of QS interference in non-polar soft coral extracts (96). The presence of lactone and furan functionality in many cembranoid diterpenes suggests they are likely candidates to be responsible for the observed interference in soft corals. The functional variability of these compounds additionally

provides a possible opportunity to investigate the structure activity relationship of a series of naturally occurring QS mimics.

1.14 Aims and Objectives:

The aim of this project was to investigate the identity and ecological relevance of QS mimics within a soft coral holobiont. Specific objectives included:

1. To confirm the presence and investigate the extent of QS activity in extracts of soft coral holobionts;
2. To determine how the structure of QS mimics affects their activity;
3. To examine the potential for QS interactions in the absence of lactone or furan containing metabolites and
4. To investigate the ecological relevance of these compounds to the soft coral holobiont and associated microbial community.

The results generated in this thesis provide valuable information on the structural requirements of QS mimics, which may be transferable to other host systems as well as pharmaceutical design.

Chapter One (this chapter) presents an introduction to QS and its potential as an alternative means of regulation of microbial communities associated with eukaryotic organisms. The increasing prevalence of antibiotic resistant bacteria has highlighted the need for alternative mechanisms for bacterial interference and control. Although QS has been linked to the regulation of bacterial associations in a number of organisms, the exact framework for understanding QS mimics, their structural plasticity and importance is still lacking. This chapter reviews the current status of knowledge of QS interactions and the structure of QS mimics. It highlights the potential for soft corals as a model system for investigation of QS due to the pre-existing knowledge of secondary metabolites in this taxon.

Chapter Two confirms the presence and investigates the extent of QS in Australian soft coral holobionts (Objective 1). Specifically, this study used QS biosensors to investigate whether interference of AHL-type QS was limited to the family Alcyoniidae, for which QS inhibition was demonstrated previously, or to soft corals that are known to produce cembranoid diterpenes.

Chapter Three describes how the structures of QS mimics affect their activity (Objective 2). Information gained previously from *D. pulchra* furanones suggests that a five membered oxygenated ring system is required for activity in QS mimics. For this reason, cembranolides and furanocembrenes were isolated to determine if they could be responsible for the observed QS interference. Structural patterns were identified that may affect the ability of these metabolites to mimic QS signals.

Chapter Four examines the potential for QS interaction in the absence of lactone or furan containing metabolites (Objective 3). Most QS interference studies have focussed on species known to produce secondary metabolites that contain either a lactone or furan ring. This study examined the assumed requirement of such functional groups for activity by using bacterial biosensors to guide the isolation of a QS active compound from *Nephthea chabroli*; a soft coral species not known to produce these metabolite features.

Chapter Five further assesses the ecological potential for these mimicking compounds by isolating and identifying QS bacteria that live in association with the soft corals *Sinularia flexibilis* and *Lobophytum compactum* (Objective 4). These two species were chosen for this study because their well characterised secondary metabolite profiles were determined to be dominated by cembranoid diterpenes capable of QS interference. However, at the start of this study, little information was available on the bacterial communities that live in the surface mucosal layer of soft corals and nothing was known about their QS abilities. The importance of these compounds to culture of relevant microbes was further investigated by the inclusion of isolobophytolide in the growth media.

Chapter Six briefly summarises the findings of these investigations. It discusses the relevance of the structural findings to the design of biotechnology products and the potential role and importance of these QS mimics to the soft coral holobiont.

Chapter 2

Quorum Sensing in Australian Soft Corals

2.1 Introduction

Quorum sensing (QS) systems are more prevalent amongst bacteria associated with mixed bacterial biofilms and macro-organisms, suggesting that possession of QS systems confers an advantage in these habitats (97). In addition to the use of QS as a mechanism to coordinate gene expression within a population, many bacteria also possess the ability to detect and respond to the QS signal molecules of other species (23). In this instance, the extracellular nature of the signalling molecules facilitates their disruption and mimicry (98). Consequently, the detection and manipulation of bacterial QS signals can perform an important role in the regulation of these mixed bacterial communities (22).

Host organisms that have evolved mechanisms of interference with QS would be able to respond to the presence of pathogenic or mutualistic bacteria quickly and reliably (34, 33). In keeping with this theory, Bauer and Robinson (38) found that interactions between hosts and bacteria were considerably affected by manipulation of QS pathways. For example, chemical interruption of QS can render some pathogenic bacteria non-pathogenic (99, 100). Similarly, such mechanisms in the host can enable manipulation of the abundance and composition of its associated bacterial community as observed in the marine alga *Delisea pulchra* (35).

The compounds responsible are termed QS mimics and have been located in a number of algae, plant and fungal species (38). QS mimics have been observed to occur widely in terrestrial plants (101, 25, 49, 50). Similarly, a previous screening by Skindersoe and colleagues (96) that demonstrated widespread QS inhibitory activity in the marine benthos, has been further supported by an in depth assessment of QS inhibition in sponges (102) and gorgonians (51). The screening by Skindersoe and colleagues (103) also indicated that, of the taxa screened, soft corals displayed the highest relative activity. All of the soft corals screened by Skindersoe were from the family Alcyoniidae, a family known to be rich in secondary metabolites (93, 87 and previous reviews in this series). The evolutionary and ecological successes of this family has largely been attributed to the bioactivity of the cembranoid diterpene class of compounds (93), however, the

specific ecological roles of the individual compounds are not well demonstrated (94). A combination of chromatography and bacterial biosensors were utilised to examine a number of soft coral species for QS interference and to determine whether this QS interference is restricted to QS inhibition by the family Alcyoniidae.

2.2 Experimental

2.2.1 Soft coral sample collection and documentation

Twenty four specimens of soft coral, representing 15 species, were collected at a depth of 1-3 m from Orpheus Island (Great Barrier Reef, Australia; latitude, 18° 36.878' S; longitude, 146° 29.990' E). Tentative identifications were performed using relevant available guides and keys. All specimens, except *Cespitularia* sp., were photographed underwater (Figure 2.1) before sampling and a taxonomic voucher sample of each was placed into 70% ethanol for reference and submitted to the Museum of Tropical Queensland.

Figure 2.1: Underwater images of soft corals species collected from Orpheus Island, Australia.

2.2.2 Soft coral extract preparation

Soft coral tissue samples were placed into plastic bags underwater and frozen within 1 hour of collection. All samples were stored at -80°C until lyophilisation. Dried coral tissue was weighed and homogenised before being exhaustively extracted. Solvents used for exhaustive extraction were, in order, dichloromethane (DCM), methanol (MeOH) and water (H_2O). For each solvent, the dried tissue was immersed in a solvent volume three times the tissue volume and sonicated in a bath for 20 min. The procedure was carried out three times before proceeding to the next polarity solvent. Extracts were combined according to solvent and concentrated via rotary evaporation before being dried under a stream of nitrogen. Three extracts of different polarity were thereby generated for each coral sample and these were stored at -20°C prior to analysis. The dichloromethane and methanol extracts were dissolved in ethanol and the aqueous extracts in water (H_2O) at 20 mg/ml and a 1 : 10 dilution at 2 mg/ml by vortexing for 30 seconds.

2.2.3 Bacterial biosensor strains and culture medium

The biosensor strains *Agrobacterium tumefaciens* A136 (104) and *Chromobacterium violaceum* CV026 (26) were used for detection of QS induction and inhibition in soft coral extracts. *A. tumefaciens* A136 utilises the QS receptor protein TraR to detect AHLs of acyl chain lengths of 6-14 carbon atoms, whereas, *C. violaceum* CV026 uses the receptor protein CivR to detect acyl chain lengths of 4-8 carbon atoms (24). *A. tumefaciens* A136 was grown on ABt media (105; Appendix D) supplemented with 4.5 µg/ml of tetracycline and 50 µg/ml of spectinomycin. *C. violaceum* CV026 was grown on LB media (106; Appendix D) supplemented with 20 µg/ml of kanamycin as previously described (107).

2.2.4 Screening for QS induction activity

All extracts and fractions were tested for the presence of QS induction activity in *A. tumefaciens* A136 and *C. violaceum* CVO26 at three times in two independent experiments. The presence of AHL type activity in soft coral extracts was detected by performing an agar diffusion assay where the biosensor was embedded in the agar (107). Briefly, *C. violaceum* was grown on LB agar plates supplemented with 8.5 µM N-hexanoyl-DL-homoserine lactone to ensure QS capability was retained in the biosensor. Single colonies were picked and grown in 10 ml liquid LB medium overnight (28°C, 180 rpm). A 1 ml aliquot of this culture was diluted 1 : 50 and again allowed to grow overnight, before being cast into 100 ml of LB agar (42°C) and poured into petri dishes. *A. tumefaciens* A136 was prepared as for *C. violaceum* CV026 with two exceptions, firstly that ABt medium (Appendix D) was used in place of the LB medium and secondly, the ABt agar was supplemented with 40 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) dissolved in N,N-dimethyl formamide. After solidification, wells (4 mm) were made in the plates and 20 µl of each extract was added. The positive control used was 20 µl of 1 mM N-hexanoyl-DL-homoserine lactone. The negative controls used were the extraction solvents (ethanol, dimethyl sulfoxide and water) at the same volume. The plates were incubated at room temperature (approximately 22°C) for 48 hours. Positive results were read as a blue colouring surrounding the wells in *A. tumefaciens* A136 and a purple coloration in *C. violaceum* CVO26 (Figure 2.2). Intensity of the response was measured as the diameter of the coloured zone and normalised to the response of the positive control (details of the normalisation process are included within Appendix D).

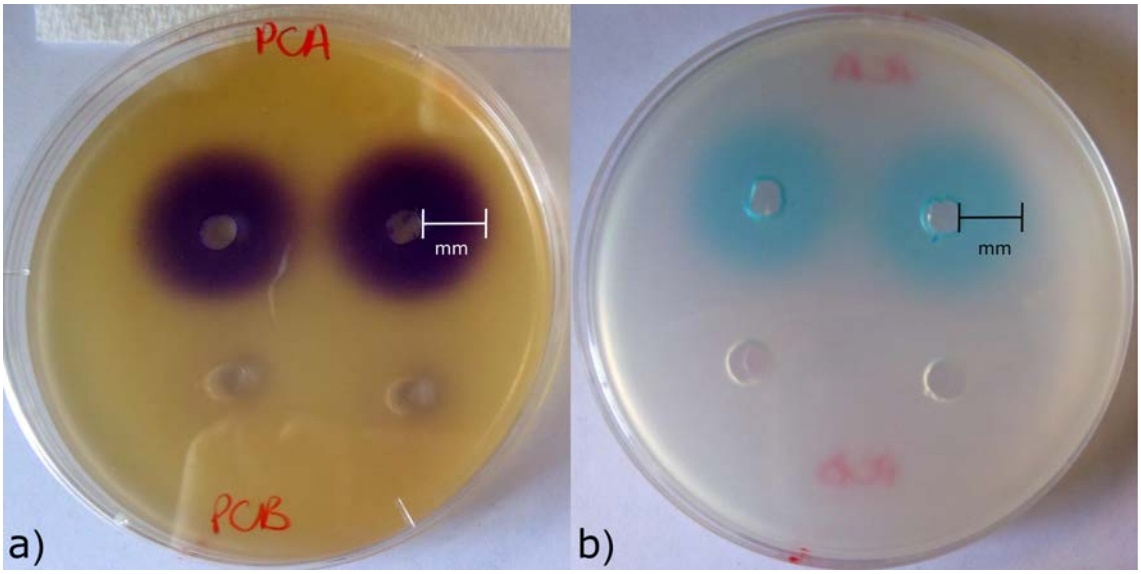

Figure 2.2: Examples of positive responses in the QS induction assay. Petri dishes containing agar embedded with a) *Chromobacterium violaceum* CV026 and b) *Agrobacterium tumefaciens* A136. Positive results were measured by the thickness (in mm) of the blue colouring surrounding the wells in *A. tumefaciens* A136 and the purple coloration in *C. violaceum* CVO26. The response to the positive control (20 nMols N-hexanoyl-DL-homoserine lactone) and place of measurement is indicated by the scale bar.

2.2.5 Screening for QS inhibition activity

Diffusion assays were performed with both *A. tumefaciens* A136 and *C. violaceum* CV026 biosensor strains as described for induction activity with the following modifications. *A. tumefaciens* A136 and *C. violaceum* CV026 are not able to QS without exogenous addition of AHLs, so in order to test for inhibition of QS 8.5 µMol N-hexanoyl-DL-homoserine lactone was added to the agar plates embedded with *A. tumefaciens* A136 or *C. violaceum* CV026. Two positive controls were chosen based on previously reported ability to inhibit QS: N-dodecanoyl-DL-homoserine-lactone (*C. violaceum* can be inhibited by long chain AHLs (26) and vanillin (108). The solvents dimethyl sulfoxide and H_2O were utilised as negative controls. The plates were incubated at room temperature for 2 days and positive results were read as inhibition of blue colouring of the plates containing *A. tumefaciens* A136 and inhibition of purple colouring of the plates with *C. violaceum* CV026. Intensity of the response was measured as the thickness of the coloured zone surrounding the well and normalised to the response of the positive control. All samples

were tested at least twice for both presence and inhibition of AHLs in three independent experiments (Figure 2.3).

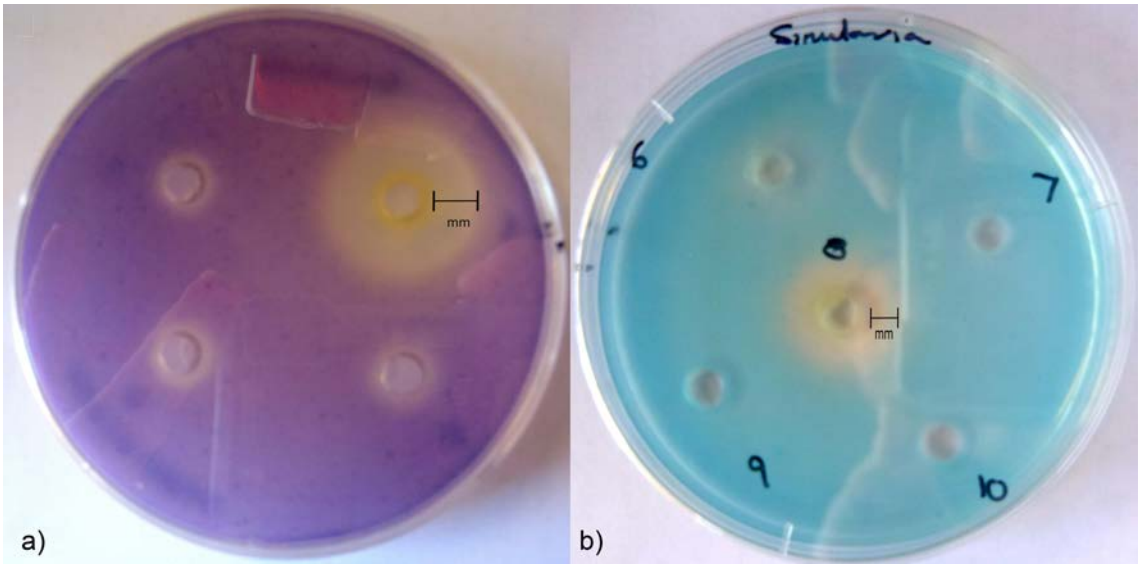

Figure 2.3: Examples of positive responses in the QS inhibition assay. Petri dishes containing agar embedded with a) *Chromobacterium violaceum* CV026 and b) *Agrobacterium tumefaciens* A136. Positive results were measured by the thickness (in mm) of the ring of inhibited of blue colouring surrounding the wells in *A. tumefaciens* A136 and the inhibition of purple colouring in *C. violaceum* CVO26.

2.2.6 Extract fractionation

To further explore the crude extract screening results, nine soft coral extracts were fractionated using reverse phase flash column chromatography. The nine soft coral extracts were chosen from five genera, representing three families: Alcyoniidae, Clavulariidae and Nephtheidae. Four (*L. compactum, P. violacea, N. chabroli* and *S. polydactyla*) had previously demonstrated activity in the unfractionated extracts (Table 2.1). An additional five species were chosen that had not demonstrated activity but were from the same genera as active samples (*L. microlobulatum, L. sarcophytoides, Lobophytum* sp., *S. flexibilis*) or represented common genera on the GBR (*Sarcophyton ehrenbergi*) (Table 2.1). The soft coral species chosen were recollected from Orpheus Island and extracted with dichloromethane as already described. ^{1}H NMR spectra and biosensor activity profiles of the crude dichloromethane extracts were compared with the previous collection

to ensure consistency in the metabolites and species tested.

Table 2.1: Summary of the patterns of QS interference activity of the dichloromethane extracts (Figures 2.7, 2.8, 2.9) of the nine soft coral species chosen for further investigation.

Family	Species	Induction of QS	Inhibition of QS
Alcyoniidae	*Lobophytum compactum*	A136	Both
	Lobophytum microlobulatum	None	CV026
	Lobophytum sarcophytoides	A136	Both
	Lobophytum sp.1	A136	Both
	Sinularia flexibilis	None	Both
	Sinularia polydactyla	None	CV026
	Sarcophyton ehrenbergi	None	Both
Clavulariidae	*Pachyclavularia violacea*	A136	A136
Nephtheidae	*Nephthea chabroli*	A136	CV026

Extracts were fractionated using flash column chromatography on RP-C18 silica cartridges (Phenomenex Strata C18-E 55 µm 70Å, 1000 mg) eluted with a stepwise 20% to 100% methanol : water gradient followed by a 1 : 1 DCM:MeOH wash (Figure 2.4). to generate 10 fractions of decreasing polarity. The resulting fractions were concentrated to dryness under a stream of nitrogen and redissolved in ethanol at 20 mg/ml and a 1 : 10 dilution to 2 mg/ml for QS screening was made as previously described.

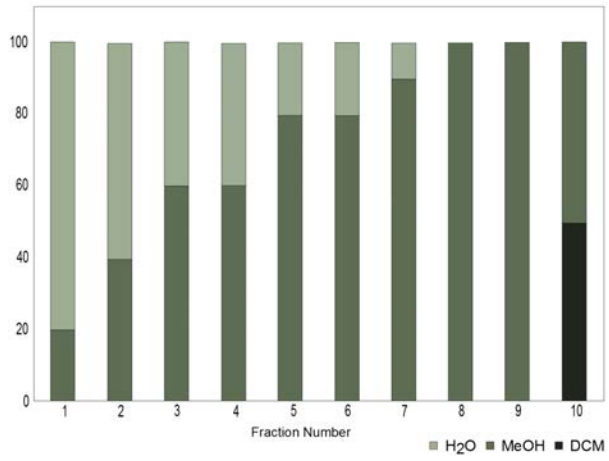

Figure 2.4: Solvent elution profile for fractionation of soft coral dichloromethane extracts through C18 SPE cartridges

2.2.7 Chemical fingerprinting of soft coral extracts

Nuclear Magnetic Resonance (NMR) spectrometry was used to generate a chemical fingerprint of all extracts and fractions. Each fingerprint sample (2 mg) was dissolved in 700 µl of deuterated solvent (DCM extracts into deuterated chloroform, $CDCl_3$, methanol extracts with deuterated methanol, CD_3OD, and water extracts into deuterium oxide, D_2O). ^1H NMR spectra were collected using a 300 MHz Bruker Avance NMR spectrometer and standard pulse parameters.

2.3 Results

Extracts of three different levels of polarity were generated from 24 samples representing 15 species (14 soft corals and 1 unidentified gorgonian). The extracts were screened for induction or inhibition of QS in *A. tumefaciens* A136 and *C. violaceum* CV026 in at least three independent experiments. Induction and inhibition of QS were both observed and were not limited to the family Alcyoniidae. Inhibition of QS was more prevalent than induction across all polarity extracts. The non-polar DCM extracts displayed the highest proportion of active species (48.9%) in comparison with the more polar extracts (44.4% for methanol extracts and 6.7% for aqueous extracts) (Figure 2.5). For both biosensors, the detected incidence of QS interference was substantially reduced at the lower dosage (Figure 2.6). For instance *C. violaceum* CV026 inhibitory activity of dichloromethane extracts dropped by almost half to 44.4% when dosed with 4 µg extracts instead of 40 µg (Figure 2.5).

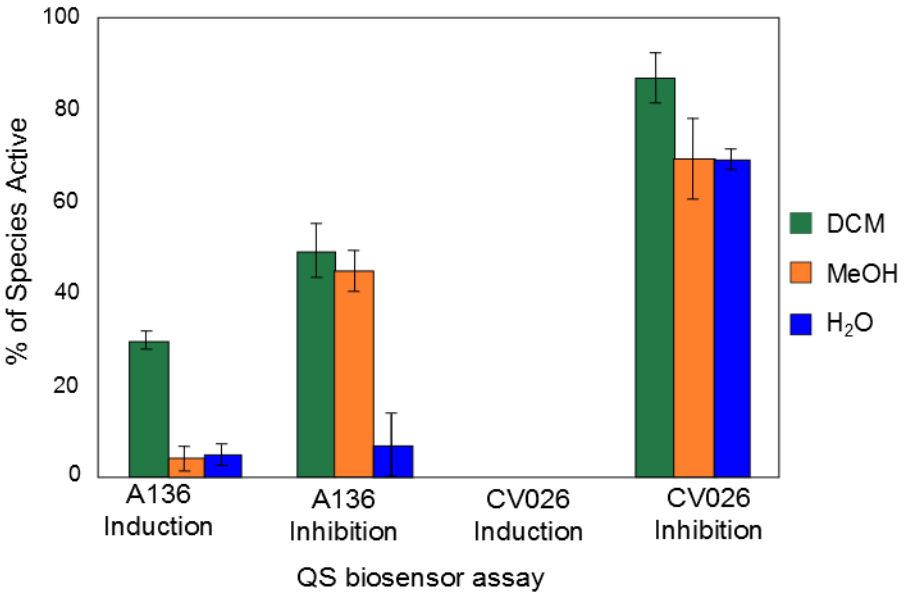

Figure 2.5: Percentage of soft coral species displaying QS induction or inhibition against *A. tumefaciens* A136 and *C. violaceum* CV026 after addition of 40 µg extract. Error bars represent the standard error from three screening efforts.

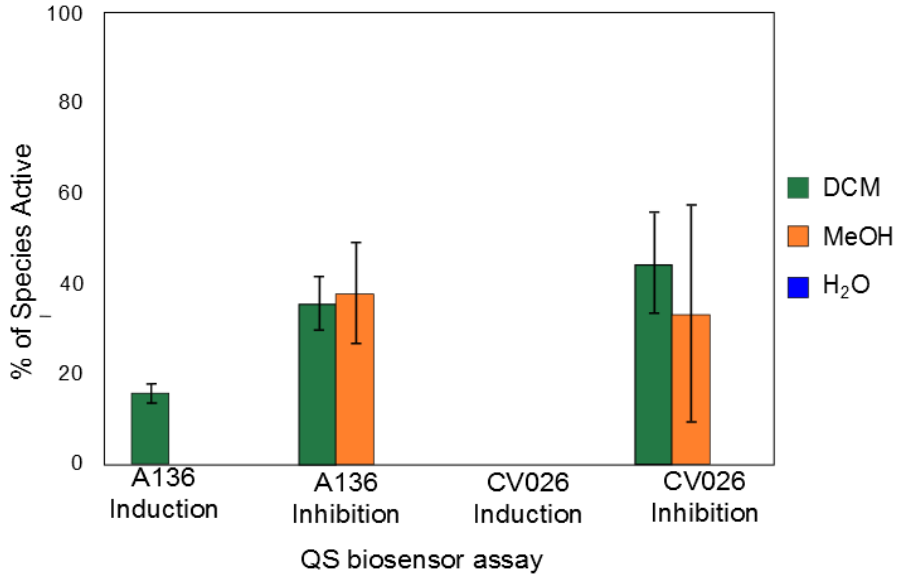

Figure 2.6: Percentage of soft coral species displaying QS induction or inhibition against *A. tumefaciens* A136 and *C. violaceum* CV026 after addition of 4 µg extract. Error bars represent the standard error from three screening efforts.

Substantial differences were seen in the patterns of activity between the two biosensors and between the type of activity (induction or inhibition) (Figure 2.5). All soft corals tested demonstrated inhibition activity to at least one of the biosensors tested with the

exception of *Clavularia* sp. and *P. violacea*. *Clavularia* sp. displayed no activity in either biosensor whereas *P. violacea* was only observed to induce QS in *A. tumefaciens* A136. Inhibition of QS in *C. violaceum* CV026 occurred across all polarity extracts, whereas QS inhibition by *A. tumefaciens* A136 was only unambiguously detected in the DCM and MeOH extracts. Interestingly, both induction and inhibition of QS was observed for the biosensor *A. tumefaciens* A136 (Figure 2.5), whereas none of the species tested, regardless of polarity or concentration, were able to induce QS in *C. violaceum* CV026 (Figure 2.5). For both biosensors, the detection of QS interference was considerably reduced at the lower dosage (4 µg) (Figure 2.6).

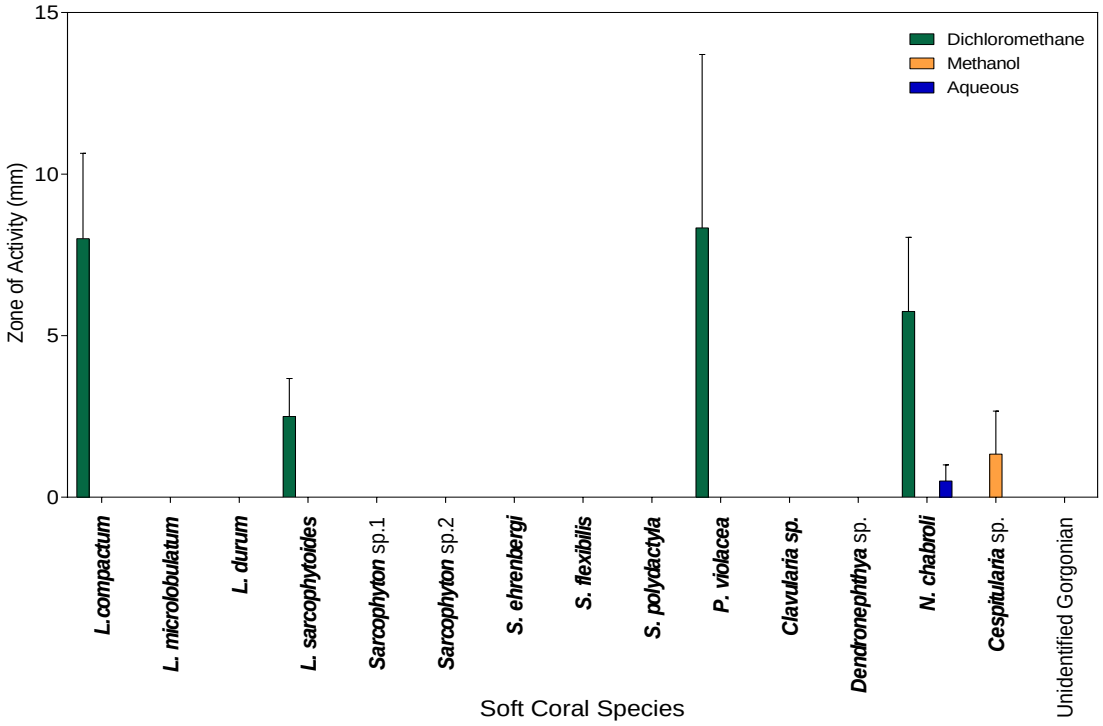

Figure 2.7: Results of the *Agrobacterium tumefaciens* A136 QS induction assay for the soft coral extracts from all polarity solvent extracts (dichloromethane, methanol and water). The bars represent positive responses, normalised to the response of the positive control (8.5 µMol N-hexanoyl-DL-homoserine lactone). Error bars represent the standard error from three screening efforts.

Induction of QS in *A. tumefaciens* A136 was observed in six extracts; primarily in the non-polar dichloromethane extracts where four species *(L. compactum, L. sarcophytoides, P. violacea* and *N. chabroli*) demonstrated strong inductive activity. In addition, induction

of QS was observed for the methanol extract of *Cespitularia* sp. and for the aqueous extract of *N. chabroli* (Figure 2.7). The largest halos of colouration were observed in the dichloromethane extracts of *L. compactum* (12 mm) and *P. violacea* (14.5 mm).

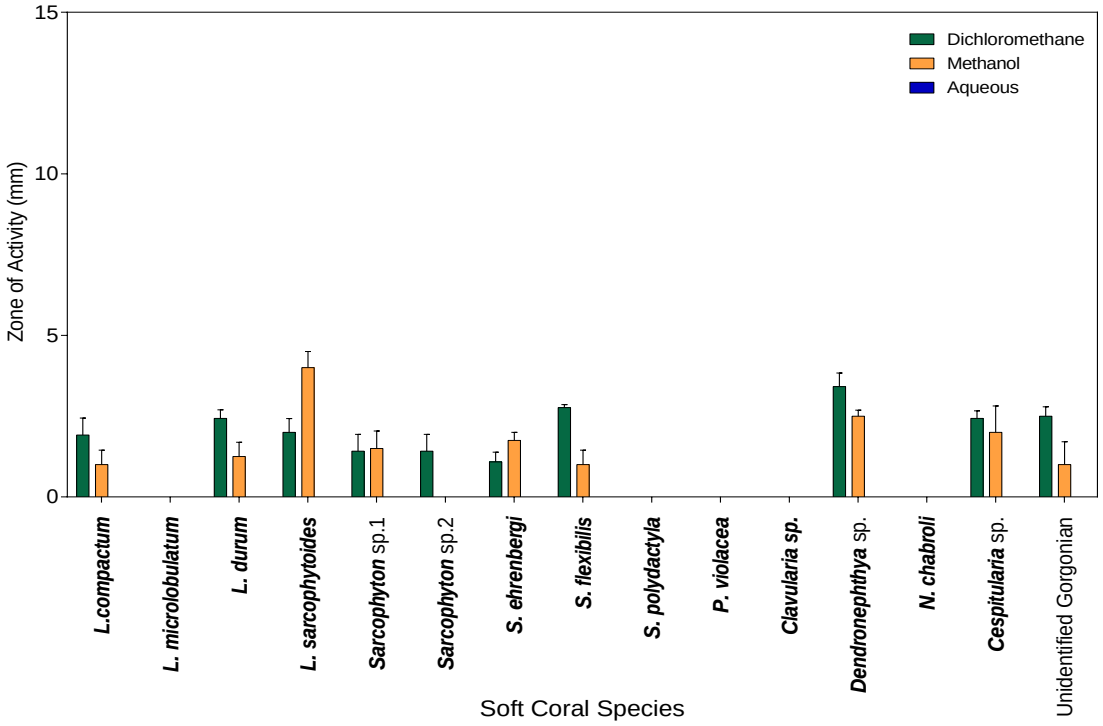

Figure 2.8: Results of the *Agrobacterium tumefaciens* A136 QS inhibition assay for the soft coral extracts from all polarity solvent extracts (dichloromethane, methanol and water). The bars represent positive responses, normalised to the response of the positive control (vanillin). Error bars represent the standard error from three screening efforts.

Inhibition in *A. tumefaciens* A136 was absent in the dichloromethane extracts of five species (*Clavularia* sp., *L. microlobulatum, S. polydactyla, P. violacea and N. chabroli*) (Figure 2.8). In contrast, inhibition of QS in *C. violaceum* CV026 was only absent from the dichloromethane extract of *P. violacea* (in addition to *Clavularia* sp.) (Figure 2.9). Interestingly, of the five species that were seen to induce QS, three species (*L. compactum, L. sarcophytoides* and *Cespitularia* sp.) were also responsible for the inhibition of QS in both biosensors (Figure 2.8, Figure 2.9). This contrasts with the polar and non-polar *N. chabroli* extracts which only inhibited QS in *C. violaceum* CV026 (Figure 2.9) and *P. violacea* which demonstrated no inhibitory activity towards QS in either biosensor (Figure 2.8, Figure 2.9).

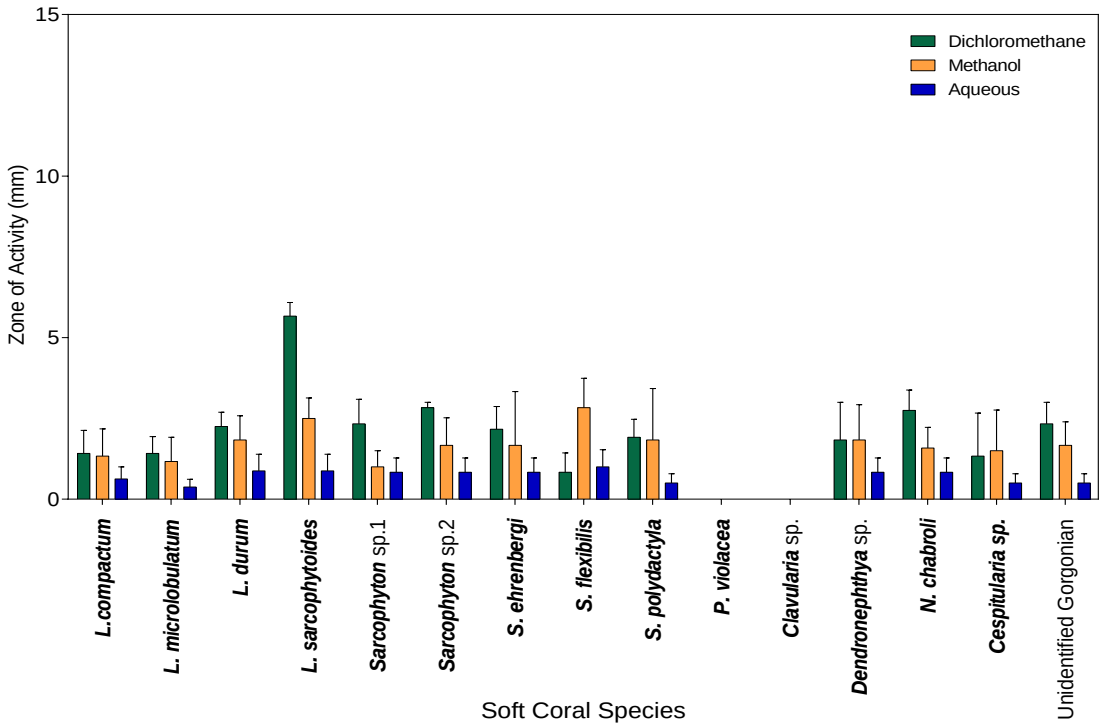

Figure 2.9: Results of the *Chromobacterium violaceum* CV026 QS inhibition assay for the soft coral extracts from all polarity solvent extracts (dichloromethane, methanol and water). The bars represent positive responses, normalised to the response of the positive control (vanillin). Error bars represent the standard error from three screening efforts

Inductive activity was greatly reduced at the lower dosage (4 µg)(Figure 2.6). Induction of QS in *A. tumefaciens* A136 (Figure 2.6) was only retained in the dichloromethane extracts of three species (*L. compactum, L. sarcophytoides*, and *P. violacea*; Figure 2.10). None of the aqueous extracts demonstrated QS induction at the lower dosage (Figure 2.6). The inhibitory activity observed against *A. tumefaciens* A136 at the lower dosage (4 µg) was only substantially determined in *S. flexibilis* when tested (Figure 2.10).

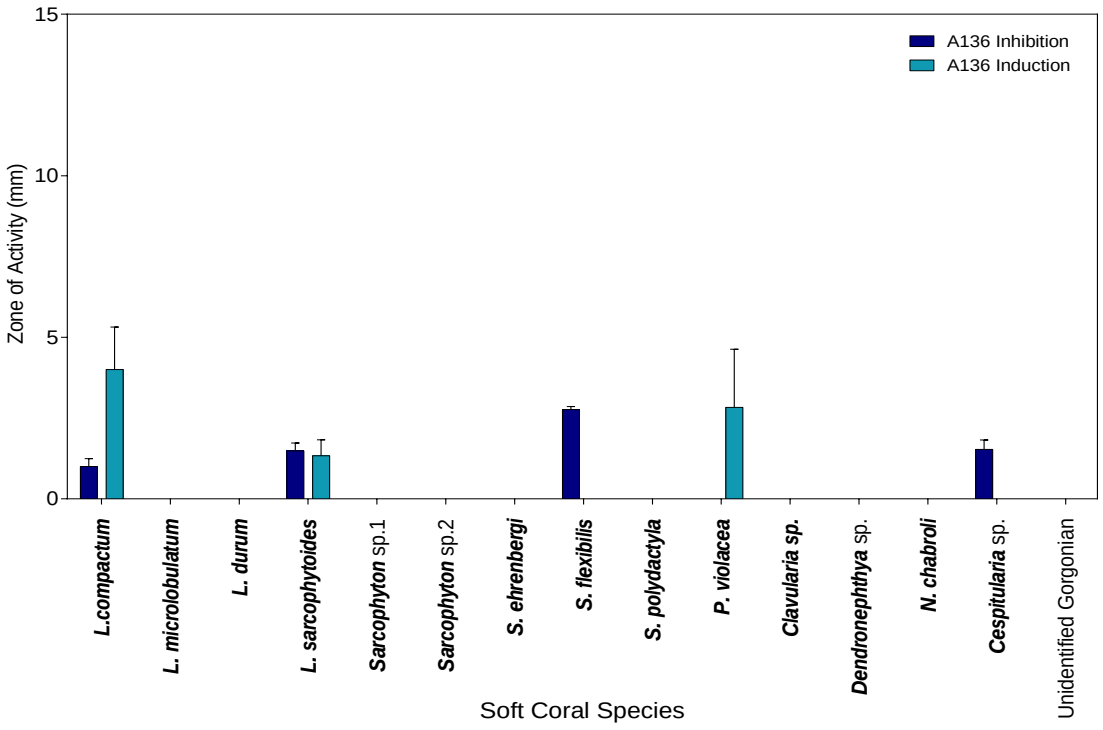

Figure 2.10: Results of the *Agrobacterium tumefaciens* A136 QS interference assays for the soft coral dichloromethane extracts. The bars represent positive responses, normalised to the response of the positive control (vanillin). Error bars represent the standard error from three screening efforts.

Inhibition and induction of QS patterns were not conserved across species for both *Lobophytum* and *Sinularia* genera. This contrasted with the consistency of the genus *Sarcophyton*. To further investigate this and to identify if the unexpected inductive activity is the result of similar chemical compounds to those causing the inhibition activity, nine soft corals from five genera were chosen for further fractionation (Table 2.1). Species chosen for fractionation displayed four profiles of activity: *C. violaceum* CV026 inhibition only (*L. microlobulatum* and *S. polydactyla*); inhibition of *A. tumefaciens* A136 and *C. violaceum* CV026 but not inductive activity (*L. durum, S. ehrenbergi*, and *S. flexibilis*); inhibition of both biosensors and induction of *A. tumefaciens* A136 (*L. sarcophytoides, L. compactum*, and *P. violacea*) and finally *A. tumefaciens* A136 inductive capability and *C. violaceum* CV026 only inhibitory capability (*N. chabroli*) (Table 2.1). Ten fractions of decreasing polarity were generated for the dichloromethane extracts of these soft corals.

2.3.1 Fraction results

For all species fractionated, at least two fractions induced QS in *A. tumefaciens* A136, regardless of whether their corresponding un-fractionated extracts were active (Table 2.2). In contrast, *C. violaceum* CV026 failed to be induced to QS by any fraction from any species, consistent with that observed during the screening of un-fractionated extracts (Table 2.2). All species that induced QS after fractionation likewise inhibited QS in at least one biosensor strain, with two exceptions: *Lobophytum* sp. and *P. violacea*. *Lobophytum* sp., which only inhibited *C. violaceum* CV026, was also consistent with un-fractionated results (Table 2.2). Similarly, *P. violacea,* did not inhibit QS in either strain, consistent with un-fractionated results. The within genera differences continued to be observed in *Sinularia* but were no longer apparent in *Lobophytum* due to the appearance of *A. tumefaciens* A136 QS induction for all species (Table 2.2).

Table 2.2: Active soft coral fractions by QS biosensor assay.

	QS Induction		QS Inhibition	
Species	**A136**	**CV026**	**A136**	**CV026**
Lobophytum compactum	4, 6-7	-	6	4-10
Lobophytum microlobulatum	6-7	-	5-8	4-10
Lobophytum sarcophytoides	6-7	-	6	4-10
Lobophytum durum	4-8	-	-	4-10
Sinularia flexibilis	3-4, 6	-	1-4, 8	1-6, 10
Sinularia polydactyla	6-7	-	7-8	4-10
Sarcophyton ehrenbergi	1-2, 7	-	2-10	4-10
Pachyclavularia violacea	6-7	-	-	-
Nephthea chabroli	3-4, 6-7	-	7	4-10

Three major bands of induction activity can be seen in the *A. tumefaciens* A136 bioassay, occurring at an elution of 60% methanol (fraction four), 80% methanol (fraction six) and 90% methanol (fraction seven) across the soft corals assayed (Table 2.2). Fraction four was active across *L. compactum, Lobophytum sp., N. chabroli and S. flexibilis* (Table 2.2), whereas fraction six was active for all species except for *S. ehrenbergi*. Similarly, fraction seven was active for all species except *S. flexibilis* (Table 2.2). Similar to the

extract responses, the two largest inductive haloes were observed for *N. chabroli* and *L. compactum*, the only species whose fractions retained their activity even at the 1:10 dilution level.

The distinct patterns of QS inductive activity observed for *A. tumefaciens* A136 strongly contrast with the broad *C. violaceum* CV026 inhibition activity observed from 60% methanol elution onwards (fractions four to ten) in seven of the nine species fractionated (Table 2.2). In the *A. tumefaciens* A136 QS inhibition bioassay, similar to the induction assay, activity was predominantly located in fractions six and seven (Table 2.2). In this bioassay, *S. flexibilis* was again distinguished by not being active in fraction seven and was the only strain to inhibit QS in fractions one to three (with activity also detected in fractions four and eight (Table 2.2). Three species demonstrated *A. tumefaciens* A136 QS inhibition activity in only one fraction*: L. compactum* (fraction six), *L. sarcophytoides* (fraction six) *and N. chabroli* (fraction seven).

2.4 Discussion

Interference with QS regulated activity was detected in extracts of three different polarities from soft corals using two different biosensors, *A. tumefaciens* A136 and *C. violaceum* CV026. Neither induction nor inhibition of QS by soft coral extracts was limited to those species from Alcyoniidae known to contain cembranoid diterpenes. Strong QS induction responses were observed for the dichloromethane extracts of *L. compactum* (Alcyoniidae), *L. sarcophytoides* (Alcyoniidae), *P. violacea* (Clavulariidae) and *N. chabroli* (Nephtheidae). Induction was also observed in the methanol extract of *Cespitularia* sp. (Xeniidae) and the aqueous extract of *N. chabroli.* Cembranoid diterpenes are well documented in eleven of the soft coral species tested and may be responsible for the observed activity in these species (91). The non-polar nature of these compounds means they would be expected to extract in the DCM fraction where the majority of induction activity was observed. The same diterpene scaffolds, however, are

not commonly known in the genera *Nephthea* and *Cespitularia* or in gorgonian corals, so a role for these compounds cannot be confirmed at this stage.

Widespread activity, across not only species but polarity barriers is indicative of active compounds of more than one structural type and raises the possibility of active compounds that are common across soft coral species. The widespread prevalence of QS inhibition as well as the presence of QS induction in the soft corals screened here are of a similar level to QS activity found across a range of marine invertebrates (96, 102, 51). For example, QS mimics have a well established role in the regulation of biofilms associated with the marine alga *Delisea pulchra* (33). Unlike the sole QS inhibition activity that was identified in *D. pulchra*, gorgonian coral extracts were found with activity for both induction and inhibition of QS as well as antibiotic activity (51). QS induction was also established in extracts of marine sponges and sponge associated bacteria (102). The *in situ* ecological role of QS activity in the holobionts of soft corals, marine sponges and gorgonians has yet to be established. Results from this study, however, highlight the need to examine both induction and inhibition of QS across the entire metabolome to generate a realistic understanding of the complexity of ecological interactions between a host organism and its associated bacteria. The presence of QS compounds in the soft coral extracts studied here could be evidence of cross communication (either microbe-microbe or coral-microbe) and may represent a mechanism of regulation of their associated microbial community.

In contrast with the initial extract testing, all of the fractionated soft corals displayed at least one active fraction in the *A. tumefaciens* A136 QS induction assay. The increase in the presence of induction activity reduced the within genera differences seen in the four *Lobophytum* samples. This may reflect an inherent increase in concentration of the active components or a decrease in complexity of the samples being tested. Soft coral extracts and fractions tested may be highly complex mixtures of compounds. For instance, dichloromethane extracts of soft corals are likely to contain primary metabolites such as lipids, sterols and other cellular components in addition to secondary metabolites. The fractions, likewise, might also contain multiple metabolites. For this reason, the

contra-intuitive activity profiles could be the result of multiple compounds that have contrasting QS regulatory activity. It follows that the potential for activity masking within extracts, a phenomenon that was previously observed in the screening of QS activity in marine sponge extracts (102) is high. Such false negatives are more likely to be undetected when only one concentration or level of complexity is tested.

Of particular note is that most of the active species that induced QS in *A. tumefaciens* A136, except *P. violacea,* also actively inhibited both *A. tumefaciens* A136 and *C. violaceum* CV026. None of the *P. violacea* fractions inhibited QS in either strain, consistent with the results from the un-fractionated extract. The QS inductive activity was generally limited to one or two bands of active fractions for each species, this pattern of activity that was not reflected in the inhibition activity. In addition, the bands of induction activity usually occurred in the same polarity fractions (80% and 90% methanol elution) for each species of soft coral tested and suggests that the inductive capability may be due to the presence of structurally similar compounds. The fractions with both forms of activity may contain compounds that have opposing activity or compound(s) that can both inhibit and induce QS systems. In contrast, the presence of the inhibition of QS in *C. violaceum* CV026 across nearly 70% of the fractions tested, suggests that the activity is either due to multiple compounds, or, the compound(s) responsible are not suited to the method of fractionation used. If the compounds become spread across multiple fractions there will be a corresponding bleeding effect in the activity.

The QS inductive responses observed in *A. tumefaciens* A136 were not replicated in *C. violaceum* CV026. In contrast, the presence of *A. tumefaciens* A136 inhibitory activity was not observed without a corresponding response in *C. violaceum* CV026, a relationship that was not reciprocal. It is possible that *C. violaceum* CV026 exhibits a greater sensitivity to inhibition by these soft coral extracts than *A. tumefaciens* A136. *C. violaceum* CV026 is known to be inhibited by long chain AHL molecules (26), whereas *A. tumefaciens* A136 may be induced to QS by an AHL with an acyl chain of up to fourteen carbons (24). It is also possible that the fractions showing only *C. violaceum* CV026 inhibition may contain trace residues of the active compound(s) but at lower

concentrations such that only *C. violaceum* CV026 shows inhibition. The differential responses of the two biosensors to the same sample highlights the advantage of using multiple biosensors. In addition, differential responses are likely to occur within a microbial biofilm and reflect the potential complexity of interactions that QS mimics can be involved in.

2.5 Conclusion

Soft coral extracts have the ability to interfere and induce QS pathways of Gram negative bacteria. The ability of soft corals to interact with QS could be important to the health and resilience of the host organism and may reflect a more widespread strategy of sessile marine invertebrates. Consequently, a decision to further purify these extracts was made in order to definitively identify the compound(s) responsible for this activity (Chapter 3).

Chapter 3

Structural Variation of Soft Coral Cembranoid Diterpenes and Quorum Sensing Interference

3.1 Introduction

The discovery of QS interference by soft coral extracts (103; Chapter 2) provides an opportunity for a structural investigation of QS mimics in a natural system. Compounds which mimic QS signal molecules are of particular interest due to both their role as inter-species or inter-kingdom communication markers and also their potential as pharmaceutical agents (41). Disruption and mimicry of QS signals are emerging as commonly employed mechanism for macro-organisms to regulate and manipulate associated microbial communities. Disruption of QS can inhibit the coordination of a pathogen's virulence attack without affecting the growth of the organism, vastly slowing any resistance selection pressure when compared to the action of antibiotics (99). Manipulation of QS could also strengthen the resilience of the associated community against pathogen invasion (22, 109).

The biological activity of many of the QS mimics that have been discovered appears to be specific to QS, suggesting that their mechanism of action is similar to the true bacterial signal molecules. In the case of Gram negative bacteria, the activity of the AHL molecules has been intensely studied; QS activation is achieved through a molecular lock and key mechanism (110). The AHL molecules bind to the QS receptor protein to form a complex that acts as a transcriptional regulator and in order for mimic compounds to specifically interfere with this system, it is hypothesised that they must also be able to bind to (or otherwise isolate) the receptor protein. The results of these studies suggest that the γ-lactone ring is required for any QS activity and that the length and functionality of the acyl side chain provides specificity (Figure 3.1) (19, 21, 111). For this reason, it has been hypothesised that the corresponding QS mimics would also contain a γ-lactone ring or homologous functionality. This hypothesis is supported by a study of the QS mimics of the red alga *Delisea pulchra*, the furanones (one of the few QS inhibitors to have been structurally elucidated), where the presence of the oxygenated ring was likewise demonstrated to be essential to their activity (Figure 3.1) (36).

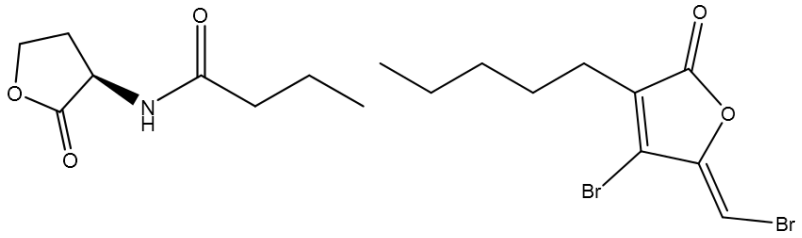

Figure 3.1: a) butyryl homoserine lactone b) halogenated furanone

Soft corals contain a number of secondary metabolites with the structural potential to mimic QS. Prime candidates are furanocembrenes (cembranoid diterpenes with fused 5-membered ether rings) and cembranolides (cembranoids that possess a fused second ring in the form of a lactone). The apparent requirement for the mimic compound to possess an oxygenated ring in the form of a lactone implicates these cembranoid diterpenes as likely sources of the QS interference identified (Chapters 2 and 3; 96). Cembranoid diterpenes are inherently variable in the presence, position and size of oxygenated ring systems (Figure 3.2) (91). In addition, variation of substituents, direction of cyclisation and the corresponding position of the isoprenoid double bonds of different diterpenes from different species of soft coral are also encountered (Figure 3.2) (91). The activity of QS across the range of soft coral species assessed in Chapter two and also by Skindersoe and colleagues (96), demonstrates both induction and inhibition of QS interference that justifies further investigation. Therefore, cembranoid diterpenes from soft corals represent a natural pool of compounds to investigate structure activity relationships.

This study aimed to assess the potential of soft coral cembranolides and furanocembrenes to interact with QS. To this end, cembranolides and furanocembrenes were isolated from soft coral species and tested for their ability to induce or inhibit QS in two bacterial biosensors, *Agrobacterium tumefaciens* A136 and *Chromobacterium violaceum* CV026. The implications of the detected variability in QS activity for the resilience of the host organism are also discussed.

3.2 Experimental

3.2.1 Screening for AHL interference

All compounds were tested for induction and inhibition of QS in the biosensors *Agrobacterium tumefaciens* A136 and *Chromobacterium violaceum* CV026 as described by Ravn and colleagues (107) and in Chapter Two. In order to generate dose response curves the following modifications were required: compounds were resolubilised in ethanol and serially diluted to generate five different concentration solutions (x10^2 μM to 1 x10^{-2} μM) for each compound. Agar containing the biosensors was poured into custom built moulds with 28 preformed wells 4 mm in diameter and after solidification of the agar, 20 μl of diluted compound (1 x10^2 μM to 1 x10^{-2} μM) was added to each well. N-heptanoyl-DL-homoserine-lactone and N-octanoyl-DL-homoserine-lactone were used as positive controls for QS induction and ethanol was used as a negative control. Positive results for QS induction were read as formation of blue colouration in *A. tumefaciens* A136 and formation of purple colouration in the plates with *C. violaceum* CV026 (Figure 2.3). QS inhibition was tested using the same custom moulds and dilution series, with the exception that a 10 μM concentration of N-hexanoyl-DL-homoserine lactone was added with the biosensor strains into the agar. Vanillin was used as the positive control for QS inhibition (50) and ethanol as the negative control. Positive results for QS inhibition were read as inhibition of blue colouration in *A. tumefaciens* A136 and inhibition of purple colouration in the plates with *C. violaceum* CV026 (Chapter 2).

3.2.2 Soft coral and soft coral metabolite selection

Soft coral metabolites were chosen to assess the effect of structural variation on QS interference. Isolobophytolide (*Lobophytum compactum*), lobolide (*Lobophytum* sp.) and sarcophine (*Sarcophyton glaucum*) all possess a five membered (γ) lactone ring (with α, β unsaturation in the case of sarcophine) (Figure 3.2; Table 3.1). Flexibilide and

Table 3.1: Functional Group Analysis of Tested Soft Coral Metabolites

#	Cembranoid Diterpene	Secondary Ring Type	Secondary Ring Position	Other Oxygen Functionality	Double Bonds	Exo methylene	-OH	Oxygenated side chain	Acetate
1	Isoneocembrene A	Absent	Absent	Absent	C4-C5, C8-C9, C12-C13, C14-C1	Absent	Absent	Absent	Absent
2	Isolobophytolide	γ-lactone	C1-C2	C12-C13 (Epoxide)	C4-C5, C8-C9	Present	Absent	Absent	Absent
3	Isolobophytolide monoacetate	γ-lactone	C1-C2	C12-C13 (Epoxide)	C4-C5, C8-C9	Present	Absent	Absent	Present
4	Lobolide	γ-lactone	C1-C14	C3-C4 (Epoxide)	C7-C8, C11-C12	Present	Absent	On C4	C20
5	Sarcophine	α, β unsaturated ɣ-lactone	C1-C14	C8-C9 (Epoxide)	C4-C5, C12-C13	Present	Absent	Absent	Absent
6	Flexibilide	δ-lactone	C1-C3	C11-C12 (Epoxide)	C7-C8	Present	C4	Absent	Absent
7	Dihydroflexibilide	δ-lactone	C1-C3	C11-C12 (Epoxide)	C7-C8	Absent	C4	Absent	Absent
8	Sinulariolide	ε-lactone	C1-C4	C12-C13 (Epoxide)	C8-C9	Present	C5	Absent	Absent
9	Pachyclavulariadiol	furan	C1-C2	C9-C12 (Ether)	C1-C2, C14-C15, C15-C16	Absent	Absent	Absent	Absent
10	Diacetyl Pachyclavulariadiol	furan	C1-C2	C9-C12 (Ether)	C1-C2, C14-C15, C15-C16	Absent	Absent	Absent	C13, C14

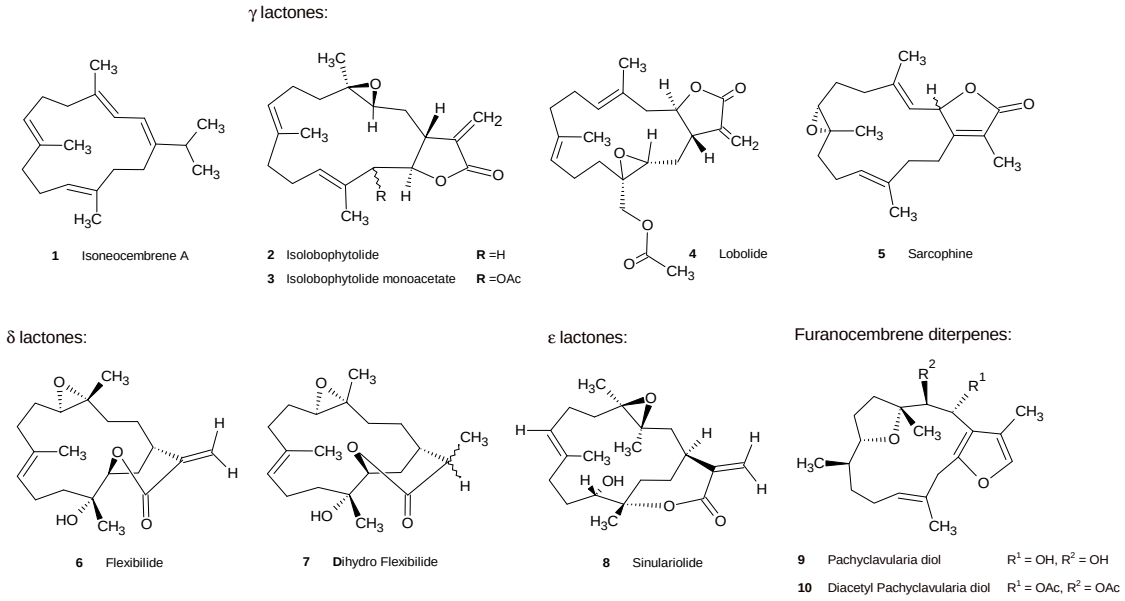

Figure 3.2: Soft coral cembranoid diterpenes tested for QS interference capability.

dihydroflexibilide from *Sinularia flexibilis* both possess six membered (δ) lactone rings, whereas sinulariolide (also from *Sinularia flexibilis*) possesses a seven membered (ε) lactone ring (Figure 3.2; Table 3.1). Pachyclavulariadiol and diacetyl pachyclavulariadiol were also examined as they contain furan rings (Figure 3.2; Table 3.1), similar to the furanones of *D. pulchra*. Finally a cembrene without the secondary oxygen ring functionality was also assessed in the form of isoneocembrene A from *Nephthea* sp. (Figure 3.2; Table 3.1).

3.2.3 Soft coral collection and extract preparation

Lobophytum compactum, *Sinularia flexibilis* and *Pachyclavularia violacea* were collected at a depth of 1-3 m from Orpheus Island (Great Barrier Reef, Australia; latitude, 18° 36.878' S; longitude, 146° 29.990' E) by the method described previously (Chapter 2). Briefly, all specimens were photographed underwater before sampling, then soft coral tissue samples were placed directly into plastic bags with seawater, transported to land, frozen within an hour at -80°C and stored prior to freeze drying. The dried coral tissue was weighed and homogenised before being exhaustively extracted with dichloromethane

(DCM). Extracts were concentrated via rotary evaporator and dried under a stream of nitrogen and stored at -20°C prior to cembranoid diterpene isolation.

3.2.4 Pure samples of cembranoid diterpenes tested: isoneocembrene A (1), lobolide (4) and sarcophine (5)

Pure samples of isoneocembrene A (**1**) lobolide (**4**) and sarcophine (**5**) were acquired from the Bowden Laboratory, Townsville Australia. Structure elucidation and purity checks were undertaken with NMR spectroscopy (Appendix) and literature comparisons (112, 113, 114, 115).

3.2.5 Isolation of isolobophytolide (2) and isolobophytolide monoacetates (3) from *Lobophytum compactum*

Vacuum liquid chromatography of active crude extracts (2 g) was performed over reverse phase C18 silica gel (Phenomenex Luna 10 µm C18 silica gel) and 10 fractions (200 ml) were collected using methanol: water 0-100% stepwise gradient for each extract. Activity was identified in the 80% methanol fraction. This fraction was subjected to RP-HPLC 60-100% MeOH gradient over 30 min (Phenomenex Gemini 3 µm NX-C18 110 Å, LC Column 30 x 4.6 mm,). Isolobophytolide (**2**) eluted at 15 min and the two isomers of isolobophytolide monoacetate (**3**) eluted at 17 min. Structure and purity was confirmed by 1D and 2D NMR experiments and comparison with literature values (116).

3.2.6 Isolation of flexibilide (6), dihydroflexibilide (7) and sinulariolide (8) from *Sinularia flexibilis*

Flexibilide and dihydroflexibilide were isolated as reported by Kazlauskas and colleagues (117). Briefly, *Sinularia flexibilis* was freeze dried and extracted with dichloromethane.

The dichloromethane extract was subjected to normal phase flash column chromatography with combinations of hexane, dichloromethane and ethyl acetate. Flexibilide (**6**) was isolated using 6 : 1 dichloromethane:ethyl acetate. Further elution of this column using 4 : 1 dichloromethane:ethyl acetate yielded a mixture of **6** and **7** followed by 3 : 1 dichloromethane:ethyl acetate which yielded pure dihydroflexibilide (**7**). Sinulariolide (**8**) was isolated separately with a method adapted from Tursch (118) in an ether partition of the dichloromethane extract. Purification of compounds was performed by HPLC. Structures were confirmed through 1D and 2D NMR experiments (Appendix B) and comparison with literature values (117, 118).

3.2.7 Isolation of pachyclavulariadiol (9) and diacetyl pachyclavulariadiol (10) from *Pachyclavularia violacea*.

Due to instability of the furanocembrenes from *P. violacea*, instead of utilising repeat fractionation, the pure compounds were generated semi-synthetically as per Bowden and colleagues (119). Briefly, lyophilised tissue was exhaustively extracted with cold (4°C) dichloromethane. The dichloromethane extract was then partitioned between hexane and aqueous methanol before normal phase flash chromatography of the aqueous methanol fraction with a hexane: ethyl acetate gradient. All fractions containing (by TLC and ^{1}H NMR) pachyclavulariadiol, diacetyl pachyclavulariadiol and the two monoacetyl pachyclavulariadiols were combined. This mixture was converted to pachyclavulariadiol (9) by incubation for 24 hours at room temperature with methanol containing 1% (w/v) potassium hydroxide. Methanol was removed under vacuum and the residue was partitioned between diethyl ether and water. The ether fraction was recovered and dissolved in hexane. Diacetylpachyclavulariadiol (**10**) was acquired by acetylation of half of the obtained pachyclavuriadiol (**9**). Acetylation was effected by incubation for 24 hours with 1 : 1 acetic anhydride and pyridine before evaporation of the solvent and retrieval via liquid partitioning between hexane and water. The semi-synthesis of monoacetyl pachyclavulariadiol was not performed as the reaction would have yielded both isomers.

Consequently the decision was made not to pursue this compound unless differences were observed between the QS interference capability of pachyclavulariadiol and diacetyl pachyclavulariadiol. Structural and purity checks were undertaken with 1D and 2D NMR experiments and comparison with literature values (119; Appendix B).

3.2.8 General characterisation methods

^{1}H - NMR (600 MHz) and ^{13}C - NMR (150 MHz) spectra were recorded with a Bruker 600 Avance spectrometer in $CDCl_3$, with TMS as internal standard. High resolution mass spectra were collected using an unmodified Bruker BioAPEX 47e mass spectrometer equipped with an Analytica model 103426 (Branford, CT) electrospray ionization (ESI) source. Analytical TLC was performed on Merck Kieselgel 60. Spots were visualized by UV light or by spraying with a 1% vanillin in acidified ethanol solution.

3.3 Results

QS interference appears to be caused by cembranoid diterpenes within the coral tissue (Figure 3.3). The effect of these compounds on QS was, however, dependent on the biosensor strain utilised. None of the cembranoid diterpenes tested demonstrated QS induction capability against *C. violaceum* CV026 (Data not shown). QS activity was observed against *A. tumefaciens* A136 over three to four orders of magnitude, with a loss of activity at higher concentrations (Figure 3.3). The ability to interact with the QS mechanism was only observed for cembranoid diterpenes possessing secondary oxygen rings, no activity was observed for isoneocembrene A (**1**). Peak QS interference occurred at approximately 1 x 10^{-5} μM (or 3 ppm; Figure 3.3).

Strength and type of QS interference was observed to correlate with the size and type of secondary oxygenated rings possessed by the metabolite (Figure 3.3). The strongest induction of QS in *Agrobacterium tumefaciens* A136 was observed from the furanoditerpenes pachyclavulariadiol and diacetylpachyclavulariadiol (Figure 3.3). Induction was

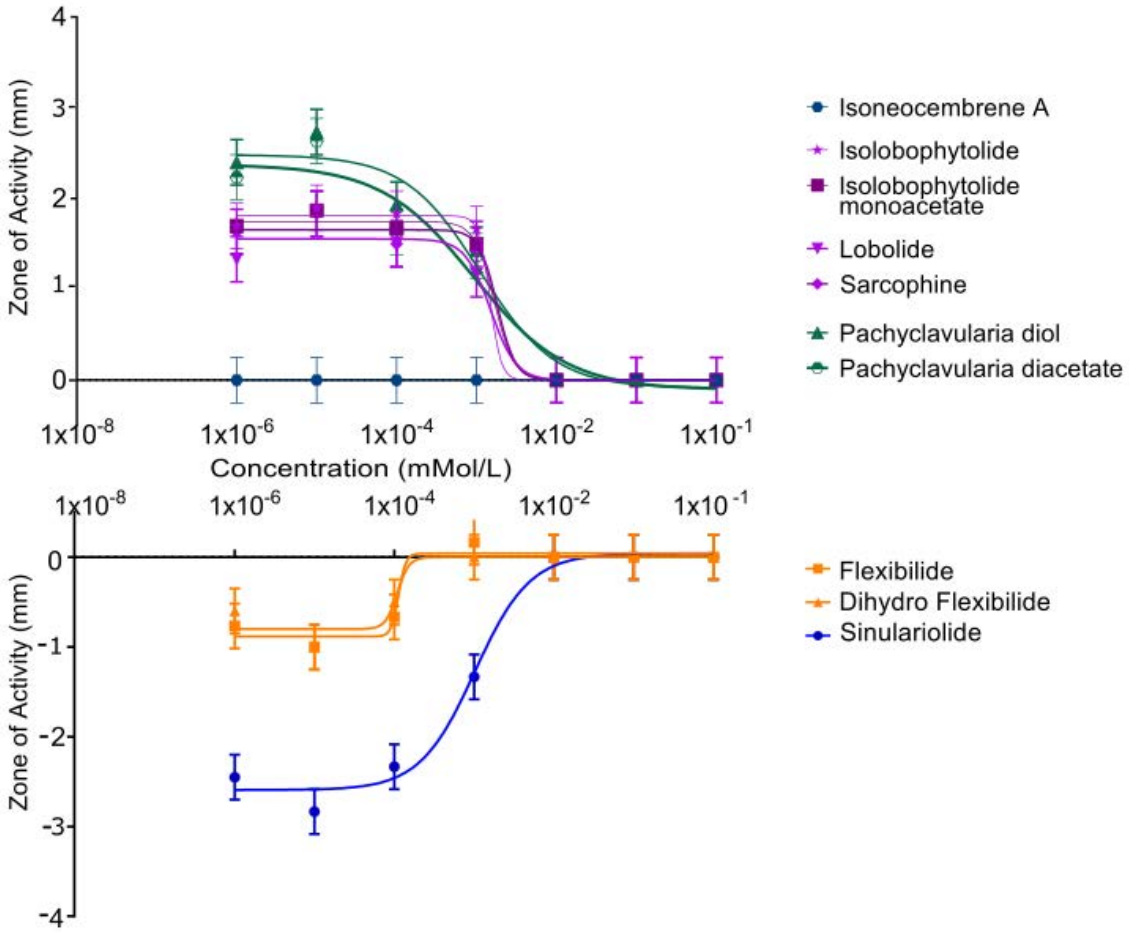

Figure 3.3: Dose response patterns of QS in *Agrobacterium tumefaciens* A136 in cembranoid diterpene compounds isolated from soft corals. A zone of activity is defined as the size in mm of either pigment production or pigment inhibition. Concentration refers to the concentration of the compound that was present in the agar wells.

also observed in the cembranolides isolobophytolide, lobolide and sarcophine that possess γ-lactone (5 membered) rings (Figure 3.3). In contrast the δ- and ε-lactones (6 and 7 membered) rings respectively possessed by the cembranolides flexibilide, dihydroflexibilide and sinulariolide demonstrated the ability to inhibit QS in this strain (Figure 3.3). QS inhibition was strongest in the ε-lactone ring represented by sinulariolide (Figure 3.3).

Other functional groups (including epoxides and exomethylenes) showed essentially indistinguishable effects on QS interference of *A. tumefaciens* A136 (Figure 3.3). The furan diacetylpachyclavulariadiol was observed to result in smaller activity zones than pachyclavulariadiol, but within the error limits (Figure 3.3). Consequently, the monoacetyl pachyclavulariadiols were not pursued for testing at this stage. Similarly, no significant

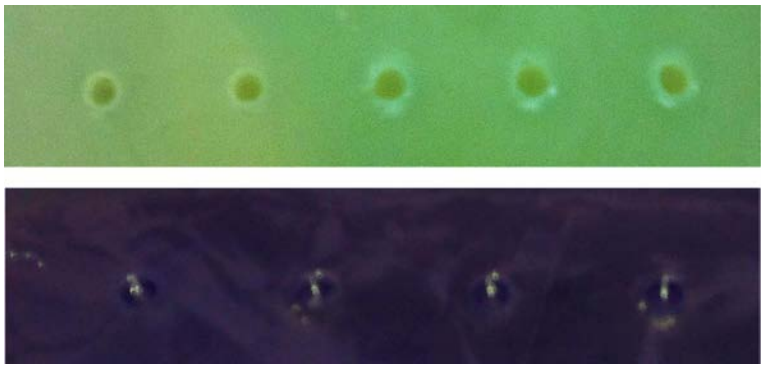

Figure 3.4: Replicate wells showing no effect on QS or growth by flexibilide against the biosensor strains *A. tumefaciens* A136 and *C. violaceum* CV026

differences were observed between isolobophytolide and isolobophytolide monoacetate. Interestingly, the furans tested appeared to have higher activities than the lactones and the presence of an exomethylene group had little effect (Figure 3.3). An epoxide moiety within the cembrene ring did not significantly alter the activity either (compare flexibilide and dihydroflexibilide; Figure 3.3). No growth inhibition zones were apparent in the biosensors for the cembranoid diterpenes at the concentrations tested. The biosensor strains were observed to grow within the walls of the test wells and no affect on the QS activity was observed at higher doses in the QS inhibition assay (Figure 3.4). Although, this could also be partially due to the lipophilic nature of the molecules tested and poor diffusion characteristics in the aqueous medium.

3.4 Discussion

This study aimed to determine the potential for cembranolides and furanocembrenes of soft corals to act as QS mimics. The results presented here clearly show that cembranolides and furanocembrenes isolated from soft corals are all, at least partially, responsible for previously observed QS interference in soft coral extracts (Chapter Two; 96) and may be acting as QS mimics within the soft coral holobiont. Cembranolides and furanocembrenes potentially represent a new structural backbone for Gram negative AI-1 QS mimic compounds. However, functional group overlap, in the form of the oxygenated ring system, between these compounds and previously elucidated QS mimic compounds

exists.

A strong relationship was observed between the presence of the type of oxygenated functional groups with the form of interference demonstrated. Those cembranoid diterpenes that contained either a furan ring or five membered lactone were capable of inducing QS in *A. tumefaciens* A136, whereas the cembranolides with larger lactone rings (six or seven membered) were seen to inhibit QS in *A. tumefaciens* A136. In keeping with our current understanding of the QS mechanism, the presence of other functional groups (epoxides, acetates or level of saturation) had minimal discernible impact on the strength of activity observed and no obvious effect on the type of activity observed with respect to *A. tumefaciens* A136. QS mimics have previously been isolated that possess a γ-lactone, however, these mimics (such as the furanones of *D. pulchra*) are often associated with QS inhibition rather than the inductive activity demonstrated here (35, 120). In the case of the furanones from *D. pulchra,* bromine substituents are also present and may be influencing the type of activity (Figure 3.1). The impact of these different functional groups on the activity of QS mimics is important if new pharmaceuticals based around this form of interaction are to be designed.

Strong evidence for the ecological role of cembranolides and furanocembranoid diterpenes as QS mimics is exhibited in the selective QS interference capacity. This reflects patterns of selectivity in AHL signal molecules. The two biosensors used in this study demonstrate different sensitivities to AHL molecules. *A. tumefaciens* A136 utilises the TraR QS receptor that responds to AHLs with acyl side chains of four to fourteen carbon atoms, whereas *C. violaceum* CV026 which utilises the CivR QS receptor, only responds to acyl side chains of six to eight carbon atoms (24). QS mimics have previously been indicated to be involved in processes that both encourage or disrupt QS phenotypes in host associated bacteria (67). Micro-organisms are important members of the coral holobiont and represent both a mechanism of resilience for the coral host as well as agents of disease (67). It is therefore likely that any chemical agents mediating this complex community would have different effects on different species.

All of the active compounds present have previously been reported to demonstrate bio-

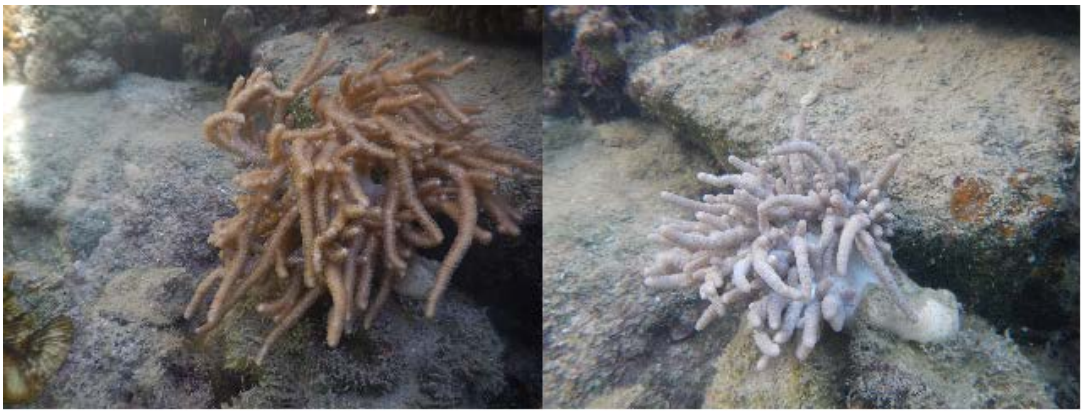

Figure 3.5: *Sinularia flexibilis* colony showing change in volume over three hours

logical activities. Flexibilide, dihydroflexibilide and sinulariolide, for example, have been identified as possessing antimicrobial activity (growth inhibition), however, this activity was primarily observed in the Gram positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* at 5 ppm and above (95). In contrast, inhibition of Gram negative bacteria only occurred at concentrations of 50 ppm and higher (95). The concentrations at which these cembranoid diterpenes demonstrate QS interference activity, however, reflect realistic *in situ* concentrations for these compounds. The peak QS active concentration occurred at 3 ppm, reflecting the concentrations of flexibilide and sarcophytoxide in the mucous and water column surrounding *S. flexibilis* and *S. crassocaule* detected by Coll and colleagues (121). In addition, this is supported by a previous suggestion that a number of natural products isolated previously and designated as antibiotic or antimicrobial compounds (by growth inhibition) were involved in cell signalling at lower ecological concentrations (8). However, it is possible that some of these QS interference compounds may be acting in either a growth inhibition or QS interference capacity within the coral colony. A feature of many soft coral species in which the sclerites are loosely packed, is a colony volume that can fluctuate rapidly. The uptake or release of water from the tissue of such soft coral colonies can be observed to reach both extremes of volume over a matter of hours (personal observation; Figure 3.5). The result of this is that any associated metabolites will show correspondingly dramatic change in concentration on a volumetric basis (Figure 3.5) over the same time period.

3.5 Conclusion

Although the producer of these compounds is still under debate, there is a strong correlation between their presence, structure and taxonomy. It is possible that these compounds are mediating interactions between the soft coral and its associated microbial community. There is clear potential for these compounds to moderate the soft coral associated bacterial communities, however, further investigation is required to elucidate their true *in situ* roles. Whether or not these compounds are QS mimics in an ecological setting, there is a lot that can be learnt from their structural variation for the design and search of QS pharmaceuticals.

Chapter 4

Quorum Sensing Without a Lactone? Quorum Sensing Induction in *Nephthea chabroli*

4.1 Introduction

QS signal mimic compounds produced by host organisms are thought to represent an evolved and stable mechanism of interaction with bacterial communities and pathogens (122, 23) and have been highlighted as potential pharmaceutical targets. Gram negative bacteria are an urgent target for this form of pharmaceuticals due to the rapidity with which resistance spreads throughout a population (2). Consequently, knowledge of the structural plasticity of these mimic compounds is vital if any pharmaceutical effort is to effectively take advantage of this system.

The primary form of QS signal molecules for Gram negative bacteria are, as previously mentioned, the acyl homoserine lactones (AHLs) of the auto-inducer one (AI-1) system. The γ-lactone ring moiety of AHLs is considered to be essential for their ability to bind to QS receptors (20). In accordance with this, it was assumed that QS mimics would likewise possess a γ-lactone or equivalent functionality (123, 120). QS mimics identified for the AI-1 system have, however, been found with variable oxygen functionality (120). Oxygenated functionality of QS mimics has included γ-lactone rings (123, 120), different sized (δ- and ε-) lactone rings (Chapter 3), and non-lactone (furan) rings (Chapter 3). QS mimics have even been found lacking oxygenated ring functionality altogether in the form of the alkylated cyclosulfanes of garlic (124) and the diketopiperizines of *Streptomyces* (125). The mechanism by which these non-lactone mimics interact with AI-1 is, unfortunately, poorly understood. The combined lack of structural knowledge of QS mimics and their mechanism(s) of interaction directly hampers the search for new pharmaceuticals (16).

The genus *Nephthea* is known to contain numerous terpenoid and steroid metabolites (126, 127). A previous study (Chapter 2) identified that the dichloromethane extract of the soft coral *Nephthea chabroli* contained strong QS inductive capacity against the QS biosensor *Agrobacterium tumefaciens* A136. Unlike the soft coral species focussed on in Chapter 3, however, *N. chabroli* is not well known to contain cembranolides or furanocembranoid diterpenes. Instead, this species is heavily dominated by the presence

of sterols. Two steroid hormones, β-estradiol and progesterone, were previously implicated as potential QS interference compounds during an in silico investigation (128). This current study investigated the observed QS induction activity of *N. chabroli* to determine the structural identity of the QS mimic(s) present. This chapter reports the isolation and identification of a new sterol, 17,22-dihydroxy 24-methylene cholesterol **1** and the known compound 24-methylene cholesterol **2** (Figure 4.1) from a *Nephthea* that also contained a number of known caryophyllene-based diterpenoid structures.

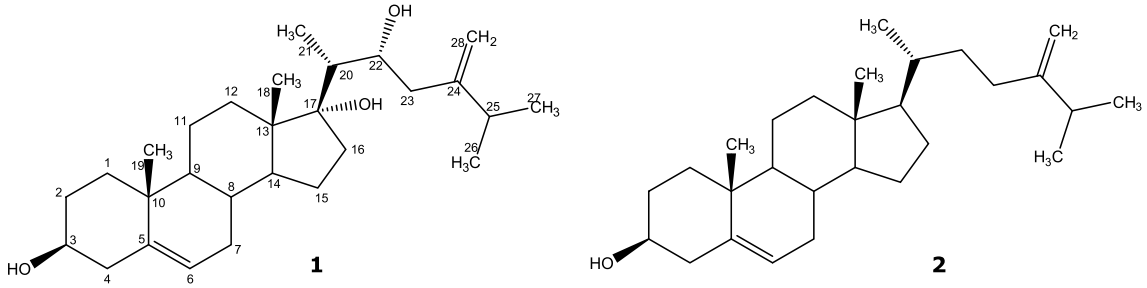

Figure 4.1: Compound **1** new polyhydroxylated sterol: 17,22-dihydroxy-24-methylene cholesterol and Compound **2** 24-methylene cholesterol structure isolated from the soft coral *Nephthea chabroli*

4.2 Experimental

4.2.1 Collection of *Nephthea chabroli*

Colonies of *Nephthea* (Figure 4.2) were collected at a depth of 1-3 m from Cattle Bay at Orpheus Island (Great Barrier Reef, Australia; latitude, 18° 36.878' S; longitude, 146° 29.990' E) by the method described previously (Chapter 2) and tentatively classified as *Nephthea chabroli*. Briefly, soft coral tissue samples were placed directly into plastic bags with seawater, transported to land and stored at -80°C prior to lyophilisation. The dried coral tissue was weighed and homogenised before being exhaustively extracted with dichloromethane. Extracts were concentrated via rotary evaporation and dried under a stream of nitrogen. Extracts were stored at -20°C prior to bioassay guided purification. Specimens were photographed underwater before sampling.

Figure 4.2: *Nephthea chabroli* colony collected from Cattle Bay, Orpheus Island.

4.2.2 Bioassay guided compound isolation

The crude dichloromethane extract (2.46 g) was fractionated to isolate the active compound(s). A vacuum-assisted normal phase silica gel column (50 mm diameter) was dry packed and prewashed with hexane, 4-6 times before use. An aliquot (60 ml) of each of the following solvents were applied to the column (in the order of increasing polarity): hexane (100%), hexane : dichloromethane (1 : 1), dichloromethane (100%), dichloromethane : ethyl acetate (9 : 1), dichloromethane : ethyl acetate (1 : 1), ethyl acetate (100%), ethyl acetate : methanol (1 : 1), methanol (100%). These eight fractions were collected, dried, weighed and subjected to the *Agrobacterium tumefaciens* A136 QS bioassay as described previously (Chapters 2 and 3) (107). Fractions displaying activity in the bioassay were pursued to identify the compound(s) of interest. Purity was assessed in each fraction through thin layer chromatography (TLC) analysis and ^{1}H NMR spectra. Fractions identified as active and containing mixtures of compounds were subjected to repeat chromatographic efforts. Fractions generated at each chromatographic stage were screened using the *A. tumefaciens* A136 bioassay in order to identify the compounds responsible for the observed QS induction. In the event of multiple active fractions TLC profiles and ^{1}H NMR spectra were utilised as the basis of decisions

to recombine or pursue fractions separately. A full flow chart of the bioactivity guided effort is included in Appendix C.

4.2.3 Quorum sensing bioassay

All fractions generated were screened for QS inductive capability against the QS bacterial biosensor *Agrobacterium tumefaciens* A136 as per Chapter 2. *A. tumefaciens* A136 was chosen for this study as QS induction for this strain was the only activity observed for the *N. chabroli* extract in the preliminary screening effort of Chapter 2. Positive results in this QS bioassay were used to guide purification methodology. Pure compounds were subjected to dose response assays as per Chapter 3.

4.2.4 17,22-dihydroxy-24-methylene cholesterol isolation

The fractions eluted with methanol from the vacuum column silica gel chromatography showed the greatest QS induction activity and were combined on the basis of TLC elution profiles and ^{1}H NMR spectra. The combined fractions were subjected to C18 silica gel (60-120) reverse phase vacuum assisted column chromatography eluted with a water: methanol gradient. Ten fractions were collected and screened for *A. tumefaciens* A136 QS induction. Activity was identified in the fraction eluted with 100% methanol. This fraction was further purified with normal phase column chromatography eluted isocratically with 75% dichloromethane : ethyl acetate to isolate compound **1** and **2** in a mixture with fatty acids. Compounds **1** and **2** were purified by isocratic HPLC with 3% isopropanol in hexane (Phenomenex Silica column, 250 mm x 4.6 mm, 3 µm, 1 ml/min). Both compounds were revealed to be sterols. Compound **1** appeared to be novel while compound **2** was identified as 24-methylene cholesterol by NMR comparison with an authentic sample.

4.2.5 General characterisation methods

Assessments of purity were undertaken with analytical TLC and NMR spectrometry. Analytical TLC was performed on Merck Kieselgel 60. Spots were visualized by UV light or by spraying with vanillin (1%) in acidified ethanol solution. ^{1}H - NMR (600 MHz) and ^{13}C - NMR (150 MHz) spectra were recorded with a Bruker 600 Avance spectrometer in $CDCl_3$ using standard pulse programs. 1D and 2D NMR selective experiments (COSY, edited HSQC, HMBC, TOCSY and NOESY) were utilised for structure elucidation.

Isolation of compounds by HPLC was conducted on an Agilent 1100 series HPLC (Agilent, USA) comprising of a solvent degasser, a binary pump, a thermostated column compartment, and a Gilson 215 autosampler (USA) equipped with a 20 µl injection loop. Separation was performed in normal phase on a Phenomenex Silica column (250 mm $\times$4.6 mm, 3 µm particle size) maintained at 28°C, with mobile phase 3% isopropanol in hexane run isocratically at a flow rate of 1 ml/min. The HPLC system was coupled to a Bruker Esquire 3000 (Bruker Daltonics, USA) quadrupole ion trap mass spectrometer (LC–MS) equipped with an electrospray ionization (ESI) interface operating in positive mode.

High resolution mass spectra were measured with a Bruker BioApex 47e FT-ICR mass spectrometer fitted with an Analytica of Branford electrospray source; briefly, ions were detected in both positive and negative mode within a mass range m/z 100-1800. Direct infusion of compound in MeOH (~0.2 mg.mL^{-1}) containing the internal calibrant CF_3CO_2Na was carried out using a Cole Palmer 74900 syringe pump at a flow rate of 180 µL.h^{-1}.

4.2.6 Molecular modelling

Molecular modelling of stereocentres was performed using a substructure of **1** consisting of the C (chair conformation) and D rings of the sterol along with the side chain. This substructure was drawn in Chem3D and subjected to an MM2 energy minimisation

(129) with and without hydrogen bonding between the two hydroxyl groups. The dihedral angles between H-20, C-20, C-22, H-22; H-22, C-22, C-23, H23a; and H-22, C-22, C-23, H23b (Figure 4.3) were measured and the calculated angles were then further optimised in ORCA (130).

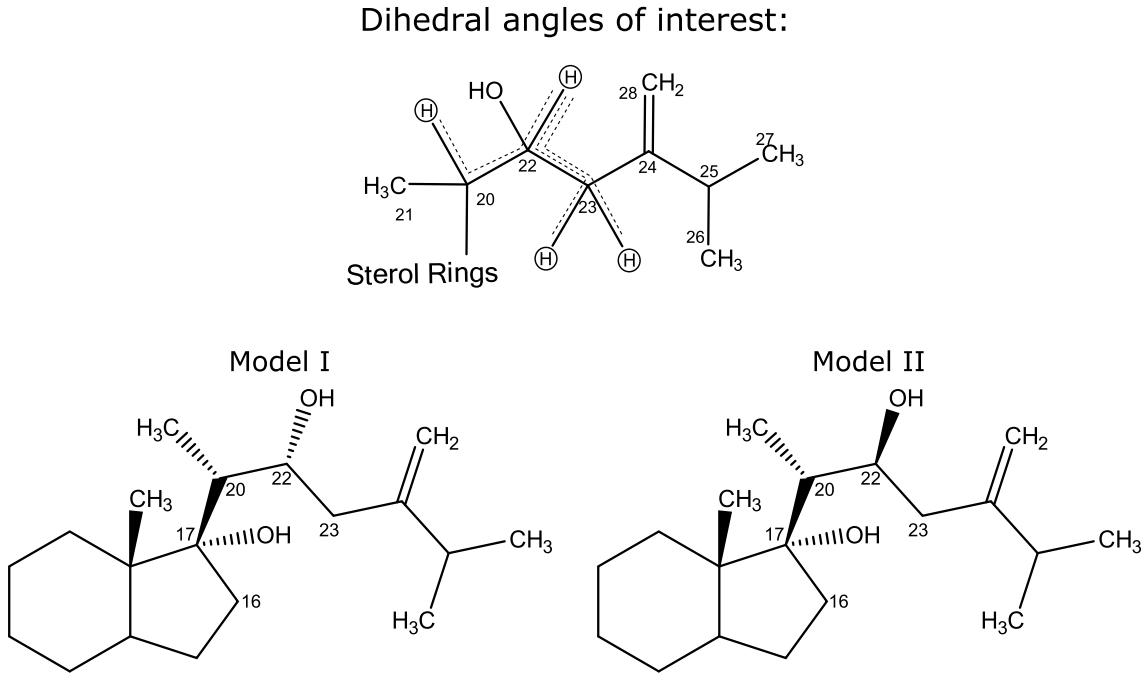

Figure 4.3: Molecular models I and II (sub-structures of compound **1**) were subjected to energy minimisation in Chem 3D and ORCA. The dihedral angles between H-22, C-22, C-20, H-20; H-22, C-22, C-23, H-23a and H-22, C-22, C-23, H-23b were measured for each model (Table 4.2).

4.3 Results

QS bioassay guided fractionation of the dichloromethane extract of the *N. chabroli* yielded one new sterol compound **1** and the known compound **2** identified as 24-methylene cholesterol by comparison with literature and a reference sample. Compound **2** demonstrated no activity in the QS biosensors assays for *A. tumefaciens* A136. Compound **1**, however, demonstrated maximum activity at a concentration of 100 nMol/L (or 4.5 ppm) (Figure 4.4).

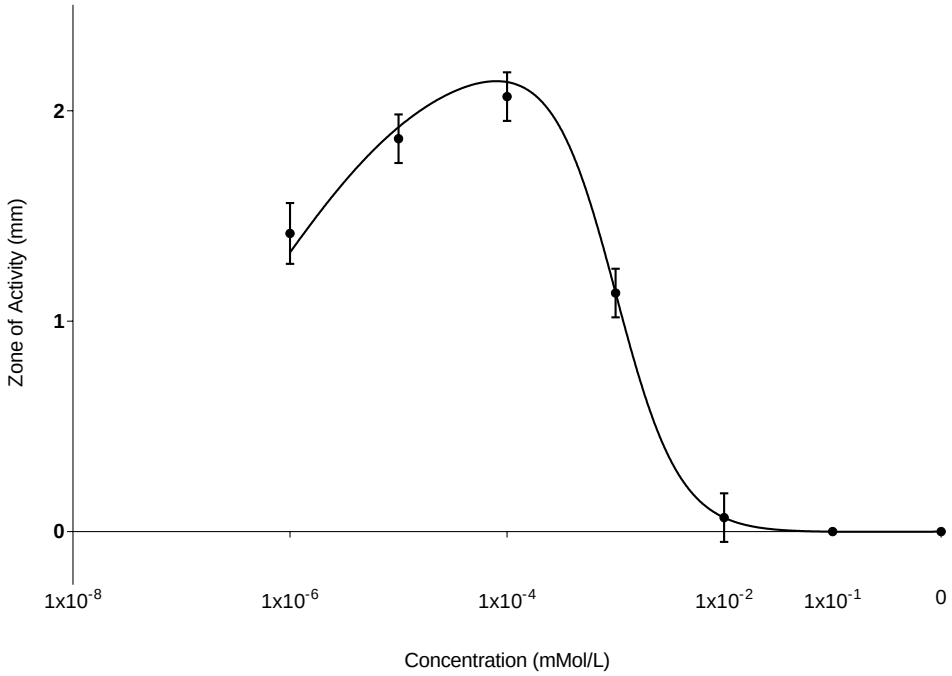

Figure 4.4: Dose response curve for new polyhydroxyl sterol from *Nephthea chabroli*. Peak QS activation occurs at 100 nM against the bacterial biosensor *Agrobacterium tumefaciens* A136.

Compound **1** was isolated as a white amorphous solid. Its molecular formula $C_{28}H_{46}O_3$ was established by HRESMS (m/z) within <1ppm (Appendix C) implying six degrees of unsaturation. Analysis of the NMR data revealed 28 carbons of which the edited adiabatic HSQC spectra indicated signals for five methyl, ten sp^3 methylene, one exo-methylene, five sp^3 methine, three sp^2 methines, four quaternary carbons and three hydroxyl groups (Table 4.1). The NMR data could therefore account for only two degrees

of unsaturation suggesting the tetracyclic nature of **1**. ^{1}H NMR data for compound **1** was typical of sterols with a multiplet at δH 5.35 for H-6 (δC 121.70) and a multiplet at δH 3.53 for H-3 (δC 70.80) (Table 4.1). All C-H ^{1}J correlations of **1** were detected in the HSQC experiment.

Table 4.1: NMR chemical shifts of compound **1**

	17,22-dihydroxy 24-methylene cholesterol	
Carbon #	^{1}H NMR	^{13}C NMR
(C-1)H_2	1.73, 1.13 m	23.4
(C-2)H_2	1.49, 1.84 m	31.5
(C-3)H	3.54, tt J=11.0, 5.0 Hz	71.6
(C-4)H_2	2.24, 2.29	42.0
(C-5)		140.5
(C-6)H	5.36, m	121.5
(C-7)H_2	1.62, 1.99 m	31.7
(C-8)H	1.50 m	32.2
(C-9)H	1.70	50.8
(C-10)		36.1
(C-11)H_2	1.48, 1.57 m	20.7
(C-12)H_2	1.71 m	32.2
(C-13)		46.9
(C-14)H	0.98	49.5
(C-15)H_2	1.08, 1.84	37.0
(C-16)H_2	1.86	37.8
(C-17)H		87.3
18- CH_3	0.75, s, 3H	14.4
19-CH_3	1.00, d J=6.0 Hz, 3H	7.43
(C-20)H	1.74 m	41.4
21- CH_3	1.01, s, 3H	19.3
(C22) H_2	4.19, ddd J=1.2, 5.0, 8.9 Hz	71.4
(C23) H_2	2.10, 2.28	40.5
(C24)		152.1
(C25) H	2.24, m	33.4
(C26) CH_3	1.06, d, J=6.7 Hz	21.6
(C27) CH_3	1.04, d, J=6.7 Hz	21.8
(C28) H_2	4.79, 4.90	109.4

Careful analysis of the ^{1}H-^{1}H COSY correlations observed for **1** led to the establishment of three partial structures shown in bold in Figure 4.5. The molecular framework of **1** was further established by an HMBC experiment (Figure 4.5). The structures and connection among A, B, C, and D rings were elucidated with the aid of the HMBC

spectrum. HMBC correlations between H_3-19 and C-1/C-5/C-9/C10 established partial structure **a**, could be connected to **b** through a quaternary carbon (C-10) (Figure 4.5). Partial structure **a** could be further linked to partial structure **b** from HMBC correlations between H-9 and C-8/C-7/C-1 (Figure 4.5). HMBC correlations between H3-18 and C-12/C-13/C-17 revealed partial structure **b** could be connected to partial structure **c** through a quaternary carbon (C-13) (Figure 4.5). On the basis of the above findings and HMBC correlations observed, the planar skeleton of **1** could be established unambiguously (Figure 4.5). The ^{1}H-^{1}H COSY correlations also established the side chain with key correlations occurring between H-22 and H_2-23 as well as H_2-23 and H-25 (δH 4.79). These structural connections were verified by HMBC correlations between H-21 and C-17/C-22/C-23 and H_2-23 and C-21/C-22/C-24/C-28.

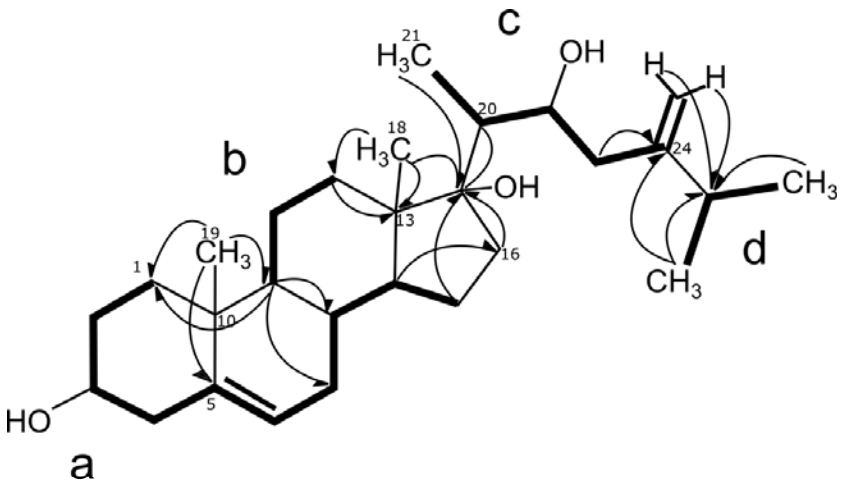

Figure 4.5: Novel sterol from *Nephthea chabroli* Partial structures **a**, **b**, **c** and **d** as elucidated by COSY ^{1}H-^{1}H correlations are indicated in bold. Key HMBC correlations are indicated by the arrows.

The conformation of the stereocentres was established through a combination of selective TOCSY and NOESY experiments. *J*-coupling constants of 1 Hz, 5 Hz and 9 Hz suggested dihedral angles of approximately 90°, 50° and 167° (Table 4.2). These coupling values were supported by selective TOCSY observations; a small correlation was observed between H-22 and H-20 but little to no correlation between H-22 and H_3-21 when H-22 was selectively irradiated. The observed *J*-couplings appeared to support the configuration of Model I (C-17 S; C-21 R; and C-22 R), where Chem3D and ORCA energy minimisations indicated dihedral angles of 89°, 50° and 167° (Model I, Figure

4.3). Although there was little difference in the calculated dihedral angles between the two models (Table 4.2).

Table 4.2: Molecular modelling results of 17,22-dihydroxy-24-methylene cholesterol, showing calculated and inferred dihedral angles and observed *J*-couplings.

dihedral angle	Model I	Model II
H-22, C-22, C-20, H-20	-89.0°	74.8°
H-22, C-22, C-23, H-23a	50.2°	-58.0°
H-22, C-22, C-23, H-23b	167.0°	-170.3°

Selective NOESY experiments, however, indicated NOe's between H-21 and H-16 (Figure 4.6), these NOE's would only be possible if the molecule was in the configuration of Model II (Figure 4.3). Therefore, on the basis of these correlations, the relative stereoconformation of the side chain of **1** was determined to be (C-17 S; C-21 R; and C-22 S).

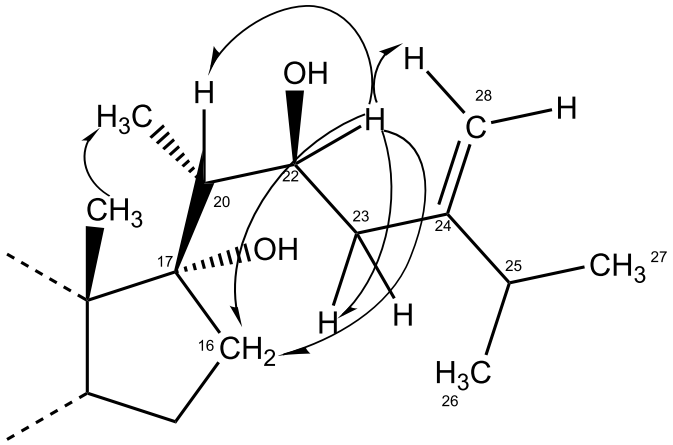

Figure 4.6: Key NOESY correlations for side chain of compound **1**

4.4 Discussion

While the non-steroidal hormones, epinephrine and nor-epinephrine have been demonstrated to interact with the auto Inducer two (AI-2) system of bacterial QS (131). This may be the first indication that a steroidal hormone could demonstrate activity in the AI-1

system. Furthermore, it occurs at concentrations that could be expected to occur within the coral tissue. Sterols are ubiquitous signalling molecules produced by eukaryotes. Although a small number of reports of bacterial sterols do occur in the literature, the majority of these reports have been treated with caution (132). The amounts of sterols detected in bacteria are generally very low (<0.3% of dry weight) and have frequently been traced to contamination by yeast, agar and other sources (132). In contrast, the sterol presented here represents ~3% of the dry weight of the coral. The signalling and coordination role of many steroidal hormones within multi-cellular eukaryotes, makes this class of compounds a likely pathway for inter-kingdom interactions through QS. If these compounds are demonstrated to act in this ecological capacity, it would substantially widens the number of organisms in which the potential for QS interaction through AI-1 may be found.

The mechanism of interaction between the sterol and QS receptor are currently unclear. Previous QS mimics have been shown to work by simulating the active site of the bacterial AHLs thereby allowing them to bind to the QS receptor. In the instance of the *Delisea pulchra* furanones this results in an increased turnover of the receptor complex which disables the downstream gene activation (36). Two possible mechanisms of action are suggested. The first is that hydrogen bonding between the hydroxyl group on the D ring and the hydroxyl group at C-22 on the side chain are effectively forming an oxygenated ring system (Figure 4.7) that is able to bind to the QS receptor TraR. Models that assumed hydrogen bonding between the hydroxyl groups were not explicitly diagnostic, favouring the R configuration at C-22 (by dihedral angles). In contrast, the NOESY experiments unambiguously favoured the S configuration at C-22, which strongly supports the proposed presence of hydrogen bonding between the hydroxyl groups and the possibility of this mechanism of action (Figure 4.7).

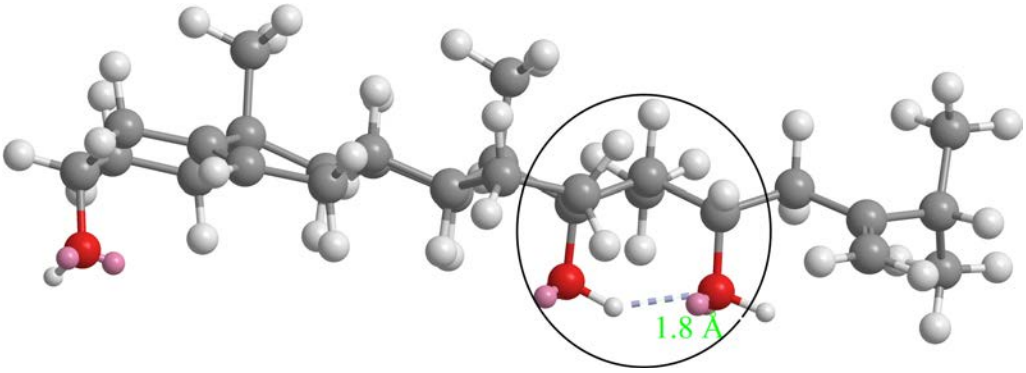

Figure 4.7: Proposed mechanism 1 demonstrating the formation of a pseudo-ring (in the circle) through H-bonding between the hydroxyl groups (indicated by the dotted line).

An alternate possibility is that the sterol is first undergoing oxidation at C-3 and C-22 to ketones, which would then enable the possibility of a retro Diels Alder reaction to occur. In this scenario, the side chain would be readily cleaved. Movement of the C-5 unsaturation into conjugation at any stage could afford a testosterone analogue (Figure 4.8). This proposal, although unlikely, is a possibility for two main reasons: firstly, the TraR receptor present in the *A. tumefaciens* A136 biosensor used in this study has been determined to be a homologue of the testosterone receptor TeiR (133). It is possible that the resulting ketone might be sufficient to bind to the receptor protein, or alternately, reduction to testosterone may occur. Secondly, similar steroidal hormones, progesterone and β-estradiol, were implicated as QS interference molecules in a previous *in silico* screening effort based on flexible docking sites of the QS receptors LuxR, LasR and TraR (128), although neither progesterone nor β-estradiol were confirmed as active in the biological screening phase of that study, it only assessed a bioluminescent LuxR screen and did not include biosensors with LasR or TraR receptors (128).

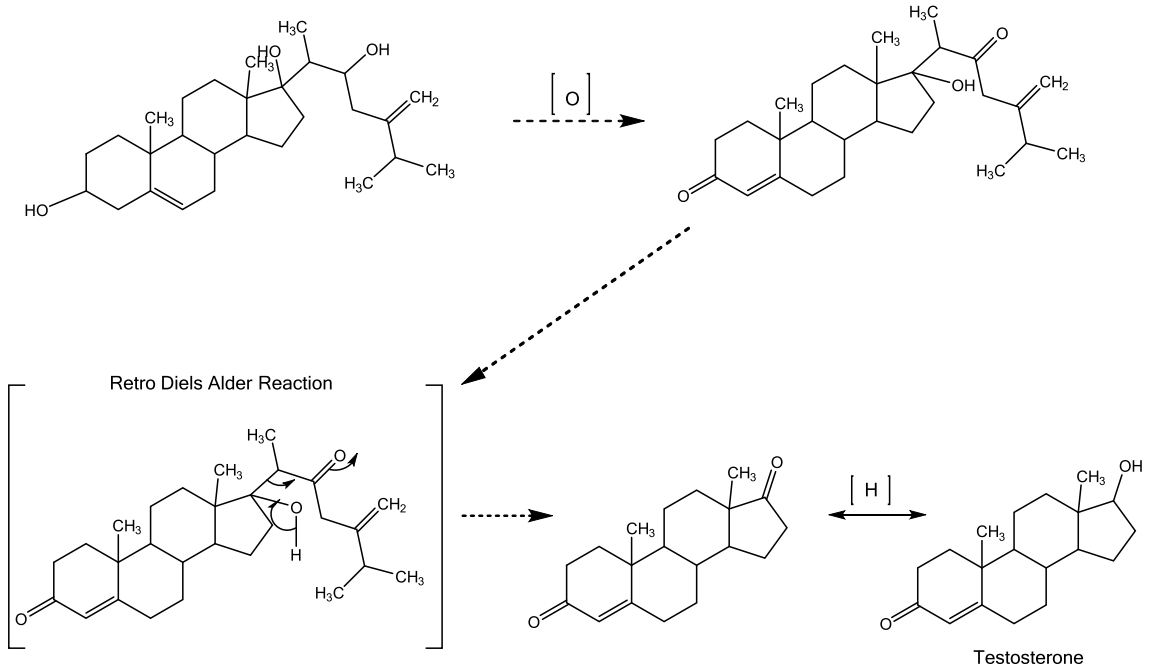

Figure 4.8: A possible pathway for conversion of compound **1** to testosterone via a retro Diels-Alder reaction.

In the first proposed mechanism, the observed QS activity would result from the poly-hydroxylated nature of the new isolated sterol. Hydrogen bonding of the hydroxy substituents at C-17 and C-22 could hold the region in a semi-rigid ring conformation that mimics a lactone in terms of its binding ability and explains why 24-methylene cholesterol would not demonstrate activity (as it lacks these functional features). Further *in silico* investigations may reveal more poly-hydroxylated steroids with similar QS interference potential.

4.5 Conclusion

The identification of a sterol as an active mimic inducing QS in *A. tumefaciens* is potential evidence of inter-kingdom communication between the host soft coral *Nephthea chabroli* and the associated bacterial community. Further, it is active in the absence of lactone or furan functionality. These two points suggest that both the number of organisms capable of QS interference as a possible mechanism of bacterial interaction

or regulation is far greater than previously imagined: a proposition with implications for both the design of QS pharmaceuticals and our fundamental understanding of bacterial regulation.

Chapter 5

Quorum Sensing Bacteria from the Surface Mucosal Layer of *Sinularia flexibilis* and *Lobophytum compactum*

5.1 Introduction:

Previous studies have established the widespread presence of AHL type QS mimics within corals, in particular within soft corals (Chapters 1 and 2; 51, 96). The chemical source of this activity has been demonstrated, at least in part, to be related to the presence of cembranoid diterpene metabolites which are common amongst Alcyoniidae soft corals (Chapter 3). Terpenes are known to act as chemical mediators for both beneficial and antagonistic interactions between organisms (134, 135, 136, 90). Furthermore, they are often credited with the ecological and evolutionary success of Alcyoniidae soft corals (134, 135, 90, 93). These biologically active compounds can be found within the tissue, mucosal layer and immediately surrounding water column. A number of ecological roles have been suggested for these compounds including interactions with microorganisms. Their location within the mucosal layer adds to the possibility of involvement in mediating soft coral associated microbial communities.

Sinularia flexibilis and *Lobophytum compactum* are common Alcyoniidae soft corals of the central Great Barrier Reef, Australia, with well characterised secondary metabolite profiles (Chapter 3; 137, 138, 117). The secondary metabolite profile of *L. compactum* is dominated by the cembranoid diterpene, isolobophytolide (138; Chapter 3). Isolobophytolide has been linked to QS, ichthyotoxicity and cytotoxicity and can be present at 10-20% of the dry weight of the colony. In contrast, *S. flexibilis* is host to a suite of cembranoid diterpenes including flexibilide and sinulariolide that have also been linked to QS, cytotoxicity and algaecidal properties (137, 95, 139). To date, the majority of studies into the ability of these compounds to interact with bacteria has focused on antibiotic effects against human pathogens or other biotechnology interests rather than *in situ* ecological roles.

Currently, very little is known about the bacterial communities that live in the SML of soft corals and their QS abilities are unknown. To definitively establish a regulatory role for AHL type QS within the holobiont, the bacteria and QS phenotypes affected by these compounds need to be conclusively demonstrated. To this end, bacterial strains from

the soft corals *S. flexibilis* and *L. compactum* were cultured from their SML and their extracts assessed for QS activity as well as the ability to inhibit QS in other bacterial strains. The role that the cembranoid diterpene, isolobophytolide, from *L. compactum* might play in influencing bacterial selection and QS activity was also assessed.

5.2 Experimental:

5.2.1 Collection *of Sinularia flexibilis* and *Lobophytum compactum* surface mucosal layer.

The surface mucosal layer (SML) was collected underwater (3 m) from 3 healthy replicate colonies of *Sinularia flexibilis* & *Lobophytum compactum* in Hazard Bay, Orpheus Island, using 50 ml needleless sterile syringes (Figure 5.1). SML samples were taken from the mid-capitulum region of the coral colony. After each SML sample was retrieved, tissue samples of each replicate were also collected for comparison of the chemical fingerprints (metabolite profiles) with samples of *S. flexibilis* and *L. compactum* collected previously (Chapters 2 and 3). Samples were maintained at ambient temperatures and processed within three hours of collection.

5.2.2 Culturing of bacterial isolates from *S. flexibilis* and *L. compactum* surface mucosal layer.

SML samples were serially diluted (10^{-2}, 10^{-3}, 10^{-4}) using autoclaved artificial seawater (Appendix D). One hundred microlitres of each dilution was spread plated in triplicate (Figure 5.1) on two types of standard media commonly used for studies of marine bacteria: 50% Marine Agar (50MA; BD, Appendix D) and Glycerol Artificial Seawater Agar (GASW; 140; Appendix D). Additionally, one medium was used that specifically selects for bacteria belonging to the genus *Vibrio*: Thiosulfate Citrate Bile Salts Sucrose Agar

(TCBS Agar; BD; Appendix D). *L. compactum* SML dilutions were also plated onto 50% Marine Agar and GASW Agar supplemented with isolobophytolide (see below). Plates were incubated at 28°C and sampled after 48 hours, 72 hours, 1 and 2 weeks. Representatives of each colony morphotype from each plate were subcultured to purity for identification and QS screening.

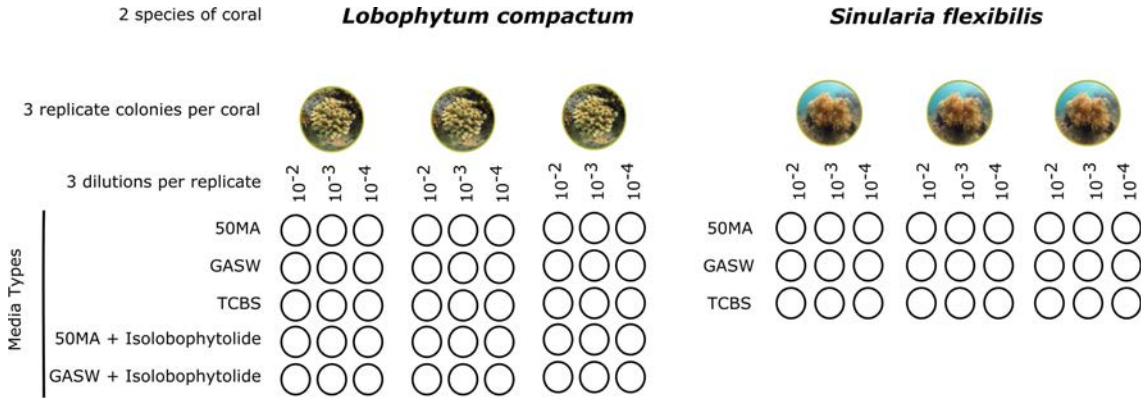

Figure 5.1: Experimental design for isolation of bacteria from the mucosal layer of *Lobophytum compactum* and *Sinularia flexibilis*.

5.2.3 Isolobophytolide extraction and purification

Isolobophytolide was isolated and purified from colonies of *L. compactum* collected from Hazard Bay, Orpheus Island as previously described (Chapter 3). The pure isolobophytolide was stored at -20°C. Pure isolobophytolide was dissolved in ethanol at 20 mg/ml for addition into media at a final concentration of 3 ppm, which is equivalent to that found in the SML and immediately surrounding water column (121). Isolobophytolide containing agar plates were stored at 4°C for no more than 48 hours before inoculation.

5.2.4 Quantification and statistical analysis of isolates

Colony forming unit estimates were calculated from dilutions that yielded between 30 and 300 colonies per plate. Differences between samples were determined using the Kruskal-Wallis statistic on all three replicates for the corresponding species, dilution

and media type (Figure 5.1). Isolate morphotype profiles were compared using a non-metric Multidimentional Scaling (nMDS) analysis. Colony morphotype profile analysis was conducted on the variables colour, size and texture. An nMDS was chosen for this anaylsis as it is used to spatially represent complex data sets containing multiple variables, large numbers of zeroes and non-normal distributions (141). Both the Kruskal-Wallis analysis of the CFU estimates and the nMDS analysis of the isolate profiles were performed using Graphpad PRISM.

5.2.5 Bacterial DNA extraction, PCR amplification and sequencing

Genomic DNA of bacterial isolates cultured from the SML of *S. flexibilis* and *L. compactum* was extracted using the Promega Wizard Genomic DNA Isolation Kit (Promega, Madison WI USA) according to the manufacturer's directions.

PCR amplification of 16S rRNA gene fragments (~1,465 bp) was performed using the primers 27F (5'-AGAGTTTCATCMTGGCTCAC-3') and 1492R (5'-GGTTACCTTGTT ACGACTT-3') (142). The PCR reactions contained the following reagents: 0.4 µM of each primers, MyTAQ (Bioline, Australia) 0.25 µL, and 1 µL of isolated DNA product in MyTAQ buffer (Bioline, Australia) to a final volume of 50 µL. Cycling conditions were an initial denaturing step of 94°C for 5 min, followed by 30 cycles at 95°C for 1 min, 56°C for 45 s, 72°C for 60 s, and a final elongation step at 72°C for 10 min. PCR products were verified by agarose gel electrophoresis and purified for sequencing using the Qiaquick PCR Purification Kit (Qiagen, Valencia, CA) according to company supplied directions. Sequencing was carried out at Macrogen (Seoul, South Korea).

5.2.6 Phylogenetic analysis of bacterial isolates.

For each soft coral species, phylogenetic trees were constructed for the recovered isolates and close relatives based on partial 16S rRNA gene sequences. Sequence fragments were

assembled using Sequencher (Version 5, Gene Codes, Ann Arbor, USA). For each isolate, the 16S rRNA gene sequence was aligned with sequences in the nr database at the NCBI (National Centre for Biotechnology Information) using the BlastN tool to obtain nearest matches (143). The closest type sequences of recognised species were also obtained using the EzTaxon database (144). Sequences of isolates and database matches were imported into Mega6 and aligned using ClustalW (145). Maximum Likelihood and Maximum Parsimony algorithms were implemented to construct trees. The resulting tree topologies were evaluated for robustness based on 1000 bootstrap replicates. The 16S rRNA gene sequences for the 72 of bacterial isolates were deposited in NCBI Genbank database under the accession numbers indicated (Appendix E). The type strain of *Thermoplasma acidophilum* was used as an outgroup for the analyses.

5.2.7 Selection of bacteria for QS screening and sample preparation

Where possible, two representatives of each morphotype were chosen for screening of QS activity. All bacteria screened for QS were grown without the presence of isolobophytolide in the medium, as the previously identified (Chapter 3) QS activity of this compound would confound results. Where bacteria had been initially isolated using media embedded with isolobophytolide, growth was attempted on the equivalent medium without isolobophytolide. Strains that were not able to be cultured without isolobophytolide were therefore not included in this screening.

QS screening was performed on acidified ethyl acetate extracts of the cell free supernatant of soft coral isolates. Extracts of the supernatant were acquired as follows: isolates were grown on 50% MA to obtain single colonies. Colonies were transferred to liquid culture (10 ml 50% Marine Broth culture at 28°C, 170 rpm) and grown to late exponential phase. Cultures were centrifuged for 10 min at 4°C at 10,000 x g to obtain the cell free supernatant (CFS). Each CFS was subjected to three subsequent extractions with acidified ethyl acetate (1% acetic acid) which were combined and concentrated to

dryness under a stream of N_2 gas. Extracts were then dissolved and made up to a concentration of 20 mg/ml with ethanol.

5.2.8 Quorum sensing induction and inhibition bioassay

Extracts were assayed using *A. tumefaciens* A136 and *C. violaceum* CV026 for both induction and inhibition of QS in the manner previously reported (Chapter 2.2.3). Analysis of QS prevalence in bacterial isolates was performed in Graphpad PRISM.

5.3 Results

5.3.1 Quantification of culturable bacteria

The estimated numbers of colony forming units in the SML of *S. flexibilis* and *L. compactum* are presented in Figure 5.2. The presented results and trends are based on the data obtained for the 50MA plates, however, there was no significant difference in the number of CFUs formed on GASW or 50MA media for either coral (Appendix E). Differences were observed between species: *S. flexibilis* resulted in a significantly higher number of colony forming units than *L. compactum* with or without isolobophytolide in the isolation medium ($H=7.200$, 2 d.f., $P=0.0036$; Figure 5.2). Interestingly, if isolobophytolide was included in the growth media, a significant increase was observed in the number of CFUs estimated for *L. compactum* (Figure 5.2).

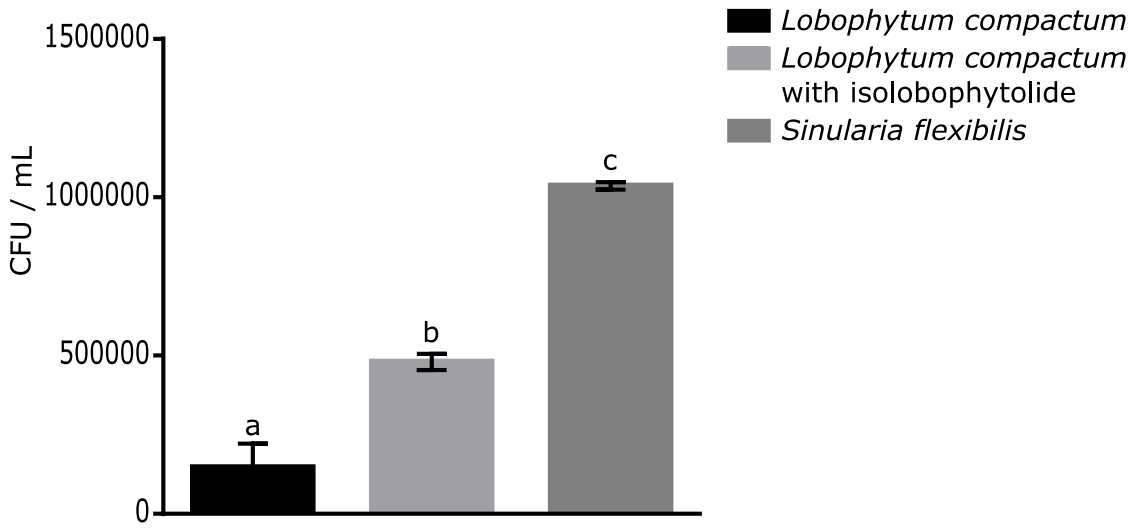

Figure 5.2: Average Colony forming units with standard error for *Lobophytum compactum* and *Sinularia flexibilis* from a 10^{-3} dilution of the SML as plated on 50% marine agar after 72 hours incubation at 28°C. The letters indicate significant difference as determined by the Kruskal-Wallis statistic (H=7.200, 2 d.f., P=0.0036).

5.3.2 Colony morphotype analysis

The number and type of cultured morphotypes differed between *L. compactum* and *S. flexibilis* (Figure 5.3). *S. flexibilis* showed little variability in the morphotype profiles of GASW or 50MA media, forming a tight cluster on the nMDS biplot (Figure 5.3). In comparison, greater variability was apparent in the morphotype profiles generated from *L. compactum* (Figure 5.3). The presence of isolobophytolide as a selection agent within the media appeared to be driving the observed variability (Figure 5.3).

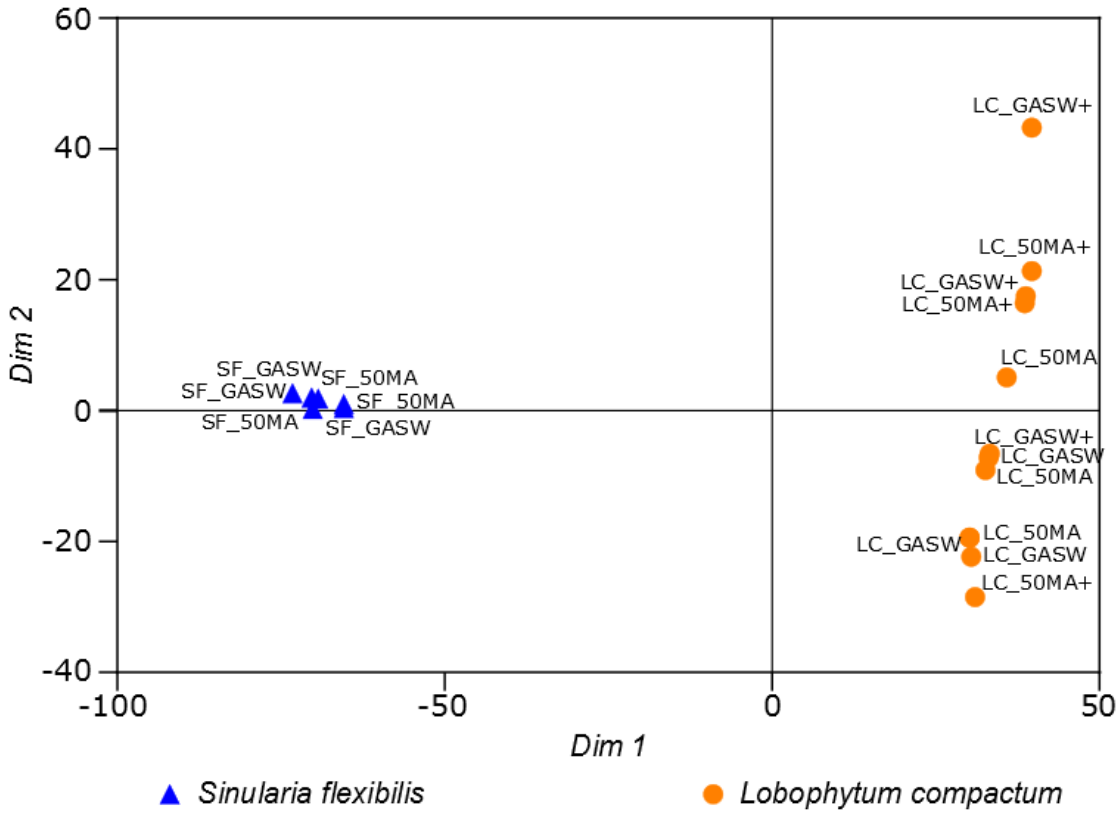

Figure 5.3: nMDS plot of bacterial isolate morphotype profiles generated from *Sinularia flexibilis* and *Lobophytum compactum*. *S. flexibilis* samples are indicated by the prefix SF whereas *L. compactum* profiles are indicated by the prefix LC. 50MA indicates a profile from a 50% marine agar plate, GASW indicates a profile from a Glycerol Artificial Seawater plate. The plus symbol indicates the presence of isolobophytolide in the isolation media.

5.3.3 Phylogenetic tree of *Sinularia flexibilis* bacterial isolates

In total, 20 bacterial isolates from *S. flexibilis* were identified through 16S rRNA gene sequencing followed by BLAST searches and construction of phylogenetic trees. The majority of the *S. flexibilis* isolates were *Gammaproteobacteria* of the genus *Vibrio* (13/20; Figure 5.4 and Figure 5.5). Other *Gammaproteobacteria* were also isolated, including one isolate belonging to the closely related genus *Photobacterium* (SF103), two isolates (SF102 and SF204) whose closest relative was a *Spongiobacter nickelotolerans* strain (Figure 5.4); and three *Alteromonas*-related strains (YSF, SFYBB and SFB10_2). Finally, one firmicute isolate (SF10T2) was identified with 99% sequence identity to *Bacillus megaterium* (Figure 5.4). Interestingly, all of the isolated *Vibrio* strains were cultured

from the general marine culture media (50MA and GASW) rather than the *Vibrio* selective medium, TCBS (Figure 5.5).

The potential of soft coral isolates from *S. flexibilis* to participate in AHL-type QS communication systems was investigated using two reporter bioassays, *A. tumefaciens* A136 and *C. violaceum* CV026. Of the isolates tested from *S. flexibilis* 55% demonstrated QS activity under the growth conditions tested. Both tested *Alteromonas* strains exhibited QS activity (Figure 5.4). The *Alteromonas* SFB10_2 strain triggered QS in both sensors strains, whereas, the *Alteromonas* YSF strain only triggered QS in *C. violaceum* CV026 (Figure 5.4). Similarly, both *A. tumefaciens* A136 and *C. violaceum* CV026 QS induction were observed for the *Endozoicomonas* - *Spongiobacter* strains (SF204 and SF102; Figure 5.4). *S*train SF204, however, also produced positive results in the inhibition of *C. violaceum* CV026 (Figure 5.4). None of the tested *Vibrio* strains showed both QS induction and inhibition, however, three *Vibrio* strains (SF208, 01SF1M10 and SF10T1) demonstrated inhibitory activity against both biosensors (Figure 5.5).

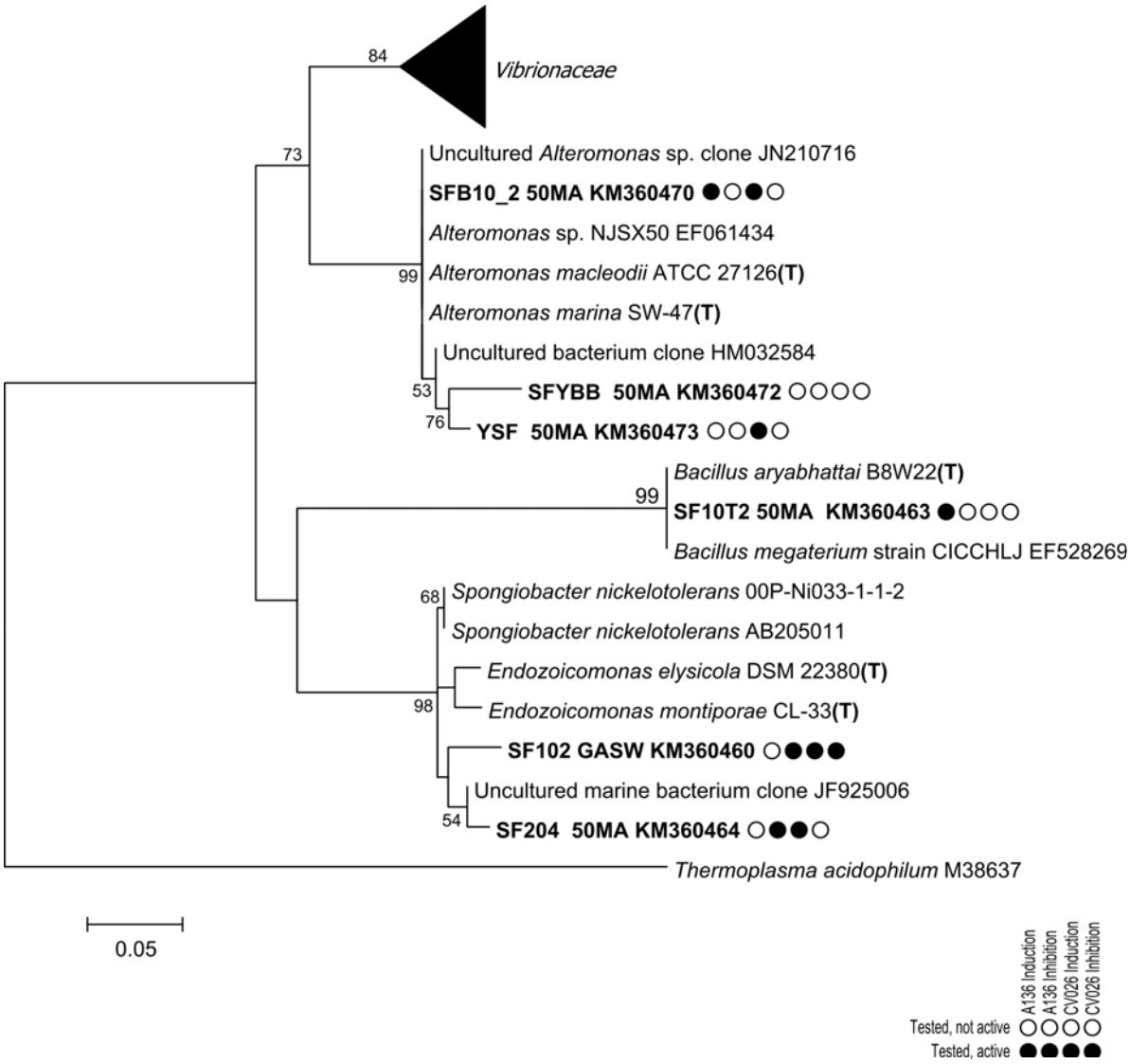

Figure 5.4: A phylogenetic tree based on partial 16S rRNA gene sequences retrieved from bacterial isolates from the mucus of the soft coral *Sinularia flexibilis*. Details of the *Vibrionaceae* are shown in Figure 5.5. The tree is based on maximum-likelihood analysis, using a 50% conservation filter. The scale bar indicates 5% estimated sequence divergence. *Thermoplasma acidophilum* was used as the outgroup for analysis. Isolated sequences and their accession numbers are indicated in bold type. Nearest matches from the NCBI database and type strains from the EZtaxon database **(T)** are also included.

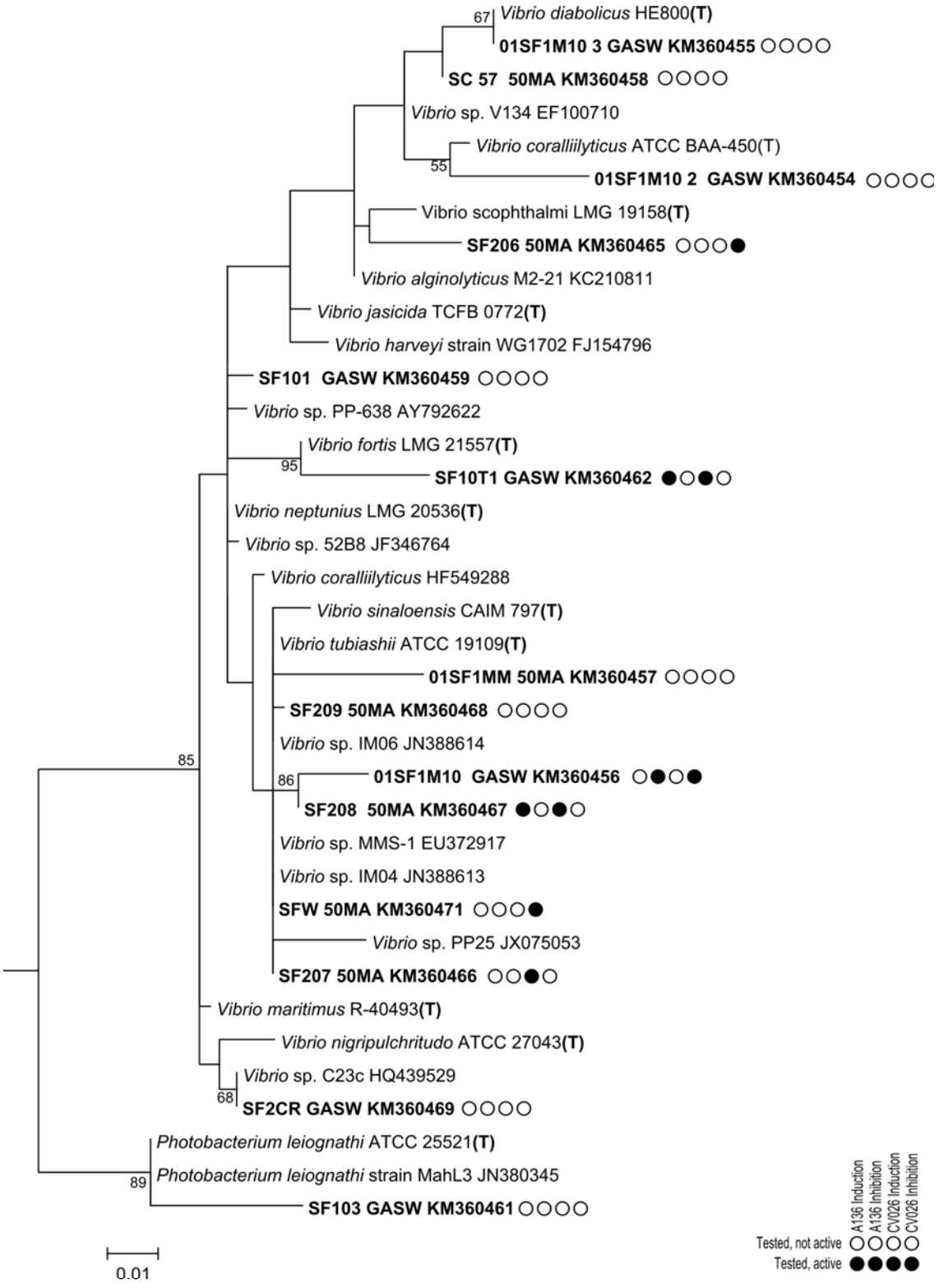

Figure 5.5: *Vibrionaceae* sub-tree tree (part of tree presented in Figure 5.4) based on 16S rRNA gene sequences retrieved from an analysis of bacterial isolates from the mucus of the soft coral *Sinularia flexibilis*. The tree is based on maximum-likelihood analysis, using a 50% conservation filter. The scale bar indicates 1% estimated sequence divergence. *Thermoplasma acidophilum* was used as the outgroup for analysis. Isolated sequences and their accession numbers are indicated in bold type. Nearest matches from the NCBI database and type strains from the EZtaxon database **(T)** are also included.

5.3.4 *Lobophytum compactum* bacterial isolates

A phylogenetic tree of partial 16S rRNA gene sequences was constructed for the *L. compactum* derived bacteria and close relatives. The isolates cultured from *L. compactum* demonstrated a number of similarities to the bacteria isolated from *S. flexibilis*. Firstly, the majority of *L. compactum* isolates were *Gammaproteobacteria* of the genus Vibrio (26/51) (Figures 5.6 and 5.7). Secondly, strains related to the genera *Photobacterium* (LC135, LC128), *Spongiobacter* (LC205) and *Bacillus* (LC305) and from the order *Alteromonadales* were isolated also from this soft coral species. In this instance, however, the diversity of *Alteromonadales*-related strains was higher with strains related not only to the genus *Alteromonas* (seven strains: LC301, LC203, LC314, LC137, LC309, LC315, LC214) but also to the genera *Pseudoalteromonas* (six strains: LC210, LC201, LC215, LC310, LC212, LC219), *Paramoritella* (LCP), *Ferrimonas* (LC131) and *Shewanella* (LC302) (Figure 5.6). The type match for strain LC205 was *S. nickelotolerans*, an uncharacterised genera and the nearest neighbour match from the NCBI nr database was an uncultured bacterium clone. For this reason, the type strain of the closely related and described Endozoicomonas elysicola strain was also included. In contrast to the *S. flexibilis* isolates, the *L. compactum* isolates also included strains belonging to the gammaproteobacterium genus *Psychrobacter* (LC127), the alphaproteobacterium genus *Erythrobacter* (LCORI) and the actinobacterium genus *Micrococcus* (LC409) (Figure 5.7).

The potential of soft coral isolates from *L. compactum* to participate in AHL-type QS communication systems was investigated using two reporter bioassays, *A. tumefaciens* A136 and *C. violaceum* CV026 (Figure 5.6 and 5.7; Appendix E). Of the tested isolates from *L. compactum,* 53.3% demonstrated QS activity at the growth conditions tested and activity was mixed between induction and inhibition. Three of the tested *Vibrio* strains (LC111, LC103 and LC208) showed inhibitory activity against both biosensors. Of the strains that were initially isolated with media containing isolobophytolide, 41% were unable to be cultured in the absence of this compound and consequently were not tested for QS activity (Appendix E).

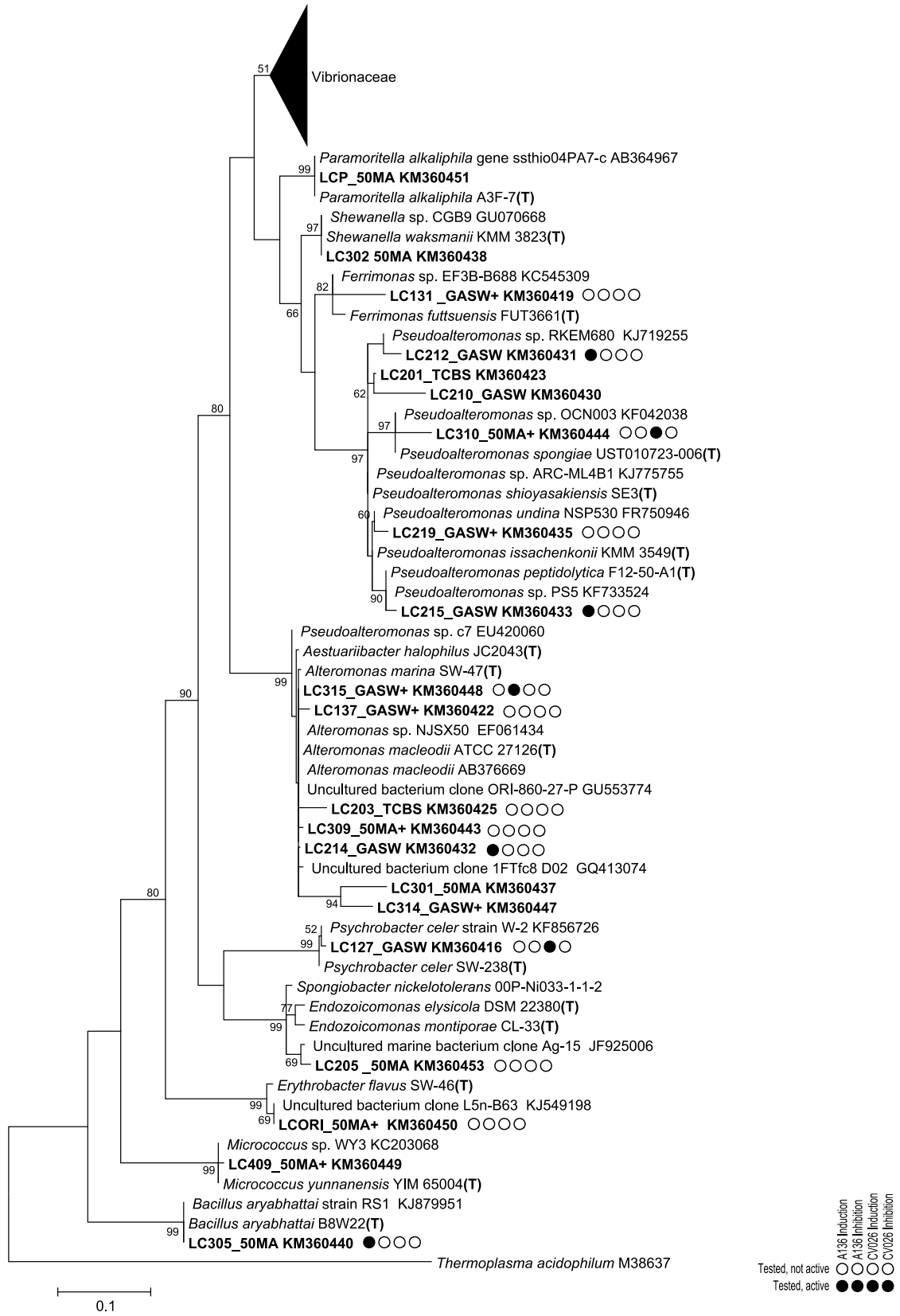

Figure 5.6: A phylogenetic tree based on 16S rRNA gene sequences retrieved from an analysis of bacterial isolates from the mucus of the soft coral *Lobophytum compactum*. Details of the *Vibrionaceae* are shown in Figure 5.7. The tree is based on maximum-likelihood analysis, using a 50% conservation filter. The scale bar indicates 10% estimated sequence divergence. *Thermoplasma acidophilum* was used as the outgroup for analysis. Isolated sequences and their accession numbers are indicated in bold type. Nearest matches from the NCBI database and type strains from the EZtaxon database **(T)** are also included.

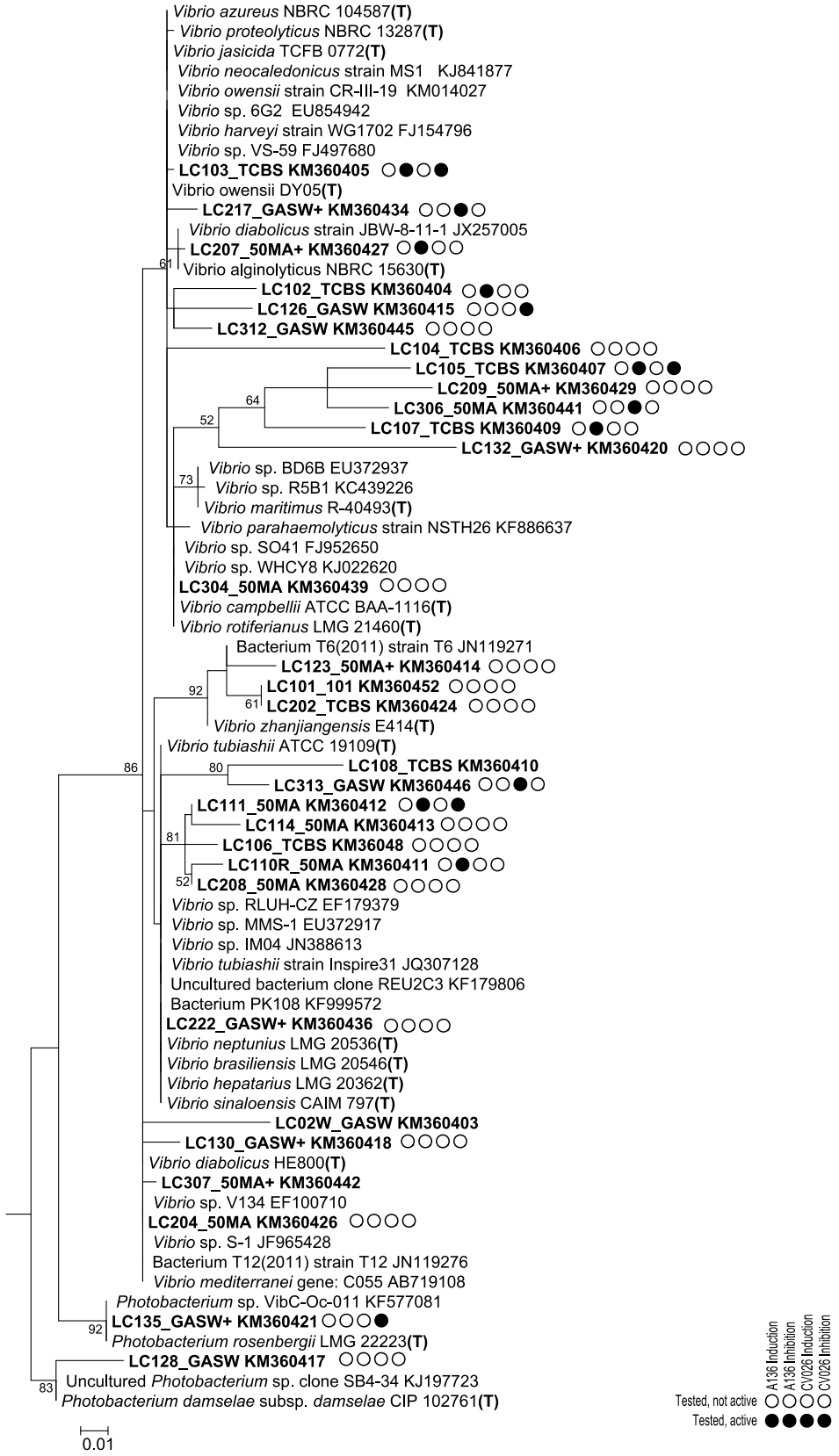

Figure 5.7: *Vibrionaceae* sub-tree tree (part of tree presented in Figure 5.6) based on 16S rRNA gene sequences retrieved from an analysis of bacterial isolates from the mucus of the soft coral *Lobophytum compactum*. The tree is based on maximum-likelihood analysis, using a 50% conservation filter. The scale bar indicates 10% estimated sequence divergence. *Thermoplasma acidophilum* was used as the outgroup for analysis. Isolated sequences and their accession numbers are indicated in bold type. Nearest matches from the NCBI database and type strains from the EZtaxon database **(T)** are also included.

5.4 Discussion

Two approaches were used to isolate culturable bacteria from the soft corals *S. flexibilis* and *L. compactum*. The first approach was to directly inoculate common media types with dilutions of the soft coral mucus. Although this technique is biased towards fast growing bacteria, it is still the most commonly used method to generate bacterial isolates (146). The second approach, conducted solely on the *L. compactum* mucus samples, involved the addition of isolobophytolide in the isolation media in order to generate a growth matrix with greater ecological relevance. Interestingly, significantly higher concentrations of CFUs as well as a larger number of morphotypes were estimated from those plates containing isolobophytolide. This result suggests that the inclusion of secondary metabolites in growth media can improve the success of culturing soft coral associated bacterial isolates.

Isolated surface mucosal layer bacteria were cultured and assessed for QS activity against two AHL type QS biosensors with differing sensitivities, *A. tumefaciens* A136 and *C. violaceum* CV026 (24). A number of strains capable of inducing and / or disrupting QS in the bacterial biosensors were isolated from *L. compactum* and *S. flexibilis* (57.5% and 57.8% of tested strains, respectively). The genera of all of the bacteria isolated, regardless of activity detected in this study, have previously been reported to possess or interact with QS systems (147, 148, 149, 150, 151, 152, 12, 153, 154). This provides support for the hypothesis that QS contributes to the regulation of the microbial communities of these corals.

The isolates generated in this study were dominated by *Gammaproteobacteria* belonging to the family *Vibrionaceae*. This held true for the non-*Vibrio* targeted media (GASW and 50MA) as well as the *Vibrio* targeted medium (TCBS) for both of the investigated coral species, a result that has been previously observed in scleractinian corals (58, 155). The 16S rRNA gene is known to be insufficient to resolve *Vibrio* strains to species level (156), however, the recovered *Vibrio* sequences clustered well into their recognised clades in the phylogenetic trees for both coral species. For both species, several *Vibrio* strains were

isolated whose sequences clustered separately from the most closely related database sequences and hence may represent novel species. Scleractinian corals have previously been recognised as harbouring a number of novel bacterial taxa (45, 65). Whilst the bacteria of Alcyonacean corals are less well studied, it is reasonable to assume a similar situation could exist.

All of the isolates generated in this study had highest sequence identity with bacterial sequences sourced from the marine environment or marine organisms and all recovered genera have previously been identified in coral mucus samples. *Spongiobacter*, although originally identified from a marine sponge, has been observed in the mucus of the gorgonian corals *Paramuricea clavata* (157) and *Gorgonia ventallina* (45) as well as the scleractinian coral *Acropora millepora* (69). *Spongiobacter* strains have been attributed a number of ecological roles, *Spongiobacter* strains from *A. millepora* demonstrated a dependence on DMSP and consequently a role in the biogeochemical sulfur cycle is postulated, whereas, *Spongiobacter* strains from the sponge *Suberites carnosus* demonstrated antibacterial activity (158). Of greatest interest to this study, however, is the QS activity detected in *Spongiobacter* strains from the sponges *Mycale laxissima* and *Ircinia strobilina* (159). Interestingly, the *Spongiobacter* strains SF102 and SF204 that were tested from *S. flexibilis* in this study showed positive responses to *C. violaceum* CV026 and not *A. tumefaciens* A136, whereas those from the sponge samples demonstrated the opposite responses (positive for *A. tumefaciens* and not *C. violaceum*) (159).

Actinobacteria produce almost 50% of all reported bioactive natural products from microorganisms (160, 161, 162, 163). Many of these natural products include antibiotic activity such as that of the new methicillin-resistant *Staphylococcus aureus* (MRSA) antibiotic thiazolyl peptide kocurin recently isolated from a sponge associated *Micrococcus* species (164). Consequently, the Actinobacterium (LC409) isolated from *L. compactum* was of significant interest, unfortunately it was one of the strains that could not be cultured without the presence of isolobophytolide and consequently was not tested for QS activity. This strain was subjected to preliminary chemical (^{1}H NMR) analysis and represents a potential target for alternative mechanisms of assessment including both

chemical and molecular analyses.

No clear QS activity patterns were observed across the tested *Vibrio* isolates under the experimental conditions. This is consistent with a previous assessment of QS in twenty nine *Vibrionaceae* strains by Tait and colleagues (150) and six *V. coralliilyticus* strains which revealed four active and two inactive strains (Appendix E). The study by Tait and colleagues (150) also demonstrated variability in the response of *Vibrio* strains with higher temperature (150). Consequently, testing the QS activity of these isolates under more than one set of growth conditions may reveal more definitive taxonomic patterns.

Many of the genes involved in pathogenicity have been linked to QS regulation (165, 166). Interestingly, 24% of the strains identified in this study had their highest sequence identity with bacterial sequences recovered from diseased marine organisms. For example, the known pathogen *Vibrio coralliilyticus* was isolated from both species although none of the sampled coral colonies displayed morphological symptoms of disease. In *Vibrio* species, AHL synthesis can be tightly regulated by environmental conditions including host-released cues and nutritional status (150). The presence of so many potential pathogens in otherwise healthy corals requires further investigation, particularly if the pathogenicity of those strains is QS regulated.

5.4.1 Conclusion

This study has identified QS bacterial strains with QS and QS interference capacity within the surface mucosal layer of both *L. compactum* and *S. flexibilis.* The presence of both QS metabolites and QS bacteria within this micro-environment is supportive of QS and cembranoid diterpenes, such as isolobophytolide, as mediators of this community. Further investigation utilizing QS mutants and/or pure metabolites may shed light upon the mechanisms by which this regulation occurs, however, to definitively establish a regulatory role for AHL type QS within the soft coral holobiont the biological source of the QS mimics, as well as the bacteria and bacterial phenotypes affected, need to be conclusively demonstrated.

Chapter 6

Synthesis and General Discussion

6.1 Brief summary of outcomes

Bacterial communities in the surface mucosal layer (SML) of coral are hypothesised to have an important role in the health and resilience of the holobiont. Quorum sensing (QS) and QS interference are mechanisms that have been implicated in the regulation of mixed bacterial communities such as those found in the SML of corals (150, 167, 168). This is because many bacteria, as well as some host eukaryotic organisms, possess the ability to detect and respond to QS compounds produced by other species (15, 19, 22, 50, 55, 97, 23). Consequently, the detection and manipulation of bacterial QS signals can present an important role in the regulation of mixed bacterial communities. The aim of this project was to gain a better understanding of the role that QS plays in regulating eukaryote associated bacterial communities using soft corals as the model system.

The thesis sections were designed first to assess the prevalence of QS activity in soft coral holobionts (Chapter 2) and then to explore the structure function relationship of soft coral cembranoid metabolites to QS interference (Chapter 3). The plasticity of this system was further scrutinised by identifying a QS mimic in the form of a sterol from the soft coral *Nephthea chabroli* (Chapter 4). Finally, to establish the *in situ* regulatory role of these compounds, QS bacteria were isolated from both *Sinularia flexibilis* and *Lobophytum compactum* as the first step toward manipulation experiments with ecologically relevant bacterial strains (Chapter 5).

In this chapter, the main findings are synthesised and discussed, taking into consideration the strengths and limitations of the performed studies. In addition, avenues for further research into the role of QS mimics in the soft coral holobiont are proposed. Finally, overall conclusions from this work is presented highlighting the importance of developing an understanding of the ecology behind QS mimics concurrent with structural studies if QS mimics are to reach their biotechnology potential.

The first objective of this thesis was to confirm the presence and investigate the extent of QS activity in soft coral holobionts. QS inhibition activity has previously been indicated

in non-polar extracts of soft corals from the family Alcyoniidae (Chapter 2). This study, however, was able to demonstrate that QS interference extended across at least three soft coral families (Alcyoniidae, Clavulariidae and Nephtheidae). It also demonstrated that QS included polar and non-polar induction activity, as well as inhibition activity (Chapter 2). This indicates that QS interference capability is widespread in soft corals from the central Great Barrier Reef, Australia (Chapter 2). These results, which mirror similar findings in algae (35) and other marine invertebrates (51, 102, 96) provide further support for the importance of QS type interactions to the soft coral holobiont.

Coral associated bacterial communities are complex and diverse. The presence of multiple active fractions in the soft coral extracts suggested that individual corals possessed multiple compounds that have the ability to interfere with QS. This complexity may reflect the capability of some bacteria to possess multiple QS systems (46), with each system regulating a different process or interaction. Furthermore, different sensitivities and responses were observed in the two QS biosensors (*A. tumefaciens* A136 and *C. violaceum* CV026) used in this study (Chapter 2 and Chapter 5). Interestingly, the QS inductive capability in the soft coral extracts was only detected in the *A. tumefaciens* A136 strain, not the *C. violaceum* strain (Chapter 2). The presence of multiple QS compounds within a single holobiont would potentially enable a larger number of interactions with different bacterial strains and / or trigger different QS responses.

A number of organisms are capable of QS interference, however, the role of cembranoid diterpenes (soft coral secondary metabolites) in QS interference was unknown. Isolation and identification of cembranoid diterpenes was therefore required to understand the relative importance of these secondary metabolites to act as QS mimics. QS mimics are a form of manipulation where non-natant signals are produced to interfere directly with QS gene expression. Cembranolides and furanocembrenes from soft corals were found to be capable of interference with Gram negative QS systems and may be acting as QS mimics within the soft coral holobiont (Chapter 3).

Cembranoid diterpenes are common secondary metabolites of Alcyonacean soft corals with a high level of structural variation (87 and previous in series; 91). This natural struc-

tural series of compounds means that soft corals represent an important model system for the investigation of QS mimics. The observed activity of the cembranoid diterpenes is, however, not unexpected given the presence of lactone and furan functional groups that have well-established activity (120). The QS interference observed in cembranolides and furanocembrenes (Chapter 3) translates to a potentially new structural backbone for Gram negative QS mimic compounds. More importantly though, the findings of this study reveal important information of the relative impact of manipulating different aspects of a QS mimics structure. In this instance, the size of the oxygenated ring had more bearing on the activity expressed than the presence or position of epoxides, double bonds or acetate groups (Chapter 3), thereby extending the structural understanding of QS mimics. The impact of these different functional groups to the activity of QS mimics is important if new pharmaceuticals based around this form of interaction are to be designed.

The discovery of a novel QS active sterol (17,22-dihydroxy-24-methylene cholesterol) in the soft coral *N. chabroli* provided an opportunity to explore QS mimics that lacked either a lactone or furan ring (Chapter 4). QS mimics that lack this functionality have been identified previously, such as the N,N-alkylated imidazolium derivatives from potato tubers (29), but their mechanisms of action are generally poorly understood (Persson et al., 2005). Molecular modelling of the QS active sterol from this study suggested that hydrogen bonding between the hydroxyl groups forms a pseudo-oxygenated ring system (Chapter 4). It is possible that this conformation is what allows the compounds to trigger QS, greatly extending the structural range of compounds that potentially confer some level of microbial regulation and consequently increase the pool of structures that can be targeted by QS investigations.

The isolation and identification of a sterol with QS interference capability (Chapter 4) also has broad implications for the prevalence of inter-kingdom communication between eukaryotic host organisms and their associated bacteria. Sterols are ubiquitous amongst eukaryotes but have rarely been attributed to bacterial production. Therefore, this is potential evidence of inter-kingdom communication between the host coral, *N. chabroli*,

or its endosymbiotic zooxanthellae with the associated bacterial community. If this is the case, this evidence offers a more complex series of interactions between host and associated bacterial communities than previously recognised.

In order to start to piece together the *in situ* roles that QS plays as a regulatory measure of coral associated microbial communities, it is essential to establish and identify ecologically relevant isolates. Culturable bacteria from two soft coral species (*S. flexibilis* and *L. compactum*) were isolated and identified and the QS capabilities of selected strains were assessed. The identification of QS mimics and QS bacteria within soft corals increased the support for QS as one of the mechanisms regulating coral associated microbial communities.

Generally, culture based investigations are limited by the number of species of bacteria capable of being cultured (169, 170). For this reason, isolobophytolide, a cembranoid diterpene that occurs in high concentrations in *L. compactum*, was included in some of the media used to isolate bacteria from this species' SML (Chapter 5). The inclusion of secondary metabolites in the growth media resulted in higher numbers of bacterial communities on the culture plates as well as increased variability in the diversity of morphotypes isolated (Chapter 5). Compounds that interfere with the cell-cell communication of selected bacterial strains while leaving others unimpeded would have a selective effect on the bacteria that are able to grow in the SML of a soft coral. The combined effect on QS activity in sensor strains (Chapter 3) and stimulation of growth of soft coral bacterial isolates (Chapter 5) implies that isolobophytolide is an important selection factor regulating the microbial community of *L. compactum*.

A common feature of QS mimic compounds previously identified from eukaryotic extracts is multiple forms of biological activity (8, 9, 120, 94). For example, metabolites isolated from garlic have been identified as possessing antimicrobial and antiviral activity as well as QS inhibition (171). Interestingly, antibiotic and antimicrobial properties have been reported previously (95, 172) for a number of the cembranoid diterpenes, including those QS active cembranoid diterpenes identified in the current study (Chapter 3). The antimicrobial activity identified in flexibilide by Aceret and colleagues (95), however, was

exhibited at concentrations at least one order of magnitude higher than those that produced QS interference in this study (Chapter 3). Rather than being incompatible, the contrasting activities could be evidence of a hormetic response. Hormesis is a feature of a number of antibiotic compounds whereby growth stimulation or cell signalling properties are exhibited at below their minimum growth inhibitory concentration. To date, however, so far activity at such low concentrations has been investigated for only a small proportion of antibiotic compounds (9, 8). More research is required to understand the potential hormetic relationships of many QS mimics, and this information is essential if therapeutics based on the QS mechanisms are to be effectively designed.

6.2 Future Directions

There is merit in understanding the mechanisms of how secondary metabolites of soft corals influence their associated bacterial communities. The ability to interfere with and potentially regulate a coral's associated bacterial community could be important to the health and resilience of the host organism and may reflect a more widespread strategy of sessile marine invertebrates. Soft corals represent only one of a number of different model systems that have been examined on the topic of QS mimics. Each model has allowed the analysis of such interactions from different aspects, further generating new hypotheses. There exists a large volume of potential research that could build on the information and materials generated in this project to further develop and enhance our understanding of how the structure of QS mimics influences the ecology of these symbioses. These include:

1. Cembranolides and furanocembrenes are only a small proportion of the secondary metabolites from soft corals with the potential for QS interference. Exploration of alternate sources of the QS activity present in soft corals by subjecting the more polar extracts to bioactivity guided assays could yield valuable information. For example, the briarene diterpenes represent a similar natural series of structural variation that would be worthy of investigation, as many of these also contain functionalised δ-lactone rings.

2. All of the cembranolides in this study had trans ring junctions between the cembrene and secondary oxygen rings. Although very few *cis* ring junctions have been discovered in cembranoid diterpenes, this configuration could reveal important structural information in other QS mimics. Investigation of the potential impacts of other forms of variation within the cembranoid diterpenes would expand on this knowledge.

3. The bacterial isolates generated from soft coral SML could be used in further studies to establish the true effect of soft coral QS mimics on ecologically relevant strains. It would be beneficial if a method can be developed to assess the QS activity of those bacterial strains that were dependent on the presence of the cembranoid diterpene isolobophytolide for growth on the media used in the present study. The effect of QS mimics on the growth and biofilm formation of wild type isolates can be tested to investigate if they display differential responses. The generation of ecologically relevant reporter strains and other quorum sensing mutants would represent a big step forward towards a mechanistic understanding of the interaction between QS mimics and ecologically relevant bacterial strains.

4. It would be interesting to perform these type of experiments with soft coral larvae to investigate how QS mimics can influence the establishment of soft coral associated bacterial communities. Molecular methods could be used to assess the overall microbial communities of soft coral mucosal layers and their response to QS mimic compounds at different life stages.

6.3 Conclusions

The outcomes of this research highlight the potential value of soft corals as a model system for both structural and ecological investigations of QS mimics. Ecologically, this research establishes a framework for the importance of QS and the identity of potential QS mimics within the soft coral holobiont. Furthermore, this detailed study on QS in soft corals provides new insights into the mechanisms that regulate soft (and

potentially hard) coral against fluctuating or undesirable bacterial infections. Although knowledge gaps still exist with respect to the *in situ* roles of these compounds within the soft coral holobiont, the presence of both QS metabolites and QS bacteria within soft corals supports the role of QS as a way of mediating soft coral associated microbial communities.

Whether or not the compounds isolated in this study are QS mimics in an ecological setting, there is a lot that can be learnt from their structural variation with respect to the design and search for new QS pharmaceuticals. This research provides necessary information on the potential for regulation of microbial communities, not only through an understanding of the complexity of the interaction, but also through a structural understanding of how QS mimic compounds are able to interfere with this system. This dual ecological and fundamental understanding is essential if QS mimics are to be adapted for successful pharmaceutical use.

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Appendix A

Soft Coral Chemical Fingerprints

Chemical Fingerprints of collected soft corals

The following are ^{1}H NMR spectra of the soft coral extracts screened for Quorum Sensing (QS) activity in Chapter 2.

Twenty four specimens of soft coral were collected at a depth of 1-3 m from Orpheus Island representing 15 species (Great Barrier Reef, Australia; latitude, 18° 36.878' S; longitude, 146° 29.990' E). All specimens were photographed underwater before sampling (Figure 2.1) and a taxonomic voucher sample of each was placed into 70% ethanol for reference. Voucher samples have been submitted to the Museum of Tropical Queensland Museum, Townsville Australia.

Samples were collected in May of 2009 (Figures A.1 - A.13) and 2010 (Figures A.14 - A.17). These spectra were collected using standard ^{1}H pulse sequences on a Bruker Avance 300 Nuclear Magnetic Resonance Spectrometer. Each extract (2 mg) was dissolved in 700 µl of deuterated solvent (DCM extracts into deuterated chloroform, $CDCl_3$, methanol extracts with deuterated methanol, CD_3OD, and water extracts into deuterium oxide, D_2O).

A.1 Chemical fingerprints from the 2009 collection

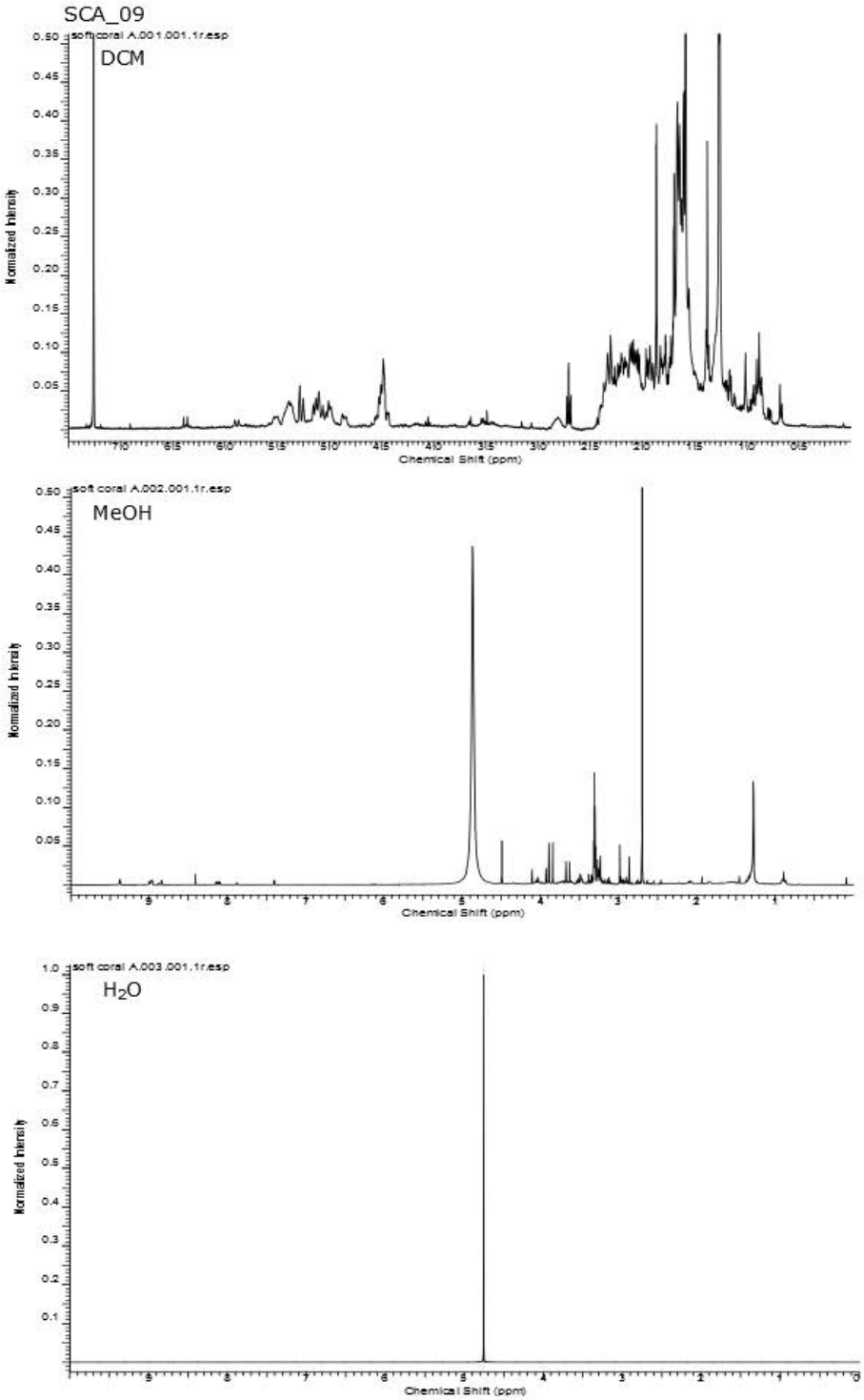


Figure A.1: ^{1}H NMR Chemical Fingerprint of the a) DCM, b) MeOH and c) H_2O extracts of soft coral A

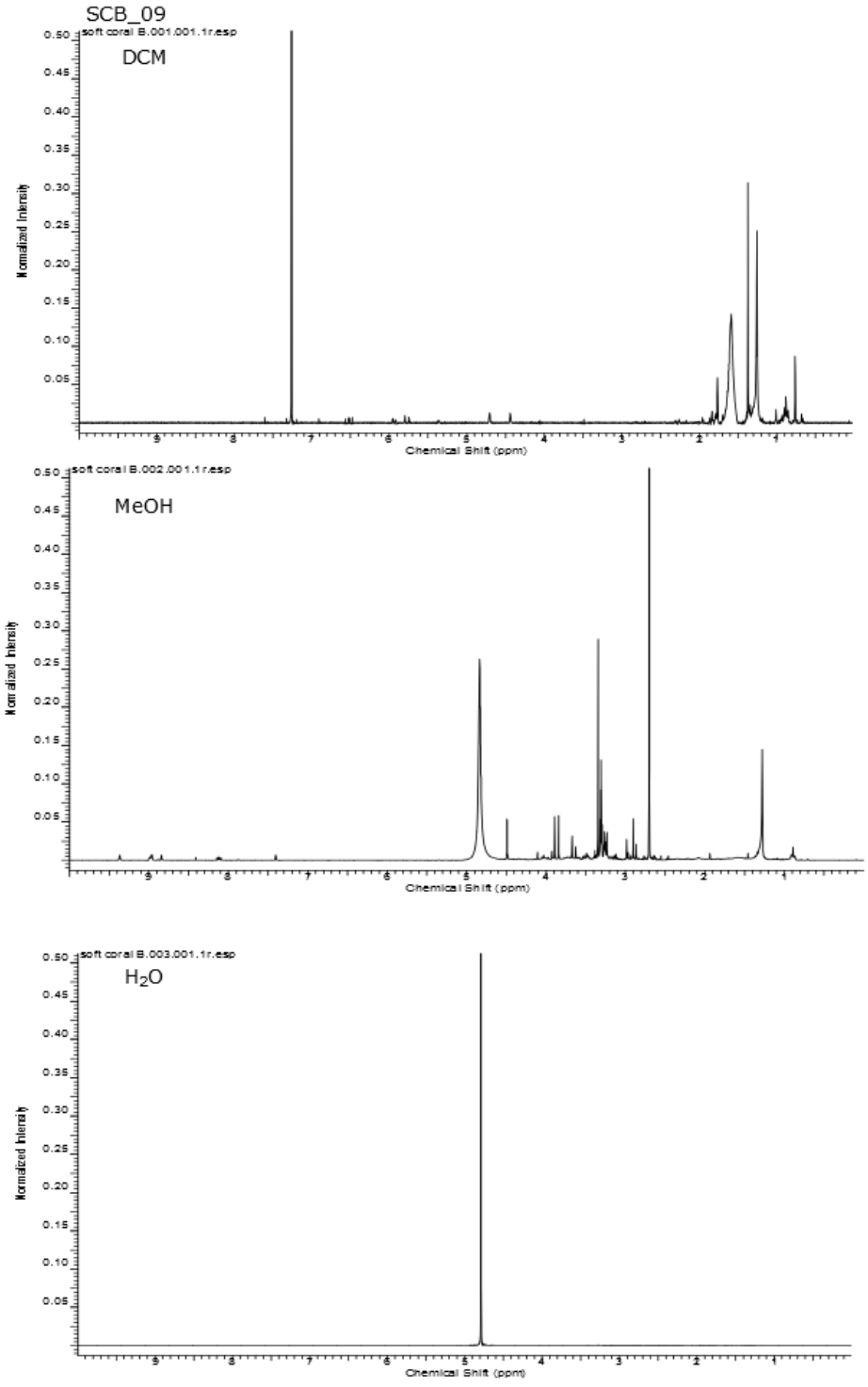


Figure A.2: ^{1}H NMR Chemical Fingerprint of the a) DCM, b) MeOH and c) H_2O extracts of soft coral B

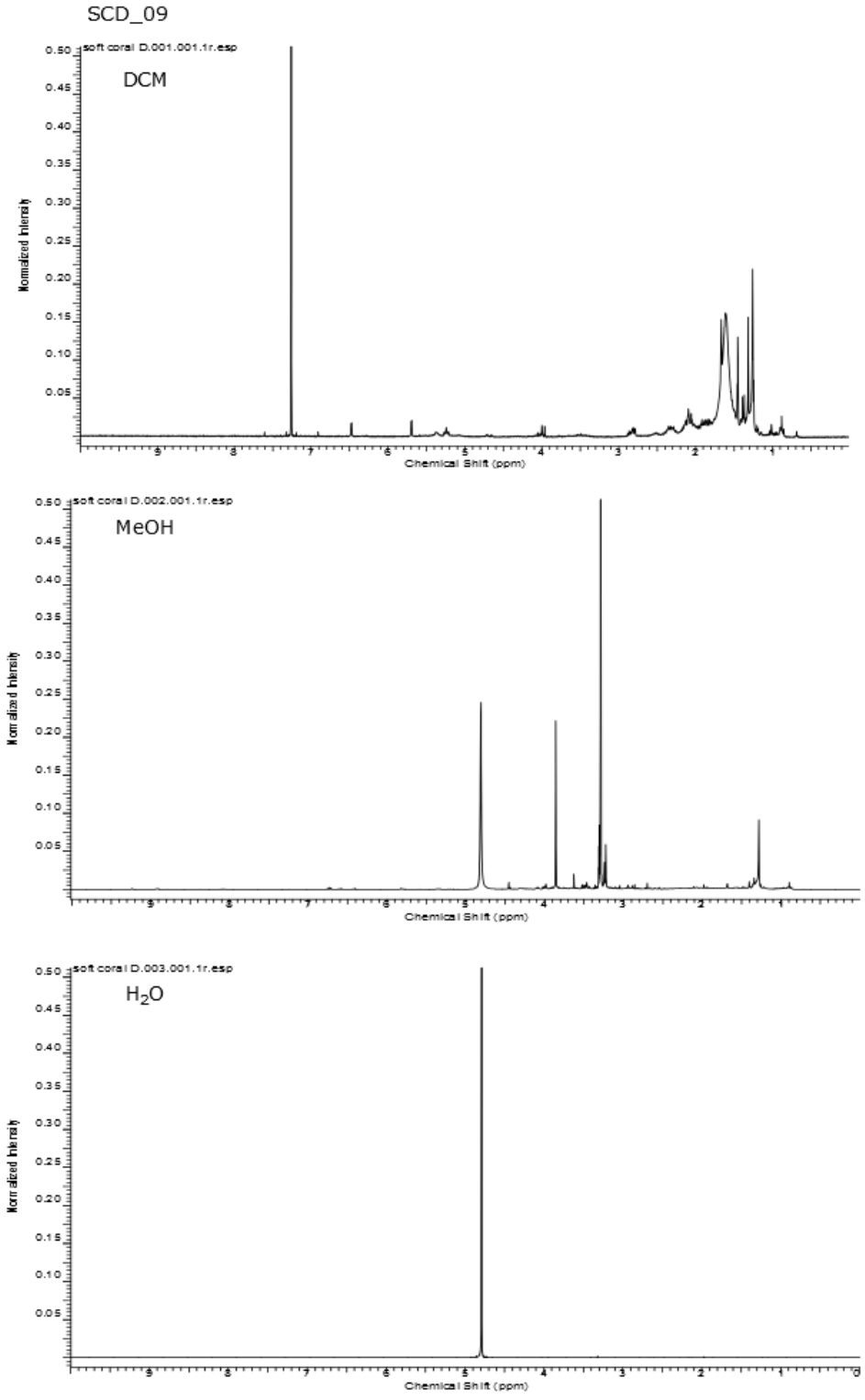


Figure A.3: ^{1}H NMR Chemical Fingerprint of the a) DCM, b) MeOH and c) H_2O extracts of soft coral D

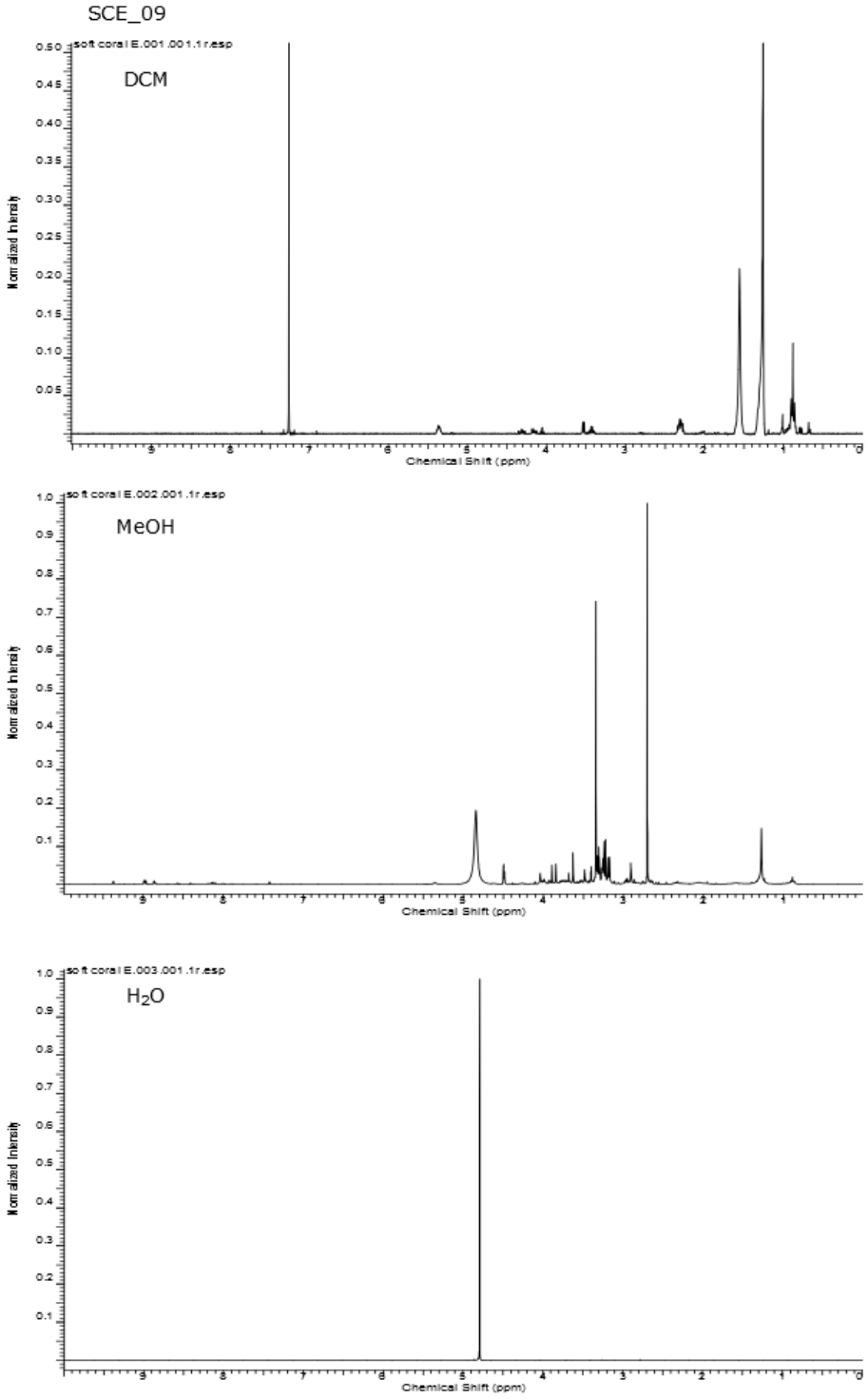


Figure A.4: ^{1}H NMR Chemical Fingerprint of the a) DCM, b) MeOH and c) H_2O extracts of soft coral E (unidentified gorgonian)

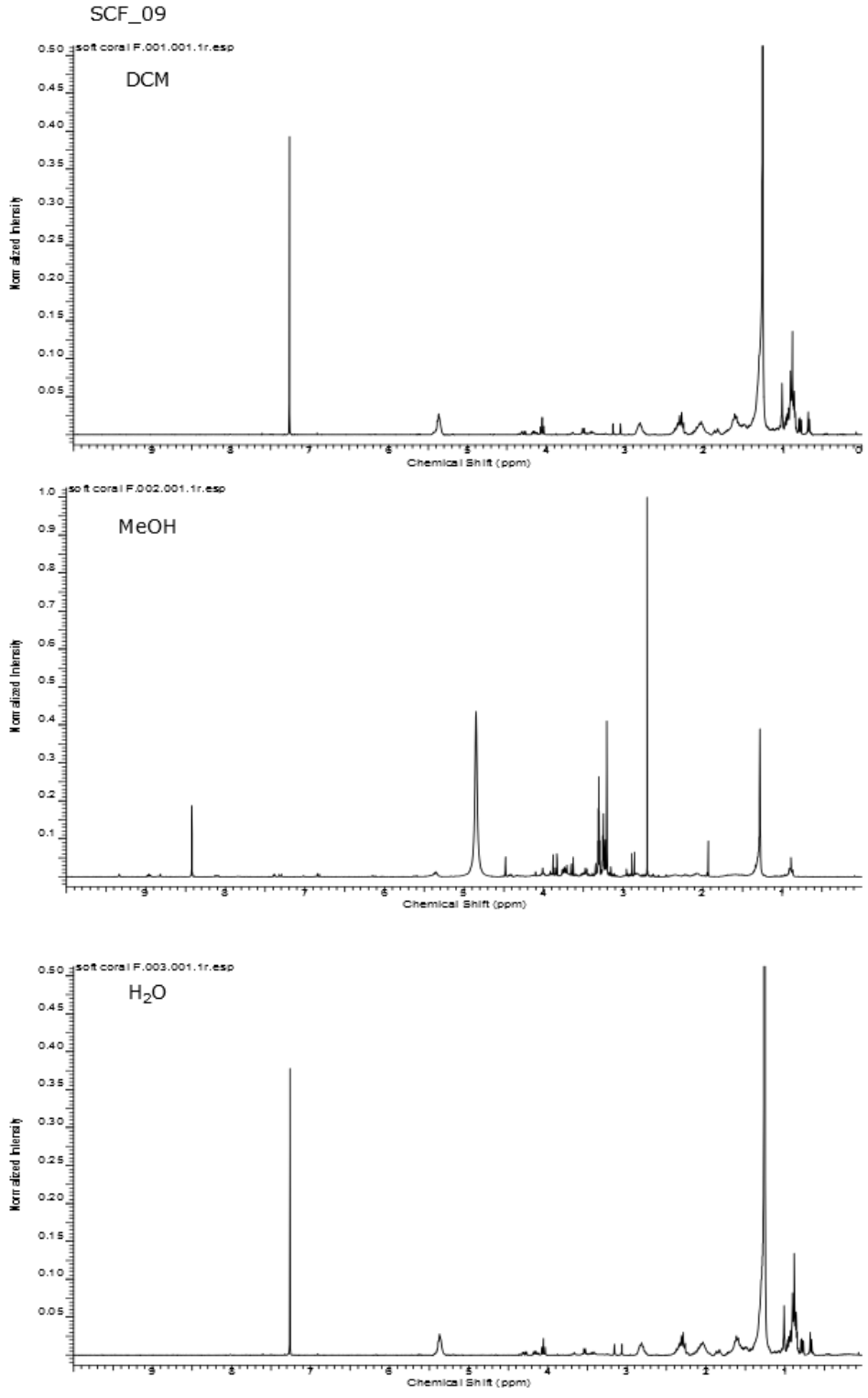


Figure A.5: ^{1}H NMR Chemical Fingerprint of the a) DCM, b) MeOH and c) H_2O extracts of soft coral F

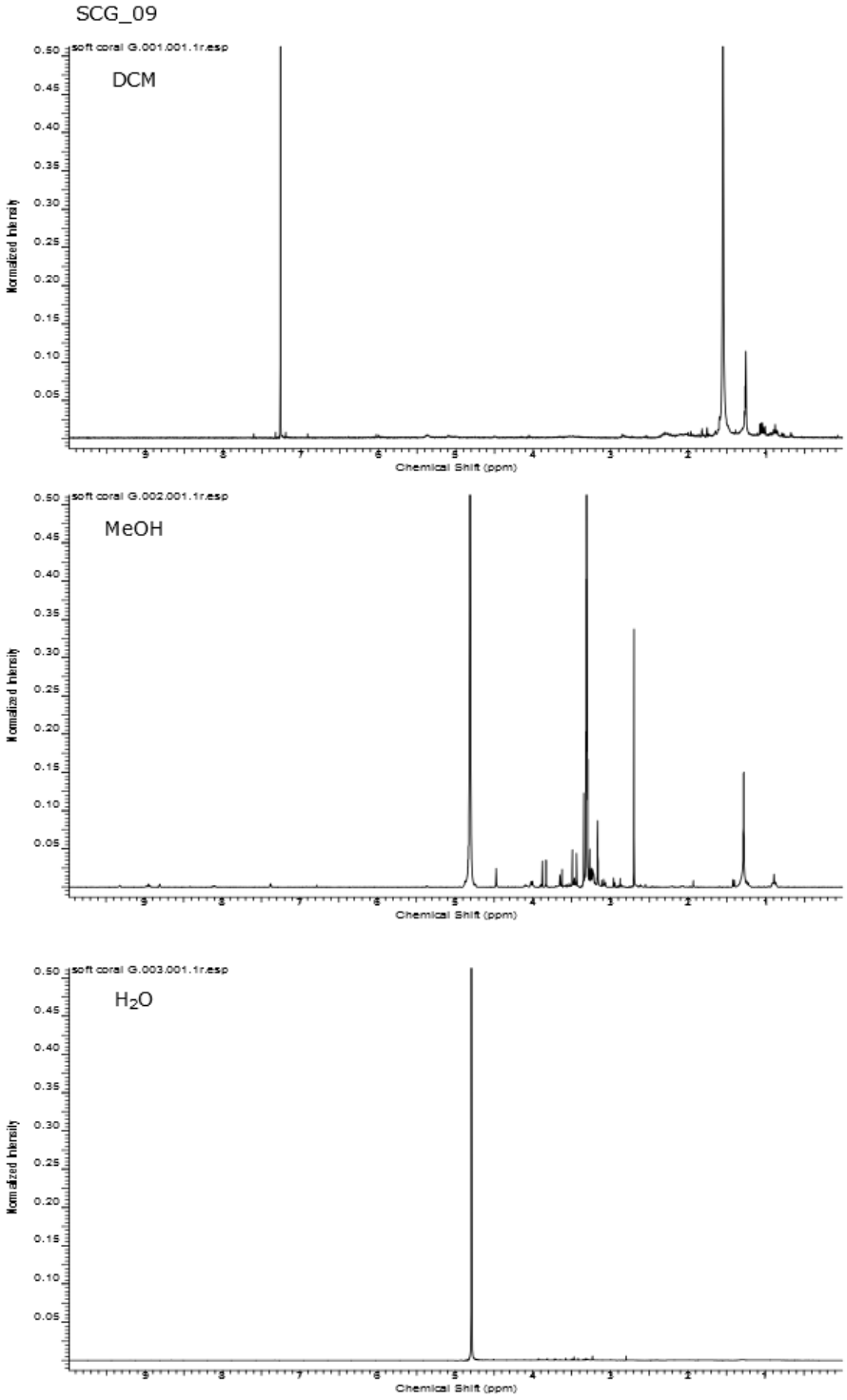


Figure A.6: ^{1}H NMR Chemical Fingerprint of the a) DCM, b) MeOH and c) H_2O extracts of soft coral G

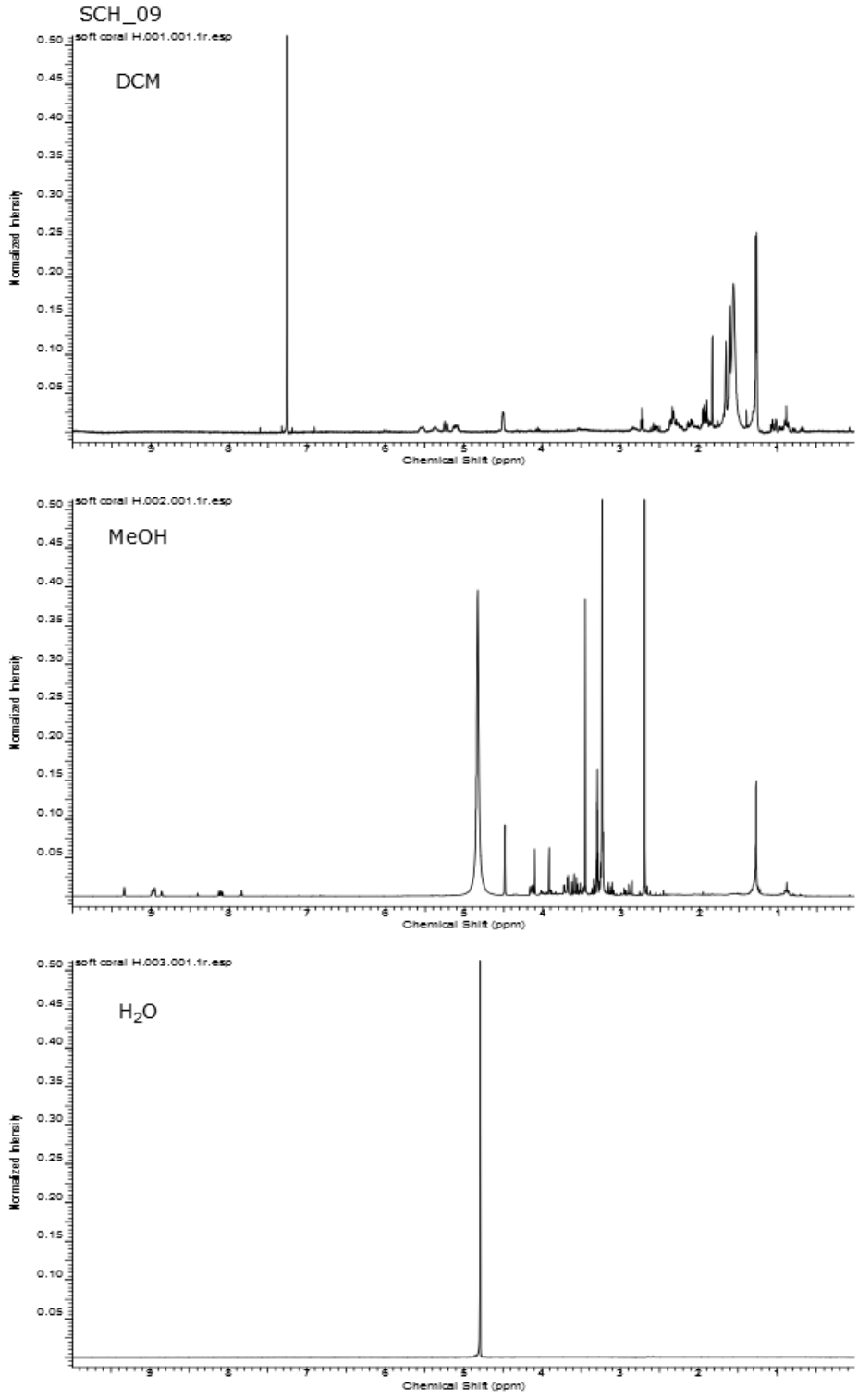


Figure A.7: ^{1}H NMR Chemical Fingerprint of the a) DCM, b) MeOH and c) H_2O extracts of soft coral H

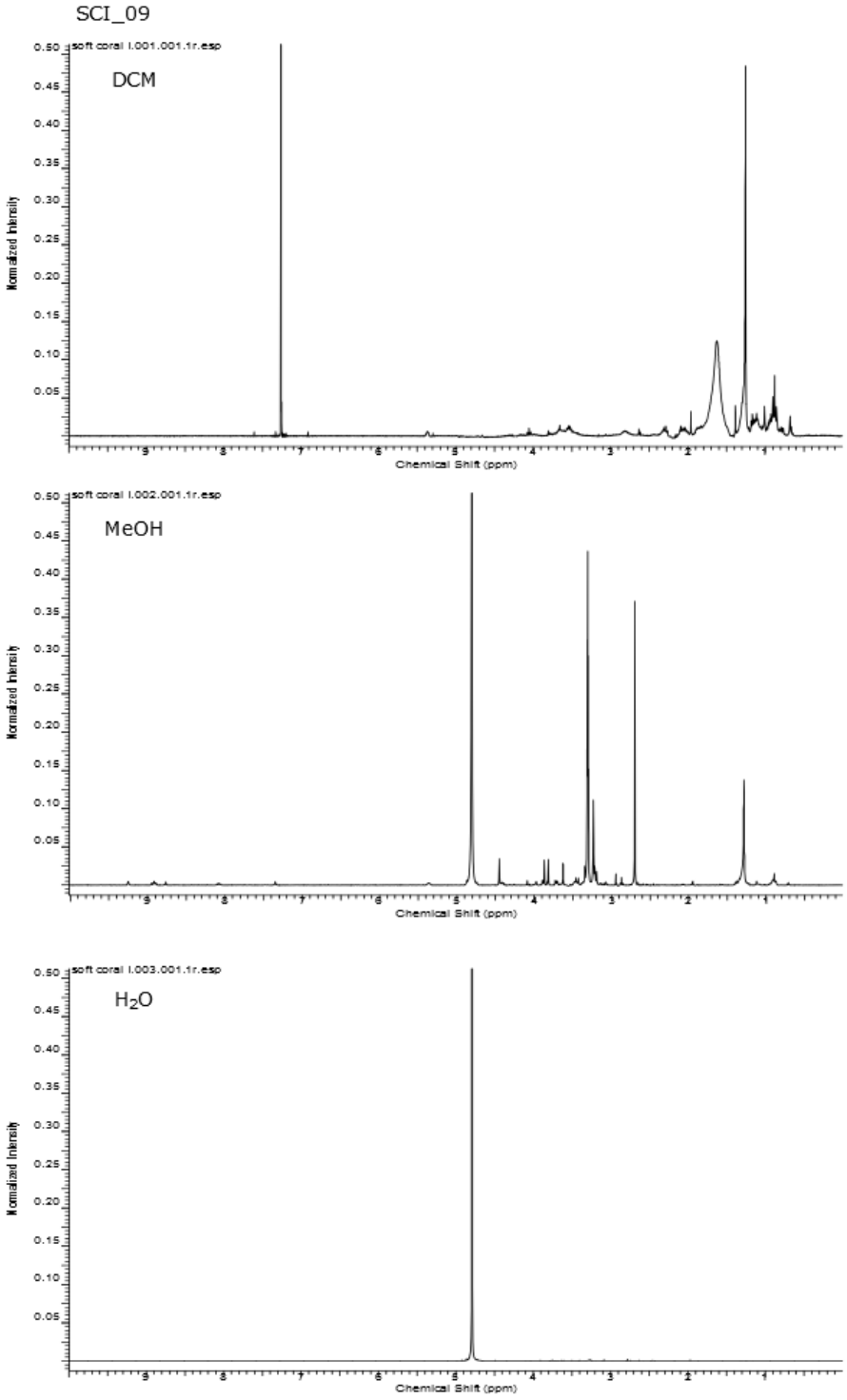


Figure A.8: ^{1}H NMR Chemical Fingerprint of the a) DCM, b) MeOH and c) H_2O extracts of soft coral I

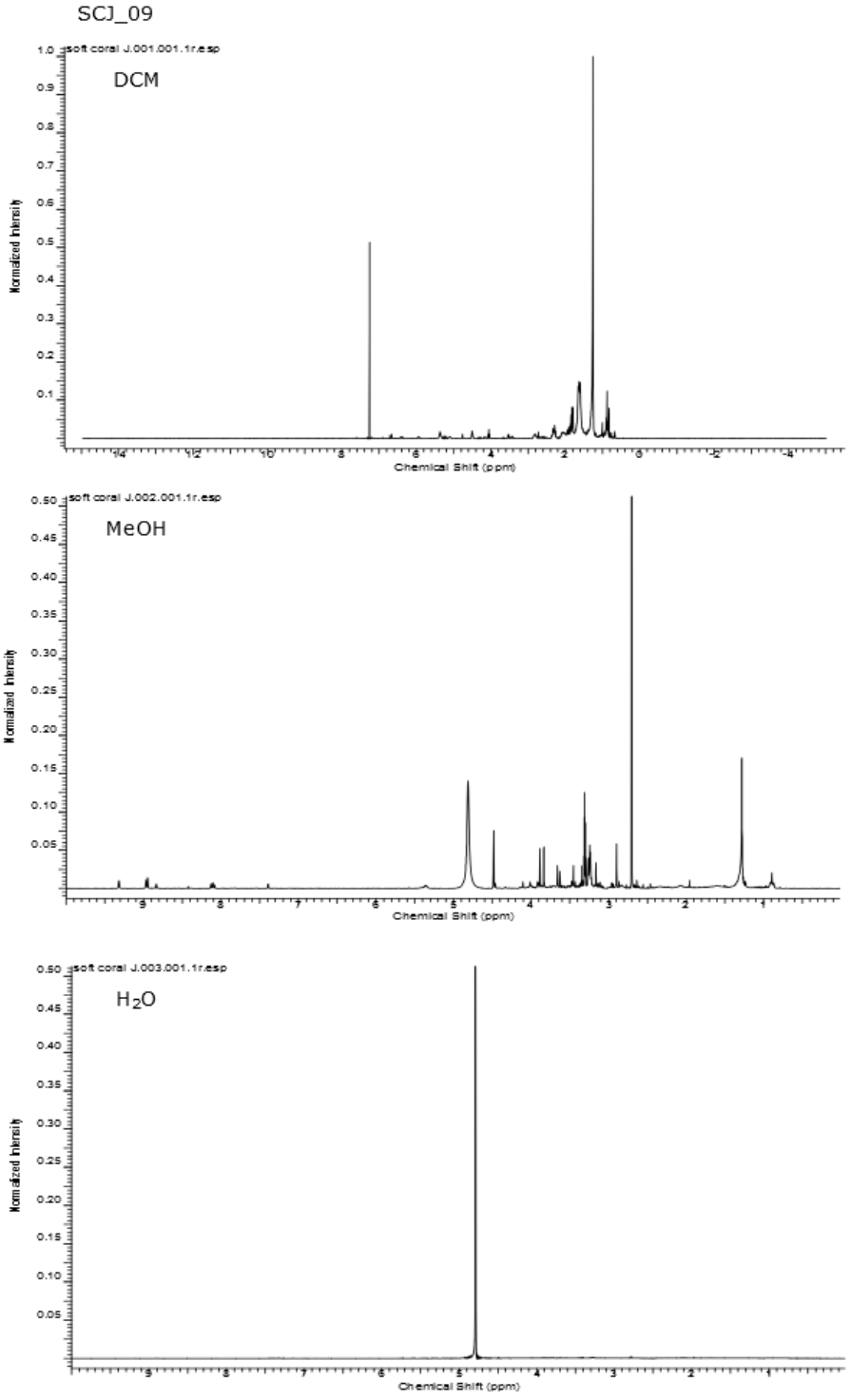


Figure A.9: ^{1}H NMR Chemical Fingerprint of the a) DCM, b) MeOH and c) H_2O extracts of soft coral J

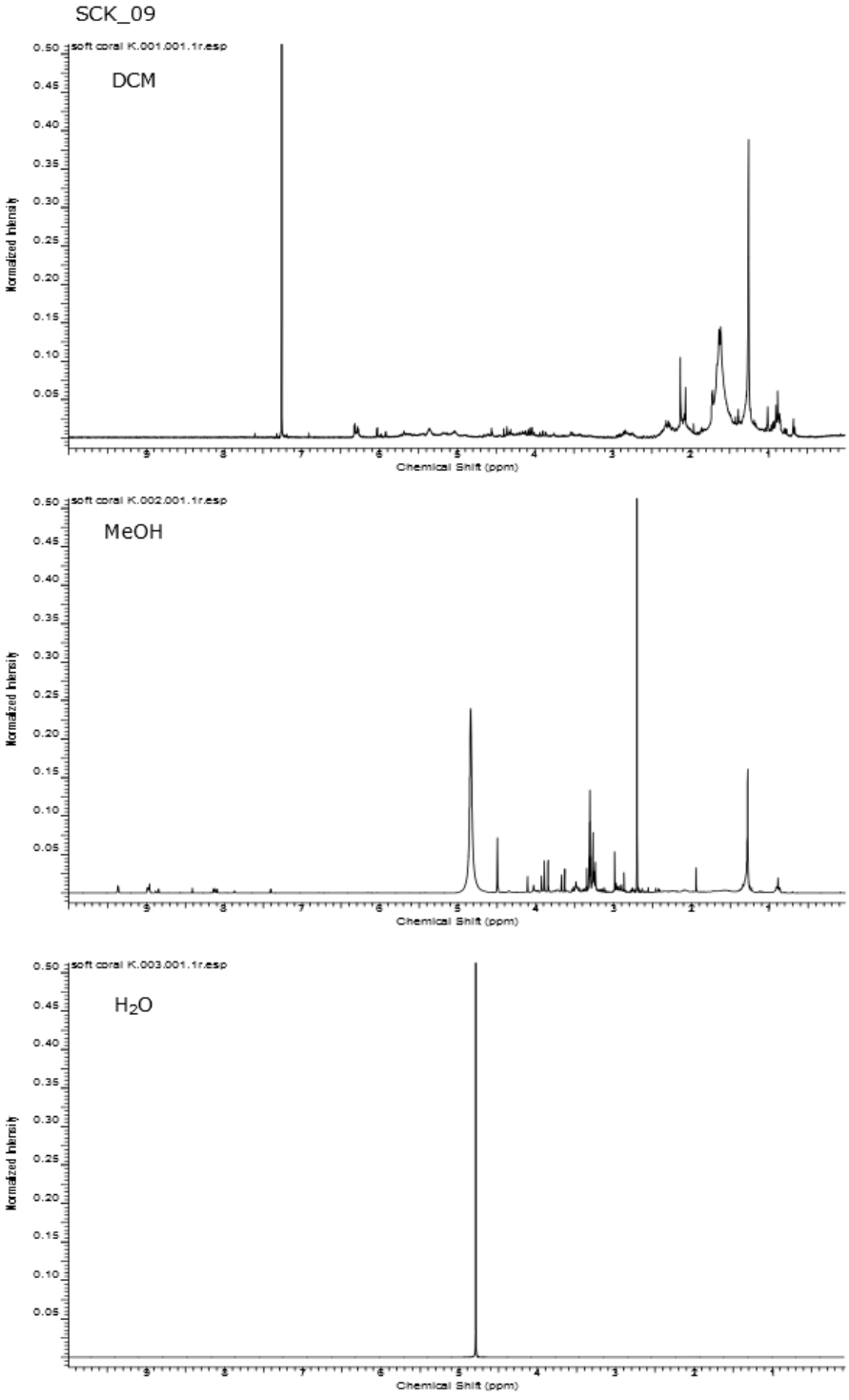


Figure A.10: ^{1}H NMR Chemical Fingerprint of the a) DCM, b) MeOH and c) H_2O extracts of soft coral K

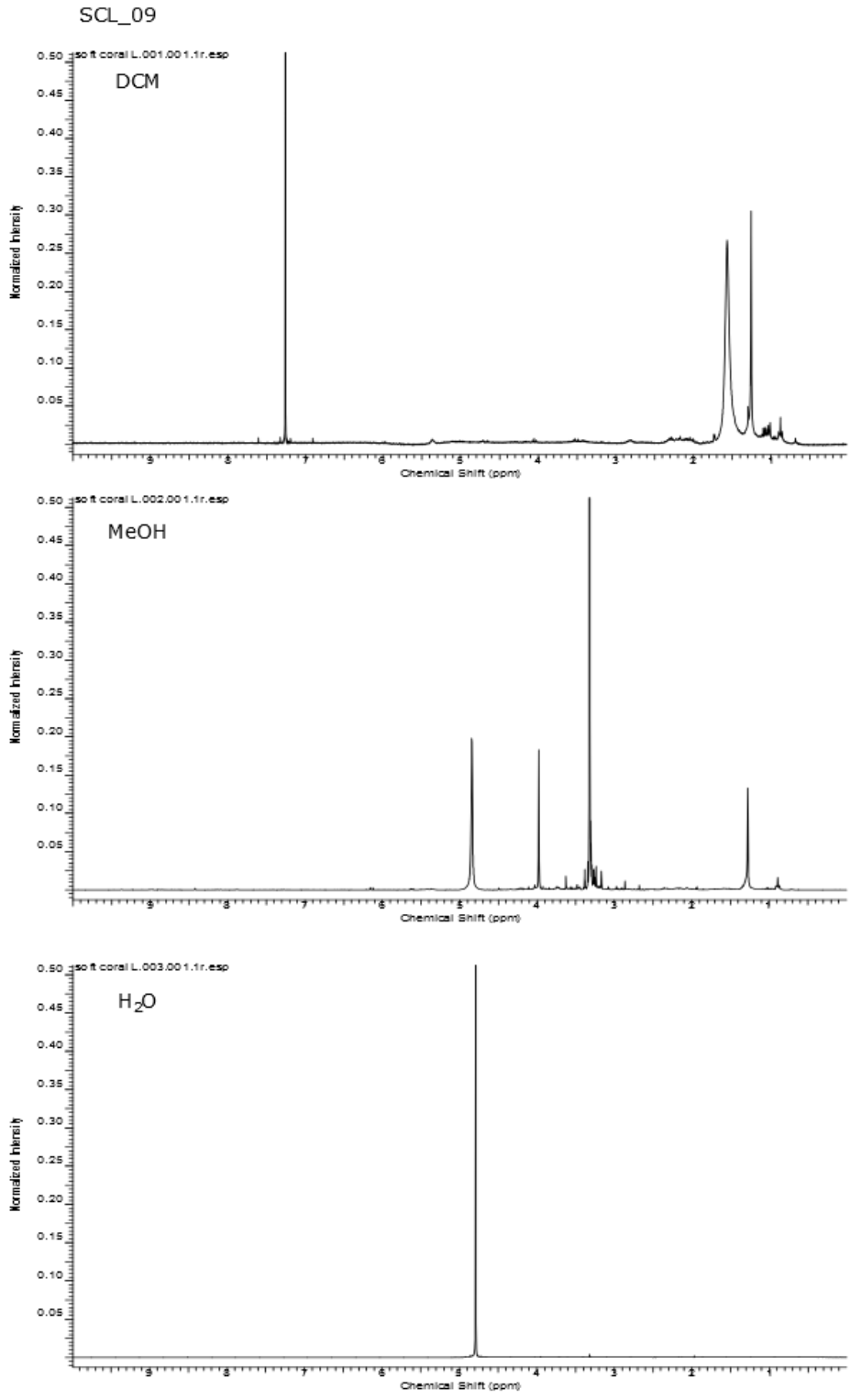


Figure A.11: ^{1}H NMR Chemical Fingerprint of the a) DCM, b) MeOH and c) H_2O extracts of soft coral L

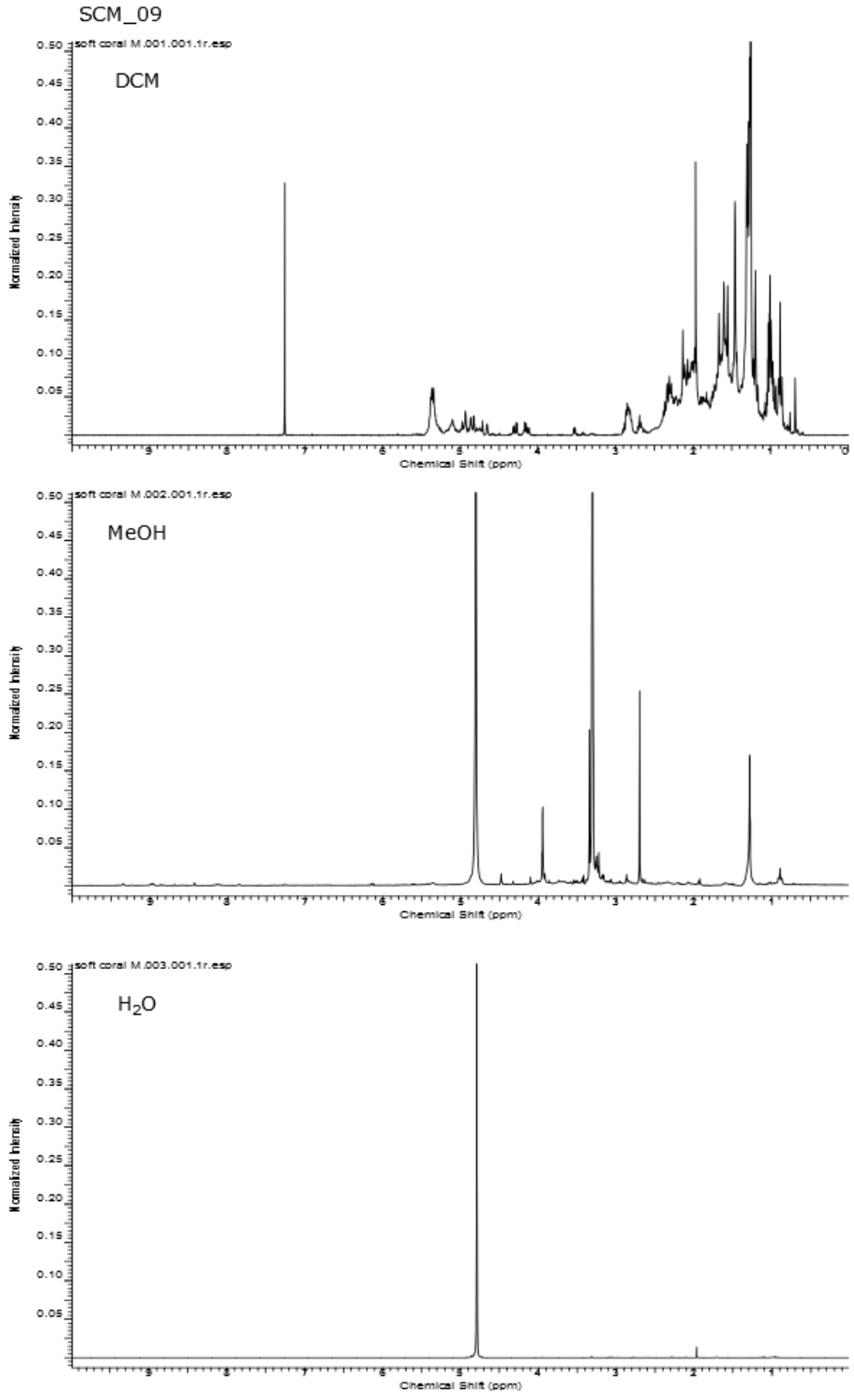


Figure A.12: ^{1}H NMR Chemical Fingerprint of the a) DCM, b) MeOH and c) H_2O extracts of soft coral M (*Nephthea chabroli*)

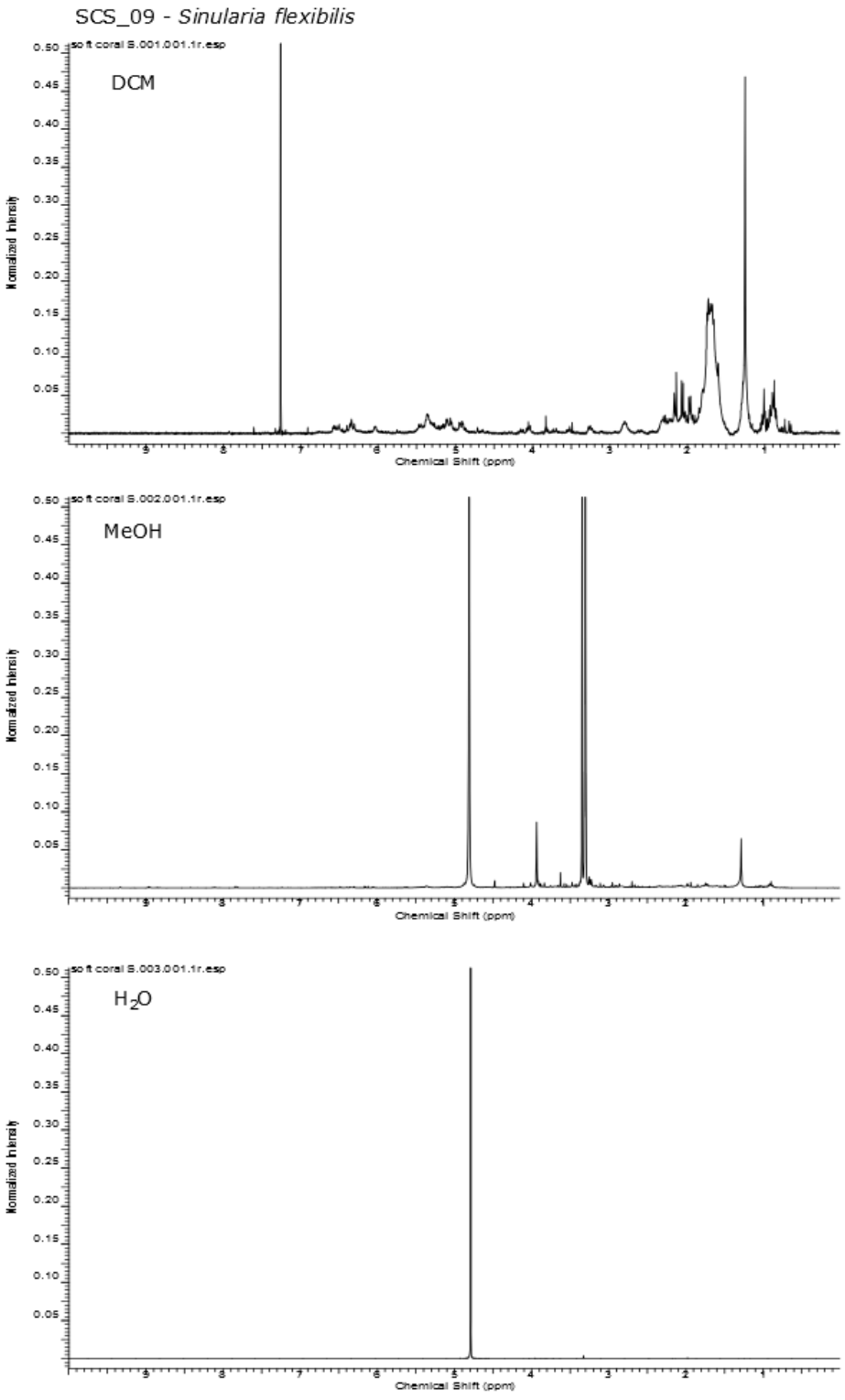


Figure A.13: ^{1}H NMR Chemical Fingerprint of the a) DCM, b) MeOH and c) H_2O extracts of soft coral S (*Sinularia flexibilis*)

A.2 Chemical fingerprints from the 2010 collection

(DCM extracts of ^{1}H NMR spectra only).

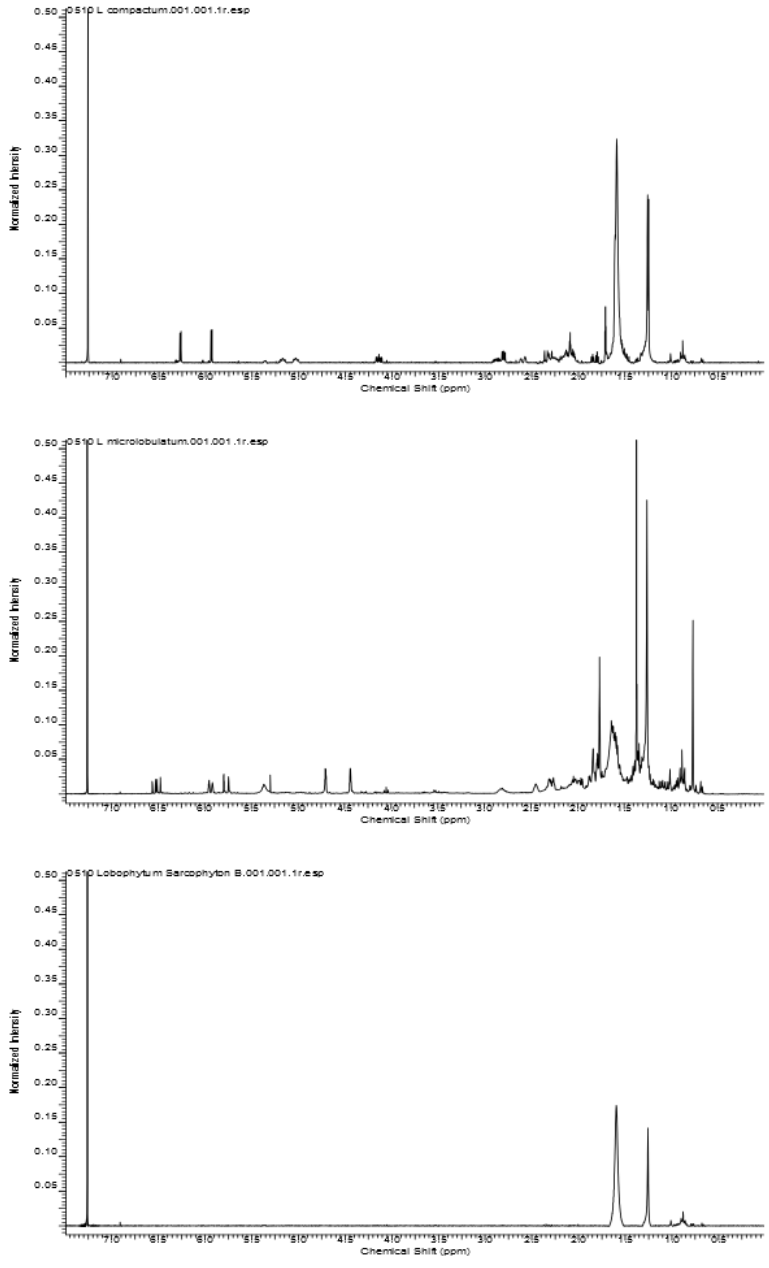


Figure A.14: ^{1}H NMR Chemical Fingerprint of the DCM extracts of a) *Lobophytum compactum*; b) *Lobophytum microlobulatum* and c) *Lobophytum sarcophytoides*.

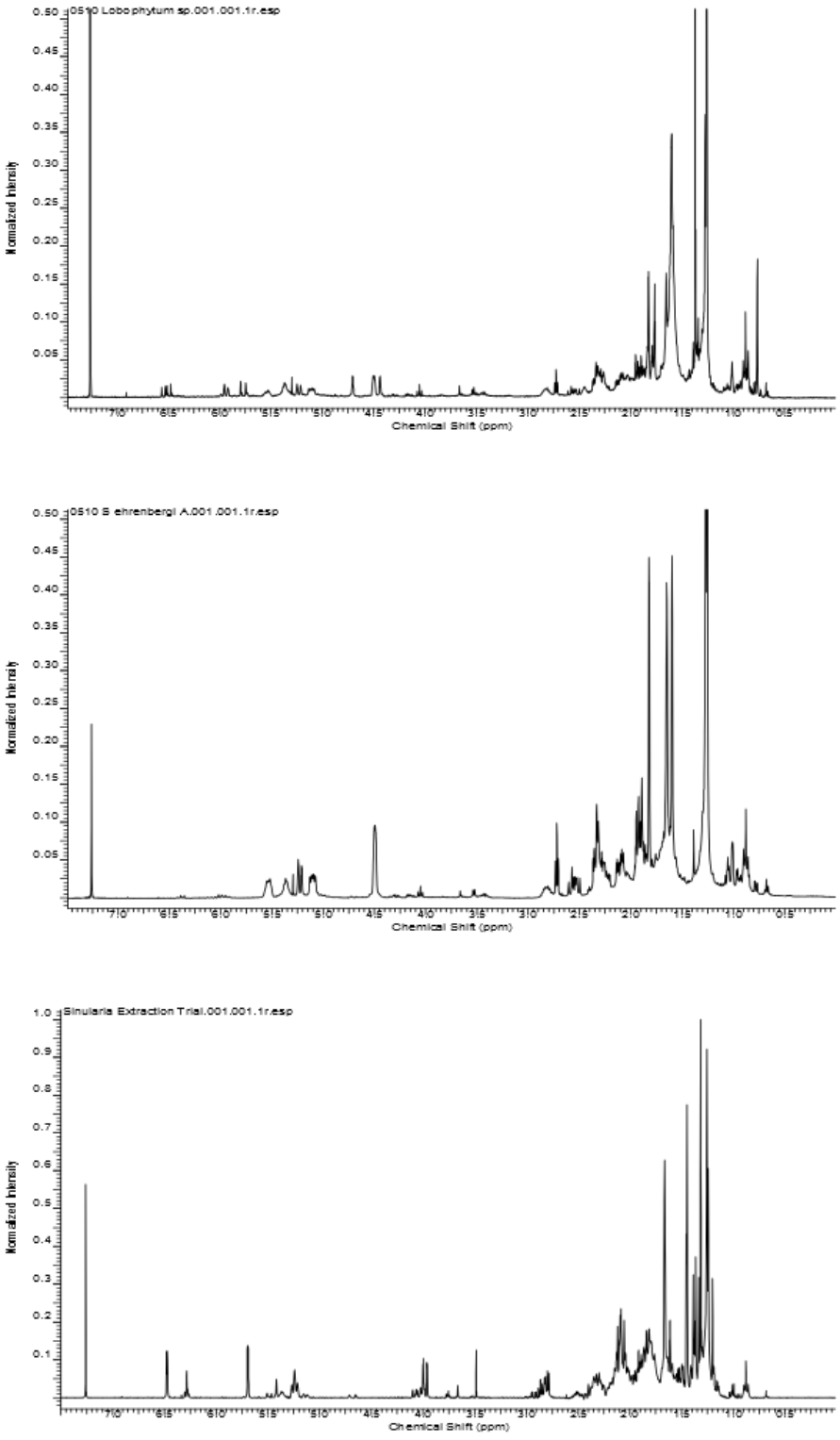


Figure A.15: SC_2010_2

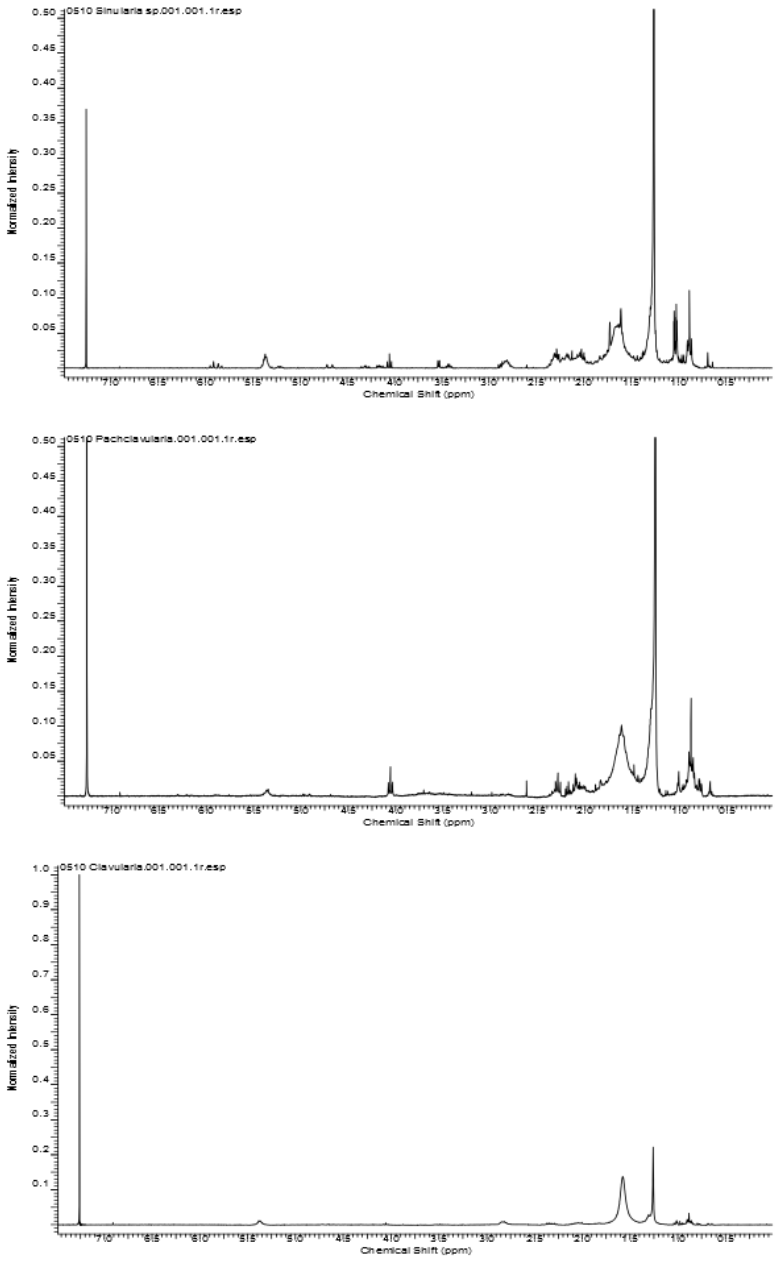


Figure A.16: ^{1}H NMR Chemical Fingerprint of the DCM extracts of a) *Sinularia polydactyla*; b) *Pachyclavularia violacea* and c) *Clavularia* sp..

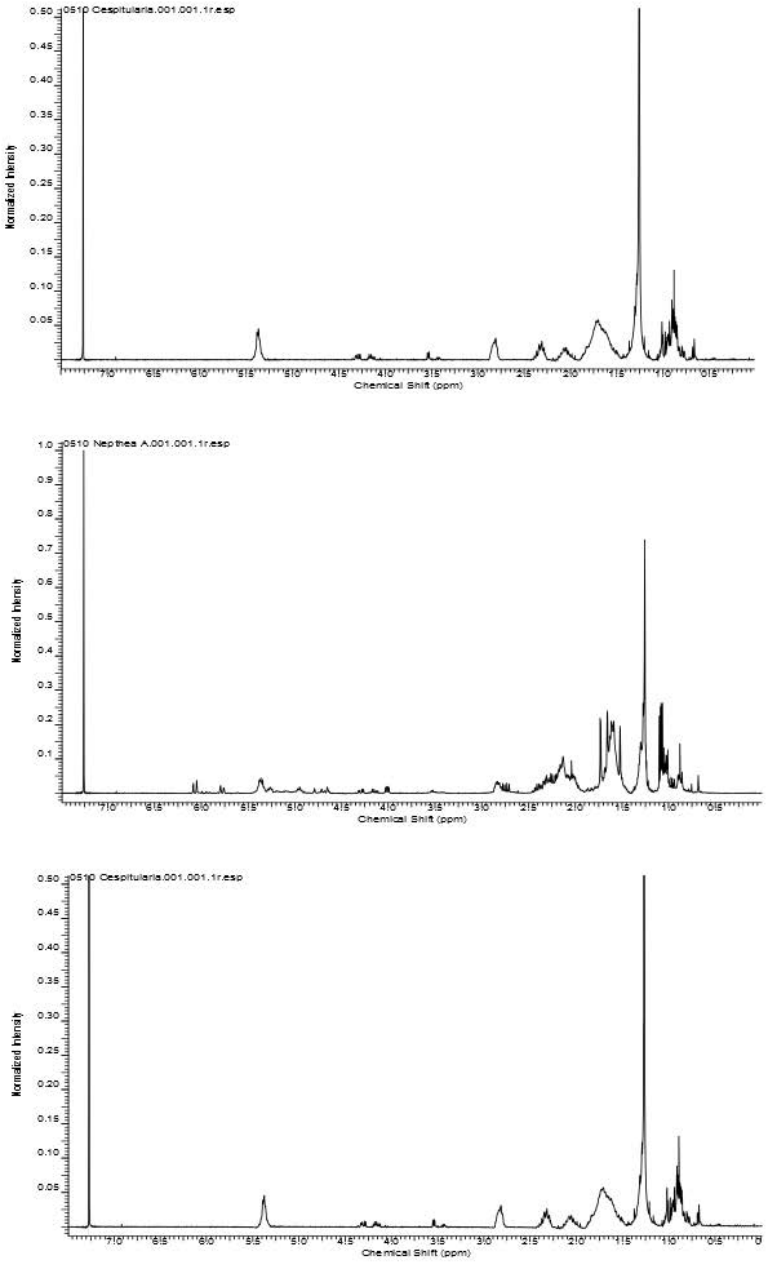


Figure A.17: ^{1}H NMR Chemical Fingerprint of the DCM extracts of a) *Cespitularia* sp.; b) *Nephthea chabroli* and c) *Lobophytum durum*.

Appendix B

Cembranoid Diterpene Purity Checks

B.1 NMR tables of isolated compounds

The following ^{1}H NMR spectra are the pure compounds tested in Chapter 3.

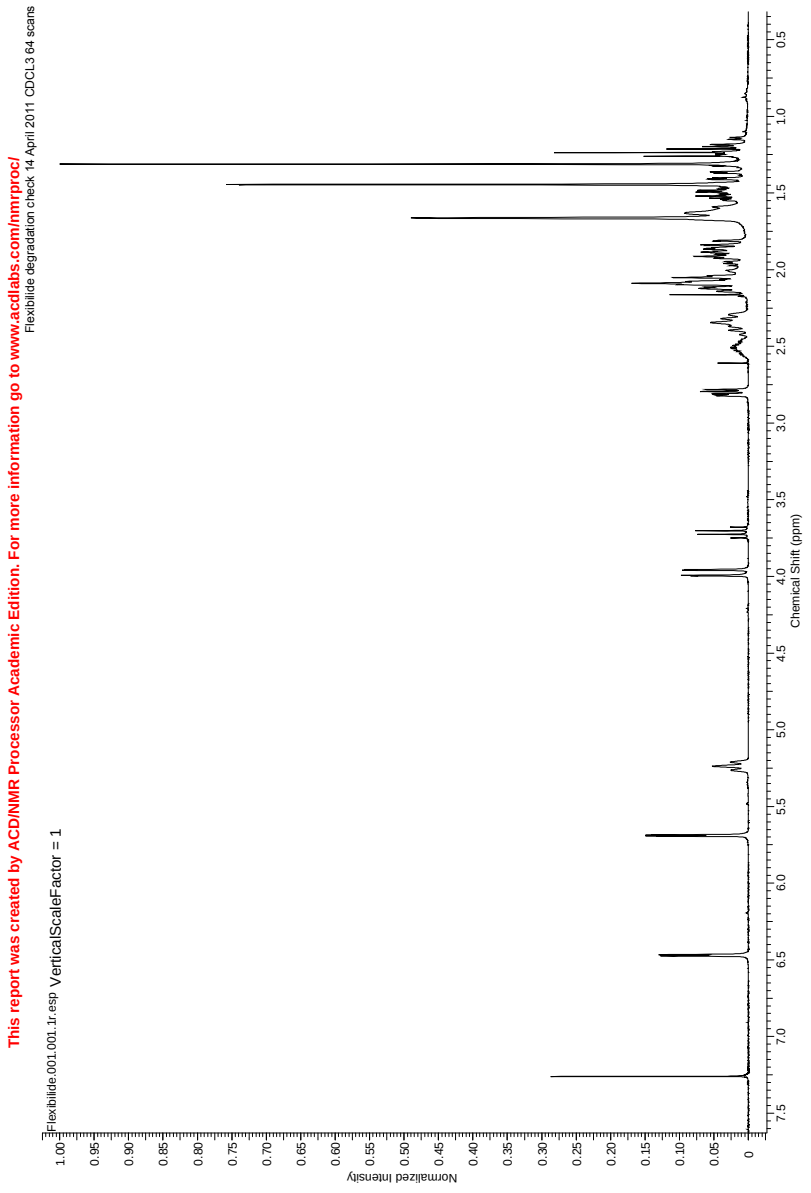


Figure B.1: ^{1}H NMR spectrum of Flexibilide

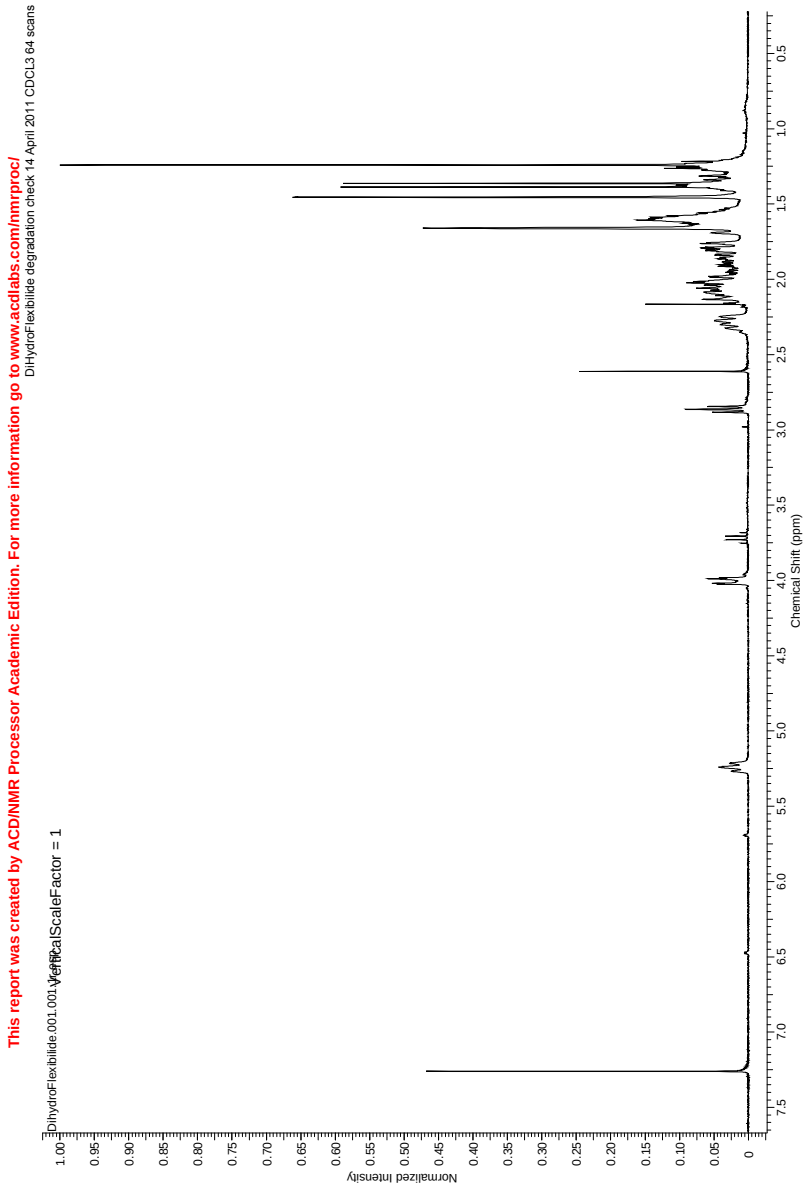


Figure B.2: ^{1}H NMR spectrum of Dihydro Flexibilide

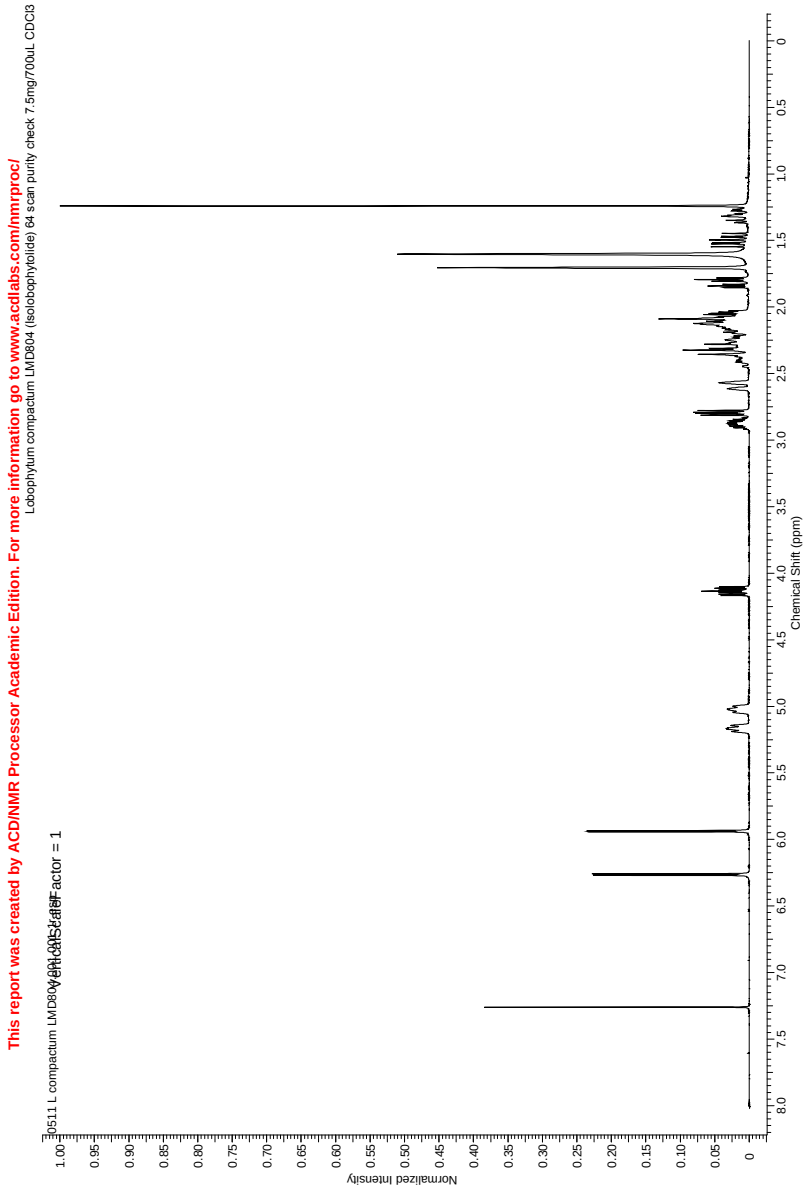


Figure B.3: ^{1}H NMR spectrum of Isolobophytolide

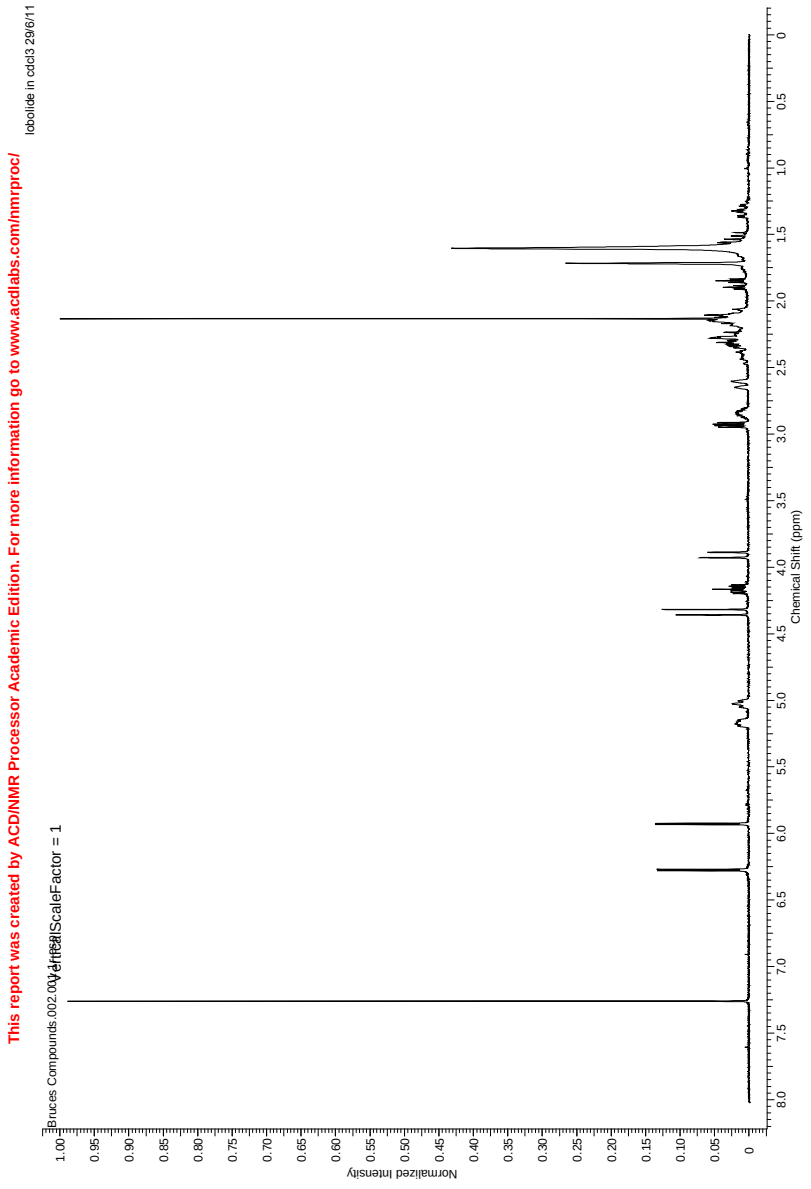


Figure B.4: ^{1}H NMR spectrum of Lobolide

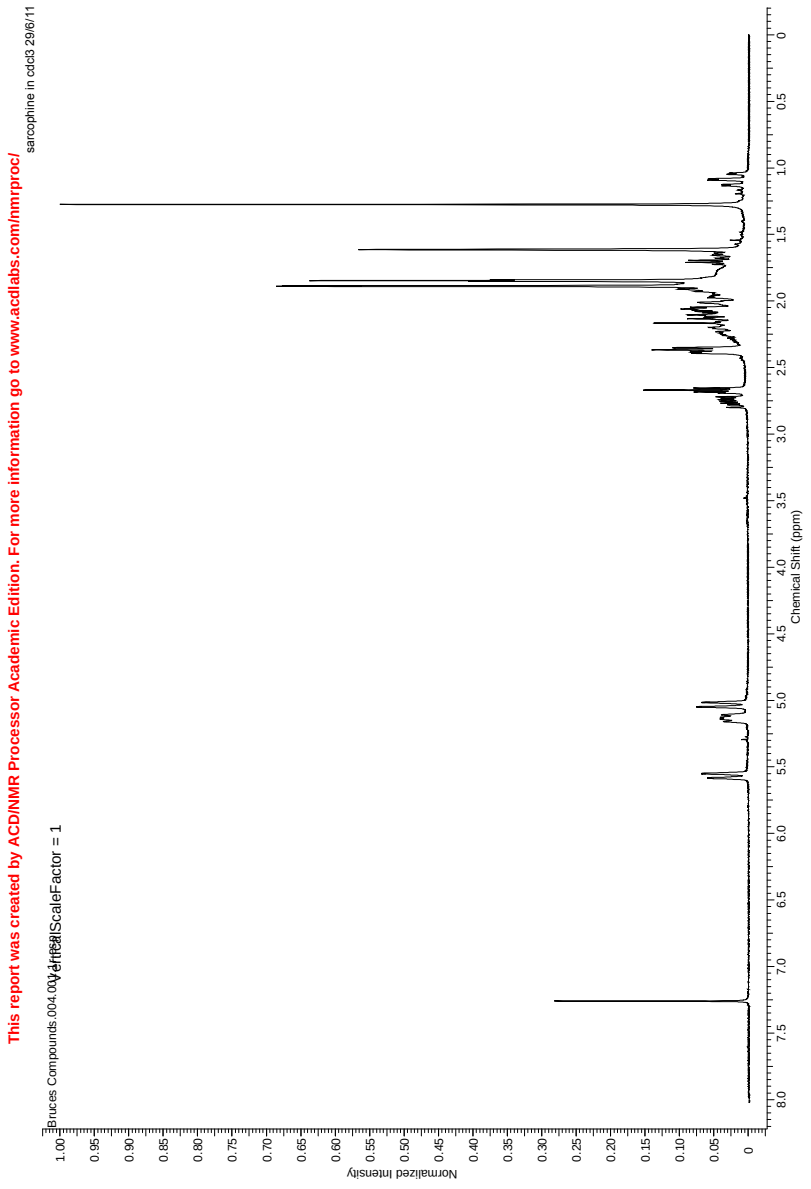


Figure B.5: ^{1}H NMR spectrum of Sarcophine

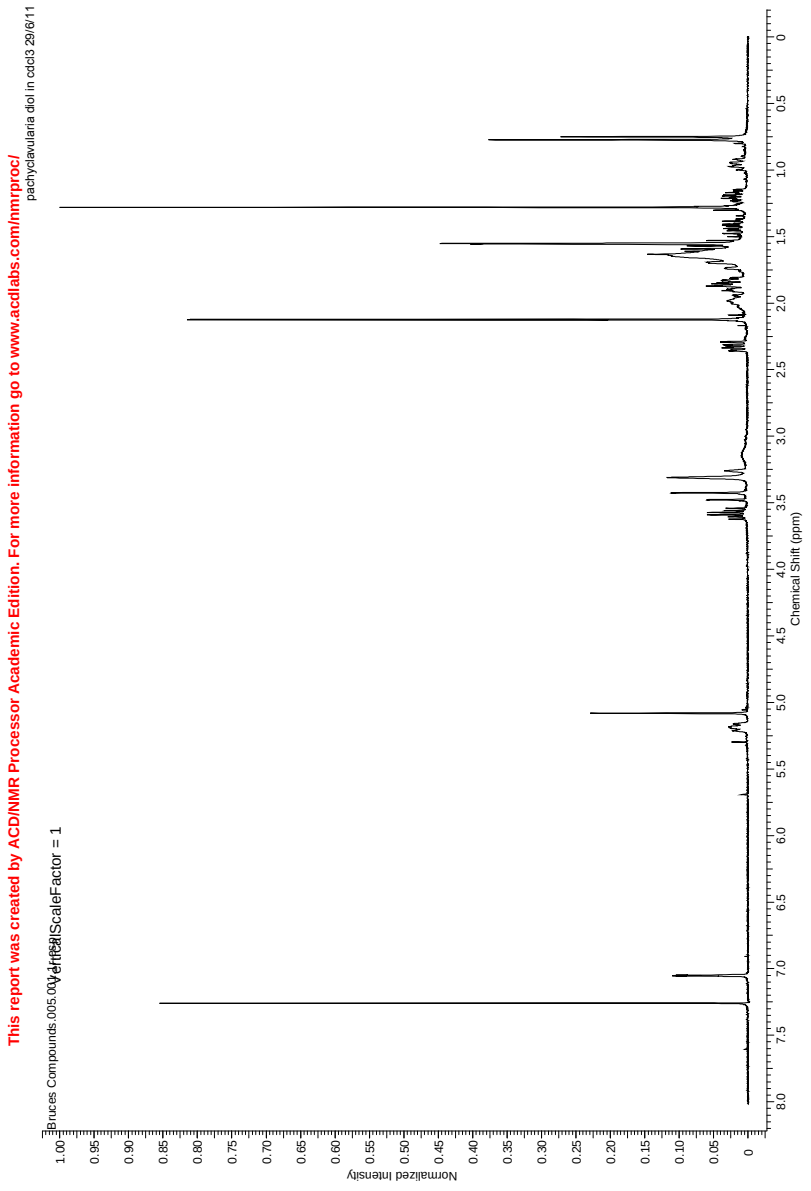


Figure B.6: ^{1}H NMR spectrum of Pachyclavulariadiol

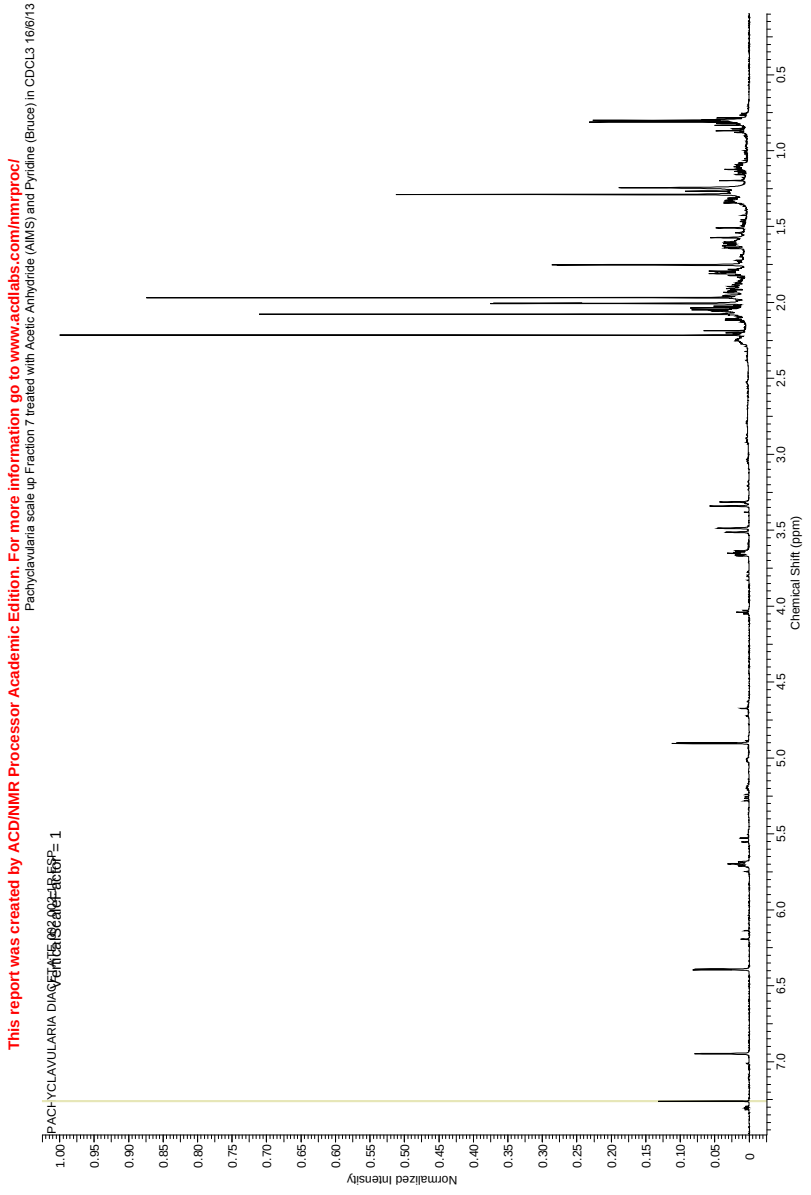


Figure B.7: ^{1}H NMR spectrum of Diacetyl Pachyclavulariadiol

Appendix C

Bioassay Guided Fractionation of *Nephthea chabroli*

This appendix contains figures and spectra detailing the isolation methodology (C.1) and chemical characterisation using the techniques NMR (C.2, C.3, C.4 and C.5) and High Resolution MS(C.6, C.7, C.8 and C.9) employed in Chapter four.

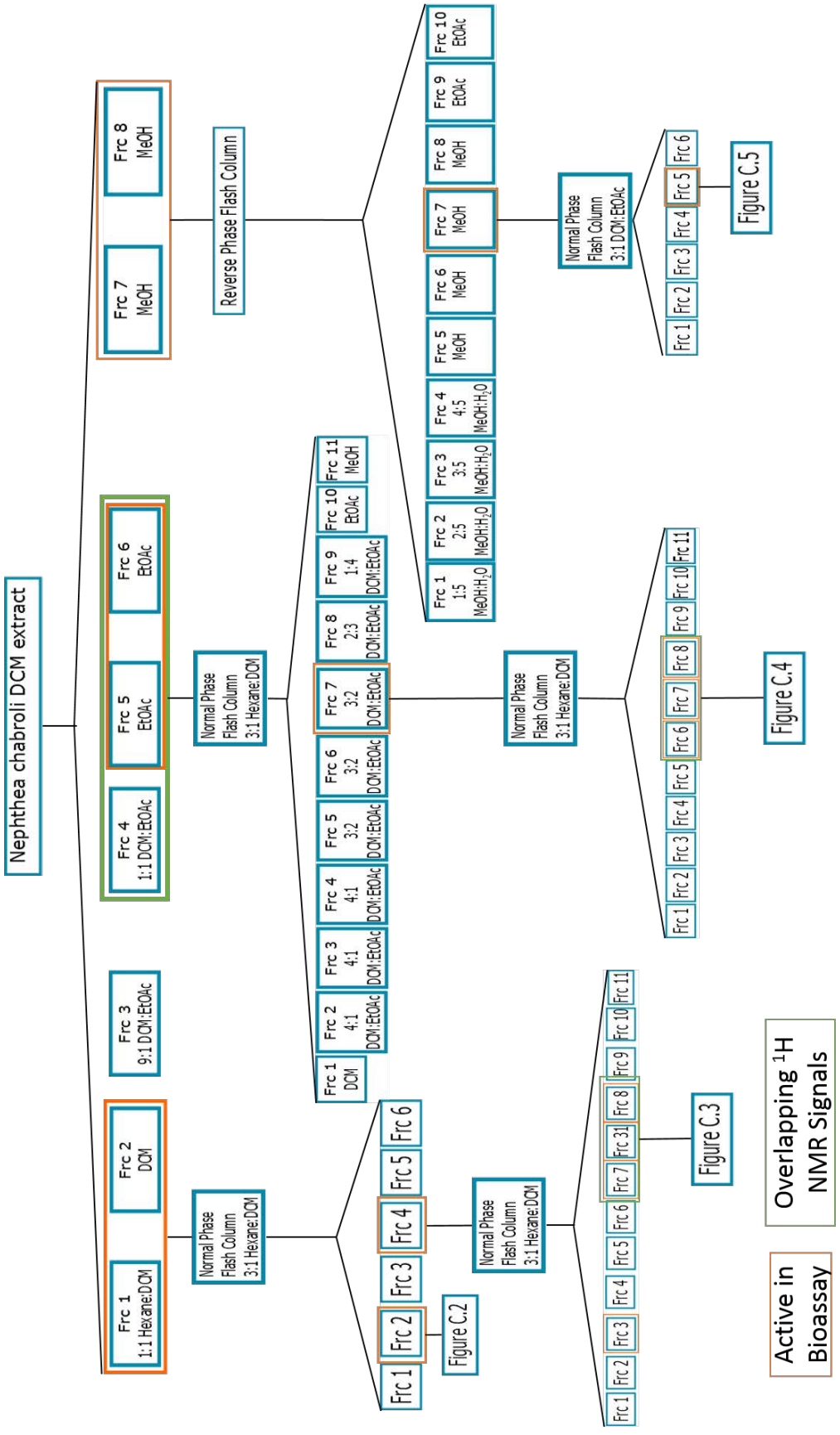


Figure C.1: Flowchart of the bioassay guided isolation pathway employed in Chapter four. The bacterial biosensor *Agrobacterium tumefaciens* A136 guided isolation of *Nephthea* compounds by being used as a screening mechanism to identify the active fraction generated at each chromatographic purification step. Active fractions were further chromatographed to isolate pure compounds.

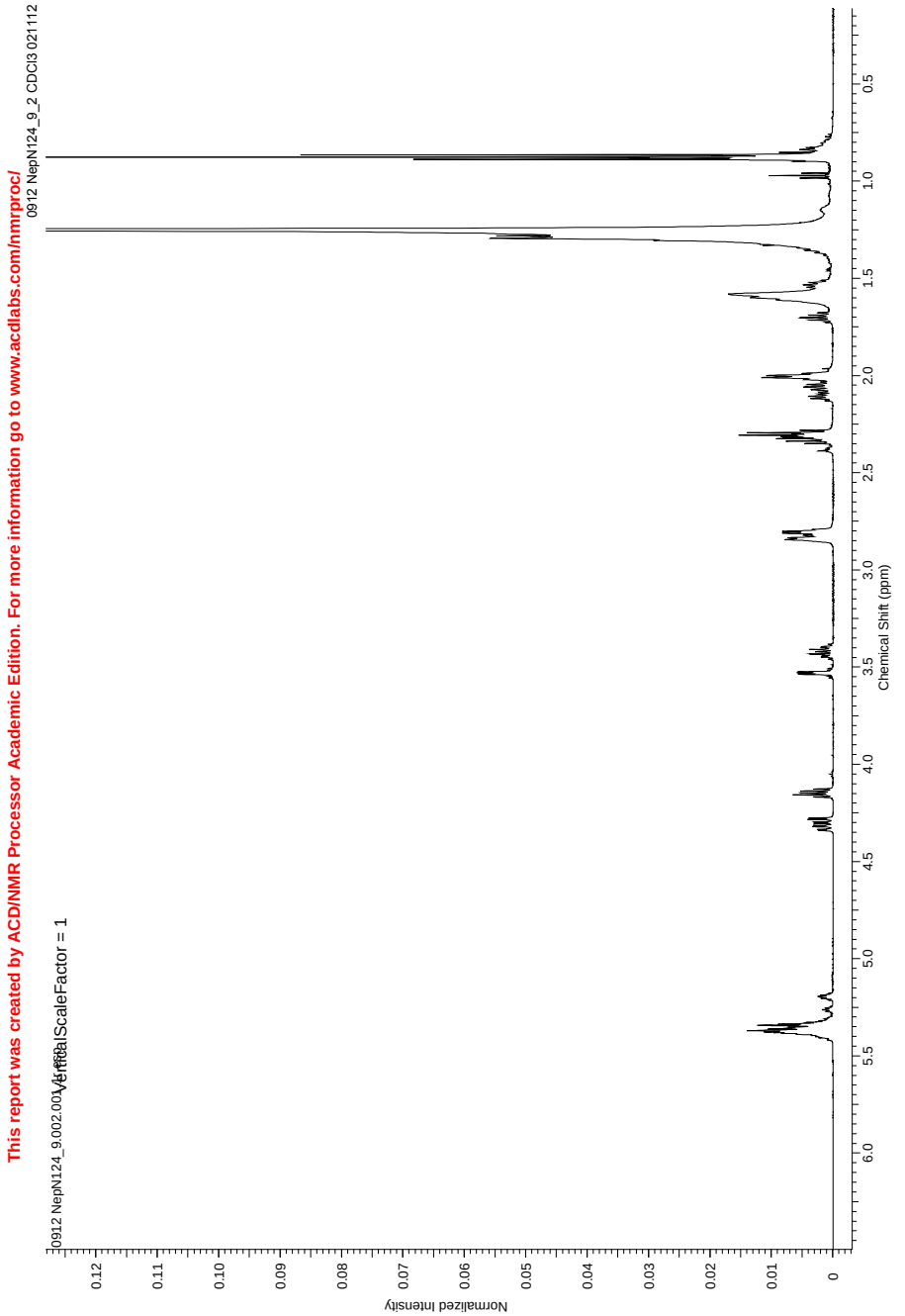


Figure C.2: ^{1}H NMR spectra of *Nephthea brassica* fraction following bioassay guided fractionation (Figure C.1). Activity in fractions one and two was followed to extinction. This fraction of triglyceride was no longer active in the assay.

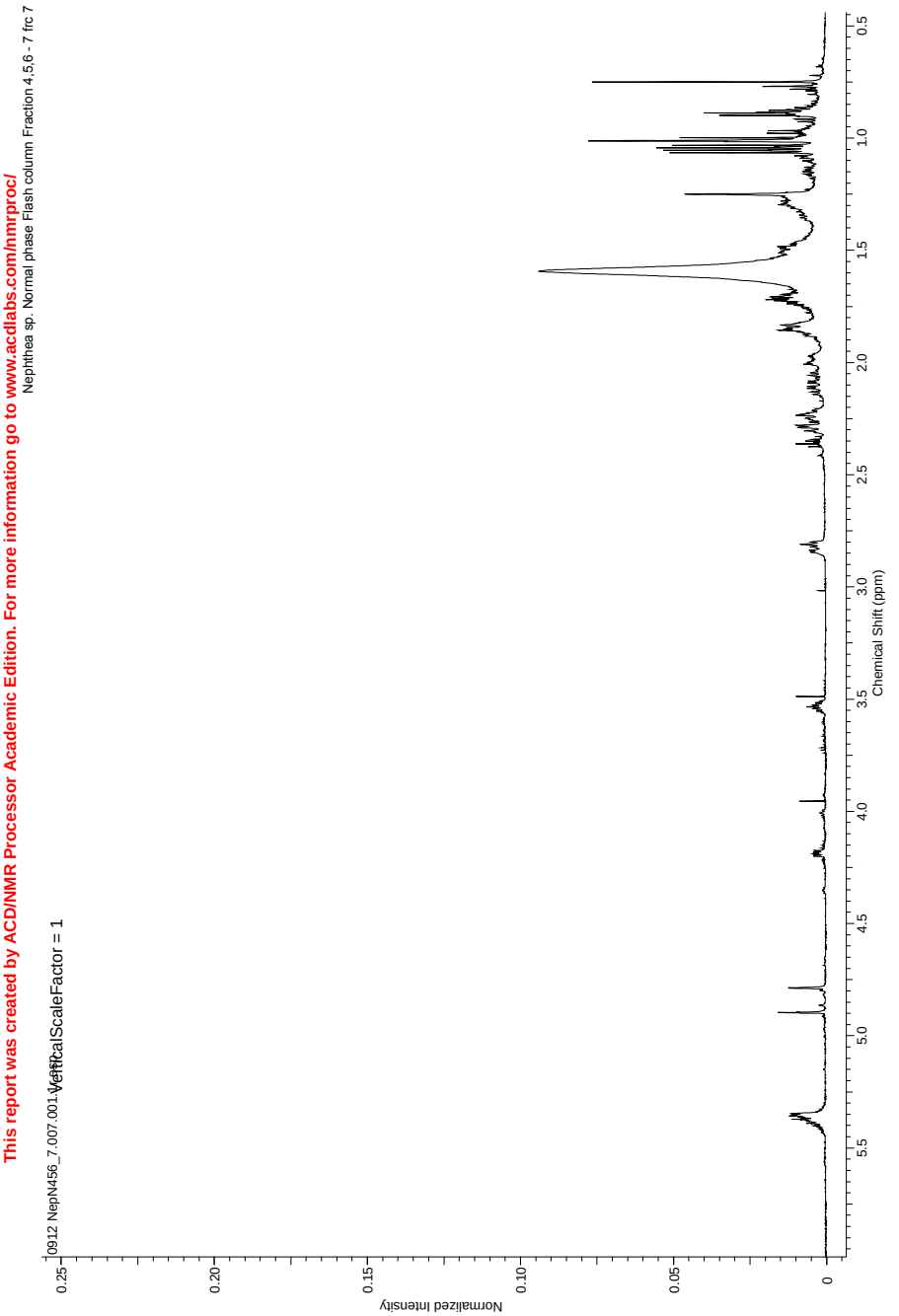


Figure C.3: ^{1}H NMR spectra of *Nephthea brassica* fraction following bioassay guided fractionation (Figure C.1).

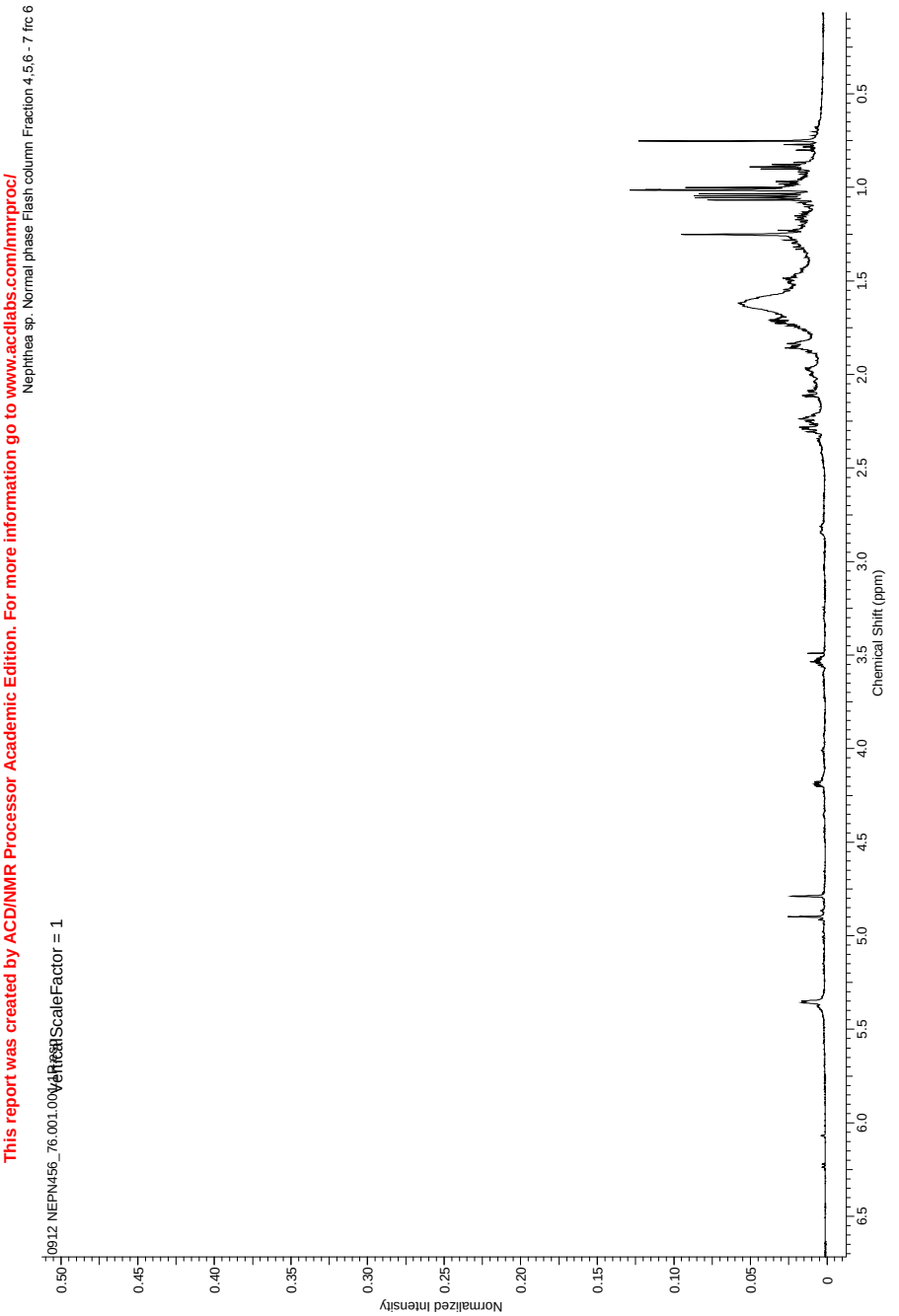


Figure C.4: ^{1}H NMR spectra of *Nephthea brassica* fraction following bioassay guided fractionation (Figure C.1).

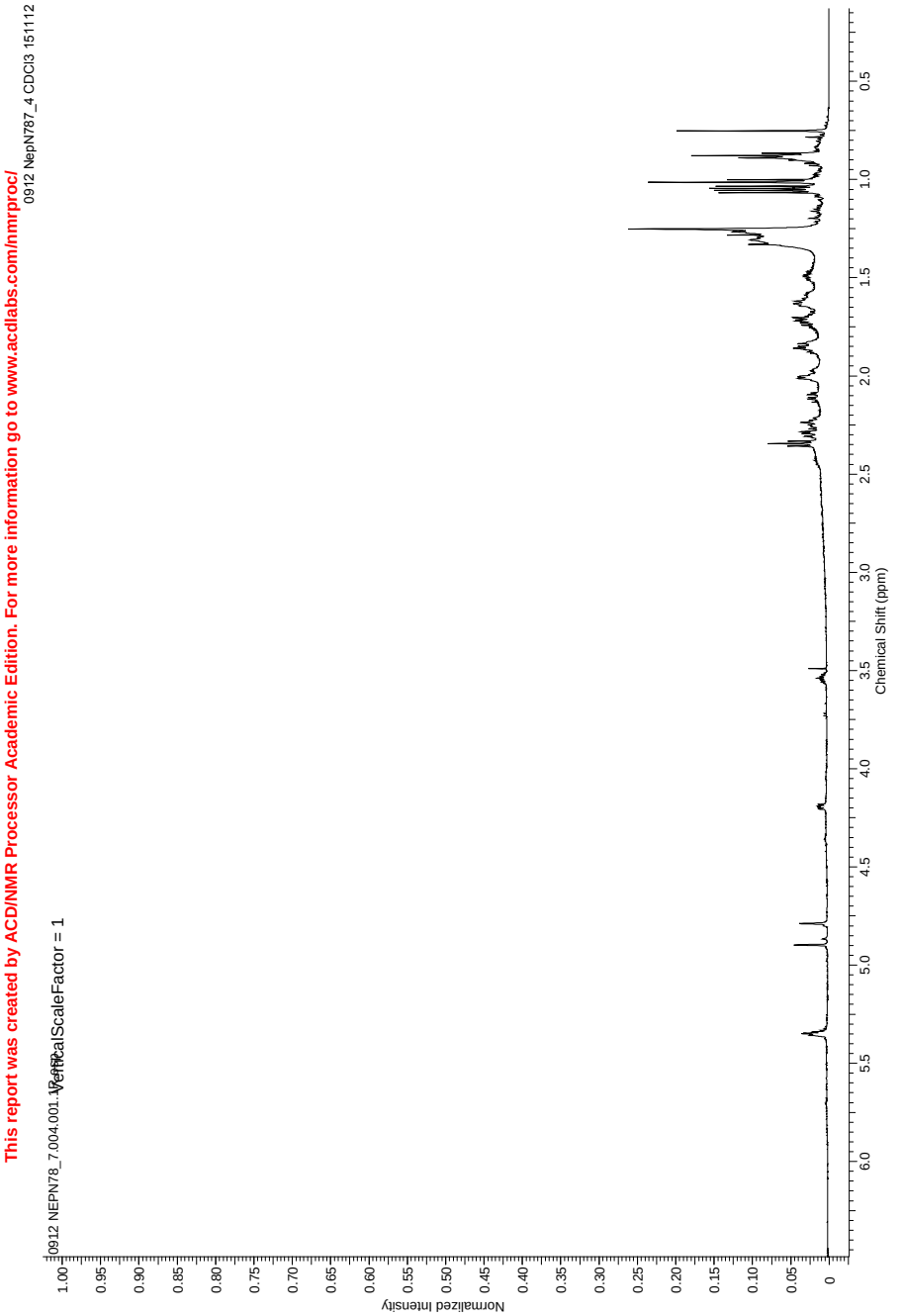
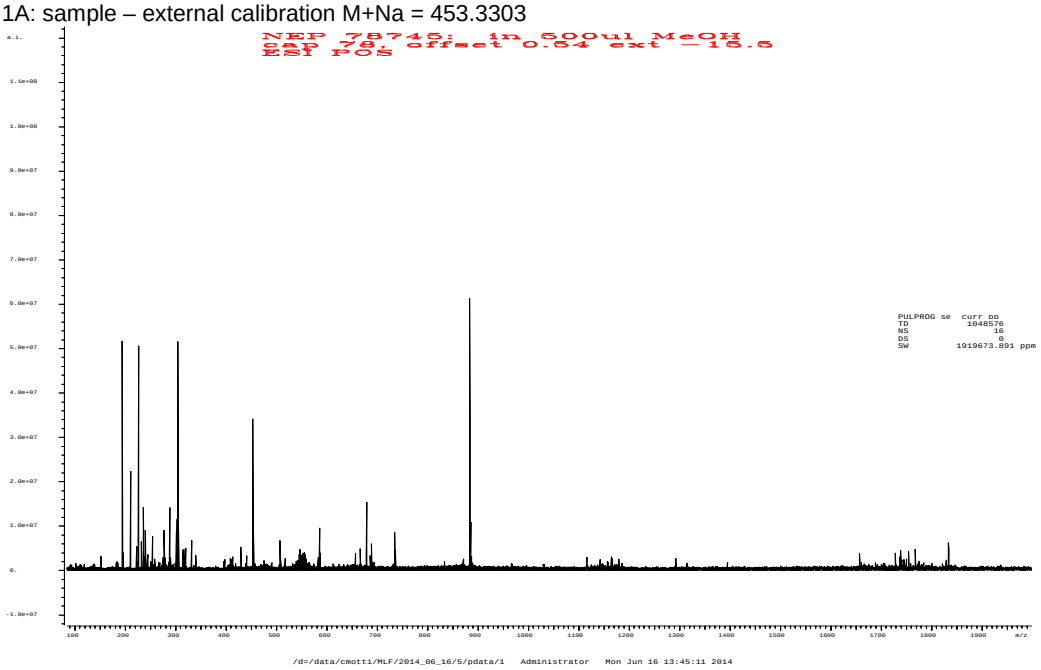


Figure C.5: ^{1}H NMR spectra of *Nephthea brassica* fraction following bioassay guided fractionation (Figure C.1).

Positive mode data

1A: sample – external calibration M+Na = 453.3303



1B: Internal calibration with STFA showing M+Na = 453.3339 (calc 453.3339 <1ppm) and 2M+Na = 883.6805 (calc 883.6786 <2ppm)

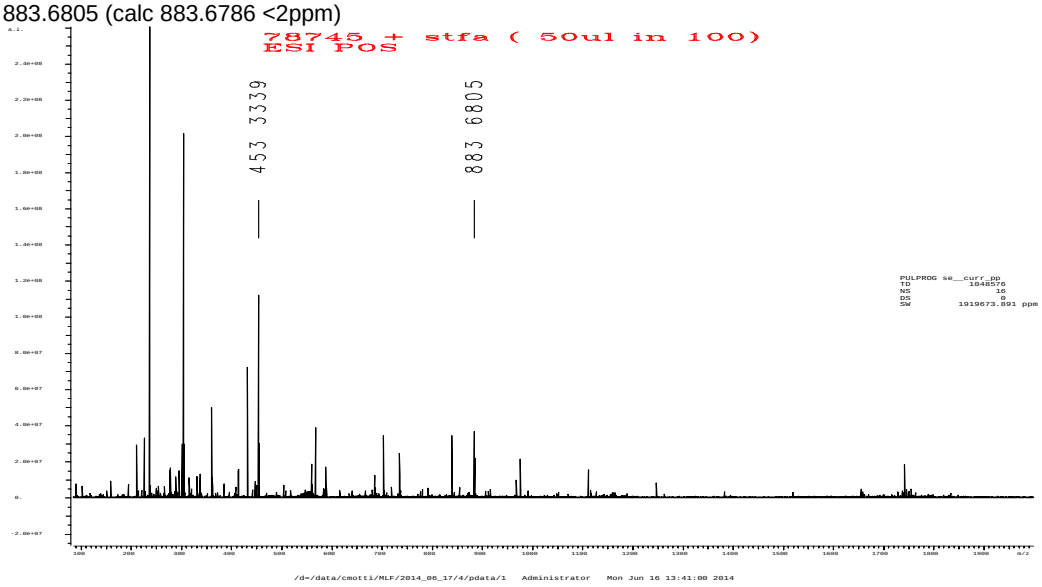
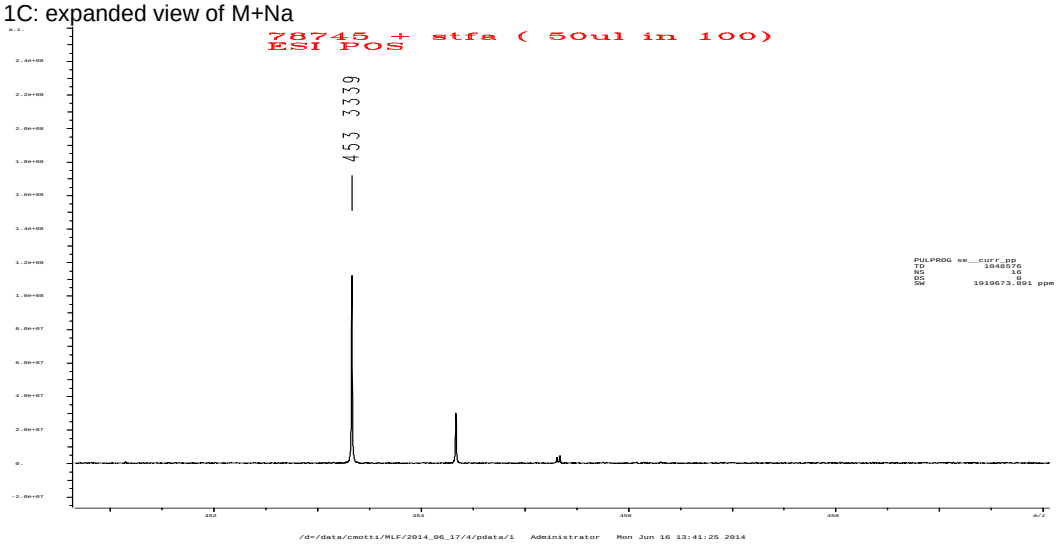


Figure C.6: Positive mode FTMS data of Compound **1**. Ions were detected within a mass range m/z 100-1800. Direct infusion of compound **1** in MeOH (~0.2 $mg.mL^{-1}$) containing the internal calibrant CF_3CO_2Na was carried out using a Cole Palmer 74900 syringe pump at a flow rate of 180 $\mu L.h^{-1}$ $^{-1}$A) displays the Molecular ion plus Na [M+Na] with external calibration. B) displays both the [M+Na] and the dimer [2M+Na] ions observed.

1C: expanded view of M+Na



1D: Expanded view of 2M+Na

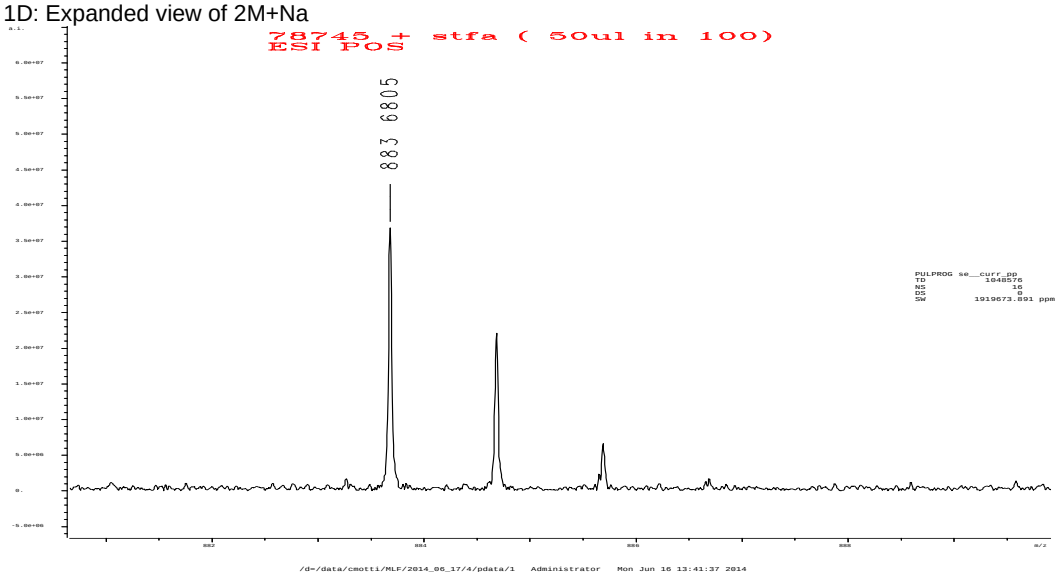


Figure C.7: Positive mode FTMS data of Compound **1**. Ions were detected within a mass range m/z 100-1800. Direct infusion of compound **1** in MeOH (~0.2 $mg.mL^{-1}$) containing the internal calibrant CF_3CO_2Na was carried out using a Cole Palmer 74900 syringe pump at a flow rate of 180 $\mu L.h^{-1}$. A) is the expanded image of the Molecular ion plus Sodium [M+Na]. B) is the expanded view of the dimer plus sodium [2M+Na].

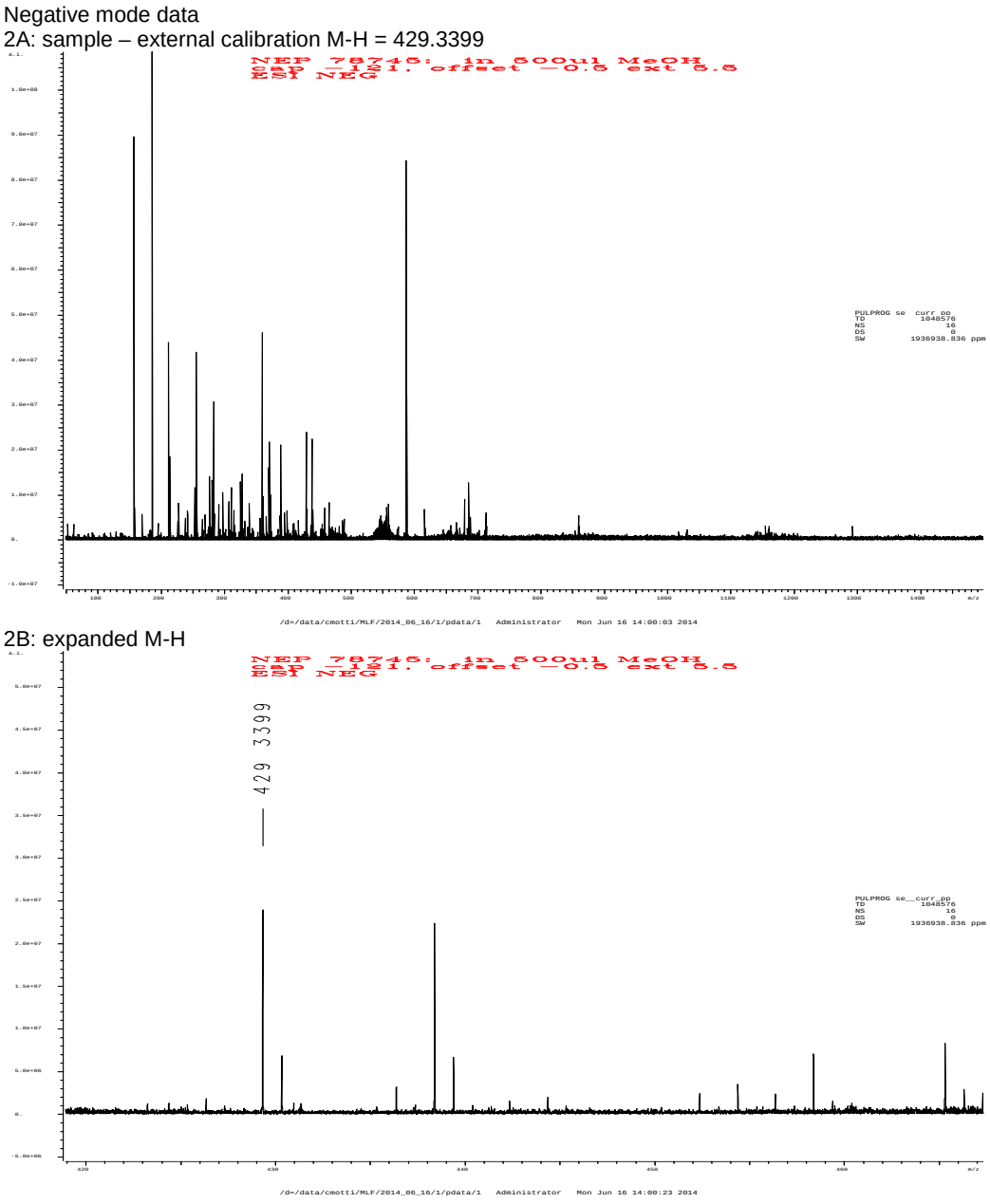


Figure C.8: Negative mode FTMS data of Compound **1**. Ions were detected within a mass range m/z 100-1800. Direct infusion of compound **1** in MeOH (~0.2 mg.mL^{-1}) containing the internal calibrant CF_3CO_2Na was carried out using a Cole Palmer 74900 syringe pump at a flow rate of 180 µL.h^{-1}A) is the full mass range indicating the Molecular ion minus Hydrogen [M-H]. B) is the expanded view of this ion [M-H].

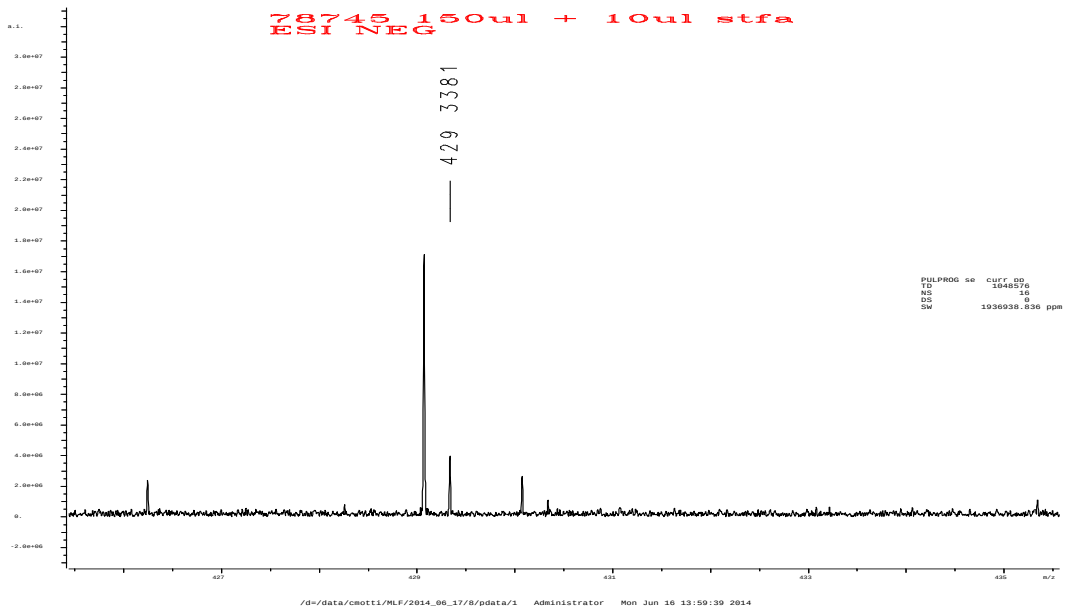


Figure C.9: Negative mode FTMS data of Compound **1**. Mass calibration of the the negative M-H ion demonstrating <2ppm agreement with predicted ion. Ions were detected within a mass range m/z 100-1800. Direct infusion of compound **1** in MeOH (~0.2 $mg.mL^{-1}$) containing the internal calibrant CF_3CO_2Na was carried out using a Cole Palmer 74900 syringe pump at a flow rate of 180 $\mu L.h^{-1}$.

Appendix D

Media Components

D.1 ABt Media:

ABt media consists is a modifcation of the AB media of Clarrk and Maaløe (105), it is made using a mixture of 100 ml A solution and 900 ml of B solution (Table D.1). Solutions are made and autoclaved separately. For ABt agar, agar (1.2 g / 90 ml) is added to B solution prior to autoclaving. Optional antibiotics (4.5 µg/mL Tetracycline and 50 µg/ml Spectinomycine) are added to media post-autoclaving once temperature has dropped to 42°C.

Table D.1: ABt Media Components

A solution	
$(NH_4)_2SO_4$	4.0 g
$Na_2HPO_4.2H_2O$	6.0 g
KH_2PO_4	3.0 g
NaCl	3.0 g
Milli Q	100 mL

B Solution	
1.0M $MgCl_2$	1 mL
0.1M $CaCl_2$	1 mL
0.01M $FeCl_3$	1 mL
Milli Q	900 mL
To be added after autoclaving	
20% (w/v) Cas-amino Acid	25 mL
20% (w/v) Glucose	25 mL
1 mg/mL Thiamine	2.5 mL

D.2 LB Agar

Table D.2: Difco™ LB Agar, Miller. Approximate Formula* Per Liter

Tryptone	10.0 g
Yeast Extract	5.0 g
Sodium Chloride	5.0 g
Agar	15.0 g

Difco™ LB Broth, Miller consists of the same ingredients without the agar.

D.3 Marine Agar:

Table D.3: Difco™ Marine Agar 2216 Approximate Formula* Per Liter

Peptone	5.0 g
Yeast Extract	1.0 g
Ferric Citrate	0.1 g
Sodium Chloride	19.45 g
Magnesium Chloride	8.8 g
Sodium Sulfate	3.24 g
Calcium Chloride	1.8 g
Potassium Chloride	0.55 g
Sodium Bicarbonate	0.16 g
Potassium Bromide	0.08 g
Strontium Chloride	34.0 mg
Boric Acid	22.0 mg
Sodium Silicate	4.0 mg
Sodium Fluoride	2.4 mg
Ammonium Nitrate	1.6 mg
Disodium Phosphate	8.0 mg
Agar	15.0 g

D.4 GASW Agar:

Table D.4: Glycerol Artificial Seawater Ingredients per litre of Agar. Final pH of the medium was 8.2. Modified from (Smith and Haysaka, 1982)

Peptone	4.0 g
Yeast Extract	2.0 g
Ferric Sulfate	0.001 g
Sodium Chloride	20.8 g
Magnesium Chloride	4.0 g
Magnesium Sulfate	4.8 g
Potassium Chloride	0.56 g
Potassium Hydrogen Phosphate (K_2HPO_4)	0.08 g
Tris	0.48 g
Glycerol	2.0 ml
Rila Salts	2.0 g
Sodium Fluoride	2.4 mg
Ammonium Nitrate	1.6 mg
Disodium Phosphate	8.0 mg
Agar	15.0 g

D.5 TCBS Agar:

Table D.5: Difco™ TCBS Agar Approximate Formula* Per Liter

Yeast Extract	5.0 g
Proteose Peptone No. 3	10.0 g
Sodium Citrate	10.0 g
Sodium Thiosulfate	10.0 g
Oxgall	8.0 g
Saccharose	20.0 g
Sodium Chloride	10.0 g
Ferric Ammonium Citrate	1.0 g
Bromthymol Blue	0.04 g
Thymol Blue	0.04 g
Agar	15.0 g

D.6 Artificial Seawater

Table D.6: Formula of Artificial Seawater (per litre) used in Chapter 5.

Distilled Water	1000 mL
Sodium Chloride	17.55 g
Potassium Chloride	0.75 g
Sodium Sulfate	0.285 g
Magnesium Chloride	5.10 g
Calcium Chloride	0.145 g

Appendix E

Isolate Data

Table E.1: Details of bacterial isolates cultured and identified from *Sinularia flexibilis* mucus. NCBI accession numbers as well as closest matches and source are included. Table continues in Table E.2.

Isolate	Acession #	Media	Dil.	Rep	hrs	Morphotype	QS tested	QS Active	QS Type	NCBI Match	NCBI Match Source	Seq Id %
01SF1M10	KM360456	GASW	10^-2	1	48	off white round rainbow ring	Y	Y	A Inh, C Inh	HF549288.1 Vibrio coralliilyticus partial 16S rRNA gene, strain VC LPI 06_210	diseased Crassostrea gigas larvae	87
01SF1M102	KM360454	GASW	10^-2	1	48	small white round	Y	N	NA	JN388613.1 Vibrio sp. IM04	Phylogenetic analysis of bacterial isolates utilizing sucralose as sole carbon source	93
01SF1M103	KM360455	GASW	10^-2	3	48	off white with clear ring	Y	N	NA	EF100710.1 Vibrio sp. V134	sea water	86
01SF1MM	KM360457	50MA	10^-4	1	72	orange	NT	NA	NA	JN388614.1 Vibrio sp. IM06	Phylogenetic analysis of bacterial isolates utilizing sucralose as sole carbon source	84
SC_57	KM360458	50MA	10^-2	3	48	small white round	Y	N	NA	EF100710.1 Vibrio sp. V134	sea water	98
SF101	KM360459	GASW	10^-2	2	48	white with rough edge	Y	N	NA	AY792622.2 Vibrio sp. PP-638	Diseased outbreak shellfish hatchery	93
SF102	KM360460	GASW	10^-3	1	48	off \white ringed	Y	Y	C Ind, C Inh, A Inh	JF925006.1 Uncultured marine bacterium clone Ag-15	Alcyonium gracillimum (coral)	90
SF103	KM360461	GASW	10^-4	2	48	off white	Y	N	NA	JN380345.1 Photobacterium leiognathi strain MahL3	Indian mackerel	92
SF10T1	KM360462	GASW	10^-3	2	72	off white round	Y	Y	C Ind, A Ind	FJ154796.1 Vibrio harveyi strain WG1702	eruptive epidemic disease in Meretrix meretrix	99
SF10T2	KM360463	GASW	10^-3	1	72	white with pink centre	Y	Y	A Ind	EF528269.1 Bacillus megaterium strain CICCHLJ Q37	Unknown	98
SF204	KM360464	50 MA	10^-2	1	48	off white not round	Y	Y	C Ind, A Inh	AB205011.1 Spongiobacter nickelotolerans	Marine Sponge	93
SF206	KM360465	50MA	10^-2	2	72	off white round	Y	Y	C Inh	KC210811.1 Vibrio alginolyticus strain M2-21	Aquatic pathogen in Epinephelus awoara	96

Table E.2: Details of bacterial isolates cultured and identified from *Sinularia flexibilis* mucus. NCBI accession numbers as well as closest matches and source are included. Continued from Table E.1.

Isolate	Acession #	Media	Dil.	Rep	hrs	Morphotype	QS tested	QS Active	QS Type	NCBI Match	NCBI Match Source	Seq Id %
SF207	KM360466	50MA	10^-2	3	48	white with clear ring	Y	Y	C Ind	JX075053.1 Vibrio sp. PP25	Probiotic protecting Ornate Spiny Lobster (Panulirus ornatus) Larvae against Vibrio owensii	96
SF208	KM360467	50MA	10^-4	3	48	off white round	Y	Y	C Ind, A Ind	JN388613.1 Vibrio sp. IM04	Phylogenetic analysis of bacterial isolates utilizing sucralose as sole carbon source	99
SF209	KM360468	50MA	10^-2	2	72	Orange cream round	Y	N	NA	EU372917.1 Vibrio sp. MMS-1	disease legion interface Montipora aequituberculata	93
SF2CR	KM360469	GASW	10^-3	2	48	cream	NT	NA	NA	HQ439529.1 Vibrio sp. C23c	reef surface biofilm - coral settlement	99
SFB10_2	KM360470	50MA	10^-3	3	72	orange cream	Y	Y	A Ind, C Ind	EF061434.1 Alteromonas sp. NJSX50	Sea Water	99
SFW	KM360471	50MA	10^-2	1	48	white	Y	Y	C Inh	EU372917.1 Vibrio sp. MMS-1	disease legion interface Montipora aequituberculata	99
SFYBB	KM360472	50MA	10^-2	2	72	yellow, round	NT	NA	NA	HM032584.1 Uncultured bacterium clone K11131_061	epibiotic strains on Trichodesmium	92
YSF	KM360473	50MA	10^-2	2	48	yellow, round	Y	Y	C Ind	JN210716.1 Uncultured Alteromonas sp. clone T028deg42	Rhopaloeides odorabile larvae	94

Table E.3: Details of bacterial isolates cultured and identified from *Lobophytum compactum* mucus. NCBI accession numbers as well as closest matches and source are included. Continues Table E.4.

Isolate	Media	Dilution	Rep	Acession #	Morphotype	QS tested	QS Active	QS Type	NCBI Match	NCBI Match Source	Seq Id %
LC02W	GASW	10^-2	2	KM360403	pale yellow, wet edge	NT	NT	NA	AB719108.1 Vibrio mediterranei C055	intestinal contents of Holothuria leucospilota	85
LC101	TCBS	10^-2	1	KM360452	orange	Y	Y	A Inh, C Ind	JN119271.1 Bacterium T6(2011) strain	Coral	99
LC102	TCBS	10^-2	1	KM360404	orange	Y	Y	A Inh	KJ022620.1 Vibrio sp. WHCY8	pond water associated with Scophthalmus maximus	96
LC103	TCBS	10^-3	1	KM360405	green	Y	Y	A Inh, C Inh	KM014027.1 Vibrio owensii strain CR-III-19	Epibiotic Vibrios on the Mediterranean invasive alga Caulerpa racemosa	95
LC104	TCBS	10^-4	1	KM360406	brown	Y	N	NA	FJ154796.1 Vibrio harveyi strain WG1702	eruptive epidemic disease in Meretrix meretrix	96
LC105	TCBS	10^-3	1	KM360407	brown	Y	N	NA	EU372937.1 Vibrio sp. BD6B	Montipora aequituberculata diseased skeleton	93
LC106	TCBS	10^-4	1	KM360408	orange	Y	N	NA	KF179806.1 Uncultured bacterium clone REU2C3	putative pathogens associated with Porites white patch syndrome	96
LC107	TCBS	10^-4	1	KM360409	orange	Y	Y	A Inh	EU372937.1 Vibrio sp. BD6B	Montipora aequituberculata diseased skeleton	94
LC108	TCBS	10^-4	1	KM360410	orange	NT	NT	NA	KC439226.1 Vibrio sp. R5B1	circulation system Narcine bancroftii	93
LC110r	50MA	10^-2	1	KM360411	Off white	Y	Y	A Inh	JN388613.1 Vibrio sp. IM04	Phylogenetic analysis of bacterial isolates utilizing sucralose as sole carbon source	97
LC111	50MA	10^-2	1	KM360412	off white agar eater	Y	Y	A Inh, C Inh	EU372917.1 Vibrio sp. MMS-1	disease legion interface Montipora aequituberculata	99
LC114	50MA	10^-2	1	KM360413	off white wet edge	Y	N	NA	JN388613.1 Vibrio sp. IM04	Phylogenetic analysis of bacterial isolates utilizing sucralose as sole carbon source	95
LC123	50MA+	10^-3	1	KM360414	Yellow centre	Y	Y	A Ind	JN119271.1 Bacterium T6(2011) strain	Coral	93
LC126	GASW	10^-2	1	KM360415	off white ringed	Y	N	NA	KM014027.1 Vibrio owensii strain CR-III-19	Epibiotic Vibrios on the Mediterranean invasive alga Caulerpa racemosa	94
LC127	GASW	10^-2	1	KM360416	orange rough	Y	Y	C Ind	KF856726.1 Psychrobacter celer strain W-2	Puffer fish digestive gland	96
LC128	GASW	10^-2	1	KM360417	off white round	Y	N	NA	KJ197723.1 Uncultured Photobacterium sp. clone SB4-34	Chilomycterus schoepfi gut	89
LC130	GASW+	10^-2	1	KM360418	custard yellow	Y	Y	C Ind	JF965428.1 Vibrio sp. S-1	sea water	95
LC131	GASW+	10^-2	1	KM360419	orange clear	Y	N	NA	KC545309.1 Ferrimonas sp. EF3B-B688	Eunicea fusca	84

Table E.4: Details of bacterial isolates cultured and identified from *Lobophytum compactum* mucus. NCBI accession numbers as well as closest matches and source are included. Continued from Table E.3, continues on Table E.5.

Isolate	Media	Dilution	Rep	Acession #	Morphotype	QS tested	QS Active	QS Type	NCBI Match	NCBI Match Source	Seq Id %
LC132	GASW+	10^-2	1	KM360420	off white round	Y	Y	A Ind	KF886637.1 Vibrio parahaemolyticus strain NSTH26	oyster	80
LC135	GASW+	10^-2	1	KM360421	white	Y	Y	C Inh	KF577081.1 Photobacterium sp. VibC-Oc-011	bacteria associated to Oculina patagonica	94
LC137	GASW+	10^-3	1	KM360422	pink centre	Y	N	NA	AB376669.1 Alteromonas macleodii	Polychaeta	9997
LC201	TCBS	10^-2	2	KM360423	orange	NT	N	NA	KJ719255.1 Pseudoalteromonas sp. RKEM680	octocoral Antillogorgia elisabethae	94
LC202	TCBS	10^-2	2	KM360424	green	Y	Y	A Inh, C Ind	JN119271.1 Bacterium T6(2011) strain	Coral	98
LC203	50MA	10^-2	2	KM360425	off white, wet edge	Y	N	NA	EF061434.1 Alteromonas sp. NJSX50	sea water	86
LC204	50MA	10^-2	2	KM360426	off white round	Y	N	NA	JN119276.1 Bacterium T12(2011) strain	Coral	99
LC205	50MA	10^-2	2	KM360453	orange cream	Y	N	NA	JF925006.1 Uncultured marine bacterium clone Ag-15 16S ribosomal RNA gene,	Alcyonium gracillimum (coral)	95
LC207	50MA+	10^-2	2	KM360427	white	Y	Y	A Inh	JX257005.1 Vibrio diabolicus strain JBW-8-11-1	North Inlet Estuary	92
LC208	50MA+	10^-2	2	KM360428	off white round	Y	Y	A Inh, C Inh	EU372917.1 Vibrio sp. MMS-1	disease legion interface Montipora aequituberculata	89
LC209	50MA+	10^-3	2	KM360429	white rough	Y	N	NA	EU854942.1 Vibrio sp. 6G2	Montastraea faveolata	89
LC210	50MA+	10^-4	2	KM360430	off white round	NT	NA	NA	KJ775755.1 Pseudoalteromonas sp. ARC-ML4B1	Mytilus edulis gut	85
LC212	GASW	10^-3	2	KM360431	orange cream round	Y	Y	NA	KJ775755.1 Pseudoalteromonas sp. ARC-ML4B1	Mytilus edulis gut	98
LC214	GASW	10^-4	2	KM360432	off white round	Y	Y	A Ind	AB376669.1 Alteromonas macleodii	Polychaeta	99
LC215	GASW	10^-4	2	KM360433	orange	Y	Y	A Ind	KF733524.1 Pseudoalteromonas sp. PS5	Paragoniolithon solubile (crustose coralline algae)	96
LC217	GASW+	10^-2	2	KM360434	white in off white	Y	Y	C Ind	FJ497680.1 Vibrio sp. VS-59	Vailulu'u Seamount	95
LC219	GASW+	10^-3	2	KM360435	colourless rough	Y	N	NA	FR750946.1 Pseudoalteromonas undina	Marine Sponge Associated bacteria	98
LC222	GASW+	10^-3	2	KM360436	off white round	Y	Y	A Ind	EF179379.1 Vibrio sp. RLUH-CZ	Seahorse	99
LC301	50MA	10^-2	3	KM360437	off white rainbow	NT	NA	NA	GQ413074.1 Uncultured bacterium clone 1FTfc8_D02	Porites	83
LC302	50MA	10^-3	3	KM360438	orange	NT	NA	NA	GU070668.1 Shewanella sp. CGB9	Haliotis diversicolor gut (cage culture)	99
LC304	50MA	10^-4	3	KM360439	off white swarm	Y	N	NA	FJ952650.1 Vibrio sp. SO41	Mytilus edulis	95
LC305	50MA	10^-4	3	KM360440	Off white wet edge	Y	Y	A Ind	KJ879951.1 Bacillus aryabhattai strain RS1	soil	99

Table E.5: Details of bacterial isolates cultured and identified from *Lobophytum compactum* mucus. NCBI accession numbers as well as closest matches and source are included. Continued from Table E.4.

Isolate	Media	Dilution	Rep	Acession #	Morphotype	QS tested	QS Active	QS Type	NCBI Match	NCBI Match Source	Seq Id %
LC306	50MA+	10^-2	3	KM360441	off white round	Y	Y	C Ind	JQ307128.1 Vibrio tubiashii strain Inspire31	bacterial pathogens form Bay of Bengal	97
LC307	50MA+	10^-3	3	KM360442	orange	NT	NA	NA	EF100710.1 Vibrio sp. V134	sea water	99
LC309	50MA+	10^-4	3	KM360443	yellow rough	Y	N	NA	EU420060.1 Pseudoalteromonas sp. C7	coral	97
LC310	50MA+	10^-4	3	KM360444	orange centre	Y	Y	C Ind	KF042038.1 Pseudoalteromonas sp. OCN003	Montipora capitata	95
LC312	GASW	10^-2	3	KM360445	off white, wet edge, rainbow effect	Y	N	NA	KJ841877.1 Vibrio neocaledonicus strain MS1	China sea	98
LC313	GASW	10^-3	3	KM360446	orange wet	Y	Y	C Ind	KF999572.1 Bacterium PK108	estuary water	98
LC314	GASW+	10^-2	3	KM360447	white ringed	NT	NA	NA	GU553774.1 Uncultured bacterium clone ORI-860-27-P_S041-043_271B07	subseafloor sediment	93
LC315	GASW+	10^-2	3	KM360448	off white	Y	Y	A Inh	EF061434.1 Alteromonas sp. NJSX50	Sea water	99
LC409	50MA+	10^-3	2	KM360449	yellow	NT	NA	NA	KC203068.1 Micrococcus sp. WY3	Cr(VI) contaminated soil	99
LCORI	50MA+	10^-3	1	KM360450	Orange	Y	N	NA	KJ549198.1 Uncultured bacterium clone L5n-B63	hydrothermal plume	99
LCP	50MA	10^-2	3	KM360451	purple black	NT	NA	NA	AB364967.1 Paramoritella alkaliphila	Marine Sand	96

Non-metric Multidimensional Scaling Analysis of Soft Coral Bacterial Isolate Morphotypes

The following pages include a copy of the spreadsheet used to analyse the isolate morphotype profiles generated in Chapter 5.

Coral	Rep	Dilution	Media	Time	blue green (4 mm)	brown (1mm)	brown orange (1.5mm)	clear swarming	colourless (4mm)	fungi black	fungi white	green (3mm)	off white clear ring no rainbow (1-3 mm)	off white clear rough ring (1-2mm)	off white cream smooth no ring (1-2 mm)	off white cream smooth no ring (3mm)	off white rainbow (2 - 3mm)	off white rough (1-3mm)	off white round pink centre (5mm)	off white round rainbow ring (4mm)	off white smooth rainbow ring (1-2mm)	off white smooth rainbow ring (2-3mm)	off white smooth round (1-2mm)
LC	1	10^2	50 MA	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	11	0
LC	1	10^2	50 MA+	48	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	32	0
LC	1	10^2	GASW	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0
LC	1	10^2	GASW+	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	13	0
LC	1	10^2	TCBS	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	1	10^3	50 MA	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0
LC	1	10^3	50 MA+	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
LC	1	10^3	GASW	48	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
LC	1	10^3	GASW+	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
LC	1	10^3	TCBS	48	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	1	10^4	50 MA	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	1	10^4	50 MA+	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	1	10^4	GASW	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	1	10^4	GASW+	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	1	10^4	TCBS	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	2	10^2	50 MA	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	26	0	0
LC	2	10^2	50 MA+	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
LC	2	10^2	GASW	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	2	10^2	GASW+	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	65	0	0
LC	2	10^2	TCBS	48	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
LC	2	10^3	50 MA	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	2	10^3	50 MA+	48	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
LC	2	10^3	GASW	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	2	10^3	GASW+	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0
LC	2	10^3	TCBS	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Figure E.1: nMDS of isolate morphotypes p1 of 9

Coral	Rep	Dilution	Media	Time	off white smooth round (3mm)	off white smooth, rough/wet edge (swarming?)	orange clear (1-2 mm)	orange cream clear zone (2mm)	orange cream with clear zone (5mm)	orange rough	orange round cream (3mm)	orange smooth wet (3mm)	orange yellow (3mm)	purple black wet (2mm)	smear colourless no rainbow	smear off white rainbow	smear orange 30mm has eaten agar	smear swarming	white rough edge (2-5mm)	white round clear ring (2mm)	white round white rings	white smooth (1mm)
LC	1	10^2	50 MA	48	0	0	1	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0
LC	1	10^2	50 MA+	48	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	1	10^2	GASW	48	0	1	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0
LC	1	10^2	GASW+	48	0	0	2	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
LC	1	10^2	TCBS	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	1	10^3	50 MA	48	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0
LC	1	10^3	50 MA+	48	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
LC	1	10^3	GASW	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	1	10^3	GASW+	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	1	10^3	TCBS	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	1	10^4	50 MA	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	1	10^4	50 MA+	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	1	10^4	GASW	48	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
LC	1	10^4	GASW+	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	1	10^4	TCBS	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	2	10^2	50 MA	48	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
LC	2	10^2	50 MA+	48	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0
LC	2	10^2	GASW	48	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
LC	2	10^2	GASW+	48	0	0	0	0	0	0	0	0	6	0	0	4	0	0	0	0	0	5
LC	2	10^2	TCBS	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	2	10^3	50 MA	48	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
LC	2	10^3	50 MA+	48	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0
LC	2	10^3	GASW	48	0	13	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
LC	2	10^3	GASW+	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	2	10^3	TCBS	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Figure E.2: nMDS of isolate morphotypes p2 of 9

Coral	Rep	Dilution	Media	Time	white solid round (1-2 mm)	yellow orange (5mm)	yellow round clear	yellow round smooth (1-2 mm)
LC	1	10^2	50 MA	48	0	0	0	0
LC	1	10^2	50 MA+	48	0	0	0	0
LC	1	10^2	GASW	48	0	0	0	0
LC	1	10^2	GASW+	48	2	0	0	7
LC	1	10^2	TCBS	48	0	2	0	0
LC	1	10^3	50 MA	48	0	0	0	0
LC	1	10^3	50 MA+	48	0	0	0	0
LC	1	10^3	GASW	48	0	0	0	0
LC	1	10^3	GASW+	48	0	0	0	0
LC	1	10^3	TCBS	48	0	0	0	0
LC	1	10^4	50 MA	48	0	0	0	0
LC	1	10^4	50 MA+	48	0	0	0	0
LC	1	10^4	GASW	48	0	0	0	0
LC	1	10^4	GASW+	48	0	0	0	0
LC	1	10^4	TCBS	48	0	1	0	0
LC	2	10^2	50 MA	48	0	0	0	0
LC	2	10^2	50 MA+	48	0	0	0	0
LC	2	10^2	GASW	48	0	0	0	0
LC	2	10^2	GASW+	48	0	0	0	0
LC	2	10^2	TCBS	48	0	1	0	0
LC	2	10^3	50 MA	48	0	0	0	0
LC	2	10^3	50 MA+	48	0	0	0	0
LC	2	10^3	GASW	48	0	0	0	0
LC	2	10^3	GASW+	48	4	0	0	0
LC	2	10^3	TCBS	48	0	0	0	0

Figure E.3: nMDS of isolate morphotypes p3 of 9

Coral	Rep	Dilution	Media	Time	blue green (4 mm)	brown (1mm)	brown orange (1.5mm)	clear swarming	colourless (4mm)	fungi black	fungi white	green (3mm)	off white clear ring no rainbow (1-3 mm)	off white clear rough ring (1-2mm)	off white cream smooth no ring (1-2 mm)	off white cream smooth no ring (3mm)	off white rainbow (2 - 3mm)	off white rough (1-3mm)	off white round pink centre (5mm)	off white round rainbow ring (4mm)	off white smooth rainbow ring (1-2mm)	off white smooth rainbow ring (2-3mm)	off white smooth round (1-2mm)
LC	2	10^4	50 MA	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0
LC	2	10^4	50 MA+	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0
LC	2	10^4	GASW	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0
LC	2	10^4	GASW+	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0
LC	2	10^4	TCBS	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	3	10^2	50 MA	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	3	10^2	50 MA+	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	39	0	0
LC	3	10^2	GASW	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	3	10^2	GASW+	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	34	0
LC	3	10^2	TCBS	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	3	10^3	50 MA	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0
LC	3	10^3	50 MA+	48	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
LC	3	10^3	GASW	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7	0
LC	3	10^3	GASW+	48	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
LC	3	10^3	TCBS	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	3	10^4	50 MA	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
LC	3	10^4	50 MA+	48	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
LC	3	10^4	GASW	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	3	10^4	GASW+	48	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0
LC	3	10^4	TCBS	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SF	1	10^2	50 MA	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SF	1	10^2	GASW	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SF	1	10^2	TCBS	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SF	1	10^3	50 MA	48	0	0	0	0	0	0	0	0	0	42	0	0	0	0	0	0	0	0	0
SF	1	10^3	GASW	48	0	0	0	0	0	0	0	0	0	28	0	0	0	0	0	2	0	0	0

Figure E.4: nMDS of isolate morphotypes p4 of 9

Coral	Rep	Dilution	Media	Time	off white smooth round (3mm)	off white smooth, rough/wet edge (swarming?)	orange clear (1-2 mm)	orange cream clear zone (2mm)	orange cream with clear zone (5mm)	orange rough	orange round cream (3mm)	orange smooth wet (3mm)	orange yellow (3mm)	purple black wet (2mm)	smear colourless no rainbow	smear off white rainbow	smear orange 30mm has eaten agar	smear swarming	white rough edge (2-5mm)	white round clear ring (2mm)	white round white rings	white smooth (1mm)
LC	2	10^4	50 MA	48	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
LC	2	10^4	50 MA+	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	2	10^4	GASW	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	2	10^4	GASW+	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	2	10^4	TCBS	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	3	10^2	50 MA	48	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
LC	3	10^2	50 MA+	48	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0
LC	3	10^2	GASW	48	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0
LC	3	10^2	GASW+	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	3	10^2	TCBS	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	3	10^3	50 MA	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	3	10^3	50 MA+	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	3	10^3	GASW	48	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
LC	3	10^3	GASW+	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	3	10^3	TCBS	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	3	10^4	50 MA	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	3	10^4	50 MA+	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	3	10^4	GASW	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	3	10^4	GASW+	48	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
LC	3	10^4	TCBS	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SF	1	10^2	50 MA	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	102
SF	1	10^2	GASW	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	98
SF	1	10^2	TCBS	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SF	1	10^3	50 MA	48	0	0	0	0	0	0	0	0	0	0	0	0	0	1	9	0	0	0
SF	1	10^3	GASW	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	23

Figure E.5: nMDS of isolate morphotypes p5 of 9

Coral	Rep	Dilution	Media	Time	white solid round (1-2 mm)	yellow orange (5mm)	yellow round clear	yellow round smooth (1-2 mm)
LC	2	10^4	50 MA	48	0	0	0	0
LC	2	10^4	50 MA+	48	0	0	0	0
LC	2	10^4	GASW	48	0	0	0	0
LC	2	10^4	GASW+	48	0	0	0	0
LC	2	10^4	TCBS	48	0	0	0	0
LC	3	10^2	50 MA	48	0	0	0	0
LC	3	10^2	50 MA+	48	0	0	0	0
LC	3	10^2	GASW	48	0	0	0	0
LC	3	10^2	GASW+	48	0	0	0	0
LC	3	10^2	TCBS	48	0	0	0	0
LC	3	10^3	50 MA	48	0	0	0	0
LC	3	10^3	50 MA+	48	0	0	0	0
LC	3	10^3	GASW	48	0	0	0	0
LC	3	10^3	GASW+	48	0	0	0	0
LC	3	10^3	TCBS	48	0	0	0	0
LC	3	10^4	50 MA	48	0	0	0	0
LC	3	10^4	50 MA+	48	0	0	0	0
LC	3	10^4	GASW	48	0	0	0	0
LC	3	10^4	GASW+	48	0	0	0	0
LC	3	10^4	TCBS	48	0	0	0	0
SF	1	10^2	50 MA	48	0	0	0	0
SF	1	10^2	GASW	48	0	0	0	0
SF	1	10^2	TCBS	48	0	0	0	0
SF	1	10^3	50 MA	48	0	0	0	0
SF	1	10^3	GASW	48	0	0	0	0

Figure E.6: nMDS of isolate morphotypes p6 of 9

Coral	Rep	Dilution	Media	Time	blue green (4 mm)	brown (1mm)	brown orange (1.5mm)	clear swarming	colourless (4mm)	fungi black	fungi white	green (3mm)	off white clear ring no rainbow (1-3 mm)	off white clear rough ring (1-2mm)	off white cream smooth no ring (1-2 mm)	off white cream smooth no ring (3mm)	off white rainbow (2 - 3mm)	off white rough (1-3mm)	off white round pink centre (5mm)	off white round rainbow ring (4mm)	off white smooth rainbow ring (1-2mm)	off white smooth rainbow ring (2-3mm)	off white smooth round (1-2mm)
SF	1	10^3	TCBS	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SF	1	10^4	50 MA	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SF	1	10^4	GASW	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8
SF	1	10^4	TCBS	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SF	2	10^2	50 MA	48	0	0	0	1	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0
SF	2	10^2	GASW	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SF	2	10^2	TCBS	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SF	2	10^3	50 MA	48	0	0	0	0	0	0	0	0	0	82	0	0	0	0	0	0	0	0	0
SF	2	10^3	GASW	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SF	2	10^3	TCBS	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SF	2	10^4	50 MA	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SF	2	10^4	GASW	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SF	2	10^4	TCBS	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SF	3	10^2	50 MA	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SF	3	10^2	GASW	48	0	0	0	0	0	0	0	0	0	106	0	0	0	0	0	0	0	0	0
SF	3	10^2	TCBS	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SF	3	10^3	50 MA	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	21	0
SF	3	10^3	GASW	48	0	0	0	0	0	0	0	0	0	0	0	7	0	0	0	0	0	0	0
SF	3	10^3	TCBS	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SF	3	10^4	50 MA	48	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
SF	3	10^4	GASW	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SF	3	10^4	TCBS	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Figure E.7: nMDS of isolate morphotypes p7 of 9

Coral	Rep	Dilution	Media	Time	blue green (4 mm)	brown (1mm)	brown orange (1.5mm)	clear swarming	colourless (4mm)	fungi black	fungi white	green (3mm)	off white clear ring no rainbow (1-3 mm)	off white clear rough ring (1-2mm)	off white cream smooth no ring (1-2 mm)	off white cream smooth no ring (3mm)	off white rainbow (2 - 3mm)	off white rough (1-3mm)	off white round pink centre (5mm)	off white round rainbow ring (4mm)	off white smooth rainbow ring (1-2mm)	off white smooth rainbow ring (2-3mm)	off white smooth round (1-2mm)
LC	1	10^2	50 MA	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	11	0
LC	1	10^2	50 MA+	48	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	32	0
LC	1	10^2	GASW	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0
LC	1	10^2	GASW+	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	13	0
LC	1	10^2	TCBS	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	1	10^3	50 MA	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0
LC	1	10^3	50 MA+	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
LC	1	10^3	GASW	48	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
LC	1	10^3	GASW+	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
LC	1	10^3	TCBS	48	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	1	10^4	50 MA	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	1	10^4	50 MA+	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	1	10^4	GASW	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	1	10^4	GASW+	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	1	10^4	TCBS	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	2	10^2	50 MA	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	26	0	0
LC	2	10^2	50 MA+	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
LC	2	10^2	GASW	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	2	10^2	GASW+	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	65	0	0
LC	2	10^2	TCBS	48	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
LC	2	10^3	50 MA	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	2	10^3	50 MA+	48	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
LC	2	10^3	GASW	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LC	2	10^3	GASW+	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0
LC	2	10^3	TCBS	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Figure E.8: nMDS of isolate morphotypes p8 of 9

Coral	Rep	Dilution	Media	Time	white solid round (1-2 mm)	yellow orange (5mm)	yellow round clear	yellow round smooth (1-2 mm)
SF	1	10^3	TCBS	48	0	0	0	0
SF	1	10^4	50 MA	48	7	0	0	0
SF	1	10^4	GASW	48	0	0	0	0
SF	1	10^4	TCBS	48	0	0	0	0
SF	2	10^2	50 MA	48	0	0	0	1
SF	2	10^2	GASW	48	0	0	0	0
SF	2	10^2	TCBS	48	0	0	0	0
SF	2	10^3	50 MA	48	0	0	0	0
SF	2	10^3	GASW	48	0	0	0	0
SF	2	10^3	TCBS	48	0	0	0	0
SF	2	10^4	50 MA	48	0	0	0	0
SF	2	10^4	GASW	48	0	0	0	0
SF	2	10^4	TCBS	48	0	0	0	0
SF	3	10^2	50 MA	48	0	0	0	0
SF	3	10^2	GASW	48	0	0	0	0
SF	3	10^2	TCBS	48	0	0	0	0
SF	3	10^3	50 MA	48	0	0	0	0
SF	3	10^3	GASW	48	0	0	0	0
SF	3	10^3	TCBS	48	0	0	0	0
SF	3	10^4	50 MA	48	0	0	0	0
SF	3	10^4	GASW	48	0	0	0	0
SF	3	10^4	TCBS	48	0	0	0	0

Figure E.9: nMDS of isolate morphotypes p9 of 9