Chemical ecological insights into metabolites of the New Zealand marine sponge, *Cliona celata*

A thesis

submitted in partial fulfilment

of the requirements for the degree

of

Master of Science (Research)

at

The University of Waikato

by

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THE UNIVERSITY OF

WAIKATO

Te Whare Wānanga o Waikato

2015

In dedication to mum, dad and Swebbiekins,

for your love, laughs and unwavering support.

In loving memory of Poppa Jack, I miss you.

xoxo

ABSTRACT

Marine sponges are known to produce secondary metabolites that have roles including chemical defence against cellular challenge. An investigation into *Cliona celata* around the Bay of Plenty (New Zealand) was conducted in response to discovery of unusual metabolites that had the potential to be biologically active. The study was designed to determine chemical variation in the sponge over small spatial and temporal scales and to determine the possible ecological triggers for the presence of these metabolites. The hypothesis was that a chemical defensive response would be elicited 24-48 hours post a cellular challenge event such as physical damage. The hypothesis was based on the fact that the sponge heals quickly when damaged with little evidence of necrosis.

Cliona celata was chosen as there is little knowledge of the species within New Zealand, and previous work showed a single specimen to produce brominated compounds, never before reported for this genus. This project examines whether the production of brominated compounds is common throughout all *C. celata* within the Bay of Plenty region or is a localised response.

A preliminary study was conducted to investigate spatial chemical variability of sponges from three locations, Leisure Island (intertidal), Rabbit Island (subtidal) (both on the coast adjacent to Mount Maunganui, Tauranga) and White Island (geothermal subtidal). A damage response experiment for Leisure Island and Rabbit Island specimens was conducted to determine chemical differences in healthy versus damaged sponges, and whether a chemical defence mechanism was utilised by the sponge species. Lastly, a stratification experiment was performed to establish whether chemical changes occur throughout the sponge or are localised to the immediate damage site.

Cliona celata identification was confirmed using spicule mounts. However, some variation between intertidal and subtidal specimens was noted with differences in spicular morphology, likely due to variations in chemistry and micro-environments.

On a small scale, extracts of each sample were prepared and analysed using Liquid Chromatography Mass Spectrometry (LCMS). Damaged and non damaged sponge specimens were subjected to an MTT assay using the human cervical cancer cell line (HeLa). But the crude extracts showed no bioactivity in this assay.

Chromatograms obtained from LCMS, for all samples contained a common set of peaks, however, variations in intensities and some minor peak variations within the sites (intertidal, subtidal and geothermal subtidal) and individual specimens were noted, and could represent interactions with different micro-environments. A lack of chemical response to damage was not expected. It is therefore hypothesised that the lack of secondary metabolite production observed, is due to cells reverting to archaeocytes to repair and re-grow the removed sponge section, a histological response rather than a chemical one.

It was hypothesised that specimens with increased levels of epibiont coverage had increased production of certain metabolites, as represented by increased intensities of some peaks in chromatograms, however, to a threshold. Once epibiont coverage of approximately 50% was met, novel chemicals were thought to be produced by the sponge. Two sponge samples did produce monobrominated and dibrominated compounds, however, these were localised events and could potentially be the response to an unidentified external stressor or epibiont coverage. The stratification experiment indicated that the isolation and production of these brominated compounds may be restricted to the pinacoderm of the sponge, possibly suggesting a transient chemical response as cells re-organise themselves in a healing process. However, further investigation is required with all experiments and a more comprehensive time series of samples needs to be taken post damage treatment.

This project provides a preliminary study into the chemical variation and chemical response to damage for intertidal and subtidal Bay of Plenty *Cliona celata*. Any hypotheses made during this thesis need to be further examined to be accepted.

ACKNOWLEDGEMENTS

A huge thank you to my mum, dad and sister, your never-ending belief in me has got me through this thesis. You have all helped me with some very stressful and hard times in the past year; I don't know where I would be without my family. Also mum and dad thank you for the much needed financial top ups, you will be getting a first class cabin on your retirement cruise. To the rest of my family; grandparents, aunties, uncles and cousins, thank you for your loving support.

Thank you to all my friends for encouraging me and forcing me to take breaks. In particular Alice and Kara you are my best friends that keep me grounded, thank you. Ashley and Gary thank you for putting up with me at home and helping me with field work. Alicia, Katie and Kirsty thanks for the

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LIST OF ABBREVIATIONS

ACN   Acetonitrile

DCM   Dichloromethane

ESI   Electrospray ionisation

EtOH   Ethanol

GPS   Global Positioning System

H_2O water

HeLa   Henrietta Lacks cells (human cervical cancer cells)

HPLC   High Pressure Liquid Chromatography

LC   Liquid Chromatography

MeOH   Methanol

MS   Mass Spectrometry

MTT   3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NMR   Nuclear Magnetic Resonance

ROV   Remotely Operated Vehicle

SCUBA   Self Contained Underwater Breathing Apparatus

TFA   Trifluoroacetic acid

UV   Ultra Violet

CHAPTER 1

GENERAL INTRODUCTION

1.1 Marine Sponges

Marine sponges (phylum Porifera) are the oldest known metazoan animals. Fossils have been found dating back more than 580 million years ago.[1] Sponges can be found in all benthic marine environments, such as tropical, subtropical, polar and deep sea. There are large populations of sponges, albeit with reduced diversity compared to marine species, found in freshwater systems.[2,3] Over 15,000 sponge species have been discovered and over 8,500 species are currently valid,[4,5] with more being found every day.[6] These soft-bodied, sessile (non-moving) invertebrates can have a large ecological impact on their environment.[7]

Sponges are classified into four classes based on their morphology.[8] Demospongiae is the largest and most diverse class with 15 orders known.[5] The Demospongiae class are described as having two to four rayed siliceous spicules, spongin protein fibres or a combination of both.[8] The Hexactinellida group (glass sponges) are formed from six rayed glass spicules and have multinucleate cells.[9] This class of sponge are typically located in deep and isolated waters such as in fiords.[2,10] The Calcarea sponges have a skeletal structure entirely composed from calcium carbonate and are predominantly found in shallow waters of tropical and temperate zones.[10] The last class, Homoscleromorpha have recently been differentiated from Demospongiae. The class was originally recognised as a subclass of Demospongiae due to similar siliceous, tetractinal-like, calthrop spicules. However, recent phylogenic studies show this class to be more closely related to Calcarea sponges.[11,12]

1.1.1 Marine Sponge Structure

Marine sponges are dynamic organisms that adapt to their current environment. They are able to expand or reduce their size based on the surrounding conditions.[13] Sponges vary considerably in morphology from thin/thick encrusters to ball, tubular, vase, and barrel shapes.[1,2] The shape of a sponge typically depends on its environment. High energy environments tend to support more compact encrusting sponges whereas low energy environments will permit more tubular/vase-like sponges to exist. Indeed the body plan of many sponges is quite flexible and the same species may have a number of morphologies dependant on the prevailing micro-environment.[14]

Marine sponges do not have organs as such, but function from a cluster of cells that form a series of canals and chambers, creating the internal body of the sponge which functions as an efficient pump (the aquiferous system) (Figure 1.1).[12,15,16] Sponges are often organised into two layers internally. The outer epidermis of the sponge is known as the pinacoderm (populated by endo- and exo-pinacocytes).[16,17] This can be a leather-like structure in those species where the pinacoderm is well formed, which helps to protect the softer body of the sponge. The inner layers of canals and chambers are known as choanoderm (layered with choanocytes).[12] An area under the pinacoderm can be elaborated to provide for a tougher outer region of the sponge and where this occurs, the sponge can be delineated by a matrix and an ectosome. It is important to note that Hexactinellids have a different structure entirely and that the degree of internal elaboration in the Demospongiae can be either simple (Asconoid) or complex (Leuconoid) (Figure 1.2).[2,12,16]
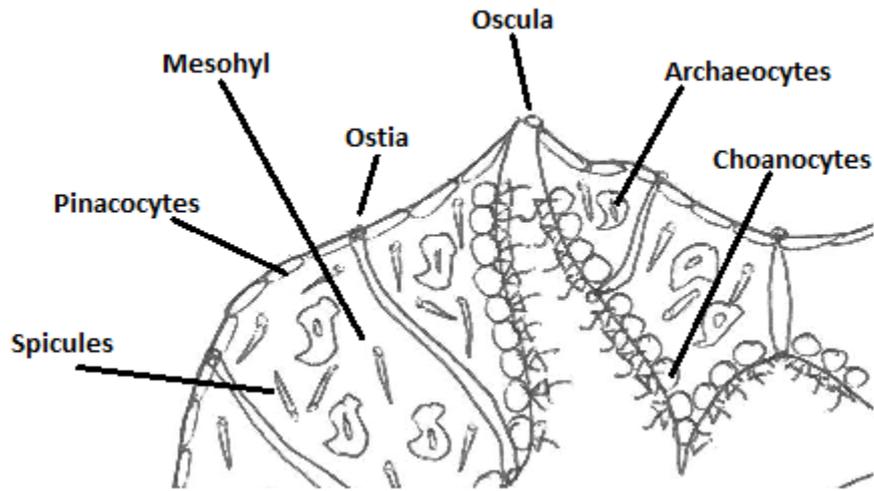
Figure 1.1: Basic anatomy of an encrusting demosponge
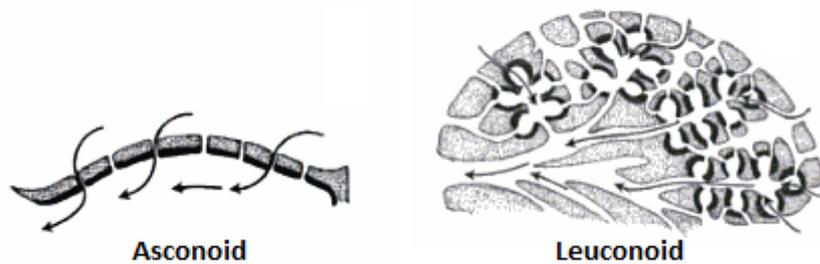
Figure 1.2: Structure of Asconoid (simple) sponge and Leuconoid (complex) sponge, adapted from Reference 8[8].

The condensed fluid filled pockets, known as mesohyl, are the spaces between inner and outer layers. This contains a number of cells such as, digestive cells, symbiotic algae, sclerocytes (spicule producing cells), collencytes (collagen producing cells), embryos and gametes for developing larvae.[2,16,17] The mesohyl spaces are supported by fibrous systems (collagen/spongin) and/or glass-like skeletal structures. The skeletal structures are identified as spicules which can be made from silica, calcium carbonate and collagen.[2,18] Large quantities of water from the surrounding water column are pumped through the chambers and canals.[8,12] Nutrients and oxygen trapped or dissolved in the water column are extracted out by the choanocytes and are utilised by the sponge.[4,9,10]

Typically, marine sponges have a coarse and fibrous texture with visible exhalant pores called oscula, which pump filtered water out of the sponge.

The inhalant pores (ostia) are smaller and much harder to visualise. Ostia pump water into the sponge through the connecting canals.[18]

Symbiotic and pathogenic microbes are commonly found in sponge tissue and can significantly contribute to the overall biomass, often to more than 40% wet weight of the sponge.[19-21] The symbiotic relationship between the sponge and microbes can lead to the production of secondary metabolites that are used for a range of functions including cellular defence.[22,23] Sponges are able to re-aggregate cells when damaged by influences such as predation or environmental factors. Thus regeneration after damage is not often an issue for sponges.[13] However, when sponge cells do die, they are quickly disintegrated, leaving no obvious sponge cell debris.[13]

The multicellular structures (Figure 1.1) that make up the body of the sponge include pinacocytes (the epidermis of the sponge); a thin layer of compact cells that line the exterior of the sponge, creating the pinacoderm as stated previously.[2,16] Archaeocytes (known also as totipotent cells) are one of the most important cells within the sponge as they are able to reversibly transform into any type of cell required in the sponge.[24] Archaeocytes ingest, digest and store food by phagocytosis,[10,18,25] which is delivered by the choanocyte collars (flagella). They also transport nutrients around the body of the sponge. Archaeocytes may form into gamete sponge cells, "building blocks" of the sponge. They are essentially stem cells that can transform into a range of cells required.[24]

Other cell types include choanocytes, these are distinctive cells that line the interior walls of the chamber and canals within sponges. Each cell has a central flagellum that is surrounded by a collar of microvilli.[4] The beating of the flagella actively pulls water and oxygen from the surrounding environment into the sponge. Nutrients are then absorbed into the body of the sponge, primarily through the choanocytes. Sclerocytes are spicule producing cells and collencytes generate collagen/spongin fibres. Spherulous cells are cells that are thought to be one of the prime cell types that manufacture secondary metabolites.[26] They frequently contain

endosymbionts, (bacteria, archaea and eukaryotes) which are also assumed to contribute to secondary metabolite biosynthesis.[20]

It is often difficult to identify a sponge by its exterior. Many sponge species have similar morphologies. However, the same species can also have variable physical characteristics.[18] Sponges can be identified by their unique skeletal structures, spicules.[8] Identification of a sponge is very important in biodiscovery and marine natural product development processes, associated with drug discovery, for example, as it provides a reference for future collections/investigations and database searching for previous studies. Patents for bioactive compounds and drug development are essential for pharmaceutical companies thus it is crucial to determine the identity of the sponge collected.

1.1.2 Ecological Functions

Marine sponges have important ecological functions that influence their surrounding environments and are often the predominant organism in that environment. However, in the past, the significance of their ecological roles has been largely overlooked.[27] Sponges are able to influence the environment by impacting substrates, for example, stabilising and protecting coral reefs in tropical areas.[27,28] Marine sponges can impact the benthic-pelagic coupling regime (the interaction of water at the sea bed and in the water column) by clearing of the near bottom water column, although the full extent of this effect in the field has not been completely investigated.[27] As sponges are often non-selective filter feeders (unless presented with an abundance of a preferred food source), water mass with high organic matter content, as well as microbes and plankton can be taken up and the flocculant carbon or ultra-plankton are thus utilised as a nutrient source for the sponges.[6,27] Lastly, sponges influence and are influenced by other organisms. In particular, symbiotic relationships with microorganisms can lead to the co-production of secondary metabolites.[27]

Sponges can reflect the chemical environment that surrounds them by the production of defensive compounds in response to cellular challenge,

brought on by stress or pathogenic attack. Evidence suggests that marine sponges could be utilised as bio-indicators of affected environments during ecological disasters such as oil spills, as they have the ability to accumulate products from the environment.[29] It is this response to environmental micro-environments that this project examines, that is, the production and release of secondary metabolites into the surrounding environments based on threatening stimuli. These secondary metabolites additionally may possess bioactive properties that could be very useful to the pharmaceutical industry.[30]

1.1.3 Symbiotic Relationships

As mentioned previously, symbiotic relationships are a common and important occurrence for marine sponges. It is often a mutualistic (both partners benefit) relationship for the sponge; where the sponge acts as the host, and the other organism is a symbiont.[20,31] Symbiosis can involve a range of different microorganisms, for example bacteria, algae, fungi, dinoflagellates and diatoms.[10,20] These organisms can be either exo- (live on the outer pinacoderm) or endosymbionts (live within the mesohyl).[31] Due to marine sponges being effective filter feeders, the concentration of food particles and nutrients within the sponge is much higher than in the surrounding water column, thus symbiotic microorganisms are able to receive nutrients and protection while living in the sponge.[21,31] Sponges on the other hand are able to utilise the microorganisms to increase regular metabolic function, for example, enhancing the efficiency to uptake nutrients, the transportation and removal of waste products, and sequestration of secondary metabolites for chemical defences,[20,32] although it is not fully understood how these symbiotic relationships are involved in the elicitation and production of secondary metabolites.[23,32]

An example of a symbiotic relationship involving metabolite production is between the marine sponge *Dysidea herbacea* and cyanobacteria.[33] A brominated secondary metabolite, 2-(2',4'-dibromophenyl)-4,6-dibromophenol, was discovered within the sponge. Separation of the cyanobacteria from the sponge tissue was completed using flow cytometry

and chemical analysis was conducted using gas chromatography and NMR spectroscopy. Upon this investigation it was found that the metabolite was exclusively found within the cyanobacterium and not the sponge itself. The sponge, however, did utilise the metabolite to benefit itself.[33]

1.1.4 **Reproduction**

Marine sponges have two modes of reproduction, asexually via budding or fragmentation and sexually (often as an hermaphrodite) (Figure 1.3).[10] Asexual budding occurs when a cluster of cells form a 'mini' sponge on the exterior of the parent sponge. The 'mini' sponge creates a teardrop shaped bud, the point of attachment to the parent sponge being the tip of the teardrop.[34] When the bud reaches a certain size, it detaches from the parent sponge to establish nearby or is transported in the current, attaches itself to a substrate and continues to grow as an adult sponge.[34]

Fragmentation occurs when the sponge is damaged and a section is removed from the parent sponge (likely to occur during a storm event).[35] As the sponge is able to regenerate itself, the sponge fragment reattaches to a substrate and continues growth to form a mature sponge.[25]

Sexual reproduction usually involves egg cells and sperm cells being produced within the same sponge (hermaphrodite). However, the production of these cells usually occurs at different times.[10] Within the mesohyl, embryogenesis (egg cell formation) occurs in archaeocytes and the choanocytes convert to male cells where spermatogenesis occurs (sperm cell formation).[10,25] Each gamete is released into the water column, where the sperm cell is consumed by the egg and fertilisation occurs. The embryo then forms into a planktonic larva which disperses into the water column. Once the larva settles onto the sea bed it attaches to a substrate where it starts to form into a new sponge.[25]

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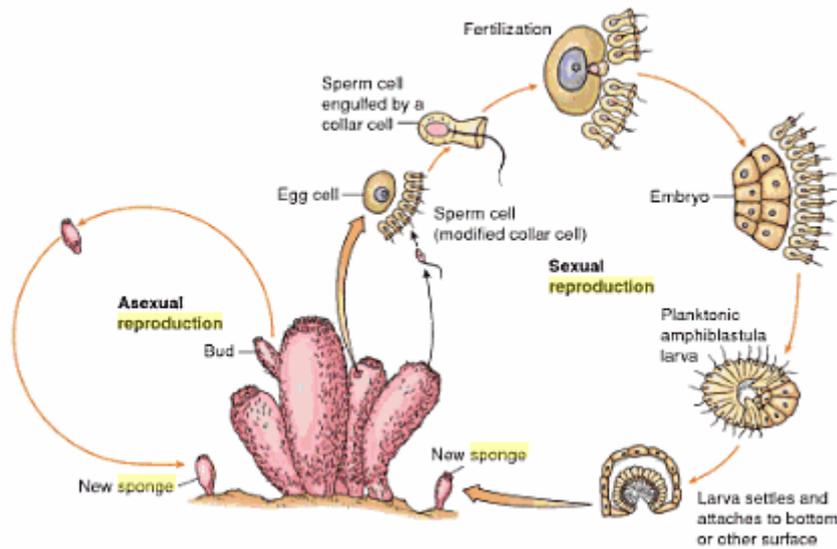
Figure 1.3: Asexual and sexual reproduction of a marine sponge, retrieved from Reference 25[25].

1.1.5 *Cliona celata*

Cliona celata (Grant 1826) is a commonly distributed temperate marine sponge around the world and New Zealand (Figure 1.4).[36-38]

Phylum : Porifera

Class : Demospongiae

Order : Hadromerida

Family : Clionidae

Genus : *Cliona*

Species : *Cliona celata*

Cliona are typically located in intertidal/subtidal temperate zones although the genus is also found in the tropics. *Cliona celata* ranges in morphology but typically has a colour variation of orange to yellow, thus it can be difficult to identify *in situ* from photographs.[39] However, one distinctive characteristic is the linear pattern of oscula along the sponge body which are larger and more visible than the ostia.[6] This is a large, boring and encrusting sponge that can reach over 1 m^2 in diameter.[8,39]

8

Figure 1.4: *Cliona celata in situ* at Site Three, Rabbit Island. Image taken by Rex Fairweather.

The alpha (boring) stage of the sponge is presented as small papillae that erupt from the rock or coral surface as the matrix of the sponge bores into the substrate. A gamma (massive) stage sponge presents as a thickly encrusting to massive sponge covering the substrate (this is what was collected during this project).[30] The exterior of *Cliona celata* is a firm and leathery texture.[39] The skeletal structure is typically limited to one class of megasclere (large spicules) – tylostyle spicules, with no microscleres (small spicules) present. Tylostyle morphology characteristically (Figure 1.5) has a bulbous head with a straight or curved shaft and a pointed tip.[8] Calcareous debris and other spicular debris can also be incorporated into the body of the sponge.[39]
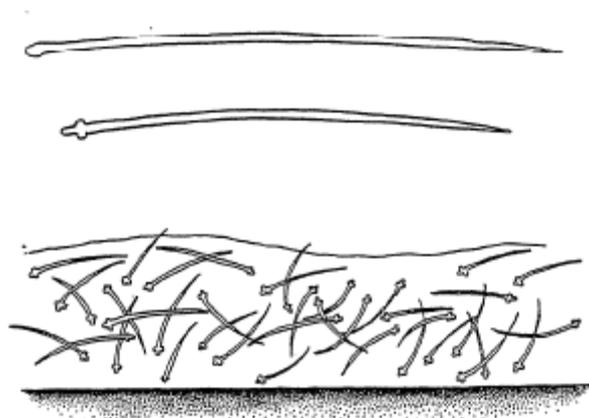
Figure 1.5: Tylostyle spicules of *Cliona celata*, adapted from Reference 39[39]

Cliona celata is typically located on calcareous substrates or coralline algae where it can use chemicals to dissolve and bore into the calcareous/coralline material.[30,39] It can largely impact these areas as the sponges can almost disintegrate the substrates, especially in coral reef environments.[40] As this sponge has been understudied in New Zealand, there is an interest in its chemical ecology.

Secondary metabolites identified from many *Cliona* species have been isolated in numerous global locations. It has been observed that specimens of *C. celata* turn purple on exposure to air for instance, possibly due to oxidising secondary metabolites.[41] One of the earliest chemical studies on *Cliona* was conducted by Bergmann, who found that poriferasterol (**2**) and clionasterol (**1**) were common sterols found within this species.[42] Since this discovery, sterols of *Cliona* have been further studied. Samples collected from Port Erin, Isle of Man were found to contain a mixture of eight different sterols, the predominant of these being cholesterol (**3**).[42]

(**1**)

(**2**)
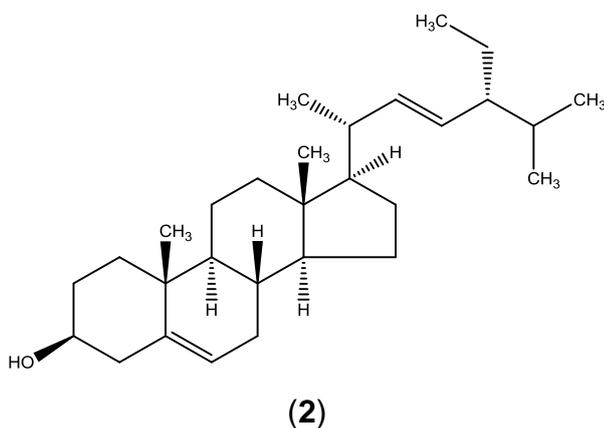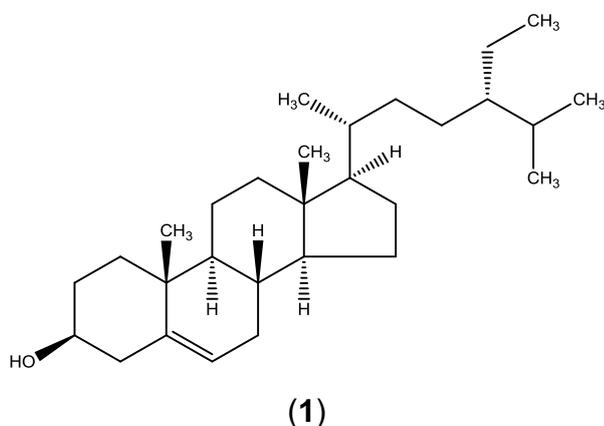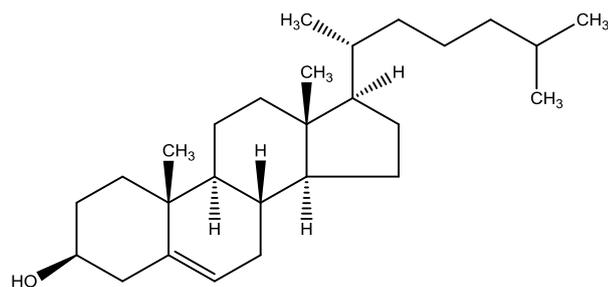
(3)

A study in British Columbia found that ethanol extracts from *C. celata* had antibiotic properties against the bacterium *Staphylococcus aureus in vitro*,[43] thus a further investigation was conducted to try and purify the crude extracts. From acetylated crude material, a high yield of pure tetracetyl clionamide (**4**), (a 6-bromotryptophan derivative) was obtained.[43] Ethyl acetate extracts of *Cliona celata* from the Korean seaside have found to have anti-inflammatory properties, further studies on how these extracts affect muscle cells are being conducted.[44] As stated above the chemical composition of *Cliona celata* in New Zealand has been largely unstudied, therefore this thesis aims to investigate the chemical variation of the sponge.
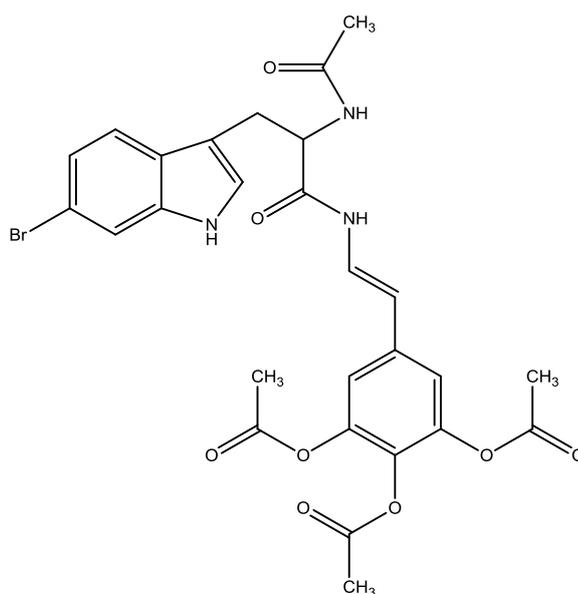
(4)

1.2 Marine Chemical Ecology

Chemical ecology can be interpreted as the science pertaining to the ecological ramifications of organisms producing and/or utilising chemical compounds then releasing them into the surrounding environment where they cause intra- and interspecific interactions with other organisms.[45-47] The chemicals produced tend to have physiological and behavioural impacts on other organisms.[48] Chemical ecological interactions can be used for a number of different reasons such as pheromone production (attracting a mate), symbiotic relationships with organisms (nutrient cycling), protection against abiotic stresses (UV and desiccation adaptations) and chemical defence mechanisms (predator deterrents).[49]

There are two different types of chemical metabolites produced in organisms. Primary metabolites are essential for the organism to live and are common to a range of different organisms. They make up the building blocks of organism cell structures. For example, primary metabolites can include compounds such as amino acids, lipids and proteins. In comparison to these compounds are the secondary metabolites, which are synthesised from the primary metabolites via specialised pathways.[49] Secondary metabolites are not essential for the life of the organism; however, they are important. These compounds are more species specific and their function is often unknown. Secondary metabolites include compounds such as alkaloids, peptides and steroids.[50] Secondary metabolites are of great interest to the natural product community due to their unusual structure and combinations of functional groups, together with their medicinal and commercial properties. Thus the chemical ecology of an organism is potentially useful (for the natural product industry) as the chemicals produced are likely to have bioactive properties and their role in nature can inform possible applications.

In the past, the majority of investigations into the chemical ecology of organisms have been conducted in the terrestrial environment.[51,52] This is due to the ease of accessibility and utilisation of this environment. An example of a terrestrial derived drug is aspirin, which originates from the bark of the Common White Willow tree that contains a secondary

metabolite called salicin (**5**). This compound was the framework for the synthetically produced salicylic acid (**6**) which was then acetylated to form acetylsalicylic acid (**7**) (aspirin).[53] Even though terrestrial environments were the main source of natural products in the past, the marine environment is now becoming the best environment for discovering secondary metabolites with pharmaceutical potential.[54]
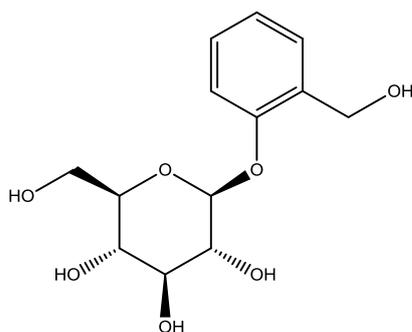
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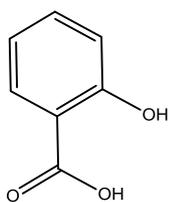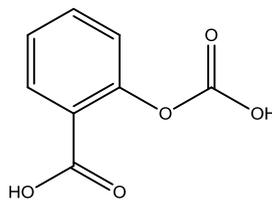
(6)   **(7)**

The marine environment contributes to more than 70% of the earth's surface.[52,55] All organisms were once derived from the oceans; thus marine organisms have had longer to adapt to their environment and produce complex metabolites.[55,56] Certain marine ecosystems have such a range of organisms that they are said to have more diversity than tropical rainforests. These include deep sea vents and tropical reefs. Therefore the chance of discovering a novel secondary metabolite is high.[52,55]

The marine environment has been understudied in the past due to access restrictions. However, with the invention of SCUBA diving and the use of dredging, submersibles and Remotely Operated Vehicles (ROVs) this restriction has been largely overcome.[47,49,51,56] Some access issues still arise as very deep depths cannot be reached with SCUBA diving and

dredging. Dredging is also a very unselective and environmentally destructive process. Even though submersibles and ROVs solved some accessibility issues, they are extremely expensive and beyond the funding of most organisations. In spite of this, marine secondary metabolites are continuously being discovered.[51,52,57]

1.2.1 Chemical Defences

As most sponges are soft sessile structures without any physical means to protect themselves, many Porifera use chemical defences as a deterrent.[6,46] The sponge can excrete secondary metabolites that are detrimental to predating organisms, pathogens or diseases. This inhibitory reaction to growth or reproductive processes of the other organism is referred to as allelopathy.[47,58] It is these metabolites that are a particular interest for marine natural product chemists and the biochemical industry. They hold the potential to be utilised as potent pharmaceuticals for diseases and illnesses such as, cancers and bacterial infections. The production of these secondary metabolites is often through sequestration (uptake) of chemicals from other organisms, symbiotic organisms or food sources.[31]

The defence mechanism of the Arctic fjord sponge, *Haliclona viscosa* was found to involve the production of a predator deterrent that stopped amphipods and starfish (*Asterias rubens*) feeding on it.[59] However, the chemical composition of the deterrent was not determined. In *Isodictya erinacea,* an Antarctic sponge, two secondary metabolites were isolated.[60] Erinacean (**8**), was produced to prevent predation by a sea star but more interestingly, erebusinone (**9**), prevented the moulting of a sponge boring amphipod (*Orchomene plebs*) which consequently increased its rate of mortality, protecting the sponge.[59,60]

(8) **(9)**
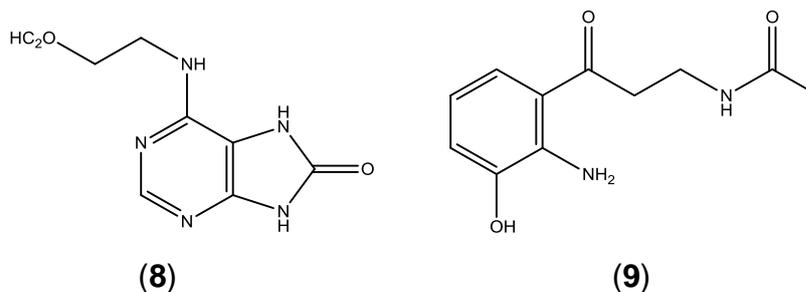
In recent years, there has been an increase in studies and evidence of chemical ecology and metabolite production within marine organisms. The metabolite production has been shown repetitively in a range of marine sponges, hence the great interest in their chemistry.[6] However, there are still a large number of organisms in the marine environment that have not been examined which could have the potential to produce extremely beneficial natural products and pharmaceuticals.[30]

1.3 Natural Products

Natural products are secondary metabolites produced by an organism but are not essential to the survival of the organism as stated previously.[49] In recent years, with advances in technology and methodology, the discovery of marine natural products for pharmaceutical and industrial applications has been increasing rapidly.[49,50] A number of these biologically active (bioactive) metabolites have been found in marine sponges, however, only a handful have been commercialised.[6] An example of a marine sponge natural product is the anti-tumour compound, halichondrin B (**10**).[55,61] This compound was originally extracted from the marine sponge *Halichondria okadai* in Japan, but was also found in a New Zealand sponge *Lissodendoryx* n.sp.1 from which the compound was extracted in bulk to source a sufficient supply for early phases of a drug development process.[52,62] A simplified analogue of halichondrin B (**10**), eribulin mesylate (**11**) was synthesised and developed as a treatment for metastatic breast cancer. Its mode of action is to bind to the tubulin in tumour cells, and stop mitosis (cellular reproduction).[51,61]

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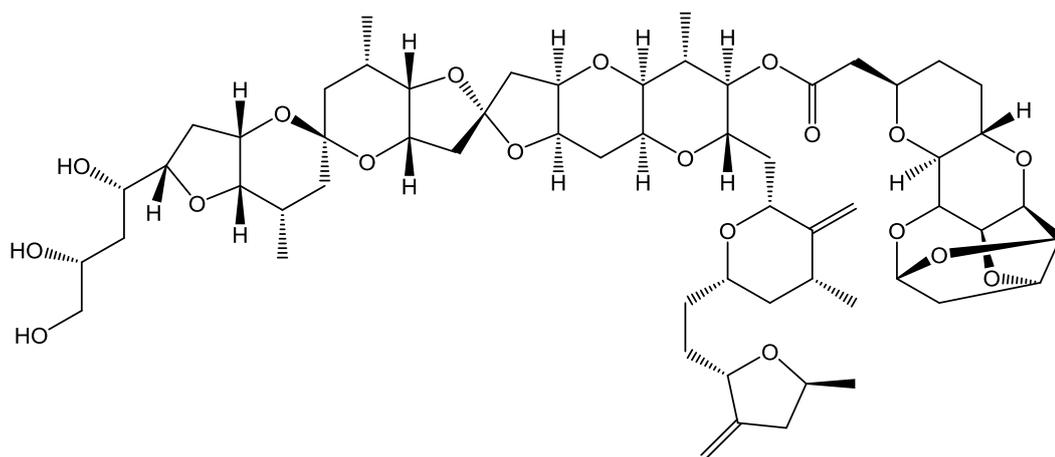
(**10**)
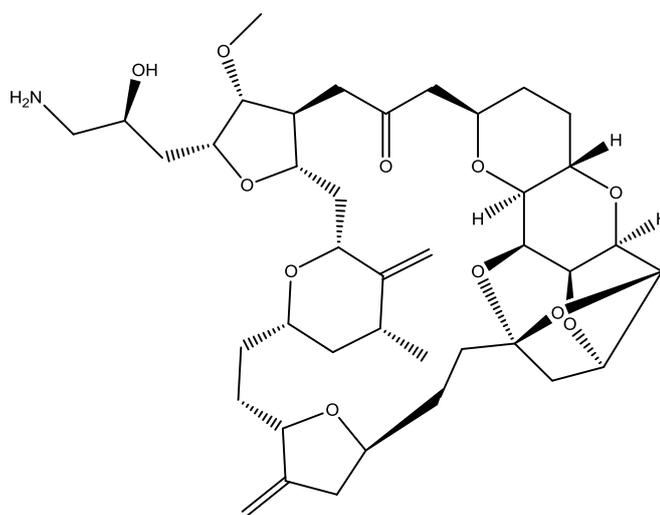
(**11**)

The marine environment is often limited by nitrogen sources and has very low levels of essential nutrients such as, amino acids and sugars. Therefore it is not surprising that marine organisms form bioactive metabolites to increase their chances of survival.[50] However, the marine environment does contain high concentrations of inorganic salts, such as the anions, bromine, chloride and sulphate, and the cations, sodium, potassium and calcium. It is common for these inorganic salts, in particular covalently bound halogens, bromide and chloride, to be incorporated into marine secondary metabolites.[50] Bromine is less available than chloride in the marine environment but it is more easily oxidised and integrated into marine organisms, thus secondary metabolites commonly have bromide functionalities, in particular bromopyrrole alkaloids (**12** and **13**).[63] Bromine is highly reactive with many other species[64] and has known antibacterial

16

properties which can prevent the formation of biofilms.[65] Therefore, other compounds containing bromine could have similar or even more effective impacts on the medicinal world. For example, members of the *Agelas* sponge genus are commonly found in the Caribbean.[66] This genus of sponges protect themselves from fish predation with brominated pyrrole containing alkaloids (**12** and **13**), and the greater the degree of bromination the more effective the repellent.[49,63,66]
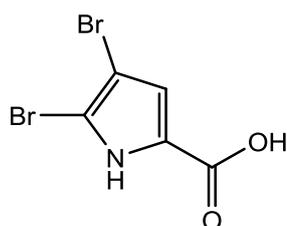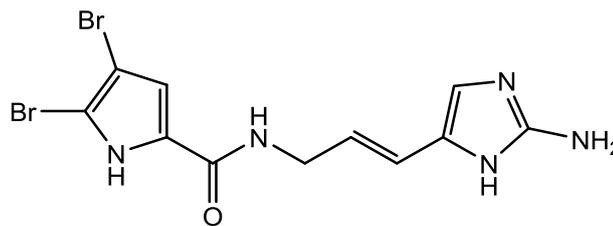
(**12**)   (**13**)

Secondary metabolites need to be very potent to be effective as they are quickly diluted once released into the water column.[55] These secondary metabolites are likely to be compounds such as polyketides, alkaloids, peptides, and a range of mixed biogenesis metabolites.[50] This assortment of chemical compounds can lead to the production of human pharmaceuticals, as metabolites have potent anti-cancer, anti-tumour, immunosuppressant, anti-inflammatory, anti-bacterial properties and so forth.[55] Some marine sponge natural product examples are outlined below.

Over 40 years ago, C-nucleosides were isolated from *Cryptotheca crypta,* a marine sponge from the Caribbean.[52,62] This discovery led to the synthesis of cytarabine (**14**), one of the first marine derived natural products. Cytarabine (**14**) is now used routinely as an anticancer treatment for leukaemia and lymphoma. Gemcitabine (**15**), a fluorine derivative of cytarabine (**14**) has also been clinically approved to treat patients with bladder, pancreatic, breast and non-small cell lung cancer.[52]

(14) **(15)**

An investigation of the biopotential of chemical extracts from the marine sponge *Dendrilla nigra* was conducted.[67] Sponge samples were collected from the Gulf of Mannar, India. The extracts were tested against human pathogens such as *Staphylococcus aureus, Escherichia coli* and *Pseudomonas* sp., and shown to have very potent antibacterial properties. Further testing showed that the sponge extracts had anti-inflammatory, anti-oxidant and anti-cancer properties. Thus the extracts will be trialled as potential pharmaceuticals.[67]

Tethya maza is a sponge located from the northeast to the southeast coast of Brazil.[68] A study to determine whether adult sponges and buds had similar chemical defences was conducted. This study showed that the chemicals produced were sterols and 62% of the chemical deterrents were similar in both adult and juvenile (buds) sponges. However, two sterols were present in the buds and not the adults and one sterol in the adults and not the buds. The differences in chemicals are thought to defence mechanisms against selected predators for each sponge type.[68]

1.3.1 Natural Product Processes

Selection methods prior to collection can be conducted to increase the chances of discovering a bioactive compound in a marine organism.[56,69] These include, field observations, chemical ecology in related species and traditional knowledge.[70] Field observations focus on examining chemical ecology factors, for example determining interactions occurring between organisms (one organism repelling another) and investigation of species without any obvious predators.[56] Often organisms within the same genus

produce chemical compounds similar to each other, thus if a known organism produces a natural product, it is likely an organism from the same genus will be worth investigating. This is the case with the *Aplysina* sponge genus, as multiple bromotyrosine alkaloids have been discovered with varying bioactivities, for example, 11-oxoarethionin (**16**) and aerophobin 1 (**17**).[71] Traditional knowledge from indigenous cultures is often a very successful technique for discovery of natural products as plant or animal derived medicines are likely to be effective due to secondary metabolite production.[70]

(**16**)

(**17**)
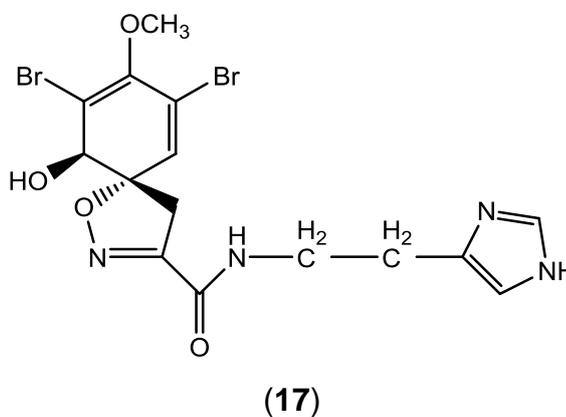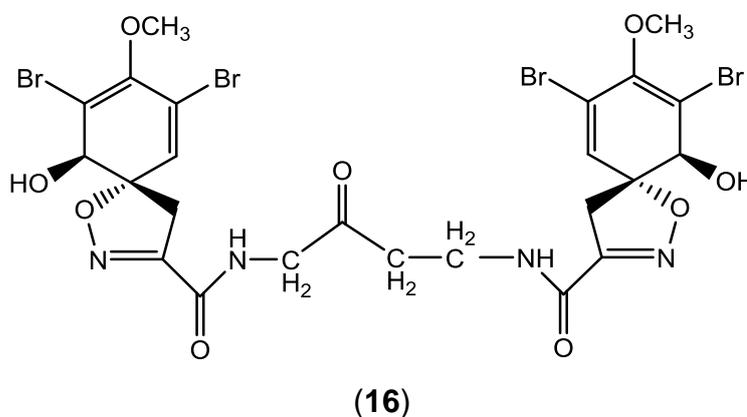
Advances in technology have made it easier to isolate and elucidate bioactive secondary metabolites from marine organism matrices. Techniques such as extraction, filtration and chromatography (reversed phase) are used to isolate the target metabolite.[72] Structure elucidation of the metabolite is achieved by spectroscopic techniques such as Nuclear Magnetic Resonance (NMR) spectroscopy and Mass Spectrometry (MS) or X-ray crystallography.[63,72] For example, the Caribbean marine sponge

Phorbas amaranthus was found to contain two novel sulphated dimeric sterols (amaroxocane A (**18**) and B (**19**)) which were isolated and identified using MS and NMR spectroscopy. Once the structure of the bioactive natural product is determined, the natural product, semisynthetic natural product or compound derived from the natural product can be subjected to further testing.[73]

(**18**)

(**19**)
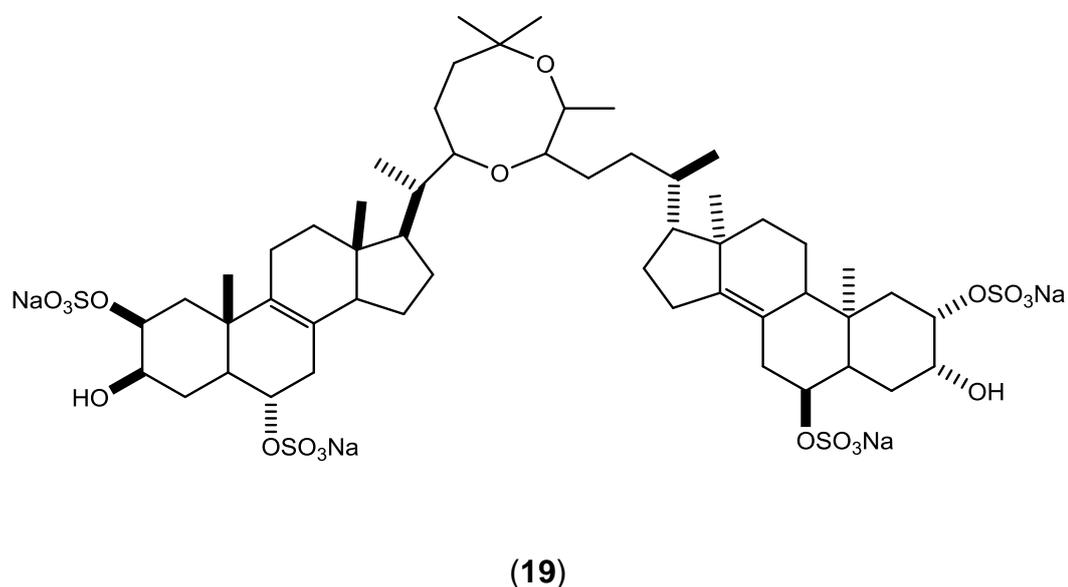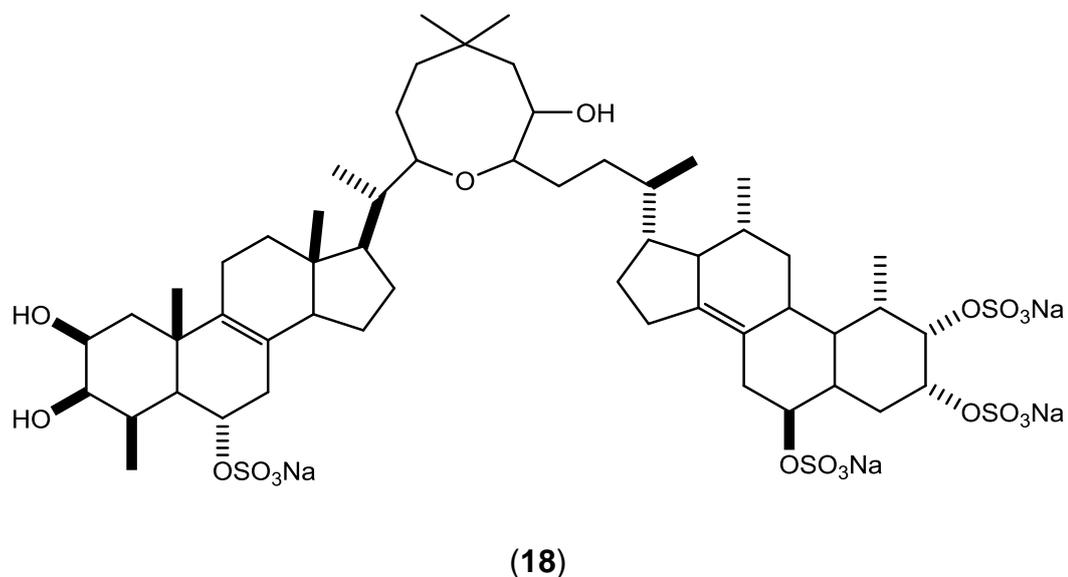
Structural purification is beyond the scope of this project, however, sponge samples were analysed by Liquid Chromatography Mass Spectrometry (LCMS). The LC separates out the chemicals detected in the sponge by running the sample through a column with C_{18} as stationary phase. Chemicals are separated by eluting at different times based on their

polarity. The sample then runs through the MS where the mass of each eluent is measured using a mass to charge ratio (*m/z*). Some isotopes will be able to be identified, for example a compound with bromine present. Bromine has two isotopes (^{79}Br and ^{81}Br), thus two approximately equal peaks will be present with a two mass unit difference. Using this technique trends and differences within the sponge samples can be categorised.

1.3.2 Marine Natural Product Limitations

There are several problems associated with the collection and production of marine natural products. These include, accessibility, obtaining enough sample for research, purification of the metabolite, and intellectual property rights.

Accessibility, as stated under the chemical ecology section, is a limitation due to depth and expense, especially when organisms of interest are benthic. In addition, in early phases of any biodiscovery process, there is a need for sufficient target material to progress bioassays.[23,74,75] For rare or uncommon species, this can be an issue, for example salicylihalamide A (**25**) has potent anti-tumour properties but the drug development process has been delayed due to the lack of sufficient supply.[76] This can be compounded if the metabolite is present in only low concentration, hence there is benefit in identifying high yielding variants or triggers that will enhance natural biosynthetic process to generate more metabolite.[74] Identifying the trigger for metabolite biosynthesis can enhance yields for early phase drug discovery and furthermore, later on it may be possible to harness the biochemical machinery for the biosynthesis process. The levels of metabolite can also be impacted by environmental conditions such as season and nutrient availability, thus sampling can be problematic.[6,74] In recent years there has been a move to cultivate sponges in a sea-based environment to harvest their secondary metabolites in larger quantities.[23] Sponges involved in this process so far include, *Mycale murryi, Latrunculia brevis* and *Polymastia croceus.* However, this has led to some issues with reproducible production of bioactive metabolites.[23]

Purification of the target metabolite can prove to be a challenge as marine organisms are a very complex matrix and as stated previously the secondary metabolite of interest tends to be in low concentration and is likely to be one elaboration on a common scaffold. It can also be difficult to remove the large quantity of salt (from sea water) from the target metabolite. With advancements in technology, separation via C_{18} silica reversed phase chromatography and structural elucidation via spectrometry and X-ray crystallography can be achieved.[72]

Lastly, the issue of intellectual property rights and traditional ownership of a natural product can impact the production of a pharmaceutical. A major issue is that countries with high biodiversity tend to be poorer, developing countries and therefore do not have the funding to investigate natural products,[56,77] whereas, wealthy countries typically have low biodiversity, but the money and resources to research natural products. Therefore, the United Nations Convention on Biodiversity (CBD) has set up guidelines to support the sharing of benefits between countries.[56,69]

1.4 Bioactivity

A bioactive response can be demonstrated when a metabolite is produced and released, that affects a targeted cell or tissue, preferably with the resultant action of inhibition of growth or a cytotoxic outcome. This can include a range of reactions such as anti-bacterial, anti-cancer, anti-tumour, anti-viral, anti-fouling and anti-inflammatory.[75] To determine whether a metabolite is bioactive, bioassays are performed.[78]

There are a range of methodologies used for bioassay; depending on the type of activity being tested. For example, an anti-bacterial assay could be executed with a paper disc diffusion method.[67] The metabolite extract is soaked into a small paper disc, this is then transplanted onto an agar plate seeded with the bacteria strain being tested against. The plate is then incubated and the diameter of inhibition is measured around the paper disc to determine the bioactive strength of the metabolite.[78] In comparison

to this, an anti-inflammatory assay can use healthy blood for instance, with a mixture of the metabolite extract and hyposaline, and a reference sample of pure water.[67] Samples are then incubated and centrifuged; a measurement of haemolysis is calculated against the reference sample (water should give 100% haemolysis) using a spectrophotometer at 520 nm. The percentage of inflammation protection in the blood determines the bioactive potency of the metabolite extract.[67]

This thesis uses the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) bioassay against a HeLa (human cervical cancer) cell line.[79] The crude extract is added to the HeLa cells and incubated. The MTT bioassay is a colourimetric test that targets a healthy cell's mitochondrial dehydrogenase. If the cell is healthy and active, the yellow MTT is reduced to purple formazan. If the cell is damaged or dead there will be no colour change. The colour change is measured using a spectrophotometer at 570 nm and the percentage of bioactivity is calculated from a control.[79] Other cell lines can be used with a MTT assay, for example the P388 murine leukaemia cell line.

It is important to note that even though a metabolite might have potent bioactivity against the targeted cell line, this does not ensure that it will make a good pharmaceutical as it may be harmful to humans in other ways, such as damaging healthy cells.

1.5 Objectives

The aim of this thesis is to gain an insight into the chemical ecology of *Cliona celata* within the Bay of Plenty, New Zealand. The project detailed herein is an investigation of *C. celata* chemistry based on preliminary research that suggests the presence of novel compounds that have potentially bioactive properties (Webb 2012, Appendix 1). As such, the work is designed to investigate the basic metabolite variability within the scope of MSc logistics, as a precursor for more in depth studies at a later time. This will be conducted by determining; whether there is any chemical variation between samples from a range of reef systems from a variety of

open coast exposure profiles: Leisure Island, Rabbit Island and White Island samples. In addition, a response to damage likely to be caused by storm events or fish predation will be mimicked by knife damage applied to the sponge. The subsequent chemical response will be investigated, and once damaged has occurred, whether there is any chemical stratification from the damage site. It is hypothesised that there will be a chemical response to damage. Bioactivity readings from a healthy and a damaged sponge will be performed. Finally the taxonomy of each sponge will be determined using spicule mounts to confirm that the species is indeed consistently *C. celata* given the sponge has a range of morphologies. This study will result in preliminary knowledge of the chemical ecology of *Cliona celata* within New Zealand waters.

This research project is a hybrid between ecological and chemical research techniques and as such the thesis style borrows from both disciplines for clarity.

1.6 Ethics statement

The organisms collected and tested in the experiments described in this thesis are marine invertebrates (sponges). These are not protected under the Animal Welfare Act, therefore, no ethical consideration needs to be conducted when removing and experimenting with these organisms. However, as we are working in the marine environment, we would like to ensure that all work performed will take into account sustainability of the environment and its organisms. This will be achieved by leaving all surrounding environments untouched and when collecting the invertebrates, small solitary organisms are fully removed (only where common), whereas encrusting organisms will have one-third of the adult invertebrate untouched and intact. Previous work suggests that the sponge individual usually recovers well from such sampling.[80]

CHAPTER 2

SAMPLING SITE AND SPICULE TAXONOMY

2.1 Sampling site

All collection sites were based within the Bay of Plenty, New Zealand, Figure 2.1 and Figure 2.2. The Bay of Plenty is located on the East Coast of the North Island of New Zealand. The Pacific Ocean within this area is temperate with temperatures averaging 14°C in August and 20-21°C in February.[81] The region is predominantly sheltered from New Zealand's prevailing swells from the west/south-west. It is also mainly sheltered from cooler currents as New Zealand's land mass blocks the prevailing westerly winds from this part of the ocean, however, strong easterly winds and outer oceanic weather events can cause significant waves and swells from the east and north east, especially during summer/autumn when south-easterly trade winds are prevalent. The water temperature is influenced by the East Auckland Current, giving it subtropical/warm temperate temperature ranges.[81]
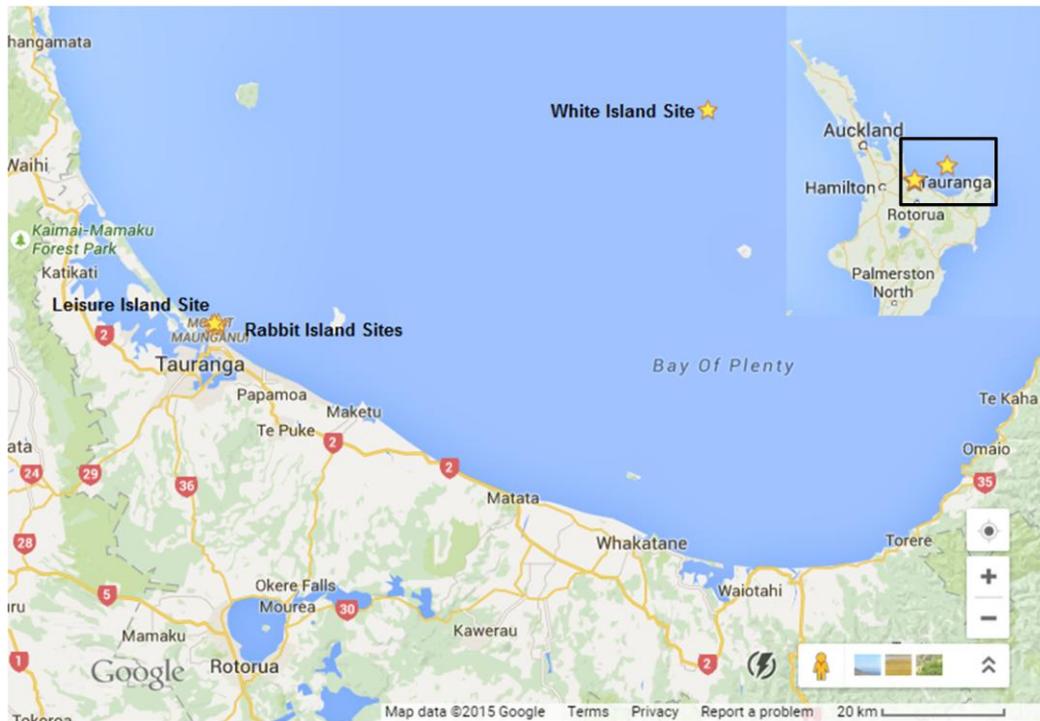
Figure 2.1: Map of sampling locations in the Bay of Plenty, New Zealand. Three sampling sites are shown; Leisure Island, Rabbit Island and White Island.

The three study locations were, Leisure Island (Moturiki) (intertidal), Rabbit Island (Motuotau) (subtidal) and White Island (Whaakari) (geothermal subtidal) (Figure 2.1 and Figure 2.2). These sites were chosen to determine preliminary chemistry and chemical variation across a range of coastal locations and over a range of depths. One extended site on each of Leisure Island and White Island was used for sampling; however, three locations on Rabbit Island were sampled to determine whether there was any localised smaller spatial scale variation in the chemistry of *Cliona*. These were respectively known as, Site One, Site Two and Site Three (named for other experimental purposes).

GPS locations for these sites were:

Leisure Island: S37.62955 E176.18543

Rabbit Island:

      Site one: S37.62713 E176.19754

      Site two: S37.62616 E176.19101:

      Site three: S37.63185 E176.19677

White Island: S37.30.721 E177.11.293
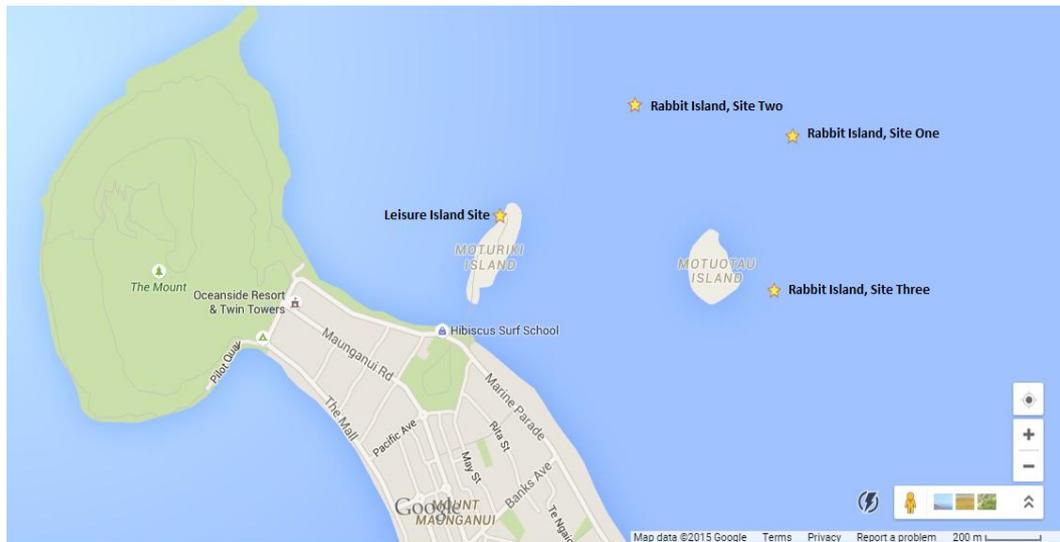
Figure 2.2: Map of Leisure Island and Rabbit Island; Site 1, Site 2 and Site 3.

Collections of *Cliona celata* made intertidally at Leisure Island were sampled from a sheer rock face that allowed for safer sampling. Samples were accessible by a ten minute swim from the Mt. Maunganui beach to the site, where snorkelling was conducted to obtain samples. Subtidal samples (Rabbit Island and White Island) were collected from known sites routinely used for monitoring by the University of Waikato Coastal Marine Field Station. These sites were all accessible by boat and collections were made using SCUBA.

The damage experiment outlined in Chapter 4 focussed on obtaining samples from Leisure Island (intertidal) and Site Three, Rabbit Island (subtidal). Finally, the study to determine whether there was any stratification change of the chemicals within the sponge, using distance from the damaged site as a factor, used samples collected from Site Three of Rabbit Island (subtidal).

2.2 Spicule Taxonomy Introduction

Due to the high diversity of sizes and shapes of spicules in Porifera, spicules can be used to help differentiate sponge species.[8] The main function of spicules is to constitute skeletal support of the sponge tissue, however, it is also thought that the spicules can be a defence mechanism for the sponge, as the skeletal shards are unpalatable to generalist predators.[66,82,83] Evidence has suggested, however, that specialist predators do not find these to be deterrents and that silica spicules can pass through the guts of predators without any long-term effects.[82]

Demospongiae characteristically have two to four rayed silica spicules, which are separated into megascleres and microscleres (Figure 2.3). The megascleres are generally monaxons (single rayed) or tetraxons (four rayed), however triaxons (three rayed) have been noted in one subclass.[8] This skeletal structure can also be supported by a protein matrix, spongin. The Clionidae family are typically excavating sponges that burrow into calcareous substrates and produce megascleres of either tylostyles or subtylostyle formations. Microscleres are either amphiasters, spirasters or thorny microxeas.[8]
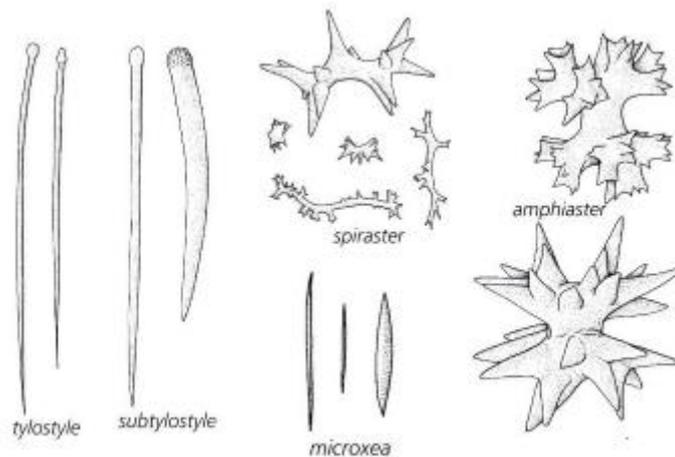
Figure 2.3: Megascleres (tylostyle and subtylostyle) and microscleres (spiraster, amphiaster and microxea) found in the Clionidae family. Note spicule sizes are not to scale. Adapted from Reference 38[38]

Cliona celata typically only produces megascleres with tylostyle morphology, and microscleres (spirasters) are present only in juvenile sponges.[8,39] The tylostyles have characteristically bulbous bases with

spindle-like shafts and a curved end. There is often a small amount of foreign microsclere spicules deposited in the sponge matrix from the ingestion of debris from the water column.[39] Work outlined in a previous MSc thesis[84] indicated that there are variants of *C.celata* species due to the differences in spicule morphology as well as suspected chemical variation. The samples in McCormack's study showed *C.celata* to have the typical tylostyles, however, other samples produced both tylostyles and subtylostyles, leading to potential variations in species.

Cliona celata sponges sampled and tested in this thesis project were initially identified *in situ* based on their morphology. To confirm that the sponges sampled were *C. celata,* spicule mounts were prepared for each individual. The most widely used method to differentiate and identify species of marine sponges is via a morphologically based classification technique [30] hence, *in situ* appearance and spicule morphology is used in this thesis.

2.3 Results

All specimens subjected to spicule analysis showed a dense disordered array of spicules within the sponge samples.

2.3.1 Leisure Island

All individuals collected from Leisure Island were subjected to spicule investigation. This comprised a collection of six sponge samples. Figure 2.4 shows a representative sample of the spicules present in intertidal *Cliona celata*. Spicule morphology shows almost exclusively tylostyles as the megascleres with no microscleres present. These tylostyles are either straight or slightly curved with rounded to globular bases and pointed tips. This spicule analysis was consistent for all intertidal samples.
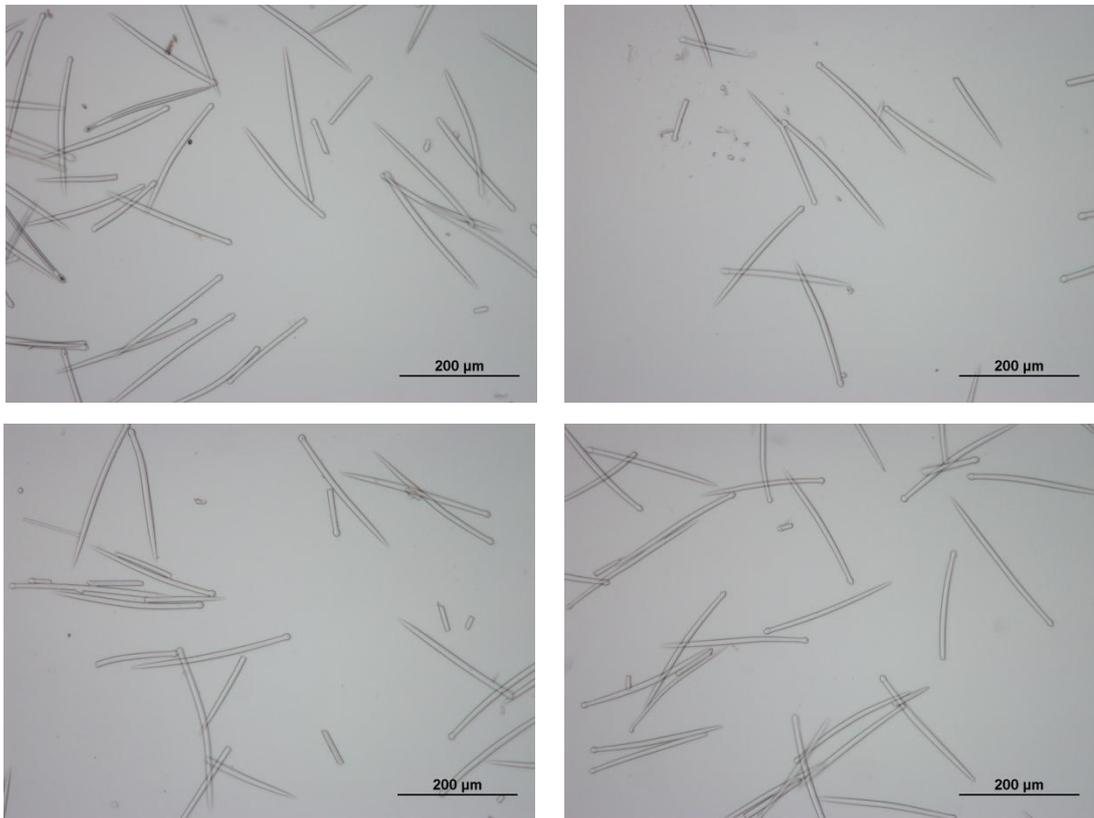
Figure 2.4: Spicule mounts showing tylostyles from a representative sample of Leisure Island (intertidal) sponges.

2.3.2 Rabbit Island

All subtidal individuals collected from Rabbit Island; Site 1 Site 2 and Site 3 were subjected to spicule investigation. This comprised a collection of 20 individual sponges. Figure 2.5 shows a representative sample of spicules

found within the subtidal Rabbit Island sponges. The predominant megascleres present are tylostyles, however, there are some slightly subtylostyle spicules present. This is consistent will all sponge specimens collected from Rabbit Island. Tylostyles are straight or curved with bulbous bases and pointed tips. The subtylostyles are straighter with curved bases and pointed tips. There were no microscleres present in any subtidal Rabbit Island sample.
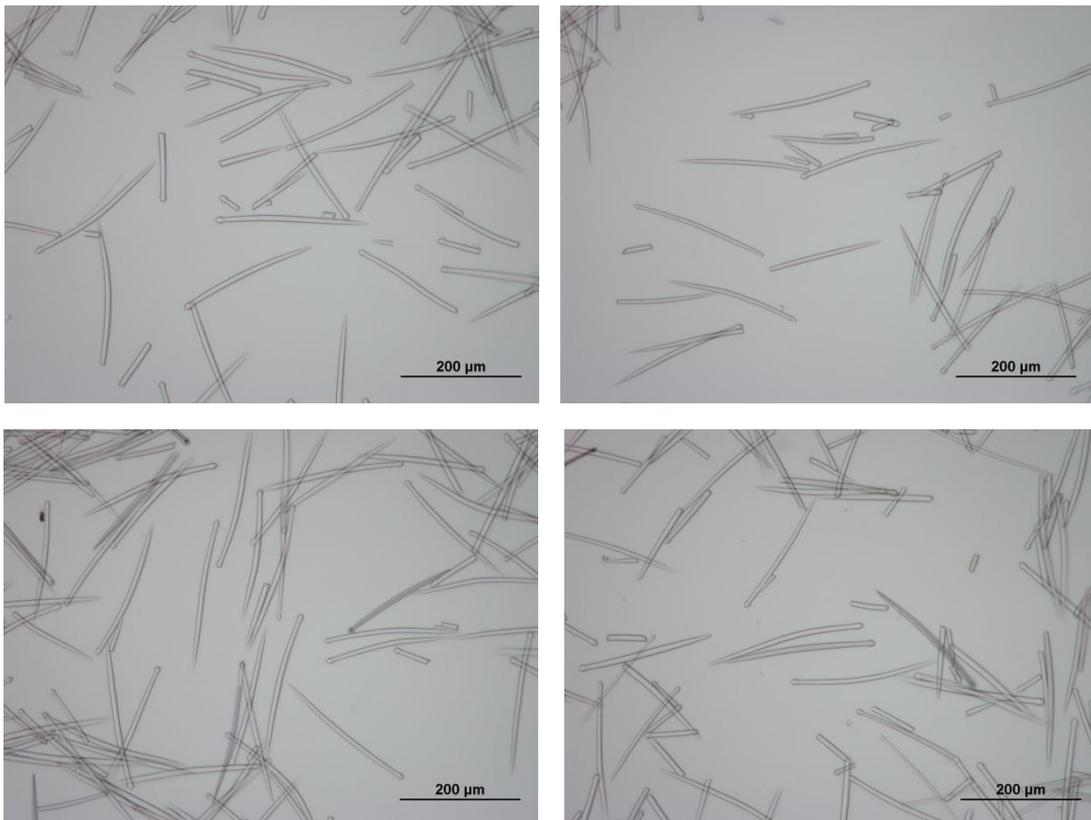
Figure 2.5: Spicule mounts showing tylostyles and some subtylostyles from a representative sample of Rabbit Island (subtidal) sponges.

Sponge number NW.1.2.3, collected from Site 1, Rabbit Island had a slight variation in spicular content from the other Rabbit Island samples. This sponge sample was clean and healthy with minimal epibiont coverage. The spicule morphology was the same (tylostyles and some subtylostyles), however, spicules were stouter than other samples. Figure 2.6 shows the spicules present in NW.1.2.3.
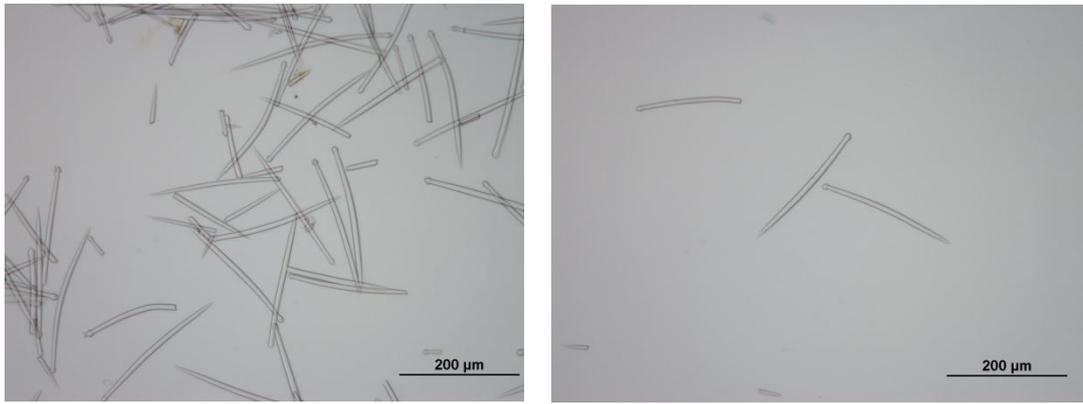
Figure 2.6: Spicule mounts from NW.1.2.3, Site 1, Rabbit Island (subtidal) showing stouter tylostyle and subtylostyle spicules.

2.3.3 White Island

One sample (NW.1.6.1) was collected from the subtidal, geothermal site at White Island and subjected to a spicule investigation. Figure 2.7 shows a representative sample of the spicules found within the sponge. Spicules within this sponge are similar to those found in samples from Rabbit Island. The predominant megascleres are tylostyles which are slightly curved with rotund bases and sharp tips. There are some subtylostyles within the sample which are straighter and have curved tips. There are no microscleres present in the sample.
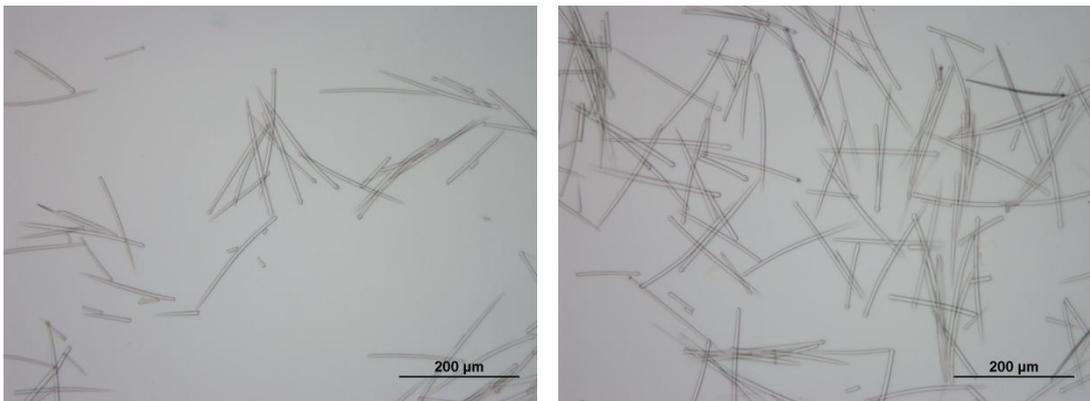
Figure 2.7: Spicule mounts showing tylostyles and some subtylostyles from NW.1.6.1, White Island (geothermal subtidal) sponge sample.

2.4 Discussion

Tylostyle spicule morphology throughout all samples was characteristic of *Cliona celata* speculation, with globular bases, straight to curved shafts and pointed tips. Intertidal specimens show exclusively tylostyle spicules, which is consistent with *C. celata* specimens sampled in other studies.[39] Rabbit Island (subtidal) and White Island (geothermal subtidal) samples showed the majority of spicules to be the expected tylostyle morphology; however, some subtylostyle spicules were present. This is consistent with the spicules found from *C. celata* in McCormack's MSc thesis.[84] Using this technique, verification that only the *C. celata* species was tested throughout the studies described in this thesis was confirmed.

It is thought that spicule morphology can change depending on the environmental conditions and can vary with seasonal changes.[14,30] For example, in environments with higher flow rates, it is believed that the sponge needs to invest in greater skeletal strength and spicules are often, longer, thinner and more flexible to be able to deal with the constant movement of water. One Rabbit Island specimen (NW.1.2.3) presented with the consistent tylostyles with few subtylostyle structure, however, the spicules were stouter than in any other specimen. This can be an indication of tougher environmental conditions.[14,30,85]

There were no microscleres present in any of the specimens collected. This indicates that all specimens are adult sponges, as previously stated only juvenile sponges have spiraster microscleres.[8]

CHAPTER 3

PRELIMINARY CHEMISTRY

3.1 Introduction

A preliminary yet detailed investigation into the metabolite chemistry of *Cliona celata* within the Bay of Plenty was first required due to limited knowledge on the subject. A range of areas within the Bay of Plenty were sampled and these included: Leisure Island (intertidal), Rabbit Island (subtidal) and White Island (geothermal subtidal) as shown in Chapter 2. These sampling areas were chosen to determine whether there was any variation in chemistry of the sponge at different locations and different marine environments. Testing at Rabbit Island was also conducted in three locations (Site 1, Site 2 and Site 3) to determine whether there was any variation within one localised area but over a smaller spatial scale. It is important to note that temporal variation is also likely to be a factor in examining variability in metabolite chemistry in sponges. Seasonal variation in micro-environments is likely to be important. For the scope of this study, however it was not possible to sample over season in an exhaustive manner, given a need to examine the chemistry from spatial analyses in detail first. This will form the basis of a further study.

The chemistry within an organism is often influenced by its surrounding environment. However, it is largely unknown how these organisms interact or vary chemically with different marine environments.[47] Sponges in particular can sequester chemicals from their environment as they are filter feeders that ingest particulates from the adjacent water column. Chemical interactions can also be more localised. The sponge can be influenced by the organisms which inhabit the same immediate area as itself or which predate on the sponge. Therefore as each individual is unique in terms of its micro-environment, there is likely to be variation of chemistry shown among all the sponges sampled. It is important to determine these spatial differences as it can give a better understanding of the ecological role

these organisms encompass, but also gives a greater insight into the potential and evolution of chemical ecology uses.[47]

Studies have shown there to be spatial chemical variation among sponge individuals.[46,76] The common shallow water Mediterranean sponge *Aplysina aerophoba* collected at several locations around the Canary Islands, Spain produced secondary metabolites, such as bromotyrosine derivatives, aerophobin-2 (**20**) and dienone (**21**) within all sponges sampled at the various locations. However, geographical variation was shown as there was a concentration difference of the secondary metabolite between these locations.[46,86,87]

(**20**)   (**21**)
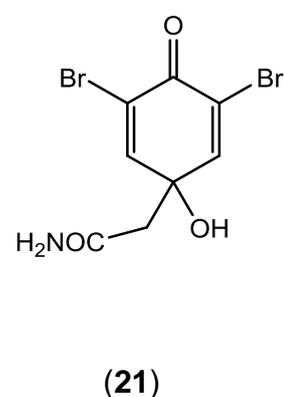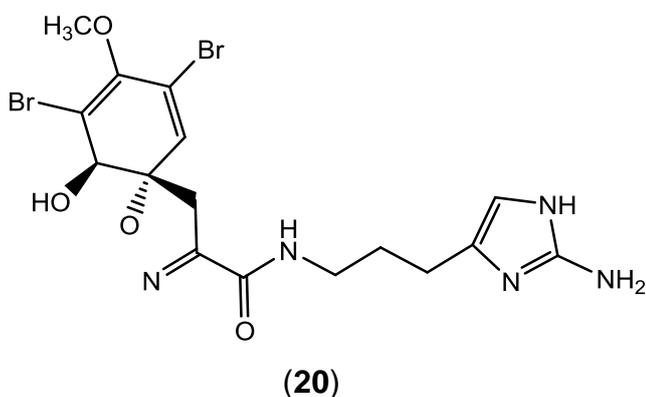
The New Zealand marine sponge, *Mycale hentscheli* produces the bioactive compounds, mycalamide (**22**), pateamine (**23**) and peloruside A (**24**)[75] depending on where it is collected from.[23] There is an indication that these compounds are likely to support the sponge through the control of predators, competitors and biofouling organisms. Evidence has shown that there is variation in the concentration of these compounds geographically and on a seasonal temporal scale. Concentration levels were found to be both site specific and seasonal with peak levels of metabolites during spring/summer.[23]
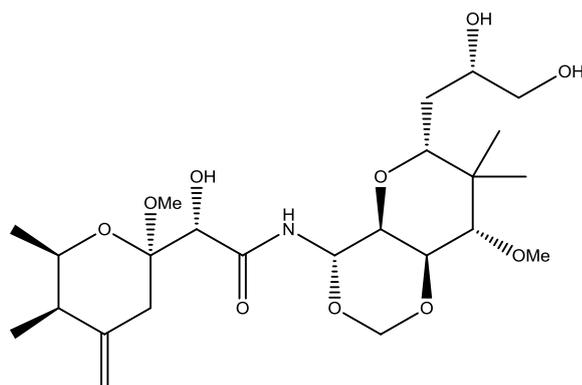
(**22**)
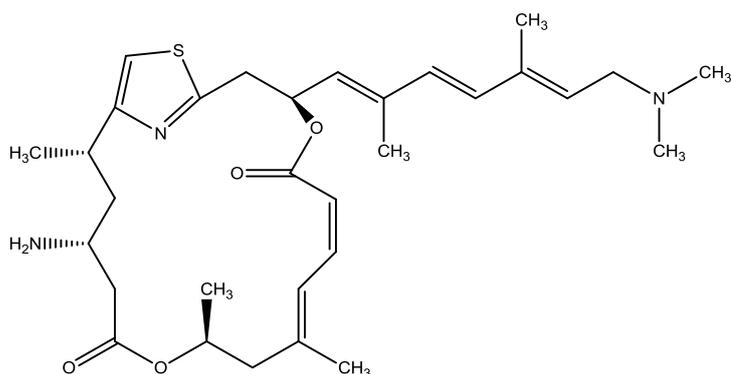
(**23**)
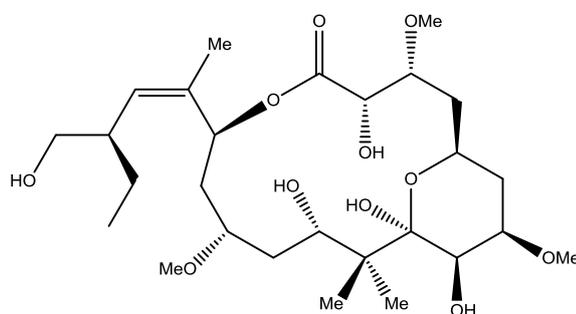
(**24**)

Another example that supports this response is the marine sponge *Haliclona* sp. found around the Southern Coast of Western Australia. The green variant of the sponge contained a potent bioactive compound, salicylihalamide A (**25**), however the brown did not.[76] The chemical is highly cytotoxic and has the potential to be an anti-tumor compound by inhibiting mammalian ATPase activity. Higher levels of salicylihalamide A (**25**) within the sponge correlated with a more southern sampling site and with lower temperatures.[76]
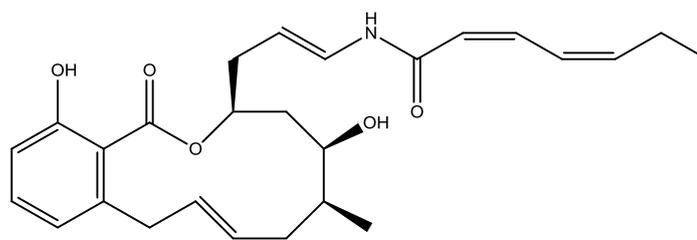
(25)

A marine biodiversity investigation in 2012 was conducted in the Tauranga Harbour. During this study, analysis showed that a sample of *Cliona celata* from Rabbit Island (subtidal), produced some interesting chemical metabolites. Mono- and dibrominated metabolites were identified in the sample by analysis of crude extract of the sponge by LCMS. As only one sample of *C. celata* was sampled at the time, it was unclear if this chemistry was consistent throughout all *C. celata* in the area or confined to the individual (Webb 2012, Appendix 1). Thus a more in depth look into the chemical ecology of this sponge is necessary, hence this study. Given evidence from research on different sponges described, it is necessary to determine whether there is any chemical variation in *Cliona celata* around the Bay of Plenty.

3.2 Results

3.2.1 Healthy versus unhealthy shallow water sponges

The first intertidal sponge sampled (Figure 3.1) had a mixture of healthy and naturally damaged tissue. Healthy tissue was dense and a bright orange colour whereas damaged tissue was a darker brown/orange with a more "honeycomb" texture. There was minimal epibiont coverage on the sample. Two subsamples were extracted (healthy (NW.1.1.2) and damaged (NW.1.1.1)). Figure 3.2 shows the second intertidal sample (NW.1.1.3) was of healthy, dense and orange tissue with minimal epibiont coverage.

Figure 3.1: Leisure Island (intertidal) sponge sample NW.1.1.1 (damage, lower half of specimen) and NW.1.1.2 (healthy, top half of specimen).

Figure 3.2: Leisure Island (intertidal) sponge sample NW.1.1.3, healthy specimen.

The damaged section of the first sponge sampled (NW.1.1.1) (Figure 3.3) showed a single Liquid Chromatography Mass Spectrometry (LCMS) predominant peak at 29.4 minutes on the chromatogram. This peak was found in no other preliminary traces. The MS trace (Figure 3.4) showed this peak to be a presumed [M+H]$^+$ ion at 538 *m/z*. Chromatograms of the

two healthy samples (NW.1.1.2 and NW.1.1.3) were overlaid onto the same LC trace (Figure 3.5). Although the chromatograms look somewhat different, there were no significant chemical differences between these two samples as the same peaks were present in both samples. However, the relative proportions of these components differed markedly indicating differences in concentrations of the metabolites.
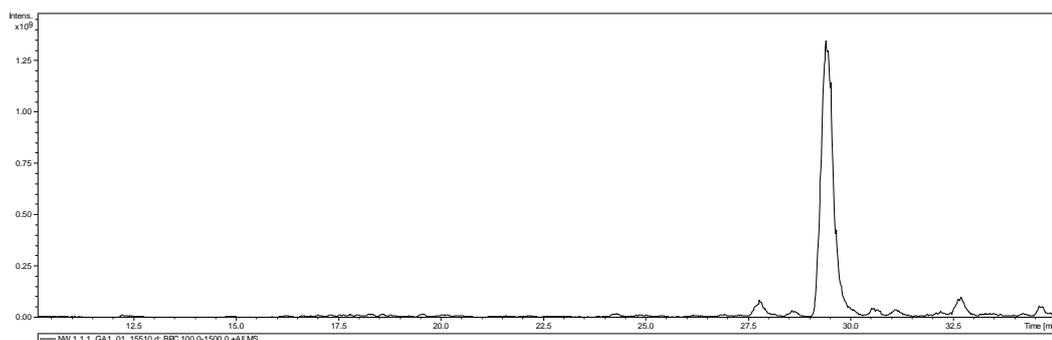
Figure 3.3: Chromatogram of NW.1.1.1 intertidal damaged sponge subsample. Elution time of 10-35 min.
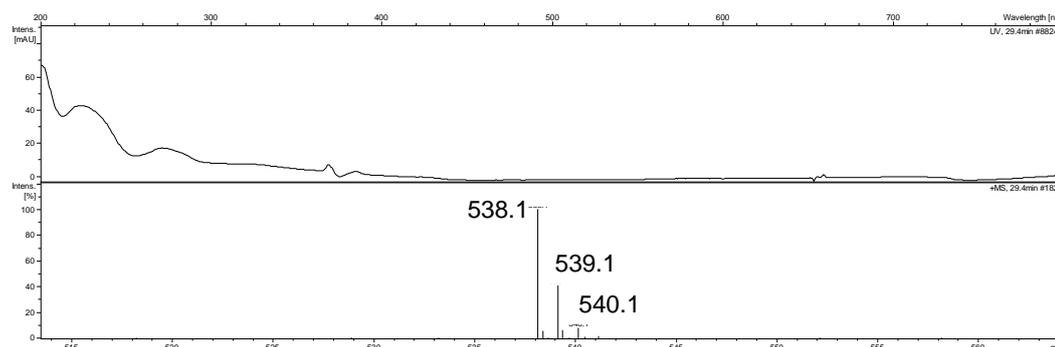
Figure 3.4: UV (top) and MS (bottom) spectra of NW.1.1.1 intertidal damaged sponge subsample at an elution time of 29.4 min, [M+H]$^+$ ion at 538 _m/z_.
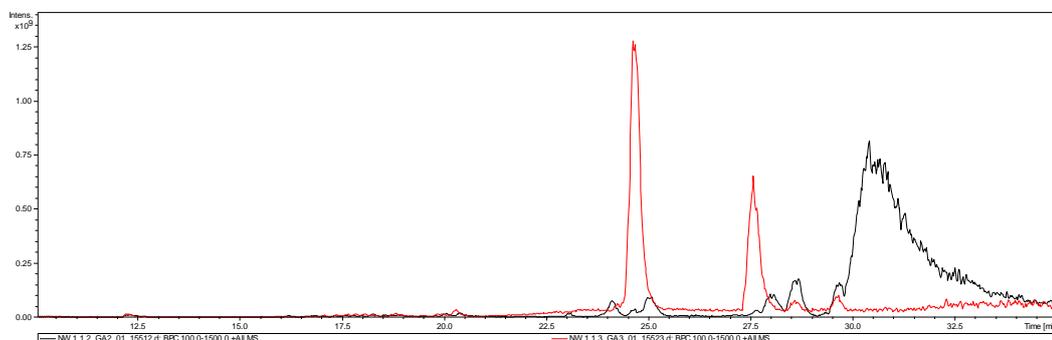
Figure 3.5: Chromatogram overlay of NW.1.1.2; (black) and NW.1.1.3; (red) intertidal healthy sponge samples. Elution time of 10-35 min.

3.2.2 Rabbit Island subtidal sponges

Rabbit Island Site One subtidal sample, NW.1.2.1 was a healthy, dense sponge with orange colouration and some epibiont coverage on ostia/oscules. NW.1.2.2 was sampled from a sponge with black colouration to the interior and exterior and a very hard texture. NW.1.2.3 came from a sponge with minimal epibiont coverage to the pinacoderm whereas NW.1.2.4 was clean; however, all samples had healthy, dense tissue with a bright orange colouration (Figure 3.6). The chromatograms of the four Site One samples overlaid are shown in Figure 3.7. The traces were all very similar but with varying proportions of the same base peaks. Similar peaks were also present in the chromatograms of samples NW.1.1.2 and NW.1.1.3.

Figure 3.6: Rabbit Island (subtidal) sponge samples with varying epibiont coverage, minimal coverage, NW.1.2.4 (left) and approximately 50% epibiont coverage, NW.1.3.3 (right).
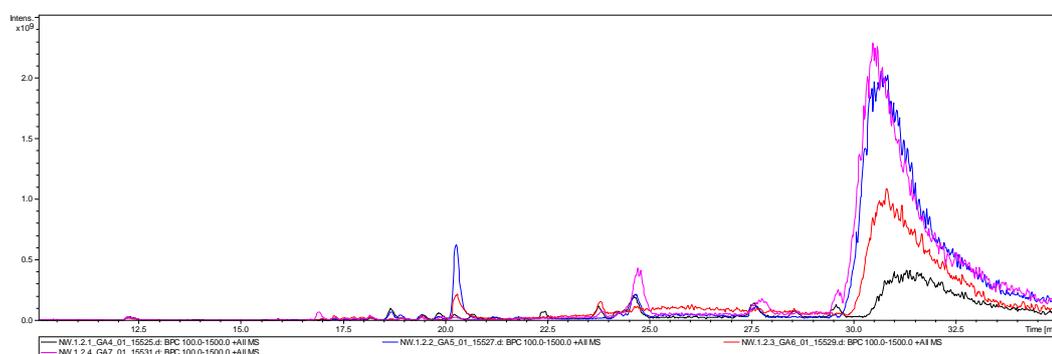
Figure 3.7: Chromatograms of NW.1.2.1; (black), NW.1.2.2; (red), NW.1.2.3; (blue) and NW.1.2.4; (pink) subtidal Site One sponge samples overlaid. Elution time of 10-35 min.

Subtidal, Rabbit Island, Site Three samples all had healthy, dense, orange tissue. However, NW.1.3.1 and NW.1.3.2 (Figure 3.8) came from thinly encrusting sponge samples with minimal epibiont coverage, whereas NW.1.3.3 (Figure 3.6 and Figure 3.9) came from a sponge with approximately 50% epibiont coverage and NW.1.4.1 was a clean sample. Figure 3.8 shows the overlaid chromatograms of NW.1.3.1, NW.1.3.2 and NW.1.4.1, the chromatograms are very similar to those of Site One and intertidal sponge samples. Again there is some variation in the proportion of peaks present but the same major peaks/components are presents in all samples.
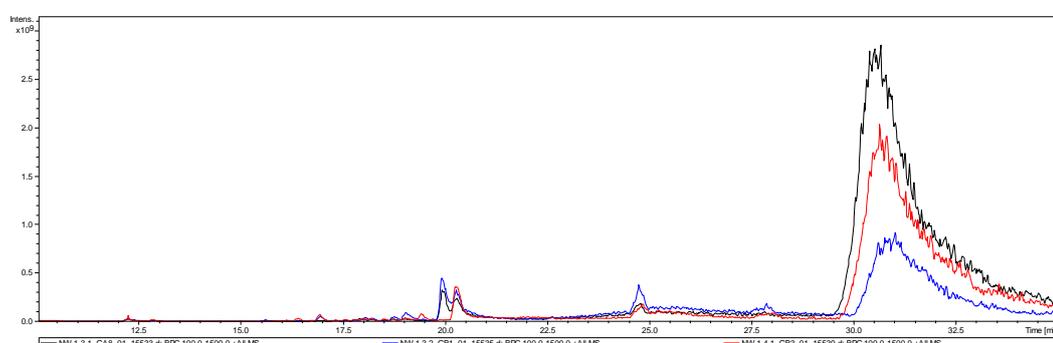
Figure 3.8: Overlaid chromatograms of NW.1.3.1; (black), NW.1.3.2; (red) and NW.1.4.1; (blue) subtidal Site 3 sponge samples. Elution time of 10-35 min.

The chromatogram of NW.1.3.3 (Figure 3.9) looks completely different from that of any another sample. There are many more peaks present, with three notable peaks unique to this sample. Figure 3.10 shows the MS spectrum of a monobrominated compound with an elution time of 21.6 min and compound mass of 408/410 *m/z*. Bromine has two isotopes, ^{79}Br and ^{81}Br so monobrominated compounds with have two peaks approximately the same size. Another monobrominated compound is present at an elution time of 21.1 min (Figure 3.11) with a presumed [M+H]$^+$ of 410/412 *m/z*. Lastly the predominant peak with an elution time 22.6 min shows a distinct 1:2:1 isotope pattern in the mass spectrum (Figure 3.12), indicating the presence of a dibrominated compound with a presumed [M+H]$^+$ of 487.9/489.9/491.9 *m/z*.

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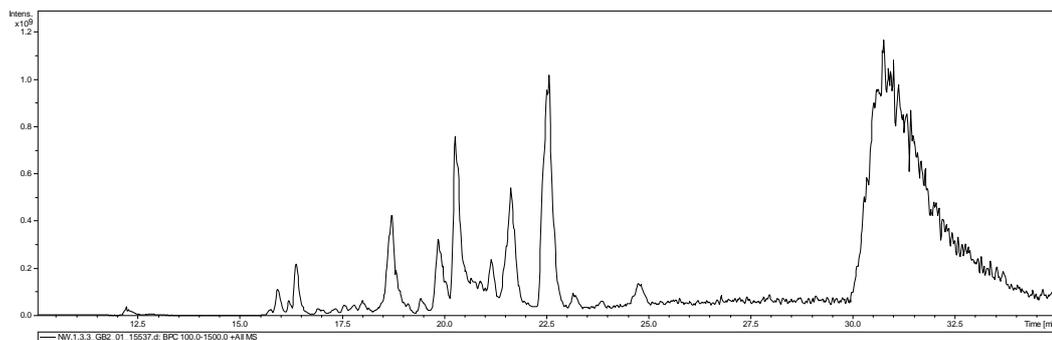
Figure 3.9: Chromatogram of NW.1.3.3 subtidal Site 3 sponge sample. Elution time between 10-35 min.
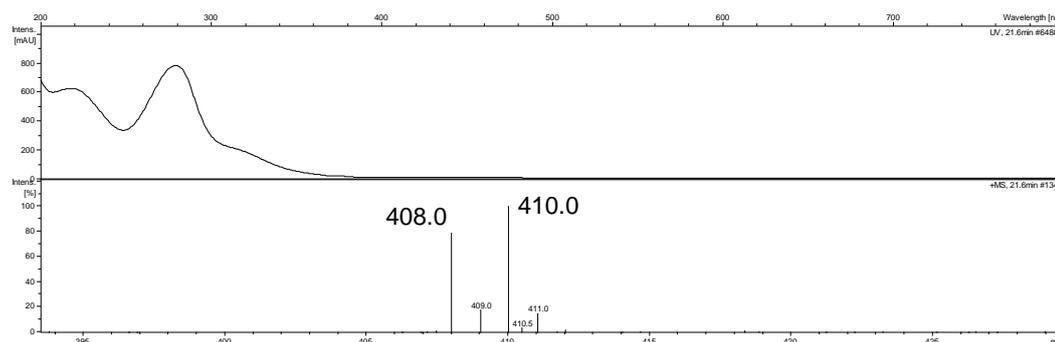
Figure 3.10: UV (top) and MS spectra (bottom) of NW.1.3.3 subtidal Site 3 sponge sample at an elution time of 21.6 min, monobrominated, [M+H]$^+$ of 408/410 *m/z*.
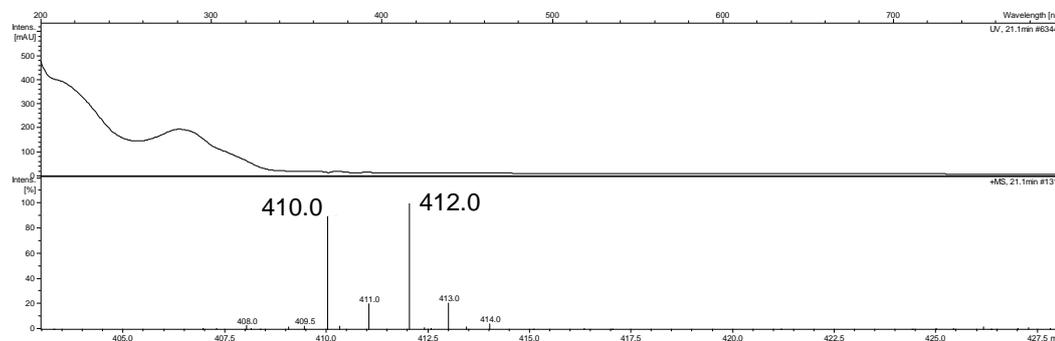
Figure 3.11: UV (top) and MS spectra (bottom) of NW.1.3.3 subtidal Site 3 sponge sample at an elution time of 21.1 min, monobrominated, [M+H]$^+$ of 410/412 *m/z*.
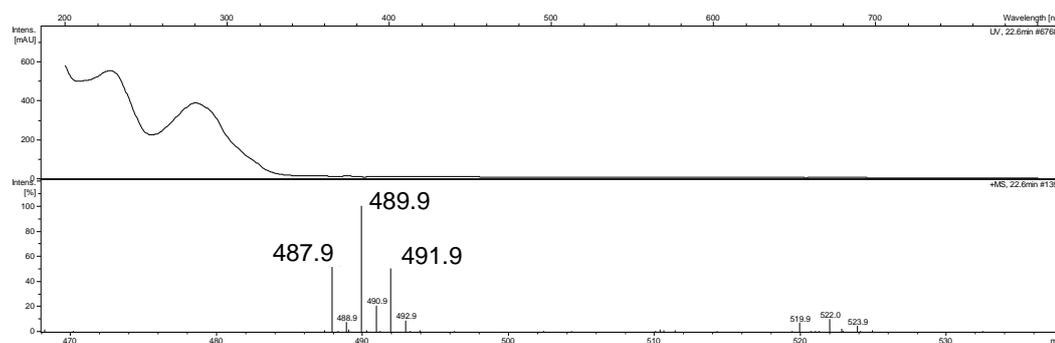
Figure 3.12: UV (top) and MS spectra (bottom) of NW.1.3.3 subtidal Site 3 sponge sample at an elution time of 22.6 min, dibrominated, [M+H]$^+$ of 487.9/489.9/491.9 *m/z*.

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Samples collected from Site Two, Rabbit Island all consisted of healthy, dense orange tissue. However, they had varying degrees of epibiont coverage, NW.1.4.2 and NW.1.4.4 had minimal epibiont coverage, NW.1.4.3 had approximately 25% epibiont coverage and lastly NW.1.5.1 had approximately 33% epibiont coverage. The chromatograms for these four samples (overlaid in Figure 3.13) all showed comparable peaks with no significant differences. The strength of the peaks however did vary with each sample. These peaks also corresponded to those found in previous intertidal and subtidal samples.
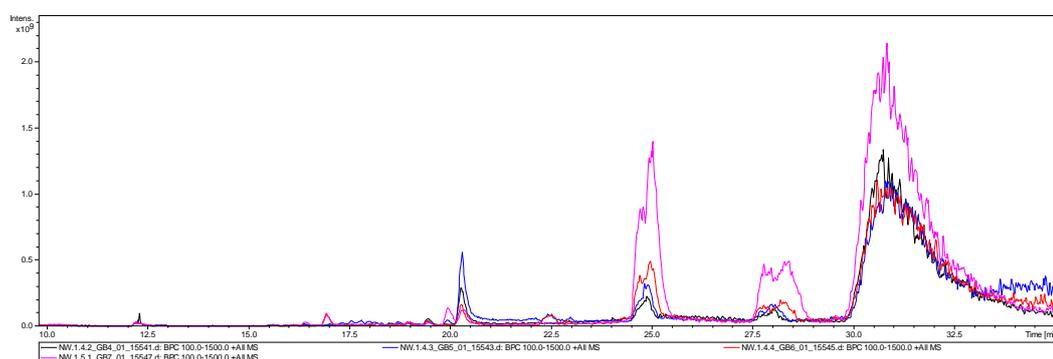
Figure 3.13: Overlaid chromatograms of NW.1.4.2; (black), NW.1.4.3; (red), NW.1.4.4; (blue) and NW.1.5.1; (pink), subtidal Site Two sponge samples. Elution time of 10-35 min.

3.2.3 White Island Geothermal/Subtidal Sample

The single geothermal subtidal sample (Figure 3.14) (NW.1.6.1) came from a thick healthy sponge with dense orange tissue. The chromatogram (Figure 3.15) shows a chemical composition with some peaks in common with the previous intertidal and subtidal samples. However, there were differences, firstly an early eluent at 16.5 min and another unique peak at 23.2 min (Figure 3.16). The mass spectrum (Figure 3.17) for the early eluting peak at 16.5 min shows a predominant peak with a [M+H]$^+$ of 556 *m/z*. The mass spectrum (Figure 3.18) of the later eluting peak at 23.2 min shows a predominant peak with a mass of 429.4 *m/z*.

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Figure 3.14: White Island (geothermal subtidal) sponge sample, NW.1.6.1.
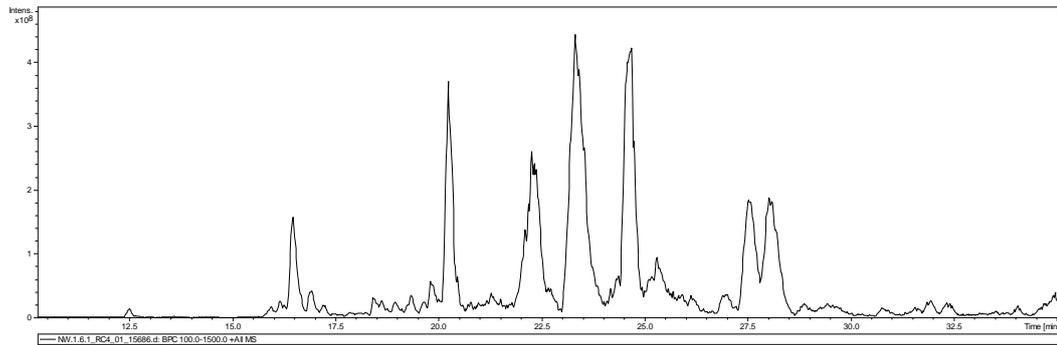
Figure 3.15: Chromatogram of NW.1.6.1 geothermal subtidal sponge sample from White Island. Elution time of 10-35 min.
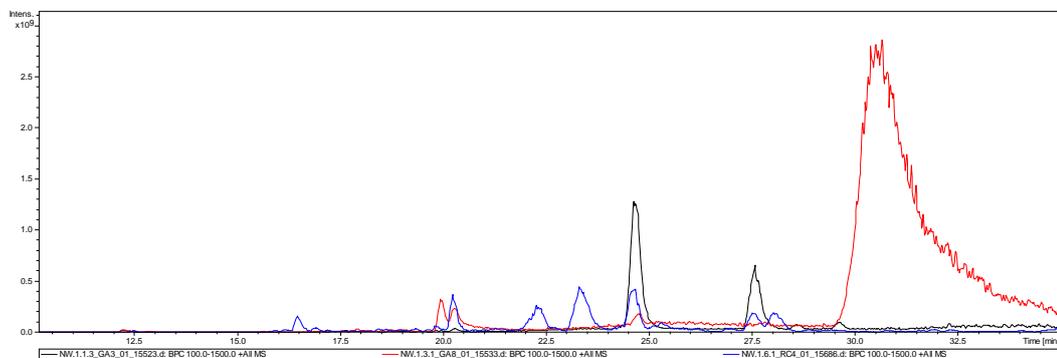
Figure 3.16: Overlaid chromatograms of NW.1.1.3 intertidal (black), NW.1.3.1 subtidal (red) and NW.1.6.1 geothermal subtidal (blue). Elution time between 10-35 min.
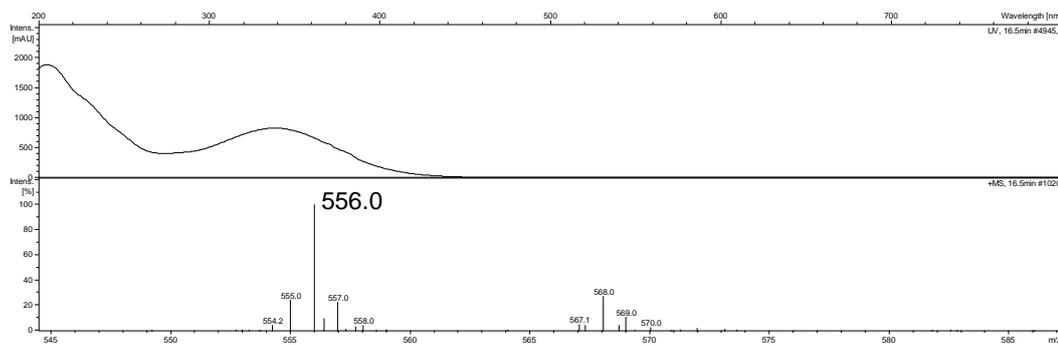
Figure 3.17: UV (top) and MS spectra (bottom) of NW.1.6.1 geothermal subtidal sponge sample at an elution time of 16.5 min, [M+H]⁺ of 556 *m/z*.
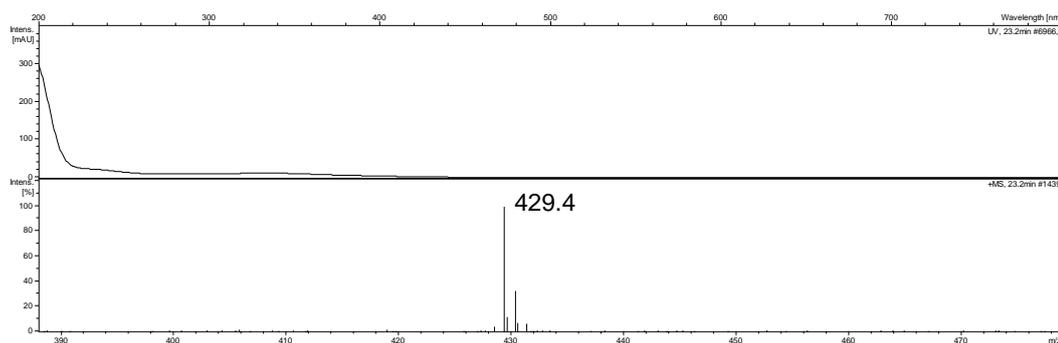
Figure 3.18: UV (top) and MS spectra (bottom) of NW.1.6.1 geothermal subtidal sponge sample at an elution time of 23.2 min, [M+H]⁺ of 429.4 *m/z*.

3.2.4 **Bioactivity: MTT assay**

A MTT assay was completed on an extract of a healthy sponge sample collected from Site Three, Rabbit Island. There was no activity shown against the HeLa cells used during this assay.

3.3 Discussion

There was minimal variation between healthy samples (NW.1.1.2 and NW.1.1.3) from the intertidal site, apart from the varying intensities of the peaks. This was to be expected as the samples inhabit the same localised environment. Any slight variations in LC peak intensities could be due to micro-habitats and the interactions with surrounding organisms.[14] The damaged section (NW.1.1.1) of the intertidal sponge contained one peak in particular that was not present in any other preliminary sample. This is potentially caused by dead tissue or a fungal infection that has overridden the specimen, changing the structural and chemical composition of the sponge. Figure 3.4 shows the mass spectrum of the damaged tissue, however, the isotope pattern gave no indication to a potential bioactive secondary metabolite.

In comparison to the intertidal sponges, the majority of subtidal samples (Sites One, Two and Three) showed the same common peaks at approximately 20, 25 and 27 min on the chromatograms. This could potentially represent the chemical 'backbone' of *Cliona celata* within the Bay of Plenty, New Zealand area. Similar chemical compounds were present regardless of different encrusting morphologies and varying epibiont coverage. As for intertidal sponges, the differences in peak intensities could be due to the individual micro-environments of each specimen.[41]

It is hypothesised that the amount of epibiont coverage on a sponge may have an effect on metabolite chemistry. Figure 3.13 shows an increase in peak intensities with increasing epibiont coverage. This could lead to a build up of compounds, with further antagonistic interaction from epibiont cover.[41] The intensities of the peaks may be useful to indicate chemical mobility in response to micro-environmental effects. The large shift in chemistry of the 50% epibiont covered sponge shown in Figure 3.9, potentially suggests that there may be a threshold over which the sponge modifies chemistry to meet defensive need. This is a novel finding and thus, very much a hypothesis, a more detailed investigation would be required to accept the hypothesis.

This was shown in subtidal Site Three sample NW.1.3.3 although this sample produced the expected common peaks, there were also unique peaks representing monobrominated and dibrominated compounds. The presence of brominated compounds is not unexpected, as previously stated (Appendix 1), bromine is readily soluble and often incorporated into secondary metabolites of marine organisms,[50,64] for example multiple bromotryptophan derivatives have been sourced from a range of marine sponges, including tetracetyl clionamide (**4**) isolated from *Cliona celata* from Vancouver Island, Canada.[43]

A search of the MarinLit database showed some matches for the brominated compounds. The monobrominated compound with a [M+H]$^+$ of 408/410 *m/z* was similar to Coscinamide (**26**) found in 2000 from a marine sponge, *Coscinodermia*.[88] Figure 3.11 monobrominated compound with a mass of 410/412 *m/z* had a MarinLit match with three other masses; however, two of these were from tunicates and one from an unknown sponge sample. The dibrominated compound from Figure 3.12 with a mass of 487.9/489.9/491.9 *m/z* had one match of similarity to an ascidian, *Didemnum* sp.,[89] however there were UV match to any of these compounds.

(**26**)
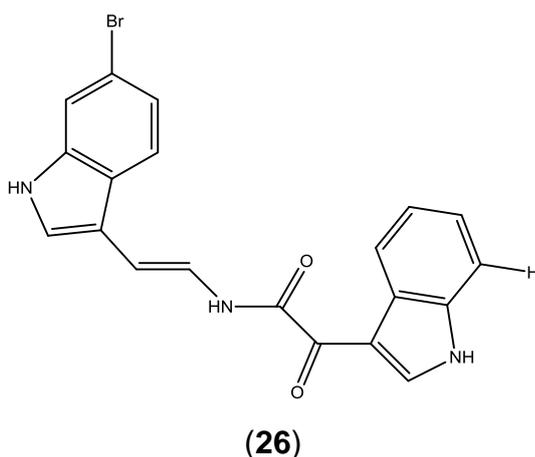
The White Island sample (Figure 3.16) does show similar base peaks to the intertidal and subtidal samples, again indicating that these could be common chemicals produced within the *C. celata* species in the Bay of Plenty. However, there were peaks present that are unique to this

geothermally influenced sample. The mass spectra (Figure 3.17 and Figure 3.18) did not show any isotope patterns that would indicate a bioactive metabolite.

The differences in peaks may be due to the interactions of the sponge with the surrounding environment.[86] Geothermal areas are likely to produce different water chemistry from intertidal and subtidal areas due to the leaching of geothermal gases and sediments into the water column, such chemical differences include higher levels of arsenic, mercury and molybdenum.[90] As sponges are filter feeders that sequester substances from their environment, it is very likely that this would influence the variations in the sponge chemistry. Also the symbionts living internally and externally with the specimen are expected to be different to those found in intertidal/subtidal locations, causing differences in chemistry.

Due to the different peak heights present in not only the three sampling locations but also between each individual site within those locations, there is the potential for spatial and micro-climate variation for *Cliona celata*.

The preliminary undamaged crude sample of *Cliona celata* tested negative for bioactivity in the MTT assay. However, this does not necessarily mean there are no bioactive metabolites within *C. celata,* as the only cell culture *C. celata* was tested against were HeLa cells. Thus preliminary crude extracts showed no bioactivity against this line. However, the sponge could potentially hold bioactivity against a number of other cancerous cell lines or other useful purposes. For example, *Cliona celata* extracts from the Gulf of Mannar, India have shown some mosquito larvicidal properties in hopes to help reduce Malaria.[40] A more in depth bioassay investigation would be required to rule out bioactivity, such as anti-microbial or anti-inflammatory assays and fractions of the crude extracts could also be tested. Previous studies have shown that crude extracts of the marine sponge, *Hymeniacidon perleve,* did not show bioactivity, however, fractions of the extract did show some anti-bacterial bioactivity.[78]

CHAPTER 4

DEFENCE AND REPAIR CHEMISTRY

4.1 Introduction

An investigation into how *Cliona celata* would react chemically from an external stressor such as knife damage was conducted within the Bay of Plenty. This was to determine whether there would be any chemical defence response and consequently a bioactive compound produced.[74] Two sites within the Bay of Plenty were sampled. These included: Leisure Island (intertidal) and Site Three, Rabbit Island (subtidal) as shown in Chapter 2. These sites were chosen due to easy accessibility for re-sampling of sponges and the detection of brominated metabolites as outlined in Chapter 3.

Marine sponges do not have a physical means to protect themselves therefore they are likely to use a chemical deterrent as a defence mechanism.[6] Mineralised skeletal structures of sponges (spicules) are also believed to reduce the dietary appeal and aggravate the digestive systems of predators,[91] however, the main defence mechanism has proven to be chemical defences, particularly in fleshy sponges. Chemicals produced are known as secondary metabolites. These secondary metabolites tend to repel or inhibit the growth of predator organisms.[6]

An example of this is shown with *Suberites domuncula*, a marine sponge collected from the northern Adriatic Sea, near Rovinj, Croatia.[19] This sponge *in situ* was considerably free of epibiont coverage. Therefore an investigation to determine whether there was any anti-bacterial activity within the sponge or its associated bacteria was conducted. Crude extracts of the sponge showed potent anti-bacterial activity against bacteria such as α-Proteobacterium and *Idiomarina* sp. Associated bacteria showed some inhibition to the growth of *Pseudomonas* sp. The sponge also was discovered to produce a perforin-like anti-bacterial

protein that helped to protect the sponge against bacterial epidermis coverage. This is a defence mechanism.[19]

It is very common for a marine sponge to produce a secondary metabolite and utilise it as a chemical defence. Therefore it is essential to investigate whether *Cliona celata* sponges around the Bay of Plenty have chemical defence mechanisms, particularly in light of the fact that *Cliona celata* is frequently observed to be healing from fish or storm induced scarring.[41]

A regeneration experiment of *Cliona celata* was conducted at high and low flow areas in Ireland.[80] Circular cores of 10 mm diameter and 1 cm deep were cut from sponge samples and regeneration rates were measured over 300 days. After 200 days high flow sponges had fully regenerated, but low flow specimens took 300 days to fully regenerate. This is likely due to high flow areas having higher food levels and the sponge requires less energy to pump water through their bodies, putting more energy into healing.[80] This experiment looked at physical regeneration rates however the following experiment hopes to give an insight into the chemical ecological processes of this sponge and the potential for a natural product.

4.2　Results

It is important to note that all damaged samples produced a bright yellow coating at the site directly where the knife damage had occurred, however this darkened once frozen (Figure 4.1 and Figure 4.2). A representative subsample of this area of the sponge was utilised for preparation of the damaged extracts.

Figure 4.1: Representative Leisure Island (intertidal) damage experiment sample, non damaged (left), 24 hr post damage (middle) and 48 hr post damage (right).

Figure 4.2: Representative Rabbit Island (subtidal) damage experiment sample, non damaged (left) and 48 hr post damage (right).

The first intertidal sponge sampled from Leisure Island was a healthy sponge with red algal epibiont coverage of approximately 50% of the sample. The sponge tissue was dense and a bright orange colour. Samples extracted were NW.1.18.1 (0 hr), NW.1.18.2 (24 hr) and NW.1.18.3 (48 hr). The chromatograms for these samples are shown overlaid in Figure 4.3. The same peaks are present in all samples. The early elution peaks between 15-20 min were all of similar size. The sample for 0 hr non damaged had two very predominant peaks present. These

were present in both 24 and 48 hr post damage chromatograms, however, on a much smaller scale.
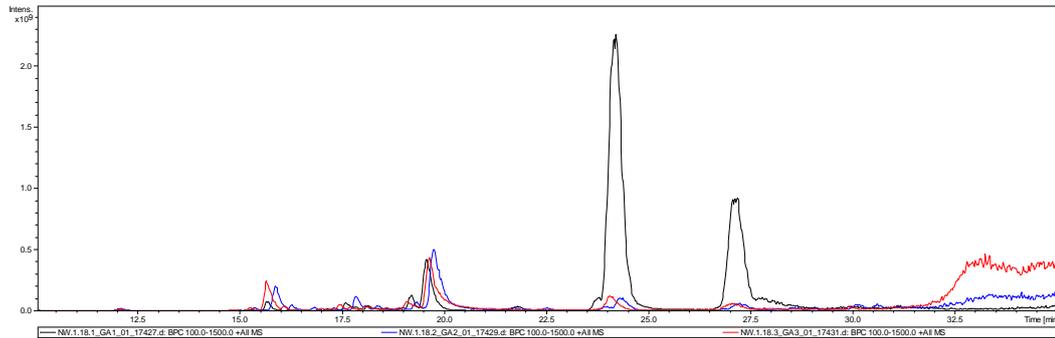
Figure 4.3: Overlaid chromatograms of intertidal sponge samples from Leisure Island, elution time of 10-35 min. NW.1.18.1; (black), 0 hr non damaged, NW.1.18.2; (blue), 24 hr damage and NW.1.18.3; (red), 48 hr damage.

Sample two was collected intertidally at Leisure Island. This was a healthy sponge with a dense, orange texture. An unknown red seaweed was imbedded into approximately 50-70% of the sample. The chromatograms are shown in Figure 4.4. The same peaks were present in all samples with variation in peak proportions. The predominant peak at approximately 19.5 min was larger in both damaged samples than in the non damaged sponge section.
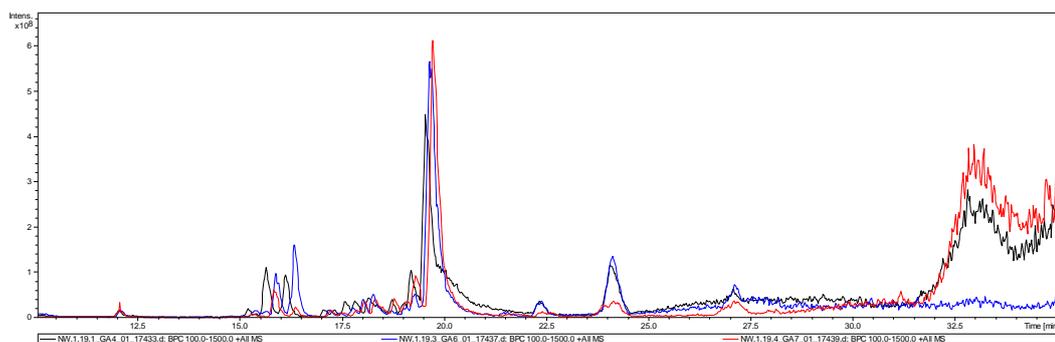
Figure 4.4: Overlaid chromatograms of intertidal sponge samples from Leisure Island, elution time of 10-35 min. NW.1.19.1; (black), 0 hr non damaged, NW.1.19.3; (blue), 24 hr damage and NW.1.19.4; (red), 48 hr damage.

The third intertidal sample collected from Leisure Island was clean with no epibiont coverage. The sponge texture was dense and fleshy with a bright orange colour. Figure 4.5 shows the chromatogram overlay of the non damaged sample (NW.1.20.1), 24 hr post damage (NW.1.20.2) and 48 hr post damage (NW.1.20.3). The peaks present were in all samples except

for the peak with an elution time of approximately 28 min in the 24 hr post damage sample (blue). This is a substantial peak but not present at the other times. All other peaks were similar but with varying proportions.
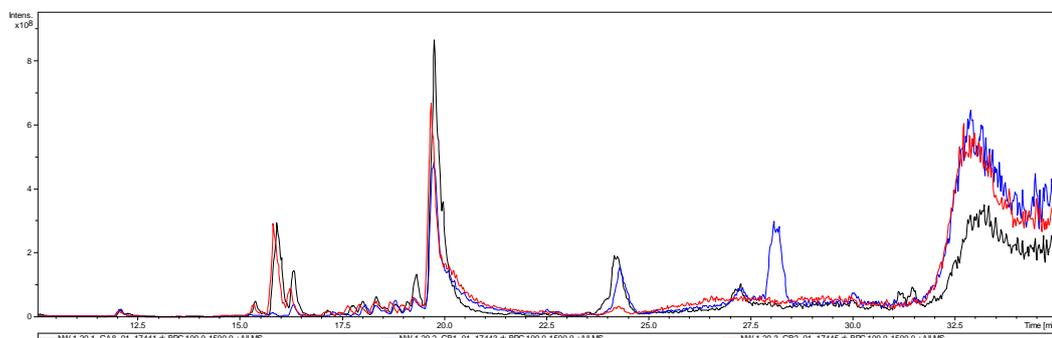
Figure 4.5: Overlaid chromatograms of intertidal sponge samples from Leisure Island, elution time of 10-35 min. NW.1.20.1; (black), 0 hr non damaged, NW.1.20.2; (blue), 24 hr damage and NW.1.20.3; (red), 48 hr damage.

The final sponge sample collected from Leisure Island had a heavy black epibiont growth on the exterior of the sponge. The body of the sponge was healthy and dense. The extract was a dark orange colour. The overlaid chromatograms for NW.1.21.1 (0 hr, non damage), NW.1.21.2 (24 hr post damage) and NW.1.21.3 (48 hr post damage) are shown in Figure 4.6. The early elution peaks (10-20 min) were present at each time, 0 hr and 48 hr post damage had similar peak heights whereas 24 hr post damage peaks were a lot smaller. There was a peak at approximately 24.5 min in NW.1.21.1, non damaged sample showed a peak that was not present in the damaged sponge samples. NW.1.21.2, 24 hr post damage showed a significant peak at approximately 29 min, this was not present in the other chromatograms.
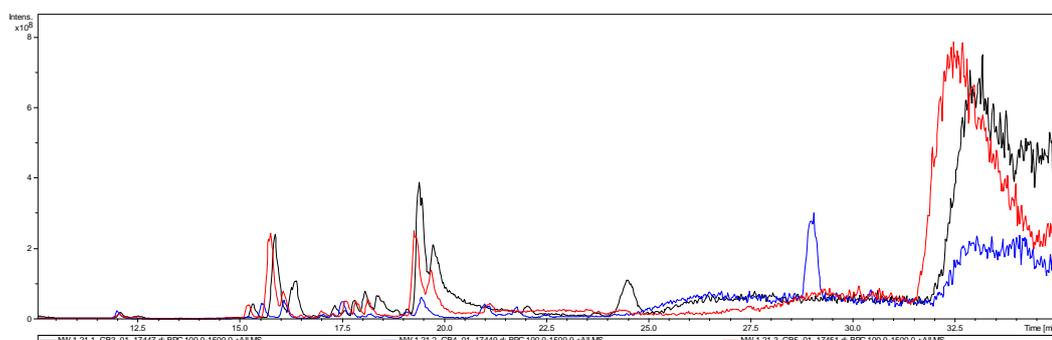
Figure 4.6: Overlaid chromatograms of intertidal sponge samples from Leisure Island, elution time of 10-35 min. NW.1.21.1; (black), 0 hr non damaged, NW.1.21.2; (blue), 24 hr damage and NW.1.21.3; (red), 48 hr damage.

Figure 4.7 and Figure 4.8 show chromatograms from samples collected from the same sponge at Site Three, Rabbit Island, a subsample taken from either end of the sponge. This sponge had healthy, dense tissue and a bright orange colour. There was approximately 33% red algae epibiont coverage to the surface of the sponge. Figure 4.7 shows the overlaid chromatograms of NW.1.8.2, 0 hr non damaged and NW.1.11.1, 48 hr post damage. During elution times from 10-20 min the same peaks were present in both subsamples, however, those in the chromatogram of NW.1.11.1 have higher intensities. Between elution times of 20-35 min, similar peaks were present in varying proportions in both samples, although NW.1.11.1 contains a peak at 28.3 min that was not present in NW.1.8.2. Figure 4.8 shows the overlaid chromatograms of NW.1.8.3, 0 hr non damaged and NW.1.12.1, 48 hr post damage. Although the peaks in Figure 4.8 were similar to those present in Figure 4.7 they were of significantly less intensity. Elution times between 10-20 min show comparable peaks and intensities between NW.1.8.3 and NW.1.12.1. At later elution times (20-31 min), NW.1.8.3 showed three distinct peaks, whereas NW.1.12.1 had no distinguishable peaks present.

Figure 4.7: Overlaid chromatograms of subtidal sponge samples from Rabbit Island, elution time of 10-35 min. NW.1.8.2; (black) 0 hr non damaged, NW.1.11.1; (red), 48 hr damage.

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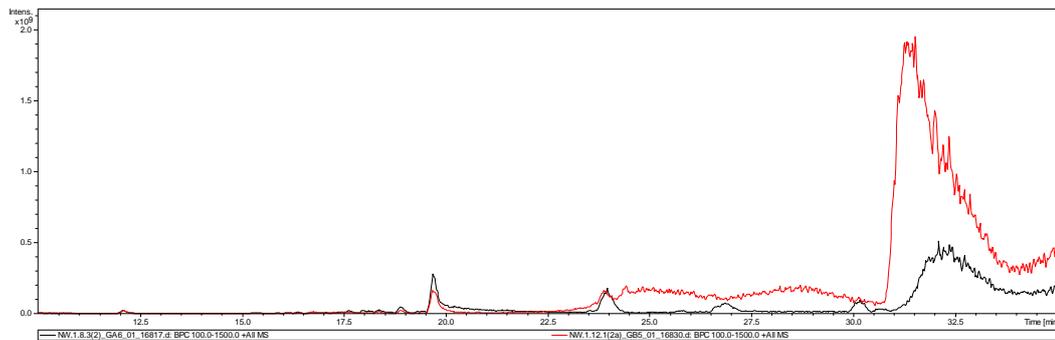
Figure 4.8: Overlaid chromatograms of subtidal sponge samples from Rabbit Island, elution time of 10-35 min. NW.1.8.3; (black) 0 hr non damaged, NW.1.12.1; (red), 48 hr damage.

Figure 4.9 and Figure 4.10 show chromatograms of samples collected from the same sponge at Site Three, Rabbit Island, a subsample from either end. The sponge collected was very clean and healthy with dense, orange tissue. Figure 4.9 shows the overlaid chromatograms of NW.1.7.3, 0 hr non damaged and NW.1.13.1, 48 hr post damage subsamples. The non damaged (NW.1.7.3) subsample shows a distinct set of peaks with a prominent peak at approximately 20 min. NW.1.13.1 has some similar peaks at early elution times (between 10-20 min), however, they are significantly less intense. The later elution times show no distinct peaks at all. Figure 4.10 shows the chromatogram overlay of NW.1.9.2, 0 hr non damaged and NW.1.14.1, 48 hr post damage. The chromatogram shows a similar pattern to that of Figure 4.9 but in varying proportions. The non damaged NW.1.9.2 has a defined peak pattern, however, NW.1.14.1 has two small peaks at earlier elution times otherwise has no distinguishable peaks.

Figure 4.9: Overlaid chromatograms of subtidal sponge samples from Rabbit Island, elution time of 10-35 min. NW.1.7.3; (black) 0 hr non damaged, NW.1.13.1; (red), 48 hr damage.

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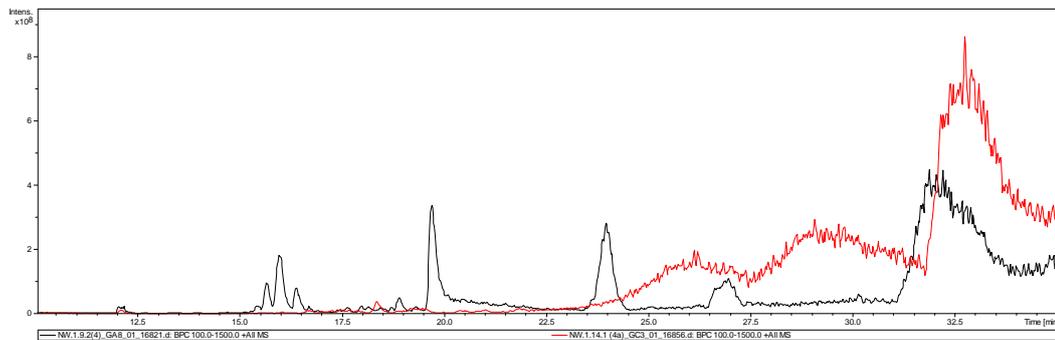
Figure 4.10: Overlaid chromatograms of subtidal sponge samples from Rabbit Island, elution time of 10-35 min. NW.1.9.2; (black) 0 hr non damaged, NW.1.14.1; (red), 48 hr damage.

Figure 4.11 and Figure 4.12 show chromatograms from subsamples of the same sponge collected from subtidal Site Three, Rabbit Island, a subsample taken from either end. This sample had healthy, dense, orange tissue with approximately 33% epibiont coverage to the exterior of the sponge. Figure 4.11 is a chromatogram overlay of NW.1.7.2, 0 hr non damaged and NW.1.15.1, 48 hr post damage. The chromatogram of the non damaged sample shows a defined set of peaks throughout the whole trace whereas the chromatogram of the subsample 48 hr post damage shows no distinct peaks and is of very low intensity. This is comparable with Figure 4.12 which are overlaid chromatograms of NW.1.7.1, 0 hr non damaged and NW.1.24.2, 48 hr post damage subsamples. The peaks present for NW.1.7.1 are similar to those shown in Figure 4.11, however, with varying proportions. The most predominant peak is at an elution time of approximately 19.5 min. The chromatogram of NW.1.24.2 is also similar to that in Figure 4.9 as there are no defined peaks and the intensities are very low.
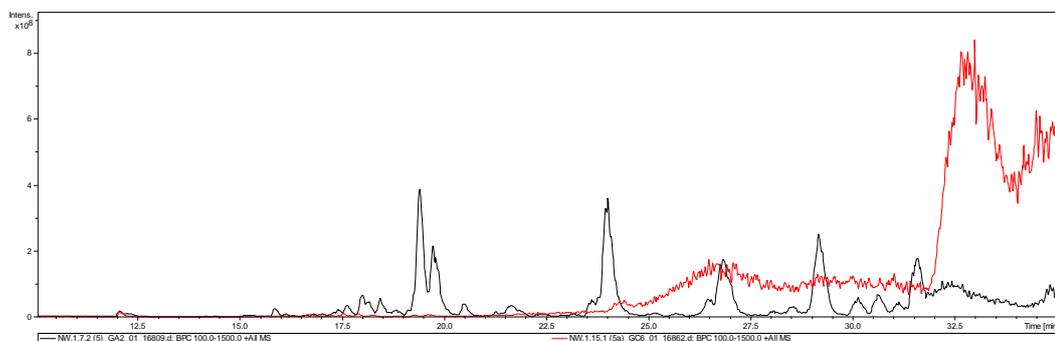
Figure 4.11: Overlaid chromatograms of subtidal sponge samples from Rabbit Island, elution time of 10-35 min. NW.1.7.2; (black) 0 hr non damaged, NW.1.15.1; (red), 48 hr damage.

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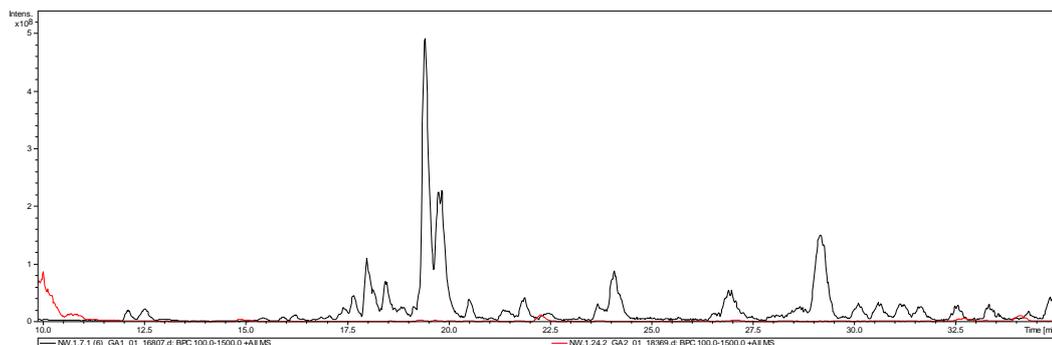
Figure 4.12: Overlaid chromatograms of subtidal sponge samples from Rabbit Island, elution time of 10-35 min. NW.1.7.1; (black) 0 hr non damaged, NW.1.24.2; (red), 48 hr damage.

Figure 4.13 and Figure 4.14 show chromatograms of subsamples from the same sponge collected subtidally from Site Three, Rabbit Island. The interior of the sponge was dense, healthy, orange tissue. One side of the sponge had minimal red epibiont coverage (Figure 4.14) while the other side had a clean exterior (Figure 4.13). Figure 4.13 shows the overlaid chromatograms of NW.1.9.1, 0 hr non damaged and NW.1.22.1, 48 hr post damage. The chromatogram of the non damaged subsample shows clear peaks with dominant ones at elution times of approximately 16.5 and 19.8 min. That of NW.1.22.1 shows some similar peaks to that of NW.1.9.1 but with significantly lower intensities. There is, however, a peak present at 30 min which is not present in that of the non damaged subsample. Figure 4.14 shows the chromatogram overlay of NW.1.8.1, 0 hr non damaged and NW.1.27.2, 48 hr post damage. Figure 4.14 of a non damaged sample is similar to that in Figure 4.13, however, with varying proportions, the predominant peak being at an elution time of 19.8 min. The chromatogram of NW.1.27.2 contains peaks of very low intensity compared to that of NW.1.8.1 with no defined peaks.

Figure 4.13: Overlaid chromatograms of subtidal sponge samples from Rabbit Island, elution time of 10-35 min. NW.1.9.1; (black) 0 hr non damaged, NW.1.22.1; (red), 48 hr damage.

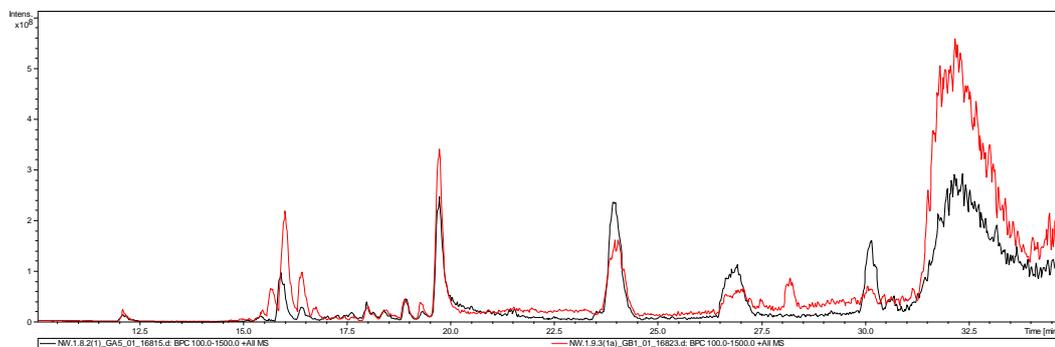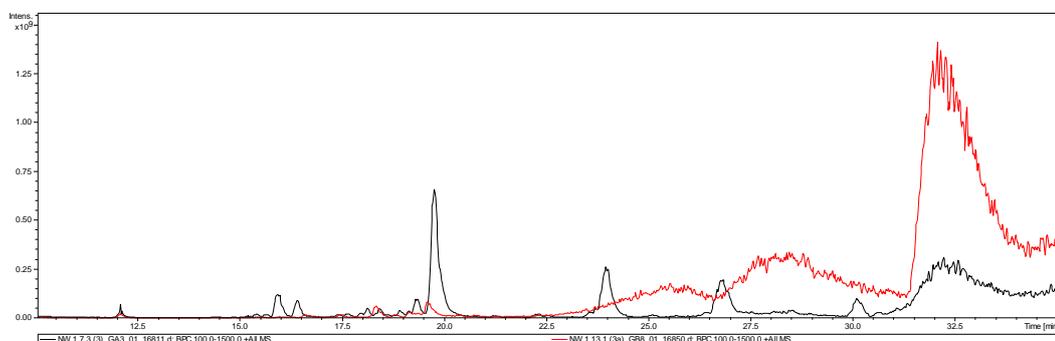**Figure 4.14: Overlaid chromatograms of subtidal sponge samples from Rabbit Island, elution time of 10-35 min. NW.1.8.1; (black) 0 hr non damaged, NW.1.27.2; (red), 48 hr damage.**

### 4.2.1 **Bioactivity: MTT assay**

A MTT assay was completed on a damaged sponge sample, 48 hr post damage occurring. The sample was collected from Site Three, Rabbit Island. There was no activity shown against the HeLa cells used during this assay.

4.3 Discussion

The bright yellow coating observed on all damaged samples at the point of direct damage is thought to be a fast response mechanism. The sponge mobilises pinacocytes and archaeocytes to the point of damage to provide a thin veneer of cellular material to seal off the affected area as a precursor to aquiferous system reconstruction and to limit infection.[24]

Samples from Site Three, Rabbit Island were the first to be collected. It was hypothesised that a lag time of 48 hours would be sufficient for the sponge to produce a chemical response. However, initial testing indicated this may be too long since no response was observed. Previous studies have suggested that metabolite variation is small over fine-scale temporal experimentation.[74] There was however a decrease in the chromatogram peak intensity from the majority of samples. The chemical compounds within the sponge have potentially leached into the environment once the damage has occurred, resulting in the sponge being unable to recover.[55] This could explain the loss of peak intensities.

In response to this finding, when the damage experiment from Leisure Island was conducted, collection times were adjusted to 24 and 48 hours post knife damage. Variations in the sample responses were present within the intertidal zone. Intertidal sample one (Figure 4.3) showed a similar response to the subtidal samples, where post damage the peak intensities decreased. Intertidal sample two (Figure 4.4) retains the same peaks throughout the experiment but to varying proportions, the specimen could potentially be adapting to its changing conditions. Whereas the two remaining intertidal sponges did present with similar pre damage and post damage peaks, but also showed new peaks 24 hr post damage. This could indicate a chemical response to the damage. However on further investigation of the mass spectra of these peaks there were no indications of a potentially bioactive metabolite.

It is thought that the yellow colouration presented on all directly damaged specimens is the regeneration of sponge tissue, creating a scab-like effect over the exposed sponge section. This is supported by the chemistry as

there is a common trend of a decrease of intensity in chromatograms throughout all damaged sponge samples. A potential hypothesis is that instead of the sponge producing a chemical defence to the damage occurring, it is possibly reverting all the cells types back into archaeocytes so the sponge can repair and re-grow instead of defend, as the "attack" is no longer present.[24] If this is the case, it is likely a different outcome would be observed in the presence of an irritant such as a fungal infection, something that represents long term damage.

From previous studies, *Cliona celata* has been found to be a slow growing sponge, however, it can rapidly regenerate when damaged.[80] This is thought to be due to encrusting *C. celata* having an optimal shape and size for metabolic process and feeding patterns and the sponge wanting to return to that size as fast as possible. Otherwise if other external stressors are placed on the specimen and it cannot recover, the sponge can potentially be destroyed.[80] Therefore, this is believed to be the case with these specimens.

CHAPTER 5

STRATIFICATION CHEMISTRY

5.1 Introduction

It was noted from Chapter 3 (samples NW.1.1.1 and NW.1.1.2) that the same sponge could have vastly different chemistry depending on its condition. Therefore the stratification experiment was conducted to determine whether the chemicals produced in response to damage are directly localised to the damage site or distributed throughout the sponge. Samples were collected from Site Three, Rabbit Island in the Bay of Plenty. Four large sponges were sampled, with two subsamples collected from each sponge, one from either end. Tissue from the directly damaged site, the pinacoderm, and (depending on the size of the subsample) 1-2 cm from the damage site and 2-3 cm from the damage site was measured for its chemical composition. This is thought to be a novel investigation due to the lack of literature on the subject.

5.2 Results

Stratification shows the change in chemistry throughout the sponge samples (Figure 5.1). Figure 5.2 and Figure 5.3 show chromatograms collected from the same sponge. This sponge had a healthy interior tissue and approximately 33% red epibiont coverage to the exterior. Figure 5.2 shows the overlaid chromatograms of NW.1.8.2, 0 hr non damaged, NW.1.11.1, direct damage site, NW.1.11.2, pinacoderm only, NW.1.11.3, 1-2 cm from the damaged site and NW.1.11.4, 2-3 cm from the damaged site. There are consistent peaks present in all subsamples but with varying proportions, however, a unique peak is present in the chromatogram of NW.1.11.4 at an elution time of approximately 25.3 min, then peaks become undefined.
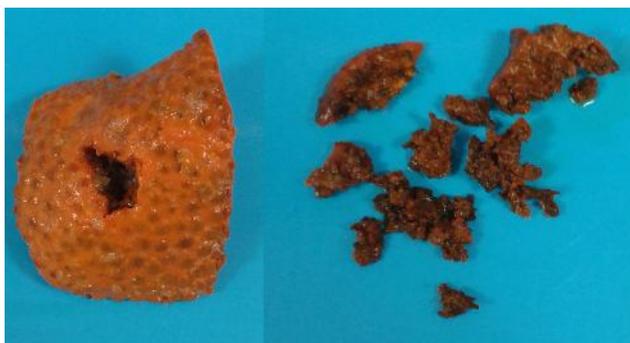
Figure 5.1: Representative Rabbit Island (subtidal) stratification experiment sample, non damaged (left) and 48 hr post damage (right)

Figure 5.3 shows the overlaid chromatograms of NW.1.8.3, 0 hr non damaged, NW.1.12.1, direct damage site, NW.1.12.2, pinacoderm only and NW.1.12.3, 1-2 cm from the damaged site. The non damaged NW.1.8.3 shows a set of defined peaks, however, in low intensity compared to those in Figure 5.2. The damaged subsamples (NW.1.12.1, NW.1.12.2 and NW.1.12.3) show a similar peak present to that of NW.1.8.3 at approximately 19.8 min with comparable peak intensities. There are no defined peaks at later elution times for these damaged subsamples.
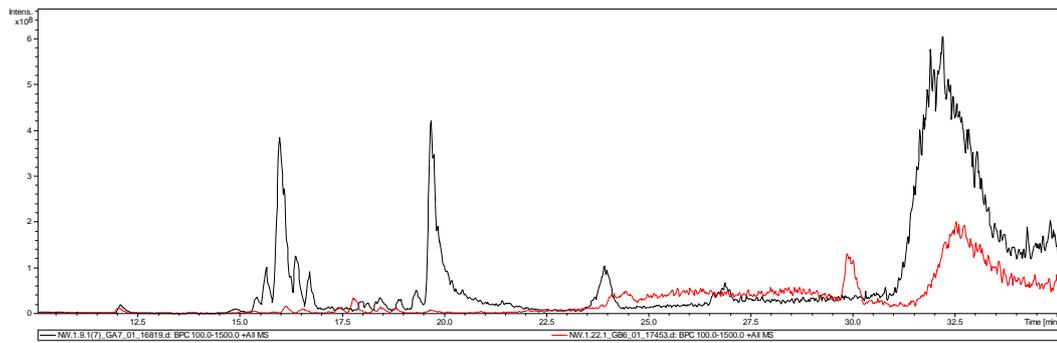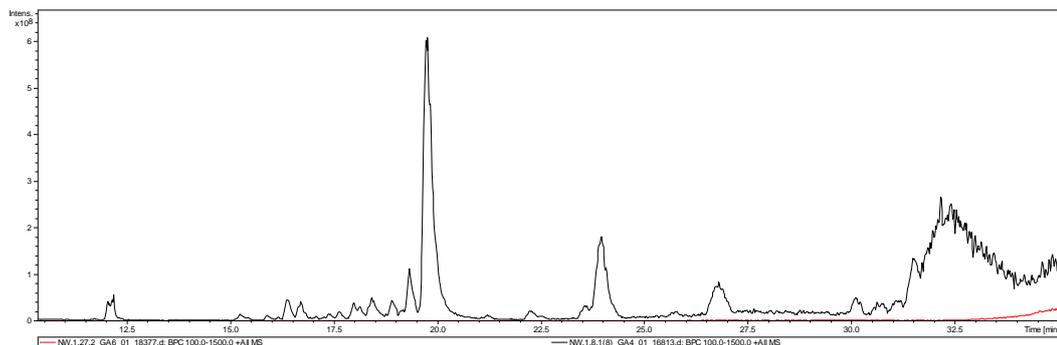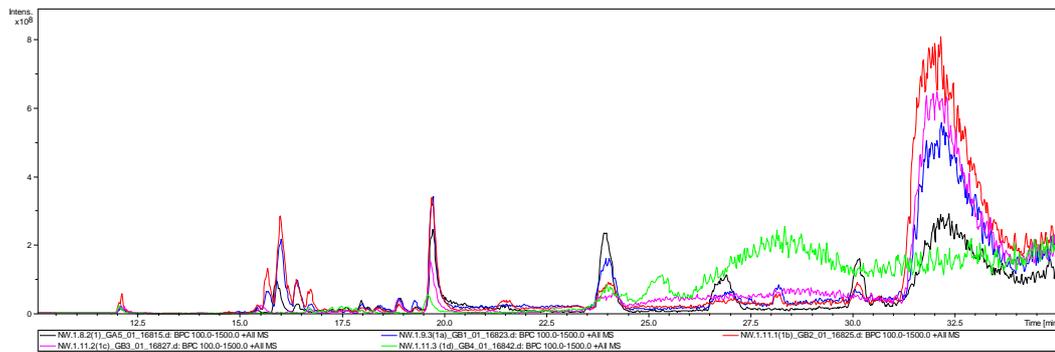
Figure 5.2: Overlaid chromatograms of subtidal sponge samples from Rabbit Island, elution time of 10-35 min. NW.1.8.2; (black) 0 hr non damaged, NW.1.11.1; (blue), direct damage site, NW.1.11.2; (red), pinacoderm only, NW.1.11.3; (pink), 1-2 cm from damaged site and NW.1.11.3; (green), 2-3 cm from damaged site. Resampling of sponge occurred 48 hr post knife damage.
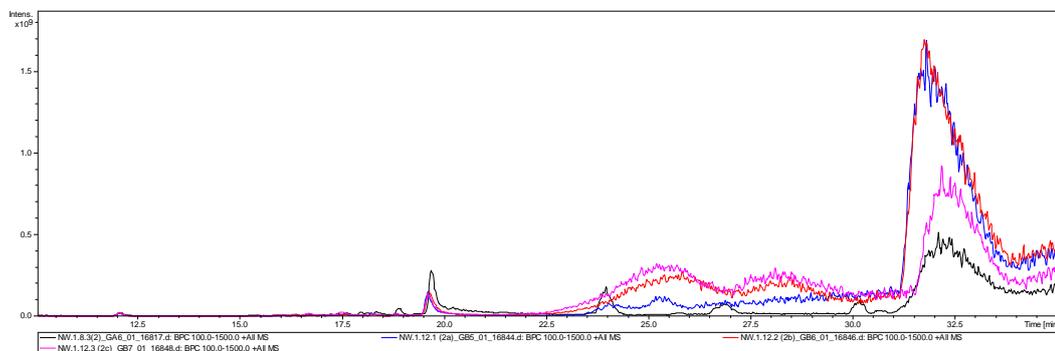
Figure 5.3: Overlaid chromatograms of subtidal sponge samples from Rabbit Island, elution time of 10-35 min. NW.1.8.3; (black) 0 hr non damaged, NW.1.12.1; (blue), direct damage site, NW.1.12.2; (red), pinacoderm only and NW.1.12.3; (pink), 1-2 cm from damaged site. Resampling of sponge occurred 48 hr post knife damage.

Figure 5.4 and Figure 5.5 show chromatograms from subsamples collected from the same sponge that had a clean and healthy interior and exterior. Figure 5.4 shows the chromatogram overlay of NW.1.7.3, 0 hr non damaged, NW.1.13.1, direct damage site, NW.1.13.2, pinacoderm only and NW.1.13.3, 1-2 cm from the damaged site. The undamaged subsample (NW.1.8.3) shows a set of peaks similar to the previous sample, peaks are well defined with a predominant peak at approximately 19.8 min. Peaks eluting between 10-20 min for the damaged samples (NW.1.13.1, NW.1.13.2 and NW.1.13.3) show some similarities to those in NW.1.8.3, however, in varying proportions. After an elution time of 20 min there are no definite peaks present.

Figure 5.5 shows the overlaid chromatograms of NW.1.9.2, 0 hr non damaged, NW.1.14.1, direct damage site, NW.1.14.2, pinacoderm only and NW.1.14.3, 1-2 cm from the damaged site. The chromatogram of the non damaged sample shows a peak layout similar to that in Figure 5.4 but with varying proportions. Also similar to Figure 5.4 in the chromatograms of the damaged samples, are peaks between elution times of 10-20 min. These are consistent with those in the non damaged NW.1.9.2, however, at later elution times defined peaks are not present with the exception of NW.1.14.2. This subsample has a set of peaks present between 20-25 min that are unique to the sample. Mass spectra (Figure 5.6 and Figure 5.7) shows brominated isotope patterns present in these peaks. The peak with an elution time of 21 min shows a distinct 1:2:1 isotope pattern in the mass spectrum, indicating the presence of a dibrominated compound with *m/z* of 408/410/412 (Figure 5.6). The peak with an elution time of 21.9 min again shows a 1:2:1 isotope pattern in the mass spectrum, indicating the presence of a dibrominated compound with a *m/z* of 487.9/489.9/490.9 (Figure 5.7).

Figure 5.4: Overlaid chromatograms of subtidal sponge samples from Rabbit Island, elution time of 10-35 min. NW.1.7.3; (black) 0 hr non damaged, NW.1.13.1; (blue), direct damage site, NW.1.13.2; (red), pinacoderm only and NW.1.13.3; (pink), 1-2 cm from damaged site. Resampling of sponge occurred 48 hr post knife damage.

Figure 5.5: Overlaid chromatograms of subtidal sponge samples from Rabbit Island, elution time of 10-35 min. NW.1.9.2; (black) 0 hr non damaged, NW.1.14.1; (blue), direct damage site, NW.1.14.2; (red), pinacoderm only and NW.1.14.3; (pink), 1-2 cm from damaged site. Resampling of sponge occurred 48 hr post knife damage.
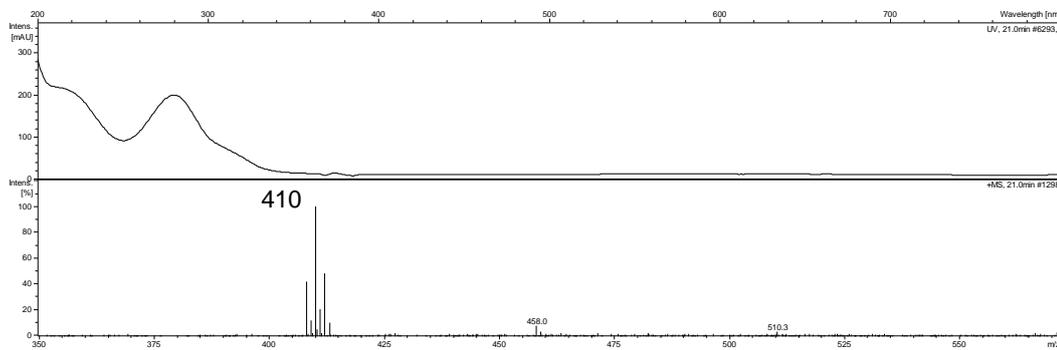
Figure 5.6: UV (top) and MS spectra (bottom) of NW.1.14.3 subtidal damaged sponge subsample at an elution time of 21 min, [M+H]$^+$ ion at 410 *m/z*.
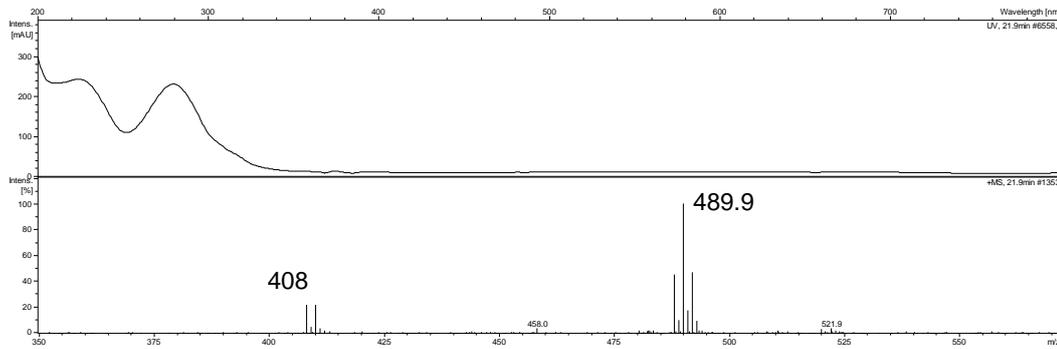
Figure 5.7: UV (top) and MS spectra (bottom) of NW.1.14.3 subtidal damaged sponge subsample at an elution time of 21.9 min, [M+H]$^+$ ion at 408 *m/z* and 489.9 *m/z*.

Figure 5.8 and Figure 5.9 show chromatograms of subsamples collected from the same sponge. This was a healthy sponge with dense, orange tissue and approximately 33% epibiont coverage. Figure 5.8 shows the overlaid chromatograms of NW.1.7.2, 0 hr non damaged, NW.1.15.1, direct damage site, NW.1.15.3, pinacoderm only and NW.1.15.4, 1-2 cm from damaged site. The chromatogram of NW.1.7.2 shows defined peaks

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throughout the entire elution time. These are consistent with peaks present in previous samples. The chromatograms of damaged subsamples, (NW.1.15.1, NW.1.15.3 and NW.1.15.4) have peaks comparable with early elution times of NW.1.7.2, however, with significantly smaller intensities. Later elution times (20-35 min) do not have any defined chemical peaks which is consistent with previous samples.

Figure 5.9 shows the chromatogram overlays of NW.1.7.1, 0 hr non damaged, NW.1.24.2, direct damaged site, NW.1.24.3, pinacoderm only and NW.1.24.4, 1-2 cm from the damaged site. The chromatogram of NW.1.7.1 shows similar peaks to that of the non damaged subsample of Figure 5.8 but with varying proportions. The predominant peak of this subsample is at an elution time of approximately 19.5 min. The chromatograms of damaged subsamples (NW.1.24.2, NW.1.24.3 and NW.1.24.4) have no distinct chemical peaks present, however, NW.1.24.1 shows some small peaks at later elution times. The mass spectra of these peaks contain no distinctive isotope patterns.
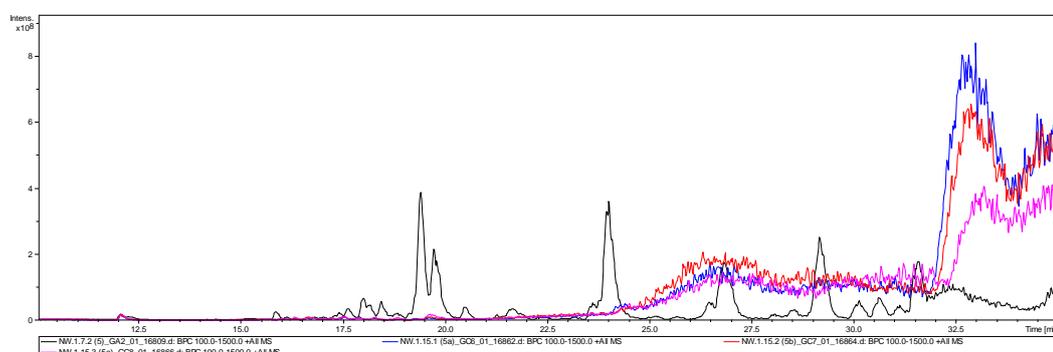
Figure 5.8: Overlaid chromatograms of subtidal sponge samples from Rabbit Island, elution time of 10-35 min. NW.1.7.2; (black) 0 hr non damaged, NW.1.15.1; (blue), direct damage site, NW.1.15.3; (red), pinacoderm only and NW.1.15.4; (pink), 1-2 cm from damaged site. Resampling of sponge occurred 48 hr post knife damage.
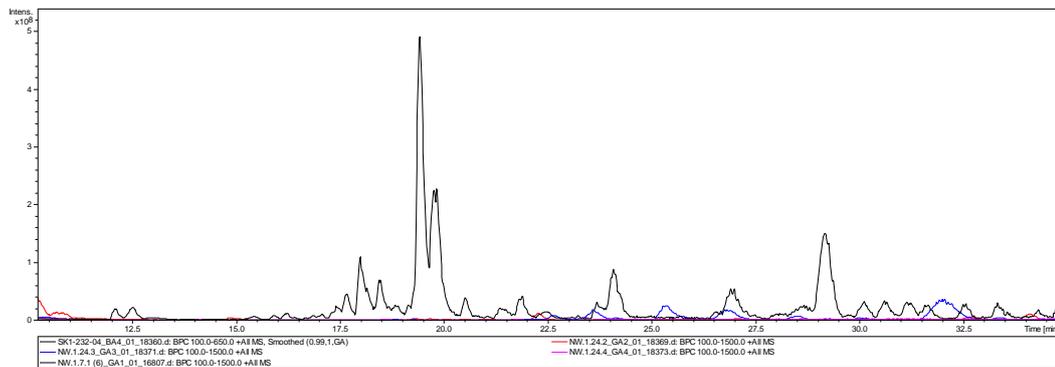
Figure 5.9: Overlaid chromatograms of subtidal sponge samples from Rabbit Island, elution time of 10-35 min. NW.1.7.1; (black) 0 hr non damaged, NW.1.24.2; (blue), direct damage site, NW.1.24.3; (red), pinacoderm only and NW.1.24.4; (pink), 1-2 cm from damaged site. Resampling of sponge occurred 48 hr post knife damage.

Figure 5.10 and Figure 5.11 show chromatograms of subsamples from the same sponge collected subtidally at Site Three, Rabbit Island. This sponge had healthy, dense and orange tissue, with one side of the exterior clean (Figure 5.10) and the other side with red epibiont coverage (Figure 5.11). Figure 5.10 shows the overlaid chromatograms of NW.1.9.1, 0 hr non damaged, NW.1.22.1, direct damaged site, NW.1.22.2, pinacoderm only and NW.1.22.3, 1-2 cm from the direct damage site. The chromatogram of non damaged subsample (NW.1.9.1) shows a clear set of peaks throughout the elution period. The chromatograms of damaged subsamples (NW.1.22.1, NW.1.22.2 and NW.1.22.3) have peaks comparable with NW.1.9.1, however, with very low intensities. At later elution times of 25-35 min peaks become indistinguishable.

Figure 5.11 shows the chromatogram overlay of NW.1.8.1, 0 hr non damaged, NW.1.27.2, direct damage site, NW.1.27.3, pinacoderm only and NW.1.27.4, 1-2 cm from damaged site. The chromatogram of NW.1.8.1 shows a predominant peak at approximately 19.9 min. Other peaks present are consistent with those found in Figure 5.10, however, with varying proportions. There are no significant chemical peaks for the chromatograms of damaged subsamples (NW.1.27.2, NW.1.27.3 and NW.1.27.4).
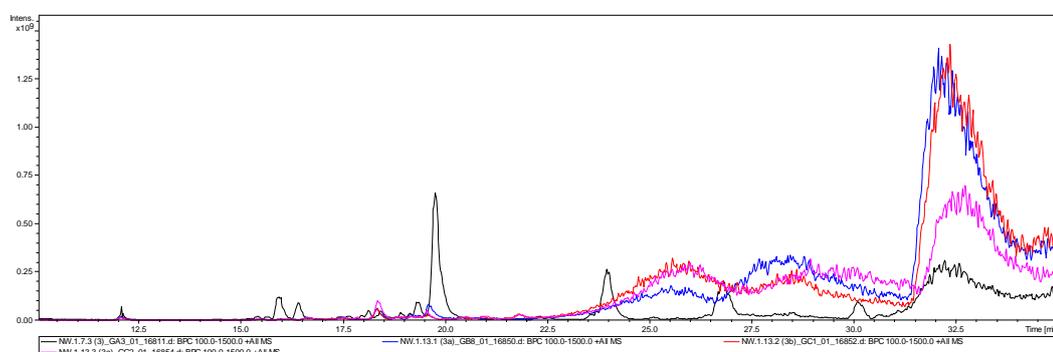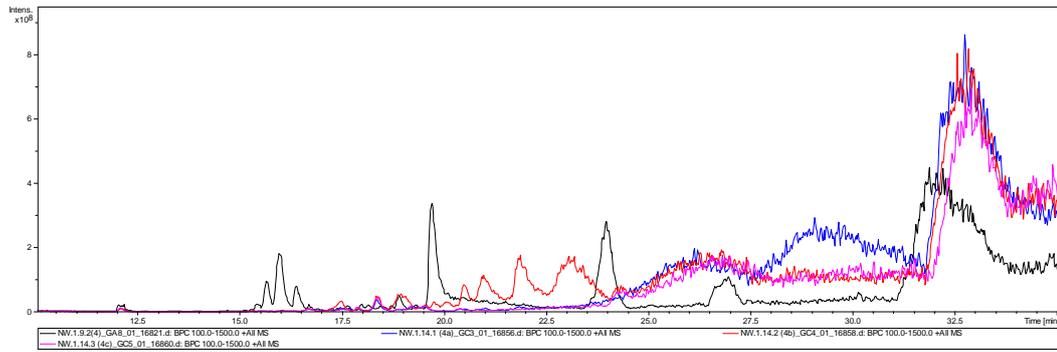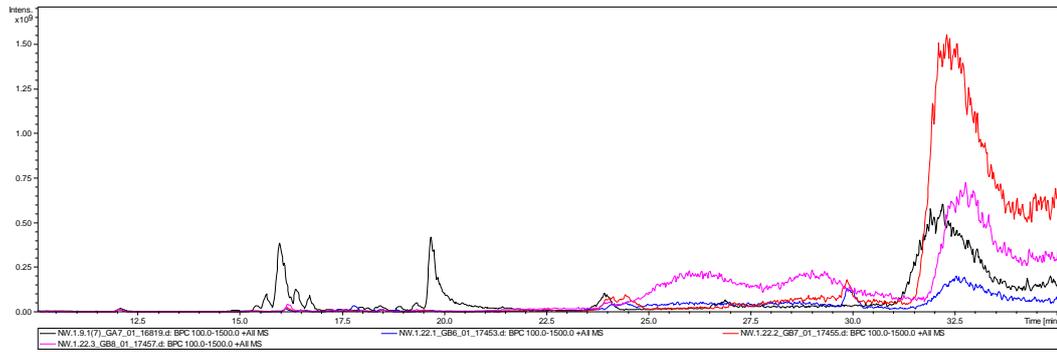
Figure 5.10: Overlaid chromatograms of subtidal sponge samples from Rabbit Island, elution time of 10-35 min. NW.1.9.1; (black) 0 hr non damaged, NW.1.22.1; (blue), direct damage site, NW.1.22.2; (red), pinacoderm only and NW.1.22.3; (pink), 1-2 cm from damaged site. Resampling of sponge occurred 48 hr post knife damage.
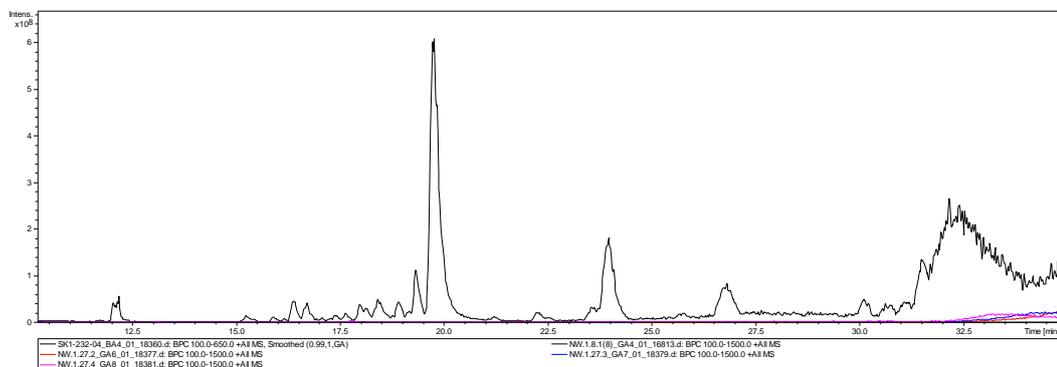
Figure 5.11: Overlaid chromatograms of subtidal sponge samples from Rabbit Island, elution time of 10-35 min. NW.1.8.1; (black) 0 hr non damaged, NW.1.27.2; (blue), direct damage site, NW.1.27.3; (red), pinacoderm only and NW.1.27.4; (pink), 1-2 cm from damaged site. Resampling of sponge occurred 48 hr post knife damage.

5.3 Discussion

Similarly to results outlined in Chapters 3 and 4, regardless of the morphological differences between the sponges sampled, there were common peaks with each individual, indicating the baseline chemical composition of *Cliona celata*. There is also evidence of chemical variation with samples and within their subsamples due to varying peak intensities with the chromatograms. Again, this is thought to be localised responses to micro-environments, such as predators targeting one section of a sponge or epibiont coverage only to one portion of the sponge, or the stratification to the environment itself, different interacting organisms present in different zones causing localised reactions to the individuals.

Previously represented in Chapter 4, the majority of specimens, once damaged, produced less intense peaks leading to undefined peaks at later elution times. This was consistent throughout all stratified subsamples. With the exception of samples shown in Figure 5.2, peaks were retained up to 1-2 cm within the sponge, but at 2-3 cm into the specimen there was a loss of defined chemistry as represented by the lack of peaks in the chromatograms of the extracts. It is uncertain why this has occurred, however, it could indicate movement of chemicals in response to a deterrent within the sponge individual. There have been previous studies that showed defence chemicals tend to be more potent in exposed sections of sponges, rather than in the base of the sponge, however this did not necessarily stop predation on the sponge.[92,93] The decreased chemical intensities within the damaged samples could lead to more evidence that cells are reverting into archaeocytes to repair and reproduce rather than defend.

The unique peaks present in the chromatograms of NW.1.14.2, Figure 5.5 showed dibrominated isotope patterns (Figure 5.6 and Figure 5.7). As previously stated, brominated compounds can indicate the production of a secondary metabolite as bromine is commonly sequestered into sponges.[50] The location of these compounds could signify brominated and/or secondary metabolites are contained and potentially produced in the pinacoderm as no other stratified samples produced a brominated

compound. This could relate to chemical concentration being higher in the tips of a sponge.[93] Further investigations are required for all hypothesises introduced as there is not enough information to accept these hypotheses.

CHAPTER 6

CONCLUDING REMARKS AND FUTURE DIRECTIONS

6.1 Concluding Remarks

This project examines the chemical variation of the marine sponge *Cliona celata* collected from the Bay of Plenty region, and whether the production of bioactive secondary metabolites was a defence mechanism. It is a necessary precursor to more in depth chemical ecological studies and work involving structural elucidation. From the results obtained, there was a trend of common peaks present throughout *C. celata* collected from all locations. However, chemical variation did occur spatially and between each individual specimen, likely due to micro-environment interactions,[14] as was shown with varying peak intensities.

No clear chemical defence was indicated from the damaging of the sponges. It was hypothesised that the response was to revert cells into archaeocytes to repair and rebuild sponge tissue, rather than chemically defend the remaining sponge. However, further investigations are essential as there is currently not enough evidence to accept this hypothesis.

In comparison to this, there are chemical changes in response to epibiont coverage observed in the data. The variations in peak intensities are hypothesised to be related to the amount of epibiont coverage on the sponge. The higher the percentage of epibiont coverage, the higher the peak intensities. Epibiont coverage is thought to trigger a release in chemical mobility, hence the more intense peaks. However, once the sponge reaches a threshold of approximately 50% epibiont coverage, it is believed to trigger the release of novel chemicals. This is thought to be a new area of study that shows small temporal and spatial changes to the

sponge metabolite chemistry in response to damage and would require a greater detailed investigation.

There was no bioactivity against HeLa cells for both non damaged and damaged specimens. Despite this, some sponge specimens produced monobrominated and dibrominated compounds that could indicate a chemical response to an external stimulus. There is currently not enough evidence to determine how or why these chemical compounds are produced. The stratification experiment indicates that these compounds could potentially be isolated/produced in the pinacoderm of the sponge, but further testing is required.

All specimens collected were confirmed to be *Cliona celata* from spicule identifications of tylostyles and subtylostyles. The slight variations in spicule morphology between intertidal and subtidal sites do not suggest a variation in the *C. celata* species, however, they do support the chemical variations of the sponges due to micro-environment interactions.

This project gives an introductory insight into the chemical variation and potential chemical defence secondary metabolites of *Cliona celata* in the Bay of Plenty, New Zealand. Although the response to damage is unexpected, there have been multiple new hypotheses made from the trends in chemical variations in sponge. This opens up a new chapter of work required to investigate sponge chemistry and metabolite production, and the ecological significance of these factors.

6.2   Future Directions

Future studies of *Cliona celata* within the Bay of Plenty region are required to further confirm hypothesises established in this thesis project that are beyond the scope of the thesis. A more in depth study of the sponges within each intertidal, subtidal and geothermal subtidal zone is required to further establish the variations within *C. celata*. Also a wider area of sampling would be required to gain a more representative insight of spatial deviation throughout *C. celata* in all of New Zealand.

As interactions with the micro-environments have the potential to be the greatest impacting factor for *Cliona celata* chemistry, an *in situ* investigation of the surrounding environment and interactions for each individual sponge would be required. Also from this, an investigation into the predominant interacting organisms would also be required to determine whether the sponge is sequestering compounds from those organisms [20]. This also leads into an investigation of the type of symbionts within the sponge and whether they are actually producing secondary metabolites rather than the sponge itself.

Previous studies have indicated that the production of secondary metabolites can be in higher quantities or that different metabolites may be produced during different seasons.[23,46,76,87] Thus it would be essential to investigate whether temporal variation is a factor for *Cliona celata* by sampling specimens during each season/month.

As the knife damage simulation did not produce the chemical defence response expected, other damage techniques should be conducted, such as microbial inoculation. With a constant attack and rather than removing a piece of sponge that it would need to repair, the sponge may be more likely to defend itself and produce a secondary metabolite. A sponge palatability to predators test should also be conducted to determine whether that has any effect on metabolite production, as there have been evidence that chemical defences are less effective with high quality tissue.[83]

The brominated compounds and any other secondary metabolites discovered need to be subjected to bioactivity assays, for example, anti-microbial, anti-inflammatory and anti-cancer assays. The *C. celata* crude extracts did not show bioactivity against the HeLa cells, however other assays should be conducted to rule out the presence of bioactive metabolites. The role of bioassays gives an insight into how the secondary metabolite may potentially be utilised.

The identification of *Cliona celata* was determined by classical morphological techniques, *in situ* identification and spicule mounts. However, in recent years other techniques, such as genetic markers, have been shown to classify sponges with a higher accuracy.[23] For example, molecular phylogeny helped with the differentiation of the classes, Homoscleromorpha and Demospongiae.[11,12] Thus, it may be desirable to utilise this type of technique for future marine sponge studies.

Any novel compounds discovered will need to be isolated and identified. This is essential for the natural product industry to ensure that compounds found are novel and patents against the structure can be established, so the production of a natural product is protected. Techniques for isolation and identification may include standard chromatographic techniques and NMR spectroscopy.

As New Zealand has high biodiversity, there are multiple marine sponge species that have yet to be studied. Therefore this experiment should be repeated on different species. This may lead to not only the discovery of a natural product but also interspecific and intraspecific variations and patterns of marine sponges in New Zealand,[47] contributing to the understanding and ecological importance of marine sponges to the marine ecosystem.

CHAPTER 7

METHODS

7.1 Field Methods

7.1.1 Intertidal methods

Intertidal sponge sampling was carried out by swimming from the shore to the north eastern side of Leisure Island (approximately ten minutes swim) in snorkelling gear with required sampling equipment and diving down 2-3 m until a suitable site with multiple *Cliona* sponges was located. A GPS reading was taken to ensure relocation of samples could occur for the later time periods. Samples were collected by free diving down and removing the encrusting sponge with a knife. Sample size was dependent on the overall size of the sponge but was typically between 50-300 g wet weight. Once the subsample of sponge was removed, it was placed into a labelled, sealable sampling bag as quickly as possible to limit exposure to oxygen and all water was removed from the bag. Each *Cliona* sample was bagged separately. Samples were placed into a catch bag while other sampling occurred. Once back on shore, samples were placed on ice until they could be frozen in the laboratory, this was for approximately 2-3 hr, during transportation from Tauranga to Hamilton. The samples were then stored in a chest freezer at -20°C.

This method was used for both the preliminary chemistry experiment and damage experiment of intertidal *Cliona celata*. The two preliminary *Cliona* samples collected in 2014 for initial chemistry readings were:

1. A sample with a mixture of healthy and damaged tissue (84 g)
2. A healthy/clean individual (179 g)

Four sponges were then sampled in 2015 for the damage experiment, these were as follows:

1. A healthy/clean individual (55 g)

2. An individual with approximately 50% red algae coverage (245 g)

3. An individual with approximately 90% black algae coverage (296 g)

4. An individual with red seaweed imbedded into approximately 50% the sponge (126 g).

These sponges were collected using the methods described above. However, after the initial subsample for each sponge was collected (time 0 hr) a section of the remaining *in situ* sponge was damaged using a knife to create a crosshatch pattern approximately 2-3 cm deep into the flesh of the sponge. This sponge section was collected 24 hr post damage and then sampling was repeated at 48 hr.

7.1.2 Subtidal Method

Subtidal *Cliona* samples were collected from various sites around Rabbit Island (Coastal Marine Field Station: Site 1, Site 2 and Site 3) and one sample was collected from White Island, which were all accessible by boat requiring SCUBA diving collections. Damage and stratification experiment sponges were collected from the Rabbit Island Site Three. One diver collected the sponge samples and the other recorded all relevant information including photo/video, GPS location, any prominent features, depth, morphology and *in situ* colour. Sample size of the sponge varied depending on the size of the parent sponge but was typically between 50-300 g wet weight. Each sponge was cut with a knife to approximately 2-3 cm deep and 5-10 cm diameter (again sample size was dependent on size of parent sponge). The subsample was removed then placed in a labelled bag and stored in the catch bag until the dive trip was concluded. A new bag was used for each sample. At the surface, water was removed from the bags and they were placed on ice. Samples were then frozen at -20°C in a chest freezer in the laboratory until chemical analysis could be conducted.

Using the previously described methods, investigative chemistry samples were collected in 2014. These included four samples from each of the Rabbit Island sites and one from White Island:

Rabbit Island Site Three

1. Two thinly encrusting healthy individuals with minimal epibiont coverage (55 and 69 g)
2. A thinly encrusting individual with approximately 50% epibiont coverage (42 g)
3. A thick and healthy individual with minimal epibiont coverage (113 g)

Rabbit Island Site 1

1. An individual with epibiont coverage (93 g)
2. An individual with black colouration to the interior and exterior to approximately 33% of the sample (18 g)
3. A clean/healthy individual with minimal epibiont coverage (59 g)
4. A clean/healthy individual (55 g)

Rabbit Island Site 2

1. Two thick healthy individuals with minimal epibiont coverage (135 and 53 g)
2. A thinly encrusting healthy individual with approximately 25% epibiont coverage (47 g)
3. A healthy individual with approximately 33% epibiont coverage (53 g)

White Island

1. A healthy/clean individual (141 g)

The damage/stratification experiments utilised samples collected from the Rabbit Island Site Three only. Four large sponges were sampled with two subsamples collected from either end of each sponge:

1. Two subsamples from a healthy thick individual with approximately 33% red epibiont coverage (125 and 101 g)
2. Two subsamples from a clean/healthy individual (90 and 125 g)
3. Two subsamples from a healthy individual with approximately 33% epibiont coverage (128 and 7 g)

4. Two subsamples from a healthy thick individual with epibiont coverage to one side (115 and 105 g)

These samples were collected using the previously described methods and then recollected 48 hr after the initial sampling. The second set of subsamples was removed from the damaged/cut section of the sponge.

7.2 Laboratory Methods

7.2.1 Extraction process

A representative subsample of each sponge (pinacoderm and body of sponge) (2 g) was manually crushed using a spatula then extracted in methanol: dichloromethane (3:1) (70-80 mL) using a stick blender (Ultra-turrax) (8000-9500 min^{-1}). The extract was filtered under vacuum through a Buchner funnel. This procedure was repeated on the unfiltered material until the liquid extract was colourless, (typically 3-4 times and 210-240 mL solvent total). Any remaining unfiltered material was discarded (Figure 7.1).

Figure 7.1: Crude extraction process of *Cliona celata*.

Liquid extracts were combined and solvents removed using a rotary evaporator with a water bath at 35°C.The crude extracts were placed into scintillation vials. The vial were placed on a heating block (approximately 25-30 °C), and solvent was removed under a gentle stream of nitrogen gas. Vials were re-weighed at room temperature and stored in a cold room until needed for chemical analysis.

7.2.2 LCMS procedure

Liquid Chromatography Mass Spectrometry (LCMS) is a technique that has the ability to separate out each sample at the same time as determining the compound masses. To achieve this, the dried sample was rehydrated with HPLC grade MeOH (500 µL) and filtered through a LabServ Millipore filter (0.2 µm). HPLC grade MeOH (2 x 250 µL) was run through the filter, total of 1 mL solvent (Figure 7.2). Each sample was then analysed on a C_{18} column (Reversed Phase; Phenomenex, Luna 5 µ C18 100 Å, 150 x 4.60 mm) with a 0.2 mL/min flow rate and a column temperature of 25 °C. Using a standard gradient with two solvents (A: water (H_2O) and B: Acetonitrile (ACN)) (HPLC grade solvents) each with 0.05% Trifluoroacetic acid (TFA) added. Detection was via a DIONEX UltiMate 3000 diode array detector coupled with an electrospray ionisation (ESI) ion trap mass spectrometer. The instrument was controlled using HyStar (Bruker Daltonics).

Figure 7.2: Crude extracts of *Cliona celata* samples, prepared for LCMS analysis.

Table 1: LCMS Gradient Profile of *Cliona celata* crude extracts

Time (min)	Solvent A (%) (Acetonitrile)	Solvent B (%) (deionised H_2O)
0	10	90
2	10	90
27	100	0
39	100	0
45	10	90

7.3   Bioassay

Bioassay on *Cliona celata* was completed by students Geoffrey Tait and Richard Hailes using the following method. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) bioassay was used to determine whether healthy and damaged sponge samples were cytotoxic to HeLa cells. The MTT assay was to determine mitochondrial dehydrogenase activity, as described by Mosmann.[79] The MTT assay is based on the mitochondrial dehydrogenases of healthy cells reducing soluble yellow MTT to insoluble purple formazan product, unhealthy or deceased cells cannot perform this reduction [79].

7.4   Spicule Mounts

A representative sample of sponge (approximately 0.2 g) was finely diced using a scalpel and placed in a 10 mL Falcon tube. Concentrated nitric acid (0.5-1 mL) was added to each sample and mixed. The sponge was left overnight to digest, allowing organic matter/spicules to settle to the bottom of the tube. Nitric acid was carefully removed (not to disturb the pellet) and each sample was washed with water (1 mL). The sample was again allowed to settle and water was removed, the water wash was repeated three times. Ethanol (1 mL) was added to each sample, mixed then allowed to settle. The majority of ethanol was removed with a pipette. The remaining pellet/ethanol mixture was pipetted (avoiding any large amounts of organic material) onto a glass microscope slide in a very thin layer, ethanol was allowed to evaporate. Once the slide was dry, Sigma-Aldrich Canada balsam or depex (sample mountants) in xylene was

carefully added to the slide, a glass cover slip was placed over the sample. Spicules were observed and photographed using x100 magnification on an Olympus 2 microscope.

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APPENDICES

Appendix 1: Preliminary Investigation from Summer Research Scholarship and Chemistry Special Topics, Webb 2012.

A single specimen of *Cliona celata* was collected from Rabbit Island (subtidal), during the summer of 2011/2012. The chemistry of the marine sponge, *Cliona celata* was investigated. LCMS analysis of crude extracts of *C. celata* indicated it contained a series of bromine containing compounds.

The separation of these bromine containing compounds from a *Cliona celata* crude extracts was conducted. This involved the fractionation of the sample on a C_{18} silica column and a LH-20 Size Exclusion column. These samples were run through the LCMS to determine the retention time, UV and mass spectra of each brominated compound. It was found that two monobrominated compounds and one dibrominated compound were present in the *Cliona celata* marine sponge.

A search through the MarinLit database was conducted for each brominated compound found in the sponge. The search determined the compounds as novel. That was the extent of the Chemistry Special Topics investigation. However further work was required to determine the ecological significance and bioactivity of the compounds, hence this thesis.