Novel New Zealand marine bioactive natural products for the control of Psa biovar 3

A thesis submitted in partial fulfilment

of the requirements for the degree

of

Master of Science (Research)

in Biological Sciences

at

The University of Waikato

by

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The University of Waikato

2016

THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

“Every country has three forms of wealth – material, cultural and biological. The first two we understand well because they are the substance of our everyday lives. The essence of the biodiversity problem is that biological wealth is taken much less seriously. This is a major strategic error, one that will be increasingly regretted as time passes” - (Wilson, 1992*).*

ABSTRACT

Natural products (secondary metabolites), produced by living organisms to confer selective advantages via chemical-ecological interactions, have had extensive use in biodiscovery programmes and commercial applications. Such compounds are a unique source of chemical and structural novelty and thus provide relevant modes of biological activity unsurpassed by synthetic compounds. The marine environment has become recognised as an important natural product resource in recent years. Increasing interest is attributed to its high biodiversity, which is complemented by an elaborate chemical diversity in marine organisms. Of particular interest are those sessile and colonial marine organisms which utilise secondary metabolites for chemical defence.

Marine natural products represent unexplored potential for control of the causal agent of bacterial canker of kiwifruit, *Pseudomonas syringae* pv. *actinidiae* (Psa) biovar 3. Very few options are available to control the highly virulent pathogen, hence there is an urgent need to find alternative products which kill or inhibit Psa. Previous natural product discovery programmes informed the chance of discovering 'hit' activity is low. To increase the chance of success, this study focused on previous biomedicinal work examining related organisms with known activity against *Pseudomonas aeruginosa* and bacterial quorum sensing processes (known as ecologically-guided discovery).

The study sought to identify bioactive compounds from marine organisms with biological activity against *P. syringae* pv. *actinidiae* biovar 3. Nineteen New Zealand

macroalgae specimens collected in summer and winter were screened for preliminary biological activity against Psa biovar 3. Five candidates (26 percent) were identified with 'hit' activity; of which 16 percent is attributed to those targeted for screening and 10 percent is attributed to those selected at random. The secondary screening phase identified the presence of four brominated ions in *Vidalia colensoi*, *m/z* 278.8, 308.8, 421.8, and 311.9, as likely to represent biologically active compounds of interest against Psa. There is currently no evidence to suggest these ions represent previously isolated compounds. Hence, *Vidalia colensoi* is the species of highest priority to further explore chemically. The elicitor screening phase informed that further considerations are to be made for an effective higher order screening method to examine the macroalgae for elicitor activity against Psa biovar 3.

In summary, the findings of this study suggest than ecologically-guided discovery approach effectively directed the selection of species with 'hit' activity against Psa biovar 3 and thus fast tracked the initial discovery phase. Secondly, the discovery of four previously unidentified brominated ions with potential biological activity against Psa suggests they may represent structurally novel compounds with commercial patentability and marketability. Lastly, the elicitor screening findings provides important preliminary information for optimisation of higher level screening process to explore elicitor activity as a mode of action against Psa biovar 3.

ACKNOWLEDGEMENTS

First and foremost, I would like to acknowledge my small supervisor army comprising of Professor Chris Battershill, Associate Professor Michèle Prinsep, Dr. Michael Clearwater, and Dr. Joel Vanneste.

Firstly, Chris. Chris is the great man behind all the great ideas. His commitment and spark for projects such as this, which explore completely novel ideas at the risk of 'failure', is a prime example that there is no great science without great passion. Perhaps his next venture could be to create a carbon copy of himself; we need more big thinkers like Chris to create more magical ideas.

Secondly, Michèle. Finding a supervisor with as much expertise as Michèle is like finding a needle in a haystack. Her interdisciplinary scientific mind constantly challenged and encouraged me to ask the bigger picture questions. She truly is the cog in the wheel which will continue to drive the future success of this project.

Thirdly, Mike. Mike was the glue holding the project (and at times a very stressed student) together. His open-door policy provided me with some of the most invaluable knowledge, advise, reassurance, and clarification. Sometimes, all I needed were a few words with Mike to keep my mind focused on the path ahead.

Lastly but certainly not least, Joel. Joel was 'icing on the cake' for this research, or in other words the 'cotyledon on the organically sourced soybean'. His passion and excitement for which he talks about *Pseudomonas syringae* pv. *actinidiae* was nothing short of inspiring. Joel's world leading knowledge and expertise within this area bridged many of the gaps between marine natural product chemistry and plant pathology.

Secondly, I would like to acknowledge both the technical and financial assistance received throughout the duration of my research. A special thanks to Janet Yu and Dierdre Cornish from Plant and Food Research; Cheryl Ward, Colin Monk, Geoff Tait, and Lynne Baxter from the University of Waikato (Hamilton); David Culliford, Rex Fairweather, and Kim Pritchard from the University of Waikato Coastal Marine Field Station (Tauranga), and Dr. Wendy Nelson from NIWA Without the advice and support of these individuals my research would certainly not have flowed as smoothly. The financial support received from ZESPRI©, Kiwifruit Vine Health Inc, the University of Waikato, the Whakatane Historical Society, and the C. Alma Baker Trust absolutely blew me away. It truly is heart-warming to know that so many external persons could also acknowledge the importance of this research right from the get-go.

Thirdly, I would like to acknowledge my family. To Linda and Kerry Browne; it is difficult to express in a few short sentences my appreciation for your five years of endless love, guidance, support, and advice during my tertiary pathway. Above all, you have taught me to work hard, be humble, and let the success be my noise. My surrogate family, Raewyn and Clive Burnell, opened up their arms and their home in Hamilton for the past two years. It is not always easy living in a town three hours away from where I studied; however, their generosity always provided me with a full tummy and a warm bed when I needed it. Lastly, I would like to acknowledge Gabriel Anderson; a man whom always had my best interests at heart. He knew when I was internally waving a little white flag and needed a rest from study; that when my head was down at my desk writing I needed to be left alone; and above all, that I needed a like-minded best friend to support me through this process. Thanks B, the best views do always come after the hardest climbs.

This thesis is dedicated to the loving memory of Nana Joan Patricia Rawlinson.

In my name is yours. I therefore share this accomplishment with you.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Opening comments

This thesis is a study of two parts. The first half explores the issue of the causal agent of bacterial canker disease *Pseudomonas syringae* pv. *actinidiae* (Psa) biovar 3 within the New Zealand kiwifruit industry. The increasing threat of evolved pathogen resistance and/ or adverse environmental effects have decreased the efficacy of the practices currently used to control Psa biovar 3. The need to explore alternative long term and sustainable control options is overdue. Examination of biomedicinal informatics from previous marine biodiscovery programmes targeting *Pseudomonas* infections in humans and bacterial quorum sensing systems has highlighted that New Zealand's virtually untapped marine resource could fulfil this need. Thus, the other part of the study explores the possibility that New Zealand marine natural products could provide a novel solution for control of Psa biovar 3. Previous natural product discovery programmes informs that the likelihood of finding target biological activity in a first phase biodiscovery programme such as this is low and likely a lengthy process. To increase the chance of success, the research is heavily focused on an ecologically-guided approach which targets the selection of certain species with promising activity against Psa biovar 3.

1.2 Biodiscovery

'Biodiscovery' or 'bioprospecting' is the process by which attributes from nature are examined for potential commercial applications. Biological features of relevance can include chemical compounds, genes and their products, materials, and whole organisms (PMSEIC: Prime Minister's Science Engineering and Innovation Council, 2005). These can have applications for industries such as agriculture, forestry, fisheries, tourism, pharmaceuticals, crop protection, botanical medicines, cosmeceuticals, nutraceuticals, biomining, biomonitoring, bioremediation, ecological restoration, industrial biomimetics, and ecotourism (Beattie, 2005).

The term 'biodiscovery' is broadly defined. There is no universally agreed standardisation, nor are there any international agreements that specifically describe the term (Jeffery *et al.*, 2008). Some countries narrowly define biodiscovery to include only the preliminary search for biological characteristics of interest, whereas others include the development and application of such resources. Hence, the commercialisation and potential economic profitability elements of biodiscovery as an entity remain undefined in an international context (Slobodian *et al.*, n.d). As per the New Zealand Biodiversity Strategy, biodiscovery in New Zealand is loosely described as 'the search among biological organisms for commercially valuable compounds, substances, or genetic material' (New Zealand Government, 2000).

Biodiscovery as a process consists of a number of phases, from the initial collection of biological material right through to the commercialisation of a product (Slobodian *et al.*, n.d). Four major phases of biodiscovery have been described by Leary (2007) (Figure 1.1). The first phase involves the initial search and collection of biological material for potential commercial applications. The initial sample size of any collected material in this phase is usually small, and in some cases surplus *ex-situ* material collected for another purpose (which is no longer needed) can be used (Jeffery *et al.*, 2008). The order by which the second and third phases of biodiscovery occur is dependent on the objectives of the programme at hand. Phase two includes the screening of organisms for sought biological activity, and phase three includes the isolation, characterisation and production of specific compounds of interest. Lastly, the fourth phase includes further developments of the promising compound to a commercially valuable and patented product (Jeffery *et al.*, 2008; Slobodian *et al.*, n.d).

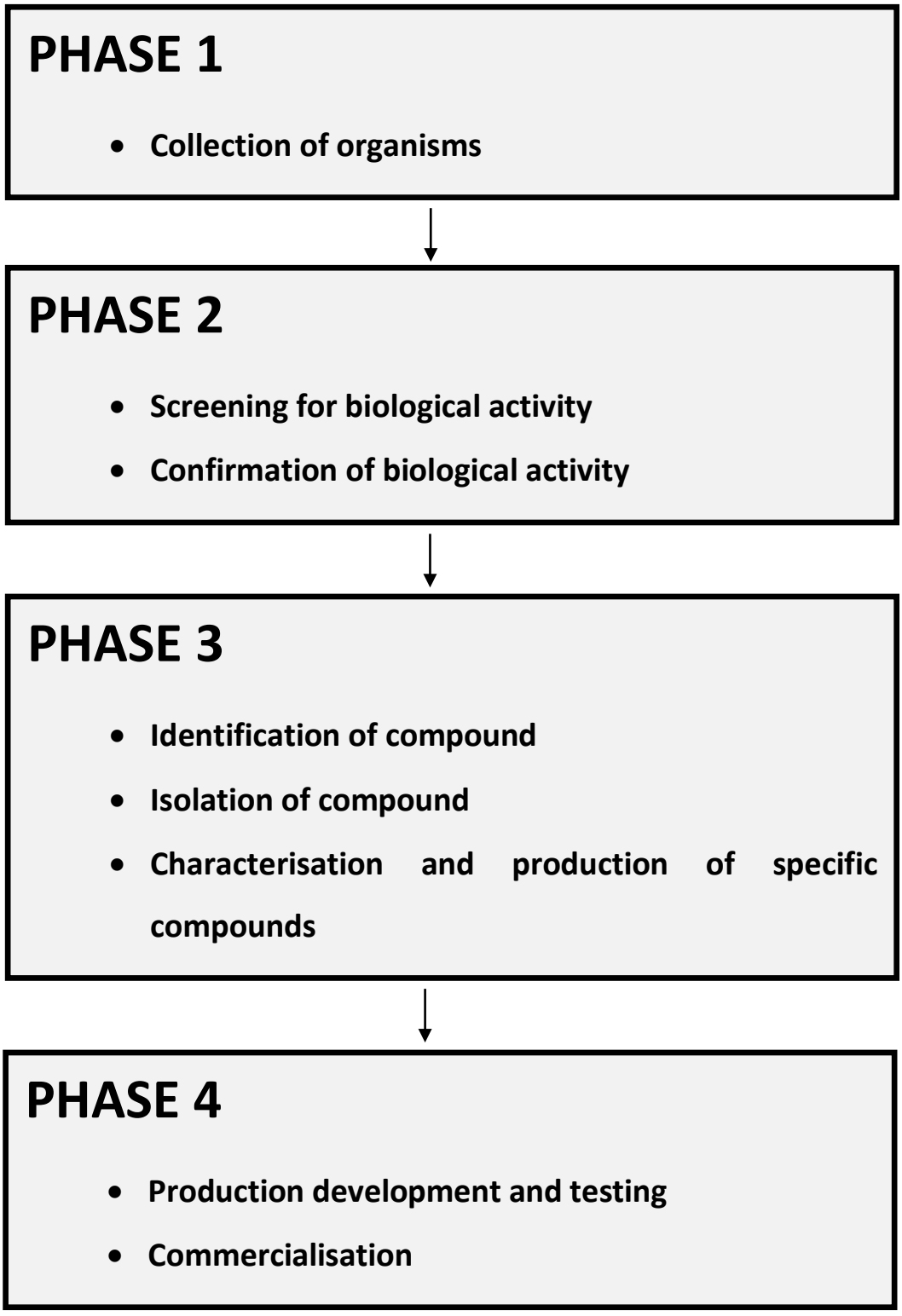


Figure 1.1. Overview of the four phases of biodiscovery. Image adapted from Leary (2007).

1.3 Natural Products

1.3.1 Role of natural products

Natural products, also referred to as secondary metabolites, are organic compounds produced by living organisms in nature (Buchanan *et al.*, 2000). Secondary metabolites are differentiated from primary metabolites in their chemical structure, function, and distribution within the plant and animal kingdom. Primary metabolites have essential functions for organism growth, development, and reproduction, whereas secondary metabolites are considered non-essential to life (Martins *et al.*, 2014). Rather, they are believed to confer selective advantages in the producing organisms through chemical-ecological interactions with other organisms in their surrounding environment (Buchanan *et al.*, 2000). These secondary metabolites can have functions as weaponry for intra- and inter- specific competition, metal transporting agents, communication agents of symbiosis, sexual hormones and pheromones, and differentiation effectors (Demain & Fang, 2000). Such roles are highly specific and thus secondary metabolites are distributed amongst limited marine and terrestrial phylogenetic groups (Buchanan *et al.*, 2000).

Natural products have been used therapeutically for thousands of years and have formed the basis of many traditional medicinal systems. The emergence of the scientific field of natural product drug research within the past century is comparatively recent (Newman *et al.*, 2000). Historical examples of early identified natural products are most notably the isolation of morphine from opium poppies (*Papaver somniferium*) first reported in 1803, and the discovery

of the first antibiotic penicillin from the terrestrial fungus *Penicillium notatum* in 1928 (Fleming, 1980). Since then, a large number of natural products have been isolated and have provided the single most productive source of leads for drug development to date (Harvey, 2008). Analysis by Newman *et al.* (2003) demonstrated that 28 percent of drugs launched into the pharmaceutical market between 1981 and 2002 were natural products or derivatives thereof. Such an extensive application is related to the characteristic chemical diversity and structural complexity of natural products which offer a rich source of structural novelty for the discovery of new classes of drugs (Hong, 2011). Furthermore, their modes of biological activity are often functionally relevant to applied targets sought in biodiscovery programmes.

1.3.2 Role of marine natural products

The marine environment has become increasingly recognised as an important and virtually untapped natural product resource in recent years. Traditional natural product biodiscovery has largely been focused on terrestrial organisms and therefore marine natural product discovery is very much an emerging field. Commercialisation of the first marine natural product dates back to the 1950s when compounds isolated from the marine sponge *Tethya crypta* led to the identification of a close analogue as an antileukaemic agent which is now marketed as Ara-C (Newman & Cragg, 2004). Commercial interest in marine natural products since this discovery has exponentially increased. In the past decade alone, numbers of approved drugs in the marine drug pipeline have more than doubled (Martins *et al.*, 2014). Increasing interest in marine natural

products is attributed to the highest biological diversity on earth being found in the marine environment (32 of the 33 animal phyla). It is therefore a location where natural product based leads are most likely to be discovered (Margulis & Schwartz, 1998; Battershill *et al.*, 2005). Furthermore, it has now been recognised that the extensive biodiversity of the marine environment is complemented by an elaborate chemical diversity in marine organisms, which have evolved to overcome the ecological pressures associated with living in such a diverse and challenging marine environment (Thakur *et al.*, 2005).

Sessile and colonial marine species, such as soft-bodied invertebrates (sponges, cnidarians, and tunicates) and algae, are a particularly rich source of potential natural product candidates (Figure 1.2). Due to their lack of physical defence structures, it is thought that these organisms survive by utilising secondary metabolites as chemical defence mechanisms. The immediate dilution of the chemicals released into the water column means that the compounds are often highly potent, targeted in their mode of action, and elicit an effect on other organisms while ensuring no harm to their own cells or biochemical systems (Haefner, 2003; Newman *et al.*, 2009). These features are of high relevance to biodiscovery and thus organisms which lack morphological defence structures are an important source to explore for novel natural product-based drugs.

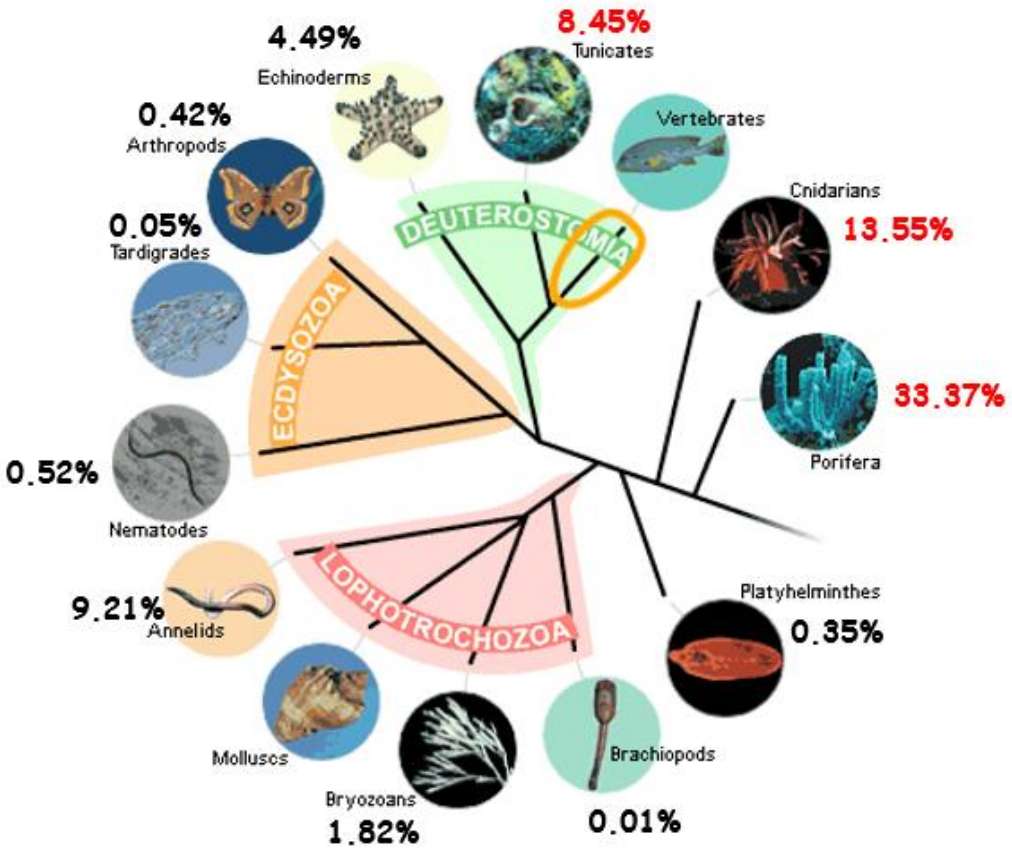


Figure 1.2. Percentage of each phyla present in the marine natural product research database 'Marinlit'. Dominating phyla (identified in red) are those which lack morphological defence structures (tunicates=8.45%, cnidarians=13.55%, porifera=33.37%). Image sourced from Munro (2013).

1.3.3 New Zealand marine natural product potential

The New Zealand marine environment represents a particularly rich and unique source of natural products. This is because the latitudinal range over which the land mass extends (sub-tropic to sub-Antarctic), combined with its long history of evolutionary isolation, has ensued a rich marine biodiversity that has a particularly high level of endemism (Gordon *et al.*, 2010; Department of Conservation, n.d). New Zealand has already contributed one marine natural product to the commercial market thus far. The drug, marketed as Halaven©, was synthesised from Eribulin, which was in turn modified from the compound

halicondrin B for use in the treatment of metastatic breast cancer. The parent molecule is a naturally occurring mitotic inhibitor with a potent mechanism of action originally found in the *Halichondria* genus of sponges (National Cancer Institute, 2012). Returns of halicondrin B from collections of a deep sea New Zealand sponge *Lissondendoryx* n.sp1, formerly identified as *Halichondria isodictyalis*, were found to be substantially higher than from its overseas relative, *Halichondria okadai* (Ledford, 2010; University of Canterbury, n.d). One tonne of *Lissondendoryx* collected by dredging, diving, and aquaculture methods enabled progression of synthesis of the compound as a more sustainable supply. (Battershill, 2016). Halaven© was approved for commercialisation in the U.S.A in 2010 after a series of pre-clinical trials and has since obtained approval in 60 countries (Eisai Co., 2015). Economic returns generated for Halaven© in 2014 were nearly NZ$467 million, and in 2015 were over $NZ600 million (Eisai Co., 2015). Less than one percent of New Zealand's extraordinarily unique marine biodiversity has been explored for its biological 'resource' potential. Thus, the story of Halaven© exemplifies the untapped economic opportunity for the discovery of novel New Zealand marine natural products (Gordon *et al.*, 2010; Department of Conservation, n.d).

1.3.4 Traditional vs ecologically-guided biodiscovery

The implementation of natural product leads in biodiscovery and development programmes, due to their perceived low success rates, has resulted in a recent decline in commercial interest over the preceding 5-10 years (Mishra & Tiwari, 2011). This is largely because previous natural product-based screening programmes failed to recognise that the sought compounds are products of evolution by natural selection in the context of ecosystem dynamics. Ecological theories were not considered relevant, nor were the emerging scientific methods associated with the concept of biodiversity (Beattie *et al.*, 2011). Subsequently, organisms were collected for screening using haphazard protocols which resulted in low success rates of relevant targets for further development (Battershill *et al.*, 2005). Furthermore, recent advances in technology-driven discovery methods has increased the pace of screening for identification of promising compounds. The cycle times of lead generation through traditional natural product based screening programmes are now considered too long or costly. As a consequence, natural products had become de-emphasised by industry until recently (Wildman, 2003).

Ecologically-guided discovery has been suggested as a method to accelerate rates of success in natural product-based screening programmes. The approach, which applies ecological theory and testable hypotheses to guide the selection of organisms to screen, is relatively novel and still in the early stages of development. Nonetheless, its importance has been increasingly recognised since Coley *et al.* (2003) showed chemical plant defence theory can increase the

probability of discovering compounds with activity in human disease targets. An increasing amount of literature has since supported the importance of this discovery approach. For example, Benkendorff *et al.* (2001) demonstrated high levels of antibiotic activity in egg masses of marine invertebrates which were previously known to have evolved chemical defence from microbial infection. There is not yet enough literature available to assess the full impacts of this approach to natural product discovery programmes. It can, however, be said that the targeted collections of biological material guided by ecological principles are a major advance on random and traditional collection methods (Benkendorff *et al.*, 2001; Beattie *et al.*, 2005). This, in turn, is likely to increase the frequency of success rates for further developments and the value of the industry as well as its resources (Beattie *et al.*, 2005).

1.4 *Pseudomonas syringae* pv. *actinidiae*

1.4.1 Global emergence

Kiwifruit (*Actinidia* spp.) crops represent a relevant economic segment of global agricultural production. In recent years, however, the viability of the global kiwifruit industry has been threatened by the causal agent of bacterial kiwifruit canker disease, *Pseudomonas syringae* pv. *actinidiae* (Psa) (Greer & Saunders, 2012). Psa was first identified, isolated, and characterised as an economically important bacterium in Japan in 1984 (Takikawa *et al.*, 1989); a short while later in 1984 in South Korea (Koh *et al.*, 1994); and in 1992 in Italy (Scortichini, 1994). It should be noted that the same disease was described in the Hunan province in China in 1984/ 1985, however, it was never validated as Psa (Fang *et al.*, 1990).

Early occurrences of Psa caused relatively minor orchard damage and had minimal economic impacts. A sudden and aggressive re-emergence of the disease, first detected in Italy in 2008, spread with devastating virulence to all major kiwifruit producing areas of the world (Scortichini *et al.*, 2012). Today, Psa is present in China, Italy, New Zealand, Japan, Korea, China, France, Portugal, Australia, Switzerland, and Turkey. The disease is now identified as the largest threat to the global production of kiwifruit (Vanneste, 2013).

Pseudomonas syringae pv. *actinidiae* is a pathogenic bacterium which colonises a kiwifruit host through infection of natural apertures (stomata, hydathodes, lenticels, trychomes), open leaf wounds, and leaf scars (Serizawa & Ichikawa, 1993). Symptoms of infection can include brown angular leaf spots surrounded by chlorotic halo, dark colouration and detachment of the floral organs (buds and flowers), flower and leaf wilting, extensive canker formation on the leaders/ canes/ trunks, cane dieback or blight in young canes, collapse of the fruit, white opalescent or dark red exudate oozing from bark, and complete plant mortality (Figure 1.3) (Vanneste *et al.*, 2011b). Symptoms are most visible in spring and autumn when temperatures are between 12–18°C. Persistent rainfall, frost, winds, and high humidity can assist the systematic movement of Psa throughout a plant (Scortichini *et al.*, 2012). Transmission of the disease can occur via infected plant material on footwear, vehicles, and animals, and by using infected orchard grafting and pruning tools (Centre for Agriculture and Bioscience International, 2015). Windborne pollen has also been suggested as a possible

vector for transmission after the pathogen was identified in pollen samples in both Italy and New Zealand (Vanneste *et al.*, 2011a).

a b c

Figure 1.3. Common bacterial kiwifruit canker symptoms associated with *Pseudomonas syringae* pv. *actinidiae*. This includes a) brown angular leaf spotting, b) oozing of dark red exudate from bark, and c) flower and leaf wilting, and cane dieback. Left image sourced from Ministry of Agricultural Food and Forestry Policies (2013). Middle and right images sourced from Everett *et al.* (2011).

Psa is taxonomically classified within the multifaceted *Pseudomonas syringae* complex which consists of more than 60 economically important pathovars (Lodovica Gullino & Bonants, 2014). Strains of Psa isolated around the globe over the past 30 years have been grouped into five populations which are distinguished by their distinct molecular, microbiological, and pathogenic characteristics (Vanneste *et al.*, 2013). These are referred to as biovars 1, 2, 3, 4, and 5 (Table 1.1).

Strains of **biovar 1** correspond to those isolated from the initial occurrence of bacterial canker disease observed in Japan in 1984 (Takikawa *et al.*, 1989) and Italy in 1992 (Scortichini, 1994). In both countries, the strain was isolated from vines of *A. deliciosa* cv. Hayward and caused minor sporadic damage. Strains of

biovar 2 have been linked to the incursion of bacterial canker disease in South Korea in 1984 (Han *et al.*, 2003a). The population has caused severe past economic losses to *A. deliciosa* cv. Hayward and at present to *A. chinensis* cv. Hort16a (Koh *et al.*, 1994; Koh *et al.*, 2010). The first outbreak of bacterial canker disease associated with strains of **biovar 3** was observed in Italy in 2008. It is suspected this strain first emerged in the province of Latina, where it spread throughout Italy before reaching France and Portugal (Vanneste *et al.*, 2011b). There is, however, now evidence to suggest that Psa biovar 3 may have originated in China around 10-30 years ago (Butler *et al.*, 2013; McCann *et al.*, 2013). The advanced bacterial canker symptoms attributed to Psa biovar 3 have caused severe economic losses in countries including Chile, China, New Zealand, France, Spain, Portugal, Japan, and Korea. Strains of **biovar 4** have been associated with bacterial canker disease in New Zealand, Australia, and France. Biovar 4 is pathogenically distinguished from other biovars; it is less aggressive and symptoms have never progressed beyond minor leaf spotting. Due to these differences (and other phenotypic, genetic, and phylogenetic differences), the taxonomic identification of biovar 4 has recently been transferred to the new pathovar *actinidifoliorum* (Cunty *et al.*, 2015). Lastly, **biovar 5** is the most recently reported strain of Psa. It was first isolated in Japan in 2005, and at present remains in a localised region. Biovar 5 has been observed to cause minor bacterial canker symptoms on vines of *A. chinensis* cv. Hort16A (Sawada *et al.*, 2014; Fujikawa & Sawada, 2016).

Table 1.1. Characteristics of biovars within the pathovar *P. actinidiae.*

Biovar	Affected countries	Virulence factor
1	Japan, Italy	High virulence toward *A. deliciosa* cv. Hayward
2	South Korea	High virulence toward *A. deliciosa* cv. Hayward
3	Italy, Chile, China, New Zealand, France, Spain, Portugal, Japan, and Korea	High virulence toward *A. chinensis* cv. Hort16A cultivar.
4	New Zealand, Australia, France	Low virulence
5	Japan	High virulence toward *A. chinensis* cv. Hort16A cultivar.

1.4.2 Psa in New Zealand

The New Zealand kiwifruit industry has been regarded as a horticultural success story (Vanneste, 2012). It is New Zealand's single largest horticultural export industry by both volume and value, and in 2015 was valued at NZ$1.015 billion (Ministry for Primary Industries, 2015). Targets to further develop the industry to NZ$3 billion by 2025 were dealt severe blows upon the incursion of Psa in 2010. Symptoms of the disease were first isolated from vines of *A. chinensis* cv. Hort16A and later from *A. deliciosa* cv. Hayward in a Te Puke orchard in the Bay of Plenty region. The symptoms comprised of brown angular leaf spotting and the wilting and browning of flowers. A number of shoots showed blackening and terminal wilting. In some cases, red exudate and young cankers were observed on infected vines of the highly susceptible *A. chinensis* cv. Hort16A (Everett *et al.*, 2011).

Genomic analysis of Psa infected kiwifruit vines in New Zealand has identified the presence of both Psa biovar 3 and 4. Psa biovar 4 is of low economic importance to the New Zealand kiwifruit industry. As previously mentioned, it is no longer classified as *Pseudomonas syringae* pv. *actinidiae* and is now pv. *actinidifoliorum*. Psa biovar 4 has a low virulence factor and somewhat trivial leaf spotting symptoms when compared to biovar 3 (Vanneste *et al.*, 2013). Due to the similarity of bacterial canker kiwifruit symptoms of biovar 4 with those caused by other *Pseudomonas* spp., combined with the lack of pre-existing bacterial diseases in New Zealand, it is thought biovar 4 may have been present in New Zealand for many years prior to its 'named' detection (Vanneste *et al.*, 2013; Cunty *et al.*, 2015). This hypothesis is supported by the relatively wide distribution of biovar 4 throughout New Zealand's North and South Island by December 2010, shortly after Psa was first detected (Vanneste *et al.*, 2013).

In comparison, Psa biovar 3 is of high economic importance to the New Zealand kiwifruit industry. Its high level of virulence and advanced bacterial canker symptoms are expected to cause the industry losses of NZ $310–410 million between 2012 and 2017 (Greer & Saunders, 2012). Within the first two years of the detection of Psa, biovar 3 had spread to 43 percent of New Zealand's total productive canopy area. By December 2015, this had increased to 89 percent (Figure 1.4) (Greer & Saunders, 2012; Kiwifruit Vine Health, 2015a). On account of its huge impact over such a short period of time, it is unlikely that biovar 3 would have been present in New Zealand for much longer than before it was first detected in November 2010 (Vanneste *et al.*, 2013).

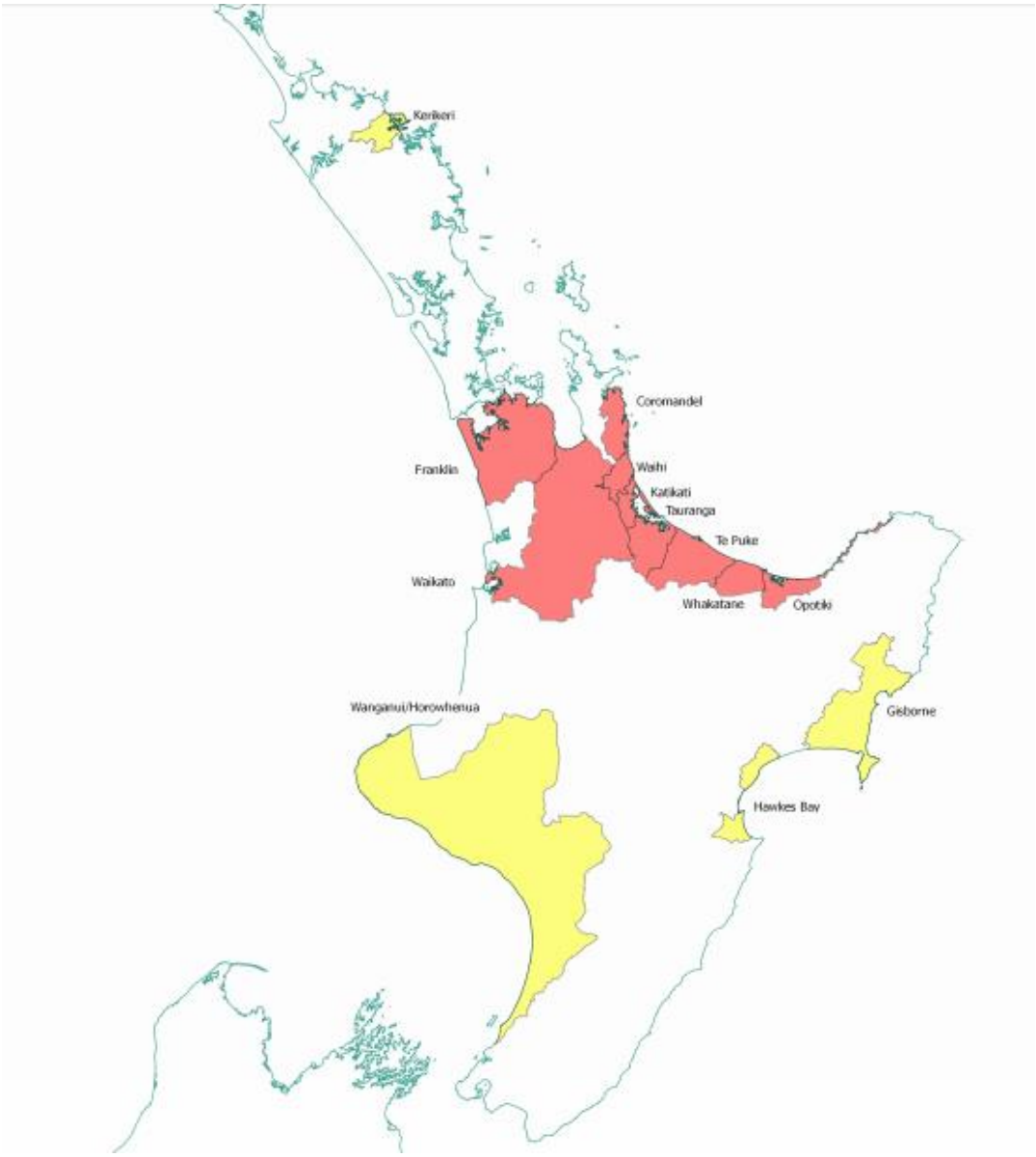


Figure 1.4. Psa biovar 3 infected regions of New Zealand as at December 2015. Red highlighted regions indicate heavily infected areas and yellow highlighted regions indicate areas with limited infection. Image sourced from Kiwifruit Vine Health (2014).

The rapid spread of Psa biovar 3 to the majority of kiwifruit producing regions of New Zealand is largely attributed to the current lack of effective control options available. On a global scale there is no current curative treatment for Psa. Thus, very little can be done to prevent the spread of Psa biovar 3 throughout a plant once it has been infected (Vanneste, 2013). There are, instead, a range of short-term preventive measures used by New Zealand kiwifruit growers to preclude bacterial canker infection. Copper- and antibiotic- based sprays are currently considered most effective by the industry. They have been shown to reduce proportions of infected leaves from 44 percent to 15 percent for the copper treated vines and by 4-7 percent for the streptomycin treated vines (Serizawa *et al.*, 1989). Their efficacies have, however, been reduced by the potential risk of Psa building antibiotic resistance to streptomycin (Han *et al.*, 2003b) and copper resistance (Goto *et al.*, 1994). Furthermore, the indiscriminate use of copper can lead to long term accumulation in New Zealand soils (Morgan & Taylor, 2004) and/ or cause phytotoxic kiwifruit plant injury (Serizawa *et al.*, 1989).

Products containing active elicitor molecules are currently used by New Zealand kiwifruit growers to stimulate plant natural defence mechanisms against Psa biovar 3. Their application has proven effective in preventing infection during seasons of high susceptibility. For instance, the foliar application of the elicitor product Actigard™ during prolonged rain events in spring has been shown to reduce leaf spotting infection on kiwifruit, and was particularly effective when used in combination with copper (Zespri & Kiwifruit Vine Health, 2015). However, the efficacy of this control is limited by the timeliness of application required to

stimulate optimal plant elicitation (10 days) (Cellini *et al.*, 2014). Excessive frequencies and/ or rates of application can also have adverse effects on kiwifruit plant growth (Zespri & Kiwifruit Vine Health, 2015).

Actinidia chinensis cv. Gold3 was introduced to the New Zealand kiwifruit market in 2012 to provide the industry with a form of resistance against Psa biovar 3. Prior to the New Zealand incursion of Psa in 2010, the Gold3 cultivar was used in a pre-commercialisation trial to widen the market window for New Zealand kiwifruit. Shortly after the discovery of Psa in New Zealand, screening for Psa resistance on the Gold3 trial orchard demonstrated it was tolerant. Subsequently, commercialisation was accelerated and in 2012 more than 2,000 grafts of Gold3 were released to New Zealand growers (Plant and Food Research, 2012). The efficacy of Gold3 as a control option is limited as it is not truly resistant to Psa. Growers are still required to use other measures for vine protection. Rather, it offers a better form of protection against biovar 3 than the highly susceptible Hort16a cultivar. The problem remains that if Psa were to evolve with virulence against Gold3, then the lack of genetic diversity amongst New Zealand kiwifruit cultivars leaves the industry at risk of a recurring pandemic.

Mechanisms which interact with bacterial quorum sensing processes have been identified as a novel strategy to control *P. syringae* pv. *actinidiae* in the New Zealand kiwifruit industry. Quorum sensing is a well-established phenomenon which enables bacterial populations to coordinate the expression of various traits in a cell-density-dependent manner. It is based on the production and

release of chemical signal molecules called autoinducers to the surrounding environment. Detection of a minimal threshold stimulatory concentration of an autoinducer leads to an alteration in gene expression, resulting in the activation of target genes specific to the needs of the bacteria (Summerfield, 2012). Quorum sensing in *Pseudomonas syringae* has been shown to regulate traits which contribute to epiphytic fitness and stages of disease development during infection (Quinones *et al.*, 2005). Molecules which interfere with bacterial quorum sensing in *P. syringae* pathogens such as Psa could therefore be used to prevent bacterial colonisation and subsequent invasion.

1.4.3 New Zealand marine natural products to control Psa biovar 3

There is evidence to suggest that certain marine organisms produce secondary metabolites with potential biological activity against *P. syringae* pv. *actinidiae* biovar 3. Examination of biomedicinal informatics demonstrates that there are certain marine species which produce metabolites with antibacterial activity when screened against the human pathogen *Pseudomonas aeruginosa* and/ or antimicrobial activity resulting in the inhibition or disruption bacterial quorum sensing processes (Solomon & Santhi, 2008; Patil *et al.*, 2010; Taylor, 2011; Bianco *et al.*, 2013). These studies provide chemotaxonomic chemical or process clues of high relevance to marine species which are likely to produce compounds of relevance for control of Psa biovar 3. This is the essence of ecologically-guided discovery and thus provides direction as to which species are of highest priority to explore in the following screening phases.

The discovery of anti-Psa bioactive compounds produced by a marine organism is of high relevance to the New Zealand kiwifruit industry. The marine origin of the natural products means that terrestrial systems would not have previously come into contact with such molecules. Thus, they are more likely to elicit strong responses and exclude pathogenic resistance; a problem which is threatening to reduce the efficacy of the current antibiotic- and copper-based spray products. Their organic nature also means the environmental effects from their use are likely to be minor when compared to current control measures. Furthermore, because the New Zealand marine environment is a particularly rich source of unique natural products, the target metabolites are more likely to be novel for commercial marketability and patentability (Battershill, 2016).

1.5 Purpose of research

The purpose of this research is to identify bioactive compounds from marine organisms which result in a decrease of incidence, inhibition, or complete kill of the causal agent of bacterial canker disease of kiwifruit, *Pseudomonas syringae* pv. *actinidiae* biovar 3. The selection of certain species for examination is addressed using an ecologically-guided discovery approach which enables the development of two key hypotheses relating to which marine species are likely to elicit compounds of relevance. The two hypotheses are:

1. Marine species or close relatives which produce secondary metabolites active against *Pseudomonas aeruginosa* (when screened in previous studies) will also produce metabolites with activity against the causal

agent of bacterial canker in New Zealand kiwifruit, *P. syringae* pv. *actinidiae* biovar 3.

2. Marine species or close relatives which produce secondary metabolites with quorum sensing inhibition/ disruption activities (when screened in previous studies) will also produce metabolites with activity against the causal agent of bacterial canker in New Zealand kiwifruit, *P. syringae* pv. *actinidiae* biovar 3.

The search for marine species with compounds of relevance against *P. syringae* pv. *actinidiae* biovar 3 is outlined in the following three experimental chapters.

- **Chapter One** outlines the examination of New Zealand marine species for compounds with biological activity against Psa biovar 3 by a series of *in vitro* screens from small scale extracts and liquid chromatography-mass spectrometry (LC-MS) analysis. Specifically, this chapter explores the hypotheses that the targeted selection of certain marine species driven by ecologically-guided discovery will increase the likelihood of the 'hit' rates of success.

- **Chapter two** outlines the examination of selected 'hit' marine species with promising biological activity against Psa by a series of *in vitro* screens from larger scale extracts and their fractions produced from subsequent fractionation, as well as LC-MS analysis.

- **Chapter three** outlines the examination of two preliminary *in vivo* elicitor bioassays to provide insight for an effective screening method which can be used to examine New Zealand marine species for compounds with elicitor activity against Psa biovar 3.

1.6 Environmental impact and permission

To ensure that this research is conducted in an environmentally sustainable manner, no marine species will be collected from a particular location where they were found to be rare and areas of collection will remain in their prior state. Previous work by Battershill *et al.* (2005) has shown that up to half of a marine organism can be excised without causing mortality which allows the lost somatic material to be regrown. Therefore, collections will be made such that the half of the organism is left intact.

Pseudomonas syringae pv. *actinidiae* biovar 3 is classed as an unwanted organism due to its pathogenicity and destructive nature on the New Zealand kiwifruit industry. Permission has been granted by the Ministry for Primary Industries (MPI) under section 52 and /or section 53 of the Biosecurity Act 1993 relating to Unwanted Organisms to work with Psa biovar 3 in the enhanced PC1 or PC2 labs at the University of Waikato and Plant & Food Research.

CHAPTER 2

PRELIMINARY SCREENING

2.1 Introduction

The preliminary screening component of this study encompasses elements of the first and second phases of biodiscovery (Figure 1.1). As previously mentioned in Section 1.2, the order by which phases two and three occur is dependent on the scope and objectives of the specific biodiscovery programme at hand. The focus of this research is to identify bioactive compounds from marine organisms which have biological activity against Psa biovar 3. Therefore, the screening of organisms for target activity (phase two) will occur prior to the identification, isolation, characterisation and production of any specific biologically active compounds (phase 3); a phase which extends beyond the scope of this thesis.

The collection of organisms to screen for relevant anti-Psa activity is performed using both an ecologically-guided (targeted) approach and a traditional (random) approach. Targeted collections of certain species is based on data from related organisms producing compounds with relevant biological activities in previous biomedicinal screens. The following paragraphs will discuss the considerations, motives, and rationale toward the selection of marine macroalgae as the first marine candidates for preliminary screening. It will also discuss in further detail the two hypotheses guiding the selection of certain species.

2.1.1 Sustainable supply considerations

Sustainable supply is an issue frequently encountered in marine natural product discovery programmes. Marine organisms have relatively low natural abundances of secondary metabolites (Brahmachari, 2015). This not generally an issue in the initial phases of discovery as only small amounts of biomass are required to screen organisms for target biological activity. The problem arises in later phases of discovery, once the target compound has been isolated and a biomass increase of several orders of magnitude is required to generate enough pure compound for its progression as a lead (Kingston, 2011; Dias *et al.*, 2012). If the natural product undergoes trials and reaches the point of commercialisation, then multi-kilogram quantities of the pure compound will be required to supply market production. This, in turn, corresponds to multiple tonnes of target organism required per annum (Brahmachari, 2015). Some biodiscovery programmes have adequate supplies of the natural product to reach commercialisation (Kingston, 2011). However, if this supply is not maintainable then the issue of overharvesting to supply the market can create wider ecosystem effects (Sandhu, n.d; Slobodian *et al.*, n.d). Hence, the sustainable supply of a natural product at a scale large enough to fulfil the needs of the both later phases of discovery and commercial application is a major issue, and is one which increases as a natural product progresses through the commercialisation pipeline. The future sustainable supply is therefore a key consideration in this study and is one which influences the initial selection of marine candidates.

With consideration to the issue of sustainable supply arising in later phases of this research, macroalgae are the first marine candidates selected to screen for biological activity against Psa biovar 3. Macroalgae are primary producers. They contain photosynthetic pigments which use available energy from the sunlight to convert carbon dioxide and nutrients into living biomass (Redmond *et al.*, 2014). The distribution and abundance of macroalgae is therefore determined by sufficient amounts of sunlight to drive photosynthesis, a firm substratum for attachment within the photic zone, and sufficient nutrients to enable growth (Lewis, 1964). The nutrient rich waters and expansive rocky shores of New Zealand's marine coastal environment provides optimal growth conditions for marine macroalgae. Hence, they are a relatively sustainable organism to explore for biological resources. Furthermore, the combined tropical, subtropical, and sub-Antarctic water current surrounding New Zealand's coastlines provides a rich selection of macroalgae species to select and screen (Wassilieff, 2012).

Aquaculture, or the farming of aquatic organisms, has been suggested as an approach to resolve the issue of supply in natural product discovery and commercialisation programmes (Martins *et al.*, 2014). Macroalgae cultivation is a well-established global industry which provides a sustained, consistent, and long term supply of algae for use in agars, alginates, carrageenans, therapeutics, agricultural fertilisers, fish feed, human consumption, and cosmetics. There are two primary methods of macroalgae cultivation. Extensive cultivation generally occurs in the wild to utilise the natural surrounding environmental conditions, whereas intensive cultivation occurs in an artificial environment for the control

of certain growth parameters. The method of cultivation is dependent on the needs of the macroalgal species and how the yields of target products may be optimised. Often both forms are harnessed in natural product aquaculture; land based pond culture and target metabolite refinement/yield enhancement can occur after a sea based phase for optimal biomass production. With these considerations in mind, macroalgae culture could potentially provide a sustained and long term supply of target metabolite for any future developments of this research.

2.1.2 Ecologically-guided selection of macroalgae species

The following paragraphs will discuss the theories behind the two hypotheses guiding the selection of certain macroalgae species for preliminary screening.

2.1.2.1 Hypothesis one: Antibacterial activity against *Pseudomonas aeruginosa.*

Therapeutic drugs have provided essential tools for the prevention and treatment of infectious human diseases throughout the past century. Paradoxically, despite their importance in modern medicine, their indiscriminate and somewhat imprudent use throughout this time has attributed to the widespread emergence and dissemination of globally resistant bacterial strains (Wright, 2015). Drug resistance is now emerging at a faster rate than we are sourcing novel therapeutic agents. It has now developed to every major antibiotic drug class (Debabov, 2013). Subsequently, commercial industries have

highlighted the importance of the potential resource of marine natural products and their desirable biological features for the development of novel drugs (see Section 1.3 for further details) (Bianco *et al.*, 2013).

Pseudomonas aeruginosa is one particularly problematic human pathogen. The bacterium can tolerate a variety of environmental conditions, which has facilitated its perseverance as a major nosocomial pathogen of immunocompromised and debilitated patients (Lister *et al.*, 2009). Selection of the most appropriate antibiotic for treatment has been complicated by the fact that *P. aeruginosa* is both intrinsically resistant to a variety of antimicrobial agents and can develop resistance during the course of treatment (Poole, 2011). This problem is further compounded by the emergence of multidrug-resistant strains of *P. aeruginosa*, which have severely limited the availability of therapeutic options (Porras-Gómez *et al.*, 2012). Consequently, there is an urgent need to develop novel therapeutic drugs for the treatment of *P. aeruginosa*.

Biomedicinal research examining marine organisms as novel drug leads for the treatment of *P. aeruginosa* has identified a range of marine macroalgae which produce metabolites with targeted bioactivity against the pathogen. Observations from these studies provide insight as to which species or their close New Zealand relatives may produce compounds with relevant activity for control of the *Pseudomonas* pathogen of relevance to this study, Psa biovar 3. Thus, New Zealand macroalgae species targeted for screening include; the red alga*, Vidalia*

colensoi (previously recognised as *Osmundaria colensoi*) based on research from Bianco *et al.* (2013) examining *Vidalia obtusiloba*; the brown alga, *Dictyota kunthii* based on research by Solomon and Santhi (2008) examining *Dictyota acutiloba*; the green alga, *Ulva* sp. based on research by Patil *et al.* (2010) examining *U. intermedia*; and the red algae, *Pterocladia lucida* and *Gigartina atropurpurea* based on research by Taylor (2011) examining *Pterocladia capillacea* and *Gigartina tenella.*

2.1.2.2 Hypothesis two: Inhibition/ disruption of bacterial quorum sensing processes

Quorum sensing is a term used to describe an intercellular communication system which enables bacteria to regulate gene expression in response to fluctuations in cell-population density (Quinones *et al.*, 2005). Quorum sensing was first discovered by Nealson and Hastings (1979) in the marine bacterial species *Aliivibro fischeri* (previously recognised as *Vibrio fischeri*), and has since been studied in a variety of bacteria (Miller & Bassler, 2001). Quorum sensing bacteria produce and release chemical signalling molecules called autoinducers which increase in concentration as a function of cell density. The autoinducers accumulate in the surrounding environment and, upon the detection of a minimal threshold stimulatory concentration by bacterial cell receptor, activate an alteration in gene expression (Summerfield, 2012).

There are a multitude of quorum sensing systems which have been detected since quorum sensing was first discovered. The majority of Gram-negative

bacteria (such as *Pseudomonas syringae*) use the LuxR-LuxI homologous quorum sensing system containing the regulatory proteins, LuxI and LuxR. The luxI homologue is involved in the biosynthesis of a specific autoinducer, acylated homoserine lactone signalling molecule (AHL). At a certain threshold AHL concentration, the autoinducer interacts with the receptor LuxR homologue. In conjunction with the RNA polymerase, the LuxR-autoinducer complex then promotes or represses the transcription of target genes (Figure 2.1) (Hentzer & Givskov, 2003; Summerfield, 2012). As stated by Hentzer and Givskov (2003), there is now evidence to suggest many opportunistic Gram-negative pathogens use quorum sensing as a regulatory mechanism for the control of virulence through the formation of biofilms (self-produced polymeric matrices). These links suggest that virulent Gram-negative bacteria such as *P. syringae* could become non-pathogenic via the inhibition or disruption of their quorum sensing communication processes (Hentzer & Givskov, 2003).

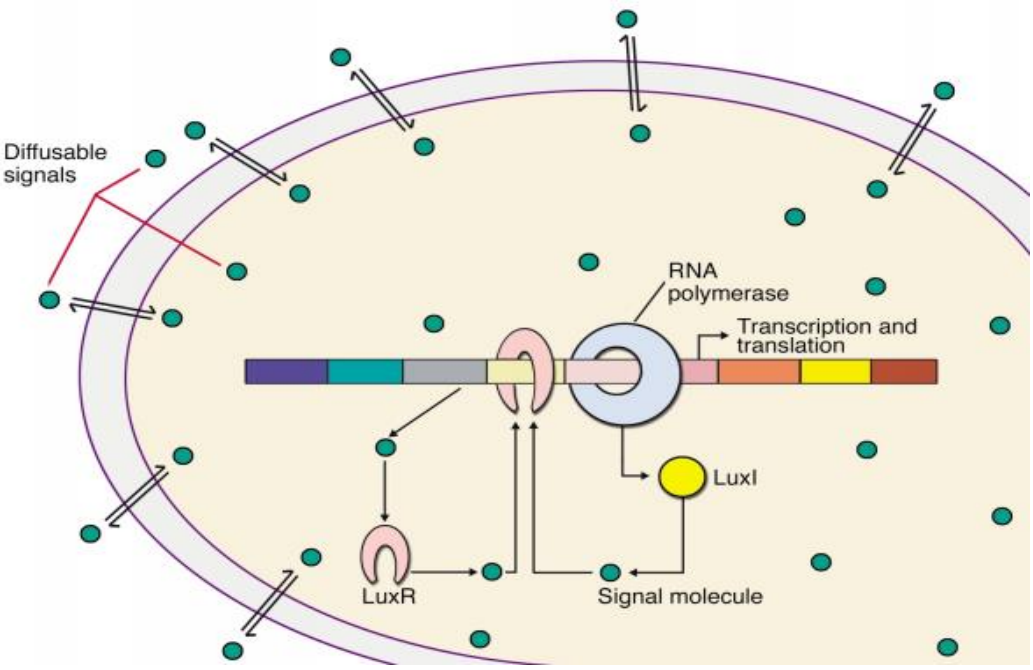


Figure 2.1. Overview of the Gram-negative LuxI-LuxR homologous quorum sensing system. Image sourced from Hentzer and Givskov (2003).

Knowledge of the links between biofilm formation to virulence factor in pathogenic Gram-negative bacteria has contributed to the increasing amount of biomedicinal studies examining marine secondary metabolites for novel therapeutic drug targets (Batista *et al.*, 2014). Marine macroalgae provide both a suitable substrate and an abundant source of nutrients to facilitate biofilm formation. However, the occurrence of a fully developed biofilm on their surface is a rare event. This is because many marine macroalgae produce secondary metabolites with specific quorum sensing inhibition or disruption properties to prevent biofilms forming on their surfaces (Jha *et al.*, 2013). The first quorum sensing inhibitory compound was in fact isolated from a red alga, *Delisea pulchra* which was shown to disrupt autoinducer signalling in the bacterial pathogen *Aliivibrio fischeri* (Givskov *et al.*, 1996). Observations from these studies provide insight as to which species or their close New Zealand relatives may produce quorum sensing disruption/ inhibition compounds with relevant activity against *P. syringae* pv. *actinidiae* biovar 3. Thus, New Zealand macroalgae species targeted for screening include; the red alga, *Asparagopsis armata* based on research by Jha *et al.* (2013) examining *Asparagopsis taxiformis*; the green alga, *Codium fragile* based on research by Batista *et al.* (2014) examining *Codium* sp; the green alga, *Ulva* sp. based on research by Egan *et al.* (2002) examining *Ulva lactuca*; and the brown alga, *Undaria pinnatifida* based on research by Barrow and Shahidi (2007) and Sano *et al.* (2008).

2.1.3 Work outlined in this chapter

The primary aim of the work outlined in this chapter is to examine 19 New Zealand marine macroalgae specimens for relevant biological activity against the causal agent of bacterial kiwifruit canker disease in New Zealand kiwifruit, *Pseudomonas syringae* pv. *actinidiae* biovar 3. It is hypothesised that using the approach whereby organisms are preselected for screening based on ecologically and taxonomically guided theory will increase the likelihood of success (higher 'hit' rates) over using a traditional discovery approach. Based on this, the second aim of the work outlined here is to identify and select 'hit' macroalgae species with promising activity for further investigation in a secondary screening phase.

2.2 Materials and methodology

2.2.1 Field collections

Nineteen New Zealand macroalgae specimens comprising 16 red, green, and brown species were selected for preliminary screening (Table 2.1). Eleven specimens were targeted using an ecologically-guided discovery approach and eight specimens were randomly selected using a traditional discovery approach. Collections of marine macroalgae were obtained from four locations around the Bay of Plenty on the eastern coastline of the North Island of New Zealand. These were: Karewa Island (37.52862S, 176.13111E); Mayor Island (37.30633S, 176.25997E); Leisure Island (37.62955S, 176.18543E); and Tauranga Harbour (37.47924S, 175.94824E) (Figure 2.2). The Bay of Plenty region is predominantly sheltered from New Zealand's prevailing westerly and south-westerly swells and from cooler sub-Antarctic currents. Strong easterly winds and oceanic weather events can cause large east or north-easterly swells, particularly in summer and autumn when the southeasterly trade winds prevail (Chappell, 2013). Climatic conditions are influenced by the warm East Auckland current. Average temperatures range from an average minimum of 15°C in August to a maximum of 21°C in February (Chappell, 2013; World Sea Temperature, 2016).

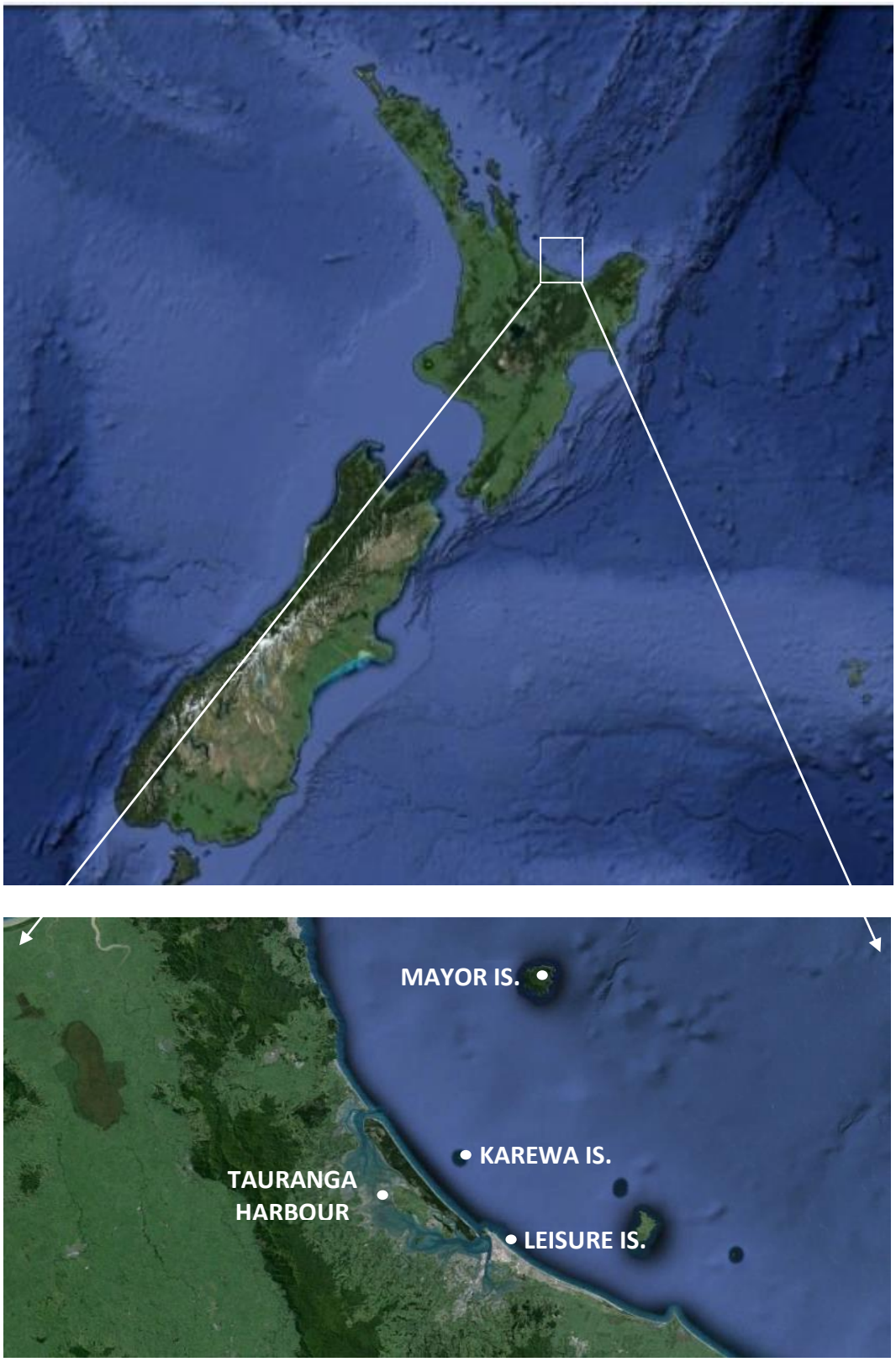


Figure 2.2. Aerial map of Bay of Plenty region in the North Island of New Zealand and four associated locations of collection. Images sourced from Google Earth.

Marine macroalgae specimens were collected in both summer and winter. Initial collections were made in the summer months November 2014-March 2015 when average ocean temperatures ranged between 17-21°C. Where possible, specimens were recollected in the winter months September-October 2015 when ocean temperatures averaged 15°C (World Sea Temperature, 2016). Some specimens were available for collection in both seasons, whereas others were only seasonally available at one time of collection. Specimens were collected subtidally or intertidally using SCUBA and snorkel methods. Samples were no larger than two kilograms wet weight each and material was taken just above the holdfast (base) of the algae to allow the regrowth of lost somatic material.

Upon collection, specimens were labelled by their assigned field identification code which consisted of: AB (initials of researcher), S (summer collection) or W (winter collection), and 1-19 (sequential collection number). For example, ABW1 can be interpreted as a winter specimen of *Pterocladia lucida* which was obtained at Karewa Island (see Table 2.1 for all specimen identification codes). Each specimen was retained in an individual zip lock bag and immediately placed into a freezer until required for further use. Ecological data including date and depth of collection, and specimen description were recorded. Site GPS coordinates were recorded using World Geometric System (WDS) 84 format in decimal degrees. All specimens were pressed, dried, and sent to New Zealand marine phycology expert Dr. Wendy Nelson at NIWA for taxonomic verification (see Appendix A.1 for further details).

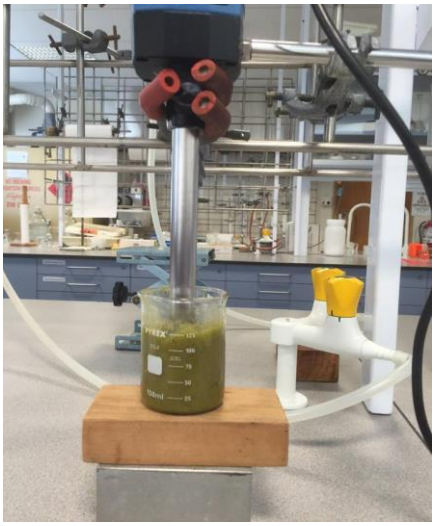
Table 2.1. Taxonomically identified macroalgae specimens including their associated field identification code, season of collection, location of collection, and selection method. Note that 'Targeted' refers to selection based on chemical ecology, bioinformatics, and/ or chemotaxonomy; 'Random' refers to selection based on its presence during collection.

Field ID Code	Species	Season of collection	Location of collection	Selection method
AB1	*Pterocladia lucida*	Summer/winter	Karewa Is.	Targeted
AB2	*Ulva* sp.	Summer/winter	Tga Harbour	Targeted
AB3	*Vidalia colensoi*	Summer/winter	Leisure Is.	Targeted
AB4	*Splachnidium rugosum*	Summer/winter	Leisure Is.	Random
AB5	*Carpophyllum plumosum*	Summer/winter	Leisure Is.	Random
AB6	*Asparagopsis armata*	Summer only	Mayor Is.	Targeted
AB7	*Ecklonia radiate*	Summer/winter	Karewa Is.	Random
AB8	*Glaphyrosiphon lindaueri*	Summer only	Leisure Is.	Random
AB8	*Carpophyllum maschalocarpum*	Summer/winter	Leisure Is.	Random
AB10	*Gigartina atropurpurea*	Summer/winter	Leisure Is.	Targeted
AB11	*Lessonia* sp.	Summer/winter	Leisure Is.	Random
AB12	*Pterocladia lucida*	Summer/winter	Leisure Is.	Targeted
AB13	*Vidalia colensoi*	Summer/winter	Karewa Is.	Targeted
AB14	*Xiphophora chondrophylla*	Summer/winter	Leisure Is	Random
AB15	*Ulva* sp.	Summer/winter	Leisure Is.	Targeted
AB16	*Dictyota kunthii*	Summer/winter	Leisure Is.	Targeted
AB17	*Codium fragile*	Summer/winter	Karewa Is.	Targeted
AB18	*Chondracanthus chapmanii*	Winter only	Tga Harbour	Random
AB19	*Undaria pinnatifida*	Winter only	Tga Harbour	Targeted

2.2.2 Small scale extraction preparation

A small representative subsample of each specimens (2g) was chopped up and homogenised in 3:1 methanol (MeOH): dichloromethane (DCM) (70-80mL) using an Ultraturrax homogeniser at speeds of 8000-9500 per min (Figure 2.3a). After 1-1.5 mins of blending, the homogenised solvent mixture was filtered through a Büchner funnel and the filtrate transferred into a round bottom flask (Figure 2.3b-c). The solid material was re-extracted 3-6 times until the liquid produced was virtually colourless (approximately 210-480 mL total solvent used). Any remaining solid material was discarded. A rotary evaporator with a water bath temperature of 35°C was used to remove solvent (Figure 2.3 d-e). Dried crude extract was resuspended (MeOH/ DCM) and transferred by pipette into a pre-weighed scintillation vial. Nitrogen (N_2) gas in a heating block set to 20-25°C was used to evaporate excess solvent. The sample was reweighed at room temperature and stored at 4°C until required for further use (Figure 2.3f).

Two duplicate extractions (2 g) were conducted on specimens initially collected in summer and one extraction (2 g) was conducted on those recollected in winter. Each extract was identified by a label with its appropriate field identification code (as stated in Table 2.1), followed by the letter ‘a’ (first 2 g extraction) or ‘b’ (second 2 g extraction). See Appendix A.2 for further details.

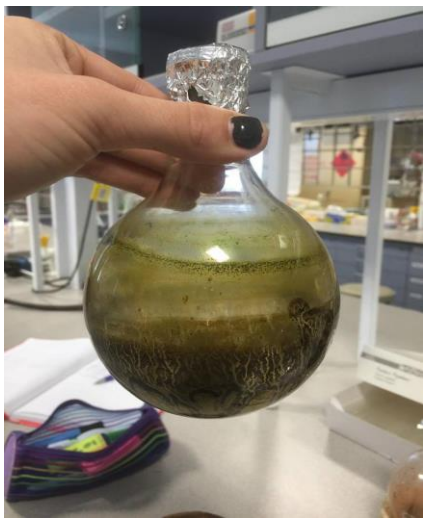


a

b

c

d



e

f

Figure 2.3. Crude extract preparation as exemplified by ABS4a (first 2 g extraction of *Splachnidium rugosum* collected in summer). This includes a) homogenisation, b-c) filtration and transferal to flask, d-e) evaporation of solvent, and f) resultant extract in scintillation vial.

2.2.3 Liquid Chromatography – Mass Spectrometry (LC-MS)

Samples 'a' from each crude extraction were screened by LC-MS using reversed-phase chromatography and electrospray mass spectrometry (positive ion).

2.2.3.1 Procedure

A subsample of each crude extract was resuspended in HPLC-grade MeOH (1000 µL) and passed through a Millipore filter (0.45 µm) into a small glass vial (1.5 mL capacity). LC-MS spectra were acquired using a HPLC system (UltiMate 3000; Dionex) directly coupled to a mass spectrometer using electrospray ionisation (ESI)-ion trap mass spectrometer (AmaZon X; Bruker Daltonics). HyStar (Bruker Daltonics) was used to control the dual system. Samples were separated on a C18 column (Luna 5µ C18, 150 × 4.6 mm; Phenomenex) using trifluoroacetic acid (TFA; 0.01%) in a standard solvent gradient of 90% H_2O to 100% Acetonitrile (ACN) with a 0.2 mL/min flow rate and a column temperature of 25°C. The UV absorption (200-800 nm) of the eluting compounds was assessed using photodiode array detection. The *m/z* of the eluting compounds was assessed using a capillary voltage of 4.5 kV for positive ions and a nebuliser pressure of 1.5 bar. Desolvation was accomplished with a nitrogen flow of 8 L/min at 225°C (Tait, 2015; Wang, 2015).

2.2.3.2 Analysis

Peaks of interest for each of the crude extracts were examined. Peaks of interest were defined as those: with a relative signal intensity of at least 15% of the total chromatogram; dominated by a single unique ion *m/z* 100-900; and with a retention time of 15-45 mins. Ions were considered identical if peaks containing the common ion varied in retention time by no more than two mins. Results of ions common in at least two crude macroalgae extracts are summarised in Table 2.2. Ions observed as unique to a single crude macroalgae extract is summarised in Table 2.3. Chromatograms for these extracts (elution time 15-45 mins) with peaks annotated with *m/z* of ions observed are provided in Appendix A.3.

2.2.4 *In vitro* bioassay of crude extracts

Crude macroalgae extracts (2 g) were screened by *in vitro* liquid assay to determine the biological activity against Psa biovar 3 at 2 and 24 hours of interaction time. Extracts were divided into three assay groups; ABS1a-ABS17a; ABS1b-17b; and ABW1a-19a. Each assay was performed in duplicate.

2.2.4.1 Procedure

Each crude extract was dissolved in MeOH (10 mL) for approximately 30 mins. An aliquot of this solution (40 µl) was diluted in sterile H_2O (360 µl) containing Psa biovar 3 (strain #10627) (4 µl) to give a 1:10 dilution ratio. After waiting an arbitrary time of 2 hours, an aliquot (100 µl) was diluted in sterile H_2O (0.9 mL). This was repeated a further four times to give 6 x 1/10 serial dilutions labelled DO (0), D1 (1:10), D2 (1:100), D3 (1:1,000), D4 (10,000), and D5 (1:100,000) (Figure 2.4). Each sample (10 µl) was transferred by pipette onto a Petri dish containing Kings B media. This was carried out in triplicate. The remaining 1:10 dilution (330 µl) was stored for 24 hours and the procedure was repeated. Each bioassay included a negative control which added distilled H_2O (40 µl) in the place of the stock extract, and a control which added MeOH (40 µl) in the place of the stock extract. All plates were placed in a 28°C incubator for 48-62 hours until results were read. The start time of each assay and time of the 2 and 24 hour interaction were recorded.

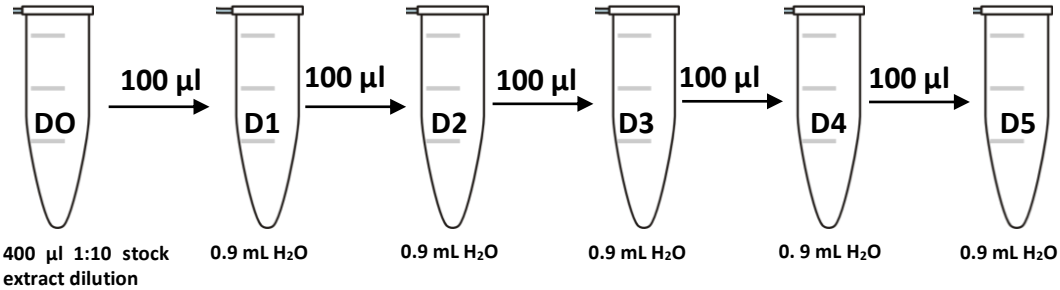


Figure 2.4. Process of dilution for each extract.

2.2.4.2 Analysis

The population of Psa biovar 3 when screened against each of the extract dilutions after 2 and 24 hours of interaction time was calculated by averaging the number of colonies present in the most diluted sample. The population was recorded in colony-forming-units per mL (cfu/mL). See Appendix A.4 for further details.

The level of biological activity of each extract when screened against Psa biovar 3 was classified as: no decrease of Psa incidence/ decrease lesser than log cfu/mL when compared to the control population of Psa biovar 3 (-); a decrease of Psa incidence greater than a log cfu/mL when compared to the control population of Psa biovar 3 (+/-); and complete inhibition with no growth of Psa biovar 3 (+). The level of biological activity for all macroalgae extracts is summarised in Table 2.4.

2.3 Results

2.3.1 Ions present in crude summer extracts

The following ions representative of peaks at the listed retention times were commonly observed in at least two of the 17 crude summer extracts and/ or the 16 crude winter extracts (Table 2.2).

Table 2.2. Ions common amongst summer and/ or winter collected small scale crude extracts.

Ion (*m/z*)	Retention time (min)	Crude extracts
301.3	24	ABS4a; ABS6a, ABS8a; ABS11a; ABS15a; ABS17a
329.3	28	ABS6a; ABS8a; ABS11a
340.3	26	ABW14a; ABW15a
389.3	37	ABS7a; ABS11a
500.4	32	ABS9a; ABS15a
507.3	40	ABS6a; ABS8a; ABS10a; ABS11a
522.2	29	ABS8a; ABS9a
544.2	34	ABS7a; ABS11a
550.5	33	ABW16a; ABW18a
586.5	36	ABW2a; ABW16a
609.3	42	ABS1a; ABS2a; ABS5a; ABS12a
623.3	44	ABS1a; ABS6a; ABS12a
641.3	40	ABS4a; ABS7a; ABS11a
704.4	38	ABS2a; ABS8a
710.5	37	ABS6a; ABS10a; ABS15a; ABS16a; ABW16a; AB18a
726.6	35	ABW16a; ABW18a
730.5	38	ABW5a; ABW7a; ABW16a

Ion (*m/z*)	Retention time (min)	Crude extracts
732.5	42	ABS2a; ABW2a; ABW4a; ABS5a; ABW9a; ABW10a; ABS16a; ABW16a; ABS17a; ABW19a
734.5	40	ABS2a; ABS5a; ABS16a; ABS17a
738.5	42	ABW2a; ABS5a; ABW5a; AWBS7a; ABS9a; ABW9a; ABW10a; ABS14a; ABW14a; ABS15a; ABS17a
741.5	38	ABS1a; ABS3a; ABS4a; ABS12a; ABS13a
741.5	38	ABW1a; ABW3a; ABW4a; ABW12a; ABW13a
743.7	41	ABS1a; ABS3a; ABW3a; ABS4a; ABW4a; ABS13a; ABW13a
754.4	35	ABS2a; ABS14a; ABS15a
758.5	37	ABS2a; ABS5a; ABW5a; ABS6a; ABW7a; ABS9a
760.5	37	ABW4a; ABS5a; ABW5a; ABS6a; ABW7a; ABS9a; ABW9a; ABS10a; ABS14a; ABW14a; ABS15a; ABW16a; ABS17a; ABW18a
763.5	36	ABS1a; ABW1a; ABS3a; ABW3a; ABW4a; ABS12a; ABW12a; ABS13a; ABW13a
768.7	37	ABW5a; ABW9a
802.3	42	ABS9a; ABS10a; ABS11a
806.7	36	ABW5a; ABW9a; ABW10a; ABW18a
808.5	37	ABS5a; ABW5a; ABS9a; ABW9a; ABW10a; ABW16a; ABW18a
836.8	38	ABW16a; ABW18a

The following ions representative of peaks at the listed retention times were observed as unique within a certain crude summer and/ or winter extract (Table 2.3).

Table 2.3. Ions unique to a summer and/ or a winter collected small scale crude extract.

Crude extract	Ion (*m/z*)	Retention time (mins)
ABS1a	433.5	36
	457.4	31
	535.3	41
ABS2a	474.4	34
ABW2a	304.3	32
	332.3	34
	602.4	36
	628.5	37
ABS3a	278.8*	27
	389.8*	18
	393.8	19
	479.9*	19
ABW3a	421.8*	18
	435.8*	20
ABS4a	279.1	28
	528.1	39
ABW4a	301.3	28
ABS5a	764.5	42
ABW5a	752.6	37
ABS7a	744.9	21
ABW7a	804.7	37
	810	37

Crude extract	Ion (*m/z*)	Retention time (mins)
ABS8a	361.2	35
	393.2	17
	437.2	17
ABS9a	782.3	36
ABW9a	522.4	33
	782.3	37
ABS11a	144.1	18
	302.1	22
ABW11a	144.1	20
	302.1	24
ABS13a	389.8*	18
	393.8*	19
	469.8*	17
ABW13a	435.8*	20
ABS14a	298.2	21
	340.3	23
ABW14a	282.3	38
	298.2	21
	787.7	43
ABS15a	726.4	32
ABS16a	586.4	34
*Isotope pattern indicates dibromination. Ion listed is the lowest mass ion of the pattern.		

2.3.2 Biological activity of crude extracts of macroalgae against Psa biovar 3

2.3.2.1 Extracts collected in summer

The following crude summer collected extracts were inactive against Psa biovar 3 at 2 hours of interaction; ABS1, ABS2, ABS4, ABS5, ABS6, ABS7, ABS8, ABS9, ABS10, ABS11, ABS12, ABS13, ABS15, ABS16, and ABS17. Extract 'a' of ABS3 did not demonstrate any biological activity in both replications, however, crude extract 'b' completely inhibited any growth. ABS14 was the only macroalgae specimen which consistently inhibited the growth of Psa after two hours (Table 2.4).

The following crude summer collected extracts consistently inhibited the growth of Psa biovar 3 in both extract 'a' and 'b' after 24 hours of interaction; ABS3, ABS6, ABS9, and ABS14. Extract 'a' of AB8 decreased the incidence of Psa in one replicate but was otherwise inactive. Similarly, only one replicate of extract 'a' of ABS7 and one replicate of extract 'b' of AB11 completely inhibited Psa. Extract 'a' of both ABS13 and ABS15 decreased the incidence of Psa, while extracts 'b' were inactive. All other crude extracts were inactive against Psa after 24 hours of interaction time (Table 2.4).

2.3.2.2 Extracts collected in winter

The following crude winter collected extracts were inactive against Psa after 2 hours of interaction; ABW1, ABW2, ABW4, ABW5, ABW7, ABW9, ABW10, ABW11, ABW12, ABW15, ABW16, ABW18, and ABW19. Extracts of ABW3 and ABW13 consistently decreased the incidence of Psa. The extract of ABW14 was the only one to consistently inhibit the growth of Psa after two hours (Table 2.4).

The following crude winter collected extracts inhibited the growth of Psa biovar 3 after 24 hours of interaction; ABW3, ABW7, ABW13, and ABW14. The extract of ABW9 demonstrated a decrease of incidence of Psa in both replicates. The biological activity of the extract of ABW11 varied; one replicate completely inhibited the growth of Psa, whereas the other replicate was inactive. The biological activity of the extract of ABW16 also differed; in one replicate it decreased the incidence of Psa and in the other replicate it completely inhibited the growth. All other extracts were inactive against Psa after 24 hours (Table 2.4).

Table 2.4. Summary of biological activity of all crude, small scale macroalgae extracts when screened against Psa biovar 3.

Season of collection		Summer								Winter			
Interaction time		2 hour				24 hour				2 hour		24 hour	
Extraction		A		B		A		B		A		A	
Replicate		1	2	1	2	1	2	1	2	1	2	1	2
AB1	*Pterocladia lucida*	-	-	-	-	-	-	-	-	-	-	-	-
AB2	*Ulva* sp.	-	-	-	-	-	-	-	-	-	-	-	-
AB3	*Vidalia colensoi*	-	-	+	+	+	+	+	+	+/-	+/-	+	+
AB4	*Splachnidium rugosum*	-	-	-	-	-	-	-	-	-	-	-	-
AB5	*Carpophyllum plumosum*	-	-	-	-	-	-	-	-	-	-	-	-
AB6	*Asparagopsis armata*	-	-	-	-	+	+	+	+	N/A			
AB7	*Ecklonia radiata*	-	-	-	-	-	+	-	-	-	-	+	+
AB8	*Glaphyrosiphon intestinalis*	-	-	-	-	-	+/-	-	-	N/A			
AB9	*Carpophyllum maschalocarpum*	-	-	-	-	+	+	+	+	-	-	+/-	+/-
AB10	*Gigartina atropurpurea*	-	-	-	-	-	-	-	-	-	-	-	-
AB11	*Lessonia* sp.	-	-	-	-	-	-	+	+	-	-	+	-
AB12	*Pterocladia lucida*	-	-	-	-	-	-	-	-	-	-	-	-
AB13	*Vidalia colensoi*	-	-	-	-	+/-	+/-	-	-	+/-	+/-	+	+
AB14	*Xiphophora chondrophylla*	+	+	+	+	+	+	+	+	+	+	+	+
AB15	*Ulva* sp.	-	-	-	-	+/-	+/-	-	-	-	-	-	-
AB16	*Dictyota kunthii*	-	-	-	-	-	-	-	-	-	-	+/-	+
AB17	*Codium fragile*	-	-	-	-	-	-	-	-	N/A			
AB18	*Chondracanthus chapmanii*	N/A								-	-	-	-
AB19	*Undaria pinnatifida*	N/A								-	-	-	-

KEY	
-	No inhibition
+/-	Decrease of incidence (>log cfu/mL)
+	Full inhibition

2.4 Discussion

2.4.1 Selection of 'hit' macroalgae candidates

Preliminary screening of 19 crude extracts of macroalgae collected in summer and winter from the Bay of Plenty region of New Zealand, enabled the selection of five 'hit' candidates with target activity against Psa biovar 3. Macroalgae were selected as 'hit' candidates if they displayed 'consistent biological activity' throughout preliminary screening. This was defined as: a decrease of incidence or complete inhibition of Psa biovar 3 in: both replicates of extracts 'a' and 'b' of the summer collections; and both replicates of extracts 'a' of the winter collections. The macroalgae which met this criteria were selected for further investigation for control of Psa biovar 3.

The brown alga, *Xiphophora chondrophylla* (AB14) was the first of the five selected 'hit' macroalgae candidates. The species was consistently bioactive against Psa biovar 3 and completely inhibited any growth after both two and 24 hours of interaction. It is noted that *X. chondrophylla* was the only species to have biological activity against Psa after two hours of interaction, which suggests it had the fastest mode of action of all screened macroalgae.

The red alga, *Asparagopsis armata* (AB6) and the brown alga, *Carpophyllum maschalocarpum* (AB9) were the second and third selected 'hit' candidates. Unfortunately, *A. armata* was not available at the time of winter collections and therefore no seasonal comparisons could be provided. However, extracts of the summer collected specimen demonstrated a consistent biological activity against

Psa by completely inhibiting its growth after 24 hours of interaction. This justifies the selection of *A. armata* as a 'hit' candidate. By comparison, the summer collected specimen of *C. maschalocarpum* completely inhibited the growth of Psa after 24 hours of interaction, whereas the specimen collected in winter decreased the incidence. This suggests that the level of biological activity of *C. maschalocarpum* against Psa could vary seasonally. Nonetheless, the consistent biological activity against Psa after 24 hours of interaction justifies its selection as a 'hit' candidate for further investigation.

The Leisure Island (AB3) and Karewa Island (AB13) samples of the red alga, *Vidalia colensoi* were both selected as the fourth and fifth 'hit' macroalgae candidates. After 2 hours of interaction with Psa, neither of the summer collected samples were active against Psa, whereas both winter collected samples decreased its incidence. This suggests that the winter collected samples of *V. colensoi* may have a faster mode of action against Psa than those summer collected samples.

At 24 hours of interaction with Psa biovar 3, the biological activity of the two samples of *V. colensoi* varied. At this time the Leisure island (AB3) sample consistently inhibited the growth of Psa, whereas the Karewa Island (AB13) sample varied in the level of biological activity between the summer collected extracts 'a' and 'b'. Differences in biological activity between the two *V. colensoi* samples may be attributed to researcher bias during the extraction preparation, whereby preference may have occurred toward one particularly active region of

the plant. If this is the case, then the biological activity of *V. colensoi* at Karewa Island against Psa may vary over a very small spatial scale (that is within the individual itself). Further investigation is needed to confirm this. It was also noted that the Karewa Island sample was fouled by sponges and other encrusting organisms, whereas the Leisure Island sample was very clean. This suggests there may be chemical-ecological factors contributing to the biological activity of *V. colensoi*. With these considerations in mind, the Karewa Island sample was selected as a 'hit' candidate to provide further comparisons between the two samples of *V. colensoi* in secondary screening.

The species *Ecklonia radiata* (AB7), *Glaphyrosiphon intestinalis* (AB8), *Lessonia* sp. (AB11), *Ulva* sp. (AB15), and *Dictyota kunthii* (AB16) all exhibited biological activity against Psa biovar 3 at some stage during the preliminary screening process. This activity, however, did not meet the criteria for 'consistent biological activity'. It either varied between seasons, between replicates, or between extracts. These species were therefore dismissed as 'hits' for further investigation in this study. The species *Pterocladia lucida* (AB1 and AB12), *Ulva* sp. (AB2), *Splachnidium rugosum* (AB4), *Carpophyllum plumosum* (AB5), *Gigartina atropurpurea* (AB10), *Codium fragile* (AB17), *Chondracanthus chapmanii* (AB18), and *Undaria pinnatifida* (AB19) were completely inactive against Psa biovar 3. Thus, they were also dismissed as 'hit' candidates for further investigation.

Selection of five 'hit' New Zealand macroalgae candidates from the possible 19 specimens demonstrates that 26 percent of screened macroalgae have target biological activity against Psa biovar 3. This is a particularly high success rate given that the percentage of organisms with relevant activity in the first phase of natural product screening programmes is often very low. Three of the 'hit' candidates were species targeted for screening and two hits were selected at random. This confirms that using an approach whereby organisms are preselected for screening based on ecologically and taxonomically guided theory does in this study increase the likelihood of success (higher 'hit' rates) over using a traditional discovery approach. However, the selection of 'non targeted' species did have some ecological base. For instance, small turfing algal species were not considered in this study but were abundant on many of the macroalgae. Hence, these may not have been entirely random in the truest sense.

Examination of data from marine macroalgae with relevant biological activities in previous biomedicinal screens enabled the development of two hypotheses to guide the selection of 11 macroalgae specimens for screening. The hypotheses suggested that marine macroalgae or close relatives which produce secondary metabolites a) active against *P. aeruginosa* and b) with quorum sensing inhibition/ disruption activities (when screened in previous studies) would also produce metabolites with activity against Psa biovar 3. As previously mentioned, three of the five 'hit' macroalgae candidates were targeted for screening. This included the two specimens of *Vidalia colensoi*, selected from research examining antibacterial activity against *P. aeruginosa* in its close relative *V.*

obtusiloba (Bianco *et al.*, 2013); and *Asparagopsis armata*, selected from research examining quorum sensing inhibition against the pathogen *Chromobacterium violaceum* in its close relative *A. taxiformis* (Jha *et al.*, 2013). The discovery of these macroalgae with relevant biological activity against Psa biovar 3 thus supports both hypotheses.

2.4.2 Preliminary information of 'hit' macroalgae candidates

Analysis of common ions present in at least two crude extracts in both summer and winter samples (where possible) provides preliminary information as to which ions present in the 'hit' candidates are likely to represent ubiquitous macroalgae metabolites or common contaminants. Ions that were common between active and inactive extracts include: *m/z* 741.5, 743.7, and 763.5 in *V. colensoi* (AB3 and AB13) AB3; *m/z* 301.3, 329.3, 507.3, 623.3, 710.5, 758.5, and 760.5 in *A. armata* (AB6); *m/z* 500.4, 522.2, 732.5, 738.5, 758.5, 760.5, 768.7, 802.3, 806.7, and 808.5 in *C. maschalocarpum* (AB9); and *m/z* 340.3, 738.5, 754.4, and 760.5 in *X. chondrophylla* (AB14). Given that these ions are common with other inactive species it can be assumed they are not likely to represent bioactive compound/s of interest. No diagnostic isotope patterns were observed for these ions.

Analysis of ions unique to a respective 'hit' candidate in both summer and winter samples provides preliminary information as to which ions are likely to represent bioactive compound/s of interest. Ions identified as unique in samples of *V. colensoi* (AB3 and AB13) provided the most informative preliminary information.

Unique ions within these samples included *m/z* 278.8, 389.8, 393.8, 421.8, 435.8 469.8, and 479.9. The ions *m/z* 389.8 and 393.8 were consistently observed in both samples at both seasons of collection. All other ions not consistently present; however, this does not necessarily mean that they were not present. Rather, the compound that it represents may not have been present in sufficient quantity to be identified as a peak (at least 15 percent of the total chromatogram). The isotope pattern of these ions is typical of a dibrominated compound, whereby three ions are each separated by two mass units and observed in the ratio 1:2:1. This suggests that potential compounds of relevance in *V. colensoi* are very likely to be dibrominated.

Ions identified as unique in the remaining three 'hit' macroalgae candidates did not provide any relevant preliminary information. Unique ions in *C. maschalocarpum* included: the ion *m/z* 522.4, which was present the extract of the winter sample only: and the ion *m/z* 782.3, which was present in extracts from both seasons. Unique ions in *X. chondrophylla* included: *m/z* 282.3, 340.3, and 787.7 which were each present in extracts from only one season; and the ion *m/z* 298.2, which was present in extracts from both seasons. Furthermore, no evident isotope patterns were observed for these ions. No ions of interest were identified as unique to *A. armata* (AB6).

2.4.3 Conclusions

There are three key points for which the preliminary screening phase of this research provides insight. Firstly, the targeted selection of certain macroalgae species in this study did work to effectively increase the rate of 'hit' organisms with relevant biological activity against *Pseudomonas syringae* pv. *actinidiae* biovar 3 (16 percent) than those that were randomly selected (10 percent). This suggests that an ecologically-guided discovery approach can in fact be used in natural product-based screening programmes to accelerate the turnover of 'hits' and thus increase commercial interest for the industry (Beattie *et al.*, 2005). Secondly, the examination of data from marine macroalgae which produce metabolites with a) antibacterial activity against *Pseudomonas aeruginosa* and b) quorum sensing inhibition/ disruption activity indicates that these species can also produce relevant activity against *Pseudomonas syringae* pv. *actinidiae* biovar 3. Lastly, LC-MS analysis indicated the extract *V. colensoi* contains ions with distinct dibrominated isotope patterns. This provides preliminary information for the secondary screening phase as to which compounds may be biologically active against Psa biovar 3.

The primary limitation for this study was the size of the subsample from which crude macroalgae compounds were extracted. Ten macroalgae specimens demonstrated biological activity effective against Psa biovar 3 at some stage of the *in vitro* screening process. Five of these were eliminated as 'hits' for further investigation due to differences in activity between extracts 'a' and 'b' or between replicates. These variations suggest that the biological activity of certain

macroalgae species may vary over a small spatial scale (that is within the individual); thus, a tw0 gram subsample may not be large enough to represent the chemistry of the macroalgae. Future work could focus on larger scale extractions of these species to determine whether their biological activity is more representative on a larger scale. Alternatively, specimens could undergo freeze drying and homogenisation prior to preparation of a small scale extraction. This approach standardises the macroalgae samples and thus can provide a more representative indication of the biological activity.

The second limitation for this study was the lack of seasonal information provided for the 'hit' macroalgae candidate *Asparagopsis armata*. Initial summer collections of *A. armata* were obtained from Mayor Island in November 2014. Due to factors beyond the control of this research, the species was unable to be sighted at the same GPS location in winter (it is an ephemeral species). Future work could therefore focus on obtaining a winter sample, preparing a small scale extraction, and screening it for biological activity against Psa biovar 3. This will provide information relating to the chemistry of the alga at a temporal scale. It may also provide relevant insight for the secondary screening phase.

CHAPTER 3

SECONDARY SCREENING

3.1 Introduction

The secondary screening component of this study encompasses elements of the second phase of biodiscovery (Figure 1.1) to further investigate marine bioactive compounds for control of the causal agent of bacterial kiwifruit canker disease, *Pseudomonas syringae* pv. *actinidiae* biovar 3. The preliminary screening phase involved the collection (phase one) and screening of 19 New Zealand macroalgae extracts (phase two) to identify five 'hit' candidates with promising activity. The secondary screening phase will therefore determine the biological activity of each 'hit' from a larger scale extract and identify any potential compounds of interest from subsequent fractionation. Collectively, this will assist in prioritisation of 'hits' for further chemical exploration in later phases of discovery. The following paragraphs will discuss the known natural product chemistry of the two 'hit' red algae, *Vidalia colensoi* (AB3 and AB13) and *Asparagopsis armata* (AB6), and the two 'hit' brown algae, *Carpophyllum maschalocarpum* and *Xiphophora chondrophylla.* This will provide insight as to which classes of compounds may be present and/ or of relevance to Psa biovar 3.

3.1.1 Rhodophyta – the red algae

The Rhodophyta, or red algae, are a phylum belonging to the single class, Rhodophyceae. This is a large group which consists of around 8,000 algal species; the majority of which are marine and predominate along the coastal and continental shelf areas of tropical, temperate, and cold water regions (Lüning, 1990; El Gamal, 2010; Guiry, 2016). The distinct red or purplish colouration of the Rhodophyta results from the dominance of various photosynthetic phycobilin pigments (phycoerythrin, phycocyanin, and allophycocyanin) (El Gamal, 2010). These pigments mask other pigments present including chlorophyll *a* (no chlorophyll *b*), *β* carotene, and a number of unique xanthophylls (El Gamal, 2010). The pigment phycoerythrin can reflect red light and absorb blue light. This enables the Rhodophyta to have the greatest light absorption capacity and thus reside at greater depths than all other marine algae. Cell walls are composed of the polysaccharides cellulose, agarose, and carrageenan, and the assimilatory product is floridean starch (Guiry, 2016).

The Rhodophyta are recognised as the most abundant source of natural products of all the marine algae (El Gamal, 2010). Secondary metabolites isolated from the Rhodophyta to date predominantly belong to the terpenoid and sterol compound classes (Faulker, 2002). The marine environment is particularly rich in halides and therefore many secondary metabolites isolated from red algae contain one or more atoms of the halogen elements; bromine (Br), iodine (I), chlorine (Cl), and fluorine (F) (McConnell & Fenical, 1977; Cabrita *et al.*, 2010). Of these, chlorine and bromine appear to be the primary halogens of secondary

metabolites, whereas iodine and fluorine are rare within chemical structures (Cabrita *et al.*, 2010). The ecological functions of halogenated compounds in marine red algae, and in fact amongst other marine phyla, are not clearly understood. They do, however, provide secondary metabolites with interesting key features and thus deliver unique environmental advantages to the producing organisms (Fenical, 1975; Cabrita *et al.*, 2010).

A review of natural product literature of the two 'hit' Rhodophyta species belonging to the *Vidalia* and *Asparagopsis* genera indicates that the majority of compounds isolated to date are halogenated (Table 3.1). Major focal points of research associated with these genera have been the discovery and characterisation of novel halogenated compounds, and their evaluation for possible biotechnological/ biomedicinal applications. Predominantly, novel halogenated metabolites isolated from *Vidalia* have been phenolic metabolites containing bromine. For example, Wiemer *et al.* (1991) identified two novel bromophenols (Viadols A and B) with demonstrated anti-inflammatory properties from *V. obtusiloba* (Figure 3.1a-b). By comparison, novel halogenated metabolites isolated from *Asparagopsis* have predominantly been alicyclic and aliphatic compounds. For example, Greff *et al.* (2014), identified two novel, highly brominated cyclopentenones, mahorone and 5- bromomahorone from *A. taxiformis* with demonstrated antibacterial activity against the human pathogen *Acinetobacter baumannii* (Figure 3.1c-d). These halogenated secondary metabolites and their biological features provide insight as to which compounds may be present and have relevant activity against Psa biovar 3. A full summary of

the natural product literature for the 'hit' Rhodophyta genera *Vidalia* and *Asparagopsis* is summarised in Table 3.1.

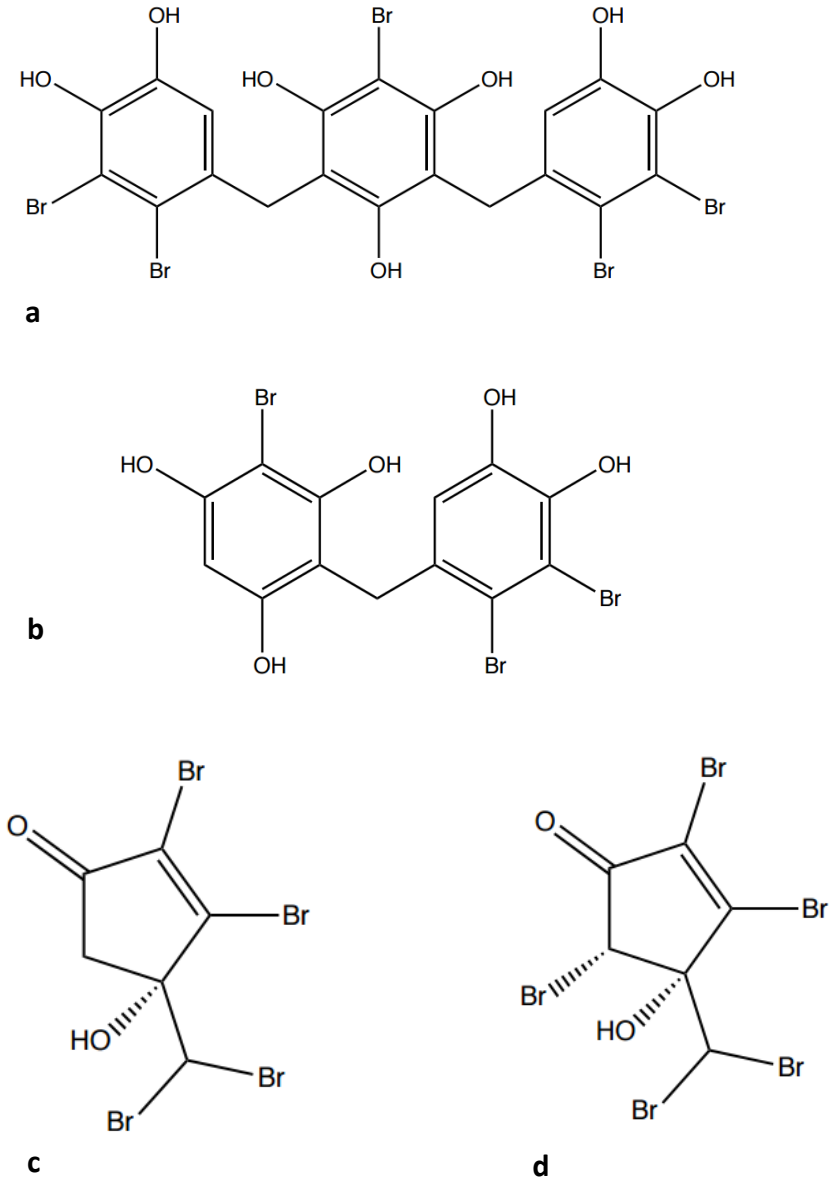


Figure 3.1. Chemical structures of brominated compounds isolated from 'hit' Rhodophyta genera, *Vidalia* and *Asparagopsis*. This includes a) Viadol A and b) Viadol B isolated from *V. obtusiloba* (Wiemer *et al*., 1991), and c) mahorone and d) 5-bromomahorone isolated from *A. taxiformis* (Greff *et al*., 2014). Structures self-produced.

Figure 3.2. 'Hit' Rhodophyta macroalgae species *Vidalia colensoi* (top) and *Asparagopsis armata* (bottom). Top image self-produced. Bottom image sourced from Guiry and Guiry (2016).

Table 3.1. Summary of natural product literature on the 'hit' marine red algae genera *Vidalia* (*Osmundaria*) and *Asparagopsis,* and/ or their reported biological activity.

Genus	Species	Isolated compound	Bioactivity	Reference (s)
***Vidalia* (*Osmundaria*)**	*V. obtusiloba*	Crude extracts	Antileishmanial	Flores Lira *et al.* (2016)
			Anti-oxidant	de Alencar *et al.* (2016)
			Anti-infective	Bianco *et al.* (2013)
			Antiviral	Romanos *et al.* (2002)
		Glycolipids	Antiviral	de Souza *et al.* (2012)
		Bromophenols*	-	Carvalho *et al.* (2006)
			Anti-inflammatory	Wiemer *et al.* (1991)
	V. serrata	Crude extracts	Antifungal	Barreto *et al.* (1997)
		Bromophenols*	Antimicrobial	Barreto and Meyer (2006)
	Vidalia sp.	Phenols*	Anticancer	Yoo *et al.* (2002)
	V. spiralis	Nitrogen containing carboxlic acid	-	Patti *et al.* (1992)
		Bromophenols*	-	Kazlauskas *et al.* (1982)
	V. colensoi	Bromophenols*	Antibacterial/ cytotoxicity	Popplewell and Northcote (2009)
Asparagopsis	*A. taxiformis*	Crude extracts	Antileishmanial	Genovese *et al.* (2009) Vitale *et al.* (2015)
			Antifungal	Genovese *et al.* (2013)
			Antimicrobial	Manilal *et al.* (2009)
		Ethylenes*	-	Abrahamsson *et al.* (1995)
	Asparagopsis sp.	Crude extracts	Antimicrobial	Vatsos and Rebours (2014)
			Antibacterial	Manilal *et al.* (2012a)
		Halogenated aliphatics*	-	Marshall *et al.* (1999)
		Halogenated aliphatics*	-	McConnell and Fenical (1977)
	A. armata	Crude extracts	Antimicrobial	Pinteus *et al.* (2015) (Salvador *et al.*, 2007)
			Cytotoxicity	Alves *et al.* (2011)
			Antioxidant	Zubia *et al.* (2009)
			Antiviral	Rhimou *et al.* (2010)
			Antibacterial	Bansemir *et al.* (2006)
		Polysaccharides	Antiviral	Haslin *et al.* (2001)
	A. taxiformis	Halogenated alicyclics*	Antibacterial	Greff *et al.* (2014)
		Halogenated aliphatics*	-	Burreson *et al.* (1976) Woolard *et al.* (1976) Moore *et al.* (1976)
		Polysaccharides	Anticoagulant	Manilal *et al.* (2012b)
	A. sandfordiana	Steroids	-	Bano *et al.* (1988)

* indicates halogenation

3.1.2 Ocrophyta – the brown algae

The Ocrophyta (brown algae), formerly referred to as the Phaeophyta, are a phylum that is placed into the single class, Phaeophyceae. There are around 265 genera of Ocrophyta, of which the majority are exclusively marine and predominate rocky coastlines in cold and temperate water regions (1500-2000 species). (Dawes, 1998; *Phaeophyta (Brown Algae)*, n.d). Only five or six genera comprise of species from freshwater habitats (Dawes, 1998). The Ocrophyta derive their brown colouration from the dominance of several brown carotenoid pigments, including fucoxanthin and neofucoxanthin (*Phaeophyta*, 2016). These pigments mask other photosynthetic pigments present including chlorophyll *a* and *c* (no chlorophyll *b*), *β*-carotenes and other xanthophylls (El Gamal, 2010; *Phaeophyta*, 2016). Carotenoid pigments do not have light absorption capacity as high as phycoerythrin, thus the Ocrophyta reside at shallower depths than the Rhodophyta. They do, however, have a higher capacity to absorb light than pigments in the Chlorophyta (green algae). Cell walls are composed of the polysaccharides cellulose, algin, and fucoidan. Assimilatory products are the polysaccharide laminarin and the alcohol mannitol (Dawes, 1998).

The Ocrophyta are a rich source of secondary metabolites. In 2007, more than 1,140 compounds from 3,000 Ocrophyta lineages were reported in the marine natural product database, Marinlit (La Barre *et al.*, 2010). Marine species belonging to the Ocrophyta produce a variety of phlorotannins which are unique to brown algae. These metabolites provide the producing organism with protection from hostile environmental conditions and defence against predation

(Cabrita *et al.*, 2010). While halogenation in the marine Ocrophyta is less common than in Rhodophyta, the rare halogen iodine is more frequent in secondary metabolites of Ocrophyta than in those of other marine algae. By comparison, the more abundant halogen chlorine occurs in less than one percent of secondary metabolites in brown algae (Cabrita *et al.*, 2010).

A review of natural product literature of the 'hit' Ocrophyta genus *Carpophyllum* indicates that compounds isolated to date are all structurally novel phlorotannins (Table 3.2). Amongst these, only four phlorotannins are halogenated and were isolated from *C. angustifolium* (Glombitza & Schmidt, 1999b) (Figure 3.3a-d). It is noted that no recent novel metabolites have been isolated from the genus *Carpophyllum*, which may be a reflection of the overall decreasing number of novel compounds isolated from the phylum Ocrophyta every year (La Barre *et al.*, 2010). In comparison, the only compound isolated to date from the *Xiphophora* genus is the novel sphingolipid, 3-epi-xestoaminol c (Figure 3.3e). This compound was shown to have antibacterial activity when screened against the causal agent of tuberculosis, *Mycobacterium tuberculosis* (Dasyam *et al.*, 2014).

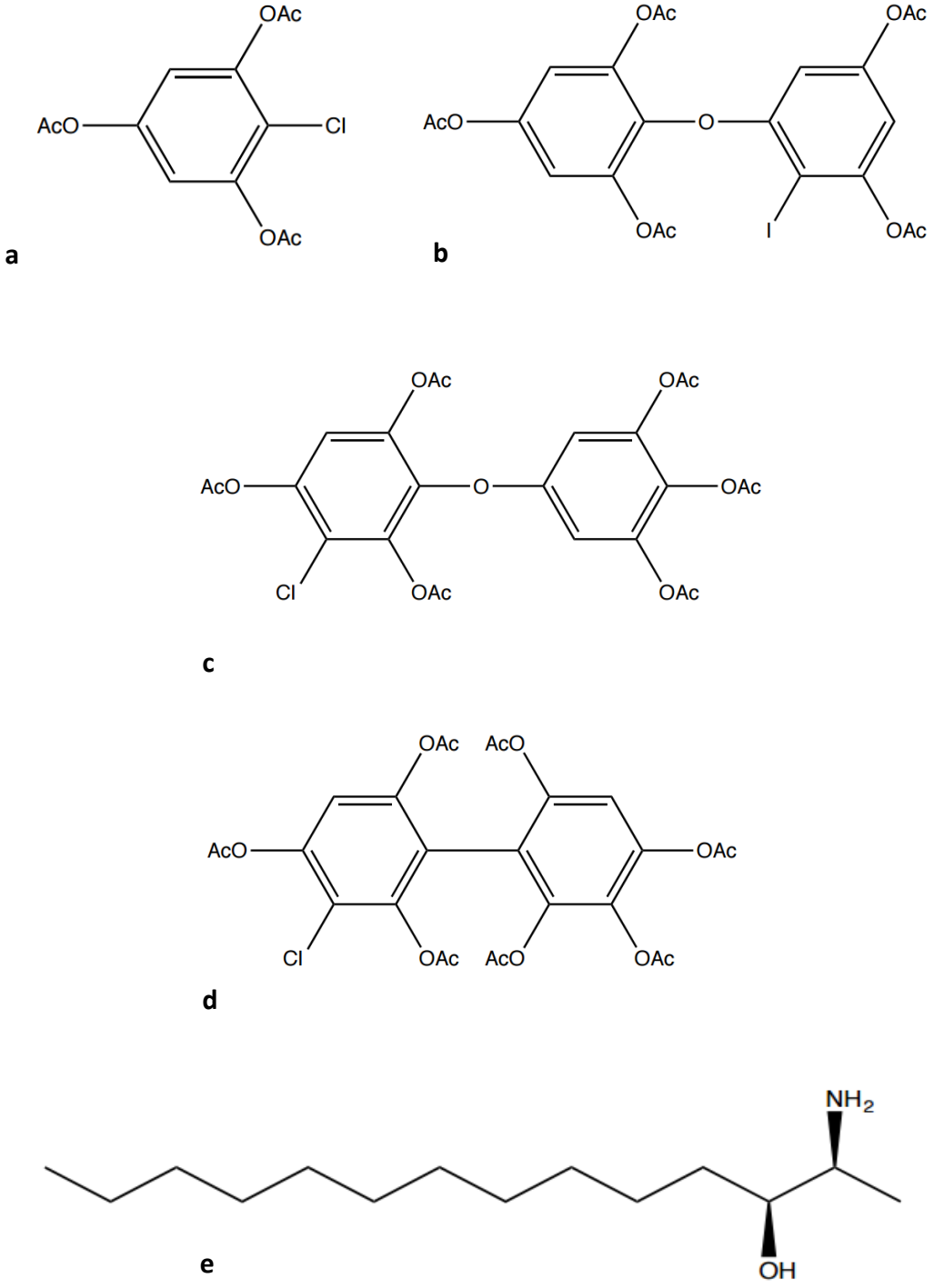


Figure 3.3. Chemical structures of brominated compounds isolated from 'hit' brown algae genera, *Carpophyllum* and *Xiphophora.* This includes a) 2-chlorophloroglucinal triacetate, b) 2-iodophlorethol pentaacetate, c) 3-chlorobifuhalol hexaacetate, d) 3-chlorodifucol hexaacetate isolated from *C. angustifolium* (Glombitza & Schmidt, 1999b), and e) 3-*epi*-xestoaminol c isolated from *X. chondrophylla* (Dasyam *et al.*, 2014). Structures self-produced.

Figure 3.4. 'Hit' Ocrophyta macroalgae species *Carpophyllum maschalocarpum* (top) and *Xiphophora chondrophylla* (bottom). Top image sourced from Marinelife (2015). Bottom image self-produced.

Table 3.2. Summary of natural product literature on the 'hit' brown macroalgae genera *Carpophyllum* and *Xiphophora,* and/ or their reported bioactivity.

Genus	Species	Isolated compound	Bioactivity	Reference (s)
Carpophyllum	*C. maschalocarpum*	Phlorotannins		Glombitza and Li (1991b) Li and Glombitza (1991) Glombitza and Li (1991a) Glombitza and Schmidt (1999a)
	C. angustifolium	Phlorotannins*		Glombitza and Schmidt (1999b)
Xiphophora	*X. chondrophylla*	Sphingolipids	Antibacterial	Dasyam *et al.* (2014)
* indicates halogenation				

3.1.3 Work outlined in this chapter

The primary aim of the work outlined in this chapter is to further examine the five selected 'hit' New Zealand macroalgae candidates for relevant activity against the causal agent of bacterial kiwifruit canker disease in New Zealand, *Pseudomonas syringae* pv. *actinidiae* biovar 3. This will be addressed by *in vitro* screening of 'hit' crude extracts prepared on a larger scale and by LC-MS analysis from subsequent separation of produced fractions. Based on this, the second aim of the work outlined here is to provide insight as to which of the 'hit' macroalgae candidates are of the highest priority to further explore chemically in later phases of discovery.

3.2 Materials and methodology

3.2.1 Large scale extraction preparation

Crude extracts of five summer collected macroalgae specimens (ABS3, ABS6, ABS9, ABS13, and ABS14) were each prepared from a large subsample (20 g). The extraction process was performed using the same procedure in Section 2.2.2, except that a larger biomass of algae (20 g) and solvent quantity (240-900 mL/ 3-9 blends) were used. Each extract was identified by labelling its vial with its respective field identification code (see Table 2.1) and the letter c (third extraction). See Appendix A.5 for further details.

3.2.2 Reversed-Phase Chromatography (C_{18}): 50g column

Crude extracts (ABS3c, ABS6c, ABS9c, ABS13c, and ABS14c) were fractionated using reversed-phase chromatography on a reversed-phase C_{18} bench column (50 g) (Figure 3.5).

3.2.2.1 Column preparation

A glass column with a Teflon tap was cleaned thoroughly with MeOH and water. After placing cotton wool into the bottom of the column, the column was prepared by mixing YMC-Pack Pro C_{18} gel with sufficient amount of MeOH to produce a slurry with pouring consistency. With the tap open, the slurry was quickly transferred to the column which was tapped gently with a hard object to release any air bubbles and facilitate even packing of C_{18} material. Additional MeOH was added to the column to resuspend non-transferred material and

wash any excess material off the sides of the column. This solvent was then flushed through until the level was 1-1.5cm above the column bed. The column was flushed again with 1:1 MeOH: H_2O (with two column volumes (100 mL)) before being flushed with distilled H_2O (two column volumes (100 mL)). Headspace pressure was applied with N_2 gas as required throughout to maintain a flow rate of no more than two drops per second while solvent was flushing through the column. C_{18} columns were stored in H_2O until required for use.

3.2.2.2 Sample preparation

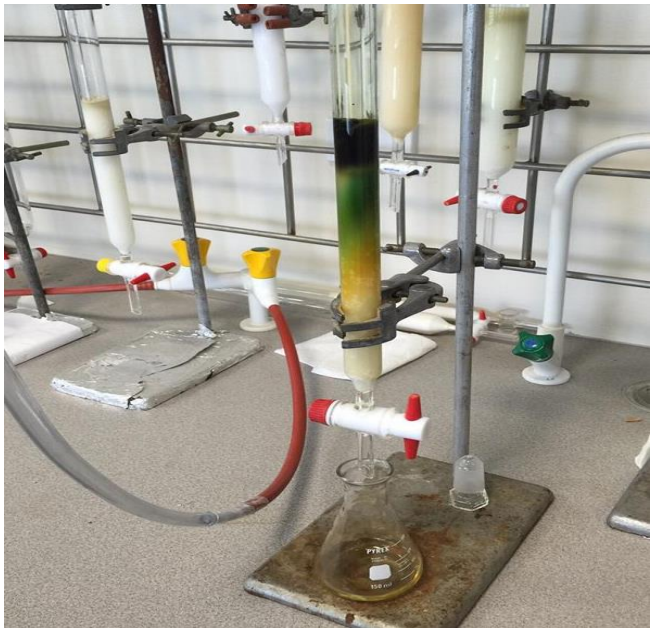
Each crude extract was prepared for fractionation by freeze drying. Samples were pre-frozen in liquid nitrogen (approximately -80°C) before being transferred to a freeze drying chamber. Samples remained in the chamber for 2-3 days or until all remaining moisture was removed so that the extract was in a solid form.

3.2.2.3 Procedure

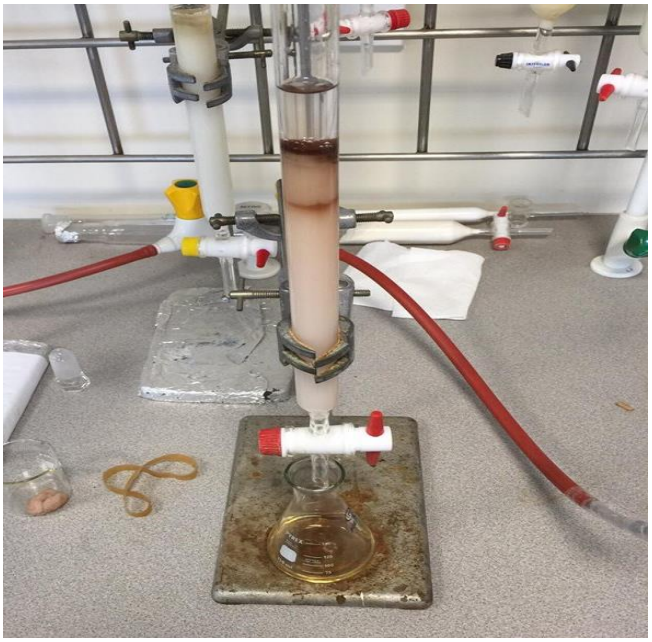
Each extract was crushed into small pieces with a metal spatula and loaded into the column via pouring. Care was taken while loading the sample to ensure that the extract did not adhere to the sides. If samples were poorly soluble in MeOH, then a few drops of DCM (HPLC grade) were added to aid solubilisation. Solvent was loaded onto the column slowly and evenly to minimise disturbance to the column bed (see Table 3.5-3.9 for solvent schemes). Headspace pressure was applied with N_2 gas as required to maintain a flow rate of no more than two drops per second while solvent was flushing through the column. Collection of

each fraction into a flask ceased when the solvent had drained down to 0.5 cm above the column bed. The process was repeated for each solvent.

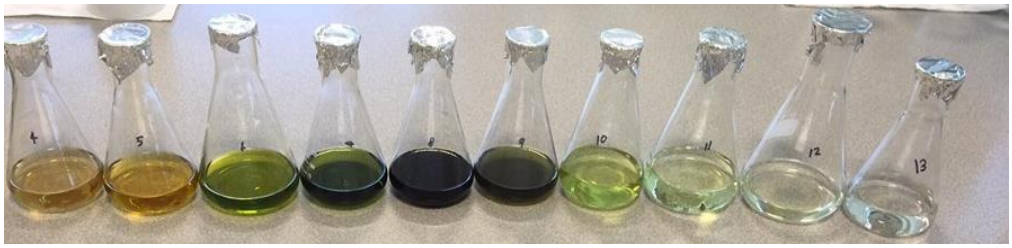
Each fraction was transferred to a round bottom flask and solvent was removed using a rotary evaporator with a water bath temperature of 35°C. Dried crude fractions were resuspended (in MeOH/ DCM) and transferred by pipette into a pre-weighed scintillation vial. Nitrogen (N_2) gas in a heating block set to 20-25°C was used to evaporate excess solvent. The sample was reweighed at room temperature and stored 4°C until required for further use. Each fraction was labelled by its respective identification code (see Table 2.1), followed by the solvent number as stated in the solvent scheme (for example, ASB14c.1-11).



a



b



c

Figure 3.5. Separation of 20g extract of ABS9a by reversed phase C_{18} column chromatography. This includes a) elution of early polar fractions with a distinctive green colour, b) elution of fractions in the non-polar region, and c) collection of all fractions produced from the C_{18} column.

3.2.3 *In vitro* bioassay of 'hit' macroalgae candidates

Crude extracts and fractions produced from subsequent chromatography separation were screened by *in vitro* liquid assay to determine the biological activity against Psa biovar 3 after 2 and 24 hours of interaction time. Extracts were divided into six assay groups; crude extracts ABS3c, ABS6c, ABS9c, ABS13c, ABS14c; fractions ABS3c.1-14; fractions ABS6c.1-11; fractions ABS9c.1-13; fractions AB13c.1-13; and fractions ABS14c.1-11. Each assay was carried out in duplicate.

3.2.3.1 Procedure

Each sample was dissolved in MeOH (10 mL) for approximately 30 mins. An aliquot of this solution (5 µl) was diluted in sterile H_2O (495 µl) containing Psa biovar 3 (strain #10627) (5 µl) to give a 1:100 dilution ratio. After waiting an arbitrary time of 2 hours, an aliquot (100 µl) was further diluted in sterile H_2O (0.9 mL). This was repeated a further four times to give 6 x 1/10 serial dilutions labelled DO (0), D1 (1:100), D2 (1:1000), D3 (1:10,000), D4 (1:100,000), and D5 (1:1,000,000) (Figure 3.6). Each diluted sample (10 µl) was transferred onto a Petri dish containing Kings B media. This was carried out in triplicate. The remaining 1:100 stock dilution (430 µl) was stored for 24 hours and the process was repeated. Each bioassay included a negative control which added sterile H_2O (5 µl) in the place of the stock extract, and a control which added MeOH (5 µl) in the place of the stock extract. All plates were placed in a 28°C incubator for 48-62 hours until results were read. The start time of each assay and time of the 2 and 24 hour interaction was recorded.

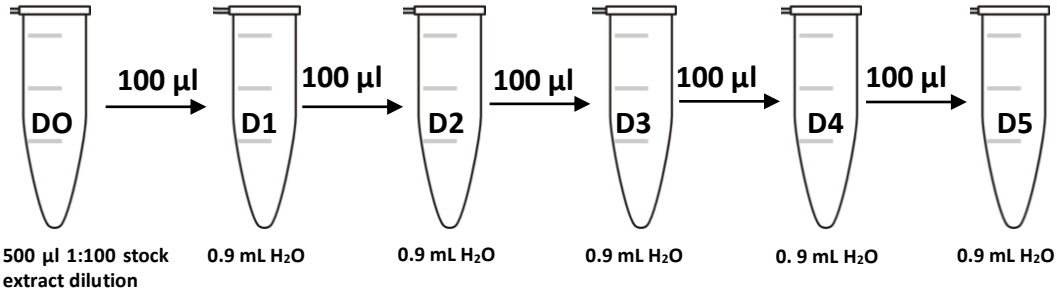


Figure 3.6. Process of dilution for each extract.

3.2.3.2 Analysis

The population of Psa biovar 3 when screened against of each crude extract dilution and subsequent fraction dilutions after 2 or 24 hours of interaction time was calculated using the same procedure outlined in Section 2.2.4. The level of biological activity for each sample is presented in a summary format in Table 3.3. Further details are shown in Appendices A.6 (20 g crude extracts) and A.7 (subsequent fractions).

3.2.4 Liquid Chromatography – Mass Spectrometry (LC-MS)

Crude extracts fractions were screened by LC-MS using reversed phase chromatography and electrospray mass spectrometry (positive ion).

3.2.4.1 Procedure

LC-MS procedure was performed using the protocol outlined in Section 2.2.3.1.

3.2.4.2 Analysis

3.2.4.2.1 Crude extracts

Peaks of interest in each of the crude extracts were examined. Peaks of interest in this study were defined as what was previously stated in Section 2.2.3.2. Individual peaks and chromatograms for these extracts eluting between 15-45 mins are provided in Appendix A.8. Ions present in these extracts are shown in Table 3.4.

3.2.4.2.2 Produced fractions

Peaks of interest eluting between 15-45 mins in biologically active fractions and adjacent fractions either side were examined. These peaks were placed into five categories based on their respective size in the base peak total chromatogram: trace (0-2 percent), small (2-30 percent), medium (31-60 percent), large (61-90 percent), and very large (91-100 percent). Individual peaks and chromatograms for these fractions eluting between 15-45 mins are provided in Appendix A.9. Peaks of interest in these fractions are shown in Tables 3.10-3.15.

3.3 Results

3.3.1 Biological activity of crude extracts of 'hit' macroalgae against Psa biovar 3

Crude extracts of ABS3c, ABS6c, ABS9c, and ABS14c all consistently inhibited the growth of Psa biovar 3 (+) at 2 and 24 hours of interaction (Table 3.3). The extract of ABS13 did not have any biological activity (-) against Psa at either 2 or 24 hours of interaction.

Table 3.3. Summary of biological activity of 20g crude extracts of 'hit' macroalgae candidates when screened against Psa biovar 3.

Season of collection		**Summer**			
Interaction time		**2 hour**		**24 hour**	
Replicate		**1**	**2**	**1**	**2**
ABS3c	***Vidalia colensoi***	+	+	+	+
ABS6c	***Asparagopsis armata***	+	+	+	+
ABS9c	***Carpophyllum maschalocarpum***	+	+	+	+
ABS13c	***Vidalia colensoi***	-	-	-	-
ABS14c	***Xiphophora chondrophylla***	+	+	+	+

KEY	
-	No inhibition
+/-	Decrease of incidence (>log cfu/mL)
+	Full inhibition

3.3.2 LC-MS spectra of 'hit' crude macroalgae extracts

The following ions representative of peaks at the listed retention times were observed in crude extracts of 'hit' macroalgae candidates (Table 3.4).

Table 3.4. Ions of interest in crude 20g extracts of 'hit' macroalgae candidates.

Crude extract	Ion (*m/z*)	Retention time (mins)
ABS3c	389.8*	16
	393.8*	19
	741.5	38
	743.7	41
	763.5	36
ABS6c	391.8	17
	741.5	38
	743.7	37
	760.5	37
ABS9c	522.2	29
	710.5	37
	732.5	42
	734.5	40
	758.5	36
	760.5	37
	782.3	36
	808.5	37
ABS13c	389.8*	18
	469.8*	17
	609.3	44
	741.5	38
	743.7	41
	760.5	37
	763.5	36
	781.7	37
ABS14c	282.2	21
	340.3	23
	732.5	42
	734.5	40
	738.5	42
	758.5	37
	760.5	37
	808.5	37

*Isotope pattern indicates dibromination. Ion listed is the lowest mass ion of the pattern.

3.3.3 Biological activity of 'hit' macroalgae fractions against Psa biovar 3

3.3.3.1 *Vidalia colensoi* (ABS3c)

The biological activity of ABS3c was separated into two groups between fractions 3.3-3.5, and fraction 3.10 (Table 3.5). Fractions 3.3-3.5, and 3.10 consistently decreased the incidence of Psa biovar 3 after 2 hours of interaction. After 24 hours of interaction, fractions 3.4 and 3.10 consistently decreased the incidence of Psa, while fractions 3.3 and 3.5 only decreased its incidence in one replicate and completely inhibited growth in the other replicate. Fractions 3.1-3.2, 3.6-3.9, 3.11-3.14 were inactive against Psa after 2 and 24 hours of interaction.

Table 3.5. Biological activity of fractions ABS3c.1-14 when screened against Psa biovar 3.

Fraction	Solvent	Volume (mL)	Colour	Mass (mg)	2 hour		24 hour	
					1	2	1	2
3.1	H_2O	50	Clear	24.2	-	-	-	-
3.2	H_2O: MeOH (1:1)	50	Yellow	278.9	-	-	-	-
3.3	H_2O: MeOH (3:7)	50	Yellow	269	+/-	+/-	+/-	+
3.4	H_2O: MeOH (1:9)	50	Yellow	96.7	+/-	+/-	+/-	+/-
3.5	MeOH	50	Yellow	51.8	+/-	+/-	+/-	+
3.6	MeOH: DCM (1:1)	50	Brown	102.8	-	-	-	-
3.7	DCM	50	Brown	33.8	-	-	-	-
3.8	MeOH	50	Yellow	9.2	-	-	-	-
3.9	H_2O: MeOH (1:1)	50	Yellow	142	-	-	-	-
3.10	MeOH	50	Pink	137.8	+/-	+/-	+/-	+/-
3.11	DCM	50	Yellow	157.7	-	-	-	-
3.12	MeOH	50	Yellow	7.5	-	-	-	-
3.13	H_2O: MeOH (1:1)	50	Pink	5.6	-	-	-	-
3.14	H_2O	75	Clear	1.8	-	-	-	-
			Total mass:	1,319				

KEY	
-	No inhibition
+/-	Decrease of incidence (>log cfu/mL)
+	Full inhibition

3.3.3.2 *Asparagopsis armata* (ABS6c)

The biological activity of ABS6c was contained in bands between fractions 6.3-6.5 (Table 3.6). Fraction 6.4 displayed the highest level of biological activity against Psa biovar 3 amongst all fractions; after 2 hours of interaction it decreased its incidence and after 24 hours completely inhibited any growth. Fractions 6.3 and 6.5 were inactive against Psa after 2 hours of interaction; however, after 24 hours of interaction both fractions consistently decreased its incidence. Fractions 6.1-6.2, and 6.6-6.11 were all inactive against Psa at both 2 and 24 hours of interaction.

Table 3.6. Biological activity of fractions ABS6c.1-11 when screened against Psa biovar 3.

Fraction	Solvent	Volume (mL)	Colour	Mass (mg)	2 hour		24 hour	
					1	2	1	2
6.1	H_2O	50	Clear	295.1	-	-	-	-
6.2	H_2O: MeOH (1:1)	50	Yellow	115.5	-	-	-	-
6.3	H_2O: MeOH (3:7)	50	Yellow	11.3	-	-	+/-	+/-
6.4	H_2O: MeOH (1:9)	50	Yellow	10.4	+/-	+/-	+	+
6.5	MeOH	50	Green	24.1	-	-	+/-	+/-
6.6	MeOH	50	Green	29.4	-	-	-	-
6.7	MeOH: DCM (1:1)	50	Yellow	21.5	-	-	-	-
6.8	DCM	50	Yellow	4.7	-	-	-	-
6.9	MeOH	50	Green	1.5	-	-	-	-
6.10	H_2O: MeOH (1:1)	50	Green	0.5	-	-	-	-
6.11	H_2O	75	Clear	0.4	-	-	-	-
			Total mass:	514.4				

KEY	
-	No inhibition
+/-	Decrease of incidence (>log cfu/mL)
+	Full inhibition

3.3.3.3 *Carpophyllum maschalocarpum* (ABS9c)

The biological activity of ABS9c was contained in fractions 9.2-9.3, and 9.7 (Table 3.7). All fractions were inactive against Psa biovar 3 after 2 hours of interaction. After 24 hours of interaction, fractions 9.2 and 9.3 consistently decreased the incidence of Psa, whereas fraction 9.7 completely inhibited the growth in one replicate and decreased its incidence in the other replicate. Fractions 9.1, 9.4-9.5, and 9.8-9.13 were also inactive against Psa after 24 hours of interaction.

Table 3.7. Biological activity of fractions ABS9c.1-13 when screened against Psa biovar 3.

Fraction	Solvent	Volume (mL)	Colour	Mass (mg)	2 hour		24 hour	
					1	2	1	2
9.1	H_2O	50	Clear	435.8	-	-	-	-
9.2	H_2O: MeOH (1:1)	50	Red	190.8	-	-	+/-	+/-
9.3	H_2O: MeOH (3:7)	50	Orange	78.5	-	-	+/-	+/-
9.4	H_2O: MeOH (1:9)	50	Yellow	44.8	-	-	-	-
9.5	MeOH	50	Yellow	36.4	-	-	-	-
9.6	MeOH	50	Green	41.6	-	-	-	-
9.7	MeOH: DCM (1:1)	50	Green	157.9	-	-	+	+/-
9.8	DCM	50	Green	64.7	-	-	-	-
9.9	MeOH	50	Green	109.4	-	-	-	-
9.10	DCM	50	Green	6.3	-	-	-	-
9.11	MeOH	50	Green	2.8	-	-	-	-
9.12	H_2O: MeOH (1:1)	50	Green	4.3	-	-	-	-
9.13	H_2O	75	Clear	9.5	-	-	-	-
			Total mass:	1,183				

KEY	
-	No inhibition
+/-	Decrease of incidence (>log cfu/mL)
+	Full inhibition

3.3.3.4 *Vidalia colensoi* (ABS13c)

The biological activity of ABS13c occurred in fractions 13.1 and 13.11-13.12 (Table 3.8). The only active fraction after 2 hours of interaction was fraction 13.1; this decreased the incidence of Psa in one replicate only and was inactive in the other replicate. After 24 hours of interaction, fractions 13.11 and 13.12 decreased the incidence of Psa in one replicate only and in the other replicate was inactive. Fractions 13.1-13.5, 13.8-13.10, and 13.13 were inactive against Psa biovar 3 at both 2 and 24 hours of interaction with Psa.

Table 3.8. Biological activity fractions ABS13c.1-13 when screened against Psa biovar 3.

Fraction	Solvent	Volume (mL)	Colour	Mass (mg)	2 hour		24 hour	
					1	2	1	2
13.1	H_2O	50	Clear	1675.4	-	+/-	-	-
13.2	H_2O: MeOH (1:1)	50	Orange	117.8	-	-	-	-
13.3	H_2O: MeOH (3:7)	50	Orange	30.3	-	-	-	-
13.4	H_2O: MeOH (1:9)	50	Orange	23.4	-	-	-	-
13.5	MeOH	50	Orange	33.2	-	-	-	-
13.6	MeOH	50	Orange	35	-	-	-	-
13.7	MeOH: DCM (1:1)	50	Green	65	-	-	-	-
13.8	DCM	50	Green	25.2	-	-	-	-
13.9	MeOH	50	Yellow	3.5	-	-	-	-
13.10	DCM	50	Yellow	1.9	-	-	-	-
13.11	MeOH	50	Yellow	1.3	-	-	+/-	-
13.12	H_2O: MeOH (1:1)	50	Yellow	2.1	-	-	+/-	-
13.13	H_2O	75	Clear	0.4	-	-	-	-
			Total mass:	1,950				

KEY	
-	No inhibition
+/-	Decrease of incidence (>log cfu/mL)
+	Full inhibition

3.3.3.5 *Xiphophora chondrophylla* (ABS14c)

The biological activity of ABS14c was contained in fractions 14.3-14.5 (Table 3.9.). After 2 hours of interaction, fractions 14.3 and 14.4 consistently inhibited the growth of Psa biovar 3, whereas one replicate of fraction 14.5 decreased its incidence and the other completely inhibited any growth. After 24 hours of interaction, fractions 14.3, 14.4, and 14.5 all consistently inhibited the growth of Psa. Fractions 14.1-14.2 and 14.6-14.11 were inactive against Psa at both 2 and 24 hours of interaction.

Table 3.9. Biological activity of fractions ABS14c.1-11 when screened against Psa biovar 3.

Fraction	Solvent	Volume (mL)	Colour	Mass (mg)	2hour		24 hour	
					1	2	1	2
14.1	H_2O	50	Clear	445.7	-	-	-	-
14.2	H_2O: MeOH (1:1)	50	Green	125.6	-	-	-	-
14.3	H_2O: MeOH (3:7)	50	Yellow	132.4	+	+	+	+
14.4	H_2O: MeOH (1:9)	50	Yellow	183	+	+	+	+
14.5	MeOH	50	Orange	53.2	+/-	+	+	+
14.6	MeOH	50	Orange	110.7	-	-	-	-
14.7	MeOH: DCM (1:1)	50	Green	134.7	-	-	-	-
14.8	DCM	50	Green	15.8	-	-	-	-
14.9	MeOH	50	Yellow	1.6	-	-	-	-
14.10	H_2O: MeOH (1:1)	50	Clear	0.2	-	-	-	-
14.11	H_2O	75	Clear	2.5	-	-	-	-
			Total mass:	1,205				

KEY	
-	No inhibition
+/-	Decrease of incidence (>log cfu/mL)
+	Full inhibition

3.3.4 LC-MS spectra of 'hit' macroalgae fractions

3.3.4.1 *Vidalia colensoi* (ABS3c)

Ions of interest in biologically active fractions of ABS3c (3.3-3.5, 3.10) and adjacent fractions (3.2, 3.6, 3.9, 3.11) displayed various distinct mono-, di-, or tetra- brominated isotope patterns (Table 3.10). The ions *m/z* 311.9 (monobrominated) and 389.8 (dibrominated) were each observed in all fractions; *m/z* 393.8 (dibrominated) was observed in seven fractions; *m/z* 421.8 and 435.8 (both dibrominated) were each observed in six fractions; *m/z* 278.8, 308.8 and 479.9 (all dibrominated) were each observed in four fractions; *m/z* 469.8 and 669.8 (both dibrominated) were each observed in three fractions; *m/z* 447.8 and 651.8 (both dibrominated) were each observed in two fractions; *m/z* 401.9, 407.9, 417.7, 419.9 (all dibrominated), and 667.8 (tetrabrominated) were each observed in one fraction.

Table 3.10. Ions of interest in biologically active fractions of ABS3c and adjacent fractions.

Ion (*m/z*)	Isotope pattern	Present in	Size
278.8	Dibrominated	3.3	Small
		3.4	Small
		3.5	Small
		3.10	Trace
308.8	Dibrominated	3.3	Small
		3.4	Small
		3.5	Small
		3.10	Trace

Ion (*m/z*)	Isotope pattern	Present in	Size
311.9	Monobrominated	3.2	Small
		3.3	Small
		3.4	Small
		3.5	Small
		3.6	Trace
		3.9	Small
		3.10	Small
		3.11	Trace
389.8	Dibrominated	3.2	Small
		3.3	Very large
		3.4	Very large
		3.5	Very large
		3.6	Small
		3.9	Large
		3.10	Very large
		3.11	Small
393.8	Dibrominated	3.3	Medium
		3.4	Medium
		3.5	Medium
		3.6	Trace
		3.9	Small
		3.10	Medium
		3.11	Trace
401.9	Dibrominated	3.10	Small
407.9	Dibrominated	3.4	Small
417.7	Dibrominated	3.10	Trace
419.9	Dibrominated	3.6	Trace
421.8	Dibrominated	3.3	Small
		3.4	Small
		3.5	Small
		3.9	Trace
		3.10	Small
		3.11	Trace
435.8	Dibrominated	3.4	Small
		3.5	Small
		3.6	Trace
		3.9	Small
		3.10	Small
		3.11	Trace

Ion (*m/z*)	Isotope pattern	Present in	Size
447.8	Dibrominated	3.9	Small
		3.11	Trace
469.8	Dibrominated	3.4	Trace
		3.5	Small
		3.10	Small
479.9	Dibrominated	3.4	Small
		3.5	Trace
		3.6	Trace
		3.11	Trace
651.8	Dibrominated	3.5	Small
		3.10	Trace
667.8	Tetrabrominated	3.10	Trace
669.8	Dibrominated	3.4	Small
		3.5	Small
		3.9	Small

3.3.4.2 *Asparagopsis armata* (ABS6c)

Ions of interest in biologically active fractions of ABS6c (6.3-6.5) and adjacent fractions (6.2, 6.6) did not display any distinct isotope patterns (Table 3.11). No ions of interest were observed in fraction 6.2. Two very early eluting ions with an *m/z* of 468.9 were observed in fractions 6.3-6.6. The ion *m/z* 564.3 and 580.3 were observed in fractions 6.3 and 6.4.

Table 3.11. Ions of interest in biologically active fractions of ABS6c and adjacent fractions.

Ion (*m/z*)	Present in	Size
468.9	6.3	Trace
	6.4	Trace
	6.6	Trace
564.3	6.3	Medium
	6.4	Trace
580.3	6.3	Medium
	6.4	Trace

3.3.4.3 *Carpophyllum maschalocarpum* (ABS9c)

Ions of interest in biologically active fractions of ABS9c (9.2, 9.3, 9.7) and adjacent fractions (9.1, 9.4, 9.6, 9.8) did not display any distinct isotope patterns (Table 3.12). No ions of interest were observed in fractions 9.4 and 9.6-9.8. The ion *m/z* 282.2 was observed in 9.1-9.3; *m/z* 468.2 was observed in 9.2 and 9.3; and *m/z* 298.2 was observed in 9.1 only.

Table 3.12. Ions of interest in biologically active fractions of ABS9c and adjacent fractions.

Ion (*m/z*)	Present in	Size
282.2	9.1	Very large
	9.2	Medium
	9.3	Medium
298.2	9.1	Small
468.9	9.2	Trace
	9.3	Trace

3.3.4.4 *Vidalia colensoi* (ABS13c)

Ions of interest in biologically active fractions of ABS3c (3.3-3.5, 3.10) and adjacent fractions (3.2, 3.6, 3.9, 3.11) were also analysed in equivalent fractions of ABS13c (Table 3.13). Ions present displayed various distinct mono-, di-, or tetra- brominated isotope patterns. The ion *m/z* 469.8 (dibrominated) was observed in all fractions; *m/z* 389.8 (dibrominated) was observed in seven fractions; *m/z* 435.8 (dibrominated) was observed in six fractions; *m/z* 515.9 (dibrominated) was observed in three fractions; *m/z* 393.8, 499.8, 527.9, 559.8 (all dibrominated) were each observed in two fractions; *m/z* 473.9, 541.8 (all dibrominated), 327.9, (monobrominated) and 667.8 (tetrabrominated) was observed in one fraction.

Table 3.13. Ions of interest in ABS13c from biologically active fractions of ABS3c and adjacent fractions.

Ion (*m/z*)	Isotope pattern	Present in	Size
327.9	Monobrominated	13.2	Small
389.8	Dibrominated	13.3	Medium
		13.4	Medium
		13.5	Large
		13.6	Small
		13.9	Small
		13.10	Small
		13.11	Small
393.8	Dibrominated	13.2	Small
		13.3	Medium
435.8	Dibrominated	13.3	Medium
		13.4	Medium
		13.5	Small
		13.9	Trace
		13.10	Trace
		13.11	Trace
469.8	Dibrominated	13.2	Small
		13.3	Large
		13.4	Large
		13.5	Medium
		13.6	Small
		13.9	Small
		13.10	Trace
		13.11	Trace
473.9	Dibrominated	13.2	Small
499.9	Dibrominated	13.9	Trace
		13.11	Trace
515.9	Dibrominated	13.2	Small
		13.3	Small
		13.4	Small
527.9	Dibrominated	13.3	Small
		13.4	Small
541.8	Dibrominated	13.2	Small
559.8	Dibrominated	13.3	Small
		13.4	Small
667.8	Tetrabrominated	13.5	Small

3.3.4.5 *Xiphophora chondrophylla* (ABS14c)

Ions of interest in biologically active fractions of ABS14c (14.3-14.5) and adjacent fractions (14.2, 14.6) did not display any distinct isotope patterns (Table 3.14). No ions of interest were observed in fraction 14.2. The ions *m/z* 298.2 and 340.3 were each observed in four fractions; *m/z* 282.2 was observed in three fractions; *m/z* 278.2, 314.2 and 398.2 were each observed in one fraction.

Table 3.14. Ions of interest in biologically active fractions of ABS14c and adjacent fractions either side.

Ion (*m/z*)	Present in	Size
278.2	14.6	Small
282.2	14.4	Large
	14.5 x 2	Trace, trace
	14.6	Small
298.2	14.3	Very Large
	14.4	Small
	14.5	Small
	14.6 x 2	Small
314.2	14.3	Small
340.3	14.3	Very large
	14.4 x 2	Medium, very large
	14.5 x 2	Small, small
	14.6 x 2	Small, small
398.2	14.3 x 2	Medium

3.4 Discussion

The secondary screening phase of this study further examined five selected 'hit' New Zealand macroalgae candidates collected from the Bay of Plenty region for control of the causal agent of bacterial kiwifruit canker disease, *Pseudomonas syringae pv. actinidiae* biovar 3. This phase was divided into two parts. The first part performed *in vitro* bioassays and LC-MS analysis of crude extracts on a larger scale, and the second part performed *in vitro* bioassays and LC-MS analysis of fractions produced from subsequent fractionation. Collectively, this provided insight as to which of the 'hit' macroalgae candidates were of the highest priority to further explore chemically in the later phases of discovery.

3.4.1 Confirmation of biological activity against Psa biovar 3 and compound information

In vivo bioassays were performed on crude extracts of 'hit' macroalgae to confirm the biological activity against Psa biovar 3 at a larger scale. Results from the bioassay provide a more clear-cut indication of the biological activity for each candidate than what was observed when bioassays were performed on smaller scale extracts. Previously, the only small scale extract of species to completely inhibit Psa after 2 hours of interaction was that of *X. chondrophylla* (AB14). Results from the large scale extraction, however, confirmed the Leisure Island sample of *V. colensoi* (AB3), *A. armata* (AB6), *C. maschalocarpum* (AB9), and *X. chondrophylla* (AB14) all completely inhibited growth of Psa. At 24 hours of interaction, the small scale extracts to completely inhibit Psa were those of the Leisure Island sample of *V. colensoi* (AB3), *A. armata* (AB6), *C. maschalocarpum*

(AB9), and *X. chondrophylla* (AB14). These results were also consistent on a larger extraction scale. The only extract of all the 'hit' candidates to exhibit less biological activity against Psa on a larger scale was the Karewa Island sample of *V. colensoi* (AB13). The small scale extract of AB13 decreased the incidence of Psa after 24 hours of interaction; however, the larger scale extract was inactive. This more clear-cut indication of the biological activity for each 'hit' macroalgae candidate may be due to the upscaled extraction, whereby a more chemically representative subsample of the alga was selected on a larger scale than on a smaller scale. Alternatively, something may have been amiss with the extract of the Karewa Island sample of *V. colensoi*. Further investigation is required to validate either suggestion. Nonetheless, the secondary screening results conclude that the Karewa Island sample of *V. colensoi* (AB13) is no longer a candidate of interest to examine for control of Psa biovar 3. It is, however, still a candidate of interest to provide comparisons with the other sample of *V. colensoi* (AB3).

Analyses of the LC-MS spectra of large scale extracts of 'hit' macroalgae were performed via a process of elimination to inform which ions are likely to represent compounds of relevance against Psa biovar 3. The preliminary screening phase provided insight as to which ions present in small scale extracts were also present in extracts of inactive macroalgae. A number of these ions were observed again in the large scale extracts (Table 3.15). These ions cannot be ruled out as being representative of a compound with biological activity against Psa; however, confirmation of their presence at a larger scale does

increase the likelihood that they are ubiquitous macroalgae metabolites or common contaminants. There were also certain ions observed at a larger scale which were not previously observed at a smaller scale (Table 3.16). Again, these ions cannot be ruled out as being representative of a compound with biological activity against Psa; however, given that they were not previously observed it is unlikely they represent any relevant compounds of interest.

The preliminary screening phase also provided insight as to which ions observed as unique in the larger scale extracts were also noted as unique at a smaller scale (Table 3.17). It cannot be assumed that these corresponding ions represent compounds with biological activity against Psa; however, their recurrence at a larger scale does increase the likelihood that they represent compounds of interest. Furthermore, the distinct dibrominated isotope pattern of both of the unique ions observed in the Leisure Island *V. colensoi* sample confirms that the compounds of interest may be brominated.

Table 3.15. Ions present in both the smaller and larger scale extracts of 'hit' macroalgae candidates which were also present in smaller scale extracts of inactive macroalgae.

Crude 20g extract	Common ion (*m/z*)
ABS3 ***Vidalia colensoi***	741.5; 743.7; 763.5
ABS6 ***Asparagopsis armata***	741.5; 743.7; 760.5
ABS9 ***Carpophyllum maschalocarpum***	522.2; 710.5; 732.5; 734.5; 758.5; 760.5; 782.3; 808.5
AB14 ***Xiphophora chondrophylla***	732.5; 734.5; 738.5; 758.5; 760.5; 808.5

Table 3.16. Ions present in larger scale extracts of 'hit' macroalgae candidates which were not previously observed in the smaller scale extracts.

Crude 20g extract	Ion (*m/z*)
ABS3 ***Vidalia colensoi***	
ABS6 ***Asparagopsis armata***	391.8
ABS9 ***Carpophyllum maschalocarpum***	-
AB14 ***Xiphophora chondrophylla***	282.2

Table 3.17. Ions present in larger scale extracts of 'hit' macroalgae candidates which were previously observed in the smaller scale extracts.

Crude 20g extract	Unique on (*m/z*)
ABS3 ***Vidalia colensoi***	389.8*; 393.8*
ABS6 ***Asparagopsis armata***	-
ABS9 ***Carpophyllum maschalocarpum***	-
ABS14 ***Xiphophora chondrophylla***	340.3
*Isotope pattern indicates dibromination. Ion listed is the lowest mass ion of the pattern.	

3.4.2 Compounds of interest against Psa biovar 3

In vitro bioassays against Psa biovar 3 were performed on each of the fractions produced from the separation of the larger scale macroalgae extracts. It should be noted that the ions observed are the $[M+H]^+$ ions. Hence, the molecular mass is -1. This is an assumption. In theory other ions such as $[M+Na]^+$ could be present; however, it is assumed that they are not.

Ions of interest in biologically active (3.3-3.5, 3.10) and adjacent fractions (3.2, 3.6, 3.9, 3.11) in the Leisure Island sample of *V. colensoi* (ABS3) indicated the presence of a variety of ions with distinct mono-, di-, or tetra- brominated isotope patterns (Figure 3.7). These brominated ions were then compared with brominated ions in equivalent fractions of the less active *V. colensoi* sample collected at Karewa Island (ABS13) to inform which of the brominated ions are of greatest interest against Psa biovar 3.

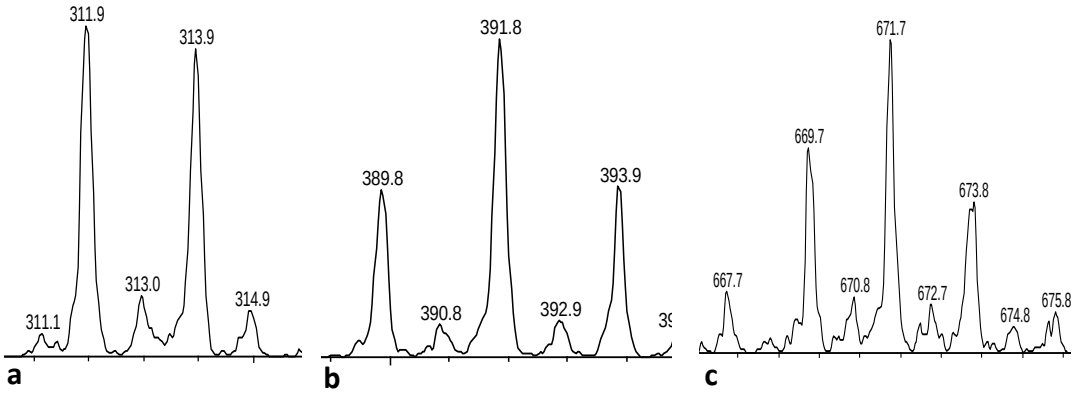


Figure 3.7. Various ions present in ABS3 with distinct a) monobrominated, b) dibrominated, and c) tetrabrominated isotope patterns.

The first ions eliminated as peaks of interest were those observed at equal relative concentrations in both samples of *V. colensoi* (ABS3 and ABS13). For

instance, the dibrominated ions *m/z* 389.8 and 393.8 were noted as present in both the smaller and larger scale extracts of ABS3 and ABS13. These ions were observed again in biologically active fractions of ABS3; however, they were also observed at the same relative concentration in some adjacent inactive fractions. Furthermore, they were observed at the same or greater relative concentration in equivalent fractions of ABS13. The dibrominated ion *m/z* 469.8 was previously identified as an ion of interest in the smaller scale extract of AB13. It was observed in some biologically active fractions of ABS3 (3.4, 3.5, 3.10); however, it was also present at the same relative concentration in most equivalent fractions of ABS13. Similarly, the dibrominated ion *m/z* 435.8 was observed in very small/ trace concentrations in biologically active fractions of ABS3 (3.4, 3.5, 3.10). It was however, also observed at equal relative concentration in adjacent inactive fractions of ABS3 (3.6, 3.9, 3.11) and at an equal or greater concentration in equivalent fractions of ABS13. Furthermore, the dibrominated ion *m/z* 667.8 was only observed in adjacent inactive fraction 3.10 of ABS3. These comparisons lead to the conclusion that the above ions in *V. colensoi* are unlikely to represent compounds of interest against Psa biovar 3.

There were certain ions only observed in some fractions of the Leisure Island sample of *V. colensoi* which were eliminated as likely compounds of interest against Psa biovar 3. For instance, the dibrominated ion *m/z* 479.9 was observed in some active (3.4 and 3.5) and adjacent inactive fractions (3.6 and 3.11) of ABS3. Given that it was not present in all active fractions and was also observed in equal relative concentrations in inactive fractions, it was ruled out as an ion of

interest. Furthermore, the dibrominated ions *m/z* 401.9, 407.9, 417.7, 419.9, 447.8, 651.8, and 669.8 were all observed at a low relative concentration in very few fractions of ABS3. Given that these ions were observed at random and in very few fractions, it is also unlikely they are likely to represent biologically active compounds of interest in *V. colensoi.*

There are certain dibrominated ions in the Leisure Island sample of *V. colensoi* which are suggested as likely to represent compounds of interest against Psa biovar 3. For instance, the dibrominated ions *m/z* 278.8 and 308.8 were both present in small relative concentrations in all biologically active fractions of ABS3 (3.3, 3.4, 3.5, 3.10) and were not observed in adjacent inactive fractions or in ABS13. These ions could therefore represent biologically active compounds of interest. The monobrominated ion *m/z* 311.9 was observed in both all biologically active and adjacent inactive fractions of ABS3. It was, however, observed at higher concentrations amongst active fractions and thus could represent a biologically active compound of interest. Furthermore, the dibrominated ion *m/z* 421.8 was observed at a low relative concentration amongst all biologically active fractions (3.3, 3.4, 3.5, 3.10) and was only in observed trace concentration in two inactive fractions (3.9 and 3.11). This ion could also potentially represent a potentially biologically active compound.

Comparisons between brominated ions observed in fractions from the Leisure Island sample of *V. colensoi* (AB3) with those observed in equivalent fractions from the Karewa Island sample (AB13) suggest that there are four ions in *V.*

colensoi which are of greatest interest for further study against Psa biovar 3. These include the dibrominated ions *m/z* 278.8 (mass 277.8), 308.8 (mass 307.8), and 421.8 (mass 420.8), and the monobrominated ion 311.9 (mass 310.9). Information provided by the previous review (Table 3.1) on *V. colensoi* indicates that various brominated compounds have been previously isolated from the *Vidalia* genera. These include a novel pentabromide, Viadol A (mass 759.9) isolated from *V. obtusiloba* (Figure 3.8a) (Wiemer *et al.*, 1991); a novel tribromide, Viadol B (mass 482) isolated from *V. obtusiloba* (Figure 3.8b) (Wiemer et al., 1991); a novel dibromine, colensolide A (mass 434.9) isolated from *V. obtusiloba* (Figure 3.8c) (Popplewell & Northcote, 2009); a novel dibromide (mass 267.9) isolated from *V. spiralis* (Figure 3.8d) (Kazlauskas *et al.*, 1982); two novel tetrabromides isolated from *V. obtusiloba* (Figure 3.8e, mass=889.5 ; Figure 3.8f, mass=889.5) (Carvalho *et al.*, 2006); and four novel dibromines (Figure 3.8g), lanosol (compound 1, mass=324), Lanosol-1'-methyl (compound 2, mass=297.7), lanosol-1'-ethyl ether (compound 3, mass=323.9), and lanosol-1,4-disulfate ester (compound 4, mass=534) isolated from *V. serrata* (Barreto & Meyer, 2006). At this stage of the research, there is no evidence to suggest that the bromination of these compounds and/ or their molecular weights coincide with the brominated ions of interest. Thus, it can be speculated that these ions could represent structurally novel compounds with biological activity against Psa. Further chemical investigation which extends beyond the scope of this study is required to validate this.

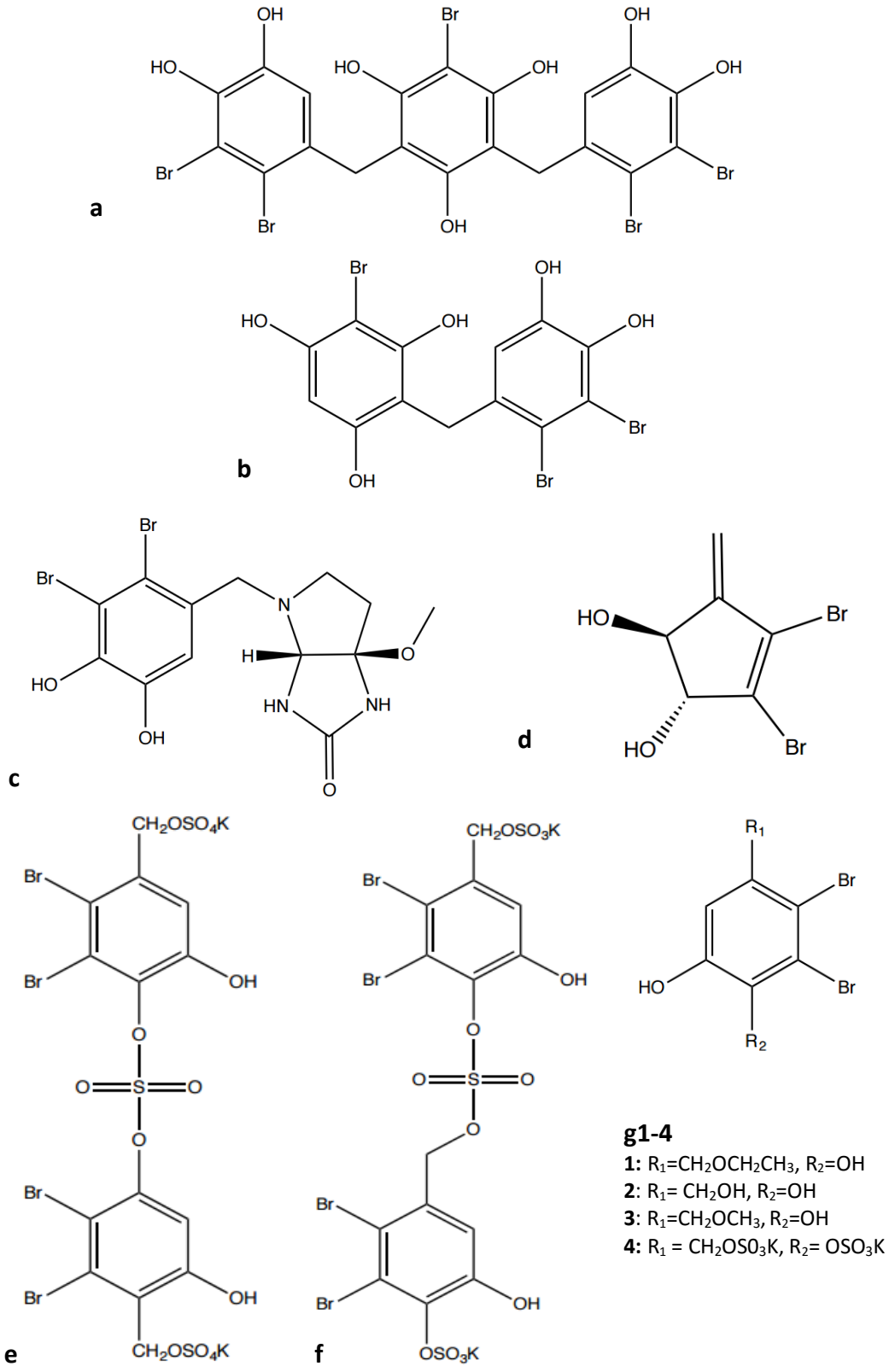


Figure 3.8. Chemical structures of brominated compounds isolated from the *Vidalia* genus to date. These include a) Viadol A; b) Viadol B isolated from *V. obtusiloba* (Wiemer et al., 1991); c) colensolide A (isolated from *V. obtusiloba* (Popplewell & Northcote, 2009); d) a novel dibromide isolated from *V. spiralis* (Kazlauskas *et al.*, 1982); e); f) two novel tetrabromides isolated from *V. obtusiloba* (Carvalho *et al.*, 2006); and g) 1. lanosol, 2. Lanosol-1'-methyl, 3. lanosol-1'-ethyl ether, 4. Lanosol-1,4-disulfate ester isolated from *V. serrata* (Barreto & Meyer, 2006).

Four ions of interest were observed in biologically active (6.3, 6.4, 6.5) and adjacent inactive fractions (6.2, 6.6) of *Asparagopsis armata* (ABS6). The ion *m/z* 469.9 was observed in trace amounts in biologically active fractions 6.3, 6.4, and 6.5. It was, however, also observed at the same concentration in the inactive fraction 6.6. Thus, it is not likely that it is representative of any compounds of interest. The ions *m/z* 564.3 and 580.3 were each observed at medium concentrations in fraction 6.3 and trace concentrations in 6.4. Given that these ions were not present in the other fraction 6.5 (which had equal biological activity against Psa biovar 3), they are also not likely to represent compounds of interest. This suggests that no ions of interest correlate with the biological activity of *A. armata* against Psa biovar 3. Furthermore, no evident isotope patterns were observed for these ions.

Three ions of interest were observed in biologically active (9.2, 9.3, 9.7) and adjacent fractions (9.1, 9.4, 9.6, 9.8) of *Carpophyllum maschalocarpum* (ABS9). The ion *m/z* 282.2 was observed in medium concentrations in the active fractions 9.2 and 9.3. It was, however, also observed at a high concentration in the adjacent inactive fraction 9.1 and not observed in the other active fraction 9.7. Thus, it is not likely representative of a compound of interest. The ion *m/z* of 468.9 was observed in trace amounts in the active fractions 9.2 and 9.3; however, it was not observed in the other active fraction 9.7. Similarly, the ion *m/z* of 298.2 was observed in only the inactive fraction 9.1. This suggests that no ions of interest correlate with the presence of biological activity in *C. maschalocarpum*

against Psa biovar 3. Furthermore, no evident isotope patterns were observed for these ions.

A number of peak ions of interest were observed in biologically active (14.3-14.5) and adjacent inactive fractions of interest (14.2 and 14.6) in *Xiphophora chondrophylla* (ABS14). The ion *m/z* of 298.2 was previously identified as an ion of interest in the smaller scale extracts of ABS14. It was observed in low and higher concentrations in active fractions 14.3 and 14.4; however, it was also observed in small concentration the inactive adjacent fraction 14.6 and not observed in the active fraction 14.5. The ion *m/z* 282.2 was observed in trace and small concentrations in active fractions 14.4 and 14.5. It was, however, also observed at low concentration in the inactive 14.6 fraction and not observed in the other active fraction 14.3. The ion *m/z* 340.3 ranged from a small to very large concentration in all active fractions; however, it was also observed at small concentration in the inactive fraction 14.6. Each of the ions *m/z* 314.2 and 398.2 were observed only in the active fraction 14.3, while the ion *m/z* 278.2 was observed only in the adjacent inactive fraction 14.6. Given that the presence of these ions does not correlate with the biological activity observed in fractions of *X. chondrophylla*, they are unlikely to represent compounds of interest for further chemical exploration. Furthermore, no evident isotope patterns were observed for these ions.

3.4.3 Conclusions

The secondary screening phase of this research provided insight as to which of the five selected 'hit' macroalgae candidates are of highest priority to further explore chemically. The first key finding was that the biological activity of each extract of 'hit' macroalgae candidate against Psa biovar 3 was much more clear-cut at a larger scale. Extracts of *Vidalia colensoi* (AB3), *Asparagopsis armata* (AB6), *Carpophyllum maschalocarpum* (AB3), and *Xiphophora chondrophylla* (AB14) all consistently inhibited the growth of Psa biovar 3 after 2 and 24 hours of interaction. In comparison, the other *V. colensoi* sample collected from Karewa Island (AB13) was inactive against Psa and thus was eliminated as a viable candidate. The second key finding was that ions of interest in biologically active and adjacent inactive fractions of *V. colensoi* (both AB3 and AB13) were associated with a variety of distinct mono-, di-, or tetra- brominated isotope patterns. Through a process of elimination, four brominated ions were identified in the Leisure Island sample of *V. colensoi* as most likely to represent compounds with relevant biological activity against Psa biovar 3. Comparison of these ions with the bromination pattern and molecular weights of those compounds previously isolated in the *Vidalia* genus led to the third key finding; that the ions of interest in *V. colensoi* may represent structurally novel bioactive compounds with patentability and commercial marketability. With these leads in mind, it is recommended that the Leisure Island sample of *V. colensoi* is of greatest priority to explore for bioassay guided fractionation in the later phases of discovery.

The primary limitation of this study was the lack of direct comparisons which could be drawn amongst biologically active fractions of each 'hit' macroalgae candidate. For instance, both fractions 3.3 (mass 269 mg) and 3.4 (mass 96.7 mg) of *V.colensoi* (AB3) were resuspended in MeOH at a set volume of 10 mL. Both fractions demonstrated a decrease of incidence of Psa biovar 3 at 2 and 24 hours of interaction. However, fraction 3.4 is diluted nearly 3 times more than fraction 3.3. Thus, it not cannot be assumed the level of biological activity between these two fractions is equal. The results were interpreted with this in mind and therefore no direct comparisons were made amongst fractions with very dissimilar masses. Further work could focus on another bioassay where each fraction is made up to the same concentration. This may provide a clearer indication of which fraction/s are most active and thus which compounds are most relevant for the control of Psa biovar 3.

The second limitation of this study was that only the summer collections of 'hit' macroalgae were examined and no seasonal comparisons were provided. The five 'hit' candidates were selected for further investigation because of their consistent biological activity against Psa biovar 3 in both summer and winter. This provided confirmation that the target activity of the alga did not vary seasonally. Thus, the priority of the secondary screening phase was to further examine 'hit' macroalgae candidates collected in summer only. Future work could focus on repeating the secondary screening phase for 'hit' macroalgae candidates collected in winter to obtain further seasonal comparisons.

CHAPTER 4

ELICITOR SCREENING

4.1 Introduction

This chapter outlines the elicitor screening phase of the study, which provides insight for an effective screening method to further examine New Zealand macroalgae species for relevant biological activity against Psa biovar 3. The preliminary screening phase of this study screened 19 smaller scale crude extracts (2 g) of New Zealand macroalgae for antimicrobial or antibacterial activity Psa biovar 3. The elicitor screening phase will therefore examine a series of preliminary *in vivo* screens as potential methods to further investigate the crude extracts for elicitor activity at a higher order of screening. It should be noted that due to time constraints, conducting the higher order screens is beyond the scope of this study. Rather, this work will provide information for optimisation of an efficient high order screening process to explore elicitation as a relevant mode of action against Psa biovar 3.

4.1.1 Plant defence mechanisms

Plants are constantly threatened by invasions from disease causing phytopathogens. Unlike animal cells, the plant cellular structure is composed of immobile cells embedded in rigid walls. The transportation of mobile defensive cells to sites of infection (adaptive immunity), as seen in animals, is therefore not possible for plant pathogen defence. Instead, plants have evolved both pre-existing and induced response mechanisms to react to and defend against attempted infection (Ebel & Mithofer, 1998). Invasion is primarily prevented by pre-existing plant defence responses. This can include physical barriers on the plant surface such as wax layers, rigid cell walls, and cuticular lipids, as well as chemical barriers such as antimicrobial enzymes and secondary metabolites (Muthamilarasan & Prasad, 2013). Pathogens which overcome these pre-existing barriers elicit an induced plant innate immunity which enables each individual cell to defend against invasion (Ebel & Mithofer, 1998). Subsequently, very few pathogens succeed in causing disease in plants (Muthamilarasan & Prasad, 2013).

Plant innate immunity is comprised of a two branched interconnected system. The first branch is associated with the recognition of microbe- or pathogen-associated molecular patterns (MAMPS/PAMPS). These are essential molecules characteristic of many microbes and can include bacterial flagellum, lipopolysaccharides, chitin, glucan, xylanase, ergosterol, and oligogalacturonides (Muthamilarasan & Prasad, 2013). MAMP/PAMPS are recognised by localised plant cell surface receptors called transmembrane pattern recognition receptors (PRRS), thereby activating MAMP/ PAMP-triggered immunity (MTI/PTI) (Newman

et al., 2013). The second branch of the plant innate immune system acts largely inside the plant cell. It uses nucleotide binding (NB) and leucine rich repeat (LRR) protein products encoded by disease resistant (*R*) gene products to stimulate effector-triggered immunity (ETI). This second defence response is effective only against biotrophic and hemibiotropic pathogens which require living host tissue to cause infection. Thus, it is not effective against those necrotrophic pathogens which kill the host during colonisation (Jones & Dangl, 2006).

Interactions between the two branched plant innate immune system and its suppression by pathogens is best represented by Jones and Dangl (2006) as a four phased 'zig-zag' model (Figure 4.1). In phase one, transmembrane pattern recognition receptors recognise PAMPS/MAMPS, thereby activating MTI/ PTI to prevent further colonisation of the pathogen. In phase two, successful pathogens deliver effector molecules (virulence factors) to suppress the MTI/PTI or otherwise enable pathogen nutrition and dispersal, resulting in effector triggered susceptibility (ETS). In phase three, a given effector is then directly or indirectly recognised by disease resistance NB-LRR proteins, in turn activating ETI. Induced disease resistance via the activation of ETI can often pass the threshold for hypersensitive cell death response (HR), which is an induced voluntary cell death response at sites of infection (Jones & Dangl, 2006; Hiemstra & Zaat, 2013). In phase 4, natural selection enables the pathogen to suppress or avoid ETI by either detaching or diversifying the recognised effector or by acquiring additional effectors. Novel NB-LRR plant alleles are then created so that ETI can again be triggered, and the process is repeated (Jones & Dangl, 2006).

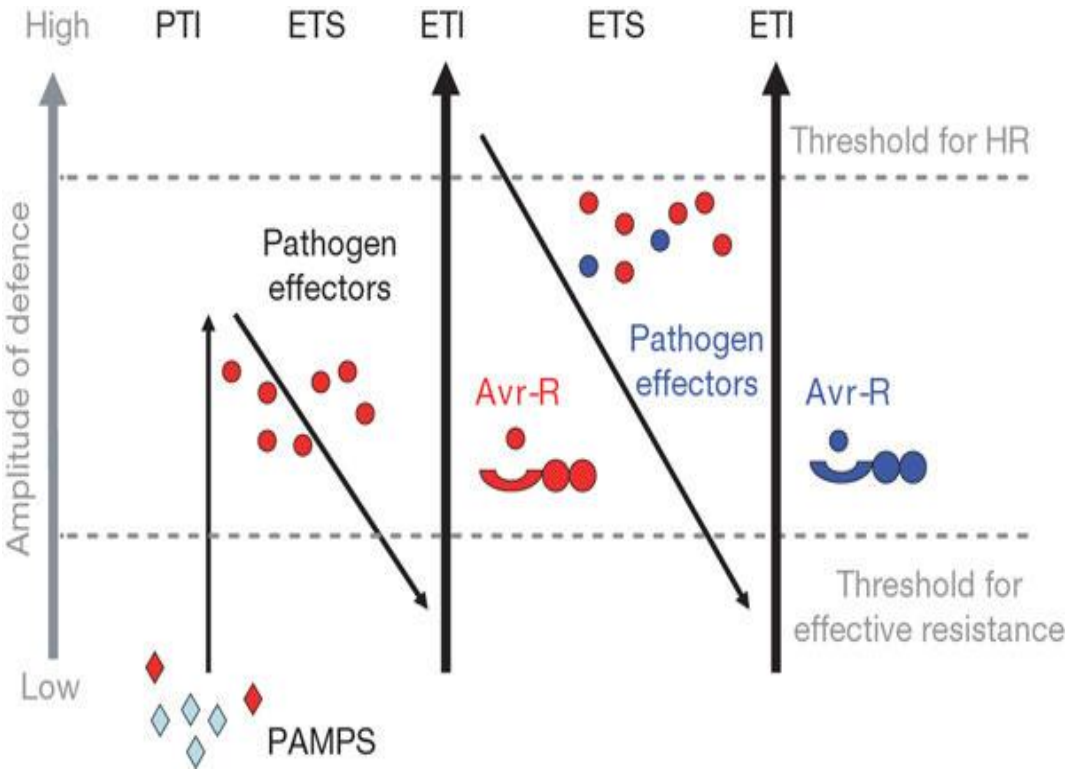


Figure 4.1. Four phased model of the plant innate immune system. Image sourced from Jones and Dangl (2006).

4.1.2 Plant elicitation

Increased understanding of plant defence responses throughout the past century has led to the recognition that signalling compounds called elicitors can stimulate plant innate immunity (Bent & Mackey, 2007). This is a term known as elicitation and is activated upon detection of microorganisms via recognition of elicitor molecules released during plant-pathogen interactions. Elicitors can be of pathogenic origin (exogenous elicitors) or they can be liberated from the targeted plant by the action of enzymes of the pathogen (endogenous elicitors) (Angelova *et al.*, 2006; Thakur & Sohal, 2012). Depending on their origin and molecular structure, elicitors are classified as either physical or chemical, biotic or abiotic, and complex or defined (Figure 4.2) (Angelova *et al.*, 2006).

Furthermore, elicitors can be classified as non-specific or specific depending on their host specificity. Non-specific elicitors can induce plant innate defence responses in both host and non-host plants, whereas specific elicitors induce defence responses only in specific host plants (University of Sydney, 2003).

Elicitors						Reported effects on
Physical Elicitors	Injury					P
	Abiotic		Metal ions (lanthanum, europium, calcium, silver, cadmium), oxalate			Pc
Chemical Elicitors	Biotic	Complex Composition	Yeast cell wall, Mycelia cell wall, Fungal spores			Pc ,F
		Defined Composition	Carbohydrates	Polysaccharides	Alginate	Pc, F, B
					LBG	F
					Pectin	Pc, F
					Chitosan	Pc
					Guar Gum	Pc
				Oligosaccharides	Mannuronate	F
					Guluronate	F
					Mannan	F
					Galacturonides	Pc
				Peptides	Glutathione	Pc
			Proteics	Proteins	Cellulase, Elic-itins, Oligandrin	Pc
			Lipids		Lipopolysaccharides	Pc
			Glycoproteins		Not characterized	Pc
			Volatiles		C_6-C_{10}	Pc

Abbreviations: P, plants; Pc, plant cell culture; B, bacterial cell culture; F, fungal cell culture.

Figure 4.2. Various types of plant elicitors. Image sourced from Angelova et al. (2006).

Elicitors can stimulate a multitude of induced plant responses which provide defence against phytopathogenic invasion. These responses can either be rapidly acting or more delayed. Rapid defence responses are those which are stimulated almost immediately after an attempted invasion. They can include changes to the permeability of the membrane, the production of reactive oxygen species (ROS), hypersensitive cell death response (HR), reinforcements to the cell well, and phytoalexin accumulation (University of Sydney, 2003; Angelova *et al.*, 2006; Thakur & Sohal, 2012). By comparison, delayed defence responses are those which are stimulated well after an attempted pathogenic invasion. They can include the construction of physical barriers such as cork cells and abscission layers and the accumulation of pathogenesis-related proteins, and systemic acquired resistance (SAR) (University of Sydney, 2003).

4.1.2.1 Phytoalexin accumulation

Phytoalexins are antimicrobial compounds of low-molecular weight. They are biosynthesised and accumulated by healthy neighbouring cells and transported to sites of infection (Yoshikawa *et al.*, 1983; Ebel, 1986). Phytoalexin accumulation in a host plant can be triggered by infection; however, it can also be triggered by elicitors and thus provide induced host resistance (Ayers *et al.*, 1976). Laminarin, a storage *β*-1, 3 glucan in brown algae (Ocrophyta) has been shown to stimulate phytoalexin (glyceollin) accumulation in soybean cotyledons against the pathogen *Phytophthora megasperma* (Keen *et al.*, 1983). Later studies have also shown it can stimulate the accumulation of phytoalexins in tobacco (*Nicotiana tabacum*) or grapevine (*Vitis vinifera*) plants (Aziz *et al.*, 2003).

Vacciplant® is a commercially produced biological fungicide which contains the active ingredient laminarin and thus stimulates phytoalexin accumulation in plants. It is registered in countries such as France to provide resistance against the causal agent of strawberry powdery mildew, *Sphaerotheca humuli* and fire blight in apples (*Malus domestica*) and pears (*Pyrus* sp.), *Erwinia amylovora* (Goemar, n.d). While Vacciplant® is not readily available or approved for use in New Zealand, a preliminary study by Kiwifruit Vine Health (2011) has shown it can significantly reduce leaf spotting in *A. deliciosa* cv. Hayward following inoculation of Psa biovar 3.

4.1.2.2 Systemic acquired resistance

Systemic acquired resistance (SAR) is a mechanism of induced plant defence which confers long-lasting host resistance to unrelated pathogens (Durrant & Dong, 2004). Ryals *et al.* (1996) states that after the formation of necrotic lesions, either in response to HR or as a symptom of pathogenic invasion, SAR is activated. The establishment of SAR involves the transportation and transduction of signals via the phloem to target tissues and, in turn, provides host resistance for several days or weeks (Thakur & Sohal, 2012). There is now a large body of evidence to suggest that salicylic acid (SA), an endogenously synthesised signalling molecule, plays an important role in inducing SAR against biotrophic and hemibiotrophic pathogens (Ryals *et al.*, 1996; Tamaoki *et al.*, 2013). For instance, levels of SA in tobacco (*Nicotiana tabacum*) and cucumber (*Cucumis sativus*) have been shown to increase exponentially after attempted pathogen invasion, which correlated with SAR (Malamy *et al.*, 1990; Metraux *et al.*, 1990).

Actigard™ is a foliar- and soil- applied commercial elicitor product used by kiwifruit growers to induce host resistance against *P. syringae* pv. *actinidiae* biovar 3. The active compound contained in Actigard™, Acibenzolar-*S*-Methyl, is a functional analogue of SA. This, in turn, induces SAR in kiwifruit and thus provides long lasting protection against Psa biovar 3 for up to three weeks (Kiwifruit Vine Health, 2015b).

4.1.3 Considerations for a macroalgae screening method

Investigation of small scale crude extracts (2 g) of macroalgae for elicitor activity against Psa biovar 3 in higher order screens requires the design of an appropriate screening method. The commercially produced elicitor, Actigard™ was selected as a reference due to its proven efficacy in providing host plant resistance against Psa. A series of calculations using Actigard™ was performed to determine a recommended resuspension volume required for each small scale extract to trigger potential elicitor activity. These were carried out as follows.

Step one of calculation: Actigard™ contains 500 g/kg of the active ingredient Acibenzolar-*S*-Methyl. The recommended rate for spray control of Psa biovar 3 by Actigard™ is 200 g/ha, of which growers use an average of 1000 L/ha (Kiwifruit Vine Health, 2015b). This equates to 200 g of Actigard™/1000 L, which is equivalent to 200 mg/L. This indicates that one litre of Actigard™ contains 100 mg of active ingredient, which is equivalent to 0.1 mg/mL.

Step two of calculation: The average weight across all small scale crude extracts was 139.2 mg. As advised by University of Waikato natural product specialist Associate Professor Michèle Prinsep, approximately 1 mg of this weight may be comprised of secondary metabolites. Therefore, if one tenth of those metabolites had elicitor activity, which was as effective as Actigard™ (assuming other compounds do not interfere with the efficacy of the elicitor), then each extract would need to be diluted in no more than 1 mL of solvent at the same rate as what is recommended for Actigard™.

A maximum resuspension volume of no more than 1 mL for each crude extract is logistically problematic for two reasons. The first issue is associated with the volume of solution required for screening. For instance, if an average kiwifruit plant has eight leaves and approximately 30 mL was required to spray each leaf, then a minimum of 240 mL is required for each plant. Diluting the available extract in the required volume would result in an active ingredient concentration approximately 240 times lower than the recommended rate for eliciting a response using Actigard™. The second issue is associated with the method of application, as 1 mL would be too viscous for use a spray treatment. Two preliminary *in vivo* bioassays were therefore established to provide insight for an effective downscaled screening method which could be used to examine the small scale extracts of macroalgae for elicitor activity against Psa biovar 3. The first bioassay was considered to examine the crude extracts of macroalgae for general relevant elicitor activity, using Actigard™ as a reference. The second bioassay was considered to examine the crude extracts of brown macroalgae

(containing the active ingredient laminarin) for phytoalexin elicitation, using Vacciplant® as a reference. Soybeans were selected as subjects in this assay; they require very small quantities of solvent and their defence response is easily identified by a red wound colouration (via the production of glyceollin).

4.1.4 Work outlined in this chapter

The primary aim of the work outlined in this chapter is to investigate the effectiveness of two preliminary *in vivo* bioassays, each examining a) elicitor activity of Actigard™ on kiwifruit plant and b) phytoalexin glyceollin elicitation of Vacciplant on soybeans. Based on this, the second aim of the work outlined here is to provide insight and recommendations on whether these assays are appropriate methods to examine crude macroalgae extracts for elicitor activity against Psa biovar 3 in higher order screens.

4.2 Materials and methodology

4.2.1 Screening method for general elicitor activity

The first preliminary bioassay was considered as a screening method to examine the crude extracts of macroalgae for general elicitor activity against Psa biovar 3. Two replicate assays were performed on clonal and open pollinated *Actinidia chinensis* cv. Hort16a plants using the elicitor product Actigard™. The Hort16a cultivar is highly susceptible to bacterial canker infection caused by Psa biovar 3. The objective the experiment was to determine the minimum concentration and/ or volume of crude extract required to stimulate plant detectable elicitation against Psa biovar 3. To test this, the experiment utilised a series of Actigard™ concentrations (full, $^1/_2$, $^1/_4$, $^1/_8$, and $^1/_{16}$ strength) and two application methods (whole plant/ single leaf). Each experiment included a non-treated/ non-inoculated treatment (negative control), a H_2O treated/ non-inoculated treatment (negative control), and a H_2O treated/ inoculated treatment (positive control) (Table 4.1).

Table 4.1. Treatment scheme for preliminary *in vivo* Actigard™ bioassay, designed to examine crude extracts macroalgae for general elicitor activity against Psa biovar 3.

	Control		Actigard™ dilutions					Spray method		Inoculation	
Treatment	**None**	**H_2O**	**Full strength**	**½**	**$^1/_4$**	**$^1/_8$**	**$^1/_{16}$**	**Whole plant**	**Single leaf**	**Yes**	**No**
1.	■							■			■
2.		■						■			■
3.		■						■		■	
4.			■					■		■	
5.			■						■	■	
6.				■				■		■	
7.				■					■	■	
8.					■			■		■	
9.					■				■	■	
10						■		■		■	
11.						■			■	■	
12.							■	■		■	
13.							■		■	■	

4.2.1.1 Preparation

4.2.1.1.1 Culture of plantlets

Clonal *A. chinensis* cv. Hort16a plantlets were produced at Multiflora Laboratories Ltd. using plant tissue culture techniques. Plantlets (3-6 cm in height) were transferred to the University of Waikato in containers on agar. Plantlets were hardened off by making small incisions on the lids of containers and leaving for 3-5 days, and then removing the lids completely for 2 days (Figure 4.3a). Agar was occasionally moistened with water using a misting bottle to prevent desiccation. Plantlets were deflasked by removing each individual out of its respective container and pricking it into a central incision in a moistened rockwool cube (Figure 4.3b-c). As much agar as possible was removed from the roots to prevent fungal growth in the rockwool.

a **b** **c**

Figure 4.3. Hardening off (a) and deflasking (b-c) process of clonal *A. chinensis* cv. Hort16a plantlets.

Seedlings of Hort16a were cultured at the University of Waikato using open pollinated seed extracted from fruit sourced from a local commercial food supplier. Approximately 500 seeds (0.685 g) were weighed and sterilised in sodium hypochlorite (0.6%) for 20-25 mins and then rinsed extensively in sterile H_2O for a further 5 mins. Sterilised seeds were spread evenly in Petri dishes on filter paper moistened with a solution of fungicide (1.25 g/L). Petri dishes were then closed, sealed with parafilm, and stored in a dark chiller at 1-4°C for a 4-6 week stratification process. Seeds were removed from the chiller and alternated between temperatures of 5°C (16 hours) and 24°C (8 hours) for 10-14 days. Seeds were then left under constant white light at 24°C to enable germination, resulting (50-75% germination rate). Germinated seeds were individually pricked into a central incision in a moistened rockwool cube.

4.2.1.1.2 Grow out of plantlets

Grow out of both the clonal and non-clonal *A. chinensis* Hort16a plantlets occurred using the same protocol. Clam trays each containing 12 rockwool cubes were transferred to a warm area out of direct sunlight in the University of Waikato greenhouse facility (Figure 4.4a). Lids were gradually opened over a 2-4 day period and then left fully open for a further 2 days. Rockwool cubes were then transferred into cell trays (one row distance apart) and placed onto a hydroponic ebb and flow system set on a 24 hour flood cycle (Figure 4.4b-c). Plantlets (10-15 cm in height) were removed from the ebb and flow system and potted into potting mix in 1 L pots. Each plantlet was staked and grown in the University of Waikato greenhouse facility (Figure 4.4d-e). Water was supplied to

plantlets using individually staked arrow drippers set on an automated timing system. Large plantlets (≥40 cm in height) were occasionally trimmed back until required for further use (Figure 4.4f).

Figure 4.4 Grow-out phase for clonal and non-clonal *A. chinensis* Hort16a plantlets. This includes a) transferal to greenhouse in crab trays, b-c) placement of rockwool trays and transferal on the ebb and flow system, d-e) transferal to pots, and f) trimming back of plantlets larger than 40 cm in size.

4.2.1.2 Inoculation procedure

Seventy eight plants (30-35 cm) of the same origin (tissue culture or seedling) were transferred to the approved Psa biovar 3 containment facility at the University of Waikato. Six plantlets were placed at random into each of the 13 labelled treatment trays (1-13) and labelled as replicates a-f (Figure 4.5). Water was supplied to plantlets using individually staked arrow drippers set on an automated timing system.

Figure 4.5. Experimental design of the preliminary elicitor experiment performed on *A. chinensis* Hort16a plants.

Preparation of the full strength Actigard™ (Syngenta) occurred by mixing sterile H_2O (1000 mL) with the recommended industry concentration of Actigard™ (200 mg/L) in a 1 L container. Five hundred mL of this was then transferred into another 1 L container and mixed with sterile H_2O (500 mL) to produce the $^1/_2$ strength Actigard™ treatment. This process was repeated a further three times to produce 5 x Actigard™ dilutions. Each Actigard™ and H_2O treatment was applied as required to both the top and underside of each leaf of each plantlet

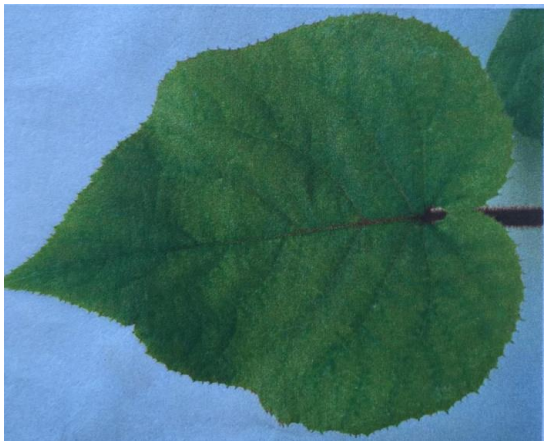
using a hand sprayer set at a constant spray strength. Spraying stopped once the leaf appeared visibly moistened.

Inoculation of Psa biovar 3 occurred 7 days following treatment application. Cultures of Psa biovar 3 (strain #10627) grown at the Plant and Food Research laboratory were scraped off agar plates and mixed with sterile H_2O (1400 mL) to produce a 10^8 cfu/mL strength liquid inoculum (clonal plantlets=1.3 x 10^8 cfu/mL, non-clonal plantlets=7.8 x 10^8 cfu/mL). The inoculum was transferred under containment to the greenhouse facility for immediate use. Inoculum was applied as required on the underside of each leaf of each plantlet using a hand sprayer at a constant spray strength. Spraying stopped once the leaf appeared visibly moistened. Plantlets in the non-inoculated treatments were temporarily relocated to the other side of the greenhouse to minimise the risk of airborne transmission of Psa biovar 3 during this time.

4.2.1.3 Analysis

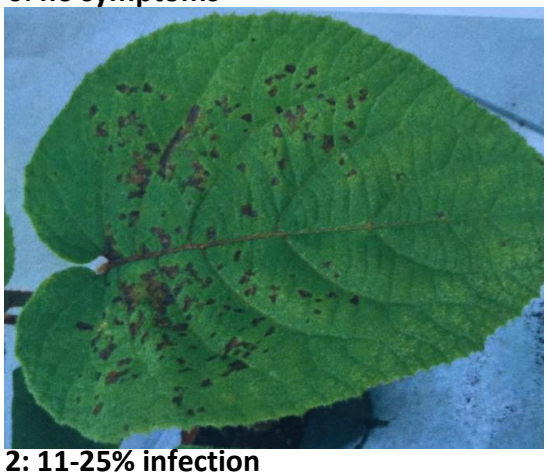
Plants were examined weekly for leaf spotting symptoms attributed to bacterial canker infection for 3 consecutive weeks following inoculation. Starting from the base of the plant and counting up, 8 leaves per plant were visually assessed and allocated an appropriate leaf spotting infection score (between 0-5) as per the Plant and Food Research disease assessment scale (Figure 4.6). Average infection scores per week are shown in a bar graph (±standard error) in Figure 4.8 (clonal plants) and Figure 4.9 (non-clonal plants). Further details are provided in Appendix A.10.

Statistical analysis was carried out using 'Microsoft Excel' and 'Statistica'. A parametric two-way analysis of variance (ANOVA) was performed in 'Statistica' to investigate the effect of the application method of Actigard^{TM} (single or whole leaf), the dilution of Actigard^{TM} (full, $^{1}/_{2}$, $^{1}/_{4}$, $^{1}/_{8}$, $^{1}/_{16}$ strength) and the interaction of the two variables on average leaf spotting infection scores for each week of assessment ($p<0.05$) ($Fstat>Fcrit$). Various statistical tests were performed which confirmed the data met homogeneity of variances and normal distribution assumptions of ANOVA. Post-hoc pairwise Duncan's mean separations tests were performed when main effects were significant ($p<0.05$) ($Fstat>Fcrit$) (Duncan, 1955).

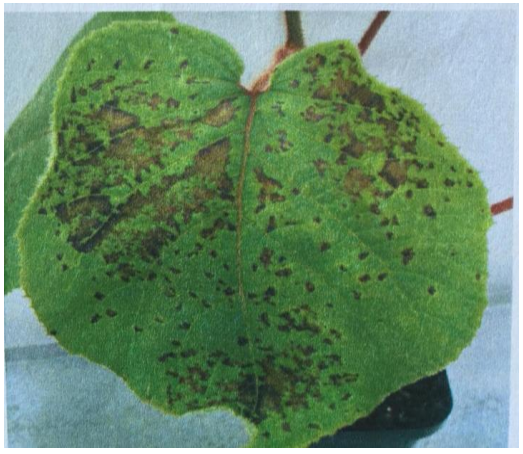


0: no symptoms

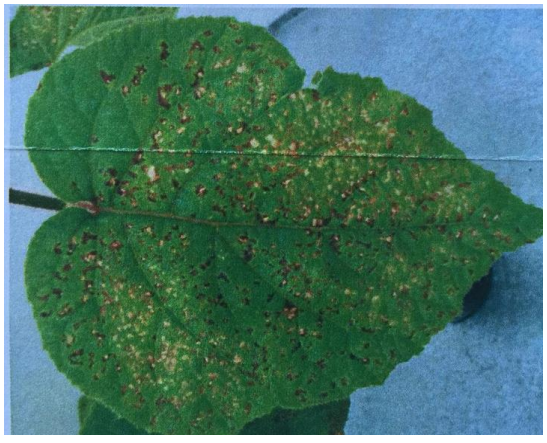
1: 1-10% infection



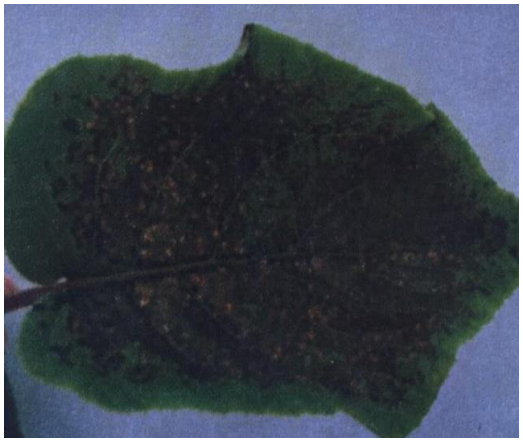
2: 11-25% infection



3: 26-50% infection



4: 51-75% infection



5: 71-100% infection

Figure 4.6. Leaf spotting disease assessment scale as specified by Plant and Food Research.

4.2.2 Phytotoxicity bioassay

A preliminary phytotoxicity bioassay was performed to determine whether any of the small scale extracts of macroalgae and/ or solvent (MeOH) had phytotoxic effects on clonal *A. chinensis* cv. Hort16a plantlets. To test this, the experiment comprised of full strength treatment and a 1:100 stock dilution treatment for each of the extracts.

4.2.2.1 Procedure

One clonal Hort16a plantlet unused from the previous bioassay was selected at random and transferred to the Plant and Food Research laboratory. A large leaf with a flat surface was labelled numbers 1-19 in a sequential order down the leaf, followed by the labelling of the undiluted treatment in the first column, and the 1:100 treatment in the second column.

Each crude extract was dissolved in MeOH (10 mL) for approximately 30 mins. An aliquot of each extract (10 µl) was transferred by pipette to its respective place on the leaf. Another aliquot (10 µl) was taken and diluted in sterile H_2O (360 µl) to give a 1:100 dilution ratio. A subsample (10 µl) was then transferred by pipette to its respective place on the leaf surface. Care was taken while pipetting to ensure that the solvent did not run off the leaf surface. The plant was left for 48 hours until the results were read.

4.2.2.2 Analysis

The treated leaf was examined for phytotoxicity symptoms at the site of extract application.

4.2.3 Screening method for phytoalexin elicitation

A preliminary bioassay adapted from Ayers *et al.* (1976) was considered as a screening method to examine the crude extracts of brown macroalgae (containing the active ingredient laminarin) for phytoalexin elicitation. Two replicate assays were performed on soybean seedlings to examine the effect of Vacciplant® (contains laminarin) phytoalexin glyceollin accumulation in soybean (*Glycine max*) seedlings. To test this, the experiment comprised of a Vacciplant® treatment (positive control), 1:10 MeOH treatment, 1:100 MeOH treatment, and a H_2O treatment (negative control).

4.2.3.1 Preparation

Soybean seeds (unknown cultivar) were sourced from a local bulk food supplier. Approximately 100 seeds were randomly selected, surface-sterilised in 0.75% sodium hypochlorite (NaOCl) for 5 mins, and rinsed extensively in H_2O for a further 5 mins. Sterilised seeds were placed in a container on moistened tissue paper and stored in a humid area for 2-3 days until emerging radicles were visible (50-60% germination rate) (Figure 4.7a). Germinated seedlings were placed on a tray above moistened potting soil (5-6 cm) and covered in a layer of moistened porous sand (1-2 cm). Seedlings were left to grow at 20-24°C in the

Plant and Food Research greenhouse facility, where they were watered daily until required for further use.

The Vacciplant® treatment (1.5 µl Vacciplant®: 1000 µl H_2O) 1:100 MeOH treatment (100 µl MeOH: 900 µl H_2O), 1:100 MeOH treatment (10 µl MeOH: 990µl H_2O), and H_2O treatment (1000 µl) were each prepared in an Eppendorf tube.

4.2.3.2 Procedure

Forty cotyledons of the same size were detached from young soybeans whose primary leaves had just expanded (14-17 days after germination) and transferred to the Plant and Food Research laboratory (Figure 4.7b). Where possible, the cotyledons were turgid, uncurled, and free of blemishes or cracks. Ten cotyledons were placed at random into a labelled treatment container and soaked in sterile H_2O for 5 mins (Figure 4.7c). Cotyledons were removed and evenly spaced on moistened tissue paper in their respective Petri dish. A section of tissue (approximately 7 x 5 x 1 mm) was then cut from the abaxial surface of each cotyledon so that the resulting wound had a flat, smooth, and wet surface that was free of discoloration (Figure 4.7d). Twenty five µl of each treatment was then transferred by pipette as required onto the respective wound. The petri dish was closed immediately after all 10 cotyledons were treated and stored in a dark incubator at 28°C. Results were read after 48 hours.

4.2.3.3 Analysis

Soybean cotyledons were scored for phytoalexin glyceolllin elicitation after 48 hours. Elicitation was considered to have occurred if the cotyledon wound displayed a red colouration from the oxidation of glyceollin (Ebel *et al.*, 1976) (Figure 4.7e), whereas a brown wound colouration indicted soybean necrosis (Figure 4.7f). Results are shown in Table 4.10.

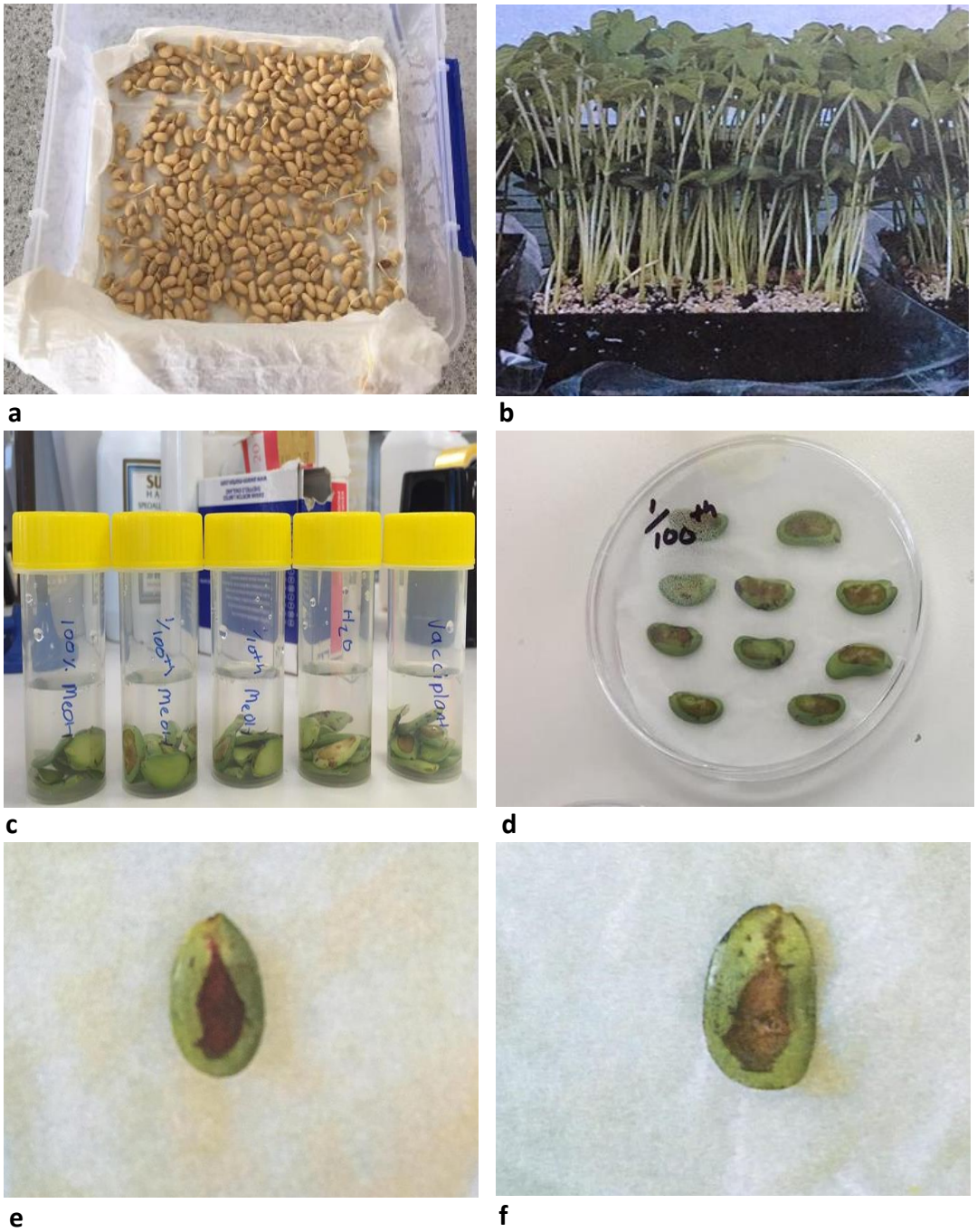


Figure 4.7. Soybean assay for phytoalexin glyceollin elicitation. This includes a) germination of seedlings, b) detachment of cotyledons from 14-17 day old soybeans, c) soaking of seedlings in sterile H_2O, d) arrangement of soybean on a Petri dish, e) red wound colouration (positive response), and f) brown wound colouration (negative response).

4.3 Results

4.3.1 Defence response of kiwifruit plants

The first of the two Actigard™ bioassays was performed on clonal *A. chinensis* cv. Hort16a plants. No leaf spotting symptoms attributed to Psa biovar 3 were observed after one or two weeks following inoculation; however, minor infection was observed after three weeks (Figure 4.8). Overall, the infection score increased as the concentration of Actigard™ decreased ($p<0.01$, $F_{stat}=12.60$) (Table 4.2). The highest infection score observed in the whole plant-applied treatment regime was the $^1/_{16}$ strength treatment (1.01±0.19), whereas in the single leaf applied regime it was the $^1/_8$ strength treatment (0.54±0.14), followed by $^1/_{16}$ strength treatment (0.50±0.12). The lowest infection score observed in both treatment regimens was the full strength treatment (whole plant-applied=0.17±0.06; single leaf applied=0). The H_2O treatment (positive control) had an average infection score of 0.17±0.11 over this time.

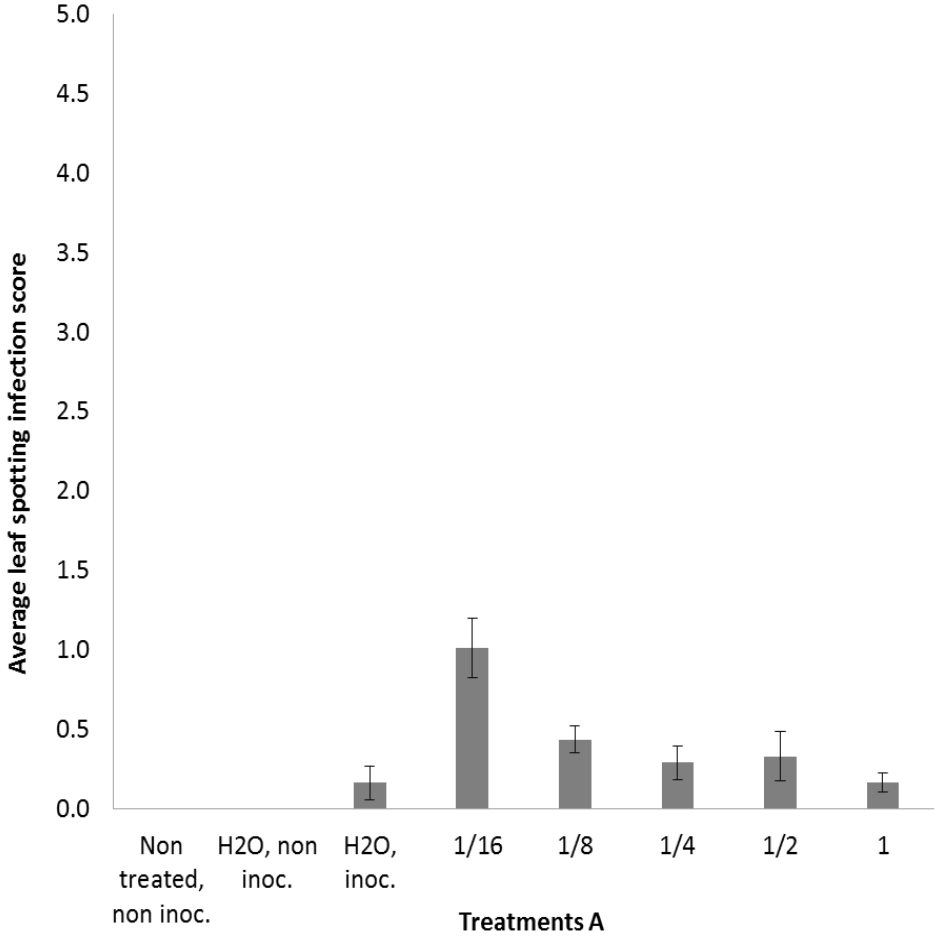
A Duncan's post-hoc analysis confirmed that at a 5 percent level of significance, there were 16 application method x concentration comparisons which statistically differed in average leaf spotting infection scores ($p<0.05$) (Table 4.3). The most noticeable trend was that the whole plant-applied $^1/_{16}$ strength treatment had a significantly higher infection score than all other inoculated treatments ($p<0.05$).

Table 4.2. Two-way analysis of variance investigating the effect of a) application method of Actigard™, b) concentration of Actigard™, and c) their interaction on the average clonal *A. chinensis* cv. Hort16a leaf spotting infection score three weeks after Psa biovar 3 inoculation (effects are significant at <0.05).

Effect	SS	Degrees of F	MS	F stat	P value
Application method	0.30	1	0.3	0.9	0.33
Concentration	21.80	5	4.40	12.60	<0.01
Application method x Concentration	6.30	5	1.30	3.70	<0.01

Table 4.3. Duncan's post-hoc analysis of multiple mean comparisons investigating the concentration of whole plant-applied (treatments A) x single leaf-applied (treatments B) Actigard™ treatments on the average clonal *A. chinensis* cv. Hort16a leaf spotting infection score three weeks after Psa biovar 3 inoculation (effects are significant at <0.05).

Effect	A 1	A ½	A ¼	A 1/8	A 1/16	B 1	B ½	B 1/4	B 1/8	B 1/16	H_2O
A 1		0.24	0.37	0.06	<0.01	0.93	0.19	0.15	0.01	0.02	0.84
A 1/2			0.73	0.45	<0.01	0.27	0.86	0.75	0.14	0.23	0.29
A 1/4				0.30	<0.01	0.40	0.63	0.54	0.08	0.14	0.43
A 1/8					<0.01	0.07	0.52	0.61	0.43	0.61	0.08
A 1/16						<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
B 1							0.21	0.17	0.01	0.02	0.90
B 1/2								0.86	0.18	0.28	0.24
B 1/4									0.22	0.34	0.20
B 1/8										0.73	0.01
B 1/16											0.03
H_2O											



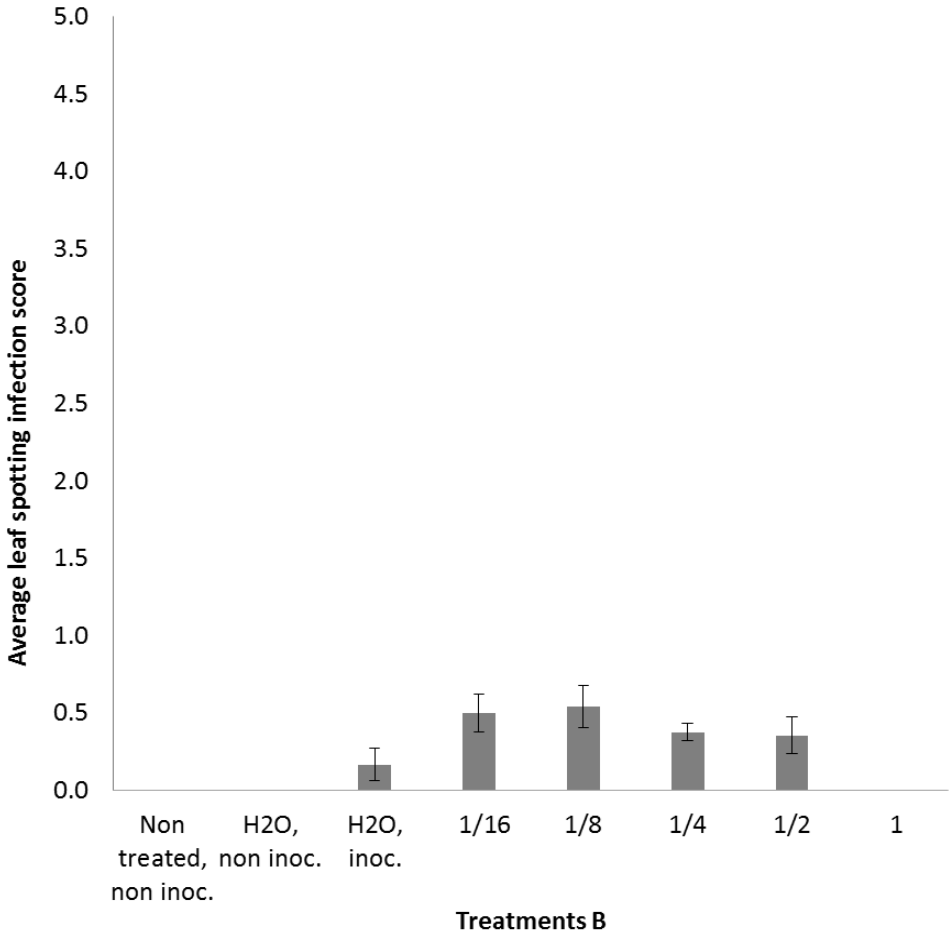


Figure 4.8. Average leaf spotting infection score observed on clonal *A. chinensis* cv. Hort16a plantlets three weeks following the inoculation of Psa biovar 3 (±standard error). Treatments A=whole plant-applied Actigard™, Treatments B=single leaf applied-Actigard™.

The second of the two Actigard™ bioassays was performed on non-clonal *A. chinensis* Hort16a plants (Figure 4.9). Leaf spotting symptoms attributed to Psa biovar 3 were observed amongst all inoculated treatments one, two, and three weeks after inoculation. Three general trends were observed over this period: a) infection scores amongst both treatment regimens increased weekly, b) average leaf spotting infection score amongst both treatment regimens increased as the concentration of Actigard™ decreased, and c) at any given concentration of Actigard™, average leaf spotting infection scores were generally higher in single-leaf applied treatments compared to those whole leaf-applied treatments.

One week observations: Overall, the infection score increased as the concentration of Actigard™ decreased ($p<0.01$, $F_{stat}=32.16$) (Table 4.4). At a given concentration, the infection score was generally higher when single leaves were treated compared to the whole plant ($^1/_8$ strength treatment regimens=$p<0.05$). However, all single leaf treatments still resulted in a significant reduction in infection rates when compared to the H_2O treatment ($p<0.05$) (Table 4.5). The highest infection score in the whole plant-applied treatment regime was the $^1/_{16}$ strength treatment (1.27±0.10), whereas in the single-leaf applied treatment is was the $^1/_8$ strength treatment (1.79±0.16), followed by the $^1/_{16}$ strength treatment (1.23±0.14). The lowest infection score in the whole plant-applied treatment regime was the full strength (0.75±0.07) and $^1/_2$ strength (0.73±0.09) treatments, and in the single-leaf applied regime was the full strength treatment (0.60±0.15). The highest overall score was in the H_2O treatment (2.15±0.15).

Duncan's post-hoc analysis confirmed that at a 5 percent level of significance, there were 30 Actigard™ application x concentration comparisons which significantly differed in their average leaf spotting infection scores ($p<0.05$) (Table 4.5). The most noticeable trend was that the single leaf-applied $^1/_8$ strength treatment had a significantly higher infection score than all other Actigard™ treatments, and the H_2O treatment had a significantly higher infection score than all other treatments (excluding the single leaf-applied $^1/_8$ strength treatment).

Table 4.4. Two-way analysis of variance investigating the effect of a) application method of Actigard™, b) concentration of Actigard™, and c) their interaction on the average non-clonal *A. chinensis* Hort16a leaf spotting infection score one week after Psa biovar 3 inoculation (effects are significant at <0.05).

Effect	SS	Degrees of F	MS	F stat	P value
Application method	2.80	1	2.75	3.22	0.07
Concentration	137.7	5	27.54	32.16	<0.01
Application method x Concentration	7.90	5	1.58	1.85	0.10

Table 4.5. Duncan's post-hoc analysis of multiple mean comparisons investigating the concentration of whole plant-applied (treatments A) x single leaf-applied (treatments B) Actigard™ treatments on the average non-clonal *A. chinensis* Hort16a leaf spotting infection score one week after Psa biovar 3 inoculation (effects are significant at <0.05).

Effect	A 1	A 1/2	A ¼	A 1/8	A 1/16	B 1	B ½	B 1/4	B 1/8	B 1/16	H_2O
A 1		0.83	0.15	0.01	0.01	0.73	0.16	0.03	<0.01	0.02	<0.01
A 1/2			0.19	0.01	0.01	0.60	0.21	0.04	<0.01	0.04	<0.01
A 1/4				0.21	0.18	0.08	1.00	0.41	<0.01	0.37	<0.01
A 1/8					0.91	<0.01	0.20	0.61	<0.01	0.66	<0.01
A 1/16						<0.01	0.17	0.55	<0.01	0.61	<0.01
B 1							0.09	0.01	<0.01	0.01	<0.01
B 1/2								0.38	<0.01	0.35	<0.01
B 1/2									<0.01	0.91	<0.01
B 1/8										<0.01	0.10
B 1/16											<0.01
H_2O											

Two week observations: All inoculated treatments displayed statistically significant increases in their infection scores between the one and two week inoculation periods (as indicated by separation of standard error bars) (Figure 4.9). Overall, the infection score increased as the concentration of Actigard^{TM} decreased ($p<0.01$, $F_{stat}=30.63$) (Table 4.6). At a given concentration, the infection score was generally higher when single leaves were treated compared to the whole plant (full, $^1/_2$, $^1/_8$ strength treatment regimens=$p<0.05$). However, all single leaf treatments (excluding the $^1/_{16}$ strength treatment) still resulted in a significant reduction in infection rates when compared to the H_2O treatment ($p<0.05$) (Table 4.7). The highest infection score in both treatment regimens was the $^1/_{16}$ strength treatment (whole plant-applied=3.10±0.32; single leaf-applied=3.41±0.28), of which the single leaf treatment had a higher score than the H_2O treatment (3.12±0.34). The lowest infection score in the whole plant-applied regime was in $^1/_2$ strength treatment (0.96±0.07), whereas in the single leaf-applied regime was the full strength treatment (1.10±0.18).

Duncan's post-hoc analysis confirmed that at a 5 percent level of significance, there were 38 Actigard^{TM} application method x concentration comparisons which significantly differed in their average leaf spotting infection score ($p<0.05$) (Table 4.7).

Table 4.6. Two-way analysis of variance investigating the effect of a) application method of Actigard™, b) concentration of Actigard™, and c) their interaction on the average non-clonal *A. chinensis* Hort16a leaf spotting infection score two weeks after Psa biovar 3 inoculation (effects are significant at <0.05).

Effect	SS	Degrees of F	MS	F stat	P value
Application method	29	1	28.97	16.54	<0.01
Concentration	268.30	5	53.65	30.63	<0.01
Application method x Concentration	82.20	5	16.44	9.39	<0.01

Table 4.7. Duncan's post-hoc analysis of multiple mean comparisons investigating the concentrations of whole plant-applied (treatments A) x single leaf-applied (treatments B) Actigard™ treatments on the average non-clonal *A. chinensis* Hort16a leaf spotting infection score two weeks after Psa biovar 3 inoculation (effects are significant at <0.05).

Effect	A 1	A 1/2	A ¼	A 1/8	A 1/16	B 1	B 1/2	B 1/4	B 1/8	B 1/16	H_2O
A 1		<0.01	0.88	0.17	<0.01	0.01	<0.01	0.07	<0.01	<0.01	<0.01
A 1/2			<0.01	<0.01	<0.01	0.52	<0.01	<0.01	<0.01	<0.01	<0.01
A ¼				0.19	<0.01	0.01	0.01	0.08	<0.01	<0.01	<0.01
A 1/8					<0.01	<0.01	0.13	0.59	<0.01	<0.01	<0.01
A 1/16						<0.01	0.05	<0.01	0.89	0.73	0.71
B 1							<0.01	<0.01	<0.01	<0.01	<0.01
B ½								0.28	0.06	0.04	0.04
B ½									<0.01	<0.01	<0.01
B 1/8										0.81	0.80
B 1/16											0.97
H_2O											

Three week observations: Highest infection scores across all inoculated treatments were observed at the three week inoculation period (Figure 4.9). Overall, the infection score increased as the concentration of Actigard^{TM} decreased ($p<0.01$, $F_{stat}=16.71$) (Table 4.8). At a given concentration, the infection score was generally higher when single leaves were treated compared to the whole plant ($^1/_2$ strength regimes=$p<0.05$). However, all single leaf treatments (excluding the $^1/_{16}$ strength treatment) still resulted in a significant reduction in infection rates when compared to the H_2O treatment ($p<0.05$) (Table 4.9). The highest infection score in both treatment regimens was the $^1/_{16}$ strength treatment (whole plant-applied=3.35±0.23, single leaf-applied=4.10±0.17), both of which had a higher score than the H_2O treatment (3.22±0.20). The lowest infection score in the whole plant-applied regime was in the $^1/_2$ strength treatment (1.40±0.30), whereas in the single leaf-applied regime was the full strength treatment (1.58±0.46).

Duncan's post-hoc analysis confirmed that at a 5 percent level of significance there were 29 Actigard^{TM} concentration x application comparisons which significantly differed in their average leaf spotting infection scores ($p<0.05$) (Table 4.9). The most noticeable trend was that the whole leaf-applied full strength Actigard^{TM} treatment had a significantly lower infection score than all other inoculated treatments.

Table 4.8. Two-way analysis of variance investigating the effect of a) application method of Actigard™, b) concentration of Actigard™, and c) their interaction on the average non-clonal *A. chinensis* Hort16a leaf spotting infection score three weeks after Psa biovar 3 inoculation (effects are significant at <0.05).

Effect	SS	Degrees of F	MS	F stat	P value
Application method	29	1	29.02	10.52	<0.01
Concentration	230.50	5	46.10	16.71	<0.01
Application method x Concentration	82.20	5	16.44	5.96	<0.01

Table 4.9. Duncan's post-hoc analysis of multiple mean comparisons investigating the concentration of whole plant-applied (treatments A) x single leaf-applied (treatments B) Actigard™ treatments on the average non-clonal *A. chinensis* Hort16a leaf spotting infection score three weeks after Psa biovar 3 inoculation (effects are significant at <0.05).

Effect	A 1	A 1/2	A 1/4	A 1/8	A 1/16	B 1	B 1/2	B 1/4	B 1/8	B 1/16	H_2O
A 1		<0.01	0.58	0.15	0.01	0.07	0.01	0.13	0.02	<0.01	<0.01
A 1/2			<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
A ¼				0.32	0.02	0.18	<0.01	0.30	0.08	0.01	<0.01
A 1/8					0.17	0.64	<0.01	0.90	0.36	0.09	<0.01
A 1/16						0.32	<0.01	0.19	0.58	0.71	0.06
B 1							<0.01	0.71	0.61	0.20	0.01
B ½								<0.01	<0.01	<0.01	<0.01
B ½									0.41	0.11	<0.01
B 1/8										0.39	0.02
B 1/16											0.11
H_2O											

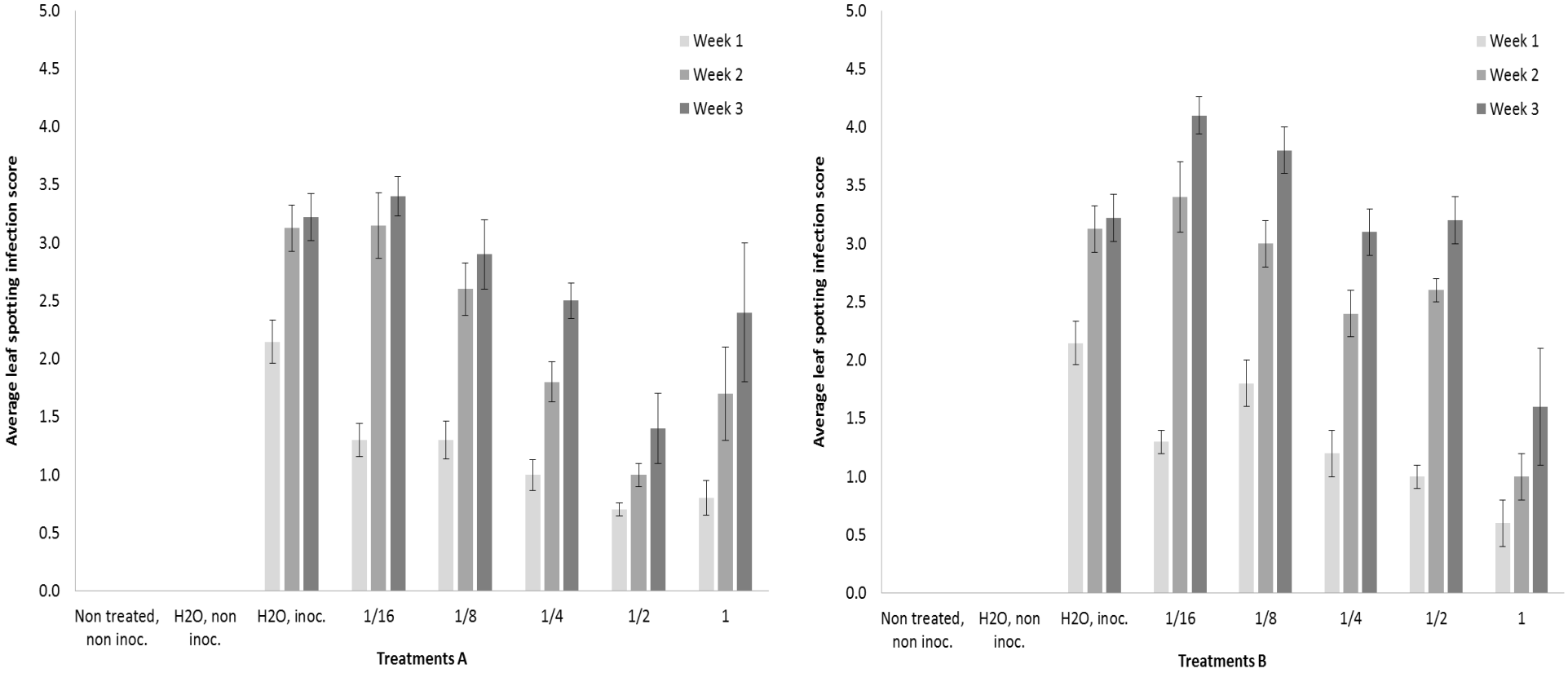


Figure 4.9. Average leaf spotting infection score observed on clonal *A. chinensis* cv. Hort16a plantlets at a one, two, and three week period following inoculation of Psa biovar 3 (±standard error). Treatments A=whole plant-applied Actigard™, Treatments B=single leaf applied-Actigard™

4.3.2 Phytotoxicity of macroalgae extracts

No small scale crude extracts of macroalgae had any phytotoxic effects on the leaf of a clonal *A. chinensis* cv. Hort16a plant at the site of extract application. See Appendix A.11 for further details.

4.3.3 Defence response of soybean

Two of the 10 Vacciplant® treated wounds of soybean cotyledons demonstrated phtyoalexin glyceollin elicitation in the first experiment (Table 4.10). The remaining eight wounds demonstrated necrosis. All cotyledon wounds in the 1:10 MeOH treatment, the 1:100 MeOH treatment, and the H_2O treatment demonstrated necrosis. In the second experiment all wounds of soybean cotyledons demonstrated necrosis.

Table 4.10. Number of cotyledon wounds which demonstrated phytoalexin glyceollin elicitation vs the number which demonstrated necrosis.

Treatment	Experiment one		Experiment two	
	Elicitation	No elicitation	Elicitation	No elicitation
Vacciplant®	2	8	0	10
H_2O	0	10	0	10
1:10 MeOH	0	10	0	10
1:100 MeOH	0	10	0	10

4.4 Discussion

The elicitor screening phase of this research sought to further examine 19 small scale extracts of New Zealand macroalgae collected in coastal waters of the Bay of Plenty region for elicitor activity against *Pseudomonas syringae* pv. *actinidiae* biovar 3. Preliminary calculations determined that the maximum resuspension volume of less than one 1 mL required to stimulate elicitation was logistically problematic. Two preliminary *in vivo* bioassays were therefore established to provide insight for an effective downscaled screening method which could be used to examine the small scale extracts of macroalgae for effective elicitor activity.

4.4.1 Screening method for general elicitor activity

The general elicitor bioassay was the first of the two preliminary *in vivo* screens. This assay was performed with consideration to the minimum concentration and/ or volume of crude macroalgae extract required to stimulate elicitation in *A. chinensis* against Psa biovar 3. Actigard™ was selected as a positive control due to its proven efficacy at reducing bacterial kiwifruit canker symptoms attributed to Psa biovar 3 (Zespri & Kiwifruit Vine Health, 2015). As previously mentioned, Actigard™ contains the active compound Acibenzolar-*S*-Methyl. This is a functional analogue of the endogenously synthesised signalling molecule salicylic acid (SA), which induces systemic acquired resistance (SAR) active against biotrophic and hemibiotrophic pathogens (Thakur & Sohal, 2012; Tamaoki *et al.*, 2013). The application of Actigard™ on kiwifruit plant activates the SAR response

and thus offers relatively long lasting host plant resistance for up to three weeks (Kiwifruit Vine Health, 2015c).

The response of clonal *A. chinensis* cv. Hort16a plantlets was affected by the low levels of leaf spotting in the H_2O treatment when compared to the Actigard™ treatments (Figure 4.8.). All the plantlets were clonally cultured, of a similar size (30-35cm), placed at random into one of the 13 treatment trays, and exposed to the same environmental conditions. Results from the experiment were compromised by the fact that the control plants showed only slight symptoms of infection and that infection only occurred in the third week. This may be attributed to the relatively low strength of inoculum (1.3×10^8 cfu/mL) applied and/ or suboptimal environmental conditions inside the greenhouse facility for the pathogen to proliferate. With these limitations in mind, a full interpretation of the efficacy of each Actigard™ treatment cannot be discussed; however, trends amongst the Actigard™ treatments can be mentioned.

Results from the whole plant-applied Actigard™ treatment regime indicate that the $^1/_{16}$ strength treatment had a significantly higher infection score than all other concentrations ($p<0.05$). In comparison, the $^1/_8$ and $^1/_{16}$ strength treatment had a significantly higher infection score than the full strength treatment in the single leaf-applied regime. These results suggest that the $^1/_2$, $^1/_4$, and $^1/_8$ strength treatments in the whole plant-applied Actigard™ regime, and the $^1/_2$ and $^1/_4$ strength treatments in the single leaf-applied Actigard™ regime, had the same

elicitation efficacy as their respective full strength treatments. When comparing the two Actigard™ application methods, whole plant-applied $^1/_{16}$ strength treatment had a significantly higher infection score than all the single leaf-applied treatments, and the whole plant-applied full strength treatment had a significantly lower infection score than the $^1/_8$ and $^1/_{16}$ strength single leaf-applied treatments. These results suggest that application of $^1/_2$ and $^1/_4$ strength Actigard™ on a single leaf had the same elicitation efficacy as the full strength application of Actigard™ on a whole plant. Further tests with an effective control treatment are required to validate this.

In the second experiment performed on non-clonal *A. chinensis* Hort16a plants, the severity of bacterial canker infection increased over a three week observation period. There were many significant differences observed amongst and between the Actigard™ treatment regimens after a one week period. After two and three weeks; however, these differences were not as significant and thus only the results from the first week are discussed. Results from the whole plant-applied Actigard™ treatment regime indicate that the two highest scoring $^1/_8$ and $^1/_{16}$ strength treatments had a significantly higher infection score than the full strength and $^1/_2$ strength treatments ($p<0.05$). This suggests that the $^1/_2$ and $^1/_4$ strength whole leaf-applied Actigard™ treatments had the same elicitation efficacy as the full strength treatment. Furthermore, the H_2O control treatment had a significantly higher infection score than all single leaf-applied Actigard™ treatments ($p<0.05$). This suggests that all whole leaf-applied

Actigard™ treatments successfully elicited a defence response in non-clonal *A. chinensis* plants.

Results from the single leaf-applied Actigard™ treatment regime indicate that the $^1/_8$ strength treatment had a significantly higher infection score than all other concentrations and that the $^1/_{16}$ and $^1/_4$ strength treatments had a significantly higher infection score the than full strength treatment ($p<0.05$). This suggest that the $^1/_2$ strength single leaf-applied Actigard™ treatment had the same elicitation efficacy as the full strength treatment. Excluding the $^1/_{16}$ strength treatment, the H_2O control treatment had a significantly higher infection score than all other single leaf-applied Actigard™ treatments ($p<0.05$). This suggests that the majority of single leaf-applied Actigard™ treatments successfully elicited a defence response in non-clonal *A. chinensis* plants.

When comparing the two Actigard™ application method regimens, the single leaf-applied $^1/_8$ strength treatment had a significantly higher infection score than all whole leaf-applied treatments, and single leaf-applied $^1/_4$ and $^1/_{16}$ strength treatments had a significantly higher infection score the whole leaf-applied full strength and $^1/_2$ strength treatments. These results suggest that the single leaf application of $^1/_2$ and $^1/_4$ strength Actigard™ had the same elicitation efficacy as the full strength application of Actigard™ when the whole plant was treated.

The overall results from the preliminary general elicitor bioassay conclude that the $^1/_2$ and $^1/_4$ strength application of Actigard™ on a single leaf can elicit the same defence response as the recommended rate of Actigard™ application when applied to the whole leaf of *A. chinensis*. This, in turn, suggests that a crude extract of macroalgae could be further diluted by a factor of two (2 mL) or four (4 mL) and applied on a single leaf to stimulate the same amount of elicitation as the recommended full strength extract in 1 mL solvent. The problem, however, remains that 30 mL of extract is still required to spray one leaf. With consideration of the six replicates, a minimum of 180 mL is needed to use this as a higher order screening method. Diluting the available extract by a factor of four would still result in an active ingredient concentration approximately 45 times lower than the recommended rate for eliciting a response using Actigard™. Thus, another screening method is required to further reduce the amount of solvent required.

4.4.2 Screening method for phytoalexin elicitation

The phytoalexin elicitation bioassay was considered as a screening method to further reduce the amount of solvent required to screen the crude extracts of macroalgae for elicitor activity. The assay was performed with consideration to examining the effect of laminarin, an active molecule contained in brown macroalgae, for phytoalexin elicitation of soybean seedlings. Soybeans were selected as subjects in this assay as phytoalexin accumulation is easily identified by a red wound colouration via the oxidation of glyceollin (Ebel *et al.*, 1976). As

previously mentioned, phytoalexins are antimicrobial compounds which are biosynthesised by healthy neighbouring cells and transported to sites of infection. Phytoalexin accumulation in a host plant can be triggered by infection; however, it can also be triggered by elicitors and thus provide induced host resistance (Ayers *et al.*, 1976). Laminarin, a storage *β*-1, 3 glucan in brown algae (Ocrophyta) has been shown to induce phytoalexin accumulation in a variety of plants including soybean (*Glycine max*) (Keen *et al.*, 1983), tobacco (*Nicotiana tabacum*) (Klarzynski *et al.*, 2000), and grapevine (*Vitis vinifera*) (Aziz *et al.*, 2003). Thus, Vacciplant® was selected as a positive control to investigate this assay.

There were a multitude of problems encountered with the soybean bioassay. Soybean seeds are not available for sowing in New Zealand. They were therefore sourced from a local bulk food supplier with no indication of the cultivar. Soybeans which germinated produced curved cotyledons which made it difficult to apply the treatment solution; they were relatively small in size and thus limited by the volume of solution which could be applied to the wound; and they took longer than expected to produce usable green cotyledons. Furthermore, the cotyledon response was not chemically analysed and thus it could not be confirmed whether the produced red wound colouration was due to the accumulation of glyceollin or due to a different compound. On account of these difficulties, it is not yet known if the soybean bioassay is a suitable higher order screening method to examine crude extracts of brown macroalgae for phytoalexin elicitation.

4.4.3 Conclusions

The elicitor screening phase of this research provides valuable insight for the design of an effective higher order screening method which can be used to examine small scale crude extracts of macroalgae for elicitor activity against Psa biovar 3. Results from the general elicitor bioassay concluded that a crude extract of macroalgae could be further diluted by a factor of two (2 mL) or four (4 mL) and applied on a single leaf to stimulate the same elicitation as the recommended full strength extract in 1 mL solvent. The limitation is that a minimum of volume of 180 mL (30 mL per leaf x 6 replicates) of solvent would still be required to utilise this as a screening method. With this consideration in mind, the soybean bioassay was considered as an elicitor screening method to examine the effect of laminarin (contained in brown algae) on phytoalexin accumulation of soybeans and thereby reduce the amount of solvent. A multitude of problems with the soybeans impeded the effectiveness of the bioassay. Thus, it could not be determined whether or not it was an effective higher order screening method to examine crude extracts of brown macroalgae for phytoalexin elicitation.

As previously mentioned, the primary limitation of the study was the lack of conclusions that could be drawn from the soybean bioassay. Soybeans which germinated produced small and curved cotyledons and thus the solution would often drop off or soak into the wound. This meant that the droplet was unable to be retained for chemical analysis. Instead, the wound was visually examined for

phytoalexin elicitation by a red colouration from the oxidation of glyceollin. Further work could focus on repeating the soybean bioassay on a cultivar which is known to produce large cotyledons with a flat surface. This would enable the cotyledon response to be chemically analysed and a quantifiable measure of phytoalexin accumulation to be obtained. This, in turn, would provide insight as to whether or not the bioassay is an appropriate screening method.

CHAPTER 5

GENERAL DISCUSSION

This study was a first phase biodiscovery programme which combined elements of plant pathology, chemical ecology, and natural product chemistry to explore a novel solution for the control of the causal agent of bacterial canker in New Zealand kiwifruit, *Pseudomonas syringae* pv. *actindiae* biovar 3. Natural products, believed to confer selective advantages in producing organisms through chemical-ecological interactions, offer a potential source of novelty and functionally relevant modes of biological activity. The marine environment is a particularly rich source of natural products. Its high biodiversity is complemented by an elaborate chemical diversity in marine organisms; particularly in those sessile and colonial marine organisms which utilise secondary metabolites for chemical defence. Thus, it was highlighted that New Zealand's rich and virtually untapped marine natural product resource could provide a novel solution for the control of Psa biovar 3. Previous biomedicinal natural product discovery programmes informed that the likelihood of finding an appropriate marine organism with target biological activity is low and is likely also a costly and lengthy process. To increase the chance of success, the study focused on the targeted collection of certain marine species based on data from related organisms producing compounds with relevant anti-*Pseudomonas* and anti-quorum sensing activities in previous biomedicinal screens.

The following chapter summarises the key discoveries of this study and the importance of these findings with insight to future 'bigger picture' work.

5.1 Summary of key findings

The first key finding from this study was that 26 percent of New Zealand macroalgae specimens (5 of the 19) screened against *P. syringae* pv. *actinidiae* biovar 3 produced metabolites with 'hit' biological activity in preliminary screening. These 'hit' candidates included *Vidalia colensoi* (AB3, AB13), *Asparagopsis armata* (AB6), *Carpophyllum maschalocarpum* (AB9), and *Xiphophora chondrophylla* (AB14). Of this 'hit' success rate, 16 percent is attributed to macroalgae that were targeted for screening using an ecologically-guided discovery approach (three of the five 'hits'), and the remaining 10 percent is attributed to those that were selected at random (two of the five 'hits'). This finding is particularly important as it suggests an ecologically-guided approach can work to effectively to guide the selection of species with metabolites of relevance and thus fast-track initial phases of biodiscovery. Furthermore, using this approach enhanced the selectively of such species to a very high degree by focusing on their relevant antibacterial and quorum sensing modes of action. This enabled the selection of a refined number of macroalgae with highly relevant target activity, in turn increasing the amount of 'hit' rates of discovery and decreasing required collection and screening efforts.

The second key finding was that the biological activity of larger scale extracts of candidate 'hit' macroalgae resulted in greater reproducibility when screened against Psa biovar 3 in the secondary screening phase. Larger scale extracts of *V. colensoi* (AB3), *A. armata* (AB6), *C. maschalocarpum* (AB3), and *X. chondrophylla* (AB14) all consistently inhibited the growth of Psa biovar 3. In comparison, the Karewa Island sample of *V. colensoi* (AB13) was inactive against Psa. Again, this finding is important as it provides confirmation that four of the five macroalgae remained as suitable candidates to further examine, whereas the other specimen was eliminated as a candidate of interest.

Chemical analysis of the remaining four 'hit' macroalgae candidates led to the third key finding; that the Leisure Island sample of *V. colensoi* (AB3) is of greatest priority to explore for bioassay guided fractionation in the later phases of discovery. Through a process of elimination of brominated ions in equivalent fractions of the Karewa Island sample of *V. colensoi* (AB13), four brominated ions were identified as most likely to represent compounds with relevant biological activity against Psa biovar 3. This includes the dibrominated ions *m/z* 278.8 (mass 277.8), 308.8 (mass 307.8), and 421.8 (mass 420.8), and the monobrominated ion *m/z* 311.9 (mass 310.9). Comparison of these ions with those compounds previously isolated in the *Vidalia* genus indicate that neither their bromination pattern nor molecular weights coincide. This, in turn, suggests that ions of interest may represent structurally novel bioactive compounds against Psa biovar 3 with patentability and commercial marketability.

5.2 Importance and direction for future research

First and foremost, the discovery of four New Zealand macroalgae candidates which produce secondary metabolites with target activity against *P. syringae* pv. *actindiae* biovar 3 is of high relevance for the New Zealand kiwifruit industry. The increasing threat of evolved pathogen resistance from the indiscriminate use of streptomycin and copper, combined with their adverse environmental effects, has reduced the efficacy of these current control practices. Consequently, there is an urgent need to develop alternative effective and sustainable measures for long term management of the pathogen. The discovery of macroalgae with relevant activity not only opens up a window of opportunity to explore a novel control solution, but it introduces options which may sustain both the New Zealand kiwifruit industry and the environment. The marine origin of the natural products means that terrestrial systems would not have previously come into contact with such molecules. They are therefore more likely to exclude pathogenic resistance. Their organic nature also means the environmental effects from their use are likely to be minor when compared to current control measures. Furthermore, a supply of macroalgae by various methods of aquaculture suggests there may be a sustainable and long term source of target metabolites at a scale large enough to fulfil the needs of further discovery phases as well commercial application. Commercialisation of this target metabolite for control of Psa biovar 3 would place the New Zealand kiwifruit industry at the forefront of agricultural control utilising natural biological processes.

Secondly, insight provided from the findings of this study has opened up a number of pathways to further examine macroalgae secondary metabolites as a novel control solution for *P. syringae* pv. *actinidiae* biovar 3. Findings from the study suggest that there are four possible novel and commercially patentable brominated compounds in *V. colensoi* (AB3) with biological activity against Psa biovar 3. The next step of the research would therefore require further chemical analysis involving bioassay guided fractionation and structural elucidation of such compounds. A chemical-ecological element of the research could occur in parallel to investigate which contributing microecological factors (that is season, depth, location, epiphytes, biofouling) trigger the secondary metabolic activity within this species. Understanding the ecological mechanisms which influence the chemical ecology of a species could, in turn, lead to consideration of scale-up aquaculture to elicit larger amounts of the target metabolites. The mode of cultivation to enhance yields of target metabolites is generated by the chemical ecology.

Thirdly, insight provided from this study has provided a platform for the implementation of ecologically-guided discovery in other agricultural areas. This includes control of other diseases, plant nutrition regulation, and regulation and development of vine and fruit growth. For instance, the causal agent of root rot in avocado, *Phytopthora cinnamomi,* could be the next disease of focus using an ecologically-guided discovery approach. Like *P. syringae* pv. *actinidiae*, *P. cinnamomi* is a Gram negative bacterium which uses quorum sensing processes

to cause disease in a host. New Zealand macroalgae species targeted for screening based on biomedicinal research examining species with quorum sensing inhibition or disruption activity included *Asparagopsis armata*, *Codium fragile, Ulva* sp., and *Undaria pinnatifida*. These candidates could also be screened for target activity against *P. cinnamomi*. Discovering natural product management solutions for these can have wider benefits for both the regional/ national economy and the natural environment.

In summary, the findings from this study highlight that an ecologically-guided discovery approach did effectively target the selection of species with relevant 'hit' activity against Psa biovar 3 and thus fast track the initial phases of biodiscovery. Future natural product discovery programmes could also adapt this approach to increase the amount of 'hit' rates of success and, in turn, decrease the required collection and screening efforts. The discovery of four potentially unidentified brominated ions of interest in the 'hit' candidate *V. colensoi* suggests they may represent structurally novel bioactive compounds against Psa biovar 3 with patentability and commercial marketability. Given their natural product origin, such compounds are also more likely to exclude terrestrial pathogen resistance and have minimal environmental effects. Lastly, the findings from the elicitor screening phase provided important preliminary information for optimisation of higher level screening process to explore elicitor activity as a relevant mode of action against Psa.

APPENDICES

A.1: Ecological Macroalgae Data

Summer Collected Specimens

Field ID code	Species	Date	Location	Depth/ method	GPS coordinates	Description
ABS1	*Pterocladia lucida*	13.01.2015	Karewa Island	5m by SCUBA	37.52862S, 176.13111E	Healthy red alga with biofouling on up to 90 percent of the specimen.
ABS2	*Ulva* sp.	11.03.2015	Tauranga Hbr	4m by SCUBA	37.47924S, 175.94824E	Healthy green cellophane-looking alga. No biofouling present.
ABS3	*Vidalia colensoi*	27.02.2015	Leisure Island	1m by snorkel	37.62955S, 176.18543E	Healthy red alga with long blades. No biofouling present.
ABS4	*Splachnidium rugosum*	27.02.2015	Leisure Island	1m by snorkel	37.62955S, 176.18543E	Healthy brown tubular alga with high water retention capacity. No biofouling present.
ABS5	*Carpophyllum plumosum*	27.02.2015	Leisure Island	2m by snorkel	37.62955S, 176.18543E	Healthy brown leathery alga with large gas bladders. Biofouling of red algae on up to 60 percent of the specimen.
ABS6	*Asparagopsis armata*	24.11.2015	Mayor Island	4m by SCUBA	37.30633S, 176.25997E	Healthy red alga with bright pink colouration. No biofouling present.
ABS7	*Ecklonia radiata*	13.01.2015	Karewa Island	13m by SCUBA	37.52862S, 176.13111E	Healthy brown leathery alga with slimy film upon touch. No biofouling present.

Field ID code	Species	Date	Location	Depth/ method	GPS coordinates	Description
ABS8	*Glaphyrosiphon lindaueri*	27.02.2015	Leisure Island	5m by snorkel	37.62955S, 176.18543E	Healthy red alga with thick, string-like blades. No biofouling present.
ABS9	*Carpophyllum maschalocarpum*	27.02.2015	Leisure Island	2m by snorkel	37.62955S, 176.18543E	Healthy brown alga with long stipes and large gas bladders. No biofouling present.
ABS10	*Gigartina atropurpurea*	27.02.2015	Leisure Island	3m by snorkel	37.62955S, 176.18543E	Healthy red and leathery alga with flat, rubbery, and ribbed blades. No biofouling present.
ABS11	*Lessonia* sp.	27.02.2015	Leisure Island	1m by snorkel	37.62955S, 176.18543E	Healthy brown and leathery alga with large blades and thick stipes. No biofouling present.
ABS12	*Pterocladia lucida*	27.02.2015	Leisure Island	1m by snorkel	37.62955S, 176.18543E	Healthy red and leafy alga. Biofouling attached to blades on up to 60 percent of the specimen.
ABS13	*Vidalia colensoi*	13.01.2015	Karewa Island	13m by SCUBA	37.52862S, 176.13111E	Healthy red alga with long blades. Biofouling present on up to 70 percent of the specimen.
ABS14	*Xiphophora chondrophylla*	27.02.2015	Leisure Island	1m by snorkel	37.62955S, 176.18543E	Healthy brown alga with thick, hard stipes. No biofouling present.
ABS15	*Ulva* sp.	27.02.2015	Leisure Island	1m by snorkel	37.62955S, 176.18543E	Healthy green cellophane-looking alga. No biofouling present.
ABS16	*Dictyota kunthii*	27.02.2015	Leisure Island	1m by snorkel	37.62955S, 176.18543E	Healthy brown alga with multiple furry blades attached to a single stipe. No biofouling present.
ABS17	*Codium fragile*	13.01.2015	Karewa Island	5m by SCUBA	37.52862S, 176.13111E	Healthy green alga with frond-like stipes and large water capacity. No biofouling present.

Winter Collected Specimens

Field ID code	Species	Collection date	Collection location	Depth/ method	GPS coordinates	Description
ABW1	*Pterocladia lucida*	08.09.2015	Karewa Island	3m by SCUBA	37.52862S, 176.13111E	Healthy red algae with biofouling on up to 60 percent of the specimen.
ABW2	*Ulva* sp.	22.10.2015	Tauranga Harbour	2m by snorkel	37.47924S, 175.94824E	Healthy green cellophane-looking algae. No biofouling present.
ABW3	*Vidalia colensoi*	01.10.2015	Leisure Island	1m by snorkel	37.62955S, 176.18543E	Healthy red algae with long blades. No biofouling present.
ABW4	*Splachnidium rugosum*	01.10.2015	Leisure Island	2m by snorkel	37.62955S, 176.18543E	Healthy brown tubular algae with high water retention capacity. No biofouling present.
ABW5	*Carpophyllum plumosum*	01.10.2015	Leisure Island	2m by snorkel	37.62955S, 176.18543E	Healthy brown leathery algae with large gas bladders. Biofouling of red algae on up to 80 percent of the specimen.
ABW7	*Ecklonia radiata*	08.09.2015	Karewa Island	3m by SCUBA	37.52862S, 176.13111E	Healthy brown leathery algae with slimy film upon touch. No biofouling present.
ABW9	*Carpophyllum maschalocarpum*	01.10.2015	Leisure Island	2m by snorkel	37.62955S, 176.18543E	Healthy brown algae with long stipes and large gas bladders. Biofouling on up to 65 percent of the specimen.
ABW10	*Glaphyrosiphon atropurpurea*	01.10.2015	Leisure Island	2m by snorkel	37.62955S, 176.18543E	Healthy red and leathery algae with flat, rubbery, and ribbed blades. No biofouling present, however some blades appeared to be bleached.
ABW11	*Lessonia* sp.	01.10.2015	Leisure Island	3m by snorkel	37.62955S, 176.18543E	Healthy brown and leathery algae with large blades and thick stipes. No biofouling present.

Field ID code	Species	Collection date	Collection location	Depth/ method	GPS coordinates	Description
ABW12	*Pterocladia lucida*	01.10.2015	Leisure Island	2m by snorkel	37.62955S, 176.18543E	Healthy red and leafy algae. Biofouling on up to 50 percent of the specimen.
ABW13	*Vidalia colensoi*	08.09.2014	Karewa Island	13m by SCUBA	37.52862S, 176.13111E	Healthy red algae with long blades. Biofouling present on up to 30 percent of the specimen.
ABW14	*Xiphophora chondrophylla*	01.10.2015	Leisure Island	2m by snorkel	37.62955S, 176.18543E	Healthy brown algae with thick, stipes. No biofouling present.
ABW15	*Ulva* sp.	01.10.2015	Leisure Island	1m by snorkel	37.62955S, 176.18543E	Healthy green cellophane-looking algae. No biofouling present.
ABW16	*Dictyota kunthii*	27.02.2015	Leisure Island	1m by snorkel	37.62955S, 176.18543E	Healthy brown algae with multiple furry blades attached to a single stipe. No biofouling present.
ABW18	*Chondracanthus chapmanii*	29.09.2015	Tauranga Harbour	1m by snorkel	37.66413S, 176.03679E	Healthy red algae with no biofouling present.
ABW19	*Undaria pinnatifida*	17.10.2015	Tauranga Harbour	0m by snorkel	37.66761S, 176.17971E	Healthy brown algae with biofouling on up to 60 percent of the specimen.

A.2: Small Scale Extraction Preparation

Extract 'a' (2 g) of Summer Collected Specimens

Sample	Date	Specimen weight (g)	Extraction weight (g)	Solvent used (mL)	Extract weight (mg)
ABS1a	11.03.2015	101.14	2.00	320	130
ABS2a	11.03.3015	545.74	2.00	385	190
ABS3a	11.03.2015	302.35	2.02	320	120
ABS4a	11.03.2015	459.18	2.00	240	140
ABS5a	11.03.2015	164.60	2.01	320	152
ABS6a	12.03.2015	171.55	2.01	280	137
ABS7a	12.03.2015	146.95	2.00	240	154
ABS8a	12.03.2015	358.86	2.00	300	84
ABS9a	12.03.2015	1384.28	2.01	210	161
ABS10a	19.03.2015	600.45	1.99	305	187
ABS11a	19.03.2015	929.35	2.00	310	195
ABS12a	19.03.2015	810.10	1.99	310	169
ABS13a	26.03.2015	311.75	2.00	450	167
ABS14a	26.03.2015	344.70	2.00	395	293
ABS15a	26.03.2015	414.00	2.01	315	196
ABS16a	26.03.2015	491.10	2.02	310	109
ABS17a	01.04.2015	1754.60	2.00	225	85

Extract ‘b’ (2 g) of Summer Collected Specimens

Sample	Date	Specimen weight (g)	Extraction weight (g)	Solvent used (mL)	Extract weight (mg)
ABS1b	16.04.2015	112.61	2.00	315	128
ABS2b	16.04.2015	544.03	2.00	310	94
ABS3b	10.04.2015	315.98	1.99	380	131
ABS4b	08.04.2015	461.18	2.01	315	110
ABS5b	31.03.2015	161.26	2.01	320	144
ABS6b	17.04.2015	181.21	2.01	225	72
ABS7b	01.04.2015	139.50	2.01	230	159
ABS8b	09.04.2015	368.90	2.00	240	84
ABS9b	16.04.2015	1379.74	2.00	390	172
ABS10b	16.04.2015	587.89	2.01	230	47
ABS11b	21.04.2015	919.74	2.00	315	134
ABS12b	21.04.2015	807.45	2.00	320	120
ABS13b	31.03.2015	290.42	2.00	475	154
ABS14b	15.04.2015	319.50	2.00	390	204
ABS15b	16.04.2015	406.50	1.99	315	67
ABS16b	09.04.2015	497.56	2.00	305	149
ABS17b	21.04.2015	1577.30	1.99	235	78

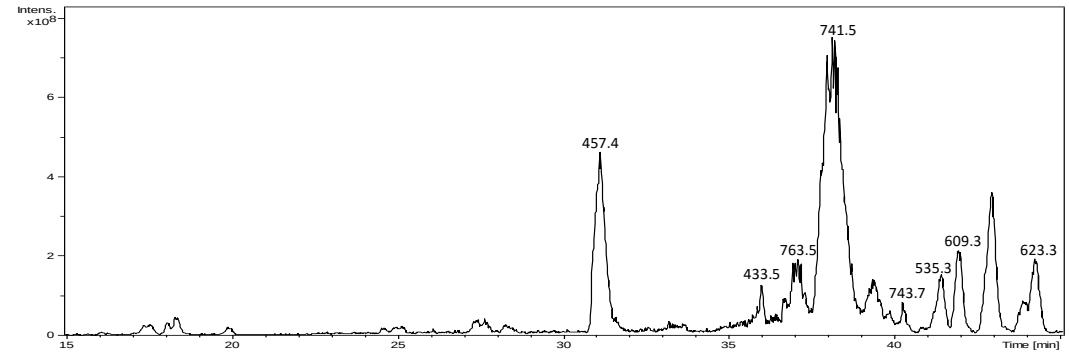
Extract ‘a’ (2 g) of Winter Collected Specimens

Sample	Date	Specimen weight (g)	Extraction weight (g)	Solvent used (mL)	Extract weight (mg)
ABW1a	08.09.2015	1000	2.03	355	144
ABW2a	17.12.2015	710.66	2.03	315	89
ABW3a	21.11.2015	365.77	2.01	305	164
ABW4a	01.10.2015	32.42	2.01	210	106
ABW5a	21.11.2015	660.20	2.00	290	155
ABW7a	24.09.2015	1000	2.03	210	171
ABW9a	11.11.2015	1237	2.03	360	149
ABW10a	06.10.2015	596.20	1.99	365	77
ABW11a	27.10.2015	868	2.02	280	218
ABW12a	06.10.2015	734	2.00	280	108
ABW13a	23.09.2015	557.80	2.00	475	154
ABW14a	06.10.2015	676.90	2.00	280	1557
ABW15a	11.11.2015	298.38	2.00	315	102
ABW16a	24.10.2015	864.27	2.01	295	175
ABW18a	05.10.2015	211.80	2.00	350	68
ABW19a	17.12.2015	236.91	1.99	295	118

A.3: LC-MS Base Peak Chromatograms of Small Scale Crude Extracts

Ion Annotation for Extract 'a' (2 g) of Summer Collected Specimens

ABS1a



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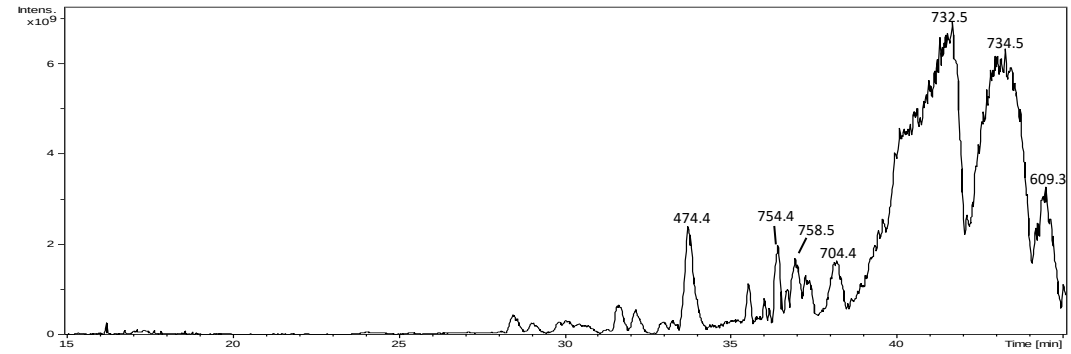

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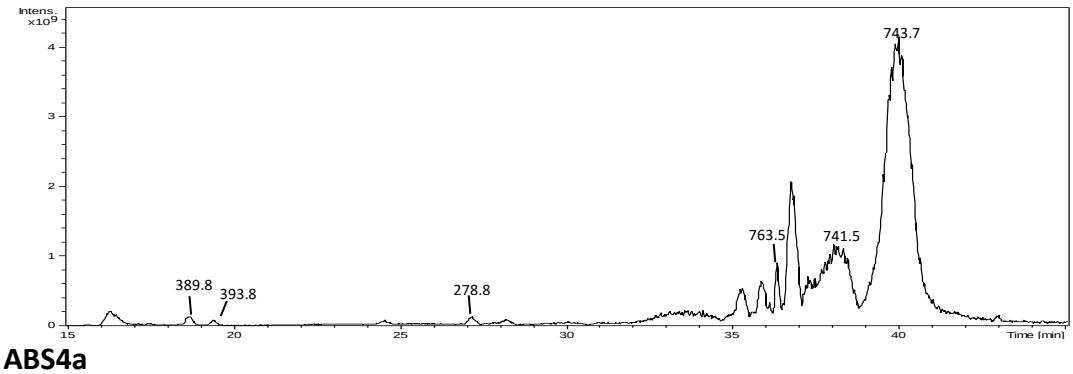

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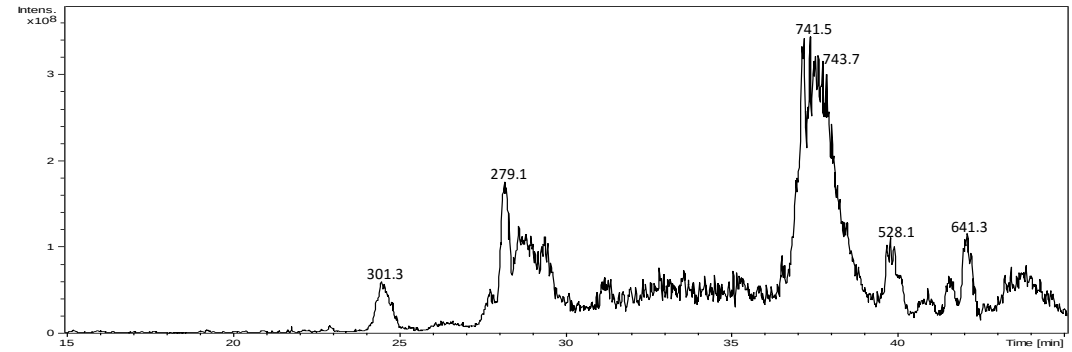

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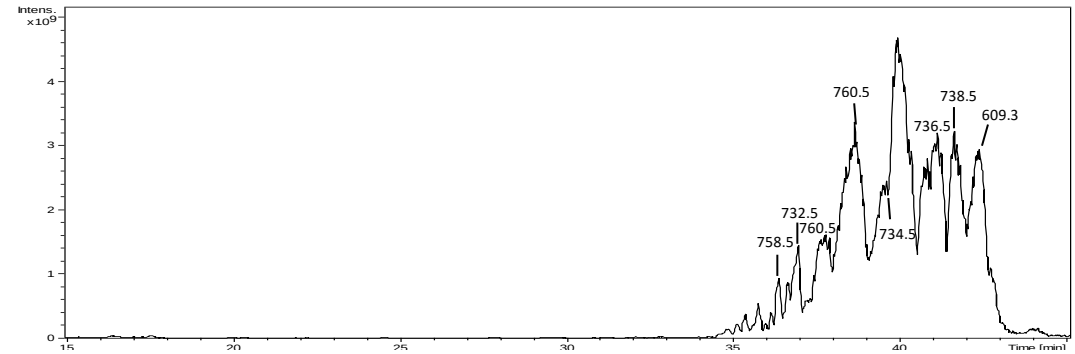

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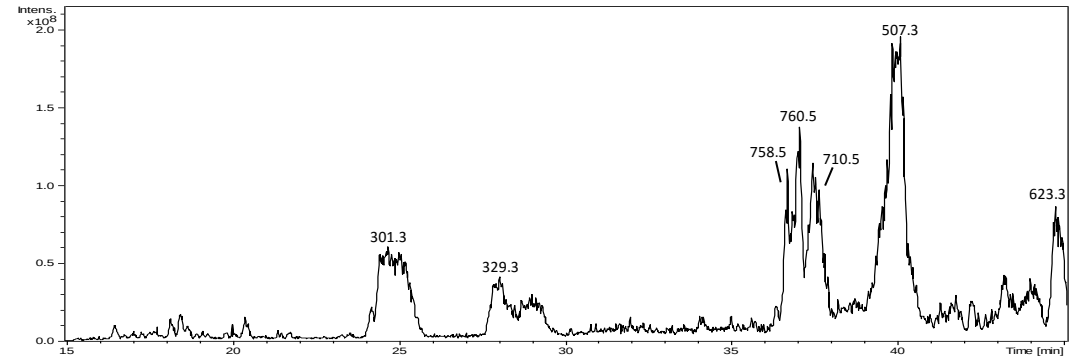

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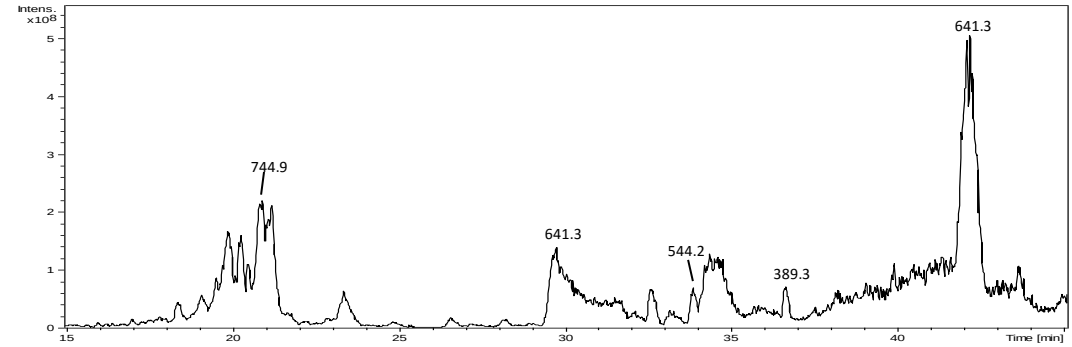

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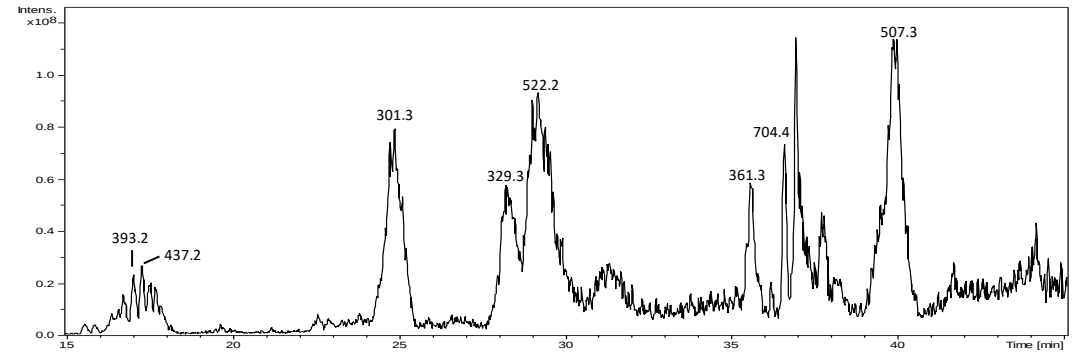

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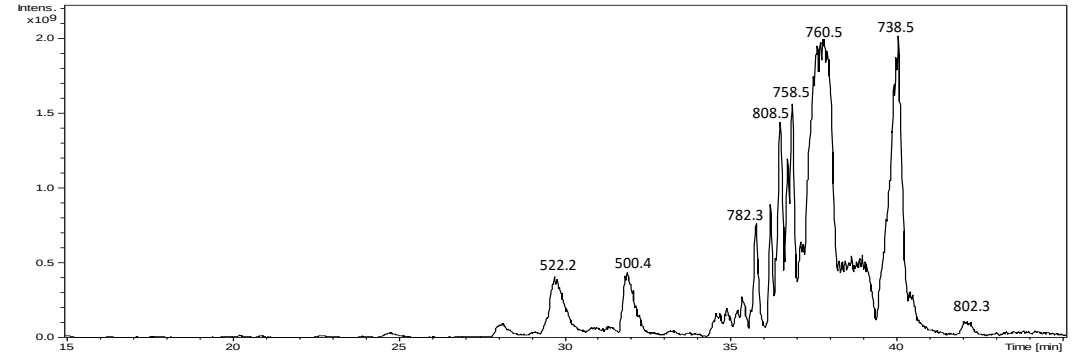

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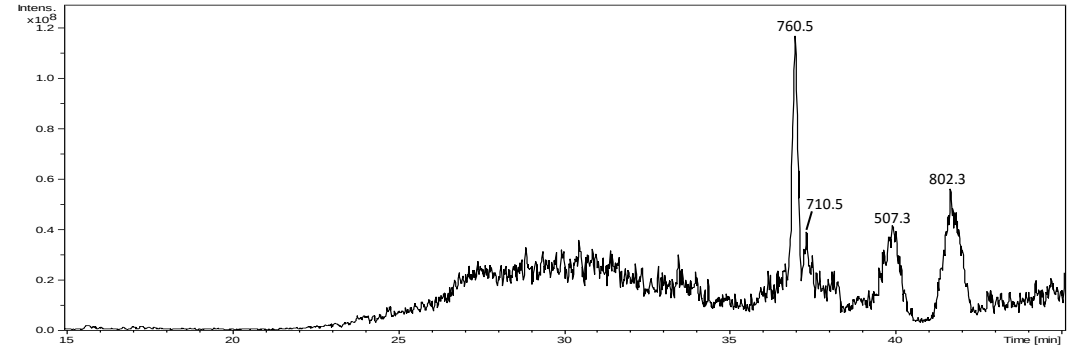

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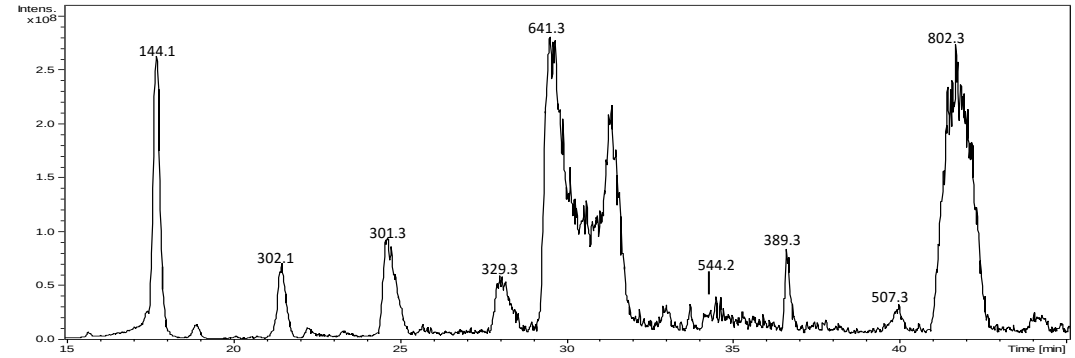

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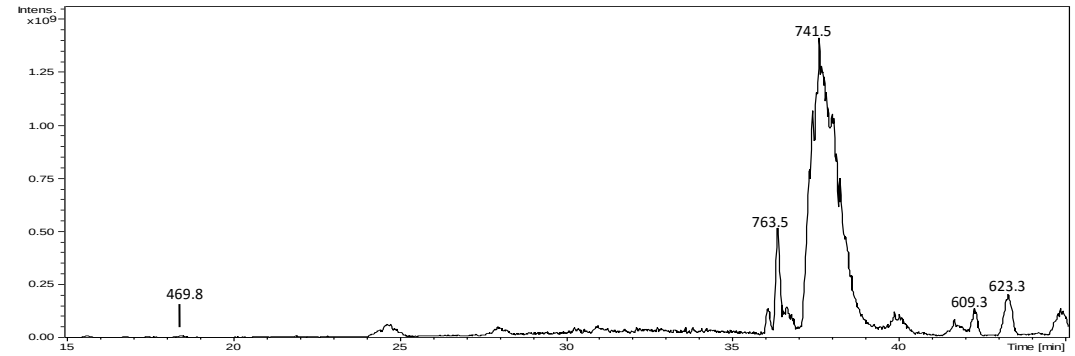

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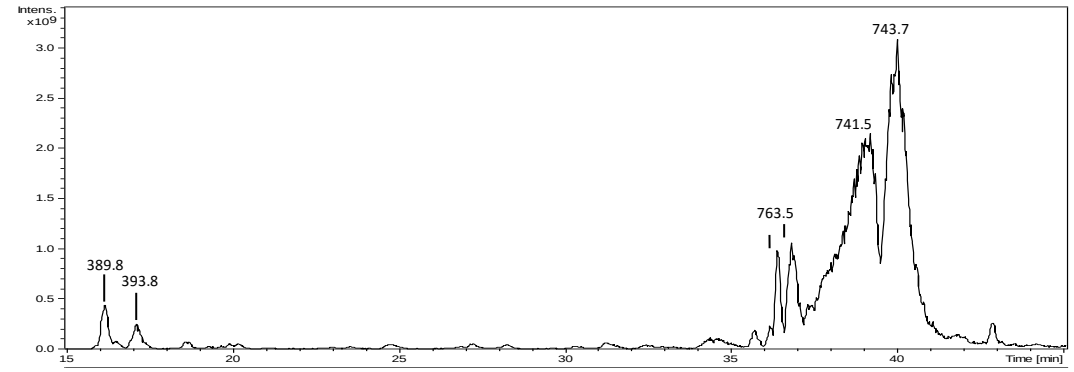

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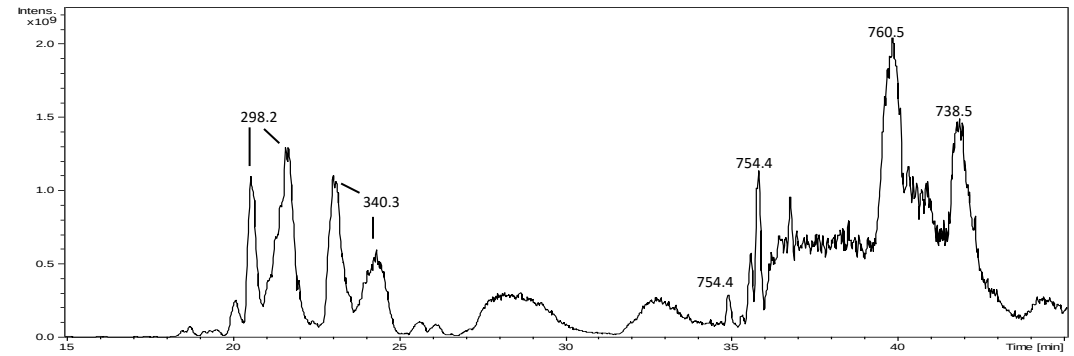

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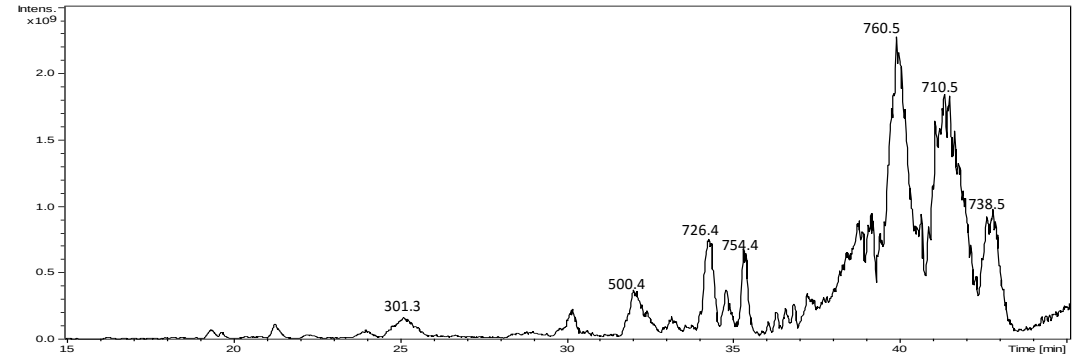

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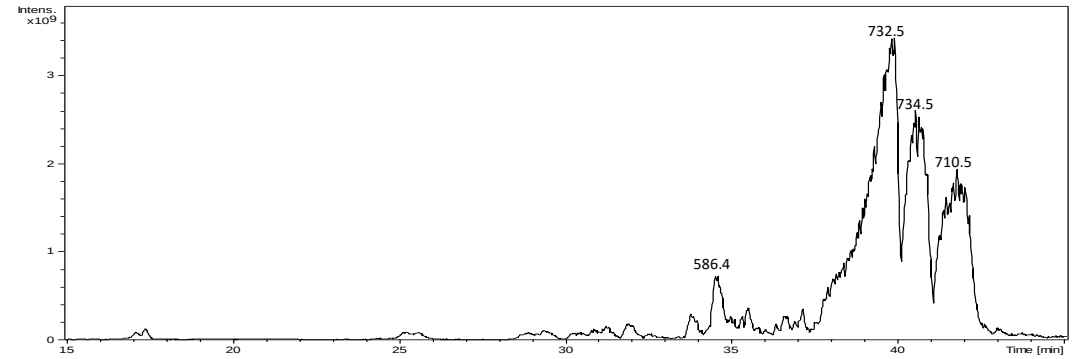

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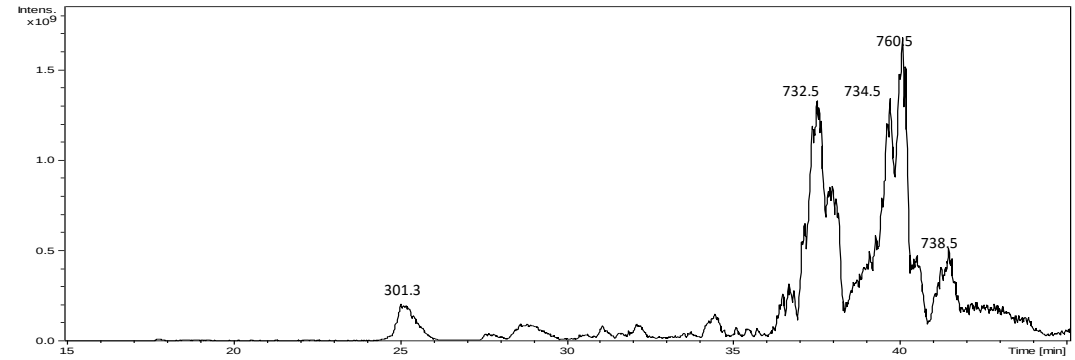
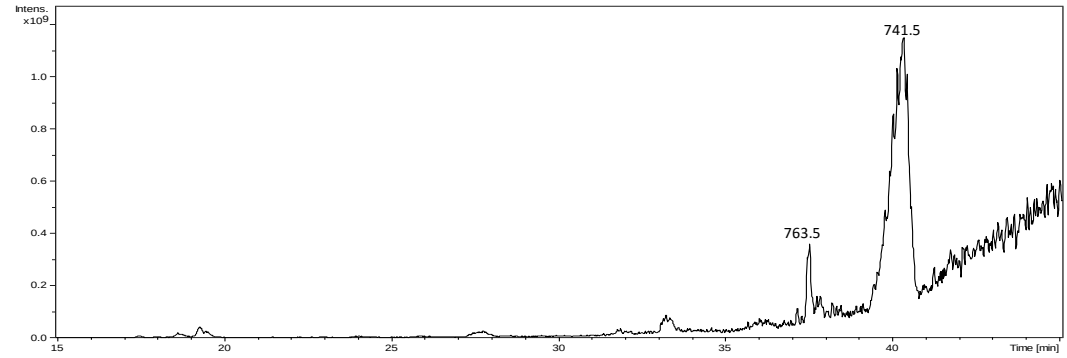


Ion Annotation for Extract 'a' (2 g) of Winter Collected Specimens

ABW1a



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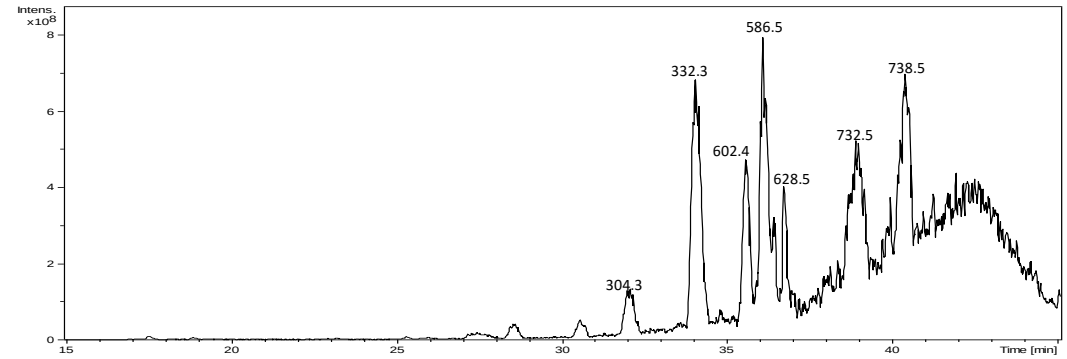

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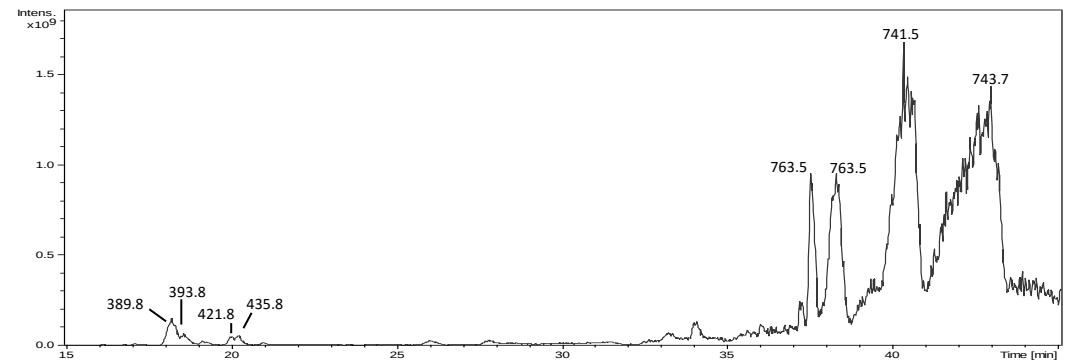

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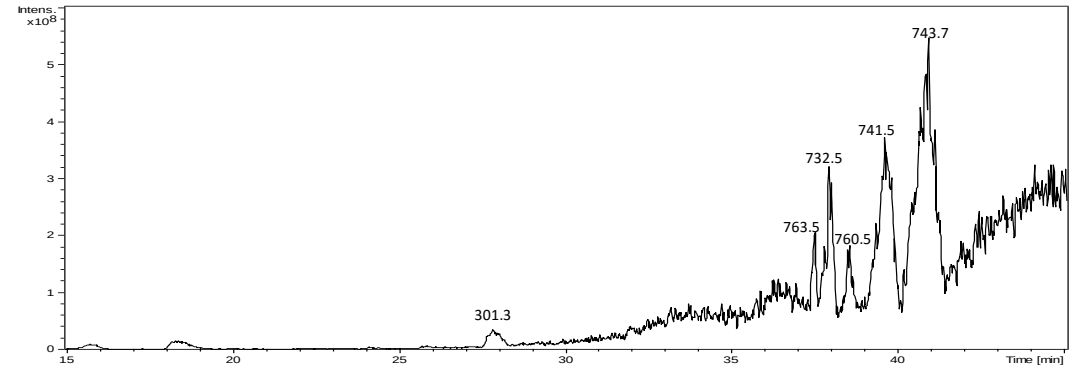
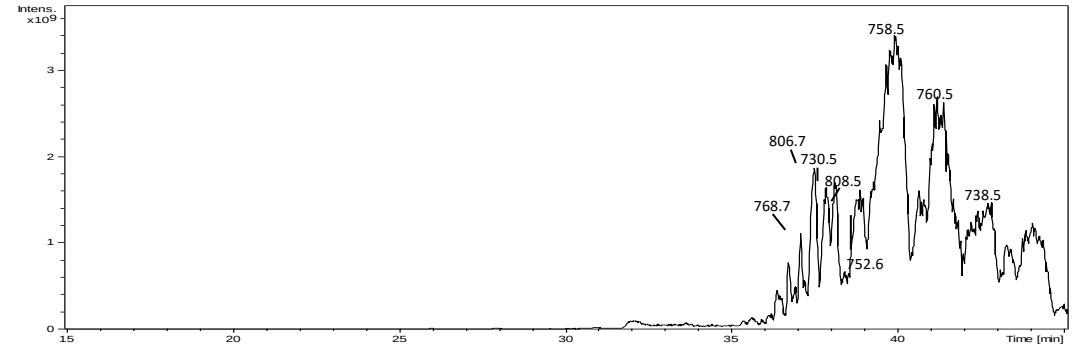


ABW5a



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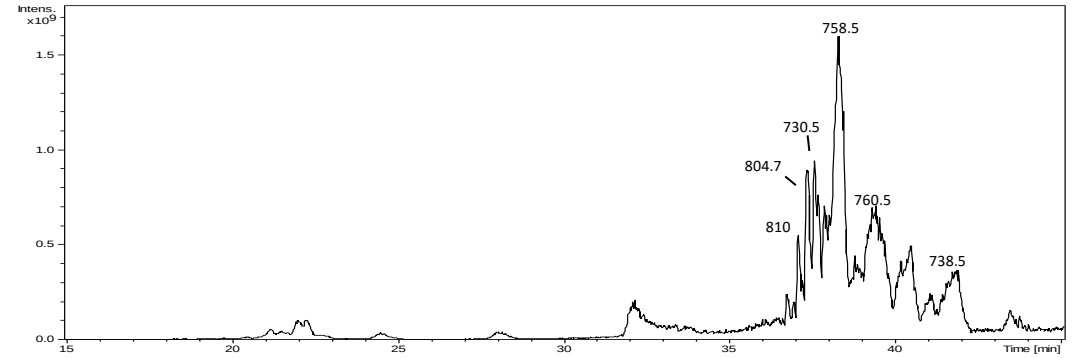

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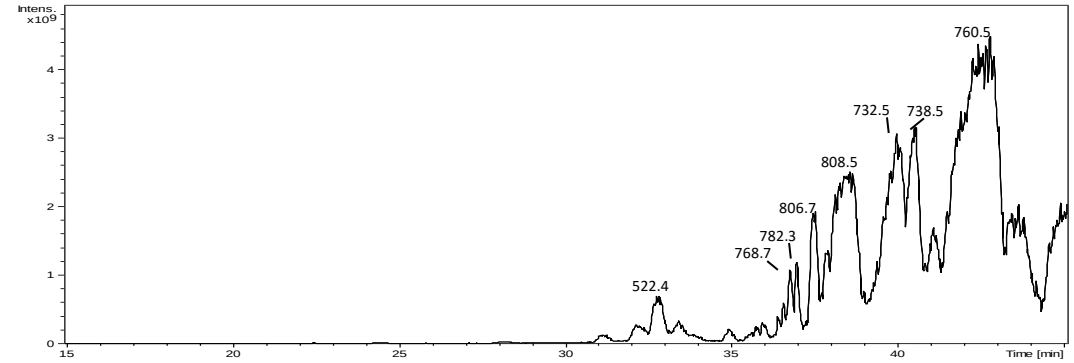

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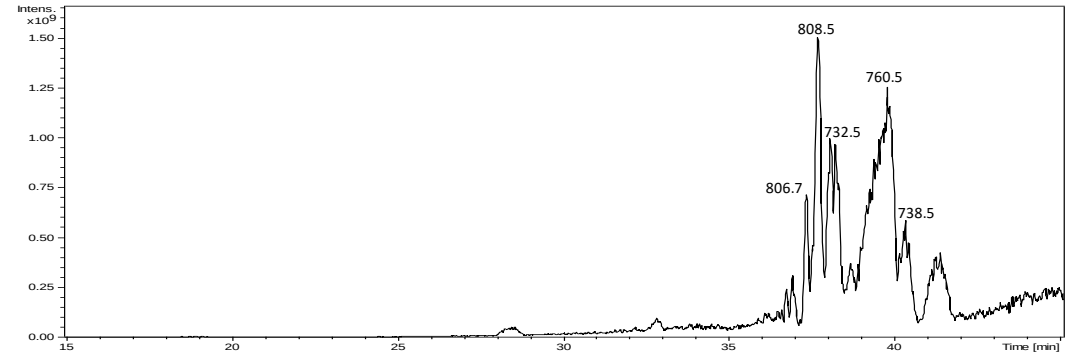

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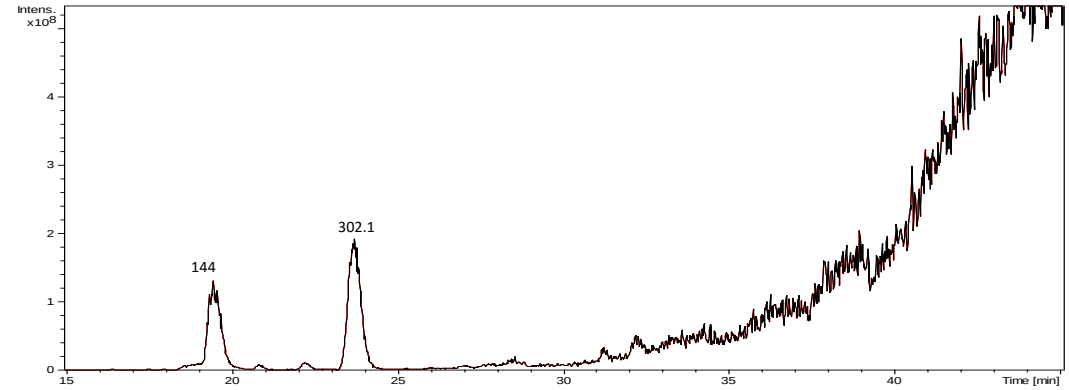

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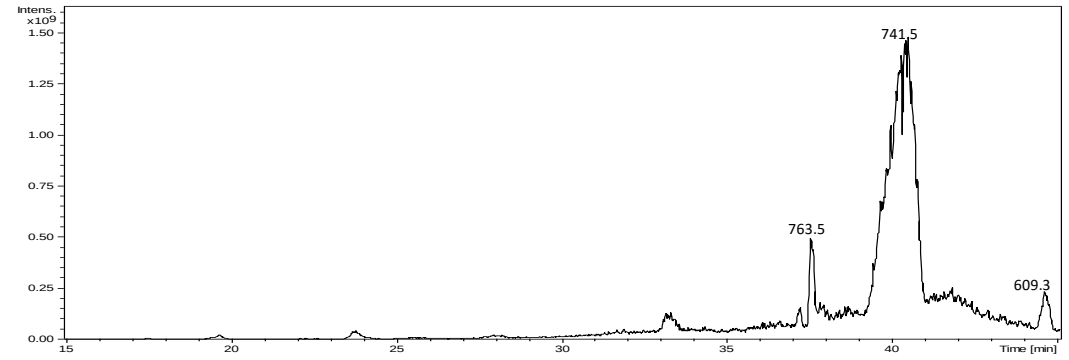

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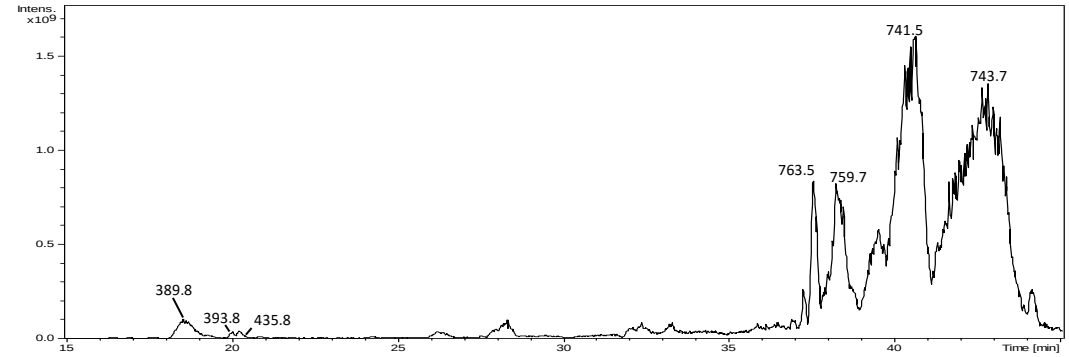

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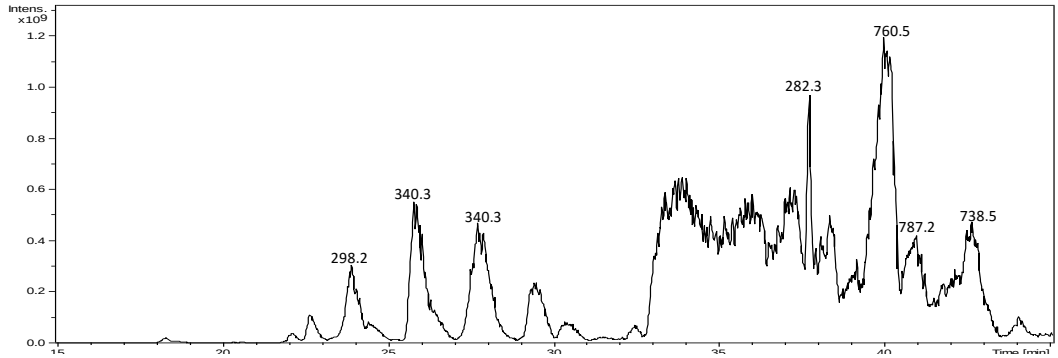
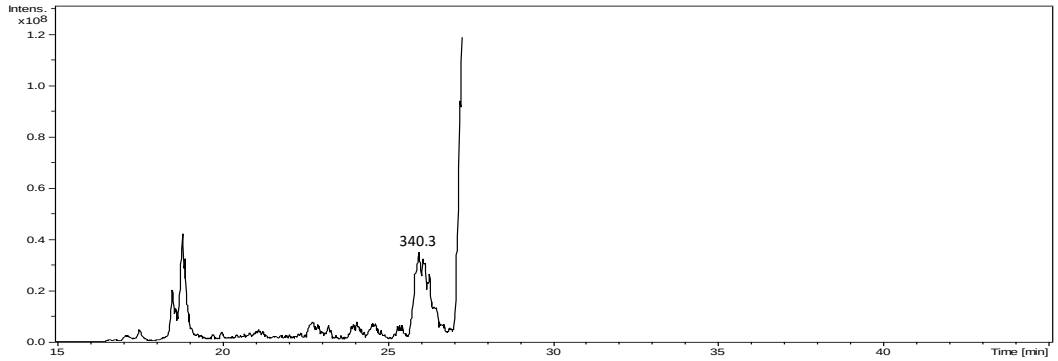


ABW15a



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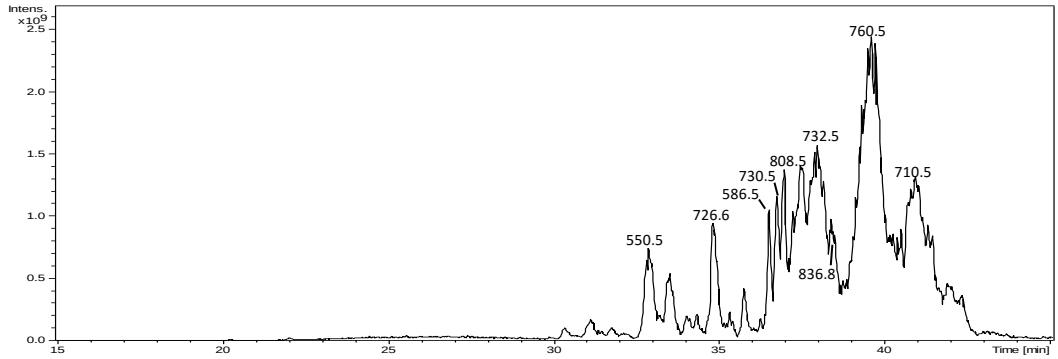
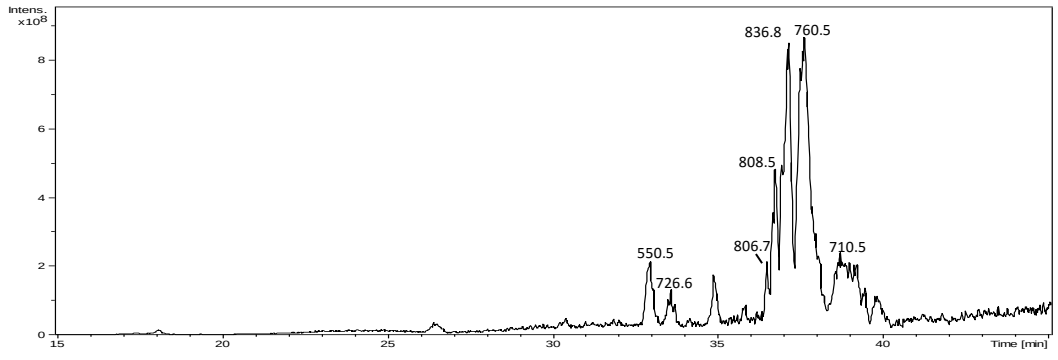
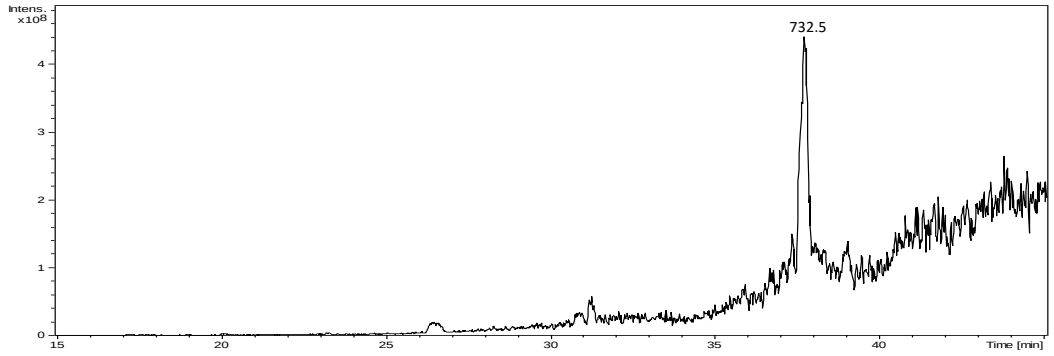


ABW18a



ABW19a



A.4: *In Vitro* Bioassay of Small Scale Crude Extracts

Extract 'a' (2 g) of Summer Collected Specimens

Sample	Summer extraction a – 2 hours				Summer extraction a – 2 hours			
	Replicate 1				Replicate 2			
	Dilution	Counts	Av.	cfu/mL	Dilution	Counts	Av.	cfu/mL
H_2O	D4	16, 16, 14	15.3	1.5×10^7	D4	6, 5, 2	4.3	4.3×10^6
10:1 MeOH	D4	15, 15, 15	15	1.5×10^7	D5	1, 2, 1	1.3	1.3×10^7
ABS1a	D4	19, 10, 16	15	1.5×10^7	D4	10, 15, 18	14.3	1.44×10^7
ABS2a	D4	8, 9, 8	8.3	8.3×10^6	D4	7, 9, 7	7.7	7.7×10^6
ABS3a	D4	5, 2, 7	4.7	4.7×10^6	D4	4, 4, 4	4	4×10^6
ABS4a	D4	17, 14, 13	14.7	1.5×10^7	D4	15, 17, 20	17.3	1.7×10^7
ABS5a	D4	11, 11, 10	10.7	10.7×10^7	D4	11, 8, 9	9.3	9.3×10^6
ABS6a	D4	7, 8, 8	7.7	7.6×10^6	D4	4, 3, 6	4.3	4.3×10^6
ABS7a	D4	18, 13, 9	13.3	1.3×10^7	D4	12, 12, 14	12.7	1.3×10^7
ABS8a	D4	11, 13, 9	11	1.1×10^7	D4	17, 7, 7	10.3	1×10^7
ABS9a	D4	5, 10, 5	6.7	6.7×10^6	D4	6, 5, 6	5.7	5.7×10^6
ABS10a	D4	9, 7, 1	5.7	5.7×10^6	D4	8, 9, 8	8.3	8.3×10^6
ABS11a	D4	15, 19, 15	16.3	1.6×10^7	D4	18, 17, 25	20	2×10^7
ABS12a	D4	12, 2, 12	8.7	8.7×10^6	D4	9, 12, 14	11.7	1.2×10^7
ABS13a	D4	2, 5, 2	3	3×10^6	D4	7, 5, 11	7.7	7.7×10^6
ABS14a	D0	0, 0, 0	0	0	D0	0, 0, 0	0	0
ABS15a	D4	3, 7, 7	5.7	5.7×10^6	D4	7, 4, 2	4.3	4.3×10^6
ABS16a	D4	8, 3, 7	6	6×10^6	D4	8, 12, 6	8.7	8.7×10^6
ABS17a	D4	4, 4, 4	4	4×10^6	D4	5, 10, 4	6.3	6.3×10^6

Sample	Summer extraction a – 24 hours				Summer extraction a - 24 hours			
	Replicate 1				Replicate 2			
	Dilution	Counts	Av.	cfu/mL	Dilution	Counts	Av.	cfu/mL
H_2O	D4	15, 7, 7	9.7	9.7×10^6	D4	7, 14, 5	8.7	8.7×10^6
10:1 MeOH	D4	15, 14, 16	15	1.5×10^7	D5	1, 2, 1	1.3	1.3×10^7
ABS1a	D4	7, 4, 4	5	5×10^6	D4	3, 6, 1	3.3	3.3×10^6
ABS2a	D4	7, 6, 3	5.3	5.3×10^6	D3	24, 26, 27	25.7	2.6×10^6
ABS3a	D0	0, 0, 0	0	0	D0	0, 0, 0	0	0
ABS4a	D4	14, 6, 12	10.7	1.1×10^7	D4	8, 9, 12	9.7	9.7×10^6
ABS5a	D4	5, 9, 9	7.7	7.7×10^6	D4	6, 7, 9	7.3	7.3×10^6
ABS6a	D0	0, 0, 0	0	0	D0	0, 0, 0	0	0
ABS7a	D4	3, 4, 6	4.3	4.3×10^6	D0	0, 0, 0	0	0
ABS8a	D4	4, 3, 7	4.7	4.7×10^6	D2	2, 1, 1	1.3	1.3×10^4
ABS9a	D0	0, 0, 0	0	0	D0	0, 0, 0	0	0
ABS10a	D4	5, 4, 3	4	4×10^6	D4	3, 4, 11	6	6×10^6
ABS11a	D4	5, 5, 4	4.7	4.7×10^6	D4	16, 9, 11	12	1.2×10^7
ABS12a	D4	1, 9, 2	4	4×10^6	D3	23, 28, 23	24.7	2.5×10^6
ABS13a	D3	3, 3, 1	2.3	2.3×10^5	D2	6, 4, 8	6	6×10^4
ABS14a	D0	0, 0, 0	0	0	D0	0, 0, 0	0	0
ABS15a	D1	4, 5, 4	4.3	4.3×10^3	D2	2, 1, 1	1.3	1.3×10^4
ABS16a	D4	2, 2, 2	2	2×10^6	D4	3, 6, 2	3.7	3.7×10^6
ABS17a	D4	1, 1, 2	1.3	1.3×10^6	D4	3, 4, 2	3	3×10^6

Extract 'b' (2 g) of Summer Collected Specimens

Sample	Summer extraction b – 2 hours				Summer extraction b - 2 hours			
	Replicate 1				Replicate 2			
	Dilution	Counts	Av.	cfu/mL	Dilution	Counts	Av.	cfu/mL
H_2O	D5	1, 3, 1	1.7	1.7×10^7	D4	7, 4, 6	5.7	5.7×10^6
10:1 MeOH	D4	6, 8, 8	7.3	7.3×10^6	D4	20, 15, 12	15.7	1.6×10^7
ABS1b	D5	2, 2, 2	2	2×10^7	D4	7, 8, 10	8.3	8.3×10^6
ABS2b	D4	6, 10, 8	8	8×10^6	D4	7, 6, 10	7.7	7.7×10^6
ABS3b	D0	0, 0 ,0	0	0	D0	0 ,0 ,0	0	0
ABS4b	D5	2, 5, 4	3.7	3.7×10^7	D4	25, 17, 36	26	2.6×10^7
ABS5b	D4	17, 15, 18	16.7	1.7×10^7	D4	23, 14, 15	17.3	1.7×10^7
ABS6b	D4	19, 13, 19	17	1.7×10^7	D4	9, 2, 10	7	7×10^6
ABS7b	D5	2, 4, 2	2.7	2.7×10^7	D4	19, 25, 16	20	2×10^7
ABS8b	D4	17, 13, 11	13.7	1.4×10^7	D5	1, 4, 3	2.7	2.7×10^7
ABS9b	D4	9, 10, 15	11.3	1.1×10^7	D4	12, 12, 13	12.3	1.2×10^7
ABS10b	D4	8, 19, 18	15	1.5×10^7	D4	20, 21, 28	23	2.3×10^7
ABS11b	D4	12, 13, 18	14.3	1.4×10^7	D4	15, 13, 17	15	1.5×10^7
ABS12b	D4	9, 18, 16	14.3	1.4×10^7	D4	21, 8, 5	11.3	1.1×10^7
ABS13b	D4	10, 14, 13	12.3	1.2×10^7	D4	9, 6, 7	7.3	7.3×10^6
ABS14b	D0	0, 0, 0	0	0	D0	0, 0, 0	0	0
ABS15b	D4	15, 14, 21	16.7	1.7×10^7	D4	20, 22, 14	18.7	1.9×10^7
ABS16b	D4	15, 11, 14	13.3	1.3×10^7	D4	8, 11, 13	10.7	1.1×10^7
ABS17b	D4	16, 13, 15	14.7	1.5×10^7	D4	11, 9 ,19	13	1.3×10^7

Sample	Summer extraction b – 24 hours				Summer extraction b - 24 hours			
	Replicate 1				Replicate 2			
	Dilution	Counts	Av.	cfu/mL	Dilution	Counts	Av.	cfu/mL
H_2O	D4	8, 8, 8	8	8×10^6	D4	6, 5, 6	5.7	5.7×10^6
10:1 MeOH	D4	8, 19, 6	11	1.1×10^7	D3	15, 15, 9	13	1.3×10^6
ABS1b	D4	3, 4, 3	3.3	3.3×10^6	D4	4, 3, 7	4.7	4.7×10^6
ABS2b	D4	17, 8, 9	11.3	1.1×10^7	D4	0, 4, 9	4.3	4.3×10^6
ABS3b	D0	0, 0 ,0	0	0	D0	0 ,0, 0	0	0
ABS4b	D4	8, 13, 17	12.7	1.3×10^7	D4	11, 7, 10	9.3	9.3×10^6
ABS5b	D4	12, 12, 9	11	1.1×10^7	D4	15, 8, 22	15	1.5×10^7
ABS6b	D0	0, 0, 0	0	0	D0	0, 0, 0	0	0
ABS7b	D4	8, 12, 5	8.3	8.3×10^6	D4	4, 6, 6	5.3	5.3×10^6
ABS8b	D4	2, 1, 2	1.7	1.7×10^6	D4	9, 3, 2	4.7	4.7×10^6
ABS9b	D0	0, 0, 0	0	0	D0	0, 0, 0	0	0
ABS10b	D4	8, 5, 2	5	5×10^6	D4	4, 6, 6	5.3	5.3×10^6
ABS11b	D0	0, 0, 0	0	0	D0	0, 0, 0	0	0
ABS12b	D3	14, 16, 12	14	1.4×10^6	D3	26, 22, 21	23	2.3×10^6
ABS13b	D4	0, 7, 4	3.7	3.7×10^6	D4	4, 7, 3	4.7	4.7×10^6
ABS14b	D0	0, 0, 0	0	0	D0	0, 0, 0	0	0
ABS15b	D4	8, 3, 7	6	6×10^6	D4	3, 7, 5	5	5×10^6
ABS16b	D4	3, 5, 4	4	4×10^6	D4	3, 4, 9	5.3	5.3×10^6
ABS17b	D4	10, 12, 13	11.7	1.2×10^7	D4	7, 8, 9	8	8×10^6

Extract 'a' (2 g) of Winter Collected Specimens

Sample	Winter extraction a – 2 hours				Winter extraction a - 2 hours			
	Replicate 1				Replicate 2			
	Dilution	Counts	Av.	cfu/mL	Dilution	Counts	Av.	cfu/mL
H_2O	D5	2, 3, 5	3.3	3.3×10^7	D5	1, 7, 5	4.3	4.3×10^7
10:1 MeOH	D4	9, 12, 6	9	9×10^6	D5	8, 9, 4	7	7×10^7
ABW1a	D5	1, 1, 5	2.3	2.3×10^7	D5	3, 3, 0	2	2×10^7
ABW2a	D5	1, 5, 0	2	2×10^7	D5	0, 1, 1	0.7	0.7×10^7
ABW3a	D2	3, 3, 2	2.7	2.7×10^4	D2	6, 4, 3	4.3	4.3×10^4
ABW4a	D5	6, 4, 3	4.3	4.3×10^7	D5	1, 4, 0	1.7	1.7×10^7
ABW5a	D5	4, 3, 2	3	3×10^7	D5	2, 2, 4	2.7	2.7×10^7
ABW7a	D5	1, 7, 5	4.3	4.3×10^7	D5	4, 3, 1	2.7	2.7×10^7
ABW9a	D5	2, 0, 3	1.7	1.7×10^7	D5	3, 1, 1	1.7	1.7×10^7
ABW10a	D5	2, 0, 2	1.3	1.3×10^7	D5	5, 2, 2	3	3×10^7
ABW11a	D5	3, 1, 0	1.3	1.3×10^7	D5	1, 2, 1	1.3	1.3×10^7
ABW12a	D5	2, 3, 2	2.3	2.3×10^7	D5	2, 0, 1	1	1×10^7
ABW13a	D0	6, 4, 0	3.3	3.3×10^2	D0	2, 1, 0	1	1×10^2
ABW14a	D0	0, 0, 0	0	0	D0	0, 0, 0	0	0
ABW15a	D5	3, 4, 1	2.7	2.7×10^7	D5	3, 0, 4	2.3	2.3×10^7
ABW16a	D5	1, 3, 4	2.7	2.7×10^7	D5	1, 3, 2	2	2×10^7
ABW18a	D5	3, 2, 1	2	2×10^7	D5	1, 0, 1	0.7	0.7×10^7
ABW19a	D5	4, 4, 1	3	3×10^7	D5	2, 3, 1	2	2×10^7

Sample	Winter extraction a – 24 hours				Winter extraction a - 24 hours			
	Replicate 1				Replicate 2			
	Dilution	Counts	Av.	cfu/mL	Dilution	Counts	Av.	cfu/mL
H_2O	D4	2, 4, 5	3.7	3.7×10^6	D4	11, 10, 14	11.7	1.7×10^7
10:1 MeOH	D4	12, 9, 6	9	9×10^6	D5	5, 5, 5	5	5×10^7
ABW1a	D4	2, 1, 0	1	1×10^6	D4	2, 3, 5	3.3	3.3×10^6
ABW2a	D4	2, 3, 1	2	2×10^6	D4	6, 4, 4	4.7	4.7×10^6
ABW3a	D0	0, 0, 0	0	0	D0	0, 0, 0	0	0
ABW4a	D4	3, 1, 1	1.7	1.7×10^6	D4	8, 4, 7	6.3	6.3×10^6
ABW5a	D4	2, 1, 1	3	1.3×10^6	D4	1, 1, 1	1	1×10^6
ABW7a	D0	0, 0 ,0	0	0	D0	0, 0, 0	0	0
ABW9a	D1	5, 1, 1	2.3	2.3×10^3	D3	1, 1, 1	1	1×10^5
ABW10a	D5	1, 1, 2	1.3	1.3×10^7	D5	1, 0, 1	0.7	0.7×10^7
ABW11a	D0	0, 0, 0	0	0	D4	4, 3, 8	5	5×10^6
ABW12a	D3	5, 5, 6	5.3	5.3×10^5	D4	2, 1, 0	1	1×10^6
ABW13a	D0	0, 0, 0	0	0	D0	0, 0, 0	0	0
ABW14a	D0	0, 0, 0	0	0	D0	0, 0, 0	0	0
ABW15a	D5	1, 2, 0	1	1×10^7	D4	5, 3, 3	3.7	3.7×10^6
ABW16a	D0	1, 0, 3	1.3	1.3×10^2	D0	0, 0, 0	0	0
ABW18a	D5	2, 0, 3	1.7	1.7×10^7	D5	1, 1, 0	0.7	0.7×10^7
ABW19a	D4	3, 5, 11	6.3	6.3×10^6	D4	3, 8, 6	5.7	5.7×10^6

A.5: Large Scale Extraction Preparation

Extract ‘c’ (20 g) of Summer Collected Specimens

Sample	Date	Specimen weight (g)	Extraction weight (g)	Solvent used (mL)	Extract weight (mg)
ABS3c	17.09.2015	239.48	20.06	745	1680
ABS6c	17.09.2015	120	20.05	420	641
ABS9c	17.09.2015	1086.43	20.03	565	1795
ABS13c	23.09.2015	242.80	20.01	640	1526
ABS14c	26.03.2015	219.30	19.9338	580	1836

A.6: *In Vitro* Bioassay of Large Scale Crude Extracts

Extract 'c' (20 g) of Summer Collected Specimens

Sample	Extraction c – 2 hours				Extraction c - 2 hours			
	Replicate 1				Replicate 2			
	Dilution	Counts	Av.	cfu/mL	Dilution	Counts	Av.	cfu/mL
H_2O	D5	5, 6, 3	4.3	4.3×10^7	D5	7, 5, 11	7.7	7.7×10^7
100:1 MeOH	D5	6, 9, 4	6.3	6.3×10^7	D5	8, 9, 4	7	7×10^7
ABS3c	D0	0, 0, 0	0	0	D0	0, 0, 0	0	0
ABS6c	D0	0, 0, 0	0	0	D0	0, 0, 0	0	0
ABS9c	D0	0, 0, 0	0	0	D0	0, 0, 0	0	0
ABS13c	D5	4, 7, 2	4.3	4.3×10^7	D5	6, 8, 7	7	7×10^7
ABS14c	D0	0, 0, 0	0	0	D0	0, 0, 0	0	0

Sample	Extraction c – 24 hours				Extraction c - 24 hours			
	Replicate 1				Replicate 2			
	Dilution	Counts	Av.	cfu/mL	Dilution	Counts	Av.	cfu/mL
H_2O	D5	7, 4, 3	4.7	4.7×10^7	D5	4, 3, 2	3	3×10^7
100:1 MeOH	D4	11, 13, 16	13.3	1.3×10^7	D5	4, 4, 4	4	4×10^7
ABS3c	D0	0, 0, 0	0	0	D0	0, 0, 0	0	0
ABS6c	D0	0, 0, 0	0	0	D0	0, 0, 0	0	0
ABS9c	D0	0, 0, 0	0	0	D0	0, 0, 0	0	0
ABS13c	D4	19, 25, 22	22	2.2×10^7	D5	0, 7, 3	3.3	3.3×10^7
ABS14c	D0	0, 0, 0	0	0	D0	0, 0, 0	0	0

A.7: *In Vitro* bioassay of C_{18} Fractions of 'Hit' Macroalgae Candidates

Fractions of ABS3c

Fraction	Fractions of ABS3c-2 hours				Fractions of ABS3c-2 hours			
	Replicate 1				Replicate 2			
	Dilution	Counts	Av.	cfu/mL	Dilution	Counts	Av.	cfu/mL
H_2O	D5	1, 2, 0	1	1×10^7	D4	4, 7, 4	5	5×10^6
100:1 MeOH	D5	3, 3, 0	2	2×10^7	D4	4, 4, 6	4.7	4.7×10^6
1	D5	2, 2, 2	2	2×10^7	D4	5, 3, 6	4.7	4.7×10^6
2	D4	9, 9, 10	9.3	9.3×10^6	D4	3, 3, 7	4.3	4.3×10^6
3	D2	2, 3, 9	4.7	4.7×10^4	D2	3, 1, 1	1.3	1.3×10^4
4	D1	1, 2, 0	1	1×10^3	D2	9, 2, 4	5	5×10^4
5	D2	1, 0, 2	1	1×10^4	D2	8, 7, 8	7.7	7.7×10^4
6	D4	12, 10, 12	11.3	1.1×10^6	D4	3, 5, 6	4.7	4.7×10^6
7	D4	10, 9, 11	10	1×10^7	D4	4, 4, 7	5	5×10^6
8	D5	1, 2, 1	1.3	1.3×10^7	D4	3, 4 , 0	2.3	2.3×10^6
9	D5	1, 3, 3	2.3	2.3×10^7	D4	4, 7, 5	5.3	5.3×10^6
10	D3	1, 5, 0	2	2×10^5	D2	6, 2, 0	2.7	2.7×10^4
11	D5	2, 1, 2	1.7	1.7×10^7	D4	3, 2, 4	3	3×10^6
12	D4	14, 14, 13	13.7	1.4×10^7	D4	4, 2, 1	2.3	2.3×10^6
13	D4	10, 9, 16	11.7	1.2×10^7	D4	2, 4, 6	4	4×10^6
14	D5	3, 2, 0	1.7	1.7×10^7	D4	8, 1, 1	3.3	3.3×10^6

Fraction	Fractions of ABS3c-24 hours				Fractions of ABS3c-24 hours			
	Replicate 1				Replicate 2			
	Dilution	Counts	Av.	cfu/mL	Dilution	Counts	Av.	cfu/mL
H_2O	D4	3, 4, 6	4.3	4.3×10^6	D4	3, 4, 5	4	4×10^6
100:1 MeOH	D4	7, 6, 8	7	7×10^6	D4	2, 3, 2	2.3	2.3×10^6
1	D3	13, 18, 18	16.3	1.6×10^6	D5	1, 1, 1	1	1×10^7
2	D5	3, 2, 2	2.3	2.3×10^7	D4	9, 12, 5	8.7	8.7×10^6
3	D2	2, 1, 0	1	1×10^4	D0	0 , 0, 0	0	0
4	D2	3, 5, 2	3.3	3.3×10^4	D0	2, 0, 0	0	0.7×10^2
5	D0	2, 1, 0	1	1×10^2	D0	0 , 0, 0	0	0
6	D4	19, 5, 8	10.7	1.1×10^7	D4	2, 4, 6	4	4×10^6
7	D5	6, 2, 0	2.7	2.7×10^7	D4	4, 5, 2	3.7	3.7×10^6
8	D5	1, 1, 1	1	1×10^7	D4	3, 6, 3	4	4×10^6
9	D5	4, 2, 2	2.7	2.7×10^7	D4	10, 7, 6	11.3	1.4×10^6
10	D1	5, 5, 2	4	4×10^3	D1	2, 1, 1	1.2	1.3×10^3
11	D5	3, 1, 0	1.3	1.3×10^7	D4	4, 8, 5	5.7	5.7×10^6
12	D5	2, 3, 2	2.3	2.3×10^7	D4	4, 4, 9	5.7	5.7×10^6
13	D4	13, 14, 10	12.3	1.2×10^7	D4	4, 4, 6	4.7	4.7×10^6
14	D4	16, 11, 16	14.3	1.4×10^7	D4	13, 8, 10	10.3	1×10^7

Fractions of ABS6c

Fraction	Fractions of ABS6c-2 hours				Fractions of ABS6c-2 hours			
	Replicate 1				Replicate 2			
	Dilution	Counts	Av.	cfu/mL	Dilution	Counts	Av.	cfu/mL
H_2O	D4	3, 2, 3	2.7	2.7×10^6	D4	3, 1, 1	1.7	1.7×10^6
100:1 MeOH	D4	5, 3, 1	3	3×10^6	D3	10, 10, 10	10	1×10^6
1	D5	0, 3, 2	1.7	1.7×10^7	D4	11, 11, 12	11.3	1.1×10^6
2	D4	7, 10, 8	8.3	8.3×10^6	D4	8, 8, 9	8.3	8.3×10^6
3	D4	6, 7, 1	7.3	7.3×10^6	D3	2, 1, 2	1.7	1.7×10^6
4	D0	8, 5, 13	8.7	8.7×10^2	D1	7, 12, 8	9	9×10^3
5	D3	7, 4, 1	4	4×10^5	D4	5, 2, 5	4	4×10^6
6	D4	8, 9, 8	8.3	8.3×10^6	D5	2, 2,0	1.3	1.3×10^7
7	D4	8, 8, 6	7.3	7.3×10^6	D4	14, 9, 14	12.3	1.2×10^7
8	D4	3, 4, 2	3	3×10^6	D4	2, 5, 5	4	4×10^6
9	D4	0, 3, 2	1.7	1.7×10^6	D3	8, 3, 5	5.3	5.3×10^5
10	D3	8, 8, 8	8	8×10^5	D4	1, 2, 1	1.3	1.3×10^6
11	D3	4, 3, 4	3.7	3.7×10^5	D3	10, 7, 0	5.7	5.7×10^5

Fraction	Fractions of ABS6c-24 hours				Fractions of ABS6c-24 hours			
	Replicate 1				Replicate 2			
	Dilution	Counts	Av.	cfu/mL	Dilution	Counts	Av.	cfu/mL
H_2O	D4	2, 5, 5	4	4×10^6	D4	9, 7, 12	9.3	9.3×10^6
100:1 MeOH	D3	11, 10, 9	10	1×10^6	D4	3, 1, 1	1.6	1.6×10^6
1	D5	1, 0, 1	0.7	0.7×10^7	D4	10, 8, 9	9	9×10^6
2	D4	7, 10, 8	8.3	8.3×10^5	D4	5, 4, 0	3	3×10^6
3	D1	6, 9, 5	6.7	6.7×10^3	D0	0, 2, 0	0.7	0.7×10^2
4	D0	0, 0, 0	0	0	D0	0, 0, 0	0	0
5	D0	2, 2, 0	1.3	1.3×10^2	D1	3, 2, 1	2	2×10^3
6	D4	11, 15, 14	13.3	1.3×10^7	D4	7, 6, 9	7.3	7.3×10^6
7	D5	1, 1, 1	1	1×10^7	D4	7, 9, 9	8.3	8.3×10^6
8	D4	2, 2, 4	2.7	2.7×10^6	D4	1, 1, 1	1	1×10^6
9	D1	6, 10, 11	9	9×10^3	D1	5, 1, 3	3	3×10^3
10	D4	2, 1, 0	1	1×10^6	D4	9, 5, 8	7.3	7.3×10^6
11	D4	5, 3, 7	5	5×10^5	D4	1, 1, 1	1	1×10^6

Fractions of ABS9c

Fraction	Fractions of ABS9c-2 hours				Fractions of ABS9c-2 hours			
	Replicate 1				Replicate 2			
	Dilution	Counts	Av.	cfu/mL	Dilution	Counts	Av.	cfu/mL
H_2O	D4	7, 9, 13	9.7	9.7×10^6	D4	9, 2, 8	6.3	6.3×10^6
100:1 MeOH	D4	6, 3, 3	4	4×10^6	D4	4, 3, 7	4.7	4.7×10^6
1	D4	11, 13, 11	11.7	1.2×10^7	D4	9, 5, 13	9	9×10^6
2	D4	7, 4, 3	4.7	4.7×10^6	D4	9, 2, 3	4.7	4.7×10^6
3	D4	3, 0, 2	1.7	1.7×10^6	D4	3, 2, 6	3.7	3.7×10^6
4	D4	8, 3, 1	4	4×10^6	D4	4, 5, 5	4.7	4.7×10^6
5	D4	2, 1, 4	2.3	2.3×10^6	D4	6, 6, 4	5.3	5.3×10^6
6	D4	8, 3, 10	7	7×10^6	D4	1, 5, 5	3.7	3.7×10^6
7	D4	8, 3, 9	6.7	6.7×10^6	D4	2, 0, 2	1.3	1.3×10^6
8	D4	3, 3, 1	2.3	2.3×10^6	D4	4, 3, 3	3.3	3.3×10^6
9	D4	6, 2, 0	2.7	2.7×10^6	D4	12, 6, 8	8.7	8.7×10^6
10	D4	1, 3, 7	3.3	3.3×10^6	D4	7, 4, 8	6.3	6.3×10^6
11	D4	3, 6, 5	4.7	4.7×10^6	D4	6, 7, 8	7	7×10^6
12	D4	6, 11, 3	6.7	6.7×10^6	D4	8, 9, 9	8.7	8.7×10^6
13	D4	5, 7, 6	6	6×10^6	D4	9, 9, 5	7.7	7.7×10^6

Fraction	Fractions of ABS9c-24 hours				Fractions of ABS9c-24 hours			
	Replicate 1				Replicate 2			
	Dilution	Counts	Av.	cfu/mL	Dilution	Counts	Av.	cfu/mL
H_2O	D4	12, 12, 15	13	1.3×10^7	D4	8, 12, 6	8.7	8.7×10^6
100:1 MeOH	D4	3, 12, 6	7	7×10^6	D4	9, 7, 10	8.7	8.7×10^6
1	D5	3, 3, 0	2	2×10^7	D5	2, 1, 0	1	1×10^7
2	D2	8, 11, 6	8.3	8.3×10^4	D2	6, 2, 1	3	3×10^4
3	D3	3, 3, 5	3.7	3.7×10^5	D3	4, 2, 5	3.7	3.7×10^5
4	D4	1, 2, 2	1.7	1.7×10^6	D3	16, 15, 14	15	1.5×10^6
5	D4	10, 3, 9	7.3	7.3×10^6	D5	2, 1, 2	1.7	1.7×10^7
6	D4	2, 2, 0	1.3	1.3×10^6	D4	5, 2, 5	4	4×10^6
7	D0	0, 0, 0	0	0	D2	3, 0, 2	1.7	1.7×10^4
8	D4	6, 5, 6	5.7	5.7×10^6	D4	3, 3, 3	3	3×10^6
9	D4	2, 5, 2	3	3×10^6	D3	26, 25, 20	23.7	2.4×10^6
10	D4	4, 6, 7	5.7	5.7×10^6	D4	12, 6, 10	9.3	9.3×10^6
11	D4	6, 14, 10	10	1×10^7	D4	9, 13, 8	10	1×10^7
12	D4	11, 12, 14	12.3	1.2×10^7	D4	10, 13, 13	12	1.2×10^7
13	D4	12, 6, 14	10.7	1.1×10^7	D4	8, 12, 6	8.7	8.7×10^6

Fractions of ABS13c

Fraction	Fractions of ABS13c-2 hours				Fractions of ABS13c-2 hours			
	Replicate 1				Replicate 2			
	Dilution	Counts	Av.	cfu/mL	Dilution	Counts	Av.	cfu/mL
H_2O	D3	17, 18, 11	15.3	1.5×10^6	D4	11, 8, 12	10.3	1×10^7
100:1 MeOH	D5	3, 0, 3	2	2×10^6	D4	5, 8, 5	6	6×10^6
1	D4	1, 2, 3	2	2×10^6	D3	2, 6, 4	4	4×10^5
2	D4	13, 13, 12	12.7	1.3×10^6	D4	3, 2, 1	2	2×10^6
3	D4	5, 2, 9	5.3	5.3×10^6	D5	1, 1, 1	1	1×10^7
4	D5	1, 3, 1	1.7	1.7×10^7	D4	18, 7, 4	9.7	9.7×10^6
5	D4	3, 2, 1	2	2×10^6	D4	1, 4, 4	3	3×10^6
6	D4	4, 4, 2	3.3	3.3×10^6	D4	11, 10, 7	9.3	9.3×10^6
7	D5	2, 1, 1	1.3	1.3×10^7	D5	7, 8, 5	6.7	6.7×10^7
8	D4	14, 5, 8	9	9×10^6	D4	22, 27, 27	25.3	2.5×10^7
9	D5	1, 2, 1	1.3	1.3×10^7	D5	7, 4, 3	4.7	4.7×10^6
10	D4	4, 8, 4	5.3	5.3×10^6	D4	5, 5, 6	5.3	5.3×10^6
11	D4	3, 3, 3	3	3×10^6	D4	9, 5, 8	7.3	7.3×10^6
12	D4	1, 2, 1	1.3	1.3×10^6	D4	3, 4, 4	3.7	3.7×10^6
13	D4	5, 3, 8	5.3	5.3×10^6	D5	2, 0, 2	1.3	1.3×10^7

Fraction	Fractions of ABS13c-24 hours				Fractions of ABS13c-24 hours			
	Replicate 1				Replicate 2			
	Dilution	Counts	Av.	cfu/mL	Dilution	Counts	Av.	cfu/mL
H_2O	D3	1, 3, 2	2	2×10^7	D4	1, 3, 2	1	1×10^7
100:1 MeOH	D5	3, 1, 3	2.3	2×10^7	D4	3, 1, 3	2.3	2.3×10^7
1	D4	1, 2, 0	1	1×10^7	D3	1, 2, 0	1	1×10^7
2	D4	10, 12, 7	9.7	9.7×10^6	D4	10, 12, 7	9.7	9.7×10^6
3	D4	2, 1, 4	2.3	2.3×10^7	D5	5, 7, 4	5.3	5.3×10^6
4	D5	19, 11, 5	11.7	1.8×10^7	D4	2, 3, 1	2	2×10^6
5	D4	3, 3, 1	2.3	2.3×10^7	D4	4, 3, 8	5	5×10^6
6	D4	0, 4, 4	2.7	2.7×10^7	D4	10, 13, 12	11.7	1.2×10^7
7	D5	1, 5, 2	2.7	2.7×10^7	D5	12, 8, 7	9	9×10^6
8	D4	0, 1, 2	1	1×10^7	D4	1, 5, 1	2.3	2.3×10^7
9	D5	2, 3, 2	2.3	2.3×10^7	D5	10, 4, 11	8.3	8.3×10^6
10	D4	0, 2, 1	1	1×10^7	D4	8, 12, 13	11	1.1×10^7
11	D4	1, 1, 5	2.3	2.3×10^3	D4	15, 7, 8	10	1×10^7
12	D4	2, 4, 1	2.3	2.3×10^2	D4	1, 1, 1	1	1×10^7
13	D4	6, 6, 6	6	6×10^6	D5	3, 3, 3	3	3×10^6

Fractions of ABS14c

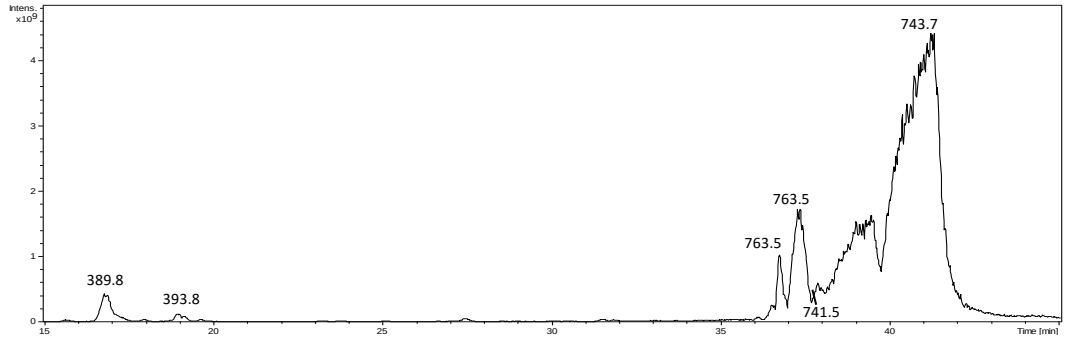
Fraction	Fractions of AB14cc-2 hours				Fractions of ABS14c-2 hours			
	Replicate 1				Replicate 2			
	Dilution	Counts	Av.	cfu/mL	Dilution	Counts	Av.	cfu/mL
H_2O	D5	1, 5, 1	2.3	2.3×10^7	D5	2, 2, 0	1.3	1.3×10^7
100:1 MeOH	D5	2, 1, 0	1	1×10^7	D5	1, 1, 1	1	1×10^7
1	D5	2, 2, 1	1.7	1.7×10^7	D5	2, 4, 1	2.3	2.3×10^7
2	D5	2, 0, 3	1.7	1.7×10^7	D5	1, 1, 2	1.3	1.3×10^7
3	D0	0, 0, 0	0	0	D0	0, 0, 0	0	0
4	D0	0, 0, 0	0	0	D0	0, 0, 0	0	0
5	D0	1, 0, 0	0.3	0.3×10^2	D0	0, 0, 0	0	0
6	D4	9, 9, 10	9.3	9.3×10^6	D4	5, 5, 7	5.7	5.7×10^6
7	D5	2, 2, 2	2	2×10^7	D4	2, 4, 1	2.3	2.3×10^7
8	D5	2, 5, 2	3	3×10^7	D5	1, 2, 2	1.7	1.7×10^7
9	D5	3, 1, 0	1.3	1.3×10^7	D5	1, 0, 1	0.7	0.7×10^7
10	D4	11, 9, 11	10.3	1.1×10^7	D4	13, 13, 18	14.7	1.5×10^7
11	D5	0, 6, 2	2.7	2.7×10^7	D5	4, 1, 3	2.7	2.7×10^7

Fraction	Fractions of AB14cc-2 hours				Fractions of ABS14c-2 hours			
	Replicate 1				Replicate 2			
	Dilution	Counts	Av.	cfu/mL	Dilution	Counts	Av.	cfu/mL
H_2O	D5	3, 2, 0	1.7	1.7×10^7	D5	3, 3, 1	2.3	2.3×10^7
100:1 MeOH	D5	3, 1, 2	1.7	1.7×10^7	D5	1, 1, 1	1	1×10^7
1	D5	2, 1, 3	2	2×10^7	D5	1, 4, 2	2.3	2.3×10^7
2	D5	2, 2, 0	1.3	1.3×10^7	D5	7, 3, 9	6.3	6.3×10^7
3	D0	0, 0, 0	0	0	D0	0, 0, 0	0	0
4	D0	0, 0, 0	0	0	D0	0, 0, 0	0	0
5	D0	0, 0, 0	0	0	D0	0, 0, 0	0	0
6	D4	4, 6, 4	4.7	4.6×10^6	D4	7, 6, 7	6.7	6.7×10^7
7	D4	12, 13, 14	13	1.3×10^7	D5	2, 1, 3	2	2×10^7
8	D5	2, 4, 4	3.3	3.3×10^7	D5	2, 0, 2	1.3	1.3×10^7
9	D4	9, 11, 9	9.7	9.7×10^7	D5	1, 0, 3	1.3	1.3×10^7
10	D4	9, 9, 12	10	1×10^7	D5	1, 2, 0	1	1×10^7
11	D5	2, 2, 0	1.3	1.3×10^7	D5	4, 1, 3	2.7	2.7×10^7

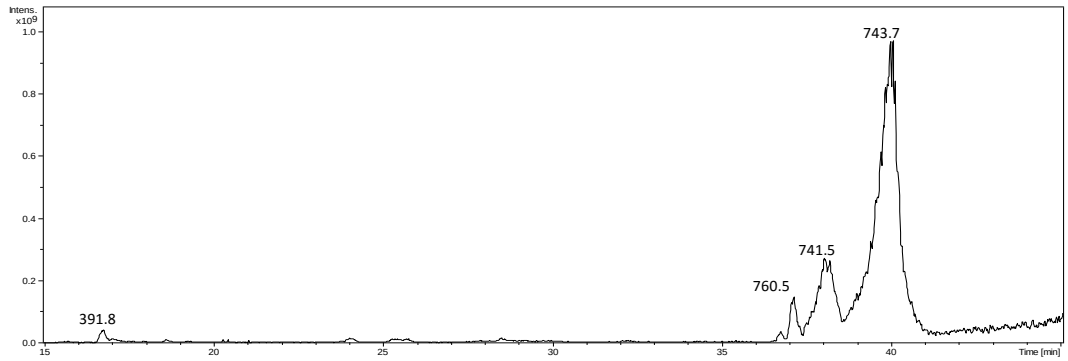
A.8: LC-MS Base Peak Chromatograms of Large Scale Crude Extracts

Ion annotation for Extract 'c' (20 g) of Summer Collected Specimens

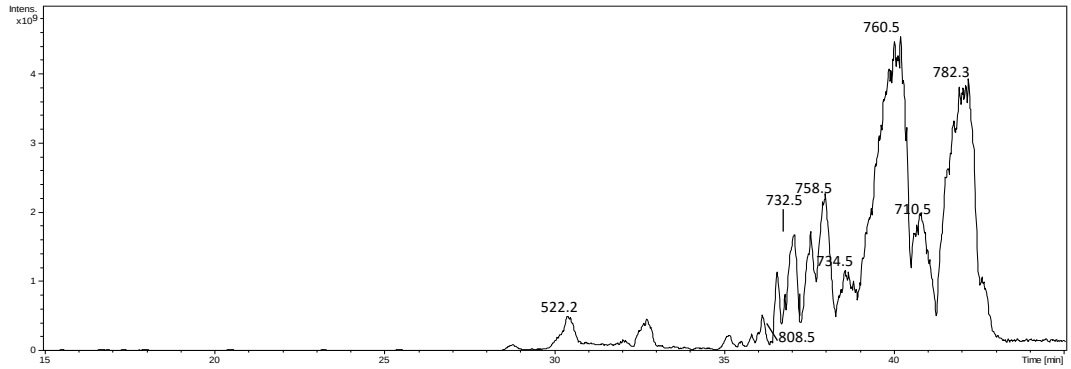
ABS3c



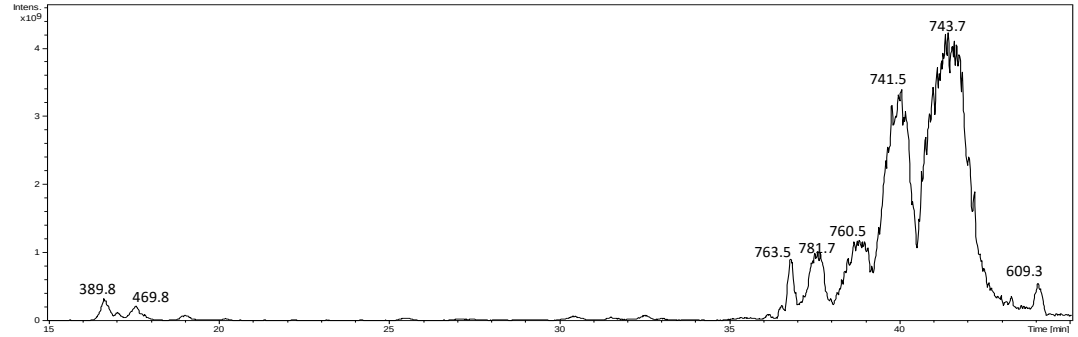
ABS6c



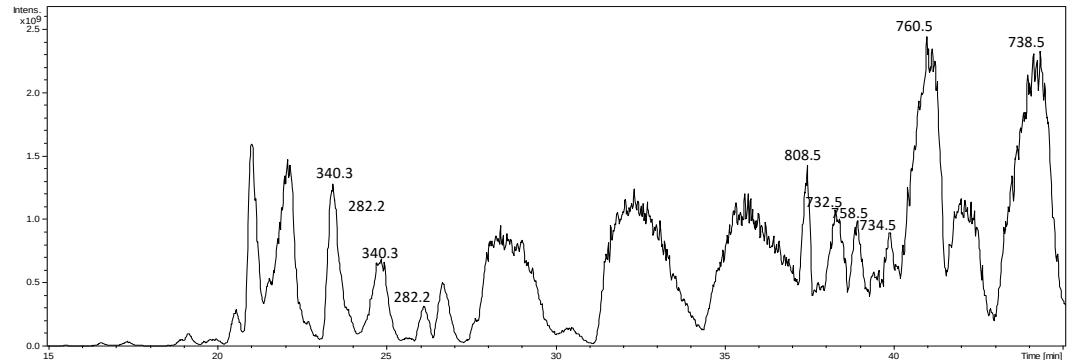
ABS9c



ABS13c



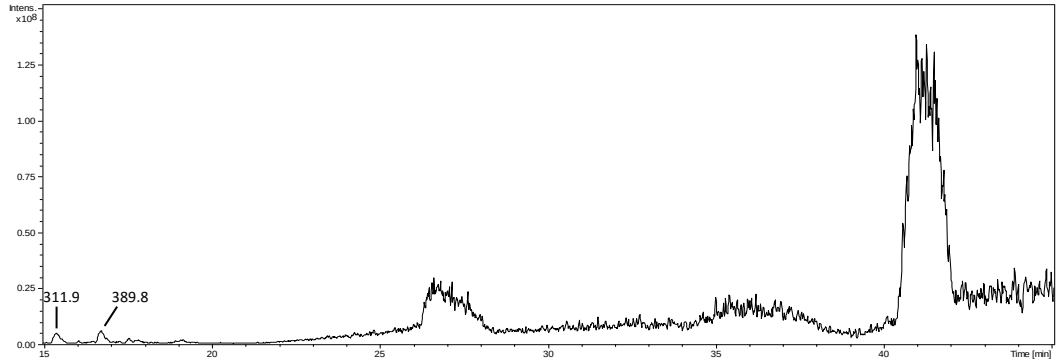
ABS14c



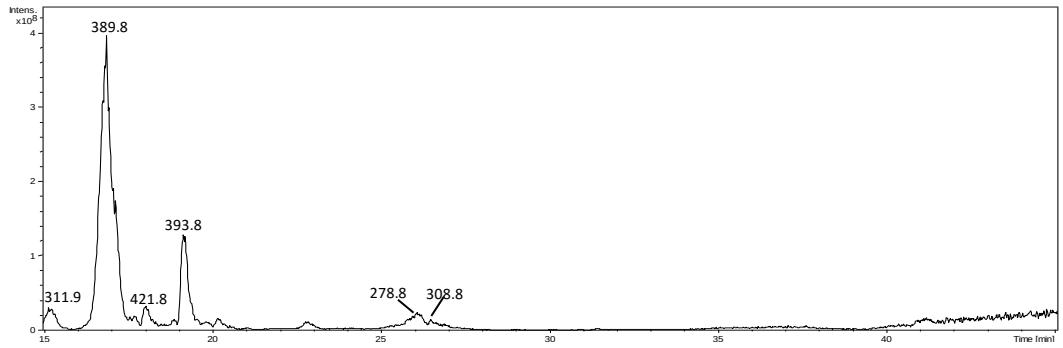
A.9: LC-MS Base Peak Chromatograms of C_{18} fractions of 'Hit' Macroalgae Candidates

Ion Annotation for Fractions of ABS3c

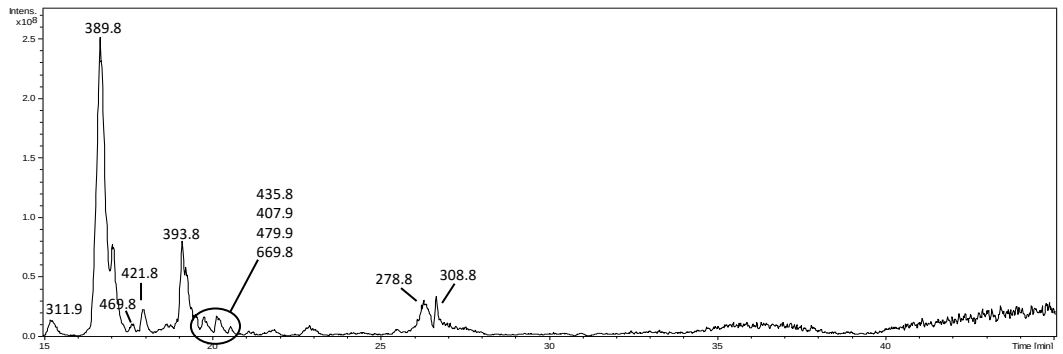
3.2



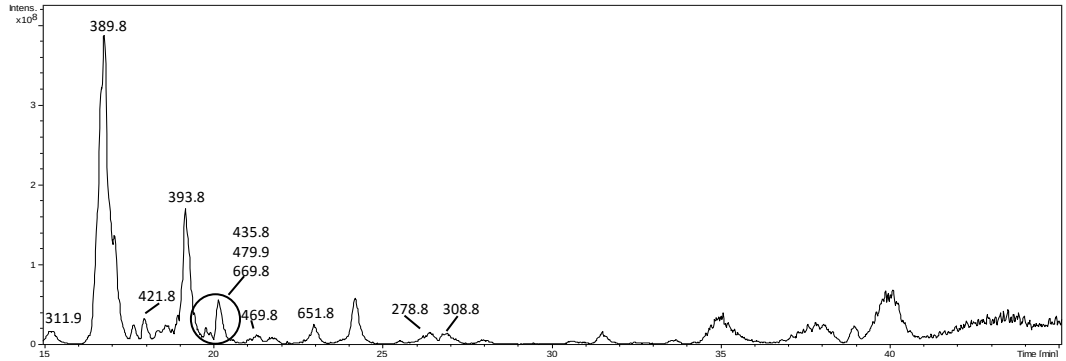
3.3



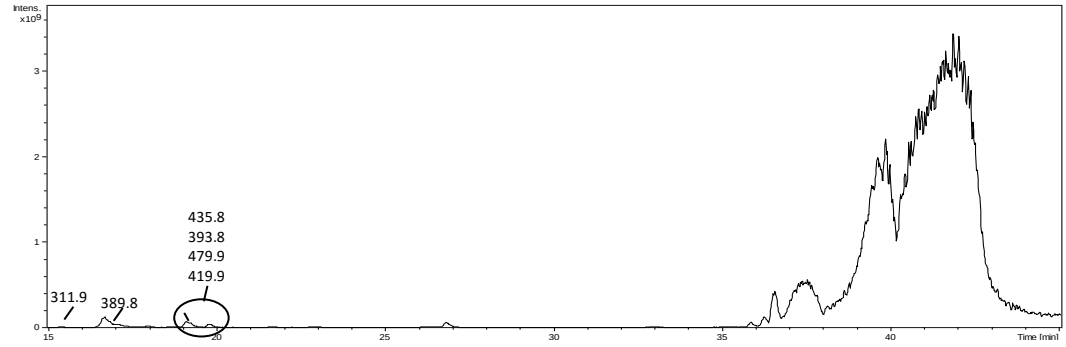
3.4



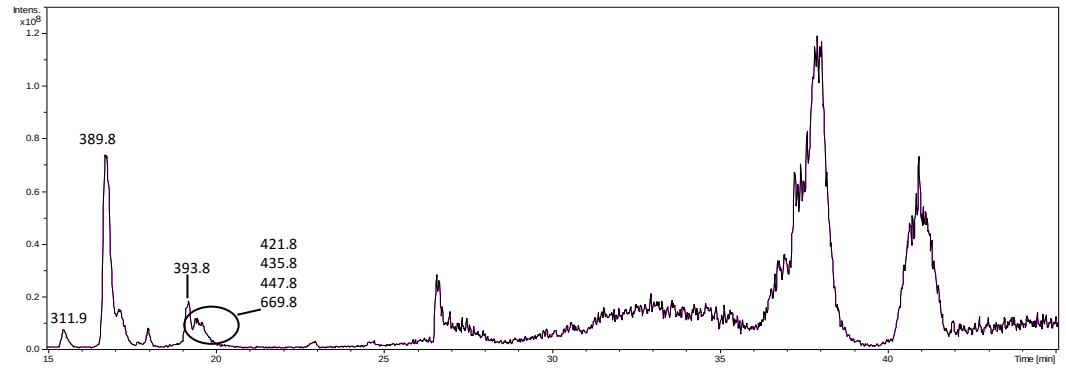
3.5



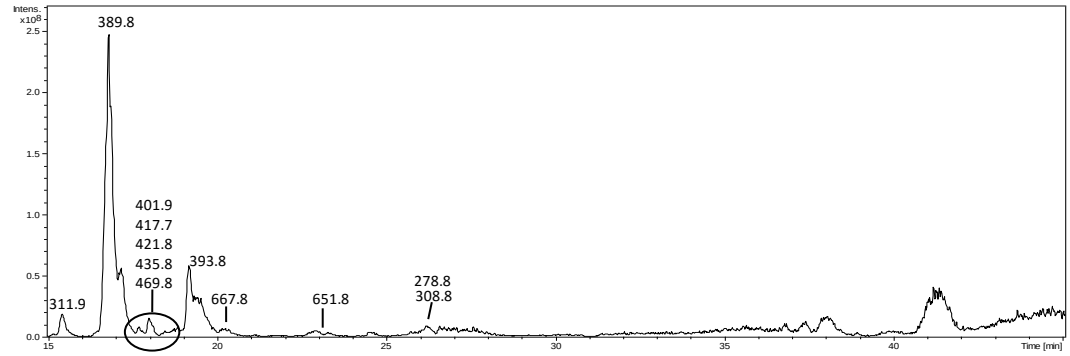
3.6


Intens.
x10^9
3
2
1
0
435.8
393.8
479.9
419.9
311.9
389.8
15
20
25
30
35
40
Time [min]

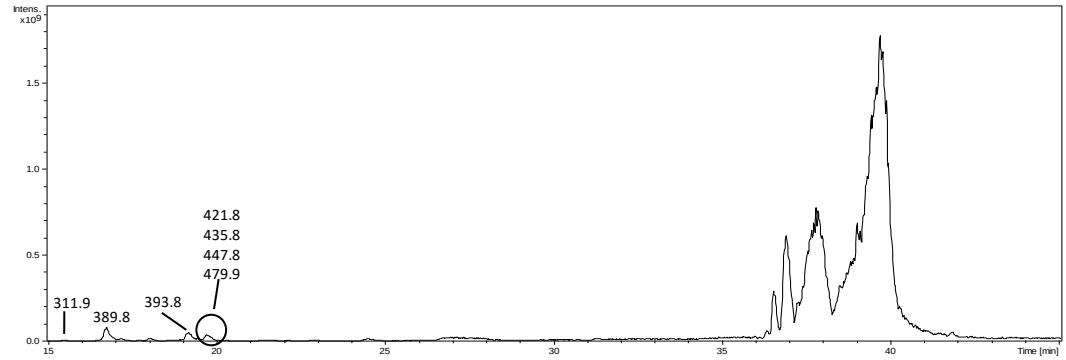
3.9


Intens.
x10^8
1.2
1.0
0.8
0.6
0.4
0.2
0.0
389.8
421.8
435.8
447.8
669.8
393.8
311.9
15
20
25
30
35
40
Time [min]

3.10

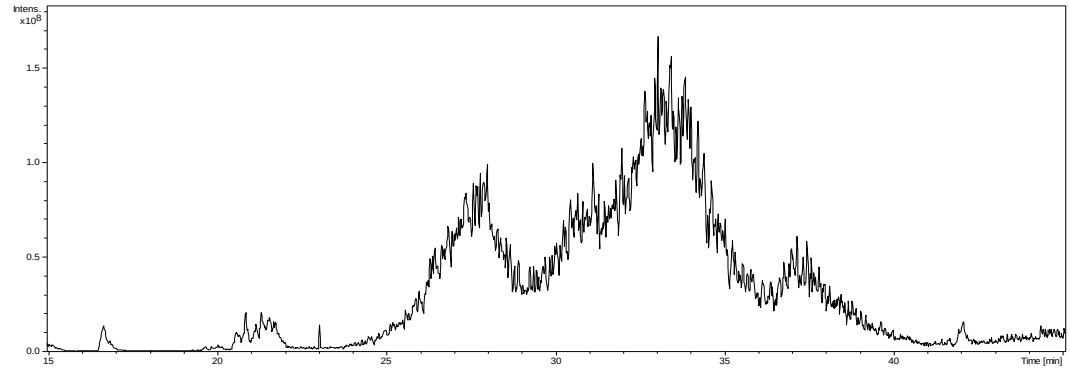

Intens.
x10^8
2.5
2.0
1.5
1.0
0.5
0.0
389.8
401.9
417.7
421.8
435.8
469.8
393.8
667.8
651.8
278.8
308.8
311.9
15
20
25
30
35
40
Time [min]

3.11

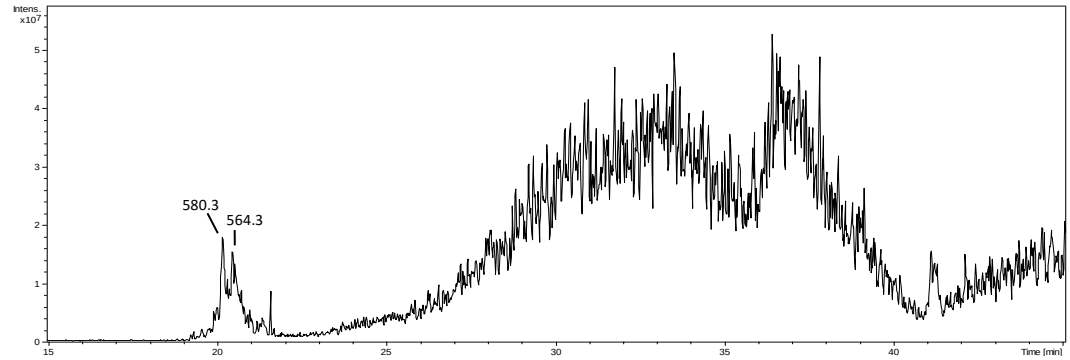

Intens.
x10^9
1.5
1.0
0.5
0.0
421.8
435.8
447.8
479.9
311.9
389.8
393.8
15
20
25
30
35
40
Time [min]

Ion Annotation for Fractions of ABS6c

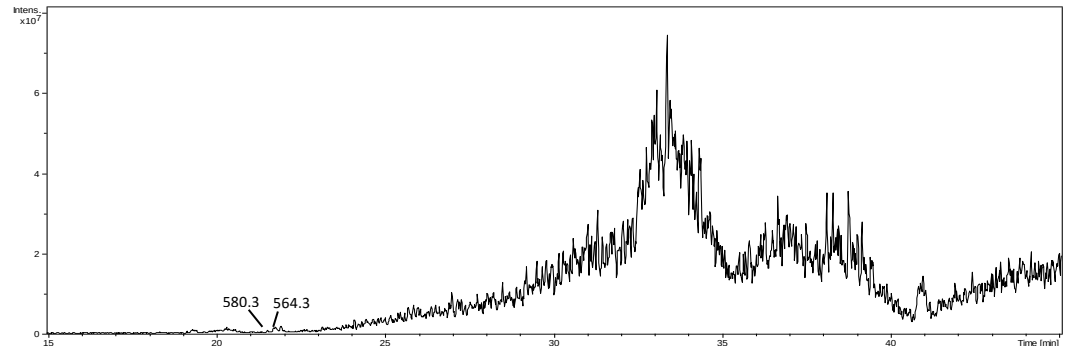
6.2



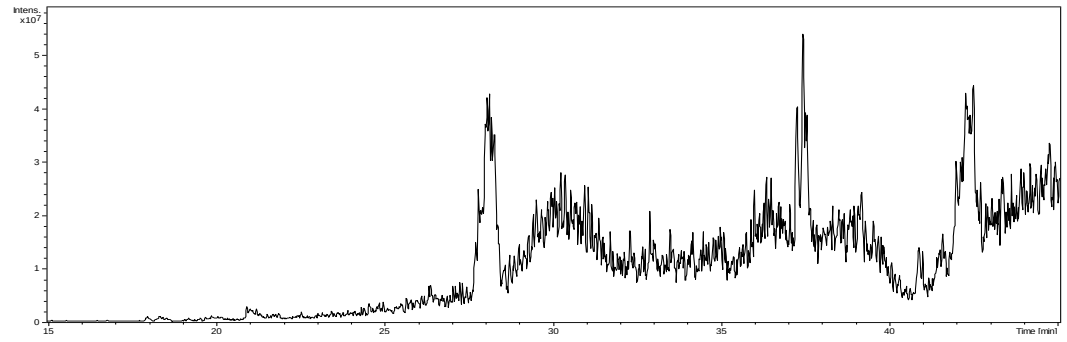
6.3



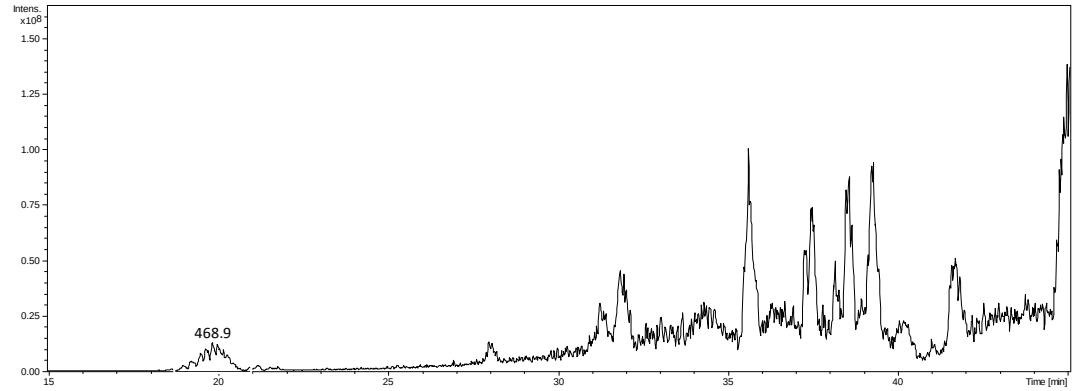
6.4



6.5

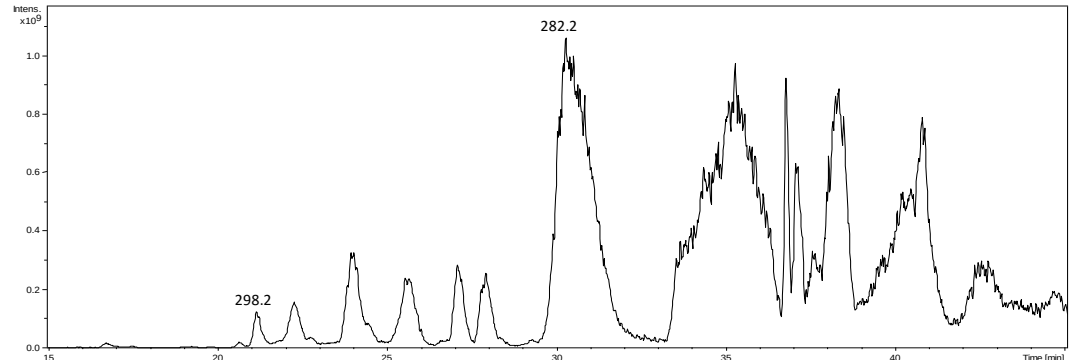


6.6

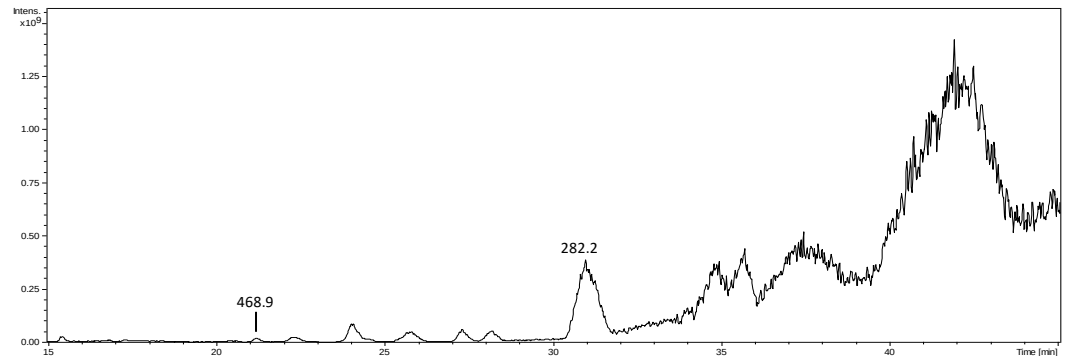


Ion Annotation for Fractions of ABS9c

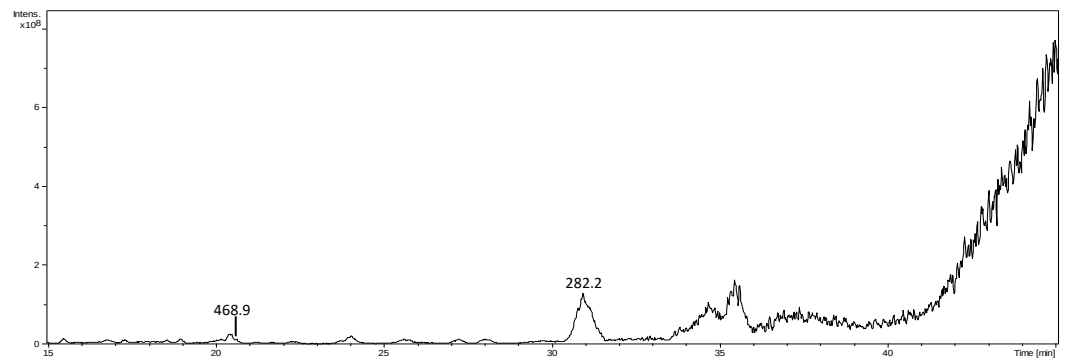
9.1



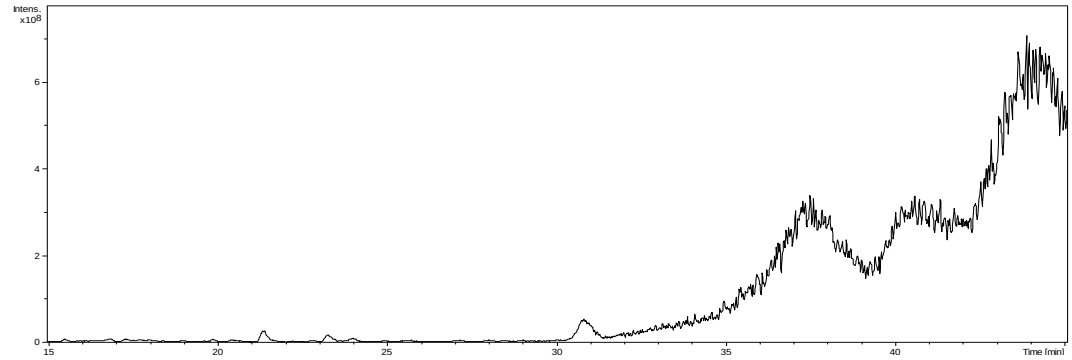
9.2



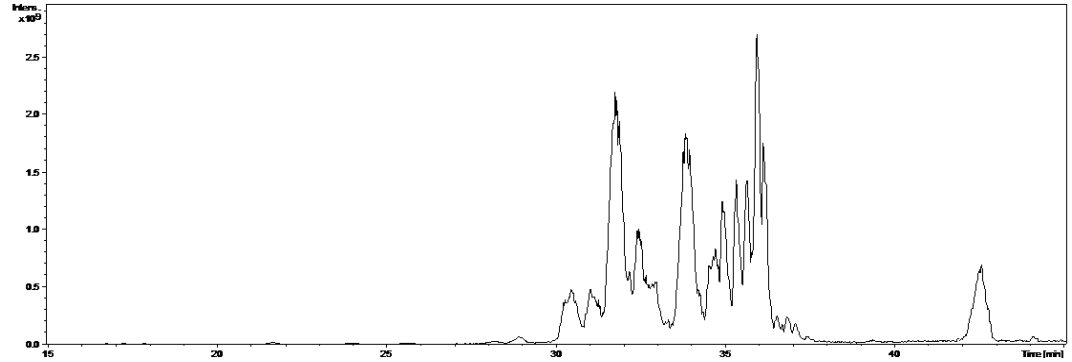
9.3



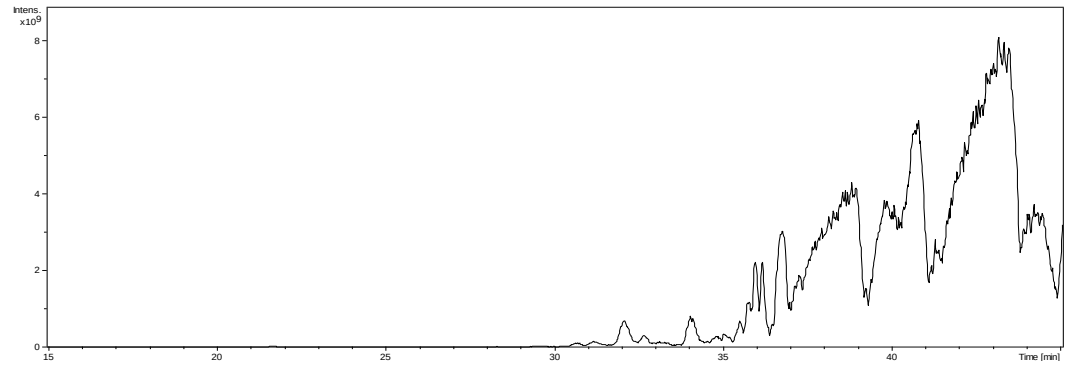
9.4


Intens.
x10^8
6
4
2
0
15
20
25
30
35
40
Time [min]

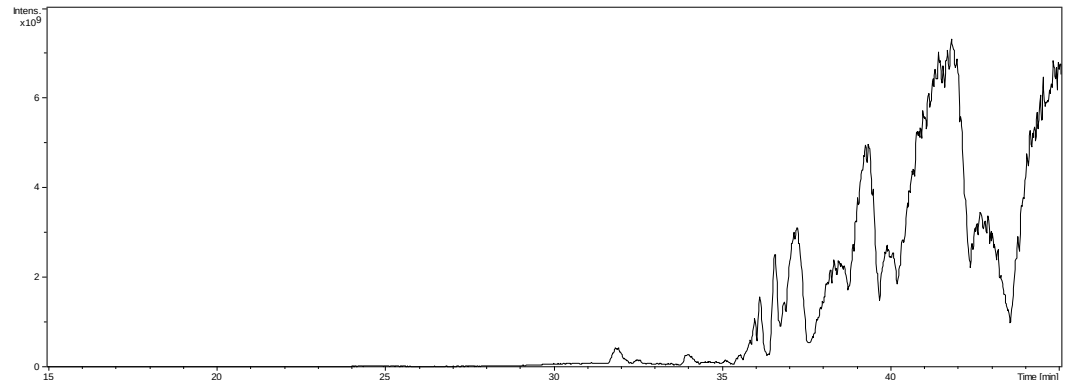
9.6


Intens.
x10^9
2.5
2.0
1.5
1.0
0.5
0.0
15
20
25
30
35
40
Time [min]

9.7

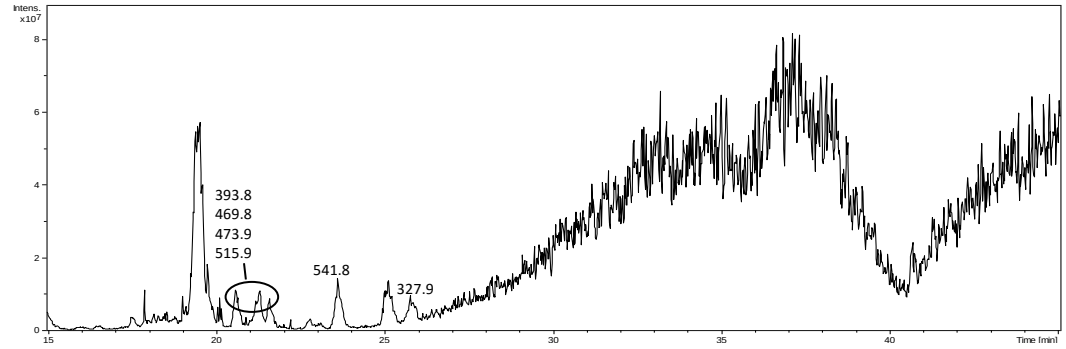

Intens.
x10^9
8
6
4
2
0
15
20
25
30
35
40
Time [min]

9.8

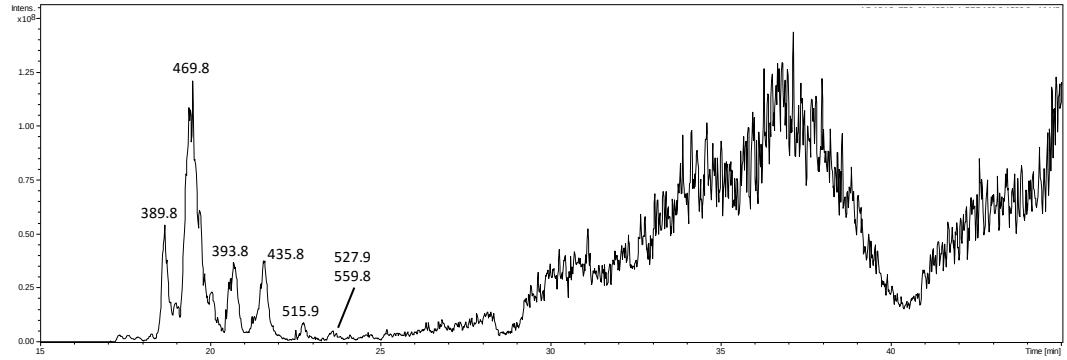

Intens.
x10^9
6
4
2
0
15
20
25
30
35
40
Time [min]

Ion Annotation for Fractions of ABS13c

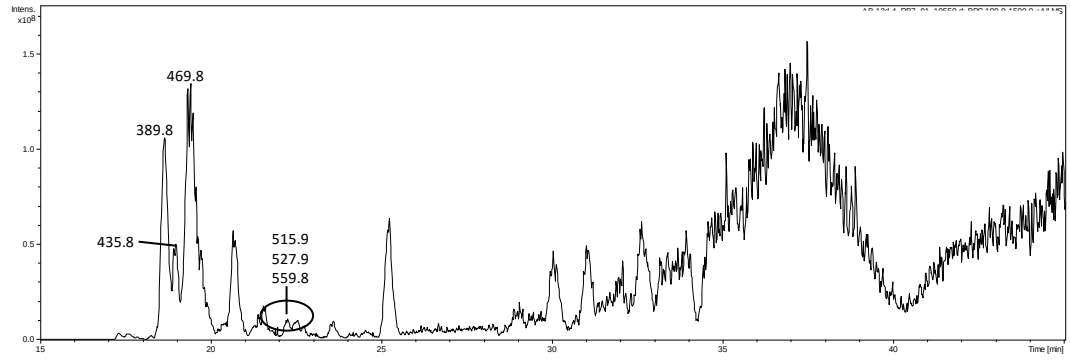
13.2



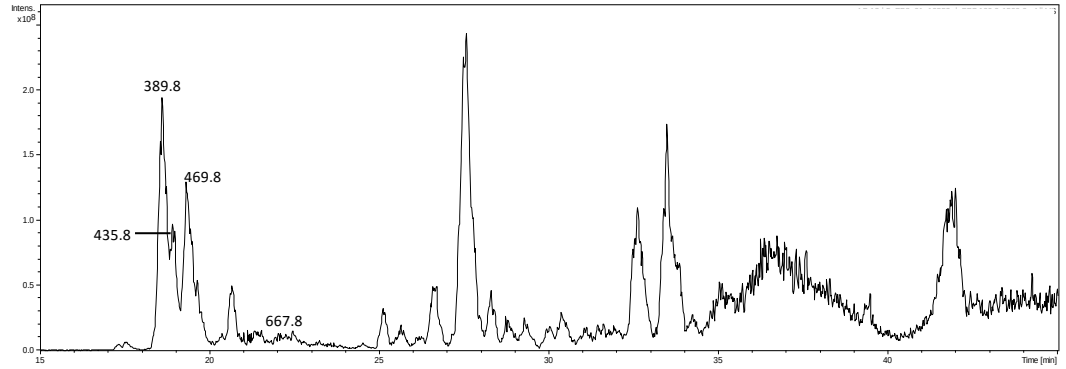
13.3



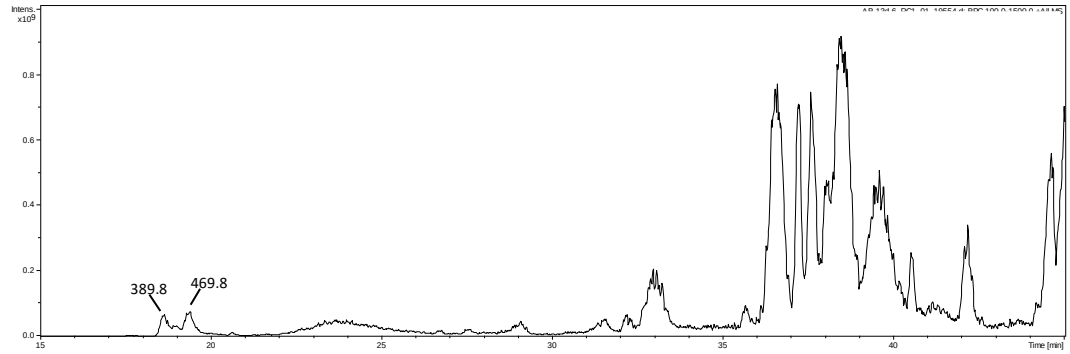
13.4



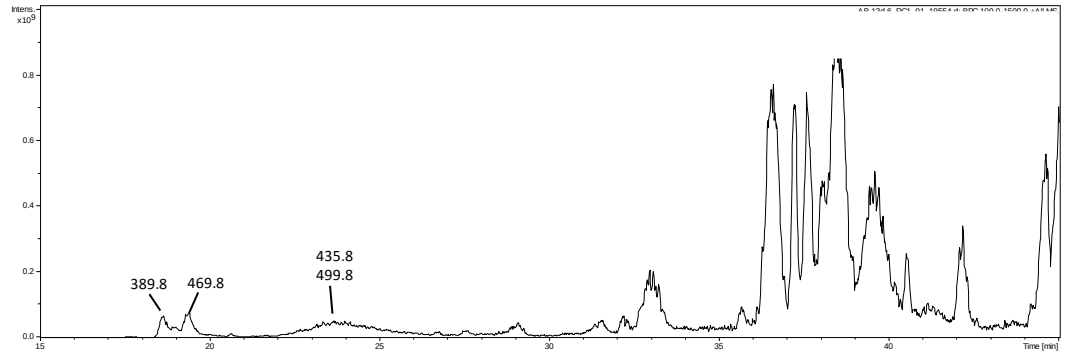
13.5



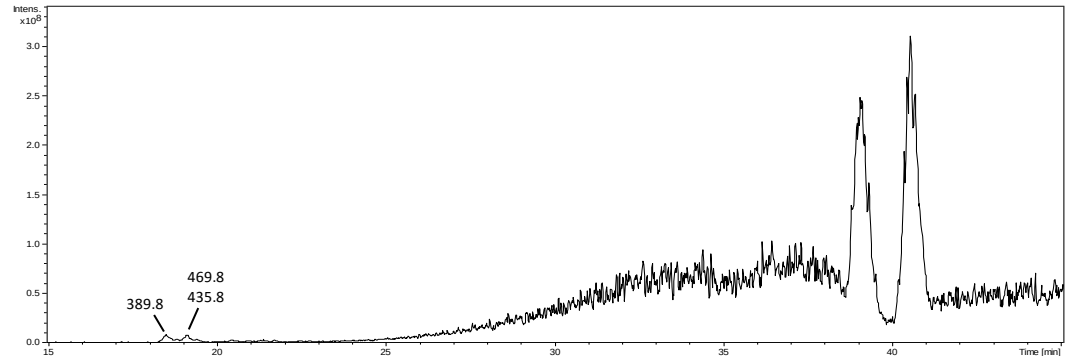
13.6



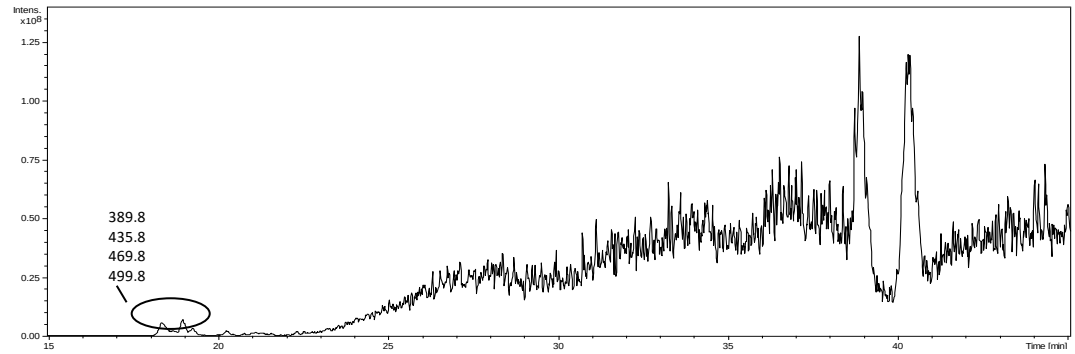
13.9



13.10

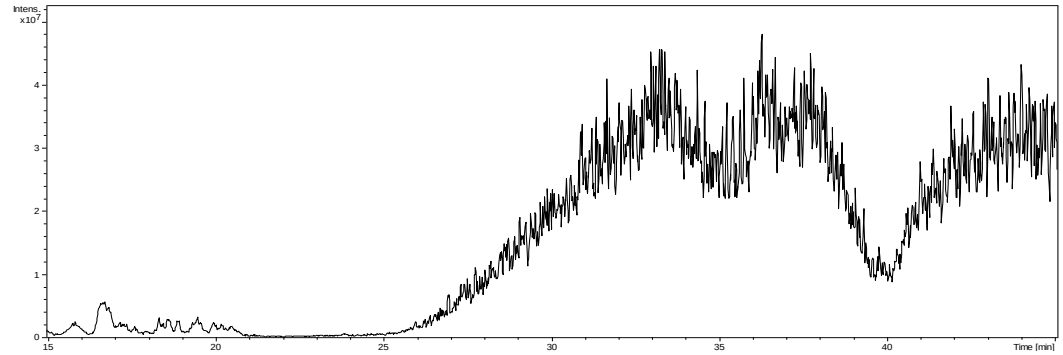


13.11

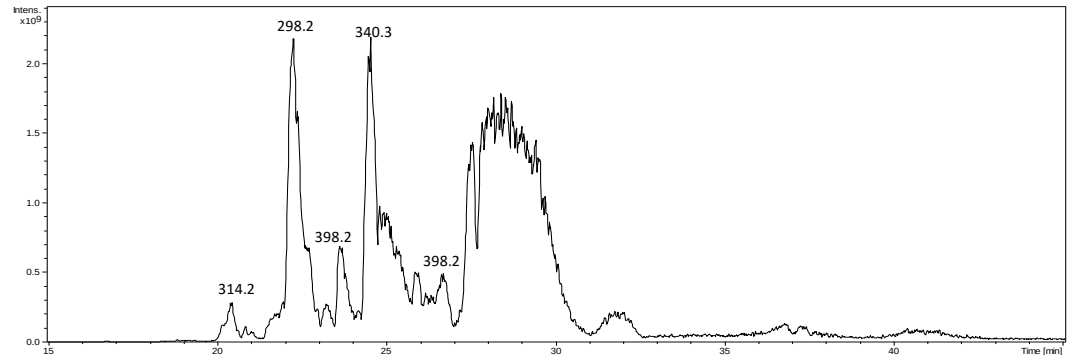


Ion Annotation for Fractions of ABS14c

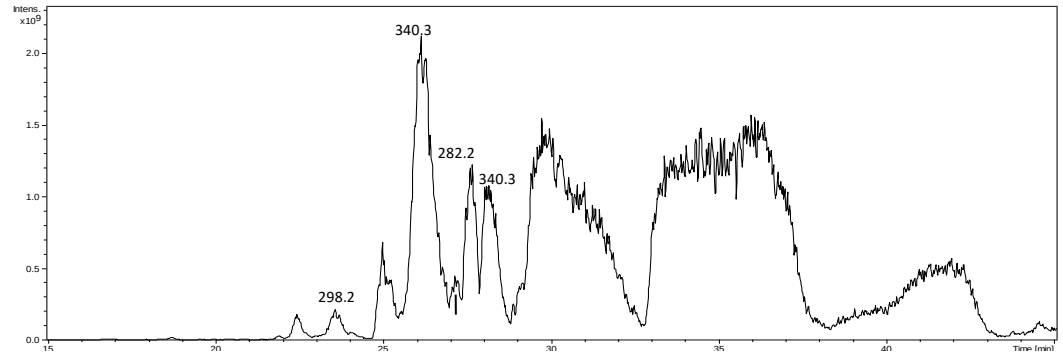
14.2



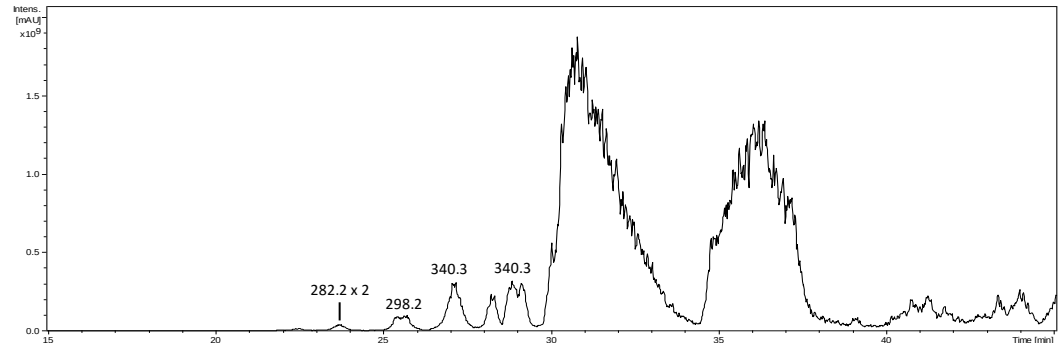
14.3



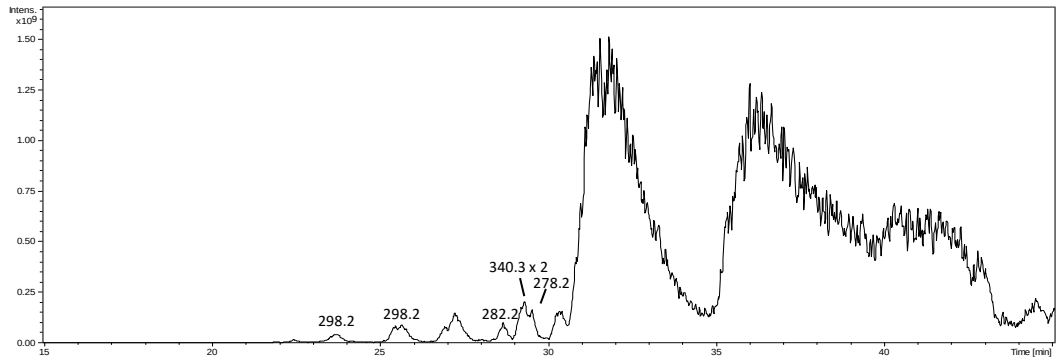
14.4



14.5



14.6


Intens.
x10^9
1.50
1.25
1.00
0.75
0.50
0.25
0.00
15
20
25
30
35
40
Time [min]
298.2
298.2
282.2
340.3 x 2
278.2

A.10: Actigard™ experiment infection scores

Clonal 'Hort16a' infection scores – week three

Treatment	Replicate	Leaf 1	Leaf 2	Leaf 3	Leaf 4	Leaf 5	Leaf 6	Leaf 7	Leaf 8
Non treated, non inoculated	Leaf 1	0	0	0	0	0	0	0	0
	Leaf 2	0	0	0	0	0	0	0	0
	Leaf 3	0	0	0	0	0	0	0	0
	Leaf 4	0	0	0	0	0	0	0	0
	Leaf 5	0	0	0	0	0	0	0	0
	Leaf 6	0	0	0	0	0	0	0	0
H_2O treated, non inoculated	Leaf 1	0	0	0	0	0	0	0	0
	Leaf 2	0	0	0	0	0	0	0	0
	Leaf 3	0	0	0	0	0	0	0	0
	Leaf 4	0	0	0	0	0	0	0	0
	Leaf 5	0	0	0	0	0	0	0	0
	Leaf 6	0	0	0	0	0	0	0	0
H_2O treated, inoculated	Leaf 1	0	0	0	0	0	0	0	0
	Leaf 2	0	0	0	0	0	0	0	0
	Leaf 3	0	0	1	1	1	1	0	0
	Leaf 4	0	0	0	0	0	0	0	0
	Leaf 5	0	0	0	0	0	0	0	0
	Leaf 6	0	0	0	1	1	1	1	0

Treatment	Replicate	Leaf 1	Leaf 2	Leaf 3	Leaf 4	Leaf 5	Leaf 6	Leaf 7	Leaf 8
Full strength Actigard, whole plant applied	Leaf 1	0	0	0	0	0	0	1	1
	Leaf 2	0	0	0	0	0	1	1	0
	Leaf 3	0	0	1	1	0	1	0	0
	Leaf 4	0	0	0	0	0	0	0	0
	Leaf 5	0	0	0	0	0	0	0	0
	Leaf 6	0	0	0	0	1	0	0	0
Full strength Actigard, single leaf applied	Leaf 1	0	0	0	0	0	0	0	0
	Leaf 2	0	0	0	0	0	0	0	0
	Leaf 3	0	0	0	0	0	0	0	0
	Leaf 4	0	0	0	0	0	0	0	0
	Leaf 5	0	0	0	0	0	0	0	0
	Leaf 6	0	0	0	0	0	0	0	0
1/2 strength Actigard, whole plant applied	Leaf 1	0	0	0	2	2	1	2	1
	Leaf 2	0	0	0	0	0	0	0	0
	Leaf 3	0	0	1	0	1	1	1	0
	Leaf 4	0	0	0	0	0	1	0	1
	Leaf 5	0	0	0	0	0	0	0	0
	Leaf 6	0	0	0	0	0	0	1	1
1/2 strength Actigard, single leaf applied	Leaf 1	0	0	0	1	0	2	2	1
	Leaf 2	0	0	0	0	1	1	0	0
	Leaf 3	0	0	0	2	2	0	1	0
	Leaf 4	0	0	1	0	2	0	0	0
	Leaf 5	0	1	0	0	0	0	0	0
	Leaf 6	0	0	0	0	0	0	0	0

Treatment	Replicate	Leaf 1	Leaf 2	Leaf 3	Leaf 4	Leaf 5	Leaf 6	Leaf 7	Leaf 8
1/4 strength Actigard, whole plant applied	Leaf 1	0	0	0	0	0	1	1	1
	Leaf 2	0	0	0	0	1	0	1	0
	Leaf 3	0	0	0	0	0	0	0	0
	Leaf 4	1	0	0	1	1	1	1	1
	Leaf 5	0	1	0	0	0	0	1	0
	Leaf 6	0	0	0	0	0	1	0	0
1/4 strength Actigard, single leaf applied	Leaf 1	1	0	0	1	0	0	0	1
	Leaf 2	0	0	0	0	0	1	1	1
	Leaf 3	0	0	0	0	1	1	0	0
	Leaf 4	0	0	0	0	0	1	1	1
	Leaf 5	0	0	0	0	1	1	0	0
	Leaf 6	0	1	0	0	1	1	1	1
1/8 strength Actigard, whole plant applied	Leaf 1	1	0	0	0	1	1	1	1
	Leaf 2	0	0	0	1	0	1	0	0
	Leaf 3	0	0	0	0	1	2	2	1
	Leaf 4	0	0	1	1	1	0	0	0
	Leaf 5	0	0	0	0	0	0	1	1
	Leaf 6	0	0	0	1	0	1	1	0
1/8 strength Actigard, single leaf applied	Leaf 1	0	0	1	0	0	0	1	0
	Leaf 2	0	2	1	0	1	2	1	2
	Leaf 3	1	1	1	0	0	1	1	1
	Leaf 4	0	0	0	0	0	1	0	1
	Leaf 5	0	0	0	1	1	0	1	0
	Leaf 6	0	0	0	0	0	1	1	2

Treatment	Replicate	Leaf 1	Leaf 2	Leaf 3	Leaf 4	Leaf 5	Leaf 6	Leaf 7	Leaf 8
1/16 strength Actigard, whole plant applied	Leaf 1	1	1	1	0	0	2	0	1
	Leaf 2	0	0	1	0	0	2	0	0
	Leaf 3	0	0	1	3	3	2	2	3
	Leaf 4	1	1	0.5	1	1	2	2	1
	Leaf 5	1	0	0	1	2	2	1	1
	Leaf 6	2	1	0	0	2	2	0	1
1/16 strength Actigard, single leaf applied	Leaf 1	0	1	0	1	0	0	2	1
	Leaf 2	0	0	0	0	0	1	1	1
	Leaf 3	0	0	0	0	0	1	0	2
	Leaf 4	0	1	2	1	0	0	0	0
	Leaf 5	0	0	0	0	0	0	0	1
	Leaf 6	1	2	2	2	0	0	1	0

Non-clonal ‘Hort16a’ infection scores – week one

Treatment	Replicate	Leaf 1	Leaf 2	Leaf 3	Leaf 4	Leaf 5	Leaf 6	Leaf 7	Leaf 8
Non treated, non inoculated	Leaf 1	0	0	0	0	0	0	0	0
	Leaf 2	0	0	0	0	0	0	0	0
	Leaf 3	0	0	0	0	0	0	0	0
	Leaf 4	0	0	0	0	0	0	0	0
	Leaf 5	0	0	0	0	0	0	0	0
	Leaf 6	0	0	0	0	0	0	0	0
H_2O treated, non inoculated	Leaf 1	0	0	0	0	0	0	0	0
	Leaf 2	0	0	0	0	0	0	0	0
	Leaf 3	0	0	0	0	0	0	0	0
	Leaf 4	0	0	0	0	0	0	0	0
	Leaf 5	0	0	0	0	0	0	0	0
	Leaf 6	0	0	0	0	0	0	0	0
H_2O treated, inoculated	Leaf 1	0	2	2	2	3	2	0	0
	Leaf 2	2	1	2	3	3	2	3	0
	Leaf 3	3	2	2	0	4	3	3	2
	Leaf 4	1	2	4	4	1	3	2	4
	Leaf 5	3	2	2	2	2	2	1	2
	Leaf 6	4	2	3	3	4	0	4	0
Full strength Actigard, whole plant applied	Leaf 1	1	1	1	2	1	1	1	0
	Leaf 2	1	0	0	1	2	1	0	0
	Leaf 3	0	1	0	1	1	1	1	0
	Leaf 4	1	1	1	2	1	1	1	0
	Leaf 5	1	0	0	1	2	1	0	0
	Leaf 6	1	1	0	1	1	1	0	0

Treatment	Replicate	Leaf 1	Leaf 2	Leaf 3	Leaf 4	Leaf 5	Leaf 6	Leaf 7	Leaf 8
Full strength Actigard, single leaf applied	Leaf 1	0	0	2	0	2	2	2	0
	Leaf 2	1	1	1	1	1	0	0	0
	Leaf 3	0	1	0	0	1	0	0	0
	Leaf 4	1	1	0	0	0	1	1	1
	Leaf 5	1	1	1	2	0	0	2	1
	Leaf 6	0	0	0	0	0	0	0	1
1/2 strength Actigard, whole plant applied	Leaf 1	2	2	1	2	1	0	0	0
	Leaf 2	0	0	0	1	1	1	1	0
	Leaf 3	0	2	0	2	1	0	0	0
	Leaf 4	0	1	0	1	1	1	1	1
	Leaf 5	1	0	0	1	1	0	1	0
	Leaf 6	1	1	0	1	2	1	2	0
1/2 strength Actigard, single leaf applied	Leaf 1	1	2	0	0	1	1	2	1
	Leaf 2	3	1	1	0	0	1	1	0
	Leaf 3	1	1	2	2	1	0	1	0
	Leaf 4	1	1	2	0	0	2	1	0
	Leaf 5	2	0	0	0	1	1	2	2
	Leaf 6	3	1	2	0	2	1	1	0
1/4 strength Actigard, whole plant applied	Leaf 1	0	1	1	1	1	2	0	0
	Leaf 2	2	1	1	2	1	0	0	1
	Leaf 3	1	2	1	1	2	1	1	0
	Leaf 4	2	1	3	1	3	0	0	1
	Leaf 5	0	0	2	2	1	1	0	1
	Leaf 6	1	1	1	1	1	1	1	0

Treatment	Replicate	Leaf 1	Leaf 2	Leaf 3	Leaf 4	Leaf 5	Leaf 6	Leaf 7	Leaf 8
1/4 strength Actigard, single leaf applied	Leaf 1	0	1	1	0	1	1	1	1
	Leaf 2	2	2	1	2	1	1	0	0
	Leaf 3	1	1	1	2	2	1	1	1
	Leaf 4	1	0	0	1	1	1	1	2
	Leaf 5	3	2	1	1	1	3	1	1
	Leaf 6	4	1	3	1	0	2	0	0
1/8 strength Actigard, whole plant applied	Leaf 1	2	1	2	1	2	1	0	0
	Leaf 2	3	3	3	0	3	2	2	0
	Leaf 3	0	1	2	2	1	2	0	0
	Leaf 4	1	3	2	1	2	1	1	1
	Leaf 5	1	0	2	1	1	1	0	0
	Leaf 6	1	1	1	1	2	1	1	1
1/8 strength Actigard, single leaf applied	Leaf 1	1	1	2	3	3	2	1	0
	Leaf 2	3	2	3	3	3	1	0	2
	Leaf 3	2	3	3	1	3	2	3	1
	Leaf 4	2	0	0	3	3	2	2	0
	Leaf 5	4	2	3	3	2	1	1	0
	Leaf 6	2	2	3	2	1	0	0	0
1/16 strength Actigard, whole plant applied	Leaf 1	2	0	1	1	2	1	2	1
	Leaf 2	1	2	3	1	0	0	0	0
	Leaf 3	0	1	1	2	2	1	1	1
	Leaf 4	2	3	1	1	1	1	2	1
	Leaf 5	2	1	1	2	2	1	1	0
	Leaf 6	0	0	3	2	2	2	2	2

Treatment	Replicate	Leaf 1	Leaf 2	Leaf 3	Leaf 4	Leaf 5	Leaf 6	Leaf 7	Leaf 8
1/16 strength Actigard, single leaf applied	Leaf 1	1	1	2	4	3	3	0	0
	Leaf 2	3	0	2	2	3	1	0	1
	Leaf 3	0	1	2	0	1	1	3	1
	Leaf 4	0	0	1	1	2	2	1	0
	Leaf 5	0	2	1	1	3	2	1	0
	Leaf 6	0	1	0	1	2	1	2	0

Non-clonal 'Hort16a' infection scores – week two

Treatment	Replicate	Leaf 1	Leaf 2	Leaf 3	Leaf 4	Leaf 5	Leaf 6	Leaf 7	Leaf 8
Non treated, non inoculated	Leaf 1	0	0	0	0	0	0	0	0
	Leaf 2	0	0	0	0	0	0	0	0
	Leaf 3	0	0	0	0	0	0	0	0
	Leaf 4	0	0	0	0	0	0	0	0
	Leaf 5	0	0	0	0	0	0	0	0
	Leaf 6	0	0	0	0	0	0	0	0
H_2O treated, non inoculated	Leaf 1	0	0	0	0	0	0	0	0
	Leaf 2	0	0	0	0	0	0	0	0
	Leaf 3	0	0	0	0	0	0	0	0
	Leaf 4	0	0	0	0	0	0	0	0
	Leaf 5	0	0	0	0	0	0	0	0
	Leaf 6	0	0	0	0	0	0	0	0

Treatment	Replicate	Leaf 1	Leaf 2	Leaf 3	Leaf 4	Leaf 5	Leaf 6	Leaf 7	Leaf 8
H_2O treated, inoculated	Leaf 1	5	4	5	4	4	5	2	4
	Leaf 2	3	3	3	5	2	3	4	4
	Leaf 3	5	2	4	4	3	3	3	4
	Leaf 4	5	5	4	5	3	1	0	0
	Leaf 5	4	4	3	4	4	1	1	0
	Leaf 6	5	5	4	4	3	2	2	2
Full strength Actigard, whole plant applied	Leaf 1	3	3	5	3	2	2	0	0
	Leaf 2	1	0	0	0	0	3	0	0
	Leaf 3	3	2	4	3	4	2	2	0
	Leaf 4	3	3	4	2	2	2	1	0
	Leaf 5	3	2	2	2	3	3	1	1
	Leaf 6	3	1	1	1	1	0	0	0
Full strength Actigard, single leaf applied	Leaf 1	3	3	0	0	1	3	0	0
	Leaf 2	2	1	1	3	0	0	0	0
	Leaf 3	1	1	0	0	1	1	0	0
	Leaf 4	3	2	0	1	1	2	1	1
	Leaf 5	3	1	1	1	3	2	2	1
	Leaf 6	1	1	1	1	1	1	0	1
1/2 strength Actigard, whole plant applied	Leaf 1	1	1	2	2	1	0	0	0
	Leaf 2	2	1	1	1	1	1	1	0
	Leaf 3	1	1	2	2	2	1	1	0
	Leaf 4	1	1	1	1	1	1	0	1
	Leaf 5	1	1	1	1	1	1	0	0
	Leaf 6	0	1	0	1	1	2	1	2

Treatment	Replicate	Leaf 1	Leaf 2	Leaf 3	Leaf 4	Leaf 5	Leaf 6	Leaf 7	Leaf 8
1/2 strength Actigard, single leaf applied	Leaf 1	3	3	3	3	1	2	1	0
	Leaf 2	5	2	2	3	4	2	2	0
	Leaf 3	4	4	4	3	3	1	2	1
	Leaf 4	5	4	3	4	4	4	2	1
	Leaf 5	3	3	4	3	2	3	3	3
	Leaf 6	5	5	3	2	2	0	0	0
1/4 strength Actigard, whole plant applied	Leaf 1	2	2	3	2	1	0	1	0
	Leaf 2	3	2	2	1	1	2	3	2
	Leaf 3	2	2	2	2	2	3	1	0
	Leaf 4	3	2	1	0	4	2	0	1
	Leaf 5	2	3	2	1	0	1	0	1
	Leaf 6	3	3	2	2	2	2	3	3
1/4 strength Actigard, single leaf applied	Leaf 1	4	4	2	1	2	2	0	0
	Leaf 2	2	3	3	4	3	3	1	0
	Leaf 3	3	3	3	3	4	2	2	3
	Leaf 4	3	3	4	3	3	2	2	2
	Leaf 5	4	4	2	5	1	0	0	0
	Leaf 6	5	4	3	4	3	3	0	0
1/8 strength Actigard, whole plant applied	Leaf 1	3	2	2	3	3	4	1	1
	Leaf 2	4	4	3	3	3	2	1	0
	Leaf 3	2	3	3	4	4	3	1	0
	Leaf 4	1	4	4	4	3	3	2	1
	Leaf 5	5	5	5	4	3	2	1	1
	Leaf 6	3	2	2	2	3	0	2	0

Treatment	Replicate	Leaf 1	Leaf 2	Leaf 3	Leaf 4	Leaf 5	Leaf 6	Leaf 7	Leaf 8
1/8 strength Actigard, single leaf applied	Leaf 1	4	4	4	5	3	3	4	4
	Leaf 2	5	4	4	4	4	1	0	0
	Leaf 3	5	5	3	3	4	2	3	2
	Leaf 4	3	2	2	3	3	2	4	0
	Leaf 5	5	5	4	5	1	1	0	0
	Leaf 6	4	2	4	5	5	3	1	0
1/16 strength Actigard, whole plant applied	Leaf 1	4	5	3	3	2	2	1	1
	Leaf 2	5	5	5	5	4	3	3	3
	Leaf 3	3	5	5	0	3	3	3	3
	Leaf 4	2	3	1	4	3	2	1	0
	Leaf 5	5	5	5	4	3	4	4	1
	Leaf 6	3	3	3	5	3	3	3	0
1/16 strength Actigard, single leaf applied	Leaf 1	3	5	4	3	5	4	1	4
	Leaf 2	5	4	2	5	4	2	2	3
	Leaf 3	3	3	3	2	3	4	2	1
	Leaf 4	2	3	3	3	4	3	2	1
	Leaf 5	3	5	3	3	5	4	4	4
	Leaf 6	5	5	3	5	4	5	5	3

Non-clonal ‘Hort16a’ infection scores – week three

Treatment	Replicate	Leaf 1	Leaf 2	Leaf 3	Leaf 4	Leaf 5	Leaf 6	Leaf 7	Leaf 8
Non treated, non inoculated	Leaf 1	0	0	0	0	0	0	0	0
	Leaf 2	0	0	0	0	0	0	0	0
	Leaf 3	0	0	0	0	0	0	0	0
	Leaf 4	0	0	0	0	0	0	0	0
	Leaf 5	0	0	0	0	0	0	0	0
	Leaf 6	0	0	0	0	0	0	0	0
H_2O treated, non inoculated	Leaf 1	0	0	0	0	0	0	0	0
	Leaf 2	0	0	0	0	0	0	0	0
	Leaf 3	0	0	0	0	0	0	0	0
	Leaf 4	0	0	0	0	0	0	0	0
	Leaf 5	0	0	0	0	0	0	0	0
	Leaf 6	0	0	0	0	0	0	0	0
H_2O treated, inoculated	Leaf 1	5	5	4	5	2	0	0	0
	Leaf 2	3	4	3	2	4	4	1	1
	Leaf 3	4	3	4	5	5	4	4	3
	Leaf 4	5	5	4	5	3	1	0	0
	Leaf 5	5	3	4	3	4	3	1	1
	Leaf 6	5	5	3	3	0	5	5	0
Full strength Actigard, whole plant applied	Leaf 1	5	5	5	4	5	5	3	3
	Leaf 2	4	0	2	1	0	0	0	0
	Leaf 3	5	0	1	4	0	0	0	0
	Leaf 4	2	2	2	4	2	0	0	0
	Leaf 5	4	4	3	3	3	4	5	5
	Leaf 6	5	5	4	3	2	1	1	1

Treatment	Replicate	Leaf 1	Leaf 2	Leaf 3	Leaf 4	Leaf 5	Leaf 6	Leaf 7	Leaf 8
Full strength Actigard, single leaf applied	Leaf 1	1	1	4	4	4	0	0	0
	Leaf 2	4	4	2	2	0	0	0	0
	Leaf 3	0	0	2	1	0	0	0	0
	Leaf 4	2	5	4	4	3	3	0	0
	Leaf 5	3	3	2	4	4	4	3	1
	Leaf 6	1	0	1	0	0	0	0	0
1/2 strength Actigard, whole plant applied	Leaf 1	3	3	2	0	1	1	1	1
	Leaf 2	3	1	0	0	0	0	1	0
	Leaf 3	2	2	3	1	2	1	0	0
	Leaf 4	4	4	4	3	3	2	1	1
	Leaf 5	3	3	0	1	1	0	0	0
	Leaf 6	2	2	2	3	0	0	0	0
1/2 strength Actigard, single leaf applied	Leaf 1	5	5	4	3	3	2	2	1
	Leaf 2	5	5	5	3	2	1	1	0
	Leaf 3	5	5	3	3	4	3	0	0
	Leaf 4	5	5	5	5	4	3	0	0
	Leaf 5	4	3	3	3	5	4	0	1
	Leaf 6	5	5	3	5	3	5	5	0
1/4 strength Actigard, whole plant applied	Leaf 1	4	4	3	3	2	1	1	0
	Leaf 2	3	3	3	2	3	2	3	3
	Leaf 3	5	4	4	3	1	0	0	0
	Leaf 4	3	5	5	3	4	2	4	1
	Leaf 5	3	3	0	2	1	0	3	3
	Leaf 6	5	3	3	4	3	2	0	0

Treatment	Replicate	Leaf 1	Leaf 2	Leaf 3	Leaf 4	Leaf 5	Leaf 6	Leaf 7	Leaf 8
1/4 strength Actigard, single leaf applied	Leaf 1	5	5	5	5	3	1	1	2
	Leaf 2	5	5	4	4	4	5	0	0
	Leaf 3	5	5	4	3	2	4	1	1
	Leaf 4	5	5	5	3	0	3	2	1
	Leaf 5	5	5	5	5	5	0	0	0
	Leaf 6	5	5	4	4	0	1	0	0
1/8 strength Actigard, whole plant applied	Leaf 1	2	2	1	4	3	3	4	1
	Leaf 2	5	5	4	1	1	5	5	5
	Leaf 3	5	5	4	4	3	3	0	0
	Leaf 4	1	4	4	4	3	3	2	1
	Leaf 5	5	5	5	4	3	2	1	1
	Leaf 6	3	3	3	4	1	1	1	1
1/8 strength Actigard, single leaf applied	Leaf 1	5	5	5	4	4	4	2	2
	Leaf 2	5	5	5	5	5	4	2	2
	Leaf 3	5	5	0	4	5	5	5	4
	Leaf 4	5	4	4	4	3	3	1	0
	Leaf 5	5	5	5	5	5	0	1	0
	Leaf 6	5	5	5	4	4	5	5	0
1/16 strength Actigard, whole plant applied	Leaf 1	4	5	3	3	2	2	1	1
	Leaf 2	5	5	5	5	4	3	3	3
	Leaf 3	5	5	4	0	4	5	3	3
	Leaf 4	4	4	3	3	4	3	1	1
	Leaf 5	5	4	5	3	5	1	5	2
	Leaf 6	5	4	4	4	3	3	1	1

Treatment	Replicate	Leaf 1	Leaf 2	Leaf 3	Leaf 4	Leaf 5	Leaf 6	Leaf 7	Leaf 8
1/16 strength Actigard, single leaf applied	Leaf 1	5	4	5	5	5	4	5	5
	Leaf 2	5	5	5	5	5	4	3	1
	Leaf 3	5	5	4	4	4	4	1	3
	Leaf 4	5	5	3	5	5	3	3	3
	Leaf 5	5	5	5	3	5	4	1	1
	Leaf 6	5	5	5	4	5	3	5	3

A.11: Phytotoxicity bioassay

Macroalgae extract	Stock solution	1:100 stock dilution
AB1	No effect	No effect
AB2	No effect	No effect
AB3	No effect	No effect
AB4	No effect	No effect
AB5	No effect	No effect
AB6	No effect	No effect
AB7	No effect	No effect
AB8	No effect	No effect
AB9	No effect	No effect
AB10	No effect	No effect
AB11	No effect	No effect
AB12	No effect	No effect
AB13	No effect	No effect
AB14	No effect	No effect
AB15	No effect	No effect
AB16	No effect	No effect
AB17	No effect	No effect
AB18	No effect	No effect
AB19	No effect	No effect

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