An investigation into the bioactivity of the red seaweed *Polysiphonia lanosa*

Waterford Institute of Technology

A dissertation submitted for the degree of Doctor of Philosophy

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Based on work carried out by Caroline Botha

Under the supervision of Professors Helen Hughes, Peter McLoughlin and Gillian Gardiner

DECLARATION

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of PhD, is entirely my own work and has not been taken from the work of others, save and to the extent that such work has been cited and acknowledged within the text of my work.

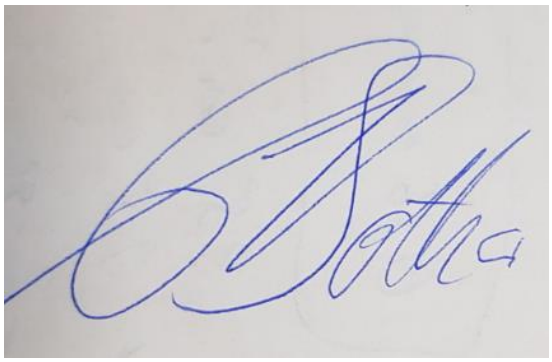

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TABLE OF CONTENTS

Page Number

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ABSSSI	Acute bacterial skin and skin structure infection
ACE	Accelerated solvent extraction
AD	Alzheimers Disease
ADP glucose	Adenosine diphosphoglucose
ANOVA	Analysis of variance
APC	Allophycocyanins
APX	Ascorbate peroxidase catalase
ASE®	Accelerated (pressurized) solvent extraction (Dionex)
BHA	Butylated hydroxyanisole
BHT	Butlyated hydroxytoluene
BIM	Bord Iascaigh Mhara
BP	Bromophenol
CA-MRSA	Community-associated MRSA
CAT	Catalase
CDC	Centers for Disease Control and Prevention (United States)
DASON	Duke Antimicrobial Stewardship Outreach Network
DCA	Deoxy cholic acid
DCM	Dichloromethane
DEAE	Diethylaminoethanol

DGLA	Dihomo -γ- linoleic acid
DHA	Docosahexaenoic acid
DLD-1	Human colon adenocarcinoma cells
DMSP	Dimethyl sulphonopropionate
DNA	Deoxyribonucleic acid
DPPH	Diphenyl-1-picrylhydrazyl
EAA	Essential amino acids
eDNA	Extracellular DNA
EPA	Eicosapentaenoic acid
EPS	Extracellular substance
FA	Fatty acid
FRAP	Ferric reducing power activity
Fts Z	Cell division protein
GAE	Gallic acid equivalent
GLA	γ-linoleic acid
GL	Glycolipid
GR	Glutathione reductase
HCT-116	Human colon carcinoma cells
HL-60	Human leukaemia, promyelocytic cells
HPLC	High performance liquid chromatography
HTS	High throughput screening

HA-MRSA	Hospital-associated MRSA
hBMSCs	Human bone marrow stromal cells
HVAB	High value added biomolecules
IAA	Indole acetic acid
IC	Inhibitory concentration
ICPMS	Inductively coupled plasma mass spectrometry
IL	Ionic liquid
IMTA	Integrated multi-trophic aquaculture
IR	Infrared
LC-PUFA	Long chain polyunsaturated fatty acid
LMWC	Low molecular weight carbohydrate
L929	Mouse connective tissue cells
MAA	Mycosporine-like amino acid
MAE	Microwave assisted extraction
MAPK	Mitogen-activated protein kinases
MBC	Minimum bactericidal concentration
MCF	Human breast adenocarcinoma cells
MDR	Multi drug resistance
MIC	Minimum inhibitory concentration
MRSA	Methicillin resistant Staphylococcus aureus
MS	Mass spectroscopy

MTT	Tetrazolium dye – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NEA	Neuroexcitatory amino acids
NICE	National Institute fo Health and Care Excellence
NMDA	N-methyl-D-aspartic acid
NMR	Nuclear magnetic resonance
ORAC	Oxygen radical absorbance capacity
PA	Polyamine
PC	Phycocyanins
PAGE	Polyacrylamide gel electrophoresis
PBP	Phycobiliproteins
PBP2a	Penecillin binding protein 2a
PD	Parkinsons Disease
PE	Phycoerythrins
PF	Polar fraction
PIA	Polysaccharide intercellular adhesion
PSM	Phenol-soluble modulin
QS	Quorum sensing
RAW	Mouse macrophage cells
ROS	Reactive oxygen species
R^2	Co-efficient of determination
Rubisco	Ribulose-bisphosphate-carboxylase/oxygenase

SAR	Structure and activity relationship
SD	Standard deviation
SFE	Supercritical fluid extraction
SOD	Superoxide dismutase
Spp.	Species
SSU rRNA	Small subunit ribosomal ribonucleic acid
TAG	Triacylglyceride
TEAC	Trolox equivalence antioxidant capacity
TLC	Thin layer chromatography
TFC	Total flavonoid content
TPC	Total phenolic content
TRAP	Trapping antioxidant parameter
TOSC	Total oxyradical scavenging capacity
UDP glucose	Uridine diphosphate glucose
UAE	Ultrasound assisted extraction
USA	United States of America
US FDA	United States Food and Drugs Adminisration
UV B	Ultraviolet B
UV-Vis	Ultraviolet-visible

Caroline Botha

An investigation into the bioactivity of the red seaweed *Polysiphonia lanosa*

ABSTRACT

This research involved an investigation into the bioactivity of an aqueous *P. lanosa* extract against a hospital isolate of MRSA. It encompassed an evaluation of the effect of abiotic stress on anti-MRSA activity, isolation and characterisation of the antibacterial compounds, MIC, MBC and BBC determination, as well as antioxidant and cytotoxicity potential assessment. Visual observations of MRSA growth permitted speculation of the mechanism of anti-MRSA activity.

The cultivation (tank storage) conditions of harvested seaweed was monitored and compared with anti-MRSA activity in a baseline study. This is the first time that an epiphytic seaweed was cultured unattached to determine the effect on antimicrobial activity. Temperature and light exposure duration conditions were investigated with retention of anti-MRSA activity confirmed for a period of 8 days post harvesting. Slight recovery in anti-MRSA activity was evident at lower temperatures but deterioration occurred for all light conditions, indicating the photosensitive nature of the bioactive compounds.

Extraction, isolation and identification of the anti-MRSA compounds was pursued using bioautography and well diffusion to ensure bioactivity against planktonic MRSA with isolation of a semi-pure extract achieved. A bromo indole with heterocyclic substitution of carboxylic acid and/or nitrile functionalities or a bromo substituted bi or bis(indolyl) structure was proposed.

MIC, MBC, BBC and DPPH IC_{50} of the crude and semi-pure *P. lanosa* extracts were determined. Exceptional DPPH radical scavenging activity of the crude *P. lanosa* extract contrasted with that of the semi-pure fraction confirming that their bioactivities were due to different compounds.

The crude *P. lanosa* extract displayed significant cytotoxicity against HT-29 colonic epithelial cells using an xCELLigence real-time cell analysis system, whilst no cytotoxicity was observed in the MTT assay using liver carcinoma HepG2 cells.

Anti-MRSA activity visualisation was explored at sub-inhibitory concentrations of crude and semi-pure *P. lanosa* extract (staphyloxanthin pigment), together with MRSA colony-spreading inhibition by crude *P. lanosa* extract.

CHAPTER 1: INTRODUCTION

1.1 Natural Products

Natural medicinal products have a long history of use. Sumarian stone tablets from 2600 BC describe the use of willow leaves to treat inflammatory rheumatic disease and the Ebers papyrus from 1500 BC details the use of plant, animal and mineral components in Egyptian medicine (Vinel & Pialoux, 2005). Other areas of the world have their own pharmacological directories such as the Chinese Materia Medica from 1100 BC and documents specifying the Ayurevic system from 1000 BC. These early texts have served as sources of some modern medicines. For example, aspirin was developed from salicin, first isolated from willow bark in 1829 (Mahdi, 2010).

However, natural products have been marginalised by the major pharmaceutical companies, as the accepted strategy for drug discovery is high throughput screening (HTS) of hundreds of thousands of small molecules against single targets, followed by medicinal chemical engineering of lead compounds. This approach has led to a much higher failure rate than predicted and has failed to supply sufficient innovative new drugs (Carter, 2011). The premise that new drug approvals equate to innovation is a false one in that the increase in the number of new drugs actually reflects relatively minor modifications of current drugs with a minority of new drugs in fact regarded as clinically superior to existing alternatives (Naci *et al*., 2015). The pharmaceutical industry is perceived as investing mainly in already established areas with a disproportionate emphasis on marketing rather than research (Health and Social Care Committee, 2018). At present, the industry faces expensive product recalls, aggressive generic competition and the patent cliff.

Re-evaluation of the drug discovery process is crucial and the pursuit of natural products as sources of drug leads is gaining momentum, albeit with challenges. For preclinical studies, hundreds of grams of highly consistent, well-characterised product are required and although natural products from renewable sources offer initial advantages, extraction protocols would need to be robust and capable of delivering sufficient quantities of product. The other challenge is whether or not there is potential for adequate diversification strategies. However, functional synthesis and biosynthetic technology in microbial systems hold the key in this respect (Carter, 2011). It has been reported that the

natural product field is still uncovering products with no structural precedent and together with new discovery methods should yield compounds with unique structural and biological properties (Pye *et al*., 2017) .

The realisation that natural products possess high molecular diversity, biochemical specificity and other unique molecular properties that distinguish them from libraries of synthetic compounds has hastened their reassessment. Computational studies have compared the physicochemical properties of representative natural, combinational and synthetic drug molecules and found that natural products have a greater number of chiral centres and increased steric complexity compared with both synthetic drugs and combinatorial libraries (Koehn & Carter, 2005). They also possess greater molecular rigidity, a larger number of solvated hydrogen-bond donors and acceptors and a greater diversity of ring systems (only one fifth of the ring systems found in natural products are represented in current trade drugs) (Carter, 2011). These facts bolster their potential for novel applications and further present diversification consideration. It has been reviewed and analysed that, despite the move away from natural products, nearly two thirds of small molecule drugs approved are natural products, natural product derivatives, synthetic mimics of natural product action or based on a natural product pharmacophore (Newman, 2007; Pye *et al*., 2017).

The issue of pure compound screening needs to be addressed to permit evaluation of natural product mixtures and it was suggested that libraries be composed of pre-fractionated natural products representing mixtures which would include the bulk of constituents, rather than the major components or peaks, so that the potential to discover novel compounds is protected (Carter, 2011). This would further reduce the evaluation lag time, as compound purification is no longer necessary.

1.2 Marine Products

Terrestrial biodiversity is exceeded by an even greater diversity of marine flora and fauna. The oceans cover 70% of the earth's surface and comprise 95% of its biosphere and represent an untapped source of novel biomolecules with unrealised pharmaceutical

potential (Cragg & Newman, 2014). However, by 1974, only two marine-derived natural products had been added to the pharmacopeia to treat human disease and it took three more decades for another marine-derived natural product to gain approval (Ebada & Proksch, 2011). The fact that most of these marine-derived drugs are targeted towards cancer treatment serves to illustrate the way in which the pharmaceutical industry has neglected infectious diseases and targeted the high profit markets of chronic disease (cancer and HIV) (Falconer & Brown, 2009). In 2016 there were 5 marine-derived compounds in Phase III, 8 in Phase II and 14 in Phase I (Mayer *et al*., 2017) and most of these are auristatin-containing antibody drug conjugates (auristatin – antimitotic activity). This is in contrast to the 2013 pipeline which identified seven FDA-approved drugs that are marine-derived, with 11 in the clinical pipeline and a larger number in the pre-clinical pipeline (Mayer *et al.*, 2013;Figure 1.1).

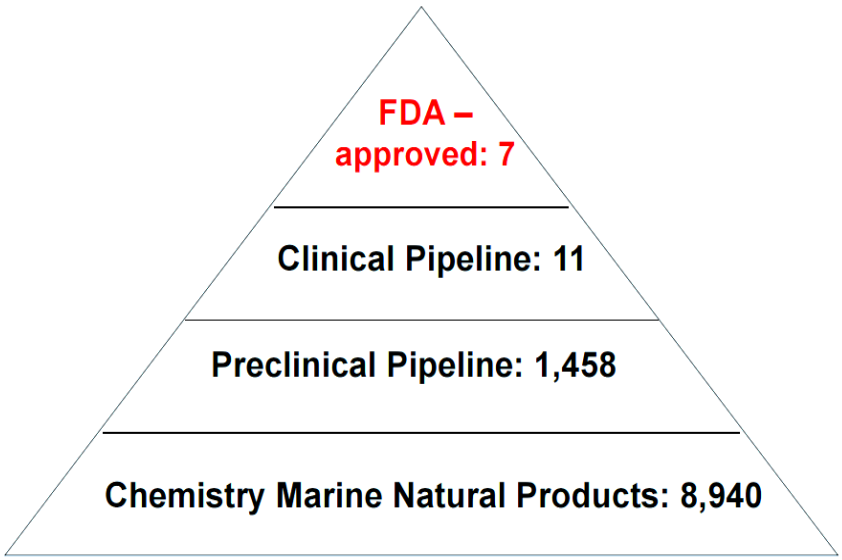


Figure 1.1: Global Marine Pharmaceutical Pipeline in 2012-2013 (Mayer *et al*., 2017).

The marine natural product (MNP) literature review (Blunt *et al*., 2018) for 2016 describes 1277 new compounds from 432 research papers, a 5% reduction from the 1340 new compounds in 429 papers reported for 2015. However, there was a 65% increase in the number of MNP-related reviews for 2016 compared to 2015, emphasising the level of interest in this field.

Pharmaceutical compounds are in most need of novel products due to the increased

incidence of antimicrobial resistance. An article by Spellberg and Shlaes (Spellberg & Shlaes, 2014) demonstrated the global urgency in pursuing new antibiotics. It reported the necessity for new treatments for bacterial infections, highlighting that this was the greatest unmet requirement due to changes in resistance patterns and treatment strategies and declining new antibacterial approvals.

1.3 Seaweed

Seaweeds or macroalgae have become established as a natural source of new compounds with biological activity that could be used as functional ingredients. Macroalgae live in complex and extreme habitats which experience changes in salinity, temperature, nutrients and electromagnetic radiation. Electromagnetic radiation includes the visible spectrum as perceived by the human eye and UV light, both responsible for photosynthesis and production of metabolites. Biotic challenges include the constant interaction with hostile micro-organisms. A substantial variety of secondary (biologically active) metabolites are produced, allowing the macroalgae to adapt quickly to these ever-changing environmental parameters (Shalaby, 2011). Compounds isolated from seaweed have demonstrated a range of biological activities, including antibacterial activity, antioxidant potential, anti-inflammatory properties, anticoagulant activity, antiviral activity and apoptotic activity (El-Baroty *et al.,* 2007; Smit, 2004).

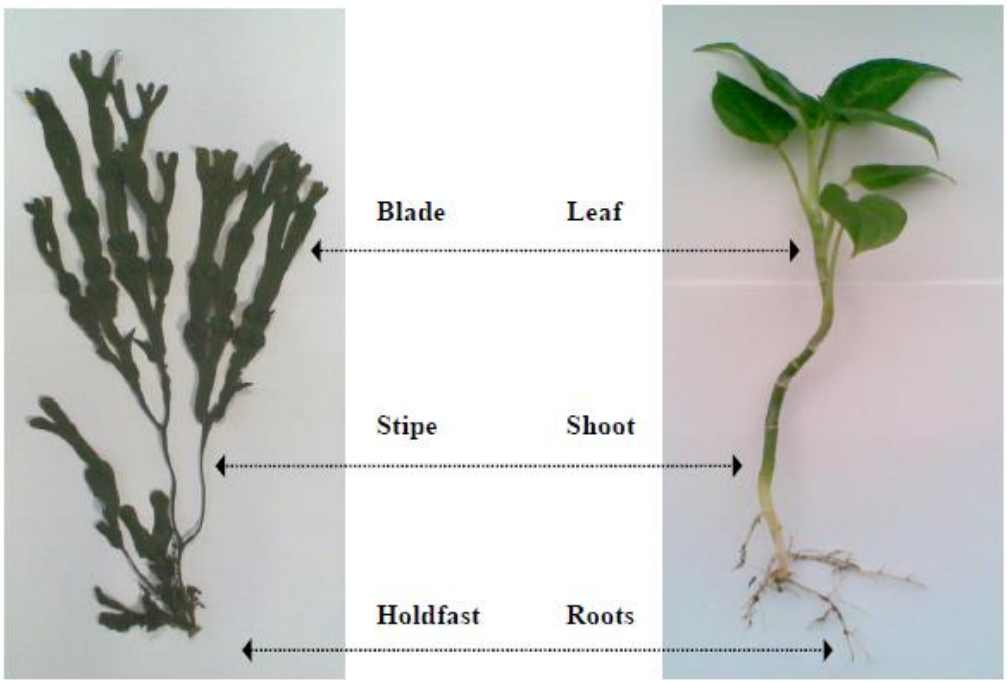
1.3.1 *Seaweed Structure*

Seaweeds are simpler in structure than terrestrial plants and consist mainly of chains of cells, attached end to end to form very thin threads or filaments. Other seaweeds can be sheet-like or tubular and can be one to numerous cells in thickness. An example of a thin sheet-like seaweed is the *Ulva* species, while *Fucus* and *Laminaria* species are comprised of thick cell walls and branches and are leather or rubber-like in texture (Lobban & Harrison, 1997), as represented in Figure 1.2.

(a) (b)

Figure 1.2: (a) *Ulva lactuca* and (b) *Fucus serratus* (Guiry & Guiry, 2019).

Seaweed shape is indicative of its biological requirements. As plants growing in water, they have no requirement for the roots, stems and leaves of land plants. Seaweeds are immersed in water which is also their nutrient supply and are able to absorb nutrients directly into their cells (Lee, 1986). Seaweeds maintain a secure attachment to rocks by the holdfast, and whereas a land plant requires a firm stem to hold its structure upright and transfer the absorbed nutrients and water through from the root to the leaves, seaweed is buoyant in the water, dispensing with this system. A rigid stem would present a disadvantage as the violent tidal motion could possibly break it. Certain species do have a stipe, a cylindrical flexible shaft rising from the holdfast bracing the rest of the plant (Lee, 1986). Many species of brown algae have air bladders to ensure that the thallus floats near the water surface so that it is able to receive the light it requires for photosynthesis. Photosynthesis in land plants is carried out in the leaves, but in seaweed, photosynthesis is performed by a complex range of various types of blades, membranes and filaments (Lee, 1986). Photosynthetic reactions are extremely efficient, using resources directly from the surrounding water medium. Figure 1.3 compares a land plant structure to that of seaweed.



(a) (b)

Figure 1.3: Comparison of seaweed plant (a) and land plant structure (b) (Ryan, 2010).

1.3.2 *Classification*

There are a number of distinguishing characteristics that permit classification of macroalgae. These include the nature of the chlorophyll(s), cell wall composition and flagellation. These features are summarised in Table 1.1 (Bocanegra *et al.,* 2009).

Seaweeds are generally classified into three main divisions based on their pigmentation as depicted by Bocanegra *et al.* (2009). Phaeophyta, or brown seaweeds, are mainly brown due to the presence of the carotenoid fucoxanthin and the primary polysaccharides which are comprised of alginates, laminarins, fucans and cellulose. Chlorophyll a and b predominate in Chlorophyta, or green seaweeds with ulvan as the major polysaccharide component, whilst the principal pigments found in Rhodophyta (red seaweed) are phycoerythrin and phycocyanin and the primary polysaccharides are agars and carrageenans (Bocanegra *et al.,* 2009). The different phytopigments in algae are linked to their specific sea habitat, as they differ in their requirement for light intensity, necessary for photosynthesis (Lobban & Harrison, 1997). Green algae are capable of absorbing large quantities of light energy and proliferate in coastal waters, whilst red and brown

macroalgae are found at greater depths, where penetration of sunlight is curtailed. The photopigments of red algae, phycocyanin, phycoerythrin and allophycocyanin, are all phycobiliproteins with phycoerythrin the most flexible, facilitating adaptation to environmental changes, as it extends the light-harvesting capability of the phycobilisome from the red region to the green region of the visible light spectrum (Johnson *et al.*, 2014), permitting habitation at lower depths.

The carbohydrates produced by photosynthesis also represent a basis for distinction of the three seaweed divisions (Bocanegra *et al.*, 2009). Chlorophyta are characterised by the starches cellulose, amylose and amylopectin, whilst the Phaeophyta have laminaran and mannitol and the Rhodophyta, floridean starch.

Table 1.1: Characteristics on which macroalgal classification are based (Bocanegra *et al.*, 2009)**.**

Division	**Common name/example of species**	**Pigments**	**Storage product**	**Structural cell wall**	**Intercellular mucilage**	**Flagella**
Chlorophyta	Green algae/ *Enteromorpha compresa*.	Chlorophylls a.b. β- carotenes, lutein, several xanthophylls.	Starch	Cellulose, xylans, mannans, ulvans.	Sulphated polysaccharides.	Present
Phaeophyta	Brown algae/ *Laminaria sp.*, *H. fusiform*, *U. pinnatifida*.	Chlorophylls a.c. β- carotenes, fucoxanthins, several other xanthophylls.	Laminaran mannitol	Cellulose, chitin.	Alginic acid/ alginates, sulphated polysaccharides, such as fucans (fucoidans).	Present
Rhodophyta	Red algae/ *P. tenera*.	Chlorophylls a.d. phycocyanins, phycoeryhtrin, α- and β- carotenes, several xanthophylls.	Floridean starch	Cellulose, xylans, mannans.	Sulphated polysaccharides as agar, carrageenans.	Not present

1.3.3 *Chemical Composition of Seaweed with a Focus on Bioactive Compounds*

Seaweed-derived compounds are found in a range of products such as food, pharmaceuticals and cosmetics, as they are rich sources of non-starch polysaccharides, minerals and vitamins (Cardozo *et al*., 2007; Darcy-Vrillon, 1993; Mabeau & Fleurence, 1993). However, the chemical composition of seaweeds depends on species, habitat, maturity and environmental conditions (Ito & Hori, 1989).

Seaweed polysaccharides cannot be completely digested by human intestinal enzymes (Jiménez-Escrig & Sánchez-Muniz, 2000). This, together with the low lipid content of seaweed, means that they have a low calorific value and have immense potential for the food industry. Seaweed is regarded as a rich source of dietary fibre (25–75% dry weight), of which water-soluble fibre comprises approximately 50–85% (Jiménez-Escrig & Sánchez-Muniz, 2000). The consumption of seaweeds is aligned to a decrease in the occurrence of chronic diseases, such as diabetes, obesity, heart disease and cancer (Southgate, 1990), which are associated with low fibre diets (Trowell, 1976). The physiological effects of the different types of fibre are linked to their physico-chemical properties (Roehrig, 1988). The viscosity of soluble fibre is responsible for slower digestion and absorption of nutrients, and reduced levels of blood cholesterol and glucose. Insoluble fibre, on the other hand, is able to increase faecal bulk and decrease intestinal transit times (Baghurst *et al*., 1996). Because of their high fibre content and the nature of this fibre, seaweeds are increasingly used as texturizing and bulking agents (Wong & Cheung, 2000), as well as beneficial food additives and supplements (Chojnacka & Saeid, 2012).

The potential applications of algal extracts are considerable and represented by those shown in Figure 1.4 (Chojnacka & Saeid, 2012). Crude extracts are complex mixtures of compounds that may have synergistic interactions and are responsible for bioactivity, whilst purified fractions may no longer retain the bioactivity pursued. The opposite effect is also possible where there may be low bioactivity in crude extracts due to the interference of other compounds such as tannins that may bind to other metabolites, masking activity.

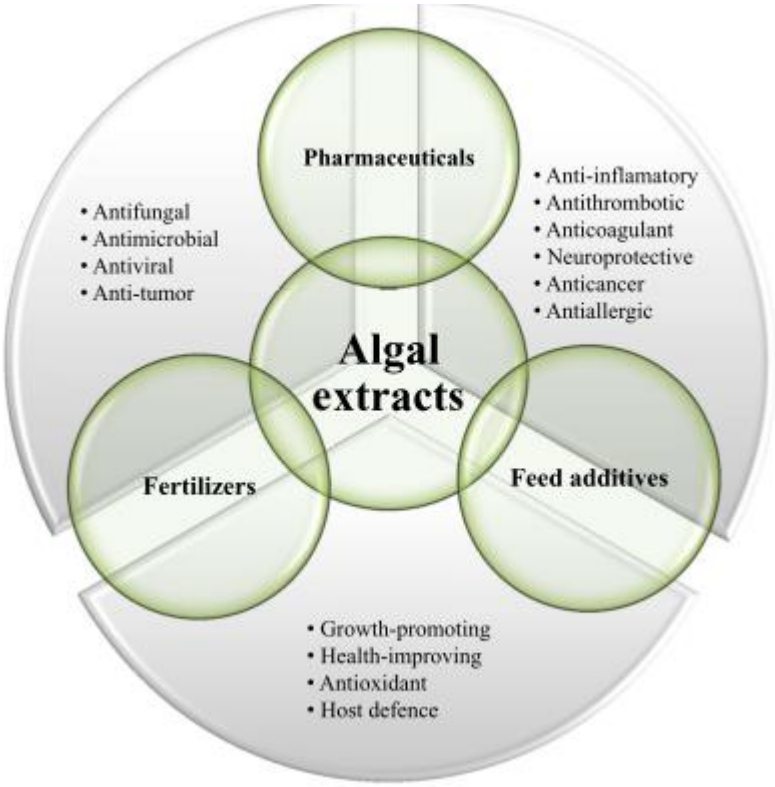


Figure 1.4: Activities of algal extracts and their applications (Chojnacka & Saeid, 2012).

This diagram displays the overlap between potential applications, in that a product destined for human pharmaceutical usage may be used in another form for animal feed or incorporated into a fertiliser to prevent fungal infection of crops. The range of bioactivity of algal extracts and their purified compounds is extensive.

The protein content of seaweed changes considerably according to season and environmental growth conditions. For example, in some brown algae species such as *Laminaria japonica*, *Hizikia fusiforme* and *Undaria pinnatifuda,* the protein content is relatively low at 7-16 g/100 g dry weight, whilst red algae such as *Palmaria palmata* and *Porphyra tenera* contain 21-47 g protein/100 g dry weight. All essential amino acids (EAA) are present in algal protein throughout the year, although concentrations may vary (Dawczynski *et al.*, 2007).

The fat content also changes between species, ranging from 1-6 g/100 g dry weight, with the composition also varying. In red algae, high concentrations of eicosapentaenoic acid are reported compared with brown seaweeds which have a different fatty acid profile

(Dawczynski *et al.*, 2007). In fact, macroalgae produce long chain polyunsaturated fatty acids (LC-PUFA) through biosynthesis (Gong *et al.*, 2011). It has further been established that, whilst differences in fatty acid composition were associated with species and environmental influences (Kumar *et al.*, 2011), levels in red algae demonstrated less variability than those in green or brown algae. Several studies comparing a range of macroalgal species (Kumari *et al.*, 2011) confirmed that fatty acid profiles were specific to subgroups, suggesting their potential use in chemotaxonomy (Stengel *et al.*, 2011). Algae grow much faster than food crops and microalgae strains with lower lipid content are able to grow as much as 30 times faster than those with a high lipid content.

Seaweed varieties have also been identified as rich sources of Vitamin C, Vitamin B-complex and Vitamin A precursors such as beta-carotene (Dawczynski *et al.*, 2007). Antioxidant benefits derived from the consumption of terrestrial plants have long been recognised but the utilisation of macoalgae for their antioxidant value is relatively new (Cornish & Garbary, 2010). Seaweed compounds which could be responsible for antioxidant activity encompass carotenoids, phenolic compounds, phycobilin pigments, polyphenols, sulphated polysaccharides and vitamins. Antioxidant production is allied to the stress-coping actions of intertidal algae and the high cellular levels of antioxidant compounds which deactivate reactive oxygen species and attenuate the hazardous effects of ultraviolet light (Dring, 2005).

Table 1.2 reveals the categories of bioactive compounds that have been identified in seaweed extracts, with each of these possessing unique bioactivity depending on the specific extraction process employed. This reflects the plethora of papers (Pangestuti & Kim, 2011; Cian *et al*., 2012; Manilal *et al.*, 2009; Sangha *et al*., 2013) dealing with seaweed-derived bioactive extracts and the multiple extraction processes used, ranging from relatively simple to more sophisticated.

Table 1.2: Biologically Active Substances Found in Seaweed Extracts (Chojnacka & Saeid, 2012).

Biologically Active Substance	Activity
Polysaccharides	Prebiotic Antimicrobial Growth-promoting Antiviral Anti-tumour Anti-inflammatory Source of soluble dietary fibres Antioxidant, Antithrombotic Anticoagulant
Proteins	Source of essential amino acids Elements of intercellular communication Antiviral Antimicrobial Anti-inflammatory Antioxidant
Polyunsaturated fatty acids	Cardiovascular support – reduction in plasma triglycerides, increase in plasma HDL-cholesterol Antibiotic Antifungal
Pigments	Antioxidant Anti-inflammatory Antiviral Neuroprotective Anti-obesity Anticancer
Polyphenols	Host defence Strong antioxidant Antimicrobial Antiviral Anti-photo aging Anti-obesity Antiallergic Anticancer

More complex molecular structures, such as glycolipids (GLs) comprising acylglycerols termed gycoglycerolipids and ceramide derivatives, termed glycolsphingolipids representing membrane lipids (Maciel *et al*., 2016) are also present in macroalgae. Novel galactoglycerolipids characterised from the red alga *Chondria armata,* have displayed antimicrobial activity (Al-Fadhli *et al.,* 2006). Oxylipins represent additional tolerance pathways to buffer oxidative stress caused by desiccation because the accumulation of lipoperoxides and carbonyls indicate that reactive oxygen species (ROS) buffering mechanisms are not effective and these oxidative markers were overproduced in low intertidal species along with reactive oxygen species production (H_2O_2) (Eckardt, 2008).

In some species the level of lipoperoxides remains high even on rehydration when ROS levels reduced. Contrasting with this are the desiccation tolerant species where no excess of lipoperoxides occur (Flores-Molina *et al.,* 2014). Bioactive proteins such as the phycobiliproteins and lectins have also been identified as having extensive pharmaceutical potential (Harnedy & FitzGerald, 2011), as have the mycosporine-like amino acids (MAAs).

1.3.4 Seaweed Ecology

Figure 1.5 represents the range of habitats at the shoreline, with the definitive zones illustrated. Different aquatic environments are defined by both spatial and temporal heterogeneity of physico-chemical parameters responsible for specific habitat variables. These include alterations in light quality and light levels (where UV-B radiation is regarded to be an important factor determining macroalgal vertical distribution), nutrient levels and composition, CO_2 availability, temperature, salinity, pH, contaminants and biotic impacts due to the distribution of grazers and endo- and epi-bionts (Stengel *et al.,* 2011).

Large varieties of seaweed are found in both the low tide (lower littoral) zone and the sub-littoral fringe of the beach, on rocky shores and in river estuaries. Smaller benthic seaweeds will attach to larger marine plants or rocks for preservation in strong sea currents (Lobban & Harrison, 1997). Smaller seaweeds can survive as epiphytes (i.e. plants that grow on other plants) as is the case with *Polysiphonia lanosa* (red seaweed) which can be found growing on either *Ascophyllum nodosum* (brown seaweed) or less frequently on *Fucus* spp (Garbary & Deckert, 2002).

The zones of Figure 1.5 represent niche preferences of individual algal species which determine distribution patterns based on environmental endurance levels and responses to biotic interactions. Seaweeds that grow on muddy or sandy beaches possess penetrating, root-like holdfasts which anchor the plant so that it is not swept off the shore.

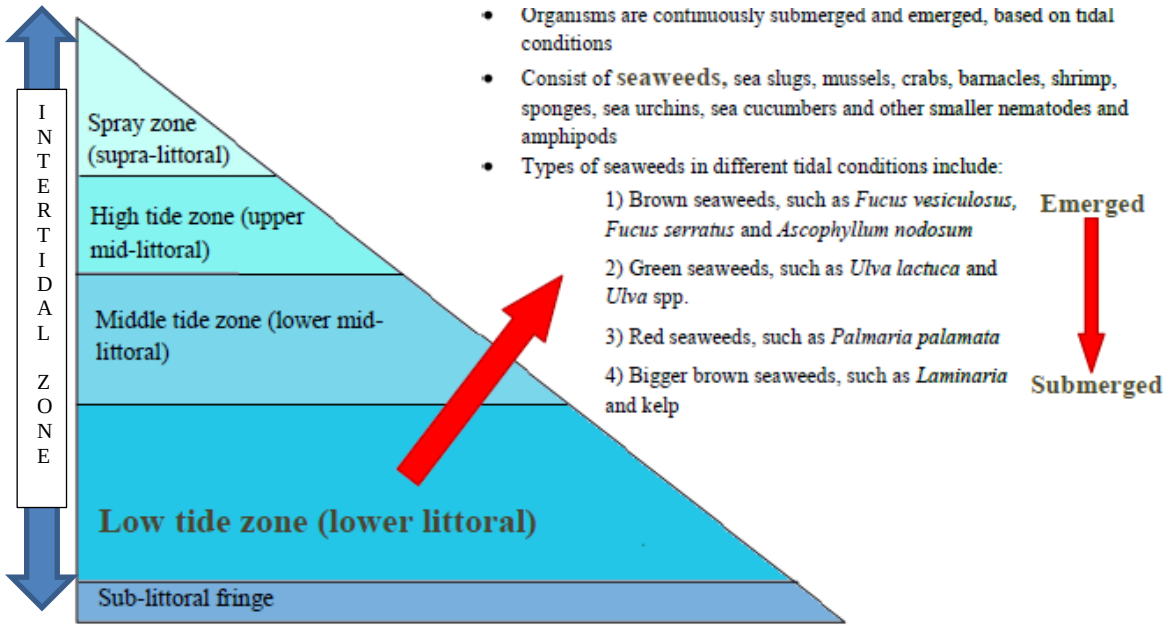


Figure 1.5: Intertidal zones and common organisms inhabiting the lower littoral zone (Hernandez-Ledesma & Herrero, 2014).

Seaweeds occupying the intertidal (littoral) zone of the beach are exposed to continuous submersion and emersion due to wave action (Lobban & Harrison 1997) and provide sheltered (from both predators and the impact of wave action) habitats for a wide range of small organisms, such as nematodes and amphipods. Brown seaweeds generally inhabit the lower intertidal region (sub-littoral) area in Figure 1.5 off the coast of Japan, Pacific North America, South Australia and the British Isles (Graham & Wilcox, 2008), while red seaweed species are commonly located in tropical and temperate waters, in the intertidal zone, nearer the lower littoral area, although they can be found much deeper, up to 250 m below the water surface (Turner, 2003). Green seaweeds constitute mainly marine seaweeds found along beaches and will bloom in nutrient-rich areas, creating problems for tourist industries in many countries.

1.4 Rhodophyta

1.4.1 *Classification and Taxonomy*

The red algae (Rhodophyta) represent a distinct eukaryotic lineage designated by the photosynthetic pigments phycoerythrins (PE), phycocyanins (PC) , and allophycocyanins (APC) aligned in phycobilisomes, and the absence of flagella and centrioles (Woelkerling, 1990). Some rhodophytes are unicellular but most species grow as filaments or membranous sheets of cells.

The Rhodophyta epitomises one of the major variant phyla of eukaryotes. Measured by divergence of small subunit ribosomal deoxyribonucleic acid (SSU rDNA) sequences within the most conservative regions, red algae are more divergent among themselves than are green algae and green plants together (Ragan *et al.*, 1994).

Rhodophytes form the dominant inhabitants along the coastal and continental shelf areas of tropical to polar regions (Luning *et al.,* 1990) and they display the widest vertical distribution in the supralittoral zone, with 445 genera such as *Bangia* and *Porphyra.* Red algae are ecologically significant as primary producers and providers of structural habitats for many other marine organisms in the primary establishment and maintenance of coral reefs. Complementary to these functions, some red algae are economically important as a source of human food and colloidal compounds found in the cell wall, known as phycocolloids. These are polysaccharides of high molecular weight comprising agar, carrageenan and alginate. For example, the carrageenans constitute a group of biomolecules composed of linear polysaccharide chains with sulphate half-esters bound to the sugar unit (Samaraweera *et al.,* 2011). The use of red algae as a food source has resulted in their extensive farming and natural harvesting in numerous areas of the world (Luning *et al.,* 1990).

Rhodophyta, together with prokaryotic Cyanobacteria, possess phycobilins rather than chlorophylls as the major constituent of their photosynthetic sensors. In Rhodophyta, R-phycoerythrin is the dominant light-harvesting pigment and imparts the characteristic red-purplish hues of these algae. R-Phycoerythrin is a large 240 kDa red phycobiliprotein isolated from red algae. It has a strong absorption peak at 565 nm and a secondary absorption peak at 492 nm (Talarico & Maranzana, 2000). These absorption peaks permit

these plants the efficient utilisation of green and blue light, which accounts for their dominance in the deeper parts of the tidal zone. With depth, the red domain of the solar spectrum is limited by both water and chlorophyll-containing phytoplankton cells, resulting in a progressive increase in the blue and green components of sunlight (Graham & Wilcox, 2008).

It is interesting to note the existence of highly toxic compounds within Rhodophyta. For example, polar fraction substances extracted from the red seaweed *Galaxaura marginata* have been shown to have neurotoxic activity (Rozas & Freitas, 2008). The same symptoms preceding death were described in both mice and rats tested with neuroexcitatory amino acids (NEA) as in those administered seaweed-derived fractions, suggesting that some polar fraction components cross the blood-brain barrier, in the same way as NEA. The seaweed-derived compounds were subjected to HPLC and mass spectrometric analysis which eliminated the possible presence of known neuroexcitatory amino acids, kainic acid, domoic acid and N-methyl-D-aspartic acid (NMDA), indicating that alternative substances extracted as a polar fraction, were neurotoxic and lethal (Rozas & Freitas, 2008).

1.5 Bioactive Compounds from Rhodophyta

1.5.1 *Low Molecular Weight Carbohydrates*

Red algae fix inorganic carbon *via* the common plant enzyme ribulose-bisphosphate-carboxylase/oxygenase (RubisCO) during photosynthesis and the consequent carbon flow into low molecular weight carbohydrates is much more disparate compared to other algal groups as is depicted in Figure 1.6. The major photosynthetic product in members of most orders of Rhodophyta is the heteroside floridoside (α-D-galactopyranosyl-(1-2)-glycerol). However, this was not found to be the case in the Florideophyceaen order Ceramiales and in more ancient lineages, such as Bangiophyceae, Porphyridiophyceae, Rhodellophyceae, and Stylonematophyceae (Yoon *et al.*, 2006) where a multitude of low molecular weight carbohydrates (LMWCs) have been identified. These include digeneaside, floridoside, D- and L-isofloridoside, glucitol, galactitol and mannitol, which function as osmolytes and compatible solutes, exhibiting stabilizing effects on enzymes,

membranes and structural macromolecules under hypersaline conditions (Ascêncio *et al.*, 2006).

In summary, members of the Ceramiales (Florideophyceae) generally synthesize and acquire the chemically related digeneaside in place of floridoside (α-D-mannopyranosyl-(1-2)-glycerate) (Kremer, 1980). The first report of digeneaside (α-D-mannopyranosyl-(1-2)-D-glycerate) was published by Colin & Augier in 1939 (Colin & Augier, 1939) and was initially isolated from the red seaweed, *Polysiphonia fastigiata* (Rhodophyceae), now classified as identical to *P. lanosa* and had its molecular structure elucidated through methylation techniques (Bouveng *et al*., 1955).

The biosynthesis and aggregation of organic osmolytes in the cytoplasm of Rhodophyta allows for the creation of low water potentials without incurring metabolic damage (Yancey, 2005), as high electrolyte levels would critically affect protein and organelle function (ribosome and mitochondria), enzyme activity, membrane integrity and structural macromolecules (Kirst, 1990). These compatible solutes vary in their chemical structure but possess some features in common: They are highly soluble (except dulcitol), in most cases have no net charges at physiological pH (except digeneaside), and are non-inhibitory at high concentrations (Kirst, 1990; Karsten *et al.*, 1996). In general, the intracellular concentrations of these organic osmolytes are actively adjusted and directly proportional to external salinity (Karsten *et al.*, 1996). With the exception of digeneaside, which has a minor role in osmotic acclimation, most LMWCs were reported to accumulate by a factor of approximately 1.5 between full and double strength seawater (Eggert & Karsten, 2010).

The relevance of these compounds is their potential contribution to bioactivity. Their function as osmolytes may allow for better diffusion or stabilisation of actual bioactive compounds or they may form active molecules by combining with other compounds during metabolic processes. In fact, floridoside is recognised as possessing anti-fouling as well as therapeutic properties (Garcia & Maarel, 2016).

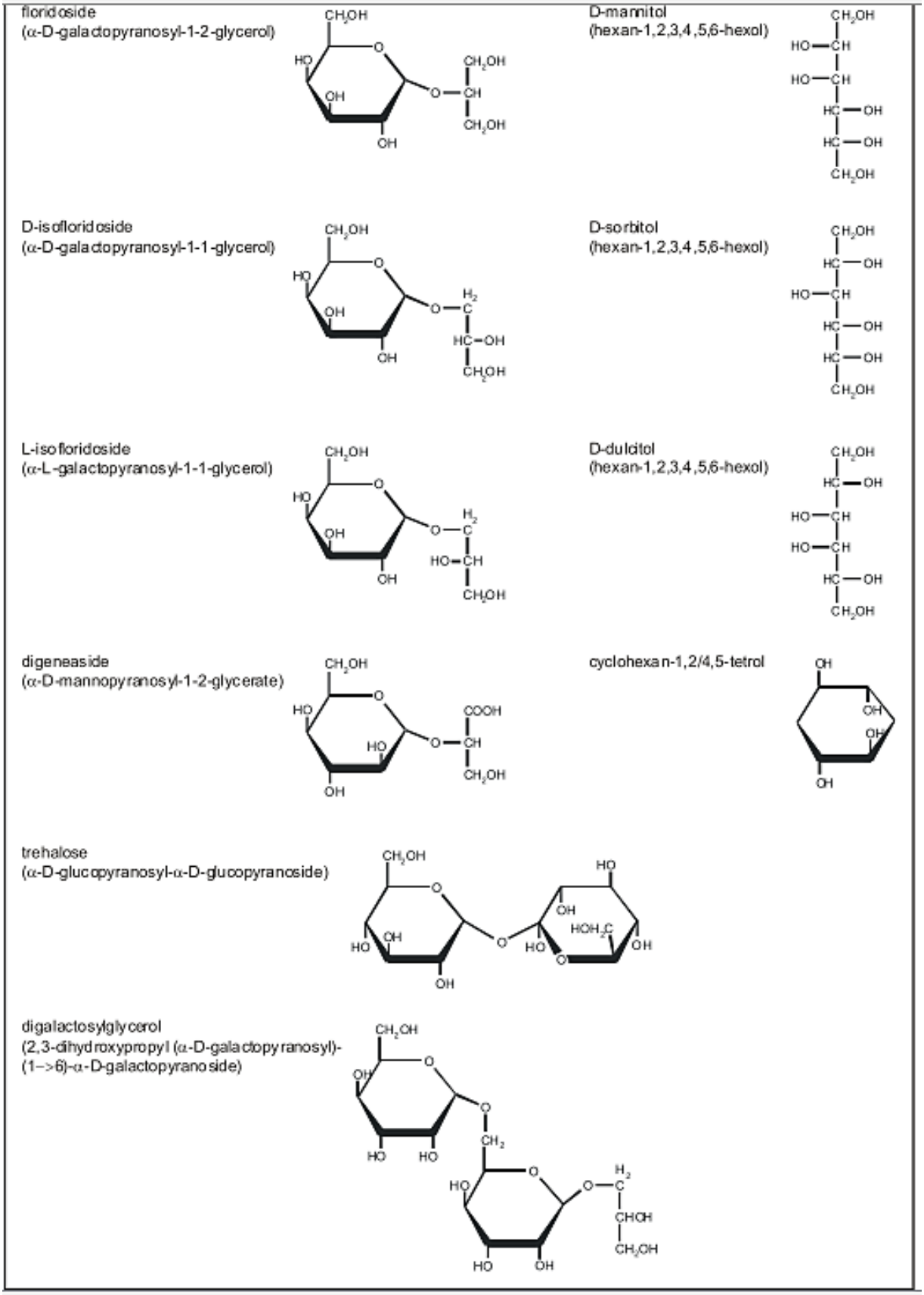


Figure 1.6: Typical low molecular weight carbohydrates found in Rhodophyta (Eggert & Karsten, 2010).

1.5.2 *Antioxidants*

Antioxidant activity is provided by a variety of compounds. Within Rhodophyta, carotenoids, phycobilin pigments, polyphenols such as flavonoids (*Palmaria palmata*), sulphated polysaccharides such as sulphated galactans (lambda carrageenan) and galactans from various Rhodophyta, porphyrin from the Porphyra species, Vitamin C from *Chondrus crispus* and Vitamin A from *Kappaphycus alvarezii* (Cornish & Garbary, 2010) represent the main groups.

Antioxidant production is allied to the stress-coping mechanisms of intertidal algae and the high cellular levels of antioxidant compounds which deactivate ROS and minimise the hazardous effects of UV light variations (Dring, 2005). The antioxidant activities observed in marine macroalgae have also been associated with potential anti-aging, anti-inflammatory, antibacterial, antimalarial, antiproliferative, and anticancer effects (Cornish & Garbary, 2010) (Koutsaviti et al., 2018) (T. Di et al., 2017).

Antioxidant activity has more recently been found to overlap with immunostimulant activity and Di *et al.* (2017) observed a strong *in vitro* antioxidant potential with significant immunostimulant activity on microphage-like Abelson leukaemia virus-transformed cell line ie. RAW 264.7 cells from extracts of *Gracilaria rubra* with a high sulphate content heteropolysaccharide (galactose and fucose) having demonstrated the greater immunostimulant effect.

1.5.3 *Halogenated Organic Molecules*

The Rhodomelacea is reported to be the largest red algal family with approximately 125 genera and some 700 species globally (Wang *et al.*, 2013). The isolation and characterisation of secondary metabolites from this family is ongoing and according to MarinLit (a marine literature database maintained by the Department of Chemistry, University of Canterbury, New Zealand) a total of 1,058 naturally occurring compounds were isolated from this family, of which 808 (76%) were halogenated (Wang *et al.*, 2013). Most of these compounds contain bromine with some containing both bromine and chlorine. Seawater contains a chloride ion concentration of 19,000 mg/L, whilst the concentration of bromide ions is 65 mg/L and this selective inclusion of bromine for the

biosynthesis of halogenated metabolites remains a definitive characteristic of the Rhodomelacea. The *Polysiphonia, Rhodomela* and *Symphycladia* genera produce halogenated phenols and aromatics as metabolites, referred to as bromophenols or haloaryl compounds. The predominance of red algae as haloaryl secondary metabolite producers is presented in Figure 1.7.

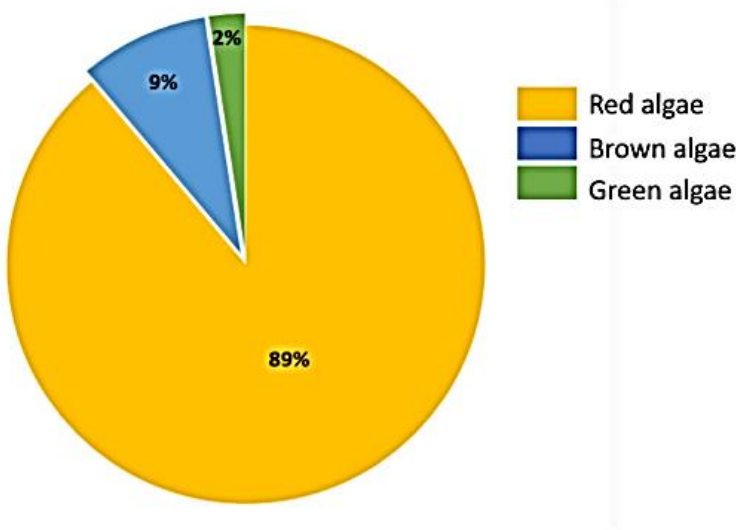


Figure 1.7: Distribution of the haloaryl secondary metabolites in macroalga by division (Jesus *et al*., 2019).

These compounds reveal a range of interesting biological activities, such as antitumour, antimicrobial, antidiabetic, and antioxidant activities (Liu *et al.,* 2011). Structure activity relationship (SAR) studies have demonstrated that the biological activity of bromophenols is dependent on the core structure and substituents. In some cases, the presence of bromine substituents seemed of little importance in terms of antioxidant effect, whereas the number of hydroxyl groups, as well as their mutual orientations contributed to antioxidant activity (Liu *et al.*, 2011). Bromines, however, were found to play a key role in anti-diabetic activity and cytotoxicity. The number of bromine substitutions was to play a role in toxicity against cancer cells, whereas the alkylation of phenol groups resulted in reduced activity. The activity towards diverse cell lines varied (Liu *et al.*, 2011). Polybromocatechols from *Neorhodomela aculeata* were found to possess anti-viral activity against two different groups of human rhinovirus (Park *et al*., 2012).

These haloaryl compounds were isolated from the Rhodomelaceae family. The origin was as follows - *Laurencia* (54 compounds), *Rhodomela* (43 compounds), *Symphyocladia* (23 compounds), *Polysiphonia* (20 compounds), and *Odontthalia* (12 compounds) (Jesus *et al*., 2019).

1.6 The *Polysiphonia* Genus

The *Polysiphonia* genus is in the Order Ceramiales and Family Rhodomelaceae. There are ~200 species worldwide and 19 of these are found on the coasts of the British Isles (Lee, 1986). Classification is usually based on microscopic features, including the number of pericentral cells, the cortication of main branches, with constriction of young branches at their base, whether the branching is dichotomous or spiral, and the dimensions of thalli. The *Polysiphonia* species exist as epiphytes and attach themselves to host plants and may themselves also act as hosts to endophytic fungi and epiphytic microalgae. These endophytic fungi are in turn being investigated as a source of novel compounds. For example, the endophytic fungus *Chaetomium globosum*, isolated from the inner tissue of *P. urceolata*, was found to produce chaetopyranin, a new benzaldehyde bioactive secondary metabolite with cytotoxic activity toward several tumour cell lines and a moderate antioxidant (Figure 1.8) (Wang, *et al.*, 2006).

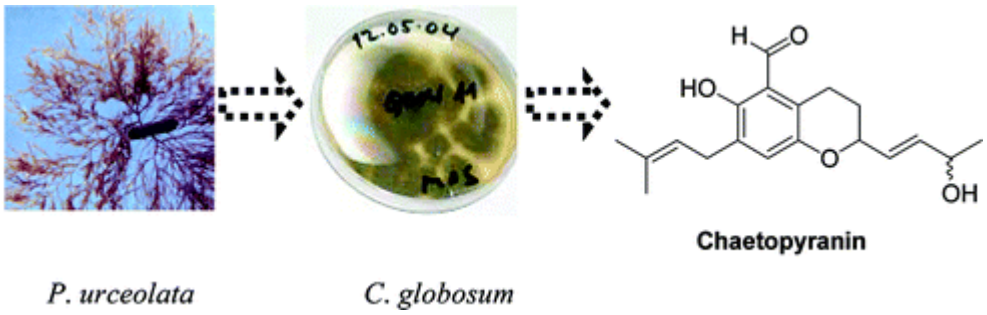


Figure 1.8: *Polysiphonia ureolata* as host to the endophytic fungus *Chaetomium globosum*, responsible for the production of chaetopyranin (Wang *et al.*, 2006).

Species of the genus *Polysiphonia*, such as *P. furcellata* C Agardh (Caccamese *et al.*,

1985), *P. lanosa* Linnaeus, *P. elongata* Hudson, *P. nigra* Hudson, *P. nigrescens* Hudson, *P. urceolata* Lightfoot ex Dillwyn (Hornsey & Hide, 1976), and *P. virgata* C Agardh (Vlachos *et al.,* 1997) have been found to contain antimicrobial compounds, and *P. hendryi* Gardner, antiviral compounds (Kim *et al.,* 1997).

Aligned to this, many investigations have focused on fatty acid profiles of *Polysiphonia* (Fleurence *et al.,* 1994). Fatty acids (FA) common to the *Polysiphonia* genus encompass PUFAs, such as arachidonic acid, with palmitic acid as the main saturated fatty acid and cis-vaccenic acid, cis-9-hexadecenoic and myristic acid as minor fractions in the fatty acid mixture (Trevor *et al.,* 1989). Fatty acids are being considered as possible therapeutic antimicrobial agents due to their efficacy, broad spectrum of activity and the lack of classical resistance to these compounds (Desbois & Smith, 2010). In particular, various long-chain polyunsaturated fatty acids (LC-PUFAs), which are found naturally at high levels in many marine organisms, including eicosapentaenoic acid (EPA; C20:5n-3), docosahexaenoic acid (DHA; C22:6n-3), γ-linolenic acid (GLA; C18:3n-6) and dihomo-γ-linolenic acid (DGLA; C20:3n-6) have shown pronounced activity against Gram-positive bacteria (Desbois & Lawlor, 2013). In addition, LC-PUFAs are also associated with anti-inflammatory properties in topical applications, accentuating their therapeutic advantage (Mullen *et al.,* 2010).

A selection of *Polysiphonia* studies are summarised below to illustrate the breadth of potential bioactive compounds produced by the genus.

1.6.1 ***Polysiphonia denudata* and *Polysiphonia denudata f. fragilis***

Prior to 2001, only fatty acids of *P. denudata* were reported but a comparative study by De Rosa *et al* (De Rosa *et al.,* 2001) investigated the chemical composition and biological activity of *P. denudata* and *P. denudata f. fragilis.* Biological activity was assessed by comparing chloroform, n-butanol and water extracts as well as associated volatile compounds against *Staphylococcus aureus, Escherichia coli* and *Candida albicans.* All fractions, except the water extracts displayed activity against *S. aureus,* although the activity was greater in *P. denudata f. fragilis* and only the n-butanol fraction from *P. denudata f. fragilis* showed activity against *E. coli.* All three extracts from *P. denudata f. fragilis* and the butanol extract from *P. denudata* revealed activity against *C. albicans.* The water fraction from *P. denudata f. fragilis* was only active against *C. albicans.* The

activity of α-O-methyllanosol (a major component of the n-butanol extract of *P. denudata f. fragilis*) was found to possess higher activity against all three microorganisms than the butanol extract itself. This observation serves to illustrate the potential presence of antagonistic components within crude extracts or that the extraction process was not optimised for the purification and separation of this specific constituent.

Cytotoxicity was also tested using *Artemia salina* (brine shrimp) with the n-butanol extract of *P. denudata f. fragilis* having strong cytotoxic activity. Volatile compounds formed part of the chloroform extract and possessed antibacterial activity (De Rosa *et al*., 2001). Volatiles are continuously emitted into the atmosphere from plants, and are known to contain defensive compounds, attractants, repellents and insecticides. The GC/MS results revealed considerable differences between the two species. Heptadecane was the main common hydrocarbon and apart from phenanthrene, all of the other hydrocarbons possessed straight chains. Phenanthrene concentrations were considered to be high for algae. Hydrocarbons and free fatty acids, common components of volatiles, may function as sex pheromones and allelopathic substances (De Rosa *et al*., 2001).

Polar constituents extracted with n-butanol possessed hydroxyl, amine and carboxyl groups. Silylation transformed these compounds into volatile ethers identified by GC/MS. The results reveal acids, carbohydrates and bromine-containing compounds as the main polar components. The GC/MS spectra further showed the presence of a range of bromophenol derivatives of benzyl alcohol. These are of particular interest, as compounds which are halogenated are known to possess antibacterial, antifungal, antiviral, anti-inflammatory, antiproliferative, antifouling, antifeedant, cytotoxic, ichthyotoxic, and insecticidal activity (Cabrita *et al*., 2010). Other potential compounds with anti-bacterial activity are the polar fatty acids, especially those which are either hydroxylated or phosphoric ester derivatives.

1.6.2 ***Polysiphonia hendryi***

High concentrations of dimethylsulphoniopropionate (DMSP) were found in a selection of macroalgae, including *P. hendryi.* This is an osmolyte and is converted to acrylic acid or acrylate depending on the pH and converted to dimethyl sulphide by the enzyme DMSP lyase when the algae is damaged (Van Alstyne *et al.*, 2001). A laboratory preference feeding experiment showed that acrylic acid deterred feeding by various predators at

specific concentrations, whilst the precursor DMSP functioned as a feeding attractant (Van Alstyne *et al.*, 2001). The localised concentrated acrylic acid has deterrent properties due to reactivity, acidity or an unpleasant taste as in the case of formic acid, used as a defence reaction among insects.

1.6.3 ***Polysiphonia urceolata***

P. urceolata has been used for the extraction of R-phycoerythrins using single-step chromatography with high yield and good purity (Liu *et al*., 2005). PE are able to absorb light in the 540-570 nm, highly fluorescent and used in immunodiagnostics. Together with PC and APC, PE is water soluble and is one of the 3 types of phycobiliprotein (PBP), extracted from cyanobacteria and red algae for a wide range of therapeutic applications (Sekar & Chandramohan, 2008). Seven new natural occurring bromophenols and previously identified structures from *P. urceolata* (Rhodomelaceae) were evaluated for their DPPH radical scavenging activity and found to be more potent than butylated hydroxytoluene (BHT), the positive control, with IC_{50} values ranging from 6.1 to 35.8 µM. These included 9,10-dihydrophenantrenes, 5,7-dihydrodibenzo[c,e]oxepine and urceolatin (Li *et al*., 2008).

1.6.4 ***Polysiphonia virgata***

Bioassay-guided fractionation of a dichloromethane *P. virgata* extract using direct bioautography resulted in isolation of a mixture of long-chain fatty acids, namely oleic acid, linoleic acid, lauric acid, and myristic acid as major antimycobacterial compounds. Oleic acid exhibited the inhibition of *Mycobacterium smegmatis* growth, whilst linoleic acid showed moderate inhibition of the growth of a clinical strain of multidrug-resistant *M. tuberculosis* (Saravanakumar *et al.*, 2008).

P. lanosa was the species investigated for this research and is discussed in more detail (Section 1.7).

1.7 ***Polysiphonia lanosa* – *Vertebrata lanosa***

Polysiphonia lanosa (Linnaeus) Tandy has a common Irish name: Olann Dhearg. Other synonyms for this species include *Vertebrata fastigiata* (Roth) Gray and *Polysiphonia fastigiata* (Roth) Greville (Guiry & Guiry, 2019).

Previous work undertaken at the Eco-Innovation Research Centre (EIRC), WIT had identified a crude aqueous *P. lanosa* extract as possessing significant activity against MRSA and provided the basis for further investigation in terms of a PhD research project. Published research articles include 'Development of a novel antimicrobial seaweed extract-based hydrogel wound dressing' (Tan *et al*., 2013) where the anti-MRSA potential of the extract is investigated in formulation as a hydrogel wound dressing.

The structure of *P. lanosa* comprises a series of tubes wrapped around a central axis to form cylindrical axes and branches. The tubes are elongated cylindrical cells surrounding a large, cylindrical 'central' cell. *P. lanosa* is easy to identify as it is a small, wiry, tufted seaweed which can grow attached to a host seaweed in the intertidal zone, Figure 1.10 (Lee, 1986). *P. lanosa*, is an example of a delicately branched, filamentous seaweed with a soft texture. It is described as 'cartilaginous, cylindrical, densely tufted, dark reddish-purple fronds, up to 75 mm in length, attached by creeping rhizoids with branches penetrating the host fronds of *Ascophyllum nodosum'* (Guiry & Guiry, 2016). The seaweed is repeatedly pseudodichotomously branched, apices pointed and widely forked. It has a large central siphon (tube) with 12-24 pericentral excorticate siphons, and the articulations are shorter rather than broad. The fact that it can have as many as 25 uncorticated pericentral cells has led to it being allocated and classified as a monotype genus, Vertebrata by some authors (Garbary & Deckert, 2002).

P. lanosa is hemi-parasitic on *A. nodosum*, (Figure 1.9), and more rarely on *Fucus vesiculosus*, never on rocks, although appearances can be deceptive as it can grow on old stalks of both species. It grows in the sheltered mid-tidal zone, generally distributed and usually abundant wherever *A. nodosum* occurs. Receptacles, air bladders, and damaged blades of this species serve as attachment sites for *P. lanosa* (Rawlence & Taylor, 1972). *P. lanosa* depends on *A. nodosum* for some amount of minerals and amino acids which it

receives directly from it (Ciciotte & Thomas, 1997). There is also a reciprocal exchange of carbon between *A. nodosum* and *P. lanosa* (Ciciotte & Thomas, 1997).

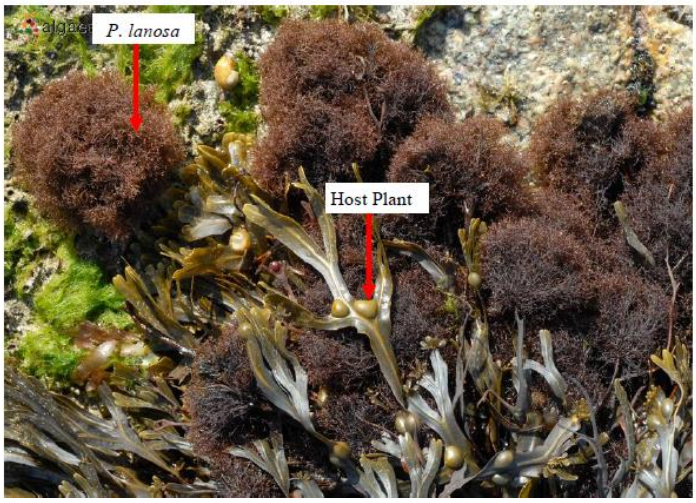


Figure 1.9: *Polysiphonia lanosa* (Guiry & Guiry, 2019).

Garbary *et al* (2009) were able to demonstrate for the first time that a physiological basis for the dependency of *P. lanosa* on *A. nodosum* existed. The exact inherent means of the photosynthetic interactions were not solved but the study showed that photosynthetic activity of *P. lanosa* declined when not attached to its host and that it also required its host for long-term maintenance of photosynthesis (Garbary *et al.*, 2014).

In 1985 two bromophenols, rhodomelol and methylrhodomelol were recovered by processing an ethanol extract of *P. lanosa* (Glombitza *et al.*, 1985). These are presented in Figure 1.10 and their respective chemical names are (2,3 dibromo-4,5-dihydroxyphenyl)methyl-trihydroxyl-dihydro-2H-furofuran-5-one and (2,3 dibromo-4,5-dihydroxyphenyl)methyl-dihydroxyl-6-methyl-dihydro-2H-furofuran-5-one.

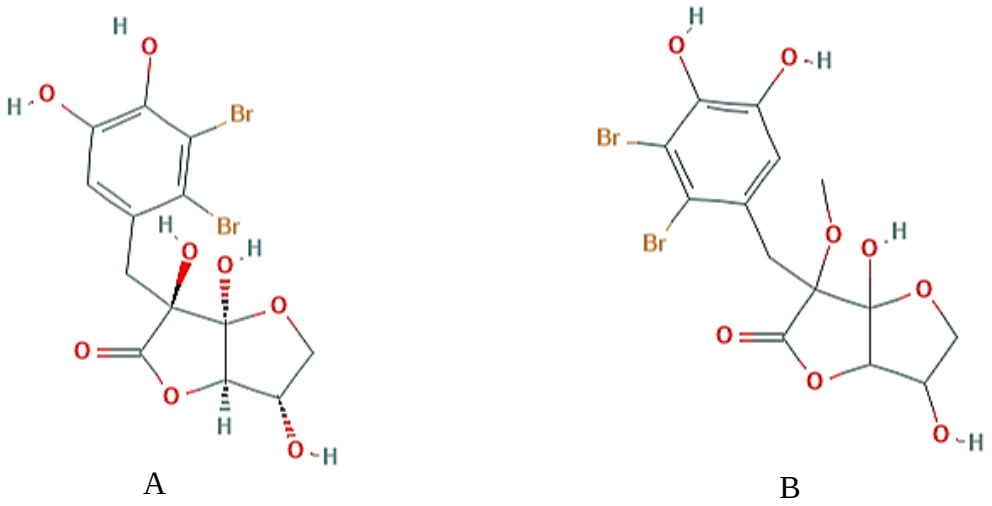


Figure 1.10: Structure of rhodomelol (A) and methylrhodomelol (B) (Glombitza *et al.*, 1985).

Bromophenols are common marine secondary metabolites, induced primarily by the ability of the phenol moiety to undergo electrophilic bromination. Red algae of the family *Rhodomelaceae* contain a large amount of various bromophenols (1%–5% dry weight) (Hodgkin *et al.*,1966) and display a number of beneficial features, including radical scavenging (Li *et al.*,2011), anticancer (Liu *et al.*,2012), anti-inflammatory (Wiemer *et al.*,1991), antimicrobial (Xu *et al.*, 2003) and α-glucosidase-inhibitory properties (Kurihara *et al.*, 1999).

In 2009, a comparative study of 24 species of red seaweed from the coasts of Brittany, found *P. lanosa* (one of two representatives of the family Rhodomelacea, the other being *Brongniartella byssoides*) to have the highest level of phenolic content which correlated with highest antioxidant activities (measured with both 2,2-diphenyl-1-picrylhydrazyl (DPPH) and reducing activity assays) (Zubia *et al.*, 2009). This study further highlighted the potential of the order Ceramiales as *Aglaothamnion pseudobyssoides* and *Hetero-siphonia plumose* represented the other two speciese providing the most active extracts.

Specific bromophenols isolated from *P. lanosa* include lanasol, lanosol methyl ether, lanosol ethyl ether and lanosol n-propyl ether. Lanosol is a known antimicrobial compound and its chemical name is 2,3-dibromo-4,5-dihydroxybenzyl alcohol. Its efficacy against both Gram-negative and Gram-positive bacteria is well-documented (Globitza *et al.*, 1974). The other three derivatives exhibited obvious cytotoxicity against

DLD-1 (human colon adeno-carcinoma) and HCT-116 (human colorectal carcinoma) cell lines (Shoeib *et al.*, 2004). The preliminary SAR supported the inference that activity is determined by the number and position of bromine substitution, as well as the number of phenolic groups and side chains (Shoeib *et al.*, 2004). The chloroform fraction was nearly 10-fold more cytotoxic than the parent methanolic extract (Shoeib *et al.*, 2004), this methanol extract having been partitioned between chloroform and water, where the different aldehyde derivatives would have varying solubilities in chloroform. This study also synthesized some derivatives and the most active compound was the synthetic compound 2,5-dibromo-3,4-dihydroxybenzyl n-propyl ether, which inhibited the proliferation of DLD-1 and HCT-116 cell lines (Inhibitory concentration to inhibit cell proliferation by 50% (IC50) = 1.72 and 0.08 µM, respectively), and arrested the cell cycle of DLD-1 cells (Shoeib *et al.*, 2004).

1.8 Aquaculture

Large quantities of seaweed are required for bioactive extraction due to the low yields of bioactive compounds obtained. However, there is an obvious limit to natural seaweed sources and seaweed aquaculture allows for the production of seaweed without impacting natural sources. Land-based agriculture provides over 10 billion tonnes per year of various products, mostly plants with decreasing arable land availability, fertiliser and fresh water for irrigation, compared to marine aquaculture which produces 100 million tonnes (Buschmann *et al*., 2017). The potential of algae (seaweeds and microalgae) as an option to guarantee food security is recognised by the European Commission where collective cultivation is anticipated to reach 56 million tonnes of protein by 2054, constituting 18% of global alternative protein market. Seaweed cultivation contributes to the global carbon, oxygen and nutrient cycles as well as reduce eutrophication and greenhouse gases (Buschmann *et al*., 2017). Algae are also able to adsorb heavy metals from solution, with some species capable of accumulating high concentrations within their cells (Malik, 2004). This bioremediation potential would assist the global challenge health, ecological, and environmental effects that excessive levels of nutrients and metals pose, resulting from population growth and increasing anthropogenic activity. The need

for alternative wastewater and industrial pollutant treatment further bolsters the requirement for massive investment in and growth of global algal aquaculture.

Marine macroalgae require sunlight, nutrients and salt water and are efficiently grown by aquaculture, contributing ~16 billion tons, valued at US$ 7.4 billion, to the global trade of aquaculture-derived production (Robinson *et al.*, 2013). This scale of production employs many thousands of people worldwide and is important in terms of future food security and sustainable blue carbon concepts. However, reliable cultivation strategies are needed to mitigate the risks and remove barriers to the growth and sustainability of the industry. The perception is that compared to terrestrial crops, seaweed cultivation is still in its infancy and, therefore, vulnerable (Buschmann *et al*., 2017). For instance, longitudinal and population-specific fluctuations in productivity can be affected by direct impacts (e.g. nutrient or temperature regimes) or ecotypic adaptations of populations to local environments, e.g. growth rates change along environmental gradients such as vertical zonation as studied for *A. nodosum* (Stengel & Dring, 1997) and wave-exposure for *Sargassum muticum* (Baer & Stengel, 2010). A further factor is that of climate change where the Earth's average surface temperature has increased by approximately 1.1°C with most of this warming experienced in the past three decades (NASA, 2020), meaning that new strains of seaweed with high thermo-tolerances will be required for the expansion of a sustainable seaweed aquaculture industry.

Figure 1.11 represents global algal output over the past six decades. It is of interest to see how the production of Rhodophyta mirrors total algal production, perhaps signifying the realisation of their potential.

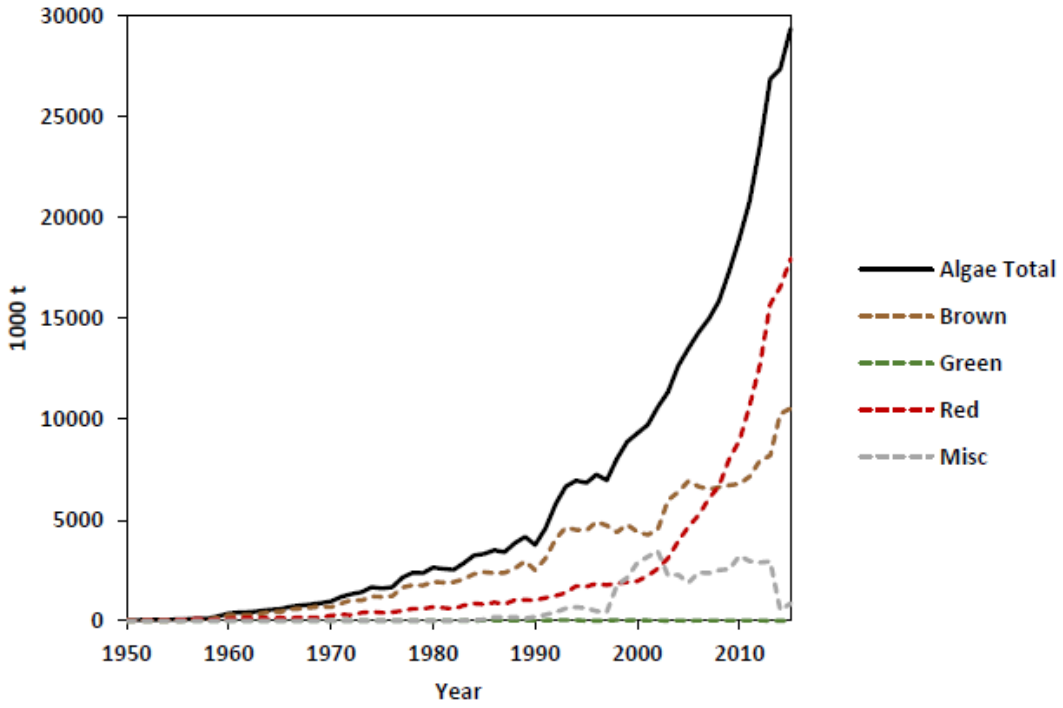


Figure 1.11: Global Algal Production since 1950 (Ross, 2017).

Natural seaweed populations are morphologically, physiologically and biochemically diverse because of genetic differentiation and ambient habitat parameters (Stengel *et al*., 2011). The strategic management of cultivated genetic seaweed resources has been identified as lacking and future demands for food will require increased usage of selectively bred stocks, improving efficient utilisation of nutrients, labour and water resources (Gjedrem *et al.*, 2012). However, selective breeding over the past two decades, as envisaged by Van Der Meer in 1990, has only been applied to a limited extent. Key strategies for genetic improvement of macroalgae for aquaculture are provided by Robinson *et al.* (2013). They conclude that, although more than 24 % of total world aquaculture production consists of seaweed (FAO, 2010), there are few published studies focussed on the genetic improvement of macroalgae. Lack of coordinated genetic management has already reduced the viability and value of some algal industries with successful genetic improvement programs targeting a few select economically important traits whilst limiting inbreeding, potentially producing large advantages for existing and developing seaweed industries (Robinson *et al.*, 2013).

Seaweeds have also been successfully incorporated into a number of demonstration and pilot-scale integrated multi-trophic aquaculture (IMTA) and nutrient bioextraction systems, where the by-products, including waste, from one aquatic species are passed on as inputs (fertilizers, food) for another. In Portugal, Matos *et al*., 2006 demonstrated that three red seaweeds, *Palmaria palmata*, *Gracilaria bursa-pastoris* and *Chondrus crispus* were effective in removing nutrients from the effluent of tank-based production of turbot (*Scophthalmus maximus*) and sea bass (*Dicentrarchus labrax*).

In comparison, Irish seaweed is predominantly hand-harvested from wild resources, mostly from the western and northern coasts. The species traditionally harvested include *A. nodosum* (*feamainn bhuí*; egg wrack*),* with ~25,000-30,000 wet tonnes harvested annually, representing the bulk of output, *Palmaria palmata* (*Creannach*, dillisk) (~30 wet tonnes), *Laminaria digitata* (*coirleach;* oar weed) (~200 wet tonnes) and maërl (8,000 wet tonnes, harvesting of which has been discontinued) (Walsh *et al*., 2013). Maerl is a hard, coral-like seaweed, pink in colour and twig-like in appearance and forms complex carpets on the seabed acting as a critical habitat for many commercial types of fish.

Aquaculture of a few select species does exist in Ireland but this has been concentrated at research- or pilot-scale. Several commercial seaweed farms do exist and production tonnages of between 50 and 90 wet tonnes of the kelps *Alaria esculenta* and *Saccharina latissimi* were recorded in 2013 (Walsh *et al*, 2013). In 2014, there were 7 existing seaweed aquaculture licenses, but in 2018, a further 17 were granted (Renwick, 2018). A 2016 report corroborates the Bord Iascaigh Mhara (BIM) figure for *A. nodosum* production at 29 000 wet tonnes and lists Irish farm cultivation of seaweed as in its infancy (Tabassum *et al*., 2017).

The bulk of Irish seaweed is exported to China for alginate extraction, used as a soil conditioner and fertiliser (*A. nodosum*), whilst the rest is utilised by small to medium sized Irish enterprises for production of cosmetics and thallasotherapy products, human food, plant growth stimulants and soil conditioners, as well as livestock and pet food supplements. BIM supports the seaweed-farming sector through the Seaweed Development Programme and the first successful sporulation of the higher value species *Porphyra umbilicalis* was conducted at a hatchery in Bantry in January 2017. A major

impediment to aquaculture expansion and progress in Ireland has been the licensing issue and in December 2016 an Independent Aquaculture Licensing Review Group was appointed (Renwick, 2018).

There are a number of Irish companies involved in developing and marketing higher value uses of seaweed. These include CyberColloids specialising in hydrocolloid chemistry and working with seaweed-derived food ingredients for many years. BioAtlanti**s,** based in Kerry, manufacture both plant strengthener products which enhance crop tolerance to stress and increasing marketable yield as well as feed additives. BioAtlantis acquired a new factory in 2014 in Tralee in County Kerry which is the largest seaweed factory in the British Isles, posessing a variety of extraction and purification technologies, together with innovative concentration and drying facilities.

In addition, international companies such as Cargill, Porto-Muinos and Maxicrop harvest high value bioactives from seaweed and marine sources for applications in animal and human health, encompassing pharmaceuticals, nutraceuticals and functional foods.

1.9 Seaweed Stressing

Macroalgae produce a variety of secondary metabolites as referred to in Section 1.3.3 which can be present continuously (constitutive defence) or induced (inducible defence), i.e. production is induced by an external agent (Pavia & Toth, 2000). In fact, the term secondary metabolite is one provoking discussion. Secondary metabolites are perceived to be compounds not involved in the basic machinery of life and, although they represent a miniscule fraction of the total organism biomass, their contribution to survival can often be comparable to metabolites involved in primary metabolism, in that they contribute to growth, reproduction and defence (Cabrita *et al.*, 2010).

The alternation of emersion and immersion periods endured by macroalgae represents immense stress. Seaweeds are generally found in parallel bands along the rocky intertidal zone as shown in Figure 1.4. Their distribution and relative abundance along the lower limits are dictated by biotic factors, such as predation and intra and inter-specific competition, whilst the upper limit is determined by abiotic factors such as UV radiation,

light, salinity, temperature, nutrient availability and air exposure. The severity and frequency of these environmental fluctuations dictate the survival strategies required to cope in these habitats (Parages *et al.*, 2014). These complicated response mechanisms are controlled by complex metabolic networks attuned to detecting stress signals which elicit the appropriate response. Molecules that may be involved in the stress response include mannitol (a sugar alcohol), polysaccharides, proline, abscisic acid, polyamines, unsaturated fatty acids, oxylipids and fatty acid desaturases (Kirst, 1990; Karsten *et al*., 1991). When ROS are produced in excess as a result of air exposure and water depletion, and the buffering buffering capacities of cells are overwhelmed, oxidation of macromolecules such as lipids and proteins takes place and may be accompanied by an increase in phycobiliproteins such as phycoerythrin, phycocyanin, and allophycocyanin which has been observed in *Pyropia orbicularis* and *Gracilaria corticata* during desiccation stress (Kumar *et al*., 2011; López-Cristoffanini *et al*., 2015).

The definition of stress requires clarification. It needs to be defined in terms of the response of an individual rather than the value of a particular environmental variable. This can be illustrated by the example that optimum temperatures for growth of tropical seaweeds may exceed the upper lethal limit of many Antarctic species. Grime (1977) defined stress as 'external constraints limiting the resource acquisition, growth or reproduction of organisms'. However, Davison and Pearson (1996) differentiate between limitation stress, such as the reduction in growth rate due to an inadequate supply of resources, and disruptive stress, where damage is caused by adverse conditions or the allocation of resources to prevent damage. Examples of limitation stress include low light and insufficient levels of inorganic nutrients whereas disruptive stress can be freezing or desiccation which cause cellular damage or require the allocation of resources to repair or prevent damage (Davison & Pearson, 1996). The stresses of salinity and desiccation are comparable because of the reduction of cellular water potential but differ in the ion uptake process, whilst the ratio between the two determines the acclimation potential.

Current research supports the hypothesis that the presence and adequacy of functional responses to desiccation in intertidal seaweeds defines their vertical position in the intertidal zone (Flores-Molina *et al.*, 2014). Representative intertidal seaweeds responded differently to desiccation, and those living higher in the intertidal regime displayed a

better tolerance to this stress. Desiccation, triggered by *in vitro* air exposure, led to a significant increase in ROS, inhibition of antioxidant enzymes, cellular alterations, and photosynthetic inactivation, mainly in the lower intertidal *Lessonia spicata* and *Gelidium rex* (Flores-Molina *et al.*, 2014). Detrimental effects observed during rehydration were also stronger in the lower intertidal species. For these reasons, it would be feasible to assume that *P. lanosa* would be extremely tolerant to desiccation stress (upper intertidal habitat).

1.9.1 *Stress Manipulation for Bioactive (secondary metabolite) Production in Macroalgae*

Environmental factors such as light, climate, depth, turbidity, UV exposure, nutrient availability, sampling location, sampling season, grazing pressure, salinity, biomass density, water motion, and temperature all impact seaweed tissue composition and there are a lack of studies in this linking these physiological changes to actual bioactivities. The first paper relating the application of abiotic stress to the production of bioactive metabolite compounds in a comprehensive selection of seaweed species was that of del Val *et al.* (2001). Standardised methanol extracts from 44 seaweed species collected from Gran Canaria were screened for antimicrobial activity against a panel of Gram-positive and Gram-negative bacteria, fungi, mycobacteria and yeasts. Twenty eight seaweed extracts were active against bacteria and six against fungi and yeasts under normal field conditions. Thirty two of the original 44 seaweeds were then cultured in bioreactors, subjected to stress and differences noted in the resulting antimicrobial activity. Two stress situations were created, one designated low stress with low density, replacement of seawater and addition of nitrogen and the other, high stress with higher stocking density and no replacement of seawater or nitrogen. Seventeen of these seaweed species exhibited activity under all three growth conditions, six of which displayed significant differences whilst, for the other species there was very little change. The results for these six species are shown in Table 1.3. Data serves to highlight the problem of comparing data across different studies and the requirement for standard protocols for all steps in any evaluation of bioactivity as subsequent as independent studies adopt various different methodologies and focus.

Table 1.3: Antimicrobial activities in different species of seaweed grown under different conditions (del Val *et al*., 2001) (n=2).

Seaweed species tested	Stress condition[1]	Microorganism activity[2]
Cymopolia barbata (Chlorophyta)	Field	Ent (+++), Sta (+++), Bac (+++), Can (++), Sac (+++), Asp (++)
	Low Stress, High Stress	Ent (+++), Sta (+++), Bac (+++), Can (++), Sac (++), Asp (+)
Dictyota sp. 1 (Phaeophyta)	Field	Sta (+++), Bac (+++), Can (+), Asp (+)
	Low Stress, High Stress	Sta (++), Bac (++)
Dictyota ciliolate (Phaeophyta)	Field	None
	Low Stress	Bac (++)
	High Stress	Sta (+), Bac (+++)
Gelidium arbuscula (Rhodophyta)	Field	Bac (+)
	Low Stress, High Stress	None
Sargassum furcatum (Phaeophyta)	Field	Bac (+++)
	Low Stress	None
	High Stress	Bac (+)
Solieria filiformis (Rhodophyta)	Field	Sta (+++), Bac (+++)
	Low Stress	Sta (+++)
	High Stress	Bac (+++)

[1]Field - seaweed collected from its natural habitat. Low Stress - cultured under low stress conditions for 10 days (low density, replacement of seawater and addition of nitrogen). High stress - cultured under high stress conditions for 10 days (higher stocking density and no replacement of seawater or nutrient nitrogen). All seaweed samples were desiccated.

[2]Microorganisms: *Asp; Aspergillus fumigatus, Bac; Bacillus subtilis, Can; Candida albicans, Ent; Enterococccus faecium, Sac; Saccharomyces cerevisiae, Sta; Staphylococcus aureus.* Antimicrobial activity classification: diameter of inhibition zone around the point of application of 25 µl of seaweed extract (+++; ≥15 mm, ++; < 15 mm, +; hazy/very hazy inhibition zone).

The interesting finding of this study was the stress-related differences in seaweed bioactivity. A range of field bioactivity was found which changed when the macroalgae were cultured under the two manipulated conditions (Table 1.3). It would appear overall that the compounds responsible for activity against *Bacillus subtilis* were more stable (or form a more substantial component of the macroalga) and were able to withstand considerable changes in conditions compared with bioactivities against other microorganisms. There was one case where bioactivity was induced by the applied stress conditions where previously there was none: *Dictyota ciliolate* displayed intermediate activity against *Bacillus subtilis* after being cultured under low stress conditions, but under high stress conditions, this was enhanced and was extended to include activity against *S. aureus.* There could be a number of reasons for this, including the fact that on harvesting, the macroalga had to adjust to new conditions and required different metabolic pathways to cope with the change. This situation was exacerbated under the high stress conditions and further activity was induced as well as 'new' activity against *S. aureus.* The reason for this observed change in activity was unknown but may have been due to the increase in compounds responsible for this specific antibacterial activity or the generation of synergistic compounds allowing for better activity as well as the formation of new compounds active against this other Gram-positive bacterium.

The other seaweed species revealed a negative response in terms of antimicrobial activity under stress conditions, in that the bioactivity was reduced or no longer evident. This could indicate that the metabolic pathways changed and that the levels of compound were no longer maintained or that the compound had degraded over the 10-day maintenance period under the stress conditions. The alteration of bioactivity induced by changing holding parameters extends the entire scope of seaweed potential and requires further investigation.

1.9.2 *Cultivation for the Production of Bioactive Molecules*

The unique ability of seaweeds to cope with harsh environments allows for the generation of novel sources of both pre-existing and unrecognised bioactive molecules, also referred to as high value added biomolecules (HVABs) with the potential to provide a range of benefits (Murray *et al*., 2013). However, the commercial exploitation of seaweed-derived

HVAB's remains limited; the main challenge is cost-effective production from sustainable sources. The potential of seaweed-derived bioactive molecules is, therefore, likely to be lost without the development of alternatives to wild harvesting. One such alternative is to cultivate the seaweed; either in its natural environment or *ex-vivo* in tanks (Gjedrem *et al*., 2012) as discussed in Section 1.8. However, in order to pursue seaweed-derived bioactives for biotechnological applications, a consistent supply is required and therein lies the paradox; the variable conditions producing the array of bioactivity within the marine environment necessitates refinement and control to ensure a viable yield of specific bioactive compounds. As a result, *ex-vivo* cultivation is more attractive, as conditions can be manipulated to achieve optimal production of the biomolecule of interest. This may also be relevant for the development of seaweed cell culture, where conditions could be manipulated for the production of a range of bioactive molecules.

To illustrate the potential possibilities that *ex-vivo* stressing of seaweeds offers in terms of inducing production of exploitable bioactive metabolites, specifically antimicrobials, it may be useful to present a hypothetical situation combining results from two studies. The first, which screened 38 seaweeds for antibacterial activity, identified a methanol extract of the red seaweed, *Laurencia papillosa*, as exhibiting maximum antimicrobial activity against both Gram- positive and -negative bacteria (Kavita *et al.*, 2014). The active compound fractionated by column chromatography was identified as a cholesterol derivative, 24-propylidene cholest-5-en-3β-ol, and may serve as a potential lead molecule for broad-spectrum drug development, as it was active against clinical isolates of *E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Shigella flexneri*. The other study investigated the effects of varying levels of photo irradiance on the lipid composition of another red alga, *Tichocarpus crinitus* (Khotimchenko & Yakovleva, 2005). The results revealed its ability to regulate lipid metabolism when grown for a period of 3 weeks under varying irradiance conditions. This alga was able to change the content of storage and structural lipids and FA composition, both in the total lipid pool and individual lipids, depending on the irradiance conditions. High light intensity (equivalent to applied stress) stimulated the accumulation of storage lipids, while low light induced an increase in structural lipids. While antimicrobial activity was not investigated, data from this study indicated the potential to manipulate the production of molecules with potential bioactivity *in vitro*. If these conditions could be applied to the *Laurencia papillosa* species

with a high yield of specific FA antimicrobial compounds, aquaculture of this species would become feasible.

As there are very few research articles dealing specifically with variations in bioactivity of macroalgae as a function of applied stress conditions, studies in which conditions responsible for a change in secondary metabolite production were identified and reviewed. The focus was on metabolites with possible bioactivity, specifically antimicrobial, as this was the main subject of the experimental chapters of this thesis. An overview of these studies was collated and is presented in Table 1.4. The table includes a range of 'compound' identities which may be involved in antimicrobial activity or indicate the onset of a stress-induced change within the macroalga examined.

Table 1.4: Manipulation of abiotic conditions to affect change in the production of specific compounds in macroalgae.

Compound(s)	Seaweed Species	Conditions for increase/method	Reversible	Duration of exposure	Other effects	Country	Reference
Phlorotannins (polyphenolics) (soluble) – act as deterrents against fish, urchins, gastropods and crustacean herbivores as well against pathogenic organisms.	*Lessonia nigrescens*	UV but also seasonal (summer only)/algal discs.	Not studied (but may be reversible)	3 days	Decreased inhibition of photosynthesis and lower DNA damage.	Chile	(Gómez & Huovinen, 2010).
MAPK-like proteins - stress indicators.	Rhodophytes - *Corallina elongata, Jania rubens.*	Desiccation imposed by low tide or high irradiance on samples.	Yes	1.5 hours	Calcification (calcium carbonate acts as a shield).	Spain	(Parages *et al*., 2014).
	Chlorophytes - *Chaetomorpha aerea, Ulva rigida*.	Low tide and high irradiance (no desiccation effect)/samples.	Yes	2.5 hours	Greater increase than for Rhodophytes (above)	Spain	
	Phaeophytes - *Dictyota dichotoma, Dilophus spiralis.*	High irradiance/samples.	Yes	3.5 hours	Higher than for Rhodophytes but lower than Chlorophytes.	Spain	
Porphyra extracts - investigated as protective solution against desiccation stress.	*Mazzaella laminariodes, Scytisiplhon lomentaria, Lessonia nigrescens.*	Spore suspensions cultured at 15 ˚C, 12:12 Light;Darkness photoperiod and 30-40 µmol photon m^{-2} s^{-1} irradiance during 12 h light period.	Yes	Development of spores recorded every 3-4 days during 20-30 days.	Extract provided compounds that protected different algal spores from desiccation stress (but only in naturally dehydrated extracts suggesting the induction of a specific stress tolerant metabolic pathway).	Chile	(Contreras-Porcia *et al*., 2012).
Various-concentrations of photosynthetic pigments, excess ROS, elevated activities of antioxidant enzymes.	*Mazzaella laminarioides, Scytosiphon lomentaria, Ulva compressa, Lessonia spicata, Gelidium rex.*	Desiccation/ blot drying and air exposure in a laboratory growth chamber at 16 ˚C and 70-80 µm photon m^{-2} s^{-1} irradiance for 4 h. A subset of dehydrated fronds were re-hydrated for 4 h as relative water content was reached.	Yes	4 hours (24 hours acclimation at 14 ±2 ˚C and 12:12 L:D photoperiod with 30-40 µmol photon m^{-2} s^{-1}).	Morphological changes Chemical changes i.e. increase in lipoperoxides except for *Scytosiphon lomentaria;* protein oxidation increased in all except *Mazzaella laminarioides* and *Scytosiphon lomentaria*.	Chile	(Flores-Molina *et al.*, 2014).

Table 1.4: Contd.

Compound(s)	Seaweed Species	Conditions for increase/method	Reversible	Duration of exposure	Other effects	Country	Reference
Various-lipid peroxidation, photosynthetic pigments, water soluble antioxidants, polyamines and fatty acids.	*Gracilaria corticata*	Desiccation/laboratory.	Yes	0, 1, 2, 3 and 4 hour monitoring (after 10 day acclimation).	Increase in chlorophyll and carotenoid concentrations initially, then gradual decrease at 3-4 hour, whereas phycoerythrin and phycocyanin increased until 2 hours; content of reduced and oxidised ascorbate increased; polyamines, putrescine and spermine increase, unsaturated fatty acids increase at expense of saturated fatty acids and monounsaturated fatty acids.	India	(Kumar *et al*., 2011).
Photosynthetic apparatus.	*Fucus vesiculosus* from the Irish Sea and the Baltic Sea.	Salinity, temperature, nutrient concentration/laboratory cultivation.	Yes	24 hour acclimation outside then cultivation inside for 5 weeks.	Optimum salinity for photosynthesis – Baltic seaweed able to grow in lower salinities (Irish sample experienced photoinhibition and tissue deterioration ; nutrient content of water increased photosynthesis and growth rates of both samples.	Ireland	(Nygård & Dring, 2008).
Hexitols, sorbitol and dulcitol (osmotic adjustment indicators).	*Stictosiphonia hookeri* sourced from 3 different regions.	Emersion-immersion cycles, salinity and temperature fluctuations/laboratory 500 ml Pyrex dishes.	ND	2-year old isolates – growth monitored weekly.	Sorbitol accumulation correlated with salinity – intracellular sorbitol concentration is a function of external salinity (also able to detect digeneaside but level unchanged).	Argentina and Chile	(Karsten *et al*., 1996).
Heat shock proteins and auxiliary anti-stress compounds.	Various.	Various.	ND	Various.	High solar stress-very high levels of mycosporine-like amino acids (commonly found in red algae) composition correlating with natural UV doses (acting as shield) with stress proteins acting after damage.	Various	(Cruces *et al*., 2012). (Review)
Polyamine metabolism changes.	*Porphyra cinnamomea*	UV-B radiation/laboratory with algal discs.	ND	3-hour acclimation.	Polyamine levels increased in response to UV-B- soluble putrescine increased by more than 200% in bound soluble spermidine and spermine by 150% and bound insoluble spermidine by more than 120%.	New Zealand	(Schweikert *et al*., 2014).

Table 1.4: Contd.

Compound(s)	Seaweed Species	Conditions for increase/method	Reversible	Duration of exposure	Other effects	Country	Reference
Phlorotannins (known to reduce fouling and attacks by pathogenic organisms).	*Ascophyllum nodosum.*	Survey of different sites for correlation between growth and phlorotannin production over a 2- year period. Adjacent annual shoots from 3 sites per study area were analysed for growth, variation in tissue N and phlorotannin content.	ND	Monitored over 2 years.	Negative relationship between phlorotannin concentration and annual growth – trade-off between growth and phlorotannin more pronounced in individuals with high N- content.	Sweden and Isle of Man	(Pavia et al., 1999).
Elatol (anti-protozoan).	*Laurencia dendroidea.*	4 separate studies with irradiance, nutrients, temperature and salinity changes.	ND	Elatol levels were determined after 1-month incubation of apical tip clones.	Irradiance had parabolic effect on elatol levels – from 3.2 mg/g alga up to 4mg/g alga and down to 2.5 mg/g alga- no significant effect. Nutrient enrichment had no effect on elatol levels. Highest levels of elatol were achieved at 20 and 25˚C, followed by 30˚C then at 15˚C. Extreme salinity (20 and 40%) resulted in low levels of elatol.	Brazil	(Sudatti *et al*., 2011).

ND – not determined

The study by Gómez & Huovinen (2010) considered the photoprotective role of phlorotannins in the kelp species *Lessonia nigrescens* and determined that phlorotannin synthesis was induced only during the summer, that artificial induction was fast and the increase was observed within three days, in support of this photoprotective role. However, there was also a positive relationship between the content of insoluble phlorotannin and the suppression of photoinhibition and DNA damage, suggestive of a primary metabolic role within the cell wall. These results allude to the interplay between UV stimulus, seasonal acclimation and intrinsic morphological-functional processes. Soluble phlorotannins (polyphenols) are known to be active against some pathogenic bacteria (Wang *et al*., 2009).

The presence and activation of two mitogen-activated protein kinase (MAPK) like proteins in a range of macroalgae were considered in the study by Parages *et al* (2014). The generation of these compounds was compared relative to their order, as MAPK-like proteins were found to be highly phosphorylated in all representative species in response to desiccation caused by low tide or high irradiance. It was found that phosphorylation was fastest in the Rhodophytes and due to desiccation, followed by the Chlorophytes where phosphorylation was due to both high irradiance and desiccation and then by the Phaeophytes where high irradiance was responsible.

Another study examined desiccation stress in the early development stages of various algal species and the severity of the effect was found to correlate with the vertical position of the algae in the intertidal zone (Contreras-Porcia *et al*., 2012). In addition, specialised metabolites were induced in *Porphyra columbina* under desiccation stress (by natural dehydration). This extract was then used for culturing sporelings of three other macroalgal species and exerted a protective effect when these were subjected to desiccation. High performance liquid chromatography (HPLC) was performed and the comparison of artificially dehydrated with naturally dehydrated extract chromatograms revealed distinct differences, although the precise individual components were not identified.

A further study highlighted the different responses to desiccation stress of macroalgae distributed at various heights in the intertidal zone (Flores-Molina *et al*., 2014). It was concluded that the presence and adequacy of functional responses to desiccation in common macroalgae dictates their altitudinal position in the intertidal zone. For example,

it was found that macroalgae dominating the upper littoral region were able to respond to desiccation (higher desiccation tolerance) with an overexpression of tolerance genes and could control their ROS production by activating antioxidant metabolism more effectively than those occupying the lower littoral regions. The upper littoral species produced less hydrogen peroxide, with lower induced levels of lipoperoxide and protein oxidation under desiccation conditions. (These differences may result in a change of bioactivity directly or change in compound level and/or influence co-factor(s) effects for bioactivity).

Gracilaria corticata inhabits the upper littoral zone and has to be prepared for desiccation which requires great energy expenditure and contributes to the slow growth of such species. The induction of polyamines was coupled with the accumulation of unsaturated FA as a response to oxidative stress due to desiccation. These FA maintained membrane fluidity during desiccation, demonstrating their role as a defence mechanism against desiccation-induced oxidative stress (Kumar *et al*., 2011). Phycobiliproteins such as PE, PC and APC also responded to the desiccation exposure with concentrations of both PE and PC increasing by 40% after 2 h of exposure and the level of APC following desiccation exposure considerably lower compared with the control.

Two samples of *Fucus vesiculosus* were compared, one from the Irish Sea and one from the Baltic Sea, to consider whether both respond in a similar way to applied stress (Nygård & Dring, 2008). The results indicated that the same amount of energy from photosynthesis was used for growth in both samples of the species at different salinities. The photosynthesis of *F. vesiculosus* in the northern Baltic was close to the minimum demand for growth, which may account for their small size. The temperature optimum for *F. vesiculosus* from the Baltic was 4–10 ˚C, while that for *F. vesiculosus* from the Irish Sea was 15–20 ˚C. The photosynthesis of Irish Sea plants was impacted less by exposure to high irradiances than that of plants from the Baltic (where the plants are more likely to be continually submerged). This demonstrates that plants of the same species from different regions possess different thermal stability due to their photosynthetic apparatus. This activity is under the control of the degree of saturation of FA in the thylakoid membrane and may signal changes in compounds responsible for bioactivity.

The adaptability of *Stictosiphonia hookeri* isolated from three climatic zones was examined to assess how physiological responses are affected by various conditions of salinity, light and temperature (Karsten *et al*., 1996). All three isolates grew well in hypo- and hypersaline media. This broad salinity tolerance is characteristic of red algae from the intertidal zone. The three isolates of *Stictosiphonia hookeri* were also able to grow well at very low irradiance as well as growing in a narrow range of temperatures. The differences in physiological response highlights the potential error in assumption that responses from algae in a single population characterise an entire species. All the tested isolates displayed similar sorbitol accumulation patterns with high concentrations correlating with high salinity in that internal sorbitol concentration increased as a function of external salinity. The accumulation of these compatible solutes permitted the generation of high intracellular osmolalities without incurring metabolic damage. These osmolytes may play a role in anti-bacterial activity as it has been found that the lower the ionic strength, the greater the bacterial killing by endogenous antimicrobials in body fluids. This could be the case with extracts displaying bioactivity, where on extraction these compounds act as synergists or antagonists (dependant on their ionic strength) for bioactivity (Zabner *et al*., 2000).

Stress protein expression (Cruces *et al*., 2012) revealed a variety of factors responsible for their induction including temperature, enhanced solar radiation and contaminants. A study was performed to determine whether these proteins operate complementarily with other protective mechanisms in two upper littoral species, *Ulva* sp and *Porphyra columbina*. However, it was found that the induction of stress proteins did not correlate with changes in photosynthetic reactions and antioxidant activity. The conclusion was made that these two seaweed genera possess opportunistic strategies associated with their small size, annual physiology and rapid photosynthetic adjustments to cope with high solar radiation. The reasons suggested for not producing these proteins was that the energy required was a limiting factor or that they are capable of maintaining high levels. The results were inconclusive, as although stress proteins were detected between 3 and 24 h, the responses did not correlate with photochemical and antioxidative response. It was noted that alternative rapid metabolic adjustments, such as the high thermo-tolerance of photosynthesis and efficient ROS scavenging with other biomolecules such as mycosporines, phenols, polyamines, etc. and morpho-functional adaptations to the

intertidal life (e.g. small size, high area/volume ratio) acting together, were responsible for protection.

A polyamine (PA) study revealed the sensitivity of these compounds (spermine, spermidine and putrescine) to UV-B light, in that they act as protectants of photosynthetic apparatus (light stress) (Schweikert *et al*., 2014). Seaweeds, like higher plants, respond with an increased activity of antioxidative enzymes when exposed to stress and earlier investigations proved that *Pyropia cinnamomea* (an upper littoral zone Rhodophyta) compensated for UV radiation by increasing PA levels, especially bound-soluble (bs) and bound-insoluble (bis) PAs. Data suggest that under UV-B radiation, an increase in free and bs PAs is favoured over an increase in bis PAs. Basic protection is continuously provided by bis PAs whereas bs PAs scavenge free radicals under acute UV-B stress. The consequence was that the levels of free PAs increased as much as bs PAs due to these also functioning as the bs precursor (Schweikert *et al*., 2014).

The last two studies cited in Table 1.4 refer to compounds known to be active against pathogens and these studies revealed different trends in terms of their production. For example, *A. nodosum* defence was secondary to growth, whereas in *Laurencia dendroidea,* elatol levels were predominantly influenced by temperature.

The changing of a cultivation parameter has the ability to affect change in bioactive compound production. Another question that may be posed is whether all secondary metabolites are biologically active? Algae represent a natural source of bioactive molecules with a broad range of biological activities such as antibiotic, antiviral, anti-tumour, antioxidant and anti-inflammatory (Chojnacka & Saeid, 2012) as outlined in Table 1.2. However, bioactivity is also dependent on the specific extraction process employed and these range from relatively simple to more sophisticated procedures. This is reflected in the large number of papers dealing with bioactive extracts, (Pangestuti & Kim, 2011; Cian, Martínez-Augustin, & Drago, 2012; Manilal *et al*., 2009; Sangha *et al*., 2013).

1.10 Extraction of Bioactive Compounds from Seaweeds

It is necessary to consider the challenges involved in the isolation of bioactive compounds from seaweed. These include variability of the source material (since an activity found in one collection may be absent in another due to origin, season, storage conditions, etc.), the difficulty of isolating the active constituents, the possibility that the active compound is a known compound (and therefore not protectable by composition-of-matter patents), and the expenses incurred on collection. The future sustainability of the natural products approach to drug discovery from marine sources depends not only on continued access to biodiversity through the environmental agencies and agreements derived therefrom, but also on using these bioresources as strategically and effectively as possible (Kingston, 2012; Morrissey & Donoghue, 2011).

Bioactivity is extract-specific so that all processes involved in the final acquisition of a sample extract contribute to its activity. Many compounds are sensitive to certain conditions; therefore, rigorous caution is required to prevent compound degradation or alteration during extraction. Another concern, once bioactivity is safeguarded by process development, is whether the activity is transferrable *in vivo*.

Sample handling and preparation have to be consistent in order to ensure that subsequent bioactive extraction is reproducible and reliable. The extraction process requires optimisation in terms of generating the precise extract composition required. Screening assays are crucial to the extraction of specific bioactive components as they dictate whether or not to pursue a process.

1.10.1 *Extraction and Purification of Bioactive Compounds from Seaweed*

Sample preparation relates to the handling of the fresh seaweed and it is essential that the sample be cleaned and rinsed of sand, salt, herbivores and epiphytes and that the time between harvesting and processing is kept to a minimum to reduce the chances of degradation. Natural product sampling is generally followed by drying in an oven, or air drying in the sun. However, this process will impact bioactivity if compounds responsible are heat sensitive or volatile. The freeze drying of seaweed samples is popular due to the

high water content and the retention or enhancement of activity (Salvador Soler *et al.*, 2007).

The extraction of compounds of interest from the non-soluble matrix in which they are implanted requires careful consideration. Pertinent factors include the polarity and stability of the desired extract and the solvent (its toxicity, volatility, viscosity and purity), the possibility of artefact formation during the extraction process, and the amount of bulk material to be extracted. Secondary metabolites are usually found within cells, so that maceration of the raw material ensuring disruption of tissue and cell integrity before extraction will affect both extraction yield and the bioavailability of extract components.

Figure 1.12 represents a typical extraction and purification method and was developed for the purification of glycolipids from *Osmandaria obtusiloba* (Souza *et al*., 2012).

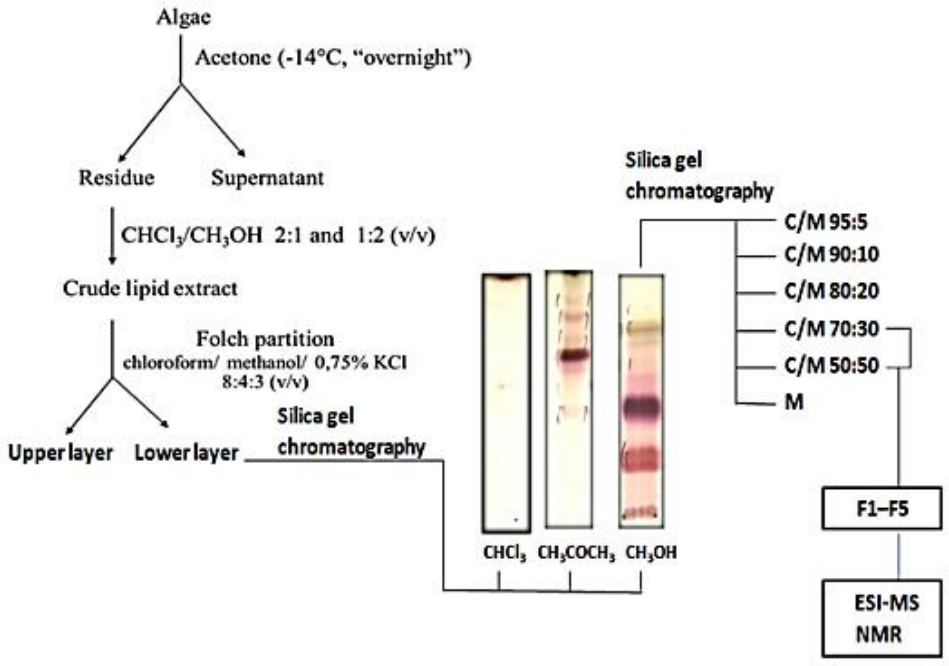


Figure 1.12: Overview of the strategy used for the purification of glycolipids from *Osmandaria obtusiloba* (Souza *et al*., 2012) with purified or partially purified fractions resolved by Thin Layer Chromatography (TLC) and visualised with iodine and orsinol-H_2SO_4 stain.

1.10.1.1 **Solvent Extraction Procedures**

Most bioactive isolation methods still feature simple extraction procedures with organic solvents of different polarity, water and their mixtures. The extraction methods using solvents include maceration, percolation, Soxhlet extraction, ultrasound-assisted extraction and turbo-extraction. Maceration can be achieved at room temperature by immersion of the material in solvent with constant stirring. This approach is advantageous as moderate extraction conditions are used but high solvent consumption, long extraction times and low extraction yields are disadvantages (Bucar *et al.,* 2013). Extraction yield is enhanced by percolation, i.e. packing the pre-soaked plant material in a container which allows the constantly controlled removal of the extract via a valve at the bottom, adding fresh solvent from the top. Soxhlet extraction is another favoured approach as it reduces solvent consumption, but the drawback is the potential degradation of thermo-labile compounds (Bucar *et al.,* 2013).

Ultrasound assisted extraction (UAE) is accomplished by immersing plant material in the extraction solvent within a glass container and placing this container in an ultrasonic bath. It decreases extraction time and increases extraction yields due to mechanical stress which causes cavitation and cellular breakdown, and has achieved growing popularity, as exemplified by the extraction of polyphenols from four *Ulva* species (Farasat *et al.*, 2014). The methanol extraction was initiated by placing the seaweed in an ultrasonic bath for 20 min. A further example is the extraction of lipids from various algal samples where freeze-dried algal samples were extracted with a chloroform/methanol (2:1, v/v) mixture at 25 °C in a sonication bath for 20 min. After sonication, the sample was filtered, collected, dried under vacuum and the residue dissolved in chloroform prior to FA and phospholipid analysis (Ragonese *et al*., 2014).

Microwave-assisted extraction (MAE) using either diffused microwaves in closed systems or focused microwaves in open systems is a common approach (Murray *et al.,* 2013). Microwave-assisted extraction works by heating the moisture inside the cells which evaporates, generating high pressure on the cell wall. This increases within the biomaterial and modifies the physical properties of the biological tissues (cell wall and organelles are disrupted) and improves the porosity of the biological matrix. This permits better penetration of extracting solvent through the matrix and improved yield of the desired compounds. Overall, MAE is more advanced than traditional solvent extraction

methods as it heats the matrix internally and externally without a thermal gradient, thereby allowing for more efficient and effective extraction of biologically active compounds (using less energy and solvent volume) (Joana Gil-Chávez *et al*., 2013). Microwave digestion has been used for the determination of total arsenic in edible seaweed samples followed by inductively coupled plasma mass spectrometry (ICPMS) (Llorente-Mirandes *et al*., 2011). A major disadvantage, however is that heat sensitive bioactive compounds may degrade or form new complexes.

Extraction with ionic liquids (ILs) together with UAE, MAE or alone as simple batch extraction of plant metabolites at room temperature or elevated temperature has received considerable attention (Bucar *et al.,* 2013). Ionic liquids also designated "designer solvents", are organic salts in the liquid state consisting of an organic cation and an organic or inorganic anion. Ionic liquids are capable of dissolving a wide variety of polar to non-polar compounds, are liquid below 100 °C, have a low vapour pressure, possess high thermal stability and low combustibility, and some are biodegradable. An example would be N-alkyl pyridium, a commonly used cation. Ionic liquids consist of at least two components that can be varied (anion and cation), ensuring that the solvent can be designed to dissolve bioactive natural products that may not be soluble in conventional solvents. An example is the pre-treatment evaluation of the red algae *Gelidium amansii* with a variety of ILs to compare sugar yields and minimise degradation to ascertain validity of this extraction method for biomass energy conversion (Malihan et al., 2014). Ionic liquids comprising 1-n-butyl-3-methylimidazolium (BMIM) cations with counterions such as chloride (≥98% [BMIM]Cl), hydrogen sulfate (≥94.5% [BMIM]HSO4) and tetrafluoroborate (≥98% [BMIM]BF4) and [BMIM] with acetate anion (≥98% [BMIM]OAc) as well as 1-n-ethyl-3-methylimidazolium chloride (97% [EMIM]Cl) and 1-allyl-3-methylimidazolium chloride (>98% [AMIM]Cl) were included in this study (Malihan et al., 2014). The ILs dissolved the lipids, leaving a carbohydrate rich solid residue and the lipids were then extracted with hexane, and the solid subjected to enzyme hydrolysis to release fermentable sugars. A selection of the IL compounds used is presented in Figure 1.13.

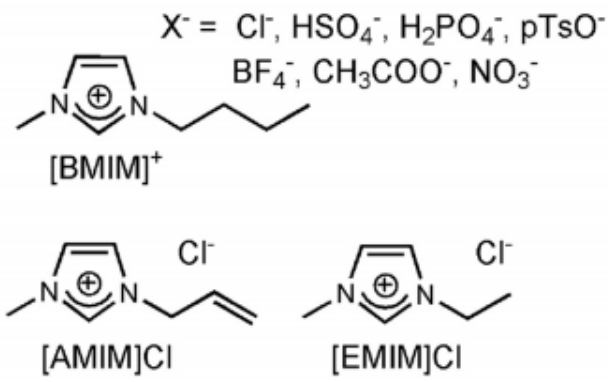


Figure 1.13: Imidazolium-based ionic liquids used as solvent IL in the biomass study by Malihan *et al.,* 2014.

Accelerated (pressurized) solvent extraction (ASE®), a patented system developed by Thermo/Dionex, removes the additional step inherent in most separations for the separation of the remaining non-soluble matter from the liquid extract, by online filtration within the automated extraction process of accelerated (or pressurized) solvent extraction (Bucar *et al.,* 2013).

Supercritical fluid extraction (SFE) involves replacing solvent extraction with extraction technologies which are less detrimental to the environment, thereby meeting increasing regulatory requirements. The current focus is on using supercritical CO_2. The use of organic solvents as modifiers for super-critical CO_2 to increase its solvating capabilities to medium- polar and polar compounds has improved the spectrum of natural product compound classes suitable for SFE (Bucar *et al.,* 2013). The red macroalgae *Hypnea charoides* was investigated as a nonconventional source of omega-3 fatty acids by extraction with SC-CO_2. Tests were conducted under mild conditions with a temperature range of 40–50°C and a pressure range of 241–379 bar. The different conditions influenced product yield, with higher temperature and pressure yielding better lipid recovery and ratio of unsaturated FA. The solubility and extractability of omega-3 fatty acids in supercritical CO_2 was also found to depend on the chain length (Cheung, 1999).

The above methods should be able to produce a bioactive extract, the extent of its purity at this stage, unknown. After generation of a bioactive crude extract, the next step is fractionation to differentiate between active and non-active fractions. This can be achieved using separation by solvent-solvent extraction or solid phase extraction.

1.10.1.2 **Separation by Solvent-Solvent Extraction**

Solvent-solvent extraction permits the distribution of the different components of the crude extract according to their solubility in various solvents or solvent mixtures. The choice of solvent is paramount and will determine whether maximum extraction is achieved. The polarity of a solvent is indicated by its polarity index and the higher the value, the more polar the solvent. Polar solvents such as methanol, ethanol and acetone are used for hydrophilic compounds and dichloromethane (DCM) and hexane are used for more lipophilic compounds. The isolation of a pure compound from hexane and chloroform-soluble fractions is perceived to be easier than from water extracts where ionisation of a compound may occur and the non-polar compounds that are extracted in hexane, benzene or chloroform are usually esters, ethers, hydrocarbons of terpenoids, sterols and fatty acids. The mixture of these compounds can then be resolved by standard chromatographic techniques over silica or alumina or by HPLC (Bucar *et al.*, 2013).

An example of the use of solvent-solvent extraction for seaweed is the extraction of antioxidants from *P. urceolata* where 100 g dried material was ground into a powder and extracted with 500 ml of a methanol chloroform (2:1v/v) solvent mixture in a Soxhlet extractor for 6 h and repeated twice. This combined organic solution was evaporated under vacuum to dryness and dissolved in 90% aqueous methanol. The solution was partitioned with 3 x 100 ml petroleum ether aliquots with the aqueous methanol evaporated under pressure yielding a semi-solid, which was then dissolved in 200 ml distilled water and further extracted successively with 3 aliquots of 100 ml ethyl acetate and 3 aliquots of 100 ml of n-butanol, respectively. These extracts were evaporated to dryness using a vacuum, yielding four products, i.e. the petroleum ether (1.4 g), ethyl acetate (1.2 g), n-butanol-soluble (1.6 g) and aqueous product (4.4 g), respectively (Duan *et al*., 2006).

If the polarity of the bioactive is unknown, the selection of an appropriate solvent system requires parallel assessment of the resultant bioactivity. Solvents generally used for the extraction of bioactives from seaweed include methanol, ethanol and acetone, but results can vary depending on the seaweed species and the nature of the bioactive compounds, which may exhibit a range of bioactivities, for example, a compound may possess both antimicrobial as well as antioxidant activity (Gupta and Abu-Ghannam, 2011; Cox, 2010).

1.10.1.3 **Separation by Solid Phase Extraction**

The components of a crude extract may also be separated based on their polarity, affinity and adsorption onto a solid phase. The solid phase is generally silica and the mobile phase is the solvent which carries the extract through the solid phase. The selection of both the stationary and mobile phases warrants considerable attention for the successful isolation of the active compounds. The various components will have different retention times on silica, providing the basis for separation. There are a variety of techniques available, including paper chromatography, thin layer chromatography (TLC) and flash chromatography (FC) as well as semi-preparative HPLC. These methods allow for the collection of fractions so that active fractions can be identified by screening for antimicrobial activity (Bucar *et al*., 2013).

An example is the processing of the red macroalga, *Haraldio phyllum* sp. Collected samples were air dried and then immersed in methanol for one month and vacuum filtered, yielding 185 g of dry residue. The evaporated residue was added to water (250 ml) and extracted with ethyl acetate. Evaporation of the organic phase gave a residue (330 mg) that was subjected to reverse phase FC (RP-18) with CH_3CN/H_2O gradient elution starting at 20:80 v/v and increasing the CH_3CN content in 5% increments so that 16 fractions of 40 ml each were collected. Evaporation of the combined fractions 11–12, produced a residue (46.5 mg) that was subjected to Si-60 FC with a hexane/ethyl acetate gradient elution. This study revealed a new indole alkaloid, named almazolone (Guella *et al*., 2006), as seen in Figure 1.14.

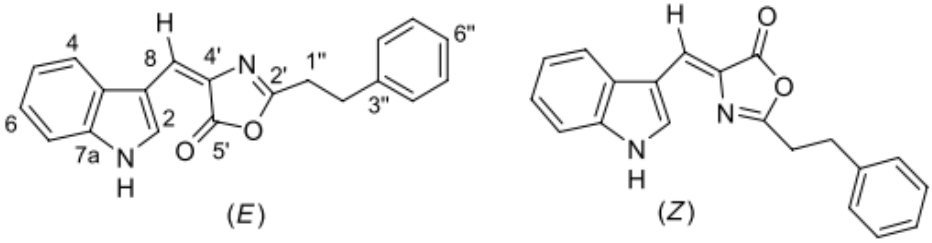


Figure 1.14: Structure of almazolone (an indole alkaloid), isolated as a 88:12 mixture of Z/E isomers (Guella *et al*., 2006).

1.10.2 *Characterisation of Purified Bioactive Compounds*

Structural characterisation of purified bioactive compounds which are possibly novel is difficult and chemical, physical and spectroscopic methods are used. The first step is to determine the molecular skeleton and this can often be assisted by referring to phytochemical literature on related genera and species. Databases, such as MarinLit, National Institute of Standards and Technology (NIST) and CHEMnetBASE may complement the search if a previously identified compound is suspected and comparisons may be drawn. However, if a novel compound is uncovered chemical, physical and spectroscopic methods represent the logical first step. Common spectroscopic methods used include UV visible (UV-Vis) spectroscopy, providing information on types of bonding in the compound, mass spectroscopy (MS), furnishing molecular mass data with fragmentation patterns of the compound, infrared (IR) spectroscopy, which clarifies the presence of different functional groups in the compound and nuclear magnetic resonance (NMR) which supplies information on the number and type of predominant carbons and hydrogens. X-ray crystallography can be performed if spectroscopic analysis is inconclusive and would provide a 3-dimensional image of the compound. Atomic Force Microscopy has been used to elucidate structural properties and molecular morphology (quaternary structure) of a purified lectin, by attaching it to a prepared mica surface (Cao *et al*., 2010).

Common hyphenated technologies include gas chromatography-mass spectrometry (GC-MS) which uses electron (or chemical) ionisation to create different fragments which are then compared with mass spectral library databases. Most natural product molecules are too large for GC-MS analysis as well as the limitation that the compounds need to be thermally stable with a low boiling point. Liquid chromatography-MS (LC-MS) is generally the preferred first option as it provides better fragmentation of large molecules with a better mass spectrum. Liquid chromatography – mass spectroscopy was employed to determine the identity of triacylglycerols (TAG), carotenoids and phospholipids from a variety of marine macroalgae - two species of green algae (Chlorophyceae), two species of red algae (Rhodophyceae) and four species of brown algae (Pheophyceae) (Ragonese *et al*., 2014). More precise data may be generated by employing a quadrupole-time-of-flight (Q-TOF) MS analyser, as different sized ions move at different velocities (Hill *et al*., 2009). These three compound classes possess a range of bioactivity, such as use as

antioxidants and in the case of phospholipids, there exists increasing support for their use in the treatment of inflammatory diseases to regulate the inflammatory reaction (Küllenberg *et al*., 2012). Another hyphenated technology is that of HPLC-NMR which has demonstrated its ability to offer valuable insight into the structure, class and the range of secondary metabolites present in a crude extract (Timmers *et al*., 2012). On- and stop-flow HPLC-NMR analyses (combined with WET 2D NMR spectra) was able to identify the major component plocamenone derived from the red alga *Plocamium angustum* and partially identified its unstable double bond isomer isoplocamenone. The crude extract exhibited potent cytotoxicity and significant antimicrobial activities (Timmers *et al*., 2012).

More powerful NMR techniques exist and provide more specific information on the compound(s) of interest. These include correlation spectroscopy (COSY) where the spin-spin coupling within the molecule is attained and provides the number of hydrogens attached to a particular carbon atom. Heteronuclear chemical shift techniques such as Heteronuclear Single-Quantum Coherence (HSQC) and Heteronuclear Multiple-Quantum Correlation (HMQC) also supply information on which particular hydrogen atom is attached to a specified carbon atom (Seo, 2015). Use was made of the HSQC spectrum for polysaccharides obtained by aqueous extraction of the red alga *Gracilaria caudata* after 2 h at 100 °C (Barros *et al*., 2013). The study concluded that this polysaccharide fraction consisted mainly of alternating residues of 3-linked-β-d-galactopyranose and 4-linked-3,6-α-l-anhydrogalactose with some hydroxyl groups substituted by methyl groups and pyruvic acid acetal. These agarans and carrageenans exhibit a broad range of bioactivities comprising antioxidant, gastroprotective, anti-inflammatory, antinociceptive, anticoagulant, anticancer, immunomodulatory, antiproliferative and antithrombogenic (Barros *et al*., 2013). Antibacterial activity of sulphated polysaccharides, common in Rhodophyta has also been reported (dos Santos Amorim *et al*., 2012).

1.11 Antimicrobial Resistance

The discovery and use of antibiotics has resulted in a decline in morbidity and mortality that would have been associated with bacterial infectious diseases before the advent of this "antibiotic era". However, the anthropogenic use of antibiotics in both the clinical setting and in related areas of human activity such as agriculture has applied a natural selective pressure on bacterial pathogens, with the resultant acquisition and diffusion of resistance (Fromm *et al.*, 2014), ultimately undermining antibiotic efficacy in the clinical context. The significance of the challenge was highlighted by the estimate that by 2050, 10 million lives a year are at risk (O'Neill, 2016). In 2016 it was reported that 700,000 people die of resistant infections every year (O'Neill, 2016). Over 80,000 severe methicillin resistant *Staphylococcus aureus* (MRSA) infections with 11,285 deaths were reported in 2011 with the caveat that MRSA is the leading cause of healthcare-associated infections (Centers for Disease Control and Prevention, 2013). In 2016, the United States (US) Congress appropriated an unprecedented $160 million of new investments for the Centers for Disease Control and Prevention (CDC) to fight antibiotic resistance which increased to $163 million for 2017, with $168 million allocated for the 2018 fiscal year (CDC, 2019). The U.S. CDC estimated that more than 2 million Americans are infected with bacteria that are resistant to antibiotics every year (FDA, 2016).

There is a range of multi drug resistant (MDR) pathogens including MDR *Mycobacterium tuberculosis*, and other hospital-linked infections such as *Acinetobacter baumannii*, *Burkholderia cepacia*, *Campylobacter jejuni*, *Citrobacter freundii*, *Clostridium difficile*, *Enterobacter* spp., *Enterococcus faecium*, *Enterococcus faecalis*, *Escherichia coli*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella* spp., *Serratia* spp., *S. aureus*, *S. epidermidis*, *Stenotrophomonas maltophilia*, and *Streptococcus pneumoniae*, the description "Superbugs" referring to microbes with enhanced morbidity and mortality as a result of multiple mutations conferring high levels of resistance to the antibiotic classes specifically recommended for their treatment. As a consequence, the therapeutic options for these microbes are reduced and the associated periods of hospital care are extended and more costly. These superresistant strains may also have acquired increased virulence and enhanced transmissibility with the suggestion that antibiotic resistance is in fact another virulence factor (Davies & Davies, 2010).

Antimicrobial resistance (AMR) within microorganisms is due to the selective pressure of evolution. There are a number of mechanisms that perpetuate the emergence and diffusion of AMR, including mutation (stochastic or mutagen-induced), gradual increased tolerance to sub-lethal concentrations of compounds, horizontal gene transfer through mobile genetic elements (plasmids, transposons and insertion sequences) and recombination events (such as those between a bacteriophage and a bacterial host) (Pendleton *et al*., 2013; Wellington *et al*., 2013).

The evidence suggests clear relationships between antimicrobial use and the emergence of multi-resistant, extensively drug-resistant and pan-drug-resistant strains (Boucher *et al*., 2009). A UK parliament paper (2018) outlined the reason that there has been no discovery of new classes of antibiotics for decades with pharmaceutical companies being more concerned about the profitability of new antimicrobial drugs combined with the need to protect new antibiotics from overuse in order to reduce the emergence of resistance (Health and Social Care Committee, 2018). This situation requires new approaches to encourage research and development in the public interest. Investment in basic scientific research is essential, but this should be accompanied by investment from pharmaceutical companies in further development and bringing products to the market. Prospective options have included changes to patent law and changes to the ways that pharmaceutical companies are reimbursed for new antimicrobial medicines. This needs to be combined with the conservation of the effectiveness of current antimicrobials by upgrading infection prevention and by taking action with respect to inappropriate and unnecessary prescribing (Health and Social Care Committee, 2018).

In 2014 only three antibiotics were granted approval by the FDA; two of which were based on vancomycin and the third based on linezolid, which was the first oxazolidinone approved by the FDA in 2000 (Wong & Rab, 2014) for the treatment of acute bacterial skin and skin structure infections (ABSSSI). However, since the release of linezolid in 2000, there have been reports of MRSA strains that are resistant to linezolid due to the acquisition of a natural resistance gene known as chloramphenicol florfenicol resistance (cfr). In June 2014, the FDA approved tedizolid phosphate (Sivextro, Cubist Pharmaceuticals) as a second-generation oxazolidinone with potentially 4- to 16-fold potency against MRSA when compared with linezolid (Wong & Rab, 2014). Favourable results from clinical trials have led to its indication for the treatment of ABSSSI in adult

patients with susceptible bacteria. In all probability, resistance genes will evolve and the cycle of manipulating current antibiotics to effect short-term success will continue. These approvals, therefore, further reveal the necessity for the pursuit of novel therapeutic strategies, based on new targets and approaches directed at preventing the emergence of antimicrobial resistance.

1.11.1 ***MRSA***

Methicillin, the first β-lactamase-resistant penicillin was introduced in 1961 and soon after, strains of *S. aureus* resistant to it were detected within the United Kingdom (David & Daum, 2010). *S. aureus* is the most frequently isolated human pathogen and a significant cause of skin and soft-tissue infections, endovascular infections, pneumonia, septic arthritis, endocarditis, osteomyelitis, foreign-body infections and sepsis (Boucher *et al*., 2009). MRSA isolates are resistant to all available penicillins and other β-lactam antimicrobials.

The genetic basis of methicillin resistance in *S. aureus* is a staphylococcal cassette chromosome mec (SCCmec) which is a mobile cassette of genes and it is the *mecA* gene that confers the resistance to β-lactams including methicillin. The peptidoglycan synthesis enzyme penicillin binding protein 2a (PBP2a) is involved in cross-linking peptidoglycan in the bacterial cell wall (Harkins *et al*., 2017). PBP2a has a decreased binding affinity for the β-lactams and the antibiotic is unable to bind to the bacterial cell wall. It has been suggested that the *mecA* gene was assembled into a gene complex containing its transcriptional regulators and incorporated into a mobile genetic element before it was assimilated by MRSA (Tsubakishita *et al.*, 2010).

Methicillin-resistant strains of *S. aureus* are capable of growth in media that include methylpenicillin and derivatives, including oxacillin, nafcillin, and methicillin. This resistance is activated by the expression of a modified penicillin-binding protein, PBP2a (Hartman & Tomasz, 1984). Alternative therapeutic strategies directed at the induction mechanism of the resistance gene (Arêde *et al*., 2012) would overcome its expression and extend the clinical utility of β-lactams for the treatment of MRSA infections.

MRSA was at one stage confined to hospitals, other health care environments and to patients frequenting these institutions. However, since the mid-1990s there has been an explosion in the number of MRSA infections in populations not associated with health care facilities (David & Daum, 2010).

Labelled as community-associated MRSA (CA-MRSA), these strains were distinct from hospital-associated MRSA (HA-MRSA) strains, with HA-MRSA strains carrying a relatively large staphylococcal chromosomal cassette mec (SCCmec) belonging to type I, II, or III containing the signature *mecA* gene found in nearly all MRSA isolates. This was the cause of resistance to many classes of non-β-lactam antimicrobials. These HA-MRSA strains seldom carried the genes for the Panton-Valentine leukocidin (PVL) although a 2011 UK report estimated that 20% of soft tissue infections were caused by PVL positive *S. aureus* and of these, 10% were PVL positive MRSA (Fogo *et al.*, 2011). The CA-MRSA isolates do carry smaller SCC*mec* elements, commonly SCC*mec* type IV or type V as well as the *mec*A gene and are are resistant to fewer non-β-lactam classes of antimicrobials and often carry PVL genes.

However, a further complication is the observation that some CA-MRSA infections are caused by HA-MRSA strains associated with the expanding common practice of complex HA-MRSA being treated in homes. The emergence of these new CA-MRSA strains has considerable implications and since 2003, the distinctions between CA-MRSA and HA-MRSA have become vague (David & Daum, 2010).

1.11.2 *MRSA Biofilms*

The challenge posed by MRSA rests not only on its antimicrobial resistance but also on its capacity to form biofilms (O'Neill *et al*., 2007). A basic definition of a biofilm is a surface–attached mode of growth where the microorganisms produce extracellular polysaccharides, lipids and proteins i.e. extracellular polymeric substances (EPSs) which provide a viscous, slimy barrier, resistant to external threats (Percival *et al.*, 2012). The surfaces may be living or non-living and biofilms are present in natural, industrial and hospital settings. The first stage of biofilm formation is adherence to a surface aided by flagella and pili in Gram-negative bacteria and surface proteins in Gram-positive bacteria

(Macia *et al*., 2014). Microbial (single or multiple species) cells inhabiting a biofilm are physiologically distinct from planktonic cells of the same organism. The bacterial cell switches to the biofilm mode of growth and is associated with a phenotypic change occurring (Sauer *et al.*, 2002). Large suites of genes controlling the metabolic state of individual cells are differentially regulated so that, genetically identical, the cells are able to diversify and respond more effectively to nutrient and oxygen stress. This change to biofilm mode may also contribute to the resistance to antibacterial compounds in the environment through drug tolerance, with or without promotion of persister cell formation (Sauer *et al.*, 2002). Furthermore, the biofilm provides a mechanism that promotes the dispersal of biofilm cells to a more habitable environment, if the stress becomes too great.

Conventional antibiotics are often ineffective against biofilms, which can require 10- to 1,000-fold-higher concentrations than their planktonic counterparts (Høiby *et al.*, 2010). This complicates therapy by conferring a high degree of innate resistance to antimicrobials, even those to which the causative bacteria are susceptible as planktonic organisms. Figure 1.15 illustrates the two ways in which a biofilm may be generated from planktonic bacteria. The top pathway shows the development of non-surface-attached biofilms. These are able to form chronic wounds, for example, on the lungs of patients with cystic fibrosis, where bacteria are not attached to a surface but embedded in mucous or other host material. The pathway below depicts surface-atttached biofilms, where bacteria are attached to the surface of biomaterials such as implants or catheters. The blue circles represent susceptible bacteria and red circles represent tolerant bacteria. (Bjarnsholt *et al*., 2013).

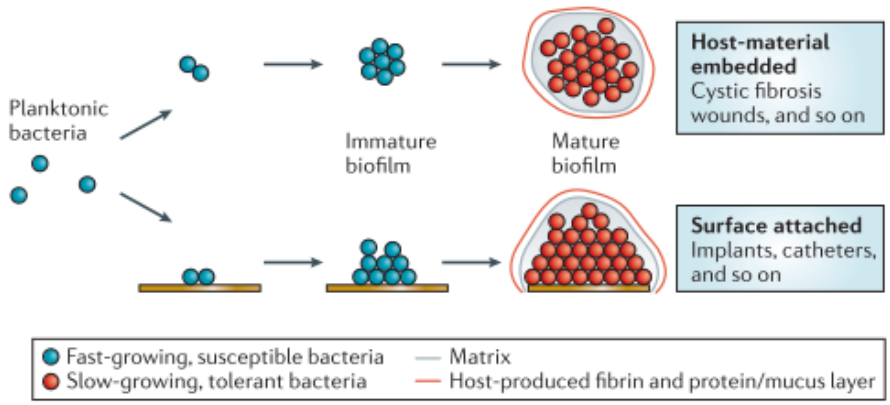


Figure 1.15: The development of bacterial biofilms from planktonic bacteria (Bjarnsholt *et al*., 2013)

The significance of biofilms in the failure of medical devices and chronic inflammatory conditions is becoming more appreciated. Increasingly, research is focusing on the association of biofilms with wound infection and non-healing of chronic wounds. It was reported as far back as 2008, that there was substantial evidence that biofilms are present in the majority of chronic wounds (Dowd *et al.,* 2008). Specialized microscopic techniques used since 2008 have allowed several research groups to demonstrate that 60% to 90% of chronic wounds have biofilms, contrasting with only 6% of acute wounds (Skrlin, 2016).

Biofilm bacteria possess molecular mechanisms for recruiting other bacteria and it is apparent that biofilms actively attempt to become polymicrobial in order to improve their survivability. The current perception is that biofilms are regarded as systems with extensive regulation due to expanded gene pool provided by the species diversity in a particular biofilm (Wolcott *et al.,* 2013). Biofilms are entities on their own, as has been accepted in other microbial environments with the recognition of bacterial social intelligence (Ben-Jacob, 2014). The biofilm entity exerts central control over the individual members to yield the activities necessary for the colony's survival. In a wound environment, biofilms require gene expression that permits attachment to the host, produces host cellular senescence to inhibit shedding, and causes local inflammation that creates plasma exudate for continuous colony nutrition (Wolcott *et al*., 2013). Microorganisms may combine their genetic resources to fulfil these requirements, so that each individual member of a biofilm need not possess all of the genes necessary to carry out each function. This has led to the proposal of functional equivalent pathogenic groups, which are frequently identified as recurring groups of microorganisms in biofilm infections. This is illustrated by the interaction between *S. aureus* and *P. aeruginosa*, where *S. aureus,* unable to produce a strong biofilm in isolation, forms a pathogenic grouping with *P. aeruginosa* and both are able to maintain chronic infection, impairing wound healing and increasing the development of antibiotic resistance (Serra *et al.,* 2015).

A biofilm is characterised by distinct subpopulations of bacteria with different metabolic states (Dakheel *et al*., 2016). The cells on the surface of the biofilm are aerobic, whilst those located deeper with reduced oxygen concentration are fermentative and dormant. These distinct matrix layers result in different selective pressures and the emergence of

antibiotic-resistant strains. Further expansion of this is that bacteria are able to respond to their neighbours in ways that fall outside strict definitions of either signalling or metabolite exchange (Short *et al*., 2014). For instance, some bacteria produce antibiotic compounds that may have a role in competition for niches or resources and it is accepted that antibiotics at sub-lethal concentrations can change the behaviour of some clinically relevant bacteria by promoting survival or infection. For example, treatment of *S. aureus* with low level of beta-lactam antibiotics was found to promote biofilm formation (Kaplan *et al*., 2012). Furthermore, many bacteria produce the metabolite indole, which is able to modulate the virulence of some bacterial pathogens that do not produce it (Lee & Lee, 2010). Indole suppresses *P. aeruginosa* virulence through interference with acyl homoserine lactone (AHL) quorum sensing, and also suppresses virulence of *S. aureus*, where it acts to repress the expression of several virulence factors including α-hemolysin and enterotoxin (Lee, Cho, *et al*., 2013).

MRSA biofilms are an important contributing factor for many treatment failures, persistent infections, increased risk of replacement of medical devices, and the morbidity and mortality of most MRSA-associated infectious diseases (Yang *et al.,* 2017).

More recent research into the extracellular matrix of *S. aureus* biofilms (Foulston *et al.*, 2014) may be considered where previous biofilm formation theories were discounted. *S. aureus* is routinely found growing as a biofilm in catheters and chronic wounds. The first premise was that *S. aureus* biofilm formation relied solely on the generation of an extracellular polysaccharide, but this polysaccharide intercellular adhesion (PIA) is no longer considered a prerequisite. Extracellular DNA (eDNA) which is released from cells by regulated autolysis is assumed to reinforce the structural stability of *S. aureus* biofilms.

Biofilm-related diseases are typified by a persistent infection that develops slowly, is rarely resolved by the immune response and which responds inconsistently to antimicrobial therapy and may include a number of conditions such as infectious kidney stones, bacterial endocarditis, and cystic fibrosis lung infections (Parsek & Singh, 2003). The pursuit of persister cells as actual targets for drug development has been adopted as exemplified by a letter by Kim *et al* in Nature – ‘A new class of synthetic retinoid antibiotics effective against bacterial persisters’ (Kim *et al.,* 2018), dealing specifically with MRSA.

The significance of biofilms cannot be underplayed in soft tissue infections and an *in vitro* model of chronic wound biofilms has been developed to test wound dressings and to assess antimicrobial susceptibilities (Hill *et al.,* 2010). Pharmaceutical companies continue to focus and commit billions of dollars on the discovery of new drugs, but less attention is directed towards designing new compounds that would prevent biofilm formation. The screening and efficacy of existing drugs as possible candidates for biofilm prevention continues rather than directive research into the molecular level interaction between bacteria and surface colonisation.

1.11.2.1 **Seaweed Extracts and Biofilm Inhibition**

Although marine algae inhabit seawater and should present a favourable environment for the formation of biofilms, they rarely occur (Jha *et al.*, 2013). Marine algae possess potent defence mechanisms to prevent biofilm formation in the form of metabolites produced to inhibit QS. It was from a red macroalga, *Delisea pulchra,* that the first QS inhibitory compound was isolated and its role in AHL regulatory systems and QS inhibition was demonstrated (Givskov *et al*., 1996). *Delisea pulchra* is known to synthesize halogenated furanones. (Manefield *et al.*, 2002). These small molecules are also known as autoinducers (AIs) and their usage allows bacteria to sense the presence of other bacteria when they gather on a surface. As the biofilm develops and cell density increases, the concentration of AIs attains a threshold, at which gene expression is altered. This QS differs between different strains of bacteria, with Gram-negative bacteria using AHLs to communicate and Gram-positive bacteria, processed oligopeptides. Other QS-mediated phenomena include sporulation, virulence factor expression, conjugation and pigment production (Bharati & Chatterji, 2013).

This has led to intensive screening of seaweeds for novel QS inhibitors with antifouling capabilities, as biofilm formation is a significant contributor to fouling in marine habitats. It is a QS-sensing-mediated process and as QS controls bacterial biofilm differentiation and maturation, its disruption may prevent microbial biofouling. Biofouling is of interest in biomedical research as it means that seaweed extracts may potentially prevent and/or disrupt biofilms in a clinical setting.

1.11.2.2 **Red seaweed and Compounds Responsible for Biofilm Inhibition**

It has been reported earlier that red algae show the highest antifouling activity among the three groups of marine macroalgae. A study, conducted in 2013 screened 30 species of seaweed with representatives from the three divisions for QS-inhibition activity against *Chromobacterium violaceum*, a reporter strain which is a mutant incapable of producing AHL and violacein, a purple pigment (Jha *et al.*, 2013). It is a versatile and easy-to-use reporter that responds to exogenous AHLs and is widely used in QS inhibition assays. A single Rhodophyta, *Asparagopsis taxiformis* was found to be positive for QS inhibition and the compound responsible was 2-dodecanoyloxyethanesulfonate ($C_{14}H_{27}O_5S$) with a calculated molecular mass of 307.4 (Jha *et al.*, 2013).

The screening for anti-fouling capability of seaweed extracts uses activity against Gram-positive bacteria to identify potential new leads. Table 1.5 describes six different approaches to such determinations (Satheesh *et al.*, 2016). These fouling inhibition processes may alternatively represent mechanisms of biofilm formation prevention as indicated by biofilm inhibition impact. This inter-relationship of biofilm and biofouling can be traced back to 1997 when it was stated that antibacterial activity against Gram-positive bacteria is a prerequisite for an anti-fouling compound screen (Hellio *et al*., 2000). This initial screening method, using a disk diffusion assay was considered simple, time- and material-efficient as it did not require removal of large quantities of seaweed from the environment (Devi *et al*., 1998).

Table 1.5: General modes of action of anti-fouling compounds on fouling organisms (Satheesh *et al.*, 2016).

Mode	Mechanism	Biofilm Inhibition Impact
Inhibitors of adhesive production/release by fouling organisms.	Prevents settlement of fouling organisms.	Positive
Inhibitors of microbe attachment.	Alters surface to prevent attachment of fouling organisms.	Positive
Quorum sensing blockers.	Affects cell-cell communication between microbial cells.	Positive
Protein expression regulators.	Alters protein expression to inhibit settlement of fouling organisms.	Positive
Blockers of neurotransmission.	Interrupts signal transduction during settlement.	Positive
Surface modifiers.	Blocks attachment of the bacteria.	Positive

1.11.3 *Current Research into anti-MRSA compounds*

The only three antibiotics approved by the FDA in 2014 were oritavancin (Orbactiv), dalbavancin (Dalvance) and tedizolid phosphate (Sivextro) to treat dangerous skin infections (Duke Antimicrobial Stewardship Outreach Network (DASON), 2014). They were all targeted at patients with skin and skin structure infections caused by drug-resistant bacteria, including MRSA, other *Streptococcus* species and *Enterococcus faecalis*. These three drugs were categorized as Qualified Infectious Disease Products (QIDP) under the Generating Antibiotic Incentives Now Act because they treat serious or life-threatening infections and bestow upon the manufacturer five additional years of marketing exclusivity before a generic product is permitted on the market (FDA, 2016). However, it can be argued that these do not represent novel products, as they are not in fact first generation discoveries, but new lipoglycopeptide and oxazolidinone derivatives.

Platensimycin, isolated from extracts of *Streptomyces platensis,* and its analogue platencin have been identified as potential agents against multidrug resistant bacteria (Allahverdiyev *et al.*, 2013). *In vitro* and *in vivo* studies have demonstrated that both of

these new antimicrobials are able to inhibit MRSA, vancomycin-resistant enterococci, and penicillin-resistant *Streptococcus pneumoniae* by targeting type II fatty acid synthesis in bacteria. Unfortunately, the pharmacokinetic profile for platensimycin is poor and requires optimisation before clinical use (Sköld, 2011).

In the past, most antibiotics were screened for by bio-prospecting soil microorganisms, but this finite resource of cultivable bacteria was over-mined in the 1960s and synthetic approaches to generate antibiotics have proved incapable of providing an alternative. It has been estimated that as much as 99% of microorganisms are not easily cultivable, but culture independent methods using total DNA of the bacterial community have currently allowed detection of many of these uncultivable organisms permitting consideration of these previously unavailable natural product resources (Martins *et al*., 2014). Ling *et al.* (2015) developed several methods to grow previously uncultured microorganisms by cultivation *in situ* or by using specific growth factors. A new antibiotic designated teixobactin was discovered in a screen of these previously uncultured bacteria. It inhibits cell wall synthesis by binding to a highly conserved motif of lipid II (precursor of peptidoglycan) and lipid III (precursor of cell wall teichoic acid). No mutants of *S. aureus* or *Mycobacterium tuberculosis* resistant to teixobactin were recovered. Ancillary to the activity against MRSA and VRE, tests in mice revealed its potential against other bacteria that cause septicaemia, skin and lung infections (Ling *et al.*, 2015).

1.11.4 *Anti-MRSA Extracts from Seaweed*

A comprehensive investigation by Tan *et al* (Tan *et al.*, 2011) established a methodology for the screening of seaweed extracts for anti-bacterial compounds and the purification of active fractions. Numerous other papers are also available in this field (Smyrniotopoulos *et al*., 2010; Oh *et al*., 2008; Stirk *et al*., 2007). Further, the study by Tan *et al*. (2011) confirmed that the efficacy of the seaweed extract was solvent-dependent, indicating that multiple components within the selected seaweed, *Ulva lactuca,* may be responsible for antibacterial activity. The spectrum of antibacterial activity for various solvent extractions differed for each solvent, with the ethyl acetate-derived extract selected as the most promising due to its consistent activity against three Gram-positive bacteria, including MRSA. A seasonal comparison found that activity was highest for samples

harvested in autumn/winter compared to samples sourced in spring/summer. This confirmed that the production of metabolites responsible for antibacterial activity depended on a variety of factors, including abiotic conditions, geographic location and physiological factors, such as growth phase and the impact of herbivore grazing (Tan *et al.*, 2011). The purified fraction was extremely potent against MRSA and a 10 µg disc loading inhibited MRSA growth. Another significant observation was that extract activity increased during storage at -20 ˚C. The results from this study then formed the basis for the development of a hydrogel wound dressing utilising the bioactive extract (Tan *et al.*, 2013). Table 1.6 presents a review of a range of extracted compounds from red seaweed and their MIC values against *S. aureus* or MRSA.

Table 1.6: Red seaweed extracts and their MIC against *S. aureus* or MRSA.

Extract source	Solvent	Compound if known	MIC µg/ml MRSA (unless other)	Reference
Various Rhodophyta	H_2O		50 000-200000 *(S. aureus)*	(Alghazeer *et al.*, 2013)
Rhodomela confervoides	MeOH	Bromophenols	70 - 140	(Xu *et al.*, 2003)
Sphaerococcus coronopifolius	DCM: MeOH, (3:1)	4 novel tetracyclic brominated diterpenes.	15 -128	(Smyrniotopoulos *et al.*, 2010)
Osmundaria serrata	Ethanol: ethylacetate (1:1)	LEE	690	(Barreto & Meyer, 2006)
Odonthalia corymbifera	Not disclosed	2,2,3,3-tetrabromo-4,4,5,5-tetra-hydroxydiphenylmethane, 2,2,3-tribromo-3,4,4, 5-tetrahydroxy-6-hydroxymethyl diphenylmethane, and 3-bromo-4-(2,3-dibromo-4,5-dihydroxybenzyl)-5-methoxymethylpyrocatechol.	25-50 *(S. aureus)*	(Oh *et al.*, 2008)
Laurencia papillosa	Methanol	24-propylidene cholest-5-en-3-β-ol.	MIC (90) 1.06 *(S. aureus)*	(Kavita *et al.*, 2014)
Laurencia chondrioides	DCM	Elatol, chamigrene-type sesquiterpenoid and similar compound.	1875 (extract) synergy. These derived compounds were not active against *S. aureus*	(Bansemir *et al.*, 2004)

1.12 Antioxidants

Reactive oxygen species are continually generated during the metabolism of living organisms and are eliminated efficiently by enzymatic and non-enzymatic defence systems. However, when subject to stressful conditions these can fail, leading to an accumulation of ROS and other free radicals, which cause irreversible damage. Oxidative stress conditions are caused by endogenous excessive formation of ROS that overwhelm the availability of antioxidants which is further impacted by external stressors. An imbalance in the antioxidant status (ROS versus defence and repair mechanisms) causes oxidative stress in humans. Among the endogenous defences are enzymes such as superoxide dismutase, catalase and glutathione peroxidase (SOD, CAT and GPX), plus vitamin E, uric acid and serum albumins. Exogenous antioxidants may have positive effects in the removal or suppression of the generation of ROS. Excessive ROS can induce apoptosis and cause damage specifically to cellular proteins, PUFA, and DNA. Approximately 200 diseases, including cardiovascular disease, cancer, atherosclerosis, hypertension, ischemia, diabetes mellitus, hyperoxaluria, neurodegenerative diseases (Alzheimer's and Parkinson's), rheumatoid arthritis, as well as aging, are exacerbated by the effects of oxidative stress (Machu *et al.*, 2015). Figure 1.16 depicts the factors and potential adverse effects associated with excessive ROS (Lachance *et al*., 2001).

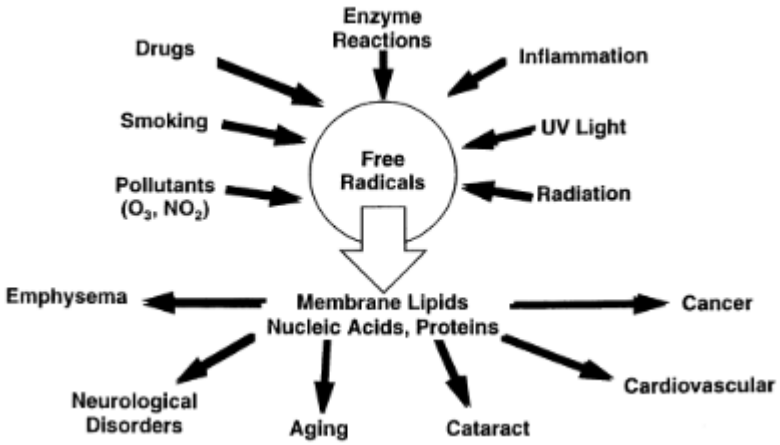


Figure 1.16: A schematic of a hypothesis recognizing the endogenous and exogenous sources of free radicals and possible adverse consequences of imbalances in free radicals (Lachance *et al*., 2001).

An antioxidant is defined as a substance that opposes oxidation or inhibits reactions promoted by oxygen or peroxides. They include tocopherols (Vit E) which act as a preservative in products such as fats, oils, food products, and soaps by slowing the progression of rancidity, or in petroleum products by inhibiting gum formation. Dietary antioxidants are added to food to inhibit oxidative deterioration as well as for the prevention of chronic diseases. Dietary antioxidants include polyphenols, Vitamins C and E, as well as carotenoids. The output of literature pertaining to antioxidants is vast and mass media has focused on the benefits of consuming natural antioxidants (Huang *et al*., 2005).

Biologically, antioxidants can be synthetic or natural and are added to products to prevent or retard their deterioration by oxygen in air. Synthetic compounds, such as butylhydroxyanisole (BHA), butylhydroxytoluene (BHT) and tert-butylhydroquinone, are currently used in processed food products but are considered potentially dangerous in terms of their toxicity and carcinogenic nature at high concentrations (Antolovich *et al.*, 2002).

A polyphenol consists of one or more aromatic rings, with one or more hydroxyl groups attached, and can be categorised into several groups, such as phenolic acids (hydroxybenzoic acids, hydroxycinnamic acids), flavonoids (flavones, flavonols, flavanones, flavanonols, flavanols, anthocyanins), isoflavonoids (isoflavones, coumestans), stilbenes, lignans, and phenolic polymers (proanthocyanidins - condensed tannins and hydrolysable tannins). Polyphenols occur mainly in fruits and beverages, such as tea, wine, and coffee, as well as in vegetables, leguminous plants, and cereals. Their concentrations in foods differ according to many factors (genetic, environmental, processing technologies). Generally, phenolic acids account for one third of the total dietary intake of polyphenols and flavonoids account for the remaining two thirds, with the most abundant flavonoids being flavanols, anthocyanins, and their oxidation products (Zern & Fernandez, 2005). Bioavailability of polyphenols differs considerably. Their primary function in plants is for protection against UV radiation and pathogens as well subsidiary roles in pigmentation, reproduction and plant growth.

The potential that antioxidants may possess antimicrobial activity necessitates examination of whether both bioactivities are, in effect, due to the same compound(s).

1.12.1 *Seaweed as a Source of Antioxidants*

Seaweeds are exposed to high concentrations and combinations of light and oxygen, which allows the generation of free radicals and other oxidative reagents. However, the absence of structural damage suggests that they produce antioxidants to protect themselves against oxidative degradation. Enzymatic protection is provided by the antioxidant enzymes, SOD, glutathione (GR,) ascorbate peroxidase (APX) and CAT, as well as ascorbic acid (José Aguilera *et al.*, 2002).

Natural antioxidants isolated from macroalgae include phlorotannins (Zubia *et al.*, 2009), pigments such as chlorophylls, carotenoids as well as the PBP, PC and APC (both pigments responsible for the colouration of Rhodophyta), vitamins and vitamin precursors such as α-tocopherol (Vit E), β-carotene (Vit A), niacin (Vit B3), thiamine (Vit B1) and ascorbic acid (Vit C), phenolics such as polyphenolics and hydroquinones and flavonoids, phospholipids particularly phosphatidylcholine, terpenoids and peptides (Pangestuti & Kim, 2011). This is borne out by the inherent stability of algal products against oxidation during storage, reflecting their capacity to neutralize superoxide and hydroxyl radicals. The main categories of plant-derived antioxidants identified in seaweeds are carotenoids, phenolics, alkaloids (Herraiz & Galisteo, 2003) and organosulphur compounds (Sahu, 2002). To date, research in the area of seaweed antioxidants has focused primarily on polyphenol compounds and carotenoids with the profile of antioxidant compounds in algae differing depending on the colour of the species. For instance, the antioxidant pigments α-carotene, β-carotene, lutein and zeaxanthin are common in both red and green algae, while brown seaweeds possess β-carotene and fucoxanthin. Fucoidan (or heterofucan, also referred to as a sulphated polysccharide), a cell wall component with potent antioxidant activity (Ngo *et al.*, 2011), is exclusively found in brown seaweeds.

In recent years, studies have demonstrated that algal extracts and fractionated composites play an important role as free radical scavengers and antioxidants in the prevention of oxidative damage in living organisms. One such study, linking antioxidant capability to anti-inflammatory potential revealed that a crude extract and semi-purified fractions isolated from the red alga *Laurencia obtusa* exhibited a high anti-inflammatory and analgesic activity compared to reference drugs in rats (Lajili *et al.*, 2015). Interestingly,

a higher polarity ethanol fraction with higher levels of both phenolics and flavonoids demonstrated superior activity. Fresh seaweeds possess labile antioxidants such as ascorbic acid and glutathione as well as more stable molecules such as carotenoids, MAA and a variety of polyphenols (Bocanegra *et al*., 2009). Fucoxanthin, one of the most abundant carotenoids found in brown algae, reportedly to exhibit preventive cancer effects through antioxidant, antiproliferative and anti-angiogenic mechanisms in both *in vitro* (cell culture) and *in vivo* murine studies (Rengarajan *et al*., 2013).

1.12.2 *Red seaweed and Antioxidant Compounds*

This is focused on the bioactivity profile of *P. lanosa* with previous studies having recorded high levels of antioxidant activity in *Polysiphonia* extracts, in particular from *P. urceolata,* from which a variety of bromophenols (BPs) have been isolated and identified as antioxidant molecules (Li *et al.,* 2007). A subsequent investigation by Zubia (Zubia *et al.,* 2009) found a high phenolic content in crude extracts (derived by solvent extraction using methanol:DCM) from *Brongniartella byssoides* and *P. lanosa* and suggested that BPs might be responsible for the antioxidant activity of these extracts.

Although the first two marine BPs were isolated from the red algae *Rhodomela larix,* later discoveries included novel BPs from diverse species of marine algae, including red, brown and green species (Liu *et al.,* 2011). It was also noted that species harvested at low tide possessed a higher level of simple BPs and that species of red algae have a relatively higher content of certain BPs, in support of their reported abundance in red algae. In fact, BPs are common marine secondary metabolites, biosynthesized in the presence of bromoperoxidases, hydrogen peroxide, and bromide (the concentration of bromide is approximately 0.65 mg/kg in both seawater and marine algae). It is assumed that BPs play a role in chemical defence and deterrence, with numerous studies recording a wide spectrum of beneficial biological activities, including antioxidant, as well as antimicrobial and cytotoxic properties (Liu *et al.,* 2011); (K. Li *et al*., 2011); (Sabeena Farvin & Jacobsen, 2013).

Until 2011, approximately 30 BPs from marine algae were identified as having antioxidant activity *in vitro* without the support of *in vivo* antioxidant studies. Bioactivity

in relation to the SAR in BPs has been presented and remains topical (Liu *et al*., 2011).

For antioxidant activity, the presence of bromine substituents appears to exert little effect, whilst the number of hydroxyl groups is obvious with their mutual orientations contributing to antioxidant activity and ortho-substitution having the greater effect (compared to para-substitution), both permitting easy quinone formation. Antioxidant activity is enhanced by conjugation to substituents such as acetyl, nitro or phenyl groups (Liu *et al*., 2011). (It should be noted that phenolic compounds usually exist as esters in nature and require hydrolysis for their identification and quantitative determination).

Table 1.7 reveals a selection of compounds, pertinent to this discussion, specifically bromophenols and their IC 50 values, as well as results for crude extracts of *Polysiphonia* species. It is obvious that the BP compounds are extremely efficient antioxidants and efforts have been made to understand the relationship of SAR, albeit in individual papers as results can vary between researchers (Duan *et al*., 2007), (Li *et al*., 2012), (Sabeena Farvin and Jacobsen, 2013).

The fact that antioxidant activity may correlate with antibacterial activity as well as cytotoxic activity provided the basis for extending the scope of this research. Potential compound/s and/or functional groups may permit clarification of the characterisation study.

Table 1.7: Antioxidant activity of various Rhodophyta-derived compounds and extracts.

Compound	Molecular formula	Molar mass	Rhodophtyta Source	IC_{50} µg/ml	Reference
1,2-bis(3-bromo-4,5-dihydroxyphenyl) ethane	$C_{14}H_8Br_6O_4$	719.55	*Symphyocladia latiuscula*	14.13 (7.34)	(Duan *et al*., 2007)
1-(2,3,6-tribromo-4,5-dihydroxybenzyl)- pyrrolidin-2-one	$C_{11}H_{10}Br_3O_3N$	441.82	*Symphyocladia latiuscula*	7.95	(Duan *et a*l., 2007)
6-(2,3,6-tribromo-4,5-dihydroxybenzyl)- 2,5-dibromo-3,4-dihydroxybenzyl methyl ether	$C_{15}H_{11}Br_5O_5$	640.62	*Symphyocladia latiuscula*	6.73	(Duan *et a*l., 2007)
2,3,6-tribromo-4,5-dihydroxybenzyl methyl sulfone	$C_8H_7Br_3O_4S$	438.76	*Symphyocladia latiuscula*	10.53	(Duan *et al*., 2007)
bis (2,3,6- tribromo-4,5-dihydroxyphenyl) methane	$C_{13}H_5Br_6O_4$	698.52	*Symphyocladia latiuscula*	5.60	(Duan *et al*., 2007)
bis(2,3,6-tribromo-4,5-dihydroxybenzyl) ether	$C_{14}H_8Br_5O_5$	650.62	*Symphyocladia latiuscula*	5.53	(Duan *et al*., 2007)
2,3,6-tribromo-4,5-dihydroxybenzyl methyl ether	$C_8H_7Br_3O_3$	388.85	*Symphyocladia latiuscula*	6.02	(Duan *et al*., 2007)
2,3,6-tribromo-4,5-dihydroxymethylbenzene	$C_7H_5Br_3O_2$	357.78	*Symphyocladia latiuscula*	5.00	(Duan *et al*., 2007)
2,3,6-tribromo-4,5-dihydroxy-benzaldehyde	$C_7H_2Br_3O_3$	370.75	*Symphyocladia latiuscula*	9.15	(Duan *et al*., 2007)
3-(2,3-dibromo-4,5-dihydroxybenzyl) pyrrolidine-2,5-dione	$C_{11}H_8Br_2NO_4$	375.88	*Rhodomela confervoides*	1.96	(Li *et al*., 2012)
Methyl 4-(2,3-dibromo-4,5-dihydroxybenzylamino)-4-oxobutanoate	$C_{12}H_{13}Br_2NO_5Na$	431.90	*Rhodomela confervoides*	2.46	(Li *et al*., 2012)
4-(2,3-Dibromo-4,5-dihydroxybenzylamino)-4-oxobutanoic acid	$C_{11}H_{11}Br_2NO_5Na$	417.8902	*Rhodomela confervoides*	2.27	(Li *et al*., 2012)
3-Bromo-5-hydroxy-4-methoxybenzamide	$C_8H_9BrNO_3$	245.97	*Rhodomela confervoides*	5.80	(Li *et al*., 2012)
2-(3-Bromo-5-hydroxy-4-methoxyphenyl) acetamide	$C_9H_{11}BrNO_3$	259.99	*Rhodomela confervoides*	5.41	(Li *et al*., 2012)
Crude DCM: MeOH (1:1)	unknown		*Polysiphonia lanosa*	2.71	*(Zubia et al., 2009)*
Crude aqueous extract	unknown		*Polysiphonia fucoides*	111.4	(Sabeena Farvin & Jacobsen, 2013)
3-(3-bromo-4,5-dihydroxyphenyl)- 2-(3,5-dibromo-4-hydroxyphenyl) propionic acid	$C_{15}H_{11}Br_3O_5$	532.80	*Polysiphonia urceolata*	11.65	(Li *et al*., 2007)
E-4-(3-bromo-4,5-dihydroxyphenyl)-but-3-en- 2-one	$C_{10}H_9BrO_3$	256.98	*Polysiphonia urceolata*	2.48	(Li *et al*., 2007)

Much research has concentrated on one specific bioactivity of *P. lanosa* extracts and this

has targeted investigation toward identifying the nature of the compounds responsible for that activity, without consideration of other potential activities. The significance of expansion of a bioactivity profile is particularly relevant considering the current interest in the repurposing of cancer treatments as antibacterial agents (Soo *et al*., 2017). The similarities between cancer cells and infectious bacterial cells include high replication rates, virulence, new mechanisms of spreading within a host, rapid development of resistance mechanisms against chemotherapeutic treatments and a tendency to become more invasive and aggressive during disease progression (Soo *et al*., 2017). The fact that an extract may possess a repertoire of compounds able to exert any range of activities, potentially of benefit in biopharmaceutical applications, should dictate and direct a more thorough evaluation and examination of these activities. This novel approach strongly influenced the work presented in this thesis – expansion of the scope of the research to encompass other bioactivity, allowing for consideration of mechanism and clarification of potential compound/s responsible.

1.13 Objectives of this Research

The overall objectives of this research was the extraction, isolation and identification of bioactive compounds from *P. lanosa,* and more specifically to:

- Enhance production and/or potency of compound(s) via holding seaweed under harvesting conditions in laboratory as well as stressing of seaweed post-harvesting, focusing on the effect of temperature and light variation on the anti-MRSA activity of crude extracts from cultivated *P. lanosa*.

- Isolate and purify a potentially novel bioactive, through a variety of separation techniques, assessing anti-MRSA activity alongside analysis to identify the nature of the active compounds and/or fraction.

- Extend bioactivity potential of the crude extract to include cytotoxicity, antioxidant capability and biofilm inhibitory capability and determine whether a crossover of activity exists which may further permit clarification of compound identity as well as support a proposed mechanism of activity against MRSA.

- Determine whether any basis for mechanism of anti-MRSA activity can be inferred.

CHAPTER 2: STRESSING *POLYSIPHONIA LANOSA* TO ENHANCE ANTI-MRSA ACTIVITY

2.1 Introduction

Secondary metabolites produced by seaweeds have a wealth of bioactivities, including anti-cancer, anti-viral and antimicrobial activity (Pal *et al*., 2014). Therefore, the unique ability of seaweeds to cope with harsh environments allows for the generation of novel sources of both pre-existing and unrecognised HVABs with the potential to provide a range of benefits (Murray *et al.,* 2013). However, the commercial exploitation of seaweed-derived HVABs remains limited; the main challenge is cost-effective production from sustainable sources. The potential of seaweed-derived bioactive molecules is, therefore, likely to be lost without the development of alternatives to wild harvesting. One such alternative is to cultivate the seaweed; either in its natural environment or *ex-vivo* in tanks (Robinson *et al*., 2013). However, in order to pursue seaweed-derived bioactives for biotechnological applications, a consistent supply is required and therein lies the paradox; the variable conditions producing the array of bioactivity within the marine environment necessitates refinement and control to ensure a viable yield of specific bioactive compounds. As a result, *ex-vivo* cultivation is more attractive, as conditions can be manipulated in order to achive optimal production of the biomolecule of interest. This may also be relevant for the development of seaweed cell culture, where conditions could be manipulated for the specific production of a range of bioactive molecules.

Stressing seaweed *ex-vivo* is one way to potentially maximise the yield of bioactive molecules (Khotimchenko & Yakovleva, 2005). Grime (1977) defined stress as 'external constraints limiting the resource acquisition, growth, or reproduction of organisms'. However, Davison & Pearson (1996) differentiate between (1) limitation stress, such as the reduction in growth rate due to an inadequate supply of resources, and (2) disruptive stress where damage is caused due to adverse conditions or the allocation of resources to prevent damage. Examples of limitation stress include low light and insufficient levels of inorganic nutrients, while disruptive stress includes freezing or desiccation, which cause cellular damage or require the allocation of resources to repair or prevent damage (Davison & Pearson, 1996). For example, increased lipid production is generally aligned to temperature stress above the optimal algal growth values with high temperatures resulting in significantly less PUFA proportions which would favour biofuel purposes. In contrast, for nutritional applications lower growth temperatures with increased PUFA proportions (Harwood, 2019) would be advantageous.

However, stress responses vary depending on the seaweed species. In one study representative intertidal seaweeds responded differently to desiccation (triggered by *in vitro* air exposure), with those living higher in the intertidal zone, such as *P. lanosa,* displaying better tolerance (Flores-Molina *et al*., 2014). Desiccation led to a significant increase in ROS, inhibition of antioxidant enzymes, cellular alterations, and photosynthetic inactivation in the lower intertidal species, *Lessonia spicata* and *Gelidium rex,* as well as exacerbating detrimental effects during rehydration (Flores-Molina *et al*., 2014). Seaweeds with higher intertidal distributions (*Mazzaella laminariodes, Ulva compressa and Scytosiphon lomentaria)* displayed greater antioxidant enzymatic activity, suggesting a higher capacity to buffer desiccation-induced ROS excess. In the low intertidal species, ROS concentrations were higher than normal and caused an overoxidation of biomolecules and photosynthetic disarray, providing an explanation (from a functional stand point) for their lower level habitat distribution in the intertidal zone (Flores-Molina *et al.,* 2014).

Previous research at WIT investigated the potential of various seaweed species as possible sources of novel antimicrobials (Tan, 2013). The red algal species *P. lanosa* was identified as particularly promising, as crude aqueous extract exhibited potent activity against MRSA. The objective of the current study was to perform a series of post-harvesting stressing investigations in order to ascertain whether this anti-MRSA activity varied under different conditions (limitation stress). The motivation for such a study was the identification of optimal conditions for maximum antimicrobial activity, which could then be replicated in a commercial setting. This study is the first to attempt to cultivate the epiphyte, *P. lanosa* alone in the absence of a host seaweed, monitoring examining the effect on a bioactive property over time.

It was previously assessed that long-term maintenance of photosynthetic processes in *P. lanosa* requires permanent attachment to *A. nodosum* (Garbary *et al*., 2014). The quantum yield (measure of photosynthetic efficiency) of the epiphyte attached to *A. nodosum*, epiphyte with *A. nodosum* but unattached and epiphyte alone was evaluated and differences revealed differences within 24 h with the attached epiphyte having demonstrated the highest maximum quantum yield, the separated epiphyte the lowest and the detached epiphyte with its host in the same vessel, an intermediate quantum yield.

The cultivation should assess the stability of *P. lanosa* post-harvest as well as determine the effects of controlled stress on its anti-MRSA activity.

2.2 Objectives

The specific objectives of this research included:

- To investigate the feasibility of *ex-vivo* cultivation of *P. lanosa*, which grows in the wild as a hemi-parasite on *A. nodosum* (and more rarely on *F. vesiculosus*).
- To record the variation of anti-MRSA activity of *P. lanosa* in laboratory tanks post-harvesting in order to generate baseline data.
- To investigate the effect of different culturing temperatures on anti-MRSA activity.
- To assess the impact of light exposure on anti-MRSA activity.
- To generate recommendations to optimise antimicrobial production from *P. lanosa.*

2.3 Experimental Procedure

2.3.1 *Bacterial Strains, Microbiological Media and Chemicals*

- Mueller Hinton Agar (MHA): Difco (Becton, Dickinson and Co., Maryland,U.S.A.).
- Brain Heart Infusion Broth (BHI): Oxoid (Basingstoke, U.K.).
- MRSA -W73365 (WIT 517): clinical isolate from Waterford University Hospital
- Chloramphenicol discs (10 µg): Oxoid (Basingstoke, U.K.).
- Chloramphenicol powder: Sigma Aldrich (Steinheim, Germany).

2.3.2 *Instrumentation*

- Hach water instrument – HQ 30d flexi with probes HDO (for oxygen) and intelliCAL pH PHC101.
- Conductivity Meter - Eutech CON 700.
- Oxygen pumps - Interpet Airvolution AV4 pumps for tanks 2 and 3 and larger Interpet Airvolution AV8 model for tank 1.
- Light source – Glo 25 W Marine Actinic Blue for salt water aquariums wavelength 400 – 580 nm, where the wavelength range of 420 – 460 nm is critical for marine environments and Glo 30 W PowerGlo superbright aquarium bulb with wavelength range 380 – 730 nm.
- Spectrophotometer - Jenway 6300.
- Incubator - Pansonic MIR – 154 – PE.
- Freeze-drier -VirTis, SP Scientific, PA, USA.
- Memmert circulating water baths.
- Priorclave tactrol 2 autoclave.
- Seawater was collected from the harvesting site in addition to seaweed.

2.3.3 *Seaweed Collection and Preparation*

P. lanosa samples were collected from Fethard-on-Sea, County Wexford, Ireland (52.39˚N, 6.94˚W), transported in a cooler box (temperature < 10 ˚C) and transferred to laboratory tanks within 3 h. The samples were collected from various locations at this site and combined to ensure representative sampling. There were three different sampling dates and the dates and harvesting conditions are presented in Table 2.1, Section 2.4.1.

The seaweed was immersed during all three experiments so that hyperosmotic stress which due to evaporation of seawater during emersion was not a consideration.

The seaweed samples were washed in seawater and dried on water absorbent paper before adding ~3 kg to tank 1 and ~1.5 kg into each of tanks 2 and 3, which correlates to a loading density of 0.0375 *m/v* (mass/volume). The tank set-up is shown in Figure 2.1. Seawater was also sampled at the same time as harvesting of the seaweed and

temperature, pH and dissolved oxygen concentrations recorded (Table 2.1) before being added to the tanks.

Table 2.1: Seawater conditions (mean values; n=3)

Date of Harvest	Purpose	Seawater Temperature (˚C)	Conductivity (mS)	Dissolved Oxygen (mg/L)	pH
3/6/2014	Baseline study	18.2	51.6	11.62	8.02
29/9/2014	Temperature study	16.8	52.8	10.14	8.90
2/3/2015	Light study	7.1	50.2	11.64	8.10

2.3.4 *Tank Holding Conditions for Baseline Study*

Three tanks were used. Tank 1 was a large, glass tank with a capacity of ~80 L, whilst tanks 2 (plastic) and 3 were smaller tanks with a capacity of 40 L. Seawater (collected at seaweed harvesting) was added to the maximum capacity of each tank. Each tank was equipped with airvolution pumps for aeration and agitation (200 L oxygen per hour). Aeration of culture tanks was essential, as without proper aeration and the resulting movement of water around the algal culture tanks, the algal material will start to deteriorate. Factors affecting algal growth include nutrient quantity and quality, light, pH, turbulence, salinity and temperature with the objective in this study to preserve conditions as closely to those at harvesting as possible. The experimental set-up is shown in Figure 2.1. The tanks were situated in a controlled environment, within a walk-in coldroom facility maintained at a temperature of 15 ˚C. This temperature was selected as it represented the average Summer temperature for seawater in a temperate zone (Nygård & Dring, 2008) and antimicrobial production from *P. lanosa* had previously been shown

to be optimal during the Summer months (Tan, 2013). Tank conditions were controlled and monitored on a 24 h basis.

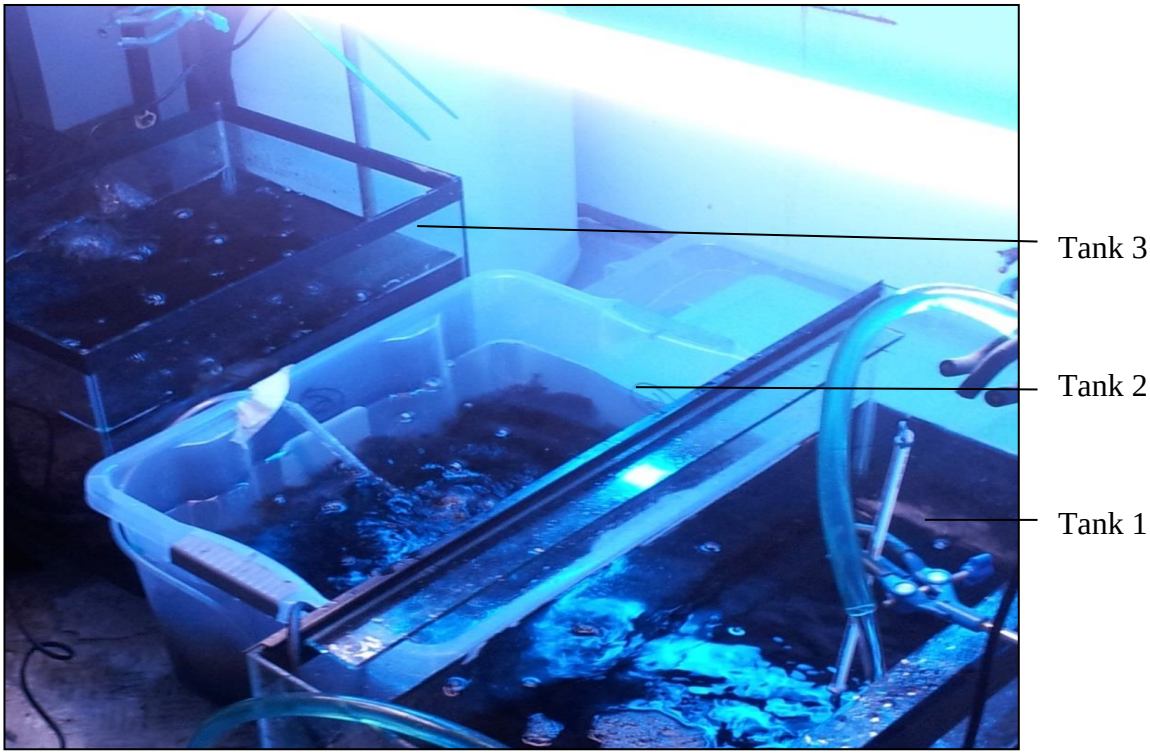


Figure 2.1: Laboratory tank set-up for baseline experiment.

All tanks were subjected to mild aeration, with the water temperature held at 15˚C and a photoperiod (daylight hours) of 16.5 h used (controlled by using a pre-set timing device). These two conditions represent typical Irish June values. Two light sources were used - one for salt water aquariums with a wavelength range of 400-580 nm at 795 lumen (Actinic bulb), and the other a superbright aquarium bulb with a wavelength range of 380-730 nm at 1330 lumen (producing more red light to improve photosynthetic growth). These sources were selected as representing the best options for simulating open water conditions for *P. lanosa* (which as a red macroalgae is particularly adapted to the absorption of blue light – 490-450 nm which is provided for by the use of the Actinic bulb). The mild aeration created turbulence in the system, mimicking natural seawater conditions that increase the transfer of oxygen across an air-water interface (in static conditions this transfer is very slow) (Howard, 1998).

2.3.5 *Experimental set-up for temperature and light studies*

The experimental set up for differed to that of the baseline study in that the seaweed was placed into 2 L beakers within temperature-controlled water baths. Each beaker held 100 g of washed, pat-dried seaweed with 1.5 L seawater (a loading density of 0.0667 *m/v*). This downscaling was necessary to ensure statistically relevant results as duplicate 'tanks' (2 L beakers) were required to corroborate each result. For the temperature study four temperatures were selected; 5, 10, 15 and 20 ˚C and for each temperature there were 2 beakers in each tank and 2 samples were collected from the middle of each beaker each day at the same time. These samples were collected after agitation of the beaker, ensuring consistent representation. An 11 h light period was selected and the tank lids were opened and closed appropriately. Water quality parameters were monitored on a daily basis to check for significant variations.

The light study was pursued in a similar manner to that of the temperature investigation, except that light exposure was varied instead of temperature. Each temperature-controlled tank (maintained at 5 ˚C) contained two beakers so that for each different light condition four samples were collected daily, two samples from each beaker, as represented by Figure 2.2. As with the temperature study, water quality parameters were monitored. All tanks were held at 5-6 ˚C (as close to harvesting temperature as possible) to ensure no acclimation other than to light occurred and this was facilitated by performing the experiment in a walk-in coldroom. Each of the four tanks (containing the beakers) was equipped with a close-fitting lid so that definite light periods could be set by closing the lid. Four different photoperiods were assessed with the same light sources used as for the baseline determination. These included constant darkness and constant light, as well as one simulating daytime hours for March (11 h) and another simulating daytime hours for May (16 h), as previous seasonal studies identified the latter as the optimal time of year for maximum anti-MRSA activity (Tan, 2013).

Samples were processed as in the baseline study and the results collated so that for each sample duplicate assays were performed (n=2), see Section 2.3.7. Collection conditions were recorded and the beakers monitored in the same way as in the baseline study (changes were recorded on a daily basis at the same time as the seaweed samples were removed).

Whilst water turbulence was supplied using oxygen pumps, the seaweed samples were not subject to the usual cycles of immersion/emersion due to tidal or weather factors so that stress associated with dessication was not a factor in this study.

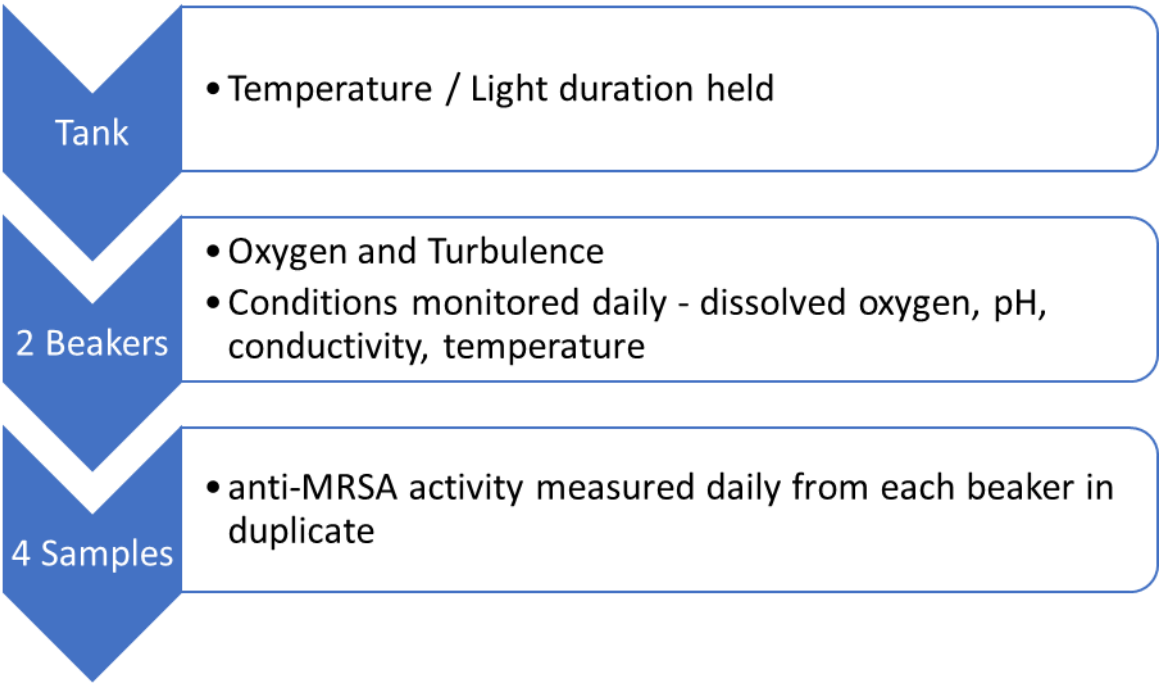


Figure 2.2: Experimental design, control of conditions and sampling – 2 samples from each of 2 beakers held under one condition in one tank for stress application.

2.3.6 *Generation of crude seaweed extracts*

For the baseline study, two seaweed samples of ~40 g wet weight each were withdrawn from each tank daily (6 samples per day) at the same time, where possible for a period of 34 days and placed into clean glass beakers. For the temperature and light study, two ~10 g separate seaweed samples were withdrawn from each beaker per day over one week. The samples were rinsed in deionised, distilled water, dried with absorbent paper, packaged in labelled brown paper envelopes and frozen immediately at -20 ˚C. Once frozen, the samples were freeze-dried within 3 weeks (VirTis freeze-drier, SP Scientific, P.A., USA). The freeze-dried samples were then blended and sieved to ensure a uniform particle size of ± 850 µm. Seaweed powders were stored under N_2 at -20 ˚C until extraction.

Approximately 1 g of the seaweed powder was extracted by stirring with distilled deionised water (1 % *w/v*) using a magnetic stirrer for 2 h at room temperature. This extraction time had previously been determined by the WIT research group as appropriate for the extraction of antibacterial compounds from *P. lanosa* (Tan *et al.*, 2011). The extracts were filtered under vacuum through 502 filter paper before being frozen at -20

˚C. Once frozen, the extracts were freeze-dried. The dry weight was recorded and the final crude extract was stored under N_2 at -20˚ C until anti-MRSA activity was assessed. The processing period was kept to a minimum, with anti-bacterial activity assessed within 8 weeks of sampling from tanks.

2.3.7 *Anti-MRSA activity of crude seaweed extract*

Anti-MRSA activity was measured using a well diffusion assay previously used at WIT (Prieto *et al*., 2012). Molten MHA (200 mL), held at 45 ˚C in a water bath, was seeded with 500 µl of an overnight BHI broth culture (18 h at 37 ˚C) of MRSA W73365, a hospital strain isolated at University Hospital Waterford. Plates of seeded MHA were then poured using a 20 ml sterile container to ensure an equal volume of medium in each plate. The plates were allowed to solidify for 30 min before wells were bored aseptically using a glass capillary tube (6 mm diameter). The freeze-dried crude seaweed extract was prepared as a stock concentration (60 mg/mL) before being diluted aseptically in Eppendorf tubes to 40 and 20 mg/mL in sterile deionised water. Then, 50 µl volumes were added to labelled wells in the MRSA-seeded plates, accounting for the well-loading of 1, 2 and 3 mg per well. The negative control was the diluent (sterile water) and chloramphenicol discs were used as positive controls.

The agar plates were incubated overnight for 18 h at 37 ˚C before being examined for zones of inhibition. The diameter of the zone of inhibition was then measured and recorded. For each of the two samples removed from each tank or beaker, the anti-MRSA activity was assessed in duplicate on two separate agar plates. Therefore, for the baseline determination 6 samples per day were assessed in duplicate plates and data presented separately for each tank, whilst for the temperature and light investigation there were a total of 4 samples, also assessed in duplicate for each well-loading with the average of

the 4 results calculated and the standard deviation used for the error bars.

Further assessment of the decline in anti-MRSA activity was carried out by closer consideration of the crude extract well loading level, with 0.5 mg increments evaluated and activity monitored over a 7-day period.

2.3.8 *Statistical Analysis*

For the baseline experiments, water quality parameter variations were measured and fitted to a linear regression model to check for slope deviation. This was applied to each of the variables independently. The anti-MRSA activity as measured by zones of inhibition were statistically evaluated using one-way ANOVA followed by a post-hoc analysis with Tukey comparison tests using Minitab 17 Statistical Software set at a 5% statistical significance level. The results were said to be statistically different if $p<0.05$. Results are presented as the mean ± standard deviation (SD).

The significance of the change in anti-MRSA activity was evaluated as a factor of time using a two sample t-test using unequal variances for the shorter term loading effect investigation.

For the stressing experiments, Minitab 17 was again used for statistical analysis. A general linear model was constructed with zone of inhibition as the response and day, temperature as well as light duration and extract loading as factors (not covariates) to determine the effect of these factors.

2.4 Results and Discussion

2.4.1 *Seawater conditions and anti-MRSA activity at time of harvesting of P. lanosa for all three studies*

Table 2.2 presents the *P. lanosa* harvesting conditions for all three studies and the initial extract activity against MRSA.

Table 2.2: Seawater conditions (mean values; n=3) (also represented in Table 2.1) and anti-MRSA activity of *P. lanosa* at harvesting.

Date of Harvest	Purpose	Seawater Temperature (˚C)	Conductivity (mS)	Dissolved Oxygen (mg/L)	pH	Diameter of Zones of Inhibition*(mm) (n=3) Well loading		
						3 mg	2 mg	1 mg
3/6/2014	Baseline study	18.2	51.6	11.62	8.02	21.0	19.0	16.5
29/9/2014	Temperature study	16.8	52.8	10.14	8.90	14.0	11.0	9.0
2/3/2015	Light study	7.1	50.2	11.64	8.10	21.5	18.5	12.5

*Diameter of zones of inhibition SD values are omitted here but represented in all later figures

2.4.2 *Baseline study*

The full range of tank conditions are presented in Table 2.3 and Figures 2.2-2.4, where the water quality parameter variations of dissolved oxygen, conductivity and pH are presented. Seawater was added to tanks to replace water lost by evaporation on days 5, 12 and 18. In considering the range of values in Table 2.3 it is necessary to recognise the natural variability of seawater. In general, the conductivity range is 39 – 50 mS, the pH range is 7.5 - 8.5 (with mariculture values of 7.8 – 8.4 being preferred) and dissolved oxygen levels > 6 mg/L (Prema, 2011).

In this study, the dissolved oxygen data reveal the greatest variation. This can be explained, at least in part, by the fact that problems were experienced with the water

circulation system in tank 1, that required unblocking on days 5, 10, 20 and 28 where the system needed to be switched off and problem resolved. This is reflected in the reduction in dissolved oxygen concentrations measured prior to these times (Figure 2.3). However, this was considered not to have affected the results, as the minimum level of dissolved oxygen required by seaweed is 5-7 mg/L (Prema, 2011), although the value on collection was 11.62 mg/L.

Table 2.3: Conditions[1] in the laboratory tanks used to hold *P. lanosa* during the course of the 34-day baseline study compared to those of the seawater at seaweed collection (n=3).

Condition	**Seawater at collection**	**Tank 1**	**Tank 2**	**Tank 3**
Temperature (˚C)[2]	14.0	14.0-16.5	14.0-15.0	14.0-16.0
pH	8.02	7.00-8.64	7.14-8.59	7.21-8.84
Conductivity (mS)	51.6	51.2-65.9	52.0-59.7	51.6-58.2
Dissolved oxygen (mg/L)	11.62	3.29-11.61	6.61-11.62	7.94-11.62

[1] Range over the 34 day study

[2] Temperature read with glass thermometer

In general, the dissolved oxygen monitoring, as depicted in Figure 2.3, revealed a substantial initial decline after harvesting, indicating the impact of factors at the tidal zone in maintaining high oxygen levels, possibly due to wave action forcing oxygen into the water.

The expectation that oxygen solubility in water declined with an increase in salinity (represented by the conductivity recorded in Figure 2.4) (Kennish, 2000) was validated by the low dissolved oxygen concentration at day 30 in tank 1, which correlated with the high conductivity value reported in this tank at the same time point. Degradation of the seaweed was also a contributory factor, as oxygen was consumed as biodegradable organic matter was released into the water (Howard, 1998).

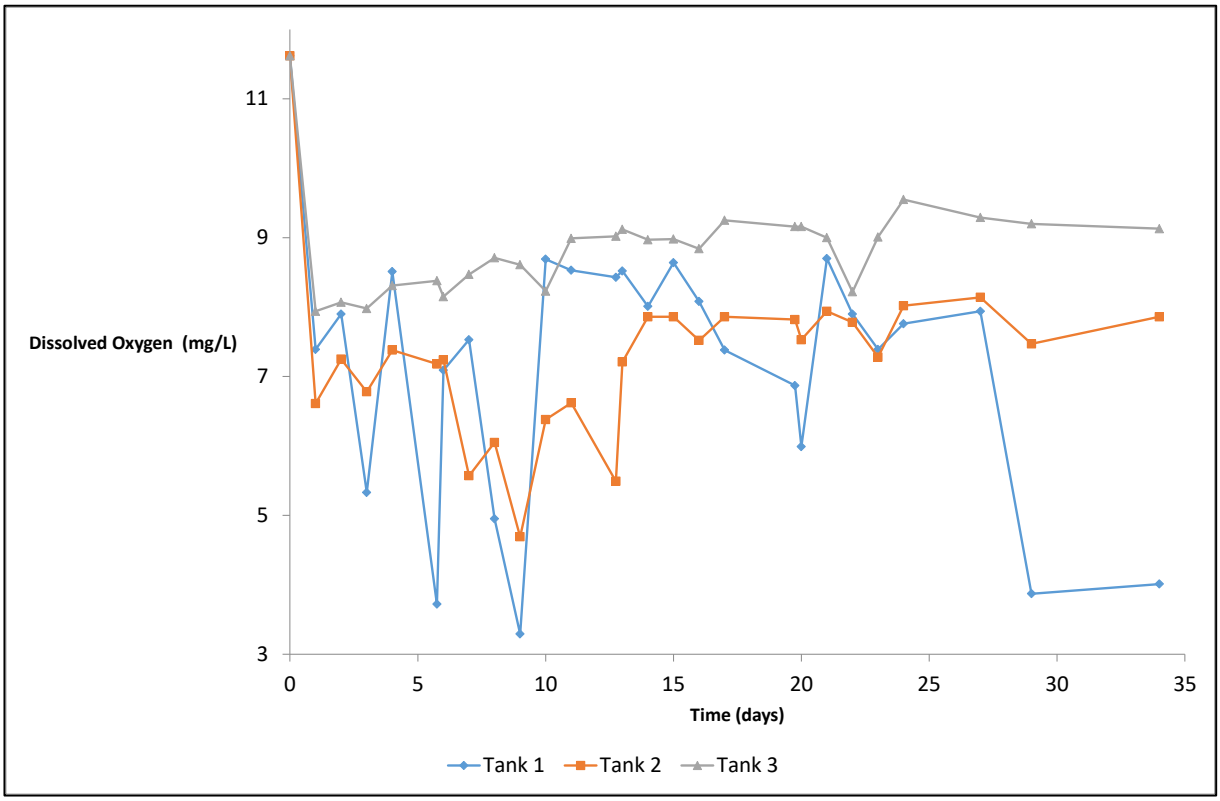


Figure 2.3: Dissolved oxygen monitoring of seawater in tanks used for the maintenance of *P. lanosa* after harvesting during the baseline study (average of 3 readings taken at the same time each day).

However, statistical analysis of the dissolved oxygen levels across all tanks, in relation to the number of days using a linear regression model and the slope deviation from zero determined that these changes were not significant up to day 25.

Conductivity is generally monitored as an indicator of water quality in aquaculture and any deviation greater than 10 % is a cause for concern, as a major fluctuation in aquatic salinity indicates a change in water quality (Kennish, 2000). Conductivity changed over time during the baseline experiment in the present study, with an overall increase observed. However, there were differences between the tanks, especially after day 1, as evidenced by an average daily rate of change of 0.407 mS, 0.227 mS and 0.173 mS in tanks 1, 2 and 3, respectively (Figure 2.4). The nearly double daily rate of change in tank 1 was possibly associated with the fact that it contained double the amount of seaweed and seawater and that the dissolved oxygen levels were not as stable as in tanks 2 and 3, due to the size of the tank.

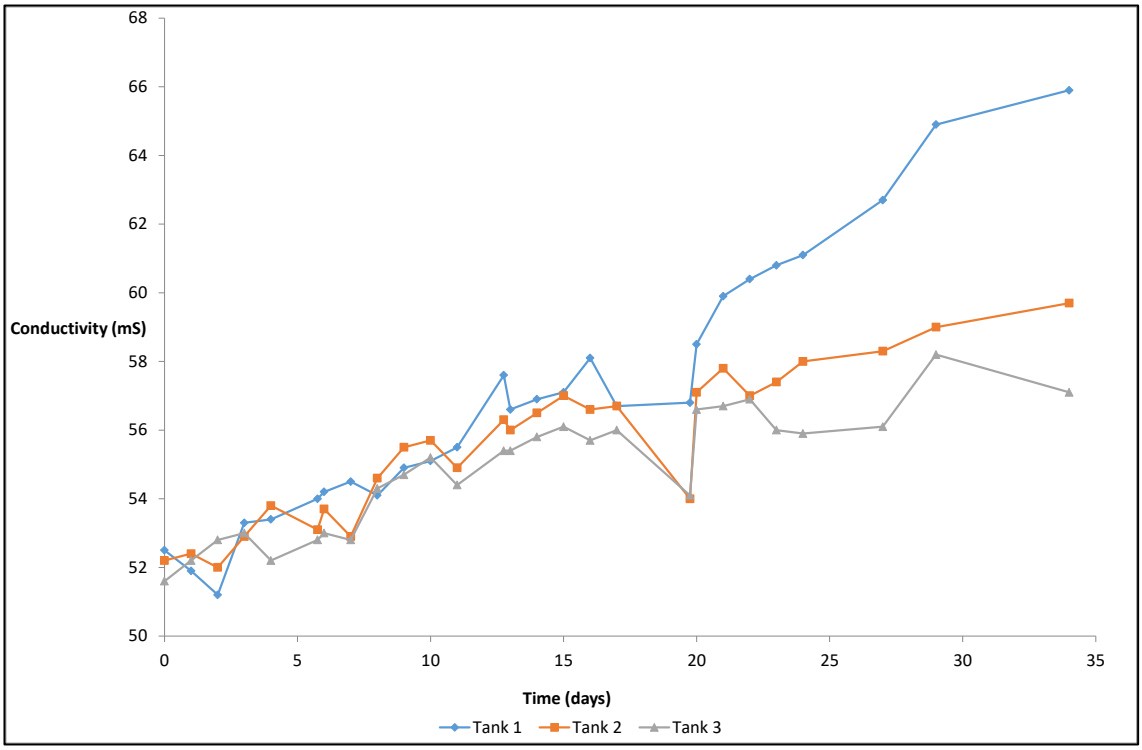


Figure 2.4: Conductivity monitoring of seawater in tanks used for the maintenance of *P. lanosa* after harvesting during the baseline study (average of 3 readings taken at the same time each day).

The increase in conductivity in all tanks after the first 21 days, as shown in Figure 2.4, was possibly linked to degradation of the seaweed and the breakdown of seaweed tissue causing excessive electrolyte leakage (Paull & Chen, 2008). This was particularly obvious in tank 1 where the conductivity measurements increased more than in tanks 2 and 3. Statistical analysis using both linear regression and Pearson correlation showed that the conductivity changed significantly over time.

The increase in conductivity was reflected in the pH data shown in Figure 2.5 where pH values also increased at day 21. Statistical analysis determined that changes in pH were significant, $p<0.05$.

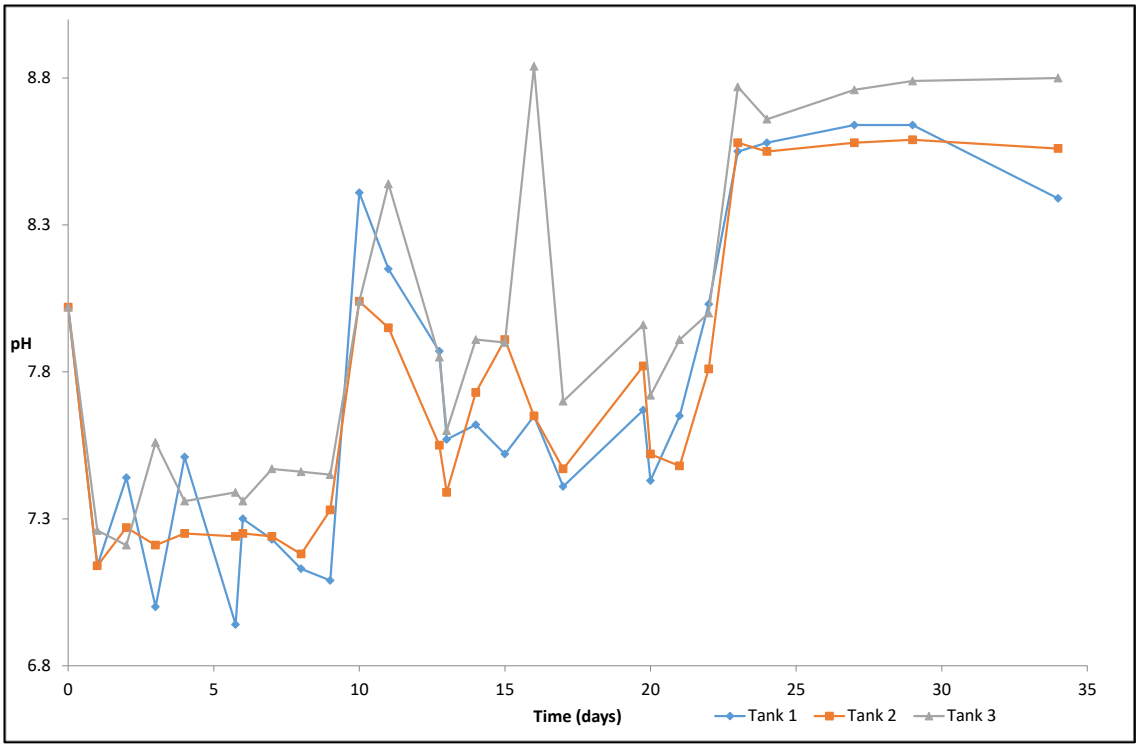


Figure 2.5: pH monitoring of seawater in tanks used for the maintenance of *P. lanosa* after harvesting during the baseline study (average of 3 readings taken at the same time each day).

The photosynthetically induced high pH indicates that the seaweed was producing oxygen and consuming carbon dioxide during the daytime photoperiod and this is evident from the data shown in Figure 2.5. These conditions remained relatively stable until day 9 when there was a major shift and the rise in pH was much greater, increasing from 7.3 to 8.0.

After this increase, the pH continued to fluctuate in all tanks, especially tank 1, until the next large increase occurred at day 21. This pH increase correlated with the degradation of the seaweed samples and perhaps represents extensive loss of seaweed structure and intercellular components, resulting in the liberation of alkaline compounds or the release of stress signalling compounds. However, in general, the pH increased at a similar rate in all three tanks, with an average daily increase of 0.040 pH units in tank 1, 0.041 in tank 2 and 0.045 in tank 3, for the initial period associated with retention of anti-MRSA activity i.e. until day 6. This similarity across all 3 tanks serves to indicate the reliability of the recorded data. It was also ascertained statistically that there was a correlation between conductivity and pH ($p<0.05$).

The temperature measurements revealed the least variability between the tanks, despite other parameters changing within the tanks and a range of 14.0-16.0 ˚C was recorded (Table 2.3). However, this was to be expected as the tanks were maintained in a temperature-controlled room.

The recording of tank conditions provides an indication of the stability of the harvested *P. lanosa* when maintained under artificial conditions. The seaweed was able to survive removal from its host plant for 36 days, but long-term maintenance of photosynthetic processes necessitates permanent attachment to *A. nodosum* (Garbary *et al*., 2014). This was previously determined by Garbary *et al*. (2014). The quantum yield (measure of photosynthetic efficiency) of the epiphyte attached to *A. nodosum*, epiphyte with *A. nodosum* but unattached and epiphyte alone revealed differences within 24 h; the attached epiphyte had the highest maximum quantum yield, the separated epiphyte the lowest and the detached epiphyte with its host in the same vessel had an intermediate quantum yield. This value was significantly higher than for the epiphyte alone (where measurements were made at 1, 3 and 7 day intervals). However, the definition of 'long-term' requires consideration as the study covered a duration of only 7 days. The association of pigmentation with independent culturing was mentioned but not measured in the Garbary *et al*. (2014) study. A reduction in pigmentation may have implications for bioactivity. For example, a correlation of phycobilin content as a function of time with anti-MRSA activity would provide additional information as to whether photosynthesis is necessary for the generation or maintenance of bioactive compounds and whether a variation of actual pigment content correlates with activity, as assessed by Paull & Chen (2008).

It is important to note that although seawater levels in the tanks were maintained by the addition of seawater collected on the day of harvesting, there was no continuous flow of fresh seawater and this may be considered an additional stress factor, as no additional nutrients were supplied and the availability of salts and minerals was finite. Dissolved nutrients in seawater include ammonium, nitrate, nitrite, phosphate and silicate. The stability of these in the collected stored seawater not assessed, with the original levels possibly reduced by assimilation by phytoplankton and bacteria.

2.4.2.1 Anti-MRSA activity of crude extract from seaweed maintained under artificial conditions

Anti-MRSA activity recorded with respect to well-loading is shown in Figures 2.6-2.8. The results for each well loading for the three tanks are presented separately. There was no significant statistical difference in anti-MRSA activity between the three tanks.

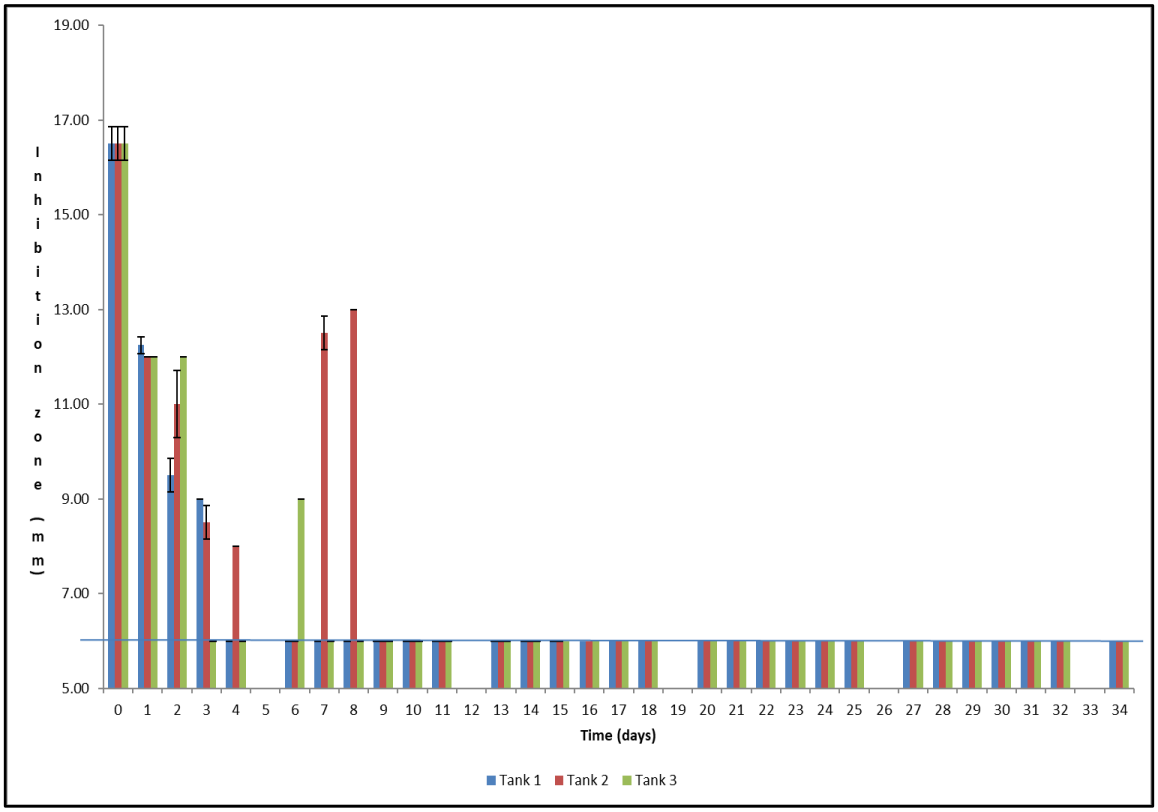


Figure 2.6: Diameter of zones of inhibition (mm) against MRSA W73365 for 1.0 mg well loading of *P. lanosa* crude extract, using the well-diffusion assay (n=2 for each tank, average of 2 samples each tested twice in duplicate) as a function of time (days) with standard error bars (standard deviation). The inhibition diameter of 6 mm is the diameter of the well and indicates no inhibition. No samples were taken on days 5, 12, 19, 16 and 33.

At the 1 mg loading rate, there was a decline in anti-MRSA activity observed during the first 3 days. This may be explained by the fact that the epiphyte had been removed from its host, as well as being removed from its natural environment, both of which may alter the composition of metabolites responsible for bioactivity.

The revival of activity in the seaweed taken from all tanks between days 4 and 8 may be due to the formation of other compounds and/or degradation products or a resurgence of defence mechanisms due to the stress of new conditions (and acclimatisation to the new environment). The revival further enhances the potential for novel approaches to the induction of bioactive compounds.

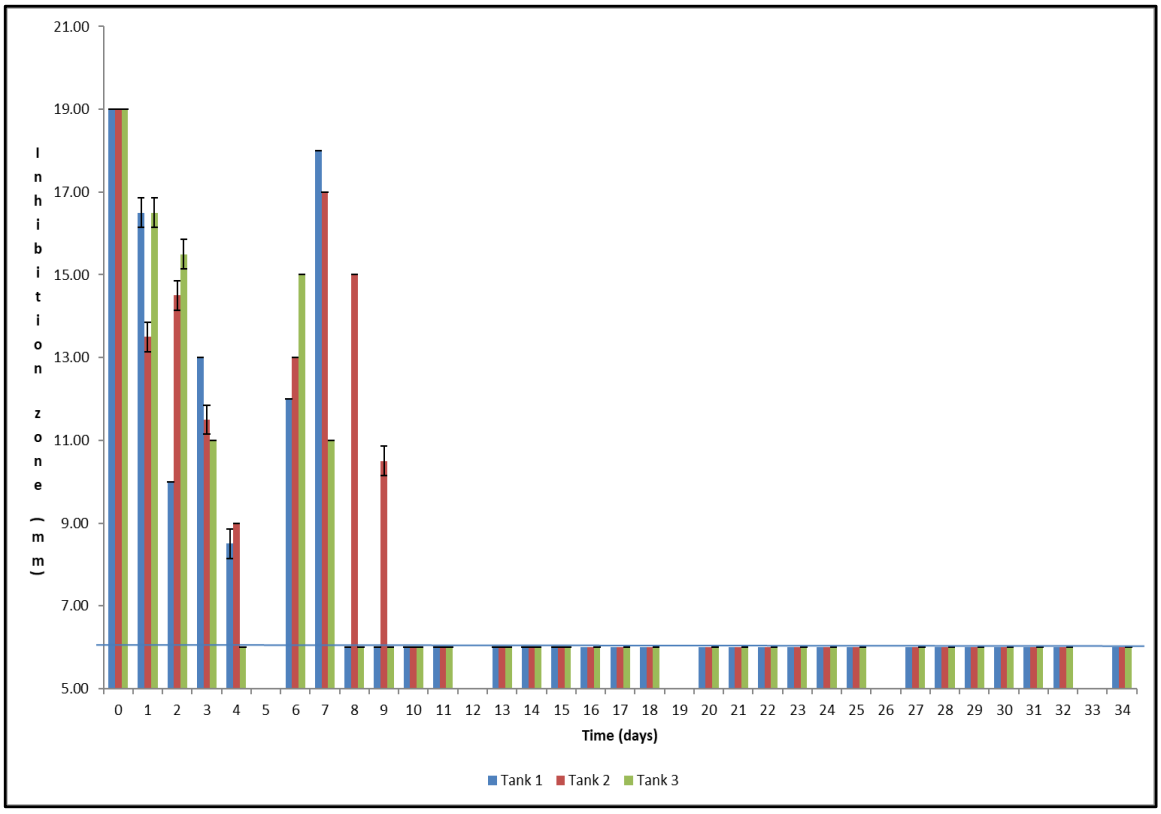


Figure 2.7: Diameter of zones of inhibition (mm) against MRSA W73365 for 2.0 mg well loading of *P. lanosa* crude extract, using the well-diffusion assay (n=2 for each tank, average of 2 samples each tested twice in duplicate) as a function of time (days) with standard error bars (standard deviation). The inhibition diameter of 6 mm is the diameter of the well and indicates no inhibition. No samples were taken on days 5, 12, 19, 16 and 33.

The inhibition zones for the 2 mg loading rate again showed a reduction of activity over the first 3 day period for all 3 tanks. In tank 3 all activity was lost by the fourth day but activity was later detected on days 6 and 7. Tank 2 samples displayed activity for a longer period i.e. until day 9, all tanks having displayed activity on days 6 and 7.

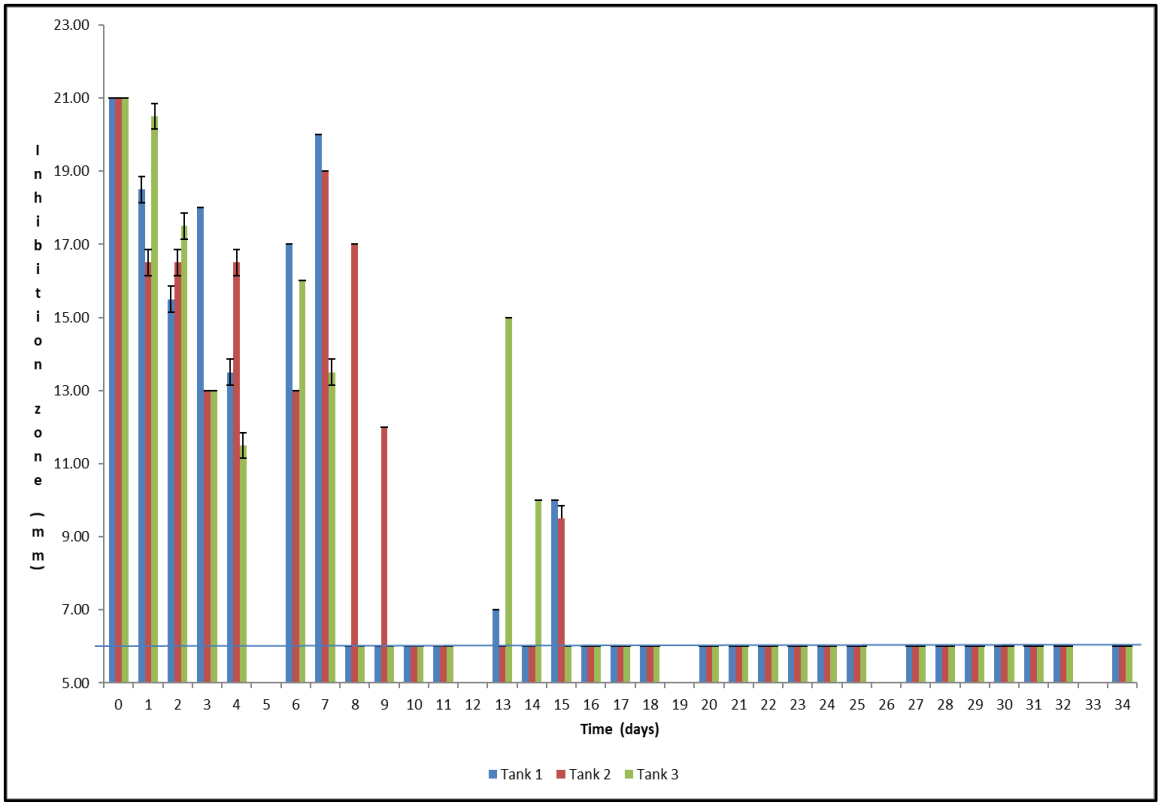


Figure 2.8: Diameter of zones of inhibition (mm) against MRSA W73365 for 3.0 mg well loading of *P. lanosa* crude extract, using the well- diffusion assay (n=2 for each tank, average of 2 samples each tested twice in duplicate) as a function of time (days), with standard error bars (standard deviation). The inhibition diameter of 6 mm is the diameter of the well and indicates no inhibition. No samples were taken on days 5, 12, 19, 16 and 33.

Anti-MRSA activity did not decrease to the same extent when 3.0 mg of *P. lanosa* extract was assayed, with activity maintained in all tanks until day 7. This was expected, as the loading rate was high and the possibility of synergistic effects between compounds maximised (there may exist a minimum level of one compound necessary to elicit activity and at this higher level of loading this requirement was fulfilled, allowing for persistence of activity). On the other hand, this prolonged activity may be explained by the fact that the sensitivity of the well-diffusion assay was optimised at this high level of loading, thereby ensuring optimal diffusion of active components into the MRSA-seeded agar. Another possibility may be that the level of crude extract (and large molecules) acted as a filter, permitting the diffusion of smaller bioactive compounds. Tank 2 again displayed better retention of anti-MRSA activity. For all three tanks, there was a loss of activity by day 10 (for 1 mg loading it was day 9), indicative of compound degradation or cessation of production of the anti-microbial compound (Heydarizadeh *et al.*, 2013). At the higher

level of loading, however, there was an observation of resumption of activity at days 13-15, which indicates that either new compounds were produced or that breakdown products with anti-MRSA activity had been generated. Another possibility is that antagonistic compound(s) were degraded due to the stress conditions.

Degradation of the seaweed (which was no longer supported by its host) may lead to formation of complexes with anti-MRSA activity i.e. as a new survival mechanism. This may be significant in light of observations by Garbary *et al.* (2014) that a reduction in quantum yield was found for *P. lanosa* when detached from *A. nodosum* and cultured independently. This may be because photosynthetic processes are reduced, perhaps resulting in a change of metabolite synthesis and the evolution of alternative anti-MRSA bioactives. It can be speculated that the generation of these 'new' compounds does not require as high a level of photosynthetic activity, especially at days 13-15.

This is further substantiated by a study by del Val *et al* (del Val *et al*., 2001) where culturing of a range of seaweeds for 10 days in bioreactors under low and high stress conditions effected changes in antimicrobial activities of crude methanol extracts. High stress was applied by utilising a stocking rate of 5 g/L without seawater exchange or nutrient addition, whilst the low stress condition employed a stocking rate of 2 g/L with a continuous flow of natural seawater and addition of a nitrogen source.

One particular species of brown seaweed, *Dictyota ciliolate,* produced new compounds capable of activity against *S. aureus* (albeit at a low level) under high stress conditions and retained previous activity against *Bacillus subtilis,* perhaps signifying two different mechanisms of action or different compounds. Another species, *Solieria filiformis,* a red highly branched algal species (similar to *P. lanosa*), maintained antimicrobial activity under all conditions, although the low stress sample only maintained high activity against *S. aureus* (potentially significant when considering the work undertaken in the present study), whilst the high stress sample retained high activity against *B. subtilis*. This may indicate that different coping strategies are invoked, as during both stress conditions, there was a change – one where there is a loss of activity when high stress is applied and one where there is a loss of activity when low stress is experienced. This may be relevant to the present investigation, in that anti-MRSA activity may not be enhanced by application

of the selected stress conditions. It could further be deduced from the del Val *et al.* (2001) study that the compounds responsible for activity against *B. subtilis* are more stable (or form a more substantial component of the macroalga) and can withstand considerable change in conditions when compared with bioactivities against other microorganisms. This was the case with *Dictyota ciliolata* which displayed intermediate activity against *B. subtilis* after being cultured under low stress conditions but under high stress conditions this was enhanced and was extended to include activity (albeit very low) against *S. aureus.* There could be a number of reasons for this, including the fact that the macroalgae had to adjust to new conditions post-harvest and required different metabolic pathways to cope with the change. This situation was exacerbated under the high stress conditions and further activity was induced as well as 'new' activity against *S. aureus.*

The reason for this observed change in activity is unknown but may be due to an increase in compounds responsible for this specific antibacterial activity or the generation of synergistic compounds, allowing for better activity, or the formation of new compounds with a different spectrum of activity. However, the del Val *et al.* (2001) study did not compare the effect of increasing seaweed extract concentrations on activity which may have yielded additional pertinent information, such as whether an increase in extract concentration was responsible for higher or lower activities against the same microorganism or a change in activity towards other microorganisms.

2.4.2.2 **Study of the effect of well-loading on anti-MRSA activity**

Figure 2.9 presents the extended assesment of the decline in anti-MRSA activity with additional well loading increments of 0.5 mg to identify the minimum levels of crude *P. lanosa* extract necessary for the observation of persistent and maximal activity.

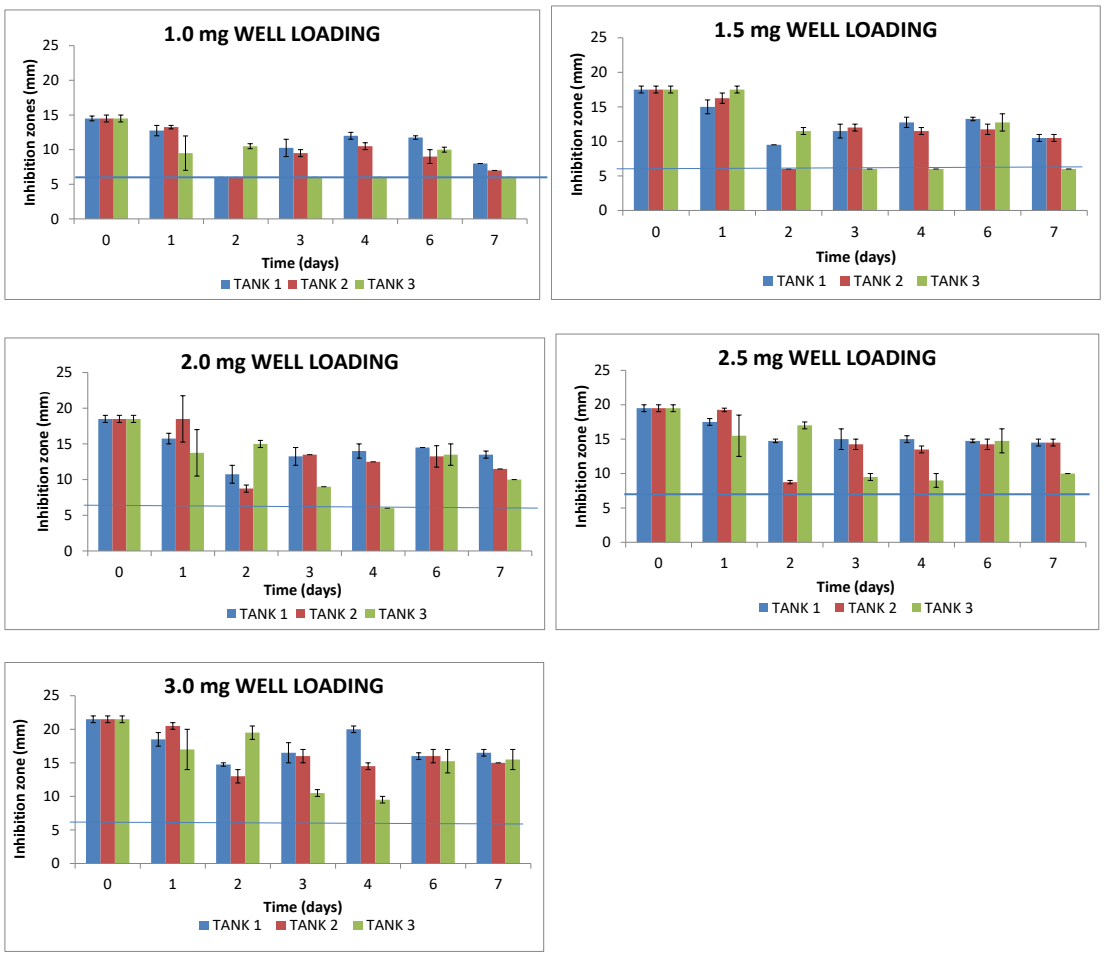


Figure 2.9: Diameter of zones of inhibition (mm) against MRSA W73365 for 1.0 - 3.0 mg *P. lanosa* crude extract well-loading, using the well-diffusion assay (n=2 for each tank) as a function of time (days) with standard error bars (standard deviation). The inhibition diameter of 6 mm is the diameter of the well and indicates no inhibition.

Comparing well-loadings of 1.0 and 1.5 mg, it would appear that the 0.5 mg increase in well-loading permitted the retention of high anti-MRSA activity in all 3 tanks after 24 h, although the reduction in activity observed after 2 days was comparable. This supports the possibility that an optimum level of active compounds is required to sustain a certain level of bioactivity. It may also substantiate the case that fewer compounds are actually affected by the detachment of the epiphyte from its host within the first 2 days (i.e. short-term effect following harvesting).

The stability of anti-MRSA activity was most evident at the higher well loading, with substantial activity retained up to day 7 for the 2.0, 2.5 and 3.0 mg loadings. The higher well loading may have masked any major changes in antibacterial activity due to changing composition as a result of compound degradation or other effects, although this may have

been a concentration effect. This was apparent when compared to the lower well loading where by day 2, there was no activity in samples from tanks 1 and 2 or from tank 2 alone for the 1 mg and 2 mg well loading, respectively, despite a resurgence of activity on days 3 and 4 (except in tank 3 samples where activity was lost). This may be related to possible instability of antimicrobial compounds responsible for the initial activity, together with new compounds produced in the stressed situation having reached higher levels and exhibiting synergistic anti-MRSA activity. (The lower well loading may, in actual fact, present a more accurate picture of the changes in antimicrobial activity, once the nature of the different compounds are realised). This is in contrast to findings for the 3 mg well loading rate, where activity was still present at greater than two thirds of the original values in all tanks at days 6 and 7. The levels of synergistic compounds or the efficacy of a sole active compound may have contributed to this retention of activity.

Average data at each time point is presented in Figure 2.10 and less anti-MRSA activity was found on day 7 than on day 0 ($p<0.05$) for all well loadings.

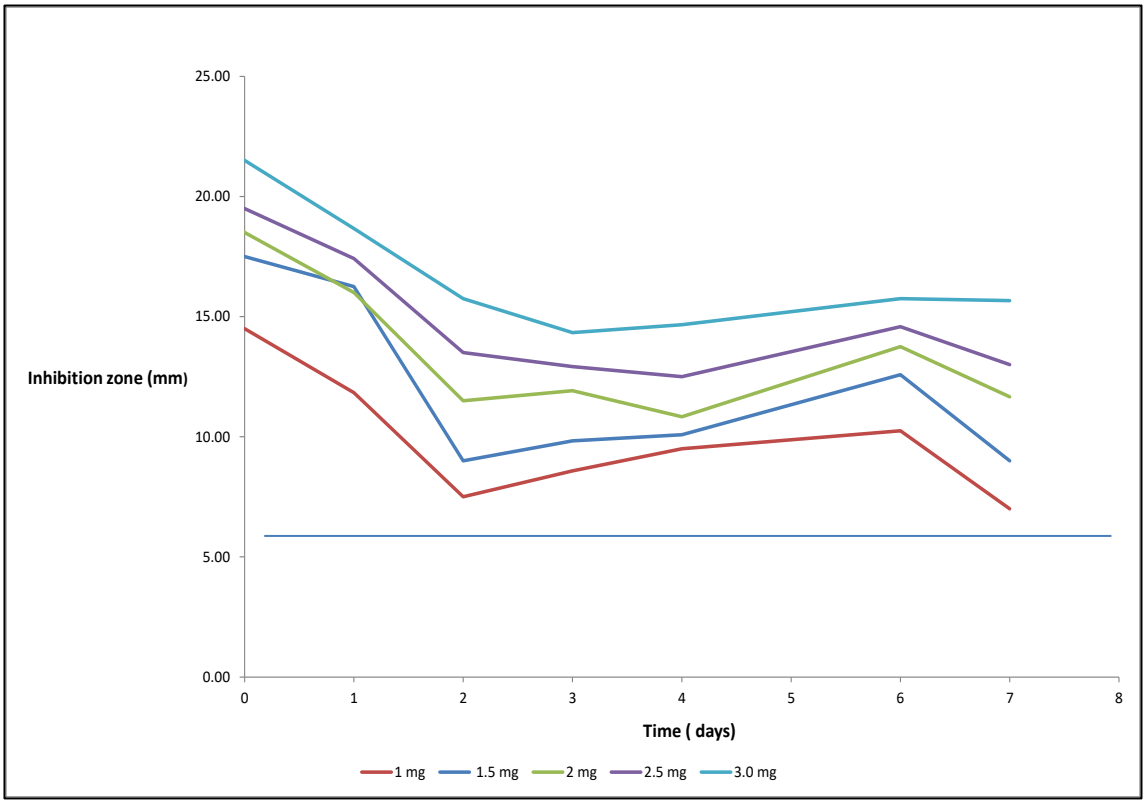


Figure 2.10: Average anti-MRSA activity of *P. lanosa* sampled from the three tanks for the 7-day post-harvest period for a subsequent loading effect analysis without the inclusion of error bars as measured by the well diffusion assay. The inhibition diameter of 6 mm is the diameter of the well and indicates no inhibition.

Overall, the data from this study shows that as the well loading rate was increased, there was a definite alleviation of the decline in antimicrobial activity, which may have implied a higher level of a variety of compounds or greater strength of the activity of a single compound (which required a certain minimum level to effect inhibition).

As regards macroalgal stability, this set of results reveals maintenance of levels of anti-MRSA activity post-harvest when *P. lanosa* was maintained for a limited time *ex-vivo* in the absence of its host. This supports the observation by Paull & Chen (2008) that post-harvest storage of *Gracilaria salicornia* fully submerged under dark conditions allowed for an extended life of 30 days. This study considered changes in algal colour, phycobilins, respiration rate, ethylene production, protein content, leakage and ion thalli content under various conditions and will be discussed in Section 2.4.3.

2.4.3 *The effect of temperature on anti-MRSA activity*

Changing temperature, salinity and light intensity are a part of the marine environment and elicit different responses from different seaweeds. Aquaculture requires high seaweed growth rates and the selection of ideal cultivars is a rigorous process. There are numerous studies comparing seaweed species and subspecies and the response of growth rate to varying combinations of abiotic conditions (Raikar *et al*., 2001;Talarico & Maranzana, 2000; Dring *et al*., 2013). The present study is novel in that it considers only the anti-MRSA activity of the crude aqueous extract from *P. lanosa* which has been removed from its host and maintained under artificial laboratory conditions and this is further explored under various temperature conditions in this section with a light period of 11 h per day.

Irish waters are considered temperate and in general seaweeds from these zones are considered more adaptable to high temperatures compared to those of colder zones (Nygård & Dring, 2008). Four temperatures were selected as representative of conditions to which *P. lanosa* may be exposed during the course of a year in Irish coastal waters; 5, 10, 15 and 20 ˚C.

2.4.3.1 **Conditions of seawater held at different temperatures**

Conductivity measurements at each temperature are shown in Figure 2.111.

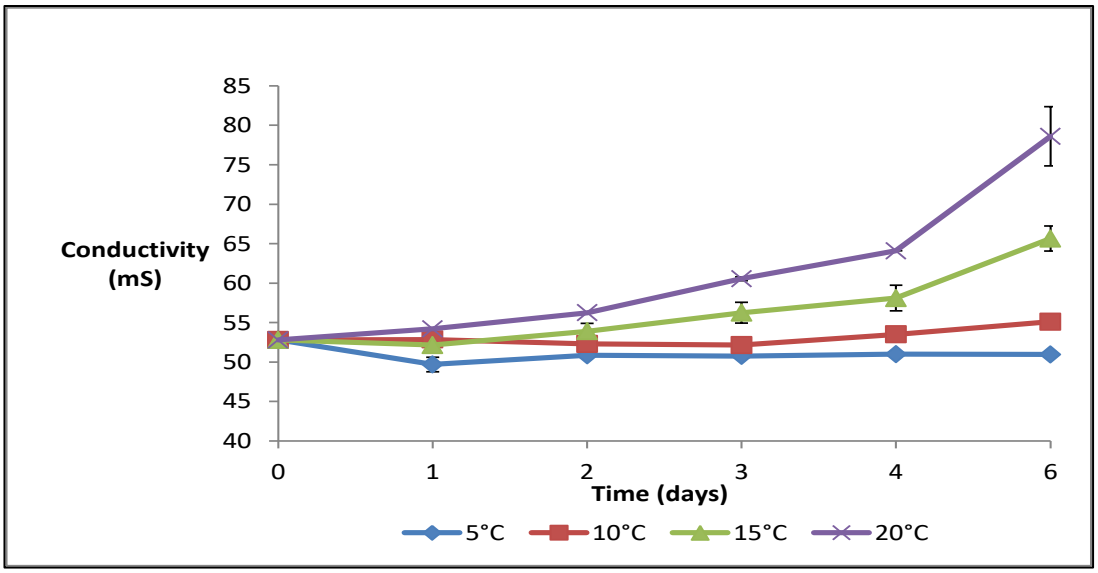


Figure 2.11: Conductivity monitoring of seawater in beakers held at different temperatures and used for the maintenance of *P. lanosa* after harvesting. Values were the mean of duplicate beakers and the value at Day 0 was the conductivity of the seawater on the day of harvesting with error bars (standard deviation) (n=2).

Values remained stable for the first 2 days for all temperatures. However, thereafter, the seawater at 15 ˚C and 20 ˚C displayed a gradual increase in conductivity up until day 4, and a more pronounced increase between day 4 and 6, especially at 20 ˚C. This is in agreement with the results of the baseline study and may reflect electrolyte leakage from the seaweed, which was associated with the breakdown of membrane integrity and subsequent seaweed degradation, as outlined in Section 2.4.2. This was possibly due to the fact that the *P. lanosa* was harvested from seawater at 16˚C and that the higher temperature of 20˚C exerted stress on the plant. This is backed up by findings from a study that examined post-harvest quality of *Gracilaria salicornia* tank-cultured in Hawaii, which showed that higher electrolyte leakage occurred at lower temperatures (10 and 12.5 ˚C) than at 15 and 20 ˚C (Paull & Chen, 2008). This was the opposite of our findings, as the natural habitat of *Gracilaria salicornia* is tropical waters with higher temperatures. However, electrolyte leakage did increase after the first 4 days even at the 15 ˚C conditions, subsequently increasing to 90 % leakage, revealing a limited tolerance for conditions outside those normally experienced *in situ*.

The pH variation of the seawater for the duration of the temperature experiment is shown in Figure 2.122.

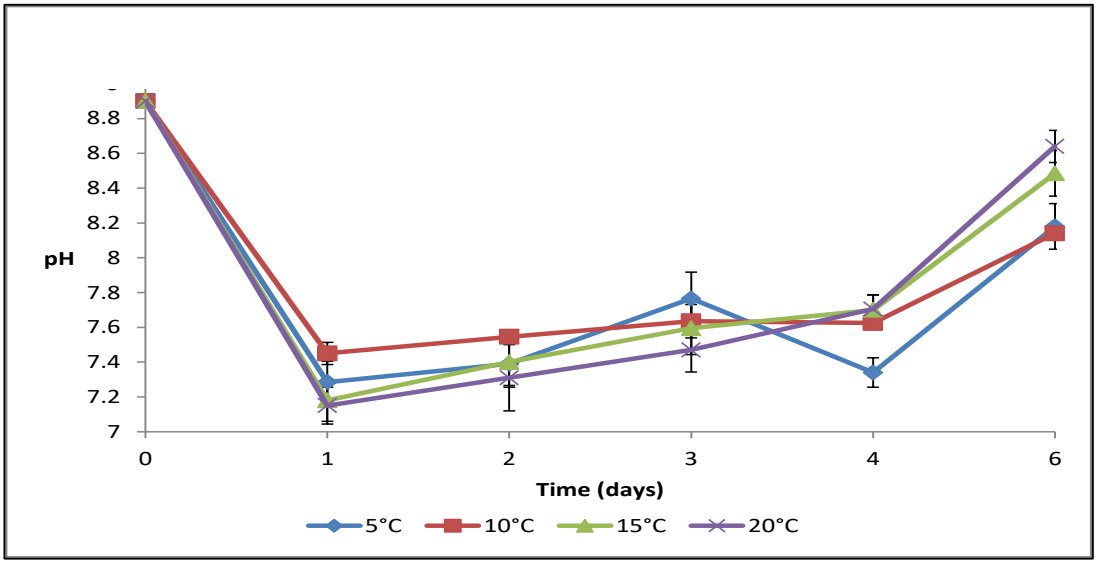


Figure 2.12: pH monitoring of seawater within beakers held at different temperatures and used for the maintenance of *P. lanosa* after harvesting. Values were the mean of duplicate beakers and the value at Day 0 was the pH of the seawater on the day of harvesting with error bars (standard deviation) (n=2).

Although the loading of seaweed was low, the pH of the collected seawater reduced considerably within the first 24 h at all holding temperatures, decreasing from 8.9 to 7.04 - 7.59. Thereafter, pH values remained below pH 7.8 across all temperatures until day 4 when they increased again. This was in agreement with findings from the baseline study. This could reflect the retention of photosynthetic processes, breakdown of the seaweed structure or the resumption of photosynthesis, as uptake of CO_2, as well as HCO_3, by marine algae will result in increased pH.

Dissolved oxygen levels, as displayed in Figure 2.133, were a different scenario, as the higher the temperature, the lower the oxygen levels, particularly up to day 3. The original dissolved oxygen value of the seawater on harvesting was 10.14 mg/L and this dropped within the first 24 h in the higher temperature seawater, whereas it remained relatively stable up to day 6 at 10 ˚C and up to day 3 at 5 ˚C, thereafter fluctuating between day 3 and 6 in the latter.

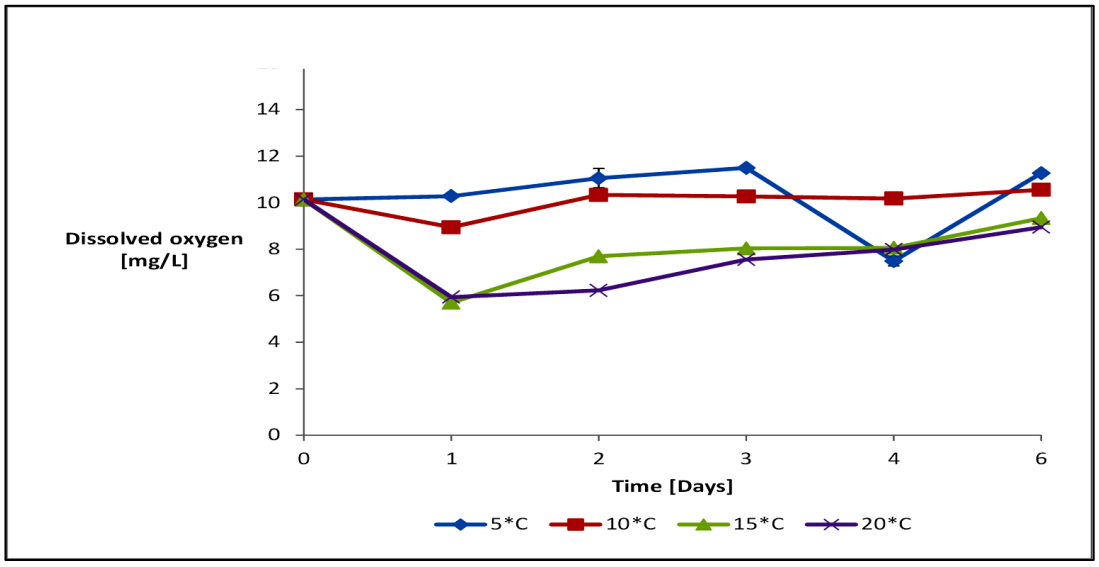


Figure 2.13: Dissolved oxygen monitoring of seawater within beakers held at different temperatures for the maintenance of *P. lanosa* after harvesting. Values were the mean of duplicate beakers and the value at Day 0 was the dissolved oxygen level of the seawater on the day of harvesting with error bars (standard deviation) (n=2).

Dissolved oxygen levels indicated recovery considerably by day 6 at all temperatures. The expectation was that dissolved oxygen levels would remain low at higher temperatures due to the decreased solubility of oxygen at higher temperatures, as well as

increasing salinity due to evaporation. The fact that oxygen levels were restored may indicate stability of conditions and that photosynthesis was resumed by *P. lanosa*. This was in agreement with data from a study by Haas *et al*. (2014) which demonstrated that the green macroalga *Bryopsis pennata* was tolerant to extremely low oxygen concentrations (as low as 2-4 mg/L) when cultivated. This was substantiated by data for the cultivation of two *Kappaphycus* species (Rhodophyta) where optimal parameters included dissolved oxygen levels of 5.43-5.46 mg/L (Zuldin & Shapawi, 2015) at culturing temperatures of 30 ˚C. This serves to illustrate that low oxygen levels of roughly similar values to those recorded in the present study at the highest temperature of 20 ˚C (mean 6.23 mg/L for the 2 beakers) were adequate for optimal culturing of seaweed by land-based farming.

It is known that seaweeds 'sense' temperature and that the temperature signal induces cellular acclimatisation responses which include changes in membrane fluidity, protein conformation and metabolic reactions and cytoskeleton depolymerisation (Murata & Los, 1997). By way of example, the degree of unsaturation of membrane lipids is the key factor affecting membrane lipid fluidity and it may be plausible that this response may be linked to compounds responsible for anti-MRSA activity and the change in lipid unsaturation critical to anti-MRSA activity.

2.4.3.2 **Anti-MRSA Activity**

Figure 2.14 shows that there was a complete loss of anti-MRSA activity on days 1 and 2 post-harvesting when *P. lanosa* was maintained at 5 ˚C. However, activity was restored on days 3 or 4, and this was more pronounced at the higher well loading of 3 mg as would be expected. At such a low temperature, photosynthesis would be impaired and cellular mechanisms in place to prevent chilling injury. This possibly prevented production of anti-MRSA compound(s) until acclimation. For example, holding the invasive red algal species, *Gracilaria vermiculophylla*, at 5 ˚C resulted in low overall growth rates regardless of light conditions (Nejrup *et al*., 2013). The study compared the growth of this invasive species with that of *Fucus vesiculosis* and *Ulva lactuca* and revealed that cellular carbon content decreased with increasing temperature for all three species. The relevance to this investigation lies in the suggestion that photosynthesis (responsible for growth) may not be necessary for the generation or protection (by the cellular

environment or other compounds) of the bioactive compounds active against MRSA.

The monitored seawater conditions revealed a drop in both pH and dissolved oxygen on day 4 whilst there was an increase in anti-MRSA activity. The latter may reflect the requirement of the seaweed for oxygen once the stress reaction was initiated between days 2 and 3. The low temperature stress response invoked may cause a cessation of production of anti-MRSA compounds and either the resumption of production of these and/or the production of new compounds once the seaweed has acclimatised to the cold conditions.

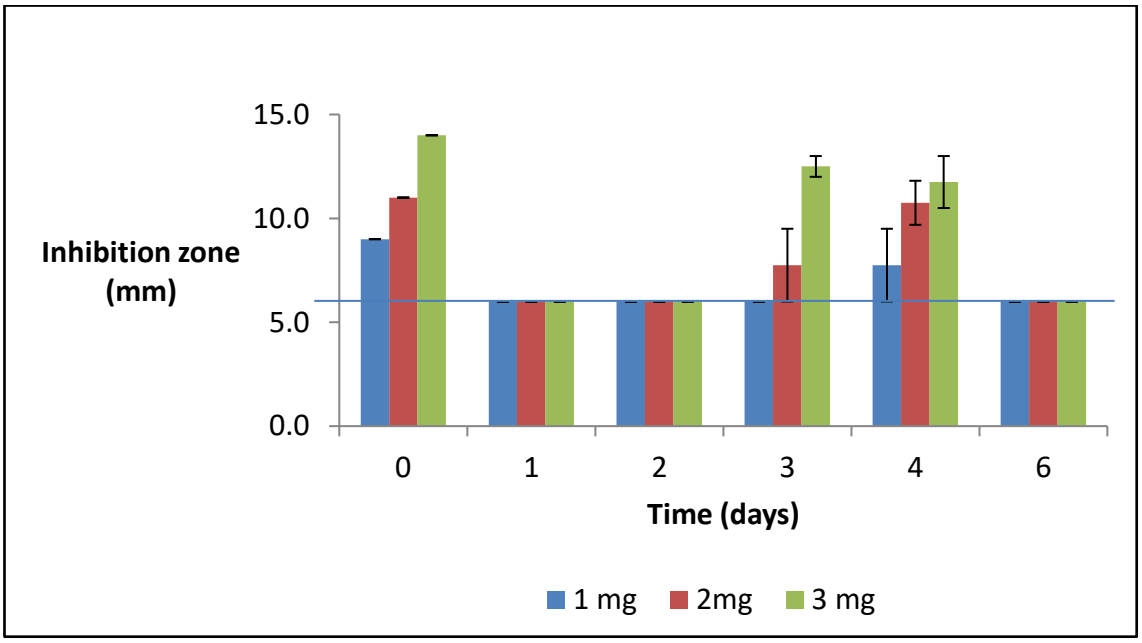


Figure 2.14: Anti-MRSA activity of extracts from *P. lanosa* held in seawater at 5 ˚C with 11 h of light per day and tested at 3 well loadings (1, 2 and 3 mg). Values were the mean of duplicate samples taken from duplicate beakers (n=4). The inhibition diameter of 6 mm was the diameter of the well and indicates no inhibition.

Figure 2.155 shows the response to a temperature of 10 ˚C. A loss of activity for all well loadings occurred by day 2, with only very low residual activity for the 3 mg loading at day 1. This was followed by recovery of observable activity during days 3-6, albeit only for the higher loading rates. Anti-MRSA activity was observed for a longer period for the 3 mg well loading and the resumption of activity required more time and the activity was less than at 5 ˚C, up to day 4, signifying less sensitivity to this temperature change. There

was an increase in activity at day 6 for both the 2 and 3 mg well loading, which was the opposite to that observed at 5 ˚C, where there was complete loss of anti-MRSA activity. This was also seen in the baseline study at 15 ˚C where there was an increase in activity at day 6 which was maintained until day 7 before loss of activity occurred. This finding may be one of the most significant of the study, as higher temperatures failed to produce activities of this level after the same time period.

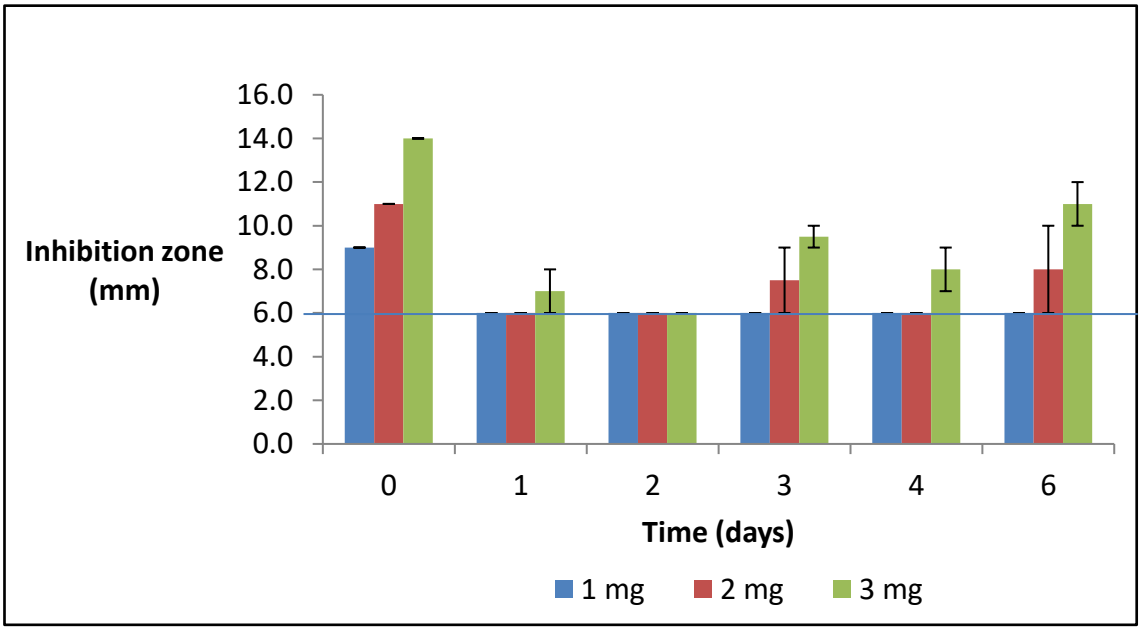


Figure 2.15: Anti-MRSA activity of extracts from *P. lanosa* held in seawater at 10 ˚C with 11 h light per day and tested at 3 well loadings (1, 2 and 3 mg). Values were the mean of duplicate samples taken from duplicate beakers, (n=4). The inhibition diameter of 6 mm was the diameter of the well and indicates no inhibition.

Figure 2.166 shows the response of *P. lanosa* when held at 15 ˚C. At the 2 and 3 mg well loading there was some residual anti-MRSA activity after one day but for the 1 mg rate, activity was completely lost. The temperature effect would be expected to be negligible at 15 ˚C, as the seawater temperature on harvesting was 16.8 ˚C so this drop in anti-MRSA activity was potentially due only to the removal of the epiphyte from its host. The subsequent recovery to almost full pre-harvest anti-MRSA activity on day 3 for both the 2 and 3 mg loading rates may also be as a result of removal from the host i.e. a stress response. However, thereafter there was a decline in activity, which may infer that some new mechanism was operating to compensate for the loss of host attachment or that the seaweed was dying. This result supported the theory that the loss of anti-MRSA activity

was due to removal of the epiphyte from its host and that after the initial considerable resumption of activity, there was insufficient induced change to sustain activity compared to the 10 ˚C holding condition, i.e. temperature was the prominent stress condition.

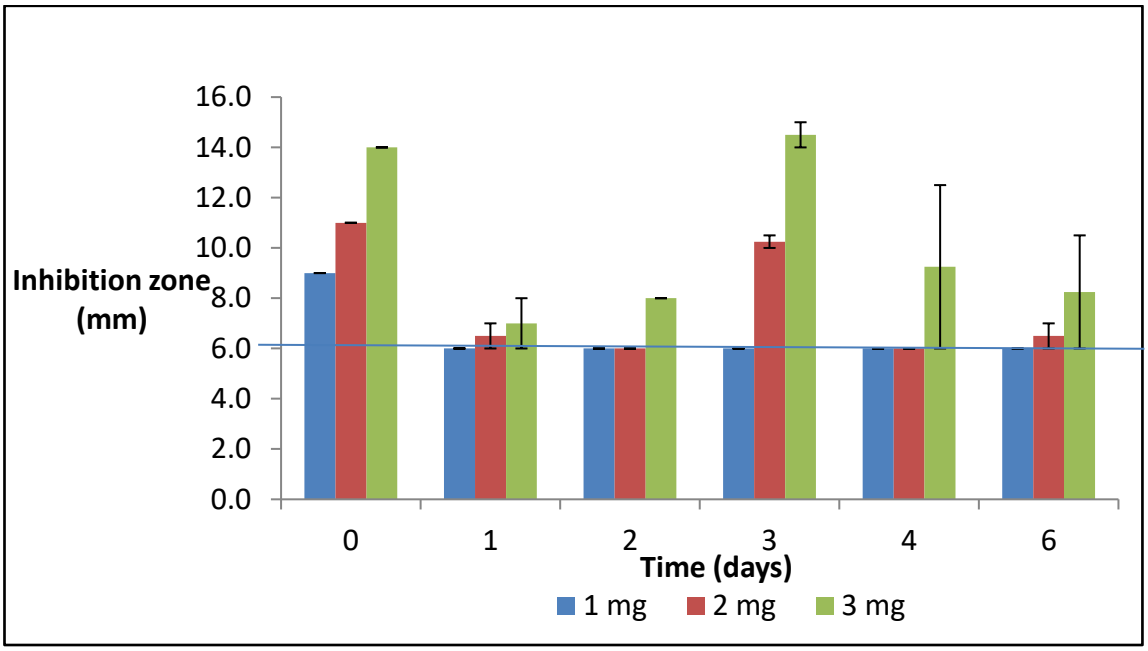


Figure 2.16: Anti-MRSA activity of extracts from *P. lanosa* held in seawater at 15 ˚C with 11 h light per day and tested at 3 well loadings (1, 2 and 3 mg). Values were the mean of duplicate samples taken from duplicate beakers, (n=4). The inhibition diameter of 6 mm was the diameter of the well and indicates no inhibition.

Figure 2.177 demonstrates that anti-MRSA activity was totally eliminated after only one day when *P. lanosa* was maintained at 20 ˚C. *Ascophylum nodosum* possesses broad thermal tolerance reflected by its wide distribution and abundance on the temperate shores of the North Atlantic Ocean, the northern western coast of Europe from Svalbard to Portugal, including eastern Greenland and limited by warmer ocean waters (Marbà *et al*., 2017). Optimum growth occurs between 10 and 17 ˚C and no *in situ* growth has been recorded above 24 ˚C. It may be that this higher temperature resulted in cessation of cellular processes in *P. lanosa* responsible for continued survival as well as for compound(s) contributing to anti-MRSA activity.

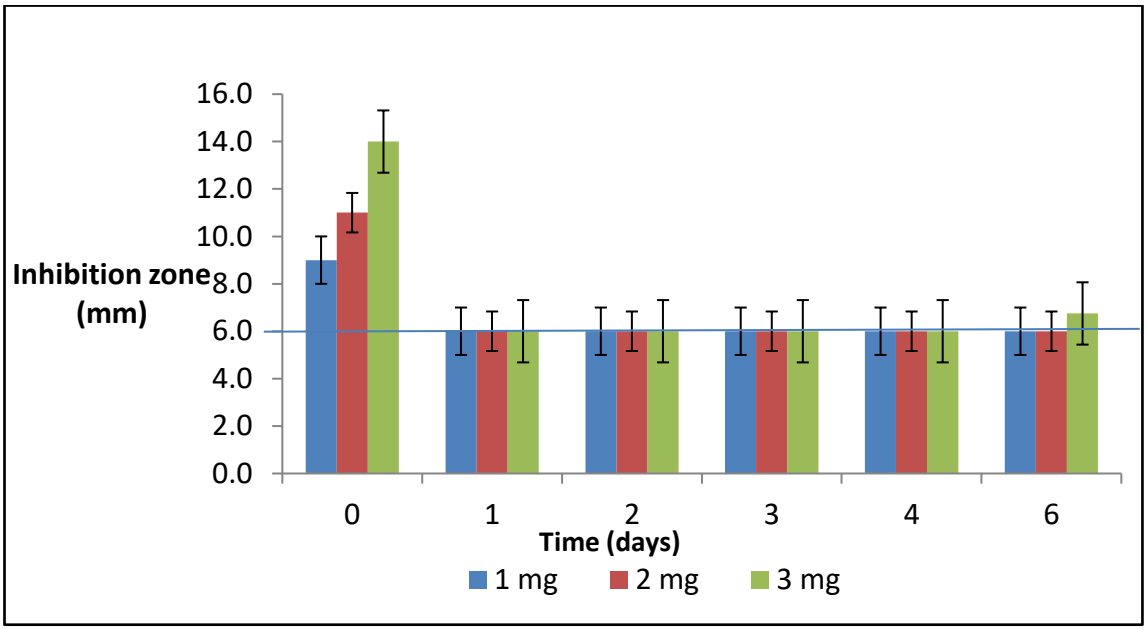


Figure 2.17: Anti-MRSA activity of extracts from *P. lanosa* held in seawater at 20 ˚C with 11 h light per day and tested at 3 well loadings (1, 2 and 3 mg). Values were the mean of duplicate samples taken from duplicate beakers, (n=4). The inhibition diameter of 6 mm was the diameter of the well and indicated no inhibition.

Overall, data from the present study demonstrated a loss of antimicrobial activity by the second day post-harvesting when *P. lanosa* was held at all of the temperatures evaluated and indicated a recovery of activity to varying levels for all temperatures except 20 ˚C.

This may allude to the nature of the anti-MRSA compounds. It is known that lipids maintain fluidity of cell membranes and it was recorded that relative polar lipid content was higher in the spring and therefore associated with a temperature increase (Goncharova *et al*., 2004). There is also the question as to whether the stress response was purely due to the process of acclimatising to the new temperature conditions and/or due to removal from the host seaweed and its natural environment.

The low temperature elicited a strong response in terms of recovered activity, as sizeable zones of inhibition were regained for the 3 and 2 mg well loadings, Figure 2.15. This was evident for both the 10 and 15 ˚C conditions. However, only at 15 ˚C at day 3 was there an indication of a slight activity increase potential. The initial increase in recovered activity was not sustained at the 5 ˚C holding condition and no activity was observed for day 6. These results may be explained by the fact that the seawater temperature on harvesting was 16.8 ˚C and that acclimation strategies had already compensated for the

large decrease in temperature and that resources were depleted and no longer sufficient to allow for sustained anti-MRSA activity. This is an unusual feature in light of the stability of algal extracts stored at low temperatures where the activity was retained or even enhanced (Tan *et al*., 2011). It would have been anticipated that the compounds within their 'normal' cellular environment would be preserved at low temperature conditions. A similar situation occurred at 15 ˚C, again supporting the possibility that the stress incurred was due to removal of *P. lanosa* from its host. Therefore, the most potentially useful result was that pertaining to the 10 ˚C holding condition, where anti-MRSA activity recovered and increased with time as observed at both the 2 and 3 mg well loadings. This could be related to a certain level of stress, where resources necessary for the production of anti-MRSA compound(s) are not used and cellular processes partially responsible for retention or generation of activity are activated by the longer exposure to this temperature. The conductivity and dissolved oxygen readings for the two temperatures were different, with 15 ˚C demonstrating higher conductivity and lower dissolved oxygen values which may indicate loss of photosynthesis when compared to the values observed at 10 ˚C. Another possible explanation for the results is that cell membrane alterations as well as metabolic changes occurred which are related to the reaction of the seaweed to the colder temperature. The explanation is that the anti-MRSA activity could be due, at least in part, to poly-unsaturated fatty acids (PUFAs), as seaweeds can accumulate these as environmental temperatures decrease, with species that inhabit cold regions possessing higher levels of PUFAs than species of higher temperature habitats (Gosch *et al*., 2012).

Figure 2.18 presents a summary of the anti-MRSA activity observed at the 3 mg loading of the *P.lanosa* extract where the seaweed was exposed to 11 h light per day as a function of temperature.

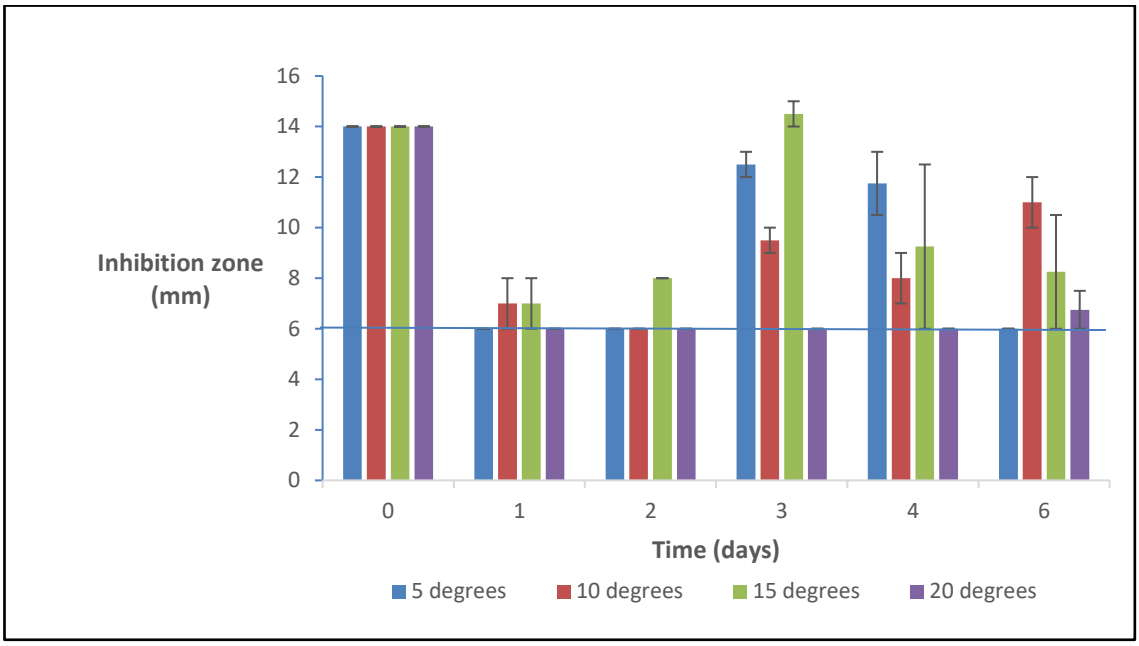


Figure 2.18: Comparative anti-MRSA activity of a 3 mg loading of extract from *P. lanosa* held in seawater with 11 h light per day as a function of temperature. Values are the mean of duplicate samples taken from duplicate beakers, (n=4). The inhibition diameter of 6 mm was the diameter of the well and indicates no inhibition.

Statistical analysis determined that all three factors (not covariates), day(time), temperature and extract loading affected the response of zone of inhibition (anti-MRSA activity) with extract loading having had a greater impact than temperature. There was only a small increase as temperature conditions changed from 5 – 20 ˚C. Furthermore, all three factors were statistically significant ($p < 0.05$) in determining anti-MRSA activity.

In 2013, Nejrup *et al.* examined the response of three seaweeds to temperature; the invasive red alga *G. vermicuphylla*, the green alga *U. lactuca* and a brown alga *F. vesiculosis* (Nejrup *et al*., 2013). They found that the response of the algae to increasing short-term incubation temperatures was affected considerably by the temperature to which the algae had been long-term acclimated. At a low short-term incubation temperature (10 ˚C), the photosynthetic rates of species that were long-term acclimated to cold temperatures were generally higher than those that were long-term acclimated to warm temperatures. The opposite was also true, as at a high incubation temperature (30 ˚C) the cold-acclimated algae generally had lower photosynthetic responses than the warm-acclimated algae (Nejrup *et al*., 2013). Another study investigating the effect of temperature on two red algal species, *Mastocarpus stellatus* and *Chondrus crispus*

demonstrated similar photosynthetic rates in fronds grown at both 5 and 15 ˚C (Dudgeon *et al*., 1995). It was therefore, considered that photosynthetic phenomena over the temperature range examined (5-35 ˚C) were genetically programmed and not influenced by environmental factors (Dudgeon *et al*., 1995). These two scenarios represent totally different temperature responses within algae, and it is not known which is applicable to the epiphyte *P. lanosa.* In light of a study by Garbary *et al*. (2014), however, it is known that *P. lanosa* reacts in terms of photosynthesis within 24 h of removal from its *A. nodosum* host, suggesting that the compound(s) contributing to anti-MRSA activity may not be those normally required for the maintenance of photosynthesis. Garbary *et al* concluded that there was little evidence for a mechanism to explain the reduction of quantum yield and electron transport rates in *P. lanosa* after detachment from *A. nodosum*. *A. nodosum* contains phytohormones which are known to modify photosynthetic processes (Tarakhovskaya *et al*., 2013), and this may also apply to its relationship with *P. lanosa.*

In all plants, temperature is a major factor controlling the rate of photosynthesis (Davison, 1991). Temperature has been found to affect the availability of inorganic carbon and the rate of carbon fixation by ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) (which is an enzyme involved in the first major step of carbon fixation, a process by which atmospheric carbon dioxide is converted by plants and other photosynthetic organisms to energy-rich molecules) and for some algal species the rate of nutrient uptake correlates with temperature. Another general trend is that algae from low-temperature environments show higher photosynthetic rates at low temperatures and have a lower optimal temperature for photosynthesis than algae from warmer environments. As mentioned in the previous paragraph, the cessation of photosynthetic processes may have directed resources into alternative metabolic pathways which permit a range of possibilities i.e. the production of more bioactive compound or other synergistic compounds, the loss of production of antagonistic compounds or the production of new compounds whilst dying, with these degradation products having a level of bioactivity.

There are limited studies on the relationship between temperature and the level of secondary metabolites in macroalgae. A parabolic response of elatol to temperature change was observed for cloned *Laurencia dendroidea J. Agardh* in a study conducted by Sudatti *et al*. (2011) where there was an increase in elatol (a sesquiterpene) levels for

both 20 and 25 ˚C from 15 ˚C followed by a reduction at 30 ˚C. This was not the case for the secondary metabolites of two other Rhodophyta species; i.e. variation in temperature between 15–25 ˚C and daylength produced no obvious change in laurinterol and dibromolaurinterol content of *L. okamurae* (Kuwano *et al*., 1998) and the levels of biologically active violacene, mertensene and compound 1 were unaffected in *Plocamium cartilagineum* when temperatures ranged from 11 to 18 ˚C (Palma *et al*., 2004). Seasonal variations in concentrations of secondary metabolites attributed to temperature changes have, however, been observed previously. For example, in *Caulerpa taxifolia* (Chorophyta species), temperature positively influenced the production of chemical defence molecules, such as caulerpenyne (another sesquiterpene with unsaturation, methyl and acetyloxy groups) and presented in Figure 2.18, which had antibiotic activity against *S. aureus* (Paul & Fenical, 1987) and compounds with cytotoxicity in mammalian cells (Leme *et al*., 1993).

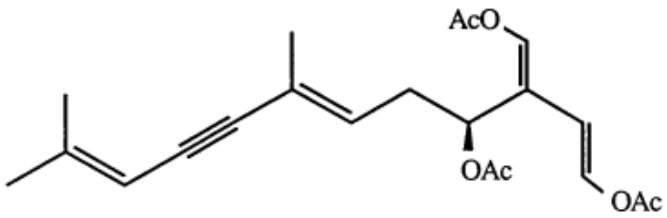


Figure 2.19: Caulerpenyne structure (Paul & Fenical, 1987)

There are a variety of processes dealing with temperature stress in seaweeds and include the following:

(1) Heat shock proteins are accumulated, which act as molecular chaperones protecting cellular proteins from mis-folding and degradation by environmental stress, including cold and heat stress. This was proposed by Henkel *et al.* (Henkel *et al.,* 2009) who suggested that the high thermal tolerance of the invasive kelp species *Undaria pinnatifida* in the northeast Pacific was due to high expression of the *hsp70* gene which encodes heat shock protein 70.

(2) The unsaturation of fatty acids increases membrane fluidity which is essential if cells are to tolerate cold stress and survive low temperatures (Los & Murata,

2004). Increasing proportions of PUFAs and, therefore, a higher degree of unsaturation in *Caulerpa racemosa* from the northern Adriatic coincided with a sharp decrease in water temperature in winter (Blažina e*t al*., 2009), allowing this species to survive winter without visible necrotic parts or chilling injuries.

(3) There was an increase in production of antioxidant proteins and detoxifying enzymes to counteract the thermal stress which disrupts the cellular homeostasis of cells and enhances the production of reactive oxygen species (ROS) (Miller *et al*., 2008).

(4) Osmolytes in seaweeds include betaines, polyols, and sugars, such as mannitol and trehalose, and amino acids, such as proline (Kirst, 1990) which are accumulated and known to exert multiple protective functions in metabolism and act as antioxidants, stabilising proteins upon heat stress.

Any of these mechanisms may be responsible for the temperature-induced variation of anti-MRSA activity in the crude aqueous extract of *P. lanosa* observed in the current study.

2.4.4 *The effect of light on anti-MRSA activity*

Another key factor which is expected to affect the production of secondary metabolites responsible for anti-MRSA activity in *P. lanosa* is the duration of exposure to light.

2.4.4.1 **Conditions of seawater held under different light durations**

Conductivity values of the seawater in which the seaweed was held were first measured and were found to increase over the course of the study, for all light exposure conditions, as shown in Figure 2.20. This was not anticipated, as the low holding temperature (5.0 ˚C) would prevent evaporation, indicating that the change must relate to the behaviour of the seaweed itself. However, conductivity values remained low compared with those observed during the temperature study. This may indicate stability of the harvested *P. lanosa* under the artificial holding conditions, as conductivity variation (>10% change) may indicate electrolyte leakage and breakdown of algal structures (Paull & Chen, 2008). Moreover, the beakers exposed to constant light underwent the greatest change in

comparison to those maintained in constant darkness, which experienced the least change. However, the former were subjected to constant light, meaning that the seaweed sample was placed under the constant stress of irradiance, perhaps forcing constant photosynthesis (without respiration) and inducing high levels of protection from light damage resulting in these changes to conductivity.

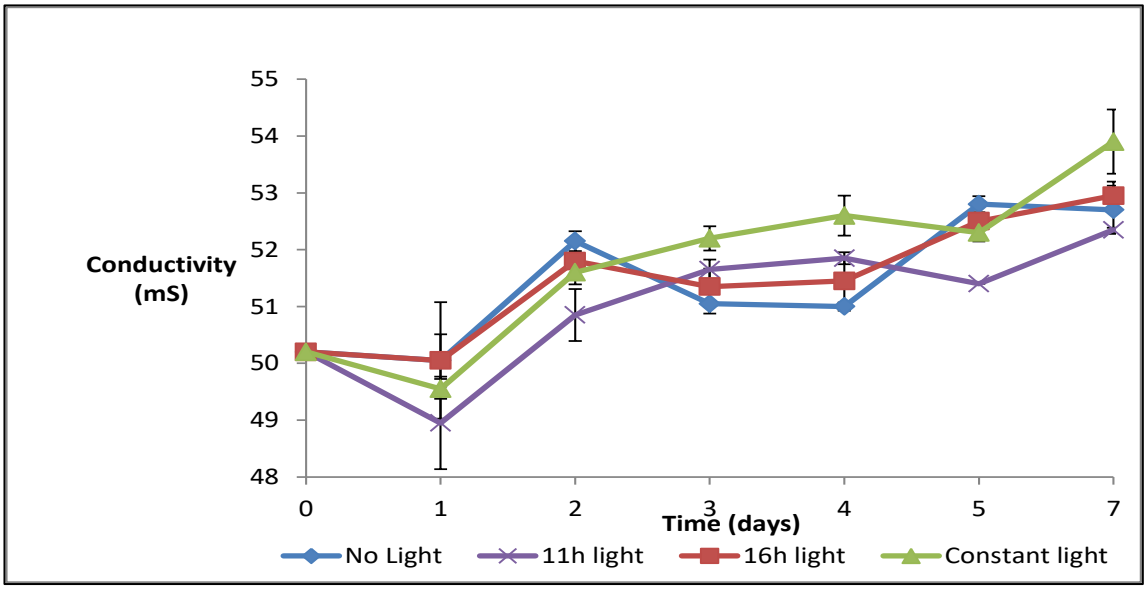


Figure 2.20: Conductivity of seawater held at different light exposures and at a temperature of 5 ˚C and used for the maintenance of *P. lanosa* after harvesting (n=2). The value at Day 0 was the conductivity of the seawater on the day of harvesting.

A study of the physiological performance of a filamentous, epiphytic red algae *Stylonema alsidii* under varying salinity gradients sampled the seaweed as an epiphyte on a green alga in the Baltic Sea (low salinity) and as an epiphyte on *Cladophora* sp from Martha's Vineyard in the North West Atlantic (high salinity) (Nitschke *et al*., 2014). Salinity is related to conductivity, with higher salinity leading to higher conductivity. It was found that levels of the polyol sorbitol accumulated with increasing salinity (5-60 mS) over a culture period of 3 days. There was a 5-fold increase for the high salinity isolate and a 10-fold increase for the low salinity isolate, in agreement with the fact that sorbitol functions as an organic osmolyte. This response to salinity stress provides insight into metabolic processes induced by changing salinity conditions. The intracellular environment and in particular, compounds such as osmolytes, could allow for stabilisation of compounds responsible for anti-MRSA activity.

As in the baseline and temperature studies, the pH of the seawater declined under all light exposure conditions compared to the intial value at harvesting (Figure 2.211). Thereafter, the overall trend was for an increase in pH during the holding period, and this was greatest for the seawater exposed to either constant light or 16 h of light per day. Those exposed to light for 11 h underwent an increase in pH but not to the same extent and the beakers with no exposure to light displayed little change in pH and remained <pH 7.8 throughout the experiment. In fact, the values for the seawater held in complete darkness and those with the 11 h exposure time are lower (0.4 pH units), especially at the end of the 7-day study. Minimal change in pH was measured in the beakers under constant darkness, probably due to lack of photosynthetic activity. Alternatively, the pH variation under the three light conditions could be due to seaweed degradation.

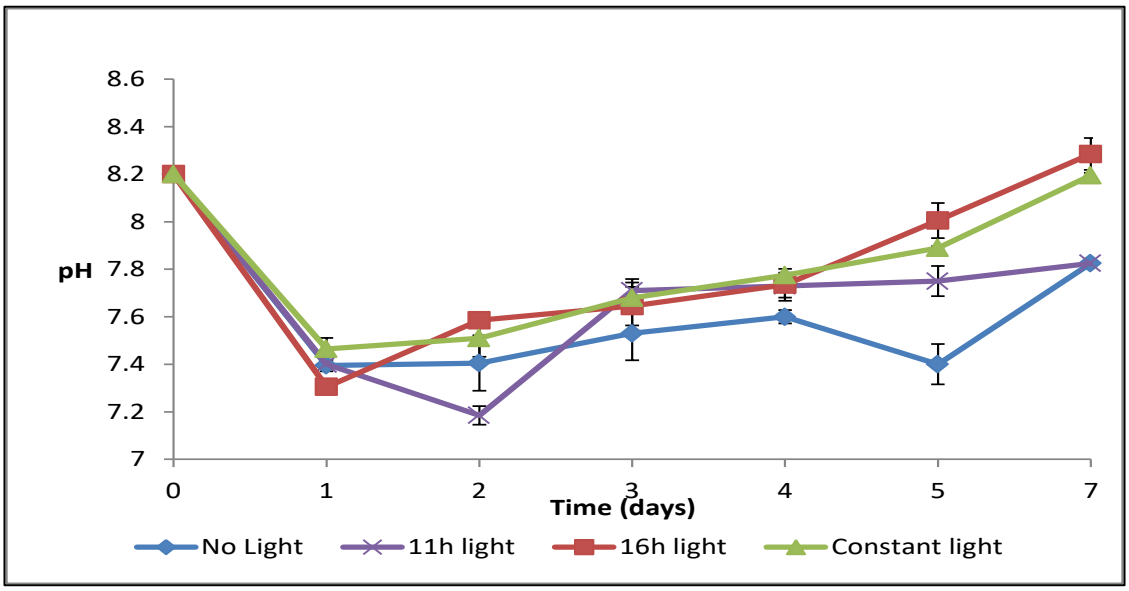


Figure 2.21: pH of seawater held at different light exposures and at a temperature of 5 ˚C and used for the maintenance of *P. lanosa* after harvesting (n=2). The value at Day 0 was the pH of the seawater on the day of harvesting.

Dissolved oxygen levels of the seawater in which the *P. lanosa* was held are presented in Figure 2.222. The values fluctuated under all light exposure conditions throughout the study period. Similar fluctuations were seen during the baseline study and were to be expected, as oxygen was provided individually to each beaker through Airvolution pumps in order to ensure that any metabolic processes would not be affected by low levels of

dissolved oxygen. Again, there was correlation between the values recorded for the beakers with 16 h of daily light exposure and those constantly exposed to light and at day 7 these were the highest values. This is perhaps indicative of either ongoing metabolic functions. The nature of these functions may relate to ongoing photosynthesis or other processes permitting seaweed survival. Algal decomposition would consume dissolved oxygen.

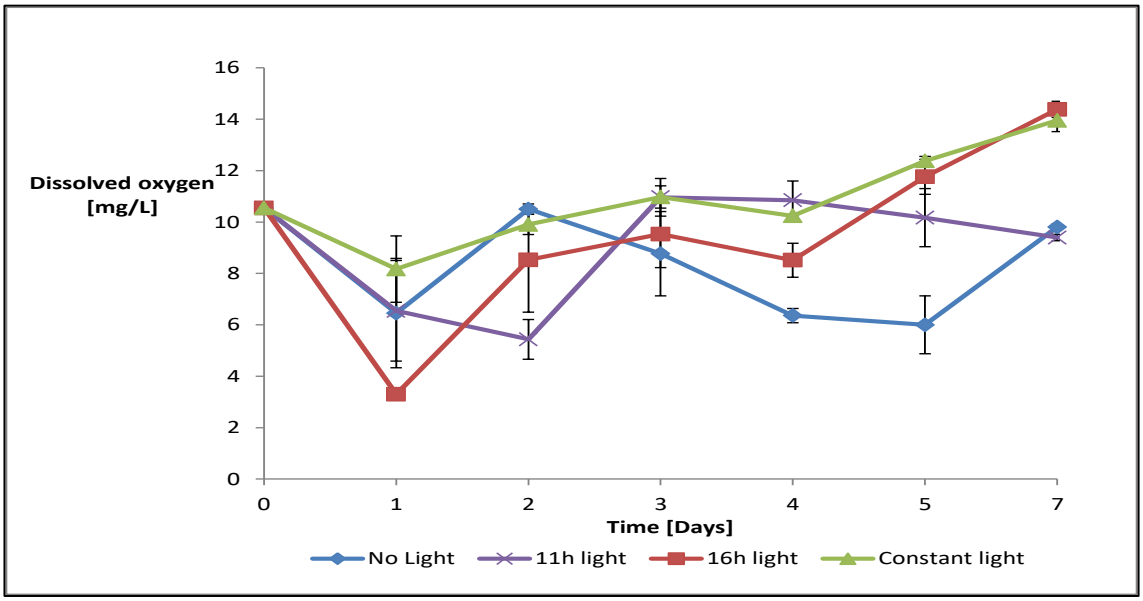


Figure 2.22: Dissolved oxygen of seawater held at different light exposures and at a temperature of 5 ˚C and used for the maintenance of *P. lanosa* after harvesting (n=2). The value at Day 0 was the dissolved oxygen level of the seawater on the day of harvesting.

2.4.4.2 **Anti-MRSA Activity**

Figures 2.23-2.26 show the results of anti-MRSA activity for crude aqueous extracts obtained from *P. lanosa* maintained under different durations of light exposure.

For seaweed maintained in complete darkness there was some indication of limited activity for both the 2 and 3 mg well loading after 24 h (Figure 2.233) and compared with the temperature study there was a delay in loss of activity, which was also observed in the baseline results. The fact that the seawater temperature at harvesting was 7.2 ˚C would have assisted the thermal acclimation of the seaweed to the artificial condition of 5 ˚C. Another possibility is that the seaweed outer structures were pre-adjusted to the

artificial temperature and that this protected the compound(s) responsible for anti-MRSA activity. The nature of the antimicrobial compounds and their associated molecules was unknown at this stage and may have contributed significantly to the protection of compound stability and efficacy. For example, it is known that antioxidants such as polyphenols can be twisted together with cell-wall polysaccharides and proteins with tight hydrophilic and hydrophobic bonds (Wijesinghe & Jeon, 2012).

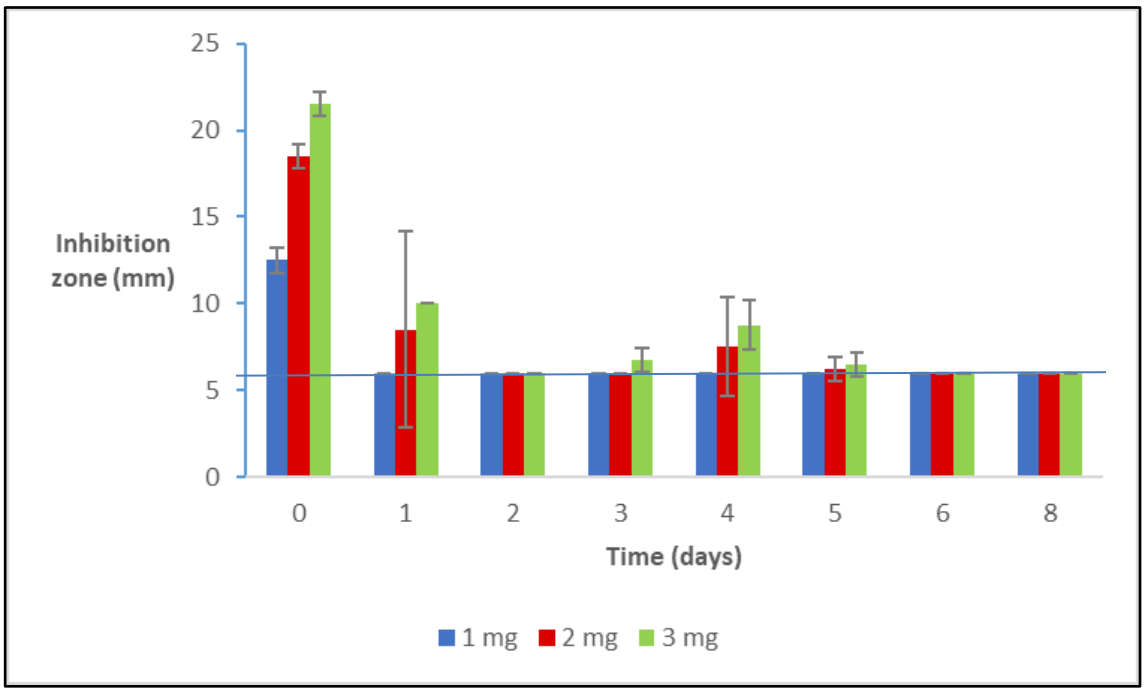


Figure 2.23: The effect of constant darkness at a temperature of 5 ˚C on anti-MRSA activity of *P. lanosa* extracts tested at 3 different concentrations (1, 2 and 3 mg), (n=4). The inhibition diameter of 6 mm was the diameter of the well and indicated no inhibition.

At day 2 anti-MRSA activity was completely lost for all extract concentrations which it gradually recovered to a minimal level, only observed for the 3 mg and to a lesser extent, the 2 mg well loading.

After maintaining the seaweed with 11 h of daily light exposure, anti-MRSA activity was also almost completely eliminated (

Figure **2.24**4). There was minimal activity for the 3 mg concentration at 2 days post-harvest but activity was completely absent at day 3 before very slight recovery. This data was particularly interesting as it reveals that at exposure for a normal photoperiod (the

same as on the day of harvesting i.e. this is an acclimated sample) there was a definite loss in activity during the 7 day post-harvest holding period.

This suggests that the simulated normal laboratory conditions do not permit a stress-induced increase in activity and/or that the removal of the epiphyte from its host dramatically affected the compounds responsible for anti-MRSA activity. The availability of light for photosynthesis did not safeguard anti-MRSA activity.

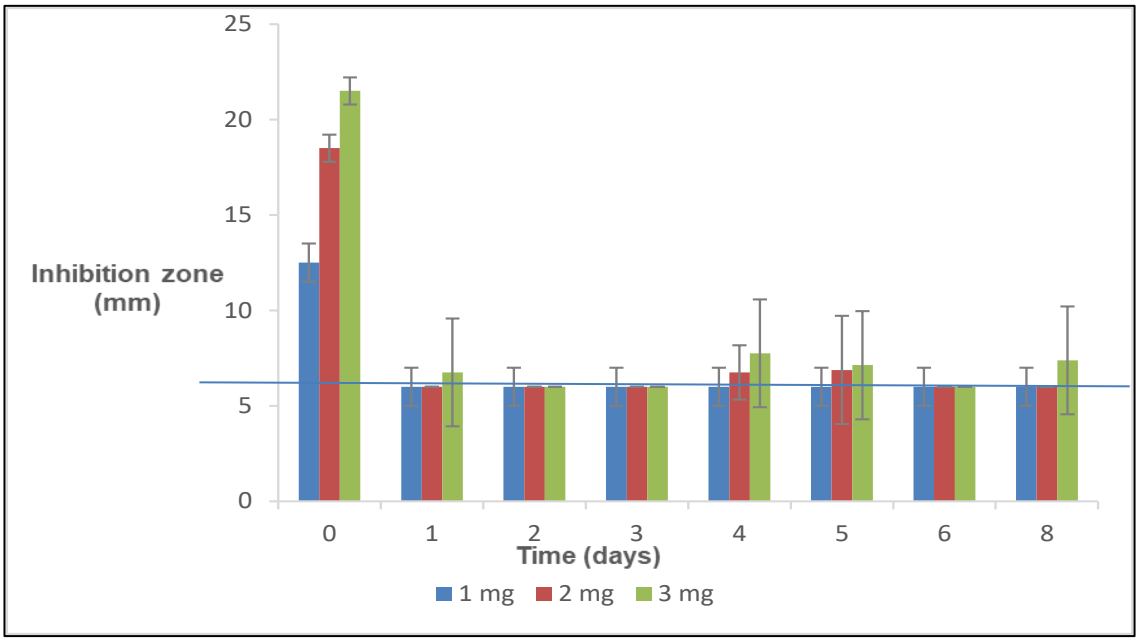


Figure 2.24: The effect of 11 hour light exposure, temperature of 5 ˚C on anti-MRSA activity of *P. lanosa* extracts tested at 3 different concentrations (1, 2 and 3 mg), (n=4). The inhibition diameter of 6 mm was the diameter of the well and indicated no inhibition.

The effect of 16 hour light exposure, Figure 2.25 revealed a more sustained retention of anti-MRSA activity for the 3 mg well loading as well as resurgence of activity at day 8. The loss of anti-MRSA activity was more gradual for the 3 mg compared to the lower loadings. This may be due to the long photoperiod and the prolonged photosynthesis induced (before harvesting the seaweed was acclimatised to an 11 h photoperiod).

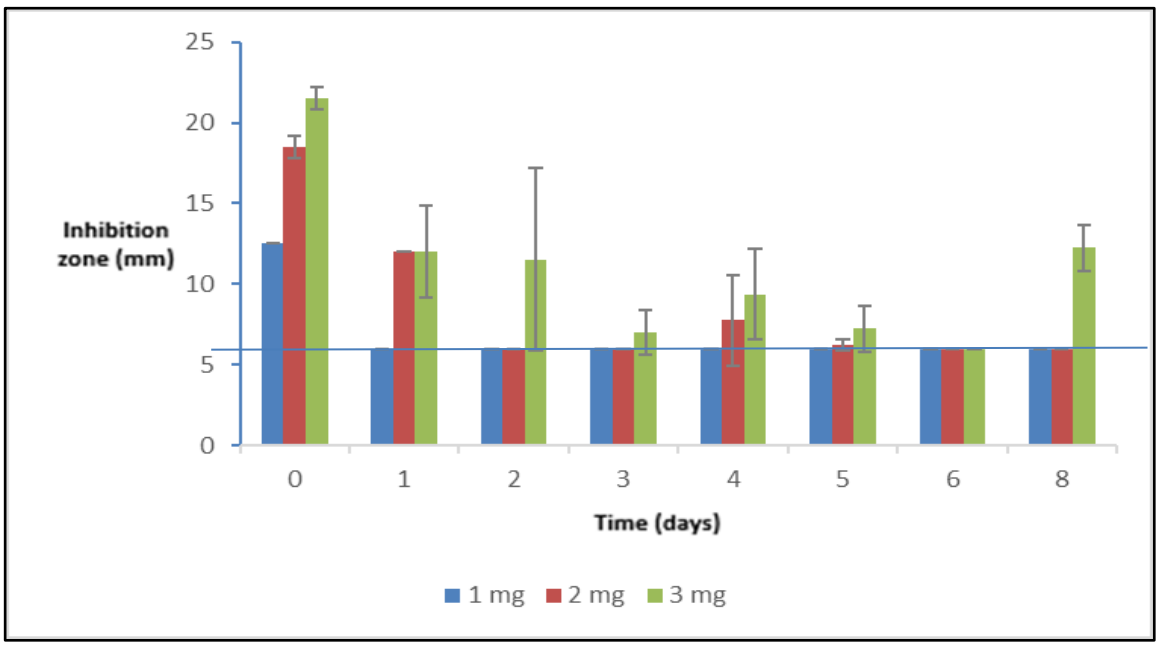


Figure 2.25: The effect of 16 hour light exposure, temperature of 5 ˚C on anti-MRSA activity of *P. lanosa* extracts tested at 3 different concentrations (1, 2 and 3 mg), (n=4). The inhibition diameter of 6 mm was the diameter of the well and indicated no inhibition.

Under constant light exposure (Figure 2.266) results were similar to those obtained with 16 h exposure (Figure 2.25). The constant light condition may have revealed very slight protection against the 2 day post-harvest loss of anti-MRSA activity, perhaps indicating that either continued photosynthesis or mechanisms induced to protect the seaweed against excessive irradiance have also impacted on the compound(s) responsible for anti-MRSA activity.

A possible explanation was that the raised level of photosynthesis (caused by longer light exposure) provided for the maintenance of anti-MRSA activity or generated more (of the same) active or synergistic compounds such as mycosporine-like amino acids (MAAs). An alternative explanation is the generation of photoprotective compounds which may be responsible for anti-MRSA activity. This could include production of MMAs (Hoyer *et al*., 2002) or a change in the level of photosynthetic pigments (Talarico & Maranzana, 2000), both are influenced by light conditions.

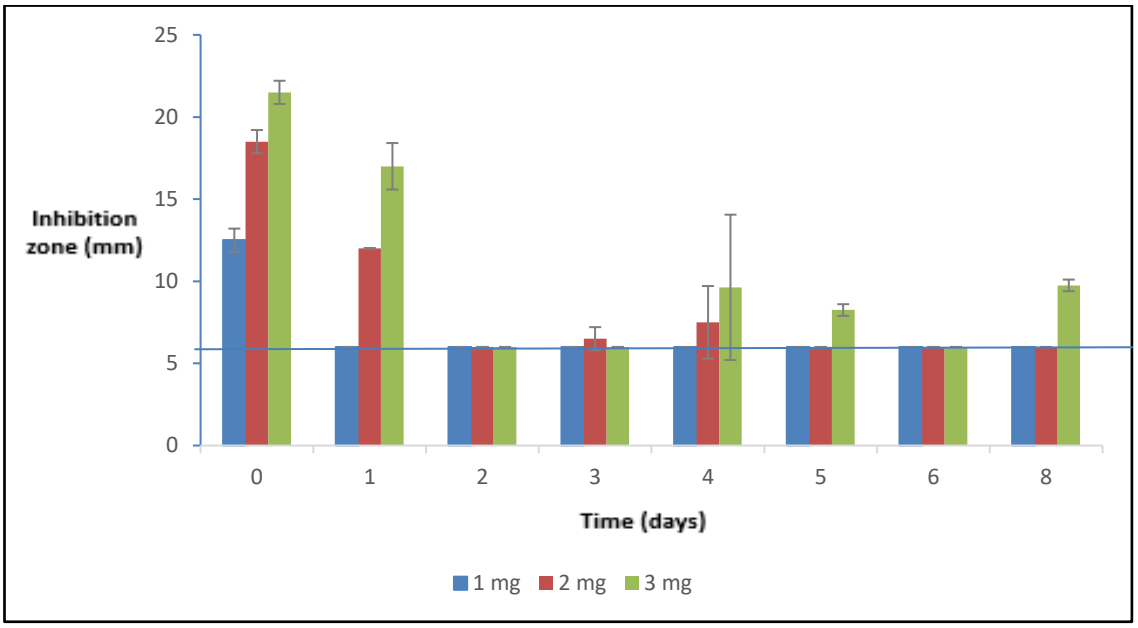


Figure 2.26: The effect of constant light, temperature of 5 ˚C on anti-MRSA activity of *P. lanosa* extracts tested at 3 different concentrations (1, 2 and 3 mg) (n=4). The inhibition diameter of 6 mm was the diameter of the well and indicated no inhibition.

These similarities may point to a limit of photosynthesis in that under the constant light condition the level has peaked and protective cellular processes have been activated accounting for similar levels of anti-MRSA activity if a certain level of photosynthesis was required for activity of the compound(s). Light studies reveal that there is considerable tolerance and lower photoinhibition for red algal species in the upper littoral zones (Gómez *et al*., 2004), indicating again that photosynthesis does not significantly affect anti-MRSA activity. Another explanation may be the presence of high concentrations of photoprotective pigments or UV-absorbing MAAs (Huovinen *et al*., 2004) affecting anti-MRSA activity. Another alternative explanation for the difference between the 16 h and constant light results was that antagonistic compound(s) may have been induced under constant light within the first day of exposure which act to mitigate the anti-MRSA activity.

The fact that there was some retention of activity for the 3 mg well loading was possible evidence of the stability and resilience of the compound(s), when present at thishigher concentration. However, as outlined above, the nature of the antimicrobial compounds and their associated molecules was unknown at this stage.

For all light conditions, there was decline in anti-MRSA activity. This was to be expected considering the findings of Garbary *et al*. (2014) who showed that a reduction in maximum quantum yield (indicative of photosynthesis) was apparent 24 h after removal of the *P. lanosa* epiphyte from its *A. nodosum* host and that there was no recovery over the 7-day cultivation period.

Overall, data obtained for the three light conditions may allude to the possibility that a minimum level of photosynthesis is required for either the generation or protection of anti-MRSA activity but at 11 h daily exposure this was not the case, which further supports the suggestion that anti-MRSA activity can be induced under stress. However, in total darkness, the anti-MRSA activity was at a much higher level after 24 h without light for photosynthesis in comparison with the 11 h result. It may be plausible that there exists two separate contributors to anti-MRSA activity i.e. some of the bioactive compounds are sensitive to light and others require a minimum level to remain active. Figure 2.27 presents a summary of the 3 mg well loading results showing the effect of light exposure variation on anti-MRSA activity over time.

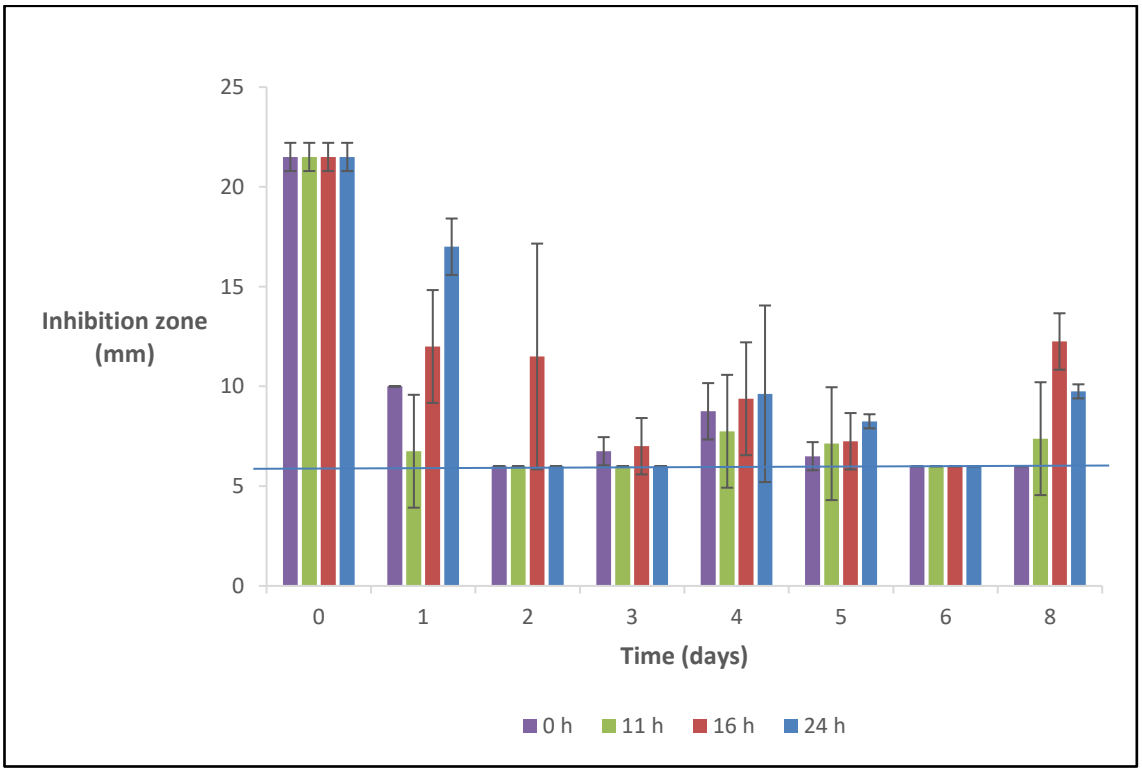


Figure 2.27: Comparative anti-MRSA activity of 3 mg loading of extract from *P. lanosa* held in seawater at 5 ºC day as a function of light exposure. Values were the mean of duplicate samples taken from duplicate beakers, (n=4). The inhibition diameter of 6 mm was the diameter of the well and indicated no inhibition.

Statistical analysis determined that day, light duration and extract loading rate all affect the antimicrobial response, with extract loading having a greater impact than light duration. All three factors were statistically significant ($p < 0.05$) in causing a change in anti-MRSA activity. The fact that the level of anti-MRSA activity was low may indicate the nature of the compounds responsible. For example, photodegradation of bromophenols is an active research field due to their increased abundance in the environment and their toxicity (Saeed *et al*., 2016) and UV light was shown to activate the photodecomposition process.

The study carried out by Nejrup (Nejrup *et al.*, 2013) found that the growth rate of the epiphyte, *G. vermiculophylla*, increased significantly with light at all temperatures, but the response was dependent on the experimental temperature. Growth rates exhibited a saturating relationship to light at all experimental temperatures and increased significantly with temperature and light, with the highest growth rate observed for a combination of 20 °C and 225 µmol photons day^{-1} s^{-1}. During the dark treatments, there was neither net growth nor appreciable loss, demonstrating that the seaweed maintained almost constant biomass, even at high temperatures, after 3 weeks of darkness. *G. vermiculophylla* had a significantly lower overall growth rate regardless of the light level in the 5 °C treatment.

Another investigation, conducted on *Tichocarpus crinitis* cultured for three weeks, investigated the effect of two levels of irradiance on lipid composition (Khotimchenko & Yakovleva, 2005). The light intensities significantly impacted the levels of storage and structural lipids under temperate conditions (although the actual temperature used was not reported). The results confirmed that *T. crinitus* was capable of regulating lipid metabolism depending on irradiance conditions, as it was able to alter the levels of storage and structural lipids and the composition of fatty acids, both in the total lipid pool and individual lipids. High light intensity stimulated the accumulation of storage lipids, while low light induced an increase in structural lipids. It is not known if a similar scenario occurs in *P. lanosa*, but if it does, then its effect on anti-MRSA activity in the present study was minimal as the activity under all light conditions was extremely low and heavily impacted compared with that observed at harvesting.

Overall, the present study was undertaken to ascertain whether stressing of freshly

harvested *P. lanosa* would allow for the retention and/or enhancement of anti-MRSA activity. The fact that the compound(s) responsible for this activity have not yet been identified or characterised prevents accurate speculation of the effects that stress conditions may exert on the chemical nature of this bioactive(s). Aligned to this is the lack of information on the location of the compound(s) within the seaweed structure, as depending on these treatments, they could either liberate or destroy the compound(s). There is an expectation that degradation and hydrolysis may occur at different pH and temperatures, which served as primary parameters for assessment, together with conductivity. Cold temperature acclimation in plants has been extensively researched, as plant membranes are stabilised against freeze-induced damage (Thomashow, 1998). Cryoprotectin has been identified as an anti-freeze protein (Hincha, 2002). Whether such molecules exist in algae is not known.

P. lanosa occupies the upper littoral zone and representative red algal species have been studied and found to possess broad salinity tolerance (Karsten *et al*., 1996). Another study compared Irish and Baltic *F. vesiculosus* plants and revealed that the Irish sea plants were less strongly affected by exposure to high levels of irradiance than those originating in Baltic waters which have lower levels of salinity (Nygård & Dring, 2008). This suggests that high salinity conditions may induce some type of a protective response in macroalgae. A further investigation into the desiccation tolerance of *G. corticata* concluded that this red algal species, an inhabitant of the upper littoral zone, was well adapted and employed a number of mechanisms to cope with its environment (Kumar *et al*., 2011). High levels of both PE and APC (both PBPs) were induced in hypersaline conditions with an increase of almost 70% and 52%, respectively from their initial concentrations under hyposaline conditions. Both high and low saline treatments induced an almost two-fold increase in the content of polyphenols and proline and in the activities of antioxidative enzymes after 6 days of exposure. This was in addition to a linear increase in the ratio of total unsaturated to saturated fatty acids. *G. corticata* was able to withstand the stress and retain the normal construction and function of the cell membrane, as evidenced by a reduction of only 25–30% in the chlorophyll pigment during the initial days of culturing under hypersaline conditions. However, a sharp decline was then observed in antioxidative enzyme activity, potentially due to the formation of ROS beyond the critical limit. Collectively, these results suggest a potential role of antioxidant

enzymes, PBPs, PUFAs and mineral nutrients in combatting salinity-induced oxidative stress in *G. corticata* and serve to illustrate the tolerance mechanisms that exist within red algae. This further highlights a range of possible compounds which are affected by stress conditions that may play a role in the anti-MRSA activity of *P. lanosa*.

Dring reported that both UV and long light exposure are linked to immediate damage specifically to D1 protein (a crucial component of the electron transport chain which acts at the reducing side of photosystem II) and cyclobutene pyrimidine dimers [solar ultraviolet (UV-B) radiation was found to induce mainly thymine dimers, which cause mutations and permanent damage if not removed by the repair-enzyme photolyase], whilst the longer term response leading to resistance is linked with the generation of MAAs (Dring, 2005). This was in contrast to the response to high temperature which may affect metabolic activity, independent of osmotic stress and involving heat stress proteins.

Various studies have been carried out to determine post-harvest conditions for the handling and storage of edible red seaweed. Paull and Chen studied various parameters, including post-harvest appearance [in particular the phycobilin (pigment) content)], electrolyte leakage, storage temperature and light, when examining optimal conditions for the extension of post-harvest life of *G. salicornia* (Paull & Chen, 2008). The findings concluded that at 22 ˚C the seaweed retained its fresh appearance for 3 days and at 15 and 20 ˚C, the colour and phycobilin content remained steady for 4 days. This compared with overnight storage at 2 ˚C which resulted in an unsightly, limp product, indicative of chilling injury, as well as a change in colour to pink within 1 day. The rate of electrolyte leakage was was higher during storage at 2, 10 and 12.5 ˚C than at 15 and 20 ˚C. The rapid increase in electrolyte leakage at 15 ˚C, together with the decline in phycobilin content at 10 ˚C, suggested significant changes in membrane integrity and chilling injury and 15 ˚C was proposed as the changeover temperature. The level of phycobilin was affected by temperature; at 10 ˚C there was a sharp drop and no recovery, whilst for 12 ˚ and 20 ˚C there was a drop followed by a slight or a good recovery, repectively and at 15 ˚C, a high level was maintained. It was concluded that seaweed stored at 16 ˚C and exposed to light had reduced loss of colour compared to that stored in the dark, but that stored in the dark at 21 ˚C had less colour change and a better appearance. Furthermore, it was determined that seaweed held fully submerged in seawater in the dark had a greatly extended post-harvest life of 30 days.

Taken together, these studies serve to illustrate the number and combination of parameters that can be considered when seeking to increase antimicrobial production in cultured seaweed. However, results from the present study suggest that maintenance of *P. lanosa* for longer than eight days under the conditions investigated for the accumulation of anti-MRSA bioactives is not feasible.

2.5 Conclusions

With regard to the stability of harvested *P. lanosa* in laboratory tanks, baseline results revealed maintenance of anti-MRSA activity which may indicate that this epiphytic macroalga is capable of being cultured for a limited time post-harvest, up to a period of 8 days when considering the 3 mg well loading results. The baseline results demonstrated that testing low crude seaweed extract concentrations was more sensitive - as the concentration increased, there was a definite stronger and more resilient anti-MRSA bioactivity, which implied a higher level of a variety of compounds or the strength of the activity of a single compound. The day 6 results for both the 2 and 3 mg well loading revealed a restoration of activity, suggesting that unknown mechanisms may exist, protecting activity or generating new anti-MRSA activity. There may be an initial readjustment of the algal metabolic pathways as most of the results revealed a loss of activity by day 4.

The temperature study revealed that maintenance of *P. lanosa* at a low temperature elicited a strong response in terms of recovered antimicrobial activity at a higher concentration. One of the most potentially useful results was that pertaining to the 10 and 15 ˚C holding condition where anti-MRSA activity was recovered over time. This coincided with observations from the baseline study where activity was found for all well loadings at day 6. This could be correlated to a certain level of stress. It suggests that the seaweed is not utilising the resources necessary for the production of anti-MRSA compound(s) but acting on cellular processes partially responsible for retention (protection) or generation thereof and suggests that the anti-MRSA compound(s) may be a lipid molecule(s) at this stage of artificial culturing.

The light exposure study revealed that with constant light, 11 h and 16 h daily exposure there was minimal (mainly temporary) restoration of antimicrobial activity at day 4 with higher concentrations of *P. lanosa* extract but this was not statistically significant. There may have been recovery of activity at 16 and 24 h light exposure which may suggest the generation of MAAs as reported by Dring (Dring, 2005) or a compound not subject to photodegradation. The fact that minimal activity was observed under dark conditions points to the possibility that the compounds involved in photosensitivity or reaction of the seaweed to light stress are not the same as those required for anti-MRSA activity. This will be discussed further in Chapter 4 when differences in minimum inhibitory concentration (MIC 80), minimum bactericidal concentration (MBC) and biofilm bactericidal concentration (BBC) are investigated.

However, the levels of activity were very low and never the same as those observed at harvesting. This may relate to the stage of growth at the time of harvesting i.e. March, when, although the seawater temperature was low (7.2 ˚C) and previous studies had reported low anti-MRSA activities at this time of year (Tan, 2013), high levels were found in this study. The impact of removal of the epiphyte was possibly highest at this time of year i.e. Springtime when the growth rates of many seaweeds are higher and would impact various constituents. It has long been recognised that fertility and spore/gamete release is affected by photoperiod, irradiance, temperature and nutrient concentration for a large number of seaweed species and this would further impact the level of secondary metabolites as the metabolic pathways adjust to another phase of the seaweed lifecycle.

Overall, these results convey a mixed message regarding the application of the limited stress conditions applied. The applied stress conditions would fall into the limitation stress category as they would not represent lethal conditions. However, the fact that *P. lanosa* was removed from its host would perhaps signify a disruptive stress condition but this had been reported previously by Garbary *et al*, 2014 when it was observed and determined that attachment to host was necessary for maximising photosynthetic processes in the epiphyte. It was not known whether the anti-MRSA compound(s) found in *P. lanosa* required photosynthetic processes to be produced or accumulated although from the results it may be argued that these processes are required for production of this specific antibacterial activity or that the compounds originate in its host, *A. nodosum*. It is plausible that the level of stress was not sufficient to effect more dramatic change in

antibacterial activity or that the two parameters investigated were not relevant elicitors of the property required. There was no major improvement in anti-MRSA activity compared to that observed at harvesting, only limited retention of activity in both the baseline and temperature studies and a drastic decrease in activity for the light study. The reason for this may be that the responsible compounds could be auxins which were found to decrease in an accelerated storage (54 º C for 14 days) investigation carried out by Stirk (Stirk *et al*., 2004) or that the compound(s) is(are) particularly susceptible to photodegradation. Anti-MRSA activity may be due to a single chemical entity with a broad spectrum of activity or many different chemical entities and changes in activity may be due to different quantities of a single compound, or the degradation of different compounds due to different conditions.

It is generally accepted that microbial and chemical decomposition can cause loss of biological activity during storage. Chemical decomposition may include hydrolysis, oxidation, polymerisation and isomerisation. Photodecomposition may have been a contributing feature in the light exposure experiments, although the major factor that contributes to chemical degradation is temperature. Environmental factors such as light and oxygen do affect rates of decomposition but temperature would be the main factor contributing to chemical degradation, with a 10 ºC increase in temperature increasing the rate of chemical reactions by an order of two to three times. The effect of temperature on the anti-MRSA activity of the crude extract is examined in Chapter 3.

Further stress investigations are required to assess their effect on antimicrobial efficacy of the crude *P. lanosa* extract. These should include a more detailed combined temperature-light study, an emersion/immersion study, as well as a salinity gradient and pH response analysis. The change in levels of PBP (pigmentation) may have allowed for a more thorough comparison of the stress situation and how *P. lanosa* was able to react to the change in conditions. Consideration should also be given to the epiphytic nature of *P. lanosa* and samples of host with epiphyte attached should be assessed in the same manner.

CHAPTER 3: PURIFICATION AND CHARACTERISATION OF ANTI-MRSA COMPONENTS FROM CRUDE EXTRACTS OF *P. LANOSA*

3.1 Introduction

The breadth of compounds responsible for antimicrobial activity found in seaweeds is constantly expanding and contributes to the challenge of any assessment. In terms of antimicrobial resistance (AMR), the significance of a novel structure will depend on the mechanism of action and whether the target organisms are able to respond and develop resistance. A large number of algal extracts have displayed antimicrobial activity. For example, fatty acids and hydroxyl fatty acids (oxylipins) (Gerwick *et al.,* 1993), macroalgal eicosanoids (biologically active C20 metabolites of arachidonic acid), glycolipids, sterols, phenolics and terpenoids. (Mellouk *et al.,* 2017; Maciel *et al.,* 2016). Some well-known algal-derived fatty acids such as lauric acid, palmitic acid, oleic acid and stearic acids act as potential antibiotic and antifungal agents, with some halogenated (with bromine, chlorine and rarely iodine) products such as diterpenes and triterpenes also possessing antibacterial activity (Cabrita *et al.,* 2010).

A marine natural product (MNP) review found 37 new compounds from red algae in eight papers, with four polybrominated C4–C8 hydrocarbons from *Plocamium australasica* including the ptilones, which possess skeletons not previously seen in algal metabolites (Blunt *et al*., 2018). Three C15-acetogenins were isolated from *Laurencia marilzae* and the absolute configuration of elatenyne was determined by the crystalline sponge method, which permits crystallographic analysis of noncrystalline compounds on a microgram scale. Halogenated indoles featured, with eleven polyhalogenated indoles from *Rhodophyllis membranacea*, including six representing the first isolation of bromo–chloro–iodo secondary metabolites. There were also four brominated indole related alkaloids from *Laurencia similis*. Overall, although there is a vast array of papers substantiating the potential of macroalgal extracts (El-din & El-ahwany, 2015; Manilal *et al*., 2009; Val *et al*., 2001; Bansemir *et al*., 2006), there are not as many studies with complete chemical profiles and pharmacological assessment.

There are many challenges associated with the isolation of bioactive compounds from seaweed. These include the variability of the source material (since an activity found at one collection point may be absent in another due to origin, season, storage conditions, etc.), the difficulty of isolating the active constituents, the nature of the extracting solvent,

the possibility that the active compound is a known compound (and therefore not protectable by composition-of-matter patents), and the expenses incurred on collection.

As all purification parameters contribute to observed bioactivity, such bioactivity may be said to be extract specific. It is necessary that a variety of extraction processes are carried out in order to maximise the efficacy of the final product, as many compounds can be sensitive to a range of conditions and so caution is required to prevent compound degradation or alteration.

Previous research at WIT demonstrated that the highest level of anti-MRSA activity was elicited by a crude water extract of *P. lanosa* (Tan, 2013). The reason for the pursuit of this research is the fact that it represents an eco-friendly extraction method. Water-soluble components would include water-soluble pigments (those responsible for the red colouration such as the various PBP), sugars and proteins. Researchers have identified *P. urceolata* as a good source of PEs due to the low concentrations of sugars in this species (Liu *et al.*, 2005) and this supports the aqueous extraction of bioactives from this genus.

However, while water-soluble extractions are preferable from an environmental perspective, the separation of water-soluble compounds presents a number of challenges. These include the abundance of salts carried over from seawater into the water extracts which complicates the subsequent isolation and separation of water soluble compounds, as well as their bioactivity (Bhakuni & Rawat, 2005). The handling of aqueous extracts of marine organisms or aqueous fractions of ethanolic/methanolic extracts can be fraught, as bacterial and fungal growth may degrade the active constituents or be responsible for false results in bioassays due to the endotoxins produced by these microorganisms (Bhakuni & Rawat, 2005), or oligosaccharides may be present which act as detergents and solubilise lipids. Enzymes comprising glycosidases, sulphatases, proteinases and various oxidases may also be activated upon homogenisation and result in changes within the bioactive compound which can affect or even destroy bioactivity (Guilhelmelli *et al.*, 2013).

Purified compounds may elicit antimicrobial activity, but the majority of literature to date deals with primary non-standardised crude solvent extraction methods (Ebada *et al*., 2008). The problem with this approach is that the actual composition of the bioactive extract is generally unknown and may contain compounds with either synergistic or

antagonistic effects. A further factor requiring consideration when extracting algal antimicrobials is the mucilage associated with the high carbohydrate levels in seaweed which may act as flocculants (Evans, 1989).

The crude extract, by virtue of its high yield and the difficulty in separating components by TLC, represents a complex matrix and it has been assessed that target compounds may represent less than 1% by weight of a crude extract (Koehn & Carter, 2005). There has been considerable advances in both separation and identification technologies. The improvement in HPLC, supercritical fluid chromatography (SFC) and capillary electrophoresis (CE) have aided resolving capabilities but the purification step can be rate-limiting in that once the correlation of the target compound to the biological activity sought is achieved there remains the problem of acquiring adequate quantities of the pure material. This situation is represented in the schematic Figure 3.1, where it is shown that several cycles of fractionation are usually required to yield sufficient sample for analysis (Koehn & Carter, 2005).

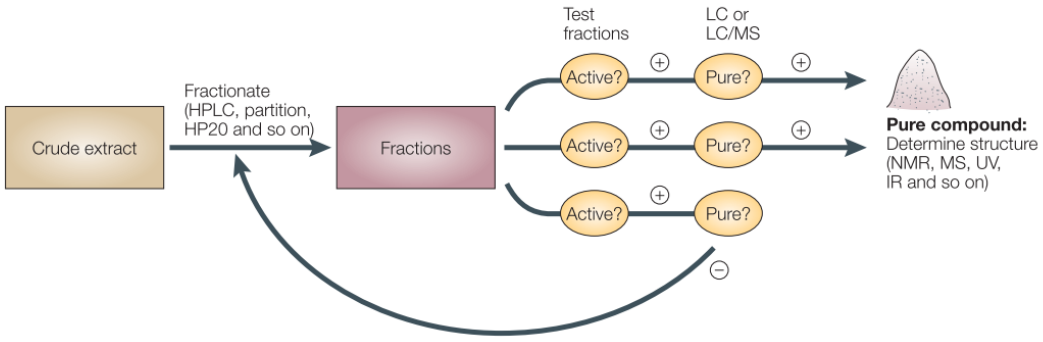


Figure 3.1: Generic schematic representation of bioassay guided fractionation (Koehn & Carter, 2005).

Pure compound screening is required in order to permit evaluation of natural product mixtures. Carter suggested libraries composed of pre-fractionated natural products, representing mixtures which would include the bulk of constituents rather than the major components so that the opportunity to discover novel compounds is preserved (Carter, 2011). This would reduce the evaluation lag time, as compound purification would no longer be necessary. However, the drawbacks of a mixed natural product fraction cannot

be overlooked, as novel molecules with non-beneficial constituents could be separated in the same extraction and purification.

One study determined that sesquiterpenes isolated from *Laurencia chondriodes* were less effective against some bacteria compared to those in the crude extract, supporting evidence that a synergistic effect of sesquiterpenes with other compounds enhanced or was responsible for the antibacterial activity (Bansemir *et al.*, 2004). Continual separation to yield a single compound from a crude extract was earlier identified as a potential problem, where only the crude extracts of *Hynea valentiae* were active against Gram positive bacteria (Sreenivasa & Parekh, 1981). This is also suggestive of synergy between more than one extracted component or it may be due to alteration of the composite chemical nature of the bioactive as a result of excessive separation steps, rendering it inactive.

In contrast, the anti-MRSA activity in an *Ulva lactuca* extract was only seen when the crude extract was separated with thin layer chromatography (TLC), causing antagonistic compounds to be separated from those with antimicrobial activity (Tan *et al.*, 2011).

The complexity of the challenge and the extract can be further illustrated by estimates of the total number of plant extract compounds which was last tallied at 1,060,000 (Afendi *et al*., 2012) so that no individual method is capable of detecting every compound present in a sample. By way of illustration the case of the natural product library may be considered - it is composed of samples that are either themselves mixtures — such as crude extracts (10–100s of components), semi-pure mixtures (5–10 compounds) or, alternatively, single purified natural products (Koistinen & Hanhineva, 2017). In the case of pure libraries, the hit-detection process is the same as that for synthetic pure libraries but for crude extracts, heterogeneity of the natural product library samples exacerbates the situation, adding two additional levels of complexity to the screening process: - initially in that once a response for the sample is detected by HTS, further cycles of chemical purification and biological assay might be necessary for identifying and isolating the active component in the mixture and, secondly, the complexity of crude or semi-pure natural-product libraries, with the chemical nature of many of the components often challenging the robustness of HTS technology (Koistinen & Hanhineva, 2017).

As with a mixed extract, the difficulty with natural product libraries is that the relative concentrations of individual components in a sample are not known precisely and may differ by orders of magnitude. A consequence is that trace components may not be high enough for detection, whilst the more concentrated components can exhibit inhibition as a result of nonspecific binding, perturbation of the assay pH or other physical properties (in effect, acting as antagonists). In addition, natural product samples can contain compounds that either fluoresce or absorb at excitation or emission wavelengths of the fluorophore (typically fluorescein), or by light scattering of insoluble components, which also affect readout of an analysis (Koistinen & Hanhineva, 2017).

3.2 Objectives

- To separate and isolate the anti-MRSA active fraction(s) from the crude *P. lanosa* aqueous extract.

- To characterise these separated active compounds using a variety of techniques. Colourimetric detection of components, comparing the anti-MRSA activity of pure molecules and assessment of solvent system suitability to achieve separation and positive bioactivity formed the first phase of the identification process. This was aligned to enzyme and heat stability, in order to aid instrumental identification techniques in the second phase of identification. Conventional instrumental identification techniques such as UV-Vis Spectroscopy, FTIR, HPLC, LC-MS and GC-MS formed the second phase of the identification process.

3.3 Experimental procedure

3.3.1 *Bacterial strains, Microbiological media, and Chemicals*

- Mueller Hinton Agar (MHA): Difco (Becton, Dickinson and Co. Maryland, USA)

- Brain Heart Infusion Broth (BHI): Oxoid (Basingstoke, U.K.)

- MRSA - W73365 (WIT 517): clinical isolate from University Hospital Warterford
- Chloramphenicol discs (10 μg): Oxoid (Basingstoke, UK)
- Sodium hydrogen carbonate: VWR Prolabo (Dublin, Ireland)
- Blank discs: Oxoid (Basingstoke, UK)
- Chloramphenicol powder: (Sigma-Aldrich Wicklow, Ireland)
- Filter paper 502 pore size 7-11 μm: (Albet, Germany)
- 2,3,5-triphenyltetrazolium chloride dye: (Sigma-Aldrich, Wicklow, Ireland)
- Analytical Reverse Phase TLC Plates RP-18W/UV: (Merck, Ireland)
- Analytical Normal Phase TLC Plates TLC Silica Gel 60 F_{254}: Merck, (Ireland)
- Analytical Aluminum oxide TLC Plates: 254 nm Fluka (Sigma-Aldrich, Wicklow, Ireland).
- Preparative Silica plates: (Merck, Ireland)
- Silica Gel: Pore size 60Å, 230-400 mesh particle size : Merck, (Ireland)
- Alumina Gel: Fluka (Sigma-Aldrich, Wicklow, Ireland)
- SiO_2 acid-washed sand: (Sigma-Aldrich, Wicklow, Ireland)
- Ethanol: (VWR, Dublin, Ireland)
- Methanol
- Chloroform
- Acetonitrile
- Hexane
- Ethyl acetate
- Acetic acid
- Acetone
- Dichloromethane

All solvents were HPLC grade and supplied by Fisher Scientific, Dublin, Ireland unless otherwise stated.

- Ethyl acetoacetate ≥ 99%: Fluka (Fisher Scientific, Blanchardstown, Dublin)
- Arabinose ≥ 99%: Sigma (Sigma-Aldrich, Wicklow, Ireland)
- Caffeine 99%: Aldrich (Sigma-Aldrich, Wicklow, Ireland)
- Acetic acid ≥ 99.7%: Scichem (Carrigtwohill, Cork, Ireland)
- 4-bromophenol 99%: Aldrich (Sigma-Aldrich, Wicklow, Ireland)
- 4-dibromoacetophenone 97%: Merck (Ireland)
- Pyridine-2-carboxylic acid > 98%: Merck (Ireland)
- Indole butyric acid ≥99.8%: Fluka (Fisher Scientific, Blanchardstown, Dublin)
- Isovaleric acid: Fluka (Fisher Scientific, Blanchardstown, Dublin)
- Proline 99%: Aldrich (Sigma-Aldrich, Wicklow, Ireland)
- Catechol 99%: Fluka (Fisher Scientific, Blanchardstown, Dublin)
- Tryptophan 99%: Aldrich (Sigma-Aldrich, Wicklow, Ireland)
- Thiamine hydrochloride ≥99%: Sigma-Aldrich (Wicklow, Ireland)
- Gallic acid 99%: Alpha Aesar (Fisher Scientific, Blanchardstown, Dublin)
- Indole acetic acid: BDH (VWR Prolabo Dublin, Ireland)
- Nicotinic acid ≥98%: Sigma-Aldrich (Wicklow, Ireland)
- Valeric acid (pentanoic acid) 99%: Merck (Sigma-Aldrich Wicklow, Ireland)
- Propionic acid ≥99%: Riedel-De-Haen (Fisher Scientific, Blanchardstown, Dublin)
- Inositol ≥99%: Sigma (Sigma-Aldrich Wicklow, Ireland)
- Prolinamide Fluka (Fisher Scientific, Blanchardstown, Dublin)
- Phenyl glycinamide: Fluka (Fisher Scientific, Blanchardstown, Dublin)
- 1-4-bromophenol ethylamine 98%: Sigma (Sigma-Aldrich Wicklow, Ireland)

3.3.2 *Instrumentation*

- Water bath: Bibby Scientific (Stone, Staffordshire, ST15 OSA, UK)
- Heidolph Laborota 4000 motor unit condenser: (Heidolph, Nurenmberg, Germany)

- Vacuubrand vacuum pump: (Heidolph, Nurenmberg, Germany)
- UV-VIS Spectrophotometer – CARY Varian 100 SCAN and UV-2401 PC, Shimadzu, Duisburg, Germany
- Varian 660-IR FTIR Spectrometer (Agilent Technologies, Cork, Ireland)
- Preparative HPLC - Varian Prostar preparative HPLC system equipped with a Varian Prostar 210 pump (10 mL head) and a Prostar 325 UV/Vis detector (Agilent Technologies, Cork, Ireland)
- Analytical HPLC system 1 - Agilent HP 1050 with a HP 1050 quaternary pump, autosampler, a variable UV-wavelength detector and controlled by HP Chemstation software
- Analytical HPLC system 2 - Agilent HP 1200 series equipped with a binary pump, Agilent 1200 series G1316B SL temperature-controlled column oven, a micro vacuum degasser with a photodiode array (PDA) detector (Agilent Technologies, Cork, Ireland)
- LC-MS – Agilent Technologies 1200 Series LC/MSD Trap XCT Ultra

3.3.3 *Methods*

Sections 3.3.3.1 – 3.3.3.5 relate to the optimisation of anti-MRSA compound extraction (and activity observation) from the crude aqueous extract whilst Sections 3.3.3.6 - 3.3.3.8 focus on the characterisation of the crude extract and its semi-pure active fraction. Section 3.3.3.6 is divided into 2 parts with TLC Methods using staining and solvent comparison followed by an examination of enzyme and heat stability. Spectroscopic techniques are covered in Section 3.3.3.7, concluding with Chromatographic techniques in Section 3.3.3.8. These analytical techniques will complement one another in achieving some definitive identification information with respect to the nature of the active extract.

It was considered necessary to explore all parameters that may contribute positively or negatively to the retention of anti-MRSA activity and that apparent changes were distinct and could be corroborated using both bioautography and well diffusion assays. This

translated into a complicated approach but ensured experimental integrity necessary to pursue the unknown but anticipated complexity of the crude extract .

3.3.3.1 **Investigation of Different Solvents to Yield Crude Extracts**

Seaweed was collected and prepared as outlined in Chapter 2, Section 2.3.3. Freeze-dried *P. lanosa* was extracted using a series of solvents with increasing polarity (hexane, DCM, chloroform, acetone, methanol, ethanol and water). Approximately 8 g of seaweed powder was extracted with 800 mL of solvent (1:100, *w/v*) and stirred with a magnetic stirrer for 2 h at room temperature. Samples were filtered under vacuum through Albet filter paper 502 and the subsequent solvent was rotary evaporated under vacuum at temperatures < 30 ˚C to yield crude dried extracts. The dried weight was recorded and the percentage yield calculated. The extracts were stored under nitrogen at -20 ˚C. These extractions were carried out 3 times on separate occasions.

3.3.3.2 **Antimicrobial Activity Assay Method Comparison**

3.3.3.2.1 Disc Diffusion Assay

The disc diffusion assay used was a standardised protocol recommended by the Clinical and Laboratory Standard Institute (CLSI) which was optimised for use with seaweed extracts at WIT. The dried *P. lanosa* extracts were dissolved in water at concentrations of 10 mg/mL with a volume of 100 µL loaded onto 6 mm blank discs, in order to obtain a final concentration of 1 mg/disc. A 100 µL aliquot of water was loaded onto a blank disc in a similar manner which served as a negative control. A 10 µg chloroamphenicol disc was used as a positive control. These steps were performed under aseptic conditions.

For this assay, MRSA was inoculated (1 %) into BHI broth and incubated overnight at 37 ˚C. The overnight culture (1 mL) was centrifuged at 13,000 rpm for 3 min. The supernatant was removed and the cell pellet was re-suspended in 1 mL of maximum recovery diluent (MRD), vortexed and centrifuged as before. The absorbance of the washed culture was then adjusted to an optical density at 625nm of ~ 0.10-0.12 [equivalent to 0.5 McFarland Standard or 10^7-10^8 colony forming units (cfu)/mL]. The adjusted culture was used within 15 min. A sterile swab was used to spread the bacterial culture onto the surface of MHA plates, rotating the plate 60˚ after each application. The diffusion discs containing crude seaweed extract were applied to the swabbed agar, along

with the positive and negative control discs. This was carried out in duplicate. The plates were incubated at 37 ˚C for 18 h, after which the zones of inhibition were measured. This procedure was performed in triplicate, on 3 separate occasions (n=3).

3.3.3.2.2 Well Diffusion Assay

A Duran bottle of 200 mL MHA was seeded with 500 µL of the MRSA culture (OD_{625nm} 0.10-0.12) and inverted gently. Plates of this seeded agar were poured and allowed to solidify. Wells were bored into the agar plates using a flamed capillary tube. A 50 µL aliquot of the diluted crude seaweed extract (50 mg/mL and 100 mg/mL) was added to each well. A 50 µL aliquot of chloramphenicol (5 mg/mL) was added as a positive control and 50 µL of sterile deionised water as a negative control. The plates were incubated for 18 h at 37 °C, after which the zones of inhibition were measured. Duplicate plates were prepared on 3 separate occasions (n=3).

(The well diffusion assay was further used to compare anti-MRSA activity of centrifuged, sonicated and a 50:50 mixture of crude extract *P. lanosa* solutions as outlined below).

3.3.3.3 **Thin Layer Chromatography**

Crude aqueous seaweed extract stock solutions of 60 mg/mL were prepared and either sonicated for 20 min or centrifuged at 13,000 rpm (16,000 rcf) for 3 min. This pretreatment was necessary as on application of crude aqueous seaweed extract stock solutions to TLC plates, the GC syringe employed to apply the sample became blocked. The samples were analysed via TLC on normal phase silica (Si) and by a well diffusion assay for anti-MRSA activity.

A number of crude aqueous seaweed extract concentrations were prepared using centrifugation (Table 3.1) and assessed by TLC on normal phase Si and reverse phase Si plates, with 10 µL of each sample spot-loaded and allowed to dry before the plates were run for subsequent analysis.

Table 3.1: Crude aqueous seaweed extract solutions and their corresponding compositions.

Concentration of crude seaweed extract solution (mg/mL)	Volume of sample components
0.2	40 µL stock + 160 µL sterile water + 40 µL methanol*
0.4	80 µL stock + 120 µL sterile water + 40 µL methanol*
0.6	120 µL stock + 80 µL sterile water + 40 µL methanol*
0.8	160 µL stock + 40 µL sterile water + 40 µL methanol*

* methanol added to assist mobility of extract on Si TLC plates

Three 0.8 mg/mL spots of extract solutions were applied to normal phase Si and reverse phase Si TLC plates using 100% methanol and 100% water as the mobile phase. Samples A, B and C were crude seaweed extracts (as outlined), with each given a pre-treatment step prior to TLC anaylsis as per Table 3.2. Sample 'A' (300 µL of the centrifuged stock and 75 µL of methanol), sample 'B' (300 µL of the sonicated stock and 75 µL of methanol) and Sample 'C' (150 µL of sample A and B mixed, with 75 µL of methanol) were prepared to evaluate the effect of acetic acid and sodium hydrogen carbonate on anti-MRSA activity.

Sodium hydrogen carbonate was selected as a potential enhancer of antibacterial activity as it was previously found to improve the antibacterial activity of ovatransferrin against *E. coli* 0157:H7 and *Listeria monocytogenes* at a loading of 100 mM (Ko *et al.,* 2008). Another reason for the addition of sodium hydrogen carbonate was its general use for the extraction of polyphenols using Folin-Ciocalteu phenol reagent. Acetic acid was chosen as a potential enhancer as it is a common modifier in HPLC extractions to enhance resolution i.e. separation (Fried & Sherma, 1996). Furthermore, the use of acetic acid is recommended when amines or carboxylic acids are run on silica TLC plates to prevent tailing. For example, addition of acetic acid to a mobile phase for chromatography of aspirin would protonate the acid form (making it neutral) and so avoid interaction with

the silica. The acetic acid also serves to convert compound salts to the acid, the salt acting as another cause of tailing or poor resolution.

Table 3.2: Samples A, B and C with their corresponding treatments of acetic acid or sodium hydrogen carbonate.

Sample	A	B	C
Acetic acid	-	0.2 % (v/v)	-
Sodium Hydrogen Carbonate	-	-	1.8 % (m/v)

where – denotes no treatment

A range of solvents were assessed as potential mobile phases (Table 3.3) for separation of the compounds in the seaweed extract. A 100 mL aliquot of the mobile phase was prepared and added to a clean TLC tank. The tank was allowed to equilibrate for 1 h before the TLC plates were placed inside. After equilibration, the analytical TLC plates were carefully inserted into the tank. The plate was removed when the solvent front reached 2 cm from the top of the plate. The plate was dried and viewed under the UV box (short wave mode) to assess separation of definitive fractions.

Table 3.3: Solvents assessed as mobile phases for TLC and their corresponding polarity index.

Solvent	Polarity
Hexane	0
DCM	3.1
Chloroform	4.1
Ethyl Acetate	4.4
Methanol	5.1
Acetone	5.1
Acetic Acid	6.2
Water	9.1

For preparative TLC, a single streak of the sample was applied across the bottom of the TLC plates using a capillary tube. The streak was allowed to dry before being run in the TLC tank.

3.3.3.4 **Bioautography**

Bioautography was carried out according to the method used by Tan (2013), except that an optimised protocol was developed. This involved adding 500 µL of the MRSA culture (OD_{625nm} 0.10-0.12) to 200 ml MHA and pouring this aseptically over a prepared analytical TLC plate (normal phase Si) which had been placed into a sterile petri-dish. After incubation for 18 h at 37 °C, 2,3,5- triphenyltetrazolium chloride dye was sprayed over the agar surface to view any inhibitory zones caused by the fractions on the TLC plate.

A number of mobile phases were investigated for separation of the pre-treated and untreated crude seaweed extracts (Table 3.4). Treatment of the crude seaweed extract was carried out with acetic acid and sodium hydrogen carbonate as outlined above before application to the TLC plates in an attempt to enhance separation and maximise the potential for identification of any anti-MRSA activity in the extract by bioautography. Both reverse and normal phase Si analytical TLC plates were used in the study.

Table 3.4: Mobile phase composition employed in the bioautography and mobile phase TLC investigation study with normal and reverse phase silica.

Mobile Phase
100% MeOH
70:30 MeOH:H_20
50:50 MeOH:H_20
30:70 MeOH:H_20
100% H_2O

3.3.3.5 **Flash Chromatography**

A normal phase silica column was prepared with approximately 30 cm height of silica. This amount of silica gel was then poured out of the column and into a beaker and hexane added to create a suspension. The column tap was closed and this suspension was poured into the column. A further 12 cm height of hexane was poured into the column above the suspension.

A vacuum was applied to the top of the column to allow for the packing of the column. The tap remained open to drain the hexane to the surface of the silica. Once the silica was packed efficiently, a layer of SiO_2 acid-washed sand was added on top of the silica to prevent the silica from splashing or being disturbed when the mobile phase and sample were added. Hexane was poured in again and the pump was turned on to ensure column stabilisation.

Centrifuged crude seaweed extract (1 mL) was added to the top of the column. This was prepared by making 10 standard stocks (50 mg in 500 µL water) which were centrifuged at 13 000 rpm for 3 min. The supernatant of each of the 10 stocks was removed using a glass Pasteur pipette and put into a sterile glass bottle and mixed. The extracts were assessed for anti-MRSA activity using various mobile phases on silica and alumina stationary phase columns which are summarised Table 3.5.

Table 3.5: Ratio of solvent systems and stationary phase used for flash chromatography.

Solvent	**Solvent 1(S1)**	**Solvent 2 (S2)**	**Ratio (S1:S2)**	**Stationary Phase (NP)[1]**
A	Chloroform	Methanol	70:30	Silica
B	Chloroform	Methanol	40:60	Silica
C	Methanol	Water	20:80	Alumina

[1]NP normal phase stationary phase

These solvent systems represent the initial solvent systems used to obtain fractions. Fractions of approximately 10 mL were collected in test-tubes, numbered and placed in test-tube racks. The solvent system was adjusted twice after collection of initial 36

fractions to increase polarity and ensure possible active components were eluted. Once collection was complete, 5 consecutive fractions were pooled at a time and rotary evaporated. The resulting dried residue was dissolved in solvent and transferred to sterile glass bottles before being evaporated under N_2, sealed and stored at -20 ºC. The weights were recorded to allow for accurate resuspension for antimicrobial activity.

Figure 3.2 summarises the methods used for the isolation of the *P. lanosa* bioactive components from the aqueous extract.

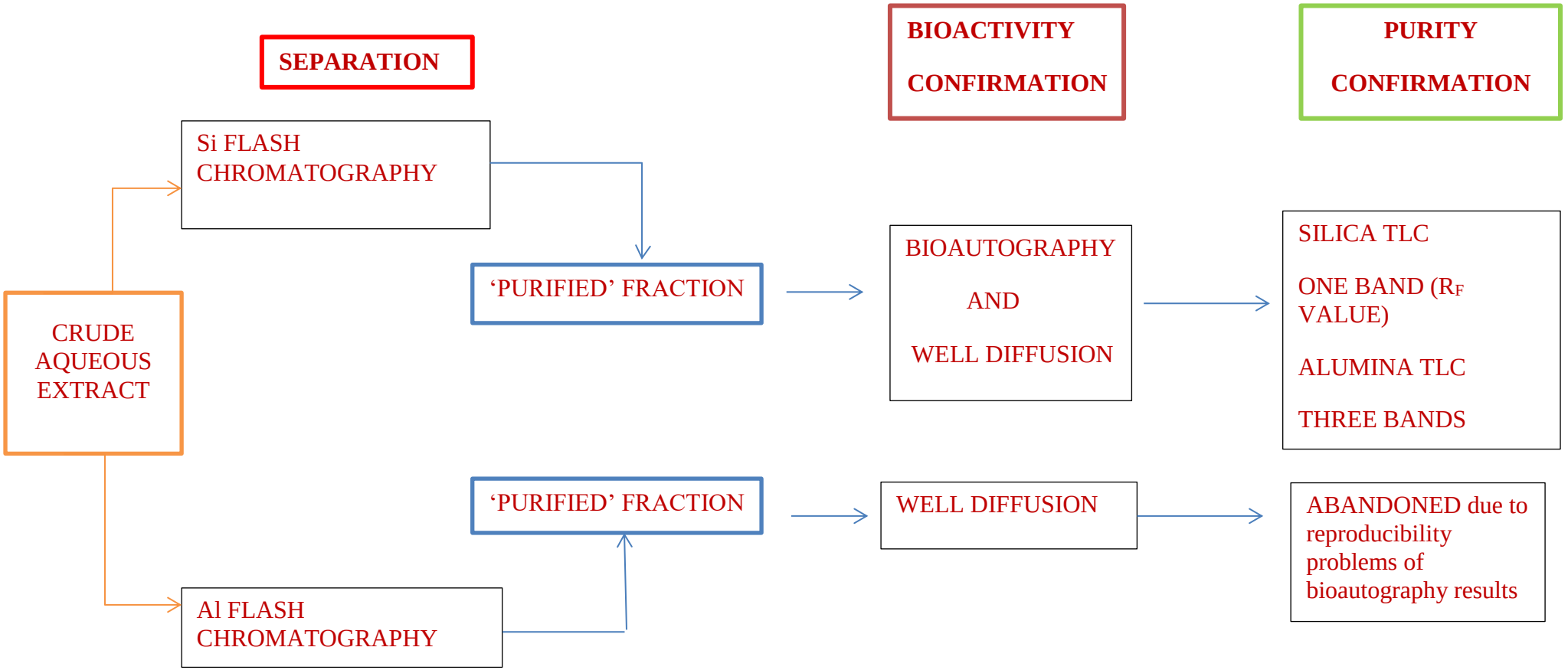


Figure 3.2: Flow diagram of methods used to isolate bioactive responsible for anti-MRSA activity from *P. lanosa*.

3.3.3.6 **Characterisation of bioactive compounds in crude aqueous *P. lanosa* extract**

3.3.3.6.1 TLC Methods – staining and solvent evaluation

The complexity of the crude aqueous extract was compared to different semi-pure fraction samples and various staining techniques were employed. Various phase compositions were also assessed. In essence, samples were applied to TLC plates as previously described in this chapter and the plates run, developed and dried before staining with the reagents summarised in Table 3.6. The results were analysed and the suitability of the solvent systems determined with reference to standard molecules. Various staining techniques (Kowalska & Cies´la, 2013) were employed and are shown in Table 3.6.

Solvent systems were selected on the basis of suitability for specific potential compound types, i.e. carotenoids, polyacetylenes, catechins, galactolipids, bromophenols and indole alkaloids. Relevant solvent systems were identified and selected to maximise the potential for positive characterisation and to ensure dissolution of these potential components of the crude *P. lanosa* aqueous extract.

Table 3.6: Reagents used for compound detection with TLC chromatography.

Staining Technique or reagent	Reagent Solution	Identification	Positive reaction
Ninhydrin	30 mg ninhydrin in 10 mL n-butanol, mixed with 0.3 mL 98% acetic acid.	Amino acids, biogenic amines, cyclic and linear peptides, cyclotides, cyclopeptides.	Red or blue zones.
Salkowski	27.6 mM $FeCl_3$ and 6.6 M H_2SO_4.	Indole derivatives.	Yellow colour for LMW* indoles.
Dragendorff	Solutions A and B are mixed with 4mL acetic acid and 20 mL water (A- 0.85 bismuth nitrate + 10 mL acetic acid +40 mL water – heated B- 8 g potassium iodide in 30 mL water).	Alkaloids -compounds containing heterocyclic nitrogen; colored zones on a white background.	Various depending on nature of amine.
Ehrlich	2 g *p*-dimethylamino benzaldehyde in 20 ml HCl + 80 ml ethanol.	Indole compounds.	Purple colour on white background.
Ferric chloride	1 g iron (III) chloride + 5 mL water diluted to 100 mL with ethanol.	Phenols.	Crimson colour on white background.
Bromocresol green	0.04 g bromocresol + 100 mL ethanol with 0.1 M NaOH until turns blue.	Carboxylic acids.	Yellow on blue background.
DPPH	0.1 mM DPPH in methanol solution.	Check for DPPH radical scavenging.	Yellow reaction on light purple background.

* LMW – low molecular weight

Various standard compounds were selected to test for mobility and anti-MRSA activity as reference compounds. These included nicotinic acid, indole acetic acid, gallic acid, thiamine, tryptophan, catechol, proline, arabinose, caffeine, iso-valeric acid, streptomycin, propionic acid, n-valeric acid (pentanoic acid), 4-bromophenol, 4-dibromoacetophenone, pyridine-2-carboxylic acid and 1-4-bromophenol ethylamine. 1 mg/mL solutions were prepared in 80 % aqueous methanol. Well diffusion assays were

carried out for positive bioautography candidates and for compounds which failed to deliver a positive bioautography result with the two mobile phases, 100% MeOH and 50:50 MeOH:H_2O.

3.3.3.6.2 Physicochemical Characterisation of the aqueous crude seaweed extract

In order to generate an extract profile, the consideration of composite stability was deemed necessary as it would allow for speculation regarding the nature of compounds responsible for anti-MRSA activity. Certain compounds could be eliminated as potential candidates based on their sensitivities to certain conditions and treatments. The sensitivity of antimicrobial compounds to proteolytic enzymes, catalase and heat was determined as outlined by O'Shea *et al.* (O'Shea *et al.,* 2009) with the following modifications; enzyme solutions were 10 mg/mL (giving a final enzyme concentration of 5 mg/mL when mixed 50:50 with the crude aqueous extract), Protease Type I and Pronase E were used with the enzymatic treatments performed at 37 °C for 2 h and lipase and β-amylase stability assays were also included as follows. The crude aqueous seaweed extract was incubated with lipase for 30 min at 37˚C and for 3 min at 20˚C with β-amylase. Heat stability was assessed by assaying the anti-MRSA activity of the crude extract by well diffusion assay (WDA) after exposure to 40, 50, 60, 70, 80, 90 or 100 °C for 30 min on a heating block.

3.3.3.7 Spectroscopic Techniques to characterise anti-MRSA compounds

3.3.3.7.1 UV-VIS Spectroscopy

A CARY Varian 100 SCAN was used to obtain spectra of fractions from flash chromatography and these were compared. A set of reference compounds were also run. UV-VIS Spectroscopy is used to suggest compound identity as compound absorbance in the UV-VIS range is dependent on compound structure with functional groups and unsaturation affecting absorbance. A distinct absorbance spectrum would significantly assist compound identification. Full spectrum (190-800 nm) scans were carried out for the semi-pure samples using the double beam spectrophotometer - UV-2401 PC

(Shimadzu, Duisburg, Germany) with methanol used to zero the instrument. It was anticipated that λ_{max} values of the compound(s) would be determined for subsequent HPLC analysis. Initially, a 20 mg/mL sample of the semi-pure extract was prepared in a 25 mL volumetric flask and ~ 4.5 mL volumes used in an appropriate cuvette, but on subsequent purification a 0.1 mL cuvette was used due to the low yields.

A comparison with a concentrated semi-pure *P. lanosa* extract sample was included as well as an inactive semi-pure *P. lanosa* extract sample.

3.3.3.7.2 FTIR

The structure and types of bonding of active samples collected from both HPTLC (high performance TLC plates) and flash chromatography were analysed using a Varian 660-IR FTIR Spectrometer (Agilent Technologies, Cork, Ireland). Infrared absorption spectra provide a molecular fingerprint of a compound or a mixture and allow for the identification of functional groups and bonding depending on the specific absorption bands. Solid samples were prepared using the KBr pressed disc technique with a prepared 10 % mix, where a pressure of 5-10 ton weight was applied for 5 min. The blank was a pure KBr disc with analysis carried out at room temperature in the mid-infrared range (4000-400 cm^{-1}) with resolution of 4 cm^{-1} and 64 scans performed. However, there was a residual oil–emulsion at the bottom of the sample bottle that necessitated the use of a liquid KBr cell. Each spectrum was displayed in terms of transmission with baseline correction incorporated. The spectra from each sample were compared and the peaks examined for key functional groups.

3.3.3.7.3 LC-MS

Fractions and purified samples were analysed with an Agilent 1200 series LC/MSD Trap XCT Ultra ion trap mass spectrometer (Agilent Technologies, Cork, Ireland). Separation was achieved using a C18 column (Agilent 5 µm, 250 mm x 4.6 mm) with a mobile phase consisting of 95:5 (*v/v*) acetonitrile: water (with 0.5 % formic acid), at a flow rate of 1 mL/min. This was run as a gradient elution which is the standard approach for efficient separation of complex samples. The eluent was analysed at various wavelengths. Ions were generated using an electrospray ionisation method, and MS spectra were collected and analysed. LC-MS combines the physical separation capability of HPLC with mass

analysis capability of mass spectrometry and this technique was expected to support and resolve the identity of the anti-MRSA compounds.

The recommended concentration of samples was 1 mg/mL for LC-MS with a micropipette used to achieve an equivalent volumetric dilution of the purified samples with methanol. A 1 µL sample volume was used.

An alternative sample preparation was used to compare the semi-pure flash chromatography (FC) sample peaks and solvent extraction with ACN adopted. ACN solvent extraction is commonly used to deproteinise biological samples and the stock aqueous crude *P. lanosa* extract (60 mg/mL) was mixed 1:3 with ACN and agitated using a vortex tube shaker before centrifugation at 4500 rpm for 5 minutes to yield a clear supernatant. This was further purified by HPTLC and analysed by LC-MS.

3.3.3.8 **Chromatographic Techniques to characterise anti-MRSA compounds**

3.3.3.8.1 **Analytical high performance-liquid chromatography (HPLC)**

HPLC conditions were optimised using two different instruments - the Agilent HP 1050 and the Agilent 1200 series. Detailed instrument set-up is provided in section 3.3.2. and was controlled by HP Chemstation software (Agilent Technologies, Cork, Ireland). The different elution conditions assessed are presented in Table 3.7.

Small molecule standards were also assessed using analytical HPLC to compare retention times and suitability of mobile phase composition.

Table 3.7: Optimised HPLC Parameters.

HPLC System	Column	Mobile phase	HPLC Conditions		
			Flow Rate **mL/min**	**λ** **nm**	**Injection Volume** **µL**
Agilent HP 1050	Symmetry C_{18}	25:75 MeOH:H_2O	1.0	220	20
Agilent 1200 Series	Symmetry C_{18}	25:75 MeOH:H_2O	1.0	220	20

Initial separation used methanol and water, whereas final separation using preparative HPLC and LC-MS analysis utilised water and acetonitrile (ACN) (with 1% formic acid) in a predetermined gradient elution. Trial runs were carried out using analytical HPLC to determine the range required for sample elution as discussed in section 3.3.3.6.4 - solvent A – 100% H_2O with 0.15 formic acid with solvent B – 95% ACN and 5% H_2O with the gradient starting at 5% A and running to 95% A.

3.3.3.8.2 Preparative high performance-liquid chromatography (HPLC)

Purification of antibacterial compound(s) using preparative HPLC was carried out following optimisation of separation conditions using both the instruments detailed in section 3.3.3.6.4. Preparative HPLC analysis was performed using EZChrom software with the different conditions used for HPLC method optimisation as above in Table 3.7. The initial 8 fractions collected from the column chromatographic separation were analysed with the optimised HPLC conditions at the concentration of 1 mg/mL. The chromatogram was compared with results from the bioautography assay in order to identify the potential antibacterial compound(s). Both isocratic and gradient elution conditions were used.

The fractions produced by flash chromatography were assessed and separation optimised

using analytical HPLC before the active fractions were further purified using a Varian Prostar preparative HPLC system, equipped with Varian Prostar 210 pump (10 mL head) and Prostar 325 UV-Vis detector (Agilent Technologies, Cork, Ireland). Separation was achieved using an ACE 5 C18 column, 5µm particle size, 100Å pore size, 15cm x 7.75mm dimension (Advanced Chromtography Technologies, Aberbeen, Scotland). The different peaks were collected and anti-MRSA activity assessed using bioautography as per Section 3.3.3.6. Injection volumes of 10 µL were used.

3.3.4 *Statistical Analysis*

Statistical analysis using one-way ANOVA with the Tukey post-hoc comparison test was used with $p<0.05$ defining statistical significance for disc and well diffusion assay assessment as well as for the well diffusion results comparing the treated and untreated crude *P.lanosa* extract, the enzyme and heat treatment results.

3.4 Results and Discussion

3.4.1 *Solvent Extraction Yields*

The solvent extraction yields are represented in Table 3.8. This data clearly demonstrated how the solvent system employed greatly effects the % yield of seaweed extract and that either water soluble compounds are more prevalent and/or that there is a higher yield of a single compound than observed for the organic solvents. The polarity of the solvents used was seen to have an effect on the overall yield of the crude seaweed extract. It is evident that overall, there was a low % yield observed with solvents of a low polarity index. Another contributory factor would be the dielectric constant of the solvent as a higher dielectric constant would correlate to a higher ability of the solvent to dissolve salts. It is worth noting the difference between the % yields obtained for methanol and acetone, even though both have a polarity index of 5.1 as there was nearly a tenfold increase in yield. The dielectric constant for methanol is 32.7 compared to that of acetone with a value of 20.7. The effect of the dielectric constant was further obvious in comparing the yields produced from water and methanol – an eightfold difference where the dielectric constant of water was three times greater than that of methanol. It would appear that the polar nature of the *P. lanosa* extracts can in part be attributed to the presence of ionised salts which were far more readily to dissolve in water compared to the other solvents.

The high yield, together with the anti-MRSA activity of the crude aqueous seaweed extract, represents an opportunity for the discovery of novel bioactive compounds using eco-friendly extraction. (Screening of the the other solvent derived extracts was assessed but together with the lower yields and lower anti-MRSA activity, this study focused solely on the crude aqueous *P. lanosa* extract).

Water-soluble components include soluble polysaccharides, proteins (such as PBP) and peptides, as well as phenolic compounds attached to sugars or proteins, glycosides, organic acids, tannins, salts and mucous (Sabeena Farvin & Jacobsen, 2013).

Different solvent systems are known to extract different compounds. This may account for the variation in the chemical composition of yields observed for each solvent system. For example, a study carried out by Shoeib *et al.*, 2004 revealed that varying lanosol

derivatives were extracted with different solvent systems (Shoeib *et al*., 2004) and reported solubility differences, for example, the 5-bromo analogue was insoluble in chloroform whilst the 2,5-dibromo aldehyde analogue was soluble.

Table 3.8: Percentage yield of crude extract from *P. lanosa* harvested 5/9/2013 from different solvents (n=2).

Solvent	Polarity	Dielectric constant	% Yield ±SD
Hexane	0	1.9	0.147 ±0.0036
DCM	3.1	9.08	0.187 ±0.0510
Chloroform	4.1	4.81	0.327 ±0.0320
Ethyl acetate	4.8	6.02	0.807 ±0.180
Acetone	5.1	20.7	0.234 ±0.0630
Methanol	5.1	32.7	2.40 ±0.450
Ethanol	5.2	24.6	1.17 ±0.790
Water	9	77.46	16.42 ±1.15

A previous study by Sabeena Farvin & Jacobsen (2013) investigating seaweed collected off the Danish coast for antioxidant activity, reported water and ethanol crude extraction yields as 14.1 and 6.8 %, respectively for a related species, *P. fucoides* (Hudson) Greville (Sabeena Farvin & Jacobsen, 2013). It would, therefore, appear that water produces significantly higher extract yields for this particular genus.

The % yield derived by aqueous extraction in the present study was significant for two reasons; firstly, it was an environmentally friendly process and secondly, the increase in polarity allowed for higher extraction yield, indicative of the abundance of water soluble compounds and their inherent polarity, present in this species.

3.4.2 *Comparison of Anti-MRSA Assay Method Sensitivity*

The selection of an appropriate assay for the assessment of antibacterial activity is important as it must be capable of displaying the range of activity i.e. sensitive to concentration gradients of activity. The fact that the assay selected was required to be robust with high sensitivity for anti-MRSA activity of aqueous crude seaweed extract was of particular concern in that the antibiotic assay of extracts in organic solvents does not reflect the antibacterial activity of marine algae under natural conditions (Valgas *et al.,* 2007).

Table 3.9 presents the comparison between disc and well diffusion for the assessment of anti-MRSA activity from crude aqueous *P. lanosa* extracts. It was found that the well diffusion assay was a more sensitive assay than the disc diffusion assay, although not all extract concentrations could be assessed by well diffusion from the 50 mg/mL stock solution, due to volume and concentration constraints (as reflected in Table 3.9). There was also a quantitative response correlated to loading level for both methods.

The hazy zones observed when using disc diffusion may relate to the incomplete diffusion of active components from the paper disc. The size of the inhibition zones for the well diffusion assay were larger and allowed for better distinction of anti-MRSA activity. Statistical analysis determined $p<0.05$, indicative of significant differences between the disc and well diffusion results but when applied to the two well diffusion results for the 2.0 mg loading this significance was not supported. This further supports the rationale for adoption of the well diffusion assay. This was also found to be the case in a study by Valgas *et al.* (Valgas *et al.,* 2007) where it was concluded that the well diffusion assay was the only suitable diffusion technique for testing aqueous suspensions of plant ethanol extracts. This study revealed that the presence of suspended particulate matter in the sample being tested was much less likely to interfere with diffusion of the antimicrobial substance into the agar from a well than from filter paper discs.

Table 3.9: Comparison of sensitivity of disc and well diffusion assays for measurement of anti-MRSA activity of crude aqueous extracts from *P. lanosa* harvested 5/9/2013 (n=3).

Disc/Well Loading (mg)	Zone of Inhibition Diameter (mm) ±SD		
	Disc	Well	
		50 mg/mL Conc stock extract	100 mg/mL Conc stock extract
1.0	none	none	none
1.5	N/A	10 ± 0.5	N/A
2.0	8.5 ± 0.5 (hazy)	11.5 ± 0.5	10.5 ± 0.5
3.0	10 ± 1.0 (hazy)	N/A	13.5 ± 0.5
4.0	13 ± 1.76 (hazy)	N/A	19 ± 1.26
5.0	15 ± 1.26 (clear)	N/A	22 ± 1.53
Chloramphenicol	30 ± 1.0 (disc-10μg)	22 ± 0.5 (soln)	22 ± 0.5 (soln)

N/A denotes not assessed due to the inability to prepare the concentration of sample required in 50 μL well loading as volume would exceed 50 μL. For the well diffusion assay, 50 μL chloramphenicol solution (5mg/mL) was added as a positive control and 50 μL of sterile deionised water as a negative control in a control well.

From the data obtained here, the well diffusion assay was employed in all further assessment of anti-MRSA activity.

3.4.3 *Thin Layer Chromatography*

Two crude seaweed extract stock solutions were prepared; one centrifuged and the other sonicated. It was evident that there was a difference between these solutions, with a larger pellet evident in the sonicated sample, as expected. Prior to analysis of the two sample preparation methods, seaweed particulates were blocking the GC syringe used for application of the seaweed crude extract to the analytical TLC plates. A solid smaller pellet formed in the centrifuged stock with a much clearer supernatant than the sonicated stock as well as less deposit, suggesting the stock should be centrifuged at 13,000 rpm for 3 min before analysis of the supernatant by TLC.

When assayed using the well diffusion assay, the centrifuged sample displayed stronger activity against MRSA than the sonicated sample and the 50:50 mixt of centrifuged and sonicated sample, both of which displayed the same level of anti-MRSA activity (data not shown). From this point onwards, the crude seaweed extract stock was centrifuged as it gave higher activity and was easier and faster to produce. A potential issue with sonication of seaweed extracts is the associated heating of the sample which could affect the integrity of bioactive/s and may explain the loss of activity observed.

The effect of various crude seaweed extract concentrations with a 10 µL loading rate was assessed on normal and reverse phase TLC plates, Figure 3.3.

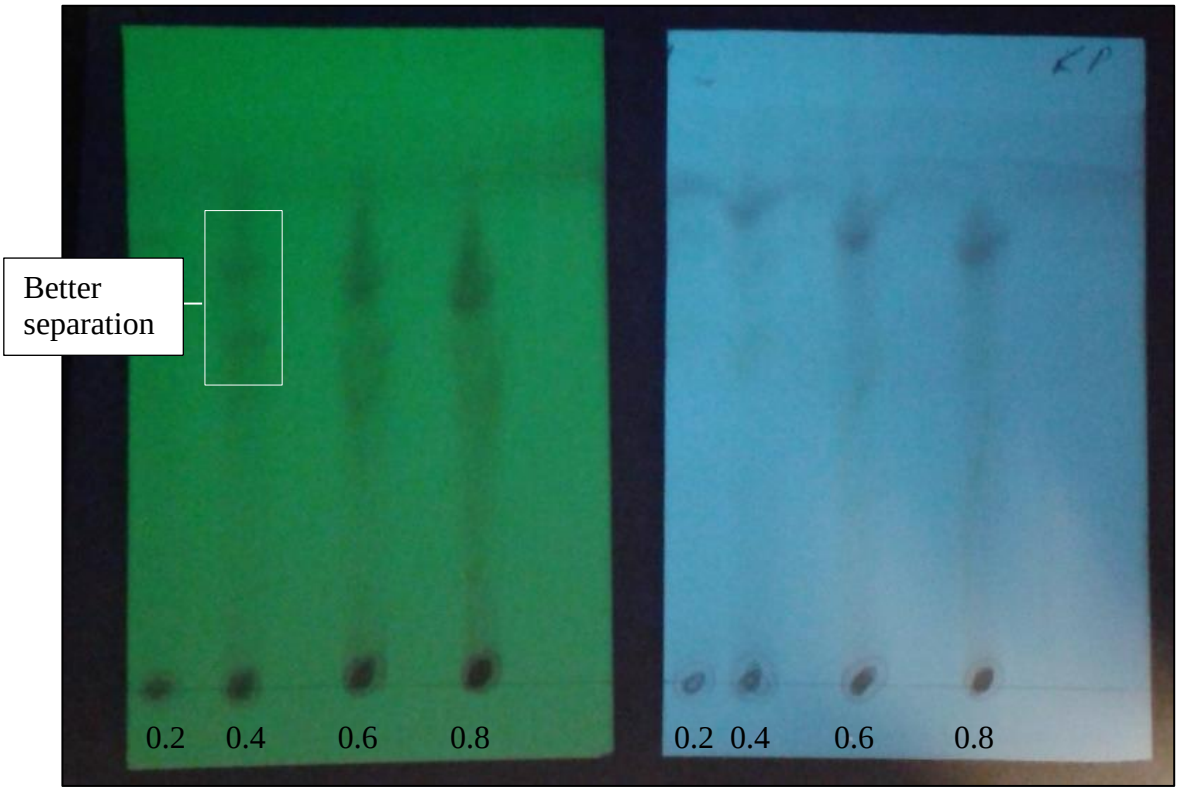


Figure 3.3: 100% MeOH mobile phase – Normal phase Si (left) & reverse phase Si (right) TLC plates with increasing concentrations of crude *P. lanosa* extract applied (mg/mL).

Figure 3.3 shows that the fractions appeared to separate further up the TLC plate as the seaweed extract concentration decreased. From this, it was clear that the concentration had an effect on visualisation of the separation of the compound. The lower the concentration of the extract loading, the less inhibition of movement and the greater the

extent of separation of components of the mixture, as is commonly seen in TLC (Costanzo, 1997). The fact that it was a crude extract, containing possibly hundreds of components, together with the probability of interaction with one another, as well as some components having similar retention (R_f) values on the TLC plate, accounts for the lack of definitive banding. However, in this investigation a positive bioautography result was crucial to identify the separated compound(s) responsible for anti-MRSA activity so a compromise was necessary, i.e. the final concentration of sample to be loaded must be suitable to allow for both visualisation of separation and a positive bioautography result.

A number of solvent systems with varying polarities were assessed for their potential as suitable mobile phases for TLC separation of the crude seaweed extract on normal and reverse phase silica analytical TLC plates (Table 3.10).

Two solvents yielded clear separation of compounds on the normal phase and reverse phase Si TLC plates. Ethyl acetate gave slight separation of red coloured components, which was expected as they are polar (expected if red water-soluble pigment such as PE), and would, therefore, have higher affinity for the polar ethyl acetate than the silica stationary phase. Hexane, dichloromethane, chloroform, acetone and acetic acid, representative of a broad polarity range (0-6.2) yielded no separation of the extract compounds due to the fact that they were less polar and were, therefore, unable to separate the component compounds.

Table 3.10: Solvent system optimisation for the separation of crude seaweed extracts on normal and reverse phase silica TLC plates (100% HPLC solvent).

Solvent	Polarity	Separation Observations
Hexane	0	No separation, spot did not move on normal Si or reverse phase Si plates.
DCM	3.1	No separation, spot did not move on normal Si or reverse phase Si plates.
Chloroform	4.1	No separation, spot did not move on normal Si or reverse phase Si plates.
Ethyl Acetate	4.4	Very slight movement of red coloured fraction up normal Si and reverse phase Si plates.
Methanol	5.1	Good separation on normal Si and reverse phase Si plates. Red coloured fraction moved further than yellow coloured fraction.
Acetone	5.1	No separation, spot did not move on normal Si or reverse phase Si plates.
Acetic Acid	6.2	No separation, spot did not move on normal Si or reverse phase Si plates.
Water	9.1	All red coloured fraction moved up normal Si and reverse phase Si plates with solvent front.

Methanol, with a polarity of 5.1 and dielectric constant of 32.7 achieved the best separation of the extract components with the red coloured components reaching the solvent front. A yellow fraction was also carried up the plate below the red fraction; indicating that the yellow compound was less polar than the red pigment as it did not migrate as far on the TLC plate. There was a lot of residual material on the spot, indicative of a large variety of immobile compounds insoluble in methanol. The separation on the reverse phase plate using the 100% methanol solvent system also showed separation of

red fractions. Therefore, methanol was the most effective pure mobile phase for use in the separation of compounds of the extract on both normal and reverse phase TLC silica plates. Methanol is a standard solvent used in liquid:liquid macroalgae extractions and separations (Kumari *et al.*, 2014).

3.4.4 *Bioautography*

An intial protocol as outlined by Tan (2013) was used to confirm anti-MRSA activity using well and disc diffusion assays. A positive control of chloramphenicol (10 μg for disc diffusion or 5 mg/mL for well diffusion) revealed a clear inhibition of the MRSA, whilst the crude aqueous extract derived from seaweed harvested in 2013 displayed no activity against MRSA. From this, an optimised modified protocol was designed. The reason for this adjustment was the different strength of anti-MRSA activity in seaweed samples harvested in 2013 and 2014 compared with seaweed collected in 2010. The MRSA overnight culture concentration was reduced from 2.0 mL to 0.5 mL per 200 mL MHA and the first positive results of strong activity using bioautography were obtained. This reduced level of MRSA within the MHA allowed for better visualisation of activity.

Optimisation of bioautography through a mobile phase investigation was extended to include solvent mixtures and Table 3.11 presents the results. A bioautography assay was carried out on each mobile phase test sample to assess for activity against MRSA. The results identify a potentially effective mobile phase composition. From Table 3.11 the optimised mobile phase for TLC analysis was identified as 100 % water or 50:50 methanol:H_2O, representing a high polarity solvent composition, indicative of highly polar bioactive compound(s).

Table 3.11: Bioautographic guided TLC optimisation on normal and reverse phase silica TLC plates.results.

TLC Plate No.	Plate Type (Phase)	Mobile Phase	Separation Quality	Bioautography Assay Results
1 & 2	Normal	100% MeOH	Poor	N/D
3 & 4	Reverse	100% MeOH	Poor	N/D
5 & 6	Normal	100% H_2O	Poor	N/D
7 & 8	Reverse	100% H_2O	Good	Good activity
9 & 10	Normal	70:30 MeOH:H_2O	Fair	N/D
11 & 12	Reverse	70:30 MeOH:H_2O	Poor	N/D
13 & 14	Normal	30:70 MeOH:H_2O	Poor	N/D
15 & 16	Reverse	30:70 MeOH:H_2O	Fair	Slight activity
17 & 18	Normal	50:50 MeOH:H_2O	Good	Good activity
19 & 20	Reverse	50:50 MeOH:H_2O	Good	Strong activity

N/D not detected, slight activity – small inhibition zone of bacterial growth, good activity – clear visible, wide inhibition of bacterial growth, strong activity – large inhibition zone

Treatment of the crude seaweed extract was carried out with acetic acid and sodium hydrogen carbonate before application to the TLC plates. The reason for this approach was that initial bioautography trials were not successful and the MRSA concentration had to be adjusted (less concentrated) to ensure an observable and reproducible result. It was reasoned that the treatments would serve to check for possible enhanced anti-MRSA activity for the extract bioautography result. The selection of sodium hydrogen carbonate as a potential enhancer of antibacterial activity was supported by a publication where it was found to improve the antibacterial activity of ovatransferrin against *E. coli* 0157:H7 and *Listeria monocytogenes* (Ko *et al.,* 2008). Sodium hydrogen carbonate has been used as a microbial disinfectant domestically as well as in food and agriculture for decades. It has been reported as active against a range of bacteria, acting as a bacteriostatic rather

than a bacteriocidal agent. In the case of *S.aureus* sodium hydrogen carbonate was found not to inhibit the bacterial growth profile (Dobay *et al*., 2018). Well diffusion and bioautography demonstrated that sodium hydrogen carbonate on its own had no inhibitory effect on the MRSA isolate selected for this work. Furthermore, a recent paper has determined the existence of two distinct MRSA phenotypes where sodium hydrogen carbonate supplementation of standard media used in standard antimicrobial susceptibility testing found one phenotype to be highly susceptible to the antistaphylococcal-lactams whilst the other remained resistant (Ersoy *et al*., 2019). Other research has investigated the impact, both enhancement and suppression of the activity of conventional antibiotics in the presence of physiological concentrations of bicarbonate, proving that there are significant clinical implications. The study concluded that tests of antibacterial potency in conventional microbiological media will potentially fail to predict *in vivo* susceptibility of antibacterial compounds that are affected by physiological concentrations of bicarbonate (Farha *et al*., 2018).

Both the sodium hydrogen carbonate and acetic acid treatments were also used in an attempt to enhance separation and maximise the potential for identification of any anti-MRSA activity in the extract by bioautography. Earlier TLC investigations showed that the bands from the extract began to form a 'crescent' just under the solvent front. The use of an amine or hydroxyl group stops this crescent formation and it was thought that the use of acetic acid would reduce or eliminate this effect.

Plate 16 exhibited the strongest activity in this bioautography investigation with the sodium hydrogen carbonate-treated extract (Figure 3.4). This may be due to the fact that the sodium hydrogen carbonate binds to the silica stationary phase allowing for enhanced availability of the active compounds to react with MRSA or to the fact that sodium hydrogen carbonate is known to act in a synergistic manner with certain antibacterial compounds (Al-Nabulsi & Holley, 2006). A combination of lactoferrin and sodium hydrogen carbonate was bacteriocidal against three strains of *E. coli* and was bacteriostatic against 5 others. The level of activity in the above assessment appeared stronger on the reverse phase plate, but was not the case when comparing the 50:50 MeOH:H_2O mobile phase in Figure 3.5. This may further reflect a variation of ionisation for the active compounds as the percentage of water decreased in the mobile phase.

The antibacterial compound(s) in *P. lanosa* was (were) assumed to be polar, based on the anti-MRSA activity observed in the water extracts versus those prepared with the more non-polar solvents. If the bioactive compound is polar, it is anticipated that it would separate on the normal phase TLC plate with a polar solvent, such as methanol due to its higher affinity for the more polar mobile phase than the silica stationary phase. Further optimisation was considered necessary to improve separation as the presence of crescents indicated incomplete separation and residual tracking of separated components. To ensure a positive result for bioautography analysis, a 0.8 mg/mL loading was selected, as separation was still evident on the TLC plate, both normal and reverse (Figure 3.3) and the response to the lower loadings of 0.4 and 0.6 mg/mL was not definitive.

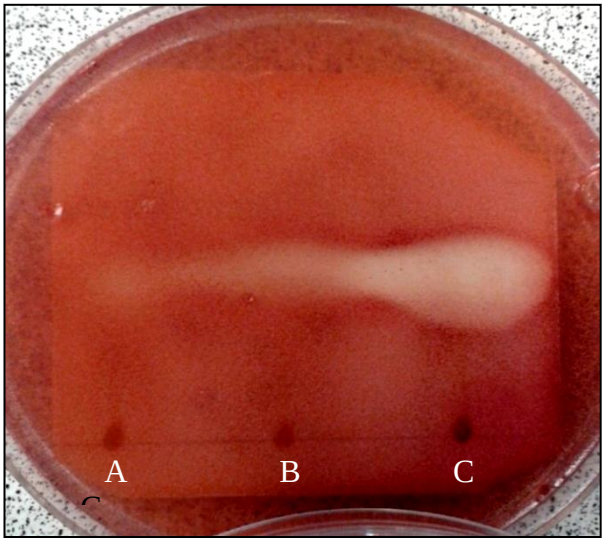


Figure 3.4: Bioautography results of plate 16 on reverse phase Si TLC plate run with 30:70 MeOH:H_2O mobile phase; untreated *P. lanosa* extract (A), acetic acid-treated extract (B) and sodium hydrogen carbonate-treated extract (C).

All of the extract spots migrated the same distance, as depicted in Figure 3.4, perhaps demonstrating the retention of structural integrity of the compounds or that carbonate anions in the sodium hydrogen carbonate-treated extract can bind divalent cations and form insoluble complexes, causing destabilisation of the bacterial membrane (Al-Nabulsi & Holley, 2006). This represents one perspective due to the difference in response between the treated and untreated extracts - variability of ionisation, partly due to mobile

phase composition as with higher water composition there appeared to be better anti-MRSA activity, specifically on the reverse phase Si TLC plate. Positive bioautography on both normal and reverse phase Si TLC plates with and without treatment of the crude extract and observed with different mobile phase composition can be attributed to the inherent stability of the anti-MRSA compound structure which may be enhanced when in an ionised solution – perhaps related to its polar nature.

Overall, this investigation pointed to polar, water-soluble compounds being responsible for the anti-MRSA activity of the seaweed extract. These compounds can include polar lipids, mono and oligo saccharides, polysaccharides, oligopeptides, amino and guandino amino acids, nucleic acids, polar carboxylic acids and glycosides, as well as PBP, coloured and highly fluorescent protein-pigment complexes which include PC, blue pigment; APC, bluish-green pigment and PE, red pigment (Pandey *et al.*, 2013).

Table 3.12 serves to illustrate a variation of R_f values (for positive bioautography) depending on the type of Si TLC plate and the composition of the mobile phase. For the normal phase Si TLC plates, the R_f value increased with increased methanol concentration, whilst for the reverse phase Si TLC plates, it decreased. This variation would be expected due to the varying polar nature of both the stationary and mobile phases. The relevance of the result lies in the fact that both stationary phases allowed for positive bioautography. This may have demonstrated the stability of the anti-MRSA compound and/or functional groups and further indicate a potential interaction between compounds as normal phase and reverse phase Si TLC plates represent two different stationary phases – normal phase Si TLC plates would possess Si-OH groups and is therefore polar so that polar compounds would be retained when a nonpolar mobile phase is used whereas on reverse phase Si TLC plates the stationary phase is non-polar permitting the preferential elution of polar compounds with a polar mobile phase.

Table 3.12: Variation of *P. lanosa* extract R_f values for midpoint of clearance zone of bioautography with respect to treatment dilution and mobile phase composition.

TLC plate	Extract Treatment	Mobile phase composition	R_f ±SD
Normal phase Si	none and both	50:50 MeOH:H_2O	0.36 ±0.08
Normal phase Si	none	80:20 MeOH:H_2O	0.76 ±0.09
Reverse phase Si	none and both	30:70 MeOH:H_2O	0.62 ±0.09
Reverse phase Si	none and both	50:50 MeOH:H_2O	0.29 ±0.06
Reverse phase Si	none and both	H_2O	0.50 ±0.09

The 50:50 methanol:water mobile phase was used on both normal phase and reverse phase Si TLC plates and resulted in positive bioautography for both the treated and untreated samples. The untreated extract displayed the highest level of anti-MRSA activity, the acetic acid-treated spot revealed less activity and the bicarbonate-treated spot displayed the least activity on the normal phase Si TLC plate (Figure 3.5).

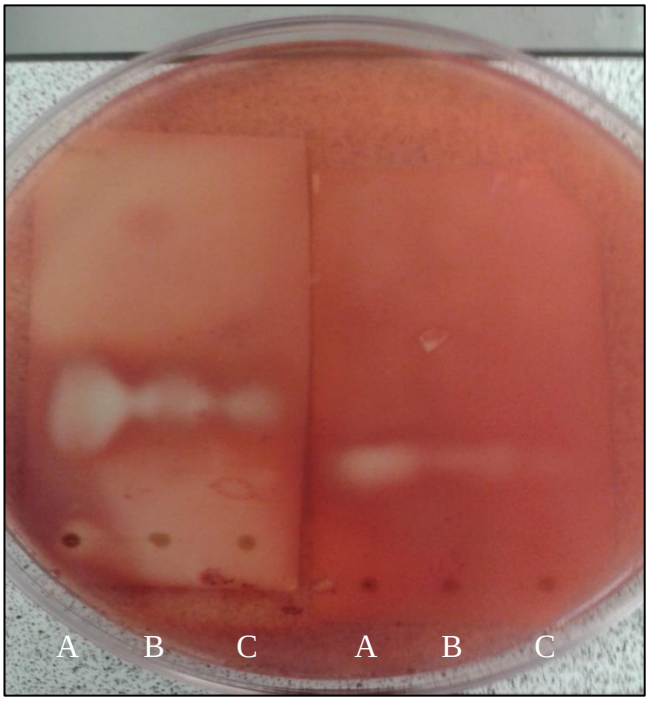


Figure 3.5: Antimicrobial activity on normal phase (left) and reverse phase (right) Si TLC plate with 50:50 MeOH:H_2O after dye application; untreated *P. lanosa* extract (A), acetic acid-treated extract (B) and sodium hydrogen carbonate-treated extract (C).

For the two treated samples, there appears to be considerable inhibition in the area around application of the spot on the normal phase Si TLC plate as seen on the left in Figure 3.5. This may demonstrate the presence of a small amount of inhibitory compounds that have diffused from the spot due to a change in extract properties with the treatments as the treatments on their own did not result in positive bioautography. The treatments may have reacted with the active compounds reducing their anti-MRSA activity and their mobility on the TLC plates (perhaps having caused deposition and dispersion close to the application zone or affected retention of other components). The reverse phase TLC run reveals a more compact zone of activity for the untreated extract and very slight inhibition for the two treated extract spots (on right of Figure 3.5). The fact that both plates showed that the untreated extract sample had the strongest anti-MRSA activity may have indicated an optimum ionisation level for the extract with 50% water. The distance of migration was also indicative of the affinity of the active compounds for the reverse phase silica. The distance of migration of the active compounds on the normal phase silica supported the selection of this stationary phase for flash chromatography of untreated crude aqueous extract.

The reverse phase TLC plate run with 100 % water as the mobile phase revealed anti-MRSA activity. The untreated extract (A) had less activity compared to the two treated extracts which revealed very strong activity as shown in Figure 3.6.

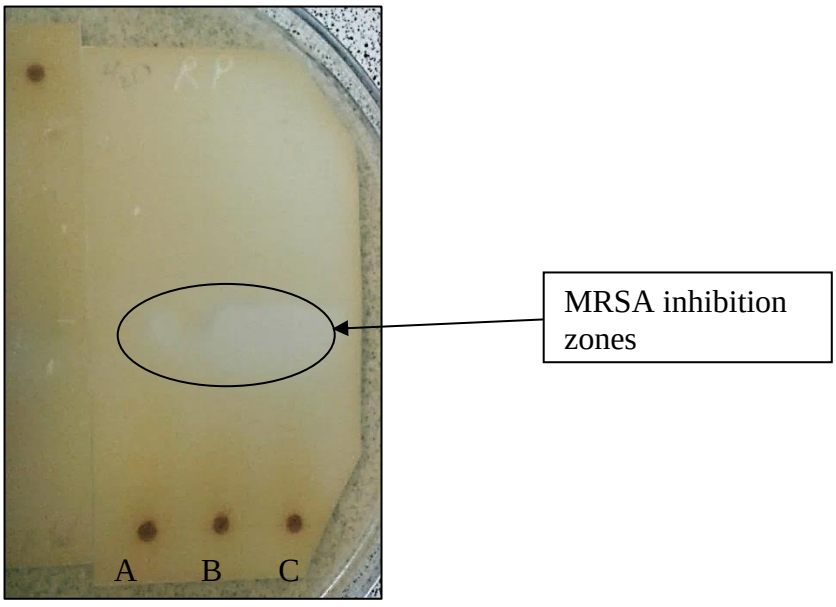


Figure 3.6: Bioautography results on reverse phase Si TLC plate run with 100 % water mobile phase untreated *P. lanosa* extract (A), acetic acid-treated extract (B) and sodium hydrogen carbonate-treated extract (C).

The differences between the reverse phase Si TLC plates shown in Figure 3.5 and Figure 3.6 suggest that the treatments may act as enhancers of activity or as modifiers for separation for reverse phase chromatography systems with the availability of water as a solvent albeit as a mobile phase having permitted a wider spread of activity. The mobile phase change from 50:50 MeOH:H_2O to 100% H_2O resulted in two changes in the resulting bioautography run: for all extracts, the active compounds moved further up the TLC plate, resulting in a higher R_f value, but the treated extracts revealed stronger MRSA inhibition; this was in contrast to the untreated extract where although the activity persisted, the zone was smaller. The reason for this was potentially due to the variable ionisation of the active compounds which were able to ionise more strongly with the treatments.or that the presence of methanol was necessary for separation of one of the components. The R_f value for the bioautography result is identical, suggestive of one active fraction despite the two treatments. The higher level of water in the mobile phase permitted better separation on the reverse phase silica plate – the R_f value increased from 0.29 to 0.50. This result further supports the hydrophilic nature of the active components of the crude extract and that the hydrophobic compounds are adsorbed on the hydrophobic stationary phase.

Figure 3.7 shows the well diffusion assay results for the treated and untreated *P. lanosa* extracts and the differences were determined to be statistically significant ($p<0.05$). Well diffusion results (n=3) obtained for no treatment varied from 29.0 – 33.0 mm, for sodium hydrogen carbonate 23.5 – 28.0 mm and acetic acid 23.0 – 26.0 mm. The untreated extract yielded greater activity, confirming the normal phase Si plate bioautography result (Figure 3.7) and indicating that these modifiers may have effected a slight detrimental change in antibacterial activity.

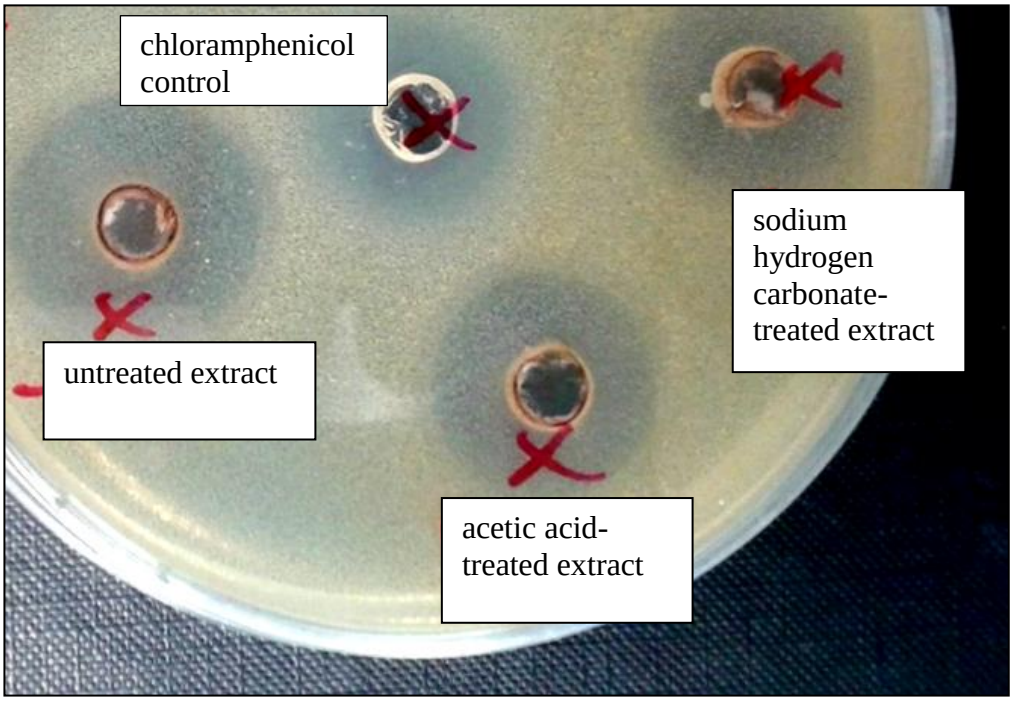


Figure 3.7: Well diffusion assay of treated and untreated *P. lanosa* extracts for anti-MRSA activity compared to a chloramphenicol positive control (n=3).

3.4.5 *Flash Chromatography*

Flash chromatography was pursued as an isolation method after preparative TLC was carried out on both 1000 and 2000 μm silica preparative TLC plates. This proved unsuccessful as very poor separation was achieved and visualisation of components indistinct with one UV absorption band. This band was scraped off and mixed with mobile phase, filtered, dried and tested for anti-MRSA activity, which proved negative. For this reason, a change in solvent composition was sought (allied to this, the use of a high water composition mobile phase represented a potential problem due to the solubility of silica in water). It was decided to substitute with chloroform, Figure 3.8, in place of water and this yielded similar deposition of extract components as that obtained with 100 % methanol mobile phase in Figure 3.3.

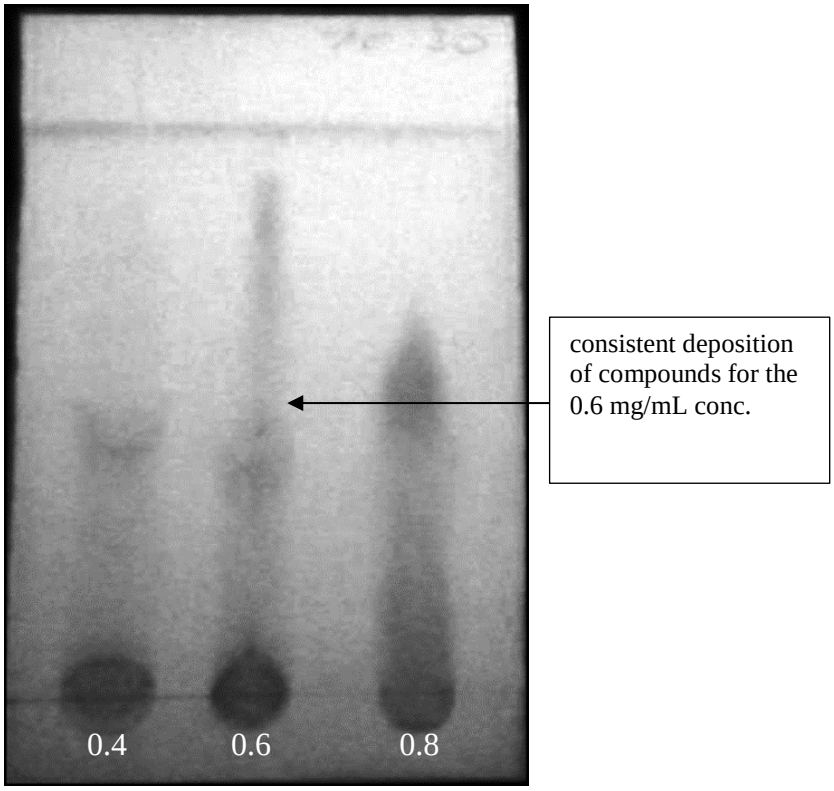


Figure 3.8: 70:30 $CHCl_3$:MeOH mobile phase on a normal phase Si TLC plate with increasing concentrations of applied spots of crude *P. lanosa* extract; left to right 0.4 to 0.8 mg/mL(greyscale).

The water soluble pigments of red algae, PBP, are polar and include PC and PE and can comprise 1-10% of dry mass of algal biomass (Hayes, 2012) and it was expected that these would have traveled up the polar plate with the more polar solvent. The rest of the extract would be retained and likely to contain less polar compounds, as there was only slight movement with the polar solvent.

The initial mobile phase utilised for flash chromatography was 70:30 chloroform:methanol and during the elution of the first fractions, the column acquired a yellow colouration. This colouration persisted during fraction collection and was interpreted as being indicative of the retention of extract components on the column which were unable to elute.

The mobile phase was changed to 40:60 chloroform:methanol with 3% (v/v) water. This was used as it was more polar for the active components but with a certain level of chloroform to aid solubilisation of less polar and possibly more lipophilic components.

Marine algae have a complex lipid composition and contain both neutral and polar lipids (Khotimchenko & Yakovleva, 2005) and these may have required a certain level of solubilisation to become desorbed from the stationary phase. This approach was successful, as the yellow components were found to move off the column. The 3% water was added to ensure the integrity of compound(s) composition requiring water for solvation.

Fractions 30–50 with the new mobile phase were yellow in colour and were pooled and the solvent removed under vacuum, anti-MRSA activity was confirmed by both well diffusion and bioautography.

In the 40:60 mobile phase investigation, this semi-pure extract spot moved with the mobile phase in demonstration of its polar nature. The crude extract spot moved less as it was retained more strongly by the stationary phase due to the complexity and concentration of composition with the comparison presented in Figure 3.9.

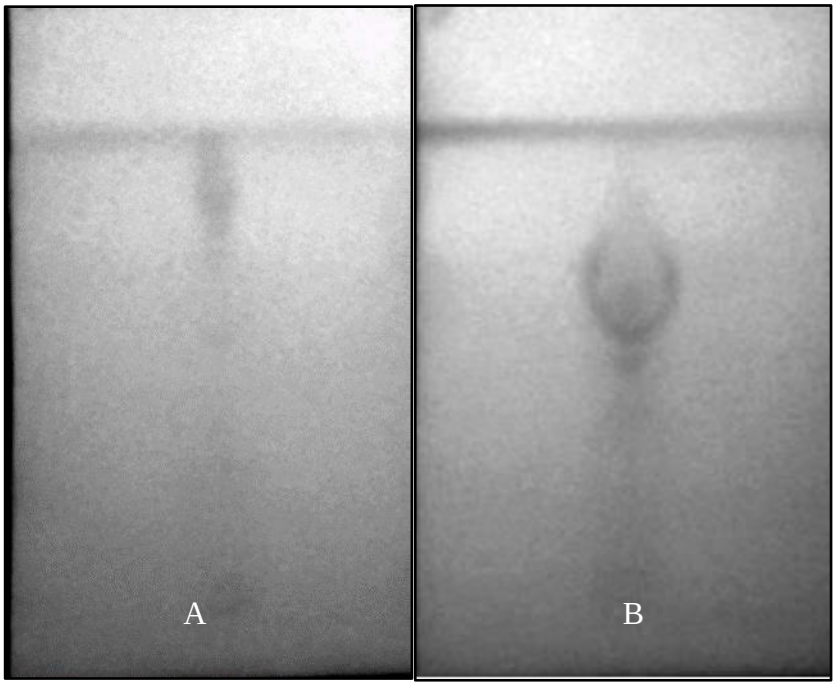


Figure 3.9: Comparison of semi-pure *P. lanosa* extract (A) with original crude extract (B) on normal phase Si TLC plates run with 40:60 $CHCl_3$:MeOH mobile phase with 3% (v/v) water added (greyscale).

A well diffusion assay was carried out on the semi-pure extract obtained by flash chromatography at 50% concentration in both water and methanol and was found to elicit definitive and sharp inhibition (diameter of 9 mm ±0.5) of MRSA W73365, regardless of whether it was diluted in water or methanol (Figure 3.10).

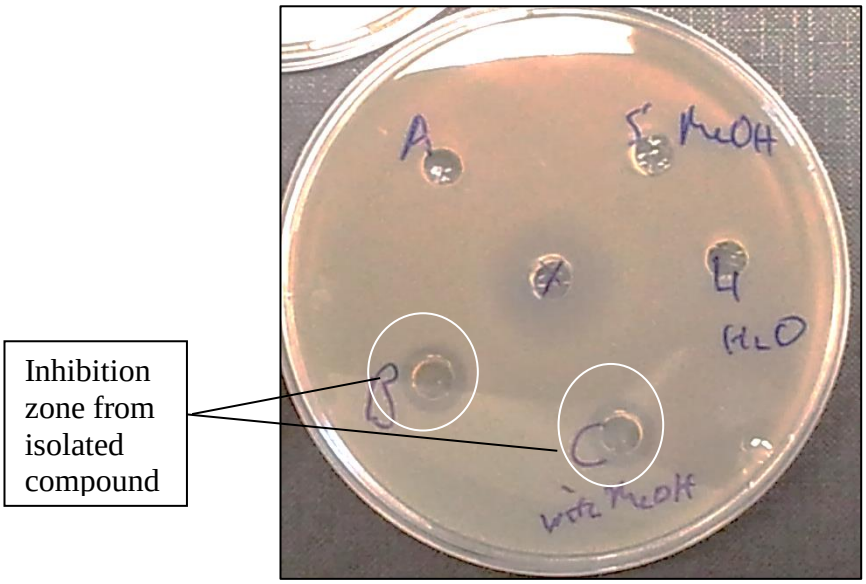


Figure 3.10: Well diffusion assay of 50% concentration of semi-pure *P. lanosa* extract purified by flash chromatography where B is an aqueous solution of the semi-pure extract and C, a methanolic solution (A - 50:50 H_2O:MeOH, 4, H_2O and 5, MeOH, solvent controls) n=3.

In the 70:30 chloroform:methanol mobile phase investigation, the crude *P. lanosa* extract spot moved less than that observed for the 40:60 chloroform:methanol mobile phase. This was assumed to be due to the fact that the 70:30 mobile phase was less polar than the 40:60 mobile phase, thereby impeding migration of the polar compounds on the plate.

Further evaluation of the purity of this semi-pure *P. lanosa* extract was assessed on analytical alumina TLC plates in order to explore the composition of the bioactive compounds. The choice of alumina plates for this analysis was due to their better suitability for separation of polar components (it is also more basic than silica and will adsorb acidic compounds). Preliminary solvent runs were carried out comparing different methanol:H_2O ratios - 50:50, 60:40 and 80:20 (Figure 3.11). There was no migration of two of the three bands with the 80:20 mixture and this was thought to be related to a requirement for a minimum level of water to move the other two components as well as the some other components of the crude extract visible under UV light. The inclusion of

a more recently harvested seaweed crude extract was to ensure consistency of extraction method as well as determine qualitatively that similar TLC results were achieved.

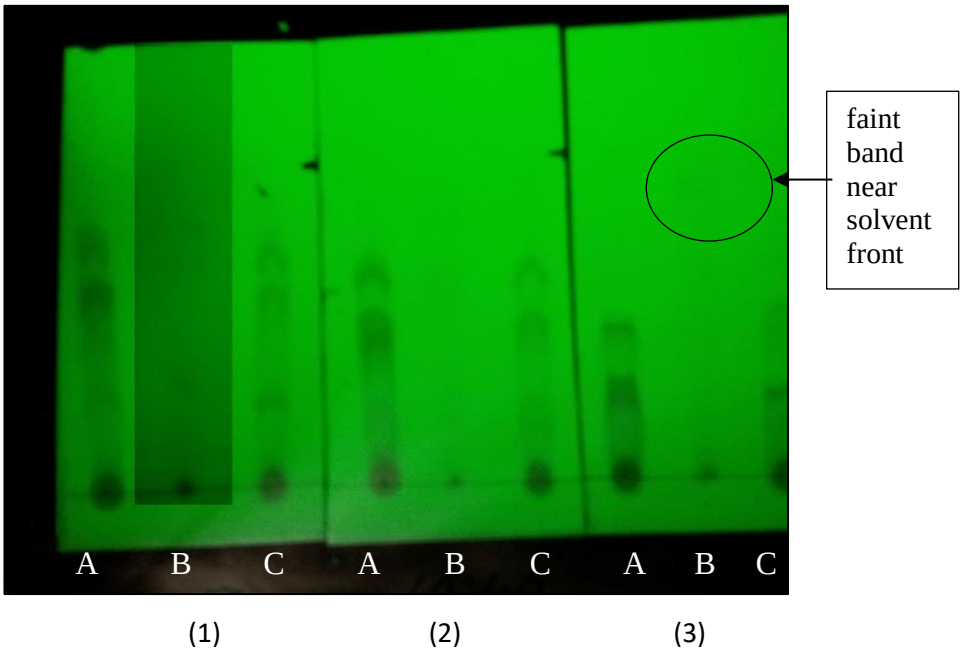


Figure 3.11: Comparison of normal phase Al TLC for newly harvested crude *P. lanosa* extract (A), semi-pure extract (B) and original crude extract (C) with 50:50 (1), 60:40 (2) and 80:20 (3) MeOH:H_2O mobile phase.

This would also account for the situation with the silica plate runs where it seemed everything including the active on the preparative plates moved with the solvent front. Another observation was that the active region using bioautography (on silica plates) with 70:30 MeOH:H_2O occurred at approximately the half way point of the solvent front. The bioactive spot (in bioautography) migrated further with increasing H_2O composition (although the position of the bioactive spot remained very similar for both 100% MeOH and 70:30 MeOH:H_2O). Again, it is possible that there exists a minimum water composition for optimum mobility and further supports the high H_2O solubility of the active components. In order to clarify this, a second TLC run was performed with a 80:20 methanol: H_2O mobile phase and is presented in Figure 3.12.

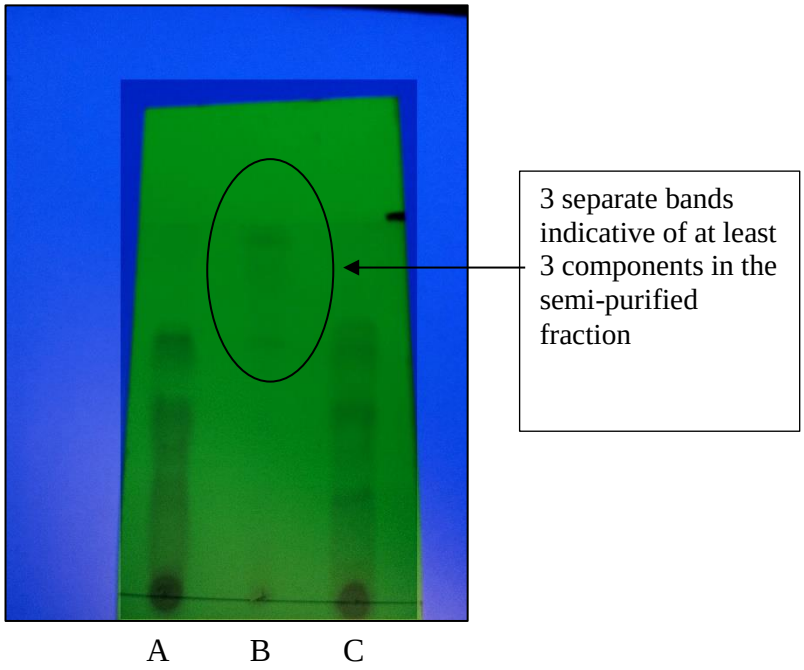


Figure 3.12: Normal phase Al TLC plate ((3) from Figure 3.13) - newly harvested crude *P. lanosa* extract (A), semi-pure extract (B) and original crude extract (C) after second run with 80:20 MeOH:H_2O mobile phase.

It was possible to visualise three fractions under UV on completion of the second run. There was one faint band next to the solvent front on the initial solvent run (indicated on the right of Figure 3.11) which was concentrated with the second run and two further bands in Figure 3.12. This indicated a minimum of three components in the isolated semi-pure fraction. These components represent only those identified under UV light and there are potentially more compounds in the bioactive semi-pure extract and this knowledge may be useful for subsequent characterisation studies.

Corroboration of the results obtained for the use of 80:20 methanol:water mobile phase is illustrated by Figure 3.13. There was distinct separation of the active fraction from the yellow coloured fraction above it and indicative R_f values were recorded as 0.67 for the non-pigmented active fraction and 0.73 for the yellow pigmented fraction. There may be a further yellow coloured compound at the solvent front.

These results provided much for consideration as it had seemed that the changes in visualisation of both the separation and bioautography were complicated. However, a

probable explanation was uncovered, which also linked up with the characterisation studies in the next section. Bis-indoles and specifically, bis(indolyl) methene is a pH responsive chromogenic molecule, containing a conjugated bisindole skeleton which acts as an efficient chromogenic-sensing molecule model. The reaction is based on the proton transfer signaling mode and in water-containing medium, the presence of acid can easily induce the proton transfer to the basic H-bond acceptor moiety, modulating the internal charge transfer state of bis(indolyl)-methene and results in dramatic colour changes (Wang *et al*., 2011). The possibility exists that there were a group of bis(indolyl) compounds in the anti-MRSA extract and depending on their functional groups, their UV-Vis spectrum varied and may have responded to changes in the mobile phase to different extents and again may also be influenced by other compounds within the semi-pure extract. There are various bis(indolyl) compounds reported in the literature as colourless (Dhumaskar & Tilve, 2012).

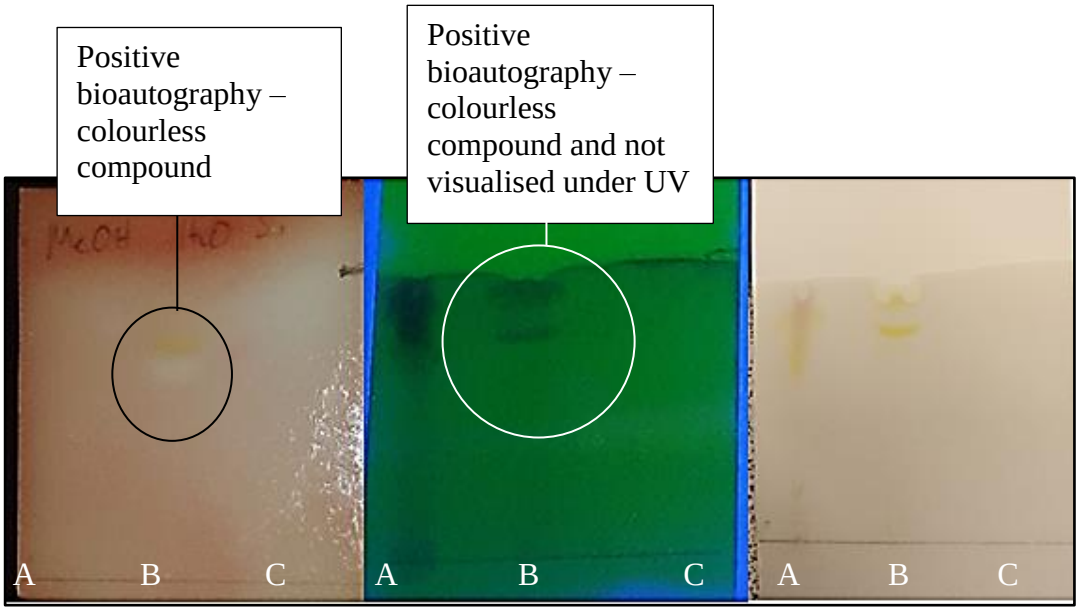


Figure 3.13: Positive bioautography result obtained for semi-pure extract run on Normal Phase Si TLC plate with 80:20 MeOH:H_2O mobile phase - crude *P. lanosa* extract (A), semi-pure extract (B) and chloramphenicol (C) (n=2) and compared to plate under UV and plate before bioautography.

3.4.6 *Characterisation of bioactive compounds*

3.4.6.1 **TLC Methods**

3.4.6.1.1 TLC method using staining techniques

Staining techniques were employed to ascertain the potential composition of the extract and the results indicated that the active semi-pure *P. lanosa* sample contained a number of different compounds at levels detectable by this technique (data not shown). The only stain which yielded a negative result was ferric chloride (phenol detection) for the semi-pure sample where the crude extract, positive. This means that the semi-pure sample contained constituents with amine or amide groups, an indole group, may have been an alkaloid, possessed carboxylic acid groups and was an anti-oxidant. The probability was that all groups were present in the semi-pure sample, either separately as different compounds and/or as functional groups of a larger molecule.

3.4.6.1.2 TLC method using specific solvent separation of potential compounds

Results of the different solvent systems suitability for compound class in terms of the mobility and resulting bioautography results for the reference samples are presented in Table 3.13. The solvent composition was anticipated as preferentially able to isolate the compound class. The crude and semi-pure *P. lanosa* extract were run with all mobile phases and did not produce positive bioautiography results (data not shown). These solvent compositions were unsuitable for isolation of the active compounds.

Table 3.13: Solvent composition specific to possible compound type, presented with mobility and bioautography assessment results

Compound	Possibility	Mobile phase	Observation (movement)	Bioautography
Carotenoids	Low	Acetone:DCM (90:10)	No movement from application spot for all 6 samples.	Positive for *IAA
Polyacetylenes	Medium	Hexane:ethyl acetate (2:1)	Slight for IAA, good for catechin.	Positive for IAA
Catechins	Medium	ACN:ethyl acetate (85:15)	Good for IAA, gallic acid, catechin.	Positive for IAA
Galactolipids	Medium	MeOH:$CHCl_3$:EtOH (20:80:1)	Slight for crude and active, good for IAA, slight for gallic acid, good for catechin.	Positive for IAA
Bromophenols	Medium	Hexane:$CHCl_3$:MeOH (1:0.5:0.1)	Good for catechin.	Positive for IAA
Indole alkaloids	Medium	hexane:ethyl acetate:IPA:acetic acid (40:20:5:1)	Slight for crude and active but excellent banding for IAA, gallic acid and catechin.	Positive for IAA

*IAA – indole acetic acid

The fact that the crude and semi-pure *P. lanosa* extracts did not produce positive bioautography results with the mobile phases listed in Table 3.13 is indicative of the complex nature of both samples and that these mobile phases were unsuitable for the separation of active anti-MRSA components from both extracts. The possibility is that either some of the active components were either separated from one another or a single synergistic molecule was removed from the mixture inhibiting activity. The compound indole acetic acid, maintained its anti-MRSA activity in all solvent systems and serves as a positive control. This result may further have eliminated indole acetic acid as a possible extract component responsible for its bioactivity although it has been reported that *P. urceolata* contained a high level of indole acetic acid (Lijun, 2006). The potential range of indoles in seaweed (Stirk *et al*., 2004), synthesised from tryptophan or indole, includes indole-3-acetic acid (IAA); indole-3-acetyl-L-aspartic acid (IAAsp); indole-3-acetyl-L-

alanine (IAAla); indole-3-acetylglycine (IAGly); indole-3-aldehyde (IAld); indole-3-acetyl-L-leucine (IALeu); indole-3-acetyl-L-phenylalanine (IAPhe); indole-3-acetyl-L-valine (IAVal); indole- butyric acid (IBA); indole-3-carboxylic acid (ICA) and indole-3-lactic acid (ILA), again revealing the reason for inconclusive detection of a single active group or compound type.

The comparison of reference compounds with the crude and semi-pure extract on normal phase Si TLC with the two optimised mobile phase compositions (Section 3.4) is presented in Table 3.14.

Table 3.14: Comparative mobility of reference compounds on TLC and bioautography R_f values bioautography to crude and semi-pure extract samples (n=2).

Sample	**Normal phase TLC (MeOH)** **R_f ±SD**		**Normal phase (50:50 MeOH:H_20)** **R_f (±SD**		**Positive Bioautography** **R_f ±SD**
	UV SW	LW	SW	LW	
Crude extract	smear		smear		0.86 ±0.015
Semi-pure extract	0.83 ±0.015	ND	dilute	ND	0.86 ±0.010
Nicotinic acid	0.88 ±0.015	ND	0.58 ±0.015	ND	
Indole acetic acid	0.88 ±0.015	ND	0.82 ±0.015	ND	0.89 ±0.010
Gallic acid	0.92 ±0.015	0.73 ±0.015	0.76 (smear)	0.79 (smear)	
Thiamine	0	0	0	0	
Tryptophan	smear	smear	smear	smear	
Catechol	0.96 ±0.02	0.93 ±0.010	0.92 ±0.012	0.96 ±0.015	
Proline	1.0	ND	ND	ND	
Arabinose	ND	ND	ND	ND	
Caffeine	0.69 ±0.015	ND	0.86 ±0.010	ND	
Iso-valeric acid	ND	ND	ND	ND	
Streptomycin	1.0	ND	1.0	ND	
Propionic acid	0.92 ±0.010	ND	ND	ND	0.79 ±0.020
n-valeric acid	0.90 ±0.011	ND	ND	ND	0.86 (v.large zone)±0.040

ND – not detected, SW – short wave UV detection and LW – long wave UV detection

The positive bioautography results are the most relevant and were observed for both the crude and the semi-pure extract as well as for indole acetic acid, propionic acid and n-valeric acid.

Positive bioautography was subsequently observed for 4-dibromoacetophenone and this was corroborated by well diffusion with an inhibition zone diameter of 12.0 mm (± 0.5 mm) in addition to inhibition zones for propionic acid of 25.0 mm (± 1.5 mm), catechol – 9.0 mm (± 1.5 mm) and gallic acid – 7.0 mm (± 0.5 mm). The fact that the well diffusion assay yielded positive results for catechol and gallic acid after negative

bioautography results could be due to interaction between these compounds and the normal phase Si TLC plate, preventing anti-MRSA activity. For example, Si is known to be acidic and would therefore retain basic compounds more strongly so that if the active groups are basic, such as amine groups, they may not be available for anti-MRSA activity.

3.4.6.2 **Physicochemical Characterisation of the aqueous crude extract**

3.4.6.2.1 Enzyme Stability

The effect of various enzyme treatments on the anti-MRSA activity of the crude *P. lanosa* extract was evaluated and the results are presented in Table 3.15.

Most bioactive compounds isolated from marine algae can be categorised into classes, such as brominated phenols, aromatics, nitrogen- heterocyclic, nitrosulphuric heterocyclic, sterols, dibutanoids, proteins, peptides and sulphated polysaccharides and it was anticipated that the enzyme study would assist in the characterisation of bioactive compound class(es).

Table 3.15: Enzyme treatment of crude extract and effect on anti-MRSA activity (n=3).

Enzyme	**Anti-MRSA activity (Zone of Inhibition in mm) ± SD**
No enzyme	12 ±1.7
Protease type 1	14 ±1.0
Pronase E	9 ±1.0
Proteinase K	11 ±1.2
α-Chymotrypsin	16 ±0.9
Pepsin	15 ±1.1
Catalase	10 ±1.0
Lipase	20 ±1.7
Amylase	20 ±1.3
Chloramphenicol	21 ±1.2

Pronase E, proteinase K and catalase effected no significant change, $p>0.05$ in anti-MRSA activity whilst protease type1, α-chymotrypsin and pepsin increased the diameter of the zone of activity for the crude *P. lanosa* extract. Overall, these results point to the involvement of protein linkages and/or proteins as at least partly responsible for anti–MRSA activity. α-Chymotrypsin is a serine protease and hydrolyses peptide bonds with a specificity for the carboxy-terminal of aromatic residues such as phenylalanine, tyrosine and tryptophan which would have supported the presence of an aromatic carboxylic acid functional group and it having potentially contributed to anti-MRSA activity in the *P. lanosa* crude extract.

The proteolytic enzymes used differ in their selectivity and another explanation for the increased activity is hydrolysis of the bound protein from the PBP, liberating the pigment structure, thereby having enhanced its activity. Another possibility is a class of macroalgal lectins, all of which have been shown to be low-molecular-weight monomeric thermostable protein molecules that possess an affinity for oligosaccharides or glycoproteins (but not for monosaccharides), and act to protect chemical composition (Harnedy & FitzGerald, 2011).

It is known that catalase breaks down H_2O_2 into oxygen and water in seaweed peroxisomes and cytoplasm (Collén *et al*., 1996) and that the liberation of hydrogen peroxide from phenols rich in hydroxyl groups (as found in tannins) has been recognised as partially responsible for the antimicrobial action of *A. nodosum* (Wang *et al.,* 2009). No significant change was observed on treatment of the crude *P. lanosa* extract with catalase.

The crude *P. lanosa* extract may have contained both bioactive sulphated polysaccharides and other polysaccharides which would be hydrolysed by amylase, thereby affecting activity. However, amylase produced a substantial increase in the anti-MRSA activity of the extract, indicating that either the smaller molecules were more active, perhaps by allowing better availability of active groups to interact with the bacteria. The breakdown of high molecular mass polysaccharides into smaller molecules may have released more active oligosaccharides. It is recognised that sulphated polysaccharides possess antibacterial activity and this has been found in other red seaweed crude aqueous extracts (dos Santos Amorim *et al*., 2012).

Antimicrobial activity was substantially increased by lipase, which breaks down constituent lipids into glycerides and fatty acids. The triacylglycerol (TAG) composition of fats or oils is important, as there are numerous TAG species due to the large number of different fatty acids on the glycerol backbone. As for bioactive glycoglycerolipids of red algae, hydroxyeicosapentaenoyl galactosyl glycerols were extracted and identified from the temperate red alga *Gracilariopsis lemaneiformis* (Gerwick *et al.*, 1993), as well as sulphoquinovosylmonogalactosyl glycerol (SQMG). This supports the suggestion that lipase may have permitted the breakdown of lipids into more active compounds, including fatty acids, thereby enhancing anti-MRSA activity that may already be associated with free fatty acids within the crude *P. lanosa* extract (Desbois & Lawlor, 2013).

The fact that there was a substantial increase in activity with both amylase and lipase may have indicated that active component groups are hindered by virtue of their attachment to a larger molecule. The proteolytic enzymes, protease type1, α-chymotrypsin and pepsin as well as amylase and lipase may have resulted in the breakdown of some compound(s) liberating biologically active IAA and/or other active small molecule compounds which contributed to enhanced anti-MRSA activity.

3.4.6.2.2 Heat Stability of *P. lanosa* Crude Extract

Heat stability can be used to complete a stability profile of the crude seaweed extract to ensure stability during processing, specifically to ensure that extraction and purification conditions involving temperature variations do not affect bioactivity of the crude *P. lanosa* extract. Table 3.16 demonstrates the relative thermal stability of the anti-MRSA compounds within the aqueous *P. lanosa* crude extract. Anti-MRSA activity was lost as temperatures increased and the differences statistically significant ($p < 0.05$). The changes observed were slight (even after 30 min at 100 ˚C). This suggests heat stable activity, whether due to stability of each of the individual active components or that the heat protection was afforded by the combination of different chemical groups of compounds.

A study by Gupta *et al.,* (Gupta *et al.*, 2010) reported that heat-treated seaweeds displayed variation in their antimicrobial activity over the range of temperatures investigated. The

research group investigated the antimicrobial activity on food spoilage and pathogenic bacteria of 3 edible brown seaweed crude methanol extracts (also at 60mg/mL concentration) with temperature fluctuations from 85 – 100 ˚C. Bactericidal activity was reduced at 85 ˚C but remained the same as non heat-treated for one of the bacteria at 95 and 100 ˚C.

Table 3.16: Effect of heating *P. lanosa* crude extract (60 mg/mL) for 30 min at various temperatures on anti-MRSA activity (n=4).

Temperature (˚C)	**Anti MRSA Activity (Zone of Inhibition in mm) ± SD**
Room temperature - 19	20.0 ±0.75
30	17.5 ±0.83
40	17.0 ±0.85
50	19.5 ±0.80
60	16.5 ±0.95
70	18.0 ±1.10
80	18.0 ±0.73
90	16.5 ±1.30
100	17.0 ±1.10

The thermal stability of the proposed bis(indolyl) based compound further supports its consideration as these compounds generally possess high melting points (Dhumaskar & Tilve, 2012). This was further supported by the enzyme study where the potential presence of thermolabile protein molecules in the form of macroalgal lectins was discussed (Section 3.4.6.2.1).

3.4.6.3 **Spectroscopic Techniques**

3.4.6.3.1 UV-VIS Spectroscopy Analysis

After identification of classes of compounds, UV-Vis spectroscopy was used to verify if this classification could be correct. The semi-pure extract (20 mg/mL) was analysed using UV-Vis spectroscopy (Figure 3.14). The λ_{max} was 331 nm and the observed absorbance of 0.322 was quite low due to the high dilution factor. The λ_{max} value of the compound was determined and will be considered later in this section as it may support functionality assignment together with the peak at 451 nm. A λ_{max} value at 451 nm may support a hypothesis that there is a conjugated polyene, as carotenes typically have values within the range of 447 – 462 nm (Olives Barba *et al*., 2006).

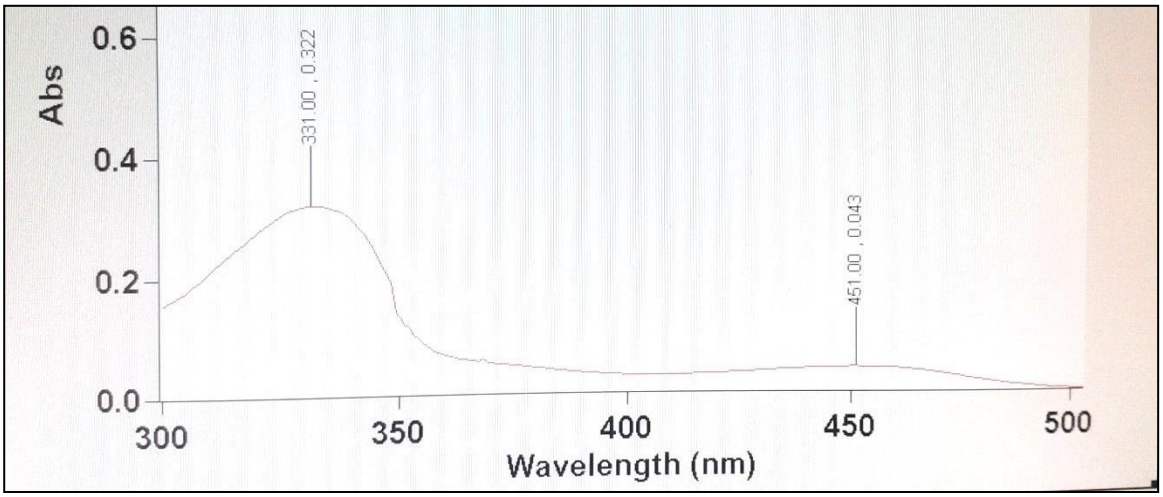


Figure 3.14: UV-Vis spectrum of the semi-pure *P. lanosa* extract from flash chromatography.

Figure 3.15, a further UV-Vis analysis where the spectra were generated for the semi-pure active (spa) and inactive extract fractions from flash chromatography. On concentrating the spa extract, a broad peak from 270-330 nm was visible. This would indicate a number of components together at 220-235 nm range. Although the assumption is made that the two peak areas of the 50% spa sample point to a variety of compounds, certain compound types do absorb in a similar manner. For example, there are two absorbance bands for flavonoids – 240-285 nm corresponding to the A-ring and 300-550 nm for the B-ring (Andersen & Markham, 2006). Acetophenone would possess significant absorption bands at of 246, 280 and 320 nm.

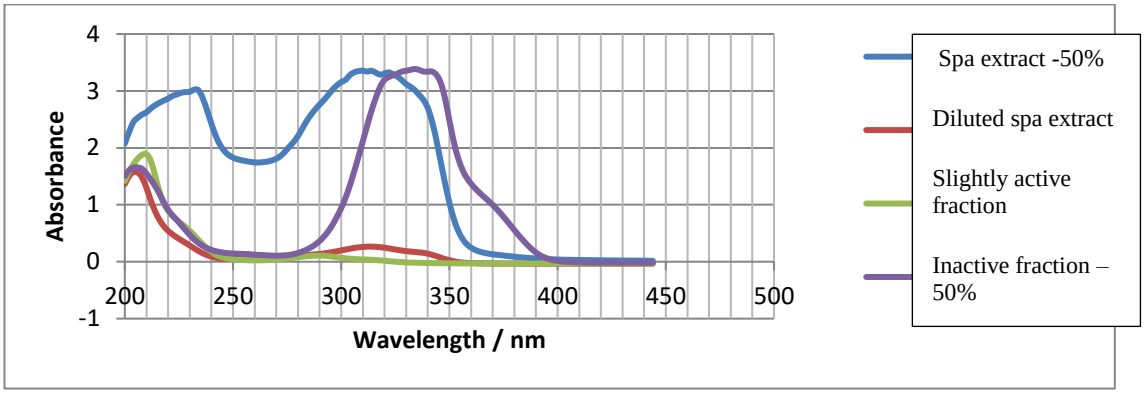


Figure 3.15: UV-Vis Spectra comparison of semi-pure active and inactive fractions.

The positive well diffusion for acetophenone also allowed for the consideration of this class of compound as a potential component of the active extract mix. However, the UV-Vis spectrum for 4-bromoacetophenone possesses maximum wavelength absorbance (λ_{max}) at 205 and 254 nm (Trivedi *et al*., 2015). Knowledge of the λ_{max} can allow for prediction of classes of compounds present in the extract. The indole chromophore is associated with absorption bands at 230, 270 and 289 nm and may be responsible for contributions to the above spectrum (X. Di *et al*., 2018). It was further observed that there was a change in the UV-Vis spectrum of the crude extract when dissolved in different aqueous solutions. A 1 % v/v HCl aqueous as well as a PBS solution resulted in the shift of the UV-Vis spectrum to higher wavelengths and this was observable in that the solutions became distinctively coloured – the PBS solution assumed a green colour whilst the acidic solution became pink. Changes in UV spectrum (MeOH) between closely related compounds have been observed and one example is that of the metabolite racemosin (bisindole alkaloid) where racemosin A UV-Vis spectrum absorption peaks of 225, 273 and 342 nm (pink amorphous powder) were observed whilst the values for racemosin B were 217, 238, 284 and 355 nm (orange-yellow amorphous powder) (Liu *et al*, 2013). As far back as 2000, brominated cresols and anisoles were identified in a *Polysiphonia* species, *P. sphaerocarpa* (sampled in Australia) so it may be possible that such 'indicator' compounds (bromocresols) are present in the crude extract (Flodin & Whit, 2000) and may corroborate the colour alteration described above. The *Polysiphonia* genus is regarded as a reliable source of bromophenols and the probability that the semi-pure extract includes these compounds was supported by the spectrum as their absorption band is typically 290 nm (± 5) (Urban *et al*., 2019).

The UV spectra of IAA can vary depending on the solvent and whether the compound is fully protonated as at pH 2 or deprotonated at pH 7 and one study found that the λ_{max} underwent a slight alteration (Carić *et al*., 2004). For example, an ethanol solution had peaks at 201, 222 and 276 nm whilst an aqueous buffered solution had no peak at 201 nm but had a peak at 220 and 275 nm. This example serves to illustrate the influence of other compounds in an extract.

A further consideration was the possible presence of carboxylic acids in the semi-pure active *P. lanosa* extract as tricarboxylic acids have been identified as common in the genus (Kamenarska *et al*., 2006) and these would not usually be observed in a UV spectrum. This was further observed in the bioautography run presented in Figure 3.13 where the zone of inhibition was below a coloured extract component and not visualised under UV or visible light.

In conclusion, the UV-Vis spectrum supported the potential presence of an indole type of molecule, perhaps a bis-indole with 2 indolinenone units with an unsaturated conjugation between the 2 indole groups. The yellow solid product permitting comparison with racemosin B (yellow) where the 2 carboxyl groups of racemosin A (pink) are lost when conjugated to form racemosin B (Liu *et al*, 2013).

3.4.6.3.2 FTIR

FTIR spectra provided information on functional groups to further explore the classification identified for the active components in the purified extract. The spectra of both the purified extract residual oil and the yellow solid on purification are represented in Figure 3.16 and 3.17, respectively. The purified extract was found to have separated on storage at – 20℃ as a yellow solid as well as an oil residue were observed on the bottom surface of the vial.

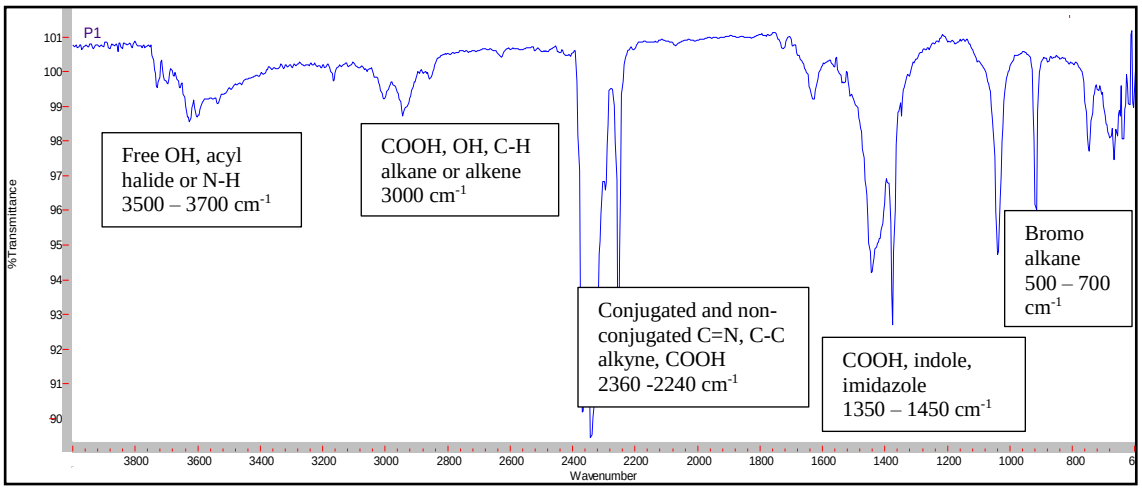


Figure 3.16: FTIR spectrum (film) for purified *P. lanosa* extract (oily residue) with possible functional group designation.

The small peaks ranging at around 3000 cm^{-1} are indicative of C-H stretches of alkenes and alkanes whilst the 2360 – 2240 cm^{-1} may indicate the presence of an indole as these peaks are observed with conjugated and non-conjugated C=N bonds with pure indole exhibiting peaks at 1456, 1352 and 731 cm^{-1}. The 2360 and 2240 cm^{-1} peaks may also suggest the presence of thio, nitrile or azido groups. Carbonyl groups would be identified by peaks in the 1300 – 1420 cm^{-1} range.

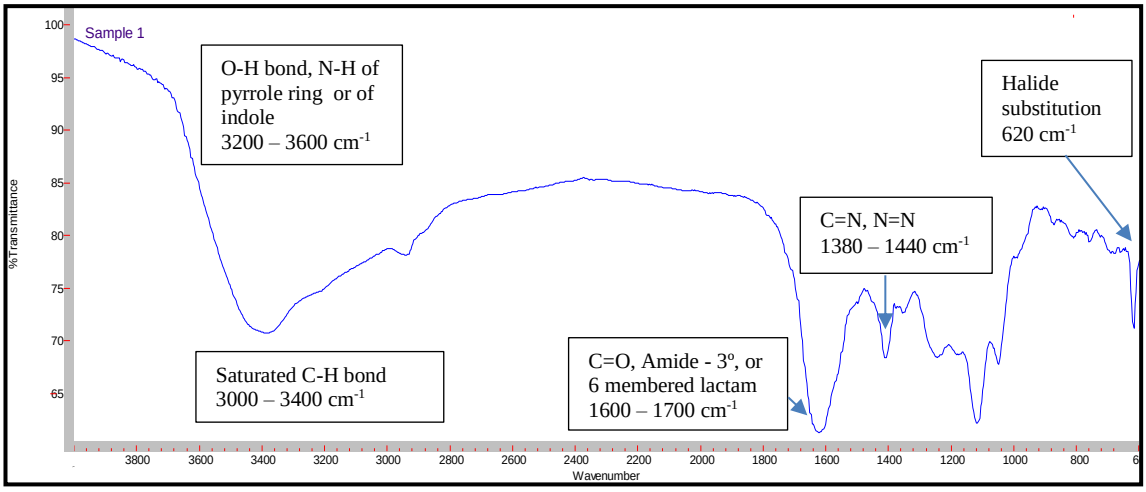


Figure 3.17: FTIR spectrum (KBr disk) of residue from purification (yellow solid) with possible functional group designation.

The spectrum in Figure 3.17 again indicated the stretching C-H bonds in the 3000 – 3400 cm^{-1} range. A strong band at 3300 cm^{-1} would indicate a phenoxyl group whilst the wide trough signals the O-H bond in a carboxylic acid, with 1610, and 1400 cm^{-1} . The peaks in the 1120 – 1640 cm^{-1} range may be comparable to those defined for 3-(2-Bromo-4-hydroxyphenyl)-5,7-dihydroxycoumarin (a lactone structure) where the following data was recorded: IR (neat) - 3346, 3118, 2829, 1678, 1608, 1573, 1495, 1463, 1426, 1285, 1259, 1206, 1125, 1030, 863, 621 cm^{-1} (Sheng *et al*., 2016) with the 621 cm^{-1} signifying the bromo substitution. More probable alternatives would be indole derivatives, either a bromoindole alkaloid as recently isolated from the marine sponge *Geodia barrette*, such as Geobarrettin A, a yellow solid with characteristic IR spectrum (KBr) - 3352, 3214, 1679, 1441, 1205, 1138, 1057, 841, 802, 724 cm^{-1} ($C_{17}H_{20}{}^{79}BrN_6O_4$) (X. Di et al., 2018) as presented in Figure 3.18 or a compound type such those synthesised from bromo indole aldehydes to yield, for example 2-(1-Ethyl-5-(methoxyphenyl)1H-indol-3-yl)-5-methyl-1H-benzoimidazole, a brown solid with characteristic IR spectrum (KBr) - 3036, 2930, 1607, 1577, 1514, 1452, 1113, 799 cm^{-1} (Lyu *et al*., 2017).

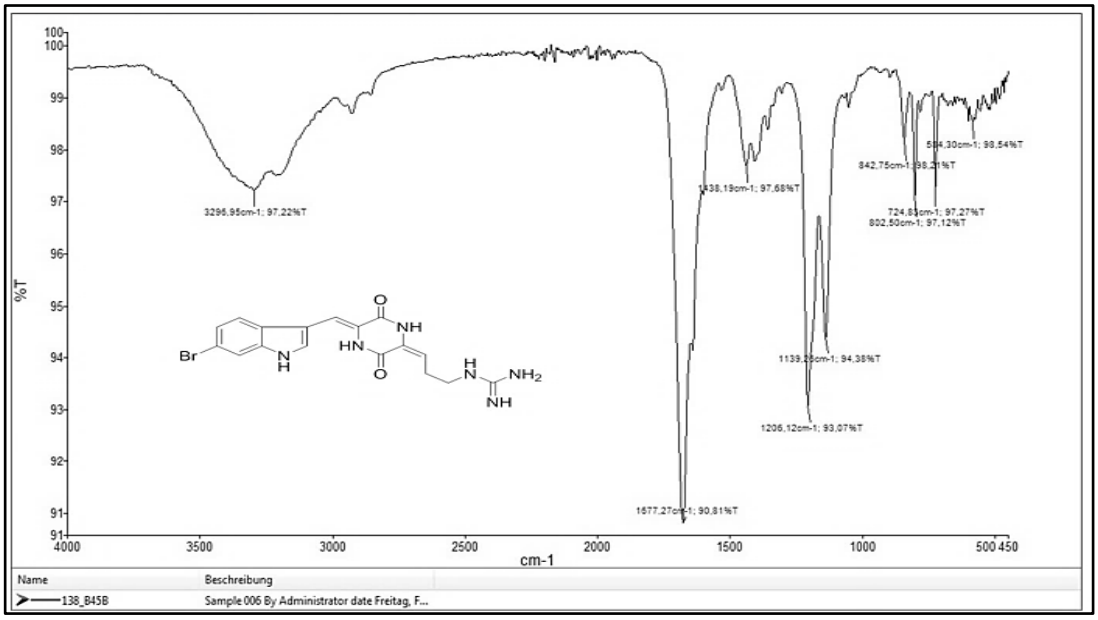


Figure 3.18: IR spectrum and structure of geobarrettin B (X. Di et al., 2018), presenting similar peaks to the semi-pure *P. lanosa* extract solid of Figure 3.17.

These spectra illustrated the possible presence of nitrogen, bromine and sulphur-containing compounds as well as carboxylic acids. The most probable compound class

would be a substituted indole, possibly with heterocyclic, carbonyl, halide and nitrile functional groups as represented in Table 3.17.

Table 3.17: FTIR peak assignment for the semi-pure fraction of *P. lanosa* extract.

Peak Value (cm^{-1})	Bond Designation (stretch)	Functional Group
3600	O-H, acyl halide or N-H	Alcohol
2800 - 3100	COOH-OH, C=H	Carboxylic, alkene
2200 - 2400	C=N, C-C (alkyne), COOH	Conjugated and non-conjugated stretching
1580 - 1640	Amide, C=O, C=C (cyclic)	Aromatic
1400 - 1500	COOH, indole or imidazole	Carboxylic, indole or imidazole
1100 - 1180	C-H in aromatic structure	Aromatic
850 - 950	C-C in pyran, indole	Unsaturated ring
650 - 750	C-halide	Halogenated compound

3.4.6.3.3 LC-MS Analysis

LC-MS analysis was first performed on the semi-pure *P. lanosa* extract produced by flash chromatography to try and get further information on molecular ions and fragmentation patterns that could enhance the information already identified from UV-Vis spectroscopy and FTIR. The components of a mixture are first separated using HPLC followed by ionisation and speparation of the ions on the basis of their mass/charge ratio. A standard gradient mobile phase was used. Targeted mass spectrometry permitted the recognition of ions of specific mass (m/z) and m/z range values of 200, 400 and 600 were selected.

The resultant chromatogram (Figure 3.19), revealed a flat baseline obtained at the start of the run and from 10 min, small peaks of 10-20 kCounts appeared. These small peaks were possibly small quantities of impurities and other compounds within the seaweed extract. This was expected, as the matrix of the original crude extract was unknown and a large number of contaminants in the semi-pure extract was plausible despite FC purification. At 10.77 min, the first major peak was observed on the chromatogram. This was ~50 kCounts in height.

The main peak of the LC chromatogram was obtained at 10.87 min and was ~140 kCounts in height. This peak was potentially the antimicrobial compound isolated by flash chromatography and the major component of the isolated fraction. This peak was not Gaussian in shape and was not very sharp which could suggest the presence of another peak within the main peak, potentially indicative of co-elution.

From 11 min to 13 min, there were more small peaks on the chromatogram. Overall, it was obvious that the sample was not 100% pure (as expected), although significant purification had occurred.

The mass spectrum for the peak at 10.77 min displayed multiple fragments and the molecular ion peak at 355 m/z had the largest molecular weight and was also of high intensity. Molecules such as octadec-9-enoic acid 2,3-dihydroxy propyl ester with a molecular mass of 356.54 were found in a fraction of *Asparagopsis taxiformis* active against pathogenic *Vibrio* strains (Manilal *et al.*, 2009).

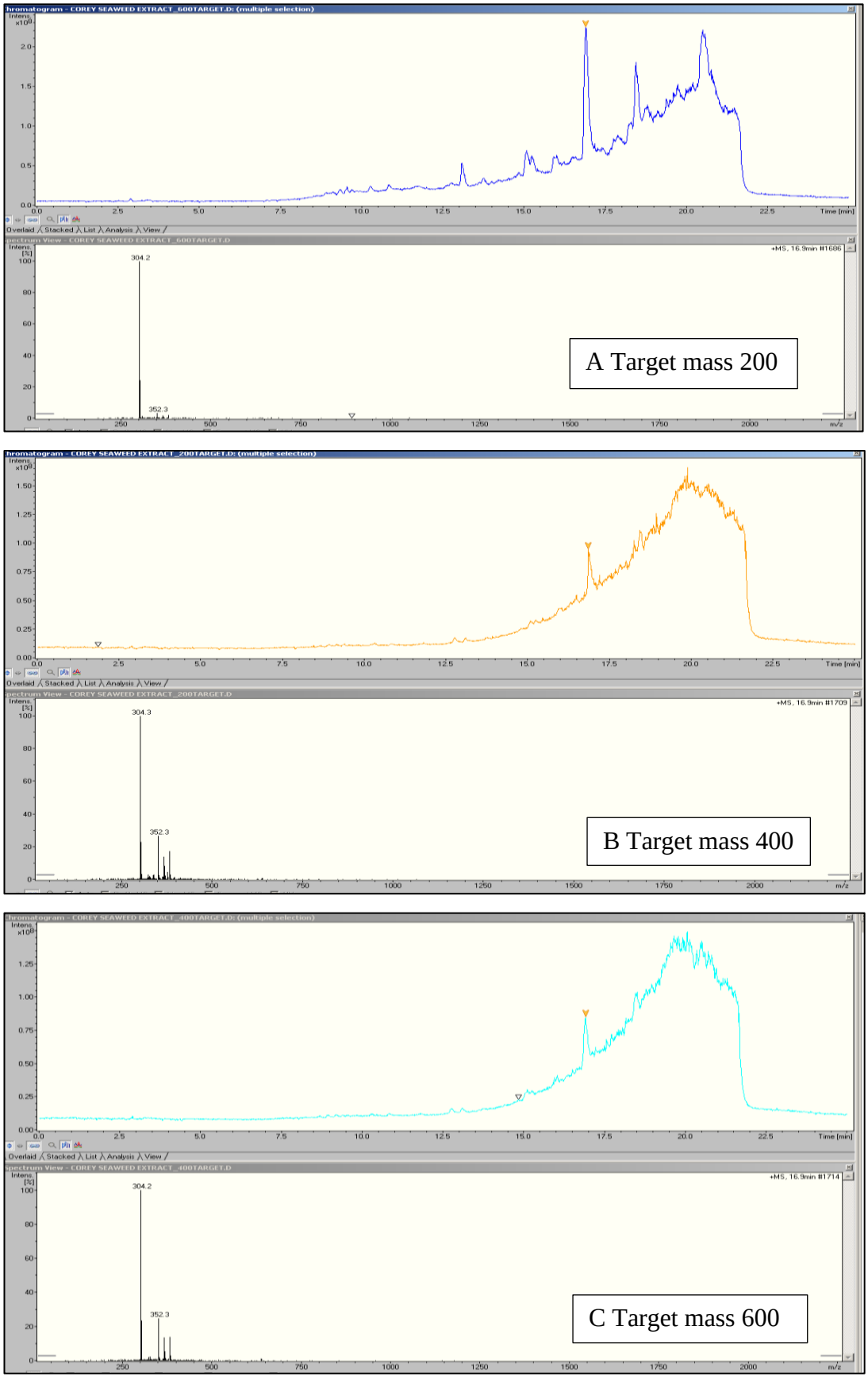


Figure 3.19: LC chromatograms and MS for Flash Chromatography semi-pure *P. lanosa* extract with specific target mass selection, A, B and C (n=2).

The base peak in this mass spectrum was at 73 m/z and the fragment formed was possibly but-2-enol (Figure 3.20).

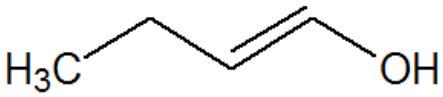


Figure 3.20: Possible fragment structure for 73 m/z peak – but-2-enol.

There was a small peak of low intensity at 45 m/z at roughly 20% in the mass spectrum. When compared with the 73 m/z peak, there was a further fragmentation of 28 m/z to obtain the 45 m/z peak. The 28 m/z fragment was considered to be a carbonyl group fragmentation and with the peak at 104 m/z a possible molecule was proposed; 2-hydroxypentenal (Figure 3.21).

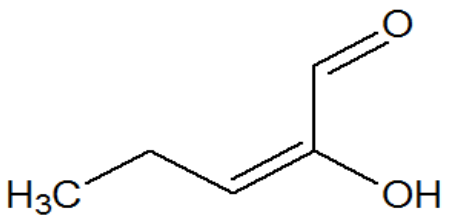


Figure 3.21: Possible structure of fragment at 104 m/z - 2-hydroxypentenal.

There was an additional small peak with an intensity of 10% with an m/z value of 117. This was possibly 4-heptanol, the structure of which is presented in Figure 3.22.

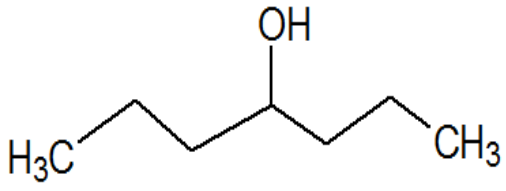


Figure 3.22: Possible structure of fragment 117 m/z – 4-heptanol.

The fragment peak at 147 m/z had an intensity of ~30% and the fragmentation between the 147 m/z and 117 m/z peaks is 30 m/z, correlated to a fragmentation of a HCOH group.

The proposed structure of the 147 m/z peak is shown in Figure 3.23, 2-ethyl-3-hydroxyhexanol.

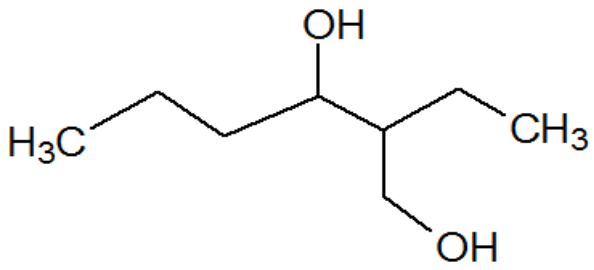


Figure 3.23: Possible structure of 147 m/z peak - 2-ethyl-3-hydroxyhexanol.

The presence of carboxyl and hydroxyl groups may account for the polar nature of the compound(s) and its/their aqueous extraction.

The most intense peak in the LC chromatogram was obtained at 10.87 min, and a distinctive mass spectrum was obtained for this peak. The fragment in the mass spectrum at 51 m/z with 25% intensity. An intense peak observed at 77 m/z, suggested the presence of a benzene ring in the compound. This fragmentation of a benzene ring from the molecule would be plentiful due to the stabilisation of the benzene ring after fragmentation through the resonance of the positive charge (Manila *et al.*, 2009).

The peak at 105 m/z was the base peak and represented the fragmentation which predominated. A possible structure for this peak was a benzene ring attached to a carbonyl group and may be phenylacetaldehyde as depicted in Figure 3.24, a common aldehyde found in algae, albeit at low % composition.

Both the benzene ring and the phenylacetaldehyde fragments would be common fragments of a range of potential algal compounds. One example would be those generated from racemosin A and racemosin B with their 2 indolinenone units (Section 3.4.6.3).

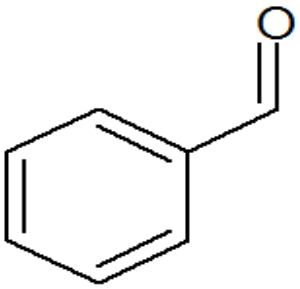


Figure 3.24: Possible structure of the 105 m/z peak – phenylacetaldehyde.

The peak at 183 m/z was of high intensity and was assumed to be a combination of the structure in Figure 3.24 and a benzene ring of 77 m/z and this is depicted in Figure 3.25.

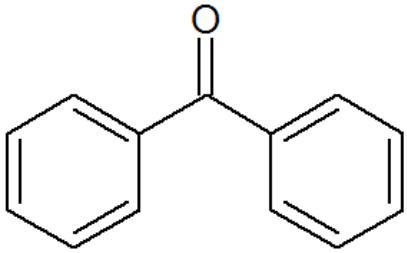


Figure 3.25: Possible structure of the peak at 183 m/z – diphenylacetaldehyde.

The 183 m/z compound as seen in Figure 3.25 is a very stable compound, stabilised by conjugation, making it readily available for fragmentation.

One previous study identified 4,5-dimethyl-1H-pyrrole-2-carboxylic acid ethyl ester (molecular mass 167), (Figure 3.26) as the major bioactive in the red alga *Aspargopsis taxiformis*, a chemical component accompanied by pentadecanoic acid and octadecanoic acid which may have contributed to bioactivity (anti-foulant molecule) as synergistic compounds (Manilal *et al*., 2010). However, another compound commonly associated with seaweed extracts and the same molecular mass is pyridine dicarboxylic acid.

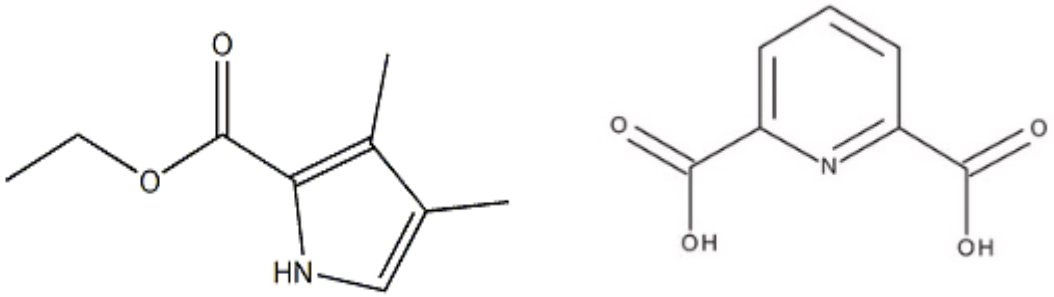


Figure 3.26: Structures of 4,5- dimethyl-1H-pyrrole-2-carboxylic acid ethyl ester (Manilal *et al*., 2009) and pyridine dicarboxylic acid, MM=167.

Previous studies elucidated 9,12-octadecadienoic acid as cytotoxic (Manilal *et al*., 2009) and that it may be involved in antimicrobial activity. Seaweeds exhibit a high level of fatty acid diversity, many of which possess potential bioactivity.

Figure 3.27 shows typical chromatograms, A, for the FC semi-pure *P. lanosa* extract with 11 characteristic peaks and B for the ACN solvent extraction *P. lanosa* sample, both of which were active against MRSA W73365. The ACN extraction was carried out as an alternative preparation of an anti-MRSA extract from *P. lanosa*. The similarity between the semi-pure and ACN derived active samples was apparent and supported by the MS data in Figures 3.28 and 3.29. There were common m/z ions in many of the chromatogram peaks. The shape of the UV and total ion current (TIC) chromatograms for both the FC and the ACN solvent extraction semi-pure *P. lanosa* extracts possess similar shape when considering Figures 3.19, 3.28 and 3.29.

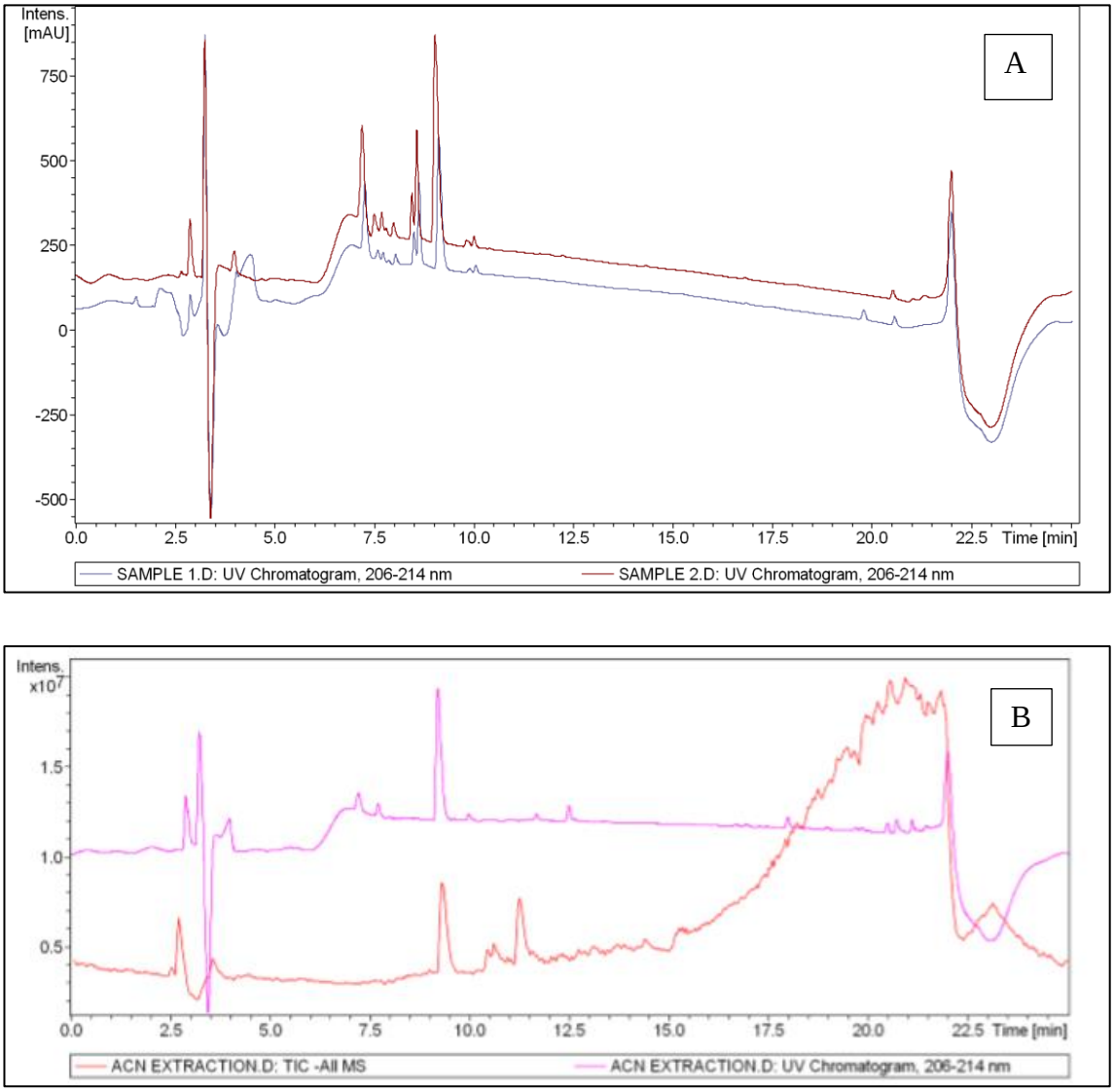


Figure 3.27: LC UV chromatograms of the Flash Chromatography semi-pure *P. lanosa* extract (A), 2 duplicate injections and the ACN solvent extraction *P. lanosa* sample (B) possessing anti-MRSA activity, including TIC for (B).

The mass spectrum for the peak in the semi-pure *P. lanosa* chromatogram with a retention of 7.1 mins is presented in Figure 3.28. The m/z value of 351.9 may have been equivalent to 3-(2-bromo-4-hydroxyphenyl)-5,7-dihydroxycoumarin with a molecular mass of 352.9 (Sheng *et al*., 2016) as has been previously discussed in the FTIR section, Section 3.4.6.3.2.

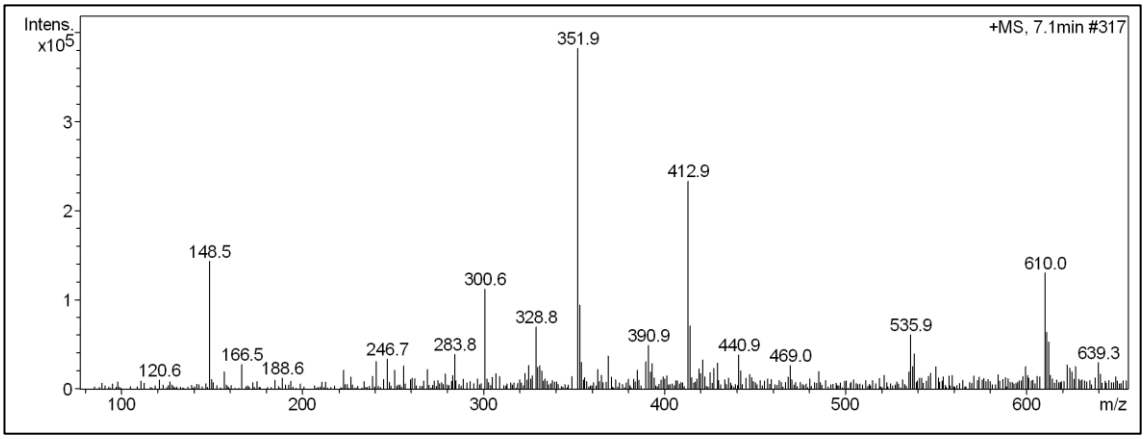


Figure 3.28: MS of Flash Chromatography semi-pure *P. lanosa* extract peak at 7.1 min.

The significance of coumarin structure as an anti-MRSA compound was supported by several research papers. Four coumarins, including 7-(1',1'- Dimethlyallyloxo)coumarin were found to possess good anti-MRSA activity against eight clinically important hospital strains with 8-Iodo-5,7- dihydroxycoumarin as the most potent derivative (Smyth *et al*., 2009). Sheng *et al* directed synthesis of such tetracyclic lactones, found in various plant species, with a fused ring system comprising coumarin and benzofuran constituent units for a variety of potential bioactivities, including anticarcinogenic, phytoestrogenic, antibacterial, antifungal, immunomodulatory, neuroprotective, anti-snake-venom, antiosteoporsis and antihepatotoxic (Sheng *et al*., 2016).

Another prospective active compound type were the indoloquinolines with alternative ESI-MS adduct information where 193.6 m/z adducts were designated m/z 413.2 [2M+H2O+2H] and m/z 428.2 [2M+ACN+H]; m/z 258.7 adduct m/z 538.4 [2M+Na]; 300.7 m/z adducts m/z 390.7 [M+ACN+Na] and m/z 412.9 [M+IsoProp+Na+H]. A compound without oxygen and with a molecular mass equivalent to a positive m/z 412.15 is (5-cyano-1-methyl-1H-indol-3-yl)-6-h-indolo[2,3-b]quinolone-9-carbonitrile ($C_{27}H_{18}N_5$) (Challa *et al*., 2017). This compound was one of two specific indolo [2,3-b]quinolines which were synthesised for their activity against MRSA and found to be extremely active. There are a number of obvious common fragments, for example m/z 428.2, 412.9, 390.9, 300.6, 193.6 and less obvious but a possibility, m/z 258.7 which was labelled in the ACN derived sample MS (Figure 3.29). The fact that there was a nitrile group may be significant as the semi-pure solid sample revealed probable nitrile groups in the FTIR analysis (Figure 3.18).

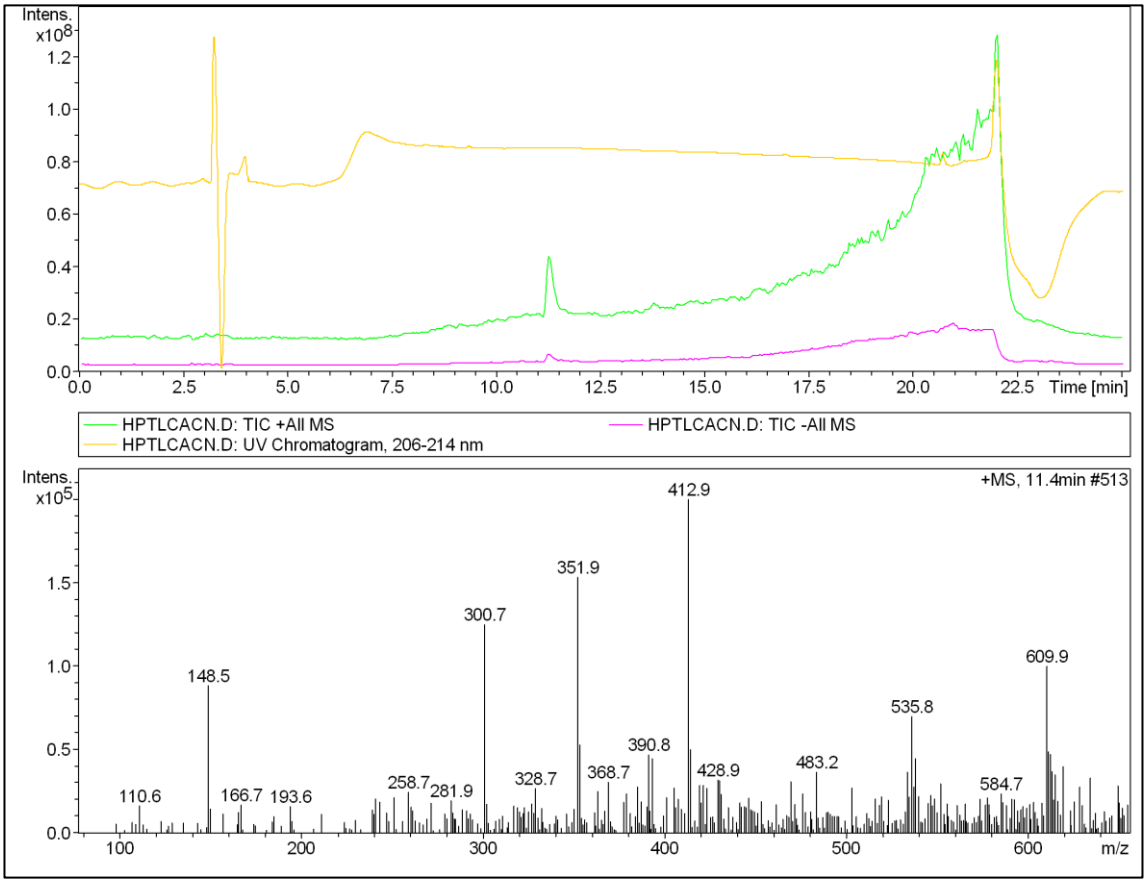


Figure 3.29: Chromatogram, TIC and MS of *P. lanosa* active compound (peak at 11.4 min) derived by ACN and HPTLC separation.

Another MS ion common to both the 18.8 and 17.4 min TIC was a 1023 ion (data not presented) which may be identified as (2R,3S,4R,6R)-6-((S)-4-((tert-Butyldiphenylsilyl)oxy)-2-methyl-3-((triethylsilyl)oxy)butan-2-yl)-2-methoxy-2-(((2S,4S,5S,6S)-6-(2-((4-methoxybenzyl)oxy)ethyl)-5-methyl-4-((tri-isopropylsilyl)oxy)-tetrahydro-2H-pyran-2-yl)methyl)-4-methyltetrahydro-2H-pyran-3-ol – an enol ether, a pale yellow oil, with HRMS (ESI) calculated for $C_{59}H_{96}O_7Si_3Na$ [(M + Na)+] 1023.6356; found 1023.6371 (Iwasaki *et al*., 2017) This product was produced during the synthesis of polycavernosides A and B which are glycosidic macrolide natural products isolated as the toxins responsible for fatal human poisoning by the edible red alga *Gracilaria edulis* (*Polycavernosa tsudai*). Polycavernosides consist of a 16-membered macrolide backbone incorporating a tetrasubstituted tetrahydropyran ring and a five-membered hemiacetal (Iwasaki *et al*., 2017).

Considering the possibility that carboxylic groups may have been present, the active compound may have been a benzoic acid ethyl ester - 4-(5-(4-Methoxyphenyl)-3-oxo-3,4-dihydropyrazin-2-yl)benzoic acid ethyl ester which is a bright yellow solid with IR characteristics cm^{-1} 3019 (Ar-CH), 1684 (C=O), 1653 (C=O) cm^{-1}; (MM = 351.1345), $C_{20}H_{19}N_2O_4$ but this was not fully supported by the FTIR data.

Figure 3.30 presents the TIC and MS of the semi-pure *P. lanosa* peak at 20.4 mins.

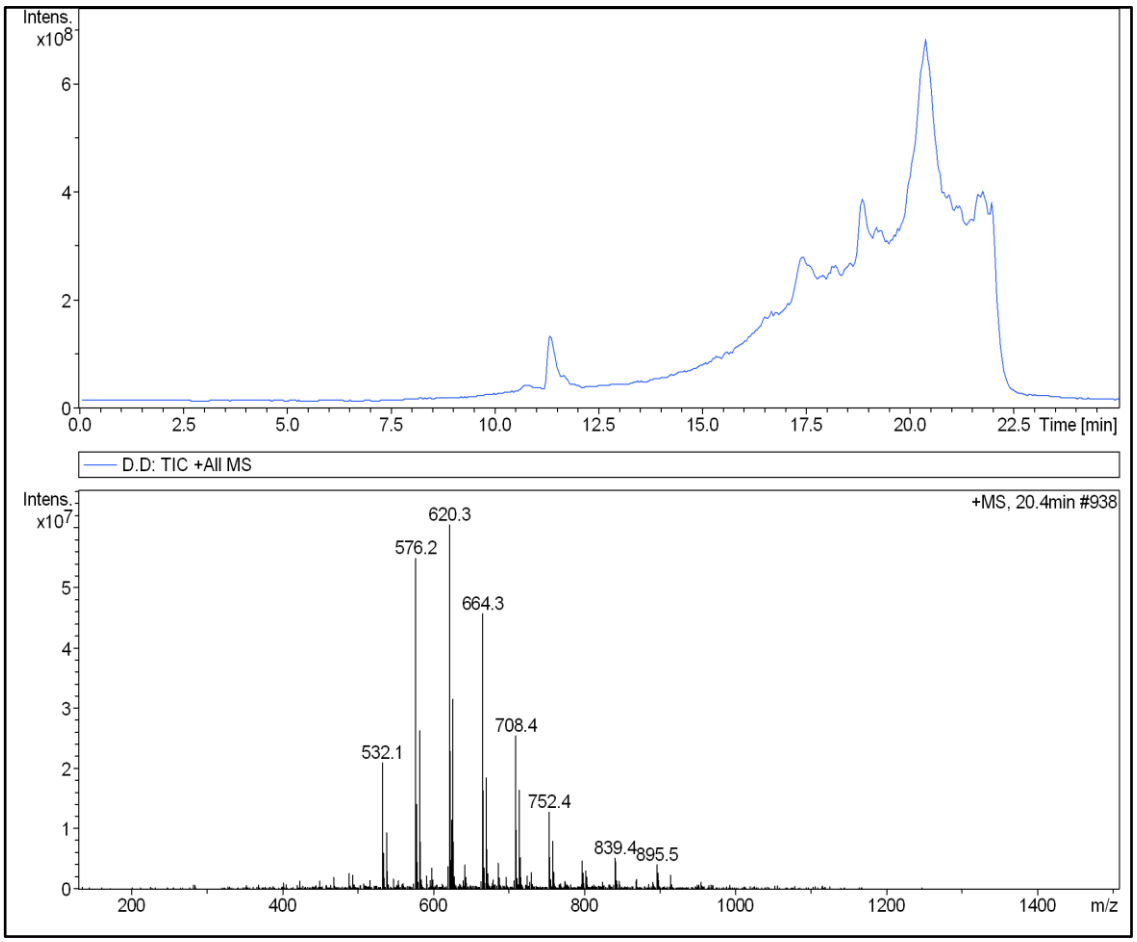


Figure 3.30: TIC and MS of Flash Chromatography semi-pure *P. lanosa* extract peak at 20.4 mins (n=2).

Figure 3.30 is significant as it allows for more specific information regarding possible fragmentation patterns. The loss of a 44 m/z value may be attributed to a fragmentation pattern equivalent to a C_2H_5N loss where a N-methyl pyrrolidine(MW=85.15g/mol) moiety was broken from a dibromoindole core – in this case there were 4 such losses – 576.2 to 532.1, 664.3 to 620.3, 708.4 to 664.3 and 752.4 to 708.4. The alternative explanation was that m/z value of 532 [M+1H] is ionised to m/z 576 [M+2Na-H] and that

this is repeated 3 times, which may have indicated 4 free carboxylic groups. Tricarboxylic acid compounds have previously been found in *Polysiphonia* species (Kamenarska *et al*., 2006). A further explanation is the loss of both of these fragments such that 2 carboxylic groups and 2 N-methyl pyrrolidine fragments are removed, which may support a proposed bi-indole or bisindole compound, with possible symmetry.

From the MS data presented, the strongest possibilities as candidate bioactives are the substituted indoles with 3 pyrrolidine structures and carbonyl and bromosubstitution or a bis-indole structure, such as 2,2-bis(6-bromo-3-indolyl)ethylamine presented in Figure 3.32 but with further functional groups. This is supported by research which has confirmed the possibility of a bis-indole structure being responsible for anti-MRSA activity and its suitability for disrupting biofilms (Campana *et al*., 2019). The natural marine alkaloid 2,2-bis(6-bromo-3- indolyl) ethylamine contains two units of indole and possesses activity against planktonic and biofilm MRSA where it was suggested that this was due to a modulation of indole-based signaling pathways with further confirmation that the presence of halogens and/or ethylamine side chain are important for the maintenance of both antimicrobial and anti-biofilm activities (Campana *et al*., 2019). This compound may be accompanied by a tricarboxylic acid candidate such as a 2,4,6 tricarboxylic acid pyridine derivative.

The vast majority of bisindoles have been isolated from marine sponges whilst bi-indoles are more commonly found in red algae species (Veale & Davies-Coleman, 2014). In order to differentiate between these two compound classes, biindoles are compounds in which the two indole rings are bonded directly to each other and bisindoles as well as trisindoles is where the two and three indole rings, respectively, are separated by any functionality and not only by a heterocyclic ring (Figures 3.31. and 3.32). Simple polybrominated and sulfur-containing indoles in addition to a novel polybrominated bi-indole thioether was isolated from the red alga *Laurencia brongniartii* collected off Okinawa, Japan with two linear bisindole enamide alkaloids, chondriamide A and B, isolated from another red alga, *Chondria sp*., unnamed, native to province of Buenos Aires, Argentina (Palermo *et al*., 1992).

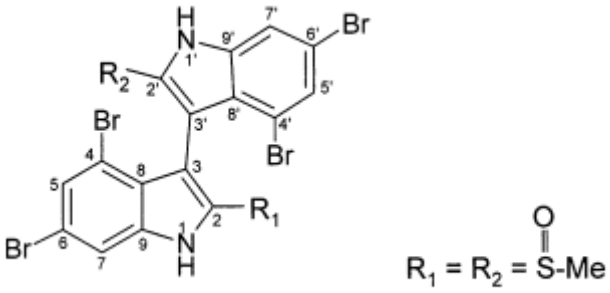


Figure 3.31: A biindole structure, 3,3-bi(4,6-dibromo-2-methylsulphinyl)indole, isolated from the Formosan red alga *Laurencia brongniartii* (El-Gamal *et al*., 2005).

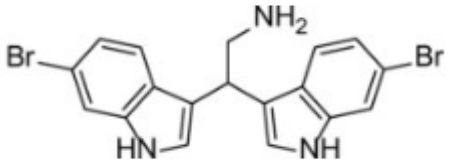


Figure 3.32: A bis(indolyl) structure, 2,2-bis(6-bromo-3-indolyl)ethylamine, isolated from the Californian tunicate *Didemnum candidum* and the New Caledonian sponge *Orina spp.* (Campana *et al*., 2019).

3.4.6.4 **Chromatographic Techniques**

3.4.6.4.1 Analytical HPLC

Analytical HPLC was used to check the purity of the active fractions generated by flash chromatography as well as after further isolation and purification phases. The absorbance of the semi-pure *P. lanosa* extract was checked at a range of wavelengths and the results compared with the results of the UV-Vis analysis where the strongest absorbance was determined at 220-235 nm but using analytical HPLC 215 nm was found to be the most favourable wavelength for detection with highest relative absorbance. This difference served to highlight problems with using UV-Vis spectra for an aqueous *P. lanosa* extract potentially possessing bis(indolyl)-type compounds which may be subject to ionisation, and alter their UV-Vis spectrum, due to extreme pH sensitivity. Figure 3.32 represents a typical chromatogram where the highest relative absorbance was achieved at 215 nm for a 10 mg/mL dilution in 50:50 MeOH:H_2O.

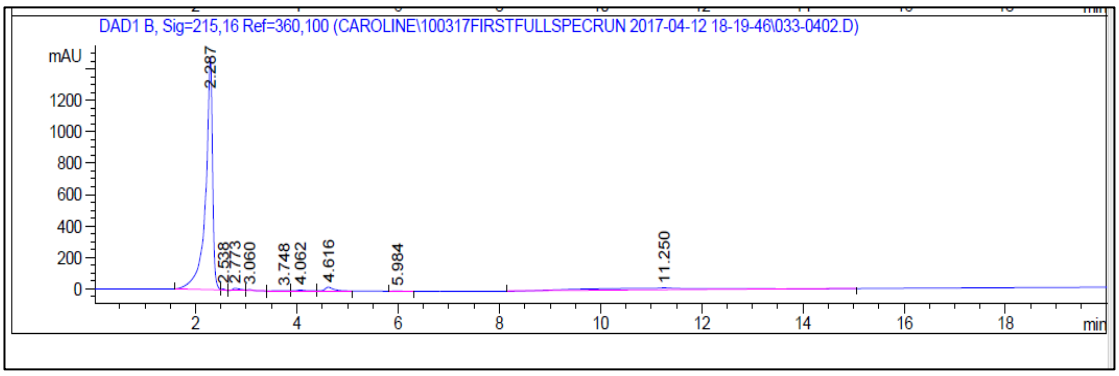


Figure 3.33: HPLC Chromatogram of Flash Chromatography semi-pure *P. lanosa* extract. Separation of 20 µL using a Symmetry C_{18} column with 25:75 MeOH: H_2O (v/v) mobile phase, detection at 215 nm.

Reference compounds soluble in 50:50 MeOH:H_2O were also run to check for relative retention times with gallic acid and n-valeric acid, possessing the same retention time with 25:75 MeOH:H_2O mobile phase. A number of standard compounds presented peaks with similar retention times as the active *P. lanosa* fractions from FC: 4-bromophenol, 4-dibromoacetophenone, isovaleric acid, propionic acid and indole butyric acid. Data not presented here.

Analytical HPLC determined the optimum mobile phase composition for gradient elution and further separation. The resulting sample retention time was 7.5 min, equating to a mobile phase composition of 66% solvent A (methanol). This served as a starting mobile phase composition for the preparative HPLC study.

3.4.6.4.2 Preparative HPLC

Preparative HPLC was pursued to isolate the active components of the semi-pure *P. lanosa* extract from flash chromatography using the analytical column on the prep HPLC instrument and the chromatograms presented in Figures 3.34 and 3.35.

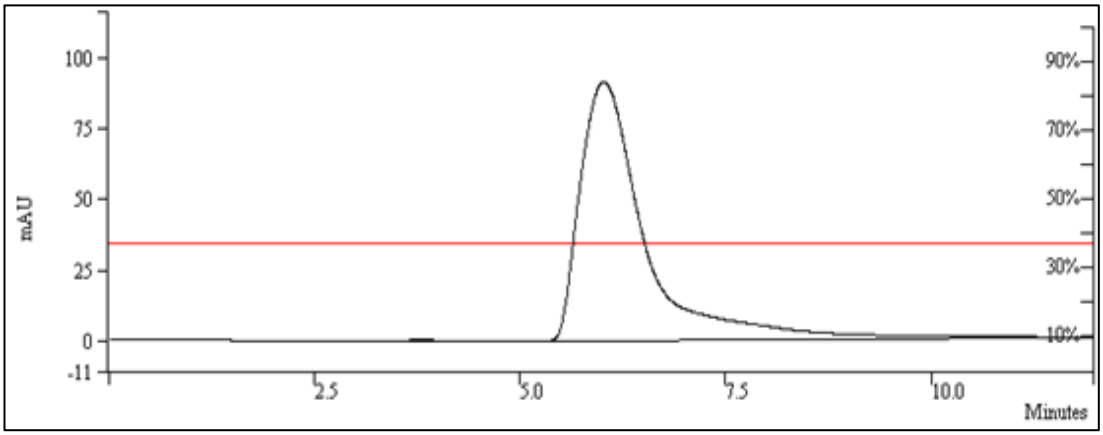


Figure 3.34: Water-soluble post-flash chromatography *P. lanosa* active fraction with absorbance at 254 nm. Column Symmetry C18 4.6 x 250 mm 35:65 MeOH: H_2O. Flow Rate 0.5 mL /min, column @ room temperature.

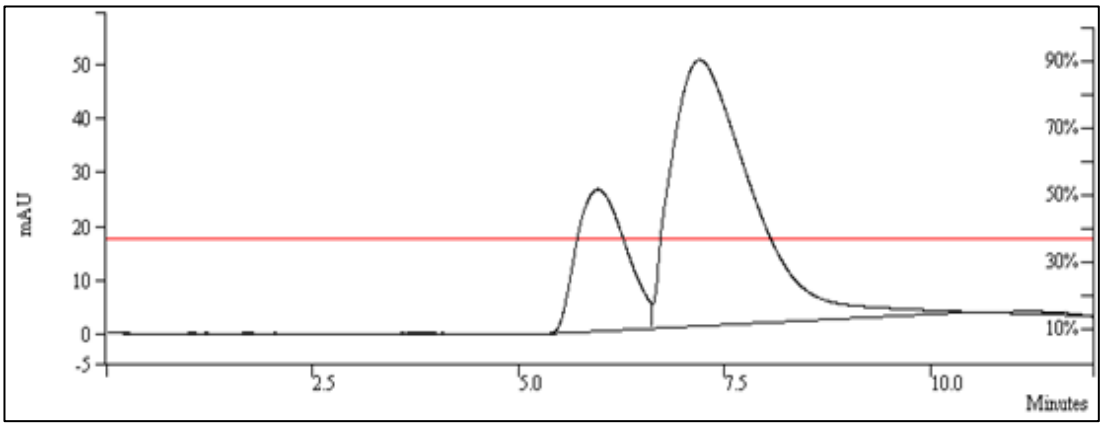


Figure 3.35: Water-soluble post-flash chromatography *P. lanosa* active fraction with absorbance at 360 nm. Column Symmetry C18 4.6 x 250 mm 35:65 MeOH: H_2O. Flow Rate 0.5 mL /min, column @ room temperature.

Although the column used was not a preparative column the level of active components with a chromophore was extremely low, as evidenced by the low level of absorbance for this 1 mg/mL solution. Analytical columns can be used for preparative purposes although the aims of the techniques are different. Generally, the preparative technique requires columns with a higher throughput capacity which undermines peak resolution as the aim is to collect and purify a compound whilst Analytical HPLC is centred on resolving individual peaks.. However, it was decided to use the analytical column after single peak resolution using Analytical HPLC due to the limited availabilty of extract sample. The favourable wavelength for detection was determined to be 254 nm on this instrument under the run conditions, and an additional wavelength of 360 nm for analysis selected

to verify the results of the UV-Vis analysis where the semi-pure *P. lanosa* active sample displayed 2 absorption bands. The two peaks at 360 nm in Figure 3.35 demonstrated that were at least 2 compounds eluting. However, when considering the UV-Vis spectrum in Figure 3.15 these would not be attributable to active compounds where the maximum absorbance was in the 210-240 nm range as well as the 300-340 nm range. This was also suggested by the visualisation of 3 bands whilst running the semi-pure *P. lanosa* extract on the Alumina TLC plates. However, the possible presence of bisindole or bi-indole structures (which may be extremely sensitive and react to changes in solvent and mobile phase) and their potential instability, would have resulted in extra peaks on elution. An example would be the bisindole chondriamide A (Figure 3.36) which is oxidised to indole-3-carboxyaldehyde and indole-3-propenoicimide when a methanol solution is stored overnight (Palermo *et al.*, 1992).

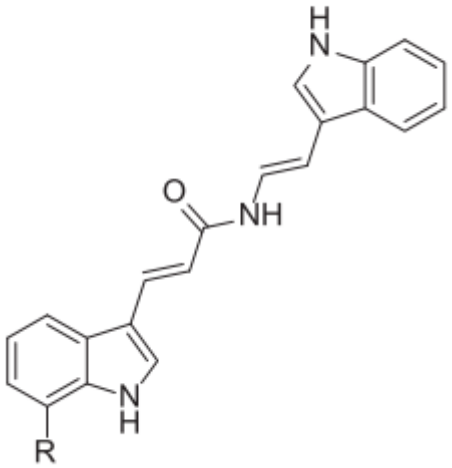


Figure 3.36: The bisindole structure chondriamide A, R=H, isolated from a Chondria species (Palermo *et al.*, 1992).

Gallic acid was also analysed using preparative HPLC and found to possess a retention time of 5.2 min i.e. identical to that of the peak of the semi-pure *P. lanosa* extract collected by FC. Subsequent inactive *P. lanosa* fractions did not possess the peak at 5.2 min and therefore the second peak observed at 6.8 min for the 360 nm spectrum may be attributed to the active compound which was supported by the UV-Vis analysis where the λ_{max} was close to 330 nm for the active fraction.

Overall, as the semi-pure *P. lanosa* extract probably contained an unknown number of compounds which were possibly capable of having reacted with another and consequently may have affected the UV-Vis spectrum, which would have undermined the significance of any observations.

3.5 Conclusions

Solvent extraction yields determined that water was extremely efficient in providing a high yield of crude *P. lanosa* extract. The well diffusion assay was also determined to be the most sensitive and reliable method for the detection of anti-MRSA activity from the crude *P. lanosa* extract samples. The optimum sample preparation method was found to be centrifugation as opposed to sonication. Further studies identified the optimum loading of the crude seaweed extract for analytical TLC as 0.8 mg/mL. The results obtained indicate that analytical TLC was the more successful method for the separation of compounds within the crude seaweed extract. The optimum mobile phase for TLC analysis was 50:50 methanol:water, and positive bioautography results were used to confirm bioactive separation.

After a comparison of crude *P. lanosa* extracts treated with acetic acid or sodium hydrogen carbonate to untreated crude seaweed extract, it was evident that the untreated sample displayed higher anti-MRSA activity, as determined by the zone clearance on normal phase silica TLC and well-diffusion. This confirmed the stability of the active compound(s) despite contrasting results with mobile phase and TLC plate change - not supported by the comparison results obtained using reverse phase silica TLC plates (It was further observed that the treated samples on the normal phase silica TLC plate did not allow for complete migration of the bioactive compound from the loading spot, suggestive of bioactive compounds retained at the spot loading location).

The transfer of TLC optimisation to flash chromatography was not possible due to retention of pigmentation on the column and an alternative mobile phase was sought. A mobile phase composition of 40:60 chloroform:methanol with 3% water (v/v) was

determined to be the most suitable mobile phase for the purification of the crude *P. lanosa* extract using normal phase silica flash chromatography.

The functional group focused staining study allowed for the conclusion that the active components were comprised of amino or amide groups, carboxylic acids, indole groups or an indole alkaloid. The semi-purified fraction would possibly have included a range of different molecules of varying compound type or the active molecule may possess several of the active groups such as an amide and/or a carboxylic acid attached to an indole alkaloid. The fact that IAA, gallic acid, propionic acid and n-valeric acid elicited a positive response in bioautography may have alluded to the activity of the carboxyl group and the length of an alkyl chain in inhibiting MRSA. Biologically active IAA is produced by hydrolysis of conjugates such as carboxyl groups, glycans, amino acids and peptides.

It is generally accepted that a variety of marine sources including red algae, sponges, tunicates, acorn worms, and symbiotic bacteria generate indole alkaloids, which represent the largest number and most complicated of the marine alkaloids. These alkaloids often possess novel frameworks not discovered in terrestrial organisms and are characterised by complexities such as halogen substituents (Franco *et al*., 2014). Indole signaling is recognised as being involved in the regulation of a repertoire of bacterial behaviours such as antibiotic resistance, virulence, and biofilm formation (Worthington et al., 2012) and there is a strong possibility that an indole alkaloid was responsible for the anti-MRSA activity in the aqueous *P. lanosa* extract.

Temperature studies indicated that the bioactive compound(s) was/were stable at temperatures up to 100 ˚C with a 30 min exposure time, indicating that these compounds may be useful in heat treated products such as wound dressings or food products requiring elevated temperatures for processing and production. An increase in activity was observed when the crude *P. lanosa* extract was digested with certain proteolytic enzymes, as well as lipase and amylase but further enzyme analysis would have to be carried out to achieve a more definitive result. Hydrolysis of components of the crude *P. lanosa* may have yielded smaller anti-MRSA active compounds such as IAA.

In consideration of the use of UV-Vis as an analytical technique, it should be highlighted that many natural product compounds possess poor UV chromophores. The approximate

λ_{max} for the semi-pure *P. lanosa* extract were 230 and 315 nm but the absorbance was weak (at 50 % concentration), again supporting the suggestion that the active compound was a poor chromophore. This lack of chromophore occurred with the compound partenolide, a sesquiterpene lactone which required a non-UV dependent detection method where mass detection was successful (Traub *et al*., 2016). An additional complication was co-elution of parthenolide (m/z=249.1) and methysticin (m/z=275.3) and resolved when using MS. UV detectors respond to nearly all compounds present in a sample with limited specificity for a specific compound of interest (Traub *et al*., 2016). This would further impact HPLC studies of the semi-pure *P. lanosa* extract as absolute purity was not assured.

The FTIR spectra yielded information regarding the semi-pure *P. lanosa* samples in that potential groups and structures were identified, such as aromatic, indole, carboxylic, halide and nitrile structures and functional groups. The FTIR data obtained does not allow for unique specification or absolute characterisation. The identification of peaks attributable to carboxylic groups was supported by a report that the composition of the polar components of the *Polysiphonia* species seems to be a suitable marker for chemotaxonomy and chemoevolution within Rhodophyta (Kamenarska *et al*., 2006), particularly tricarboxylic acids. The FTIR data supported the possibility of a heterocyclic conjugated indole with halide substitution.

LC-MS fragmentation patterns did provide information to support the case of a bromoindole in that there were 4 subsequent 44 m/z losses, indicative of either a C_2H_5N as in the case of a break in a pyrrolidene compound or the loss of carboxylic groups or a combination of both. The fact that there were 4 identical fragment losses may further have been indicative of a symmetrical arrangement, perhaps that of a bi-indole or bisindole structure.

CHAPTER 4: BACTERIAL INHIBITION AND ANTIOXIDANT STUDIES WITH P. LANOSA EXTRACTS

4.1 Introduction

As the anti-MRSA activity of crude and semi-pure *P.lanosa* extracts determined thus far using qualitative well diffusion and bioautography assays required accurate quantification, Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were investigated. MIC is defined as the lowest concentration of an antimicrobial agent that will inhibit the visible growth of the bacteria after overnight incubation whilst MBC determines the lowest concentration of the antimicrobial compound that will prevent ≥ 99% the growth of the bacteria when subcultured on fresh media. In addition, as these classic bacterial susceptibility assays cannot be interpreted and transferred to therapeutic guidelines when biofilm infection occurs, a biofilm disruption assay was also included. A variety of *in vitro* biofilm models exist (Stiefel *et al*., 2016) with the microtitre plate method representing one of these.

This biofilm analysis method involves filling a 96-well plate with sterile culture broth, inoculating with the relevant biofilm-forming bacterial strain, and incubating. Biofilm formation takes place as a ring around the well. After rinsing of wells to remove planktonic cells, the biofilm is stained with crystal violet and dissolved in a solvent for quantification of the biomass by measuring the absorbance or optical density. The main advantages of this method are its ease, rapidity and reproducibility. However, the use of crystal violet, which stains both dead and viable cells, undermines this approach as there is no relationship between biomass and biofilm viability. For this reason, 2,3,5-triphenyltetrazolium chloride (TTC) can be used to confirm the presence of viable cells (Mosmann, 1983).

Another method for biofilm measurement uses the traditional approach of staining with crystal violet or safranin to stain and quantify the total biomass comprising bacteria (both live and dead) and extracellular polymeric substances (EPS), by binding to negatively charged molecules (Stiefel *et al*., 2016). However, this method requires a secondary technique to distinguish actual biofilm inhibition.

The differentiation of viable and dead bacteria in biofilm assays can be acheived by monitoring their metabolic activity, for example, ATP using the BacTiter-Glo assay; or respiratory electrons by tetrazolium salt or membrane integrity by live/dead staining with

SYT09 and propidium iodide; or ability to grow by measuring colony forming units (CFU) (Stiefel *et al.*, 2016). However, there is a lack of standardisation of biofilm assays, hence restricting the clinical application of data obtained for antimicrobial compounds.

A 2016 research article (Chu *et al.*, 2016) examined the inhibitory effect of berberine (a natural isoquinoline alkaloid, $C_{20}H_{18}NO_4^+$, molar mass 336.367 g/mol) on MRSA biofilm formation. Safranin was used in combination with acridine orange (a DNA-binding dye) as a fluorescent biofilm biomass indicator which stains all microbial cells within a biofilm, live or dead, with absorbance measured at 530 nm. The negative control showed an OD_{530} of 1.794, whereas experimental groups treated with berberine at 1, 2, 4, 8, 16, 32, and 64 μg/mL showed OD_{530} values of 1.779, 1.734, 1.696, 1.327, 0.793, 0.343, and 0.293, respectively, indicating anti-biofilm activity. Glucose was also added to the culture medium to encourage biofilm formation. The study then used confocal laser scanning microscopy (CLSM) to confirm the effect of berberine against the live biofilm cells. It was further determined that the MIC of berberine against MRSA was 128 μg/mL, with no inhibitory effect at concentrations at 1 to 64 μg/mL i.e. the range that had anti-biofilm activity (Chu *et al.*, 2016).

It is anticipated that biofilm cells are able to grow in the prescence of antimicrobials even above the MIC for planktonic cells, so that the MIC for biofilm cells will be higher than that for planktonic cells (Lewis, 2001) despite the results obtained in study by Chu *et al*. (2016) outlined above. However, in most biofilm susceptibility analyses, bactericidal effectiveness of the antimicrobial is determined rather than growth inhibition and there are few studies on antimicrobial diffusion through a biofilm. A biofilm may in fact be heterogeneous and diffusion restricted if areas of inconsistency exist, making some biofilm susceptibility assay results questionable. The preferred option is, therefore, to measure the numbers of CFU from a growing biofilm after it is dislodged. An antibiotic (not inactivated by the bacterial cells) that has an MIC for biofilm cells similar to that for planktonic cells will diffuse well throughout the biofilm (Lewis, 2001). Much research has focused on the anti-fouling properties of macroalgae (Dahms & Dobretsov, 2017) with less emphasis extending this capability to biofilm formation associated with pathogenic microorganisms or MDR pathogens. An extensive study by Busetti *et al* included the determination of Minimum Biofilm Eradication Concentration (MBEC) for a brown alga crude extract (Busetti *et al*., 2015).

This chapter will consider anti-MRSA activity alongside antioxidant activity with an appraisal of whether flavonoid compound and/or gallic acid equivalent assays may assist possible characterisation.

Natural antioxidants are being actively sought to prevent oxidative degradation of lipids present in raw or processed foods as well as to undermine the role of ROS in carcinogenesis, artherosclerosis and ageing in humans as discussed in Section 1.12. Potent antioxidant compounds have been isolated and identified from a wide range of seaweeds. These include sulphated polysaccharides, pigments, phlorotannins, sterols, catechins, phenols and proteins (Sabeena Farvin & Jacobsen, 2013) with this antioxidant activity potentially aligned to antimicrobial activity.

There are a variety of antioxidant assays (Huang *et al*., 2005) and no single assay can be considered as the benchmark, as each possesses certain limitations.

Specific bioactive compounds such as phenolics, flavonols and flavonoids with polyphenols and anthocyanins, common in seaweed are particularly active and able to reduce DPPH, so this assay was considered well-suited to seaweed extracts.

Total phenolic content (TPC) is commonly used, as the majority of the antioxidant capacity of polar solvent derived seaweed extracts (polyphenols being typically polar) may be attributed to their polyphenol content, making it the primary method employed for the assessment of antioxidant activity of seaweeds. The mechanism behind the TPC assay involves the reduction of Mo^{6+} (yellow) to Mo^{5+} (blue) by an antioxidant compound. Therefore, the Folin–Ciocalteu (F–C) reagent, not only measures total phenols but will react with any reducing agent, in fact measuring the total reducing capacity of a sample (Ainsworth & Gillespie, 2007). Inhibition could occur as a result of oxidants competing with the F–C reagent or air oxidation after the sample is made alkaline so that it is necessary to ensure that the F–C reagent is added before the alkali. The TPC assay is subject to interference from sulphites, reducing sugars, and amino acids and has a reduced accuracy for lipophilic compounds and it is, therefore, not an entirely accurate measurement of total phenols (Singleton *et al.*, 1999).

The term antioxidant ‘activity’ as applied to antioxidants can have a variety of meanings and the consensus is that data obtained using *in vitro* methods cannot be extrapolated to

in vivo effects and clinical trials to test benefits of dietary antioxidants have produced mixed results. Antioxidant molecules in food are recognised as having a wide range of functions, many of which unrelated to the ability to absorb free radicals (Hafting *et al*., 2015).

Balboa *et al.* (2013) reviewed a range of chemical reaction based assays for antioxidant determinations in brown seaweeds and concluded that they are excellent preliminary tools, although more advanced methods would be required to deliver a complete comprehension of the antioxidant effects of seaweed extracts.

The DPPH radical scavenging assay measures the ability of an extract or a sample to reduce the DPPH, a nitrogen centred radical (purple) into a non-radical form (yellow) either by hydrogen atom transfer (HAT) or single electron transfer (SET). The colour change can be monitored by a spectrophotometer so that the percentage of remaining DPPH is proportional to the antioxidant concentration. The concentration that causes a decrease in the initial DPPH radical concentration by 50% is defined as half maximal inhibitive/effective concentration (IC50 or EC50). The lower the IC50 value of the sample, the higher its antioxidant capacity.

The limitation of the DPPH scavenging assay is that the DPPH radical is unlike any naturally occurring radical. For example, DPPH IC50 values have ranged from 297.7 µg/ml in a 100 % methanol extract to 0.125 µg/ml in a 60 % methanol extract prepared from *Himanthalia elongata* (Rajauria *et al.*, 2013). This further illustrates the effect of solvents in the extraction of antioxidant components from a seaweed source, as different compounds as well as various levels of compounds will be extracted according to their solubility and the polarity of the extracting solvent.

In relation to antioxidant assays as applied to seaweed extracts, Foti *et al* (Foti *et al,*. 2004) found that adventitious acids or bases present in the solvent may dramatically influence the ionization equilibrium of phenols and cause a reduction or an enhancement, respectively, of the measured rate constants. This renders the DPPH assay much less chemically sound as a valid assay for antiradical activity. Despite this, the free radical scavenging assay using the DPPH radical remains a preliminary test for the analysis of the antioxidant potential of extracts and is used extensively as it permits high throughput

screening with a high sensitivity for the detection of active ingredients, even at low concentrations (Foti *et al*,. 2004).

A further observation was that some compounds react in a fast manner with the DPPH radical and these include ascorbic acid, caftaric acid and caffeic acids (Parsek & Singh, 2003). This study compared many polyphenols and flavonols in 15 % ethanol in water solutions. For polyphenols such as (+)-catechin and (-)-epicatechin, the reaction times were recorded as 120 and 180 min respectively. Flavonols also vary in their reaction times with the DPPH radical and for kaempferol it was 6 min, while myricetin needed 66 min to decrease radical concentration by 50%. For this reason, a time point of measurement was essential to ensure validity of readings and rigour of assay (Parsek & Singh, 2003). The determination of total flavonoid content (TFC) was considered in order to broaden the possibility of such compounds being responsible for any of the bioactivities assessed.

4.2 Objectives

- To determine the MIC, MBC and Biofilm Bactericidal Concentration (BBC) of the crude and semi-pure *P. lanosa* extract, with novobiocin as a positive control. Biofilm prevention is a necessary extension of this investigation to ascertain the full capability of the seaweed extracts, both crude and semi-pure.

- To assess whether the components of the *P. lanosa* semi-purified fraction exhibited antioxidant activity in addition to anti-MRSA activity, thus serving as an indication of their chemical nature and in particular determining whether polyphenols were responsible for both bioactivities. This was complemented by the determination of Total Phenolic Concentration (TPC) and Total Flavonoid Concentration (TFC).

This represents a novel approach in that the bioactivity profiles were used to track changes as a result of purification steps, coupled with the comparison of anti-MRSA activity of the original crude and its subsequent semi-purified sample.

4.3 Experimental Procedure

4.3.1 *Bacterial Strains, Microbiological Media and Chemicals*

- Mueller Hinton Agar (MHA): Difco MHA Ref -225220 (7.6 g per 200 ml deionised water)
- Brain Heart Infusion Broth (BHI): Oxoid (Basingstoke,U.K.) (7.4 g per 200 ml deionised water)
- MRSA - W73365 (WIT 517): clinical isolate from University Hospital Waterford
- Maximum Recovery Diluent (MRD): Oxoid (Basingstoke,U.K.) (1.9 g per 200 ml deionised water)
- 2,3,5-triphenyltetrazolium chloride (TTC): Sigma-Aldrich (Wicklow, Ireland)
- Phosphate buffered saline (PBS) tablets: Sigma-Aldrich (Wicklow, Ireland) (one tablet dissolved in 200 ml deionised water equivalent to 0.01 M phosphate buffer, pH 7.3 – 7.5)
- 2,2-diphenyl-1-picrylhydrazyl (free radical) powder, 95%: Alfa Aesar Fisher Scientific (Ballycoolen, Dublin 15)
- Butylated hydrotoluene (BHT): Ducheta Biochemie (Haarlem,Thê Netherlands)
- Ascorbic acid (AA) Ducheta Biochemie (Haarlem,Thê Netherlands)
- Methanol HPLC grade: Fischer Scientific (Ballycoolen, Dublin 15)
- Quercetin dihydrate: Sigma (Wicklow, Ireland)
- Gallic acid: Sigma-Aldrich (Wicklow, Ireland)
- Aluminium chloride: Fluka, Lennox Supplies (Naas Road, Dublin 12)
- Potassium acetate: Merck (Damstadt,Germany)
- Sodium bicarbonate: Sigma-Aldrich (Wicklow, Ireland)
- Folin-Ciocalteu's reagent AGB Scientific (Dublin, Ireland)
- Filtrospur S 0.2 µm syringe filter unit: Sarstedt; Lennox Supplies (Naas Road, Dublin 12)

4.3.2 *Instrumentation*

- Microtitre plate reader – Bio-tek Instruments, Incorporated µQuant
- Labnet autoclavable biopipette

4.3.3 *Methods*

4.3.3.1 **Preparation of crude seaweed extracts**

Crude *P. lanosa* extracts (one normal and four light stress-derived samples) were prepared at a final concentration of 100 mg/ml in sterile, deionised water, with centrifugation employed to ensure dissolution as outlined in Section 3.3.3.7. The final concentration of extract in the first well for MIC determination was, 2.5 mg/ml (as 50 µL was loaded into each well equivalent to 5 mg/mL, and 50 µL MHB was added, making the final concentration equivalent to 2.5 mg/mL). The seaweed extract solution was filtered through a 0.2 µm syringe filter. The concentration of the semi-purified extract for the first well was 0.25 mg/ml after a 10 mg/mL stock solution had been prepared. The first well was serially diluted across the microtitre plate rows, resulting in twelve dilutions for all extract samples.

4.3.3.2 **MIC Determination**

The MIC method was based on a CLSI (formally known as NCCLS) standard protocol for Antimicrobial Susceptibility Testing Agar and Broth Dilution method to determine the minimum inhibitory concentration (MIC) of antimicrobial substances *(*Cavalieri *et al*., 2005). The first step was the preparation of a bacterial culture, by inoculating MRSA (1%) into BHI broth and incubating at 37 $^{\circ}$C overnight, followed by centrifugation of 1 ml of culture at 13,000 rpm for 2 min. The supernatant was removed and the cell pellet suspended in 1 ml of sterile MRD. This was then vortexed for ~30 s and centrifuged again at 13,000 rpm for 2 min with removal of the supernatant and resuspension of the cell pellet in 1 ml of sterile MRD followed by mixing using the vortex for ~30 s. The absorbance of 0.5 McFarland standard (equivalent to $10^7 – 10^8$ CFU/mL) at 625 nm was measured and yielded an OD value between 0.09-0.110. A dilution of the washed,

overnight culture of the bacteria was prepared in MHB and the OD at 625nm (optimum for MHB dilutions) measured in a sterile cuvette and carefully altered in order to obtain a suspension of approximately $10^7 – 10^8$ CFU/mL. The adjusted bacterial suspension was used within 30 min of preparation.

For the determination of MIC, serial dilution of the crude and light stressed extracts was carried out in the microtitre plate from well 1-12 so that a 2.5 – 0.0050 mg/mL dilution range was tested. For the semi-pure sample, the initial concentration was 0.25 mg/mL. Each microtitre plate had three replicate rows of the extract, one row serving as the extract control (50 µL extract at appropriate dilutions + 50 µL MHB) with 50 µL bacterial culture added to the remaining two sets. A negative control well (100 µL MHB) was prepared, as well as bacterial control wells (50 µL adjusted bacteria + 50 µL MHB). Two serial dilutions of novobiocin were included as a positive control, starting from 20 µg/mL. The contents of each well were mixed on delivery of the final component and the microtitre plates incubated at 37 ºC for 18 h.

The absorbance was then measured at 625nm and the MIC was the lowest concentration of antimicrobial agent that completely inhibited growth of the bacteria in the well. A reduction of ≥ 80% bacterial growth compared to the bacterial growth well was considered a valid result (Greff *et al*., 2014; Tan, 2013).

The absorbance of the sample was determined by accounting for the broth control and the absorbance of the extract control and subtracting these from the absorbance of the extract-treated bacterial culture dilution using Equation 4.1. The percentage reduction of bacterial growth was then calculated by subtracting the absorbance of the extract-treated sample from that of the untreated bacterial culture dilution and dividing it by the absorbance of the untreated bacterial culture dilution and converting the answer to a percentage using Equation 4.2.

Equation 4.1 $$\text{Absorbance}_{(\text{sample})} = \text{Abs}_{(\text{extract+bact})} - \text{Abs}_{(\text{extract})} - \text{Abs}_{(\text{broth control})}$$

Equation 4.2 $$\%\ \text{reduction} = \frac{\text{Abs}_{(\text{bact})} - \text{Abs}_{(\text{sample})}}{\text{Abs}_{(\text{bact})}} \times 100\%$$

MIC data, based on absorbance readings were analysed and the MIC value for an 80% reduction in bacterial cells recorded. The reason for selecting an 80% reduction was to maximise the potential for biofilm development. A 50 µL aliquot of TTC solution (4 mg/mL) was added to each well to check for live bacterial cells and the plates incubated at 37 ^{o}C for 3 h (TTC turns red in the presence of live bacterial cells).

This step was added to confirm the MIC result, as well as to ensure sterility of the extracts and to check for biofilm elimination in the BBC determination.

4.3.3.3 **MBC Determination**

A 50 µL aliquot of the content in the well with no bacterial growth (100% inhibition) was removed and spread plated onto MHA plates followed by incubation at 37 ^{o}C for 18 h. The plates were examined and those without bacterial growth with the lowest concentration of antimicrobial agent indicated the MBC value.

4.3.3.4 **BBC Determination**

The supernatants were gently removed from the wells exhibiting bacterial growth by tilting the 96-well plate at a 45° angle and pipetting liquid from the edge of wells to a new 96-well plate, as well as spread-plating all bacteria-containing samples at 10^{-6}, 10^{-8} and 10^{-10} dilutions on MHA medium (a random 3 wells from bacteria-only controls were selected) and all blanks (media blanks and treatment blanks) were plated un-diluted to confirm their sterility. The wells of the 96-well plate were washed gently with 200µL PBS by gently stirring the well contents and removing the PBS at an angle from the wells using the multichannel pipette. A 110 µL aliquot of MRD was added to all wells and the multichannel pipette was used to scrape the bottoms of all wells carefully to detach all bacteria, and pipetted up-and-down three times to obtain a homogeneous solution. A 100 µL aliquot was transferred to a fresh 96-well plate. This was repeated for all columns, changing tips between each column of samples. The absorbance was read at 625nm against an MRD blank with a closed lid (to avoid contamination). All bacteria-containing samples were spread-plated at 10^{-6}, 10^{-8} and 10^{-10} dilutions on MHA medium (picking the

same three wells from bacteria-only controls, as for MIC determinations), whilst all blanks were plated un-diluted.

MIC data, based on absorbance readings were analysed, MBC data processed, based on cell viability counts of algal extract+biofilm cells, and BBC was assessed by using the absorbance reading using a UV-Vis spectrophotometer to yield the minimum treatment concentration to yield 99.9 % biofilm growth prevention (comparing untreated and treated biofilm plates). These assays were carried out in duplicate on three separate occasions (n=3).

4.3.3.5 **DPPH radical scavenging assay – Microtitre Plate Method**

A 0.01 mM methanolic solution of DPPH was prepared. Positive control stock solutions of ascorbic acid and BHT were prepared, 2.0 mg/mL and 2.4 mg/mL respectively. Stock solutions of the crude aqueous seaweed extract and semi-pure derivative were prepared at a concentration of 10 mg/mL. Serial dilutions of the standards, the extract and semi-pure derivative were prepared in eppendorf tubes, resulting in dilution ranges of 200 µg/mL – 0.020 µg/mL; 240µg/mL – 0.024 µg/mL; 1000 µg/mL – 0.1 µg/mL. respectively (13 dilutions), so that for each dilution, the average % IC related to triplicate microtitre wells. An aliquot of 100 µL of each was transferred to three wells of a microtitre plate and 100 µl of the DPPH solution added. The contents were mixed on addition of the DPPH solution using clean, sterile pipette tips for each addition. Blank control wells contained 200 µL methanol. The plates were covered in tinfoil and left in darkness at room temperature for 30 min. The absorbance was then measured at 517 nm using a UV-Vis spectrophotometer. The results were calculated and interpreted using Graph pad prism 5 – non-linear fit of log inhibitor concentration versus normalised response (variable response) according to Equation 4.3.

Equation 4.3

$$\text{DPPH radical scavenging capacity \%} = \left[1 - \left(\frac{Abs_{(sample)} - Abs_{(sample\ blank)}}{Abs_{(control)}} \right) \right] \times 100\%$$

where $Abs_{(control)}$ was the absorbance of the control (DPPH solution without sample), $Abs_{(sample)}$ was the absorbance of the test sample (DPPH solution plus test sample) and $Abs_{(sample\ blank)}$ was the absorbance of the sample only (sample without any DPPH solution). The results are presented as IC 50 concentrations where there is 50 % inhibition of the DPPH radical present. The experiment was repeated 3 times (n=3).

4.3.3.6 **Total Phenolic Content - Microtitre Method**

The total phenolic content (TPC) of the *P. lanosa* extracts was measured using a modified Folin-Ciocalteu method (Singleton *et al.*, 1998). Gallic acid was used as a standard and a calibration curve plotted in order to determine the TPC of the extracts in a microtitre plate format.

A saturated sodium carbonate solution was prepared by dissolution of 20 g sodium carbonate in 100 mL of deionised water. The Folin-Ciocalteu (FC) reagent was diluted 1:1 with methanol. The change of ionic species (reagent and/or product) solubility in the F-C reaction is correlated to concentrations of methanol (final mixture concentration) and carbonate (initial stock solution concentration).

A gallic acid (GA) stock solution (10 mg/mL) was prepared by dissolution of 100 mg gallic acid in 10 mL methanol. This was serially diluted to yield concentrations of 1.0, 2.5, 5.0, 10.0, 25.0 and 50.0 µg/mL gallic acid. The *P. lanosa* extract samples were prepared as 1 mg/mL solutions in methanol.

A 1 mg/mL solution of the crude extracts as well as a semi-pure sample from HPTLC were prepared by serial dilution from a 100 mg/mL stock solution into methanol, as explained in Section 4.3.3.1. A semi-pure sample was included also at a concentration of 1 mg/mL.

Reaction mixes were prepared by pipetting 40 µL of sample or standard into each well, with the addition of 60 µL saturated sodium carbonate solution before the addition of 100 µL of F-C reagent.

The microtitre plates were covered with tinfoil and left to stand at room temperature for 30 min.

Absorbance at 765nm was then read and a calibration curve plotted allowing for the calculation of TPC for the sample solutions. The experiment was carried out in triplicate.

4.3.3.7 **Total Flavonoid Content – Microtitre Plate Method**

The total flavonoid content of the *P. lanosa* extracts was ascertained using quercetin as a standard to obtain a calibration curve and employing a modified method for microtitre plate analysis.

A 10% aluminium chloride solution, a 1 M potassium acetate solution and a standard quercetin solution 1 mg/L in methanol were prepared.

A 1 mg/mL solution of each of the crude seaweed extracts was prepared by serial dilution from a 100 mg/mL stock solution into methanol, as explained in Section 4.3.3.1. Reaction mixtures were prepared by pipetting and adding 75 µL of standard or test samples + 75 µL of methanol + 15 mL 10% aluminium chloride + 15 µL potassium acetate solution into the wells of 96 well plate in triplicate for each standard and sample, which were incubated in darkness at room temperature for 30 min. The blank solution was 75 µL methanol and 15 µL potassium acetate (without aluminium chloride). The absorbance was read at 415nm in a plate reader. The calibration curve was plotted for quercetin and the total flavonoid content determined as quercetin equivalent in mg/mL. This determination was carried out in triplicate.

4.3.4 *Statistical Analysis*

Data was analysed using Graphpad Prism version 5.01 employing one-way ANOVA followed by a post-hoc analysis using Tukey's multiple comparison test where required. A 5% statistical significance level ($p<0.05$) was selected and results were designated statistically different if $p<0.05$.

4.4 Results and Discussion

4.4.1 *MIC, MBC and BBC of P. lanosa extracts*

The bacterial loading of the MRSA culture used in the MIC, MBC and BBC assays was 2.3-2.74 x 10^7 CFU, falling within the range recommended by CLSI (Cavalieri *et al*., 2005).

Table 4.1 shows the MIC found to reduce bacterial growth by 80 % (MIC (80)) and MBC for the different *P. lanosa* seaweed extracts.

Table 4.1: MIC (80) and MBC results for crude *P. lanosa* extract and the semi-pure sample (n=3).

Sample	**MIC (80) ± SD** **µg/mL**	**MBC ± SD** **µg/mL**
Novobiocin[a]	0.0977 ±0.0332	0.195 ±0.065
Crude extract	625 ±266	2500 ±633
Semi-pure	67.5 ±22.3	250 ±63.3

[a] – control antibiotic

[b] - greater than 2500 µg/ml - highest concentration assayed and no inhibition was detected for these samples

The MIC (80) values represent the lowest concentration of an antimicrobial that inhibits the growth of a planktonic culture, in this case MRSA, by 80 % and this is often used for preliminary studies of natural products with pharmaceutical potential (Greff *et al*., 2014).

The value for novobiocin was extremely low, being almost 700 times less than the semi-pure *P. lanosa* extract derivative and of the order of 7000 times less than the crude extract.

The MIC values obtained were compared with those obtained in other seaweed studies (Table 4.2). MIC results for Rhodophyta aqueous extracts varied between 50 and 200 mg/mL (Alghazeer *et al*., 2013) against *S. aureus* , which were higher than for the crude aqueous extracts assessed here (625 µg/mL - >1250 µg/mL , whilst for the semi-pure sample, the MIC was 67.5 µg/mL). This supports the fact that *P. lanosa* possesses

promising aqueous-derived compounds active against pathogenic bacteria. An anti-fouling study carried out by Hellio *et al.* (Hellio *et al*., 2000) found that a DCM and ethanol extract of *P. lanosa* was active against *S. aureus* with a MIC of 48 μg/mL. These studies demonstrate the disparity of results gained from two screening studies, probably associated with different *S. aureus* strain and their susceptibility to the extract. The anti-fouling study was focused on the actual function of the metabolites under normal oceanic conditions, whereas the Alghazeer *et al*. study was aimed at the discovery of novel compounds active against pathogenic bacteria. In addition, the anti-fouling study found no anti- *S. aureus* activity in extracts from *Ascophyllum nodosum*; this may be significant as it is the host species for *P. lanosa* and suggests that the metabolites responsible for anti-MRSA activity are derived entirely from *P. lanosa*.

Table 4.2: Comparison of literature MIC and MBC values of seaweed extracts with those determined in the current study for *P. lanosa*.

Extract source	Solvent	Compound if known	MIC (µg/mL) MRSA (or other bacterium)	MBC (µg/mL)	Reference
Various Rhodophyta	H_2O		50 000-200000 *(S. aureus)*	nd	(Alghazeer *et al*., 2013)
Rhodomela confervoides	MeOH	Bromophenols	70 - 140	nd	(Xu *et al*., 2003)
Sphaerococcus coronopifolius	DCM:MeOH, (3:1)	4 novel tetracyclic brominated diterpenes	15 -128		(Smyrniotopoulos *et al*., 2010)
Osmundaria serrata	Ethanol: ethylacetate (1:1)	LEE	690	220	(Barreto & Meyer, 2006)
Odonthalia corymbifera	Not disclosed	2,2,3,3-tetrabromo-4,4,5,5-tetra-hydroxydiphenylmethane, 2,2,3-tribromo-3,4,4, 5-tetrahydroxy-6-hydroxymethyl diphenylmethane, and 3-bromo-4-(2,3-dibromo-4,5-dihydroxybenzyl)-5-methoxymethylpyrocatechol	25-50 *(S. aureus)*	nd	(Oh *et al*., 2008)
Laurencia papillosa	Methanol	24-propylidene cholest-5-en-3-β-ol	MIC (90) 1.06 *(S. aureus)*	nd	(Kavita *et al*., 2014)
Laurencia chondrioides	DCM	Elatol,chamigrene-type sesquiterpenoid and similar compound	1875 (extract) synergy These derived compounds were not active against *S. aureus*	nd	(Bansemir *et al*., 2004)
P. lanosa	H_2O	Unknown	[8]78.1 (crude)	1250	(Tan, 2013)
P. lanosa	H_2O	Unknown	[8]MIC (80) 625 (crude) [8]MIC (80) 67.5 (semi-pure)	2500 (crude) 250 (semi-pure)	This study

nd – not determined

* - MRSA strain – W73365 (University Hospital Waterford)

Regarding purified extracts, an antibacterial bioassay-guided study by Xu *et al*. isolated two novel bromophenols, with three known compounds from the methanolic extract of the marine alga, *Rhodomela confervoides* and determined that bis (2,3-dibromo-4,5-dihydroxybenzyl) ether was the most active against five strains of bacteria with the MIC recorded as 70 µg/mL (Xu *et al*., 2003). Other bromophenol MIC values against *S. aureus* strains were 140 µg/mL for 3-bromo-4-(2,3-dibromo-4,5-dihydroxyphenyl) methyl-5-(hydroxymethyl)-1,2-benzenediol, with MIC values of 70-140 µg/mL for 3-bromo-4-(2,3-dibromo-4,5-dihydroxyphenyl) methyl-5-(ethoxymethyl)-1,2-benzenediol, and an MIC of 70 µg/mL for 3-bromo-4-(2,3-dibromo-4,5-dihydroxyphenyl) methyl-5-(methoxymethyl)-1,2-benzenediol as well as an MIC of 70 µg/mL for 4,4′-methylenebis(5,6-dibromo-1,2-benzenediol) (Xu *et al*., 2003). These values are less than those obtained in current study for the crude *P. lanosa* extract but comparable with that of the semi-pure *P. lanosa* extract (67.5µg/mL).

Another study isolated tetracyclic brominated diterpenes from the organic (DCM: MeOH, 3:1) extract of the Rhodophyta *Sphaerococcus coronopifolius*, collected from the rocky coasts of Corfu Island. The structures of the new natural products were elucidated and the isolated metabolites were evaluated for their antibacterial activity against a panel of bacteria including 2 strains of MRSA with MIC values in the range of 15–128 µg/mL (Smyrniotopoulos *et al.*, 2010). These values were again comparable to those determined for the semi-pure *P. lanosa* extract in the current study.

An earlier study compared the MIC and MBC values of lanosol (2,3-dibromo-4,5-dihydroxybenzyl alcohol) ethyl ester (LEE) and found the mean bactericidal and fungicidal values (690 ± 150 µg/mL) were higher than the bacteriostatic and fungistatic results (270 ± 70 µg/mL), indicating that LEE from *Osmundaria serrata* was more effective at slowing microbial growth than killing the microbes, including a MRSA strain (Barreto & Meyer, 2006), as is the case in the current study. The effect on biofilms was examined by transmission electron microscopy and it was reported that LEE was responsible for bacterial cell deformity, increase in cell size and induced slime in various bacterial biofilms (Barreto & Meyer, 2006). It has been well documented that *P. lanosa* is rich in brominated phenolic compounds (Liu *et al*., 2011).

A further investigation by the same research group (Kavita *et al.,* 2014) screened

methanol extracts of 38 seaweeds for activity against a range of bacteria and found a Rhodophyta, *Laurencia papillosa* (Ceramiales, Rhodomelaceae, Rhodophyta) to exhibit the highest antibacterial activity. The active fraction was isolated using column chromatography and the acive compound identified by GC-MS and determined to be 24-propylidene cholest-5-en-3-β-ol, molecular weight (m/z) 426 and molecular formula $C_{30}H_{50}O$. This study is representative of the traditional approach and further investigated the MIC (Inhibition concentration to reduce bacterial population by 50% (IC 50) and IC 90) of the compound against various bacteria and found these were lowest against *S. aureus* – IC 50 value of 0.53 µg/mL and IC 90 value of 1.06 µg/mL.

A further consideration is the MRSA strain that is used to determine the MIC. A 2009 study (Smyth *et al.*, 2009) comparing the antibacterial activity of a range of coumarin structures against a range of MRSA strains, found that 8-iodo-5,7-dihydroxycoumarin was the most potent antibacterial agent against the selected MRSA strains. It exhibited good bioactivity against seven of eight MRSA strains, with a MIC value of >100 µg/mL for EMRSA-15 and a much lower value of 6.25 µg/mL for EMRSA-16. MICs of 50, 12.5 and 25 µg/mL were obtained against the Irish-1, Irish-2 and the distinct strain of MRSA, respectively. This study serves to illustrate the differences of antibacterial susceptibility within strains of MRSA.

In terms of therapeutic application, normally MIC(100) would serve to prevent planktonic pathogen growth and correlate to successful treatment in individuals with a responsive immune system. For all *P. lanosa* samples evaluated in the current study, the MIC value was less than the MBC value, as expected. The amount of antibiotic required to inhibit growth is not always the same as the amount required to actually kill the organism and the MBC is usually several dilutions greater than the MIC although this can depend on the mode of action. This is significant with respect to immunocompromised individuals where the hosts natural defense mechanisms are impaired and successful treatment depends upon achieving bactericidal concentrations of antibiotic (Pankey & Sabath, 2004). Furthermore, continuous release of antibiotics from drug delivery systems is vital so that the MIC(100) level is maintained as release of sub-MIC levels may stimulate biofilm formation. As well as this risk, in clinical practice biofilms are heterogeneous

populations and the presence of some strains increases the rate of gene transfer and in particular, with *S. aureus* increases the rate of horizontal plasmid transfer (Venkatesan *et al*., 2015). The presence of *Staphylococcus sp*. in a biofilm resulted in increased antibiotic resistance via enhanced mutation frequencies (Savage *et al*., 2013).

Table 4.3 presents the results of the biofilm inhibition limit as determined using TTC to stain viable MRSA cells surviving in the microtitre plates after the MIC and MBC assays.

Table 4.3: Biofilm inhibition limit as detected by addition of TTC to treatment wells after removal of non-adherent (planktonic) contents (n=3).

Treatment	**Biofilm Inhibition** **Concentration for positive TTC reaction ± SD** **(presence of viable bacteria)** **µg/mL**
None	All untreated were positive
Novobiocin[a]	0.0977 ± 0.0488
Crude	168 ± 84.0
Semi-pure	125 ± 63.0

a- control antibiotic

It was assumed that viable and biofilm cells would be identified by TTC for sub-toxic concentrations of the *P. lanosa* extract, as toxic concentrations would have killed the bacteria before it could form a biofilm. However, the MIC measured here was for 80 % inhibition and accounts for the fact that a number of persister and viable bacterial cells would still be present in the wells used for determining this value. In Table 4.3, it was observed that novobiocin-treated MRSA at its MIC(80) of 0.0977 µg/mL allowed for a positive TTC reaction, indicative of viable cells in the well. Figure 4.1 presents a microtitre plate confirming MIC results for the crude extract dilutions by the addition of TTC which, in the presence of viable bacteria undergoes a colour change from colourless to dark red.

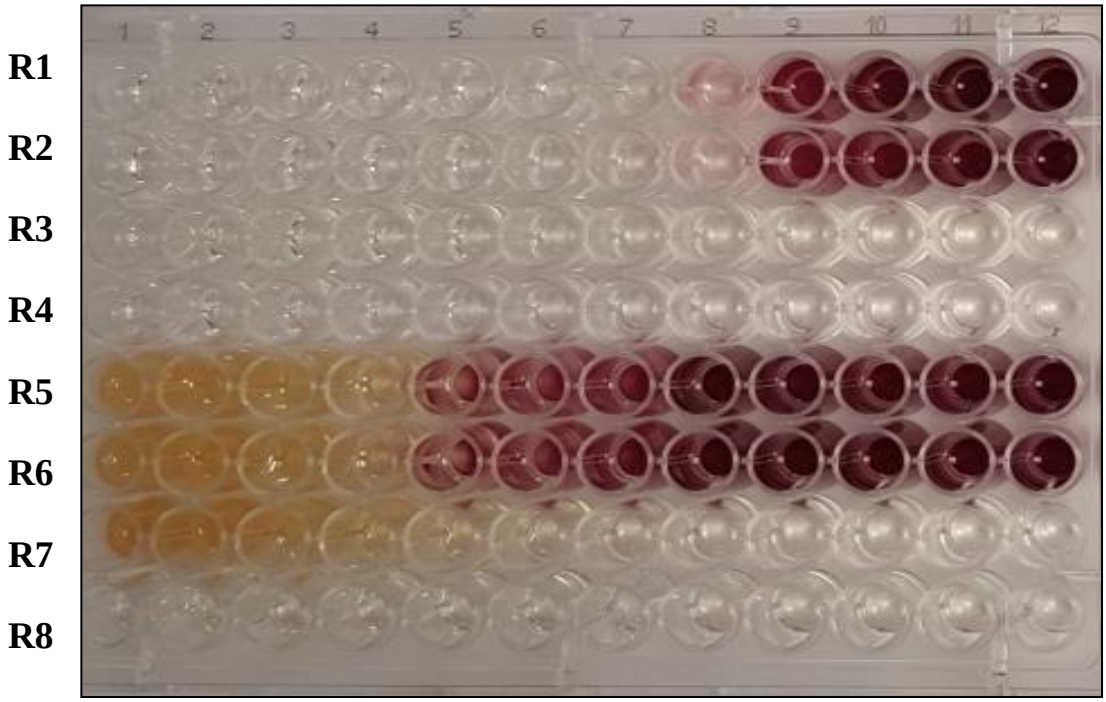


Figure 4.1: Microtitre plate showing addition of TTC to microtitre plate after reading of absorbance to confirm MIC results for the crude *P. lanosa* extract dilutions and the positive control Novobiocin. R1 and R2 are duplicate positive controls (novobiocin) with R3 and R4 negative controls with media and diluent whilst R5 and R6 were the crude extract treatment of MRSA. R7 was the crude extract dilution series displaying no bacterial contamination. R8 was the solvent serving as diluent.

The biofilm bactericidal concentration (BBC), defined as the lowest concentration of extract that killed 99.9% of the cells recovered from a biofilm culture compared to a control, was also used to evaluate the effect of the six extract samples on biofilm-growing bacteria. These results are presented in Table 4.4.

Table 4.4: Biofilm bactericidal concentration of *P. lanosa* extract sample treatments on biofilm-growing MRSA culture (n=3).

Treatment	**BBC ±SD**
	µg/mL
Novobiocin[a]	0.0391 ±0.0123
Crude	337 ±119
Semi-pure	250 ±83

[a] – control antibiotic

As regards the BBC for both novobiocin and the crude *P. lanosa* extract, lower concentrations than the MIC(80) were required, whilst the semi-pure extract sample was needed at a higher concentration than its MIC(80) value of 67.6 μg/mL. This may allude to synergistic biofilm activity within the crude extract which was absent from the semi-pureextract samples

It was anticipated that the exopolysaccharide matrix and biofilm structure would reduce diffusion and act as a primary barrier, preventing the entry of polar and charged antimicrobials as it has been reported that alginate and extracellular DNA, which are components of the extracellular matrix, have exhibited antibiotic-chelating activity (Macia *et al.*, 2014). However, the above results demonstrate that novobiocin and the crude extract were able to overcome this barrier and exert a higher level of activity on the biofilm formed than on the planktonic cells. This activity was exaggerated further when comparing the BBC results with the MBC results with the value pertaining to the crude extract sample 337 μg/mL versus 2500 μg/mL. This activity against biofilm cells suggests that either a synergistic action was responsible or that there was an extremely active compound within the extract capable of dealing with persister cells or with an alternative mechanism of dealing with surface colonisation. *S. aureus* achieves this particular effect through secretion of large amounts of surfactant, the phenol-soluble modulins (PSMs), constituting most of *S. aureus* secretions. These PSMs are also recognised as another virulence factor contributing to spreading motility and involved in surface colonisation, biofilm maturation, phagosome escape, general pathogenicity, and some also have antimicrobial properties (Pollitt & Diggle, 2017).

This activity was in contrast to that of the semi-pure extract sample where a 4-fold increase in concentration (from 67.5 μg/mL to 250 μg/mL) was necessary to overcome the biofilm cells. Lewis has postulated that for effective treatment of persister cells (Lewis, 2007), a single molecule would not be feasible and proposed that a pro-antibiotic compound should be initially benign, enabling a bacterial enzyme to convert it into a reactive antiseptic compound once inside the cytoplasm. This active molecule, being polar, would not be exported and adhere covalently to many bacterial targets, killing the cell. Multidrug resistant (MDR) efflux would be inhibited by this irreversible (covalent)

binding of the active antibiotic molecule to cellular targets. The higher concentration of viable and persister cells and the consequent requirement for supplementary levels of active antibacterial agent to achieve biofilm cell inactivation was the most probable cause for this. There would also be an increased possibility for the development of a complex biofilm particularly in light of research establishing the existence of two biofilm cell phenotypes and that some clinical isolates are able to change from PIA dependent biofilms to PIA independent (proteinaceous) biofilms (Dakheel *et al*., 2016). This situation would contribute to the higher requirement for active material. It was assumed that the biofilm cells were able to grow in the prescence of antimicrobials, even above the MIC for planktonic cells, so that the MIC for biofilm cells will be higher than that for planktonic cells (Lewis, 2001). This is, however a simple assumption, and despite the multidrug tolerance of biofilms (due to persister cells) being responsible for 65% of all cases of infections in the developed world, it is receiving minimal focus and an agreed universal strategy as applied to biofilms is not in force (Lewis, 2007).

Very few studies have extended the bacterial effects of seaweed extracts and compounds beyond that of primary inhibition, either by disk diffusion, bioautography or MIC and MBC. The effect on biofilms, apart from those associated with marine applications and biofouling of seaweed species themselves, has in the most part relied on indirect methods to imply mechanisms such as Quorum Sensing Inhibition (QSI).

However, for plant-derived extracts there have been a number of biofilm studies. A 2013 study (Lee, Park *et al*., 2013) found that quercetin and tannic acid in the methanol extract of *Alnus japonica* inhibited the formation of biofilms (crystal-violet biofilm assay method) in three strains of *S. aureus* in a dose-dependent manner. This study is significant as it revealed that one and/or two common plant components can induce gene expression change inhibiting biofilm formation at very low concentrations in pathogenic *S. aureus* strains whilst not preventing planktonic bacterial growth. This change in gene expression effect may further be the reason for the reduction of the MIC(80) 625 µg/mL result compared to the BBC 337 µg/mL result for the crude *P. lanosa* extract and that one specific compound in the extract may be responsible for such an effect.

It is worth reiterating that MIC and MBC values represent the result of an *in vitro* test in

which the fixed, static concentration of an antibacterial agent is being tested against an initially fixed concentration of bacteria in an aqueous medium which does not correspond with the *in vivo* situation, where antibacterial and bacterial concentration in various body fluids and tissues may fluctuate widely (Pankey & Sabath, 2004). Furthermore, there exists the presumption that *in vitro* bactericidal is superior to bacteriostatic action in the treatment of Gram-positive bacterial infections, but in actual fact these terms relate to strict laboratory conditions and not relevant in the clinical treatment of the great majority of Gram-positive bacterial infections (Pankey & Sabath, 2004).

4.4.2 *Antioxidant Studies*

4.4.2.1 **DPPH radical scavenging assay**

The results of the DPPH radical scavenging assay are presented in Table 4.5.

Table 4.5: Antioxidant capacity of *P. lanosa* extracts assessed as DPPH radical scavenging values, IC 50 - µg/mL of extract required to reduce DPPH radical by 50% (n=3).

Sample	**IC 50 ± SD µg/mL**
Crude extract	59.06 ±19.49
Semi-pure	377.2 ±21.52
BHT	26.06 ±1.15
AA	4.41 ±0.465

AA – ascorbic acid, BHT – butylated hydroxytoluene as antioxidant controls

Statistical analysis determined that all results were statistically significant, $p<0.05$ indicative of significant difference between DPPH IC 50 values for each sample.

The *P. lanosa* crude extract exhibited excellent antioxidant activity, 59.05 when compared to the two positive controls, BHT and AA where the values 26.06 and 4.41 respectively were determined . The results further revealed that the *P. lanosa* crude extract possessed greater DPPH radical scavenging capability than the semi-pure derivative and

was in effect approximately six times more effective. This may signal the existence of a variety of compounds with this capability or the abundance or exceptional effect of one or a group of similar compound types. The possibility existed was that there were synergistic compounds contributing to this activity. The purification process used to derive the semi-pure extract may have affected the activity by removing compounds which contribute to the stability of active compounds or the antioxidant compounds may themselves be prone to degradation or it may simply be that different compounds are responsible for antioxidant and anti-MRSA activity. The different polarity of the mobile phase (40:60 chloroform:methanol with 3 ml H_2O, see Section 3.4.5), may have removed the stronger antioxidant compound(s).

Apart from solvents and compound solubility, other preparative details can affect the composition of extracts. For example, sonication is known to preferentially assist in the extraction of polyphenols (D'Alessandro *et al*., 2012) but on its own may negatively affect the extraction of sulphated polysaccharides (Fidelis *et al*., 2014), thereby either enhancing or negatively affecting antioxidant values. The premise exists that polar solvents efficiently extract a series of polar compounds, such as polyphenols bound to sugars or proteins, phlorotannins, saponins, glycosides and organic acids, whilst a non-polar solvent such as hexane will extract metabolites of low polarity like hydrocarbons, fatty acids, acetogenins, terpenes (halogenated or non), carotenoids and tocopherols. Water-soluble polysaccharides, proteins and organic acids would also have been extracted when using water as the single extractant. The crude *P. lanosa* sample was an aqueous extract and as such would contain polar compounds in a complex matrix, whilst the semi-pure extract should contain relatively less components. The fact that the semi-pure sample revealed little radical scavenging activity using the DPPH assay supports the premise that purification based on anti-MRSA activity did not overlap with antioxidant activity.

The antioxidant results are comparable to those in literature (Table 4.6). For example, in a comparative study, the hexane extract of *Caulerpa racemosa* (Chlorophyta) demonstrated the lowest IC 50 value of 90.00 (highest antioxidant activity) whilst the IC 50 value for the methanol extract of *Turbinaria ornata* (Phaeophyta) was the highest (280.67 ± 1.15 μg/mL) (Chia *et al*., 2015). This was considered unusual as antioxidant activity is usually associated with the presence of bioactive compounds such as phenolics,

flavonols and flavonoids, which are usually extracted with more polar solvents. Polyphenols and anthocyanins scavenge DPPH via the donation of hydrogen, reducing DPPH (DPPH-H). The DPPH radical scavenging activity of the three seaweeds in the Chi *et al*. study considerable and methanol was generally the most suitable solvent for the extraction of polyphenolic compounds due to its ability to inhibit the action of polyphenol oxidase which would cause the oxidation of polyphenols. Methanolic extracts also generally possess high total phenolic content (TPC), as phenolic compounds are typically more polar compounds. It would, therefore, appear that the antioxidant activity was due to other types of compounds present in the hexane extract and may in fact be attributed to other phytochemicals soluble in hexane such as Vitamin A (Chia *et al*., 2015).

Table 4.6 compares the antioxidant activity of various *Polysiphonia* extracts and compounds with those obtained for the *P. lanosa* extracts evaluated in the current study.

Table 4.6: Antioxidant activity of various *Polysiphonia* species extracts and compounds compared to that obtained for *P. lanosa* extracts in the current study.

Compound name	Molecular formula	Molar mass	Rhodophtyta Source	IC_{50} µg/ml	Reference
3-(3-bromo-4,5-dihydroxyphenyl)- 2-(3,5-dibromo-4-hydroxyphenyl)propionic acid	$C_{15}H_{11}Br_3O_5$	532.80	*Polysiphonia urceolata*	11.65	(Li *et a*l., 2007)
E-4-(3-bromo-4,5-dihydroxyphenyl)-but-3-en- 2-one	$C_{10}H_9BrO_3$	256.98	*Polysiphonia urceolata*	2.48	(Li *et al*., 2007)
Crude DCM:MeOH (1:1)			*Polysiphonia lanosa*	2.71	(Zubia *et al*., 2009)
Crude aqueous extract			*Polysiphonia fucoides*	111.4	(Sabeena Farvin & Jacobsen, 2013)
Crude ethanol extract			*Polysiphonia fucoides*	7.5	(Sabeena Farvin & Jacobsen, 2013)
Crude aqueous extract			*Polysiphonia lanosa*	59.06	This study
Semi - pure extract			*Polysiphonia lanosa*	377.2	This study

Typical BHT value EC50 10.7µg/ml

It is evident that the type of solvent influenced the selectivity of phenolic compounds. For instance, for the *P. fucoides* ethanol extract which demonstrated a much higher antioxidant activity than the aqueous extract (Sabeena Farvin & Jacobsen, 2013), it may be that ethanol precipitates most of the proteins and leaves some of the reversibly bonded phenolic compounds in solution (ethanol releasing the polyphenols from polyphenol-protein complexes). A study by Wang *et al.* (Wang *et al*., 2009) found that water was inferior to polar organic solvents in extracting polyphenolic compounds. However, only on characterisation of the extracts can this be confirmed. Other active components in different seaweed extracts may have synergistic effects on the scavenging activities,

which can give contradictory results, so the assumed role of polyphenols as being mainly responsible for scavenging abilities may be overestimated.

One antioxidant study reported that some enzymatic extracts of *Ecklonia cava* and *Sargassum coreanum* (both Phaeophyta) exhibited only weak DPPH radical scavenging activities, even though they contained the same level of TPC as other extracts with higher antiradical activities (Heo *et al*., 2005). This again indicates one of two scenarios, where firstly the particular polyphenol extracted was not a good radical scavenger or secondly, that other unknown compounds such as low-molecular-weight polysaccharides, proteins or peptides could also contribute to the scavenging effect. The ethanolic extract of the red algae *P. fucoides* contained significantly higher levels of phenolic compounds when compared to other extracts (Sabeena Farvin & Jacobsen, 2013) suggestive of higher levels of polyphenols in this Rhodophyta species.

Structurally, the DPPH radical-scavenging activities of the bisphenols are stronger than those of the monophenols and corroborates the observation that free-radical-scavenging activity increased significantly with the number of hydroxyl groups in related molecules (Duan *et al.,* 2007). In considering the pure compounds listed in Table 4.6, it has been demonstrated that the radical scavenging activity of phenolic compounds depends upon their unique phenolic structure and the number and location of the hydroxyl groups, with compounds having the second hydroxyl group in the ortho or para position revealing higher activity than when it is in meta position. Antioxidant activity as defined by IC 50 in the DPPH radical scavenging assay, may be attributable to the number of hydroxyl groups in the molecule as well as the presence of a 3,4-dihydroxy-2,5,6-tribromobenzyloxy unit or derivative (Liu *et al.,* 2011). Previous studies have reported that caffeic acid with two hydroxyl groups was a more efficient antiradical compound than the monohydroxyl counterpart coumaric acid, and the same situation exists with gallic acid, a trihydroxyl phenol, which was more potent than protocatechuic and gentisic acid, its dihydroxyl counterparts. Thus, the radical scavenging activity of the various extracts differs with phenolic combinations.

Furthermore, if the components responsible for antioxidant activity are indeed bromophenols it has been reported that both ortho-dihydroxylation and bromine atom substitution are significant factors in DPPH radical scavenging activity for this class of compound (Li *et al*., 2011).

Other studies explored the antioxidant activity of common seaweed compounds. This included a study of the antioxidant activity of phycoerythrobilin derived from *Porphyra sp*. where the antioxidant activity of natural pigments was found to depend on their structural features such as porphyrin ring, phytyl chain and extended system of conjugated double bonds (Pangestuti & Kim, 2011). Antioxidant activity was found also in the protein extract, and specifically some PBP such as C-phycocyanin (CP) and allophycocyanin (Chojnacka & Saeid, 2012). Other components such as sulphated polysaccharides are also recorded as playing an important role as free-radical scavengers and antioxidants for the prevention of oxidative damage in living organisms (Costa *et al.*, 2010). Until a definitive characterisation of compounds present in our crude aqueous *P. lanosa* extract is achieved, various possibilities remain.

4.4.2.2 **Total Phenolic Content**

Table 4.7 present the determined TPC values after the calibration curve generated for TPC had $r^2 = 0.9972$.

Table 4.7: Total Phenolic Content results for *P. lanosa* extract samples as gallic acid equivalent (GAE) (n=3) with $p < 0.05$ for crude extract in comparison to all other samples..

Sample	**Gallic acid equivalent ± SD (GAE)mg/g dry weight**
Crude	57.84 ±3.390
Semi - pure	86.60 ±6.880

Statistical analysis determined $p<0.05$ for crude compared to semi-pure *P. lanosa* extract., The results indicated that the semi-pure sample contained higher levels of GAE, represented by a 49% increase compared to the original crude extract.

It has been inferred that phenolic compounds are generally more soluble in polar organic solvents than in water and effective recommended extractants are aqueous mixtures of methanol, ethanol and acetone suggestive of the reason for low TPC values. A Danish

study determined the TPC of *P. fucoides* to be 0.2127 mg/g dry weight (Sabeena Farvin & Jacobsen, 2013), equivalent to approximately 0.2% whereas the figure obtained here was 57 mg/g, equivalent to 5.7%. This latter value is considerably higher than that previously determined within our research group where the TPC was determined to be 4.7 – 14.7 mg/g dry weight GAE for *P. lanosa*, depending on the extraction method (Ryan, 2010). Many researchers have determined a positive correlation between TPC and antioxidant activity of different seaweed extracts (Wang *et al*., 2009) but this was clearly not the case here as the crude extract had higher DPPH activity than the semi-purified fraction. There may be a variety of compounds responsible for the strong antioxidant activity in the crude extract with minimal contribution of polyphenols to this bioactivity or the type of phenol in the crude extract possessed strong DPPH activity. The semi-pure sample may possess anti-MRSA polyphenols which exhibit low antioxidant activity in the DPPH assay.

Since seaweed extractions with low TPC also demonstrated good antioxidant results, it can be assumed that co-extracted active compounds such as pigments, tocopherols, proteins, peptides and polysaccharides contributed to the overall antioxidant properties (O'Sullivan *et al*. 2011; Farvin and Jacobsen 2013).

Furthermore, if the components responsible for antioxidant activity are indeed bromophenols it has been reported that both ortho-dihydroxylation and bromine atom substitution are significant factors in DPPH radical scavenging activity for this class of compound (Li *et al*., 2011).

A previous study had reviewed the reactivity of over 80 compounds to the F-C reagent and determined that compound classes including all tested phenols, thiols and proteins as well as vitamin derivatives were reactive (Everette *et al*., 2010).

4.4.2.3 **Total Flavonoid Content**

The calibration curve for TFC is presented in Table 4.8 summarises the results of this analysis on the seaweed extracts. The calibration curve $r^2 = 0.9993$.

Table 4.8: Total Flavanoid Content results for *P. lanosa* extract samples as quercetin equivalent mg/g dry weight (n=3).

Sample	Quercetin equivalent ±SD mg/g dry weight
Crude	0

The crude extract had no detectable flavonoid content. Seaweeds are a rich source of phenolic compounds such as phenolic acids, flavonoids, and phenolic diterpenes which can be preferentially extracted once an extraction strategy has been established. Flavonoids are a major subclass of polyphenols and are generally accepted as direct contributors to antioxidant activity and polarity may be an important consideration: less polar flavonoids (e.g., isoflavones, flavanones, methylated flavones, and flavonols) are extracted with chloroform, dichloromethane, diethyl ether or ethyl acetate, while flavonoid glycosides and more polar aglycones are extracted with alcohols or alcohol–water mixtures. Glycosides would have increased water solubility and aqueous alcoholic solutions would be suitable extracting solvents. Polyphenols can occur as glycosides, possessing several phenolic hydroxyl groups on their ring structures which act as effective ROS scavengers. The above results suggest that the compounds responsible for DPPH activity are not flavonoids and are potentially phenols not reactive in the TFC reaction mix.

The results of all of the individual investigations performed in this chapter are presented together in Table 4.9. The radical scavenging activity of the crude *P. lanosa* extract was approximately six times stronger than that of the semi-pure derivative, reflecting that the purification process did not extract the antioxidant compounds and that compounds responsible for the antioxidant activity were not the same as those responsible for anti-MRSA activity. The antioxidant activity did not correlate with the TPC and TFC results in that the TPC was lower for the crude extract compared to that of the semi-pure. These results suggested that flavonoids were not responsible for this activity, but as discussed in Section 4.1., the contact time for DPPH interaction can affect a true reflection of the

results as some compounds react more slowly than others. This may reflect the complexity of compounds extracted in the semi-pure *P. lanosa* extract and should be a consideration in subsequent investigations.

Table 4.9: Result summary for discussion and comparison.

Sample	**MIC(80)** **µg/mL**	**MBC** **µg/mL**	**BBC** **µg/mL**	**IC50 DPPH** **µg/mL**	**TPC** **GAE/ mg/g**	**TFC** **Quercetin/ mg/g**
Crude	625	2500	337	59.06	57.84	0
Semi-pure	67.5	250	250	377.20	86.60	nd

nd - not determined

As far back as the 1980s, the red algae of the family Rhodomelaceae, specifically the genera *Rhodomela, Odonthalia* and *Polysiphonia* were reported to contain several kinds of bromophenols and 2-bromo-3,4-dihydroxyl benzaldehyde, a normal constituent in some red algae like *Rhodomela teres* and *P. urceolata*, showed higher DPPH radical scavenging activity than a positive control phloroglucinol (Yan *et al.,* 1998), (Sabeena Farvin & Jacobsen, 2013). The study carried out by Yan (Yan *et al*., 1998) determined that the extraction solvents chloroform, acetone and ethyl acetate were more effective than the polar methanol at extracting compounds responsible for DPPH antioxidant results from *P. urceolata.* The reaction mix was left to stand for 60 min and results rechecked after a further 30 min, allowing for optimum reaction rates for all possible components. This may reflect the results here in that the semi-pure extract may possess an extremely active antioxidant which was not detected in the time frame of 30 min.

The compounds responsible for radical scavenging activity in the DPPH assay are more prevalent in the crude *P. lanosa* extract than in the semi-pure active anti-MRSA sample.The reason for better antioxidant activity in the crude *P. lanosa* extract may be connected with the TPC value and the existence of a strong antioxidant phenolic compound which is not present in the semi-pure sample – despite a lower TPC value for the crude extract compared to the semi-pure sample. The TFC value for the crude *P. lanosa* extract was 0 eliminating quercetin and/or its derivatives as being responsible for antioxidant activity in this extract. This is in contrast to another study (Chun *et al.*, 2003)

where quercetin was found to possess greater antioxidant activity than quercetin-3-O-glucuronide and rutin (positive control). It was reported that the antioxidant capacity as determined by the superoxide radical scavenging assay (SRSA) of phenolic compounds decreased in the following order: quercetin > anthocyanins (cyanidin glucoside, cyanidin 3- rutinoside, peonidin 3-glucoside, and peonidin 3-rutinoside) > rutin > chlorogenic acid with ascorbic acid, tocopherol, and polyphenols, exerting synergistic effects on antioxidant activity (Chun *et al.*, 2003). Furthermore, it may be plausible that the higher level of polyphenols in the semi-pure fraction are able to enhance activity against planktonic MRSA.

The TFC result pertaining to the crude *P. lanosa* extract with 0 quercetin equivalents has eliminated quercetin as responsible for the biofilm inhibition in both the crude and semi-pure extracts and indirectly supported the possibility of alternative compounds as contributing to the bioactivities explored in this study.

The presence of alternative active components in the crude *P. lanosa* extract may have synergistic effects on the scavenging activities and these unknown compounds such as low-molecular-weight polysaccharides, proteins or peptides may be responsible for the scavenging effect.

A possible purified compound structure may be one of the marine inspired halogenated phenazines which were investigated in 2015 (Garrison *et al.*, 2015). Furthermore, quinoline is water soluble and some of its derivatives are extremely potent antioxidants as well as possessing activity against *S. aureus* (Orhan Puskullu *et al*., 2013). It had been previously reported that bis (indolyl) methane compounds having 6-bromo piperanol and biphenyl groups exhibited better antibacterial activity than the standard drug Amoxiclav against *S. aureus* (Praveen *et al*., 2010). The radical scavenging activity of these bis(indolyl) methane compounds on DPPH radicals was found to increase with concentration with compounds possessing pyrazole, fluorene and 4-benzoic groups demonstrating maximum activity whilst those possessing quinoline and piperanol groups were less potent than the standard BHT (Praveen *et al*., 2010). The fact that carboxylic acid functional groups or compounds may be responsible for strong radical scavenging activity for DPPH radicals again suggested that these same bis(indolyl) methane

compounds may also be active against MRSA although SAR studies have identified that halogens and a ethylamine side chain is essential for the antimicrobial and antibiofilm activities of the 2,2-bis(6-bromo-3-indolyl) ethylamine bisindole series (Campana *et al*., 2019). It may, therefore, be plausible that the bromo derivatives were partially purified and were responsible for the activity against planktonic MRSA and to a lesser extent biofilm cells whilst the carboxylic acid derivatives were lost with the resulting decline in DPPH radical scavenging activity. Halogenated quinoline structures have been derived with potent activity against biofilm persister cells (Huigens, 2018) which further supports the possibility that the *P. lanosa* extract may include such a molecule.

4.5 Conclusions

The results of this study have shown that the crude *P. lanosa* extract may possess a compound or compounds more capable of attacking a biofilm than planktonic bacteria. The semi-pure sample indicated a low level of anti-biofilm activity on an existing biofilm and a stronger concentration than the MIC(80) was required for biofilm inhibition. This was in contrast to the crude *P. lanosa* extract which resulted in better inhibition of biofilm cells compared to normal planktonic MRSA.

There may also exist a component molecule which was present in both the crude and semi-pure *P. lanosa* extract that when better isolated (or without antagonists) may act in the same way as quercetin – as a gene expression modifier preventing or restricting biofilm formation (Lee *et al.*, 2013). This component molecule would possibly have not been a quercetin derivative as the TFC for the crude extract yielded a negative result.

There is no standard *in vitro* biofilm model and due to the multiple anti-biofilm mechanisms that have been elucidated to-date, there is no standard biofilm prevention assay. As far back as 2001 this was apparent when Lewis (Lewis, 2001), in his review, put forward a proposed assay based on the fact that cell aggregates restrict access of host defense components and that together with the production of persister cells may be viewed as a model of a recalcitrant biofilm infection. Lewis proposed a susceptibility test to determine if any persisters are present, followed by an assessment of whether the biofilm under study was capable of restricting penetration across the exopolymer surface.

Lewis' proposed assay represents a complicated assessment but would permit improved assessment of biofilm prevention and disruption capabilities. The view that biofilm formation is in fact another virulence factor and influenced and prompted by antibacterial activity further supports this proposed approach.

The TPC and TFC results were not able to contribute to the characterisation of potential compound structure as discussed above. The compound may indeed be a bromophenol but not one with a high IC 50 value due to its structural properties, which in turn may actually favour its activity as an antibacterial. The likelihood is that the crude extract contained a large number of antioxidant compounds including pigments (such as anthocyanin), tocopherols, proteins, peptides and polysaccharides, all contributing to the overall DPPH radical scavenging activity.

It may therefore be plausible that the bromo derivatives were partially purified and were responsible for the activity against planktonic MRSA and to a lesser extent biofilm cells whilst the carboxylic acid derivatives were lost with the resulting decline in DPPH radical scavenging activity. The characterisation studies of Chapter 3 indicated the possibility of carboxylic acid groups and/or N-methyl pyrrolidene fragment from the 44 m/z losses observed in the MS spectra which further supports the potential contribution of the carboxylic acid group to the DPPH radical scavenging in the crude *P. lanosa* extract which may not be present in the semi-purified anti-MRSA active sample.

This chapter represents a novel approach to the consideration of anti-MRSA activity aligned to that of antioxidant activity and an appraisal of whether flavonoid compound and/or gallic acid equivalent assays would be useful in inferring possible characterisation.

CHAPTER 5: CYTOTOXIC ACTIVITY OF *P. LANOSA* EXTRACTS AGAINST INTESTINAL EPITHELIAL CELLS AND LIVER HEPATOCELLULAR CARCINOMA CELLS

5.1 Introduction

Cellular assays may contribute to a better understanding of the bioavailability of seaweed extract components, membrane permeability and mechanism of activity. In particular, cytotoxicity studies are warranted given the current interest in the repurposing of cancer treatments as antibacterial agents (Soo *et al*., 2017). The reverse may also be considered, where antibacterial agents, such as those present in the *P. lanosa* extracts evaluated in this thesis, are assessed as potential anti-cancer agents.The similarities between cancer cells and infectious bacterial cells include high replication rates, virulence, new mechanisms of spreading within a host, rapid development of resistance mechanisms against chemotherapeutic treatments and a tendency to become more invasive and aggressive during disease progression (Soo *et al*., 2017). The current consensus is that cancer cells are able to use cell-to-cell communication in the same way as QS operates in bacterial populations, permitting co-ordination of strategies against the host cells.

Cytoxicity assays of seaweed extracts are also warranted in their own right considering the discovery of novel marine-derived compounds with anti-tumour cytotoxic activity (Lefranc *et al*., 2019). For example, Shoeib *et al*. (2004) screened methanolic extracts from 33 species of British marine algae for *in-vitro* cytotoxic activities using DLD-1 (human colon adenocarcinoma) cells. The methanol extract of *P. lanosa* was the only one found to have an IC50 value of less than 50 μg/mL and the chloroform fraction of this methanol extract was found to be significantly more active than the parent methanol extract. This serves to illustrate the complexity of isolating the specific active compound that is targeted for purification. It is known that *P. lanosa* is rich in brominated phenolic compounds (Liu *et al*., 2011) and a variety of structures have been identified and tested against DLD-1 cells using the 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay (Shoeib *et al.*, 2004).

However, with regard to aqueous extracts, only that from *Sargassum vulgare* reported cytotoxic activity (IC50 18.7±3.8 μg/mL), as it was generally considered that organic extracts with different constituents would possess better permeability of the cell membrane in comparison with the hydrophilic nature of aqueous extracts (Guedes *et al*., 2013). This was confirmed by Shoeib *et al* when only the chloroform fraction of *P. lanosa* was found to be tenfold more cytotoxic than the original methanol extract (Shoeib *et al.*,

2004) and the aqueous extract determined to possess an IC50 greater than 50 µg/mL. Wang *et al*. (2008) tested the anti-proliferative potential of aqueous extracts of 12 algal species from Hong Kong in both HL-60 (promyelocytic leukemia) and MCF-7 (breast cancer) cells and determined that the extracts of *Hydroclathrus clathratus* and *Padina arborescens* inhibited cell growth and were also less toxic to normal cells (Guedes *et al*., 2013), highlighting the potential of macroalgal aqueous extracts.

However, when conducting cytotoxicity testing, one of the challenges is that any potential chemotherapeutic agent requires both anti-apoptosis effects in normal cells and pro-apoptotic effects in cancer cells. Apoptosis is defined as a type of cell death in which a number of controlled molecular steps precede cell death. There may also be an overlap with respect to antioxidant and cytotoxic activity as it is accepted that oxidative stress is implicated in the process of carcinogenesis through damage to cellular molecules such as proteins, lipids and nucleic acids.

In addition, the assumption is often made that it is the phenolic compounds within seaweed extracts that are responsible for antioxidant activity. However, various antioxidative assays carried out with seaweed species containing low total phenolic content revealed good antioxidative effects. This indicates that some other co-extracted active compounds, such as pigments and tocopherols in the ethanolic extracts, or sulphated polysaccharides, proteins or peptides in water extracts, may also contribute to antioxidant properties and, therefore, justify further investigation (Sabeena Farvin & Jacobsen, 2013). The same situation may exist when considering the cytotoxic properties of aqueous seaweed extracts. For example, *P. fucoides* and *Fucus serratus* demonstrated the highest radical scavenging activity, reducing power, inhibition of oxidation in a liposome model system as well as in fish oil and were high in phenolic content, however, the monophenols present in these species did not account for these activities (Sabeena Farvin & Jacobsen, 2013).

The substantive objective of this thesis was to explore the capacity of the *P. lanosa* extract to inhibit MRSA but in light of the above, it was also considered relevant to assess anti-cancer activity. In fact, the crossover of various bioactivities such as anti-MRSA, cytotoxicity and antioxidant capabilities from different seaweed species may need to be assessed in more detail where information is available so that alignment may be reviewed and potential compounds reconsidered.

Two different approaches were adopted for this study of the cytotoxicity of the aqueous crude *P. lanosa* extract:

The first used HT-29 colonic epithelial cells and studied whether the crude extract samples possessed any toxic effects on this cell line by employing the xCELLigence real-time cell analysis system (Roche Applied Science, West Sussex, UK). The xCELLigence technology is a real-time cellular biosensor, which measures the net adhesion of cells to high-density gold electrode arrays printed on custom-designed E-plates. The cytotoxicity of an extract is determined by taking impedance (cell index - CI) measurements over time. Proliferation of cells or cell spreading results in enhanced electrical impedance, resulting in a higher cell index (CI) value (Ozdemir & Ark, 2014). The HT-29 cell line is widely used in biological and cancer research due to its ability to differentiate and simulate real colon tissue.

The second approach used HepG2 liver hepatocellular carcinoma cells and the traditional MTT assay. The HepG2 cell line has been used extensively to ascertain the toxic effects of a wide range of chemicals and drugs as well as environmental carcinogens (Carney & Settivari, 2013). The MTT assay was first described by Mosmann in 1983 (Mosmann, 1983) and is based on the ability of a mitochondrial dehydrogenase enzyme in viable cells to cleave the tetrazolium rings of the pale yellow MTT and form dark blue formazan crystals, which are largely impermeable to cell membranes, resulting in their accumulation in healthy cells. Solubilisation of the cells by the addition of a detergent liberates solubilised crystals and the number of surviving cells is directly proportional to the level of the formazan product created. This cleavage of MTT is evident in all living, metabolically active cells but not in dead cells or erythrocytes. The amount of formazan generated is directly proportional to the cell number. The colour can then be quantified using a simple colorimetric assay and results read on a spectrophotometer.

5.2 Objectives

- To carry out preliminary cytotoxicity testing to ascertain whether the aqueous crude *P. lanosa* extract possessed potential anti-cancer activity.

- To determine whether the the aqueous crude *P. lanosa* extract was toxic to normal cells.

5.3 Experimental Procedure

5.3.1 *Cell – lines, growth medium and ancillary requirements*

- HT-29 colonic epithelial cell line (ATCC, LGC Standards, Middlesex, UK)
- HepG2 liver hepatocellular carcinoma (ThermoFisher Life Technologies)
- McCoy's 5A modified medium
- Foetal bovine serum (cell culture medium)(10%)
- RPMI-1640 with 2mM glutamine and normal glucose (1.5 g/L)
- Trypsin (Sigma)
- Phosphate buffered saline tablets (Sigma)
- Sterile nylon syringe filter cartridges (0.45 µm)(Filtropur, Hildesheim, Germany)
- Trypan blue (0.04%) (Sigma)
- DMSO (Sigma)
- Deoxycholic acid (DCA) (Sigma)
- Thiazolyl blue tetrazolium bromide (MTT) (Sigma)
- Sterile microtitre plates
- Haemocytometer
- pH meter – Mettler MP 220
- UV/VIS Spectrophotometer – CARY Varian 100 SCAN
- xCELLigence real-time cell analysis system (Roche Applied Science, West Sussex, UK)

5.3.2 *Preparation of crude seaweed extracts*

The initial freeze-dried crude *P. lanosa* extract was diluted with sterile deionised water to produce a stock solution of 60 mg/mL which was then diluted under sterile conditions in Eppendorf tubes to determine the concentrations necessary for treatment. The MTT assay further compared the extract prepared in water to that prepared in PBS. PBS was included to assess the extract stability when exposed to ions at neutral pH. For example, it is known that peptide structure can change in a saline environment which may affect

activity (Torres et al., 2018). Initial screening of the *P. lanosa* extract determined that a positive response was observed at treatment levels of 0.5 and 1 mg/mL in the MTT assay whilst the impedance study assessed concentrations of 1, 2 and 3 mg/mL.

5.3.3 *Impedence Study with HT-29 cells*

5.3.3.1 Assessment of the effect of crude seaweed extract on the pH of cell culture medium

Various concentrations of crude seaweed extract (1, 2 and 3 mg/mL) were added to 1 mL McCoy's 5A modified medium supplemented with 10% fetal bovine serum (CC medium) and the pH measured using a pH meter to assess solution suitability for cell line growth. This was carried out as a safeguard to ensure cell growth was not affected by an alteration in cell culture medium pH – data not presented but pH was unaffected by addition of seaweed extract.

5.3.3.2 Growth and maintenance of HT-29 colonic epithelial cells

The HT-29 colonic epithelial cells (a tumour cell line that is also used as a cell culture model) were grown in cell culture medium. All cells were routinely maintained in 75 mL tissue culture flasks and incubated at 37 ˚C in a humidified atmosphere with 5 % (*v*/*v*) CO_2. Cells were subcultured when they reached ~ 90% confluence. These conditions are routinely used for cell culture studies.

5.3.3.3 Cytotoxicity testing of crude seaweed extract on HT-29 cells

The optimal concentration of HT-29 cells for use in assays was first determined by serially diluting HT-29 cells to final concentrations of 40,000, 20,000, 10,000, 5000, 2500, 1250 and 625 cells/mL in CC medium and seeding onto xCELLigence E-plates in triplicate. The E-plates were then inserted into the xCELLigence system and incubated in a humidified atmosphere with 5% CO_2 at 37 ˚C for 24 h.

Impedance (CI) measurements were taken every 30 min. CI values were plotted against time and the doubling time and slope of each curve was determined for each cell concentration. The optimal cell density is that with the greatest slope in combination with the shortest doubling time, indicating that the cells were in the exponential phase of growth.

The HT-29 cells must be growing exponentially prior to extract exposure. The optimal concentration of HT-29 cells was first determined. A density of 5.1 x 10^6 cells/mL had the greatest slope in combination with the shortest doubling time, indicating that the cells were in the exponential phase of growth. As the HT-29 cells must be growing exponentially prior to extract exposure, this seed number was used in subsequent cytotoxicity assays (Ozdemir & Ark, 2014).

Cells were then seeded into the wells of the E-plates at a density of 5.1 x 10^6 cells/well which was determined as described, above in CC medium. The E-plates were inserted into the xCELLigence system and incubated for 20–24 h with 5 % CO_2 at 37 °C. Cell index (CI) readings were taken every 20 min. Wells containing HT-29 cells were then treated with varying concentrations of aqueous *P. lanosa* crude extract – 0 (CC medium only), 1.0, 2.0 and 3.0 mg/ mL and the E-plates were incubated for a further 72 h, with CI readings taken every 20 min. A 10 % solution of ethanol, a recognised cytotoxic agent served as a positive control. Each treatment was applied to 4 wells of an E-plate with the experiment carried out in duplicate. The mean of 8 results was used to calculate CI.

5.3.4 *MTT Assay with HepG2 Cells*

5.3.4.1 Growth and maintenance of HepG2 Liver hepatocellular carcinoma cells

The HepG2 liver hepatocellular carcinoma cells (adherent epithelial-like cells that grow as monolayers and in small aggregates) were grown in RPMI-1640 modified medium (2mM glutamine) supplemented with 10% fetal bovine serum (CC medium). All cells were routinely maintained in 75 mL tissue culture flasks and incubated at 37 ˚C in a humidified atmosphere with 5 % (*v*/*v*) CO_2. Cells were passaged when they reached ~ 60-80% confluence. (These conditions are routinely used in cell culture studies).

5.3.4.2 Cytotoxicity testing of crude seaweed extract on HepG2 Liver hepatocellular carcinoma cells – MTT assay

Technique accuracy was initially assessed with a concentration of 8.5 x 10^5 HepG2 cells/ mL using DCA as the positive control to ensure that there was correlation between DCA concentration and cell viability in microtitre plates. (The results revealed that this

concentration of 8.4 X 10^5 cells/mL was suitable for the assay in that DCA as a positive control was able to affect cell viability in line with literature recommendation – Section 5.4.2.1.).

HepG2 cells were harvested from growth cultures by discarding the growth medium, rinsing with PBS and adding 5 mL 10 % trypsin in sterile PBS. The flasks were placed in the incubator for approximately 10 min allowing for the detachment of the cells, after which the trypsin was neutralised by adding growth medium (with 10% foetal growth serum) at a ratio of 1:1. This was pipetted up and down to achieve a single cell suspension and transferred to a universal tube. The cells were then centrifuged at 2000 G for 3 min. The supernatant was discarded and the cells resuspended in 5 mL fresh growth medium.

The cells in suspension were counted to determine cell density. This was accomplished by removing 50 µl of cell suspension from the tube and transferring to a 500 µl Eppendorf tube. A 50 µl aliquot of trypan blue dye was added and mixed. A coverslip was applied to a haemocytometer and a 20 µl drop applied to the edge of the coverslip and by capillary action the solution was drawn across one counting area. This process was duplicated on the opposite side of the haemocytometer. Using 20x magnification the number of viable cells were counted (bright cells) as compared to the stained blue non-viable cells (the ideal number of cells to be counted should be > 100 to ensure accuracy).

The concentration of viable cells was then calculated by combining the sum of the number of viable cells from both areas and dividing by 2 and multiplying this by the dilution factor (2) multiplied by the haemocytometer correction factor (10^4).

The cell suspension required for the wells of the 96 well microtitre plate had to be 10 times higher than the final number of cells required per well. A multi-channel pipette was used to add 100 µl cell suspension (1.5 X 10^4 cells per well) to each well of the 96 well plate which was diluted with 100 µl cell culture medium so that the total well volume was 200 µl. Each treatment can occupy a column of wells so that a maximum of 12 samples may be run on each plate. For the experiment, 1 column was used for the untreated control (medium only), another row for each of the positive control dilutions (DCA) with remaining rows used for seaweed extract samples.

These seeded plates were placed in the incubator at 37 ºC (95% air, 5% CO_2) for a period of 48 h. The growth medium was then discarded by tipping the 96-well plates upside-down and drying on sterilised tissue. Aliquots of 100 µl of each test treatment was added to to each well (1 column per treatment) and incubated for 24 h at 37 ºC. The treatments were removed after incubation and wells washed 3 times with sterile PBS and 200 µl of medium added. The 96 well plates were then placed in the incubator at 37 ºC for 48 h. An aliquot of 100 µl was removed from each well using the multichannel pipette and 15 µl of 5 mg/mL MTT in PBS added to each well. The plates were incubated at 37 ºC for 4 h. The plates were removed from the incubator and 100 µl of solubilizing buffer was added using a multichannel pipette. The plates were incubated for 18 h at 37ºC and were then read at 570 nm on the spectrophotometer (CARY Varian 100 SCAN). For each sample, the mean value over 8 readings (1 column) was calculated. The cell survival results were then presented as a percentage of the untreated control. The assay was performed 3 times (n=3). As a quality control measure standard intra-plate deviation should be < 3% and intra-assay variation < 7 %.

5.3.5 *Statistical Analysis*

Statistical analysis using one-way ANOVA determined statistical significance with analysis of variance $p<0.05$ for each of the data sets, 0-4 mg/mL extract concentrations.

5.4 Results and Discussion

5.4.1 *Cytotoxicity testing of crude seaweed extract on HT-29 cells*

It was first determined that the pH of the CC medium was not affected by the addition of the various *P. lanosa* extract concentrations, as a pH range of 7.2-7.4 is required for optimum cell culture growth. Data not presented.

The xCELLigence real-time cell analysis system was then used to obtain data on any potential toxic effects of various crude *P. lanosa* extract concentrations on intestinal epithelial cells. The xCELLigence technology is a real-time cellular biosensor, which measures the net adhesion of cells to high-density gold electrode arrays printed on custom-designed E-plates. The strength of cellular adhesion is influenced by a myriad of

factors that include cell type, cell viability, growth, migration, spreading and proliferation.

Cytotoxicity of the crude aqueous extract of *P. lanosa* was assessed by taking impedance measurements over time (Figure 5.1). Proliferation of cells or cell spreading results in enhanced electrical impedance, resulting in a higher cell index (CI) value (Ozdemir & Ark, 2014). The addition of the *P. lanosa* extract produced an immediate decrease in the CI, and this was sustained over time. This may indicate a cell membrane effect which may be promising if this was evident in the MRSA – extract interaction. Rapid *in vitro* bactericidal activity can be indicative of disrupted integrity of the cytoplasmic membrane which may equate to a mechanism to which no resistance can be developed (Koh *et al.*, 2013).

In Figure 5.1, the -24 h timepoint was the beginning of a stabilisation run, with only medium and cells in the system and the 0 h timepoint represented the time of addition of the extract. This was to confirm that conditions were suitable for cell proliferation prior to seaweed extract addition.

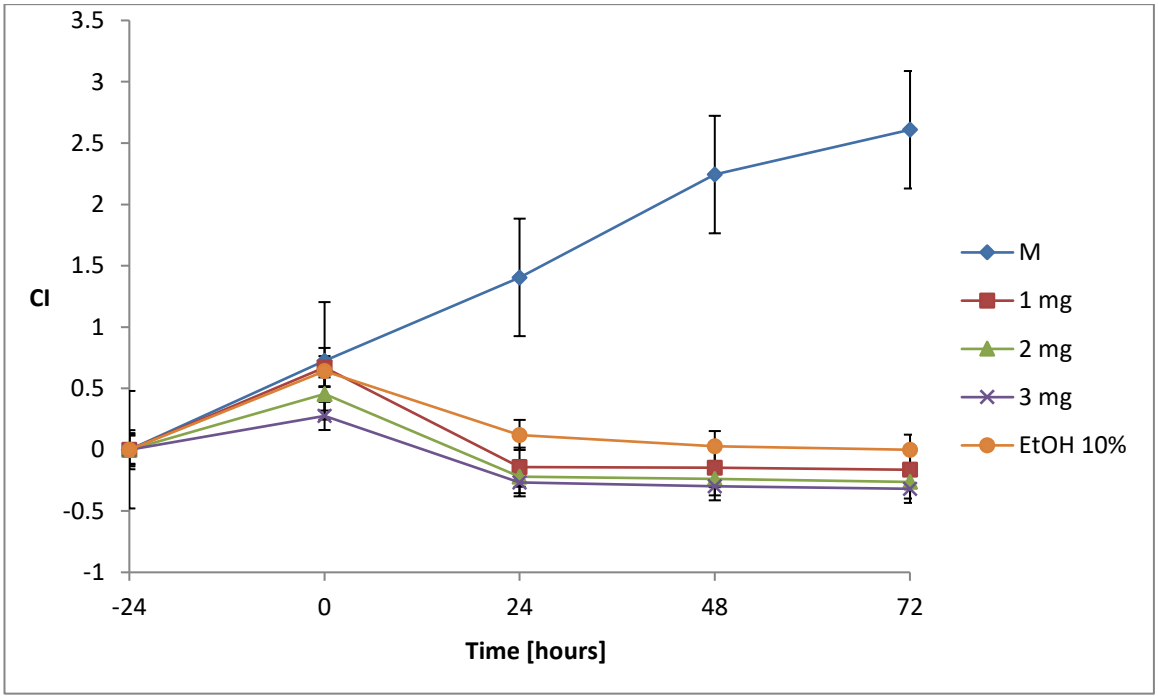


Figure 5.1: HT-29 cell index (CI) relative to exposure time to crude *P. lanosa* extract at exposure concentrations 0 – 3.0 mg/mL compared to a 10% ethanol positive control (where M=cell culture medium). Graph presenting data at 24 h intervals (n=8).

Closer examination of the data for 6 h intervals further revealed that the onset of cytotoxicity was extremely rapid. The cells were instantly affected on exposure to the extract and the effect was correlated to concentration as represented by cell index. After 6 h of contact, the 1 mg extract concentration was as effective as the 10% ethanol control at inhibiting cell proliferation. The higher *P. lanosa* extract concentrations showed very similar reductions in CI at this same 6 h timepoint, after different CI values were observed at the 0 h starting point, indicating that the maximum effect was achieved at this point and suggestive of saturation of cells with extract. In fact there was a levelling off of the CI readings after this point for all three seaweed extract concentrations, with readings at and after 18 h concentration-dependent.

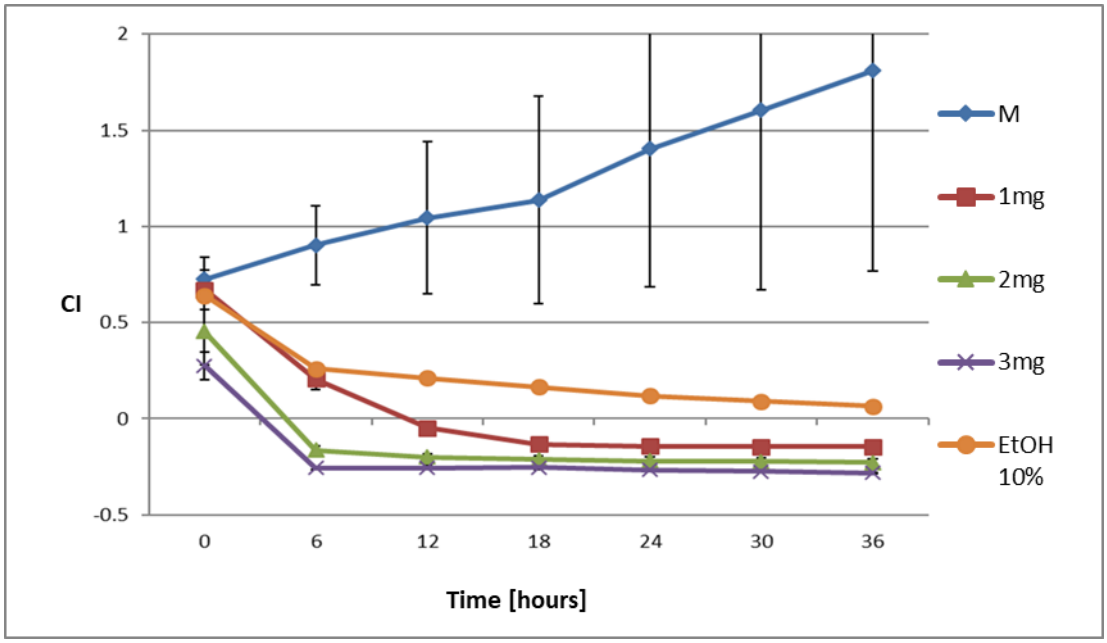


Figure 5.2: HT-29 cell index (CI) relative to exposure time to crude *P .lanosa* extract at exposure concentrations 0 – 3.0 mg/mL compared to a 10% ethanol positive control (where M=cell culture medium). Graph presenting data at 6 h intervals (n=8).

Statistical analysis using one-way ANOVA determined statistical significance with analysis of variance $p<0.05$ for each of the data sets, 0-3 mg/mL extract concentrations. Interestingly, it was previously found that some bioactive compounds such as fatty acids, bromophenols and maharones, as well as possessing anti-MRSA activity, also displayed

anti-tumour activity (Desbois *et al.*, 2009; Shoeib *et al.*, 2004; Manilal *et al.*, 2010). This is important, as on fractionation and purification, different compounds may be separated and the possibility of overlap in both antibacterial activity and cytotoxic activity exists.

A previous study at WIT (Tan, 2013) investigated cytotoxicity of the *P. lanosa* extract using L929 cells (mouse fibroblast cell line), as the extract was intended for use in wound dressings and this cell line was most appropriate. It was found that the crude aqueous extract affected the viability of L-929 cells at concentrations as low as 20 µg/mL while a sharp increase in cell viability observed when the L-929 cells were exposed to 5 mg/mL of the *P. lanosa* extract, suggesting that some compounds in the seaweed extracts may also be cell-promoting. This observation of different patterns of cell viability at different extract concentrations demonstrated that the extract may contain a number of compounds that may be both toxic and growth promoting to the L-929 cells, concluding that separation and isolation of the components was required. As only crude seaweed extracts were assessed for cytotoxicity, this further increased the need for a purified bioactive compound to eliminate any potential antagonsitic or synergistic effects between the 'contaminating' crude seaweed components.

A 2014 study by Namvar *et al* examined the effect of *Gracillaria coricata* (red), *Sargassum ilicifolium* (brown) and *Ulva fasciata* (green) seaweed extracts against five human cancer cell lines using the MTT assay in addition to considering their reducing activity (FRAP). The results revealed that the methanolic extracts of all three seaweed species revealed no toxicity to normal Vero (a commonly utilised mammalian cell line derived from the normal kidney cells of an African green monkey) but inhibited the proliferation of all five cancer cell lines, including the HepG2 and HT-29 cell lines. It was further noted that the HT-29 cell line was the most resistant to the cytotoxic effect of all three seaweed extracts at a treatment level of 300 µg/ml (Namvar *et al.*, 2014).

Different cancer cell lines display a range of responses to cytotoxic anticancer drugs and it was reported as far back as 1984 (Finlay & Baguley, 1984) that colon cancer cell lines, including HT-29, were more resistant to DNA intercalating-drugs (including Actinomycin D, Doxorubicin, Daunorubicin, Ametantrone, Bisantrene, Mitoxantrone, Amsacrine, Nitracrine) whilst breast cancer and leukaemia cell lines were more sensitive based on IC (50) values. For cytosine arabinoside (AraC), an anti-metabolite, HT-29 was found to be the most resistant cell line and required at least 30 times the concentration of AraC to demonstrate the same

degree of inhibition as for the leukaemia Jurket cell line (235nM compared to 6.4 nM) (Finlay & Baguley, 1984). This may further demonstrate the potential strength of activity of the crude *P. lanosa* extract found in the current study.

A more recent study investigated the effect of quercetin against HT-29 cell proliferation and found that quercetin (120 µmol L^{-1}) exerted potent anti-proliferative effects by quercetin-induced apoptosis which, it was suggested, may be mediated via the mitochondrial pathway (Li & Gao, 2013). Quercetin and dimethylquercetin have been identified as components of aqueous extracts of various red algae, including *Gracilaria beckeri* and *Gelidium pristoides* (Olasehinde *et al*., 2019).

5.4.2 *MTT Assay*

5.4.2.1 **Cytotoxicity testing of crude seaweed extract on HepG2 cells**

A preliminary assay using DCA a positive control was performed in order to ensure technique reproducibility. The results are presented in Figure 5.3. A concentration of 125 µM DCA caused a 13 % decrease in the viability of the HepG2 cells, whilst the 250 µM level resulted in a 96 % reduction. This outcome is in line with good practice guidelines, where for colorectal cancer cell lines it is recommended that there is a reduction in cell survival from 75 % to 5 % with 250 µM and 500 µM DCA, respectively (Mosmann, 1983).

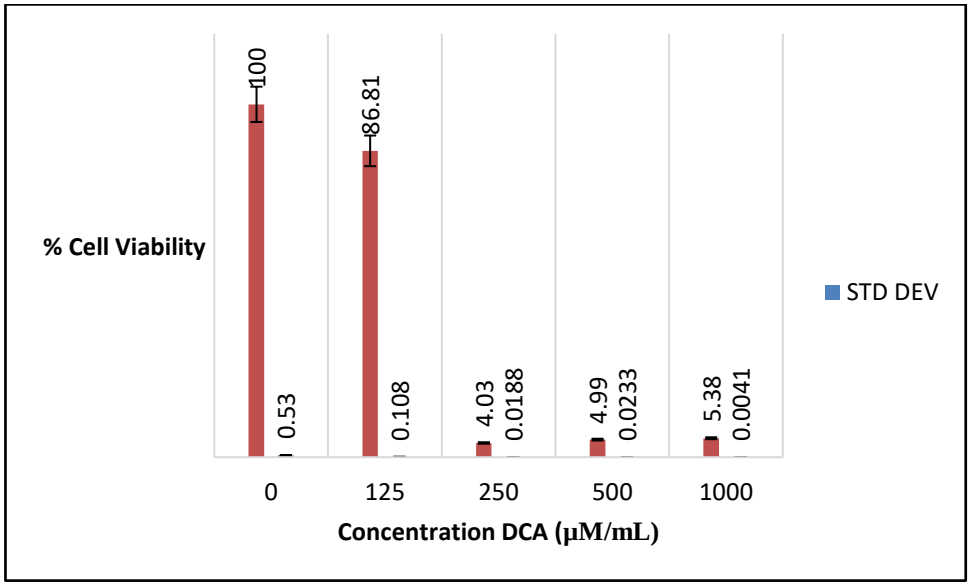


Figure 5.3: Effect of DCA as a positive control in decreasing the viability of the HepG2 cells (n=3).

The results for the treatment of HepG2 cells with crude *P. lanosa* extract using the MTT assay are shown in Figure 5.4. These are in contrast to those obtained by the real time assay using the xCELLigence technology with HT-29 cells. The seaweed extracts appeared to have promoted cell viability with the 0.5 mg/mL treatment level yielding the strongest increase. However, statistical analysis determined the variance of these results as not significant ($p > 0.05$) between the various treatments. At the higher concentration of 1 mg/mL, the effect was reduced in all the crude extract samples tested - the filtered and unfiltered extract prepared in PBS as well as that in deionised water. This supports the previous assumption that the activity of the crude *P. lanosa* extract against MRSA was unaffected by slight changes in the composition of the aqueous stock solution. A further observation was that the filtering of the extract through a 0.45 µm nylon syringe filter did not compromise its activity – also previously observed with respect to anti-MRSA activity.

The result can be further interpreted that the crude *P. lanosa* extract possessed no cytotoxic compound(s) able to affect the HepG2 cell line and suppress cell viability.This may be significant, in considering again the results of the Namvar study, where it would have been expected that the extract would have caused the opposite effect as HepG2 cells were determined to more sensitive to cytotoxic activity (Namvar *et al.*, 2014).

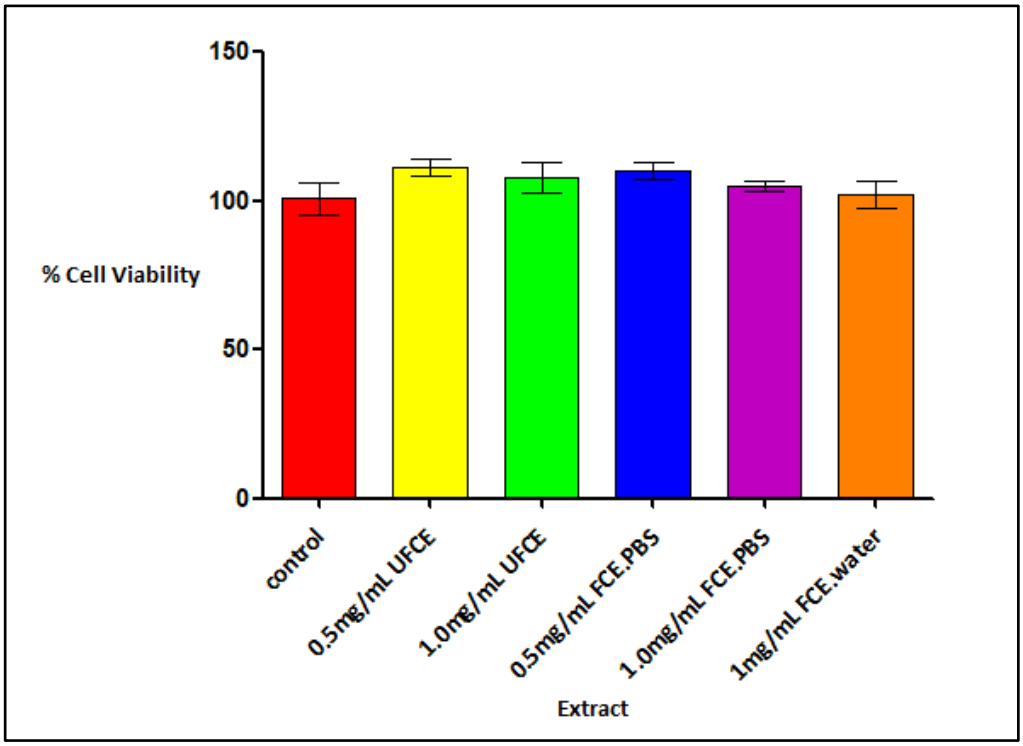


Figure 5.4: Extract treatment effect on HepG2 cell viability with *P. lanosa* extract coded UFCE – unfiltered crude extract in H_2O and FCE – filtered crude extract in PBS buffer (pH =7.3) (n=3).

The statistical analysis points determined that the treatment had no effect at all on the cell line. However, as the cells were in contact with the treatment for 24 h and one explanation is that on initial contact one component of the extract was able to promote permeability of the cell membrane enabling compounds that affect cell proliferation to integrate within the cell cytoplasm and activate factors promoting cell viability.

The use of macroalgal extracts as plant growth promoters and for enhancement of polyphenols and flavonoid content of vegetables has been documented (Lola-Luz *et al.*, 2013). Seaweed extracts act as biostimulants as they contain phytohormones such as auxins, cytokinins, gibberelins, abscisic acid and ethylene (Craigie, 2011). This presence of plant growth regulators, particularly cytokinins (of the type identified by Stirk *et al.*, 2003 who used immunoaffinity chromatography, HPLC-MS, and single ion measurements to investigate five green, seven brown, and 19 red species of seaweeds and found all 31 species contained isoprenoid cytokinins, principally cis- and trans-zeatins, their ribosides and O-glucosides, and isopentenyl adenine and -adenosine) are responsible for plant aging delay, mitosis induction, stimulation of chloroplast maturation and growth

of shoot and lateral buds. Auxins are responsible for elongational growth of plant tissues and apical dominance, cell division, plant movements and plant aging and occur at roughly ten times the concentration of cytokinins. The predominant auxin was indole acetic acid (IAA), although various conjugates are commonly detected in seaweed extracts such as carboxyl groups, glycans, amino acids and peptides (Stirk, 2006). These types of compounds may occur in the *P. lanosa* extract permitting cell proliferation of HepG2 cells.

Algal extracts have been investigated for the stimulation of various human cell lines. A recent study tested extracts from two red algae – *Plocamium lyngbyanum* and *Ceramium secundatum* (collected from Carraroe, Co. Galway and Finavarra, Co. Clare, Ireland), *in vitro* and *in vivo* for their osteogenic (promotion of new bone formation by osteoblasts) potential (Carson *et al*., 2018). *In vitro*, the growth of human bone marrow stromal cells was significantly greater in the presence of the extracts (Carson *et al*., 2018). (This study was unable to isolate or identify any of the seaweed compounds responsible and initial chemical analysis conducted showed protein to be present in each extract, along with approximately 12–15 separate HPLC peaks in the active ultrafiltered fraction).

5.5 Conclusions

Initial consideration of these results was promising in that the crude *P. lanosa* extract was active against one cancer cell-line recognised as resistant to a range of therapeutic agents whilst having no effect on a cell line used in toxicity testing – an ideal combination of effects. The crude *P. lanosa* extract displayed considerable cytotoxicity against HT-29 colonic epithelial cells with the xCELLigence real-time cell analysis system with the level of cytotoxicity observed greater than that found with 10% ethanol which was used as a positive control. This indicated that the crude seaweed extract contained compounds that are toxic to this cancer cell-line at loadings of 1 mg/mL and would normally signal a potentially new therapeutic agent. However, it must also be considered that HT-29 cells are used as an intestinal cell line for a range of food additives (such as probiotic adhesion) and as the *P. lanosa* crude extract kills the cells, ingestion may harm normal intestinal

cells. Further studies would have to be carried out with the purified bioactive compound to determine if this level of cytotoxicity was due to the bioactive anti-MRSA component or other components found in the crude *P. lanosa* extract and also to conduct toxicity studies at lower concentrations.

The above was in contrast to the results of the MTT assay conducted with liver carcinoma cells, which may corroborate previous findings by our group (Tan, 2013). The extracts, both those diluted in water and those in PBS proved that the crude *P. lanosa* extract is non-toxic to HepG2 cells. The possibility that the extract may have activated cell growth would require further study with a longer incubation period, as sufficient contact and assimilation time may be required for the crude *P. lanosa* extract compounds to activate cell growth, supported by statistical analysis. The above differences may prove significant as they supported the possibility of membrane permeability variations between the cell lines as well as having indicated alternative mechanisms employed by the compound(s) of the crude *P. lanosa* extract.

CHAPTER 6: SUPPLEMENTARY ANTI-MRSA ACTIVITY OBSERVATIONS FOR *P. LANOSA* EXTRACTS

6.1 Introduction

This supplementary study was considered relevant and of value for inclusion in this thesis as it was hoped to provide information on the potential mechanism responsible for the observed anti-MRSA activity which may also support the proposed active compound designation. Bacterial virulence factors represent alternative targets to affect inhibition of the infection pathogenesis. Possession of specific mechanisms to cause infection differentiate pathogenic bacteria from nonpathogens. As this study relates to two virulence factors not normally assessed in seaweed studies and due to the level of investigation, it offers another perspective for future studies. Assessment of the effects of sub-inhibitory concentrations of the *P. lanosa* extract was considered important, as this is a commonly used approach to investigate possible modes of activity and to assess whether an extract or compound may possess activity pertaining to anti-virulence factors. This was supplemented by the examination of colony spreading inducement of MRSA with and without crude *P. lanosa* extract.

Bacterial pathogens often employ motility mechanisms for host colonization. *S. aureus* had traditionally been viewed as non-motile, but in 2007 it was demonstrated to move over soft agar. It was observed that the colony spreading of *S. aureus* was due to multiple layers of growing cells and for this reason was compared to sliding (Kaito & Sekimizu, 2007). Colony spreading only occurred on soft agar plates and the presence of water was required. It was suggested that colony spreading represents another virulence factor permitting *S. aureus* to effectively enlarge colonised areas on host tissue surfaces as well as on other surfaces (Kaito & Sekimizu, 2007).

Preliminary investigation of the inhibition of these two virulence factors was carried out. This is a novel exploratory field which is currently receiving attention in order to explain the invasive nature of MDR infections.

6.1.1 *MRSA Virulence Factor - Staphyloxanthin*

An alternative approach to the control of MRSA is to avoid the antibiotic (inhibiting cell growth) approach where interference with cell growth exerts the selective pressure to

develop resistance, and to block bacterial virulence factors (Lee *et al*., 2003). This antivirulence approach holds much potential as these antivirulence compounds could be administered in combination with antibiotics or other bacteriocides to enhance treatment efficacies in MDR infections. In effect, virulence factors play a pathological role in bacterial colonization and invasion but are not essential for survival.

Virulence factors in *S. aureus* include staphyloxanthin, α-haemolysin; (a 33-kDa pore-forming water soluble monomer protein that has cytolytic, haemolytic and dermonecrotic activities), enterotoxins, antibiotic resistant biofilms and quorum sensing (Wang *et al.*, 2011). A variety of plant-derived compounds have been found to reduce the virulence of *S. aureus* without affecting its growth. These include thymol (Qiu *et al*., 2010), a para-cymene-derived compound primarily found in thyme, oregano and tangerine peel which reduced enterotoxins and α-hemolysin production; luteolin (a flavone) (Qiu *et al*., 2011), a polyphenolic compound found in high levels in edible plants such as celery, parsley, broccoli, onion leaves, carrots, green peppers, cabbages, apple skins and chrysanthemum flowers, and chrysin (Wang *et al*., 2011) found to decrease only α-haemolysin production ; and fisetin (Dürig *et al*., 2010), 5-deoxyquercetin or (2-(3,4-dihydroxyphenyl)-3,7-dihydroxychromen-4-one hydrate) and oleic acid (cis-9-octadecenoic acid) (Lee *et al*., 2012) which inhibited biofilm formation. An increase in oleic acid concentration effected a decrease in the adherent bacterial population, whereas the opposite effect was observed with the planktonic population. Overall, the total bacterial counts remained stable, again supporting the necessity to differentiate between planktonic and biofilm inhibition.

A considerable amount of data exists in relation to antivirulence strategies and in particular, the inhibition of staphyloxanthin production in *S. aureus* (Song *et al*., 2009, Sakai *et al*., 2012, Lee *et al*., 2013). Staphyloxanthin is a carotenoid, an orange-red triterpenoid pigment located in the cell membrane. It has numerous double bonds which can react with, and thus deactivate, the reactive oxygen species (ROS) generated by neutrophils and macrophages produced by the host immune system making *S. aureus* resistant to innate immune clearance (Song *et al*., 2009). Staphyloxanthin is necessary for infectivity and it has been demonstrated that bacteria lacking staphyloxanthin are non-pigmented, subject to neutrophil attack (Clauditz *et al*., 2006) and unable to produce disease in mouse skin and systemic infection models (Liu *et al*., 2009).

A number of studies have identified molecules capable of inhibiting staphyloxanthin production in *S. aureus* which weakens the bacterium and renews its susceptibility to antibiotics and these are presented in Table 6.1.

Table 6.1: Staphyloxanthin inhibitor studies

Compounds Tested	Source	Other Virulence Factor(s) affected	Reference
Halogen-substituted phosphonosulfonates and bisphosphonates.	Synthesised	Only pigmentation considered - diphenyl ether phosphonosulfonates most potent.	(Song *et al*., 2009)
3 phosphonosulfonates but not those based on human squalene synthase (SQS) (cholesterol lowering drugs).	Synthesised	Also inhibited haemolytic activity.	(Liu *et al*. 2009)
2 flavonoids including flavone.	Sigma-Aldrich	Only flavone produced white colonies but 8 including flavone inhibited haemolytic activity.	(J. H. Lee *et al*., 2012)
32 commercially available indoles.	Sigma-Aldrich	5-fluoroindole, 5-iodoindole,6-fluoroindole, 7-Benzyloxyindole (7BOI), 7-chloroindole, and 7-bromoindole reduced pigmentation with concentrations as low as 0.1 mM (22.33 μg/mL), 7BOI abolished staphyloxanthin production and produced colourless *S. aureus* cells. Indole and 7BOI both reduced the haemolytic activity of *S. aureus.*	(J. H. Lee, Cho, *et al*., 2013)
4 known inhibitors of lipid metabolism, cerulenin, dihydrobisvertinol, xanthohumol and zaragozic acid and two anthraquinones, 6-deoxy-8-*O*-methylrabelomycin and tetrangomycin.	Institution's Natural Product Library	All reduced pigmentation at sub-inhibitory level.	(Sakai *et al*., 2012)
Rhodomyrtone [6,8-dihydroxy-2,2,4,4-tetramethyl-7-(3-methyl-1-oxobutyl)-9-(2-methylpropyl)-4,9-dihydro-1H-xanthene-1,3(2H)-di-one], an acylphloroglucinol.	Isolated from *Rhodomyrtus tomentosa* (Aiton) Hassk. leaves	Reduced pigmentation at sub-inhibitory level.	(Leejae *et al*., 2013)
Myricetin-3,5,7,3′,4′,5′-hexahydroxyflavone a flavonoid commonly ingested through human diets such as fruits, vegetables, tea, berries and red wine.	Sigma-Aldrich	Antibiofilm properties and anti-hemolytic activity (the importance of the hydroxyl group in position 3 of the flavonol C-ring for the antivirulence activity of Myr was supported).	(Silva *et al*., 2017)
Sesquiterpene farnesol, a plant metabolite.	Wako	Inhibited biofilm formation and plasma coagulation.	(Kuroda *et al*., 2007)

6.1.2 *Phenotypic Evaluation of MRSA Colony Morphotype with and without crude extract*

The visualisation of antibacterial activity is often an aid in determining the effects of any novel extract or purified compound. As outlined above, *S. aureus* can be induced to spread as a colony in soft agar (Kaito & Sekimizu, 2007) and here the effect of the crude *P. lanosa* extract in inhibiting colony spreading was investigated.

Spreading has been associated with the *agr* quorum sensing (QS) system as well as with production of the *agr*-dependent phenol soluble modulins (PSMs), which act as surfactants (Pollitt & Diggle, 2017). The situation at present is that *S. aureus* spreading has been well defined and depends on these PSM surfactants (Tsompanidou *et al*., 2011).

In order to enhance this visualisation, a KOH-aniline blue technique originally developed for the fluorescent staining of fungi in association with plant tissues was adopted (Hood & Shew, 1996). The fluorochrome binds to glucans and polysaccharides in the agar matrix which are metabolised by the bacterial cells and in so doing enhances the visualisation of colony morphology.

Whilst this is essentially an extension of the bioactivity profile of the crude *P. lanosa* extract as it seeks to assess visually obvious effects on the MRSA strain, W73365, a hospital strain isolated at UHW. The possibility exists that the crude *P. lanosa* extract possessed specific anti-virulence factor activity which would also account for its biofilm inhibition properties, permitting confirmation of mode of action. However, the effect of the crude extract on the active spreading capacity of MRSA was considered more significant as these observations may assist the interpretation of how *S. aureus* initiates infections.

6.2 Objectives

- To explore whether a mechanistic investigation would assist in the identification of possible compound type for the active components in the crude *P. lanosa* extract.

- To capture visual images for the inhibition of MRSA strain, W73365 by the crude *P. lanosa* extract .

6.3 Experimental Procedure

6.3.1 *Bacterial strains, microbiological media and chemicals*

- Mueller Hinton Agar (MHA): Difco MHA Ref -225220 (7.6 g per 200 ml deionised water)
- Brain Heart Infusion Broth (BHI): Oxoid (Basingstoke, U.K.) (7.4 g per 200 ml deionised water)
- MRSA - W73365 (WIT 517): clinical isolate from University Hospital Waterford
- Maximum Recovery Diluent (MRD): Oxoid (Basingstoke, U.K.) (1.9 g per 200 ml deionised water)
- Quercetin dihydrate: Sigma (Wicklow, Ireland)
- Lactic acid (90 %): Merck Sigma-Aldrich (Wicklow, Ireland)
- Sodium lactate (98 %): Lancaster (Morecombe, England)
- Arginine: Merck Sigma-Aldrich (Wicklow, Ireland)
- Aniline Blue (water soluble): Clin-tech, VWR (Ballycoolin, Dublin)
- Di-Potassium Hydrogen Phosphate: Scientific and Chemical Supplies Ltd (Sci Chem) (Bilston, West Midlands, UK)

6.3.2 *Experimental Procedure*

6.3.2.1 **MRSA Virulence factor - Staphyloxanthin**

MRSA was cultured in BHI with the addition of subinhibitory concentrations of various additives, as well as crude and semi-pure *P. lanosa* extract samples. The compounds included lactic acid, arginine, arginine and lactic acid as the cultivation of MRSA in a defined medium buffered at pH 5.0 with physiological levels of arginine (4 mM concentration) and lactic acid (50 mM concentration). The crude and semi-pure *P. lanosa* extract samples were prepared at 50 % of their MIC (80) values (313 and 33.5 µg/ml, respectively). After 24 h incubation, the cultures were streaked onto MHA plates. The plates were incubated at 37℃ for 24 h and compared and examined. The experiment was repeated 3 times.

6.3.2.2 **Phenotypic Evaluation of MRSA Colony Morphotype with and without crude *P. lanosa* extract**

MRSA was cultured as previously described in Section 2.3. TSB soft agar medium was prepared by the addition of 0.25 % agar. This was supplemented with a 0.05% aniline blue solution (prepared in 0.067 M K_2HPO_4 and adjusted to pH 8.5) and 1 % glucose before pouring the medium into petri dishes. A 0.05 mL aliquot of crude *P. lanosa* extract stock solution (60 mg/mL solution equivalent to 3 mg crude *P. lanosa* extract) was added to 20 ml of soft agar as the experimental plate whilst the control contained no crude extract. The petri dishes were allowed to dry for 2 h. Morphology was assessed using a spot test on this soft TSB agar where 50 µl of MRSA culture was applied to the centre of the agar plates which were then incubated at 37℃ overnight. The plates were photographed after 18 h incubation. The experiment was repeated 3 times.

6.4 Results and Discussion

6.4.1 *MRSA Virulence factor – Staphyloxanthin*

The MRSA strain, W73365 was able to grow in the defined medium, possibly indicating the presence of the arginine catabolic mobile element (ACME) within the MRSA (Thurlow *et al*., 2013). This is considered to be a key factor for the virulence of USA300-community acquired (CA) MRSA, emerging as responsible for epidemic SSTIs in North America in 2014 (Thurlow *et al*., 2013). The streaked plates revealed one potential mechanism of activity of the crude *P. lanosa* extract in that pigmentation was lost when MRSA was cultured with crude extract but this was not the case with the semi-pure extract. Production of staphyloxanthin was completely inhibited and the resultant streak almost colourless when compared to the control streak and semi-pure extract streaks, Figure 6.1. The crude *P. lanosa* extract may have possessed a compound(s) able to affect staphyloxanthin pigment production whilst the semi-pure *P. lanosa* extract did not.

Alternatively, the explanation is that MRSA was induced to produce staphyloxanthin in the presence of a component or group of components of the semi-pure sample, indicative of a gene activation function. This latter hypothesis has not been previously proposed and may present another complicated and elaborate characteristic of MRSA – an already virulent form is able to enhance its viability in the presence of certain activator compound(s) so that some or all virulent factors are available in its arsenal, dependent on elicitor effector compound(s) interaction.

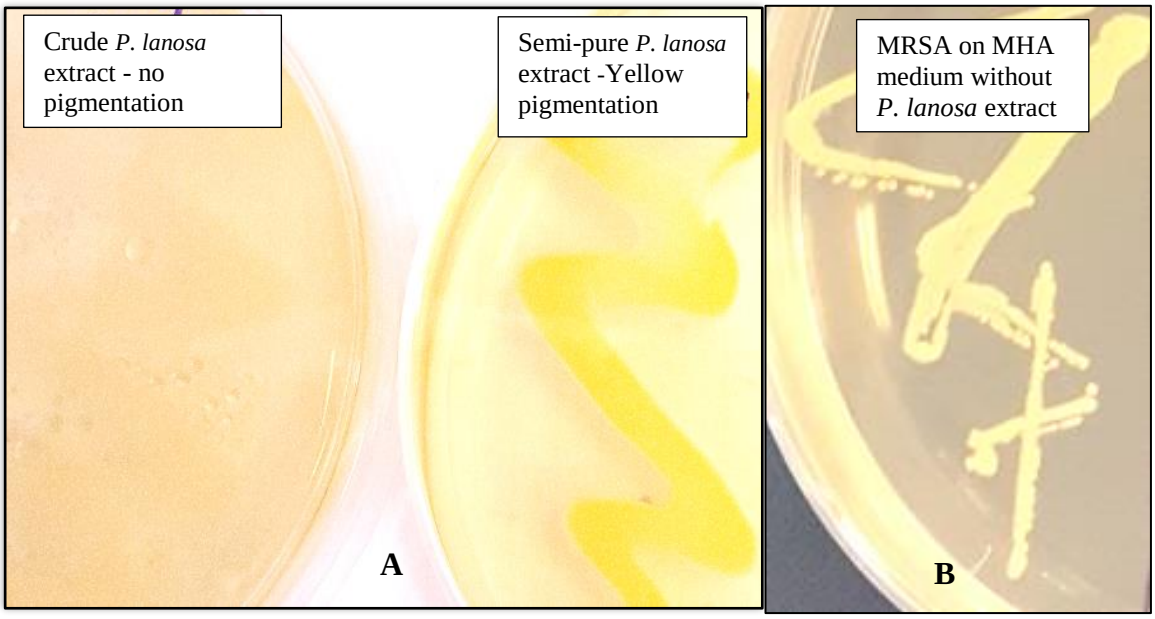


Figure 6.1: Comparison of MRSA pigmentation resulting from incubation with sub-inhibitory concentrations of crude and semi-pure *P. lanosa* extract (A) with MRSA streak on MHA (B).

6.4.2 *Colony spreading and visual characteristics*

The MRSA strain did produce colony spreading on TSB medium, resulting in a blanket type spreading as shown in Figure 6.2.

The effect of the crude *P. lanosa* extract inhibition was obvious, as it appeared that the bacteria did attempt to disperse by spreading but were unable to proceed due to the presence of antibacterial compounds. The crude *P. lanosa* extract was effective at preventing colony spreading of the hospital MRSA isolate at a treatment level of 0.15 mg/mL (3 mg in 20 mL).

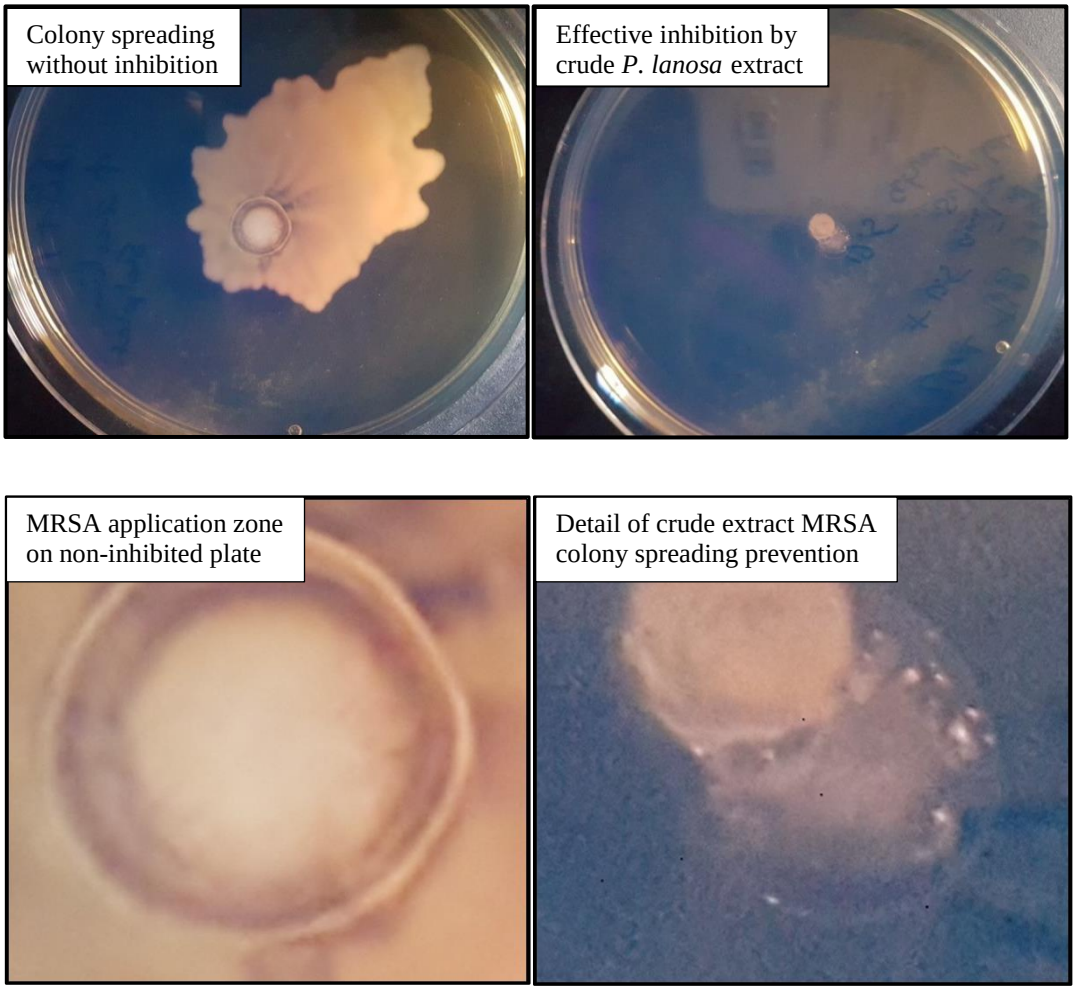


Figure 6.2: MRSA colony spreading inhibition by crude *P. lanosa* extract (0.15 mg/mL treatment level) and detail of application point.

The nature of the residue on the surface of the soft agar indicated that the bacterial cell surface was affected and contents of cells were released as there appeared to be different densities of material. This may infer that lysis of the bacterial cells had taken place and in reference to the inhibition of staphyloxanthin as revealed in the previous section, Section 6.4.1 that even with this protective pigment, the cells were directly exposed to the effect of all antibacterial components of the crude *P. lanosa* extract. This clearly demonstrated the ability of one or more of the component compounds of the crude *P. lanosa* extract to diffuse into the cells.

The white colour at the site of application of MRSA together with the circular 'barrier' on the control plate is of interest as it appears to represent a grouping of cells that may actually be physically separated. It is particularly significant when considering the

staphyloxanthin situation as the lack of colour is indicative of bacterial cells without this virulence factor and it would appear that only after the initial contact (adhesion) do the cells acquire the slight cream coloured pigmentation which may be a prerequisite for colony spreading. This 'colouration switch' type of colony spreading is distinctive and to the authors knowledge, has not been noted previously. A recent paper (Dakheel *et al*., 2016) showed images of 25 MRSA isolates and none demonstrated this property and the overall description within this paper was given as a variety of complex architectural features, including a layer of highly 'autoaggregated cells at the centre of each colony, mounted on transparent layers of adherent cells with irregular margins along the edges with some colonies exhibiting circular or vertical lines radiating from the centre, giving them a bloom-shaped appearance'. The uninhibited colony spreading image in Figure 6.2 resembles a blanket spreading out from the application area, almost with slight folding of the bacterial colony, possibly due to the high volume of bacterial growth.

The assumption made here is that the cells are planktonic (as opposed to a biofilm community) and another hypothesis is that there may be a coexistence of explorer cells and builder cells as was observed for *Paenibacillus vortex* with these two morphotypes possessing distinct functions in colony formation (Roth *et al*., 2013). This would serve as an explanation for a rapid translocation and colonization in changing environments. One such example would be the ability of explorers to swarm through areas containing high levels of antibiotics, moving the colony through stressful niches, and further capable of reverting to the mixed culture composition permitting colonisation in a new area (Roth *et al*., 2013).

It was observed that the colony spreading of *S. aureus* was due to multiple layers of growing cells and for this reason is similar to sliding (Kaito & Sekimizu, 2007). Colony spreading only occurred on soft agar plates and the presence of water was required (overdrying the plates inhibited the spreading property) and it was suggested by Kaito & Sekimizu that teichoic acids act as surfactants to facilitate colony spreading and represent another virulence factor and that the feature of colony spreading allows *S. aureus* to effectively enlarge colonised areas on host tissue surfaces as well as on other surfaces (Kaito & Sekimizu, 2007). It was initially assumed that the crude *P. lanosa* extract may function to undermine the colony spreading by interacting with the techoic acids of the cell surface of the bacteria, again reflecting the potential polar nature of the compound(s)

responsible. However, another reason for activity may be interference with the *agr* gene and the production of PSMs, necessary for motility as the *agr* system functions to disperse biofilms by inducing the expression of proteases and PSMs resulting in a shift from a biofilm state to a planktonic state of growth (Peschel and Otto, 2013).

This apparent membranolytic activity of the crude extract may also indicate the presence of triterpene glucosides. Triterpene glucosides extracted from sea cucumbers were examined in terms of their SAR and their membranolytic activity was determined to be a function of the structural feature of the glycoside. The presence of an 18-lactone as the aglycone with at least one oxygen group near it was of critical significance for biological activity of glycosides bearing 9 double bonds (Popov *et al*., 2017). Glycosides with 7 double bonds in their aglycone structure with absence of 16-ketogroup were more active than those with presence of a 16-ketogroup. The characteristics of the attached glycone structure are also critical for the bioactivities of the sea cucumber triterpene glycosides with a tetrasaccharide chain optimal for modification of the cellular membrane. Sulphation of the sugar chain further enhanced the effect against membranes (Popov *et al*., 2017). The body walls were found to contain the main hydrophilic and membranolytic glucosides, hinting at their polar nature.

Another potential group of compounds with the advantages of membrane-active antimicrobials are retinoids which are fast killing with low probability of developing resistance, and anti-persister activity. However, the major obstacle for developing retinoids as therapeutics is their potential cytotoxicity which remains an obstacle (Kim *et al*., 2018). Kim *et al* have identified a specific chemotype of membrane active synthetic retinoids that are relatively selective for bacterial membranes and exhibit a high level of activity towards MRSA persister cells, due to the presence of carboxylic acid and phenolic groups which are able to bind persistently to the bacterial membrane. This persister activity is currently recognised as superseeding the biofilm-centric studies and the development of appropriate antibiotics for persisters remains a significant challenge (Kim *et al*., 2018). However, the 3 promising compounds were highly toxic to human hepatoma HepG2 cells. This was not the case for the crude *P. lanosa* extract where the presence of both carboxylic acid and phenolic groups has been proposed for the structure of the active compound(s) isolated from *P. lanosa* in Chapter 3.

6.5 Conclusions

It was anticipated that assessing the effect of the *P. lanosa* extracts on staphyloxanthin would assist the characterisation of the active compounds in the crude *P. lanosa* extract. The range of compounds exhibiting anti-staphyloxanthin activity include Rhodomyrtone [6,8-dihydroxy-2,2,4,4-tetramethyl-7-(3-methyl-1-oxobutyl)-9-(2-methylpropyl)-4,9-dihydro-1H-xanthene-1,3(2H)-di-one], which is an acylphloroglucinol, extracted with ethanol. This compound was reported as the first antibiotic to achieve substantial changes in membrane architecture by binding to phospholipid head groups in the outer membrane, causing distortion of the lipid structure without intercalating between membrane lipids (Saeloh *et al*., 2018). A group of small molecule indoles, specifically the BOI (benzyloxyindole) completely eradicating pigmentation of MRSA (as was observed with subinhibitory concentration of the crude *P. lanosa* extract, and so do halogen-substituted and phosphonosulphonates and bisphosphonates and flavone. The possibility is again highlighted that the active compound possesses an indole group, with or without a substituted halogen and sulphonate group. This is further supported by the knowledge that indole suppresses *P. aeruginosa* virulence through interference with AHL quorum sensing, as well as suppressing virulence of *S. aureus*, where it acts to repress the expression of several virulence factors including α-hemolysin and enterotoxin (Lee *et al*., 2013). It has been found that many bacteria produce the metabolite indole (Kaplan *et al*., 2012), which is able to modulate the virulence of some bacterial pathogens that do not produce it (Lee & Lee, 2010).

The crude *P. lanosa* extract was effective in preventing colony spreading of the hospital MRSA isolate, W73365 at a treatment level of 0.15 mg/ml. This effect may be due to the synergistic effect of many different compounds or a specific compound group or a single compound. The requirement for further purification and characterisation still remains. The leakage of cellular contents has been linked to the ability of long alkyl chains that intercalate into the lipid bilayer like those of the common antibacterial cationic quaternary amine compounds which have antibacterial activity.

The motility experiment was useful in that the impact of the crude extract was visual and that the colony spreading nature of the MRSA was observed. The fact that *S. aureus* can be actively motile marks a considerable change in perspective as it was assumed that it is

introduced into the host through a break in tissue barriers, being non-motile (Pollitt & Diggle, 2017)

However, if it is motile in tissues and in association with other virulence factors, *S. aureus* would be equipped to make its own entry into a host (the factors related to spreading such as the *agr* gene and PSMs currently being studied targeted with inhibitors). An example of a motile pathogen, *Neisseria meningitidis* uses its motility mechanism (type IV pili) together with virulence factors to escape the nasal cavity and cause deep infections such as meningitis and septicaemia. *S. aureus* motility has been used to explain situations where it has entered tissues away from the site of the original infection (Pollitt & Diggle, 2017). The challenge of these *in vitro* motility studies is their application *in vivo*. Despite this, it has been suggested that motility could be a target for novel therapeutics and vaccines.

The proposed membranolytic mechanism of the crude *P. lanosa* extract was significant for two reasons – firstly resistance strategies were undermined and secondly it supports the hypothesis that hydrophilic functional groups such as carboxylic acids may have been responsible for activity – these groups bond to the hydrophilic lipid heads of the membrane bilayer of the bacteria, permitting disruption of the membrane by either other compounds or the lipophilic portion of the compound. The presence of carboxylic groups was confirmed by the characterisation studies, specifically by LC-MS and FTIR.

CHAPTER 7: CONCLUSIONS AND FUTURE WORK

7.1 Conclusions

The manipulation of abiotic conditions for the post-harvesting storage of *P. lanosa* in laboratory tanks to define anti-MRSA activity revealed some retention and possible restoration in activity for the higher well loadings. This investigation was novel in its approach and determined that this epiphytic macroalga is capable of being cultured unattached to its host seaweed for a limited time post-harvesting, up to a period of 8 days with maintenance of anti-bacterial activity representing a significant result. The results demonstrated anti-MRSA activity compared to values at harvesting, with only limited retention of activity in both the baseline and temperature studies and a drastic decrease in activity for the light study. It may be argued that there are issues with respect to consistency of anti-MRSA activity within *P. lanosa* once harvested. Statistical analysis of both the temperature and light duration studies determined that all three contributing factors, temperature or light, and day and well loading affected anti-MRSA activity, with extract well loading having the greatest impact. The well loading was an obvious factor as visualistion of antimicrobial activity and it's measurement was recorded as the result. This was, however, a novel approach aimed at tracking anti-MRSA activity post-harvesting, without and with the application of stress conditions.

The isolation, extraction and characterisation study allowed for the development of specific methods optimised for the aqueous crude *P. lanosa* extract such as the well diffusion assay, TLC, bioautography and flash chromatography. The high yield resulting from aqueous extraction may have contributed to the complex observations, potentially due to a range of component compounds. The staining study pointed to the active components being comprised of amino or amide groups, carboxylic acids, indole groups or an indole alkaloid. The semi-purified *P. lanosa* fraction would possibly have included a range of different molecules of varying types or the active molecule may possess several of the active groups such as an amide and/or a carboxylic acid attached to a indole alkaloid. The fact that indole acetic acid, gallic acid, propionic acid and *n*-valeric acid elicited a positive response in bioautography may have indicated the contribution of the carboxyl group and the alkyl chain length to anti-MRSA activity. It is generally accepted that a variety of marine sources including red algae, sponges, tunicates, acorn worms, and symbiotic bacteria generate indole alkaloids, and these represent the largest number

and most complicated of the marine alkaloids. These alkaloids often possess novel frameworks not discovered in terrestrial organisms and are characterised by complexities such as halogen substituents. The FTIR data obtained identified peaks attributable to carboxylic groups. This was corroborated by the fragmentation pattern generated by LC-MS where there were 3 losses of a 44 m/z value ion, and may have indicated 4 free carboxylic groups or alternatively, loss of C_2H_5N such as in the fragmentation of a pyrrolidine from a dibromoindole core.

The FTIR spectra also yielded specific information in that a nitrile group was a possible functional group which may represent a novel active compound type. The FTIR data further supported the possibility of a heterocyclic conjugated indole with halide substitution due to peaks observed in the 600-850 cm^{-1} region. The strongest possible compound candidate is a bi-indole or bisindole halogenated compound with carboxylic acid substitution.

The initial bioactivity studies with respect to antibacterial activity demonstrated that the crude *P. lanosa* extract may possess a compound(s) with the ability to attack biofilms as well as planktonic bacteria. This is significant as biofilm formation is regarded as a virulence factor for MRSA and may be associated with other anti-virulence factors – the crude *P. lanosa* extract possessed multiple anti-virulence activities, again highlighting its unique potential.

The compounds responsible for radical scavenging activity in the DPPH assay were more prevalent in the crude *P. lanosa* extract than in the semi-pure anti-MRSA derivative. The nature of the compounds were unknown at this stage but on characterisation the reasons for the distinction between the IC 50 values may become apparent as SAR analysis would assist in explaining the variations. The antioxidant activity did not correlate with the TPC and TFC results as the TPC result was lower for the crude *P. lanosa* extract compared to that for the semi-pure. This result further correlated to the staining observation that revealed a negative result for the presence of phenols in the crude extract. An alternative explanation may be that the specific phenol, if present, was undetectable using this method or that it was part of a larger molecule.

Compound(s) in the *P. lanosa* semi-pure fraction may include a bromophenol but not one

with a high IC 50 value due to its structural properties, which in turn may actually favour its activity as an antibacterial. This is corroborated by the overall results in Table 4.8 where the light stressed extract samples display poor anti-oxidant and antimicrobial activity, indicating that partial purification has been successful. The likelihood is that the crude extract contains a large number of antioxidant compounds such as pigments, tocopherols, proteins, peptides and polysaccharides, all contributing to the overall radical scavenging activity. The main metabolite class produced by Rhodophyta and particularly Ceramiales are phenolic compounds, particularly sulphated or non-sulphated bromophenols, which have displayed a wide range of bioactivities.

The cytotoxity investigation yielded contrasting results where the crude *P. lanosa* extract displayed intense levels of cytotoxicity to HT-29 colonic epithelial cells using the xCELLigence real-time cell analysis system whilst the MTT assay found that the crude extract, diluted both in water and in PBS, was not cytotoxic to the HepG2 cell line. The period of 24 h permitted interface of compound(s) and cell membrane. This represented a novel approach, contrasting the real-time assay with the traditional MTT assay and revealed the cytotoxic potency of the crude extract against HT-29 colonic epithelial cells, (a cell line recognised as resistant to a range of chemotherapeutics). This investigation further supported the theory that completely different assays are able to yield information that requires careful scrutiny and consideration in order to reveal new possibilities and potential novel applications for crude extracts. This was a novel approach but, due to the reconsideration of anti-cancer drugs as potential treatments for bacterial infections, this expansion of bioactive potential may become more prevalent. The inhibition of staphyloxanthin pigment at sub-inhibitory concentrations of crude *P. lanosa* extract revealed another aspect of the activity of this extract. Whether this occurs in conjunction with associated virulence factors such as haemolytic activity will require further study. The fact that blocking the staphyloxanthin biosynthetic pathway is now viewed as a potential effective strategy for complicated *S. aureus* infections supports further exploration of this activity in the *P. lanosa* extract. Synthesis evaluations for compounds displaying this activity are currently receiving attention.The motility observation also revealed a new perspective for discussion and hypothesis with this property gaining much consideration when examining the connection between virulence and colony spreading.

The observation that bacterial cell lysis may have occurred on contact with the *P. lanosa* extract is further significant in terms of AMR and this motility capability.

This work and its results and observations may complement the evolving understanding of MRSA virulence as well as offer novel strategies for MRSA treatment. The aqueous crude *P. lanosa* extract has a wide range of bioactivities and isolation of the active compounds responsible for each bioactivity would allow for specific SAR analysis, maximising its potential against the challenge of MRSA infections and other biopharmaceutical applications. The potential application of further anti-virulence studies is discussed in the next section and this approach to undermining such pathogens should not be disregarded.

7.2 Future Work

The study of *P. lanosa* anti-MRSA activity as a response to abiotic stress represented a novel and preliminary approach in determining whether the stress conditions would alter the production of metabolites contributing to anti-MRSA activity. Other parameters associated with seaweed aquaculture including the dessication cycles may be considered in later studies and although the results of light and temperature stress were not encouraging – a molecular approach, identifying secondary metabolite pathways and the chief conditions governing their induction would contribute to more applicable research.

Future research potential would include:

- Extension of the abiotic stress investigations to encompass a more intense combined temperature-light (4 levels of irradiance and 5 temperatures) study, an emersion/immersion study, as well as a salinity gradient and pH response analysis.
- Macroalgae have evolved a range of innate and induced defense mechanisms to control bacterial attack. It is necessary to consider that virulence factors are present in marine bacteria, activated by environmental conditions and that these virulence factors ensure the survival of opportunistic pathogens and that macroalgae possess any number of defense mechanisms. For this reason, a study investigating the culturing of *P. lanosa* under biotic stress and the resultant

antibacterial activity may be a feasible proposition such ashe cultivation of *P. lanosa* in the presence of MRSA (cell and cell-free supernatant).

- Extension of the tank cultivation study with epiphyte attachment to host to reduce reliance on harvesting of seaweed from natural habitats.

Further purification and identification of the bioactive components is critical to all future work. This would require completion of the specific separation and characterisation of the targeted active compounds. On elucidation of a lead identity it would be necessary to understand its mode of action against the specific target. This would comprise secondary testing in which molecular and cellular techniques are normally applied and although would constitute a major challenge, would be mandatory in both pharmaceutical and cosmeceutical industries. Complementary investigations may include:

- pH stability studies would be required to check the vulnerability of the anti-MRSA components to ionisation. This may be significant and relate to the mechanism of activity, as penetration of the bioactive into the cell membrane can depend on charge. In fact the charge of cell membrane components (such as the presence of techoic acid and D-alanylated techoic acid) can affect a variety of *S. aureus* properties, such as colony spreading, colonisation of and adhesion to human cells, all of which in turn affect pathogenicity (Kaito & Sekimizu, 2007). (Many antibiotic compounds possess polycationic regions for the purpose of interacting with bacterial membranes).
- More specific extractions should be carried out on the freeze-dried seaweed sample, for example that of polyphenols to allow for determination of whether these components are synergistic or antagonistic to anti-MRSA activity. This can be extended to the extraction of lipids and pigments (using published extraction methods (Arunkumar *et al*., 2014;Al-Fadhli *et al*., 2006;Vanitha & Chandra, 2012)), again contributing to the extract profile and relating it to the enzyme stability results. For example, free fatty acids and oxylipins (oxygenated derivatives of fatty acids) are being assessed for use as biopharmaceuticals, either on their own or in synergy with anti-infectives and their inherent advantage lies in their broad spectrum of activity, high potency, relative safety and lack of inducible resistant activity by pathogens.

- The potential synthesis of the bioactive compound(s) would be crucial in overcoming the low yields associated with bioprospecting seaweed from natural habitats.

The bioactivity profile of *P. lanosa* has been considerably extended during the course of this research and requires further definition:

- The mechanism of antibacterial activity is crucial, particularly whether the bacteria is capable of a resistance response. A time-kill study, using an EtBr assay to assess cytoplasmic membrane disruption may be carried out, and further investigated using a SYTOX green assay as well as SEM analysis supplemented by consideration of the haemolytic activity of the extract (Koh *et al*., 2013). (An assessment of whether the extract displays quorum-sensing inhibitory activity as discussed in section 1.10.2. would complement its application potential (Busetti *et al*., 2014) to include biofilm prevention and/or as an enhancer to conventional antibiotic therapy).
- The effect of *P. lanosa* extracts in modulating other MRSA virulence factors would further extend their serious consideration as novel compounds against AMR. For example, α–haemolysin is a significant virulence factor, with cytolytic, haemolytic and dermonecrotic activities. In fact, it has been reported that the production of this water soluble protein, controlled by the *agr* and a related gene is responsible for hypervirulence in certain MRSA strains, particularly in severe skin infections and diabetic foot ulcers.
- WIT has been active in the pursuit of novel seaweed wound dressings and inclusion of *P. lanosa* extract with a range of proven bioactivities against MRSA would support such an application.

Personal Reflection on Future Possibilities

I would like to offer some recommendations for future projects related to my own research which could potentially be investigated at WIT:

Fasttrack PCR for MRSA infections (wound culture from skin and soft tissue infection) to determine the strain and/or search for specific virulence genes. This would support tailored therapeutic intervention such as compounds to inhibit expression of these genes.

The explosion of diagnostic methods for the identification of Covid-19 will surely result in strain tracking technologies that can be applied to AMR organisms such as MRSA. (This may further represent a strain surveillance or data-tracking project).

This may also include sequencing to assess sodium hydrogen carbonate susceptibility, again permitting application of a dressing containing inhibitory levels of this compound to reduce the microbial virulence potential of diverse microbes that cause infection and affect the level of host susceptibility to the microbes.

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APPENDIX : RESEARCH OUTPUTS

LIST OF OUTPUTS

PUBLISHED ABTRACTS

Poster Presentation

Botha, C., McLoughlin, P., Gardiner, G., Tan, S. P., and Hughes, H. (2015, January). Optimisation of post-harvesting conditions for enhancement of anti-MRSA activity in a *Rhodophyta* species. In European Journal of Phycology (Vol. 50, pp.177-177), Oxon, England: Taylor & Francis Ltd.

POSTER AND ORAL PRESENTATIONS

- **2014** 3rd Annual Limerick Postgraduate Research Conference

Title: Novel compound from seaweed extract with bioactivity against MRSA.

- **2015** 4th Annual Limerick Postgraduate Research Conference

Title: Optimisation of antimicrobial compound extraction from red seaweed with bioactivity against MRSA.

- **2015 NutraMara Conference**

TITLE: Optimisation of antimicrobial compound extraction from red seaweed with bioactivity against MRSA.

- **2015** 6th European Phycological Congress - Algae bring life to the world - 23-28 August, Novotel Hotel, London

TITLE: Optimisation of post-harvesting conditions for enhancement of anti-MRSA activity in a *Rhodophyta* species.

ORAL PRESENTATION

- **2015** 4th Annual Limerick Postgraduate Research Conference

TITLE: Optimisation of post-harvesting conditions for enhancement of anti-MRSA activity in a *Rhodophyta* species.

WIT RESEARCH DAY 2015, 2016 AND 2017 – POSTER PRESENTATIONS

Awarded Best Poster in Department of Science – 2015

TITLE: Optimisation of post-harvesting conditions for enhancement of anti-MRSA activity in a *Rhodophyta* species.

Awarded Best Image – 2016

TITLE: Pattern-forming *Paenibacillus*

Paenibacillus alvei grown on trypic soy agar revealing intricate non-conforming pattern.

Completed SeaSmart School – Multidisciplinary Ship-based Training aboard RV Celtic Voyager – April 2014