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Exploring the Potential of Two Arctic Marine Bacteria for the Production of Bioactive Metabolites

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Abstract

The number of compounds being isolated from the marine environment is increasing, and there is a great potential for discovering new marine derived drug candidates. Improved collection techniques has strengthened bioprospecting on a wider diversity of marine microorganisms. The focus on microorganisms has led to the realisation that many of the natural products originally isolated from macroorganisms, are metabolic products produced by their associated microorganisms. This, and the fact that most marketed antimicrobial drugs originate from microorganisms, motivated the work conducted as part of this thesis.

In this study, two Arctic marine bacteria of the genus *Leifsonia* and *Polaribacter* were studied. The "One Strain-Many Compounds" (OSMAC) approach was utilised when cultivating the bacteria, in an attempt to trigger the bacteria into activating different metabolic pathways and producing compounds with interesting chemistry and bioactivity. Seven different cultivation treatments were used, varying different parameters e.g. media composition and temperature. The secondary metabolites secreted by the cultivated bacteria were harvested, extracted and prefractionated. The fractions were screened for antibacterial activity, inhibition of biofilm formation and anticancer activity. The bioactivity screening resulted in eight active fractions. Dereplication of the active fractions gave several candidates that could be responsible for the observed bioactivity. The results from this thesis give a valuable starting point for further research on cultivation of Arctic marine bacteria, with the purpose of producing bioactive secondary metabolites.

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Abbreviations

Abs	Absorbance
BHI	Brain Heart Infusion Broth
BPI	Base Peak Intensity
C1-C7	Cultivation Treatment 1-Cultivation Treatment 7
CFU	Colony Forming Unit
D-MEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DWP	Deep-Well Plate
EDTA	Ethylenediaminetetraacetic Acid Disodium Salt Dihydrate
E-MEM	Earle's Minimal Essential Medium
ESI	Electrospray Ionisation
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
F1-F6	Fraction 1-Fraction 6
FBS	Fetal Bovine Serum
G-/G+	Gram-Negative/Gram-Positive
HPLC	High-Performance Liquid Chromatography
LC	Liquid Chromatography
LS	*Leifsonia*
m/z	Mass-to-Charge
MC	Media Control
MH	Mueller Hinton Broth
MIC	Minimum Inhibitory Concentration
MNP	Marine Natural Product
MQ	Milli-Q Ultrapure Water
MS	Mass Spectrometer/Mass Spectrometry
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt
n.d.	Not Dated
NP	Natural Product
OSMAC	One Strain-Many Compounds
PAINS	Pan Assay Interfering Compounds
PB	*Polaribacter*
PBS	Phosphate-Buffered Saline
PIA	Polysaccharide Intracellular Adhesion
QToF	Quadrupole Time-of-Flight
RL	Rhamnolipid
Rpm	Revolutions Per Minute
RPMI	Roswell Park Memorial Institute Medium
TSB	Tryptic Soy Broth
UPLC	Ultra Performance Liquid Chromatography
UV	Ultra Violet
WHO	World Health Organization

1 Introduction

1.1 Natural products

Natural products (NPs) are substances produced by a living organism. One common way of subdividing the NPs is into primary and secondary metabolites. Primary metabolites are compounds that are necessary for the immediate survival and growth of the organism. Secondary metabolites, on the other hand, are not necessary for the survival, growth or reproduction of the organism, but can offer advantageous properties by acting as e.g. defence molecules. The compounds are often unique for a set of species within a phylogenetic group (Sarker & Nahar, 2012b). In this thesis, the focus will be on the secondary metabolites produced and secreted into the culture medium by cultivated Arctic marine bacteria.

NPs have evolved to interact efficiently with their biological targets. Because of this, NPs have proven to be good starting points for drug discovery (Montaser & Luesch, 2011). NPs have been the most prolific source of active ingredients for drugs, and have given rise to drugs within many different classes, e.g. anti-cancer, anti-infective and anti-diabetic. An advantage with NPs is that they are, on average, better absorbed (has higher oral availability) compared to synthetic drugs (Harvey, 2008). NPs are often architecturally more complex, contain more ring structures and have higher molecular weight than synthetic compounds (Henkel, Brunne, Müller, & Reichel, 1999). These structural features make NPs capable of interacting with biological targets with high specificity and potency (Hansen & Andersen, 2016). Figure 1 gives an overview of all approved drugs from 1981-2014, as well as the origin of the drug, illustrating that many of the drugs either are NPs or have some connection to NPs, through a NP pharmacophore or by being mimics of NPs. The purely synthetic drugs (S) have only contributed with 27% of the new drugs from 1981-2014 (Newman & Cragg, 2016).

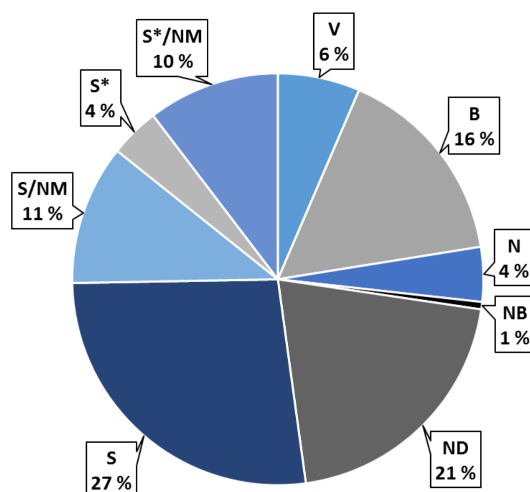

Figure 1: Sources of new approved drugs from 1981-2014 (n=1562). B = Biological macromolecule, N = Unaltered natural product, NB = Botanical drug (defined mixture), ND = Natural product derivative, S = Synthetic drug, S/NM = Synthetic drug/mimic of natural product, S = Synthetic drug (NP pharmocophore), S*/NM = Synthetic drug (NP pharmocophore)/mimic of natural product, V = Vaccine. Modified from reference (Newman & Cragg, 2016, p. 631).*

1.2 The marine environment

More than 70% of the Earth's surface is covered by the oceans. The marine environment is a diverse and ever changing habitat, ranging from the deep-sea trenches with low temperatures and high pressure, to the epipelagic zone with access to sunlight (National Weather Service, n.d.). Marine organisms have generated the ability to adapt to this hostile environment by producing specialized chemical compounds (Svenson, 2013). Compared to the terrestrial environment, the marine environment has many unique characteristics: High salinity, high hydrostatic pressure and low concentrations of organic matter. Because of this, organisms in the marine environment are metabolically and physiologically different from organisms inhabiting other habitats (Imada, 2013).

1.2.1 Marine bioprospecting

Bioprospecting is the search for biologically active substances from nature, with the potential of being developed into a product that should be commercially and scientifically profitable, and valuable to the community (Capon, 2001). The Norwegian Government is focusing on marine research, and in a national strategy from 2009 they defined marine bioprospecting as: *"Targeted and systematic search for components, bioactive compounds or genes within marine organisms"* (Regjeringen, 2009, p. 13). The goal of marine bioprospecting is to discover compounds that are applicable as products or in processes, and that can be relevant for different fields and industries, e.g. human medicine, animal feed, oil and gas (Regjeringen, 2009). This

thesis will focus on the use of marine natural products (MNPs) as lead compounds with the potential of being further developed into commercially available pharmaceutical products.

1.3 Marine natural products

For a long time, NPs have been a traditional source of drug molecules, especially those from terrestrial plants and microbes. The interest in the marine environment was put on hold until better collection technologies, such as scuba diving, emerged (Gerwick & Moore, 2012; Molinski, Dalisay, Lievens, & Saludes, 2009). In the later part of the twentieth century, the interest in marine biodiversity for exploration, extraction and commercialisation has grown (Demunshi & Chugh, 2010). Much less is known about marine organisms than terrestrial ones, and the marine environment is considered largely unexplored with regards to NP discovery (Sarker & Nahar, 2012b). Nevertheless, the marine environment with its rich biodiversity has afforded researchers with a wealth of novel bioactive compounds, some of which have been developed into drugs (Molinski et al., 2009). There are several success stories: One of the most known examples of drugs from the sea is the peptide ziconotide, marketed under the trade name Prialt®. Prialt® was approved in 2004 in the United States for treatment of chronic pain. The peptide was isolated from the tropical marine cone snail *Conus magus*, who uses the peptide to paralyse its preys (Bowersox & Luther, 1998; Molinski et al., 2009). Another known drug with marine origin is the antitumor compound trabectedin, marketed as Yondelis®, which was isolated from the tropical sea squirt *Ecteinascidia trubinata.* The drug was approved in 2007 by the European Commission for treatment of soft tissue sarcoma (Aune, Furuta, & Pommier, 2002; Molinski et al., 2009). Each year, more and more MNPs are reported, increasing from 332 in 1984 to 1378 in 2014 (Blunt, Copp, Keyzers, Munro, & Prinsep, 2016). Figure 2 shows the steep increase in the discovery of new MNPs, which has accelerated greatly, especially in the last decade (2001-2010) (Mehbub, Lei, Franco, & Zhang, 2014).

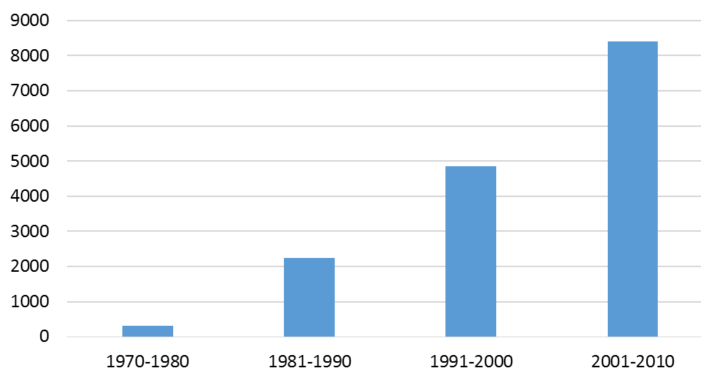

Figure 2: The number of new compounds isolated from marine organisms per decade from 1970 to 2010. Modified from reference (Mehbub et al., 2014, p. 4541).

Many of the compounds from the ocean have no terrestrial analogues and have unique structures and biological activities. This makes them especially interesting in the search for new human medicines (Jaspars et al., 2016). Uncommon functional groups, arising from e.g. halogenation, are often incorporated in the MNPs, which is a special trait for these molecules (Rocha-Martin, Harrington, Dobson, & O'Gara, 2014; Villa & Gerwick, 2010). Another important aspect with regards to the potency of MNPs is the fact that they are diluted by the surrounding water, and because of this evolution has favoured the production of particularly potent MNPs, in order for them to exert the desired effect on their target (Newman, Cragg, & Battershill, 2009).

1.3.1 Marine natural products from microorganisms

The interest in bioprospecting of smaller organisms like marine bacteria and fungi for MNP drug discovery has increased. This has provided new chemistry, but also the realization that many compounds previously isolated from macroorganisms actually were metabolic products from their associated microbes (Gerwick & Moore, 2012; Piel, 2009). In a review by Gerwick and Moore, it was predicted that associated microorganisms were the actual producers of about 80% of the approved agents and agents in clinical studies (as of 2012) (figure 3). Several of the approved marine derived drugs from macroorganisms were predicted to be produced by bacteria, with examples being Cytarabine, Vidarabine and Trabectedin (Gerwick & Moore, 2012).

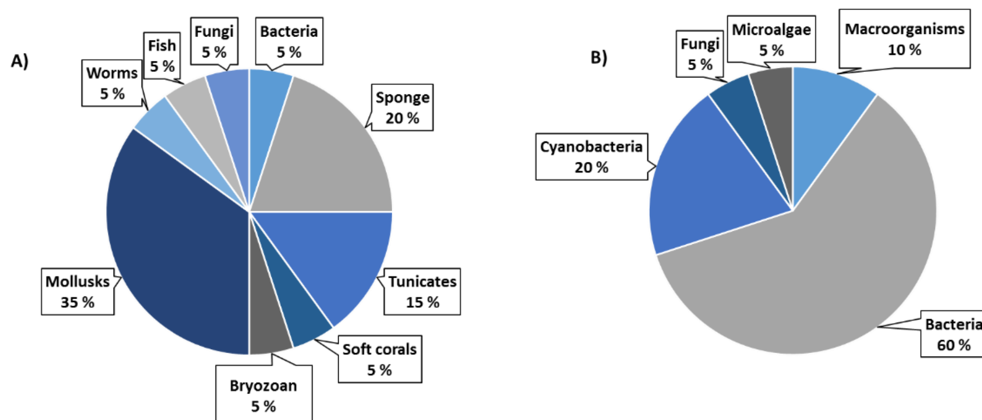

Figure 3: The collected sources (A) and predicted biosynthetic sources (B) of marine derived or inspired drugs and clinical trial agents. Based on a total of 20 agents that are either approved drugs or in clinical trials as of 2012. Modified from reference (Gerwick & Moore, 2012, p. 90).

There has been a great increase in the number of new metabolites reported from marine microorganisms (677 in 2016 vs. 493 in 2013), but the number reported for marine bacteria has not increased much (164 in 2016 vs. 158 in 2013) (Blunt et al., 2016). One advantage with bioprospecting on microorganisms is that cultivation can aid in overcoming the supply issue that is often associated with NP drug discovery from macroorganisms. Cultivation makes it possible to produce large amounts of the source of the NP, and therefore sufficient amounts of the NP for isolation and further testing and development (Gulder & Moore, 2009; Hansen & Andersen, 2016). There are examples of success stories from MNP drug discovery from microorganisms as well, one of the most known being the proteasome inhibitor Salinosporamide A, isolated from the marine bacterium *Salinispora tropica*. Salinosporamide A, also known as Marizomb, is in phase 1 human clinical trials for the treatment of multiple myeloma (Gerwick & Moore, 2012; Potts et al., 2011). The potential of marine microorganisms as producers of bioactive NPs appears to be massive.

1.4 Bacteria

1.4.1 The microbial growth cycle

Bacteria growing in an enclosed vessel, like in a batch culture, can not grow exponentially forever. Eventually a negative feedback between growth rate and one or several parameters in the vessel will lead to a steady number of cells, and will ultimately result in a reduction in cell number. These parameters include nutrient availability, accumulation of waste products, pH changes, cell density, and dissolved oxygen concentration. The microbial growth cycle begins with a lag phase: A period straight after inoculation, before growth has started. In this phase, the bacteria synthesise the enzymes that are needed for growth in the particular medium.

Following the lag phase comes the exponential phase, where the bacteria are dependent on available resources to sustain exponential growth. The rate of exponential growth can vary a lot, and is highly influenced by environmental factors like temperature and availability of nutrients. In the stationary phase, the above listed limitations to growth expansion has been reached, causing the bacterial number to enter a steady state where there is not an increase nor a decrease in cell number (Madigan, Martinko, Stahl, & Clark, 2012, p. 151-156). Figure 4A demonstrates the different phases of the microbial growth cycle. Temperature and other environmental factors have considerable influence on the growth rate of bacteria, both in their natural habitat and in batch cultures. The temperatures where different bacteria grow can vary greatly and are normally reflected by the conditions in the natural habitat of the organism. Bacteria are often classified based on the temperature range in which they are able to grow (figure 4B). Psychrophiles have low temperature optima, mesophiles have midrange temperature optima, while thermophiles and hyperthermophiles have a high temperature optima (Madigan et al., 2012, p. 160-166).

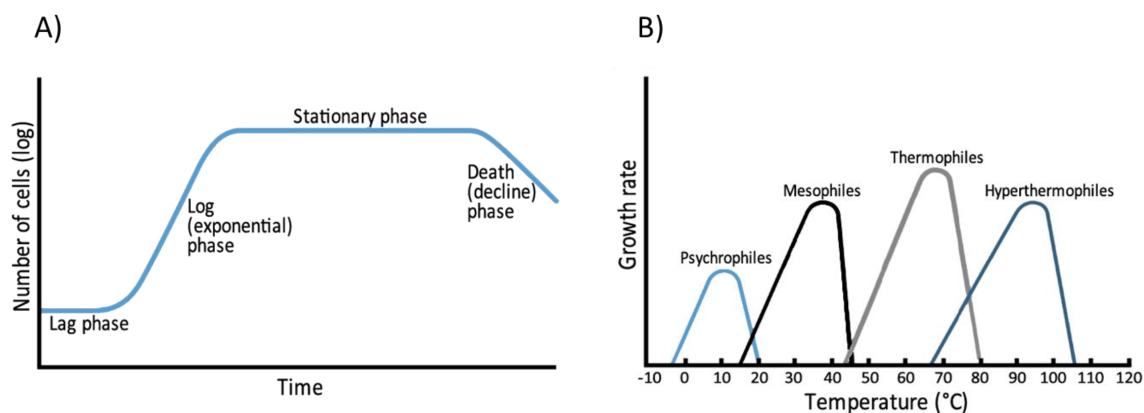

Figure 4: A) The different phases of the microbial growth cycle. Modified from reference (Madigan et al., 2012, p. 153). B) Classification of microorganisms, based on the ranges of temperature in which they are able to grow. Modified from reference (Madigan et al., 2012, p. 163).

1.4.2 Marine bacteria

The average temperature of the oceans is 5°C, and at the depths the temperatures are normally constant around 1-3°C. The microorganisms that grow in the marine environment are generally psychrophiles with temperature optima of 15°C or lower. Psychrophiles produce enzymes that function optimally at low temperatures, and are inactivated at moderate temperatures. Seawater contains dissolved halogen atoms, with about 3% sodium chloride. Many of the marine bacteria are therefore also halophiles, meaning that they require sodium chloride to grow optimally (Madigan et al., 2012, p. 169-171). One of the main limitations of bioprospecting on marine

microorganisms is the lack of knowledge regarding their nutritional needs and environmental requirements. Using standard laboratory techniques, only a small proportion of the microbial diversity of the marine environment is captured (Joint, Mühling, & Querellou, 2010). The vast majority of marine bacteria still remain to be cultured under laboratory conditions, and it is estimated that only 1% of the bacteria present in the sea has been cultured (as of 2012), and that many major lineages still have not been successfully brought to culture (Gerwick & Moore, 2012). The problem is two-sided: In order to acquire more knowledge about cultivation of these organisms, they have to be grown in laboratory cultures, but to grow the organisms in laboratory cultures, more knowledge is needed about their cultivational needs (Joint et al., 2010). In this thesis, two Arctic marine bacteria, one from the genus *Leifsonia* and one from the genus *Polaribacter,* were cultivated.

1.4.2.1 The genus Leifsonia

The genus *Leifsonia* consists of rod shaped, Gram-positive bacteria, where some species can form filament structures. The colonies formed are normally circular and often have a white to light yellow colour (figure 5A). Members of the genus *Leifsonia* occur in different habitats, and they are often found on plants or in soil. Many different unnamed or preliminarily identified organisms exhibiting high 16S rRNA gene sequence similarities to the identified *Leifsonia* species have been discovered in various aquatic environments (Goodfellow et al., 2012, p. 907-909). Table 1 shows the biological classification of *Leifsonia* with the data provided by Marbank.

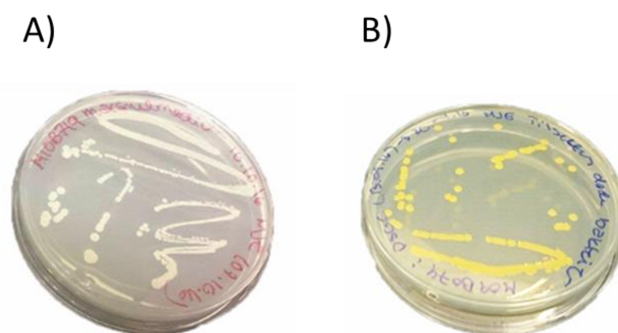

Figure 5: The bacteria cultivated in this thesis. A) Bacteria of the genus Leifsonia streaked on FMAP agar plate. B) Bacteria of the genus Polaribacter streaked on FMAP agar plate. Photo: Marte Jenssen.

1.4.2.2 The genus Polaribacter

Bacteria of the genus *Polaribacter* grow as rods, curved rods or as filaments. They are nonmotile, Gram-negative, heterotrophic and aerobic. Some of the species are psychrophilic or mesophilic, and grow well in marine media or media that have been supplemented with sodium

chloride. The colonies produce yellow, orange, salmon or pink pigments (figure 5B). All strains have been isolated from marine habitats, and some isolates have been able to grow at temperatures of 10°C or lower (Krieg et al., 2010, p. 255-256). Table 1 shows the biological classification of *Polaribacter* with the data provided by Marbank.

Table 1: The biological classification of the bacteria used in this thesis: Polaribacter and Leifsonia. Information obtained from Marbank.

Kingdom	Bacteria	Bacteria
Phylum	Bacteroidetes	Actinobacteria
Class	Flavobacteria	Actinobacteria
Order	Flavobacteriales	Actinomycetales
Family	Flavobacteriaceae	Microbacteriaceae
Genus	*Polaribacter*	*Leifsonia*

1.5 The One Strain-Many Compounds approach

The OSMAC (One Strain-Many Compounds) approach is defined as: *"Systematic alterations of easily accessible cultivation parameters in order to increase the number of secondary metabolites available from one microbial source*" (Bode, Bethe, Höfs, & Zeeck, 2002, p. 619). Examples of parameters that can be altered are aeration, culture vessel, temperature, pH and media composition. Media composition can have a great impact on the production of microbial compounds (Bode et al., 2002), and small changes in the cultivation conditions have shown to alter the metabolic profile of various microorganisms (Höfs, Walker, & Zeeck, 2000). The genetic potential of microorganisms is substantial, but only a fraction of the biosynthetic diversity of the microbes is seen under normal laboratory cultivations. This is because, under routine laboratory cultivations, only a part of the biosynthetic genes of microbes are transcribed, which limits the chemical diversity of microbial compounds that are discovered (Schroeckh et al., 2009). Using the OSMAC approach, one tries to tackle this challenge by testing different cultivation conditions, hoping to trigger the organisms into expressing more and different genes (Marmann, Aly, Lin, Wang, & Proksch, 2014). Every biosynthetic pathway can be influenced at transcriptional, translational and protein level (enzyme inhibition or activation), resulting in a vast number of possible NPs (figure 6) (Bode et al., 2002). In nature, these regulations allow the organisms to survive under changing environmental conditions (Firn & Jones, 2000).

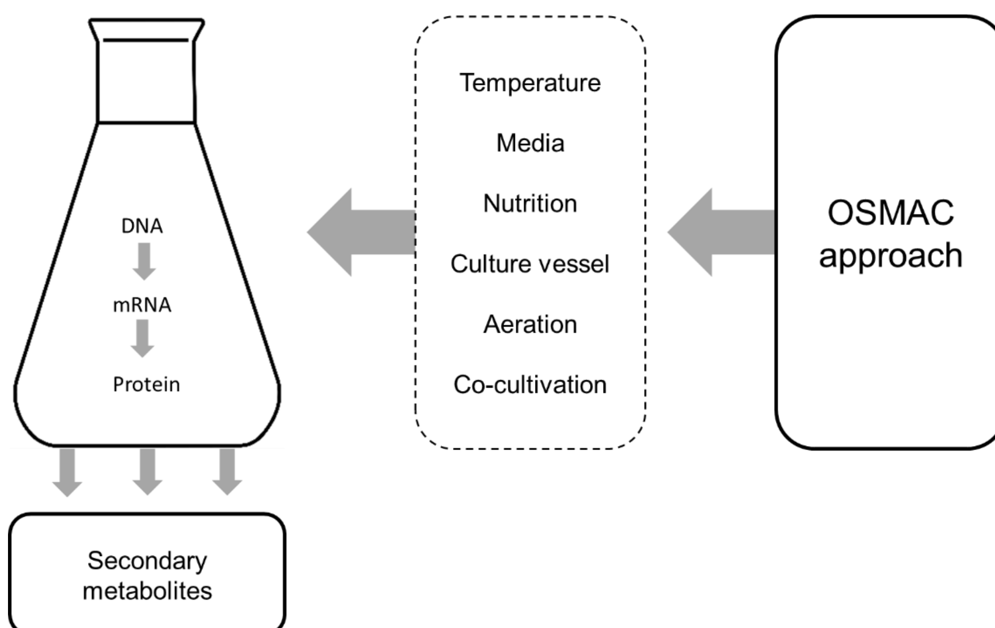

Figure 6: Illustration of the use of the OSMAC approach in an attempt to alter the biosynthetic pathways on transcriptional, translational and protein level, leading to a production of a variety of secondary metabolites. Inspired by reference (Bode et al., 2002, p. 621).

One approach for overcoming the limitations of chemical diversity of microbes is mimicking their natural ecological situations. In nature, microorganisms co-exist in complex microbial communities. In these communities, the microorganisms rely on their production of bioactive secondary metabolites to defend themselves, to fight for the limited resources and to communicate with the other organisms. Co-cultivation of two or more different microorganisms in laboratory scale is one approach to mimic this. In 2014, Marmann and co-workers published a review named "Co-Cultivation – A Powerful Emerging Tool for Enhancing the Chemical Diversity of Microorganisms", underlining the use of co-cultivation to increase the diversity of secondary metabolites produced by microorganisms during *in vitro* fermentation (Marmann et al., 2014). The approach has shown to enhance production of compounds that are not detected, or detected in smaller amounts, in cultures of single organisms (Slattery, Rajbhandari, & Wesson, 2001; Trischman, Oeffner, de Luna, & Kazaoka, 2004).

2 Workflow and background

2.1 Workflow

The work in this thesis was performed at Marbio, and their methods were the foundation of the experiments conducted. Marbio is a high throughput analytical platform within the area of NP drug discovery. Figure 7 shows an overview of the practical work for this thesis, and the background for the individual steps will be presented in the following sections.

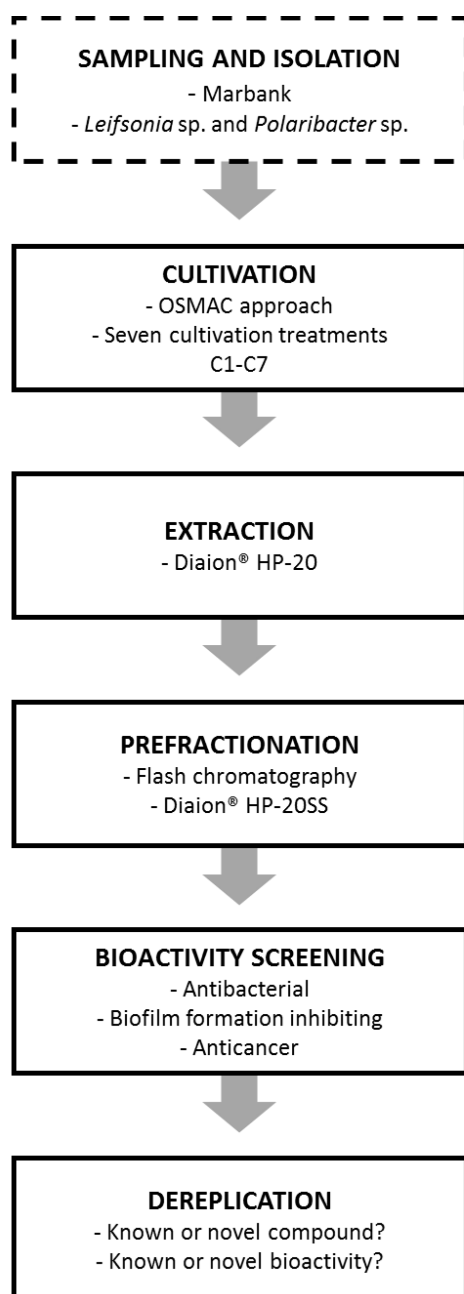

Figure 7: Workflow for the work conducted in this thesis. The bacteria were sampled and isolated by Marbank. The bacteria were cultivated using the OSMAC approach, the secreted secondary metabolites were extracted and the extracts were prefractionated using flash chromatography. The fractions were screened for antibacterial, biofilm formation inhibiting and anticancer activity. In the end, the active fractions were dereplicated.

The steps following dereplication are normally isolation, structure elucidation and bioactivity profiling of the compounds, but there is a long way from here to a commercially available product. The compound, now referred to as a "lead", has to be optimised for its target through chemical synthesis of lead analogs, and has to go through pre-clinical and clinical trials to assess its safety and efficiency in treatment. These studies normally takes several years, and in the last phase the drug has to be approved by the appropriate authorities (e.g. Food and Drug Administration) and marketed (Rang, 2006, p. 43-45).

2.2 Background

2.2.1 Cultivation and extraction

In this thesis, two Arctic Marine bacteria were cultivated under seven different cultivation treatments (utilising the OSMAC approach). Microorganisms produce a complex mixture of NPs, and these have to be extracted from the culture after cultivation (Seidel, 2012). In this project Diaion® HP-20, a polyaromatic resin, was used for the extraction of secondary metabolites from the bacterial cultures. The resin is recommended for the adsorption of solute molecules with molecular weights lower than 20-30 kilodaltons (Sterner, 2012). Diaion® HP-20 is based on a styrene-divinylbenzene matrix and is used to adsorb hydrophobic compounds like biomolecules (Sigma-aldrich, n.d.-a).

2.2.2 Prefractionation of extracts

A crude extract consists of a complex mixture of compounds. To reduce the complexity of the extract, it is often necessary to fractionate it into fractions with compounds of similar traits, like polarity or molecular size (Sarker & Nahar, 2012b). Prefractionation of extracts to less complex mixtures enhances the hit rates by increasing the concentration of the active molecules (Gerwick & Moore, 2012). It is important not to generate too many fractions, so the target compound is spread to the degree where its quantity becomes too low to be detected or display activity in bioactivity screening (Sarker & Nahar, 2012b).

Liquid chromatography (LC) is a technique that can be used to separate compounds in a mixture. The compounds are flushed through a column, and the interaction of the compounds with the stationary phase and the mobile phase determines the retention times of the different compounds (Reid & Sarker, 2012). In this study, flash chromatography was used for the prefractionation of the crude extracts. Benefits with flash chromatography is high sample capacity, and low costs (Bucar, Wube, & Schmid, 2013), but it is not expected to give the same resolution or reproducibility as high-performance LC (HPLC) (Stevens & Hill, 2009). The latter

point is however not too important in prefractionation, as a positive hit will nominate the fraction for isolation of the bioactive compound(s) using HPLC. The stationary phase used in this work was Diaion® HP-20SS, a polyaromatic adsorbent used for the separation of hydrophobic compounds and biomolecules (Sigma-aldrich, n.d.-b).

2.2.3 Bioactivity screening

At Marbio, extracts, fractions and pure compounds can be screened for different bioactivities, in both biochemical and cellular screenings. Examples of bioactivities that can be screened for are anticancer, antibacterial, biofilm formation inhibition, immunomodulation, anti-diabetes and antioxidant (Svenson, 2013). In this study, the flash fractions were screened for antibacterial, biofilm formation inhibiting and anticancer activities.

2.2.3.1 Infectious diseases and antibacterial activity screening

Infectious diseases are caused by pathogenic organisms. Bacteria are known agents of many human diseases, like tuberculosis which is caused by *Mycobacterium tuberculosis (WHO, 2017c)* and abdominal problems caused by *Escherichia coli* (WHO, 2016b). According to the World Health Organization (WHO), antibiotic resistance is one of the biggest threats to global health today, and the levels of antibiotic resistance is rising to dangerously high levels in all parts of the world. Many infections, like tuberculosis and gonorrhoea are becoming harder to treat because the antibiotics used to treat them are becoming less effective. In addition to changing the use of the current antibiotics, the development of new antibiotics is necessary (WHO, 2016a). On the 27th of February 2017, WHO published the first ever list of antibiotic-resistant “priority pathogens”, the bacteria that pose the greatest threat to human health. The organisms on the list were divided into three priorities: critical, high and medium. Among others, *E.coli* and *Pseudumonas aeruginosa* were placed as a critical priority, and *Staphylococcus aureus* were considered high priority (the complete list can be seen in table 2). The list was made in order to help prioritise the research and development of new antibiotic treatments (WHO, 2017b).

Table 2: WHO global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. Modified from reference (WHO, 2017b, p. 5).

Priority	Pathogen (Gram-stain)	Resistance
Critical	*Acinetobacter baumannii* (G-)	Carbapenem
	Pseudomonas aeruginosa (G-)	Carbapenem
	*Enterobacteriaceae** (G-)	Carbapenem, 3rd generation cephlaosporin
High	*Enterococcus faecium* (G+)	Vancomycin
	Staphylococcus aureus (G+)	Methicillin, Vancomycin
	Helicobacter pylori (G-)	Clarithromycin
	Campylobacter (G-)	Fluoroquinolone
	Salmonella sp. (G-)	Fluoroquinolone
	Neisseria gonorrhoeae (G-)	3rd generation cephalosporin, Fluoroquinolone
Medium	*Streptococcus pneumoniae* (G+)	Penicillin
	Haemophilus influenza (G-)	Ampicillin
	Shigella sp. (G-)	Fluoroquinolone

**Enterobacteriaceae include: Klebsiella pneumonia, Escherichia coli, Enterobacter sp., Serratia sp., Proteus sp., and Providencia sp., Morganella sp.*

The method used in this thesis for the antibacterial activity screening is based on the EUCAST (The European Committee on Antimicrobial Susceptibility Testing) discussion document E.Dis 5.1 (EUCAST, 2003). The antibacterial activity of the fractions was tested on five known human pathogens: *S.aureus, E.coli, Enterococcus faecalis, P.aeruginosa and Streptococcus agalactiae*.

2.2.3.2 Biofilm related infections and biofilm formation inhibiting activity screening

Many types of bacterial infections are biofilm-related. Known examples are cystic fibrosis lung infection, chronic wounds and implant associated infections. These types of infections are a significant cause of morbidity and mortality. Biofilms are more tolerant to antibiotics and immune responses, compared to bacteria in planktonic form, which often lead to chronic infections (Wilkins, Hall-Stoodley, Allan, & Faust, 2014; Wu, Moser, Wang, Høiby, & Song, 2014). *Staphylococci* are common cases of infections that have biofilm production as an important virulence factor. Production of biofilm is dependent on the synthesis of polysaccharide intracellular adhesin (PIA), and the enzymes involved in PIA synthesis are encoded by the ica operon. Biofilm production is significantly increased in the presence of glucose (Agarwal & Jain, 2013; Cafiso et al., 2004).

The fractions produced in this thesis was screened for biofilm formation inhibiting activity against *Staphylococcus epidermidis*, in a spectrophotometric assay. This type of

spectrophotometric screening, using crystal violet to dye the biofilm, is a common method for measuring biofilm production and inhibition (Martínez Díaz et al., 2015; O'Toole, 2011), see figure 8.

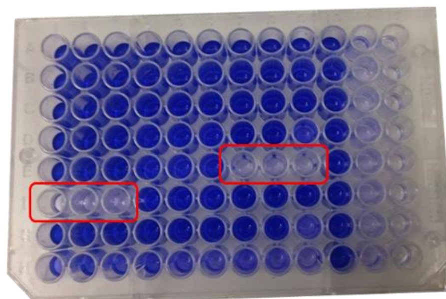

Figure 8: Biofilm dyed with crystal violet in biofilm formation inhibiting screening. The red field indicates inhibition of biofilm formation. Photo: Marte Jenssen.

2.2.3.3 Cancer and anticancer activity screening

In 2012, 14.1 million new cancer cases were diagnosed and 8.2 million people died from cancer. By the year 2025 it is expected that 19.3 million new cancer cases will be diagnosed each year, meaning that this is an increasing problem. The most common kinds of cancer are lung, breast, colorectal, stomach and liver cancer (WHO, 2013; WHO 2017a). Cancer is a term for a large group of diseases and is one of the leading causes of death. It is the uncontrolled growth of cells that can invade and spread to distant sites of the body (WHO, 2017a).

In this thesis, fractions were screened for anticancer activity in an Aqueous One Solution Cell Proliferation assay, often called the MTS reduction assay. Proliferation assays are used to measure cell growth over time and measure the effects of compounds on the cell growth. The Aqueous One Solution contains a tetrazolium salt (yellow colour), called MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt). Only metabolically active/living cells are able to reduce the salt to a formazan product, which has a dark purple colour (see figure 9). The amount of surviving cells is proportional with the level of formazan product produced (Promega, 2012). Formazan absorbs radiation at 490 nm, and the effect is measured spectrophotometrically.

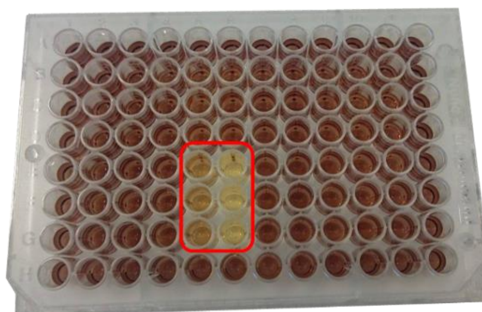

Figure 9: Microtiter plate with human melanoma cancer cells (A2058), after incubation with Aqueous One Solution. The red field indicates dead cancer cells that are unable to reduce MTS (yellow colour) to formazan (dark purple colour). Photo: Marte Jenssen.

2.2.4 Dereplication

Dereplication is an important step in the bioprospecting pipeline. The goal of dereplication is to identify and avoid further work on active substances that are already known. An efficient dereplication procedure should be incorporated early in the pipeline, to minimise the efforts put into the sample (Perez-Victoria, Martin, & Reyes, 2016). At Marbio, the bioactivity data is used to guide the dereplication process, so fractions with confirmed bioactivity are nominated for dereplication. Through this work, the research group seeks to evade replication of work that has previously been conducted, and can instead focus on finding novel compounds, or known compounds with novel bioactivities. One of the difficulties with the dereplication process is the presence of so-called pan assay interfering compounds (PAINS), which are compounds that have broad and non-specific bioactivities and often give false positives in bioactivity screenings. Some of these compounds are well known and investigated, and because of their non-specific activity, they are not interesting with regards to drug discovery (Baell & Holloway, 2010; Camp, Davis, Evans-Illidge, & Quinn, 2012). The field of PAINS has been extensively studied throughout the years (Aldrich et al., 2017; Baell & Holloway, 2010; Bisson et al., 2016), and is obviously a great problem in numerous screening programs. Through dereplication, the presence of these types of compounds can be detected and the sample can be removed from the pipeline (Hansen & Andersen, 2016).

In this study, an Ultra-Performance-LC-Quadrupole-Time-of-Flight Mass Spectrometer (UPLC-QToF-MS) was used for the dereplication. As a simplified description, the MS can be divided into five parts: The sample inlet, ion source, mass analyser, detector and the data system. The sample inlet is where the sample is introduced to the system. In the system used in this study the sample was introduced in liquid form, as it elutes from the UPLC column. In the ion source the sample molecules are converted into gas phase ions. There are several different ionisation methods, and in this study electrospray ionisation (ESI) was used: The sample is

sprayed out of a fine capillary with high voltage potential, charged droplets are expelled into the ionisation chamber, where they are subjected to a drying gas (nitrogen) which evaporates the solvent. The evaporation continues until solvent-free sample ions are left in gas phase. ESI can be used to produce both positive and negative ions. The ionised sample is then moved into the mass analyser, where the ions are separated according to their mass-to-charge (*m/z*) ratios. As with ion sources, there are several different mass analysers. In this study, the Time-of-Flight (ToF) mass analyser was applied. ToF is based on the velocities of the ions, which in turn depends on their masses. If two ions are created at the same time, with the same energy, the lighter ion will travel faster than the heavier ion, and hit the detector faster. The detector is an ion counter, which produces a current that is proportional to the number of ions that strikes it. The signal from the detector is transferred to a recorder, that sends the information to the computer system, where the mass spectrum is produced (Lampman, Pavia, Kriz, & Vyvyan, 2010, p. 418-434).

It is common to couple the MS to a liquid chromatography (LC) unit, and one of the most used hyphenated techniques today is High-Performance LC (HPLC)-MS. When coupling a HPLC (separation technique) to a MS (analytical technique) the sample can be separated on the column, and then analysed based on their mass spectral data. The MS gives information about the molecular weight and the fragmentation pattern of the molecules (Sarker & Nahar, 2012a). In this study, the MS was coupled to a UPLC system. The active fractions from the bioactivity screenings were compared to the inactive fractions, in order to identify compounds only present, or present at a higher concentration, in the active fraction. Possible elemental compositions were calculated and used to search databases like The Dictionary of Marine Natural Products. Figure 10 shows the features of a typical LC-MS system, and the continuing process with dereplication.

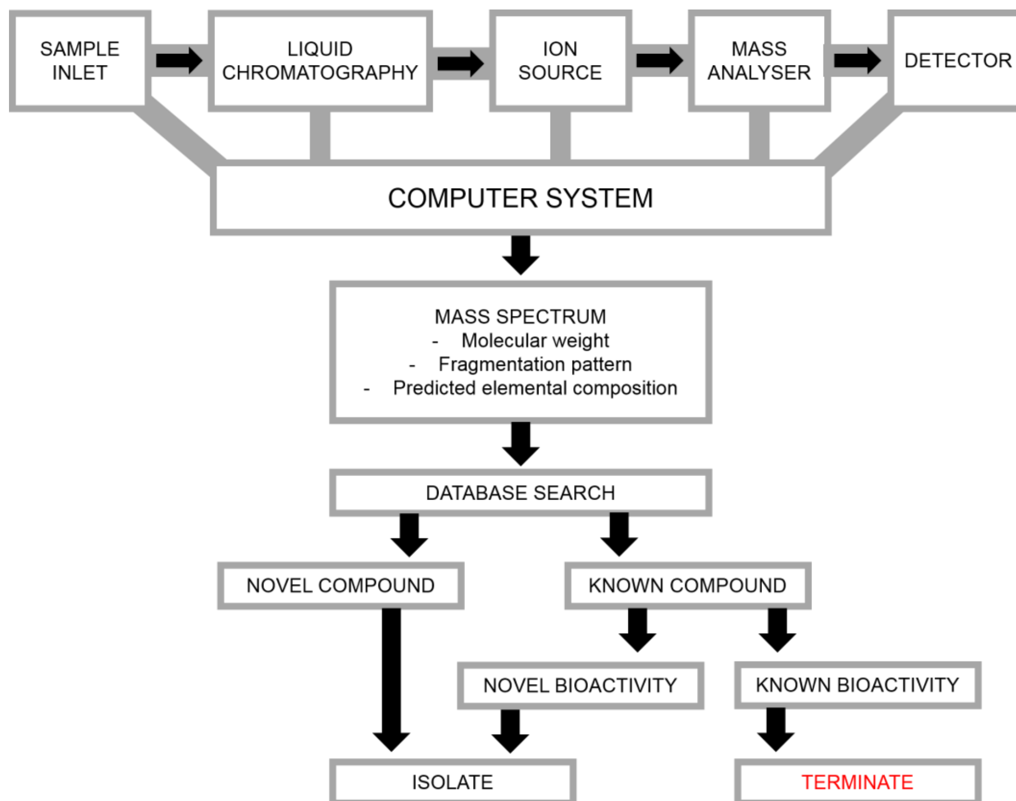

Figure 10: The components of a LC-MS system, inspired by reference (Silverstein, Webster, & Kiemle, 2005, p. 2), and the process of dereplication.

3 Aim of the thesis

The overall aim of this thesis was to investigate how altering cultivation parameters affected the production of bioactive fractions from cultures of two Arctic marine bacteria. The three key objectives were to:

1. Cultivate the two bacteria under seven different cultivation treatments
2. Screen the fractions from the bacterial cultures for antibacterial activity, inhibition of biofilm formation and anticancer activity
3. Dereplicate the active fractions in an attempt to identify the active compounds

4 Materials and methods

4.1 Sampling and storage

Materials

Table 3: The products/equipment used in sampling and storage.

Product/Equipment	Product ID/Equipment specifications	Distributor (Country)
***Polaribacter* sp.**	M09B074	Marbank, Institute of Marine Research (Norway)
***Leifsonia* sp.**	M10B719	Marbank, Institute of Marine Research (Norway)
Difco Marine Broth	279110	Becton, Dickinson and Company (New Jersey, USA)
Peptone from casein, enzymatic digest	82303	Sigma-Aldrich (Missouri, USA)
Filtered sea water	5 µm pore size, ceramic membrane filter 0.2 µm, UV (Ultra Violet) filter	Norwegian College of Fishery Science, UiT (Norway)
Glycerol	G5516	Sigma-Aldrich (Missouri, USA)
Milli-Q Ultrapure water		Merck KGaA (Germany)
Sanyo Labo Autoclave		Sanyo Electric Co. (Japan)
Stuart Heat Stir SB162		Stuart Equipment (UK)

Method

Two marine bacterial strains from the Marbank collection were used in this project; M09B074 from the genus *Polaribacter* and M10B719 from the genus *Leifsonia*. The bacteria will be abbreviated PB for *Polaribacter* and LS for *Leifsonia* throughout the thesis. PB was isolated from an invertebrate of the family *Sabellidaet*, collected the 17th of May 2009 in the Barents Sea, diving at Bjørnøya (74.3737N, 19.1984E). LS was isolated from the intestine/stomach of a hagfish (*Myxine glutinosa*), collected the 16th of April 2010 in the Norwegian Sea region on a benthic trawl in Hadselfjorden (68.5025N, 15.0046E). The bacteria were classified down to genus level by Marbank, based on 16S rDNA analysis (species not determined).

The isolates were stored in FMAP medium with 30% glycerol at -80°C. FMAP medium was prepared with 15 g/L Difco Marine broth and 5 g/L peptone in filtered seawater and Milli-Q Ultrapure water (MQ) with the ratio 3:7. Glycerol was added to the FMAP medium, and the solution was autoclaved (120 minutes, 121°C). The bacterial stock was prepared by plating the bacteria (from freeze stock isolates stored at -80°C), followed by incubation for 3-7 days at 10°C. A single colony was picked and transferred to 5 mL FMAP medium and incubated at 10°C for 2-3 days at 300 rpm (revultions per minute). From the culture, 500 µL was transferred

to cryo tubes together with 1 mL of FMAP with 30% glycerol. The isolates were stored at -80°C.

4.2 Preparation of cultivation media

Materials

Table 4: The products/equipment used in the preparation of cultivation media.

Product/Equipment	Product ID/Equipment specifications	Distributor (Country)
Sea salts	S9883	Sigma-Aldrich (Missouri, USA)
Malt extract	70167	Sigma-Aldrich (Missouri, USA)
Yeast extract	Y1625	Sigma-Aldrich (Missouri, USA)
Peptone from casein, enzymatic digest	82303	Sigma-Aldrich (Missouri, USA)
Iron(II) sulfate heptahydrate	1.03965	Merck KGaA (Germany)
Potassium bromide	221864	Sigma-Aldrich (Missouri, USA)
Dextrose	D9434	Sigma-Aldrich (Missouri, USA)
Casein hydrolysate	22090	Sigma-Aldrich (Missouri, USA)
Potassium chloride	1.04935	Merck KGaA (Germany)
Potassium dihydrogen phosphate	1.04871	Merck KGaA (Germany)
Corn flour		Risenta AB (Sweden)
Sodium nitrate	S5506	Sigma-Aldrich (Missouri, USA)
Magnesium sulphate heptahydrate	63138	Sigma-Aldrich (Missouri, USA)
Milli-Q Ultrapure water		Merck KGaA (Germany)
Sanyo Labo Autoclave		Sanyo Electric Co. (Japan)
Stuart Heat Stir SB162		Stuart Equipment (UK)

Method

LS and PB were cultivated in four different media. The media composition with relative nutritional levels are displayed in table 5. All media were prepared with MQ and autoclaved for 120 minutes at 121°C before use.

Table 5: Contents of the media used for cultivation of PB and LS. The relative nutritional levels of the different media are included. The two high nutrition media used, DVR_1 and DVR_2, are the same except for the addition of iron(II) sulphate heptahydrate and potassium bromide to DVR_2.

Media names	Nutritional level	Chemical	Amount
DVR_1	High	Sea salts	40.0 g/L
		Malt extract	6.667 g/L
		Peptone from casein, enzymatic digest	11.111 g/L
		Yeast extract	6.667 g/L
DVR_2	High	Sea salts	40.0 g/L
		Malt extract	6.667 g/L
		Peptone from casein, enzymatic digest	11.111 g/L
		Yeast extract	6.667 g/L
		Iron(II) sulphate heptahydrate (8 g/L stock)	0.0444 g/L
		Potassium bromide (8 g/L stock)	0.0444 g/L
DSGC	Intermediate	Dextrose	4.0 g/L
		Casein hydrolysate	3.0 g/L
		Sea salts	40.0 g/L
Corn flour medium	Low	Corn flour	1.0 g/L
		Sea salts	40.0 g/L
		Sodium nitrate	3.0 g/L
		Potassium dihydrogen phosphate	0.75 g/L
		Potassium chloride	0.25 g/L
		Magnesium sulphate heptahydrate	0.25 g/L

4.3 Seven cultivation treatments of the marine bacteria

Materials

The four different media described in table 5 were used for cultivation of the marine bacteria LS and PB.

Table 6: The products/equipment used in the seven cultivation treatments of the marine bacteria.

Product/Equipment	Product ID/Equipment specifications	Distributor (Country)
***Polaribacter* sp.**	M09B074	Marbank, Institute of Marine Research (Norway)
***Leifsonia* sp.**	M10B719	Marbank, Institute of Marine Research (Norway)
Infors HT Multitron Pro		Infors HT (Switzerland)
Sanyo Labo Autoclave		Sanyo Electric Co. (Japan)
Branson 3510 Ultrasonic Cleaner		Emerson Industrial Automation (Missouri, USA)
Herasafe biological safety cabinet	Class II	Thermo Fisher Scientific (Massachusetts, USA)

Method

Both bacteria were grown under seven different cultivation treatments, named cultivation treatment 1 - cultivation treatment 7 (C1-C7) (table 7). For each treatment, the bacteria were cultivated in four 1 L Erlenmeyer culture flasks with 250 mL medium (unless stated otherwise) in each flask. Preparation of the cultures was performed in a class II safety cabinet. To each culture flask, 370 µL of bacterial stock (See section 4.1 "Sampling and storage") was added. The flasks were sealed with aluminium foil during the cultivation. In the following section, the different cultivation treatments will be described with greater detail. Media controls (MCs) were also prepared for DVR_1, DVR_2, DSGC and corn flour medium, with 250 mL medium.

Table 7: Overview of the cultivation parameters used for the seven different cultivation treatments (C1-C7) that were conducted on PB and LS.

Cultivation treatment	Medium	Additional altered parameters	Temperature and shaking	Time of cultivation (days)
C1	DVR_1		10°C, 140 rpm	8
C2	DVR_2		10°C, 140 rpm	8
C3	DVR_1	Co-cultivation	10°C, 140 rpm	8 as monocultures, 4 days as co-cultures
C4	DVR_1	Cold-treatment	1-10°C, no shaking during cold treatment 4°C, 140 rpm during storage	12 days before cold-treatment, 3 days during cold-treatment
C5	DSGC		10°C, 140 rpm	14
C6	DSGC	Addition of dead bacteria	10°C, 140 rpm	8 days before addition, 6 days after addition
C7	Corn flour		10°C, 140 rpm	19

4.3.1 Normal conditions: C1, C2, C5 and C7

The cultivation treatments C1, C2, C5 and C7, did not have any additional altered parameters, but different media were used for the different cultivations (table 8). The bacterial cultures were incubated at 10°C with 140 rpm shaking. The incubation time of the cultures, before adding resin, varied. This variation was based on the time it took to obtain a visually dense bacterial culture (see table 8). Because of low density, eight culture flasks were made with corn flour cultures (C7), giving a total volume of 2000 mL, double of all the other cultures.

Table 8: Time of incubation for the cultures without any additional parameters altered (C1, C2, C5 and C7). The relative nutritional levels of the different media are included.

Cultivation treatment	Medium	Level of nutrition of media (relative)	Time of incubation before addition of resin (days)
C1	DVR_1	High	8
C2	DVR_2	High	8
C5	DSGC	Intermediate	14
C7*	Corn flour	Low	19

**The cultures prepared with corn flour medium (C7) were prepared with a total volume of 2000 mL, double of all the other cultures.*

4.3.2 Co-cultivation: C3

In treatment C3, the two strains were co-cultivated. The cultures were first grown as monocultures in DVR_1 medium, until dense cultures were obtained. Then (8 days after starting cultivation) 1% of PS was added to the LS, and reverse. The mixed cultivation continued for 4 days before resin was added. The cultures were kept at 10°C and 140 rpm for the entire cultivation.

4.3.3 Cold-treatment: C4

Both strains, grown in the DVR_1 medium, were cold treated by moving the cultures in and out of a -20°C freezer room (C4). Before starting the cold treatment, the cultures were grown for 12 days at 10°C and 140 rpm (dense cultures obtained). In the freezer room, the culture temperature was decreased to ~1-2°C. The cultures were then placed in room temperature (~21°C), where the temperature was increased to 10°C before putting them back into -20°C. In total, ten rounds in and out of the freezer room was conducted over 3 days. During the cold-treatment the cultures were not shaken. The cultures were incubated at 4°C and 140 rpm overnight during the cold-treatment period. Resin was added on the last day of cold-treatment. After adding resin, the cultures were incubated at 10°C and 140 rpm, until extraction.

4.3.4 Cultivation with dead marine bacteria: C6

For cultivation treatment C6, both strains were cultivated in DSGC medium for 8 days, before dead Arctic marine bacteria, *Leeuwenhoekiella* sp. (Marbank collection ID: M09W024) grown in M19 medium was added to the cultures (contents of the M19 medium are listed in Appendix 1). The bacteria was killed by first autoclaving (121°C for 120 minutes), followed by sonication for 10 minutes. Dead bacteria, 1% of the total culture volume, was then added to the cultures. The cultures were incubated for 6 days, before resin was added. For the entire time of cultivation, the cultures were kept at 10°C and 140 rpm.

4.4 Extraction from the bacterial cultures

Materials

Table 9: The products/equipment used in the extraction of secondary metabolites.

Product/Equipment	Product ID/Equipment specifications	Distributor (Country)
Methanol	34860	Sigma-Aldrich (Missouri, USA)
Agar	A1296	Sigma-Aldrich (Missouri, USA)
Difco Marine Broth	279110	Becton, Dickinson and Company (New Jersey, USA)
Peptone from casein, enzymatic digest	82303	Sigma-Aldrich (Missouri, USA)
Filtered sea water	5 µm pore size, ceramic membrane filter 0.2 µm, UV filter	Norwegian College of Fishery Science, UiT (Norway)
Diaion® HP-20	13607	Sigma-Aldrich (Missouri, USA)
Milli-Q Ultrapure water		Merck KGaA (Germany)
Cheesecloth filter, fine mesh		Dansk Hjemmeproduktion (Denmark)
Whatman® qualitative filter paper, grade 3	1003-090	Sigma-Aldrich (Missouri, USA)
Biofuge Pico Heraeus		Thermo Fisher Scientific (Massachusetts, USA)
Rotary Evaporator (Rotavapor)	Laborota	Heidolph Instruments GmbH & Co. (Germany)

Method

For extraction of the secreted secondary metabolites, Diaion® HP-20 resin was added to the cultures, 40 g/L medium. Before addition, the resin was activated by soaking it in 100% methanol for minimum 30 minutes, before it was washed extensively with MQ. Small amounts of MQ was used to add the resin to the cultures. All cultures were incubated with resin for 3 days before the extraction was performed. Cultures and controls were extracted using the same method. Before starting the extraction, a 400 µL sample was taken from the cultures (not MCs) as a DNA storage sample (see 4.4.1 "Treatment of DNA storage samples"). To check for contamination, the cultures were plated on FMAP agar before extraction. The plates were stored at 10°C and checked for growth after a few days of incubation. FMAP agar was prepared with FMAP medium with 15 g/L of agar added before autoclaving.

The cultures with resin were vacuum filtered using cheesecloth filters. To remove the culture medium, it was poured over the filter with the resin beads remaining in the culture flask. Next, the resin was washed with 200 mL of MQ and filtered. To extract the secondary metabolites

from the resin, 100 mL of methanol was added to the resin followed by incubation for minimum 1 hour with gentle shaking. After incubation the extract was filtrated through a Whatman filter. Methanol was added again, 100 mL, to the resin for another round of extraction, with minimum 15 minutes of incubation. The extract was again filtrated, into the same flask as the last extraction. From the extract, a 400 µL sample was taken for a MS analysis storage sample. The MS sample was stored at -20°C. The extracts were dried under pressure at 40°C using the rotavapor, and stored at -20°C until use. A total of 14 extracts were made, seven from each bacterium (C1-C7). The extracts were named according to their bacterium (PB or LS) and cultivation treatment (C1-C7). Example: LS with cultivation treatment C1 was named LSC1.

4.4.1 Treatment of DNA storage samples

Before extraction, 400 µL of culture was transferred to an Eppendorf tube and centrifuged for 5 minutes at 13 000 rpm. The supernatant was decanted, and the pellet washed with 1 mL autoclaved MQ. The sample was again centrifuged for 5 minutes at 13 000 rpm, and the supernatant decanted. The pellet was stored at -20°C for possible identity check of the cultures.

4.5 Prefractionation of the extracts

Materials

Table 10: The products/equipment used in the prefractionation of extracts.

Product/Equipment	Product ID/Equipment specifications	Distributor (Country)
Methanol	34860	Sigma-Aldrich (Missouri, USA)
Acetone	34850	Sigma-Aldrich (Missouri, USA)
Diaion® HP-20SS	13615-U	Sigma-Aldrich (Missouri, USA)
Milli-Q Ultrapure water		Merck KGaA (Germany)
Rotary Evaporator (Rotavapor)	Laborota	Heidolph Instruments GmbH & Co (Germany)
Biotage® HPFC SP4 Flash Purification System		Biotage (Sweden)
Biotage® SNAP Cartridge KP-Sil (10 g)	FSK0-1107-0010	Biotage (Sweden)
Universal Shaker SM 30		Edmund Bühler GmbH (Germany)

Method

Prefractionation of the extracts, using the Biotage Flash system, was performed to produce six fractions from each extract (MCs were not fractionated). The extracts were dissolved in 90% methanol before adding 2 g of Diaion® HP-20SS column material. If an extract consisted of more than 1.5 g material, it was divided in two, and 2 g column material was added to each

part. Further, the extracts with column material was dried using the rotavapor. When the sample was completely dry it was added to a prepacked SNAP column (see 4.5.1 "Preparation of SNAP columns"), and run on the Biotage system with MQ, methanol and acetone mobile phases (table 11). The flow rate was 12 mL/minute and each fraction was collected for two minutes. From the prefractionation, 27 tubes were obtained. These tubes were pooled, resulting in six fractions (table 12). The fractions are abbreviated fraction 1-fraction 6 (F1-F6) throughout the thesis.

Table 11: Mobile phase gradient used with the Biotage flash system for the prefractionation of the extracts.

Time (minutes)	MQ (%)	Methanol (%)	Acetone (%)	Fraction number
0-6	95	5	0	1-3
6-12	75	25	0	4-6
12-18	50	50	0	7-9
18-24	25	75	0	10-12
24-36	0	100	0	13-18
36-42	0	50	50	19-20
42-54	0	0	100	21-27

Table 12: The 27 flash tubes were pooled, resulting in six fractions (F1-F6).

Fraction	Flash tube
F1	1, 2, 3
F2	4, 5, 6
F3	7, 8, 9
F4	10, 11, 12
F5	13, 14, 15
F6	16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27

4.5.1 Preparation of SNAP columns

The SNAP 10 g cartridges used in prefractionation were pre-packed with Diaion® HP-20SS column material (adsorbent); 6.5 g of the adsorbent was pretreated by soaking in methanol for minimum 20 minutes, then washing extensively with MQ. The column material was added to the cartridges using a vacuum manifold, and was stored with MQ at 4°C until further use.

4.6 Stock solution preparation

Materials

Table 13: The products/equipment used in stock solution preparation.

Product/Equipment	Product ID/Equipment specifications	Distributor (Country)
Dimethyl Sulfoxide	D4540	Sigma-Aldrich (Missouri, USA)
Milli-Q Ultrapure water		Merck KGaA (Germany)
Syncore® Polyvap		Büchi (Switzerland)
Heto PowerDry® PL9000 Freeze Dryer		Thermo Fisher Scientific (Massachusetts, USA)

Method

After prefractionation, the fractions were dried using Polyvap and dissolved in dimethyl sulfoxide (DMSO) with concentrations varying between 10 mg/mL to 80 mg/mL (depending on the amount of material after drying). The fractions were stored at -20°C until further use. From the MC extracts, stock solutions with a concentration of 40 mg/mL in DMSO were prepared and stored at -20°C. For bioactivity screening, deep-well plates (DWPs) were prepared with all the fractions and MCs. The fractions and MCs were frozen in the DWPs, and freeze-dried until completely dry. The fractions were dissolved in autoclaved MQ with 2.5% DMSO to a concentration of 1 mg/mL. The plates were stored at -20°C when not in use, and at 4°C when in use (maximum one week). When screening for different bioactivities, the fractions were taken from these plates (termed test-DWP throughout the thesis).

4.7 Bioactivity screening of fractions and media controls

All fractions and MCs were screened for antibacterial activity, inhibition of biofilm formation and anticancer activity. The work was conducted in a class II safety cabinet. First, all fractions were screened using one concentration (50 µg/mL) in the primary screening. The active fractions were retested in a secondary screening with several concentrations, to confirm activity and to check for dose-response activity. All screenings were performed in 96-well microtiter plates, and absorbance measurements were used for evaluation of results. The different bioactivity screenings will be described in greater detail in the following sections.

4.7.1 Antibacterial activity screening

Materials

Table 14: The products/equipment used in the antibacterial activity screening.

Product/Equipment	Product ID/Equipment specifications	Distributor (Country)
Mueller Hinton broth (MH)	275730	Becton, Dickinson and Company (New Jersey, USA)
Brain heart infusion broth (BHI)	53286	Sigma-Aldrich (Missouri, USA)
Sodium chloride	S5886	Sigma-Aldrich (Missouri, USA)
Blood agar plates		University hospital of North Norway (UNN), (Norway)
Luria-Bertoni plates		University hospital of North Norway (UNN), (Norway)
Glycerol	G5516	Sigma-Aldrich (Missouri, USA)
Gentamycin (10 mg/mL)	A 2712	Merck KGaA (Germany)
Milli-Q Ultrapure water		Merck KGaA (Germany)
Staphylococcus aureus	ATCC® 25923	LGC Standards (UK)
Escherichia coli	ATCC® 25922	LGC Standards (UK)
Enterococcus faecalis	ATCC® 29212	LGC Standards (UK)
Pseudomonas aeruginosa	ATCC® 27853	LGC Standards (UK)
Streptococcus agalactiae	ATCC® 12386	LGC Standards (UK)
Heated Incubator MIR-262		Panasonic Healthcare (Japan)
Incubator Unimax 1010		Heidolph Instruments GmbH & Co (Germany)
Victor Multilabel Counter		Perkin Elmer (Massachusetts, USA)
Herasafe biological safety cabinet	Class II	Thermo Fisher Scientific (Massachusetts, USA)

Method

4.7.1.1 Preparation of the test bacteria

The antibacterial activity of the fractions and MCs was screened against five bacterial test strains (table 15). The test bacteria were stored in the same medium that they were grown in with 10% glycerol at -80°C. When in use, the bacteria were kept on blood agar plates (maximum 1 month), with re-streaks every second week for maintenance of the bacteria. When the screening was to be performed, a scoop of bacteria was transferred into 8 mL growth medium, and allowed to grow overnight at 37°C. From the overnight cultures, 2 mL was transferred into 25 mL fresh cultivation medium. The cultures were incubated with shaking at 37°C for the time period stated in table 15 to reach 0.5 McFarland turbidity (1.0 x 10^8 bacteria/mL). After incubation, the bacterial solution was further diluted 1:1000 in the appropriate cultivation medium.

Table 15: The test bacteria used in the antibacterial activity screening, their cultivation media, and incubation time needed to reach 0.5 McFarland turbidity.

Bacterial strain	Medium for cultivation	Incubation time (second day)
S.aureus	MH	2.5 h
E.coli	MH	1.5 h
E.faecalis	BHI	1.5 h
P.aeruginosa	MH	2.5 h
S.agalactiae	BHI	1.5 h

4.7.1.2 Preparation of the 96-well microtiter plates

Fractions and MCs were taken from the test-DWP and diluted with autoclaved MQ to the chosen concentrations. In the primary screening, all fractions and MCs were screened at a final concentration of 50 µg/mL (in duplicates). In the secondary screening, the active fractions were screened at 50, 25, 10 and 1 µg/mL (in duplicates). From the fraction/MC, 50 µL was transferred to a 96-well microtiter plate, one plate per bacteria to be tested. From the prepared 1:1000 bacterial solutions, 50 µL was added to the fractions/MC, making the total dilution of the bacterial solution in the screening 1:2000 (from 0.5 McFarland turbidity). Positive and negative controls for the screening were also included on the plates. Negative control was prepared with 50 µL medium and 50 µL autoclaved MQ. Positive control was prepared with 50 µL autoclaved MQ and 50 µL bacterial suspension. All the plates were incubated at 37°C for 20-24 hours.

4.7.1.3 Reading of plates and evaluation of results

After 20-24 hours of incubation at 37°C, the plates were first checked visually for growth inhibition. Absorbance (Abs) of the microtiter plates was then measured at 600 nm. Software used was WorkOut 2.5 (dasdaq, England). Threshold Abs_{600} values were used to define the fractions either active, questionable or inactive:

- Active ≤0.05
- Questionable 0.05-0.09
- Inactive ≥0.09

Fractions deemed active were retested in the secondary screening.

4.7.1.4 Gentamycin control

Gentamycin controls were performed routinely, as a control for normal growth of the bacteria, and as a control for the assay. The control was performed in final concentrations ranging from 16 to 0.01 µg/mL in order to determine the minimum inhibitory concentration (MIC) of

gentamycin for the test bacteria. In a 96-well microtiter plate, 50 µL gentamycin control was added to 50 µL bacterial solution. The plate was incubated overnight at 37°C. The MIC values were evaluated visually by looking for growth inhibition. If the MIC values for gentamycin were more than one titer step outside of the reference values (table 16), the screening had to be run again.

Table 16: Reference MIC values and CFU ranges for the test bacteria used in the antibacterial screening.

Test bacteria	**Reference MIC values for gentamycin (µg/mL)**	**CFU ranges**
S.aureus	0.25	0.5-3x10^5 CFU/mL
E.coli	0.50	0.5-3x10^5 CFU/mL
E.faecalis	10.00	0.5-3x10^5 CFU/mL
P.aeruginosa	0.50	3-7x10^4 CFU/mL
S.agalactiae	4.00	0.5-3x10^5 CFU/mL

4.7.1.5 Control of colony forming unit

Control of colony forming unit (CFU) were also performed routinely, as a control for steady growth of the bacteria. After 1.5/2.5 hour incubations on the second day of the screening, the bacterial solution was diluted in 0.9% sodium chloride solution, first 1:100 times two, and then 1:10 times two. The two last dilutions (1:100 000 and 1:1 000 000) were plated in two parallels on Luria-Bertoni plates with 100 µL culture, and incubated overnight at 37°C. The number of colonies was counted, and the CFU was calculated and checked against the standard CFU ranges (table 16).

4.7.2 Biofilm formation inhibiting activity screening

Materials

Table 17: The products/equipment used in the biofilm formation inhibiting activity screening.

Product/Equipment	Product ID/Equipment specifications	Distributor (Country)
Tryptic soy broth (TSB)	1.05459	Merck KGaA (Germany)
Dextrose (Glucose)	D9434	Sigma-Aldrich (Missouri, USA)
Ethanol	24106	Sigma Aldrich (Missouri, USA)
Blood agar plates		University hospital (UNN) Tromsø, (Norway)
Crystal violet	1.15940	Merck KGaA (Germany)
Glycerol	G5516	Sigma-Aldrich (Missouri, USA)
Milli-Q Ultrapure water		Merck KGaA (Germany)
Staphylococcus epidermidis	ATCC 35984	University hospital (UNN) Tromsø, (Norway)
Staphylococcus haemolyticus	Clinical isolate 8-7A	University hospital (UNN) Tromsø, (Norway)
Heated Incubator MIR-262		Panasonic Healthcare (Japan)
Incubator Unimax 1010		Heidolph Instruments GmbH & Co (Germany)
Victor Multilabel Counter		Perkin Elmer (Massachusetts, USA)
Herasafe biological safety cabinet	Class II	Thermo Fisher Scientific (Massachusetts, USA)

Method

4.7.2.1 Preparation of test bacteria

The fractions were screened for biofilm formation inhibiting activity against *S.epidermidis*. *S.haemolyticus* was used as a control of a non-biofilm forming bacterium. Both bacteria were stored in tryptic soy broth (TSB) medium with 10% glycerol at -80°C. When in use, the bacteria were kept on blood agar plates (maximum one month), being re-streaked every second week. One scoop of each bacterium was inoculated in 5 mL of TSB and incubated overnight at 30°C. After overnight incubation, the cultures were diluted 1:100 in TSB with 1% glucose (inducer).

4.7.2.2 Preparing 96-well microtiter plates

Fractions and MCs were taken from the test-DWP and diluted with autoclaved MQ. In the primary screening, all fractions and MCs were screened at a final concentration of 50 µg/mL (in triplicates). In the secondary screening, the fractions were screened at 50, 25, 10 and 1 µg/mL (in duplicates). From the test DWP with fractions and MCs, 50 µL was transferred to a 96-well microtiter plate. From the bacterial suspension of *S.epidermidis* (in TSB with 1% glucose), 50 µL was added to all the wells with fractions or MCs. Positive control for the

screening was 50 µL *S.epidermidis* culture and 50 µL autoclaved MQ. Negative control for the screening was 50 µL *S.haemolyticus* culture and 50 µL autoclaved MQ. A medium blank with TSB and 1% glucose was also included in the screening, with 50 µL medium and 50 µL autoclaved MQ. The microtiter plates were incubated at 37°C overnight.

After the overnight incubation, the plates were visually examined for growth inhibition to check that antibacterial activity was not misinterpreted as biofilm formation inhibiting activity. The bacterial suspension was then poured out of the plate, and the wells were rinsed with water. To fixate the biofilm to the bottom of the wells, the plates were incubated at 55°C for 1 hour. After fixation the biofilm was coloured, by adding 70 µL 0.1% crystal violet solution to all the wells, and incubated for 5 minutes. The crystal violet solution was then poured off and the plate was again rinsed with water. The plates were incubated again at 55°C for about 1 hour or until the plates were dry.

4.7.2.3 Reading plates and evaluating results

To all the wells, 70 µL 70% ethanol was added, and the plates were incubated for 10 minutes at room temperature. Absorbance was measured for the microtiter plates at 600 nm. Software used was WorkOut 2.5 (dasdaq, England). The fractions were considered active if the measured Abs_{600} was below 0.25.

4.7.3 Anticancer activity screening

Materials

Table 18: The products/equipment used in the anticancer activity screening.

Product/Equipment	Product ID/Equipment specification	Distributor (Country)
Earle's Minimal Essential Medium (E-MEM) with 20 mM HEPES	F4315	Merck KGaA (Germany)
Roswell Park Memorial Institute medium (RPMI) 1640	FG 1383	Merck KGaA (Germany)
Dulbecco's Modified Eagle Medium (D-MEM), high glucose, GlutaMAX™ Supplement, HEPES	32430027	Thermo Fisher Scientific (Massachusetts, USA)
Non-essential amino acids (NEA) (100x)	K 0293	Merck KGaA (Germany)
L-Alanyl-L-Glutamine (200 mM)	K 0302	Merck KGaA (Germany)
Gentamycin (10mg/mL)	A 2712	Merck KGaA (Germany)
Sodium Bicarbonate solution (7.5%)	L 1713	Merck KGaA (Germany)
Sodium Pyruvate (100 mM)	L 0473	Merck KGaA (Germany)
CellTiter 96® Aqueous One Solution Reagent	G358B	Promega (Wisconsin, USA)
Fetal Bovine Serum (FBS)	S0115	Merck KGaA (Germany)
A2058	ATCC® CRL-11147™	LGC Standards (UK)
MRC-5	ATCC® CCL-171™	LGC Standards (UK)
Trypsin (1:250)	27250018	Thermo Fisher Scientific (Massachusetts, USA)
Triton™ X-100	T8787	Sigma-Aldrich (Missouri, USA)
Sanyo CO_2 Incubator MCO-18AIC		Panasonic Biomedical (Japan)
DTX 880 Multimode Detector		Beckman Coulter (California, USA)
Herasafe biological safety cabinet	Class II	Thermo Fisher Scientific (Massachusetts, USA)

Method

All fractions were screened for anticancer activity against the human melanoma cancer cell line A2058. In the primary screening, all fractions were tested in one concentration to check for activity. The fractions that were considered active were subjected to secondary screening at different concentrations against A2058, and a normal human fibroblast cell line (MRC-5) as a control for toxicity.

4.7.3.1 Maintenance of the cell cultures

A2058 was cultivated in D-MEM medium with additions, and MRC-5 was cultivated in E-MEM with additions (table 19). The cell cultures were split when cell density was 70-80%, to maintain the cells in a monolayer. When splitting the cells the growth medium was discarded, and the cells were washed with phosphate-buffered saline (PBS) for about 1 minute before trypsinating (0.25% in PBS) for maximum 15 seconds (see Appendix 2 for preparation of PBS and trypsin solution). Trypsin was discarded and the cultures were incubated at 37°C for 3-6 minutes. When the cells were detached from the bottom of the flask, the cells were resuspended in 10 mL growth medium. A new culture flask was prepared with fresh medium, and transferring sufficient amounts of resuspended cells to reach a cell density of 70 – 80% before the next round of splitting. The cells were kept at 37°C and 5% CO_2.

Table 19: Cultivation media (with additions) used for cultivating the different cell lines. RPMI-1640 was used when adding fractions to the cells. D-MEM was used when maintaining the A2058 cells in culture, and E-MEM was used when maintaining MRC-5 cells in culture. MRC-5 was included as a control for toxicity.

RPMI-1640	D-MEM	E-MEM
10% FBS 0.01 mg/mL Gentamycin 1% L-Alanyl-L-Glutamine	10% FBS 0.01 mg/mL Gentamycin 1% L-Alanyl-L-Glutamine	10% FBS 0.01 µg/mL Gentamycin 1% L-Alanyl-L-Glutamine 1% NEAA 1% Sodium Pyruvat 1% Sodium bicarbonat

For screening, 100 µL cell suspension was seeded out in 96-well microtiter plates. The resuspended cells that had been trypsinated were seeded with approximately 2000 cells per well (A2058) and 4000 cells per well (MRC-5). The cells were diluted in appropriate medium, to reach the wanted cell densities, and added to the microtiter plates. The plates were incubated for 24 hours at 37°C, 5% CO_2.

4.7.3.2 MTS cell proliferation assay, reading and evaluation of results

After 24 hours of incubation, the cells were ready for the addition of fractions/MCs. The fractions and MCs were taken from the test-DWP and diluted in RPMI (table 19). In the primary screening, all fractions and MCs were screened with a final concentration of 50 µg/mL (in triplicates). In the secondary screening, the active fractions were screened at 50, 25, and 10 µg/mL (in duplicates). Before fractions/MCs were added to the cells, the cell medium was removed. Then, 100 µL fraction/MC (diluted in RPMI) was added to the cells. Negative controls for the plates were made with RPMI medium. Cells treated with 0.5% triton were used as a positive control. The plates were further incubated for ~72 hours at 37°C and 5% CO_2.

After 72 hours of incubation, 10 µL Aqueous One Solution was added to each well, and the plates were further incubated for 1 hour. Absorbance was measured at 485 nm, using the DTX 880 Multimode Detector and the Multimode Analysis Software (Beckman Coulter, USA). Cell survival was calculated (equation 1). Fractions that gave cell survival of less than 50% were considered active, and were further tested in the secondary screening against both A2058 and MRC-5.

$$\text{Survival (\%)} = \frac{(\text{AbsF-AbsP}) \times 100}{(\text{AbsN-AbsP})}$$

Equation 1: Calculation of cell survival (%) in MTS cell proliferation assay. Absorbance fraction (AbsF) is the average of Abs_{485} measured in wells with fractions, Absorbance positive control (AbsP) is the average of Abs_{485} measured in wells with positive control, and Absorbance negative control (AbsN) is the average of Abs_{485} measured in wells with negative control.

4.8 Dereplication of active fractions from bioactivity screening

Materials

Table 20: The products/equipment used in the dereplication of active fractions.

Product/Equipment	Product ID/Equipment specifications	Distributor (Country)
HPLC glass vials		Waters (Massachusetts, USA)
VION ® IMS QToF		Waters (Massachusetts, USA)
Acquity UPLC PDA Detector		Waters (Massachusetts, USA)
Acquity UPLC Column Manager		Waters (Massachusetts, USA)
Acquity UPLC I-Class Sample Manager FTN		Waters (Massachusetts, USA)
Acquity UPLC I-Class Binary Solvent Manager		Waters (Massachusetts, USA)
ACQUITY UPLC BEH C18 Column, 130Å, 1.7 µm, 2.1 mm X 100 mm	186002352	Waters (Massachusetts, USA)
Methanol LC-MS Ultra CHROMASOLV®	14262	Thermo Fisher Scientific (Massachusetts, USA)
Milli-Q Ultrapure water		Merck KGaA (Germany)
Formic acid 99% ULC/MS	069141	Biosolve B.V. (Netherland)
LiChrosolv® Acetonitrile Hypergrade for LC-MS	1.00029	Merck KGaA (Germany)

Method

The fractions that were deemed active in the secondary bioactivity screenings were further analysed using UPLC-QToF-MS. Before injection, the fractions were diluted 1:20 in 80% methanol and transferred to HPLC glass vials. The fractions were taken from the DMSO stock solutions (see section 4.6 "Stock solution preparation"). The injection volume used was 1 µL,

and the samples were run on the Acquity UPLC class system, with a C18 column, followed by the Vion IMS QToF. All samples were run on ESI+ mode, and complex samples were also run on ESI- mode. The following mobile phases were used:

- Mobile phase A: MQ + 0.1% formic acid
- Mobile phase B: Acetonitrile + 0.1% formic acid

The UPLC gradient of the mobile phases is listed in table 21, and the ESI+/- parameters for the Vion IMS QToF are listed in table 22.

Table 21: Mobile phase gradient of mobile phase A (MQ + 0.1% formic acid) and mobile phase B (Acetonitrile + 0.1% formic acid) used on the Aquity UPLC system connected to the MS.

Time (minutes)	Flow rate (mL/minute)	Mobile phase A (%)	Mobile phase B (%)
0	0.450	90	10
12	0.450	0	100
13.50	0.450	0	100

Table 22: Parameters utilised for the Vion IMS QToF in ESI+ and ESI- during the dereplication process.

Parameters	ESI+/ESI-
Mass range	50-2000 *m/z*
Capillary voltage	0.80 kV
Cone voltage	30 V
Source temperature	120°C
Desolvation temperature	450°C
Cone gas flow	50 L/h
Desolvation gas flow	800 L/h
Low collision energy	6.0 eV
High collision energy	15-45 eV
Scan time	0.2 s

The active fractions were compared to the inactive fractions eluted before and the inactive fractions eluted after the active fraction (from the prefractionation) to see if there were compounds only present, or present in higher concentrations, in the active fractions compared to the inactive ones. When a possible candidate responsible for the observed activity was detected, the software Waters UNIFI was used to calculate a possible elemental composition based on the accurate mass and the isotopic pattern of the compound. The calculated elemental compositions were used to search databases, primarily the Dictionary of Marine Natural Products, the Dictionary of Natural Products and ChemSpider.

5 Results

5.1 Extraction from the bacterial cultures

The two bacterial isolates, LS and PB, were grown under seven different cultivation treatments (see table 7). After cultivation, secreted secondary metabolites were harvested and extracted from the culture medium using Diaion® HP-20 resin and methanol, obtaining 14 extracts, seven from each isolate. The weight of the extracts varied between 400-3300 mg (figure 11). The weight was higher for the cultures grown in the high nutrition media DVR_1 and DVR_2 (C1-C4). For treatment C1-C4, the extracts produced from the LS cultures weighed more than the PB counterparts, while the weight was quite similar for the two strains for the intermediate nutritional medium DSGC cultures (C5-C6) and the low nutrition corn flour medium cultures (C7).

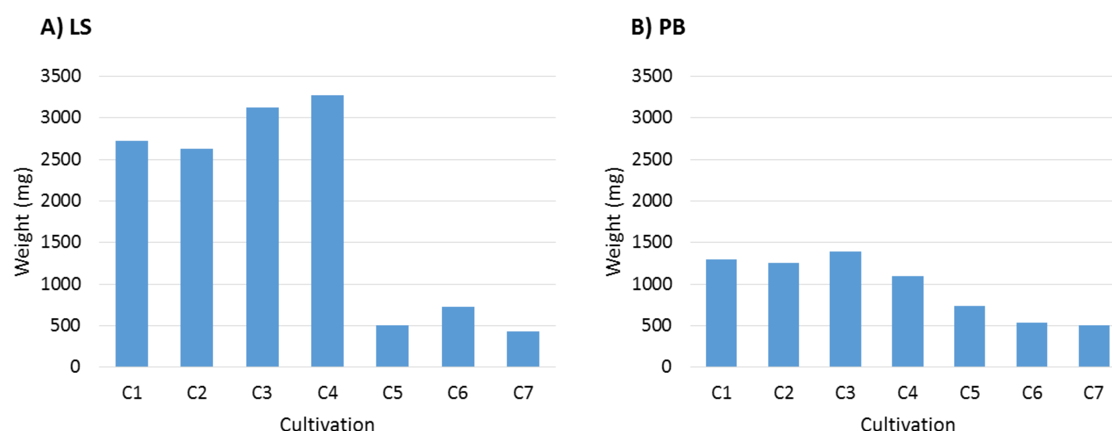

Figure 11: Weight of the extracts from the seven different cultivation treatments (C1-C7) prepared from A) LS and B) PB. The secreted secondary metabolites were harvested with Diaion® HP-20 resin, and extracted with methanol. The extracts were prepared from 1 L of bacterial culture, except for the two extracts from the corn flour cultures (C7) which were prepared from 2 L of bacterial culture.

Extracts of the media controls (MCs) were prepared for each medium used (figure 12). The two high nutrition media DVR_1 and DVR_2 gave extracts with the highest weights, approximately 890 mg from 250 mL of medium, while DSGC gave approximately 225 mg from the same volume. The low nutrition corn flour medium produced an extract of approximately 44 mg.

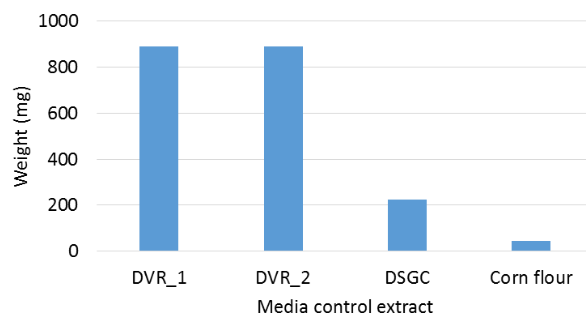

Figure 12: Weight of media control extracts for each medium used in the cultivation of LS and PB. Media components were harvested with Diaion® HP-20 resin and extracted using methanol. The weight is based on extraction of 250 mL of medium.

5.2 Prefractionation of the extracts

The extracts were prefractionated using flash chromatography. Each extract was fractionated into six fractions, giving a total of 84 fractions for further bioactivity screening. Figure 13 and figure 14 show the weight of the fractionations from LS and PB, respectively. Generally, there was higher weight of the first three fractions (F1-F3) than in the last three (F4-F6), especially for F5 and F6 that had very low weights, ranging from 2-58 mg.

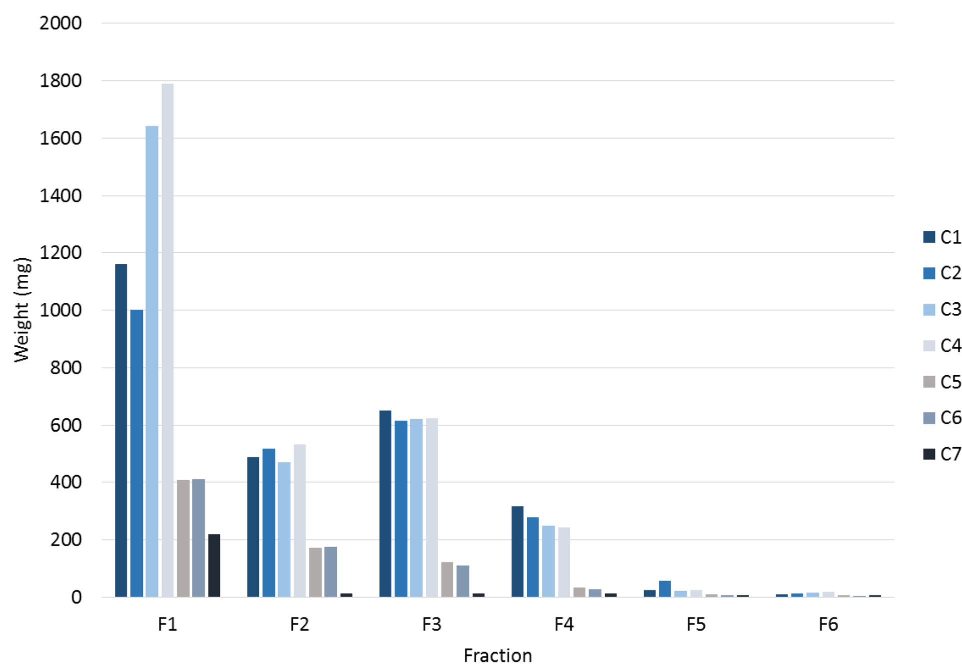

Figure 13: Weight of the fractions (F1-F6) from the extracts made from the seven different cultivation treatments (C1-C7) of LS. The extracts were fractionated using flash chromatography with Diaion® HP-20SS columns and mobile phase gradient ranging from polar (MQ) to non-polar (acetone).

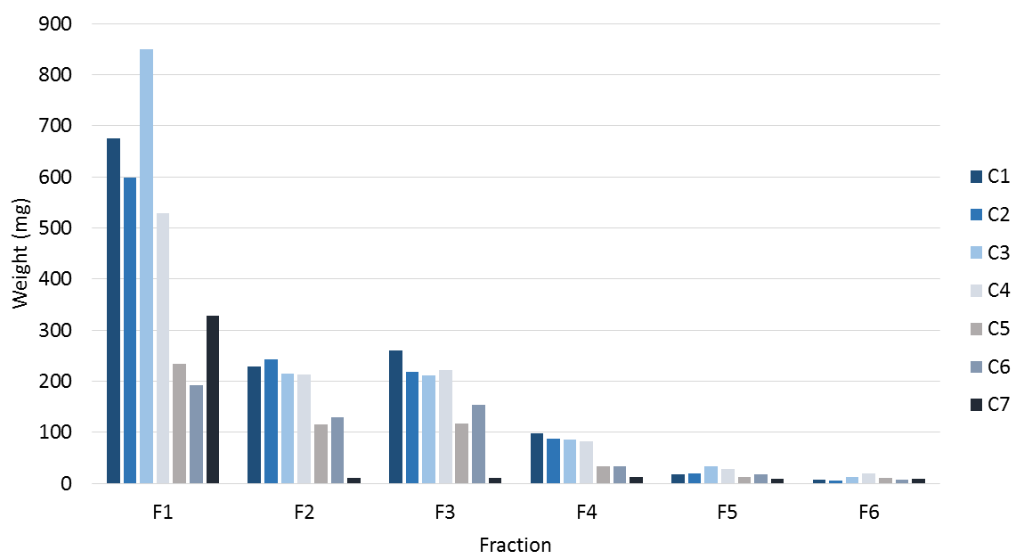

Figure 14: Weight of the fractions (F1-F6) from the extracts made from the seven different cultivations (C1-C7) of PB. The extracts were fractionated using flash chromatography with Diaion® HP-20SS columns and mobile phase gradient ranging from polar (MQ) to non-polar (acetone).

5.3 Bioactivity screening of fractions and media controls

In total, 84 fractions were prepared and screened for antibacterial activity, biofilm formation inhibiting activity and anticancer activity. No activity was observed for fractions 1, 2 or 3 (F1-F3) for any extracts, so these will not be further mentioned. Table 23 shows a compilation of the results from the primary screenings for all the fractions F4-F6. Fractions deemed active in the primary screening were further tested in a secondary screening with different concentrations. At Marbio, cut-off values are used in the primary screenings to decide which fractions/extracts are interesting for further screening. In this project, the cut-off values were used as guidance and to limit the amount of samples to be further investigated. The cut-off values are listed under the results from the individual bioactivity screenings. In total, eight fractions were deemed active from LS, and six from PB after the primary screenings. Some of the fractions were active in more than one of the bioactivity screenings. None of the MCs displayed any activity in the primary bioactivity screenings (no further work was conducted on the MCs).

Table 23: Results from the primary bioactivity screenings of the fractions (F4-F6) from the seven cultivations (C1-C7) of LS and PB. The fractions were screened for antibacterial activity, biofilm formation inhibiting activity and anticancer activity.

LS	Fraction	Anti-bacterial	Biofilm inhibiting	Anti-cancer	PB	Fraction	Anti-bacterial	Biofilm inhibiting	Anti-cancer
C1	F4				**C1**	F4			
	F5					F5			
	F6			+		F6			+
C2	F4				**C2**	F4			
	F5					F5			
	F6		+			F6			+
C3	F4				**C3**	F4			
	F5		+	+		F5	+		+
	F6					F6	+		+
C4	F4				**C4**	F4			
	F5					F5			
	F6					F6			
C5	F4				**C5**	F4			
	F5			+		F5			
	F6					F6			
C6	F4			+	**C6**	F4			
	F5	+		+		F5	+		
	F6					F6			
C7	F4			+	**C7**	F4			
	F5		+	+		F5		+	+
	F6					F6			

+ indicates active fraction, blank indicates not active fraction. Blue indicates activity in one screening, green indicates activity in several screenings.

5.3.1 Antibacterial activity screening

5.3.1.1 Primary screening

All fractions were screened for antibacterial activity with a concentration of 50 µg/mL against five known human pathogenic bacteria: *S.aureus*, *E.coli*, *E.faecalis*, *P.aeruginosa* and *S.agalactiae*. Four fractions were deemed active with Abs_{600} levels of 0.05 or below (cut-off value), see table 24.

Table 24: Active fractions from the primary antibacterial activity screening, conducted on the fractions (50 µg/mL) obtained from the LS and PB cultures. Fractions were incubated with the test strains, S.aureus, E.coli, E.faecalis, P.aeruginosa and S.agalactiae for ~24 hours. Normal Abs600 values for the test strains when uninhibited: S.aureus 0.23-0.30, E.coli 0.35-0.40, E.faecalis 0.25-0.30, P.aeruginosa 0.46-0.56, S.agalactiae 0.19-0.23.

Active fraction	Abs_{600}	Inhibited strain
LSC6F5	0.05/0.04	*E.faecalis / S.agalactiae*
PBC3F5	0.05	*S.aureus*
PBC3F6	0.04	*S.aureus*
PBC6F5	0.04	*S.agalactiae*

5.3.1.2 Secondary screening

The active fractions from the primary antibacterial activity screening were retested on the bacteria that they were active against with the following concentrations: 50, 25, 10, 1 µg/mL. Antibacterial activity was confirmed for all fractions, and for some of the fractions dose-response activity could be observed. From LS, one fraction (LSC6F5) was active against both *S.agalactiae* and *E.faecalis* (figure 15). This fraction was active in the three highest concentrations against *S.agalactiae*, and against *E.faecalis* it displayed dose-response activity.

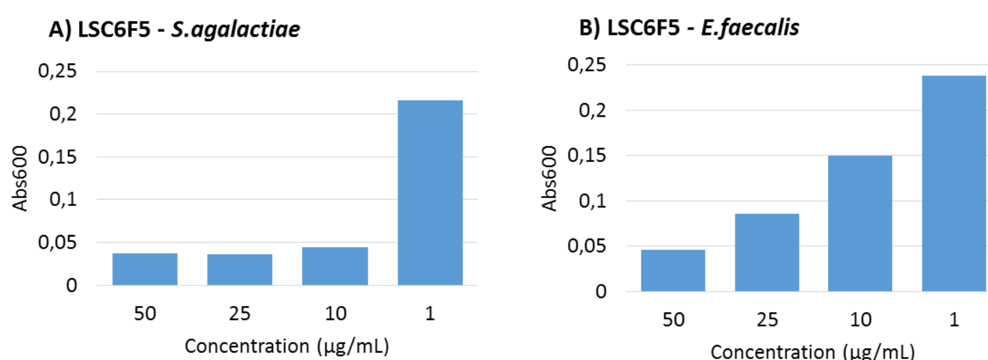

Figure 15: Results from the secondary antibacterial activity screening of the active fraction obtained from LS. LSC6F5 with activity against A) S.agalactiae and B) E.faecalis, after ~24 hours incubation. The retest was conducted at 50, 25, 10 and 1 µg/mL.

For PB, three fractions were retested and the activity was confirmed (figure 16). Two of the fractions originated from the same extract, PBC3, and were deemed active against *S.aureus*. PBC3F5 was active at the two highest concentrations, while PBC3F6 was active at the three highest concentrations. PBC6F5 was active against *S.agalactiae* at the two highest concentrations.

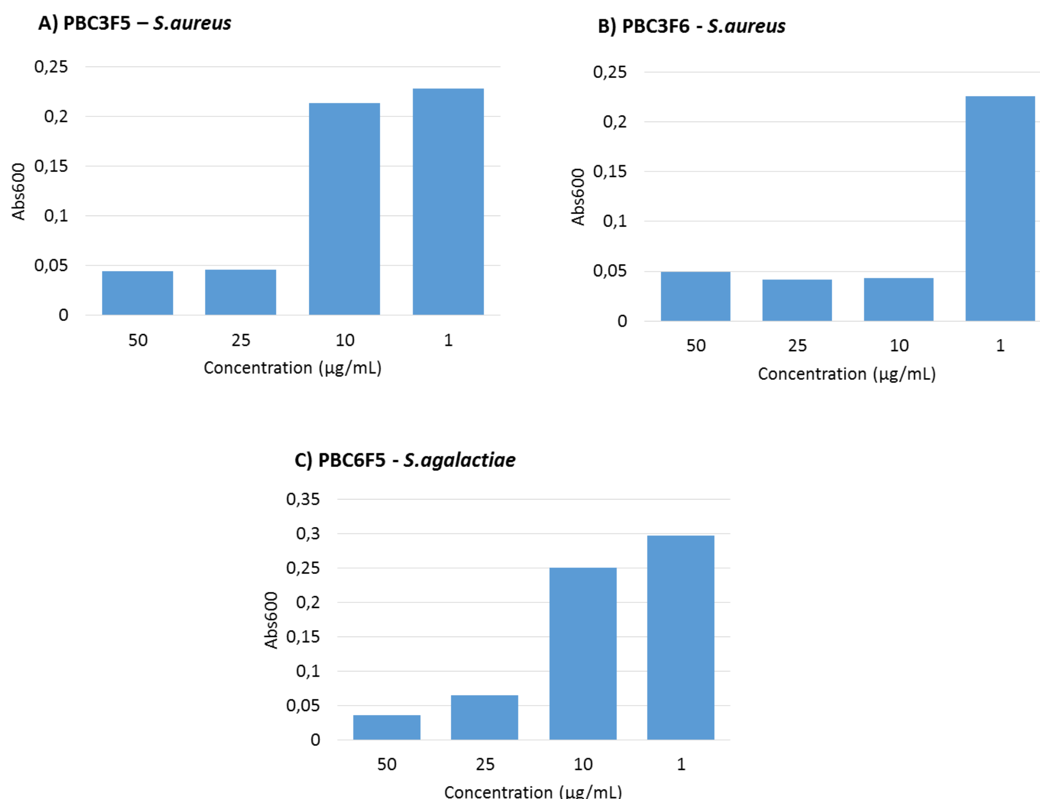

Figure 16: Results from the secondary antibacterial screening of the active fractions obtained from PB: A) PBC3F5 with activity against S.aureus, B) PBC3F6 with activity against S.aureus and C) PBC6F5 with activity against S.agalactiae. The retest was conducted at 50, 25, 10 and 1 µg/mL and the incubation period was ~24 hours.

5.3.2 Biofilm formation inhibiting activity screening

5.3.2.1 Primary screening

All fractions were screened for inhibition of the biofilm formation of *S.epidermidis,* with the concentration 50 µg/mL. Fractions that gave Abs_{600} of 0.25 or lower were considered active. Before fixating the biofilm the bacterial cultures were checked for growth inhibition, and none of the active fractions seemed to influence the growth of *S.epidermidis*. One fraction with Abs_{600} of 0.26 was also included in further screening for biofilm formation inhibiting activity. Four fractions displayed activity, three from LS, and one from PB (table 25).

Table 25: Active fractions from the primary biofilm formation inhibiting activity screening against S.epidermidis, conducted on the fractions (50 µg/mL) obtained from the LS and PB cultures. Incubation for ~24 hours. Normal Abs_{600} values for S.epidermidis when uninhibited was ~0.9.

Active fraction	Abs_{600}
LSC2F6	0.06
LSC3F5	0.06
LSC7F5	0.21
PBC7F5	0.26

5.3.2.2 *Secondary screening*

All fractions that were deemed active in the primary screening for biofilm formation inhibiting activity were retested in multiple concentrations: 50, 25, 10 and 1 µg/mL. In the secondary screening, none of the fractions gave Abs_{600} measurements beneath the cut-off value (0.25). Two fractions, LSC3F5 and PBC7F5, displayed some activity, with Abs_{600} values ~0.3 at the highest concentration (figure 17), and were nominated for dereplication. The fractions LSC2F6 and LSC7F5 lost all activity, and the work on the fractions was terminated (results not shown).

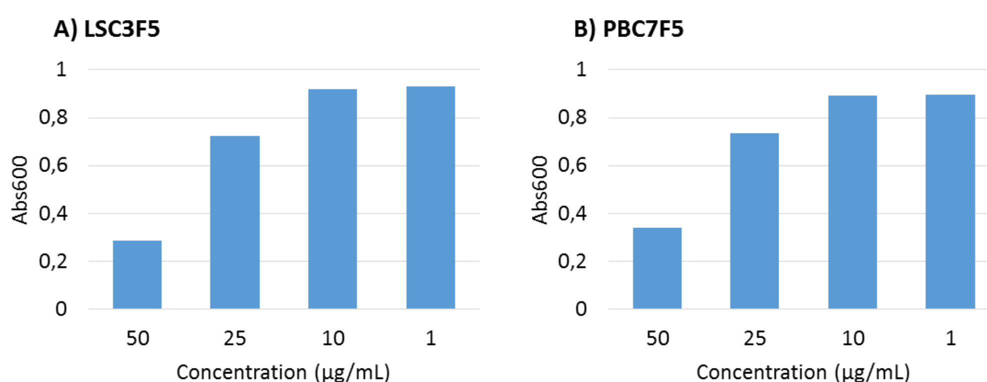

Figure 17: Results from the secondary biofilm formation inhibiting activity screening of the active fractions obtained from LS and PB cultures. Two fractions had confirmed bioactivity against biofilm formation of S.epidermidis: A) LSC3F5 and B) PBC7F5, after ~24 hours of incubation. The retest was conducted at 50, 25, 10 and 1 µg/mL.

5.3.3 Anticancer activity screening

5.3.3.1 *Primary screening*

All fractions were screened for anticancer activity against the human melanoma cancer cell line A2058 at 50 µg/mL, in a MTS cell proliferation assay. Twelve fractions were deemed active with cell survival lower than 50% (cut-off value). Five of the active fractions came from PB, and seven from LS extracts (table 26).

Table 26: Active fractions from the primary anticancer activity screening (MTS cell proliferation assay) against A2058, conducted on the fractions (50 µg/mL) obtained from LS and PB cultures. Incubation for ~72 hours.

Active fraction	Cell survival (%)	Active fraction	Cell survival (%)
LSC1F6	48	**LSC7F5**	14
LSC3F5	33	**PBC1F6**	41
LSC5F5	24	**PBC2F6**	44
LSC6F4	37	**PBC3F5**	15
LSC6F5	1	**PBC3F6**	4
LSC7F4	40	**PBC7F5**	21

5.3.3.2 Secondary screening

The fractions that were active against A2058 in the primary screening were further tested against both A2058 and a normal human fibroblast cell line (MRC-5) in the following concentrations: 50, 25 and 10 µg/mL. The MRC-5 cell line was included as a test for general toxicity. From the 12 active fractions in the primary screening, six fractions were confirmed active against A2058 in the secondary screening, two originated from LS (figure 18), and four from PB (figure 19). For the fraction LSC6F5, a dose-response activity was observed against both A2058 and MRC-5. The same was observed for LSC7F5, though the fraction was more active against A2058 than MRC-5.

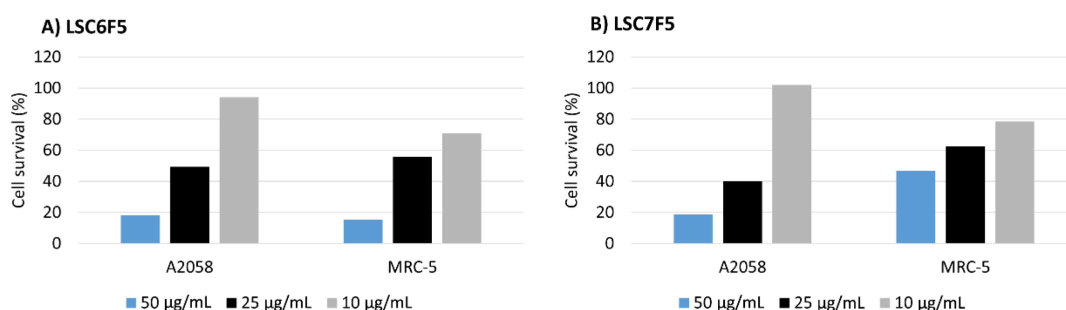

Figure 18: Results from the secondary anticancer activity screening (MTS cell proliferation assay) of the active fractions obtained from LS cultures, against A2058 (malignant) and MRC-5 (non-malignant): A) LSC6F5 and B) LSC7F5. The retest was conducted at 50, 25, and 10 µg/mL and the incubation period was ~72 hours. MRC-5 included as a test for toxicity.

Generally, for the four active fractions from PB (figure 19), a dose-response activity could be observed against the A2058 cancer cells, while a low general activity is seen against the normal MRC-5 cell line at all concentrations.

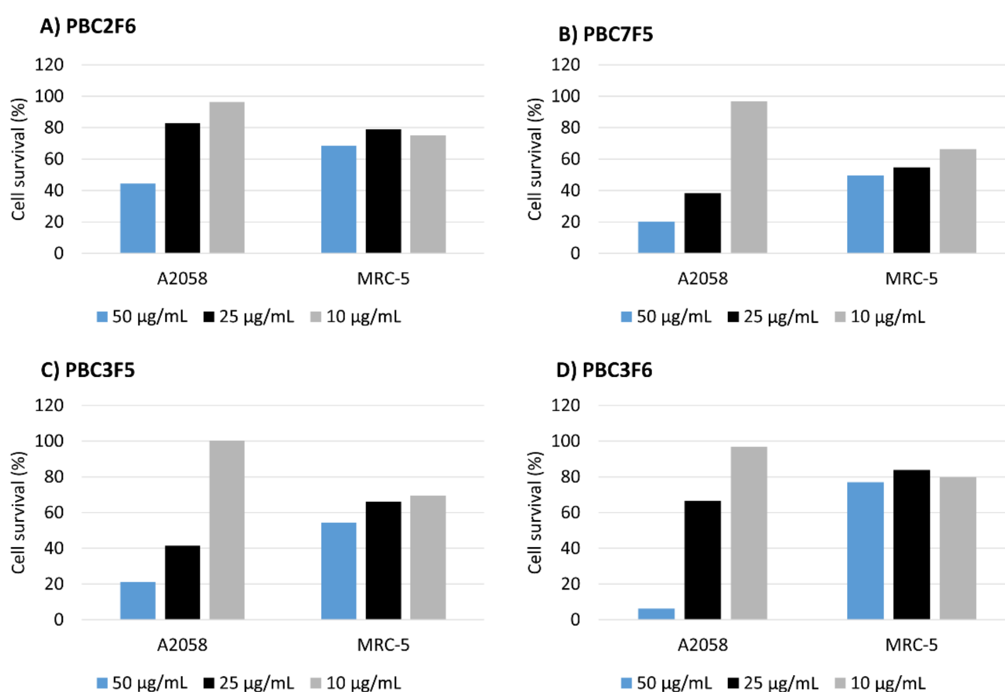

Figure 19: Results from the secondary anticancer activity screening (MTS cell proliferation assay) of the active fractions obtained from the PB cultures, against A2058 (malignant) and MRC-5 (non-malignant): A) PBC2F6, B) PBC7F5, C) PBC3F5 and D) PBC3F6. The retest was conducted at 50, 25 and 10 µg/mL and the incubation period was ~72 hours. MRC-5 included as a test for toxicity.

Three fractions were considered "questionable" after the secondary screening, because they were not as active as in the primary screening, and the cell survival at 50 µg/mL concentration was slightly above 50%. Three fractions had lost all activity in the secondary screening, with cell survival above 90% for all concentrations (results not shown). The work on the questionable fractions and the fractions that lost activity was terminated after the secondary screening. Figure 20 shows the results of the three fractions that were deemed questionable in the screening. The fractions gave cell survival of ~60% at 50 µg/mL for A2058, and the activity against the MRC-5 cells was quite similar, except for fraction LSC1F6, which did not display any activity against MRC-5 at any concentrations.

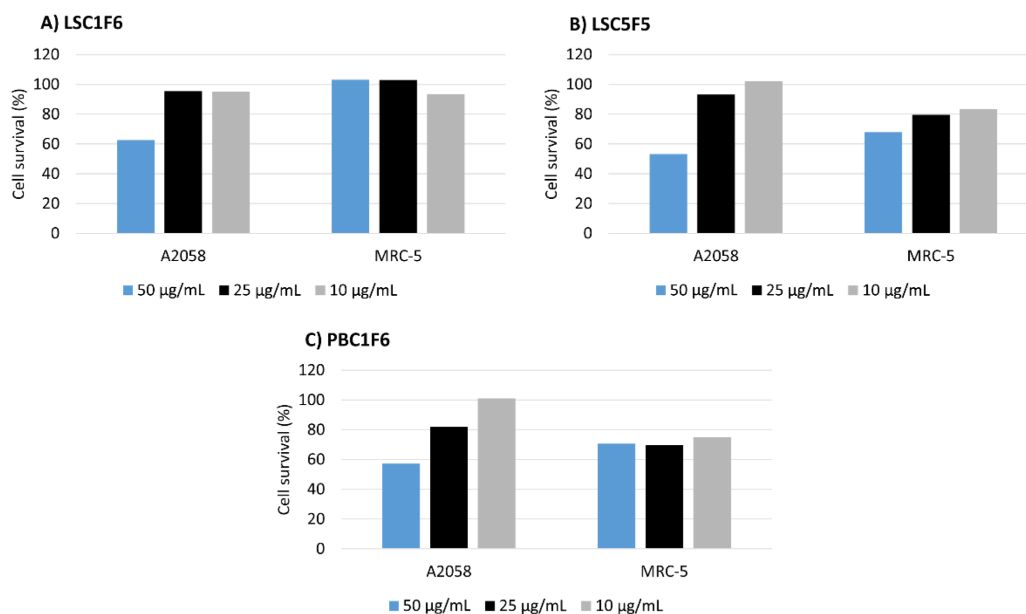

Figure 20: Activity of the three fractions that were considered questionable in the secondary anticancer activity screening (MTS cell proliferation assay) against A2058 (malignant) and MRC-5 (non-malignant): A) LSC1F6, B) LSC5F5 and C) PBC1F6. The fractions were tested at 50, 25, 10 and 1 µg/mL with ~72 hours of incubation. MRC-5 included as a test for toxicity.

5.4 Dereplication of active fractions from bioactivity screening

The fractions that were active in the secondary bioactivity screenings were nominated for dereplication using UPLC-QToF-MS. Some of the fractions were deemed active in more than one screening, as displayed in table 27. In the dereplication, the active fractions were compared to the inactive fractions eluting before and after the active one, in order to find compounds that were only present, or present at higher concentrations, in the active fraction. These compounds are candidates for the bioactivity of the fraction. All samples were run in positive ESI mode, unless stated otherwise. In the following sections, the results from the UPLC-QToF-MS analysis and the database searches obtained during the dereplication process will be presented as five cases.

Table 27: Active fractions from the secondary screenings for antibacterial activity (active against A2058), biofilm formation inhibiting activity and anticancer activity. The fractions that were considered active in the secondary screenings were nominated for dereplication.

Active fraction	Antibacterial activity	Biofilm formation inhibiting activity	Anticancer activity
LSC3F5		+	
LSC6F5	+		+
LSC7F5			+
PBC2F6			+
PBC3F5	+		+
PBC3F6	+		+
PBC6F5	+		
PBC7F5		+	+

+ indicates activity, blank indicates no activity. Blue indicates activity in one screening, green indicates activity in several screenings.

5.4.1 Case 1 – One clear candidate – LSC3F5

Fraction LSC3F5 was deemed active in the biofilm formation inhibiting activity screenings. From the Base Peak Intensity (BPI) chromatograms for the active (LSC3F5) and inactive fractions (LSC3F4 and LSC3F6), there was especially one peak that stood out in the active fraction (figure 21). This peak, with retention time 7.03 and a *m/z* of 400.2467 was investigated, resulting in an elemental composition calculated to be $C_{24}H_{33}NO_4$. Searching the Dictionary of Marine Natural Products gave two hits: Aspochalasin C and Phomasetin.

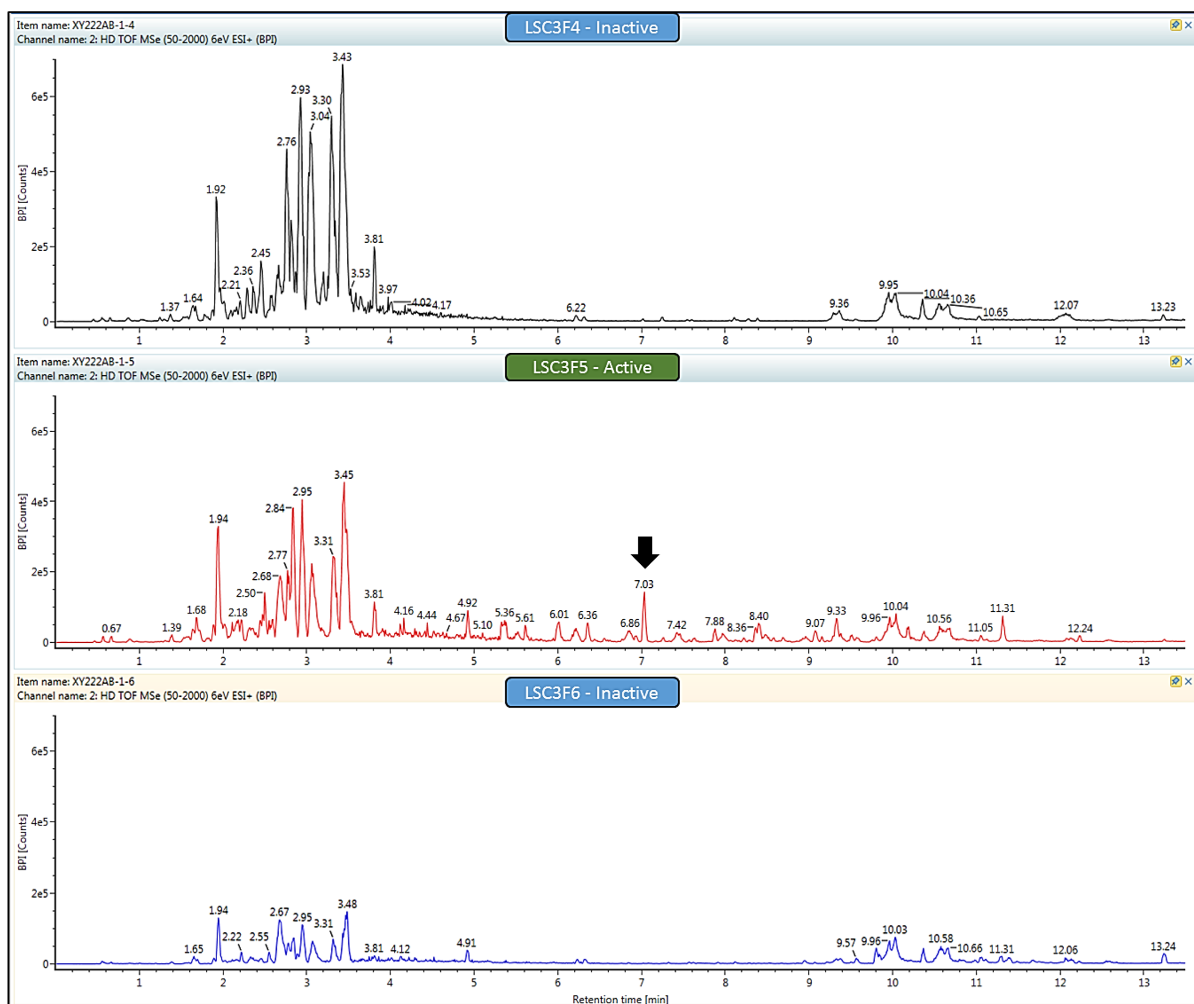

Figure 21: BPI chromatogram ESI+ (m/z 50-2000) for the comparison of fractions LSC3F4 (inactive), LSC3F5 (active) and LSC3F6 (inactive) from LS in the dereplication process. The arrow indicates the compound with retention time 7.03 (m/z 400.2467) that was further investigated.

5.4.2 Case 2 – Interfering compounds – LSC7F5 and PBC7F5

Two fractions from the low nutrition corn flour cultures, one from each bacterium, were deemed active in the bioactivity screening and they were nominated for dereplication. Fraction LSC7F5 was deemed active in the secondary screening for anticancer activity, and fraction PBC7F5 was deemed active in both the anticancer and the biofilm formation inhibiting activity screening. In both fractions, the presence of phosphocholines was observed. These compounds have been observed at numerous occasions at Marbio, and the elemental composition and retention time in this study were consistent with previous findings. Two phosphocholines were detected in both fraction PBC7F5 and LSC7F5 (figure 22 and figure 23). Table 28 provides more information about these compounds based on the information obtained from the database searches with the predicted elemental compositions of the phosphocholines.

Table 28: The calculated elemental compositions for the discovered phosphocholines (in fractions LSC7F5 and PBC7F5) were used for database searches. For the compound with retention time 7.89 there was one phosphocholine hit. Several hits for phosphocholines were found for the compound with retention time 8.38, three were included for illustration.

Retention time (minutes)	*m/z*	Predicted elemental composition	Results from data search
7.89	520.3393	$C_{26}H_{50}NO_7P$	1-linoleoyl-sn-glycero-3-phosphocholine
8.38	496.3392	$C_{24}H_{50}NO_7P$	Multiple hits for phosphocholines: • Glycerol 1-alkanoate 3-phosphocholines • 1-Palmitoyl-sn-glycero-3-phosphocholine • 2-Palmitoyl-sn-glycero-3-phosphocholine

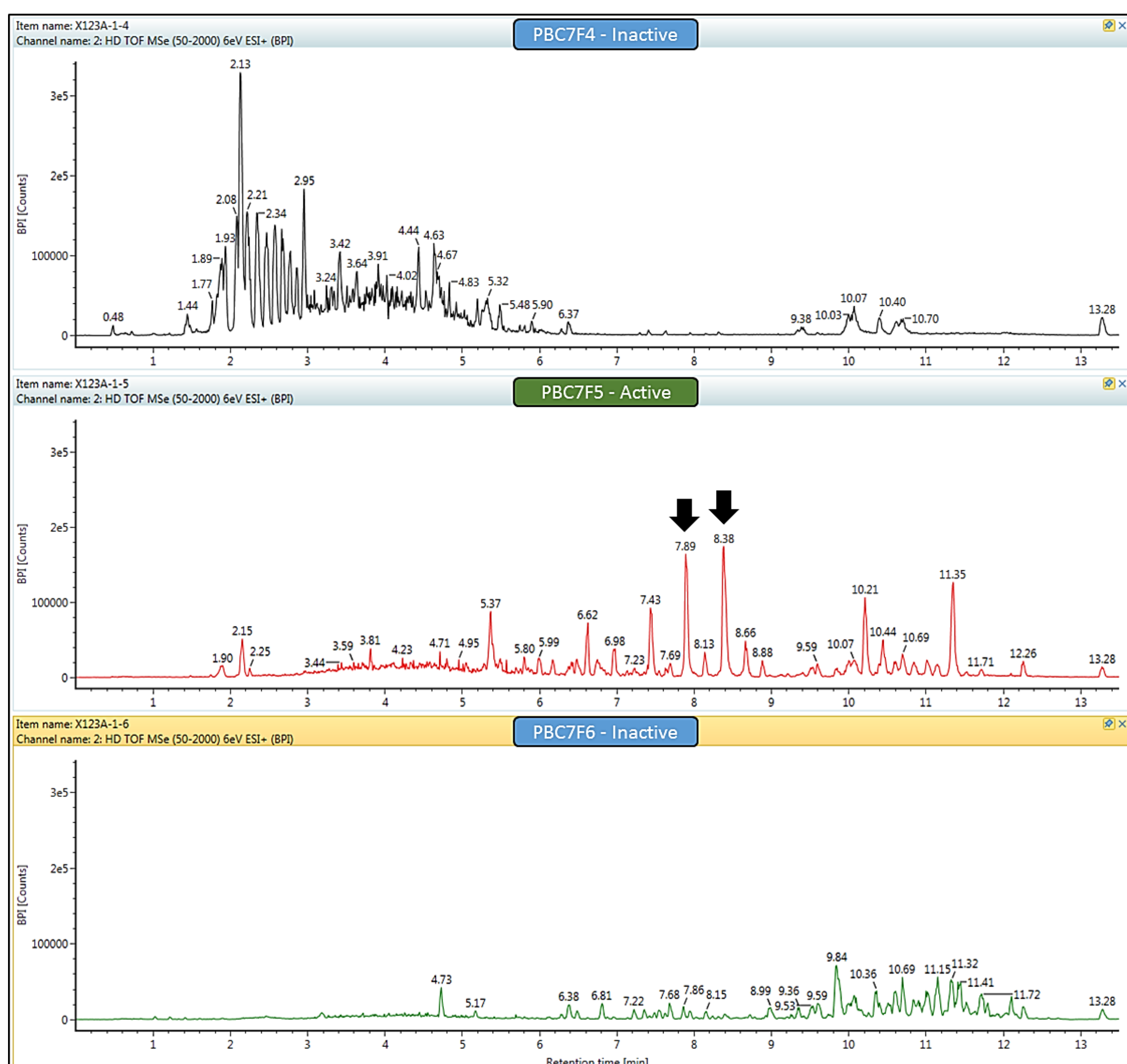

Figure 22: BPI chromatogram ESI+ (m/z 50-2000) for the comparison of fractions PBC7F4 (inactive), PBC7F5 (active) and PBC7F6 (inactive) from PB in the dereplication process. The arrows indicate the compounds that were presumed to be phosphocholines (retention times 7.89 and 8.38).

In fraction LSC7F5 another compound, in addition to the phosphocholines, was investigated. The compound eluted at 4.38 minutes, with m/z 430.1611 (indicated with a red arrow in figure 23). The elemental composition was calculated to be $C_{21}H_{23}N_3O_7$. This compound can also be seen in the inactive fraction LSC7F4 (retention time 4.35), but at lower levels. The elemental composition $C_{21}H_{23}N_3O_7$ was used to search the Dictionary of Marine Natural Products, resulting in two hits: Eurypamide C and Serratiochelin A.

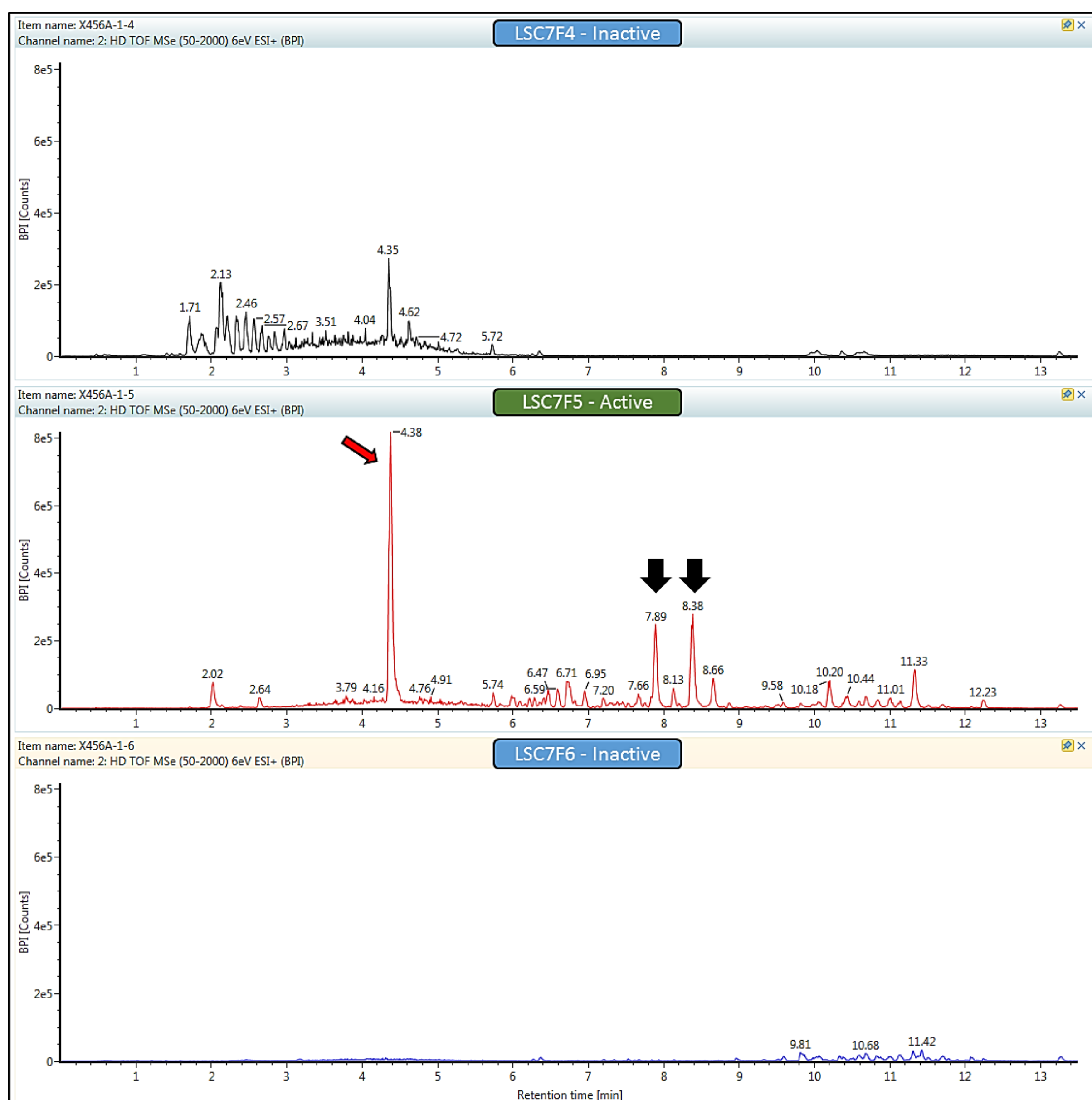

Figure 23: BPI chromatogram ESI+ (m/z 50-2000) for the comparison of fractions LSC7F4 (inactive), LSC7F5 (active) and LSC7F6 (inactive) from LS in the dereplication process. The black arrows indicate the compounds that were presumed to be phosphocholines, and the red arrow shows the peak of the compound with retention time 4.38 (m/z 430.1611).

5.4.3 Case 3 – Complex samples – PBC3F5 and PBC3F6

Two fractions from the same extract, PBC3F5 and PBC3F6, displayed activity in both the anticancer and antibacterial activity screenings. The fractions were analysed in positive ESI, and the chromatograms obtained can be seen in figure 24. In this case the chromatograms were complicated to interpret, and there were many similarities between the two active fractions, giving many candidates that could be responsible for the bioactivity. The arrows in figure 24 show examples of candidates: One compound with retention time 5.73 and one at 6.32 minutes. The elemental compositions were calculated and used in database searches. When searching for the compound with the retention time 5.73 (calculated elemental composition $C_{23}H_{22}N_2O_2$) there were no hits in the Dictionary of Marine Natural Products, nor in the Dictionary of Natural Products. The same was observed with the compound eluted at 6.32 (calculated elemental composition $C_{28}H_{33}N_3$), where no hits were found in the above-mentioned databases. Since the fractions were complex, they were also run on negative ESI mode, but the run did not provide any information that was used further (results not shown).

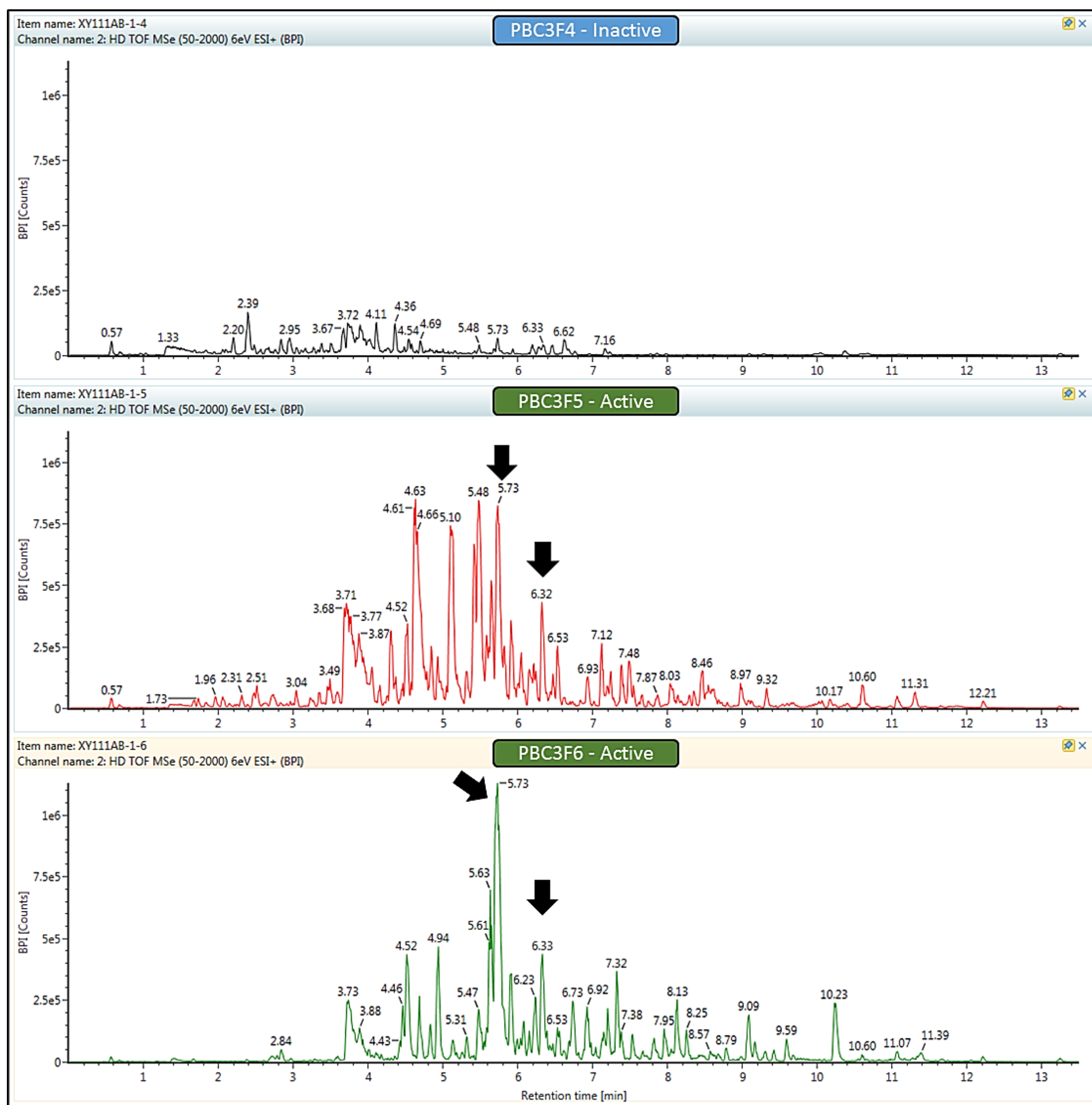

Figure 24: BPI chromatogram ESI+ (m/z 50-2000) for the comparison of fractions PBC3F4 (inactive), PBC3F5 (active) and PBC3F6 (active) from PB in the dereplication process. The arrows indicate compounds (retention times 5.73 and 6.32) that were further investigated.

5.4.4 Case 4 – Related compounds – PBC2F6

PBC2F6 was deemed active against A2058 cells in the anticancer activity screening. In this case, seven compounds stood out during the analysis (figure 25). It was discovered that there could be some type of relationship between these compounds, because the calculated elemental compositions were similar. In addition, the UV data for the compounds was examined and seemed to be similar.

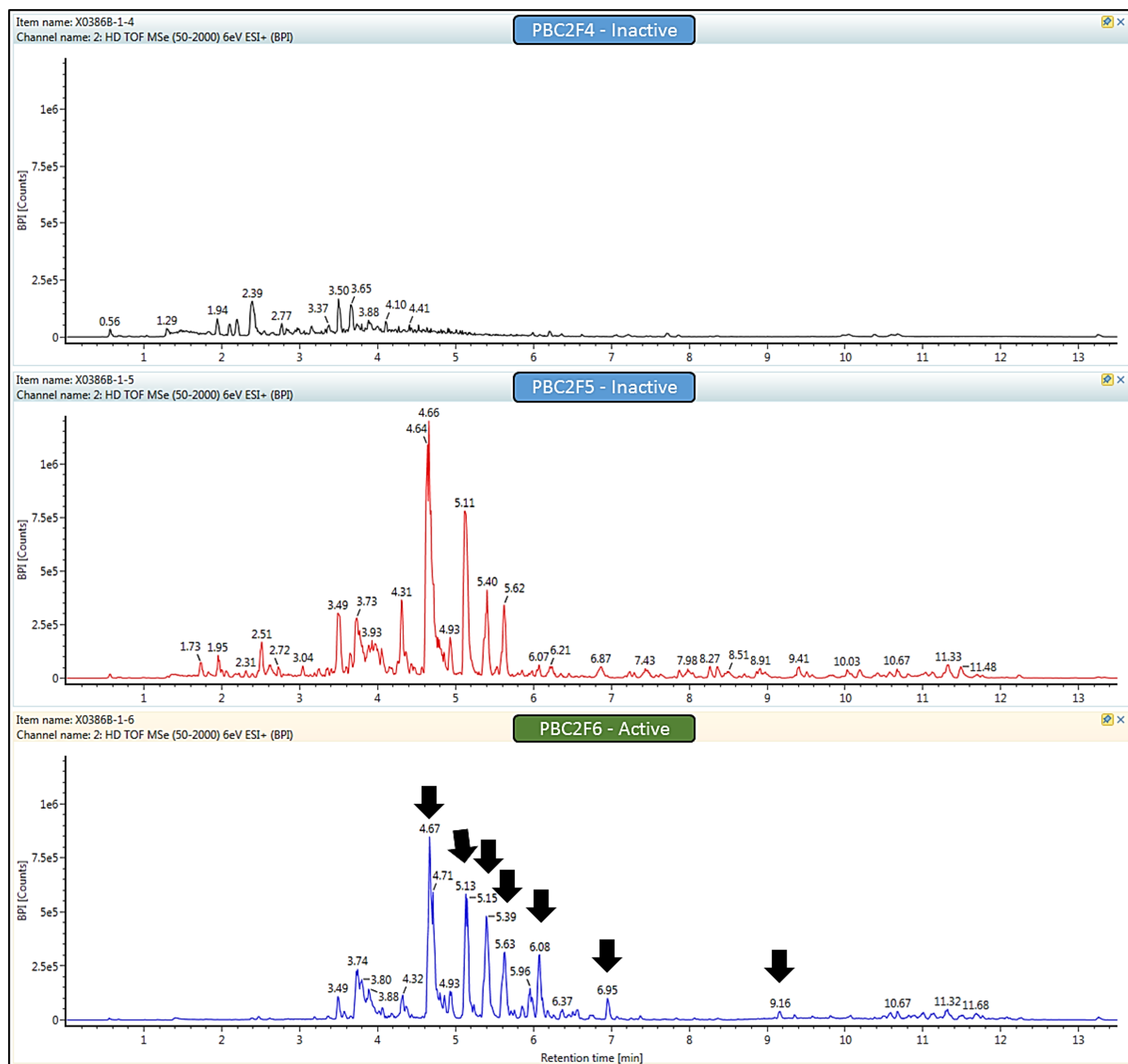

Figure 25: BPI chromatogram ESI+ (m/z 50-2000) for the comparison of fractions PBC2F4 (inactive), PBC2F5 (inactive) and PBC2F6 (active) from PB in the dereplication process. The arrows indicate compounds that were further investigated as a group of related compounds.

All the seven elemental compositions (table 29) were used to search the Dictionary of Marine Natural Products. For one of the compounds, $C_{21}H_{24}N_2$, there were seven hits in the database (no hits for the rest of the compounds), all from a family of compounds called hapalindole-type natural products.

Table 29: Retention time, m/z and predicted elemental composition of seven compounds detected in the active fraction PBC2F6.

Retention time (minutes)	*m/z*	Predicted elemental composition
6.08	381.2485	$C_{27}H_{28}N_2$
5.63	333.2315	$C_{23}H_{28}N_2$
5.39	365.2051	$C_{23}H_{28}N_2S$
5.13	319.2169	$C_{22}H_{26}N_2$
4.67	305.2015	$C_{21}H_{24}N_2$
6.95	426.2916	$C_{29}H_{35}N_3$
9.16	326.3775	$C_{22}H_{47}N$

5.4.5 Case 5 – Interesting compounds – LSC6F5 and PBC6F5

Both isolates, LS and PB, were grown in the intermediate nutritional medium DSGC with dead bacteria of the genus *Leeuwenhoekiella* added to the cultures after some time growing as monocultures. Fraction LSC6F5 was deemed active in the anticancer activity screening and the antibacterial activity screening, while fraction PBC6F5 was active in the antibacterial activity screening. During the analysis, six compounds stood out and were present in both active fractions (table 30). The compounds were found to have quite similar retention times, ranging from 8.94 minutes to 10.41 minutes, and their fragmentation patterns and elemental compositions appeared to be similar. Six rhamnolipids (RLs) were predicted to be present in the active fractions. Some of the compounds were also found in the inactive fractions, but generally at lower levels. These compounds are currently being worked with at Marbio, but from a different marine bacterial species, and several of the compounds are confirmed to be RLs by structure elucidation.

Table 30: The retention time, m/z and calculated elemental compositions of the compounds suspected of being RLs in fractions LSC6F5 and PBC6F5.

Name used in this thesis	Retention time (minutes)	*m/z*	Calculated elemental composition
RL1 *	8.94	527.3190	$C_{26}H_{48}O_9$
RL2	9.71	699.3923	$C_{34}H_{60}O_{13}$
RL3 *	9.74	553.3349	$C_{28}H_{50}O_9$
RL4	10.08	701.4082	$C_{34}H_{62}O_{13}$
RL5 *	10.10	555.3505	$C_{28}H_{52}O_9$
RL6	10.41	581.3685	$C_{30}H_{54}O_9$

**This RL has been previously isolated at Marbio and it has been confirmed as a RL by structure elucidation.*

The fragmentation patterns and calculated elemental compositions from this study matches the previous findings at Marbio. Figure 26 shows the BPI chromatograms of active fraction LSC6F5 and the inactive fractions LSC6F4 and LSC6F6. The same compounds were found in

fraction PBC6F5 (results not shown). The elemental compositions were calculated for all the possible RLs (table 30). All RLs were calculated as sodium-adducts.

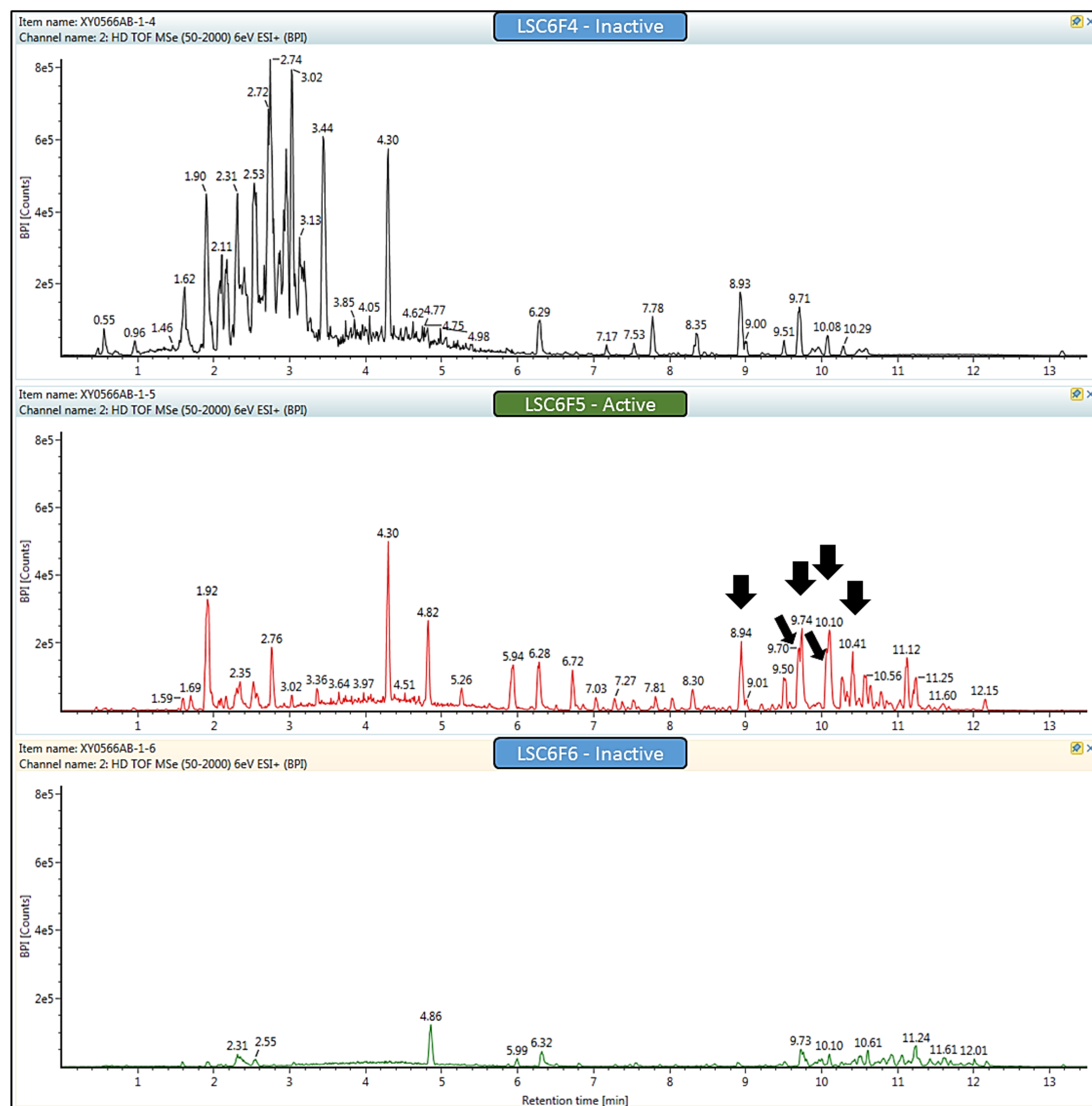

Figure 26: BPI chromatogram ESI+ (m/z 50-2000) for the comparison of fractions LSC6F4 (inactive), LSC6F5 (active) and LSC6F6 (inactive) from LS in the dereplication process. The same compounds were investigated in fraction PBC6F5. The arrows indicate the predicted RLs. The same compounds were discovered in PBC6F5 (results not shown).

5.5 Bioactivity as a function of cultivation conditions

The aim of the study was to investigate whether changing the cultivation conditions for PB and LS could trigger the bacteria into producing different secondary metabolites, and to see how this affected the bioactivity. All the results from the bioactivity screenings and the dereplication of the active fractions have been reported in the previous sections. Here, the bioactivity results (the fractions that were considered active through both the primary and the secondary

screening) are put together with the cultivations. Table 31 and table 32 shows a compilation of the activity observed in fractions, coupled to what cultures these fractions originated from, for LS and PB, respectively. Three of the seven cultures prepared with LS gave fractions that displayed bioactivity. C3 gave one fraction with biofilm formation inhibiting activity, C6 gave one fraction that was active in both the antibacterial and anticancer activity screenings and C7 gave one fraction displaying anticancer activity. No active fractions originated from C1, C2, C4 or C5 from LS.

Table 31: Bioactivity observed for the fractions prepared from all seven cultures (C1-C7) from LS after the secondary bioactivity screenings. The fractions were screened for antibacterial activity, biofilm formation inhibiting activity and anticancer activity.

LS	Culture conditions	Antibacterial activity	Biofilm formation inhibiting activity	Anticancer activity
C1	High nutrition			
C2	High nutrition, added bromide and iron			
C3	High nutrition, co-cultivation with PB		+	
C4	High nutrition, cold-treatment			
C5	Intermediate nutrition			
C6	Intermediate nutrition, added dead marine bacteria	+		+
C7	Low nutrition			+

+ indicates one active fraction from the culture, blank indicates no active fractions from the culture. Blue indicates activity in one screening, green indicates activity in several screenings.

From the seven cultures prepared with PB, four gave fractions that displayed bioactivity (table 32). C2 gave one fraction with activity against the cancer cell line A2058. C3 gave two active fractions, and both fractions were active in both antibacterial and anticancer activity screening. C6 gave one active fraction that was deemed active in the antibacterial activity screening. The low nutrition culture C7 gave one active fraction that was active with both biofilm formation inhibiting activity and anticancer activity. No active fractions were discovered form C1, C4 or C5 from PB.

Table 32: Bioactivity observed for the fractions prepared from all seven cultures (C1-C7) from PB after the secondary bioactivity screenings. The fractions were screened for antibacterial activity, biofilm formation inhibiting activity and anticancer activity.

PB	**Culture conditions**	**Antibacterial activity**	**Biofilm formation inhibiting activity**	**Anticancer activity**
C1	High nutrition			
C2	High nutrition, added bromide and iron			+
C3	High nutrition, co-cultivation with LS	++		++
C4	High nutrition, cold-treatment			
C5	Intermediate nutrition			
C6	Intermediate nutrition, added dead marine bacteria	+		
C7	Low nutrition		+	+

+ indicates one active fraction from the culture, ++ indicates two active fractions from the culture, and blank indicates no active fractions from the culture. Blue indicates activity in one screening, green indicates activity in several screenings.

6 Discussion

The aim of the study was to utilise the OSMAC approach in an attempt to trigger two Arctic marine bacteria into producing secondary metabolites with interesting chemistry and bioactivity. The bacteria were cultivated under seven different cultivation treatments, and the secreted secondary metabolites were subsequently harvested and extracted from the cultures. Further, the extracts were prefractionated, and screened for antibacterial activity, biofilm formation inhibiting activity and anticancer activity. The active fractions were dereplicated by UPLC-QToF-MS analysis followed by database searches. The latter step was done in an attempt to identify the compounds responsible for the observed bioactivities.

6.1 Cultivation, extraction and prefractionation

Two marine bacteria (LS and PB) were chosen for cultivation. LS is classified under the class *Actinobacteria*, the bacterial class that seems to be one of the most investigated from the marine environment with regards to bioactive compounds (Manivasagan, Venkatesan, Sivakumar, & Kim, 2013). PB is a bacterium within the class *Flavobacteria*, which appears to be a less investigated class of bacteria. The bacteria were both cultivated under seven different cultivation treatments (figure 27). Four different media, with varying nutritional levels, were used for cultivation. The DVR_2 medium consists of the same components as the DVR_1 medium, but with the addition of bromide and iron, which are important marine nutrients. These two media were tested to investigate how small differences in media composition could affect the bioactivities displayed by the cultures. Media composition can have great impact on the molecules that are produced. Some compounds induce production of secondary metabolites while others supress it, but the preferences differs between species (Bode et al., 2002).

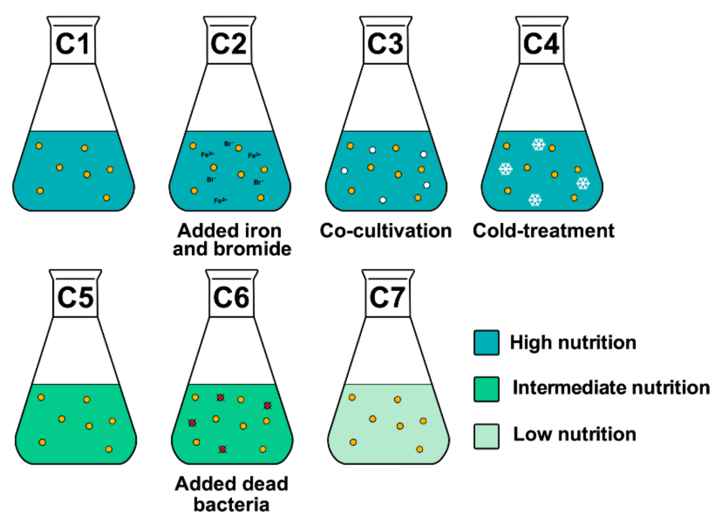

Figure 27: The seven different cultivations treatments of LS and PB (C1-C7). DVR_1 (C1,C3 and C4) and DVR_2 (C2) were the high nutrition media, DSGC had intermediate nutrition and corn flour medium (C7) had low nutrition. As can be seen in the figure, several other cultivation parameters were altered for cultivation treatments C2, C3, C4 and C6.

In addition to varying the medium used for cultivation, for some of the cultures other parameters were altered in an attempt to trigger the bacteria into activating different metabolic pathways (figure 27). C4 was cold-treated, to observe how the bacteria responded to temperature changes, with the temperature being decreased to almost freezing point several times. C3 and C6 were supplemented with additional bacteria, in an effort to trigger the bacteria into producing compounds of antibacterial nature to out-compete the intruder (supplemented bacteria). In the C3 cultures, PB was added to LS and vise versa. Before extracting the cultures, they were plated to check if both bacteria had survived the cultivation. In both cases, the supplemented bacteria could not be detected on the plates, indicating that the primary bacteria had outgrown them. In C6, dead bacteria (*Leeuwenhoekiella* sp.) was added to the cultures after some time as monocultures to see how the bacteria responded to the addition of dead bacteria. It should be made clear that the supplemented bacteria in C3 and C6 was not of the same species, and studies have shown that the species chosen for co-cultivation has great influence of the compounds that are produced (Trischman et al., 2004). The topic of co-cultivation will be further discussed in section 6.4 "Bioactivity as a function of cultivation conditions". There will always be several other parameters that can be tested when cultivating bacteria with the goal of activating different metabolic pathways. The choice of cultivation treatments in this thesis (C1-C7) display quite a broad spectrum of conditions, with alterations in media, temperature and by addition of bacteria.

The weight of the extracts from the LS cultures, especially for the high nutrition cultures, were higher compared to the PB cultures. For C5-C7 the weight of the extracts was quite similar

between the two bacteria. Based on the weight of the media control (MC) extracts (figure 12), it is apparent that the resin used is efficient in extracting media components, especially for the high nutrition media DVR_1 and DVR_2. Because of this, one can not be certain that high extract weights reflects high production of secreted metabolites, or that simply the media components are also being extracted. Therefore, LS is not necessarily a better producer of secreted secondary metabolites or biomass. It might be that PB is producing more secondary metabolites, which generally are low molecular weight compounds, which are better adsorbed by the resin, while the LS extracts contain higher amounts of media components. In order to say anything certain about what strain is the better producer, or what conditions gave the best production of secondary metabolites, one would have to measure the growth of the bacteria (biomass production) and the secretion of metabolites for each culture.

The extracts were prefractionated, giving six fractions (F1-F6) from each extract. Only fractions F4-F6 were deemed active in the bioactivity screenings, inconveniently, these were also the fractions with the lowest weights. Because of the low weight of fractions, it is probable that the active compound is present in too low concentrations to attempt an isolation, and that new cultures would have to be started and processed to get enough material for isolation. When starting new cultures one can not be certain that the compound of interest will be produced, so it is important that the parameters used for cultivation are as similar as possible to the original culture. Since fractions F1-F3 were the ones with the highest weights, it could be that they were too complex, so that the activity of possibly active compounds was masked in the bioactivity screenings. Thus, one option would be to produce more fractions from these in an attempt to unmask some of the possible bioactive molecules. It has been observed at Marbio that the most hydrophilic fractions rarely display bioactivity, and the same was observed in this project. One reason for this could be that there are large amounts of water-soluble compounds that are too polar to penetrate the cell membranes and reach intracellular targets. Because of this, it is not recommended to fractionate the most hydrophilic fraction (F1) any further.

6.2 Bioactivity screening of fractions

The antibacterial properties of the fractions were analysed against five human pathogenic bacterial strains, and their ability to inhibit biofilm formation of *S.epidermidis* was also studied. In addition, the fractions were screened for anticancer properties against the cancer cell line A2058. To determine whether a fraction was active or not, cut-off values were used. This filtering is necessary at Marbio, as a high-throughput screening platform, to quickly select what samples to focus on, and which to terminate. The cut-off values were used as a guidance in this

thesis, to limit the number of fractions to prioritize for further work. Figure 28 shows the number of fractions at each step. The number of fractions terminated after the primary screening was high, with only 14 out of 84 fractions being active in the primary screening.

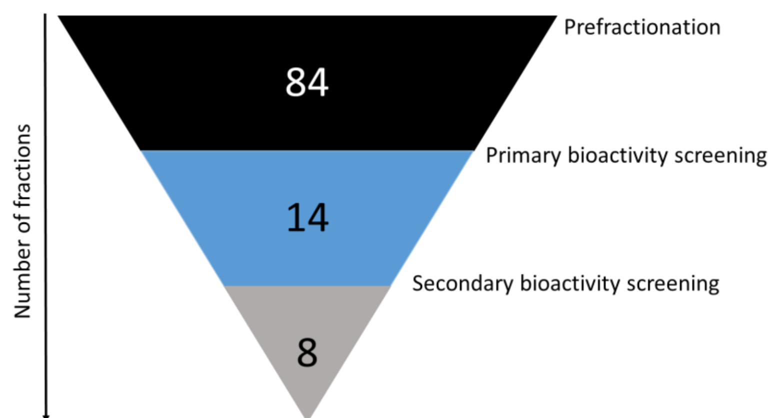

Figure 28: Number of fractions in the workflow from the start, after the primary bioactivity screening and after secondary bioactivity screening.

The results from the bioactivity screenings were not always reproducible, as some of the fractions that were active in the primary screenings did not display activity (or displayed less activity) in the secondary screenings (figure 29). This was particularly observed for the biofilm formation inhibiting activity screening and the anticancer activity screening, where 50% of the fractions no longer displayed activity in the secondary screening. These screenings were performed multiple times in an attempt to obtain reproducible results, but the results were non-consistent. The antibacterial activity screening was the first bioactivity screening to be performed, and it was performed without having to freeze/thaw the fractions in-between the screenings. Here, there was a clear correspondence between the results in the primary and the secondary screening (see figure 29). Generally in the bioactivity screening, the most stabile results were obtained when using fresh plates of fractions in MQ and DMSO, and conducting the primary and secondary screenings without having to freeze and thaw the plates in-between screenings. When the fractions were dissolved in MQ with 2.5% DMSO, and stored for some time (less than one week), the precipitation of dark pigmented compounds was observed in the test-DWPs. It is highly likely that this precipitation had an effect on the activity of the fractions. Cycles of freezing and thawing can have effects on the degradation and precipitation of compounds (Kozikowski et al., 2003). One possibility would be to prepare new plates for each screening. In this way, the fractions could be dissolved in MQ/DMSO on the day of the screen, and the fractions would be completely "fresh" for the screening. The downside of this is that

preparation of plates takes time, having to add each sample individually, freezing, freeze drying and then dissolving the fractions.

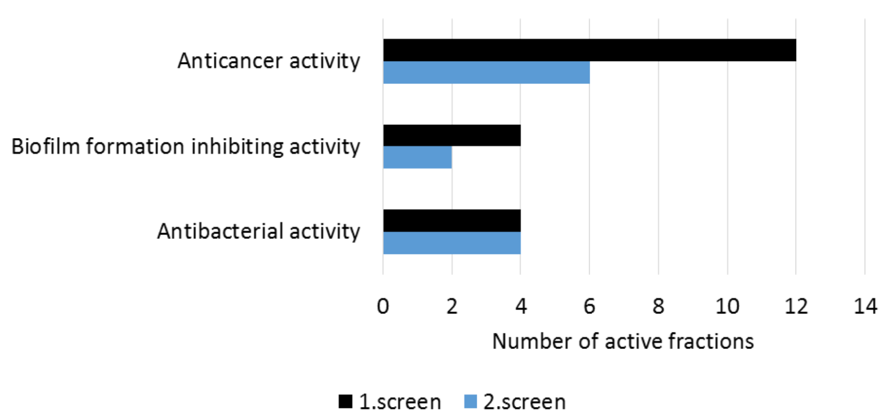

Figure 29: Number of fractions deemed active in the primary and secondary bioactivity screenings. All fractions were screened for anticancer, biofilm formation inhibiting and antibacterial activity.

None of the fractions displayed activity against the Gram-negative test strains in the antibacterial activity screening. This is consistent with observations at Marbio, where there are generally discovered more activity against the Gram-positive bacterial strains, compared to the Gram-negative ones. There is a massive problem with resistance observed for many Gram-negative bacteria, and the need for new antibiotics against these organisms is growing. One reason why Gram-negative bacterial infections are difficult to treat is the presence of porins and efflux pumps in their outer membrane, which can limit the influx and increase the outflux of antibiotics used during infection (Neelam & Harsimran, 2016). Because of this, it is important to find new antibiotics that can tackle the resistance of Gram-negatives. One approach could be to co-cultivate the marine bacteria with Gram-negative bacteria, to see if this could trigger the production of compounds that are selectively active. There are however also several human pathogens that are Gram-positive, and many of these bacteria, among them *S.aureus* and *S.pneumoniae*, are causing global resistance challenges (Woodford & Livermore, 2009), so the need for new antibiotics against Gram-positive bacteria is also urgent. Of the 12 pathogens/groups of pathogens listed in the WHO priority pathogen list (table 2) nine are classified as Gram-negative and three are Gram-positive (WHO, 2017b). With the global threat of antibiotic resistance, and seeing that the possibility of treating infections is essential in many aspects of health care (e.g. surgeries), it is becoming especially important to focus the research towards finding new antibiotics.

Six fractions were confirmed active against the A2058 cancer cell line. The fractions deemed active in the primary screening were also screened against the non-malignant lung fibroblast

cell line MRC-5 as a test for general toxicity. Generally, it seemed that the fractions displayed some activity against the MRC-5 cells, but more activity against the cancer cells. Many of the cancer drugs on the market today are cytotoxic compounds, also affecting the normal cells in the body (Ambili, 2012). The goal is to have a drug with greater effect against the cancer cells than the normal cells, so the treatment is effective enough without destroying the normal cells in the body. Activity as seen for fraction PBC3F6 (figure 19) is preferable: The activity of the fraction is considerably higher against the cancer cells than the normal cells, and a dose-response activity is observed (the activity decreases with decreasing concentration). Even if the compound of interest display some activity against normal cells, it is common to optimise compounds to give properties more suitable for its target (e.g. cancer cells). This process is called lead optimisation, where analogues of the active compound is produced and screened with the goal of producing a well-suited drug (Flannery, Chatterjee, & Winzeler, 2013).

As can be seen in the results (table 27), many of the active fractions were active in more than one bioactivity screening. Of the eight fractions that were considered active in the secondary screenings, four fractions were active in two bioactivity screenings and four were active in only one bioactivity screening. Activity in multiple bioactivity screenings can be considered a negative feature, since it might be a sign of non-specific inhibition. It could be that the active compound is destroying the cell membrane, and is in this way able to affect different types of cells. Another possibility is that it is not the same compound that is producing the activity in the different bioactivity screenings. Using biochemical (cell-free) assays in addition to cell-based assays (as used in this study) is a good way to further characterise whether or not the activity is specific, and to learn more about the actual activity of the different compounds. Biochemical assays are *in vitro* based methods that measures the activity of fractions/compounds towards a specific biological molecule, for example an enzyme (Arkin et al., 2017, p. 102).

6.3 Dereplication of active fractions from bioactivity screening

The final task of the project was to conduct the dereplication analysis, aiming at identifying the compound(s) within the active fractions responsible for the observed bioactivity. Isolation and structure elucidation can be very time and resource consuming, which is why effective and thorough dereplication is an important part of NP drug discovery. There is however no guarantee that the isolated compound is responsible for the observed biological activity detected in the screening (Wagenaar, 2008). The information you get from the dereplication process is used to decide whether the work on the sample is terminated, or that the compound of interest

is isolated and worked with further. In this study, eight active fractions were analysed on the MS and compared to the inactive fraction eluted before, and the inactive fraction eluted after in the prefractionation process.

As highlighted in the introduction, PAINS are molecules with broad and non-specific activity that might mask the activity of interesting compounds, leaving the interesting compounds un-detected. Two of the active fractions contained phosphocholines (LSC7F5 and PBC7F5), compounds that have been encountered several times at Marbio, and are considered PAINS (Hansen & Andersen, 2016). Because of their non-specific activity, and the fact that phosphocholines have been thoroughly investigated, these compounds are not considered relevant for drug discovery and the work on such fractions is normally terminated.

For fraction LSC7F5 (displaying anticancer activity) another compound, in addition to the phosphocholines, was investigated. The compound gave the highest peak in the chromatogram, and it was present in higher concentration in the active fraction compared to the inactive fractions. Searching the Dictionary of Marine Natural Products gave two hits: Eurypamide C and Serratiochelin A. The eurypamides are cyclic isodityrosines, like the antibiotic vancomycin. Cyclic isodityrosines have been reported with several bioactivities. Eurypamide C was originally isolated from a marine sponge, and has no reported bioactivity (to my knowledge) (Ito, Yamanaka, Kutsumura, & Nishiyama, 2003; Rami Reddy, Harper, & Faulkner, 1998). It would be interesting to isolate the compound for several reasons: Even though the compound has been isolated from a sponge, as previously mentioned, the actual producers of many isolated bioactive molecules (isolated from macroorganisms) are often associated bacteria. Therefore, isolation would say something about the origin of the compound, and even though it is a known compound, it might have a newly discovered bioactivity (anticancer). Serratiochelins are siderophores, compounds produced by bacteria that are responsible for acquiring iron from the environment (Seyedsayamdost et al., 2012). Since the compound has been mostly investigated for its iron-acquiring properties, it does not seem like it has been thoroughly investigated for other bioactivities. Serratiochelin A have no reported anticancer activity (to my knowledge). Further investigation would be interesting for both possibilities (Eurypamide C and Serratiochelin A), but the fact that none of the compounds have reported bioactivities makes it even more probable that the phosphocholines (with several reported non-specific bioactivities) were responsible for the observed activity of the fraction.

Fraction LSC3F5, active in the biofilm formation inhibiting activity screening, had one peak that clearly differed from the active fraction and the inactive fractions. The calculated elemental

composition was $C_{24}H_{33}NO_4$, which gave two hits in the Dictionary of Marine Natural Products: Aspochalasin C and Phomasetin. To my knowledge, none of these molecules have been isolated from bacteria before, and in the literature the compounds are referred to as fungal metabolites. The Aspohalisins, also called cytochalasins, have displayed different interesting bioactivities, among them antibacterial activity (Betina, Micekova, & Nemec, 1972; Gebhardt et al., 2004). With the possibility of discovering new bioactivities (inhibition of biofilm formation), this compound would be interesting for isolation and further investigation. Phomasetin is also a fungal metabolite, found moderately active against immunodeficiency virus integrase (Singh et al., 1998). If this is the actual compound responsible for the observed activity, it would be displaying a novel bioactivity as well. In order to say more about this compound, it would have to be isolated, have its structure elucidated, and be retested for bioactivity as a pure compound.

Two fractions from the same extract, PBC3F5 and PBC3F6, were deemed active in both the anticancer and the antibacterial activity screenings. Since the fractions displayed similar activity, and were adjacent fractions from the prefractionation, it is highly probable that the active compound(s) were the same in both fractions. The chromatograms were difficult to interpret with many similar peaks in both fractions, but two candidates were suggested for the observed bioactivities: $C_{23}H_{22}N_2O_2$ and $C_{28}H_{33}N_3$. Searches using the Dictionary of Marine Natural Products, and the Dictionary of Natural Products gave no hits. The compounds are good candidates for the observed bioactivity, and continuous work with isolation, structure elucidation and further bioactivity screening is recommended for the possibility of finding novel bioactive compounds.

Fraction PBC2F6 was deemed active against the A2058 cancer cells. Here, a group of seven compounds were detected in the fraction and investigated. The compounds were similar with regards to elemental compositions and UV data, which led to the hypothesis that this might be a group of compounds with some type of relation, e.g. as degradation products or as biosynthetic intermediates. One of the seven compounds gave hits in the Dictionary of Marine Natural Products (no hits for the other six compounds). All hits were hapalindole-type NPs, previously isolated from different cyanobacteria (Kim et al., 2012; Richter et al., 2008). The hapalindole-type NPs have displayed a wide range of bioactivities, among them anticancer activity (Richter et al., 2008). Being that the compounds previously have been isolated from cyanobacteria, it could either be that the compounds of interest are not from this group of compounds, or that the compounds are in fact (also) produced by other bacteria (non-cyanobacteria). Since the compounds have displayed a wide range of bioactivities, it would be interesting to see if some

of the compounds in this fraction are new variants of these NPs. The next steps would be to isolate the compounds, get their structures determined and then screening for different bioactivities (bioactivity profiling).

In case five of the dereplication, fractions LSC6F5 and PBC6F5 were predicted to contain different rhamnolipids (RLs) (see table 30). LSC6F5 was deemed active in the anticancer activity screening and in the antibacterial activity screening, while PBC6F5 displayed antibacterial activity. These metabolites are produced by different bacteria, with *P.aeruginosa* being the most commonly found producer (Chrzanowski, Ławniczak, & Czaczyk, 2012). RLs have been reported to display several different bioactivities, among them antimicrobial properties (Abdel-Mawgoud, Lépine, & Déziel, 2010). RL1, RL3 and RL5 have previously been isolated at Marbio, from a different marine bacterium, and their structures were confirmed by structure elucidation (nuclear magnetic resonance spectroscopy). RL2, RL4 and RL6 would have to be isolated for an absolute confirmation, but the fragmentation pattern and elemental compositions strongly indicates that these are in fact RLs. Literature searches strengthened this hypothesis (figure 30). In a mini-review by Abdel-Mawgoud and co-workers the structures and elemental compositions of known RLs are listed (Abdel-Mawgoud et al., 2010), and all six predicted elemental compositions from this thesis (table 30) are present in this list.

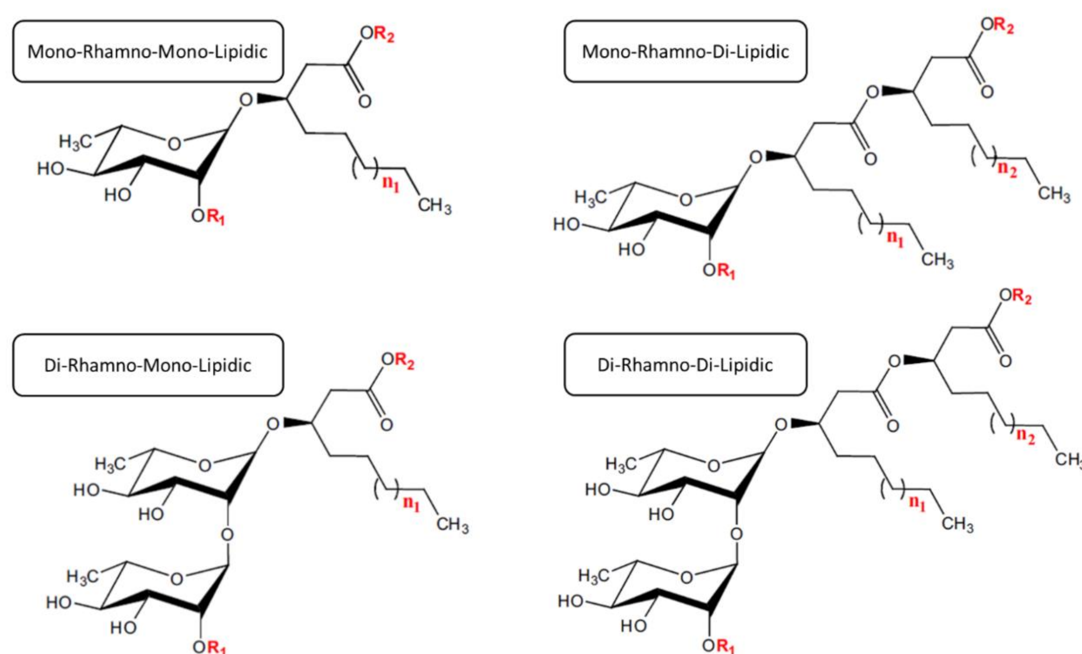

Figure 30: Chemical structure of different identified rhamnolipid congeners. Modified from reference (Abdel-Mawgoud et al., 2010, p. 1325). Rhamnolipids are glycolipids, made up of one or more rhamnose parts and one or more lipidic parts. From the elemental compositions calculated for the RLs predicted in this study, they appear to be Mono-Rhamno-Di-Lipidic (RL1, RL3, RL5 and RL6) and Di-Rhamno-Di-Lipidic (RL2 and RL4). R_1, R_2, n_1 and n_2 are variables that differs in the RLs that have been identified.

The RLs were detected in fractions from both PB and LS, but from the same cultivation treatment (C6) where dead *Leeuwenhoekiella* was added. This gave reason to believe that the RLs originated from the *Leeuwenhoekiella* sp. culture. One of the drawbacks with cultivating several bacteria together is that one can not be certain of which bacterium is the true producer of the active compound(s). The culture with dead *Leeuwenhoekiella* was plated before inoculation with LS and PB, and no growth was observed on the plates, strongly indicating that the bacteria were dead before addition to the cultures. If this bacterium was the producer of the RLs, they must have been produced before the addition to the PB and LS cultures. A control prepared with dead *Leeuwenhoekiella* was analysed on the MS to search for the RLs. In the control, the three previously detected RLs were re-discovered (RL1, RL3 and RL5), indicating that the RLs were in fact produced by the *Leeuwenhoekiella* bacterium. In cultivation C5, both LS and PB were cultivated in DSGC, but without the addition of the dead bacteria. Fractions from these two cultures were also analysed to see if the RLs could have been produced by the monocultures. In the LSC5 fractions RL3 and RL5 were detected. No RLs were discovered in the PBC5 fractions. Based on all this data, it is suggested that both LS and *Leeuwenhoekiella* produced RLs under these cultivation treatments, while it did not seem that PB were able to produce RLs. To my knowledge, neither LS or *Leeuwenhoekiella* have been reported as RL producers previously. Since RL2, RL4 and RL6 were not detected in the *Leeuwenhoekiella* culture, it is possible that LS and PB were capable of modifying these from RL1, RL3 and RL5 when co-cultivated. Further work on the RLs will be conducted at Marbio.

6.4 Bioactivity as a function of cultivation conditions

From the bioactivity screenings conducted in this study, it was clear that some of the cultivations were more successful in producing bioactive fractions (containing bioactive compounds) compared to others (figure 31). Between the two strains it seemed to be quite even, with three active fractions from LS and five from PB after the secondary screenings. For both strains, activity was observed in fractions from cultivations C3, C6 and C7, and for PB there was also one active fraction from C2. It seems that the supplementation of a different bacteria had an impact on the production of bioactive molecules from the marine bacteria, since both bacteria produced active fractions from these cultures (C3 and C6). These two cultivations contributed with 63% of the fractions considered active after the secondary screenings (see figure 31). It should be emphasised, as previously mentioned, that one can not be certain of what organism is the true producer of the active compound(s) in a co-cultivation, as was observed with the RLs that were encountered in this study. There are several studies on co-

cultivation as a means of triggering the expression of otherwise silenced genes. It is performed as an attempt to mimic the natural ecological situation for the microorganisms, where they persist in complex microbial communities. Co-cultivation has led to production of compounds that were not detected in the corresponding monocultures. This was observed when co-cultivating different bacterial strains, all isolated from the same algae, *Ulva california* (Trischman et al., 2004). In the antibacterial activity screening, all active fractions originated from C3 and C6, and one of two fractions deemed active in the biofilm formation inhibiting activity screening originated from C3, which is an indication that the supplementation of bacteria to the cultures influenced the activity against bacterial growth and biofilm formation. The results from this thesis indicates that co-cultivation and addition of dead bacteria triggers LS and PB to activate different metabolic pathways, compared to the corresponding monocultures.

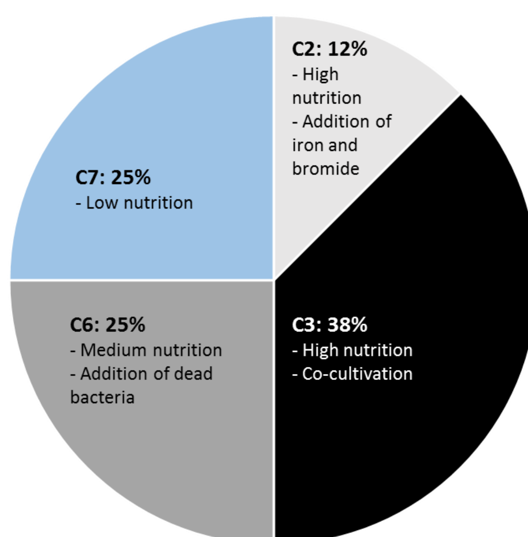

Figure 31: Cultivations providing fractions confirmed active in the secondary bioactivity screenings. Eight fractions were confirmed active. C1, C4 and C5 did not provide any confirmed active fractions.

From the low nutrition cultures (C7) both bacteria produced active fractions. This indicates that stressing the bacteria with low nutrition can induce the production of different bioactive metabolites. Another explanation is that specific components in the corn flour medium (low nutrition) triggered the bacteria to alter their metabolic pathways. Lastly, PBC2 provided one fraction with confirmed anticancer activity. C2 were the cultures where bromide and iron were added to the culture medium, indicating that this addition triggered PB to altering its synthesis of metabolic products. Further work could be to test the addition of other common marine nutrients (e.g. chloride and potassium) to investigate how this affects the cultures of Arctic marine bacteria. Fractions from the cultures C1, C4 and C5 did not display activity reaching the

cut-off values used in this thesis. C1 and C5 were cultures grown in high and intermediate nutrition medium, respectively, where no additional parameters were altered. It also appeared that stressing the bacteria with low temperatures (C4) did not induce the production of bioactive molecules, at least none that were detected in the bioactivity screenings in this project. There is always the possibility that there could be compounds with bioactivities that are not being screened for.

The main findings in the work of this thesis was that small alterations in cultivation parameters can have a considerable influence on the bioactivity displayed from the cultures. From the results, it is obvious that the biosynthetic production of the bacteria is highly dynamic and influenced by the cultivation conditions. The most successful cultivations, in terms of providing bioactive fractions, were those where additional bacteria was supplemented, the low nutrition cultures and the PB culture where bromide and iron was added to the medium. In this study, only two bacteria were cultivated and seven different cultivation treatments were conducted on the bacteria. The parameters that can be altered are endless, which makes the potential of NP drug discovery from cultivated bacteria massive.

7 Conclusions and further work

Two Arctic marine bacteria were successfully cultivated under seven different cultivation treatments, utilising the OSMAC approach. Extracts were prepared and prefractionated, and the fractions were screened for different bioactivities. There were several active fractions, with antibacterial activity, biofilm formation inhibiting activity and anticancer activity. The dereplication of the active fractions gave possible candidates responsible for the observed activity. For further work on compounds of interest, new bacterial cultures have to be started and processed to isolate these compounds in sufficient amounts for structure elucidation and bioactivity screening.

The thesis has demonstrated that LS and PB are capable of producing different bioactive compounds. It also showed that the cultivation conditions affected the compounds produced by the bacteria, and the bioactivity of the fractions produced from the cultures. With these regards, utilising the OSMAC approach was successful in this project. The possibilities with the OSMAC approach are endless. Testing different cultivation parameters and gaining more knowledge on what physiological conditions activate genes in charge of secondary metabolism would make the approach even more efficient, and will provide interesting bioactive compounds from Arctic marine bacteria in the future.

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9 Appendix

Appendix 1

Table 33: The products used for preparation of the M19 medium used in the Leeuwenhoekiella culture.

Product	Product ID	Distributor (Country)
Filtered sea water		Norwegian College of Fishery Science (Norway)
D-Mannitol	63560	Sigma-Aldrich (Missouri, USA)
Peptone from casein, enzymatic digest	82303	Sigma-Aldrich (Missouri, USA)

For the cultivations C6, both strains (LS and PB) were cultivated in DSGC medium and dead bacteria of the *Leeuwenhoekiella* sp. was added. *Leeuwenhoekiella* was grown in M19 medium, which contained the following:

- Filtered sea water 50%
- MQ 50%
- D-Mannitol 20 g/L
- Peptone from casein, enzymatic digest 20 g/L

The media components were mixed and the solution was autoclaved.

Appendix 2

Table 34: The products used for preparation of PBS and trypsin solution used for maintenance of the cells in the anticancer activity screening.

Product	Product ID	Distributor (Country)
Potassium chloride	1.04935	Merck KGaA (Germany)
Potassium dihydrogen phosphate	1.04871	Merck KGaA (Germany)
Sodium Chloride	S5886	Sigma-Aldrich (Missouri, USA)
Sodium phosphate dibasic dihydrate	30412	Sigma-Aldrich (Missouri, USA)
Trypsin (1:250)	27250018	Thermo Fisher Scientific (Massachusetts, USA)
Ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA)	E1644	Sigma-Aldrich (Missouri, USA)

Phosphate Buffered Saline (PBS) was used to wash the cells of cell medium before trypsinating. The PBS was prepared with the following ingredients:

- Potassium chloride 0.2 g/L
- Potassium dihydrogen phosphate 0.2 g/L
- Sodium chloride 8 g/L
- Sodium phosphate dibasic dihydrate 2.16 g/L

The buffer was prepared with MQ and autoclaved before use at 121°C for 120 minutes.

The trypsin solution used to loosen the cells from the culture flasks was prepared with the following ingredients:

- Trypsin 25 g/L
- EDTA 5 g/L

PBS was added up to one liter, and the solution was sterile filtered (0.2 µm filter) and 50 mL of the sterile filtered solution was added to 450 mL PBS to reach 0.25% trypsin and 0.05% EDTA.

Appendix 3

Poster presented at the BIOPROSP 2017 conference in Tromsø, Norway, March 8-10.

UiT THE ARCTIC UNIVERSITY OF NORWAY

Exploring the Potential of Two Arctic Marine Bacteria for the Production of Bioactive Metabolites

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/ INTRODUCTION

The one strain many compounds (OSMAC) approach focuses on that alterations of cultivation parameters can change the number and types of secondary metabolites that are produced by a microbial source. The theory is that many gene clusters are silenced under standard cultivation methods and by changing the cultivation conditions, one microorganism can produce a diversity of secondary metabolites by activating these gene clusters (1,2).

/ AIM OF STUDY

The aim of the study was to isolate bioactive compounds from two marine bacteria that could be possible candidates for further investigation for new antibacterial compounds. In this work, two Arctic marine bacteria were cultivated under different conditions, and their secondary metabolites were screened for antibacterial activity. The bacteria were collected at two different geographic locations: Outside of Bjørnøya (*Polaribacter* sp.) and in Hadselfjorden (*Leifsonia* sp.).

/ 1. WORKFLOW

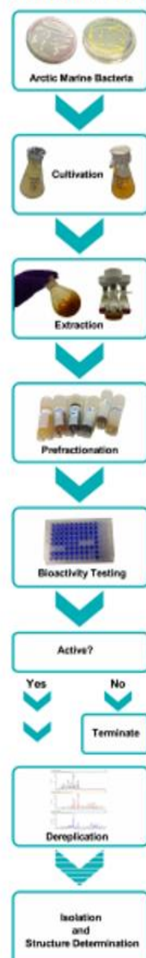

Figure 1: Workflow of the study. Secondary metabolites were extracted using Diaion HP-20 resin and 100 % methanol. The extracts were fractionated using FLASH chromatography with pre-packed Diaion HP-20SS columns. The next steps will be to isolate interesting compounds and determine their structures.

/ 2. CULTIVATION USING THE OSMAC APPROACH

In an attempt to trigger the bacteria into producing interesting secondary metabolites, the bacteria were cultivated using different conditions:

- Co-culturing
- Changing media
- Adding dead bacteria
- Cold treatment

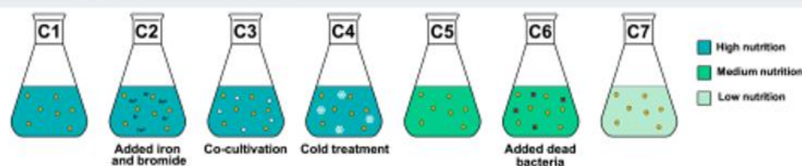

Figure 2: The bacteria were cultivated in seven different culturing conditions.

/ 3. ANTIBACTERIAL ACTIVITY

All fractions were screened for antibacterial activity against:

- *Staphylococcus aureus*
- *Escherichia coli*
- *Enterococcus faecalis*
- *Pseudomonas aeruginosa*
- *Streptococcus* gr.B

	C1	C2	C3	C4	C5	C6	C7
Leifsonia sp.	-	-	-	-	-	+	-
Polaribacter sp.	-	-	+	-	-	+	-

Table 1: Results from antibacterial activity screening.
+ indicates activity, - indicates no activity.

The antibacterial activity screening showed activity in three extracts, all of which either alive or dead bacteria had been added to the cultures. Activity was found against the gram positive test strains:

- *S.aureus*
- *E.faecalis*
- *Streptococcus* gr.B

All active fractions were retested with different concentrations, and the activity was confirmed.

/ 4. DEREPLICATION

The active fractions were compared to inactive fractions, in order to find possible candidates that could be causing the bioactivity of the fraction. The fractions were analysed with a UHPLC-IMS-QToF. Dereplication was performed to evaluate whether possible active compounds were known, novel, or known with novel bioactivity.

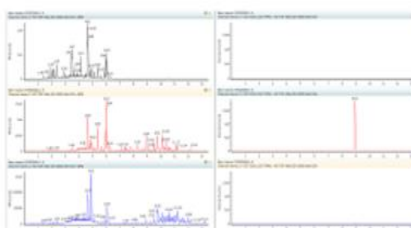

Figure 3: Left: Base Peak Intensity (BPI) chromatogram showing the active fraction (in the middle) and the fractions eluted before and after, both inactive. Right: Ion chromatogram for m/z 527.3191, a possible candidate for the antibacterial activity.

/ 5. CONCLUSIONS AND FURTHER WORK

- **Two Arctic marine bacteria were cultivated, using seven different culturing conditions**
- **The antibacterial screening indicates that bioactivity was expressed by adding bacteria**
- **Preliminary dereplication results show some possible candidates for the antibacterial activity**
- **We will try to isolate the possible active compounds and elucidate their structures, and confirm bioactivity**

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