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Bioactivity Potential of an Arctic Marine Diatom Species Cultivated at Different Conditions

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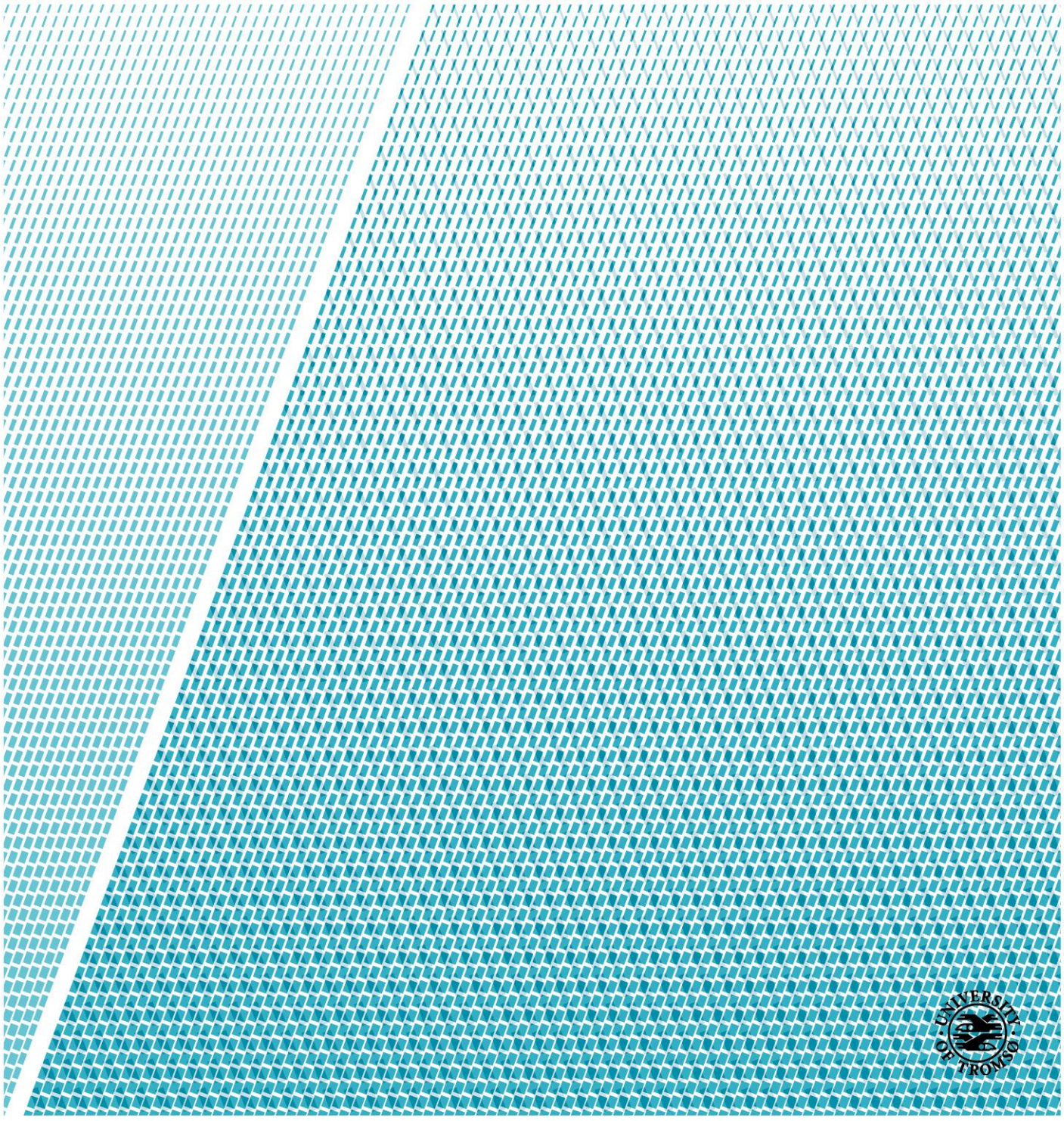
Master thesis in Marine Biotechnology (May 2018)

60 credits



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Ida Elvedal

ABSTRACT

Microalgae have proven to contain a vast amount of beneficial, high value compounds like proteins, lipids and powerful antioxidants as well as some interesting bioactive compounds. Nevertheless, microalgae are severely underrepresented in conjunction with marine bioactive natural product discovery. This thesis aims to unlock bioactivity potential of a microalgae species from the most abundant and diverse group of microalgae, namely the diatoms, by bioassay guided isolation. This diatom species is isolated from northern Arctic waters, where research on bioactivity potential in diatoms are poorly investigated.

Five samples of raw biomass from a diatom species cultivated at five different conditions were extracted, fractionated through FLASH chromatography and screened in five different bioassays; an antibacterial assay against five bacteria strains (*Staphylococcus aureus, Escherichia coli, Enterococcus faecalis, Pseudomonas aeruginosa and Streptococcus agalactiae*), an anti-biofilm assay (against *S. epidermidis* biofilm-formation), a MTS cell viability assay with three different cell lines (human melanoma A2058, human colon carcinoma HT29, and human pulmonary fibroblast MRC-5), a cellular antioxidant activity assay (with a THP-1 cell line) and an anti-inflammatory assay (with a HepG2 cell line). Some selected samples were fractionated further by HPLC chromatography and screened again for anti-biofilm and anti-inflammatory properties. Bioactivity was detected in all assays, and interestingly, some variation was observed within the assays for the different cultivation conditions. This indicated that the metabolite bioactivity profile of the diatom might have changed due to the varying pre-experiment cultivation conditions. The results demonstrate the huge bioactivity potential of diatoms, and that modification of cultivation conditions might be used to our advantage to obtain bioactive fractions with a different range of activities.

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ABBREVIATIONS

BPI	Base peak intensity
BSA	Bovine Serum Albumin
D-MEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
E-MEM	Earle's minimal essential medium
FBS	Fetal bovine serum
FFAs	Free fatty acids
HPLC	High pressure liquid chromatography
LPS	Lipopolysaccharide
MH	Mueller-Hinton broth
MIC	Minimum inhibitory concentration
MS	Mass spectrometry
NCFS	The Norwegian College of Fishery Science
NMR	Nuclear magnetic resonance
n.d.	No date
NP	Natural product
PBS	Phosphate-buffered saline
PMA	Phorbol 12-myristate 13-acetate
PUAs	Polyunsaturated aldehydes
PUFAs	Polyunsaturated fatty acids
ROS	Reactive oxygen species
RPMI	Roswell park memorial institute medium
TBS	Tris buffer saline
TSB	Tryptic soy broth

1 INTRODUCTION

1.1 NATURAL PRODUCT DISCOVERY IN THE MARINE ENVIRONMENT

The ocean covers 70% of the earth's surface, contain areas with extreme temperatures ranging between 350°C and all the way down to -1,5°C (Lindequist, 2016) and light cannot reach too far below the surface. In the deepest abyss the pressure can reach 100 MPa and the high salinity offers challenges. Still, life somehow managed through evolution to develop strategies to withstand these harsh conditions and even thrive. So, how do marine organisms and marine microorganisms like microalgae handle these conditions, in addition to other important factors like predators and competition for resources? This is where the production of important protective secondary metabolites/bioactive compounds comes into the picture. These natural products (NPs) can be highly effective as inhibitors of physiological properties in prey, predators and competitors (Haefner, 2003). In the search of novel biomolecules for the production of drugs and pharmaceuticals, these metabolites are extremely important, both directly as potential drug candidates and indirectly as a starting point where some additional synthetic modifications are necessary for activity optimization. Scientists are continuously searching for novel bioactivity to oppose some of the biggest problems humanity faces today, like the rise of antibiotic resistance due to the constant clinical use of antibiotics and important diseases like diabetes, HIV and cancer. Compared to the terrestrial environments the marine environments are poorly explored, but might hold bioactive compounds efficient in lower concentrations due to the high dilution rate in the sea water once excreted. Marine derived NPs are therefore thought to have a higher potency than terrestrial derived NPs (Haefner, 2003). In 2016 about 30.000 compounds from the marine environment were described and since 2008 about 1000 new compounds have been discovered every year (Lindequist, 2016) due to the ever increasing development of new and clever technologies for rapid and efficient discovery of new molecules. It is worth mentioning the few important "life-savers" of marine derived drugs which exist on the market today, namely Cytarabine, Vidarabine, Trabectedin, Eribulin Mesylate, Brentuximab Vedotin, Omega-3-Acid Ethyl Esters, Iota-Carrageenan and Ziconotide. These drugs are used in the treatment of important conditions; leukemia, herpes virus infection, soft-tissue sarcoma, refractory metastatic breast cancer, Hodgkin and systemic large cell lymphoma, hypertriglyceridemia

and common cold, respectively. The latter is, in addition, to be the only drug in which can treat viral conjunctivitis (pink eye). The last drug (Ziconotide) is a widely used analgesic agent which is considered safer that morphine (Mondal & Dalai, 2017).

1.2 BIOPROSPECTING

Bioprospecting is a process to discover NPs such as bioactive secondary metabolites. Bioprospecting may have many definitions, but in this paper, it will be defined as: "a process where it's desirable to find bioactive compounds in the marine environment and to give new knowledge about the activity and the molecule that can have a commercial potential in pharmacy or other usages".

A bioprospecting pipeline may be divided into four main steps: 1: collection and, if needed, cultivation of marine organisms, 2: extraction and isolation, 3: screening and structure identification of potential active compounds, and finally 4: product optimization and commercialization. Bioprospecting is a good method to identify potentially bioactive molecules, however there are some challenges. Some common challenges are collection of enough material, identification of the true producer of a discovered molecule and cultivation of the desired microorganism. It is estimated that only 0,001 - 1% of marine microorganisms are cultivable in the lab (Jaspars et al., 2016). Taxonomic identification of the organism under investigation is crucial, so that it can be re-collected if more material is needed (Querellou, 2010). In the long run, and if you want to develop a product, going out to collect an organism for a steady income of your compound is in most cases not feasible due to the vast amounts required. In the worst-case scenario, species can be extinct in certain areas due to too heavy collection, potentially disturbing entire ecosystems. This is why, in the end, synthetic production is a necessity in most cases. This secures a steady supply of the compound(s), and additional molecular modifications can be made. This is often required, for example, to reduce the toxicity of your compound (Harvey, Edrada-Ebel, & Quinn, 2015). In a way, one can say that bioprospecting is used to look for inspiration in nature, or the marine environment in this case, for development of novel bioactive molecules.

Often, it's difficult to know the exact source of discovered NPs from a marine organism due to the complexity of tiny animals and microorganisms living in symbiosis with the organism. NPs from marine organisms are most frequently isolated from invertebrates such as sponges,

tunicates, bryozoans and molluscs. However, through looking at the structure of these compounds, it is revealed that a numerous of these NPs have conspicuously structural similarities to compounds known to be produced by microbes, e. g. bacteria and microalgae. This suggests that exo- and endobiotic microorganisms accompanying the marine organisms are in fact the true producers of these compounds (Imhoff, Labes, & Wiese, 2011; Proksch, Edrada, & Ebel, 2002). This is substantiated by the many studies proving that NPs from sponges originates from symbiotic bacteria and microalgae (Proksch et al., 2002). Haefner 2003 also states that the majority of discovered NPs are thought to be of microbial origin.

Searching for bioactive compounds can be done in many ways, e.g. by bioassay-guided isolation, chemistry-guided isolation and genomic mining. However, the focus of this thesis was only on bioassay-guided isolation, and the others will not be further discussed. In bioassay-guided isolation, extracted and fractionated samples can be tested on various bioassays, for example antibacterial assay, antibiofilm assay, anti-cancer assay etc. to search for novel bioactivity.

Structure identification through dereplication is an important step in a bioprospecting pipeline to avoid spending unnecessary time and money on further isolation of molecules which already have been discovered and described. Structure identification may be done with the help of mass spectrometry (MS) or nuclear magnetic resonance (NMR). These techniques are important dereplication tools. MS is a technique where the need for pure samples or isolated compounds are unnecessary. Still, the structure can be determined to some degree, making MS a highly time-saving procedure (Svenson, 2013), and is the dereplication tool of choice in this thesis.

The main focus in this thesis was on the two middle steps of a bioprospecting pipeline; extraction and isolation and screening and structure identification of potential active compounds, see figure 1.

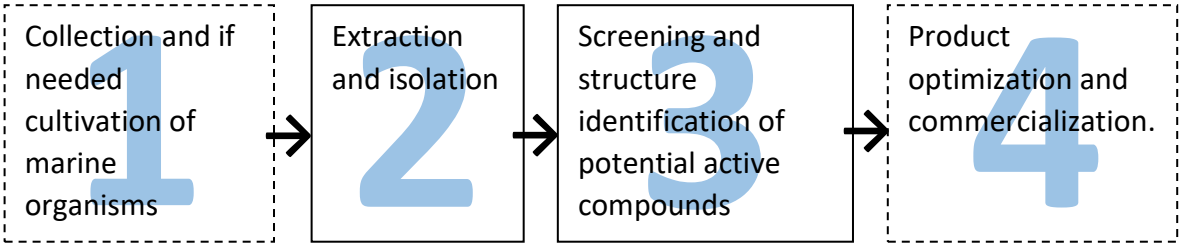

Figure 1: The four steps of a bioprospecting pipeline, whereas the two middle steps are the focus in this thesis.

1.3 MICROALGAE

Microalgae, or phytoplankton, also including the cyanobacteria, are tiny, single-celled, photoautotrphe eucaryotic (Talero et al., 2015) organisms which produces almost half of the world's oxygen through photosynthesis and serves as the fundamental nutrition unit for most life in the ocean (Armbrust, 2009).

These single-celled microorganisms often exist together in larger colonies and possess the ability to live and thrive basically everywhere where there is water or moist surfaces and a sufficient light supply, i. e. in both marine and terrestrial ecosystems (Mata, Martins, & Caetano, 2010). They also constitute the group of living organisms, both in marine and terrestrial communities, with most species diversity (Irigoien, 2004). However, even though many species are described, and the number is increasing yearly, only a small fraction have actually been investigated (Talero et al., 2015).

1.4 THE DIATOMS

The most abundant and diverse group of microalgae are the diatoms (Kooistra, 2007). They can exist both as single cells or as a chain of connected cells (Kooistra, 2007) and have an enormous species diversity with genomes that can differ as much as those of mammals and fish (Bowler et al., 2008). They exist in both saline and in fresh water (Sumich, 1999) and are described to "live inside tiny glass houses" which are essentially made up of silicon dioxide (silica) (Drum & Gordon, 2003; Munn, 2011). These tiny houses of silica are split into two parts, and that's how the diatoms got their name, since diatoms namely means "cut in half" in Greek (Armbrust, 2009). Their symmetry can be either radial (termed centric) or bilateral (termed pennate) (Munn, 2011) and they grow fast by dividing, capable of doubling their biomass every day. However, because diatoms are large in size in comparison to other microalgae, it takes longer to acquire enough nutrients for rapid growth and hence they grow slower (Morel & Price, 2003). The diatoms also have communication capabilities where they can send chemical signals between and within cells to protect themselves from predators (Vardi et al., 2008).

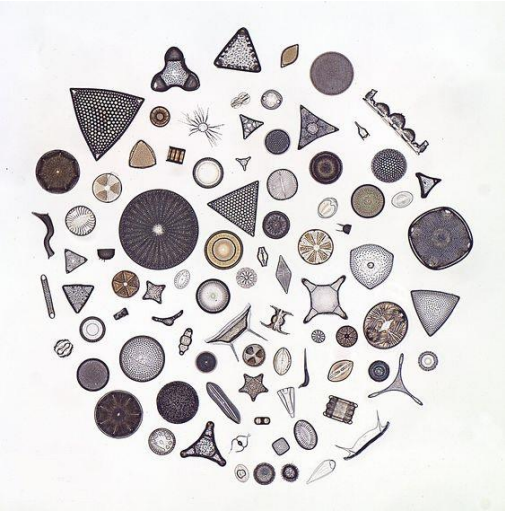

Figure 2: Some of the many shapes and appearances of diatoms. Retrieved from (Sangerhausen, n.d.).

Their importance for all life is unavoidable due to their vast production of oxygen and organic carbon. They are estimated to be responsible for as much as 20 % of earth's photosynthesis and their production of organic carbon serves as the basic unit of marine food webs. In addition, diatoms are conserved as petroleum when it falls to the sea floor, making it a valuable energy source for us humans (Armbrust, 2009).

1.4.1 Diatoms in the Arctic

In the Arctic, along the sea ice edge where sufficient sunlight and nutrients are available, microalgae communities develop, and diatoms tend to dominate these communities. Since glaciers and permafrost limits photosynthesis on land, diatoms are crucial sustainers, not only for marine ecosystems, but also for the terrestrial ecosystems (Armbrust, 2009), see figure 3.

Figure 3: Diatoms and other microalgae and microorganisms live on and in the sea ice and function as the basic nutrition unit for all marine life, also sustaining the terrestrial ecosystems. Retrieved from (PolarBearsInternational, 2017).

When the polar night has ended and sunlight returns to the northern and southern hemisphere, germination of diatoms and other microalgae in resting stages is triggered by the returning light in areas of nutrient upwelling (Richard Andre Ingebrigtsen, 2017; Munn, 2011). This leads to an exponential increase of microalgae biomass causing a massive spring bloom (Richard Andre Ingebrigtsen, 2017). This happening might be the most important annual marine event due to the immense conversion of nutrients and photons to readily available energy for all marine life (Richard Andre Ingebrigtsen, 2017).

Diatoms from the Arctic is poorly investigated for biodiscovery purposes (Richard A Ingebrigtsen, Hansen, Andersen, & Eilertsen, 2016). However, there are two major factors that makes diatom NP production unique in this context. Firstly, diatoms above the Arctic circle have the capacity to rapidly adapt to the polar day/night, where they either receive sunlight all day and all night or no sunlight at all for a couple of months (Eilertsen & Degerlund, 2010). As a response to long periods of darkness, cold temperatures or lack of nutrients some species of diatoms can form resting spores and other species can lower their metabolism (Smetacek, 1985). These physiological changes makes them able to survive the polar night and thus emerge in massive blooms in spring time (Munn, 2011). Secondly, northern diatoms are psychrophilic microorganisms, i. e. tolerant of the extreme cold temperatures of the Arctic waters. These conditions might attribute to the production of unique molecules with novel, cryptic bioactivity.

1.4.2 Bioactivity in microalgae

Microalgae are severely underrepresented compared to marine bacteria, porifera, molluscs, seaweeds and other marine organisms in the search of novel, bioactive marine compounds (Richard A Ingebrigtsen et al., 2016). This is rather controversial as most marine derived drugs are thought to originate from microorganisms like microalgae, as mentioned earlier. One compound proven to originate from microalgae however, is Brentuximab vendotin, containing a slightly modified version of dolastatin-10 produced by cyanobacteria (Mondal & Dalai, 2017). This compound targets a membrane protein on the surface of Hodgkin's lymphoma cells and are used in the treatment of Hodgkin and systemic large cell lymphoma (Mondal & Dalai, 2017).

Nevertheless, a wide range of bioactivities have been discovered in microalgae in the last 50 – 60 years (Borowitzka, 2013). This includes antibacterial-, antibiofilm-, anticancer-,

antioxidative- and anti-inflammatory activities. Other found bioactivities are antifungal, antiviral, antihypotensive (Borowitzka, 2013), antiprotozoal and antiplasmodial (Sanmukh et al., 2014) but these will not be further discussed.

With the rise of antibiotic resistance, there is a constant need for new antibiotics. Compounds produced by microalgae like free fatty acids (FFAs), oxylipins and photosynthetic pigments or their derivatives, show promising antibacterial activities (Smith, Desbois, & Dyrynda, 2010). For example, FFAs released by microalgae rapture have the capacity to kill any nearby prokaryotic organism, and hence protect the remaining population/colony of microalgae (Smith et al., 2010). Another example is chlorophyll derivates, which have proven to have activity against both gram positive and gram negative bacteria (Hansen, 1973; Jørgensen, 1962).

There are few studies done on the antibiofilm potential of microalgae. However, two examples worth mentioning are: biofilm inhibition of the highly relevant biofilm producing bacteria *Pseudomonas aeruginosa* from a *Chlamydomonas sp.* extract (Nithya et al., 2014), and inhibition of binding of the bacterium *Helicobacter pylori* to porcine gastric mucin from polysaccharides isolated from *Chlorella* and *Spirulina* (Loke, Lui, Ng, Gong, & Ho, 2007). *P. aeruginosa* is estimated to be the causal agent of 10 – 20 % of all hospital-acquired infections (Ikeno et al., 2007), and *H. pylori* is an important human pathogen which thrives in the low pH values in our stomachs and can, in some cases, cause serious infections (Tomb et al., 1997).

Several species of microalgae express anticancer, antioxidative and anti-inflammatory activities. In table 1, retrieved from Talero, Garcia-Mauriño et al. 2015, some examples of microalgae retrieved compounds with these activities are listed.

*Table 1: An overview of some discovered compounds with anticancer, antioxidant and anti-inflammatory bioactivities in different species of microalgae. Diatoms are marked with *. Retrieved from (Talero et al., 2015).*

Compound	**Source**	**Activity**
CAROTENOIDS		
β-Carotene	*Dunaliella salina* and *Haematococcus* sp.	Anticancer, antioxidant and anti-inflammatory
Astaxanthin	*Haematococcus pluvialis, Chlorella zofigiensis* and *Chlorococcum* sp.	Anticancer, antioxidant and anti-inflammatory
Lutein	*Dunaliella salina, Chlorella sorokiniana* and *Chlorella prothecoides*	Anticancer, antioxidant and anti-inflammatory
Violaxanthin	*Dunaliella tertiolecta* and *Chlorella ellipsoidea*	Anticancer and anti-inflammatory
Zeaxanthin	*Synechocystis* sp. and *Chlorella saccharophila*	Antioxidant and anti-inflammatory
Fucoxanthin	*Phaeodactylum tricornutum** and *Isochrysis* sp.	Anticancer, antioxidant and anti-inflammatory (Peng, Yuan, Wu, & Wang, 2011)
FATTY ACIDS		
Eicosapentaenoic acid (EPA)	*Tetraselmis* sp.	Anti-inflammatory
Docosahexaenic acid (DHA)	*Tetraselmis* sp.	Anti-inflammatory
Docosapentaenoic acid (DPA)	Nannochloropsisoculata	Anti-inflammatory
GLYCOLIPIDS		
Monogalactosyldiacylglycerol (MGDG)	*Gymnodinium, Mikimotoi, Stephanodiscus* sp* and *Pavlova lutheri*	Anticancer and antioxidant
Digalactosyldiacylglycerol (DGDG)	*Stephanodiscus* sp*	Anticancer and antioxidant
Sulfo-quinovosyl-acyl-glycerol (SQAG)	*Stephanodiscus* sp*	Anticancer and antioxidant
POLYSACCHARIDES		
Sulphated extracellular Polysaccharide	*Phaeodactylum tricornutum**	Anti-inflammatory
Sulphated polysaccharide B-(1,3)-glucan	*Chlorella stigmatophora* and *Chlorella vulgaris*	Anti-inflammatory Anticancer
Sulphated polysaccharide	*Tetraselmis suecica*	Anti-inflammatory
Sulphated polysaccharide	*Isochrysis galbana*	Anticancer
Sulphated polysaccharide	*Porphyridium* sp.	Anticancer and anti-inflammatory
Sulphated polysaccharide	*Gyrodinium impudicum*	Anticancer and anti-inflammatory
Extracellular polysaccharide s-Spirulan	*Arthrospira platensis*	Anticancer
PROTEIN AND PEPTIDES		
Phycobiliproteins	*Spirulina platensis* and *Porphyridium* sp.	Anticancer, antioxidant and anti-inflammatory
Peptides	*Chlorella pyrenoidosa* and Cyanobacteria	Anticancer, antioxidant and anti-inflammatory
OTHER COMPOUNDS		
Amides	*Lyngbya majuscula*	Anticancer
Quinones	*Calothrix* sp.	Anticancer
Phenolic compounds	*Spirulina maxima, Chlorella ellipsoidea* and *Nannochloropsis* sp.	Antioxidant
Tocopherols	*Porphyridium* sp.	Antioxidant

1.4.3 Advantages in terms of bioprospecting

Microalgae reproduce quickly in the right conditions and are thus easy to grow in a high rate (Talero et al., 2015) compared to higher plants (de Morais, Vaz, de Morais, & Costa, 2015). As mentioned before, most microalgae are autotrophic and thus the only requirement for cultivation are inorganic compounds, such as CO_2 and salts, and solar energy (de Morais et al.,

2015). They also have a high content of valuable compounds in their biomass (Gong, Hu, Gao, Xu, & Gao, 2011).

In bacteria and fungi, it seems like various cultivation conditions can cause metabolic pathways to be turned on and off, and consequently trigger one microbial strain to produce various compounds (Bode, Bethe, Höfs, & Zeeck, 2002). This has successfully been done through, amongst others, the "One Strain Many Compounds" (OSMAC) approach, where several metabolites were obtained from systematic alternation of cultivation parameters of one single organism (Bode et al., 2002). Like bacteria and fungi, microalgae have an extraordinary ability to change their intracellular environment as a response to changes in the external environment, i.e. adapt and change in harmony with the external environment (de Morais et al., 2015). This might be explained by looking at their harsh natural conditions (high salinity, poor light conditions, high/low temperatures, high concentration of predator organisms, lack of nutrients etc.) where rapid adaption is a necessity for self-protection and thus survival. It is desirable to gain more knowledge about this field so that we can use it to our advantage, e. g. reveal how modifications of growth conditions may change/unlock metabolic pathways in microalgae for production of applicable molecules, as done for the marine bacteria.

Light is especially essential in this context. Light provides photons necessary for photosynthesis and hence the microalgae's ability to grow, produce compounds and reproduce. Different irradiances of light have proven to have an effect on diatoms growth rate and cell protein content, as shown in an experiment with the diatom *Phaeodactylum tricornutum* (Chrismadha & Borowitzka, 1994). In addition, increasing light levels during cultivation of microalgae can both reduce and enhance different kinds of fatty acid production like PUFA's and EPA's (Grima, Camacho, Pérez, & Sánchez, 1994) and affect antioxidant production (de Morais et al., 2015).

Nutrient composition is also an important factor which can be manipulated in a series of ways, for example with lack/excess of important nutrients, bacteria presence, mass cultivation vs small scale etc. These various conditions can potentially trigger the microalgae to produce NPs for self-protection. Lack/excess of CO_2 supply have for example proven to have an effect on growth rate, where microalgae cultured with CO_2 have a significantly higher growth rate than those cultured without (Chrismadha & Borowitzka, 1994).

Another factor to take into consideration is temperature. In another experiment, six different diatom species were cultivated at two different temperatures (0.5°C and 8.5 °C). The results showed a general higher chemical diversity of the diatoms at the lowest temperatures (Huseby et al., 2013). Huseby, Degerlund et al. 2013 states that "The reason why diversity increases at lower temperature may lie in the fact that the species we tested can be found both in temperate water (Norwegian coast) and all the way up to the Arctic and as such they have the ability to mobilize alternative metabolic pathways in order to adapt to the lowered temperatures.

1.4.4 Future prospects

Today, microalgae are mainly used in aquaculture and health supplements much due to their high content of high value biomass compounds like proteins, lipids, and powerful antioxidants. Especially the carotenoids B-carotene and astaxhantin which can be used as natural colorants in feed/food or cosmetics (Christaki, Bonos, Giannenas, & Florou-Paneri, 2013; Markou & Nerantzis, 2013). However, they possess a huge commercial potential as pharmaceuticals due to their high content of bioactive molecules which includes proteins, polysaccharides, lipids, vitamins, enzymes, sterols, and other high-value compounds with pharmaceutical and nutritional importance (Priyadarshani & Rath, 2012). These compounds can either be contained in the algal biomass or excreted into the growth medium. However, lack of knowledge and technology are setting some boundaries for full and economic feasible utilization of these microorganisms (Talero et al., 2015). However, it is expected that the frequency of novel drug discovery in microalgae compared to other microorganisms can be higher due to the late commencement of NP discovery in microalgae (Olaizola, 2003).

2 AIM OF THE THESIS

The aim of this thesis was to get a closer insight in the bioactivity potential of Arctic diatoms by testing them in a wide selection of bioassays, and to investigate whether varying cultivation conditions had an effect their bioactivity profile. The main objectives were to:

- Collect and fractionate raw biomass from the same diatom species cultivated at different conditions.
- Identify bioactivity through bioassay guided isolation in antibacterial-, anti-biofilm-, anticancer-, cytotoxicity-, anti-inflammatory- and antioxidant assays.
- Identify active compounds by dereplication.

3 METHODOLOGY

3.1 THE BIOPROSPECTING PIPELINE STEP 2 (EXTRACTION AND ISOLATION)

3.1.1 Extraction

To perform experiments on bioassays it is crucial to have your raw materials extracted to withdraw as much components as possible into a liquid solution from the solid material. Different kinds of solvents are used for this purpose, some of the most common ones are acetonitrile, methanol, dichloromethane or water. Once extracted, the samples can further be tested on bioassays directly or by first having their complexity reduced over more fractions through for example FLASH chromatography or high pressure liquid chromatography (HPLC).

3.1.2 Isolation

3.1.2.1 Liquid chromatography

Liquid chromatography is a method used to separate a sample of interest into smaller fractions with similar chemical properties. This is done by the use of a stationary phase (solid phase) and a mobile phase (liquid phase). The sample of interest are separated according to its affinity for the stationary phase versus the mobile phase. Many different stationary and mobile phases are available, which can enable customized separation (Betancourt, 2017). In FLASH chromatography, also called medium pressure chromatography (Rubin, 2011), a suitable hydrophobic column (stationary phase) and a pressurized gas is used to drive the efflux of solvents (mobile phase) through the column. With the mobile phase, the sample is separated into twenty-seven 13 mm glass tubes, see figure 4.

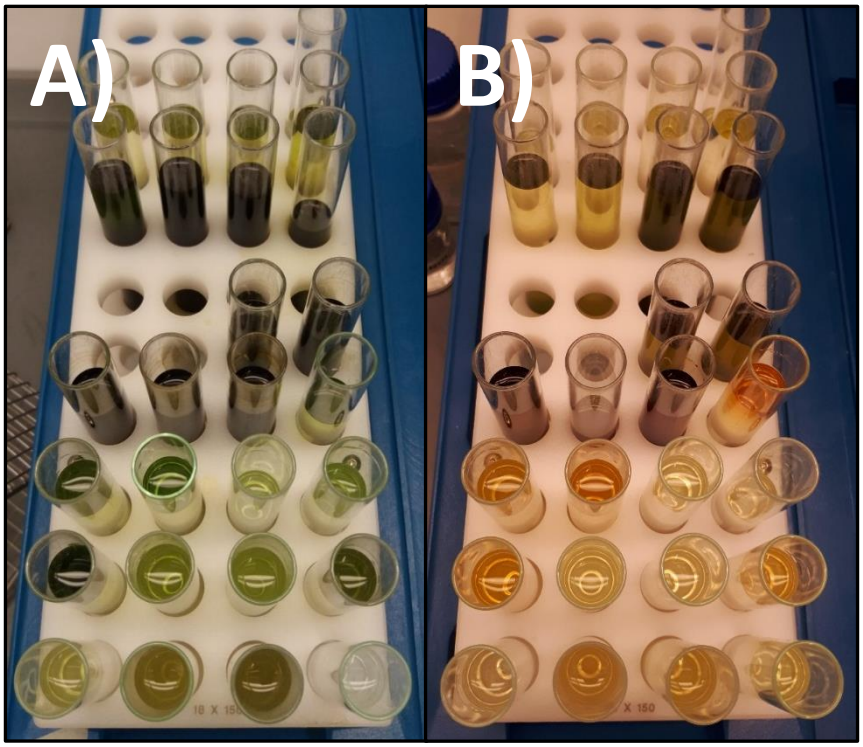

Figure 4: Two examples of microalgae extracts separated through FLASH chromatography into twenty-seven collection tubes. A) Organic microalgae extract. B) Aqueous microalgae extract. Photo: Ida Elvedal.

Mobile phases frequently used for this purpose are dichloromethane/hexane, ether/hexane, hexane/ethyl acetate, and dichloromethane/methanol, respectively (Rubin, 2011). The column material (stationary phase) used in this paper is Diaion HP-20SS, which is a polyaromatic adsorbent with large pores and a large surface area with a high affinity for aromatic and organic/hydrophobic compounds (Sigma-Aldrich, 2018). It consists of porous polystyrene polymer resins which separates molecules by size and hydrophobicity (Sigma-Aldrich, 2018).

For secondary metabolite isolation, high performance liquid chromatography (HPLC) is considered the most versatile and robust method (Hanssen, 2014). The sample of interest is separated by the use of high pressure to generate flow (the mobile phase) through a packet column (the stationary phase) (Bio-Rad, 2018). Several columns can be applied with different kinds of mobile phases, including normal phase-, size exclusion-, ion exchange- and reversed phase (RP) columns, where the latter is most frequently used in in conjunction with secondary metabolite isolation (Hanssen, 2014). There are several different kinds of RP columns, and the C18 column is the most frequently used (Hanssen, 2014), and is also the column of choice in this thesis. C18 columns consists of an octadecyl carbon chain (C18) bound to silica.

3.2 THE BIOPROSPECTING PIPELINE STEP 3 (SCREENING AND STRUCTURE IDENTIFICATION OF POTENTIAL ACTIVE COMPOUNDS)

3.2.1 Screening

3.2.1.1 Antibacterial bioassay

Antibiotic resistance is a major concern these days, and the search for novel antibiotics are a necessity. An antibacterial assay can be used to screen the sample under investigation for activity towards a bacteria strain of choice. Upon activity discovery, a minimum inhibitory concentration (MIC) assay can be used to determine the smallest concentration in which the sample of interest is active, i.e. the bacteria strains' susceptibility to the given sample (EUCAST, 2003).

Initially, a known concentration of the bacteria strain in exponential phase are inoculated with a known concentration of the sample to detect activity. Activity is determined based on an OD measurement of the assay. If active samples are detected, these can be tested in a dilution series to determine the MIC value. It's optimal to use both gram positive and gram negative bacteria strains because of their fundamental differences in terms of cell wall composition, which can affect their survivability against potential active compounds. Gram negative bacteria will in many cases be more resilient and robust, while the gram positive bacteria will be more fragile. In addition, if a sample is only active against gram positive bacteria, the mode of action will most likely be due to cell wall synthesis. This will give a good insight in the samples potential activity spectrum.

3.2.1.2 Anti-biofilm formation assay

Biofilm is the term for a mixture of microorganisms which have bonded together by the formation of extracellular polymeric substances (EPS) in an irreversible layer on a surface, resulting in a small community which can grow, secret diverse substances, exchange gene material and communicate with each other via quorum sensing (Donlan, 2002). Biofilm can exist everywhere where there is water, sufficient nutrients and a suitable surface to stick onto. It may for example be present as plaque on your teeth or the slippery layer on rocks in lakes, the sea etc. From a medical perspective, there are many challenges with biofilm because it can grow on animal and human tissue as well as on medical devices such as catheters and

pacemakers (Vidyasagar, 2016). In most cases, for bacteria to be able to cause infections in our body, they have to be organized in biofilm (Aalehaeger, 2010). This is why it is of clinical importance to obtain more knowledge of how to, not only remove biofilm, but also how to prevent biofilm from even arising at all.

In the assay used in this thesis, biofilm formation of it is desired to find activity against *S. epidermidis* biofilm formation. *S. epidermidis* is cultivated, triggered to produce biofilm by adding glucose to the medium. Prevention of biofilm formation is measured by colouring the biofilm in the wells with crystal violet, dissolving it in methanol and thereafter measuring the absorbance. The wells with more biofilm will have a stronger colour, thus affecting the light absorbed. An example of coloured biofilm in an anti-biofilm formation assay is depicted in figure 5.

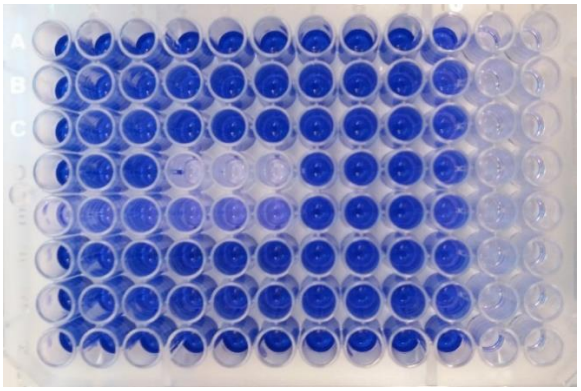

Figure 5: Biofilm formation coloured with crystal violet and dissolved in ethanol. Clear wells are indicating no biofilm growth. The clear wells to the right is a non-biofilm forming bacterium control and a medium blank control. Photo: Ida Elvedal.

3.2.1.3 MTS cell viability assays

This is an assay to test whether a sample of interest has any effect on cell viability, i.e. cell proliferation or toxicity (Riss et al., 2016). This is useful in many settings, for example to test potential commercial compounds for cytotoxicity or for activity discovery against cancer cells. Cell lines used in MTS cell viability assays in this thesis are human melanoma cell line A2058 (ATCC CRL-11147^{TM}, LGC Standards, UK) human colon carcinoma cell line HT29 (ATCC-HTB-38^{TM}, LGC Standards, UK) and human pulmonary fibroblast cell line MRC-5 (ATCC CCL-171^{TM}, LGC Standards, UK).

Melanoma is a very topical type of skin cancer. It only occurs in 4 % of skin cancer incidences, but is still responsible for the most skin-cancer related deaths due to its capacity to metastasize to several parts of the body (Roomi et al., 2006). Colon cancer is well understood, yet, it is the second most common cause of cancer-related deaths because the cancer cells remains resistant to existing therapy (O'Brien, Pollett, Gallinger, & Dick, 2007).

The assay is conducted by exposing a desired cell line with a sample of interest in microtiter wells. After three days of incubation Aqueous One Solution Reagent (AQOS) is added to the cells. The key elements in AQOS is the salt 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and the electron coupling reagent phenazine ethosulfate (PES) (Promega, 2012). MTS are transferred to a formazan product in metabolic active cells (see figure 6) which will give the microtiter wells a dark purple colour. Dead cells will not reduce MTS and these wells will therefore remain yellow, which is the original colour of AQOS. Next the absorbance can be measured in a spectrophotometer and compared to control wells for calculation of cell survival.

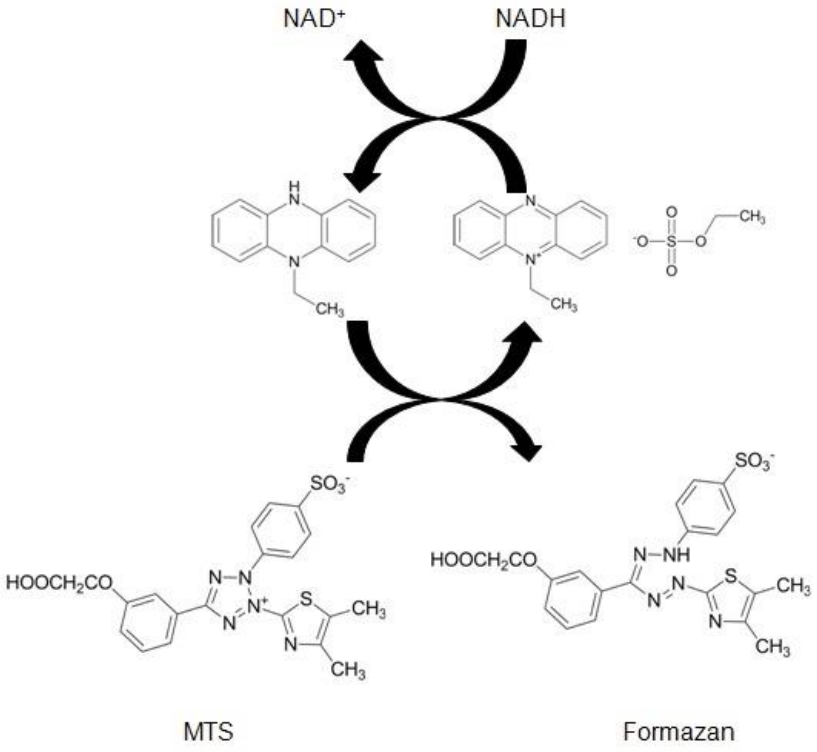

Figure 6: The electron coupling reagent PES picks up an electron from NADH in the cytoplasm and subsequently uses it to reduce MTS to an aqueous soluble formazan. NADH is oxidized to NAD+ in the process. Figure retrieved from (Riss et al., 2016).

3.2.1.4 Anti-inflammatory assay

Inflammation occurs when there is some kind of injury to cell tissue, causing the body to immediately attempt to eliminate the responsible factor, which may cause redness, heat, swelling and pain. Anti-inflammatory drugs are desired to reduce these symptoms, contribute to an enhanced immune response, prevent disease and assist the healing process (de Morais et al., 2015).

When pathogenic gram negative bacteria are discovered by the immune system, normal free floating monocytes are attracted to the area of infection and differentiate into macrophages so they can attach to the surrounding tissue (Genin, Clement, Fattaccioli, Raes, & Michiels, 2015). Once differentiated they can secret pro-inflammatory cytokines like tumor necrosis factor-α in the onset of inflammation (TNF-α) (Genin et al., 2015).

In the assay conducted, human monocytic THP-1 cells derived from the blood of a patient with acute leukemia (Tsuchiya et al., 1980) were used to mimic normal monocytes too see if a sample of interest can inhibit lipopolysaccharide (LPS) induced expression of TNF-α. The THP-1 cells are differentiated into macrophages by exposure to Phorbol 12-myristate 13-acetate (PMA), added the samples of interest and subsequently triggered to produce TNF-α through exposure to LPS. LPS is a crucial component of the gram negative bacterial cell wall, thus making the cells believe that a gram negative bacteria is present (Genin, Clement et al. 2015 and Bosshart and Heinzelmann 2016). To be more precise, LPS is an endotoxin which induces septic shock syndrome and stimulates the production of inflammatory compounds such as TNF-α (Chun et al., 2007). Expressed TNF-α can be measured by using an enzyme-linked immunosorbent assay (ELISA). With this assay, picogram quantities of cytokines, in this case human TNF-α, is measured. Antibodies are attached to the bottom of a 96-well plate and unspecific binding sites are blocked with a bovine serum albumin (BSA) blocking buffer. Samples of interest and standards are added and TNF-α binds to the antibodies. A conjugated biotin human TNF-α antibody binds the TNF-α/antibody complex and consequently to Extravidin conjugated to alkaline phosphatase which in turn reacts with pNPP substrate (Hanssen, 2009), see figure 7 for more details.

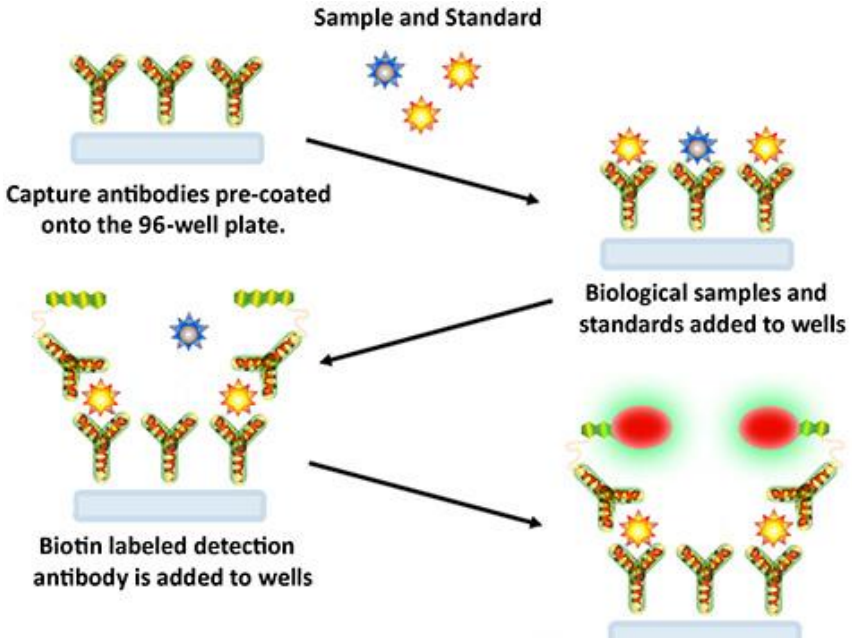

Figure 7: Antibodies are attached to the bottom of a 96-well microtiterplate and biological samples of interest are added and a TNF-α dilution series (standard) are added in a parallel control well. Next a conjugated biotin labelled human TNF-α antibody binds the TNF-α/antibody complex and thus to Extravidin conjugated alkaline phosphatase. This reacts with pNPP substrate and makes a yellow colour which can be spectrophotometrically measured. Figure retrieved from: (Sigma-Aldrich, 2015).

This makes a yellow colour which can be measured in 405 nm and is parallel to expressed TNF-α in the sample (Hanssen, 2009). The TNF-α value and a LPS control can subsequently be used to calculate percent inhibition of the cell line. Figure 8 is an example of how this assay might look like. The blank wells indicates TNF-α inhibition and thus inhibition of inflammation.

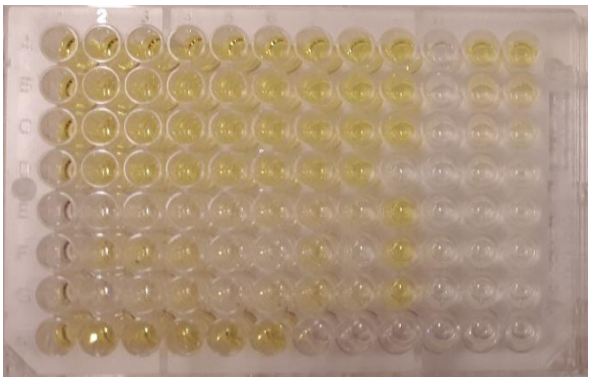

Figure 8: Anti-inflammatory and subsequently ELISA performed. Yellow wells are indicating expressed TNF-α. Blank wells are indicating TNF-α inhibition. The four last wells are control wells. Photo: Ida Elvedal.

3.2.1.5 Cellular Antioxidant Activity assay (CAA assay)

This method comprises testing of fractions of interest for antioxidative properties. Antioxidants are important in the protection of our cells against free radicals. Free radicals are highly reactive atoms or molecules which normally are beneficial, but can be highly destructive when overproduced (see figure 9). When discussing free radicals, the group of highest concern might be the reactive oxygen species (ROS), which, when overproduced, causes oxidative stress which again can lead to cell structural damages like membrane-, lipid-, protein- and DNA damage (Valko et al., 2007). These type of damages are involved in many serious diseases, including Alzheimer's disease (Qin et al., 2006), Parkinson's disease (Zhang, Dawson, & Dawson, 2000), cardiovascular diseases (Dhalla, Temsah, & Netticadan, 2000), chronic inflammation and cancers (Brieger, Schiavone, Miller Jr, & Krause, 2012).

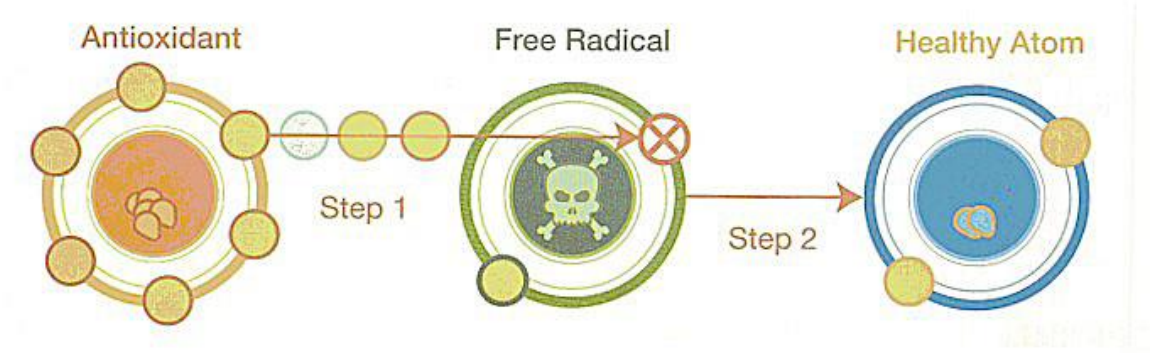

Figure 9: Antioxidants protects healthy atoms from free radicals by donating one electron, thus preventing the free radical from oxidizing the healthy atom. Figure retrieved from (Walker, 2016).

In this assay, samples of interest can efficiently be screened for their potential content of antioxidants through a HepG2 (ATCC WB-8065, LGC Standards, UK) cellular assay. This are liver cells isolated from a patient with hepatocellular carcinoma (Wolfe & Liu, 2007). These cells are exposed to the samples of interest, a 2',7'-Dichlorofluorescin diacetate (DCFH-DA) probe, and thereafter a mix of 2,2'-Azobis-2-methyl-propanimidamide and dihydrochloride (AAPH) where the latter have the capacity to generate free radicals (Cayman, 2014). DCFH-DA diffuses into the cell and are thus deacetylated to DCFH, which is more polar and thus remains within the cell. DCFH is further oxidized to DCF by peroxyl radicals generated by AAPH which emits a fluorescent light. The fluorescence is thus a proportional measurement for the level of oxidation in the cells (Wolfe & Liu, 2007). Lower levels of oxidation therefore indicates potential antioxidants present in the samples tested.

3.2.2 Structure identification of potential active compounds

3.2.2.1 Mass spectrometry

MS is a powerful scientific tool where the element composition of a molecule can be determined and information about the structure can be provided. MS systems are highly sensitive, selective, accurate and has a high throughput capability due to the three key elements: the ionization source, the mass analyser and the detector (Awad, Khamis, & El-Aneed, 2015). The sample of interest is converted to a gas-phase and ionized by the ionization source, the content is analysed by the mass analyser and the so called mass-to-charge ratio (m/z) is measured by the detector (Awad et al., 2015). The m/z ratio is the measured mass of the molecules put up against the applied charge from the ionization. Choice of ionization technique can affect the obtained data, and it is therefore crucial to choose ionization source carefully depending on your sample of interest and the purpose of your experiment (Awad et al., 2015). The mass spectrometer used in this thesis for dereplication is called Ultra-Performance-LC-Quadrupole-Time-of-Flight (UPLC-QToF-MS) with electrospray ionization (ESI) as the ionization source.

4 SAMPLE BACKGROUND

The samples explored in this thesis comprises of five biomass samples of raw material from the same diatom species cultivated in pre-experiments at different conditions, as shown in table 2. Sample 1 is further referred to as S1, sample 2 is further referred to as S2 and so on.

Table 2: Sample 1 to sample 5 (S1 – S5) and their pre-experiment cultivation conditions; treatments, cultivation volumes, culture compositions and places of cultivation (ferrosilicon factory in Finnfjord and the Norwegian College of Fishery Science (NCFS)).

	Treatments	**Cultivation volumes**	**Culture compositions**	**Places of cultivation**
S1	Light level 8	630 L photobioreactor	Monoculture	NCFS
S2	Exposed to UV for 30 minutes and subsequently 35 minutes. Light level 8.	630 L photobioreactor	Monoculture	NCFS
S3	Exposed to UV for 2 hours. Light level 8.	630 L photobioreactor	Monoculture	NCFS
S4	Light level 6.	630 L photobioreactor	Co-culture with another diatom species	NCFS
S5	Fed with CO_2 smoke and fermented in a refrigerator for 48 hours. 1200 watt LED and natural light.	6000 L photobioreactor	Monoculture	Finnfjord

For sample S1, S2, S3 and S4, a pure algae culture was obtained by single-cell isolation by micropipette. Once isolated in a monoculture, the cultures were cultivated in 630 liter photobioreactors with UV sterilized natural sea water and additional nutrients; substral (0.25 mL/liter^{-1}), silicate (1 mL/liter^{-1}) and soil extract (1 mL/100 liter^{-1}). Thereafter the cultures were treated according to table 2. After treatment, the samples were harvested and the raw material were stored at -23°C until further work.

The sample S5 originates from a diatom cultivation project, which is a collaboration between UiT and Finnfjord AS, a ferrosilicon plant in Finnfjord. Here 6000 L photobioreactors have been installed for cultivation of diatoms. A diatom monoculture was cultivated in a photobioreactor with the addition of factory smoke. The factory smoke contained, amongst others, CO_2, NO_x gasses and microsilica. The diatoms were cultivated in filtrated seawater with the addition of an excess of silicate (Na_2SiO_3 X $9H_2O$, Merck, Germany) and substral (Scotts Celaflor GmbH & Co. KG, Germany). After this treatment the algal biomass was harvested with a continuous centrifuge and fermented in a dark incubator at 7°C for 48 hours before stored at -23°C until further work.

Bear in mind that all the photobioreactors have been open in non-sterile environments during the treatments, and therefore a certain degree of contamination is to be expected.

5 WORKFLOW

All experiments in this thesis were conducted at Marbio which is a purification and screening platform. This is a high-throughput analytical laboratory where marine extracts are processed and analysed through chromatography, spectroscopy and biochemical- and cellular assays for potential discovery of secondary metabolites with new desired activities with clinical relevance for commercial partners (Svenson, 2013).

The samples in this thesis were extracted and fractionated into eight FLASH fractions, and subsequently screened for bioactivity in three screens before attempted dereplicated.

In the 1st screen, eight FLASH fraction from each sample were tested in an antibacterial-, anti-biofilm-, anticancer- (with human melanoma cell line A2058), anti-inflammatory- and antioxidant assays. S5 was, in addition, tested in an anticancer assay with human carcinoma cell line HT29 and a cytotoxicity assay with normal pulmonary fibroblasts cell line MRC-5. An overview off all activity in the first screenings are presented in the appendix (table 32).

In the 2nd screen, active FLASH fractions of S5 was prioritised to determine minimum inhibitory concentration (MIC) values in the antibacterial-, anti-biofilm and anticancer assays. In addition, active fractions in the anti-inflammatory assay were further refractionated into forty new HPLC fractions and screened for bioactivity again.

In the 3rd screen, selected active fractions in the anti-biofilm assay were further refractionated into forty new HPLC fractions and screened for bioactivity again.

By dereplication, selected active FLASH fractions from the anti-inflammatory assay and active HPLC fractions from the anti-biofilm assay were analysed by UPLC-QToF-MS to search for possible compounds responsible for detected bioactivity.

6 MATERIALS AND METHODS

6.1 EXTRACTION

From sample 1 – 4 (S1 – S4) only one extract was obtained; one organic extract, and from sample S5 two extracts were obtained; one aqueous and one organic extract.

6.1.1 Extraction of samples S1 – S4

Table 3: The equipment and solvents used for extraction of S1-S4.

Equipment	**Distributor, country**
Heto PowerDry PL9000	Thermo Fisher Scientific, USA Massachusetts
Whatman® qualitative filter paper, grade 3, 1003-090	Sigma-Aldrich, Missouri, USA
Rotavapor (Heidolph, Laborota 4002)	Heidolph Instruments GmbH & Co, Germany
Solvents, ID	
Methanol, 34860	Sigma-Aldrich, Missouri, USA
Milli-Q Ultrapure water	Merck KGaA, Germany

The raw biomasses were freeze-dried, grinded and stored at - 23°C. The powder was extracted with 20 mL 80 % MeOH per gram sample for 24 hours in 7°C, and then vacuum filtrated through a Whatman grade 3 filter and dried in the rotavapor. Remaining sample on the filter paper was extracted with about 10 mL 80 % methanol per gram sample over 24 hours in 7°C, vacuum filtrated and dried again.

6.1.2 Extraction of sample S5 – separation of supernatant and pellet

Table 4: The equipment and solvents used for extraction of S5.

Equipment	**Distributor, Country**
Multifuge 3S-R, centrifuge	Thermo Fisher Scientific, USA, Massachusetts
Heto PowerDry PL6000 Freeze Dryer	Thermo Fisher Scientific, USA, Massachusetts
Heto PowerDry PL9000 Freeze Dryer	Thermo Fisher Scientific, USA, Massachusetts
Whatman® qualitative filter paper, grade 3, 1003-090	Sigma-Aldrich, Missouri, USA
SC250EXP SpeedVac® Concentrator	Thermo Fisher Scientific, USA, Massachusetts
Solvents, ID	
Milli-Q Ultrapure water	Merck KGaA, Germany
Dichloromethane, 34856	Sigma-Aldrich, Missouri, USA
Methanol, 34860	Sigma-Aldrich, Missouri, USA

To obtain one aqueous and one organic extract the raw biomass material was added milli-Q (about 10 times the dry sample weight) after freeze-drying. The sample was then extracted in 7°C overnight and centrifuged for 30 minutes at 4000 rpm and 5°C. The supernatant was

separated from the pellet and the pellet was dissolved in 150 mL of milli-Q water. This centrifugation and separation step was then repeated.

Both the supernatant (aqueous extract) and the pellet were freeze-dried (Heto PowerDry PL6000 for pellet and Heto PowerDry PL9000 for supernatant) and the pellet was ground. Dichloromethane and methanol (1:1) was added to obtain a volume equivalent to 10:1 ratio with the pellet. Next the sample were extracted in 7°C for three hours (shaken carefully by hand every hour to expedite the extraction process). Thereafter the sample was vacuum filtrated by using a humidified Whatmann nr. 3 filter with the solvent (dichloromethane and methanol 1:1 ratio) and dried in a rotavapor. The remaining dry substance left on the filter was extracted again for one hour in 7°C with another 170 mL of solvents. The filtration process was repeated and the liquids (organic extract) were dried as much as possible to a viscous mass in the rotavapor.

6.2 PREFRACTIONATION THROUGH FLASH CHROMATOGRAPHY

Table 5: The equipment and solvents used for prefractionation of S1-S5.

Equipment	**Distributor, country**
Biotage®SNAP Cartridge KP-Sil 10 g, FSK0-1107-0010	Biotage, Sweden
Biotage® HPFC SP4 Flash Purification System	Biotage, Sweden
Heidolph, Laborota 4002	Heidolph Instruments GmbH & Co, Germany
Syncore® Polyvap	Büchi, Switzerland
Solvents, ID	
Dianon® HP-20SS, 13615-U	Sigma-Aldrich, Missouri, USA
Methanol, 34860	Sigma-Aldrich, Missouri, USA
Milli-Q Ultrapure water	Merck KGaA, Germany
Acetone, 34850	Sigma-Aldrich, Missouri, USA
Hexane, 34859	Sigma-Aldrich, Missouri, USA

6.2.1 FLASH columns (stationary phase)

For preparation of FLASH columns, 5.5 g and 6.5 g of column material were used for the aqueous extract and the organic extracts respectively. The columns were made by adding the column material and subsequently activated with 90 % MeOH for about 20 minutes, followed by replacing the MeOH with milli-Q water and then vacuum filter the solution using a 10 g plastic syringe attached to a vacuum manifold. Readily made columns are depicted in figure 10A.

6.2.2 Preparation of samples

The samples S1 – S4 were dissolved in 10 mL 90 % methanol in the rotavapor at 45°C with no vacuum, and column material was added (same amount as the dry sample weight). The samples were next dried in rotavapor under vacuum.

From the aqueous extract of S5 (figure 10B), 1.51 g was weighed out in two glass tubes, about 0.75 g in each tube. The samples were then solved in 4 mL of MeOH in each tube and 1.5 g of column material and 2 mL of milli-Q water were added to each tube respectively. Some paper towel was then attached to the top of the tube and the tubes were put in the SpeedVac vacuum centrifuge to dry overnight.

From the organic extract of S5, 1.52 g was weighed out and dissolved in 60 mL of hexane (40 mL for each gram). The hexane/organic extract mix was then poured into a separating funnel with about 50 mL of 90% MeOH, and the top phase was discarded (waste phase). This step was then repeated. Next 2.02 g of column material was added to the extract (bottom phase) and it was dried in a rotavapor to a dry mass (figure 10C).

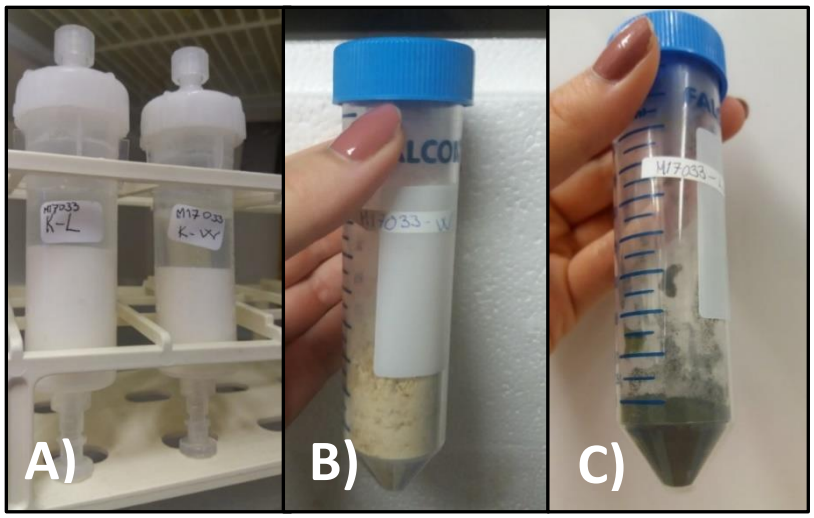

Figure 10: A) Prepared columns (stationary phases) for FLASH chromatography. B) Aqueous extract of S5. C) Organic extract of S5 prepared for FLASH chromatography.

6.2.3 FLASH chromatography

One FLASH column at the time with the appropriate amount of column material were attached to the FLASH Purification System. One sample at the time was poured into their respective columns and the sample was separated into 27 tubes with 24 mL in each tube. The mobile phases comprised of a gradient of water (weak solvent) and methanol and acetone (strong

solvents) with a flow rate of 12 mL/min. Three and three tubes were pooled in one common tube, except the final six which all were pooled in the last tube, making eight tubes in total.

Table 6: The collection of fractions, time eluted and gradients of milli-Q water, methanol and acetone.

Fraction	Time (minutes)	MilliQ (%)	Methanol (%)	Acetone (%)
1 (1-3)	0 – 6	95	5	0
2 (4-6)	6 – 12	75	25	0
3 (7-9)	12 – 18	50	50	0
4 (10-12)	18 – 24	25	75	0
5 (13-15)	24 – 30	0	100	0
6 (16-18)	30 – 36	0	100	0
7 (19-21)	36 – 42	0	50	50
8 (22-27)	42 – 54	0	0	100

Subsequently the tubes were then dried in the Polyvap with vacuum at 43°C. These eight FLASH fractions are further referred to as F1, F2.., and so on to F8. An overview of all the samples with their associated FLASH fractions are depicted in table 7. The FLASH fractions will further be referred to according to the table. To separate the organic extract from the aqueous extract in F5, these will be further referred to as "L" (organic extract) and "W" (aqueous extract).

Table 7: Names of FLASH fractions after prefractionation of sample S1-S5.

	S1	S2	S3	S4	S5 Organic extract	S5 Aqueous extract
FLASH fraction 1	S1-F1	S2-F1	S3-F1	S4-F1	S5-L-F1	S5-W-F1
FLASH fraction 2	S1-F2	S2-F2	S3-F2	S4-F2	S5-L-F2	S5-W-F2
FLASH fraction 3	S1-F3	S2-F3	S3-F3	S4-F3	S5-L-F3	S5-W-F3
FLASH fraction 4	S1-F4	S2-F4	S3-F4	S4-F4	S5-L-F4	S5-W-F4
FLASH fraction 5	S1-F5	S2-F5	S3-F5	S4-F5	S5-L-F5	S5-W-F5
FLASH fraction 6	S1-F6	S2-F6	S3-F6	S4-F6	S5-L-F6	S5-W-F6
FLASH fraction 7	S1-F7	S2-F7	S3-F7	S4-F7	S5-L-F7	S5-W-F7
FLASH fraction 8	S1-F8	S2-F8	S3-F8	S4-F8	S5-L-F8	S5-W-F8

6.3 PREPARATION OF STOCK SOLUTIONS

Table 8: The equipment and solvents used for dissolution with DMSO.

Equipment	Distributor, country
Universal shaker SM – 30 CONTROL	Edmund Bühler GmbH, Germany
Solvent, ID	
DMSO, D4540	Sigma-Aldrich, Missouri, USA

The dried samples were added DMSO to a concentration of 40 mg/mL and put on a shaker for about 2 hours for dissolution. Due to insufficient material in some of the fractions, a lower

concentration were made to secure enough material, see table 9. Next they were transferred to 1.8 mL cryo tubes and stored at 7°C.

Table 9: diluted FLASH fractions (F) in DMSO in cryo tubes of sample S1-S5. The colouration indicates the following concentrations: 40 mg/mL (none/white), 20 mg/mL (blue), 10 mg/mL (green) and 4 mg/mL (yellow).

	S1	**S2**	**S3**	**S4**	**S5-L**	**S5-W**
F	1	1	1	1	1	1
F	2	2	2	2	2	2
F	3	3	3	3	3	3
F	4	4	4	4	4	4
F	5	5	5	5	5	5
F	6	6	6	6	6	6
F	7	7	7	7	7	7
F	8	8	8	8	8	8

Deep well plates were thereafter prepared with a dilution of all the fractions in milli-Q water as shown in table 10. All first screenings are normally conducted with a FLASH fraction concentration of 100 µg/mL, but unfortunately, the DMSO could not be removed from the wells due to lack of equipment. To avoid exceeding the DMSO tolerance in the bioassays, the FLASH fraction concentrations were not adjusted for in the bioassays in this thesis. Which means that in all first screenings, samples of 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL and 0.1 mg/mL had a concentration of 100 µg/mL, 50 µg/mL, 25 µg/mL and 10 µg/mL respectively.

Table 10: FLASH fractions/DMSO in milliQ water (F) in the deep well plate of sample S1-S5. The colouration indicates the following concentrations: 1 mg/mL (none/white), 0.5 mg/mL (blue), 0.25 mg/mL (green) and 0.1 mg/mL (yellow).

	S1	**S2**	**S3**	**S4**	**S5-L**	**S5-W**
F	1	1	1	1	1	1
F	2	2	2	2	2	2
F	3	3	3	3	3	3
F	4	4	4	4	4	4
F	5	5	5	5	5	5
F	6	6	6	6	6	6
F	7	7	7	7	7	7
F	8	8	8	8	8	8

6.4 ANTIBACTERIAL ASSAY

Table 11: The equipment, bacteria strains, mediums and reagents used in the antibacterial assay.

Equipment	**ID**	**Distributor, country**
Hera safe biological safety cabinet	Class II	Thermo Fisher Scientific, Massachusetts, USA
Blood agar plates		SUMP, Media kitchen, UNN
96-well microtiter plates	734-2073	VWR International AS, Pennsylvania, USA
Incubator MIR-262		SANYO Electric Co., Ltd., Japan
Victor Multilabel Counter		Perkin Elmer, Massachusetts, USA
Bacteria strains		
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
Mediums		
Mueller Hinton broth (MH)	275730	Becton, Dickinson and Company, New Jersey, USA
Brain Heart Infusion broth (BHI)	53286	Sigma-Aldrich, Missouri, USA
Reagents		
Milli-Q Ultrapure water		Merck KGaA, Germany
Gentamycin	A 2712	VWR International AS, Pennsylvania, USA

The FLASH fractions were diluted with milli-Q water in a deep well plate to a concentration of 1/5 of the diluted deep-well plate (see table 10).

The strains are stored at -80°C in 10 % glycerol, and when in use, they are plated out on blood agar plates and incubated at 37°C overnight. Subsequently the colonies can be directly used in the assay, or stored upside down in 7°C with a maximum storage time of one month. For maintenance, the bacteria strains have to be re-streaked onto new blood agar plates every second week.

6.4.1 1st screen

This assay was conducted in a safety cabinet. For *S. aureus, E. coli* and *P. aeruginosa* a scoop of the bacteria from the blood agar plates were added to 8 mL of MH-broth. The same was done for *E. faecalis* and *S. agalactiae*, but with 8 mL BHI-broth. All the tubes were then incubated at 37°C overnight.

The next day the diluted fractions were distributed in 96-well microtiter plates in two parallels with 50 µl fraction in each well, except to the control wells. The bacteria suspensions were

standardized to achieve viable bacterial density and distributed to the plates, 50 µl in each well containing the fractions, as well as to a positive control and to a gentamycin control (in two parallels).

Controls:

- Negative control: 50 µl medium and 50 µl milli-Q water
- Positive control: 50 µl bacteria suspension and 50 µl milli-Q water
- Gentamycin control: a dilution series of gentamycin with all the bacteria stains

The plates were incubated at 37°C overnight, and finally the OD were measured at 490 nm.

6.4.2 2nd screen

Due to findings of bioactivity in S5-L, the bioassay was conducted again with the active fractions against *S. agalactiae* to confirm activity and to check the MIC value. The assay was performed with FLASH fraction concentrations of 100 µg/mL, 50 µg/mL, 20 µg/mL and 10 µg/mL.

6.4.3 Evaluation of results

The software used was WorkOut 2.5 (dasdaq, England). The parallels were compared and the control wells were checked to secure reliable results. The gentamycin control was used to control that the bacteria strains' growth and resilience were normal. The bacteria was added to a dilution series of gentamycin from 16 µg/mL to 0.01 µg/mL, and standard gentamycin inhibition values are shown for all the strains in table 12. If bacteria strains within the assay would have gentamycin values outside these ranges they would be deemed not usable.

Table 12: Standard gentamycin inhibition values for S. aureus, E. coli, E. faecalis, P. aeruginosa *and* S. agalactiae *and the bacteria strains respective Gram type.*

	S. aureus	***E. coli***	***E. faecalis***	***P. aeruginosa***	***S. agalactiae***
Gram type	+	-	+	-	+
Standard gentamycin inhibition values	0,06 µg/mL – 0,13 µg/mL	0,25 µg/mL	4 µg/mL – 8 µg/mL	0,13 µg/mL – 0,25 µg/mL	2 µg/mL

Fractions were determined active with an OD under 0.05, questionable with an OD between 0.05 and 0.09 and inactive with an OD over 0.09 at 490 nm in both the first and the second screen.

6.5 ANTI-BIOFILM FORMATION ASSAY

Table 13: The equipment, bacteria strains, mediums and reagents used in the anti-biofilm formation assay.

Equipment	**ID**	**Distributor, country**
Hera safe biological safety cabinet	Class II	Thermo Fisher Scientific, USA (Massachusetts)
Incubator Unimax 1010		Heidolph Instruments GmbH & Co, Germany
96-well microtiter plates	734-2073	VWR International AS, Pennsylvania, USA
Incubator MIR-262		SANYO Electric Co., Ltd., Japan
Victor Multilabel Counter		Perkin Elmer, Massachusetts, USA
Bacteria strains		
Staphylococcus haemolyticus	Clinical isolate 8-7A	University hospital (UNN) Tromsø, Norway
Staphylococcus epidermidis	ATCC 35984	University hospital (UNN) Tromsø, Norway
Medium/Reagents		
Glycerol	G5516	Sigma Aldrich, Missouri, USA
Tryptic soy broth (TSB)	105459	Merck KGaA, Germany
Blood agar plates		SUMP, Media kitchen, UNN
Glucose	D9434	Sigma Aldrich, Missouri, USA
Crystal violet	115940	Merck, New Jersey, USA
Ethanol	20823.362	VWR International AS, Pennsylvania, USA

6.5.1 1st screen

The experiment was conducted in a safety cabinet.

A scoop of *S. haemolyticus* and *S. epidermidis* cultivated on blood agar plates were inoculated with 5 mL TSB and incubated with shaking overnight at 200 rpm and 37°C. The freezer stock of these bacteria are stored at -80°C in TSB medium containing 20 % glycerol.

The FLASH fractions were diluted with milli-Q water in a deep well plate to a concentration of 1/5 of the diluted deep-well plate (see table 10). Next the fractions were added to 96-well microtiter plates, 50 µl in each well in three parallels. Thereafter, 100 mL TSB with 1 % of glucose was prepared in an autoclaved Erlenmeyer flask. *S. epidermidis* diluted 1:100 in TSB with 1 % glucose was then added to the wells, 50 µl in each well, except the medium control and the negative control.

Controls

- Medium control: 50 µl TSB with 1 % glucose and 50 µl milli-Q water
- Non-biofilm formation control: 50 µl *S. haemolyticus* diluted in TSB with glucose 1:100 and 50 µl ddH_2O.

- Positive control: 50 µl *S. epidermidis* diluted in TSB with glucose 1:100 and 50 µl milli-Q water

The plates were then incubated at 37°C overnight.

Next the plates were directly observed with the naked eye for inhibition of bacterial growth. This is important to separate the bacteria growth inhibition from the actual biofilm formation inhibition. The bacteria suspension were thereafter removed and the wells were washed with tap water. Thereafter the biofilm was fixated in 65°C for one hour and 70 µl of 0.1% crystal violet was distributed to each well. After 5 minutes the plates were washed with tap water two times before set to dry for one hour at 65°C. Once dried, 70 µl of ethanol was added to all the wells and the plates were stirred for 10 minutes to dissolve the crystal violet. In the wells the biofilm can be distributed unevenly and therefore, the crystal violet is dissolved in ethanol to secure an even distribution to get appropriate absorbance measurements. The OD was then measured in a Victor Multilabel Counter.

6.5.2 2nd screen

Due to activity in S5-L and S5-W, a second screen was conducted with the active fractions to confirm activity and to check the MIC value. The same method as for the 1st screen was performed with fraction concentrations of 100 µg/mL, 50 µg/mL, 25 µg/mL and 10 µg/mL for S5-L and with concentrations of 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL and 1 µg/mL for S5-W.

6.5.3 Evaluation of results

As biofilm formation has a tendency to vary, three parallels were made. If one fraction had a significant deviation from the others it was eliminated. *S. haemolyticus* was used as a non-biofilm forming bacteria control.

Fractions from both the first and second screening were determined active with an OD under 0.25, questionable with an OD between 0.25 and 0.30 and inactive with an OD over 0.30 at 490 nm.

6.6 WORKING WITH CELLS

6.6.1 Step 1: Preparation

Normal aseptic technique is applied for working with the cells in this thesis. For splitting of the cells and plating them in microtiter plates, the cell lines always have to be checked in a microscope to make sure they are healthy and thus provide reliable results.

6.6.2 Step 2: Seeding of cells in microtiter plates for bioassays.

Firstly, two solutions; Phosphate-Buffered saline (PBS) and Trypsin, have to be made for washing the cells and loosen the adherent cells from the tissue culture (TC) flasks, respectively. Preparation of these solutions can be found in the appendix, page 76.

Next, PBS, trypsin and medium are preheated to 37°C to avoid shocking the cells with a low temperature. TC flasks containing the cell line of interest are checked in a light microscope and then brought to a disinfected flow cabinet with all necessary equipment (see step 1). The medium is poured off and the adherent cells are washed with 10 mL PBS and thereafter 5 mL trypsin. The cells are then incubated at 37°C and 5 % CO_2 until they loosen from the wall of the flask (suspension cells like THP-1 does not stick to the flask wall and thus no treatment with trypsin is needed) and afterwards resuspended in 10 mL medium and mixed well. A small amount of the cell suspension are mixed with trypan blue, and by the counting cells in a Bürker counting chamber the cell concentration can be estimated. Trypan blue penetrates the membrane of dead cells and thus colouring them blue, making it possible to count only living cells. Cell suspension and medium are thereafter mixed to obtain the desired density for the relevant assay (see table 14 under headline “6.6 Working with cells”.) and seeded out in microtiter plates, 100 µl in each well. The plates are thereafter incubated at 37°C and 5 % CO_2 for about 24 hours.

Table 14: All the cell lines used in this thesis as well as their origin, medium requirements, type and concentrations in the respective bioassays.

Cell line	**Origin**	**Medium**	**Type of cell line**	**Concentration in bioassay**
A2058, ATCC CRL-11147	Human melanoma	D-MEM with 10 % FBS and 10 µg/mL gentamycin	Adherent cells	2 x 10^4 cells/mL for MTS viability assay
HT29, ATCC-HTB-38	Human colon carcinoma	RMPI-1640 with 10 % FBS and 10 µg/mL gentamycin	Adherent cells	2 x 10^4 cells/mL for MTS viability assay
MRC-5, ATCC CCL-171	Normal pulmonary fibroblasts	E-MEM with 10 % FBS, 1 % Non-Essential Amino Acids (NEAA), 1 % L-Alanyl-L-Glutamine, 1 % Sodium Pyruvate, 2 % Sodium Bicarbonate and 10 µg/mL Gentamycin	Adherent cells	4 x 10^4 cells/mL for MTS viability assay
THP-1, ATCC TIB-202	Monocyte-like cell line from an acute monocytic leukemia patient	RMPI, low endotoxin level < 0,5 EU/ml with 10 % FBS ultralow endotoxin and 10 µg/mL gentamycin	Suspension cells	10^6 cells/mL for anti-inflammatory assay
HepG2, ATCC WB-8065	Human liver cancer cell line	E-MEM medium with 1 % NEAA, 1 % Sodium Pyruvate, 1 % L-Alanyl-Glutamine and 10 % FBS.	Adherent cells	80 x 10^4 cells/mL for antioxidant assay (CAA)

6.7 MTS CELL VIABILITY ASSAY

Table 15: The equipment, sterilizers, cell lines, medium components and reagents used in the MTS viability assay.

Equipment	**ID**	**Distributor, country**
Herasafe biological safety cabinet	Class II	Thermo Fisher Scientific, Massachusetts, USA
Sanyo CO_2 incubator MCO-18AIC		Panasonic biomedical, Japan
Light microscope		Leica Microsystems, Germany
Bürker Counting chamber		Sigma-Aldrich, Missouri, USA
Victor Multilabel Counter		Perkin Elmer, Massachusetts, USA
Sterilizers		
Ethanol	20823.362	VWR International AS, Pennsylvania, USA
Cell lines		
A2058 from Human melanoma	ATCC CRL-11147	LGC Standards, UK
HT29 from Human colon carcinoma	ATCC-HTB-38	LGC Standards, UK
MRC-5 from normal lung fibroblasts	ATCC CCL-171	LGC Standards, UK
Medium components		
Dulbecco's Modified Eagle Medium (D-MEM)	32430027	Thermo Fisher Scientific, Massachusetts, USA
Roswell Park Memorial Institute medium (RPMI) 1640	FG 1383	Merck KGaA, Germany
Fetal Bovine Serum (FBS)	S 1810	VWR International AS, Pennsylvania, USA
Gentamycin (10 mg/mL)	A 2712	Merck KGaA, Germany
L-Alanyl-Glutamine	K 0302	VWR International AS, Pennsylvania, USA
Reagents		
Trypan blue		Sigma-Aldrich, Missouri, USA
CellTiter 96® Aqueous One Solution Reagent	G 358B	Promega, Wisconsin, USA

6.7.1 1st screen

This procedure were conducted for the human melanoma cell line A2058 with FLASH fractions from S1-S5. In addition, FLASH fractions of S5-L and S5-W were tested against the human colon carcinoma cell line HT29 and the non-cancerous, normal lung fibroblasts cell line MRC-5.

The first screen were conducted according to step 1 – 2 under headline "working with cells". See table 14 under headline "6.6 Working with cells" for mediums and required cell density for the relevant assays. FLASH fractions from the deep well plate were added to the fraction wells with concentrations of 100 µg/mL, 50 µg/mL and 25 µg/mL (see table 10) and incubated in 37°C and 5% CO_2 for three days.

Old medium was thereafter carefully removed from the wells and desired samples were added in three parallels with 90 µl RMPI with 10 % FBS and 10 µl sample. One negative control were made with 100 µl RMPI with 10 % FBS (100 % viability). After three days of incubation, 10 µl AquousOne were added in each of the wells. After one hour of incubation in 37°C and 5% CO_2 the plates were checked in a light microscope and the absorbance is measured at 490 nm in a Victor plate reader.

6.7.2 2nd screen

Due to activity in the first screen, a second screen was performed with a dilution series for S5-L-F6 on A2058 with FLASH fraction concentrations of 50 µg/mL, 25 µg/mL and 10 µg/mL to confirm activity and to check the MIC value. The procedure was performed the same way as for the first screening.

6.7.3 Evaluation of results

The parallels and the controls were checked for reliable results. FLASH fractions were determined active with a cell survival of under 50 %, questionable with a cell survival between 50 and 60 % and inactive with a cell survival over 60 %. Percent cell survival was calculated based on the OD values of the average of the fraction wells, positive control and negative control as shown in the equation below. Negative control is a standard number of 0.15 which is equivalent to 100 % dead cells.

$$\frac{(OD\ Fraction\ parallel\ average - OD\ Positive\ control)}{OD\ Negative\ control - OD\ Positive\ control}\ x\ 100\ \%$$

6.8 ANTI-INFLAMMATORY ASSAY

Table 16: The equipment, cell lines, mediums and reagents used in the anti-inflammatory assay.

Equipment	**Distributor, country**
Tissue Culture Plate, 96 well	Corning, New York, USA
Light microscope	Leica Microsystems, Germany
Multifuge 1S	Thermo Fisher Scientific, Massachusetts, USA
Bürker counting chamber	Sigma-Aldrich, Missouri, USA
Sanyo CO_2 incubator MCO-18AIC	Panasonic biomedical, Japan
Cell line/medium, ID	
THP-1 cells	ATCC TIB-202
RPMI 1640, low endotoxin < 0,5 EU/mL	Biochrom
Gentamycin (10 mg/mL), A2712	Merck, New Jersey, USA
FBS ultralow endotoxin, S1860-500	Biowest
Reagents, ID	
Trypan blue	Sigma-Aldrich, Missouri, USA
PMA, P1585	Sigma-Aldrich, Missouri, USA
PBS with low endotoxin levels, D8537	Sigma-Aldrich, Missouri, USA
LPS, L2630	Sigma-Aldrich, Missouri, USA

This experiment was conducted with standard aseptic technique. The THP-1 cells are suspension cells and were directly transferred to centrifuge tubes and centrifuged at 150 g in 5 minutes. The medium was then poured off, and the pellets were dissolved in 30 mL new RPMI medium. From this point, the cell concentration was estimated with trypan blue as for the other cells.

Next 50 ng/mL PMA was added to differentiate the monocytes to macrophages and the solution was distributed to microtiter plates, 100 µl in each well. The plates were then incubated at 37°C with 5 % CO_2 for two days.

Subsequently the medium was removed from the wells and each well were washed with 100 µl PBS with low levels of endotoxins before 100 µl of new medium with no PMA was added. The cells were then set to incubate at 37°C with 5 % CO_2 until the next day.

Next the medium was removed from all the wells and 80 µl RPMI medium was added to each well, except the controls.

Controls:

- LPS control: 1 ng/mL LPS diluted in RPMI medium.
- Cell control: 100 µl cell suspension

The FLASH fractions from the deep-well plate were distributed in two parallels in each well with concentrations of 100 µg/mL, 50 µg/mL and 25 µg/mL (see table 10) and then incubated

at 37°C and 5 % CO_2 for one hour. Thereafter diluted LPS with RPMI with a concentration of 1 ng/mL was distributed to the wells and the plates were incubated at 37°C and 5 % CO_2 for six hours and thereafter stored at -80°C freezer.

6.8.1.1 Detection of TNF-α with ELISA

Table 17: The equipment and reagents used in the ELISA.

Equipment	**ID**	**Distributor, country**
Nunc Maxisorp 96F-well ELISA plate	735-0083	VWR International AS, Pennsylvania, USA
AquaMax® Microplate Washer		Molecular devices, California, USA
Plate shaker TiMix 5 Nr. 6166 BX 00188		Edmund Bühler GmbH, Germany
DTX880 plate reader		Beckman Coulter, California, USA
Reagents		
Anti-Human TNF alpha Purified	14-7348-85	eBioscience, San Diego, CA
Bovine Serum Albumin (BSA)	A2153	Sigma-Aldrich, Missouri, USA
Tween®20	P1379	Sigma-Aldrich, Missouri, USA
Human TNF alpha recombinant protein	14-8329-63	eBioscience, San Diego, CA
Anti-Human TNF alpha Purified	14-7348-85	eBioscience, San Diego, CA
Anti-Human TNF alpha Biotin	13-7349-85	eBioscience, San Diego, CA
ExtrAvidin®- Alkaline Phosphatase	E2636	Sigma-Aldrich, Missouri, USA
Phosphatase substrate (pNPP)	P5994	Sigma-Aldrich, Missouri, USA

Firstly, two buffers had to be prepared, one TBS buffer and one 1 M diethanolamine buffer. Preparation of these can be found in the appendix, page 76 and 77. From the TBS buffer, a wash buffer (TBS with 0.05 % Tween 20), a blocking buffer (TBS with 2 % BSA) and an assay diluent (TSB with 1 % BSA) were made.

All incubation steps were done on a shaker in room temperature, except the last one.

Nunc Maxisorp 96F-wells were coated with a concentration of 2 µg/mL Anti-Human TNF alpha Purified in TBS, 100 µl to each well, and stored at 7°C overnight.

The washing buffer were connected to the AquaMax® Microplate Washer. The Nunc Maxisorp 96F-well plates from the day before were thereafter washed and dried in the microplate washer. Next the blocking buffer was distributed in the plates, 200 µl in each well and incubated on a shaker for one hour. Next the plates were washed with wash buffer again. Samples and LPS control from the anti-inflammatory assay were diluted 1:20 in the wells, and the cell control was diluted 1:2. A standard curve was made with diluted TNF-α to 1000 pg/mL in assay diluent in two parallels.

Controls:

- LPS control: diluted 1:20 in assay diluent
- Cell control: diluted 1:2 in assay diluent
- Standard curve (TNF-α dilution series): 1000 pg/mL – 500 pg/mL – 250 pg/mL – 125 pg/mL – 62.5 pg/mL – 31.25 pg/mL – 15.625 pg/mL – 0 pg/mL

The dilution series was made by adding 200 µl TNF-α to the top well, taking 100 µl of this to the next well and pipetting 4-5 times up and down, then 100 µl of this to the next well and so on. The final well was blank.

The plates were then incubated for about 2 hours and further washed again. Anti-Human TNF alpha Biotin was diluted to 3 µg/mL in assay diluent and 100 µl was distributed to each well. The plates were incubated for another hour, washed again and subsequently added a mix of ExtrAvidin®- Alkaline Phosphatase and assay diluent (1:20000), 100 µl in each well. Thereafter the plates were incubated for 30 minutes. Meanwhile a mixture of 1 mg/mL pNPP in the 1 M dietanolamin buffer was prepared, and after the final wash (soak wash) of the plates this solution was distributed in the wells, 100 µl in each. The plates were finally left for about 40 minutes before the absorbance was measured in a DTX880 at 405 nm.

6.8.2 Evaluation of results

LPS is used to trigger TNF-α production in the cells, and the LPS control is thus a measurement of full TNF-α production. To evaluate the results, a standard curve was made in excel based on the OD values of the TNF-α dilution series. From this, based on the average OD value of the fractions, the TNF-α value was found. Once found, the TNF-α value needs to be multiplied with twenty, since the fractions are diluted twenty times. An example is shown in figure 11.

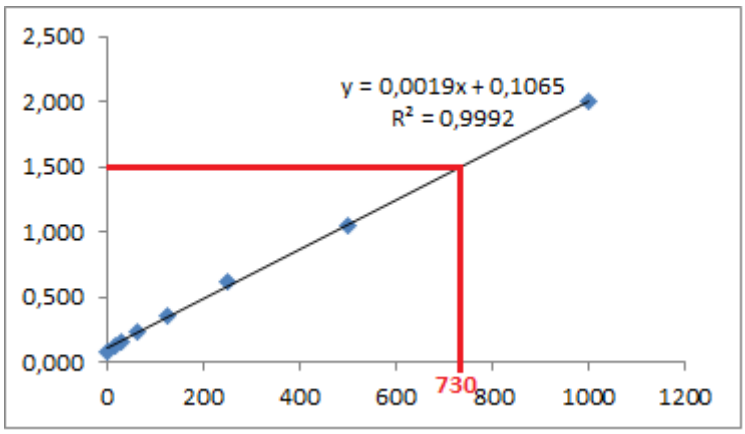

Figure 11: Example of a standard curve made based on a TNF-α dilution series. If an average OD value of a fraction is 1500 the TNF-α value is 730 x the dilution factor.

Example: if the OD value was 1500 at 405 nm, then based on the standard curve, the 20 x diluted TNF-α value is 730. The true TNF-α value would thus be 730 x 20 = 14,600.

Further this value can be put in the equation below with the OD value of the LPS control, and percent inhibition of the cell line can be calculated with the use of the equation below. Active fractions were determined with a TNF-α inhibition of over 50 %, questionable between 40 and 50 % and inactive under 40 %.

$$100 - \left(\frac{TNF - \alpha\ value}{LPS\ control}\right) x\ 100\ \%$$

6.9 CELLULAR ANTIOXIDANT ACTIVITY ASSAY

Table 18: The equipment, cell line, medium components and reagents used in the CAA assay.

Equipment	**ID**	**Distributor, country**
Sanyo CO_2 incubator MCO-18AIC		Panasonic biomedical, Japan
Light microscope		Leica Microsystems, Germany
Black microtiter plate with optical bottom (Costar)	734-1609	VWR International AS, Pennsylvania, USA
Victor Multilabel Counter		Perkin Elmer, Massachusetts, USA
Cell line/medium components		
HepG2	ATCC WB-8065	LGC Standards, UK
Earle's Minimal Essential Medium (E-MEM)	F 0325	VWR International AS, Pennsylvania, USA
Fetal Bovine Serum (FBS)	S 1810	VWR International AS, Pennsylvania, USA
Non Essential Amino Acids (NEAA)	K 0293	Merck KGaA, Germany
Sodium Pyruvate (100 mM)	L 0473	Merck KGaA, Germany
L-Alanyl-Glutamine	K 0302	VWR International AS, Pennsylvania, USA
Reagents		
DCFH-DA	35847	Honeywell, New Jersey, USA
Methanol	34860	Sigma-Aldrich, Missouri, USA
Luteolin (17 mM)	10004161	Cayman, Michigan, USA
Milli-Q Ultrapure water		Merck KGaA, Germany
Hanks' Salt Solution w/o Phenol red, BIOCHROM^{AG}	L2035	VWR International AS, Pennsylvania, USA
AAPH (200 mM)	82235	Cayman, Michigan, USA

E-MEM growth medium was made with 1 % NEAA, 1 % Sodium Pyruvate, 1 % L-Alanyl-Glutamine and 10 % FBS.

The cell line HepG2 was seeded in E-MEM growth medium in black costar plates. For concentration of cells in the wells and method see step 1 and step 2 and table 14 under headline "6.6 Working with cells". The cells were thereafter controlled under a light microscope, DCFH-DA (489.27 g/mol diluted to 20 mM in methanol) was diluted to 31.2 µM in E-MEM growth medium and luteolin was diluted with milli-Q water to a concentration of 1250 µg/mL. Next the wells were washed with 100 µl PBS and the medium/DCFH-DA mix was added to all the wells; 90 µl to the sample wells and 80 µl to the control wells. Thereafter 10 µl of each fraction from the deep well plate were transferred to the wells in two parallels to a final fraction concentration of 100 µg/mL, 50 µg/mL and 25 µg/mL (see table 10). Then diluted luteolin was added (antioxidant control).

The plates were incubated at 37°C and 5 % CO_2 for one hour, washed again with 100 µl PBS and added salt solution/AAPH mix. For samples S1 – S4 AAPH was diluted in salt solution to a concentration of 0.8 mM and for S5-L and S5-W AAPH was diluted to a concentration of 0.6 mM. From this, 100 µl was added to each well, except negative control where 100 µl salt solution with no AAPH was added.

Controls:

- Negative control: Hanks' Salt Solution and milli-Q water
- Positive control: Hanks' Salt Solution and milli-Q water with 600 µM AAPH
- Antioxidant control: 50 µg/mL luteolin.

The fluorescence was thereafter measured in a Victor Multilabel Counter at 485/520 nm and the plates were incubated again for one hour before the fluorescence was measured once more.

6.9.1 Evaluation of results

The parallels and the controls were checked to secure reliable results. Fractions were determined active with under 70 % oxidation, questionable with between 70 and 80 % oxidation and inactive with over 80 % oxidation. Percent oxidation were calculated by using the equation below.

$$\frac{(OD\ Reading\ 2 - OD\ Reading\ 1) - Negative\ control}{(Positive\ control - Negative\ control)}\ x\ 100\ \%$$

6.10 REFRACTIONATION OF SELECTED BIOACTIVE FLASH FRACTIONS

Table 19: Equipment, program and solvents used in refractionation of S1-F7, S2-F7, S2-F8, S3-F7, S4-F7 and S5-L-F3 with prep HPLC-MS.

Equipment	**Distributor, country**
Column: XTerra® Shield RP18 Prep Column, 125Å, 10 µm	Waters, Massachusetts, USA
600 Controller2996 photodiodide array detector	Waters, Massachusetts, USA
3100 mass detector	Waters, Massachusetts, USA
2767 sample manager	Waters, Massachusetts, USA
Flow splitter	Waters, Massachusetts, USA
Prep degasser	Waters, Massachusetts, USA
515 HPLC pump	Waters, Massachusetts, USA
SC250 Express SpeedVac Concentrator	Thermo Fisher Scientific, USA, Massachusetts
Heto PowerDry PL6000 Freeze Dryer	Thermo Fisher Scientific, USA, Massachusetts
Program	
MassLynx V4.1	Waters, Massachusetts, USA
Solvents, ID	
Acetonitrile, 34851	Sigma-Aldrich, Missouri, USA
Formic acid, 56302	Sigma-Aldrich, Missouri, USA
Methanol, 34860N	Sigma-Aldrich, Missouri, USA
Milli-Q Ultrapure water	Merck KGaA, Germany

From S1-F7, S2-F7, S2-F8, S3-F7, S4-F7 and S5-L-F3, 1 mg was taken out and mixed with 80% methanol for a total injection volume of 500 µl. Subsequently the samples were separated with the prep HPLC-MS to 40 fractions with a gradient of mobile phases:

- Mobile phase A: Milli-Q ultrapure water with 0.1 % formic acid
- Mobile phase B: Acetonitrile with 0.1 % formic acid

A table over instrument parameters of the prep HPLC-MS can be found in the appendix (table 30).

Table 20: The mobile phase gradient used during refractionation of S1-F7, S2-F7, S2-F8, S3-F7, S4-F7 and S5-L-F3.

Time (min)	**Flow (mL/min)**	**Mobile phase A (%)**	**Mobile phase B (%)**
Initial	6,00 mL/min	30	70
30	6,00 mL/min	0	100
40	6,00 mL/min	0	100

Each HPLC fraction had a total volume of 6 mL. This was divided on three deep well plates and dried in the SpeedVac centrifuge for about one and a half hour. Subsequently the deep well plates were frozen in -80°C before freeze-dried and dissolved in 500 µl of milli-Q water.

S1-F7, S2-F7, S3-F7 and S5-L-F3 were then tested again for bioactivity in the anti-biofilm assay and S2-F8 and S4-F7 in the anti-inflammatory assay.

6.11 STUCTURE IDENTIFICATION OF SELECTED SAMPLES (DEREPLICATION)

Table 21: The equipment, instrument components and solvents used in dereplication with UPLC-QToF-MS.

Equipment	**Distributor, Country**
Biofuge centrifuge	Thermo Fisher Scientific, Massachusetts, USA
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
Solvents, ID	
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., Netherlands
LiChrosolv® Acetonitrile Hypergrade for LC-MS, 1.00029	Merck KGaA, Germany

Dereplication was conducted three times: 1) extracted samples of S1 – S4, 2) one active (S3-F8) and one inactive (S1-F8) FLASH fraction from the anti-inflammatory assay and 3) apparent active HPLC fractions from the anti-biofilm assay:

1) Extracted samples of S1 – S4 were analysed by UPLC-QToF-MS. A scoop of the samples were taken out and added to Eppendorf tubes. Then 300 µl 90 % methanol was added and the samples sonicated for about 5 minutes in an ultrasound water bath. Next the samples were centrifuged at 13.000 rpm for 3 minutes to collect unwanted particles in the bottom of the tube. Finally 200 µl of each sample was transferred to HPLC glass vials and checked on UPLC-QToF-MS. However, only degradation products of chlorophyll were detected, and there is no further presentation of this in the results.
2) The FLASH fraction S3-F8 which expressed activity in the anti-inflammatory assay were analysed on the UPLC-QToF-MS as well as an inactive FLASH fraction (S1-F8) to be able to identify peaks responsible for the bioactivity. The FLASH fractions were diluted to 2 mg/mL in 100 % methanol in HPLC glass vials before analysation.
3) HPLC fractions 9 and 10, 18 and 19, 24 and 25 of FLASH fraction S2-F7 showed possible activity in the anti-biofilm formation assay. From these, 300 µl of pure sample was added to HPLC glass vials and run through the UPLC-QToF-MS.

All the samples were analysed on a Vion® IMS QToF with a C18 1.7µM reverse column in the ESI+ mode, as shown in table 21, with the use of two solvents:

- Solvent A: Milli-Q water with 0.1 % formic acid, pH 3.75
- Solvent B: Acetonitrile with 0.1 % formic acid, pH 3.75

Instrument parameters used can be found in the appendix in table 31.

Table 22: UPLC gradient conditions used by UPLC-QToF-MS dereplication analysis.

Time (min)	**Flow rate (mL/min)**	**Solvent A gradient (%)**	**Solvent B gradient (%)**
0.00	0.450	90.0	10.0
12.00	0.450	0.0	100.0
13.50	0.450	0.0	100.0

7 RESULTS

7.1 YIELD OF EXTRACTED SAMPLES

The weight of the raw material from S1 – S5 (see table 2) after freeze-drying and extraction, as well as the calculated yield is shown in table 23.

Table 23: Percent dry extracted yield of freeze-dried raw material of sample S1-S5-L. S5-W was not further extracted.

Sample name	Raw material weight in mg (after freeze-drying)	Extracted sample weight in mg (after drying in rotavapor)	% yield
S1	4051.6	1548.7	38.2
S2	6234.2	716.7	11.5
S3	3721.2	491.1	13.2
S4	4246.9	1018.1	24.0
S5-L	16910.0	4330.0	25.6
S5-W	9.30	-	-

7.2 YIELD OF FLASH FRACTIONS

The extracted samples (S1 – S5, see table 2) were fractionated into eight fractions by FLASH chromatography. The yield of all the FLASH fractions is shown in table 24.

Table 24: Percentage yield after FLASH fractionation of sample S1, S2, S3, S4, S5-L and S5-W.

Samples	Amount of samples used in FLASH fractionation (mg)	The total dry-weight (mg) of all FLASH fractions	% yield of extract
S1	1550	977.4	63.06
S2	720	577.1	80.15
S3	490	282.1	57.57
S4	1020	853.9	83.72
S5-L	1520	672.9	44.27
S5-W	1510	1208.2	80.01

7.3 ANTIBACTERIAL ASSAY

7.3.1 1st Screen

An antibacterial assay was performed on five bacteria strains; *S. aureus, E. coli, E. faecalis, P. aeruginosa* and *S. agalactiae* with eight FLASH fractions from all samples (S1 – S5, see table 2). Fractions were deemed active based on an activity threshold with an OD under 0.05 at 490 nm, questionable based on the questionable threshold (OD = 0.05 – 0.09 at 490 nm) and inactive with an OD over 0.09 at 490 nm. According to the activity threshold, activity were only detected in the *S. agalactiae* bacteria strain, and questionable activity were detected in both *S. agalactiae* and *E. facealis*. No particular activity were detected against the other bacteria strains (*S. aureus, E. coli, P. aeruginosa*). In addition to take the activity threshold into account, differences between the samples were emphasised by comparing the FLASH fractions to evaluate variation in bioactivity that might have emerged as a consequence of each samples cultivation condition. The OD values of the FLASH fractions against *S. agalactiae* and *E. facealis* are depicted in figure 12 and 13, respectively. The other bacteria strains are not presented below. However, the results of *S. aureus* can be found in the appendix, page 78.

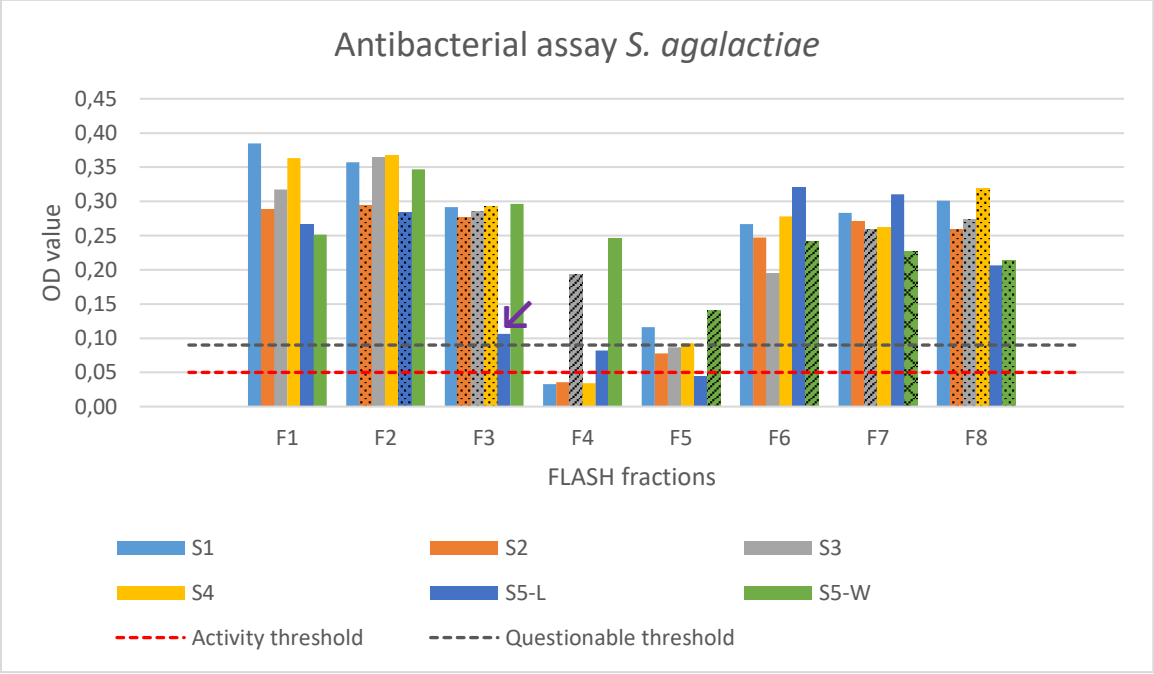

Figure 12: Comparison of activity of all the FLASH fractions from S1 – S5 with concentrations of 100 µg/mL (plain), 50 µg/mL (dots), 25 µg/mL (stripes) and 10 µg/mL (X-es) in an antibacterial assay towards S. agalactiae. *The activity threshold is marked with a red line at OD = 0.05 and the questionable threshold is marked with a grey line at OD = 0.09. The arrow marks a drop in OD in S5-L-F3 compared to the other F3 fractions.*

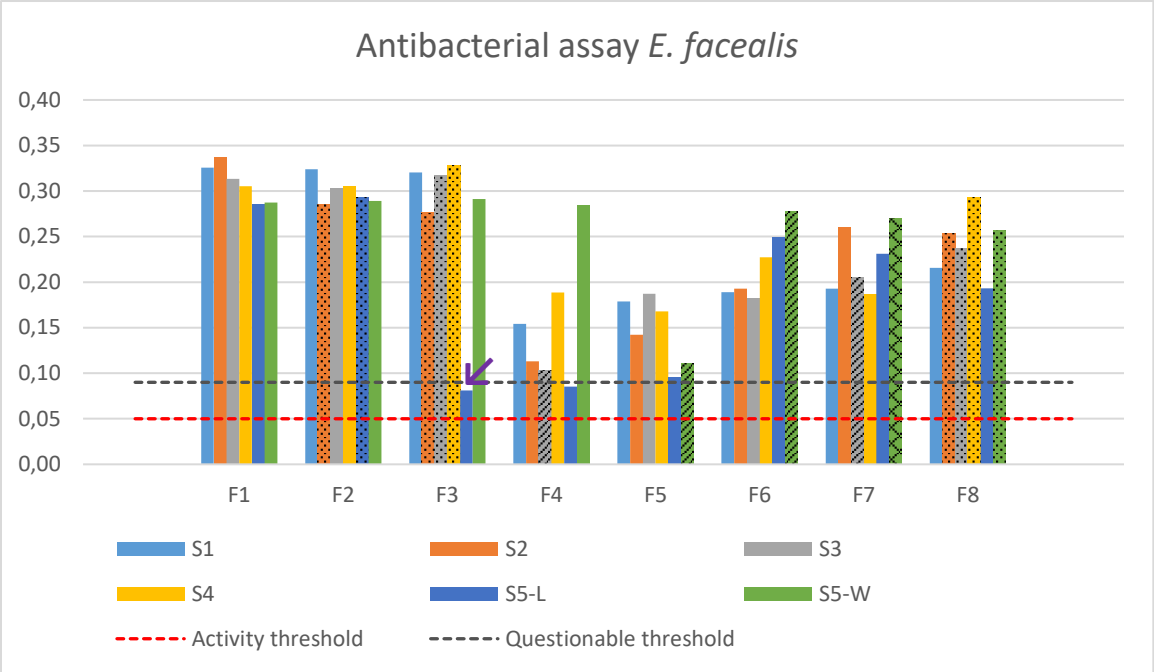

Figure 13: Comparison of activity of all the FLASH fractions from S1 – S5 with concentrations of 100 µg/mL (plain), 50 µg/mL (dots), 25 µg/mL (stripes) and 10 µg/mL (X-es) in an antibacterial assay towards E. facealis. *The activity threshold is marked with a red line at OD = 0.05 and the questionable threshold is marked with a grey line at OD = 0.09. The arrow marks a drop in OD in S5-L-F3 compared to the other F3 fractions.*

Based on the activity thresholds in the figures, four fractions were determined active against *S. agalactiae*. In addition four samples against *S. agalactiae* and two samples against *E. facealis* were determined questionable. Sample S5-L-F3 stood out from all the other F3 fractions towards both bacteria strains and are marked with an arrow in both figures.

In addition, S3-F4 and S5-W-F5 were considered presumably active against *S. agalactiae* and *E. facealis*. These fractions were almost under the questionable threshold, but with a lower concentration (25 µg/mL) than the other active fractions (100 µg/mL). Compared to the trend of the other samples it is also likely that they have similar activities.

7.3.2 2nd Screen

Only one of the active samples (S5-L-F5) was prioritised for evaluation of its MIC value in a second screen against *S. agalactiae.* The fraction concentrations were diluted to concentrations of 50 µg/mL, 25 µg/mL, 10 µg/mL, and 1 µg/mL and tested again the same way as for the first screen. The MIC value were set based on the activity threshold with an OD below 0.05. The MIC value was thus 50 µg/mL, see figure 14.

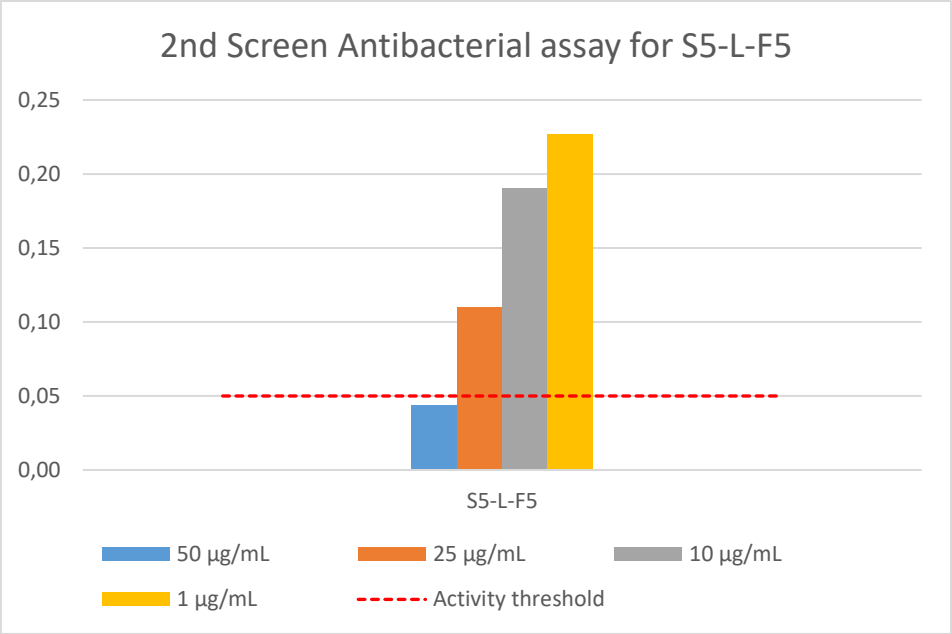

Figure 14: OD values of S5-L-F5 diluted to concentrations of 50 µg/mL, 25 µg/mL, 10 µg/mL and 1 µg/mL and tested again in a second screen towards the S. agalactiae *bacteria strain.*

7.4 ANTI-BIOFILM FORMATION ASSAY

7.4.1 1st Screen

An anti-biofilm assay was conducted with the eight FLASH fractions from all the samples (S1 – S5, see table 2) against the biofilm-producing bacteria *S.epidermidis*. The fractions were determined active based on an activity threshold of OD < 0.25, questionable with an OD between 0.25 and 0.30 and inactive with an OD > 0.30 at 490 nm. In addition, differences between the samples were emphasised by comparing the FLASH fractions to evaluate variation in bioactivity that might have emerged as a consequence of each samples cultivation condition. A comparison of all eight FLASH fractions from the different samples with their OD values are depicted in figure 15.

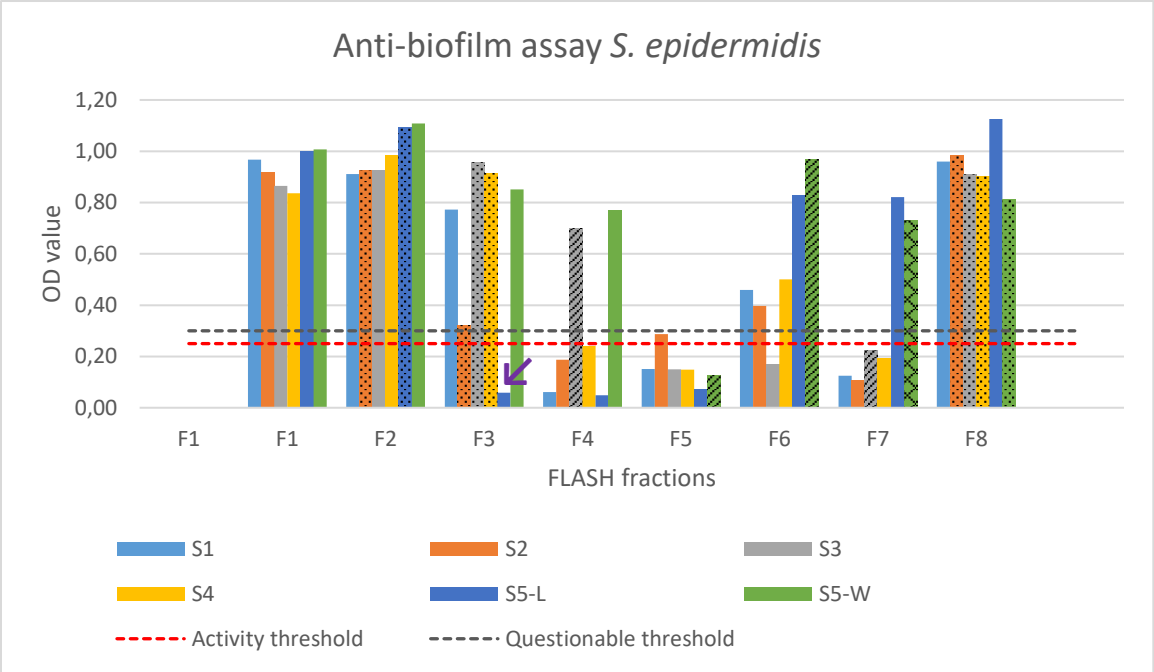

Figure 15: All FLASH fractions from S1 – S5 with concentrations of 100 µg/mL (plain), 50 µg/mL (dots), 25 µg/mL (stripes) and 10 µg/mL (X-es) and their OD values tested in an anti-biofilm assay against S. epidermidis *biofilm formation. The activity threshold is marked with a red line at OD = 0.25 and the questionable threshold is marked with a grey line at OD = 0.30. The arrow marks a drop in OD in S5-L-F3 compared to the other F3 fractions.*

Based on the activity thresholds in the figures, fifteen fractions were determined active and one fraction was determined questionable. In addition, S2-F3 were considered presumably active due to its proximity to the activity threshold in spite of its low concentration (50 µg/mL). Mentionable deviation within the FLASH fractions could be observed for F3, F4, F6 and F7. As for the antibacterial assay against *S. agalactiae* and *E. facealis,* S5-L-F3 showed a drop in OD value compared to the other F3 fractions, as marked with an arrow in figure 15.

S5-L-F3, S5-L-F4 and S5-W-F5 seemed to have killed the bacteria because little bacteria growth were observed in the wells with the naked eye after the bacteria were incubated with these fractions.

7.4.2 2nd Screen

Only active fractions of S5 were prioritised for evaluation of MIC values in a second screen against *S. epidermidis* biofilm production. The active S5-L fractions (S5-L-F3, S5-L-F4 and S5-L-F5) were screened again with concentrations of 100 µg/mL, 50 µg/mL, 25 µg/mL and 5 µg/mL. S5-L-F3 and S5-L-F4 appeared to have a MIC value of 25 µg/mL and S5-L-F5 appeared to have a MIC value of 50 µg/mL based on the activity threshold, see figure 16.

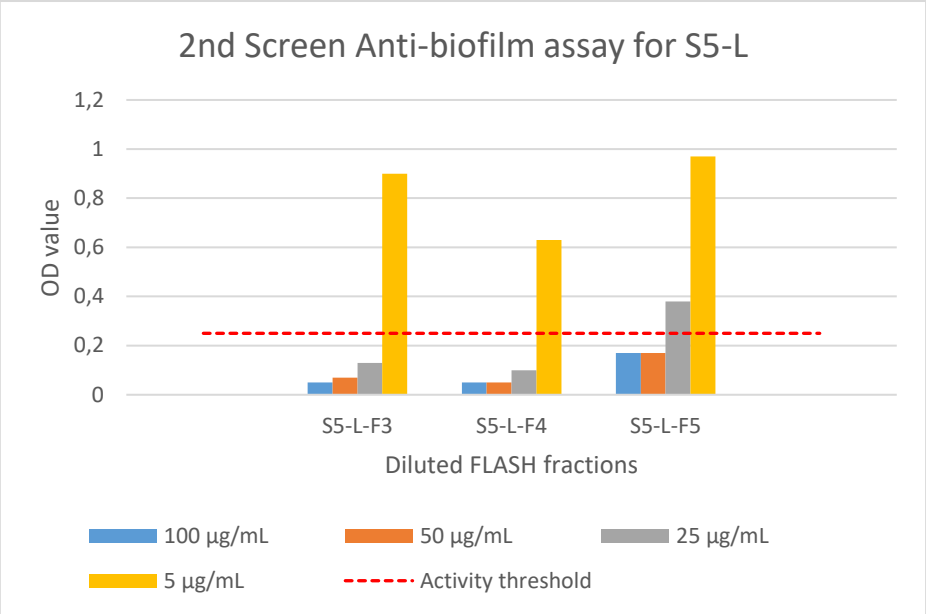

Figure 16: OD values of S5-L-F3, S5-L-F4 and S5-L-F5 diluted to concentrations of 100 µg/mL, 50 µg/mL, 25 µg/mL and 5 µg/mL and tested again in a second screen against biofilm formation of S. epidermidis*. The MIC value was determined based on the red activity threshold line (OD under 0.25).*

The active S5-W fraction (S5-W-F5) were screened again with concentrations of 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL and 1 µg/mL. The MIC value of S5-W-F5 was 12.5 µg/mL, see figure 17.

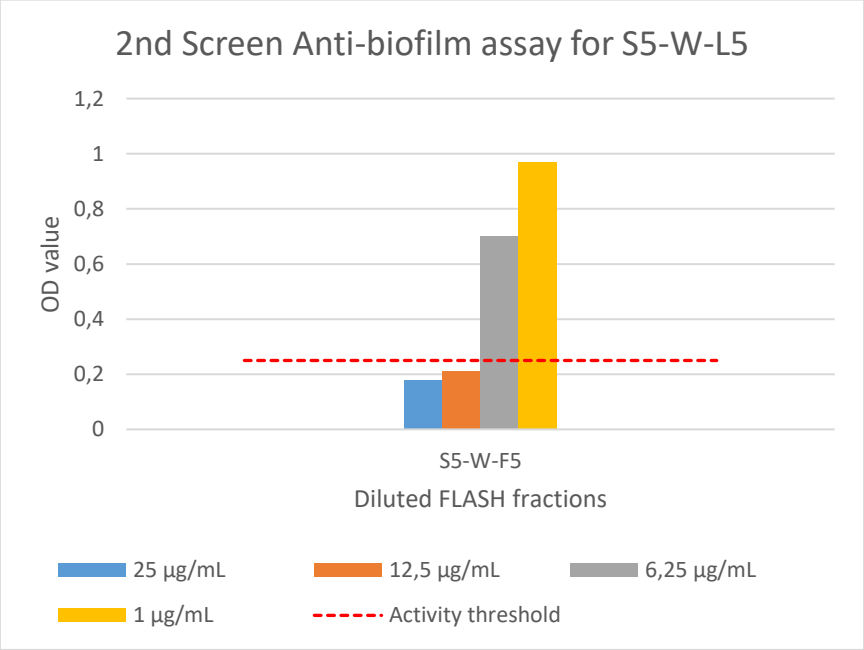

Figure 17: OD value of S5-W-L5 diluted to concentrations of 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL and 1 µg/mL and tested again in a second screen against biofilm formation of S. epidermidis*. The MIC value was determined based on the red activity threshold line (OD under 0.25).*

7.4.3 3rd Screen

Fractions expressing activity in the antibacterial assay, such as F4 and F5 in most samples, were not considered as further candidates for anti-biofilm formation compounds. See comparison of the assays in table 25.

Table 25: Comparison of bioactivity results in the antibacterial assay for S. agalactiae (S. a) *and* E. facealis (E. f) *and the anti-biofilm formation assay for* S. epidermidis (S. e) *with active fractions (+), presumably active fractions ((+)), questionable fractions (+/-) and inactive fractions (-). The yellow circles marks selected fractions for further refractionation by prep HPLC-MS.*

	S1			**S2**			**S3**			**S4**			**S5-L**			**S5-W**		
	S. a	***E. f***	***S. e***	***S. a***	***E. f***	***S. e***	***S. a***	***E. f***	***S. e***	***S. a***	***E. f***	***S. e***	***S. a***	***E. f***	***S. e***	***S. a***	***E. f***	***S. e***
F1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F3	-	-	-	(+)	-	-	-	-	-	-	-	-	-	+/-	+	-	-	-
F4	+	-	+	+	-	+	(+)	(+)	-	+	-	+	+/-	+/-	+	-	-	-
F5	-	-	+	+/-	-	+/-	+/-	-	+	+/-	-	+	+	-	+	(+)	(+)	+
F6	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
F7	-	-	+	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-
F8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

With this in mind, four FLASH fractions (S2-F7, S3-F7 and S4-F7) were selected to be separated through prep HPLC-MS to forty new HPLC fractions and tested again in a third screen. The results from the screening of HPLC fractions S2-F7, S3-F7 and S4-F7 are depicted in figure 18, 19 and 20 respectively.

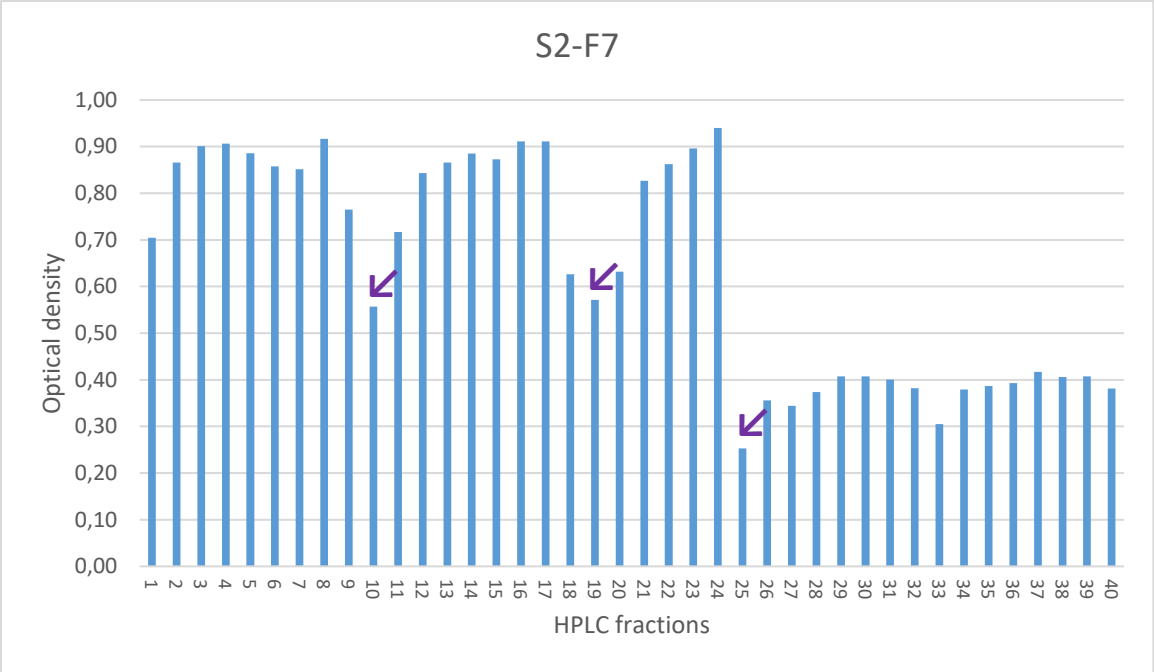

Figure 18: The OD values of the HPLC refractionated FLASH fraction S2-F7 screened against S. epidermidis *biofilm formation. HPLC Fraction 10, 18 and 25 seemed to be standing out as possible active fractions. The affected bacteria growth in well 26 – 40 were most likely not due to active fractions since less bacteria growth also were observed in the positive controls.*

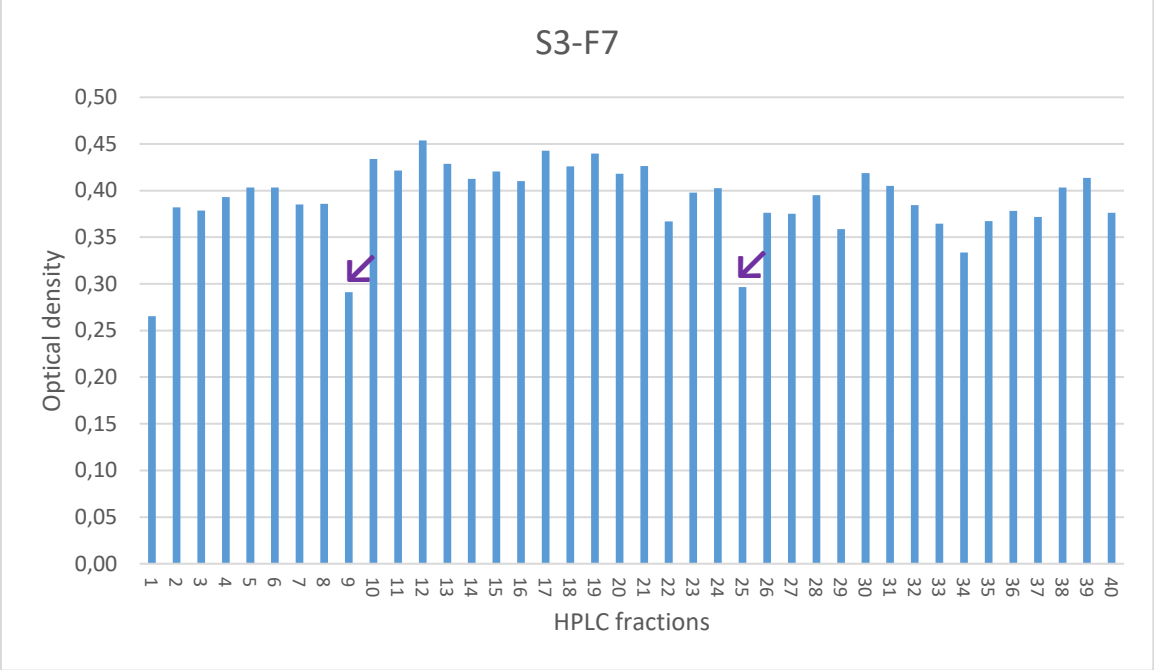

Figure 19: The OD values of the HPLC refractionated FLASH fraction S3-F7 screened against S. epidermidis *biofilm formation. HPLC Fraction 9 and 25 seemed to be standing out as possible active fractions.*

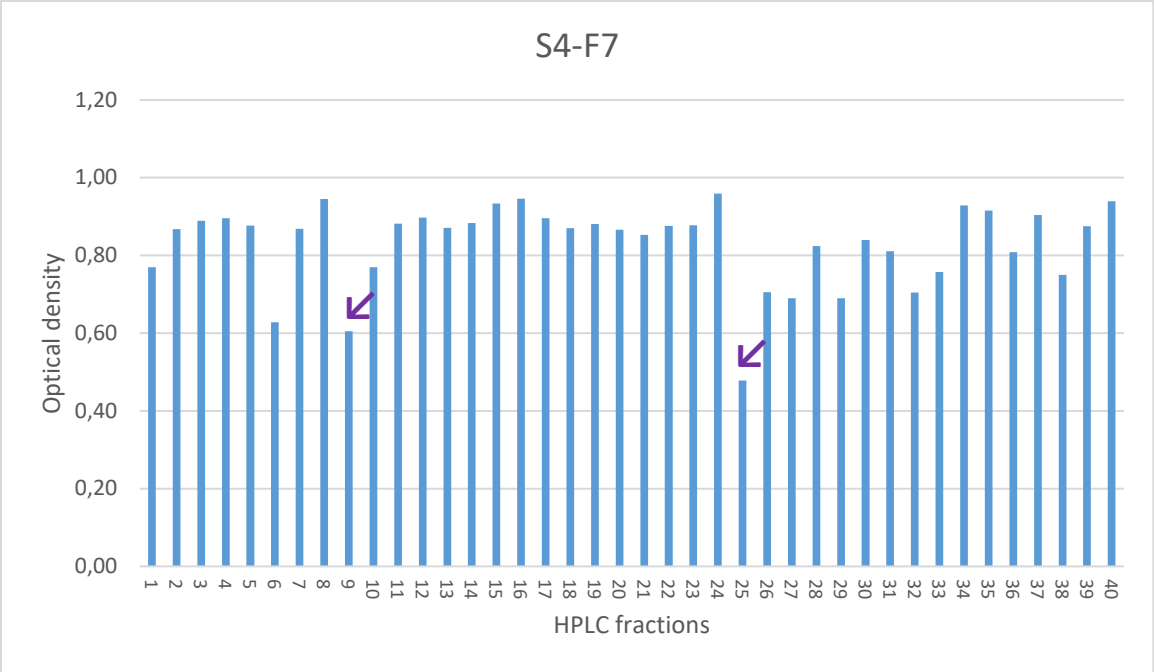

Figure 20: The OD values of the HPLC refractionated FLASH fraction S4-F7 screened against S. epidermidis *biofilm formation. HPLC Fraction 9 and 25 seemed to be standing out as possible active fractions.*

In the chromatograms obtained from the prep HPLC-MS refractionation of S2-F7, S3-F7 and S4-F7, one molecule stood out in a peak in fraction 9 in all fractions. Figure 21 shows the chromatogram of S2-F7. This supports the apparent activity of HPLC fraction 9 in the 3rd screen in the anti-biofilm formation assay against *S. epidermidis* biofilm production.

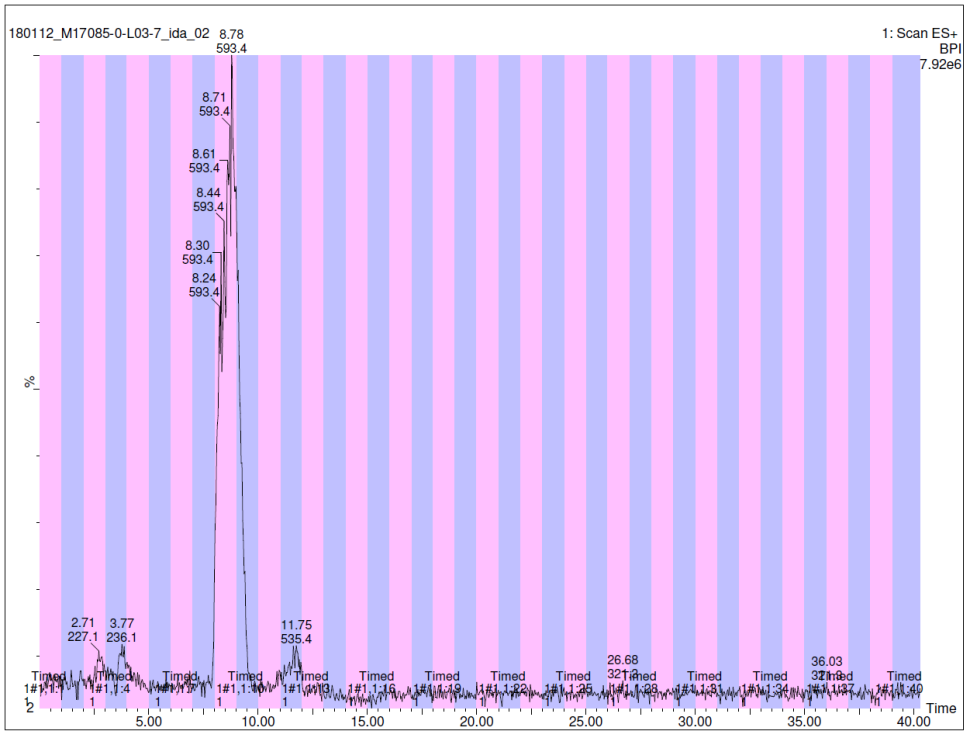

Figure 21: Base peak intensity (BPI) chromatogram of FLASH fraction S2-F7 from prep HPLC-MS refractionation. This fraction had a high intensity peak with m/z 593.4, with most of its contents in fraction 9.

7.4.4 Dereplication

Fraction 9 from S2-F7 was further analysed by UPLC-QToF-MS. Figure 22 depicts the MS spectrum of this fraction.

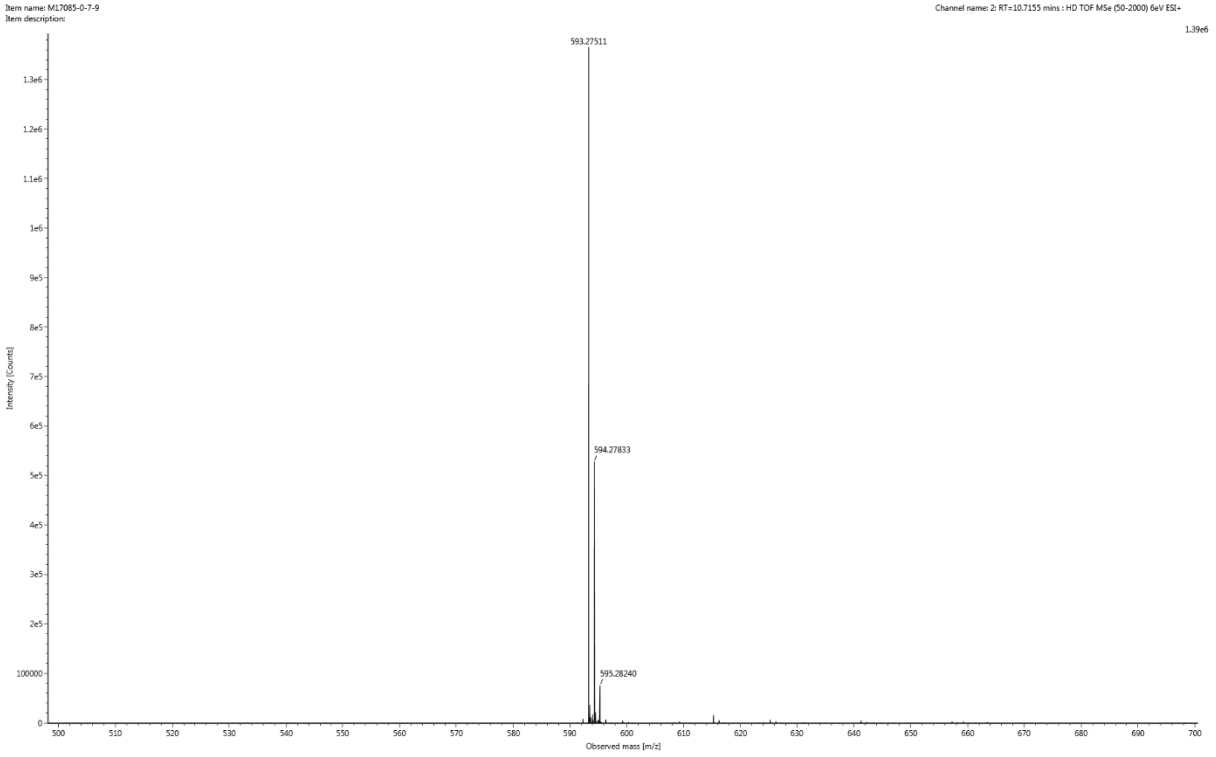

Figure 22: MS spectrum of the peak eluted in fraction 9 in the prep HPLC-MS chromatogram at retention time 10.72 with an m/z of 593.27511.

The UPLC-QToF-MS screen revealed a mass of 593.27511. Further investigation revealed that the chemical composition of this molecule most likely were: $C_{35}H_{36}N_4O_5$. This appeared to possibly be pheophorbide-a, which is a degradation product of chlorophyll as the structure is quite characteristic for chlorophyll.

7.5 MTS CELL VIABILITY ASSAYS

7.5.1 1st Screen

All eight FLASH fractions from all samples (S1 – S5, see table 2) were screened for activity against the cancer cell line A2058 (human melanoma). Fractions were determined active based on an activity threshold of a cell survival rate less than 50 %, questionable between 50 and 60 % cell survival and inactive over 60 % cell survival, see figure 23.

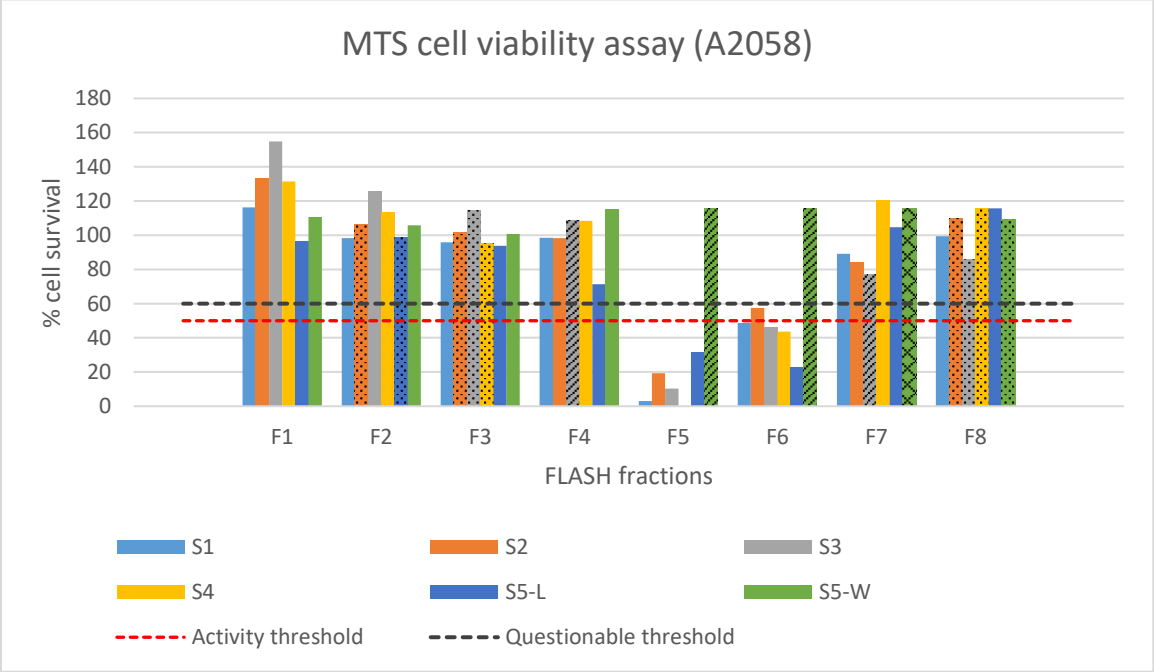

Figure 23: All FLASH fractions from S1 – S5 with concentrations of 100 µg/mL (plain), 50 µg/mL (dots), 25 µg/mL (stripes) and 10 µg/mL (X-es) and their activities tested in MTS Cell Viability assay against cell line A2058. The activity threshold is marked with a red line at % cell survival = 50 % and the questionable threshold is marked with a grey line at % cell survival = 60 %.

According to the activity threshold, eight FLASH fractions were determined active and one FLASH fraction (S2-F6) was determined questionable with an activity of 57.56 % cell survival. Unlike the bacterial assays, no particular deviation was observed in the same FLASH fractions from the different samples. Hence, no change in bioactivity could be associated with the different cultivation conditions, with the exception of the aqueous extract which appeared to not have any activity towards the cell line in any fractions.

Sample S5-L and S5-W were additionally screened for activity against a human colon carcinoma cell line HT29 (see figure 24) and a non-cancerous, normal lung fibroblast cell line MRC-5 (see figure 25) with a concentration of 100 µg/mL for S5-L and 25 µg/mL for S5-W.

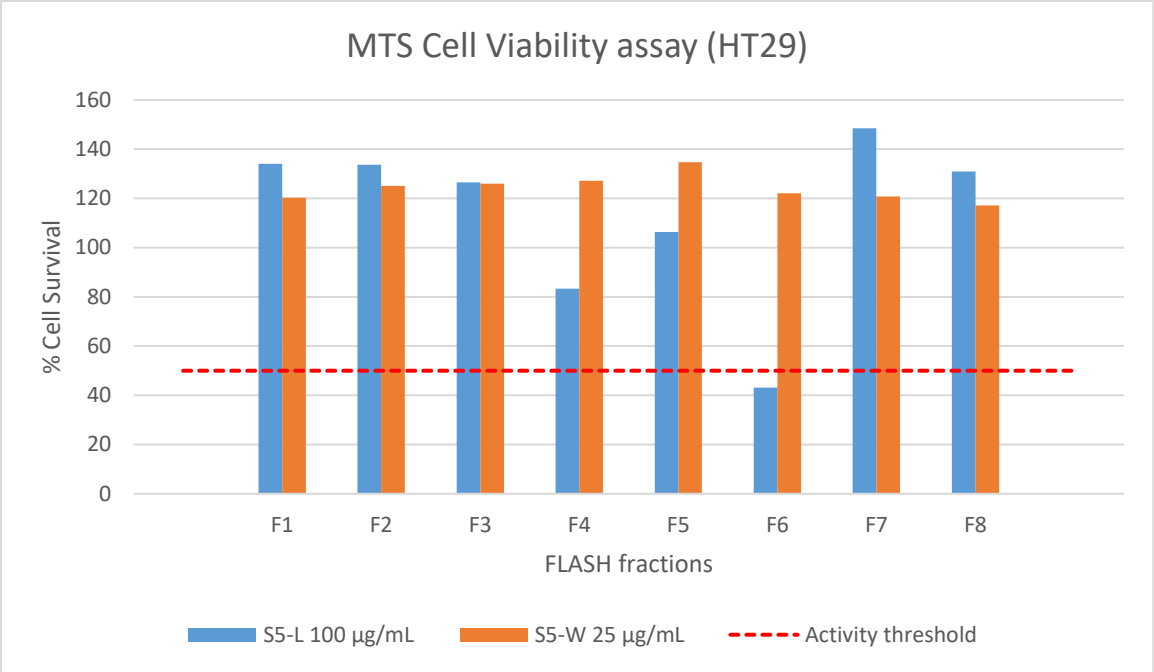

Figure 24: Sample S5-L and S5-W with concentrations of 100 µg/mL and 25 µg/mL respectively and their percent inhibition of cell survival in a screen against cell line HT29. The red line is the activity threshold at cell survival = 50 %. FLASH Fractions under this line were considered active.

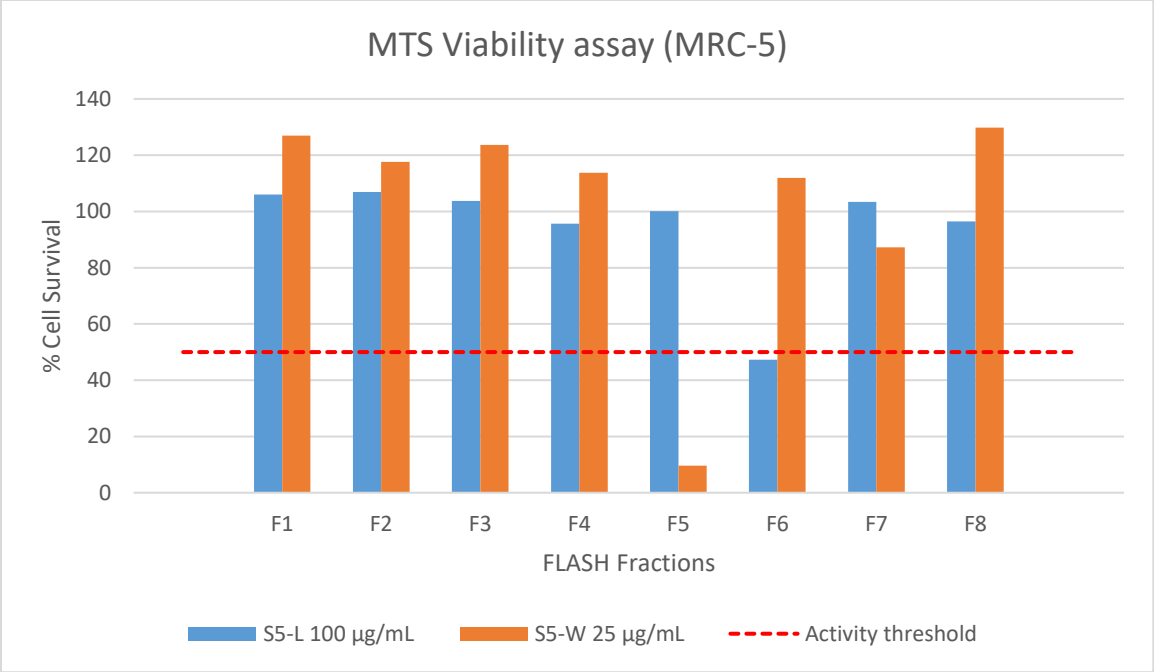

Figure 25: Sample S5-L and S5-W with concentrations of 100 µg/mL and 25 µg/mL respectively and their and their percent inhibition of cell survival in a screen against cell line MRC-5. The red line is the activity threshold at cell survival = 50 %. FLASH Fractions under this line were considered active.

7.5.2 2nd Screen

The fraction expressing activity in all cell lines (S5-L-F6) was prioritised for evaluation of its MIC value in a second screen against human melanoma cancer line A2058. S5-L-F6 was screened again with concentrations of 50 µg/mL, 25 µg/mL and 10 µg/mL, but no activity was detected.

7.6 ANTI-INFLAMMATORY ASSAY

7.6.1 1st Screen

All the FLASH fractions from all the samples (S1 – S5, see table 2) were screened for anti-inflammatory properties in a monocyte-like THP-1 cell line triggered to produce TNF-α by LPS exposure. Fractions were determined active according to an activity threshold with TNF-α inhibition of > 50 %. No fractions were considered questionable. In addition, differences between the samples were emphasised by comparing the FLASH fractions to evaluate variation in bioactivity that might have emerged as a consequence of each samples cultivation condition. All the FLASH fractions activity ranges from each sample are depicted in figure 26.

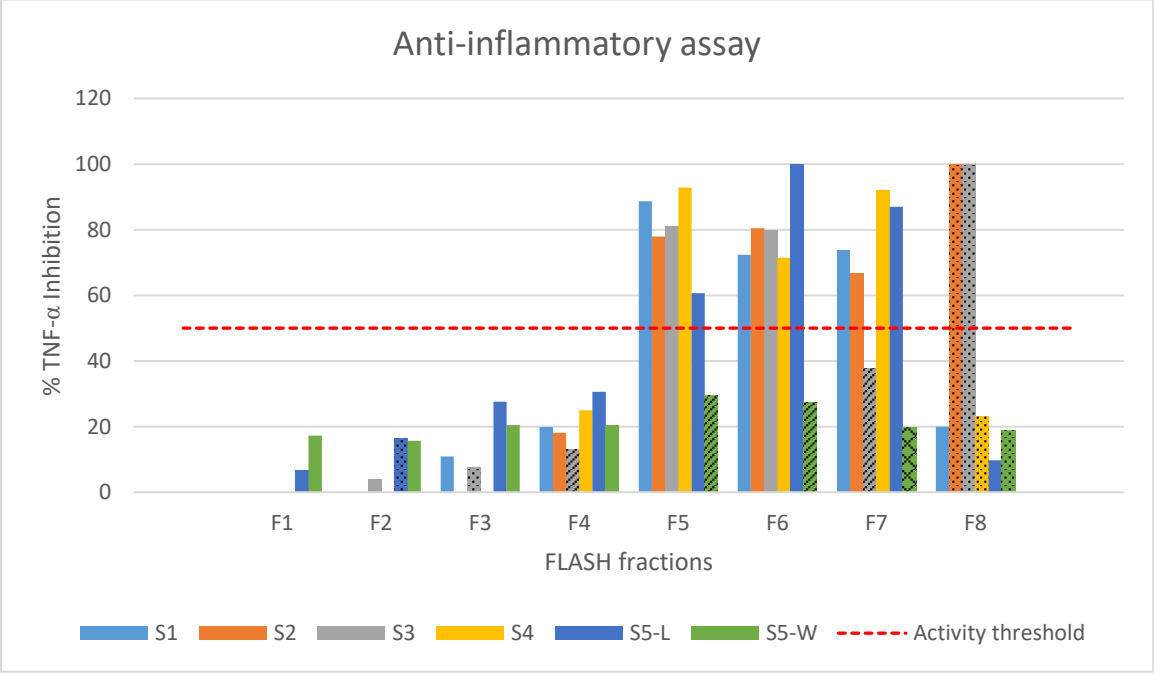

Figure 26: FLASH fractions with concentrations of of 100 µg/mL (plain), 50 µg/mL (dots), 25 µg/mL (stripes) and 10 µg/mL (X-es) with their percentage inhibition of TNF-α in the anti-inflammatory assay. Fractions were determined active with a TNF-α inhibition value > 50 % (marked with a red activity threshold line), and inactive with a TNF-α inhibition < 50 %.

Sixteen fractions were determined active according to the activity threshold. S3-F7 was very close to the activity threshold and considered presumably active due to its low concentration (25 µg/mL) and the trend of the other fractions in this sample. The same could be observed for the aqueous extract in F5, F6 and F7. A conspicuous trend could be observed in fraction F8, where S2 and S3 appeared to have a considerably higher percentage of TNF-α inhibition compared to the other F8 fractions.

7.6.2 2nd Screen

To obtain an anti-inflammatory drug it is desirable to have a compound that is not cytotoxic. The MTS cell viability assay can give an idea of what compounds can be cytotoxic. The result from MTS viability assay towards the A2058 cell line are compared to the anti-inflammatory assay in table 26.

Table 26: Comparison of MTS viability assay (MTS) against cell line A2058 and anti-inflammatory properties (AI). FLASH fractions expressing activity against cell line A2058 are marked active (+) with a cell survival < 50 %, questionable (+/-) with a cell survival between 50 and 60 % and inactive (-) with a cell survival > 60 %. FLASH fractions with anti-inflammatory properties are marked active (+) with a TNF-α inhibition > 50 %, questionable (+/-) with TNF-α inhibition between 50 and 40 % and inactive (-) with a TNF-α inhibition < 40 %. In addition S3-F7 was considered presumably active ((+)). S5-W had no activity and is thus not included in the table. The yellow circles marks selected fractions for further refractionation by prep HPLC-MS.

	S1		**S2**		**S3**		**S4**		**S5-L**	
	MTS	AI	MTS	AI	MTS	AI	MTS	AI	MTS	AI
F1	-	-	-	-	-	-	-	-	-	-
F2	-	-	-	-	-	-	-	-	-	-
F3	-	-	-	-	-	-	-	-	-	-
F4	-	-	-	-	-	-	-	-	-	-
F5	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**
F6	**+**	**+**	**+/-**	**+**	**+**	**+**	**+**	**+**	**+**	**+**
F7	-	**+**	-	**+**	-	(+)	-	**+**	-	**+**
F8	-	-	-	**+**	-	**+**	-	-	-	-

Based on these results, 1 mg of fraction S2-F8 and 1 mg of S4-F7 were separated further to forty fractions through prep HPLC-MS and tested again for anti-inflammatory properties. Unfortunately, no particular bioactivity were detected. This could be due to little material separated through the prep HPLC-MS. However, the prep HPLC-MS chromatogram and the results from the assay of S4-S7 are depicted in figure 34 and 35, respectively in the appendix.

7.6.3 Dereplication

The inactive fraction S1-F8 (treated with no particular external factor) and the active fraction S3-F8 (treated with two hours of UV) were analysed by UPLC-QToF-MS to look for compounds present in the active fraction as opposed to the inactive fraction, which could hence be responsible for the bioactivity. The chromatograms for both fractions were similar. However one peak with retention time 10.49 minutes was more prominent in S3-F8 as marked with arrows in figure 27. The analysis showed that this compound had an m/z of 609.2711. The compound eluted in two peaks (the other at 10.20 min), indicating that two isomers of the compound was present.

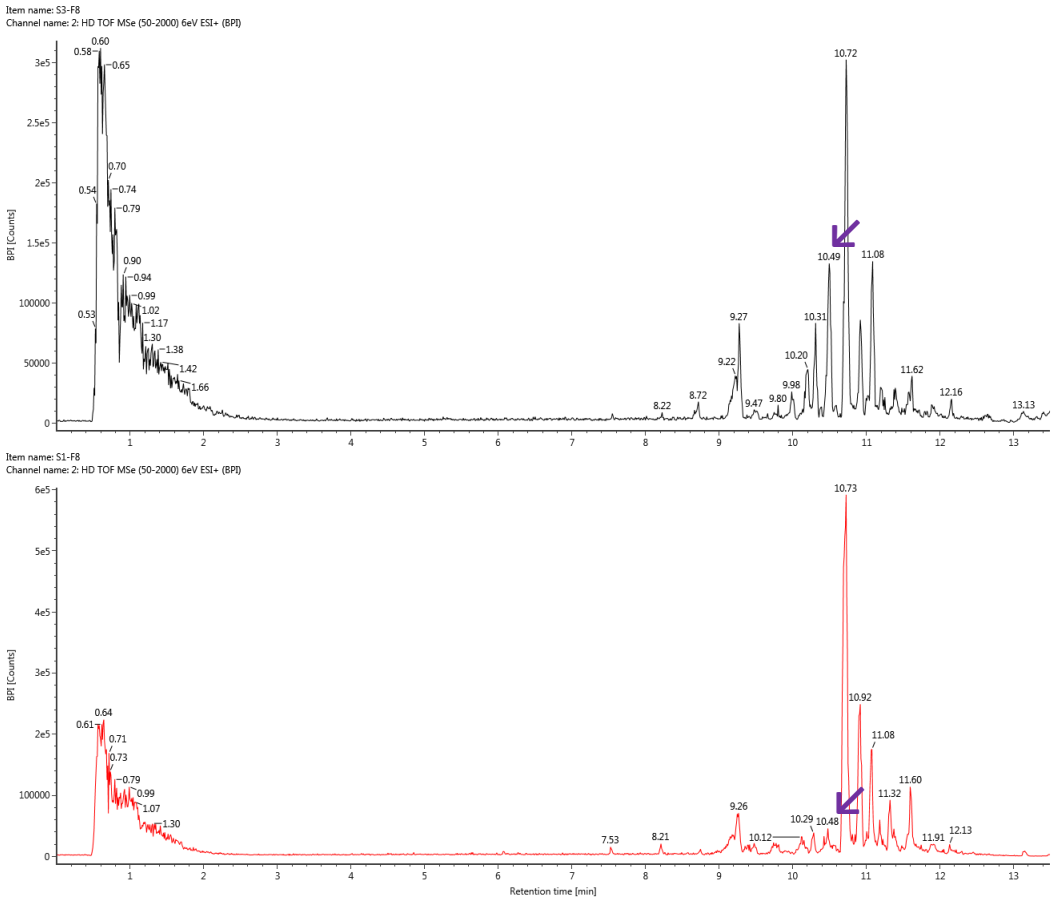

Figure 27: Comparison of ESI+ chromatograms for S1-F8 (inactive) and S3-F8 (active). The chromatogram depicts the Base peak intensity (BPI) of compounds at different retention times. The arrow marks one peak present in the chromatogram for S3-F8 and not present in the chromatogram for S1-F8 with an m/z of 609.2711.

The MS spectrum of the peak with retention time 10.49 and 10.20 from S3-F8 are depicted in figure 28.

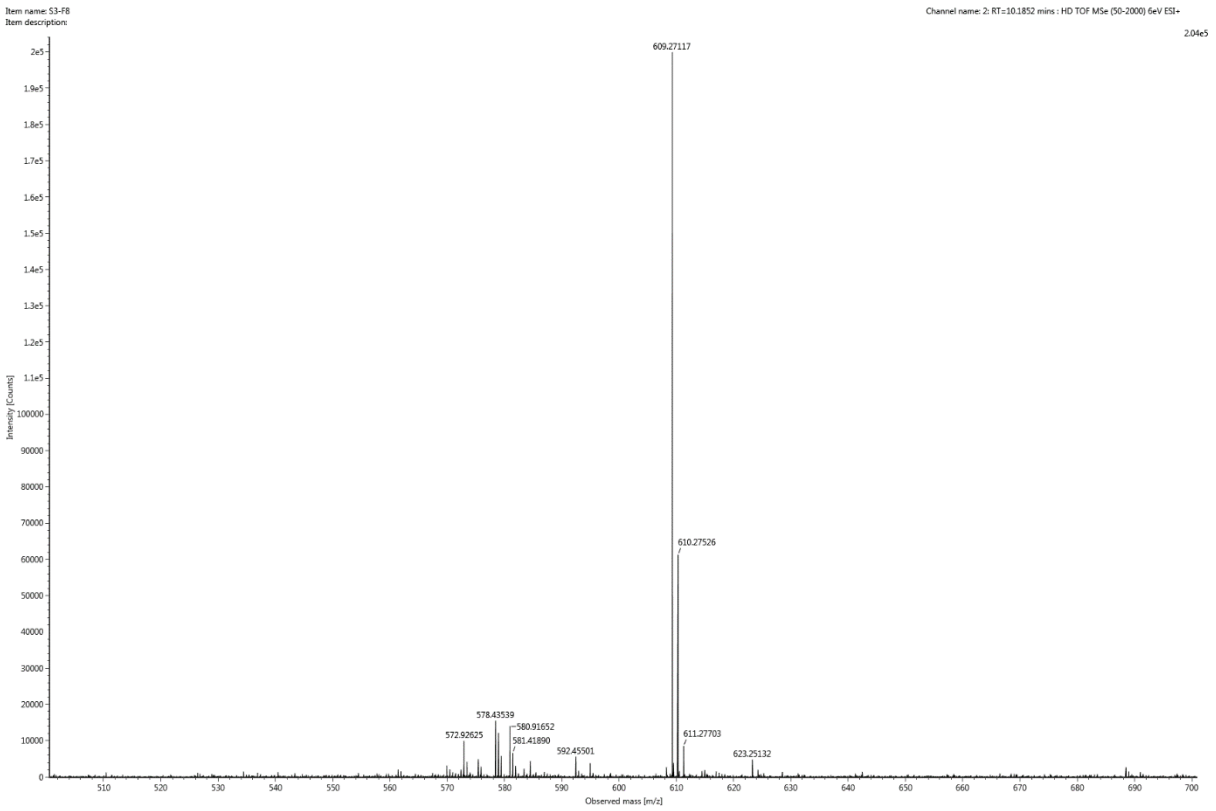

Figure 28: MS spectrum of the peak from the ESI+ chromatogram with retention time 10.49 with an m/z of 609.2711.

Further analysis revealed that the chemical composition of the molecule eluted most likely were: $C_{35}H_{36}N_4O_6$. The mass was 608.2635. This is most likely a degradation product of chlorophyll, as the structure is quite characteristic for chlorophyll. It was possible that this also was phaerophorbide-a, but with an extra hydroxyl-group attached.

7.7 CELLULAR ANTIOXIDANT ACTIVITY ASSAY

All the FLASH fractions from all the samples (S1 – S5, see table 2) were screened for antioxidant activity using a DCFH-DA probe inserted into human liver cancer cells (HepG2 cell line) with a concentration of 80,000 cells/well. The fractions were determined active according to an activity threshold of less than 70 % oxidation of the probe, questionable between 70 and 80 % oxidation of the probe, and inactive over 80 % oxidation of the probe. In addition, differences between the samples were emphasised by comparing the FLASH fractions to evaluate variation in bioactivity that might have emerged as a consequence of each samples cultivation condition. Percent oxidation with the FLASH fractions are depicted in figure 29.

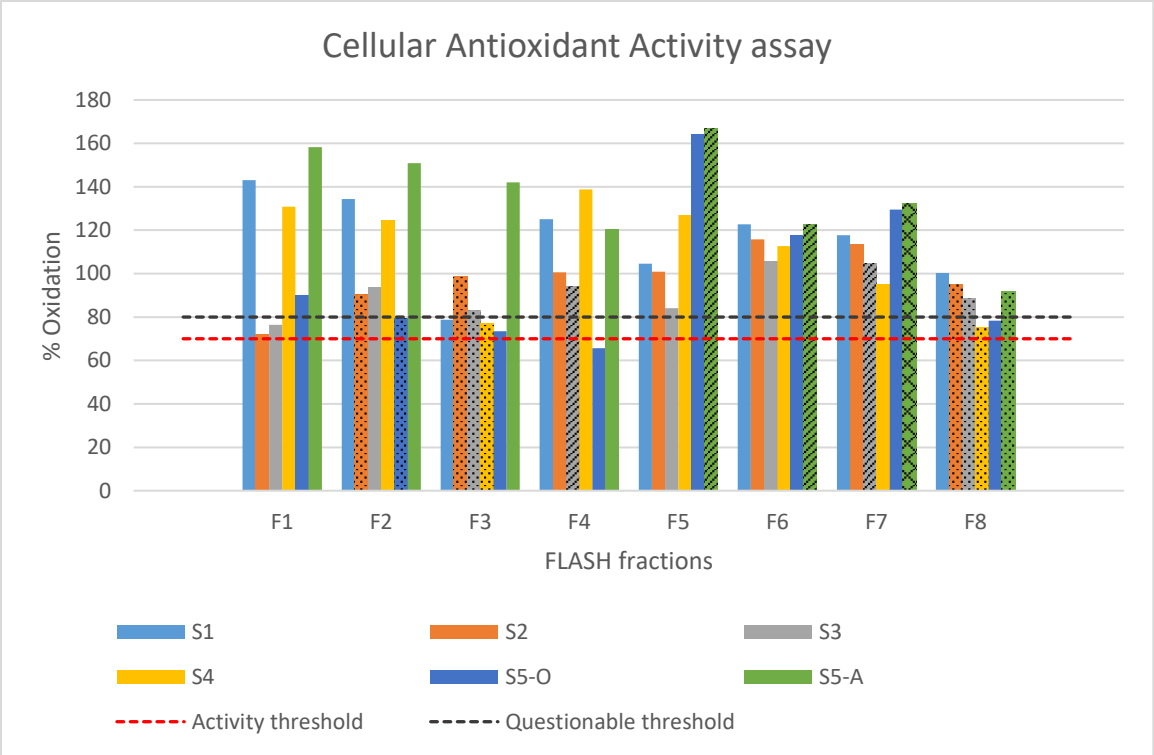

Figure 29: FLASH fractions from sample S1-S5 with concentrations of 100 µg/mL (plain), 50 µg/mL (dots), 25 µg/mL (stripes) and 10 µg/mL (X-es) and their activities in cellular antioxidant activity assay. Fractions were determined active with an oxidation < 70 % (under the red activity threshold line) and questionable between 70 and 80 % (between the grey questionable threshold line and the red activity threshold line).

Only one fraction (S5-L-F4) could be determined active according to the activity threshold. S1-F3, S2-F1, S3-F1, and S5-L-F3 were determined questionable. In addition, several fractions were determined presumably active due to their lower concentrations and yet proximity to the activity threshold, see table 27. S2, S3 and S5-L appeared to have more activity in the fractions F1, F2 and F4 compared to the other samples.

Table 27: Active (+), presumably active ((+)), questionable (+/-) and inactive (-) FLASH fractions in CAA assay.

	S1	**S2**	**S3**	**S4**	**S5-L**	**S5-W**
F1	-	**+/-**	**+/-**	-	-	-
F2	-	(+)	-	-	(+)	-
F3	**+/-**	(+)	(+)	(+)	**+/-**	-
F4	-	-	(+)	-	**+**	-
F5	-	-	-	-	-	-
F6	-	-	-	-	-	-
F7	-	-	(+)	-	-	-
F8	-	(+)	(+)	(+)	**+/-**	(+)

A summary of all first screenings in the bioactivity assays for all the FLASH fractions are presented in the appendix, page 80.

8 DISCUSSION

The main aim of this thesis was to examine bioactivity in five samples from the same diatom species cultivated at five different conditions, and to get a closer insight into whether these various external factors during cultivation were able to affect the diatoms bioactivity profile. S1 was cultivated with no particular external factor, S2 with UV irradiance for 30 minutes and subsequently 35 minutes, S3 with UV irradiance for 2 hours, S4 with a co-culture with another diatom species and S5 with CO_2 containing factory smoke and 48 hours of fermentation. Figure 30 is a simplified depiction of the samples, see table 2 for more details.

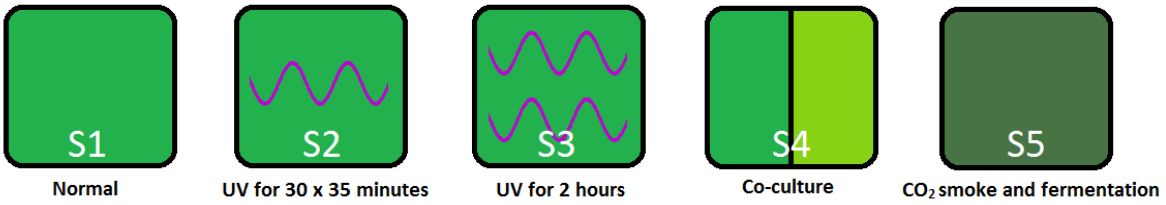

Figure 30: A simplified overview of all the samples (S1 – S5) and their respective pre-treatments.

Bioactivity were examined through five different bioassays (antibacterial, anti-biofilm, MTS viability, anti-inflammatory and antioxidant) with different degrees of fractionation of the samples. In addition, structure identification of selected bioactive compounds were attempted with the use of a UPLC-QToF-MS.

This approach is similar to the OSMAC approach were several various metabolites successfully have been obtained from a single microbial strain by systematic alternation of cultivation parameters (Bode et al., 2002). Bioactivity in diatoms are yet poorly explored, and few diatom-derived secondary metabolites have been reported (Richard A Ingebrigtsen et al., 2016). Northern Arctic marine diatoms, as the one under investigation in this thesis, is particularly poorly investigated for bioactivity purposes (Richard A Ingebrigtsen et al., 2016), hence, these diatoms are particularly interesting in this context.

The samples showed some degree of bioactivity in all bioassays conducted in the first screenings (antibacterial-, anti-biofilm-, MTS viability-, anti-inflammatory- and antioxidant assay), and interestingly, some variety between the samples were observed. However, whether these variations were a direct consequence of the cultivation treatments could not be determined for sure. Examples of other possible interfering factors could be:

- Contamination during cultivation, due to open photobioreactors.
- Bioactive molecules produced by other microorganisms.
- Bioactive molecules produced by the other diatom species in S4.
- Life-cycle changes in the diatoms upon harvest (Richard Andre Ingebrigtsen, 2017).
- Signal molecules in the cultivation seawater (many molecules can exist in the cultivation photobioreactors since natural sea water is used for cultivation. In the presence of certain molecules, the diatoms can presumably change their behavior and hence NP production) (Richard Andre Ingebrigtsen, 2017).
- False positives in the bioactivity screening.
- Pan assay interfering compounds (PAINS) (Compounds interfering with the assay readouts, such as metal chelation, redox cycling, and protein reactivity (Baell, 2016)).

DMSO could not be removed from the wells due to lack of equipment. DMSO can affect bioassays in several ways, for example, it can be toxic to bacteria (Wadhwani et al., 2009), reduce cell proliferation and have anti-inflammatory effects (de Abreu Costa et al., 2017) and even stimulate cell growth and cell transformation (Wen, Tong, & Zu, 2015). Due to a low amount of some FLASH fractions, the concentration of the stock solution in 100% DMSO where also low. To avoid exceeding the DMSO tolerance in the bioassays, some fractions where tested at lower concentrations. This was considered when evaluating the screening results.

8.1 EXTRACTION EFFECTS

Sample 5 (S5) were separated into one aqueous (W) and one organic extract (L) and samples 1 – 4 (S1 – S4) were extracted to one organic extract before FLASH fractionation. Little activity was detected in the aqueous extracts compared to the organic extracts, with the exception of two fractions that stood out; the activity of fraction 5 in S5-W (S5-W-F5, cons. 50 µg/mL) against *S. epidermidis* biofilm formation and cytotoxicity towards the MRC-5 cell line. The fraction was highly active in these assays, and activity was confirmed in a second screen in the anti-biofilm formation assay with a MIC value of 12.5 µg/mL. In the anti-biofilm formation assay, all F5 fractions from all the samples appeared to have activity. Therefore, it was likely that the same compound responsible for bioactivity was present in all fractions, and hence

nothing unique to the aqueous extract. In the MTS viability assay towards the MRC-5 cell line however, only the aqueous extract from S5 appeared active, and an active metabolite present in this fraction could therefore be something unique for the aqueous extract.

8.2 ANTIBACTERIAL AND ANTI-BIOFILM FORMATION ACTIVITY

In the antibacterial assay, a general observation was that fraction F4 and F5 from all the samples, with the exception of the aqueous extract, showed some degree of activity towards the *S. agalactiae* bacteria strain and the *E. facealis* bacteria strain compared to the other fractions. S3-F4 and S5-W-F5 exceeded the activity threshold and did thus not appear active against both bacteria strains. Still, due to their lower concentrations (25 µg/mL) and proximity to the activity threshold, they were presumed active.

With the exception of S5-L-F3 none of the samples seemed to have FLASH fractions that stood out significantly from one another. S5-L-F3 however had a higher activity than the other F3 samples. This was only the case for *S. agalactiae* and *E. facealis* presented in the results, see figure 12 and 13, page 45 and 46 respectively. The other bacteria screened in the antibacterial assay (*S. aureus, E. coli* and *P. aeruginosa*) did not show the same trend. Because S5 was cultivated with CO_2 containing smoke and subsequently fermented, this activity could be secondary metabolites produced as a result of these treatments.

There was a total of fifteen active fractions (+) and one questionable (+/-) fraction towards *S. epidermidis* in the anti-biofilm assay distributed in fractions F3, F4, F5, F6 and F7 according to the activity threshold. By comparing the fractions to look for bioactivities that could be a consequence of cultivation treatments, an interesting distribution of activities could be observed for fraction F3, F6 and F7. In fraction F3, sample S2 (30 x 35 minutes UV exposure) and S5-L (CO_2 fed and fermented) were conspicuous. Regarding S3, activity could not be explained by cultivation conditions as S3 (cultivated with UV for 2 hours) would be expected to have similar activities in some degree. As for the antibacterial assay, activity in S5-L-F3 clearly stood out from the other F3 fractions, as marked with an arrow in figure 15. In fraction F6, the sample treated with 2 hours of UV (S3) were the only sample with activity according to the activity threshold, and an apparent slight correlation between UV exposure and biofilm inhibition could be observed, see figure 15. In fraction F7, there is apparent activity in all fractions except both the S5 fractions. This could indicate that the cultivation modification of

this sample reduced production of the compound responsible for this bioactivity. Moreover, the late-fraction activities, like F7, could be due to FFAs. As mentioned before, increasing light levels can both reduce and enhance different kinds of fatty acid production like PUFA's and EPA's (Grima et al., 1994) and these can have antibacterial activities (Smith et al., 2010). S5-L-F3, S5-L-F4 and S5-W-F5 seemed to appear active because of bacteria killing and not specifically biofilm inhibition. This was because little bacteria growth in general were observed for these fractions. This is also consistent with the antibacterial assay where these fractions appeared to have some degree of activity against both *S. agalactiae* and *E. facealis*.

8.3 ANTICANCER ACTIVITY AND CYTOTOXICITY

The organic samples in fractions F5 and F6 in the MTS viability assay on the human melanoma cell line (A2058) showed activity with no particular deviation between the samples. This indicated that the cultivation conditions did not contribute to secondary metabolite production with anticancer activities. However, this activity in all the samples could indicate general anticancer properties of this diatom species. By comparing this with the results towards the non-cancerous cell line (MRC-5) with S5-L, fraction 5 did not appear cytotoxic towards this cell line, and could thus be investigated as a potential anticancer candidate in further experiments. Compared to table 1, microalgae compounds have earlier proven to have promising anticancer activities.

S5-L-F6 showed activity towards all the cell lines (A2058, HT29 and MRC-5). In spite of that, activity was absent in the second screen towards cell line A2058. As mentioned under the "extraction effects" headline, the activity expressed in S5-W-F5 towards cell line MRC-5 stood out. Other than the possibility of being a false positive, it is possible that this compound responsible for the activity/cytotoxicity could have a particular selectivity against MRC-5, and not the other cell lines.

8.4 ANTI-INFLAMMATORY- AND ANTIOXIDATIVE ACTIVITY

The assay with the highest number of bioactive FLASH fractions in the first screen was the anti-inflammatory assay with as much as sixteen active fractions and one presumable active fraction according to the activity thresholds. The activity was mostly found in F5, F6 and F7

from all the samples with the exception of the aqueous extract (S5-W). What stood out here was the bioactivity of F8 in the two samples treated with the most amount of UV (S2 and S3).

In the antioxidant assay, only one fraction expressed activity according to the activity threshold, namely S5-L-F4. Other than this there were several questionable and presumably active fractions. Because of this, it would not be expedient to elaborate on activity according to the activity thresholds, and possible active fractions will only be discussed on the basis of the general activity trend. In general, S1, S4 and S5-W show little activity compared to the other samples, with the exception of fraction 6 (F6) and fraction 8 (F8) where all the samples show a similar degree of activity. The UV treated samples (S2 and S3) as well as the CO_2 fed and fermented sample S5-L seemed to stand out in fractions F1, F2 and F4. Regarding the possible activity of S5-L in several of the assays, with the exception of the activity being a result of the specific cultivation treatments, it is important to consider that this sample was cultivated at a much bigger scale than the other samples. Therefore, a higher degree of foreign substances and microorganisms was expected to be present in this sample. These could be the true triggering factors of bioactivity in the bioassays, either directly or indirectly by interfering with other microorganisms or the diatom species.

The activities in the UV-treated samples, not only in the anti-inflammatory- and antioxidant assays, but also the antibacterial and the anti-biofilm assay, could be secondary metabolites excreted from the diatoms as a response to UV radiation stress, or a consequence of damage to the cells due to the high amounts of energy emitted from the UV light. Another suggestion is that this could be metabolites with the ability to absorb UV for protection of the organism (Cleaves & Miller, 1998).

In an UV experiment with microalgae it is shown that microalgae can produce anti-inflammatory and antioxidative compounds as a response to UV light (Al-Rashed, Ibrahim, El-Gaaly, Al-Shehri, & Mostafa, 2016). Here, the two microalgae species *Spirulina platensis* and *Dunaliella salina* showed a significant increase in carotenoids, flavonoids and phenolics after UV-B radiation. As shown in table 1, several carotenoids have anti-inflammatory and antioxidant abilities. In addition, flavonoids have the ability to reduce formation of pro-inflammatory mediators such as reactive oxygen species, prostaglandins and leukotrienes (Robak & Gryglewski, 1996), and phenolic compounds from microalgae have shown to have antioxidant activity (Safafar, Van Wagenen, Møller, & Jacobsen, 2015).

However, here the microalgae were treated with UV for 72 hours as opposed to S2 and S3 which were treated for 30 x 35 minutes and 2 hours, respectively. This scarce time exposure to UV light could be a contributing factor to the low activities in the antioxidant assay.

Further, polar benthic pennate diatoms have proven to have a relative high tolerance for UVR compared to other microalgae species (Wulff, Roleda, Zacher, & Wiencke, 2008; Zacher, Wulff, Molis, Hanelt, & Wiencke, 2007), which can be a result of the high sunlight exposure in the polar day months. Arctic diatoms could, for this reason, have a unique capacity to produce protective secondary metabolites to prevent oxidation or damage due to UV stress factors. Even though the general activity in the antioxidant assay conducted in this thesis was limited, it is important to keep in mind that oxidation is a complex process, and one type of antioxidant assay alone is not enough to eliminate this hypothesis. For further investigation, more experiments needs to be conducted in other kinds of antioxidant assays.

8.5 Refractionation and dereplication of anti-biofilm formation fractions

Samples refractionated on the prep HPLC-MS to be tested again in a third screen against *S. epidermidis* biofilm formation were chosen based on the results from both the antibacterial assay and the anti-biofilm formation assay. In the search for compounds with biofilm inhibitory properties, it is important to identify and eliminate compounds with bacteria growth inhibition properties at an early stage. These properties can be difficult to separate from one another in the anti-biofilm formation assay. Other than checking the wells directly for inhibition of bacteria growth with the naked eye, an antibacterial assay can be used as a guideline. The antibacterial properties of fractions towards *S. agalactiae* and *E. facealis* in the antibacterial assay were therefore compared to the fractions with anti-biofilm formation properties towards *S. epidermidis* for selection of fractions for the third screen. In addition, all these bacteria are gram positive bacteria, and thus have some susceptibility similarities due to their similar cell wall composition. On the basis of this, S1-F7, S2-F7, S3-F7 and S5-L-F3 were selected for refractionation by prep HPLC-MS and subsequently retested in the anti-biofilm formation assay (third screen).

From this screen, possible bioactivity could be observed for HPLC fraction 9/10 and 25 in all the samples, and fraction 18 in S2-F7. The drop in OD in fraction 26 – 40 in S2-F7 were most

likely not a consequence of bioactivity because the positive control revealed that there was less bacteria growth in these wells in general.

Further investigation of the prep HPLC-MS chromatogram showed high intensity for the peak eluted in fraction 9 for all selected samples separated in the prep HPLC-MS. Further structure elucidation by UPLC-QToF-MS revealed a mass of 592.27 Da with a high possibility of having the chemical formula: $C_{35}H_{36}N_4O_5$. From a search in the database Dictionary of Marine Natural Products, phaeophorbide-a (figure 31) appeared as a possible molecule. This is a degradation product of chlorophyll. It can be recognized as a chlorophyll derivate due to the characteristic shape of chlorophyll (Hosikian, Lim, Halim, & Danquah, 2010).

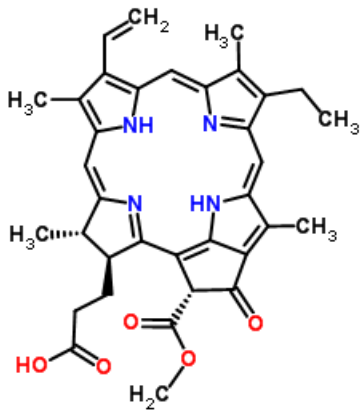

*Figure 31: **Phaeophorbide-a**; the molecule most likely to be responsible for the anti-biofilm formation properties in HPLC fraction 9 of FLASH fraction S2-F7. The figure is retrieved from ChemSpider.*

8.6 Dereplication of anti-inflammatory FLASH fractions

In an attempt to identify the compound responsible for activity in the UV treated FLASH fractions (S2-F8 and S3-F8) in the anti-inflammatory assay, S3-F8 and the inactive fraction S1-F8 (normal cultivation) were analysed and compared by UPLC-QToF-MS. In the ESI+ chromatograms obtained (see figure 27), one peak stood out with a high intensity in S3-F8 and was not detectable in S1-F8. Further investigation gave a high chance of this molecule to have the chemical formula: $C_{35}H_{36}N_4O_6$, with a mass of 608.26 Da. A search in the database Dictionary of Marine Natural Products, gave the hit: Phaeophorbide a; 10-Hydroxy. This indicated that the responsible compound for the anti-inflammatory bioactivity could be, like for the molecule found from S2-F7, phaeophorbide-a, but with an extra hydroxyl group attached. A possible molecule is depicted in figure 32.

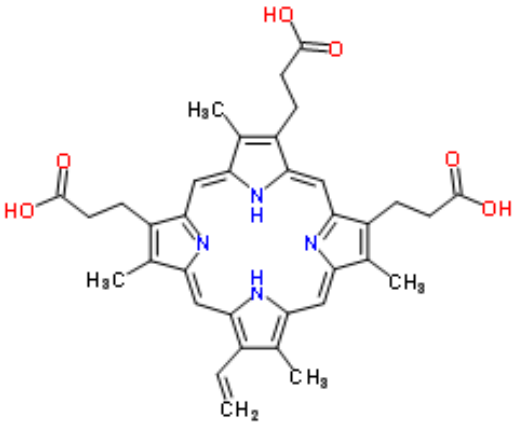

Figure 32: ***Phaeophorbide a; 10-Hydroxy****; the molecule most likely to be responsible for anti-inflammatory properties of FLASH fraction S3-F8. The figure is retrieved from ChemSpider.*

There are few studies of the bioactivity potential of phaeophorbide-a. In one study, phaeophorbide-a was shown to enhance antibacterial activity of berberine, isolated from two berberine species, towards a resistant *S. aureus* bacterium (Stermitz et al., 2000). It has also been reported that Na-phaeophorbide-a can prevent leg swelling and inhibit bone destruction in osteomyelitis models in rats (Goto et al., 2011). To make this model, the same bacteria, *S. aureus*, was used to trigger osteomyelitis in the rats. Osteomyelitis is a bone infection usually caused by a bacterial infection. This could be due to biofilm inhibition. As mentioned in the introduction; for bacteria to be able to cause infections in our body, they have to, in most cases, be organized in biofilm (Aalehaeger, 2010). As a comparison, results from the antibacterial assay for *S. aureus* showed a drop in OD value in fraction F7 that was not detected for the other bacteria strains, see appendix, page 78. This supports the possibility that phaeophorbide-a was present in fraction 7.

There is not to my knowledge any reported biofilm activity of phaeophorbide-a from before, and there is a possibility that this bioactivity was due to other effects. For example, fraction 1, 9 and 25 were all in the edge line of the microtiter plate, and drop in biofilm production could therefore be due to an edge-effect. An edge-effect is an issue concerning the outer wells in a microtiter plate. This could occur due to pipetting errors or the fact that these wells are more exposed to evaporation that the inner wells. This changes the concentration of the different components in the wells, directly affecting the robustness of the applicable assay (Jaquith, 2014).

Nevertheless, degradation products of chlorophyll has been proven to have bioactivity in earlier experiments in Marbio aswell, with non-specific and generally weak activity in several assays (Unpublished results). Potency and specificity are two major factors in drug development (Baell, 2016), and these compounds would therefore most likely, for this reason, be terminated for further investigation in a bioprospecting pipeline.

The low specificity could potentially be supported by looking at the results of the anti-inflammatory assay with the HPLC fractions of S4-F7 (sample with cocultivation). Also here, a distinct peak could be observed in fraction 9 obtained in the prep HPLC-MS chromatogram (see figure 34 in appendix), as for the other F7 fractions separated in the prep HPLC-MS. By looking at this fraction in the second screen conducted with the anti-inflammatory assay (see figure 35 in appendix), there could be some indication of weak activity also here, demonstrating potential low specificity.

These factors substantiates the importance of being aware of these degradation products of chlorophyll as potential frequent occurrences in bioassays when using microalgae for bioactivity discovery purposes.

8.7 OTHER POSSIBLE COMPOUNDS RESPONSIBLE FOR BIOACTIVITY

Table 1 is a comprehensive overview over several compounds derived from microalgae with different kinds of activities. Many of these compounds have their origin in chlorophyll. Chlorophyll is known to be a valuable source of bioactive compounds (Hosikian et al., 2010). One compound present in chlorophyll is the powerful carotenoid fucoxanthin. This is normally one of the major pigments in diatoms together with chlorophyll a and c (Munn, 2011). As shown in table 1 this powerful carotenoid has anti-cancer, antioxidant and anti-inflammatory bioactivities. In addition it has also proven to have anti-obesity, anti-diabetic and antimalarial bioactivities (Peng et al., 2011). A general trend of the bioassays conducted in this thesis is the frequent occurrences of bioactivities in the late fractions. Fucoxanthin is fat soluble, can could therefore potentially be responsible for some of these activities.

My findings of general bioactivity in diatoms are supported by an earlier experiment conducted at Marbio. Here, twenty-one different microalgae species were screened for bioactivity, whereas seven of these were diatoms. They were screened for antibacterial, anti-biofilm-, anticancer-, anti-inflammatory-, antioxidant- and anti-diabetes activities, and the

only species expressing bioactivity belonged to the diatom-group. They were confirmed active in the anti-biofilm-, anticancer- and anti-inflammatory assays (Lauritano et al., 2016), which are also the assays with the highest number of active fractions in this thesis.

9 CONCLUSION

The bioactivity potential of the poorly investigated Arctic marine diatoms was investigated in this thesis by bioassay guided isolation in a wide selection of bioassays. Five samples with the same diatom species cultivated at different conditions were tested. It was desired to give a valuable insight in the general bioactivity of this diatom species and additionally in how cultivation conditions could be used to trigger variations in the bioactivity profile of the same diatom species, as successfully done with marine bacteria through the OSMAC approach. The bioactivity potential was investigated through antibacterial-, anti-biofilm formation-, anticancer-, cytotoxicity-, anti-inflammatory- and antioxidative assays.

The results demonstrate the broad bioactivity potential of Arctic diatoms, and that cultivation modifications could be used to modify the bioactivity profile, as some clear differences were observed between the samples. This thesis also emphasises the importance of providing enough material in a bioprospecting pipeline to enable full investigation. Through dereplication, it became clear that degradation products of chlorophyll have the capacity to frequently emerge as hits in the bioassays. It is therefore of great importance that these compounds are terminated at an early stage.

Further work needs to be conducted on the metabolic plasticity of diatoms as a consequence of external factors. Not only by the cultivation modifications applied for the diatoms in this thesis, but also other factors such as seasonal seawater variations, life cycle changes and different signal molecules. With this, elimination of uncertainties around the true triggering factors of diatom NP production could be provided.

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11 APPENDIX

Preparation of PSB and trypsin for cell line assays.

Table 28: Reagents used in preparation of PBS and trypsin.

Reagents	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 dehydrate	30412	Sigma-Aldrich, Missouri, USA
Milli-Q Ultrapure Water		Merck KGaA, Germany
Trypsin (1:250)	27250018	Thermo Fisher Scientific, Massachusetts, USA
Ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA)	E1644	Sigma-Aldrich, Missouri, USA

PBS:

PBS is made with 0.2 g/l potassium chloride, 0.2 g/l potassium dihydrogen phosphate, 8 g/l sodium chloride and 2.16 g/l sodium phosphate dibasic dehydrate. The buffer was prepared with milli-Q ultrapure water and autoclaved at 121°C for 120 minutes.

Trypsin:

Trypsin is made with 25 g/l trypsin (1:250) and 5 g/l Ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA). The solution was made with 0.25 % trypsin and 0.05 % EDTA in sterile filtered PBS.

Preparation of TBS buffer and 1 M Diethanolamine buffer for ELISA assay:

Table 29: Reagents used to make TBS buffer and 1 M Diethanolamine buffer.

Reagents	ID	Distributor, country
Tris	93352	Sigma-Aldrich, Missouri, USA
NaCl	S5886	Sigma-Aldrich, Missouri, USA
Dietanolamine 99,0 %	D8885	Sigma-Aldrich, Missouri, USA
$MgCl_2$	M0250	Sigma-Aldrich, Missouri, USA
HCl	30721	Sigma-Aldrich, Missouri, USA

TBS buffer:

TBS buffer was made with 1.21 g Tris, 8.8 g NaCl and 1 L of deionized water, and adjusted to pH 7.4 with HCl.

1 M Diethanolamine buffer:

1 M Diethanolamine buffer was made with 100 mg $MgCl_2$, 97 mL Diethanolamine and 1 L of deionized water, adjusted to pH 9.8 with HCl.

Instrument parameters used in prep HPLC-MS

Instrument parameters of the prep-HPLC during separation of all FLASH fractions are shown in table 30.

Table 30: Instrument parameters of prep-HPLC.

Source parameters	**Positive ion mode (ES+)**
Source temp.	120°C
Desoviation temp.	300°C
Desoviation gas flow	650 L/hr
Cone gas flow	5 L/hr
Cone	42 V
Capillary	3.00 kV

Instrument parameters UPLC-QToF-MS

Instrument parameters of the UPLC-QToF-MS during dereplication are shown in table 31.

Table 31: Instrument parameters of the UPLC-QToF-MS.

Source parameters	**ESI+**
Capillary voltage (kV)	0.80
Cone voltage (V)	30
Cone gas flow (L/h)	50
Desolvation gas flow (L/h)	800
Temperature desolvation (°C)	450
Temperature source (°C)	120
Low mass (*m/z*)	50
High mass (*m/z*)	2000
Low collision energy (eV)	6.0
High collision energy (eV)	15-45

Antibacterial assay, *S. aureus*

Figure 33 depicts the antibacterial results of all FLASH fractions towards *S. aureus* in the first screen. The drop in OD value in F7 supports the theory that phaeopbhorbide-a was present in this fraction.

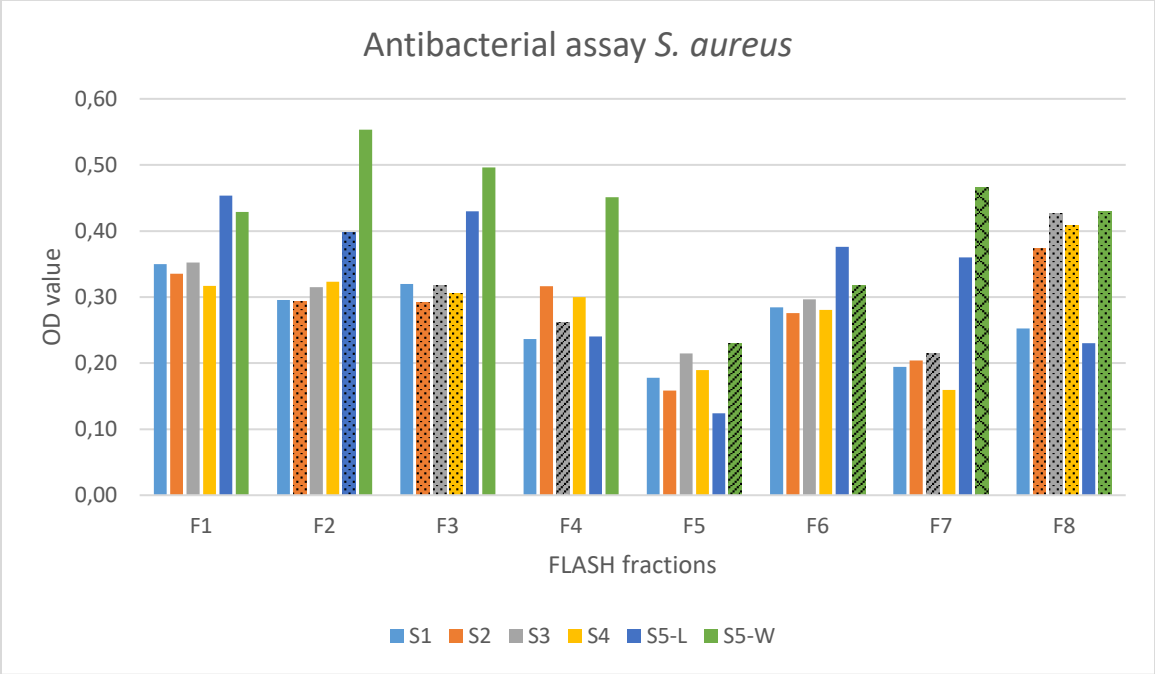

Figure 33: All FLASH fractions from S1 – S5 with concentrations of 100 µg/mL (plain), 50 µg/mL (dots), 25 µg/mL (stripes) and 10 µg/mL (X-es) and their appurtenant activities tested in an antibacterial assay against S. aureus.

Prep HPLC-MS chromatogram of S4-F7

The prep HPLC-MS chromatogram from separation of S4-S7 into forty fractions is depicted in figure 34.

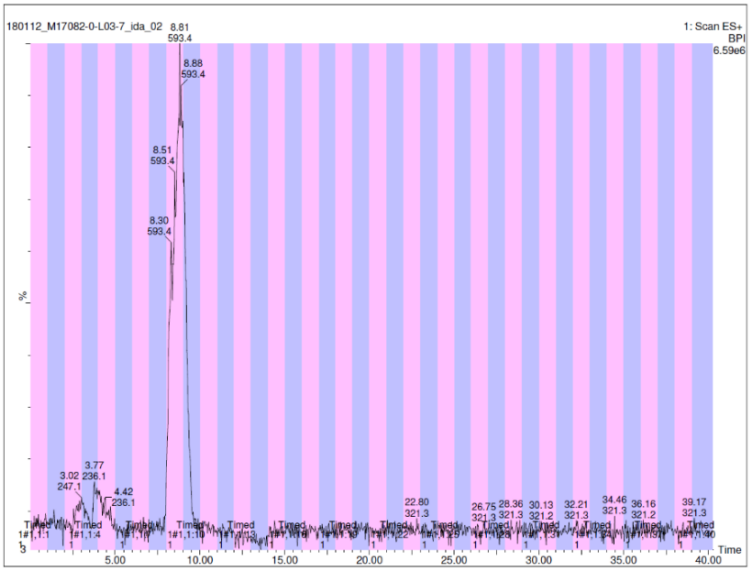

Figure 34: Chromatogram (percent intensity and time eluted) from HPLC refractionation of S4-F7.

Anti-inflammatory assay with HPLC fractions of S4-S7.

Figure 35 depicts the results from the anti-inflammatory assay with HPLC fractions of S4-S7. The arrow marks HPLC fraction 9, which could have some degree of activity due to the presence of phaeophorbide-a.

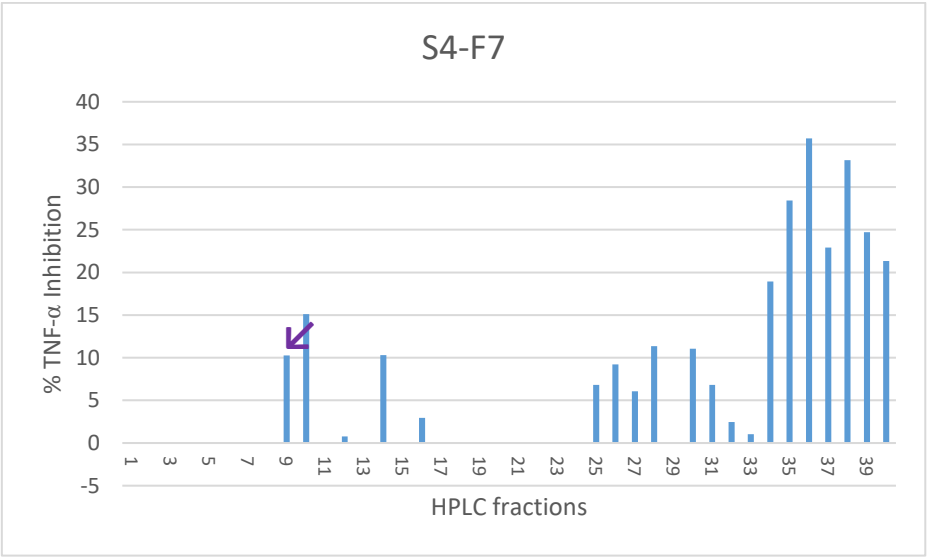

Figure 35: HPLC fractions from S4-F7 and their percentage TNF-α inhibition values in the anti-inflammatory assay.

Summary of activity of all FLASH fractions from the first screenings.

Table 32: Comparison of the activities of the FLASH fractions in the first screenings towards the antibacterial- (with S. agalactiae (S. a) *and* E. facealis (E. f)*), anti-biofilm- (with* S. epidermidis (S. e)*), MTS viability- (with A2058, HT29 and MRC-5), anti-inflammatory- (with THP-1 cell line) and antioxidant assay (with HepG2 cell line). Active (+), presumably active ((+)), questionable (+/-) and inactive (-). Blank wells means not tested.*

	Antibacterial		Anti-biofilm	MTS viability assay			Anti-inflammatory	Antioxidant
	S. a	*E. f*	*S. e*	A2058	HT29	MRC-5	THP-1	HepG2
S1-F1	-	-	-	-			-	-
S1-F2	-	-	-	-			-	-
S1-F3	-	-	-	-			-	+/-
S1-F4	+	-	+	-			-	-
S1-F5	-	-	+	+			+	-
S1-F6	-	-	-	+			+	-
S1-F7	-	-	+	-			+	-
S1-F8	-	-	-	-			-	-
S2-F1	-	-	-	-			-	+/-
S2-F2	-	-	-	-			-	(+)
S2-F3	-	-	(+)	-			-	(+)
S2-F4	+	-	+	-			-	-
S2-F5	+/-	-	+/-	+			+	-
S2-F6	-	-	-	+/-			+	-
S2-F7	-	-	+	-			+	-
S2-F8	-	-	-	-			+	(+)
S3-F1	-	-	-	-			-	+/-
S3-F2	-	-	-	-			-	-
S3-F3	-	-	-	-			-	(+)
S3-F4	(+)	(+)	-	-			-	(+)
S3-F5	+/-	-	+	+			+	-
S3-F6	-	-	+	+			+	-
S3-F7	-	-	+	-			(+)	(+)
S3-F8	-	-	-	-			+	(+)
S4-F1	-	-	-	-			-	-
S4-F2	-	-	-	-			-	-
S4-F3	-	-	-	-			-	(+)
S4-F4	+	-	+	-			-	-
S4-F5	+/-	-	+	+			+	-
S4-F6	-	-	-	+			+	-
S4-F7	-	-	+	-			+	-
S4-F8	-	-	-	-			-	-
S5-L-F1	-	-	-	-			-	-
S5-L-F2	-	-	-	-			-	(+)
S5-L-F3	-	+/-	+	-			-	+/-
S5-L-F4	+/-	+/-	+	-			-	+
S5-L-F5	+	-	+	+			+	-
S5-L-F6	-	-	-	+	+	+	+	-
S5-L-F7	-	-	-	-	-	-	+	-
S5-L-F8	-	-	-	-	-	-	-	+/-
S5-W-F1	-	-	-	-	-	-	-	-
S5-W-F2	-	-	-	-	-	-	-	-
S5-W-F3	-	-	-	-	-	-	-	-
S5-W-F4	-	-	-	-	-	-	-	-
S5-W-F5	(+)	(+)	+	-	-	+	-	-
S5-W-F6	-	-	-	-	-	-	-	-
S5-W-F7	-	-	-	-	-	-	-	-
S5-W-F8	-	-	-	-	-	-	-	-