Master thesis for the degree of Master of Pharmacy

Isolation and characterization of antibacterial compounds from marine organisms

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Preface

This project has been possible to complete thanks to good collaboration between Marbio and the Institute of Pharmacy at the University of Tromsø. The project was performed in the period from October 2009 to May 2010.

First and foremost I would like to thank my supervisors Terje Vasskog (UIT), Espen Hansen (Marbio) and Jeanette Hammer Andersen for their support, help and outstanding guidance during my master period. A special thanks to Espen for his patience and the help I received in interpreting the results. Thank you to all three of you for responding to my questions quickly and helpfully.

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Last but not least I would like to thank my mother and father, Parul and Satish Thanki, my aunt and uncle, Asha and Janak Thanki, and my two beloved brothers Hitesh and Harit Thanki for all the love, support, care and patience during my studies and my whole life. I would not have come so far and be the person I am if not for you. I love you all.

Meera Thanki

Abstract

Marine bioprospecting is a promising field of research that has gained interest worldwide for the past few decades, and can be characterised as a systematic and purpose oriented search for bioactive compounds or genetic material from marine sources. Several compounds have been isolated and some have found their way into the commercial market. The main aim of this master thesis was to investigate the antibacterial activity of bioactive compounds in the extracts of the marine ascidian *Halocynthia pyriformis.* Two other marine organisms *Metridium senile* and *Parastichopus tremulus* were also screened for antibacterial activity, but they were purified in two other parallel projects.

Aqueous and organic extracts from *H. pyriformis* were purified using preparative HPLC and screened for antibacterial activity. The antibacterial activity was mostly found in the organic fractions against the Gram-positive bacteria *E.faecalis, S. aureus* and MRSA. The active fractions from the primary screening were subjected to a secondary screening where they did not display any clear activity. Three of the fractions showed some activity and was pooled and refractionated and later subjected to a third screening. The active fractions were analysed by ESI-TOF and ESI-Q without obtaining any results. A dose-response test was carried out to investigate the potency of the raw-extract. Due to weak results it was decided to fractionate the organic extract of *H. pyriformis* once again before it was subjected to a new primary screening. This time the fractions were concentrated and three of them displayed antibacterial activity and were subjected to MS-analyses in positive and negative mode.

The results from the MS-analyses indicated that one of the fractions contained two active compounds with the *m/z* values 269.2094 and 505.4248. The calculated elemental composition proposed the empirical formula $C_{16}H_{28}O_3$ for the molecular weight 268.2021. A search in databases containing known bioactive compounds proposed four different compounds with the same empirical formula. The elemental composition of the *m/z* value 505.4248, proposed two empirical formulas $C_{32}H_{56}O_4$ and $C_{31}H_{54}N_4$. None of these two formulas gave any hits in the database over known bioactive compounds. This could indicate that this is a novel compound. It was impossible to know the identity of these

compounds without analysing them in NMR. NMR analyses require large amounts of pure compounds which was difficult to obtain in this thesis.

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Abbreviations

ACN	Acetonitrile
AMU	Atomic mass unit
BHI	Brain Heart Infusion
BPI	Base Peak Index
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
ESI	Electrospray Ionization
HPLC	High performance liquid chromatography
ISA	Iso-Sensitest Agar
LC-MS	Liquid chromatography-mass spectrometry
MeOH	Methanol
MH	Mueller Hinton II
MIC	Minimum Inhibitory Concentration
MP	Mobile phase
MRSA	Methicillin resistant *Staphylococcus aureus*
MS	Mass spectrometry
NaCl	Sodium chloride
NMR	Nuclear magnetic resonance
OD	Optical density
Q	Quadrupole
QqQ	Triple quadrupole
Rpm	Rounds per minute
TOF	Time-of-flight
UPLC	Ultra Performance Liquid Chromatography
UV	Ultraviolet

1 Introduction

1.1 Marine bioprospecting

From the beginning of time nature has always provided mankind with many essentials, ranging from materials to create equipment and tools, to food and medicine. All through history the latter has come from terrestrial sources, but lately certain aspects of medical sciences have turned their eyes towards the marine environment.

Roughly 2/3 of the earth is covered by the ocean and this gives a potential great enhancement to our medical supply. Many marine organisms living there have been forced to adapt to extreme conditions such as low temperatures, high pressure and low nutrient levels, and because of this some of them have developed different survival strategies that involve bioactive molecules which are different from what can be found in terrestrial organisms. These bioactive molecules represent interesting developmental opportunities in several human medicinal fields.

Marine bioprospecting can be characterized as a systematic and purpose-oriented search for bioactive compounds or genetic material in marine organisms, for further research, innovation and commercialisation. This type of research has become easier to perform due to knowledge about the marine environment, and better availability of resources, which makes it less difficult to collect marine organisms from areas with extreme conditions. Several areas outside the Norwegian coast are yet to be explored and scientists are eager to take on that challenge. About 15000 marine natural products [1] have been isolated and some of them have even found their way into the commercial market and many more are still to come due to the relative infancy of the field.

1.2 MabCent-SFI, Marbank and Marbio

MabCent-SFI is a centre established by the research council of Norway March 2007. It is a Centre for Research-based Innovation (CRI) for marine bioactivities and drug discovery and is situated in Tromsø. Their main focus is to screen and analyze for compounds that have antibacterial, anti-inflammatory, antitumor, anti-diabetic activities as well as enzymes and antioxidants in Arctic and sub-Arctic marine organisms from north Norwegian waters.

MabCent and Marbio collaborates tightly with Marbank which is a marine biobank that organizes the sampling and storage of marine organisms and produces extracts for screening, so they can be used for further research, innovation and commercialisation. The actual screening is performed by Marbio which is an automated/high-throughput laboratory screening for bioactive compounds in marine organisms.

1.3 Chordata

The Phylum Chordata is a line of animals which has a chorda dorsalis (notochord or "ryggstreng" in Norwegian) at some point in their life cycle. Chordata differ from other phylums in four profound features. Firstly there is a central nervous system on the animals back that is developed like a continuous tube-shaped string, the neural tube, which becomes the spine; secondly the foetus's skeletal structure, the notochord, lies under the neural tube. Thirdly the digestive tract lays under the notochord, and its upper part is shaped like a respiratory organ. Fourthly the big pulsating blood vessel and heart is placed on the same side as the abdomen (www.snl.no 08.05.2010)

1.4 Halocynthia pyriformis

Halocynthia pyriformis is of the phylum Chordata (subphylum-Tunicata), in the class Ascidiacea. The ascidians are sessile and can be colonial or solitary animals. This class is characterized by a thick gelatine or leather like tunic which surrounds the ascidians. They have two branchial openings or siphons, one for the feeding current to enter and the other

one where the waste is removed. Ascidians have few enemies due to the cellulose like tunic that make them tough to digest [2, 3]. The picture of *Halocynthia pyriformis* is shown in figure 1.

Figure 1: *Halocynthia pyriformis*. The picture is used with permission from Sten-R. Birkely, Marbank

H. pyriformis is commonly found along the coast in the northern parts of Norway, especially around Bodø, but has also been registered as far south as Stavanger. This ascidian is a solitary one, but it also appears in colonies. It can appear in red, orange or even white colour, and their body can reach a height of 10 cm. It has leather like cap with small unbranched tags in small groups. *H. pyriformis* is commonly called a Sea peach and it can become as big as a grapefruit.

In addition to *H. pyriformis* this master thesis also included antibacterial screening of two other marine organisms, which were purified in other parallel projects. The first, *Metridium senile* (figure 2), is of the phylum Cnidaria, in the class Anthozoa. It is commonly found along the entire Norwegian coast and can have an orange, yellow or white colour. The second, *Parastichopus tremulus* (figure 3), is of the phylum Echinodermata, in the class Holothuroidea. It is widespread along the entire Norwegian coast. *P. tremulus* has a beautiful red colour on the back and is white on the abdominal side [2].

Figure 2: ***Metridium senile.***
The picture is used with permission from Sten-R. Birkely, Marbank

Figure 3: ***Parastichopus tremulus.***
The picture is used with the permission from Robert A. Johansen, Marbank.

1.5 Known bioactive compounds

Tunicates, the subphylum of Chordata, have according to the literature, yielded compounds with cytotoxic characteristics. Ecteinascidin-743 (ET743) [4, 5] (Trabectedin ®) was isolated from the tunicate *Ecteinascidia turbinate* and is an anticancer agent now approved in Europe for advanced soft tissue sarcoma. Dehydrodidemnin B (Aplidin ®) [6, 7] is another compound with antitumor effects [8], which is undergoing phase II clinical trials [9]. Didemnin B (depsipeptide) and Dolastin 10 (nitrogen-containing peptides), and Phthalascidin are other compounds with antitumor activity isolated from tunicates [8].

From the ascidian body there has been isolated highly sulphated dermatan sulphates. They consist of the same backbone structure [4-α-L-IdceA-1→3-β-D-Ga1NAc-1]$_n$ but can differ when it comes to sulphation substitutions. These compounds have high amount of 2-O-sulphated α-L-iduronic acid residues but can have different type of sulphation of the N-acetyl-β-D-galactosamine. *Halocynthia pyriformis* has 4-O-sulphation of the N-acetyl-β-D-galactosamine residue and it causes their anticoagulant activity [10].

Eudistomins are cyclic peptides which have substituted condensed oxathiazepine ring systems and they are isolated from Eudistoma *Olivaceum* a colonial Caribbean tunicate. The peptide has shown antiviral activity against Herpes simplex virus type (HSV-1) [11].

From tunicate species (*Didemnum*) it has been isolated alkaloids with an unusual decahydroquinoline skeleton. They may have the potential of being lead compounds against malarial diseases [11].

1.6 Choice of methods

MabCent-SFI has a set of established methods that are used for sample preparation, fractionating and screening for bioactivities of materials from marine organisms. The methods are developed in such manner that they ensure the possibility of finding a wide range of compounds, regardless of how small or big they are. The experiments in this thesis are based on the methods established by MabCent-SFI.

The sample of *Halocynthia pyriformis* was freeze-dried and extracted first with water and subsequently with organic solvents (a mixture of dichloromethane and methanol). This gives a rough separation of polar and non polar compounds. The organic and the aqueous extract were further purified by high performance liquid chromatography (HPLC).

The fractions obtained from the HPLC purification were screened for antibacterial activity. A series of Gram-positive and Gram-negative bacteria were exposed to the fractions. After incubation the inhibition of bacterial growth was determined both visually and by UV-spectroscopy. The bacterial strains used in the screening cause serious infections in humans.

Fractions were subjected to primary and secondary screening and the re-fractionated fractions were subjected to third screening. The fractions which showed activity after the primary and the third screening were analysed by LC-MS, using two different mass separators. Electrospray was used to ionize the samples, and accurate mass of the ions was obtained to find the accurate molecular weight and the elemental composition of the active compounds. By comparing this information to databases one can find out if the compound is already known. This process of identifying and eliminating known bioactive compounds is called dereplication. The compounds not found in the databases are interesting candidates for further structure elucidation.
The flow chart gives a simple overview of the process from extraction to LC-MS analysis. This is illustrated in figure 4.

1.7 Extraction

The extractions were performed to obtain different compounds from the freeze-dried material. The solvent or mixture of solvents used in the extractions was chosen based on the compounds one wanted to extract. Water was used as a solvent to extract the polar, hydrophilic compounds, and a mixture of non polar solvents (dichloromethane and methanol) was used to extract non polar lipophilic compounds.
When screening for unknown bioactive compounds from the marine samples, the different properties of the compound when it comes to size, shape, polarity, elemental composition and so on, are not known. Therefore the selectivity of the extraction solution should be as wide as possible. That is why Marbio has chosen to perform an aqueous extraction followed by an organic extraction on the same sample of freeze-dried material.
The water soluble compounds can be bound to membranes, located in membrane pockets or be protected by lipophilic substances. This can cause a problem which can be solved by pulverizing the material after freeze-drying it, and this will destroy some of the lipophilic pockets where the polar compounds usually get trapped. A different solution can be carried out by mixing water with an organic solvent like methanol for instance. This will dissolve the lipophilic pockets and extraction of the polar compounds will be possible.

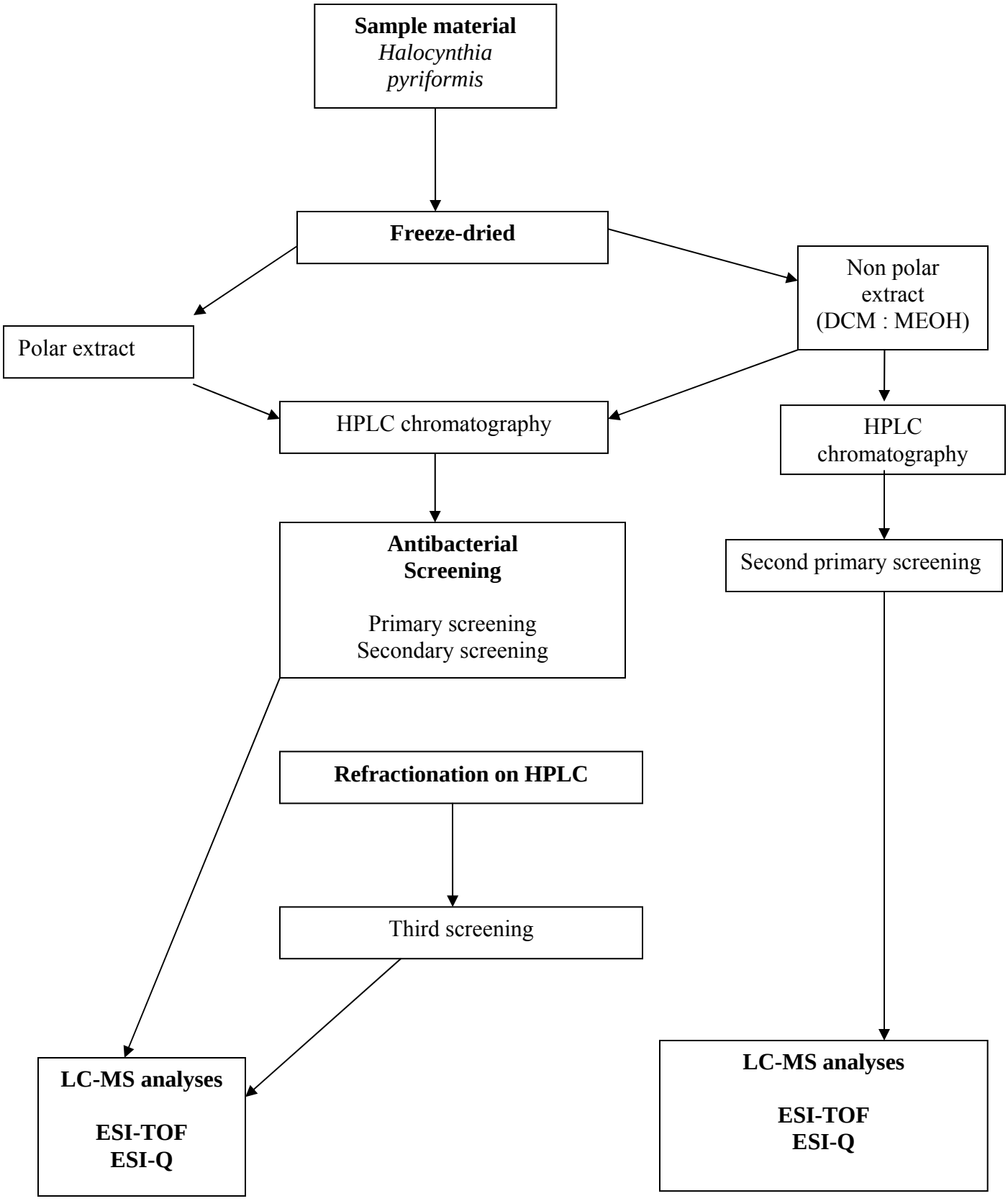

Figure 4: Simple overview of the process from extraction to LC-MS analysis.

1.8 Antibacterial screening

Bacteria are classified as Gram positive or Gram negative, where the main difference lies in their cell wall composition. The cell wall of both bacteria consists of a strong peptidoglycan layer, but this layer is thicker in Gram positive and slightly thinner in Gram negative bacteria. Further more, the Gram negative bacteria contain a lipopolysaccharide layer in their outer membrane, which makes their cell wall composition more complex than Gram positive bacteria. Some antimicrobial agents have a better effect on Gram positive bacteria than Gram negative bacteria, because of their less complex cell wall structure [12].

Unnecessary use and excessive consumption of antimicrobial agents is closely linked to the development of resistance. Bacteria have several different means to obtain and share resistance. Among these are mutation and horizontal gene transfer, the latter of these can happen by plasmids, transposons and lysogenic bacteriophage [13]. This has led to poor or slow treatment of infectious diseases. In spite of this knowledge antibiotic discovery is not favoured by most pharmaceutical companies due to many factors, two of those factors are that it is time-consuming and technically difficult to produce a novel antibiotic [14].

The bacteria used in the bacterial screening were *Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and Methicillin-resistant *Staphylococcus aureus* (MRSA).

In hospitals, MRSA is the most common resistant bacteria, and it was identified around 1970 in Europe and a decade later in the United States [15]. Later the reports of community-acquired MRSA infections in adults started to grow. The main difference between hospital acquired MRSA and the community-acquired MRSA is that the former is multidrug resistant. Some bacterial strains such as *E. faecalis* and *P. aeruginosa* are naturally resistant to many antimicrobial agents. Even though linezolid, which is a rather new antibiotic, is effective against this strain, some reports on linezolid-resistant strains have been obtained [16]. *S.aureus* and *E.coli* is known to cause several of the bacterial infections in the community. The latter cause urinary tract infections and clinical experience show that treatment with the antibiotic Co-trimoxazole has been effective, simple and cheap. However co-trimoxazole-resistant strains have been reported the last

few years [13]. This shows that antibiotic treatment of infectious diseases is complex and the big challenge lies in the control of use and further research and development of novel antibiotics.

Enterococcus faecalis

E. faecalis is gram positive cocci, capable of both aerobic and anaerobic respiration. They occur naturally in the gut and the infections are endogenously acquired but can also be the result of cross-infection from hospitalized patients. They cause urinary tract infections and can, in individuals with a suppressed immune system or after surgery, cause severe septicaemia [17].

Escherichia coli

E. coli is a gram negative rod which is capable of anaerobe respiration and can grow at temperatures up to 44 ºC. The ones that occur naturally in the gut are involved in digestion. The other ones that have virulence factors cause diseases like urinary tract infection, septicaemia, neonatal meningitis and diarrheal diseases. They spread by contact and via the faecal-oral route and it can also be food-associated [17].

Pseudomonas aeruginosa

P. aeruginosa is a gram negative rod capable of aerobic respiration and can not grow anaerobically except in the presence of nitrate. The bacteria are opportunistic pathogens which mean that they can and will infect most part of the body if the growth conditions allow it. Among other diseases it can cause skin infections, burns, pneumonia, urinary tract infections and septicaemia. They spread by contact either directly or indirectly from moist areas where they like to grow [17].

Staphylococcus aureus

S. aureus are gram positive cocci which are capable of both aerobic and anaerobic respiration. Their natural occurrence in humans is on the skin, in the nose and perineum. They are the common cause of several skin infections and this result in an intense inflammatory response. The bacteria are spread by contact with another person or it could

also be spread by airborne routes. Among other serious infections they are capable of causing urinary tract infections, bacteremia and lower respiratory tract infections [17].

Methicillin-resistant *Staphylococcus aureus*

MRSA are gram positive cocci which are resistant to methicillin and beta-lactame antibiotics. They produce beta-lactamases, enzymes that break down the beta-lactam ring, and the antibiotic effect is destroyed. Because of the resistance, the infections caused by this strain (they cause the same infections as *S. aureus*) is harder to treat [17].

1.8.1 Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration (MIC) is a technique which is used to determine the lowest concentration of different antibacterial agents necessary to inhibit visible growth of microorganisms after overnight incubation. MIC tests are used to confirm resistance, and are used as a tool to determine the activity of new antibacterial agents [17, 18].

A known concentration of bacteria is inoculated with different concentrations of the desired antibiotic agent. Visible clouding indicates that the antibacterial agent has not worked and there is a visible growth of bacteria. Such clouding will not take place if the antimicrobial agent is successful in inhibiting or killing the bacteria. The MIC value is determined by the lowest concentrations of the antibiotic (μg/ml) where no bacterial growth is visible.

1.9 High Performance Liquid Chromatography

HPLC is a chromatographic technique which separates non-volatile mixtures into their individual components. The principle is that the sample components are carried by a mobile phase through a column that contains a stationary phase, where they separate and elute one by one at the end of the column. They immediately enter a detector where they are measured. The recorded peaks are displayed on a chart called a chromatogram. The peak area indicates the quantity of a component, and the retention time, the time it takes the substance to get through the column, can give an indication of the components

identity, although this indication should not be trusted completely as several different compounds might have the same or similar retention times on the same HPLC-system.

1.10 Reversed phase columns

There are several different HPLC columns in use which are used to separate substances within a wide polarity and mass range. The most widely used are the so-called reversed phase columns where the sorbent in the columns are hydrophobic and the substances will undergo hydrophobic interactions with the stationary phase. These interactions diminish if you change to a column with shorter hydrophobic groups on the stationary phase.
The mobile phase used in reversed phase HPLC is an aqueous buffer solution mixed with organic solvents such as methanol or acetonitrile. The organic solvent must be miscible with water.
UPLC which is short for Ultra Performance Liquid Chromatography is a technique based on the same principle as HPLC, but the speed, sensitivity and the resolution are increased. The heightening of all of these is due to one parameter, decreased particle size. This is an improvement of the technique and is very useful.

1.11 Mass spectrometry

Mass spectrometry is a technique where the molecular mass of compounds is measured. The compounds undergo ionization and then they are separated according to their mass to charge ratio (*m/z*) in a mass separator. The amounts of ions are counted in the detector.
In this thesis liquid chromatography-mass spectrometry with electrospray ionization (ESI) was used with time-of-flight (TOF) and quadrupole (Q) mass separators.

1.12 Liquid Chromatography Mass Spectrometry

LC/MS, also called HPLC/MS, is an analytical technique used worldwide, which combines liquid chromatography (separation technique) and mass spectrometry (analytical technique). Very basically put the former technique is highly efficient when it comes to separating non-volatile mixtures into their component substances, without being able to identify them, and the latter, as explained above, can identify the single substances, but have some limitations when it comes to dealing with mixtures. However, some modern MS-instruments can even separate two ions with exactly the same mass from each other based on differences in the shape of the molecules. The combination of these two techniques gives almost unlimited possibilities when it comes to separating complex mixtures and then identifying the individual substances.

Electrospray ionization (ESI) is a soft ionization method, meaning it mostly produces pseudo-molecular ions without fragmenting them further, and the method takes place under atmospheric pressure. ESI can be used to ionize large or small compounds, including proteins, peptides and many polar substances. The basic principle behind electrospray ionization is that the sample solution passes through a stainless steel capillary connected to a high voltage power supply and with the help of heated nitrogen gas, an aerosol consisting of many charged droplets is formed. A nebulizer gas (i.e. nitrogen) will evaporate neutral solvent molecules from the surface of the charged droplets, leading to reduced distance between the charges. This in turn will lead to repulsion between the same charges. After a while the surface tension of the liquid can no longer compete with the Coulomb repulsion, also known as Coulomb explosion, within the droplets, and leads to disintegration of the droplets. These explosions will continue to happen until most of the solvent is evaporated and only ions remain in their gaseous phase ready to enter a mass separator of choice [19].

The principle behind the time-of-flight (TOF) mass filter is that molecules with identical kinetic energies will travel at different speed according to their masses. Molecules that possess higher masses will travel at a lower speed, and use more time to reach the detector. Ions are pushed by an electrical pulse through a field-free linear flight tube. The flight time, the time an ion uses to reach the detector, is used to calculate the *m/z*. The TOF instruments have some limitations, and the major one is that they have a limited

dynamic range, meaning that the detector, after registering an ion, needs time to reset itself (deadtime about 5 nanoseconds). If there is a high number concentration of ions arriving at the detector, some will remain undetected during the dead time. Another limitation is that small differences in kinetic energy of ions with identical *m/z* values will compromise the resolution of the instrument. This problem can be solved by using reflectrons which evens out the differences in kinetic energy, because the ions with high kinetic energy will penetrate deeper and thus spend more time in the reflectron and vice versa. Use of several reflectrons will improve the resolution even further. Another aspect influencing the resolution is that the ions are located at different distances to the pusher. The latter issue can be resolved by focusing the ions to a narrow beam by electrical lenses so that the ions undergo orthogonal acceleration.

The main advantages of TOF (figure 5) are unlimited mass range, high resolution, fast and efficient analyses and the small amount of sample required for detection. The elemental composition of the analyte is calculated based on the accurate mass and the isotopic distribution. The accuracy of the mass measurements can be improved by using internal mass calibration which means performing reference scans throughout the analysis. The ion source has two probes, a reference and an analyte probe. A reference scan is performed every 10.-15. seconds to correct the analyte scans [20].

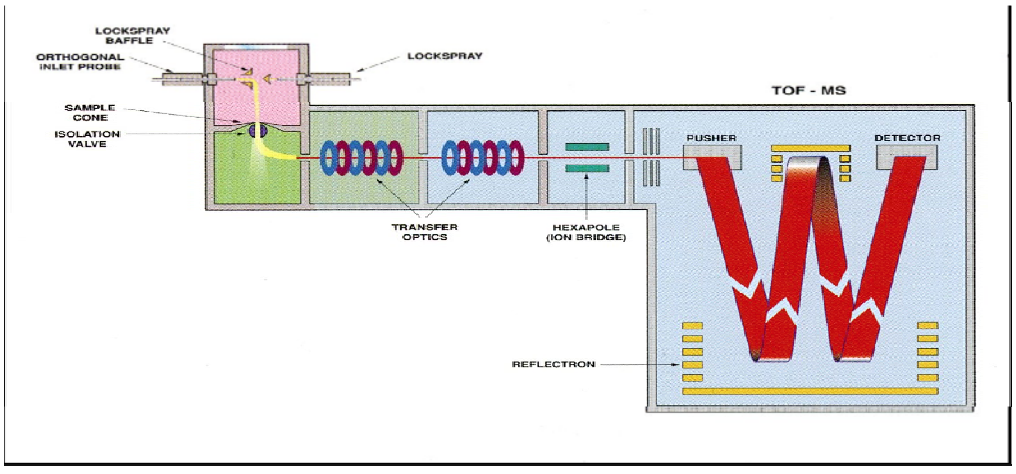

Figure 5: Schematic drawing of ESI-TOF-MS with double reflectors (www.waters.com).

As the name indicates the quadrupole is a mass analyser made up by four cylindrical rods, which are paired off diagonally in their charge. An alternating current is applied between the electrically connected pairs, and in addition a direct current voltage is applied at the top of the alternating current. This causes the charge to alternate between positive and negative with very high frequency and in this way ions are guided through the axis of the quadrupole. Adjusting the currents to specific values allow the selection of specific ions with a certain *m/z* ratio to pass through the quadrupoles. This can be used to select one specific ion or scan a wide range of ions. Thus this is useful in situation where you are looking for known compounds and test samples with unknown content. A drawback is that the quadrupole looses some sensitivity when scanning for a wide range of ions, but then again it is advantageous when looking for one specific compound or in quantitative analyses. The quadrupoles are also the most common mass separators used for LC-MS ESI-QqQ instruments (figure 6). The main drawback for the quadrupole mass analysers is that they have low mass resolution.

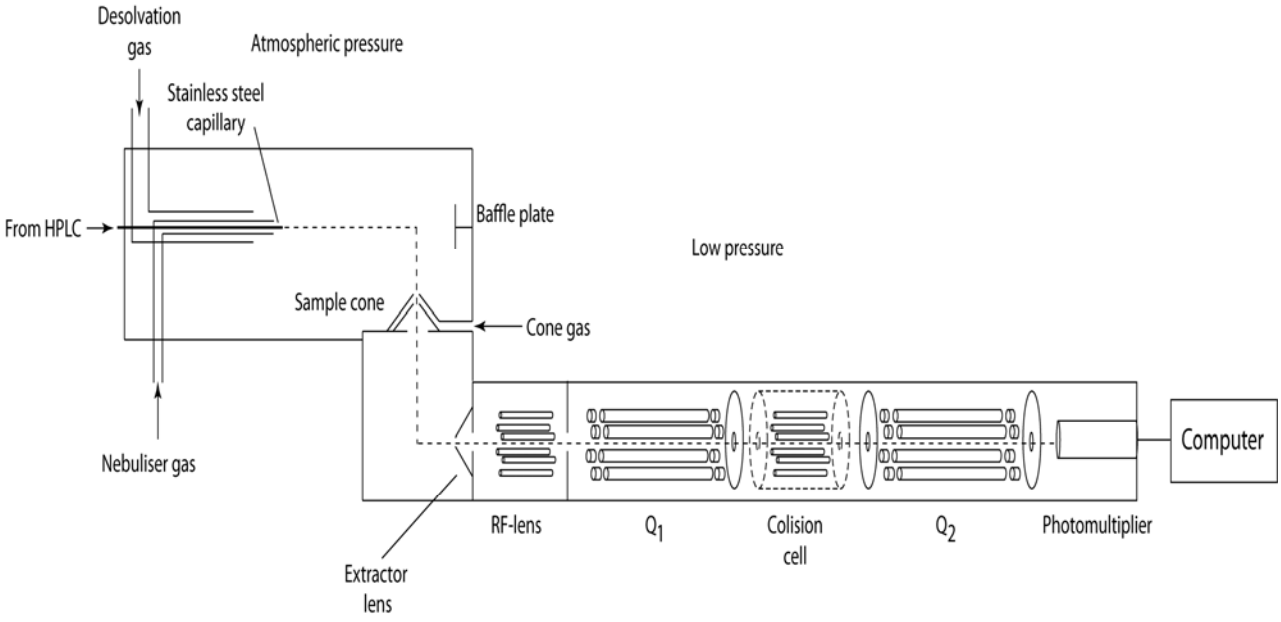

Figure 6: Schematic drawing of LC-MS ESI-QqQ. Used with permission from Terje Vasskog.

1.13 The aim of the thesis

The aim of this master thesis was to investigate antibacterial activity of bioactive compounds in extracts from *H. pyriformis.* In addition two other organisms named *Metridium senile* and *Parastichopus tremulus* were screened for antibacterial activity.
Two different kinds of extractions were performed, one with water and the other one with a mixture of DCM and MeOH at a 1:1 ratio. This was done to get a wider range of compounds extracted that was subjected to screening. The antibacterial activity tested against five different bacterial strains included both Gram-positive and Gram-negative bacteria. Finally it was attempted to purify and identify known bioactive compounds searching in databases that could contain information about these compounds.

2 Material and methods

2.1 Sampling and storage

Halocynthia pyriformis, identification number M09038, was collected in Porsangerfjorden May 29th 2009 at 20 meters depth by divers. The marine organism was stored at -20 °C until processing.

2.2 Freeze-drying

2.2.1 Materials

IKA A11 Basic	IKA Works; Staufen, Germany
Freeze drier, Heto PowerDry PL 9000	Thermo Waltman, USA

2.2.2 Method

The frozen sample was cut in pieces of approximately 1 cm^3 on a wooden trey and placed in glass containers. After the sample was weighed, it was immediately stored at -20 °C. The sample was freeze-dried for three days and then crushed and pulverized, and transferred to Duran bottles and weighed.

The extractions were performed with two different methods, one for the aqueous extract and the other for the organic extract as described in the next chapter.

2.3 Extraction and HPLC fractionation of aqueous extracts

2.3.1 Materials

Rotary evaporator, Laborata 4002	Heidolph Nürnberg, Germany
Freeze drier, Heto PowerDry PL 9000	Thermo Waltham, USA
22 µm Millex GS filter	Millipore Billerica MA, USA
Shaker Heidolph Titramax	Heidolph Nürnberg, Germany

Waters 600 Controller	Waters MA, USA
Waters 2996 Photodiode Array Detector	Waters MA, USA
Atlantis Prep C-18 (10x250 mm; 10 µm)	Waters MA, USA
MassLynx v.4.1 software	Micromass Manchester, UK
Waters 600E Multisolvent Delivery System	Waters MA, USA
Waters Prep Degasser	Waters MA, USA
Waters 2767 Sample Manager	Waters MA, USA
Milli-Q Water	Millipore Billerica MA, USA
Dichloromethane	Merck Darmstadt, Germany
Methanol	Merck Darmstadt, Germany
Ethanol	Merck Darmstadt, Germany
Acetonitrile	Merck Darmstadt, Germany
Hexane	Merck Darmstadt, Germany
Formic acid	Merck Darmstadt, Germany

2.3.2 Method

The Duran bottle containing the freeze-dried material was added Milli-Q water approximately ten times its dry weight. The total amount of Milli-Q water added was 860 mL, but it was added in smaller amounts such as 250, 210, 150, 250 mL to the freeze-dried material and the suspension was shaken vigorously between each step. The suspension was shaken once more before it was placed in the refrigerator at 5 °C over night.

The suspension was shaken once more the next day before it was divided in two, and transferred to two centrifugation bottles and centrifuged for 30 minutes at 4000 rpm at 5 °C. The supernatant was decanted and stored, while the pellet was re-extracted with 430 ml Milli-Q water (approximately half of what was added the first time) and shaken vigorously before it was placed in the refrigerator at 5 °C for 30 minutes. The suspension was centrifuged once again at 5 °C for 30 minutes. The two supernatants were pooled and freezed at -20 °C. This was done to reduce the time of freeze-drying. Afterwards they were freeze-dried for three days. The pellet was stored in the freezer at -80 °C.

The freeze-dried extracts were pulverized and stored in centrifugation tubes at -20°C after being weighed. Afterwards, 211.8 mg of the freeze-dried extract was transferred to a 13 mL polystyrene centrifugation tube and 2 mL Milli-Q water was added. This was mixed for 90 minutes at 100 rpm. Then 1 mL of the aqueous phase was transferred to a new centrifugation tube and 4 mL 96% ethanol was added, the mixture was shaken on a mini shaker and kept over night at -20 °C.

The sample was taken out of the freezer and kept at room temperature for 5-10 minutes, and centrifuged at 5 °C for 30 minutes at 4000 rpm. The supernatant was transferred to a 25 mL rotary evaporation flask and vacuum evaporated at 40 °C down to approximately 2 mL at 70 mbar pressure. The sample was divided on two 1 mL centrifugation tubes and the volume adjusted to 1 mL with Milli-Q water. Both tubes were centrifuged for 30 minutes at 13000 rpm. The supernatants were filtered through 0.22 µm filters into test tubes, mixed and then adjusted to 2x1 mL with Milli-Q water.

The aqueous extracts were ready to be fractionated and two injections of the extract were injected. The flow rate was 6 ml/min and the eluent was collected over a time span of 40 minutes resulting in 2x40 identical fractions. The mobile phase gradient is shown in table 1.

Table 1: Mobile phase gradient of the aqueous extract M09038

Flow (ml/min)	Tid (min)	MP A %	MP B%	Curve
6	0	95	5	-
6	3	95	5	6
6	30	50	50	6
6	35	5	95	6
6	40	5	95	6
6	41	95	5	6
6	50	95	5	6

2.4 Extraction and HPLC fractionation of organic extracts

2.4.1 Materials

Rotary evaporator Laborata 4002	Heidolph Nürnberg, Germany
FreezHeto Powerdry PL9000	Thermo Waltman, USA
Centrifuge Multifuge 3 S-R Heraeus	Heraeus Instruments Newport Pagnell, England
Whatman no 3 filter	Whatman Maidstone, England
Millex GS filter	Millipore Billerica MA, USA
Waters 600 Controller	Waters MA, USA
Waters 2996 Photodiode Array Detector	Waters MA, USA
XTerra® Prep RP_{18} (10x300 mm; 10 mm)	Waters MA, USA
MassLynx v.4.1 software	Micromass Manchester, UK
Waters 600E Multisolvent Delivery System	Waters MA, USA
Waters Prep Degasser	Waters MA, USA
Waters 2767 Sample Manager	Waters MA, USA
Milli-Q Water	Millipore Billerica MA, USA
Dichloromethane	Merck Darmstadt, Germany
Methanol	Merck Darmstadt, Germany
Acetonitrile	Merck Darmstadt, Germany
Hexane	Merck Darmstadt, Germany
Ethanol 96 %	Merck Darmstadt, Germany
Formic acid	Merck Darmstadt, Germany

2.4.2 Method

The remaining pellet after the aqueous extraction had been stored in the freezer, and was now freeze-dried and pulverized before it was transferred to a Duran bottle. The pulverized material was added a mixture of DCM and MeOH (1:1) at a 10:1 ratio. About

200 mL of this mixture was added to the sample in two rounds. The sample was shaken vigorously and the suspension was left over night at 5 °C.

The suspension was vacuum filtered, and the extraction was repeated and the suspension was stored at 5 °C for 30 minutes. The filtrate was vacuum evaporated using a rotary evaporator. This was done at 40 °C and atmospheric pressure (1000 mbar) and the pressure was gradually reduced to 180 mbar. A viscous liquid of approximately 10-20 mL remained in the evaporation flask. This was transferred to 3 test tubes and stored in the freezer (-20 °C).

The test tube containing the organic extract was stored at room temperature for a couple of minutes. Approximately 100-300 mg was weighed and transferred to a test tube of 13 mm diameter and 3 mL hexane and 3 mL of 90 % ACN were subsequently added. The mixture was shaken vigorously between each addition. The test tube was centrifuged at 300 rpm for 3 minutes, and the ACN phase was transferred to an evaporation flask.

Another 3 mL of 90 % ACN was added to the hexane phase and stirred vigorously. The centrifugation was repeated and the two ACN phases were pooled in the same evaporation flask. All of this was done in two parallels. The pooled ACN phases were vacuum evaporated at 40 °C. The pressure was gradually reduced to 180-150 mbar. The remaining 2 mL were divided on two centrifugation tubes and the volume was adjusted to 1 mL in each tube, and then stored at -20 °C.

The organic extracts were left at room temperature and centrifuged at 13000 rpm for 30 minutes. The liquid phase in both were pooled and transferred to one tube. The volume was divided between two test tubes and adjusted to 1 mL with 90 % ACN, and they were ready to be fractionated. Two injections of the extract were injected. The flow rate was 6 mL/min and the eluent was collected in one-minute fractions over a time span of 40 minutes, resulting in 2x40 identical fractions. The mobile phase gradient used is shown in table 2.

Table 2: Mobile phase gradient for the organic extract M09038

Flow (ml/min)	Tid (min)	MP A %	MP B%	Curve
6	0	80	20	-
6	2	80	20	6
6	30	0	100	6
6	40	0	100	6
6	41	80	20	6
6	50	80	20	6

2.5 Re-fractionation of active fractions

2.5.1 Materials

Waters 600E Multisolvent Delivery System	Waters MA, USA
Waters Prep Degasser	Waters MA, USA
2767 Sample Manager	Waters MA, USA
Waters 2996 Photodiode Array Detector	Waters MA, USA
Symmetry prep C18 7 µm; 7.8x300 mm	Waters MA, USA
MassLynx v.4.1 software	Micromass Manchester, UK
MeOH	Merck Darmstadt, Germany
Formic acid	Merck Darmstadt, Germany
Milli-Q water	Millipore Billerica MA, USA
ACN	Merck Darmstadt, Germany

2.5.2 Method

The aqueous fractions from M09038-0-W01 did not display any clear activity and was not re-fractionated. Active fractions from the second screening were subject to re-fractionation. Fraction no 24, 25 and 26 from M09038-0-L01 (three deep well plates) were pooled and dissolved in 200 µL MeOH 80 % and shaken for approximately one hour, and then transferred to test tubes. Another 100 µL MeOH 80 % was added to the same wells and shaken for approximately 30 minutes and transferred to the same test tube. The test tube was vacuum centrifuged for one hour. The volume was 475 µL.

Approximately 100 µL pure ACN was added to the test tube. The volume injected into the HPLC column was 575 µL. This was to ensure that the lipid soluble substances would dissolve and not make aggregates with water. The flow rate was 4 mL/min. The mobile phase gradient used is shown in table 3. Afterwards the 40 fractions were divided on three deep well plates. The three plates were vacuum centrifuged for 1.5 hours, frozen at -20 °C and freeze-dried for two days.

Table 3: Mobile phase gradient for the re-fractionated fractions

Flow (ml/min)	Tid (min)	MP A %	MP B%	Curve
4	0	30	70	-
4	30	0	100	6
4	40	0	100	6

2.6 New Primary fractionation

Based on the poor dose-response results it was decided to fractionate the organic extract M09038 once again into 40 fractions by a different HPLC machine than the one used earlier. The procedure was the same and the gradient used is shown in table 2. The column used was Xbridge™ Prep C18 5 µm 10x250 mm. This time the fractions were more concentrated before they were subjected to new primary screening and ESI-MS.
The two other marine organisms labelled M09036-0-L01 and M09037-0-L01 underwent the same sample preparation as M09038-0-L01. The marine organism M09037-0-L01 was not subjected to new primary screening.

2.7 Solubilisation of HPLC fractions

Organic fractions were dissolved in 7.5 µL DMSO and shaken at 400 rounds per minute (rpm) for two hours. Each fraction was added 750 µL autoclaved Milli-Q water before shaking them for another 30 minutes. Another round of 750 µL autoclaved Milli-Q water was added to the fractions and they were once again shaken for 30 minutes.

Aqueous fractions were dissolved in 750 µL autoclaved Milli-Q water and shaken as above for 30 minutes before adding another round of 750 µl autoclaved Milli-Q water followed by two hours of shaking.

2.8 Antibacterial screening

2.8.1 Materials

Microtiter plate	Nunc Roskilde, Denmark
Shakeincubator – Heidolph Incubator 1000	Heidolph Nürnberg, Germany
Blood plates	Media kitchen, UNN
Iso-Sensitest-Agar plates (ISA)	Media kitchen, UNN
Victor 3 TM 1420 Multilabel Counter	Perkin Elmer Instruments; Shelton, USA
Brain Heart Infusion (BHI)	Oxoid, England
Mueller Hinton II (MH)	Dickinson and Company, France
DMSO	Sigma Aldrich München, Germany
Autoclaved Milli-Q water	Millipore Billerica MA, USA
Autoclaved NaCl	Merck

2.8.2 Method

The bacterial strains used in the antibacterial screening were *E. faecalis, E. coli, P. aeruginosa and MRSA.* Each bacterial strain was plated out on separate blood plates. These were incubated in the refrigerator at 5 ºC for 3 days before they were used.

Day 1: Cultivation of bacteria

The bacterial strains were inoculated in 8 mL cultivation media table and incubated at 37 ºC over night.

Day 2: Experimental procedure

From the bacterial strains from the previous day 2 mL was transferred to 25 mL of fresh media for enrichment in order to achieve exponential growth. The bacterial suspensions were incubated at 37 ºC and shaken for 1.5 to 2.5 hours depending on the time needed to enter the logarithmic phase of the growth curve as seen in table 4. The bacterial strains were cultivated until their growth reached a turbidity of 0.5 McFarland standard ($1.0x10^8$ bacteria/mL).

Table 4: Bacteria, cultivation media, incubation period and bacterial density

Bacteria	Cultivation media	Inc. period	Density of bacteria
E. faecalis	BHI	1,5 h	0,5-3x10 CFU/ml
E. coli	MH	1,5 h	0,5-3x10 CFU/ml
P. aeruginosa	MH	2,5 h	3-7x10 CFU/ml
S. aureus	MH	2,5 h	0,5-3x10 CFU/ml
MRSA	MH	2,5 h	0,5-3x10 CFU/ml

For each bacterial strain, 50 µL HPLC fraction and 50 µL bacterial suspensions were added to a 96-well microtiter plate as shown in figure 4. The bacterial suspensions were diluted 1:100 and finally 1:10 in cultivation media before adding 50 µL to each fraction. This has to happen within 30 minutes to maintain viable cell density. The plates were incubated at 37º C over night.

N	1	1	9	9	17	17	25	25	33	33	P
N	2	2	10	10	18	18	26	26	34	34	P
N	3	3	11	11	19	19	27	27	35	35	P
N	4	4	12	12	20	20	28	28	36	36	P
N	5	5	13	13	21	21	29	29	37	37	P
N	6	6	14	14	22	22	30	30	38	38	P
N	7	7	15	15	23	23	31	31	39	39	P
N	8	8	16	16	24	24	32	32	40	40	P

Figure 4: HPLC fractions added to the assay plate in parallels. N (negative control) and P (positive control).

The negative control consisted of 50 µL cultivation media + 50 µL autoclaved Milli-Q water and was added to column number 1. The positive control consisted of 50 µL autoclaved Milli-Q water + 50 µL bacterial suspension and was added to column number 12. The antibacterial agent gentamicin was used as control for the setup and precision

between the tests (MIC). A series of dilution were made with the following concentrations of gentamicin: 30µg/mL- 20 µg/mL - 16µg/mL - 8 µg/mL - 4µg/mL - 2µg/mL - 1µg/mL - 0.5µg/mL – 0.25µg/mL – 0.12 µg/mL – 0.06µg/mL – 0.03µg/mL. It was not made a series of dilution of the HPLC fractions because the concentration of potential antimicrobial compounds is unknown.

To estimate the number of bacteria the suspensions had to be diluted to approximately $1x10^2$-$1x10^3$ bacteria/mL. The suspensions were diluted with 0.9 % NaCl in the following manner: 1) 1:100, 2) 1:100, 3) 1:10, 4) 1:10. 100 µL from dilution number 4 was plated out on ISA plates in two parallels and incubated at 37 ºC over night.

Day 3: Results

Visible clouding indicates bacterial growth and inactive (I) fractions. Wells that do not show visible clouding indicate inhibition of bacterial growth and active (A) fractions. The assay plates were subject to a photometric reading at optical density (OD) at 600 nm. The photometric values for the active (A), questionable (Q), or inactive (I) fractions were defined as following with an absorbance at: A <0.05 Q 0.05-0.09 and I >0.09

Active fractions from the first screening were subject to a secondary screening also called a re-test. This was done to confirm the fractions as active and to find dose-response relationship in 1:1, 1:2 and 1:4 dilutions as shown in figure. If the fractions were confirmed as active in this second screening, a third screening was performed on re-fractionated fractions. The method used during the second and the third screening was the same as the first screening.

N	A (U)	A (1:2)	A (1:4)	E (U)	E (1:2)	E (1:4)	I (U)	I (1:2)	I (1:4)		P
N	A (U)	A (1:2)	A (1:4)	E (U)	E (1:2)	E (1:4)	I (U)	I (1:2)	I (1:4)		P
N	B (U)	B (1:2)	B (1:4)	F (U)	F (1:2)	F (1:4)	J (U)	J (1:2)	J (1:4)		P
N	B (U)	B (1:2)	B (1:4)	F (U)	F (1:2)	F (1:4)	J (U)	J (1:2)	J (1:4)		P
N	C (U)	C (1:2)	C (1:4)	G (U)	G (1:2)	G (1:4)	K (U)	K (1:2)	K (1:4)		P
N	C (U)	C (1:2)	C (1:4)	G (U)	G (1:2)	G (1:4)	K (U)	K (1:2)	K (1:4)		P
N	D (U)	D (1:2)	D (1:4)	H (U)	H (1:2)	H (1:4)	L (U)	L (1:2)	L (1:4)		P
N	D (U)	D (1:2)	D (1:4)	H (U)	H (1:2)	H (1:4)	L (U)	L (1:2)	L (1:4)		P

Figure 5: HPLC fractions added to assay plate in U (undiluted), 1:1, 1:2 and 1:4 dilutions in the secondary screening. The letters represents the different fractions.

2.9 Dose-response of organic extract

A dose-response test was carried out to calculate how potent the organic raw-extract of M09038 was.

The organic extract M09038-0-L01 was taken out of the freezer and left at room temperature. About 500 mg was transferred to a 13 mm diameter test tube and then 3 mL hexane and 3 mL 90 % ACN was added. The test tube was shaken between each addition. The mixture was centrifuged at 300 rpm for three minutes. This was performed in two parallels. The lower phase (ACN) was transferred to a new test tube. The upper phase (hexane) was added 3 ml 90 % ACN and shaken before centrifuged for further three minutes. The test tube containing the ACN phase was vacuum centrifuged for a couple of hours until it was dry.

The dried sample was dissolved in 200 µL DMSO and shaken on a mini shaker until it was fully dissolved. Later a series of dilutions were made. The concentrations of the dilutions were: 500-250-100-50-25-12.5-10-5-2.5-1-0.5-0 µg/mL. The dilutions were further subjected to antibacterial screening.

2.10 LC-MS analyses

2.10.1 Materials

Waters Quattro Premier XE MS	Waters MA, USA
Waters Nano Acquity UPLC	Waters MA, USA
Waters Acquity UPLC BEH C18, 1.0x150mm, 1.7µm	Waters MA, USA
Waters Micromass LCT Premier	Waters MA, USA
XTerra ® MS C18 1.0x50mm; 3.5 µm	Waters MA, USA
MassLynx v.4.1 software	Micromass Manchester, UK
Acetonitrile	Merck Darmstad, Germany
Milli-Q water	Millipore Billerica MA, USA
Formic acid	Merck Darmstadt, Germany

2.10.2 Method

LC-MS analyses were performed using two different mass separators, Time-Of-Flight and Quadrupole, both equipped with an electrospray ion source. In addition to the active fractions, some fractions that did not display any antibacterial activity was also subjected to MS analyses to check if it was possible to find the same substances that might be found in the active fractions.

The active fraction 15 from M09038-0-L01-2426 and active fractions 30, 31 and 33 from the new primary screening were subjected to ESI-TOF and ESI-Q analyses. The inactive fractions tested were 14 and 16 from M09038-0-L01-2426 and 28, 29, 32, 34 and 35 from M09038-0-L01 was also tested. The conditions for MS-analyses on ESI-TOF and ESI-Q are listed in table 5. The mobile phase gradient used for ESI-TOF and ESI-Q are listed in tables 6 and 7.

Table 5: Conditions for MS-analyses for the organic extract M09038

Conditions	ESI-TOF	ESI-Q
Cappilary (kV)	2.6	3.3
Cone (V)	35	50
Temperature desolvation (ºC)	300	250
Temperature source (ºC)	120	100
Cone gas flow (L/hr)	5	11
Desolvation gas flow (L/hr)	550	98
Mass range	100-1500	50-1000

Table 6: Gradient used in ESI-TOF

Flow (µL/min)	Tid (min)	MP A %	MP B%	Curve
200	0	95	5	-
200	9	30	70	6
200	11	5	95	6
200	19	5	95	6
200	20	95	5	6

Table 7: Gradient used in ESI-Q

Flow (µL/min)	Tid (min)	MP A %	MP B%	Curve
50	0	100	0	-
50	45	0	100	6
50	46	100	0	6
50	55	100	0	6

3 Results

The main focus of this thesis was antibacterial activity of the marine organism *Halocynthia pyriformis,* with identification number M09038. In parallel to this two other organisms were studied in other projects which focused on antiviral and anticancer activities. These other organisms were also included in the antibacterial screening and their respective data is presented in this thesis. All three organisms are listed in table 8 with their individual identification number. In the following text the results of these are presented in the tables but not commented further in sampling and storage, but their antibacterial activity is addressed in the screening results.

Table 8: The identification numbers and the species these numbers represent.

Identification number	Marine organisms
a) M09036	*Metridium senile*
b) M09037	*Parastichopus tremulus*
c) M09038	*Halocynthia pyriformis*

3.1 Sampling and storage

The marine organism was weighed and cut in pieces before it was freeze-dried. The total weight of the organism was 954.73 g and the dry weight after freeze-drying was 104.26 g. Table 9 shows the total weight of the organism and the dry weight after it was freeze-dried. The dry weight is also shown as percentage of the total weight.

Table 9: Total weight and dry weight of the marine organisms. Dry weight is the weight of the samples after freeze-drying. Dry weight is also shown as the percentage of the total weight.

Extracts	Total weight (g)	Dry weight (g)	Dry weight (%)
M09036	1500	152.54	10.2
M09037	995	87.31	8.8
M09038	954.73	104.26	10.9

The freeze-dried material was subjected to an aqueous extraction followed by an organic extraction. This resulted in an aqueous and an organic extract which was freeze-dried, and weighed. Table 10 shows the weight of the freeze-dried material that resulted from the extractions.

Table 10: The weight of the freeze-dried extracts after the aqueous and organic extractions.

Extracts	Aqueous (g)	Organic (g)
M09036	45.37	75.41
M09037	31.91	51.76
M09038	*	52.33

***** Data missing.

3.2 UV chromatograms of the aqueous and the organic fractions

UV-chromatograms of the aqueous and the organic fractions from all 3 marine organisms were obtained after the HPLC purification.

The UV chromatogram of the aqueous fractions of M09036-0-W01 (figure 7) showed the injection top after about two minutes that lasted about a minute. In the following 10 minutes there were a few smaller peaks and then nothing much until about 38 to 40 minutes. In the UV chromatogram of the aqueous fractions of M09037-0-W01 (figure 7) showed the injection top and then the curve leveled off. The chromatogram of M09038-0-W01 (figure 7) showed an injection top followed by a couple of peaks in the following 10 minutes.

After the injection top there were some smaller peaks detected between the retention times of 20 to 30 minutes in the UV chromatogram of the organic fractions of M09036-0-L01 (figure 8). The UV chromatogram of M09037-0-L01 (figure 8) showed an injection peak and two smaller peaks the retention time of 25 to 27 minutes. In addition to the standard injection peak the UV chromatogram of M09038-0-L01 (figure 8) a couple of peaks were detected with between retention times between 15 to 32 minutes.

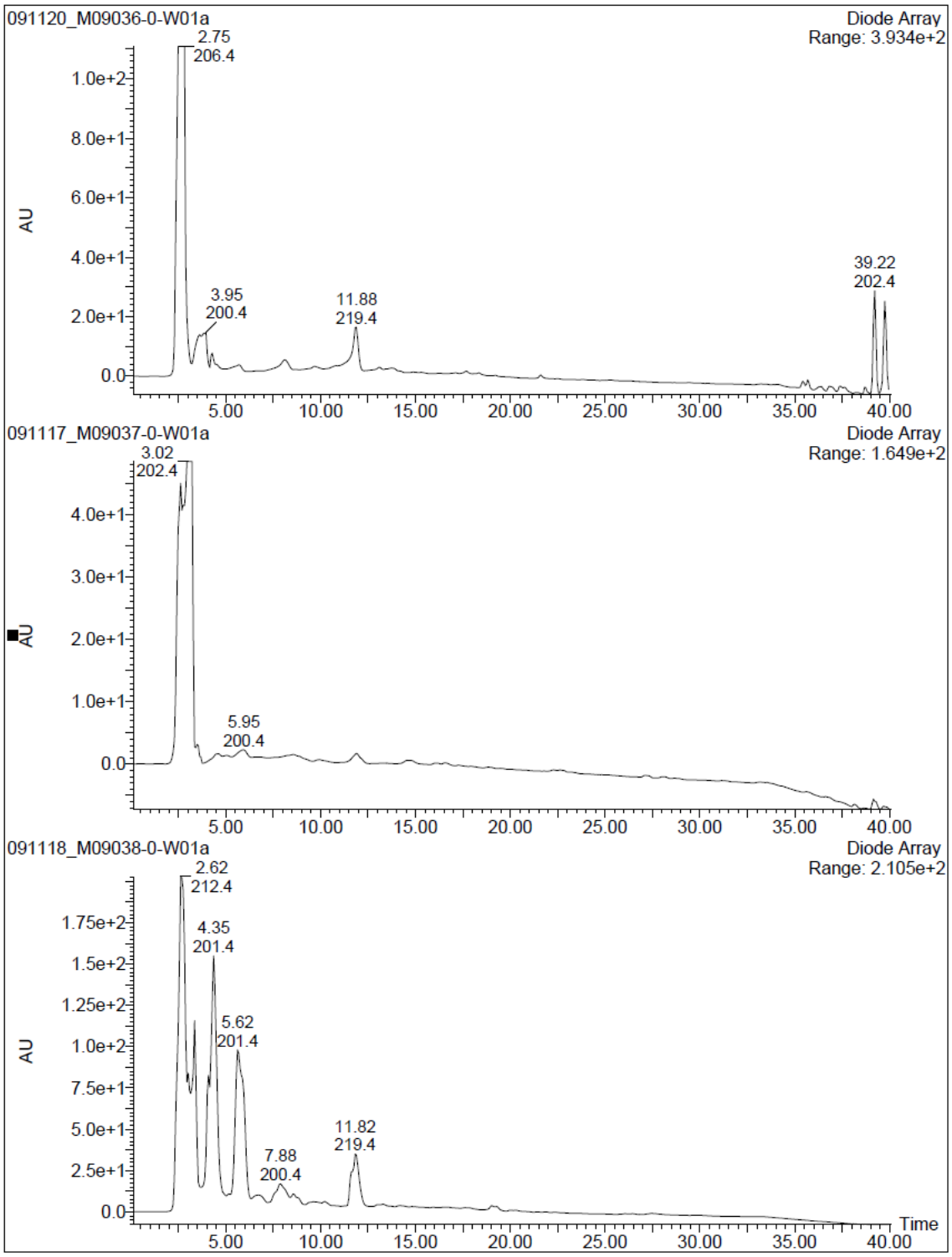

Figure 7: The UV-chromatograms of the aqueous fractions in the following order M09036-0-W01 (top), M09037-0-W01 (middle) and M09038-0-W01 (bottom) are shown in this figure.

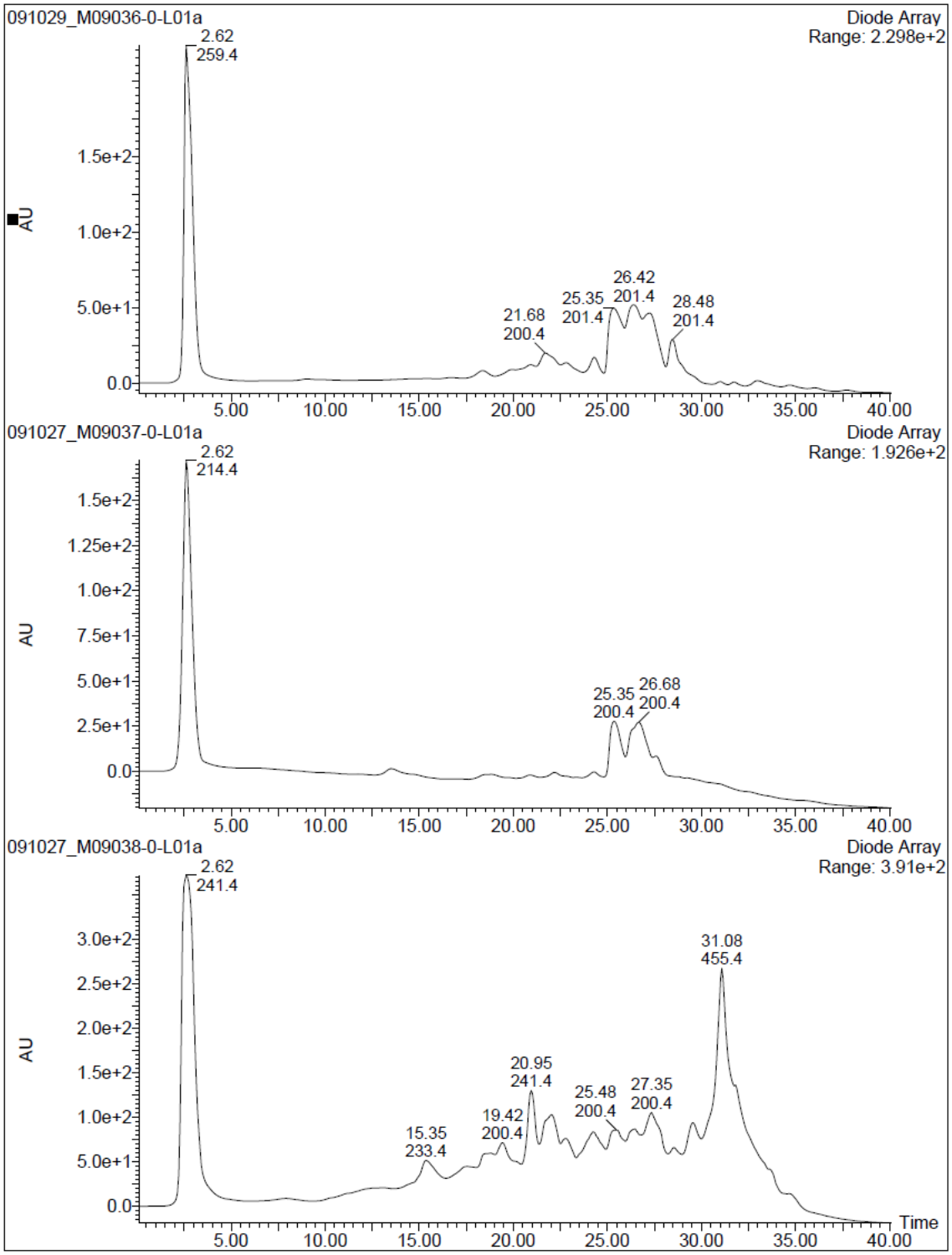

Figure 8: The UV-chromatograms of the organic fractions in the following order M09036-0-L01 (top), M09037-0-L01 (middle) and M09038-0-L01 (bottom) are shown in this figure.

3.3 Antibacterial screening

The aqueous and the organic extracts were fractionated into 40 fractions each by HPLC. These fractions were screened for antibacterial activity against five bacterial strains named *E. faecalis, E. coli, P. aeruginosa, S. aureus and* MRSA. In any fraction where there is a drop in the OD, there is some degree of antibacterial activity. Because of time constraints and the need for high throughput there has been defined certain limits that defines the activity as interesting to study further. When the OD values were below 0.05 for the fractions they were defined as active. Values between 0.05 and 0.09 were considered as borderline cases which showed some activity. Values above 0.09 were considered inactive. The OD was measured at 600 nm.

3.4 Primary screening of the aqueous and organic HPLC fractions

3.4.1 Aqueous HPLC fractions

Fraction 38 from M09036-0-W01 (figure 9a) displayed activity against the bacterial strain *E. faecalis*, and some activity against MRSA and *S. aureus.* Fraction 38 was tested in the secondary screening to confirm the antibacterial activity. The aqueous fractions from M09037-0-W01 (figure 9b) and M09038-0-W01 (figure 9c) did not show activity against any of the five bacterial strains.

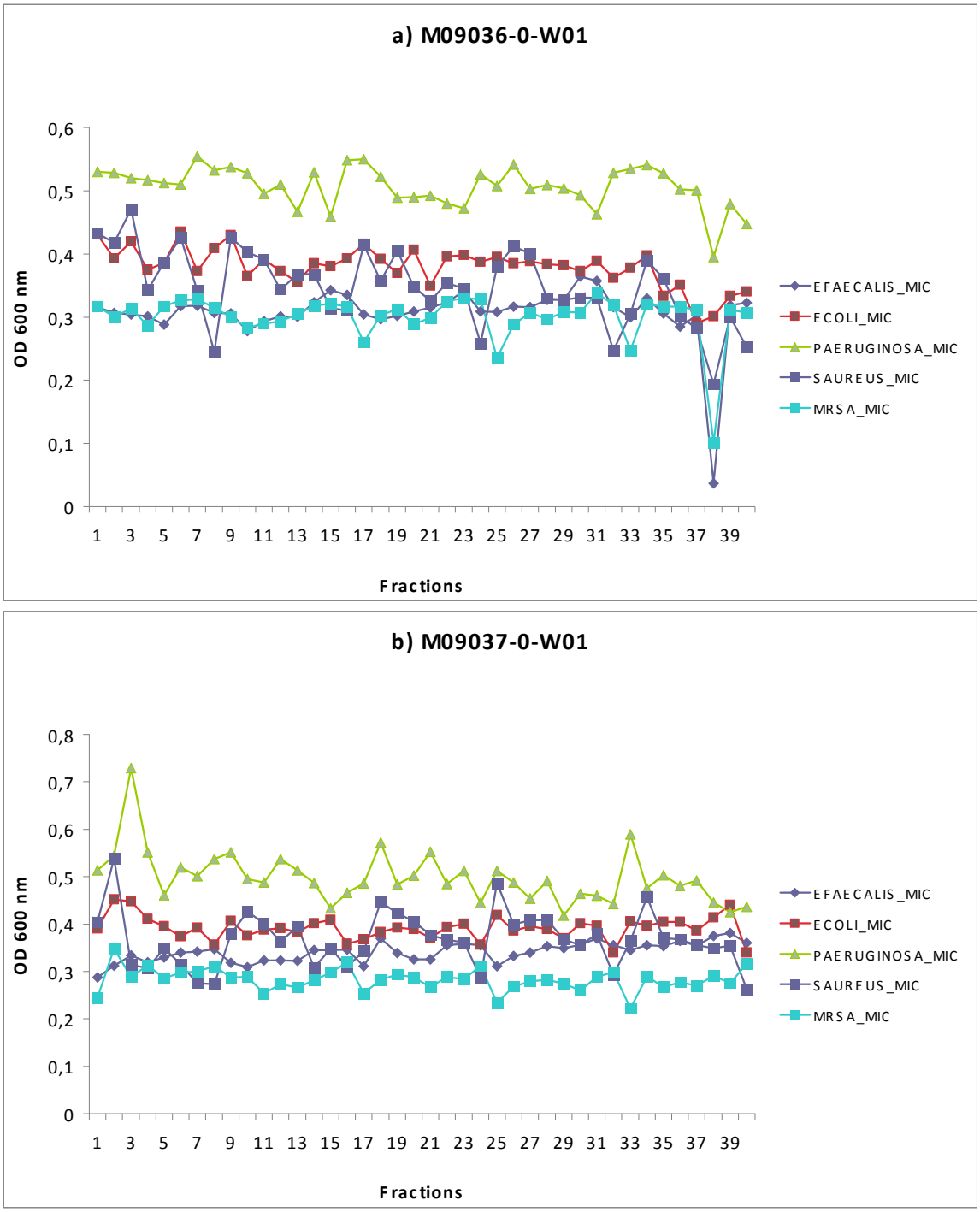

Figure 9: Primary screening: antibacterial activity of the aqueous extracts a) M09036-0-W01, b) M09037-0-W01 and c) M09038-0-W01, against five bacterial strains (*E. faecalis, E. coli, P. aeruginosa, S. aureus and MRSA*). If the OD at 600 nm was below 0.05 the fractions were considered active.

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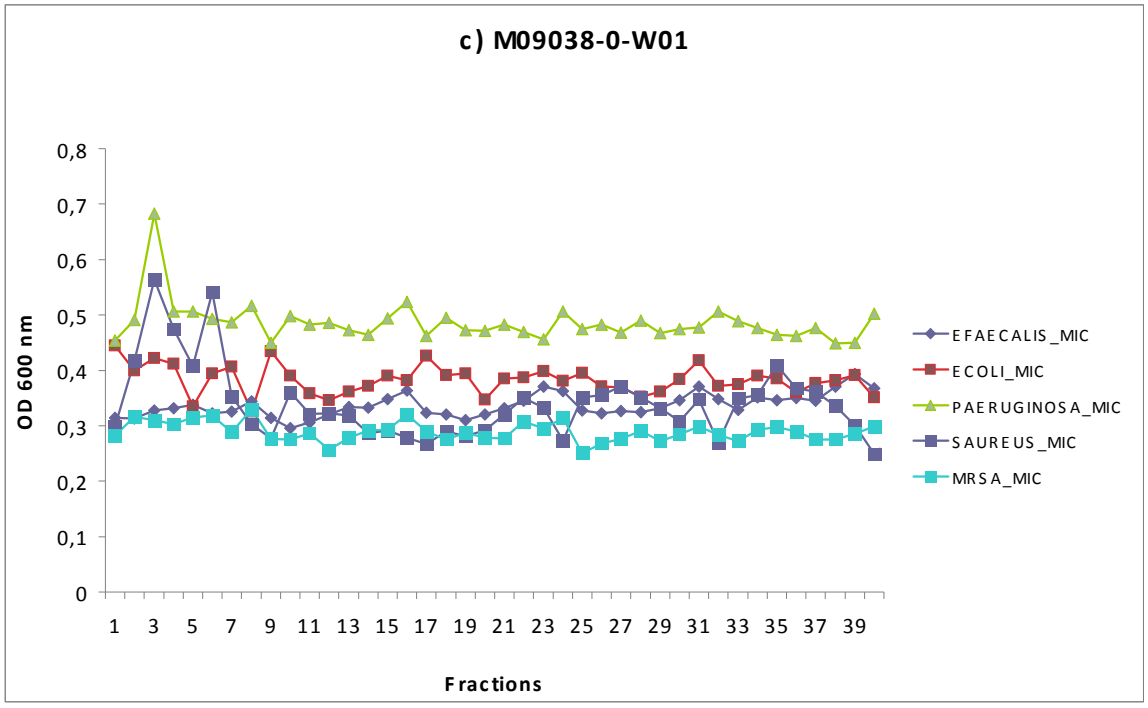
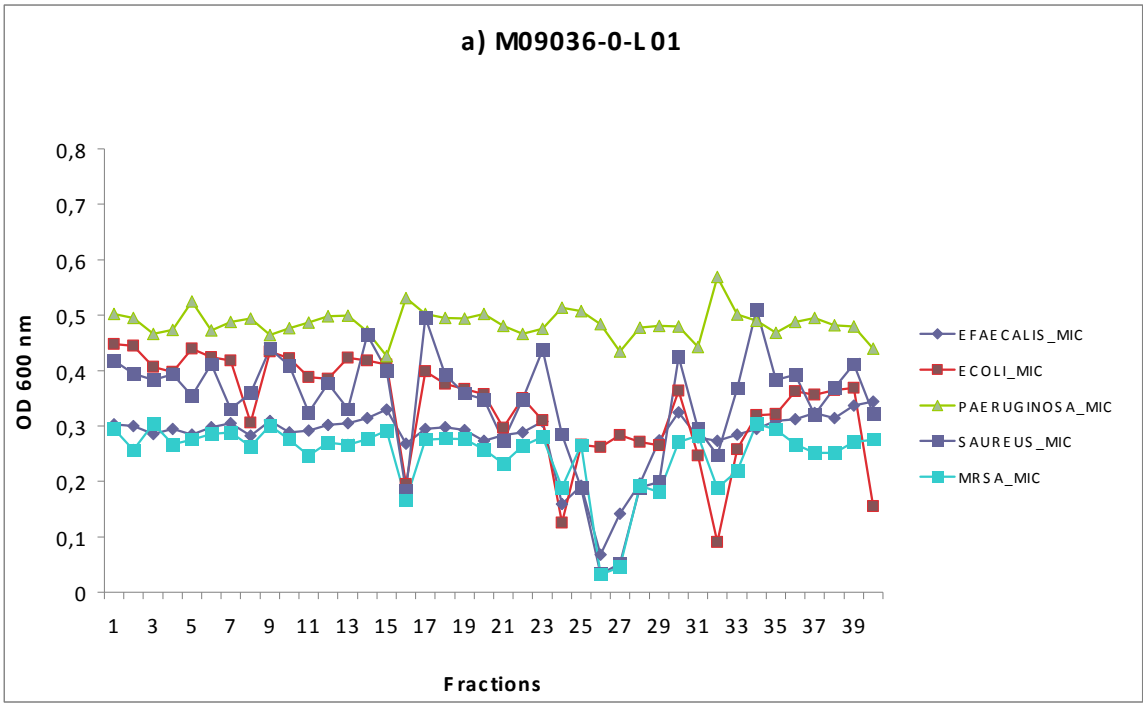


Figure 9 continued

3.4.2 Organic HPLC fractions

Fraction 26 and 27 from M09036-0-L01 (figure 10a) displayed antibacterial activity. Fraction 26 was considered active against *S. aureus* and MRSA, but also showed some activity against *E.faecalis.* Fraction 27 was considered active against MRSA, but also displayed some activity against *S. aureus*. Some other fractions displayed antibacterial activity as well but were above the threshold to be defined as active. Fractions from M09037-0-L01 (figure 10b) showed no activity. Fraction 26 from M09038-0-L01 (figure 10c) was considered active against MRSA, and showed some activity against *S. aureus*. Fractions 24 and 25 displayed some activity against *E. faecalis* although the activity was slightly above the defined limits. The fractions 26 and 27 from M09036-L01 and the fractions 24, 25 and 26 from M09038-0-L01 were subjected to a secondary screening.



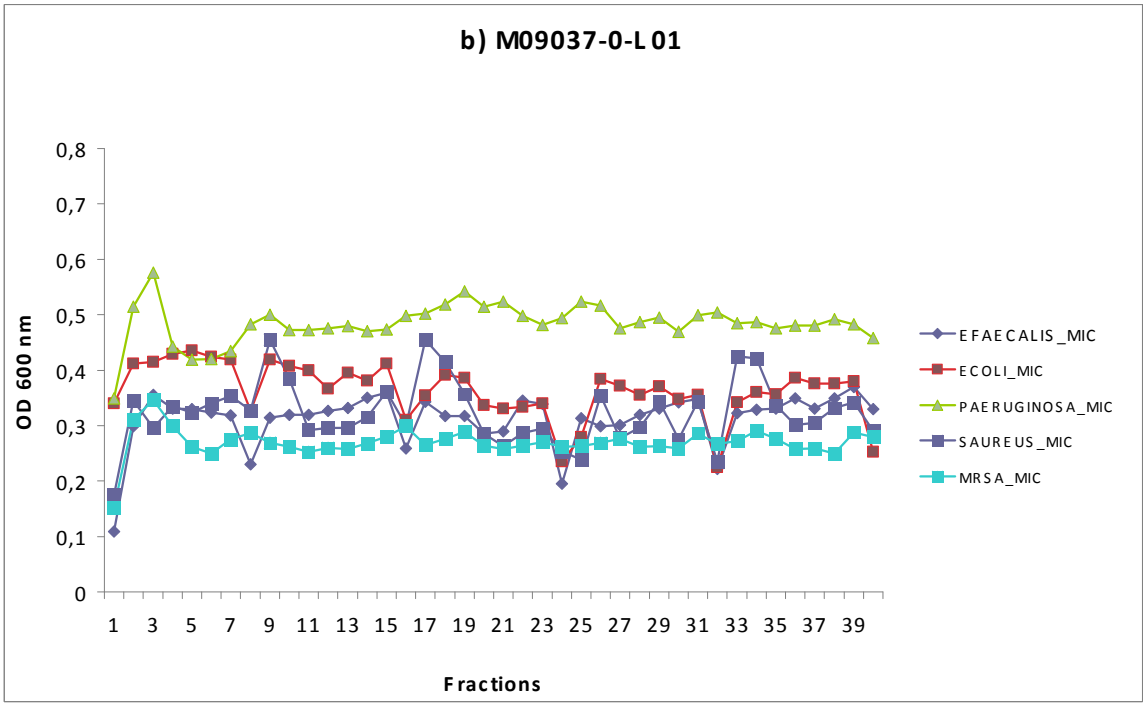


Figure 10: Primary screening: antibacterial activity of the organic extracts a) M09036-0-L01, b) M09037-0-L01 and c) M09038-0-L01, against five bacterial strains (*E. faecalis, E. coli, P. aeruginosa, S. aureus and MRSA*). If the OD at 600 nm was below 0.05 the fractions were considered active.

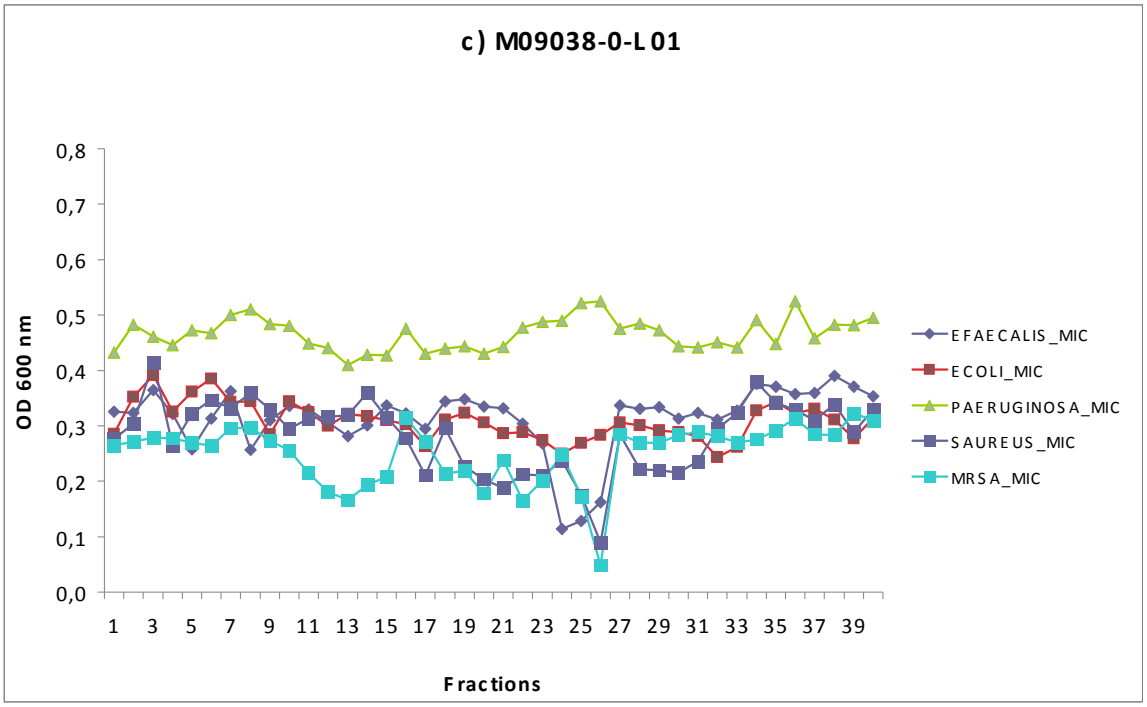


Figure 10 continued

3.5 Secondary screening of the organic fractions

In order to confirm the activity from the primary screening, the fractions were retested in dilutions of 1:1, 1:2 and 1:4, against the bacterial strains *E. faecalis*, *S. aureus* and MRSA. The fractions retested were 26 and 27 from M09036-0-L01 (figure 11a) and fraction 38 from M09036-0-W01 (results not shown) and fractions 24, 25 and 26 from M09038-0-L01 (figure 11b).

None of the fractions displayed any clear activity against the bacterial strains in any of the dilutions. This is demonstrated in figure 11a for the fractions 26 and 27 from M09036-0-L01, and in figure 11b for the fractions 24, 25 and 26 from M09038-0-L01. The fractions were tested against the bacterial strain *E. faecalis*. Fractions 24, 25 and 26 from M09038-0-L01 were selected for further purification even though they did not show

any clear activity. The three fractions were pooled and re-fractionated and then subjected to a third screening.

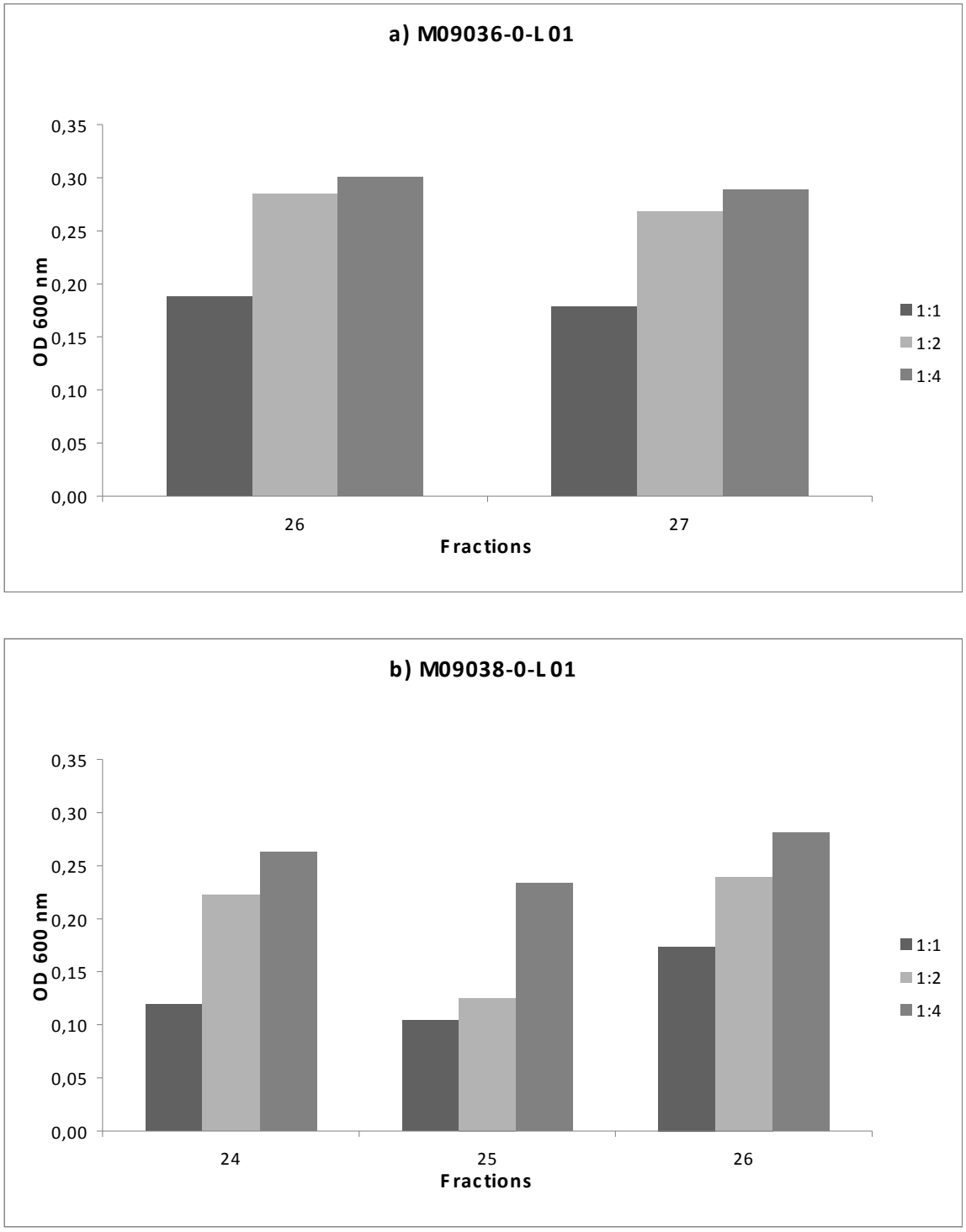


Figure 11: Secondary screening: antibacterial activity of the organic fractions a) M09036-0-L01 fraction 26 and 27, b) M09038-0-L01 fraction 24, 25 and 26 against the bacterial strain *E. faecalis*. If the OD at 600 nm was below 0.05 the fractions were considered active.

3.6 Third screening of purified fractions

Fractions 24, 25 and 26 from M09038-0-L01 were pooled and re-fractionated into 40 new fractions, and labeled M09038-0-L01-2426. The re-fractionation was performed in an attempt to isolate the active compounds. The fractions underwent a third screening against the five bacterial strains mentioned. Fraction 15 showed activity against *E. faecalis* and *S. aureus* as shown in figure 12.

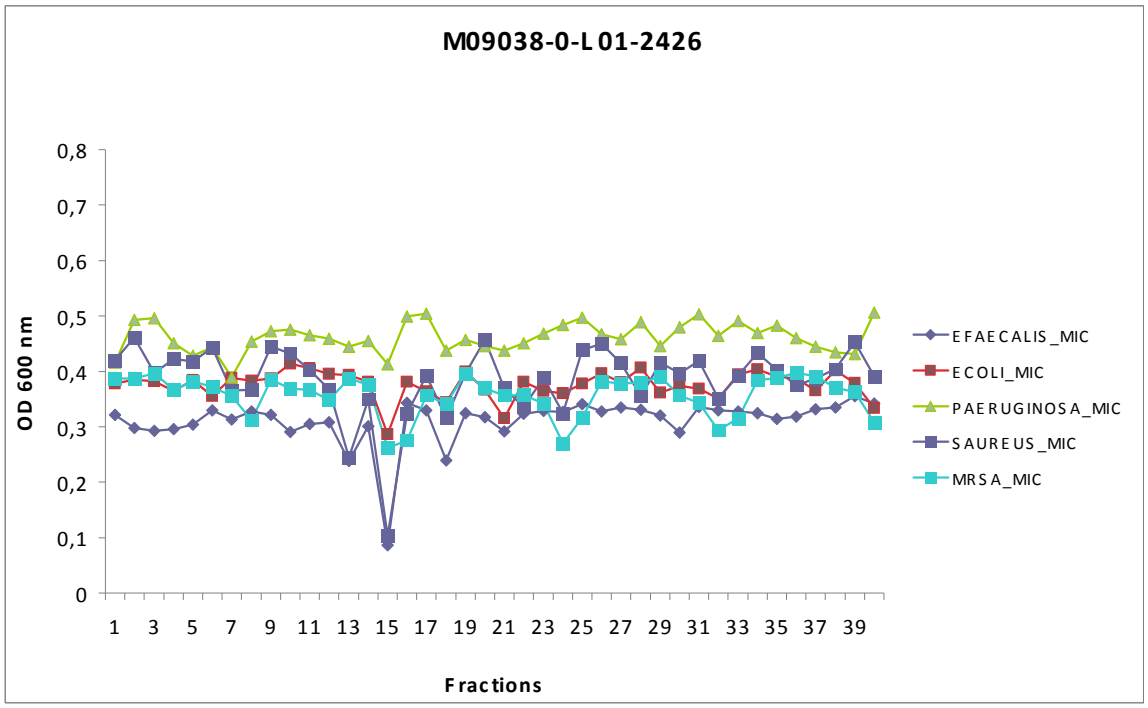


Figure 12: Third screening: antibacterial activity of the purified fractions against five bacterial strains (*E. faecalis, E. coli, P. aeruginosa, S. aureus and* MRSA).

3.7 New primary screening of the organic extract M09038

The organic extract M09038 was purified once again by HPLC (figure 13), the fractions were concentrated before they were subjected to primary screening. This time fraction 30 showed some activity against *E.faecalis*, fraction 31 showed some activity against *E. faecalis* and MRSA, but was considered active against *S. aureus.* Fraction 33 displayed some activity against MRSA.

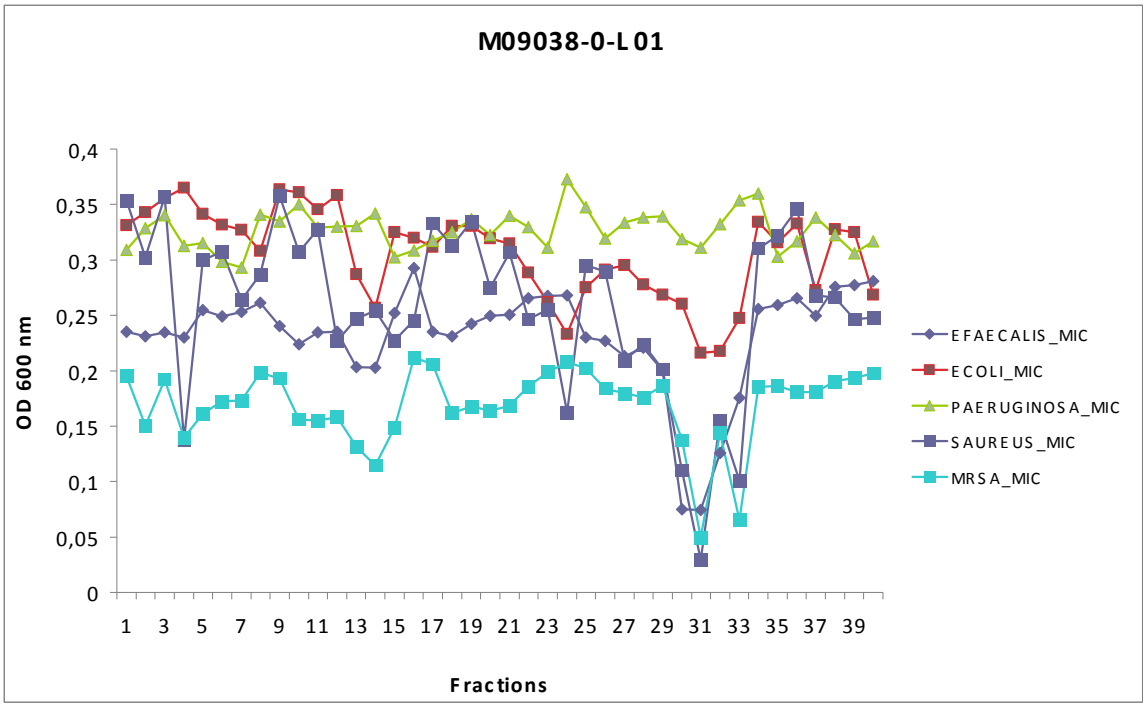


Figure 13: antibacterial activity found in new primary screening of the organic extract M09038-0-L01 against five bacterial strains (*E. faecalis, E. coli, P. aeruginosa, S. aureus and MRSA*). If the OD at 600 nm was below 0.05 the fractions were considered active.

There was also performed a dose-response screening on the organic raw-extract M09038 without purifying it to check for possible activity. The raw-extract showed weak activity and the results are not presented in this thesis.

3.8 LC-MS-analyses

The fraction M09038-0-L01-2426-15 was analysed by LC-MS using both time-of-flight and quadrupole instruments. No compounds were found that could explain the bioactivity in this fraction. Some peaks were detected but they were weak and they were also present in a number of inactive fractions. Based on the lack of good signals in the MS and the weak dose-response effect of the extract it was decided to purify the organic extract M09038 once again by HPLC. The organic extract was subjected to primary screening, but this time the fractions were more concentrated. The active fractions 30, 31 and 33

were analysed directly on ESI-TOF and afterwards with ESI-Q without being re-fractionated.

In ESI-TOF the fractions 30, 31 and 33 were analysed in positive and negative mode. There were signals detected in fraction 33. Figure 14 shows BPI (Base Peak Index) in positive and negative mode of this fraction where two interesting peaks were detected in the positive mode. Their respective *m/z* values were 505.4248, which eluted after 8.93 minutes, and 269.2094, which eluted after 9.27 minutes. The last peak had the highest intensity.

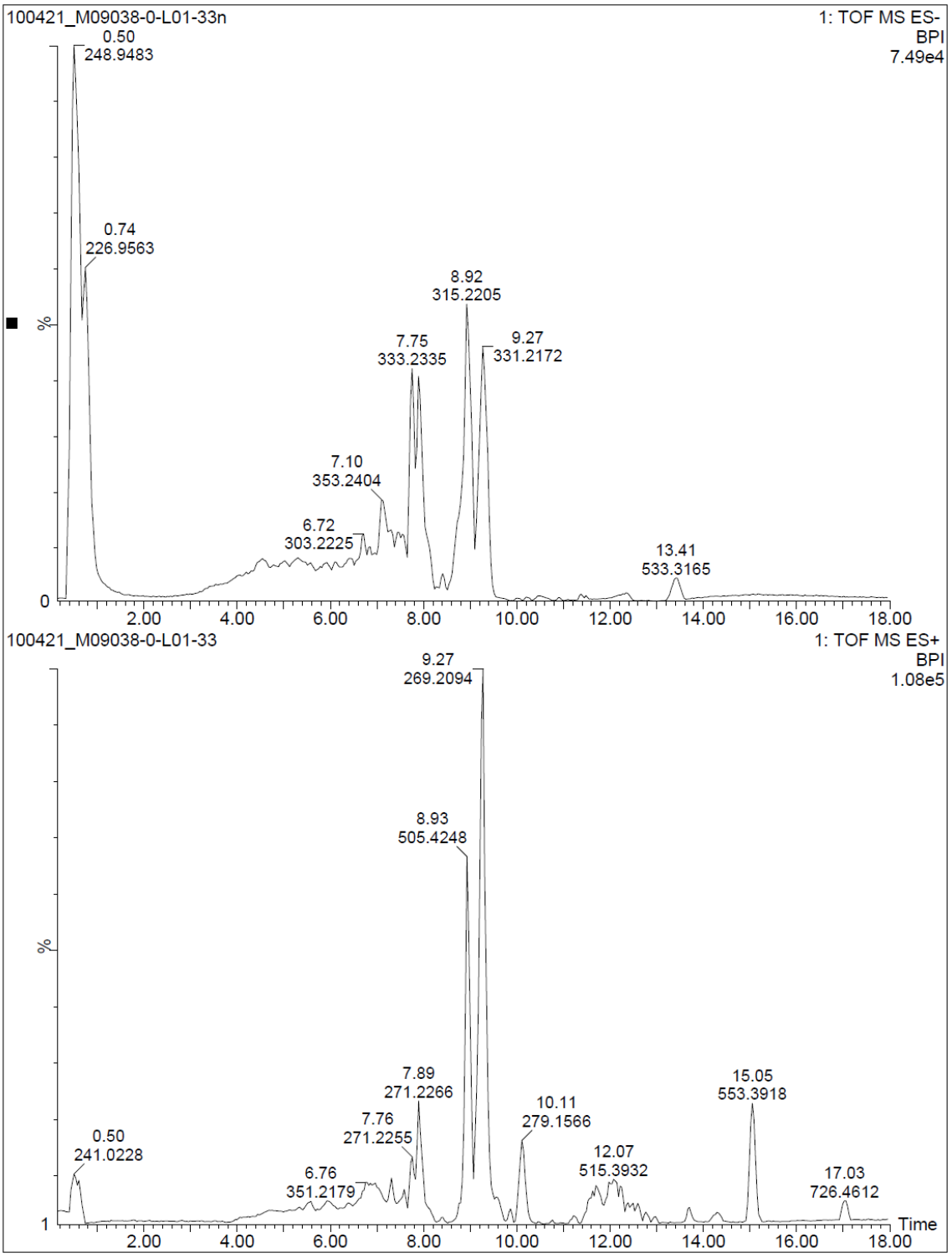


Figure 14: BPI of *m/z* 505.4248 and *m/z* 269.2094 in negative and positive mode. Signals were detected in positive mode.

Figure 15 shows the ESI-MS spectrum of the peak with the *m/z* value 269.2094 in positive mode. This value was assumed to be the protonated molecular ion; hence the molecular weight of this compound was 268.2021 amu. To confirm this, the spectrum was searched at higher *m/z* values for possible adducts which would support this conclusion. When searching the spectrum a gap of 22 amu was found between the *m/z* values of 269.2094 and 291.1926 which indicated the sodium adduct $[M+Na]^+$. Another gap of 41 amu was found between the *m/z* values of 310.2395 and 269.2094 that could be the adduct containing acetonitrile and a proton $[M+ACN+H]^+$. The *m/z* value 310.2395 was an even number which gave a stronger indication that the adduct contained ACN because even numbers indicate the presence of a nitrogen atom. The gap between *m/z* values 332.2197 and 269.2094 was 63 amu indicated the adduct containing ACN and a sodium $[M+ACN+Na]^+$. The *m/z* value 332.2197 was also an even number which meant that a nitrogen atom was present. The elemental composition of the compound with molecular weight 268.2021 amu was calculated to be $C_{16}H_{28}O_{3.}$

The Dictionary of Marine Natural Products is a database which contains isolated compounds from plants, animals and microorganisms. The empirical formula $C_{16}H_{28}O_3$ was used to search the database and there were four possible compounds that matched the formula.

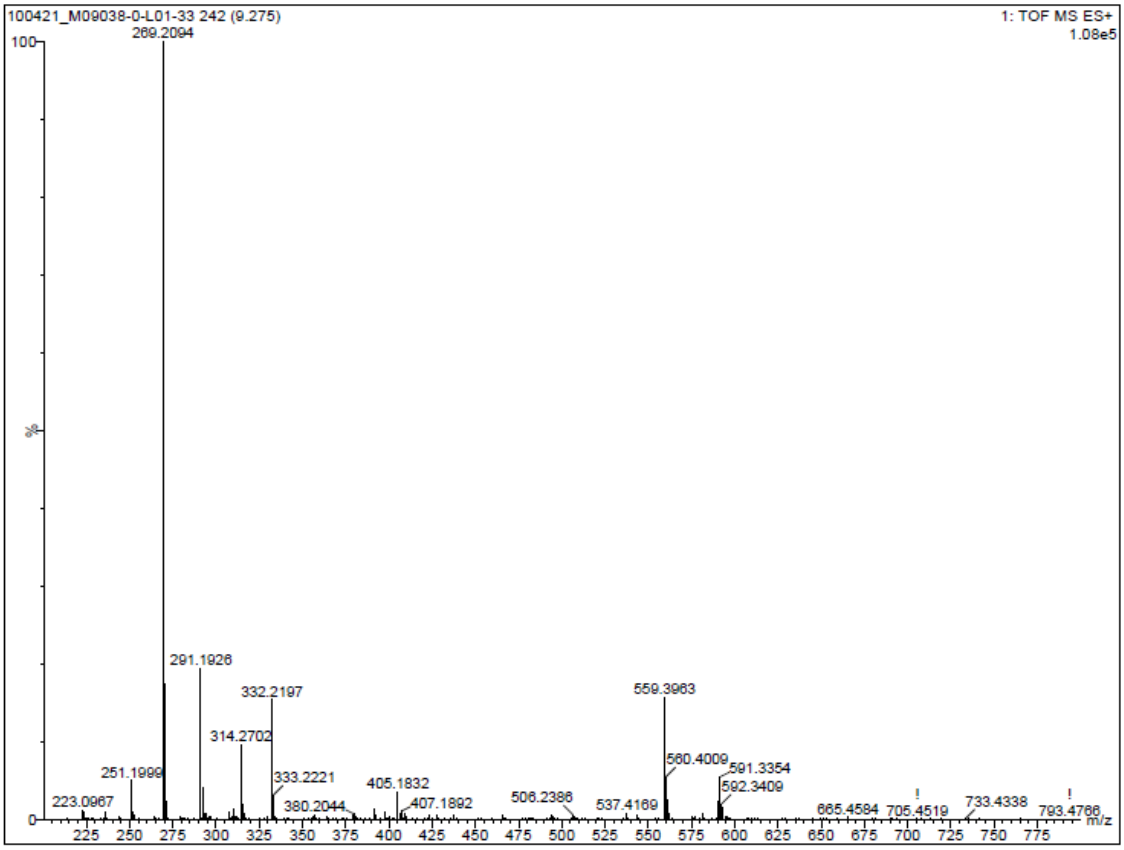


Figure 15: ESI-TOF spectrum of the peak where the highest *m/z* value was 269.2094. This was from fraction 33 in positive mode.

In the negative spectrum of the peak eluating after 9.27 minutes (figure 16), a signal correlating to an adduct with a formate ion ($[M+FA-H]^-$) was detected at *m/z* 313.2044. This finding strengthens the conclusion that 268.2024 is the molecular weight of the compound.

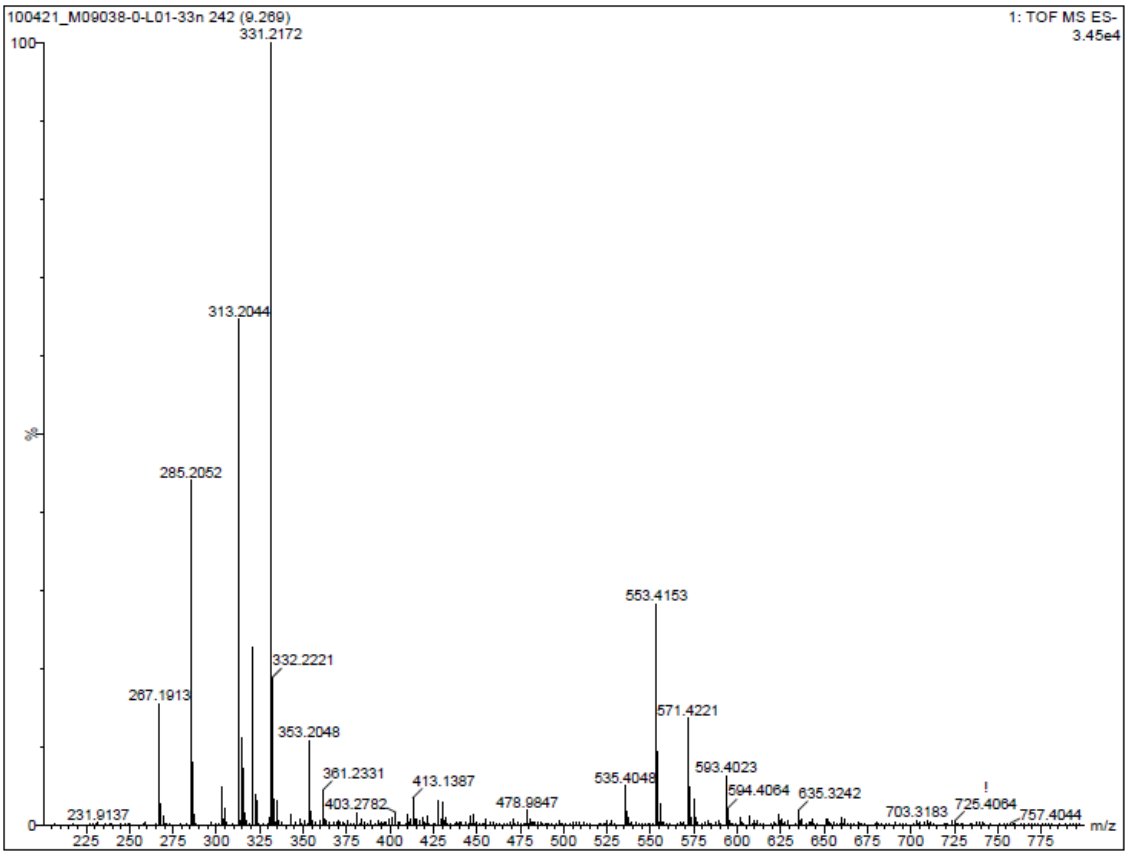


Figure 16: ESI-TOF spectrum of the peak eluating after 9.27 minutes in fraction 33 in negative mode.

Figure 17 shows the ESI-MS spectrum of the other peak with the *m/z* value 505.4248 in positive mode. There were not found any adducts in the spectrum which could confirm that 505.4248 was the protonated molecular ion $[M+H]^+$. If it was assumed that the peak was $[M+H]^+$, the molecular weight would be 504.4175 amu which gave the proposed empirical formula $C_{32}H_{56}O_4$, and if the peak was the $[M+Na]^+$ adduct the molecular weight would have been 482.4356 amu, and this gave the proposed formula $C_{31}H_{54}N_4$. None of these empirical formulas gave any hits when searching in The Dictionary of Marine Natural Products.

In the negative spectrum of the peak eluating after 8.93 minutes (figure 18), a signal at *m/z* 539.4361 might indicate that there is an adduct with a chloride ion present ($[M+Cl]^-$), supporting the conclusion that the molecular weight of the compound is 504.4175 amu.

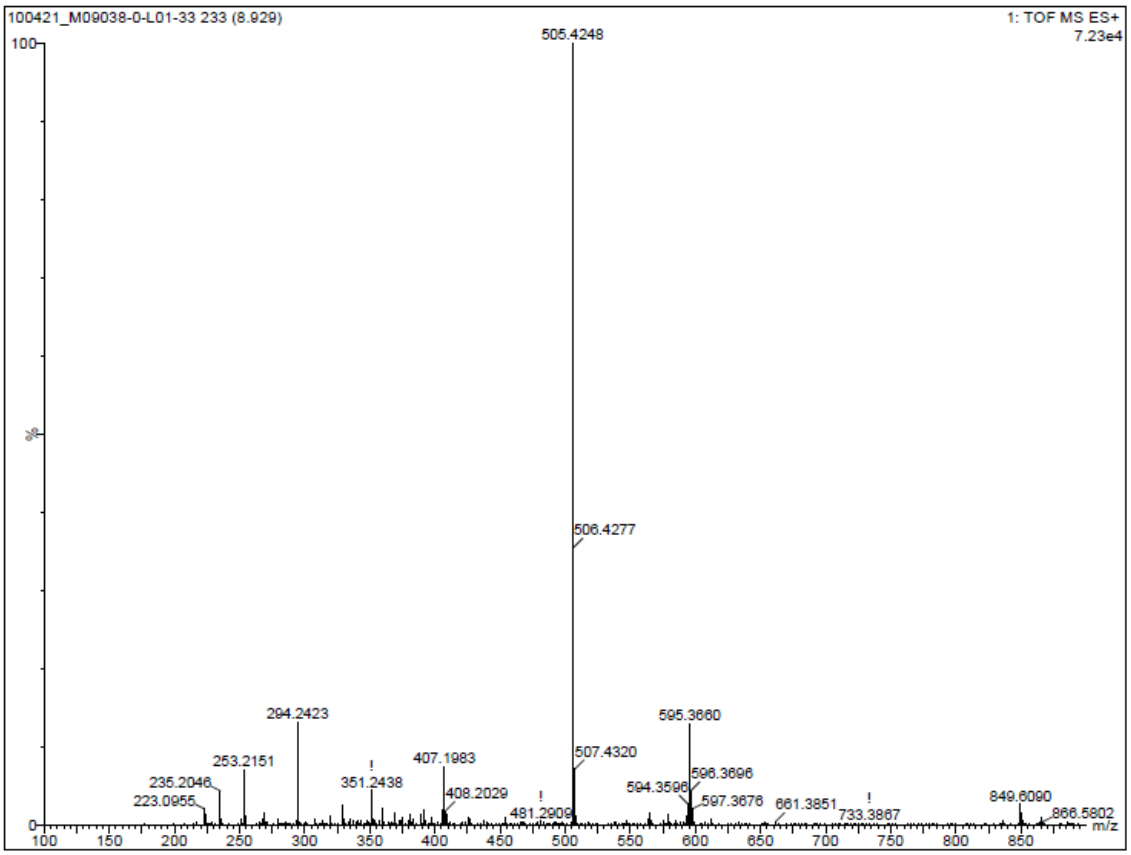


Figure 17: ESI-TOF spectrum of the peak where the highest *m/z* value was 505.4248. This was from fraction 33 in positive mode.

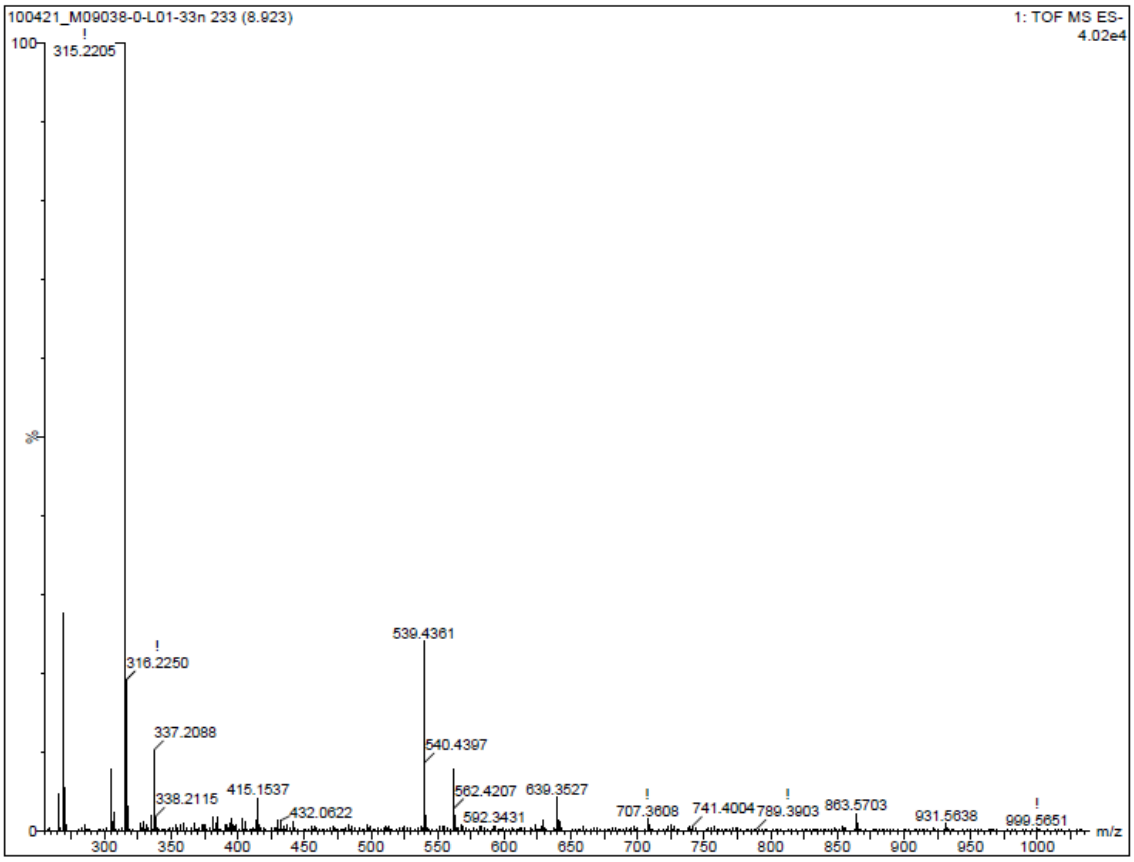


Figure 18: ESI-TOF spectrum of the peak eluating after 8.93 minutes in fraction 33 in negative mode.

In the ESI-Q analysis of the three fractions 30, 31 and 33 there was an *m/z* peak in fraction 33 as well. The assumed protonated molecular ion had an *m/z* value of 268.8 amu which matched the *m/z* value 269.2094 found in ESI-TOF. The *m/z* peak 310.2395 representing the adduct $[M+ACN+H]^+$ was also found in ESI-Q and had a stronger intensity than in ESI-TOF. The *m/z* value of 505.4248 was not detected in the quadrupole analysis.

4 Discussions

4.1 Antibacterial screening

The evolution of antibiotic-resistant bacteria has made it necessary to look for potent antibacterial agents in new places like the marine environment. A marine organism named *Halocynthia pyriformis* (M09038) from the phylum Chordata, in the class Ascidiacea, was screened for antibacterial activity. *H. pyriformis* is a marine invertebrate most commonly found in the northern parts of Norway at 20 meters of depth. In addition two other marine organisms named *Metridium senile* (M09036) and *Parastichopus tremulus* (M09037) was screened for antibacterial activity, although the focus of this thesis is on the antibacterial activity found in *H. pyriformis.*

The aqueous and organic fractions from all 3 marine organisms were screened for antibacterial activity against five bacterial strains *E. faecalis, E. coli, P. aeruginosa, S. aureus* and MRSA, where *E. faecalis, S. aureus* and MRSA are Gram-positive bacteria and *E. coli* and *P. aeruginosa* is Gram-negative bacteria. The fractions tested had some degree of antibacterial activity whenever there was a drop in the OD values, but the need for high throughput screening makes it inefficient to investigate every single drop unless the activity is within the defined limits of what is considered interesting for further studies. The fractions screened in this thesis displayed more activity against Gram-positive bacteria possibly because of their less complex cell wall making them more susceptible to inhibition.

There were found more antibacterial activity in the organic fractions than in the aqueous fractions. A possible explanation could be the difficulties with the isolation and purification of water-soluble compounds because of the abundance of salts from sea water [21]. The UV-chromatograms obtained from the HPLC purifications showed generally more signals in the UV-chromatograms of organic fractions than in the aqueous fractions which could confirm that the antibacterial compounds present can be of a more lipophilic character. Fraction 38 from M09036-0-W01 displayed clear antibacterial activity and its respective UV-chromatogram did detect a signal that indicates the

presence of a substance. This could be a compound which is more polar and thus better soluble in the aqueous fractions.

A secondary screening gives an opportunity to check for false positives. Dilutions are made to see if the activity could be rediscovered when the fractions are further purified. The organic fractions 24, 25 and 26 from *H. pyriformis* that showed activity in the primary screening were tested in secondary screening where the fractions were diluted 1:1, 1:2 and 1:4. None of the fractions showed any clear activity in any of the dilutions based on the limits set by MabCent, but it was possible to notice that they had some effect on the bacteria. The normal procedures at Marbio would be to end the screening process when there is not found activity in the 1:2 dilutions. The reason for this is that it is difficult to rediscover the bioactivity after the re-fractionation of the fractions. The concentration of the active compounds in the fractions might be too low to have a clear effect. To investigate this further these 3 fractions were pooled and re-fractionated into 40 new fractions to investigate if they showed activity in the third screening. Fraction 15 showed a clear drop in bacterial growth and it was subjected to MS-analyses.

A dose-response test on the raw-extract was performed but showed weak activity. This result was unexpected because the fractions of the organic extract did display antibacterial activities. The raw-extract is a complex mixture of several compounds and the active compounds may be bound to membranes and therefore unable to display activity, and also an interaction between the molecules in the extract could mask the antibacterial effect of the extract.

The organic extract of *H. pyriformis* was once again purified by HPLC but this time the fractions were more concentrated before being subjected to a new primary screening in the hope of detecting more activity and to confirm the authenticity of the results from the primary screening. Fraction 30, 31 and 33 did in fact display clear antibacterial activity within the defined limits, which confirmed that the active compounds have to be more concentrated in order to inhibit the bacteria more efficiently.

H. pyriformis has been tested for antibacterial activity before in two other studies. In the study performed by Margey Tadesse et al 2008 [22], the antibacterial activity was mostly found in the aqueous fractions, and the other study performed by H. Lippert et al 2003, found antibacterial activity in the crude organic extract of *H. pyriformis* [23]. The

differences between bioactivity found in this thesis and the other studies could be caused by several factors. The same marine organisms are most likely collected from different geographical areas and different time of the year which forces them to produce different metabolites that are needed for their protection. Another reason for why these findings are not comparable with the activity found in this thesis is due to the different extractions and screening methods used in all three studies.

4.2 MS- analyses

Mass spectrometry is a technique where the molecular mass of compounds in relation to their charge is measured. The purpose of this technique is to find the molecular formula of possible active compounds. In order to achieve this, it is essential to first find a signal that is assumed to be the molecular ion and then with the help of adducts confirm that it is, and by doing so the molecular weight is found, and then the molecular formula can be calculated.

Fraction 30, 31 and 33 from the new primary screening of the organic extract M09038 were analysed on ESI-TOF and ESI-Q where the analysis of fraction 33 gave a BPI chromatogram which contained two peaks that were interesting. These had the *m/z* values 505.4248 and 269.2094. Both of these *m/z* values were investigated and their possible empirical formulas were calculated. The molecular weight of 268.2021 amu gave the empirical formula $C_{16}H_{28}O_3$. When searched in The Dictionary of Marine Natural Products the empirical formula suggested a couple of candidates that had the same empirical formula as the possible bioactive compound. It is difficult to know the identity of the compound without further analyzing it in NMR, and this requires large amounts of pure substance.

The *m/z* value 505.4248 amu was assumed to have either the empirical formula $C_{32}H_{56}O_4$ or $C_{31}H_{54}N_4$. Neither one of the empirical formulas gave any hits in the Dictionary of Marine Natural Products, which means that these formulas may represent a new compound. It is impossible to know the identity of the compound without analyzing it by NMR.

If there had been obtained any strong *m/z* peaks of the protonated molecular ion 505.4248 amu in quadrupole it had been possible to fragment the compound and it could have been analysed using triple quadrupole. The *m/z* value 505.4248 amu was not detected in quadrupole but showed a clear peak in TOF. The explanation for this can be that quadrupole loses some sensitivity when scanning for a wide range of ions and have a low mass resolution, whereas TOF can analyse an unlimited mass range and have a high mass resolution.

As there are two different unique compounds present in the active fraction, it is impossible to conclude if both compounds are bioactive, or if just one of them are. Another possibility is that there is a synergistic effect between the two compounds. To answer this question, the two compounds have to be isolated and tested individually in antibacterial bioassays.

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