UiT The Arctic University of Norway

Faculty of Health Sciences

Using High-Resolution Mass Spectrometry to identify lipids in marine bacterial biomass

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Master's thesis in Pharmacy, FAR-3911, May 2023

Acknowledgment

The work presented in this thesis was performed at the Department of Pharmacy (IFA) and Department of Chemistry at UiT the Arctic University of Norway. It was performed for the Natural Products and Medicinal Chemistry Research Group at IFA in collaboration with Norstruct at the Department of Chemistry. BMFishfeed is the name for this collaborated project. The work was carried out from September 2022 to May 2023.

I thank my main supervisors, Terje Vasskog and Peik Haugen, for your support and our exciting and motivating discussions. Both of you always found time to help me and to get me back on track when I felt lost. You are passionate and eager for your work, which has been valuable to me.

I also want to thank Maria Wilhelmsen Hoff and Njål Rauø for everything you have taught me in the laboratory and the fun conversations we have had between and during laboratory sessions. You have included me in your projects which have given me a lot more depth when working on this thesis.

Thank you, Magnus A. Antonsen, for the friendly competition during the master thesis. And thank you for sharing your experiences with your project and discussing my obstacles with me.

Finally, I would like to thank Anne Sofie B. Haukefer for listening to my frustrations and always making me laugh during lunch break. It has meant a lot for morale.

Gøril Laugsand, May 2023

Abstract

Background: BMFishfeed is a cooperative project since 2021 between UiT the Arctic University of Norway, NORCE Stavanger and the University of South-Eastern Norway where the goal is to develop a lipid-rich bacterial meal that can be utilized as fish feed in the aquaculture industry. Carbon-rich waste from an omega-3 production site is used as nutrition for marine bacterial cultures, which are fermented and fed to promote lipid production in the bacteria. Alternatively, propionic acid (PA) is utilized as nutrition. The biomass is then dried to form a bacterial meal. Lipidomics was used to analyze the bacterial meal using liquid chromatography and mass spectrometry (LC-MS) in this thesis. The goal was to identify the lipid profile/lipidome in the bacterial meal. The original method for lipid extraction, Soxhlet extraction, was time and solvent consuming, and there was a need for a more efficient extraction method. Different extraction techniques were performed and compared to find the most efficient method concerning lipid yield.

Method: Sonication followed by direct extraction in DCM:MeOH was compared to Soxhlet extraction and direct extraction in DCM, providing a higher lipid yield. Biomass from a mixed microbial culture (MMC) was accumulated and harvested before it was sonicated to break down the cell walls. The sonicated biomass was then lyophilized to remove the water before extraction of lipids with dichloromethane and methanol (DCM:MeOH) as extraction solvent was performed. The lipids were dried to calculate dry weight before it was solved in isopropanol as preparation before Ultra High-Performance Liquid Chromatography – Mass Spectrometry (UHPLC-MS) analysis. The analysis was carried out in positive ionization mode to identify as many lipids as possible. The analysis data was managed through Thermo Fisher Scientifics data acquisition software called AcquireX. Lipids were identified using the data from AcquireX in the LipidSearch software. PHA precipitation was examined in different solvents as PHA is incompatible with the LC-MS system.

Results: All lipids of interest, TGs, PLs, and WEs, were identified in the biomass. The majority of identified lipids were TGs, and PLs were identified in most lipid samples. Only a few WEs were identified in the biomass. Several other lipids, such as MGs, DGs, and ceramides, were also identified, but their relevance remains unclear. The essential fatty acids DHA and EPA were present in many lipids.

Conclusion: Sonication before lipid extraction provided a higher lipid yield, probably because the membranous PLs become more exposed and accessible for the extraction solvent. Additionally, it saves solvent and time. No proper method for PHA precipitation was found and should be further investigated. Further examination of the lipidome is needed, though several lipid classes were identified during analysis.

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Abbreviations and glossary

3HB	3-hydroxybutyrate
ACN	Acetonitrile
ADF	Aerobic dynamic feeding
BM	Bacterial meal
CG	Crude glycerol
Ch	Cholesterol
CID	Collision induced dissociation
DCM	Dichloromethane
DG/DAG	Diacylglycerol
DHA	Docosahexaenoic acid
DO	Dissolved oxygen
EPA	Eicosapentaenoic acid
ESI	Electrospray ionization
F/F	Feast and famine
FA	Fatty acid
GF	Glycerol fraction
HCD	High-energy collisional dissociation
HPLC	High-performance liquid chromatography
LC-MS	Liquid chromatography and Mass spectrometry
LIT	Linear quadrupole ion trap
m/z	Mass-to-charge ratio
m	Mass
MeOH	Methanol

MG/MAG	Monoacylglycerol
MMC	Mixed bacterial culture
MS	Mass spectrometry
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
P(3HB)	Poly-3-hydroxybutyrate
PA	Propionic acid
PC	Phosphatidylcholine
PG	Phosphatidylglycerol
PHA	Polyhydroxyalkanoates
PL	Phospholipid
PO2	Partial pressure of oxygen
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
RT	Retention time
S/N	Signal-to-noise ratio
SBR	Sequence batch reactor
Sludge	Marine mud
TCA	tricarboxylic acid
TG	Triacylglycerol
UHPLC/UPLC	Ultra high-performance liquid chromatography
VFA	Volatile fatty acid
z	Charge

1 Introduction and theory

1.1 Background

The project's theme is to find ways to sustainably manage marine resources from aquaculture and fisheries. After the main product is removed from the animal (typically the fillet from a fish), side stream materials (fish cuttings, blood, guts, crab shell, etc.) are collected. The side streams from the industry are not yet utilized to their total capacity, and there is a need to improve the methods we have available to process and utilize them. In this work, we are exploring one potential new technology based on the capacity of some bacteria to store energy in the form of lipids. Especially bacteria from high-latitude marine ecosystems are suited for storing essential long-chained polyunsaturated fatty acids (PUFAs), commonly known as Omega-3 oils. Production of PUFAs can be obtained by feeding side stream materials from omega-3 production to a mixed microbial culture of mainly marine bacteria in a manner that promotes lipid storage. The biomass from this process can be collected and dried into a lipid-rich bacterial meal (BM) (Figure 1). Feed additives typically contain a high protein level. Still, BM enriched with lipids is yet to be studied as a feed additive and could present a novel opportunity as a new source for lipids to be used in aquaculture (1).

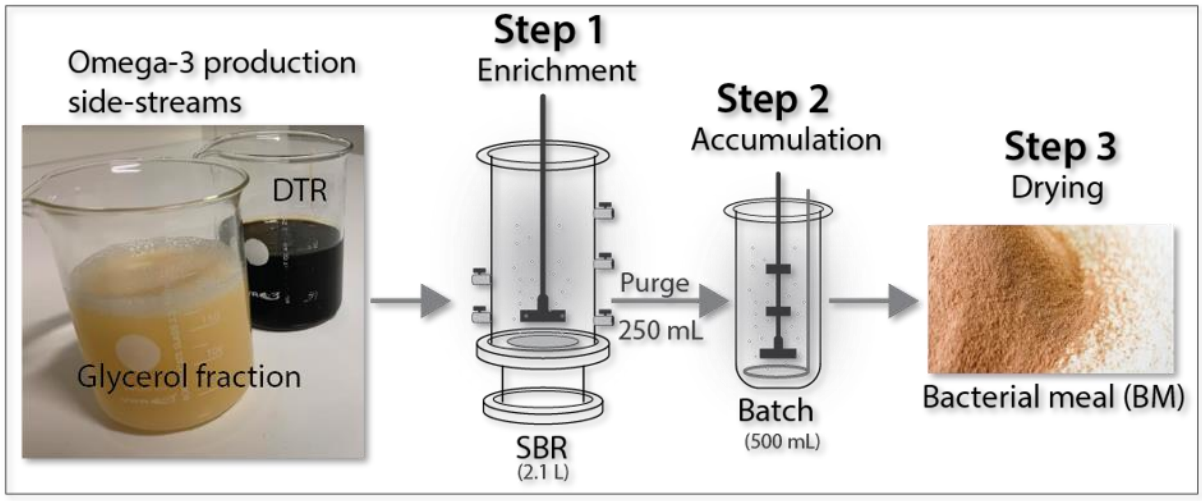

Figure 1 Steps involved in the BMFishfeed project.

1.2 Lipids

Lipids represent one of the four main classes of biological macromolecules, i.e., carbohydrates, proteins, nucleic acids, and lipids. Lipids are found everywhere in nature and can have a wide range of functions, from energy storage molecules in living tissues to acting as waterproofing agents. Lipids may be defined as fatty acids (FAs) and their derivates, and compounds that relate functionally or biosynthetically to these. Lipids are not necessarily required to have much in common structurally or functionally (2). Finally, in many textbooks and articles, the term lipid has been described as a compound readily soluble in organic solvents. Still, many lipids known today may also be soluble in water.

1.2.1 Phospholipids

Phospholipids (PL) contain FAs and a phosphate group bound to glycerol. The FAs provide a non-polar group on the phospholipid, while the phosphate group is polar. Because of this, a phospholipid is amphophilic, which makes it suitable for lipid bilayers. They act as building blocks for the lipid bilayers in cell membranes and therefore play a vital role in living organisms. Phospholipids with only one bound FA are referred to as lysophospholipids (Figure 7). The polar phosphate group may vary and decides which class of phospholipid is present. Some main classes of phospholipids are phosphatidylcholine (PC) (Figure 2), phosphatidylethanolamine (PE) (Figure 3), phosphatidylserine (PS) (Figure 4), phosphatidylglycerol (PG) (Figure 5) and phosphatidylinositol (PI) (Figure 6).

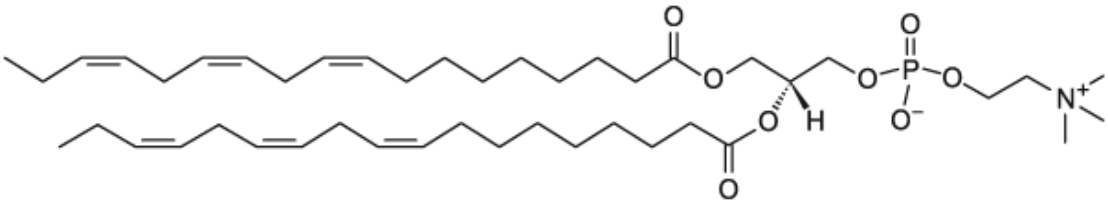

Figure 2: 18:3 (Cis) PC, 1,2-dilinolenoyl-sn-glycero-3-phosphocholine. Photo credit: avantilipids.com

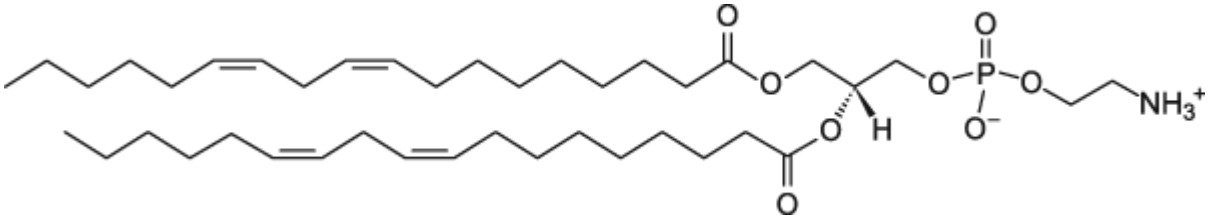

Figure 3: 18:2 PE, 1,2-dilinoleoyl-sn-glycero-3-phosphoethanolamine. Photo credit: avantilipids.com

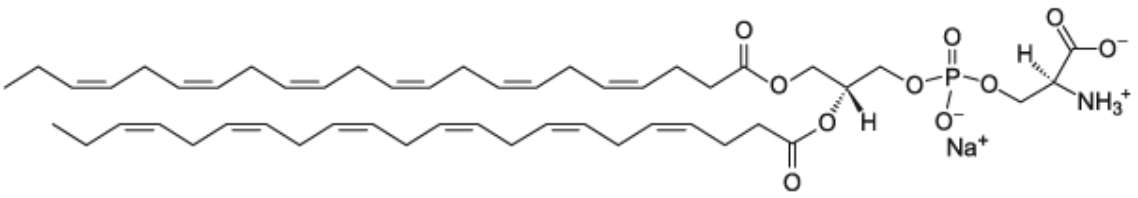

Figure 4: 22:6 PS, 1,2-didocosahexaenoyl-sn-glycero-3-phospho-L-serine (sodium salt). Photo credit: avantilipids.com

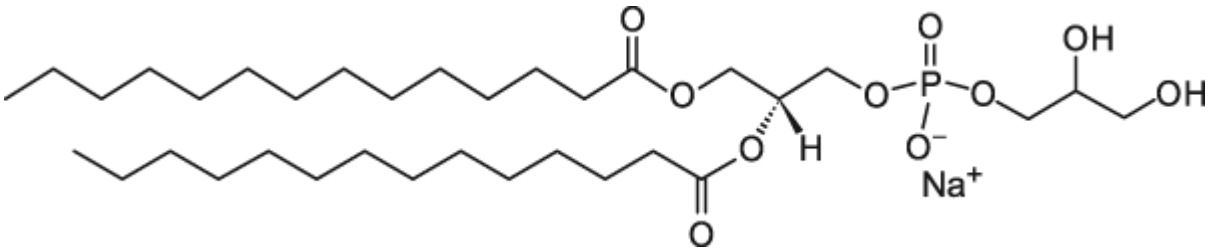

Figure 5: 14:0 PG, 1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt). Photo credit: avantilipids.com

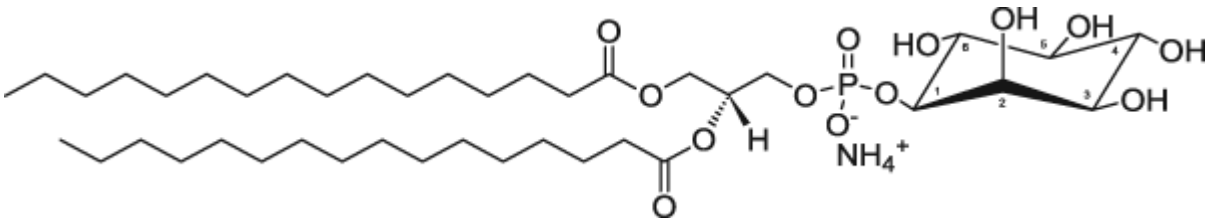

Figure 6: 16:0 PI, 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-myo-inositol) (ammonium salt). Photo credit: avantilipids.com

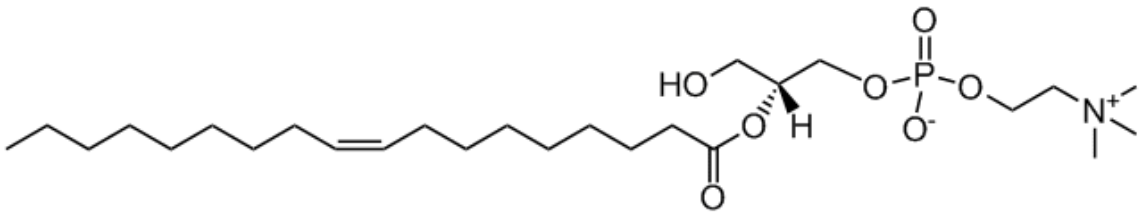

Figure 7: 2-18:1 Lyso PC, 1-hydroxy-2-oleoyl-sn-glycero-3-phosphocholine. Photo credit: avantilipids.com

1.2.2 Glycolipids

Glycolipids consist of one or two FAs and a polar carbohydrate group, such as a monosaccharide or oligosaccharide, bound to a glycerol molecule. The bonds between glycerol and the FAs are ester bonds, while an ether bond links the glycerol to the carbohydrate group (2). The glycolipids are amphiphilic and often found in lipid bilayer membranes functioning as membrane stabilizers. MGDG is one example of a glycolipid, and an example of the structure is presented in Figure 8.

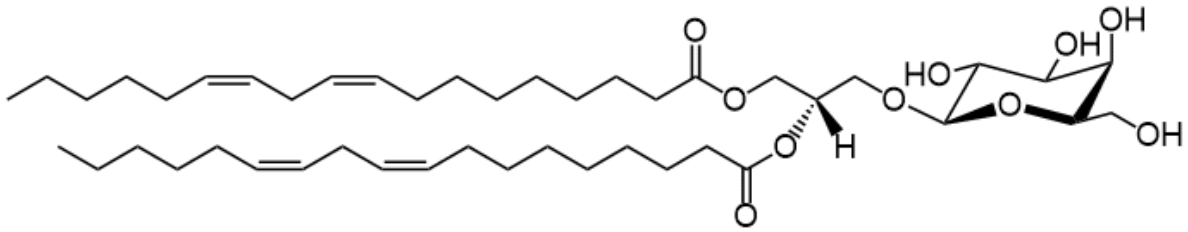

Figure 8: 18:2 MGDG (synthetic), monogalactosyldilinoleoylglycerol. Photo credit: avantilipids.com

1.2.3 Acylglycerols

An acylglycerol contains a glycerol bound to one, two or three FAs via ester bonds. Acylglycerols are named after the number of FAs, whereas the names are monoacylglycerol (MG), diacylglycerol (DG) (Figure 9) and triacylglycerol (TG). The ester bonds break due to hydrolysis performed by the organism containing acylglycerols, and the organism is left with free FAs and glycerol, which can be used as energy.

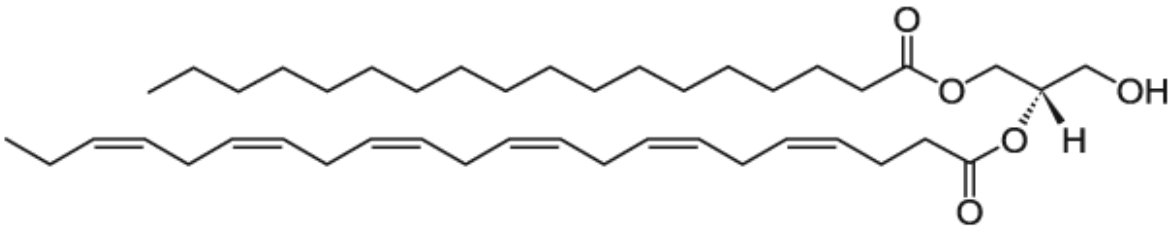

Figure 9: 18:0-22:6 DG, 1-stearoyl-2-docosahexaenoyl-sn-glycerol. Photo credit: avantilipids.com

1.2.3.1 Monoacylglycerols

MGs are constructed by one FA that is esterified to a glycerol molecule. In bacteria, they serve as energy storage for bacteria and are important precursors involved in the biosynthesis of lipids. MGs also act as signaling molecules. MGs can be metabolized into an FA and glycerol, which can be further metabolized to produce adenosine triphosphate (ATP), one of the main energy currencies in bacterial cells.

1.2.3.2 Diacylglycerols

DGs consist of two FAs bound to a glycerol molecule. In bacteria, DGs contribute to lipid biosynthesis by being transformed into phospholipids such as PEs and PGs. DGs are often found in cell membranes, acting as an agent for maintaining stability and fluidity. They can also act as signaling molecules, contributing as an activating agent for signaling processes within the bacterial cell.

1.2.3.3 Triacylglycerols

TGs consist of three FAs esterified to each carbon in glycerol (Figure 10). The FAs can be of different lengths and grades of saturation and therefore give rise to various kinds of TGs (3).

Variations within TGs happen because of the wide range of different FAs. Generally, FAs of animal, plant and microbial origin contain carbon atoms of even numbers in straight chains. The FAs have a carboxyl group at one end of the molecule and may contain 2 to 80 carbon atoms, depending on which group of microorganisms they originate from. FAs usually contain 14 to 22 carbon atoms in animal tissue (2). Bacteria have been stated to contain odd-numbered long-chained FAs, often those of 15, 17 and 19 carbons (4). FAs can be divided into subclasses depending on whether they are fully saturated or not degree of saturation they exhibit, and which substituents they may contain. The substituents can be a methyl or hydroxyl group along the chain, or a cyclopropane ring at the opposite side of the carboxyl group (2).

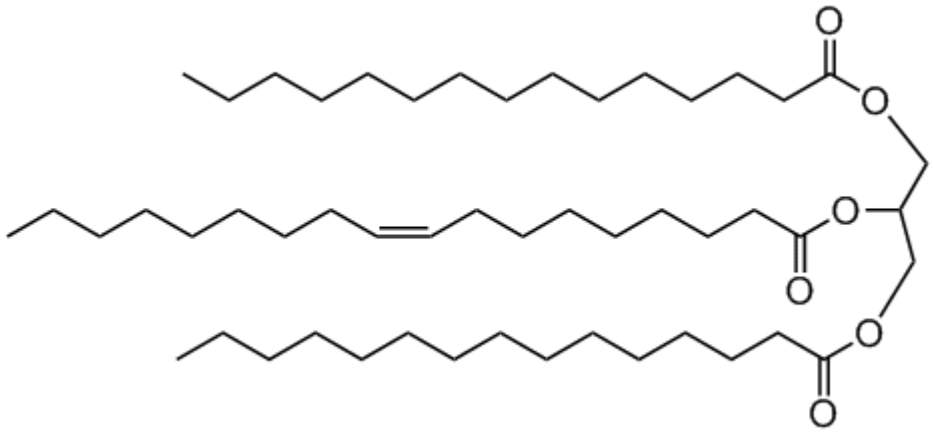

Figure 10: 15:0-18:1-15:0 TG, 1,3-dipentadecanoyl-2-oleoyl-glycerol. Photo credit: avantilipids.com

1.2.4 Wax esters

WEs and TGs have FAs in common, but only one FA is bound to a fatty alcohol instead of glycerol in wax esters. The fatty alcohols are similar in structure to FAs, though they contain a hydroxyl group instead of a carboxyl group. WEs might function as coating layers, candles, and lubricants. The biosynthesis of WEs includes the conversion of FAs to fatty alcohols via reductases before being linked to an FA (3).

1.2.5 Polyhydroxyalkanoates

PHAs are natural biopolymers containing hydroxy-fatty acids with various lengths of carbon chains within each FA. PHAs are produced by certain bacteria and are used as energy and carbon reserves (5). The FAs are esterified to each other by utilizing the hydroxy group and the carboxylic acid. The properties of PHA vary depending on the length of the FAs and the length of the polymer. Over 300 bacterial species have been documented to synthesize over 150 different PHAs (5). Poly-3-hydroxybutyrate (P(3HB)) is the most found PHA, constructed by 3-hydroxy butyric acid as the monomer (Figure 11).

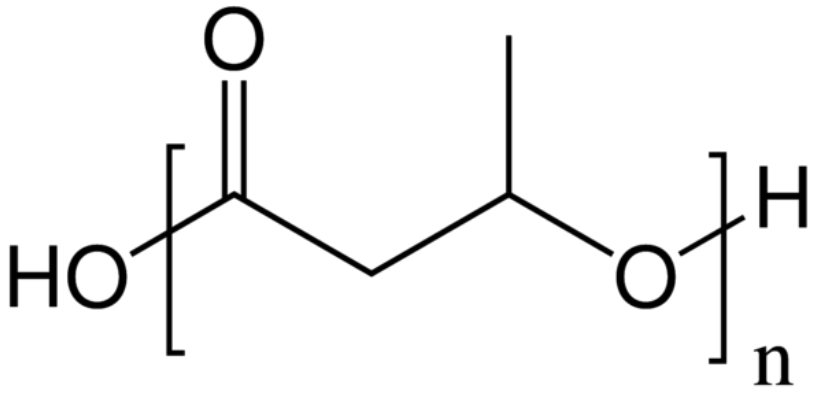

Figure 11: Poly(3-hydroxybutyric acid) (P(3HB)). Photo credit: sigmaaldrich.com

When the nitrogen supply is compromised, the cells cannot grow at the regular rate they would normally do. This is when the production and storage of PHA occur (5). In the cell cytoplasm of bacteria, PHAs are stored as granules. The production can occur by different mechanisms, and one of them is synthesizing glycerol to poly-3-hydroxybutyrate (P(3HB)) via intermediates like pyruvate and acetyl-CoA. Acetyl-CoA depends on nitrogen to enter the tricarboxylic acid (TCA) cycle. If a limited amount of nitrogen is present, nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) will accumulate in the bacterial cell. This causes acetyl-CoA not to enter the TCA cycle and instead be synthesized to 3-hydroxybutyrate (3HB), which can be synthesized further to P(3HB) (5).

Volatile fatty acids (VFA) such as propionate and butyrate can also be transformed into intracellular PHA in bacteria. Glycerol can be converted into VFAs, which are short-chained FAs with six or fewer carbon atoms. This transformation typically happens in an anaerobe digester before being fed to the bacteria. The VFAs will then be utilized by the bacteria to produce PHA (6). One of the common VFAs produced is propionic acid (PA). PA can also be fed directly to the bacteria to produce intracellular PHA. In a proposed representation of PHA production from *R.eutropha*, PA is transformed to PHA through a series of intermediates such as Acyl-CoA and (*R)*-2-hydroxybutyryl-CoA (7).

1.3 Energy storage in bacteria

1.3.1 Bacterial fermentation

Fermentation may describe several processes and is commonly related to transforming sugars into a new product via metabolism. Here, the focus is on bacterial fermentation in a fermentor. A fermentor is a specialized bioreactor that grows and maintains microorganisms, such as bacteria and yeast. Bacterial fermentation happens in a controlled manner, and the strategy for fermentation may vary. The most common strategies are batch, fed-batch, and continuous culture. In batch culture, a fixed amount of nutrition is added to the bacterial culture, which allows them to grow until all nutrition is consumed. Harvest happens after consumption. In fed-batch cultures, nutrition is added in a controlled manner, allowing the culture to grow longer than a batch culture. Since the addition of nutrients is controlled, obtaining yields of higher concentration and consistency is often possible. In continuous culture, nutrition is fed to the culture while harvesting happens simultaneously. Because of this, the bacteria are in a steady-state condition. This allows for steady production over a more extended period and higher consistency of yields.

1.3.2 Lipid storage

It has been known for decades that bacteria can store hydrophobic compounds, such as lipids and carbohydrates intracellularly. Storing these compounds is a strategy for the bacteria's survival needs in challenging environments. Bacteria must store lipids because they can be used as energy later when it is no longer available in the environment. The lipids are often found in intracellular inclusion bodies in bacteria. The inclusion bodies are usually seen as discrete structures that might be enclosed in a mono- or bilayer membrane, but they may also exist without a boundary layer (3). It is known that most procaryotic cells can accumulate at least one kind of storage compound. Lipids are excellent storage compounds as they do not affect osmosis, are often not soluble in water, and contain many calories. The most crucial storage reserves are polyhydroxyalkanoates (PHAs), triacylglycerols (TGs) and wax esters (WEs) (3).

Carbon, nitrogen and phosphor are vital for bacteria to grow, and a steady supply of oxygen is needed for aerobic bacteria. If the nitrogen source is limited while an excess of carbon is available, the stimulation of lipid accumulation increases. When the supply of nitrogen increases, the process of cell growth activates instead of storage (3). TGs are stored in bacteria for energy and carbon reserves, and accumulation happens parallel to respiration

processes in the bacteria. When glycerol is consumed with nitrogen present, it is synthesized into TGs through the *ex novo* pathway (8). TGs serve as excellent energy storage for bacteria, compared to PHAs and carbohydrates, since the energy yield produced by the oxidation of TGs is much greater than the others. This may provide the bacterial cell with a higher degree of autonomy and independence when available energy from the environment is compromised (9). The number of TGs stored compared to WEs varies with the bacterial strain and which carbon source is used to feed the bacteria. Gram-positive bacteria, known as actinobacteria, accumulate TGs to a high degree because of their specialization to do so. WE synthesis and accumulation occur mainly in gram-negative bacteria (9), and it usually happens with an external carbon source available and a limitation of nitrogen. WE production usually is lower than PHAs and TGs, but it highly depends on the bacterial strain and the environment (3). Microorganisms that produce high amounts of lipids, higher than 20% of their cellular dry weight, are referred to as oleaginous microorganisms. This term may overlap some bacterial species if they fit the description.

1.3.3 Mixed microbial cultures

A common strategy for the production of PHA and TG in mixed microbial cultures (MMCs) is aerobic dynamic feeding (ADF), with feast and famine (F/F) cycles (10). F/F is an exchange between excessive and limited access to carbon, which causes a selection of bacteria depending on their abilities to store carbon (5). This enrichment strategy has been tested in several variations to optimize production, where the time of access to nitrogen and carbon available for the MMC varies. Variations in pH, temperature and salinity might also be applied, which has been shown to lead to the enrichment of extremophile oleaginous microorganisms (11). ADF is often performed in a sequence batch reactor (SBR) equipped to monitor and control the parameters of the MMC. The oxygen level in the mixture (PO2) indicates what is happening with the bacteria. If the oxygen level is low, the bacteria are in an oxygen-consuming state. If the oxygen level is high, the bacteria are not in an oxygen-consuming state. pH is also measured and adjusted if needed. The MMC is fed with a carbon-containing substance, and a source of nitrogen and phosphor is required to ensure growth. A stirring device should be in the reactor to ensure proper mixing. In total, this is known as bacterial culture fermentation (5). The SBR combines batch culture and continuous culture fermentation process, as described in section 1.3.1. It differs from continuous culture since no steady-state is present because the microbial growth is carried out in the same manner as in batch culture.

1.3.4 Accumulation

In this thesis, accumulation refers to the process of promoting lipid accumulation in bacteria, performed in a bioreactor. Accumulation aims to saturate the bacteria with carbon to produce lipids without further cell growth. Because of this, no nitrogen is fed to the system. The saturation happens by feeding the bacteria in pulses with crude glycerol (CG) until there is no visible or seemingly significant change in PO2 after feeding. When PO2 is at saturation level, harvesting of the biomass is done.

1.4 Lipid analysis

Lipid analysis and its methods are essential for many clinical, quality control and research applications (2). Finding appropriate analytical methods for lipids has been a technical challenge to analysts since lipids vary greatly in structure and properties. Advances in technology have improved earlier challenges regarding selectivity, sensitivity, and resolution. These advances have especially been made in mass spectrometry, and lipid inventory of biological systems have been implemented to an "-omic-centric" view. Thus, the term lipidomics has been introduced. Before performing lipidomics, proper sample preparation and purification of the intracellular lipids must be exercised (12).

1.4.1 Sonication

Sonication utilizes high-frequency sound waves to disrupt and break apart biological material, such as bacterial cell membranes. The aim is to access the intracellular and membranous contents of the bacteria to perform further processing and analysis of the contents. The sonication happens in a container where the bacteria is suspended in the liquid. The sound waves alternate between high and low-pressure zones, which inflicts cell vibration, breaking the structures apart. The intensity and duration of sonication can be adjusted to ensure proper breakage of the cell membranes without causing damage to the bacteria's components. Sonication often produces a lot of heat, and it is essential to cool down the sample during the sonication to avoid denaturing and breakage of heat-sensitive components. Sonication is a highly efficient method for accessing intracellular and membranous components in e.g., bacteria.

1.4.2 Soxhlet extraction

Soxhlet extraction is a method for extracting lipids from solid material. The principle is to use a volatile organic solvent, the extraction solvent, to soak the solid material in a thimble, followed by drainage caused by a siphon mechanism. The extraction solvent is heated in a distillation flask on a heat source and evaporates into a condenser, which leads the condensed extraction solvent into the extractor where the thimble is located. Drainage from the extractor happens because of the siphon mechanism, and the extraction solvent returns to the distillation flask and can be reused as a continuous technique. See Figure 12 for the Soxhlet setup. The method is simple to execute and requires little training. A few drawbacks are that the extraction requires a long time, and much extraction solvent is wasted, both for the extraction itself but also for proper cleaning of equipment. The extraction solvent is often expensive to dispose of and often of environmental disadvantage (13).

Figure 12: Soxhlet extraction apparatus. Photo credit: Sigmaaldrich.com

1.4.3 Liquid chromatography

Liquid chromatography is a method for separating substances and is often done before further analysis. It uses a liquid (mobile phase) to transport the analytes along a solid compound (stationary phase) in a chromatography column. Separation of the analytes happens because of intermolecular interactions between the analyte and the mobile phase and stationary phase. In reverse-phase chromatography, the mobile phase has polar properties, while the stationary phase has non-polar properties. Different analytes will interact to varying grades with the phases and reach an equilibrium, and their speed through the column will vary. This results in variations in time spent in the column, which is measured as retention time (RT). The separated analytes can be transferred directly to an ion source for further mass analysis (14) or be collected separately for further use. See Figure 13 for the liquid chromatography setup and path flow.

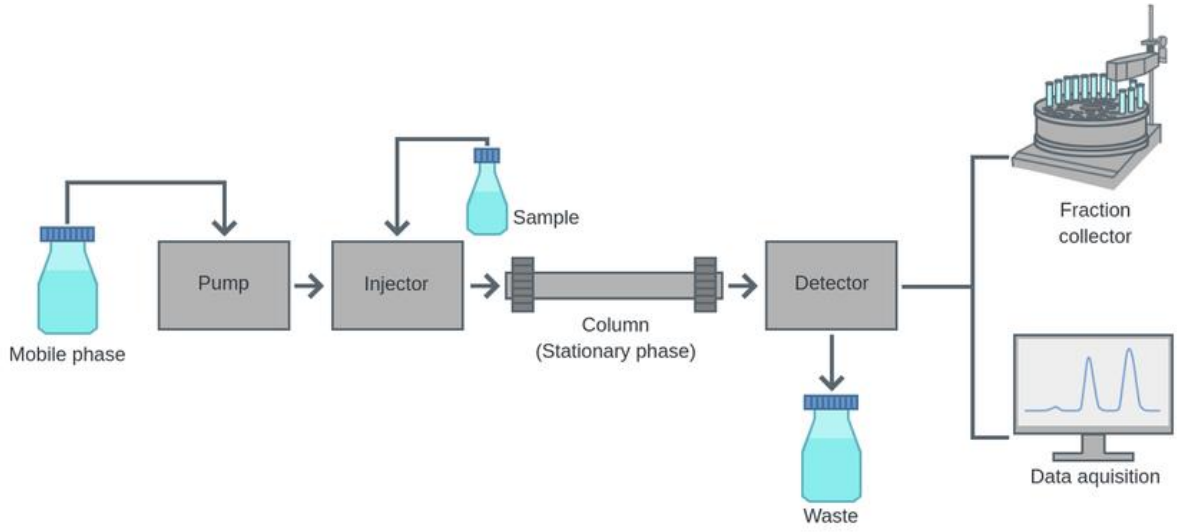

Figure 13: Path flow in liquid chromatography. Photo credit: theory.labster.com

1.4.3.1 Stationary phase and mobile phase in reverse phase chromatography

The stationary phase in a reverse-phase liquid chromatography column consists of packed non-polar particles, usually silica with covalently bonded alkyl chains. The alkyl chains may vary in length and properties, whereas some are straight chains and others can have phenyl groups attached. The non-polar stationary phase interacts with the analyte through non-polar interactions, such as van der Waals and pi-pi (π-π) interactions. The stationary phase's affinity is strongest to analytes with less polar properties, making them elute later than those with more polar properties.

In reverse-phase chromatography, it is common to use two different mobile phases during a chromatographic separation, where one is weak, and one is strong. The weak mobile phase often contains a very polar aqueous mixture which elutes the most polar analytes. The strong mobile phase often contains an organic solvent mixed with water, which can also interact with non-polar analytes and break the interactions between the non-polar analytes and the stationary phase. When performing the chromatographic separation, a gradient elution program is often used where a bigger portion of the total mobile phase is weak initially, and the proportion of the strong mobile phase gradually increases. The gradient elution program is used if the separation of analytes with isocratic elution is inadequate, which means that the mobile phase composition remains constant throughout the separation. The goal is to improve the separation of the analytes and to prevent them from eluting at the same time. The polar

analytes will elute first and have a shorter RT, followed by the non-polar analytes, which will have a longer RT.

Several other factors than mobile phase properties influence the RT and how well the chromatographic peaks are separated from each other. Some of these are flow rate, the particle size of the stationary phase, temperature, pH, and column dimensions. The flow rate, column dimensions and particle size affect the pressure in the column, and the aim is to ensure that the analytes have enough time to interact with the stationary phase. If the particles are smaller, more surface area is available, and a lower flow rate increases the interaction time. This will decrease the risk of fronting, which is when the analyte elutes before being able to interact with the stationary phase. When adjusting the pH in reverse-phase chromatography, a charge might be applied to analytes, making them interact more strongly with the mobile phase, causing a shorter RT. This is relevant if two of the analytes elute at approximately the same time, but only one can be charged under given conditions. Charging this analyte will increase the separation. A higher temperature decreases the mobile phase's viscosity, reducing backpressure in the column and decreasing the risk of tailing.

There is a distinction between High-Performance Liquid Chromatography (HPLC) and Ultra High-Performance Liquid Chromatography (UHPLC), mainly the particle size and pressure. In HPLC, the particle size is between 3-5 µM, and the pressure is 2000-4000 PSI, compared to 1.5-3 µM and 6000-20 000 PSI, respectively, in UHPLC. UHPLC provides a better signal-to-noise ratio (S/N) and increased resolution due to narrower chromatographic peaks.

1.4.4 Mass spectrometry

Mass spectrometry is used in quantitative and qualitative substance analysis, requiring advanced and expensive equipment. The principle utilizes different ions' acceleration in a magnetic or electrostatic field. The acceleration depends on the mass (m) and charge (z), and the molecular ions and fragments are measured in the unit m/z. Molecular ions are ionized analytes, and these can be further fragmented.

1.4.4.1 Ion source

The analytes can be injected directly from a liquid chromatographer, and the first process happening in the mass spectrometer is the ionization of the analytes. Electrospray ionization (ESI) is often employed as an ionization technique combined with liquid chromatography. Charged analytes solved in the mobile phase are transformed into aerosols, and a charge is

added to the analytes by utilizing a given voltage which can be negative or positive. The liquid in the aerosol will eventually evaporate with the help of drying gas, e.g., nitrogen, creating charged analyte molecules steered into the mass analyzer with the help of an electric field. Depending on the voltage's charge, the analytes will be either deprotonated or protonated. Adducts such as Na^+, NH_4^+ and $HCOO^-$ can be formed if other ions are present in the mobile phase. ESI is a soft ionization technique, meaning that primarily molecular ions are formed without further fragmentation. Fragments might be formed depending on the energy applied in the ionization source.

1.4.4.2 Mass filter

The quadrupole is often used as a mass filter in MS, consisting of four parallel cylindrical metal rods. In the quadrupole, the ions are exposed to an electric field that will steer analytes of a specific m/z value through an ion detection system. The analytes move in trajectories according to the electrical field set to the quadrupole, and the analytes with unstable trajectories are filtered out. The quadrupole will have pre-set settings in its radiofrequency to ensure that wanted analytes of a given m/z range pass through in a stable trajectory. A transmission range of 50-2000 m/z is possible, but a lower range is more common. All analytes with a m/z within this range are further transferred in the mass spectrometer.

The quadrupole might also be used as a mass analyzer in MS analysis. The ions of preferred m/z can be monitored and scanned from the quadrupole.

1.4.4.3 Mass analyzers

A linear quadrupole ion trap (LIT) consists of a set of quadrupole rods encapsulated in a ring electrode and is confined by two endcap electrodes. As the name ion trap suggests, it can trap and hold ions before ejecting them for analysis or further fragmentation. Both positive and negative charges along the rods will keep the ions in a stable trajectory within the ion trap. The end cap electrodes function as a wall with a stopping potential that prevents ions from escaping. The ions can be ejected based on a given m/z range to the ion detection system for fullscan analysis, or only selected ions might be ejected. The LIT can also function as a collision cell to further fragment the ions. In this case, a buffer gas is present within the LIT and serves as a collision gas if fragmentation is performed.

A C-trap is another known ion trap equipped by the Orbitrap instrument. It co-functions with the Orbitrap by holding the ions until the orbitrap is ready to receive them for ion detection.

While holding the ions, the C-trap performs so-called micro scans as a control function. The scans are of its ion contents and are utilized in the Orbitrap scan, providing better S/N.

The Orbitrap is among the newest mass analyzers on the market and is used because of its high-resolution analysis. The Orbitrap instrument can be used for various applications, such as metabolomics, lipidomics, proteomics, and environmental analysis. As the name suggests, the concept is ions trapped in orbit controlled by an electrostatic field created by a difference in voltage between an inner and outer electrode. The inner electrode is referred to as a spindle. The orbitrap operates at high vacuum levels, which is critical to ensure accurate detection and analysis of ions. The orbitrap is shaped uniquely (Figure 14), and its structure contributes to the orbiting of ions. The ions move at a given oscillation frequency along the z-axis, depending on their m/z value (15). The measurement accuracy depends on the number of oscillations of the ions. The Orbitrap has a large dynamic range, allowing for the analysis of both high and low-abundance compounds in a single experiment. The Orbitrap can acquire many measurement points per unit of time, which is useful in analyzing large complex samples. The high resolution is of great advantage while performing analysis since it is easier to distinguish between near isobaric compounds, and the software suggests far fewer molecular compositions, which increases the reliability of the analysis (16).

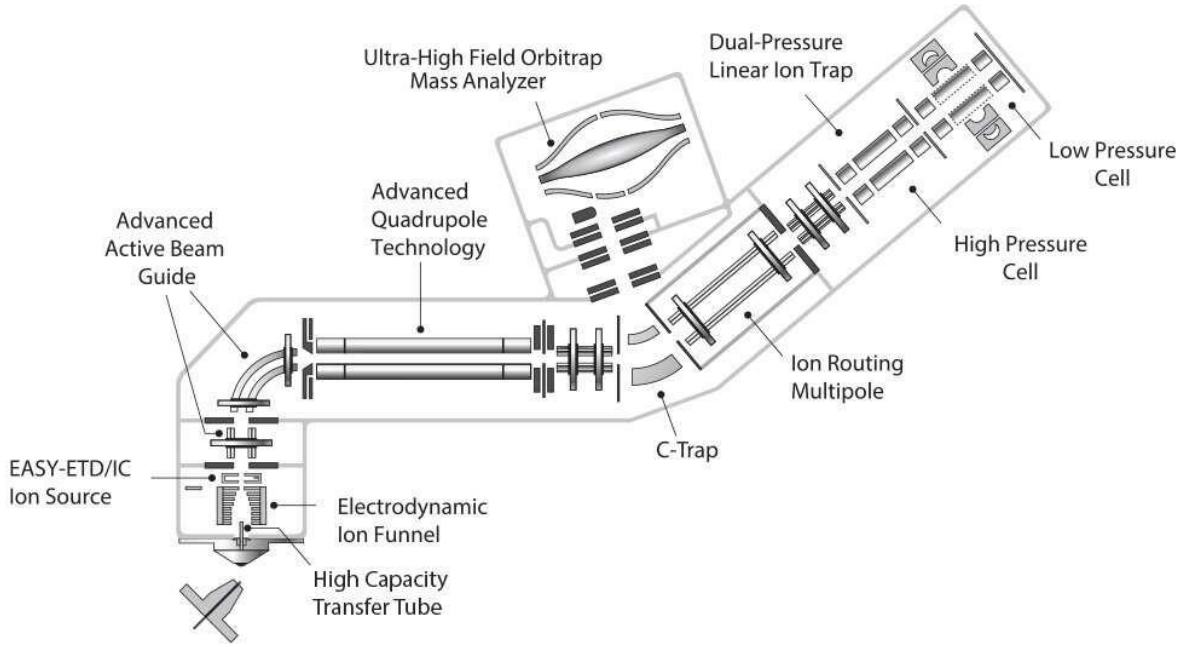

Figure 14: Orbitrap Tribrid Mass Spectrometer. Photo credit: Thermofisher.com

1.4.4.4 MS^n fragmentation

To be able to look closely into which chemical structure the analyte has, one often must perform fragmentation of the molecule ion. This means breaking it into smaller fragments characteristic of that specific analyte. The fragmentation pattern might be compared to a fingerprint, and isobaric compounds can have the same m/z but a different fragmentation pattern. Which fragments are made depends on which kind of fragmentation method is used. A low-energy fragmentation method might only be able to cleave the ion at weaker bonds, like C-O and N-O, while high-energy fragmentation can break C-C bonds. The separate ions must be isolated from other ions in the sample before fragmenting.

Different manufacturers of mass spectrometry equipment might use the same terminology for different methods. In this thesis, Thermo Fischer Scientifics terminology is used. Two common techniques for fragmenting are collision-induced dissociation (CID) and high-energy collisional dissociation (HCD), whereas the first is of low energy. In CID-fragmentation, the ions are accelerated by an electrical potential while colliding with an inert gas, e.g., helium or nitrogen. The collision gas collides with the ions, which transfers some of its kinetic energy to internal energy for the ions. When the internal energy reaches a certain threshold, weaker intermolecular bonds break, which leaves fragments. In HCD, an increased amount of acceleration energy is applied to the ions. One collision with the collision gas might be enough to break bonds, and the remaining ions will retain kinetic energy, predisposing them to further collisions and fragmentation. In CID-fragmentation, only a set m/z will fragment, which leads to no further fragmentation of its fragment. In HCD, other m/z will also be allowed to fragment, which, together with the increased energy, leads to more extensive fragmentation patterns in the mass spectra.

In the Orbitrap ID-X Tribrid (Thermo Fisher Scientific, Waltham, MA, USA) instrument (Figure 14), both CID fragmentation and HCD fragmentation happen. Ions trapped in the C-trap can either be ejected directly to the Orbitrap for detection or sent to the ion traps for further fragmentation. HCD occurs in the ion-routing multipole. The fragments can either be ejected back to the C-trap and Orbitrap for analysis or undergo in-depth fragmentation by CID in the linear ion trap. Detection of the multiple fragments is often performed in the linear ion trap as it is less time consuming, and the Orbitrap itself is better suited for fullscan analysis. Linear ion trap scans are of relatively low resolution as a trade-off for the high scan speed.

1.4.5 Lipid identification

1.4.5.1 Lipidomics

In the lipidomics field, the aim is to measure the lipid inventories of biological systems, often referred to as the lipidome. The analyte's nature influences their extractability and ionization, and which instruments are best fitted to perform the analysis. Lipidomics is performed using mass spectrometry after chromatography separation (LC-MS). The detection depends not only on their ionization properties but might be interfered with by other compounds in the sample. The lipid structures might compete for ionization, and they can interact with one another through gas-phase reactions, and the latter might lead to suppressed signals in the mass spectrum. Lipid structures that suppress other lipid signals can be removed before analysis to improve the results (12). With help from LC-MS, the lipids can be identified and quantified, giving us a tool to understand the diversity of the given lipidome better.

1.4.5.2 AcquireX

AcquireX (Thermo Fisher Scientific, Waltham, MA, USA) is a data collection software program made by Thermo Fisher Scientific that allows for complex samples to be analyzed. The data generated through AcquireX can then be further utilized for lipidomic analysis. When running the analysis, it is common to perform a MS^n scan of a pooled sample, the ID sample, to identify which lipids are present in the samples. Usually, this must be performed several times to obtain the samples' complete overall lipid profile. The program does this by performing MS^n on the most intense peaks before excluding them from the next ID Sample run. Then the program performs MS^n on the most intense remaining peaks, and so on. This ensures that all lipids of even low intensity are included when lipid identification is performed in the later samples where fullscan analysis is performed. This also saves a lot of run time by increasing the efficiency. To reduce the noise caused by impurities and contamination in the solvents and UHPLC system, AcquireX makes exclusion lists from blank samples.

1.4.5.3 LipidSearch

Lipidsearch is a lipid database created by Thermo Fisher Scientific to help perform lipid identification and quantification. The database contains more than 1.5 million lipid ions, which include fragmentation patterns and lipid adduct ions. A group-specific ID algorithm optimized for targeted identification is used, and for untargeted identification, a comprehensive ID algorithm is used. Neutral loss scans and precursor ion scans are used by the group-specific algorithm to identify lipids based on their FAs or polar head groups. A

combination of database matching and product ion scans are used by the comprehensive ID algorithms to identify lipids (17).

2 The aim of the thesis

The aim of the project is to perform high-resolution mass spectrometry to characterize the lipid profile/lipidome in lipid-rich bacterial biomass from a mixed microbial culturing process. Before mass spectrometry, the sample is prepared and separated using lipid extraction with dichloromethane and methanol, and liquid chromatography.

To accomplish the primary goal, several sub-goals were set:

- Test which lipid extraction method produces the highest yield of extracted lipids from a mixed microbial culture
- Test methods of separating PHA from the other lipids because of incompatibility with LC-MS
- Investigate the occurrence of phospholipids, triacylglycerols, and wax esters in the mixed microbial culture to obtain an overview of the lipidome
- Investigate the occurrence of DHA and EPA in the lipids

3 Materials and methods

3.1 Chemicals

Table 1 Chemicals used for accumulation, lipid extraction, method testing, and LC-MS analysis.

SUBSTANCE	PURITY	CAS-NUMBER	SUPPLIER
2-PROPANOL	≥ 99.9 %	67-63-0	VWR International, Leuven, Belgium
ACETONITRILE	≥ 99.9 %	75-05-8	VWR International
AMMONIUM FORMATE	≥ 99.9 %	540-69-2	Merck KGaA, Dramstadt, Germany
DICHLOROMETHANE	≥ 99.9 %	75-09-2	VWR International
FORMIC ACID	≥ 99.9 %	64-18-6	Thermo Fisher Scientific, Waltham, MA, USA
HEPTANE	≥ 99.9 %	142-82-5	Merck KGaA
METHANOL	≥ 99.9 %	67-56-1	VWR International
MILLI-Q WATER			Millipore Corporation, Molsheim, France
PROPIONATE	≥ 99.9 %	137-40-6	VWR International
SODIUM CHLORIDE	≥ 99.9 %	7647-14-5	VWR International

3.2 Materials

Table 2 Materials used for accumulation, lipid extraction, method testing, and LC-MS analysis

DESCRIPTION	NAME OF EQUIPMENT	SUPPLIER
BIOREACTOR FOR ACCUMULATION	Minifors 2	Infors HT, Bottmingen, Switzerland
CENTRIFUGE	Heraeus Multifuge X3R	Thermo Fisher Scientific
EVAPORATOR	Rotavapor R-3	BÜCHI Labortechnik AG, Flawil, Switzerland
FILTER	Whatman filter papers 1, 90 mm, Cat No 1001 090	Merck KGaA
FREEZE DRYER	Labconco FreeZone Freeze Dry System, Bench Top	Labconco Corporation, Kansas City, MO, USA
GLASS PIPETTES	Disposable Glass Pasteur Pipettes 150 mm	VWR International
LC-MS VIALS	Amber MRV Screw Top Vial 12x32mm	Waters, Milford, MA, USA
SONICATOR	Vibra Cell VCX 750	SONICS AND MATERIALS, INC., Newtown, CT, USA
TEST TUBES 50 ML	VWR Centrifuge tube 50 mL, Cat No 21008-240	VWR International
THIMBLE	Whatman Extraction thimbles 603, Cat No 10350211	Merck KGaA
VACUUM PUMP	Vacuum Pump V-700	BÜCHI Labortechnik AG, Flawil, Switzerland

3.3 Bacterial fermentation

3.3.1 Sequence batch reactor

The sequence batch reactor (SBR) is where all biomass was harvested from, and it was operated by personnel at the Department of Chemistry, UiT. The main components in the SBR system are a 2.1-liter vessel, a motor and an impeller for stirring, an air valve, a withdrawal valve, an acid/base pump, a carbon pump, a mineral solution pump, a nitrogen pump, and a purge pump. These components are turned on/off by relays controlled by custom-made software (by KGS Systemer AS, Tromsø) and a computer. Parameters that were monitored daily during a 30-day period of the SBR process include pH, temperature, dissolved oxygen (DO), total organic carbon (TOC), ammonium nitrogen ($NH4^+$–N), mixed liquid suspended solids (MLSS) (g/L), settled sludge volume (SSV) (mm), sludge volume index (SVI) (SSV/MLSS), feast to famine ratio (F/F) and food to biomass ratio (F/M).

The parameters varied throughout the 30-day run, and the SBR was regularly reset because of unwanted filamentous growth (likely a mix of bacteria and fungi) in the reactor. The carbon source used in this project was a CG side stream fraction called glycerol fraction (GF). In December, the carbon source was changed from GF to commercial propionic acid (PA) to prevent unwanted filamentous growth. The SBR was continuously run with 12-hour cycles where F/F was carried out to promote lipid accumulation. Feast was initiated by adding carbon and was completed when all carbon was consumed. This was seen by a sharp increase in DO (Figure 15) and a low TOC. Famine was initiated by the addition of nitrogen via ammonium of pH 7. Dissolved oxygen (DO) in the SBR was measured through the process and indicated the bacteria's state.

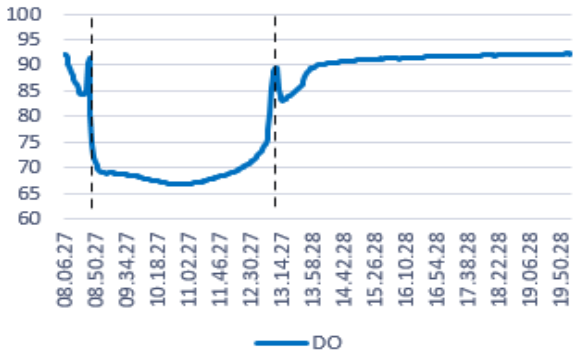

Figure 15 The y-axis is the percentage of dissolved oxygen (DO), and the x-axis is the timestamps for a typical SBR cycle set up to run from 08.00. Typical F/F cycle, where DO is dissolved oxygen. DO decreases by adding carbon at 08.50 and increases when all carbon is consumed.

A given amount of the bioreactor content was purged at the end of each cycle, between 50-250 mL. The main component in the vessel was a mineral solution composed of KH_2PO_4, allylthiourea, EDTA, sea salt, trace element solution (premade by personnel at the Department of Chemistry, Uit) and H_2O. Before the feast, the MMC is laid to settle to the bottom of the SBR. 50% of the mineral solution in the SBR is removed before a new mineral solution replaces it. The MMC should be able to flocculate when settling, which is the aggregation of particles to form larger clumps called flocs (Figure 16). Flocs are well suspended in the mineral solution, and the MMC is at risk for caking without flocculation. Caking is the formation of a dense layer at the bottom and can be hard to re-suspend in the mineral solution. For each startup of the SBR, it was assumed that it had to run for about one month before the environment within the reactor was stable. The purge was still withdrawn from the beginning of the SBR process and was utilized for accumulation even if the environment was not entirely stable.

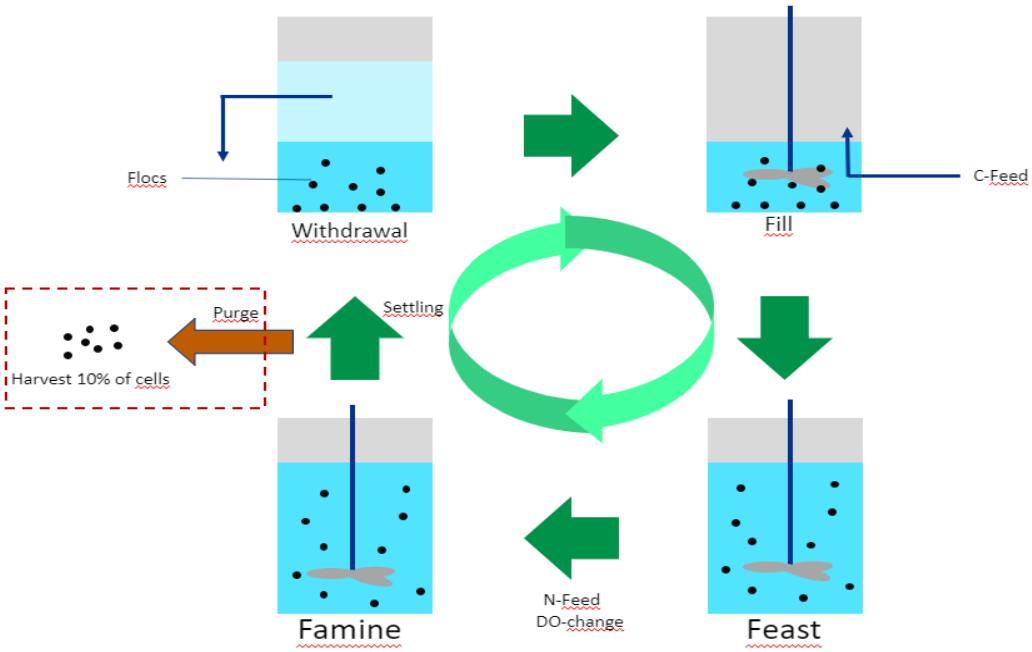

Figure 16 SBR cycle.

3.3.2 Accumulation

250 mL of the SBR content (purge) was collected after each cycle. Accumulation was executed after two or four withdrawals of purge had been collected, and the biomass could settle at the bottom of the collection flask. Excess fluid was removed until a volume of 500 mL was left in the collection flask. The 500 mL was transferred to a 2-liter lab-scale fermentor, Minifors2 (Infors HT, Bottmingen, Switzerland), and 500 mL of mineral solution was added. The accumulation was inoculated with GF (typically 12-50 C-mmol/liter) during the first three runs before changing the carbon source to PA. The mixture was stirred at 150 rpm, supplied with 0.5-1.5 L*min^{-1} oxygen and temperature regulated to 19 °C. The parameters during accumulation are given in Table 3. The bacteria were fed in pulses with GF/PA until there was no visible or seemingly significant change in the dissolved oxygen level in the mixture (DO) after feeding (Figure 17). When saturated, harvesting of the biomass was done. The mixture was transferred into a 1000 mL beaker, and the biomass was left to settle. The excess fluid was removed from the beaker by manually pipetting and centrifuging (after being moved to four 50 mL test tubes). The biomass was then frozen at –80 °C, lyophilized, and stored at –80 °C before further processing.

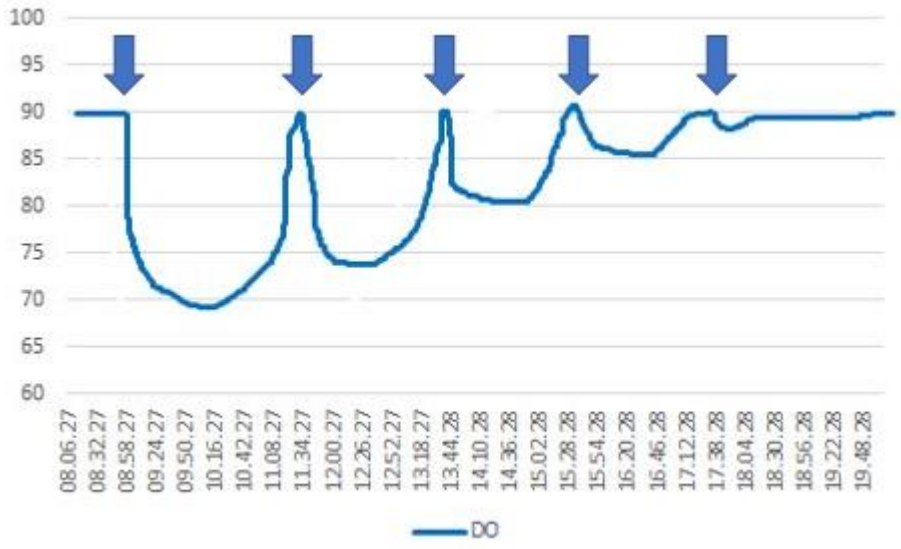

Figure 17 Ideal accumulation DO trend.

Table 3 Parameters during all accumulations (ACC 1-6) performed. pH and PO2 were measured during accumulation and airflow and stirring were set to a specific value. The carbon source was changed from GF to PA, and the amount of added carbon source varied. The number of inoculations was 1 or 2, and the lipid extraction method changed due to challenges with extraction (see section 3.4).

PARAMETER	ACC 1	ACC 2	ACC 3	ACC 4	ACC 5	ACC 6
PH	8.3	6.9	7.2	7.7	8	7
MAX PO2 WHEN STABLE	90.8%	85.8%	93.8	90.7%	55.9%	82.5%
AIRFLOW	0.4 L/min	0.4 L/min	0.82 L/min	0.5 L/min	1 L/min	1 L/min
STIRRING	150 rpm	150 rpm	150 rpm	150 rpm	150 rpm	170 rpm
CARBON SOURCE	GF	GF	GF	PA	PA	PA
GF ADDED	2.3 mL	2.3 mL	2.2 + 4.6 mL	-	-	-
PA ADDED	-	-	-	50 mL	80 mL	25 mL
TIMES INOCULATED	2	2	2	1	1	2
EXTRACTION METHOD	Soxhlet	-	-	Soxhlet	Soxhlet, sonication, direct extraction, DCM+MeOH	Sonication

3.4 Lipid extraction method development

The following three methods were compared to examine total lipid extraction yields, including PHA

1. Soxhlet extraction in DCM
2. Direct extraction in DCM
3. Sonication followed by direct extraction in DCM

All the different methods were performed with lyophilized biomass that was accumulated within a week before. The same biomass was utilized for the three methods to ensure a sound basis for comparison. The biomass was accumulated and lyophilized as described in section 3.3.2. The biomass was crushed until homogeneous before use. The solvent used for extraction was dichloromethane (DCM) in all the extractions. Whatman filter papers were used since they are DCM-proof. The goal was to analyze all the samples at a 1 mg/mL concentration. All methods were performed in only one parallel because of the available time, biomass, and equipment. See Figure 18.

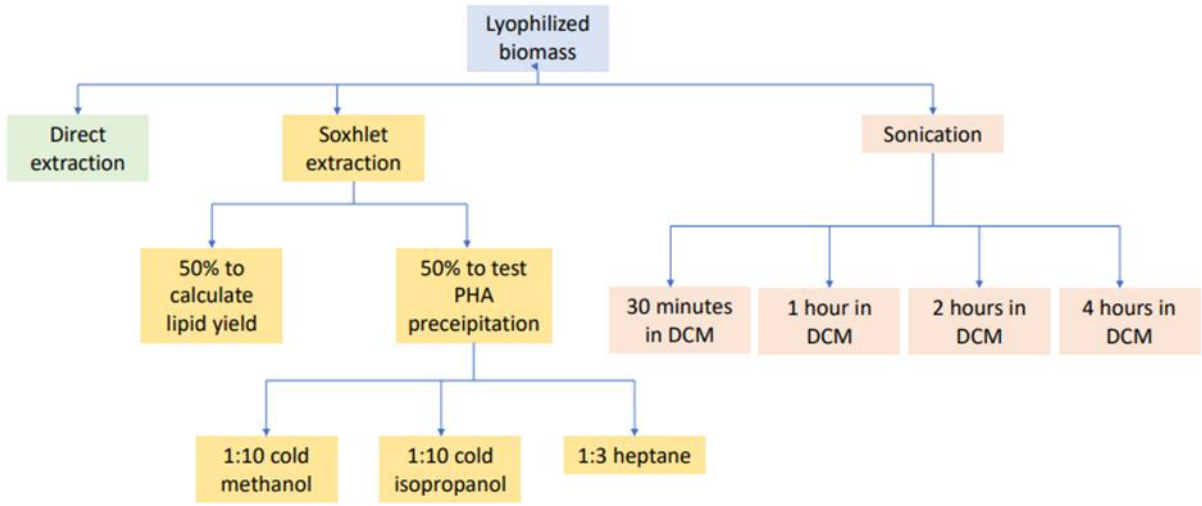

Figure 18 Flowchart describing the different lipid extraction methods tested and which anti-solvents were tested when examining precipitation of PHA.

3.4.1 Soxhlet extraction

The equipment used in the extraction was rinsed twice in DCM and once in MeOH. Homogenized biomass from accumulation was defrosted and added to a thimble for extraction. The amount varied between runs, but a 0.5-1 g dry weight was used. The thimble with biomass was weighed before extraction and placed in the Soxhlet extractor. The equipment was set up with a Dimroth condenser, sealed, and placed in a mantle. 200 mL of DCM was filled in a 500 mL round bottom flask as the extraction solvent, and three boiling stones were added. The mantle was turned on and set to around 40 °C. Great attention was paid to the first cycles to ensure no leaking and an appropriate cycle time of about seven minutes (18). If the cycle time got too high, the temperature would be increased, and vice versa. The extraction went on for five hours. After the extraction, the mantel was turned off, and the extract (DCM and lipids) was left to cool down. The thimble was removed from the thimble chamber, and leftover extract in this chamber was poured into the round bottom flask (18, 19, 20). Half of the extract was vaporized in a rotavapor at room temperature at 200 mbars until dry to weigh and calculate lipid yield. In the other half, most DCM (until it was left with 5-10 mL of extract) was vaporized in a rotavapor at room temperature with a pressure of 200 mbars. The remaining extract was stored at room temperature before attempting to precipitate PHA (see section 3.5).

Lipids in Sample 1 and 2 were extracted by Soxhlet extraction.

3.4.2 Direct extraction

Direct extraction was performed by suspending 1-1.5 g homogenized biomass in 150 mL DCM, which was put on magnetic stirring in an Erlenmeyer flask for four hours. Vacuum filtration with a Büchner flask was used to remove the remaining biomass, and the extract was transferred to a pre-weighed round bottom flask. DCM was evaporated using a rotavapor at room temperature with a pressure of 200 mbars before weighing the remaining lipids in the round bottom flask to calculate the yield.

3.4.3 Sonication

Sonication was performed using a Sonics Vibra Cell VCX 750, and the aim was to break down cell walls to increase the exposure of DCM to membranous and intracellular lipids. Sonication was performed by suspending homogenized biomass in 50 mL of distilled water in a 100 mL beaker. The probe sonicator was set to sonicate for 30 minutes with pulsating intervals of 30 seconds on and 5 seconds off. The amplitude was set to 40% of 750 W. About

1 g of biomass was sonicated at a time, and a total of 3.6381g was used in this experiment, which resulted in four sonication sessions. Centrifugation was performed at 4000 rpm at 4 °C for 6 minutes, and the supernatant was pipetted off before lyophilizing the biomass.

3.4.3.1 Extraction after sonication

The lyophilized biomass was divided into four fractions. The biomass fractions were suspended in 150 mL DCM in four separate Erlenmeyer flasks and put on magnetic stirring. The fractions were left on stirring for 30 minutes, 1 hour, 2 hours, and 4 hours to compare if the extraction times made a difference in lipid yield. After the given extraction times, all fractions were immediately filtrated with vacuum filtration with a Büchner flask to terminate the extraction process. The extracts were transferred to four separate pre-weighed round bottom flasks. DCM was evaporated using a rotavapor at room temperature with a pressure of 200 mbars before weighing the remaining lipids in the round bottom flasks to calculate the yield.

Because of a lack of PLs in the analyzed lipid samples, it was suggested to try a mixture between DCM and MeOH as an extraction solvent to obtain more PLs in the lipid sample. This method has been used for lipid extraction from, e.g., algae, and it is a modification of the Folch extraction method (21). The homogenous sonicated biomass was suspended in 150 mL DCM:MeOH (2:1) in an Erlenmeyer flask and put on magnetic stirring for 30 minutes. Vacuum filtration with a Büchner flask was used to remove the remaining biomass, and the extract was transferred to a pre-weighed round bottom flask. DCM and MeOH were evaporated using a rotavapor at room temperature with a pressure of 200 mbars before weighing the remaining lipids in the round bottom flask. The yield from the DCM:MeOH extraction was not considered when comparing lipid extraction yields. The lipids were stored at -20 °C before being solved in isopropanol to obtain a 2 mg/mL concentration. 1 mg/mL was used in former analyses, but the intensity of signals was weaker than expected. It was decided to try a higher concentration to see if the signals would improve. Lipids in Sample 4 were extracted by sonication followed by direct extraction in DCM:MeOH.

3.4.4 Lipid yield results

Table 4 represents lipid yields after each extraction method. The table does not include the yield of when lipid extraction was performed using DCM:MeOH as the extraction solvent. This is because the yield of sonication followed by direct extraction in DCM was already calculated beforehand in Table 4, and this method already provided the highest yield. Adding

MeOH as an extraction solvent was performed to increase the number of extracted PLs, not to increase lipid yield.

All lipid samples in Table 4 are included in Sample 3.

Table 4 Lipid yield results after performing Soxhlet extraction, direct extraction, and sonication followed by direct extraction in DCM. The calculated lipid yield was highest for sonication, followed by direct extraction in DCM.

METHOD	BIOMASS START (G) (AFTER SONICATION FOR THE SONICATED SAMPLES)	LIPID WEIGHT (G)	YIELD	CONCENTRATION WHEN ANALYZED
SOXHLET	1.7794	0.1928	10.84 %	1 mg/mL
DIRECT	1.0025	0.099	9.88 %	1 mg/mL
SONICATOR 30 MIN	0.6782	0.183	26.98 %	1 mg/mL
SONICATOR 1 HOUR	0.7273	0.166	22.82 %	1 mg/mL
SONICATOR 2 HOURS	0.6891	0.145	21.04 %	1 mg/mL
SONICATOR 4 HOURS	0.9249	0.177	19.14 %	1 mg/mL

3.4.5 Final method

Sonication followed by direct extraction in DCM is the preferred method for lipid extraction. This is because of a greater lipid yield calculated in Table 4. Time and solvent are saved, and the method involves less health risk involved for operating personnel. MeOH should be added to the extraction solvent in a 2:1 ratio of DCM:MeOH. This is because lipids of a polar character, such as PLs, are extracted to a higher degree by using MeOH in the extraction solvent. Since there were no problems using 2 mg/mL instead of 1 mg/mL, a concentration of 2 mg/mL is recommended to obtain stronger signals of the lipids in the LC-MS analysis. The final two analyses, for Sample 4 and 5, were performed with a concentration of 2 mg/mL.

This is the final protocol based on the results from Table 4. Lipids in Sample 5 were extracted by this final protocol.

1. Perform accumulation as described in section 3.3.2 but exclude lyophilization and freezing the sample at the end of the procedure.
2. Divide the accumulated biomass into suited containers for sonication (e.g., 100 mL glass beaker) to obtain a concentration of about 30 g/L.
3. Sonicate for 30 minutes with pulses of 30 seconds on and 5 seconds off with an amplitude of 40% of 750 W.
4. Transfer sonicated biomass to a 50 mL falcon tube and centrifuge for 6 minutes with a speed of 4000 rpm at 4 ℃.
5. Pour off supernatant (the biomass should stay behind as a firm mass in the tube).
6. Freeze at –80 °C, lyophilize and store at –80 °C before further processing.
7. Transfer 1 g of lyophilized, homogenous biomass into an Erlenmeyer flask
8. Add 100-150 mL of DCM:MeOH 2:1 and put on magnetic stirring for 30 minutes.
9. Filtrate with a Whatman filter using vacuum filtration.
10. Transfer to a pre-weighed round bottom flask and evaporate solvent with a rotavapor with a pressure of 200 mbars and at room temperature. Increase the temperature to 40 ℃ if necessary.
11. Weigh lipids and solve in isopropanol to achieve a 2 mg/mL concentration.
12. Use a filter syringe to filtrate away solid lipids and analyze on the UHPLC-MS
13. Store solid lipids for further analysis of, e.g., PHA (depends on the content of the solid lipids) on GC-MS

3.5 PHA precipitation

Since PHA is incompatible with the LC-MS for analysis, separating it from the rest of the lipids was important. A Ph.D. student connected to the project, Maria Wilhelmsen Hoff, performed a literature search to find the most suitable method for lipid extraction and PHA precipitation. It was tested by Maria Wilhelmsen Hoff but with a focus on PHA precipitation from lipids from single strained bacteria. The protocol had to be tested in this thesis to retain all other lipids than PHA in solution.

3.5.1 Background: PHA in single-strained bacteria

Earlier research projects regarding PHA production in single-strain bacteria have been carried out and are continued by Maria Wilhelmsen Hoff. Certain arctic single strains collected by MarBank are known to produce PHA in literature. The monomers in PHA occurs in different carbon chain length, and which length occurs in the different strains are described in literature. The aim is to confirm that the arctic single strains produce PHA of the given monomer. If correct, single strains could be mixed to create a synthetic MMC where the lipid profile would be more predictable. The single strains are fed with category three salmon oil, which is considered a waste material since it is not suited for human consumption. The sample preparation for the single strained bacteria is identical to the biomass from the SBR after accumulation, but the focus is on PHA instead of the whole lipidome. Lipidomics with LC-MS has been performed for the single strain samples (as a part of Sample 3), but the results were not directly relevant to this thesis. A specific method for sample preparation and analysis of PHA is described within the research project and will not be included in this thesis.

3.5.2 PHA precipitation: the original method

After performing lipid extraction for Sample 1, the extract was added dropwise to ice-cold MeOH for precipitation of PHA. It was then transferred to a DCM-proof centrifuge tube to be centrifuged at 3000 rpm until the precipitant formed a dense layer at the bottom. The extract, i.e., the supernatant, was dried in a preweighed 250 mL round bottom flask by the rotavapor at 200 mbars (18, 19), but this time in a water bath at 40 °C since MeOh has a higher boiling point than DCM. Removing the last drop of fluid in the round bottom flask was difficult. The waste flask was emptied to achieve complete dryness, and the pressure was lowered to 150 mbars for a few minutes. The dried lipids were stored at –20 °C before being dissolved in a calculated amount of isopropanol to achieve a 1 mg/mL concentration before analysis. Only a

few lipids were obtained during this run since it was later discovered that adding the extract dropwise to ice-cold MeOH would lead to the precipitation of most lipids, not only PHA. This means that the supernatant after centrifugation would contain little to no lipids.

3.5.3 PHA precipitation, change of protocol.

The protocol was corrected, and the method for precipitation of PHA was altered. Instead of the previous method, MeOH was added dropwise to the extract until precipitation occurred. It is believed that PHA would be the first of the lipids to precipitate because of its non-polar structure as a polymer. This was performed on the second run of Soxhlet extraction, of which lipids of Sample 2 were extracted. A substance in the extract precipitated as expected but separating it from the supernatant was difficult because of its apparently similar density to DCM. Centrifuging the sample was attempted, but no proper sedimentation was obtained. Alternative methods for separation were considered, e.g., the use of filter paper or filter syringe, but these had to be compatible with DCM. No options were available on the extraction day, so the total extract was dried in the rotavapor at room temperature at 200 mbars before being stored at –20 °C before analysis. Because of this seemingly failed attempt of PHA precipitation, it was decided to explore alternative ways to separate PHA from the rest of the lipids.

3.5.3.1 Dissolution in isopropanol

Before analysis of Sample 2, the extracted lipids were attempted to be solved in isopropanol to achieve a concentration of 1mg/mL. Still, some of the lipids stayed solid in isopropanol. Based on earlier experience, PLs and TGs dissolve in isopropanol at room temperature at this concentration. A filter syringe was used to separate the solid lipids from the supernatant before analysis. It is unknown which lipids stayed solid in isopropanol, but it might be PHA.

3.5.4 PHA precipitation, further method development

50% of the Soxhlet extract from section 3.4.1 was used to test how to precipitate PHA most efficiently. The extract was divided into three separate fractions. Methanol (22, 23), isopropanol, and heptane (24, 25) were attempted to use as anti-solvents to hopefully precipitate PHA at a 1:10, 1:10, and 1:3 ratio, respectively (Figure 18). No literature suggested isopropanol, but it was still examined due to the solid lipids in isopropanol, as described in section 3.5.3.1. Each of the anti-solvents was added to each fraction gradually until something precipitated and made sure that it kept precipitated. If not, more anti-solvent was added until the precipitant remained stable and unsolved. Vacuum filtration with a

Büchner flask was used to collect what is believed to be precipitated PHA on filter paper. The extract was transferred to a pre-weighed round bottom flask for evaporation before solving the lipids in isopropanol to obtain a 1 mg/mL concentration. The lipids were analyzed on LC-MS as a part of Sample 3. The content on the filter papers was dissolved in DCM before Maria Wilhelmsen Hoff performed further analysis.

3.6 Lipid analysis

The lipid analysis methods were the same for all lipid samples, but the sample concentration was increased from 1 mg/mL to 2 mg/mL in Sample 4 and 5 (Table 5).

Table 5 Overview of sample dates and concentrations.

SAMPLE NAME	ACCUMULATION BATCH	CONCENTRATION
SAMPLE 1	ACC 1	1 mg/mL
SAMPLE 2	ACC 4	1 mg/mL
SAMPLE 3	ACC 5	1 mg/mL
SAMPLE 4	ACC 5	2 mg/mL
SAMPLE 5	ACC 6	2 mg/mL

3.6.1 Chromatography method

Lipid separation was performed on a Waters Acquity UPLC® BEH C18 1.7µm 2.1 x 100 mm (Waters, Milford, MA, USA) reverse phase column. Mobile phase A consisted of 50%/50% ACN/MilliQ-water, and mobile phase B consisted of 49.5%/49.5%/1% 2-propanol/ACN/MilliQ-water. Both mobile phases contained 0.01% FA and 1mM NH_4FA, because ammonium from NH_4FA is required for TGs to form ammonium adducts in the mass spectrometer. TGs are non-polar and do not ionize efficiently, and therefore it is necessary to add protons or charge-carrying species to facilitate ionization. This improves the ionization efficiency and sensitivity of the analysis. The separation was run on a gradient elution program, illustrated in Figure 19, and described in Table 6. The column temperature was set

to 60 °C, and the injection volume for each sample was 2 µl. The flow rate was set to 0.6 ml/min for the analysis, and the total runtime was 30 minutes.

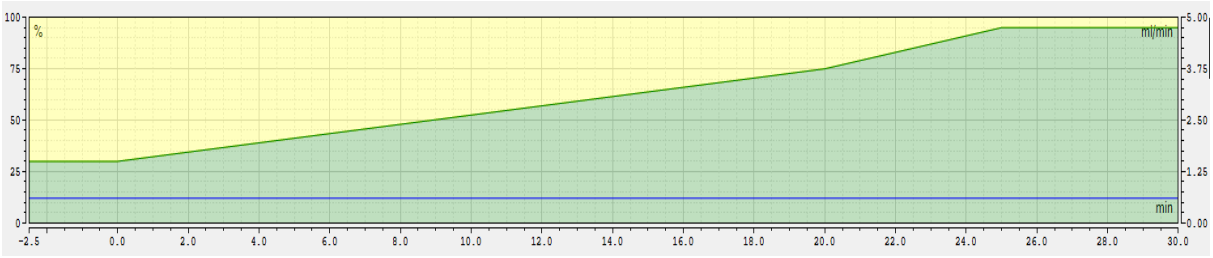

Figure 19 Flow gradient for chromatography method. Yellow is the % of mobile phase A and green is the % of mobile phase B. The y-axis is % of each mobile phase, and the x-axis is the time (-2.5 to 30 minutes).

Table 6 MS workflow in AcquireX.

No	Time	Flow [ml/min]	%B	Curve
1	-2.500	Equilibration		
2	-2.500	0.600	30.0	5
3	0.000	0.600	30.0	5
4	*New Row*			
5	0.000	Run		
6	0.000	0.600	30.0	5
7	20.000	0.600	75.0	5
8	25.000	0.600	95.0	5
9	*New Row*			
10	30.000	Stop Run		

3.6.2 Mass spectrometry method

An Orbitrap ID-X Tribrid MS (Thermo Fisher Scientific, Waltham, MA, USA) with electrospray ionization in positive mode was used to perform the MS analysis. The ion source settings are summarized in Appendix 1.

3.6.2.1 Fullscan method

The m/z scan range was set to m/z 250 to 1500 to ensure that all lipids of interest were scanned. The MS resolution was set to 120 000 FWHM, and ionization was set to positive mode.

3.6.2.2 MS^n method

Ions from the ID sample with an intensity above the set threshold were fragmented with HCD-fragmentation ($ddMS^2$ OT HCD) in the ion routing multipole before detection. PCs require a further fragmentation step compared to the other PLs since only the head group is

fragmented off during the HCD-fragmentation. The head group with an m/z of 184.0733 is identified during Targeted Mass Trigger, and the precursor ion is further fragmented with CID ($ddMS^2$ OT CID) to identify the FAs. Only one of the TGs FAs is fragmented off during HCD-fragmentation as a neutral loss. Another FA is further fragmented off with CID to identify the remaining FAs. This happens because the Targeted Loss Trigger identifies by m/z that the precursor ion is a TG. See Figure 20 for the MS^n workflow.

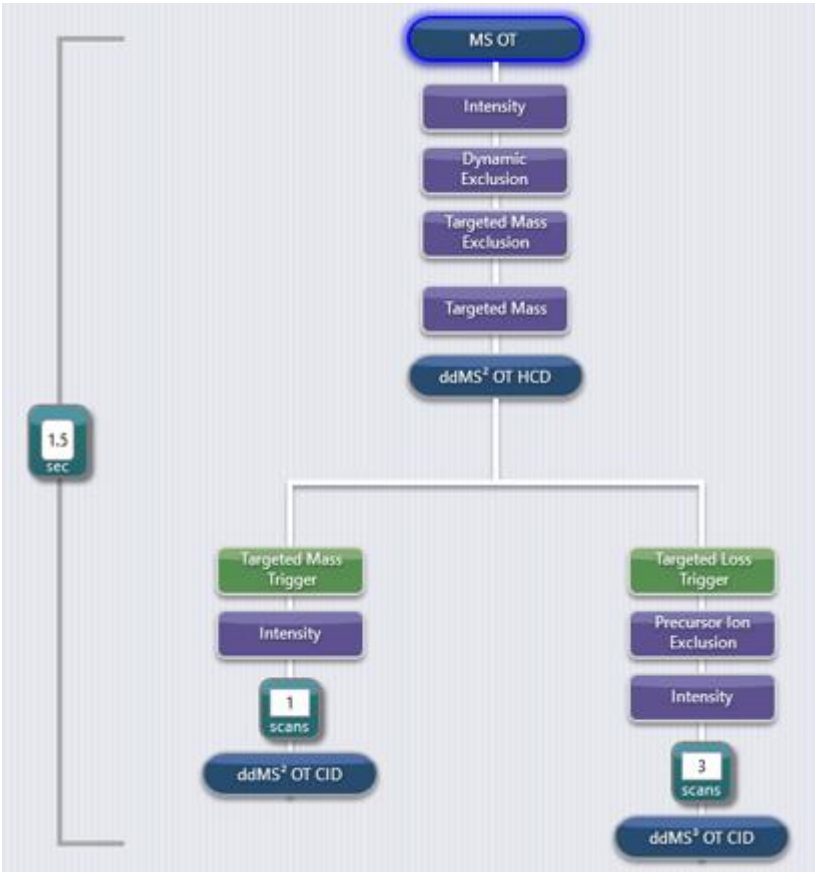

Figure 20 MS^n workflow.

3.6.3 AcquireX and Lipidsearch

In AcquireX, the blank samples were analyzed first with fullscan analysis to create an exclusion list. The exclusion list reduces noise caused by impurities and contaminants in the samples. The ID samples were analyzed with MS^n analysis afterward to identify all lipids in the samples. At last, the lipid samples were analyzed with fullscan analysis to determine which lipids exist in each lipid sample. The MS^n-spectra for the lipids were already identified from the ID samples. The RAW files and data from AcquireX were imported to Lipidsearch, where the lipids were identified and sorted.

The amount of data is immense in these kinds of analyses, and within the time and scope of this thesis, it is only possible to present a fraction of the data. Therefore, a selection of the data and results was made. The lipids of interest were TGs, WEs, and subclasses of PL, e.g., PC and PE. In the main results, only these lipid classes are included. PC and PE seemed to be about 75% of the total number of PLs and will therefore be the focus of PLs in the graphs from LipidSearch. MGs, DGs, and ceramides are also interesting but will be discussed separately from the TGs, WEs, and PLs. Lipids identified with identification grade A or B are those of most certain identification, and other grades will be excluded from the results. Definitions of the identification grades are given in Appendix 2. The manual filter used for inclusion criteria was (TotalGrade = "A" or TotalGrade = "B") and (ClassKey = "TG" or ClassKey = "PC" or ClassKey = "WE" or ClassKey = "PE"). When looking at the presence of the PUFAs docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), an additional criterion of LipidMolec.contains("22:6") or LipidMolec.contains("20:5") were added to find the lipids containing them in their molecular formula.

The parameters in LipidSearch were set as default. Retention time tolerance (Rt Tol (min)) was set to 0.05 min, and Retention time correction tolerance (Rt Correction Tol (min)) was set to 0.5. The S/N threshold was set to 3.0, and the Intensity ratio threshold was set to 1.5. The valid peak rate threshold was set to 0.5.

4 Results and discussion

4.1 Limitations concerning the biomass harvested from the SBR

It was not considered which state the SBR was in when biomass was purged for accumulation. It would be relevant and interesting to look at lipid content during the different stages in the SBR, but the main goal of this thesis is to develop a proper method for lipid extraction. The consequence is that the harvested biomass from the different accumulation runs might differ in lipid content and quality. The lipid profile results between samples might therefore not be entirely comparable.

4.2 Accumulation

In earlier work from the summer of 2022, an airflow of 0.4-1 L/min, stirring at 150-170 rpm, and a temperature of 19 ℃ was suggested. The airflow has varied between 0.4 and 1 L/min during the work in this thesis and has shown many different results in PO2. The maximum PO2 in each performed accumulation was presented in Table 3 and varied between 55% and 94%. No work has been performed during this thesis to examine changes in PO2 due to various factors. The amount of biomass should make a difference in PO2, but this has not been closely monitored. The biomass amount varies from batch to batch, depending on how dense the biomass in the SBR is and how much collected purge is available. If the amount of biomass is too low compared to the airflow, the measured PO2 might not decrease when adding carbon since the sensor is saturated by oxygen. The purged biomass has also been stored in a refrigerator for several days before accumulation, and it is not certain which state the bacteria are in. A lower PO2 should be visible if few bacteria are viable and can consume carbon and oxygen. The biomass might also be of high weight but of low quality. The biomass quality will be low if the portion of actual carbon-consuming bacteria in the biomass is low compared to filament occurrence and other possible non-consuming organisms in the SBR. Other organisms, such as fungi and microalgae, have been found in the SBR before the work of this thesis.

The amount of carbon source added to the mixture has also varied throughout this work. The amount of GF added was 2.3 mL, except for the last batch, when 4.6 mL was added at the second feeding. The amount of PA added had some variations based on the observed amount of biomass from the purge. A lot of biomass was purged during ACC 5, and the decision was made to increase the amount of PA from 50 mL to 80 mL. Ideally, several feedings should

have been performed during the accumulations, but it was time-consuming for the PO2 to stabilize and indicate that all carbon was consumed. This means that it is possible that the bacteria was not adequately saturated, which might have changed the results regarding the lipid profile. The bacteria should be able to consume the carbon quickly after addition since the purge happens at the end of famine in the SBR.

It is not easy to pinpoint exactly what causes differences in PO2 during accumulation and lipid classes during lipid analysis. It will be easier to examine and monitor when the SBR is running more stable and fewer changes in parameters and environment are made. The different parameters should be examined separately on the same purge sample to ensure no variation within the biomass**.**

4.3 Lipid extraction method development

4.3.1 Soxhlet extraction vs. direct extraction

The lipid yield was 1 % lower for direct extraction than Soxhlet extraction, but some lipids might have been lost when a failed filtration was initiated after the direct extraction. Since there was no vacuum filter available at the laboratory, it was tested to filter with just gravity. This did not work well since the DCM distributed itself with the lipids all over the filter paper. The DCM evaporated from the paper and left the lipids there. The filtration was immediately stopped when this was discovered, and the filter was pushed into the Erlenmeyer flask to dissolve the lipids from the paper. Some of the lipids may have gotten lost as it was a little difficult to insert the filter into the flask, and it was visible that some of the lipids remained on the gloves and tweezers. Because of this, it is reasonable to believe that the actual lipid yield was higher than calculated. The filtration method was changed to vacuum filtration. Even if the yield was lower for direct extraction than with Soxhlet extraction, it was decided to change the protocol to direct extraction since a lot less solvent was used. It is also possible to do several extraction parallels since no special equipment is needed. Only one Soxhlet setup is available at the lab where the work of this thesis was carried out. Direct extraction reduces the personnel's health risk since less solvent is used.

4.3.2 Sonication vs Soxhlet extraction and direct extraction

The lipid yield of sonication followed by direct extraction in DCM was about 10% higher than both Soxhlet and direct extraction, providing a total yield of about 22%. It must be considered that this yield is calculated with the biomass weight given in Table 4, which is the

weight of the lyophilized biomass after sonication. This was 3.0095g. The biomass before sonication was 3.6381g, which gave a yield of about 18%. Most likely, some of the hydrophilic contents of the biomass were lost to the water matrix while sonicating, which would explain why the biomass weight is lower. The biomass had also been transferred between several containers, which has caused a loss of biomass due to residue left behind in previous containers. Because of this biomass loss during the total process of sonication and extraction, one might conclude that the actual calculated yield is lower than the theoretical yield. Despite this, the yield was still doubled compared to Soxhlet extraction and direct extraction. This shows that sonication followed by direct extraction in DCM is the best method considering lipid yield. Later it was revealed that DCM:MeOH was the best extraction solvent compared to lipid extraction with DCM as the extraction solvent considering the number of PLs extracted.

There was no real difference in yield regarding the duration of extraction in DCM after sonication. It must be considered that the experiments were conducted using only one parallel of each parameter being tested, so the results should be looked at with a critical eye. Extraction of lipids in DCM should be a very effective and immediate method, which should explain why the yield does not seem to increase with increased duration. In both Soxhlet and direct extraction, DCM needs to break the cell wall before extraction, which is probably why it requires more time than the sonicated samples.

One advantage of sonication, in this case, is that the biomass does not need to be lyophilized after accumulation since the matrix used with the probe sonicator is water. Water is used both in the SBR and during accumulation, so it will not be as time-consuming as when this experiment was conducted (the biomass was lyophilized before sonication). This means the samples could be sonicated directly from the SBR if needed. The sonication and extraction would take ca 1.5 hours, compared to the Soxhlet, which quickly takes 6-7 hours.

4.3.3 Lipid content results

The aim was to analyze all samples at a concentration of 1 mg/mL to compare the lipid profiles in the samples. The presented result in this section is from the analysis performed before adding MeOH as an extraction solvent and are the same as in Sample 3. Lipids identified with identification grade A or B are of interest because their identification is highly reliable, and the lipid classes of most interest are TGs, PLs, and WEs. LipidSearch identified a total of 332 lipids, and 183 fit the criteria (section 3.6.3). All of these 183 lipids were identified as TGs. When applying the 22:6 as a filter for moleculeID to find lipids containing DHA in their molecular formula, 41 of 183 TGs contained DHA. Though, when looking at the molecular formula of the TGs, some of the TGs did not contain DHA after all. It is not certain why LipidSearch still identified these TGs as DHA-containing TGs. Figure 21 represents TGs identified with identification grade A or B. No WEs and PLs were identified with identification grade A or B in Sample 3. Each lipid sample within Sample 3 is named s1-s10 (Figure 21). s1, s2, s3, and s4 are lipids from sonication followed by direct extraction for 30 minutes, 1 hour, 2 hours, and 4 hours, respectively. s6 is lipids from direct extraction, and s9 is from Soxhlet extraction. s7 and s8 are lipids from attempted PHA precipitation in isopropanol and methanol, respectively. S10 is from the ID sample. s5 is lipids from single strained bacteria (see section 3.5.1), whereas the lipid extraction was carried out by Maria Wilhelmsen Hoff, a PhD student connected to the project. Lipids in s5 should have been excluded from this analysis as they are irrelevant for the method development. This will be further discussed in section 4.5.1.

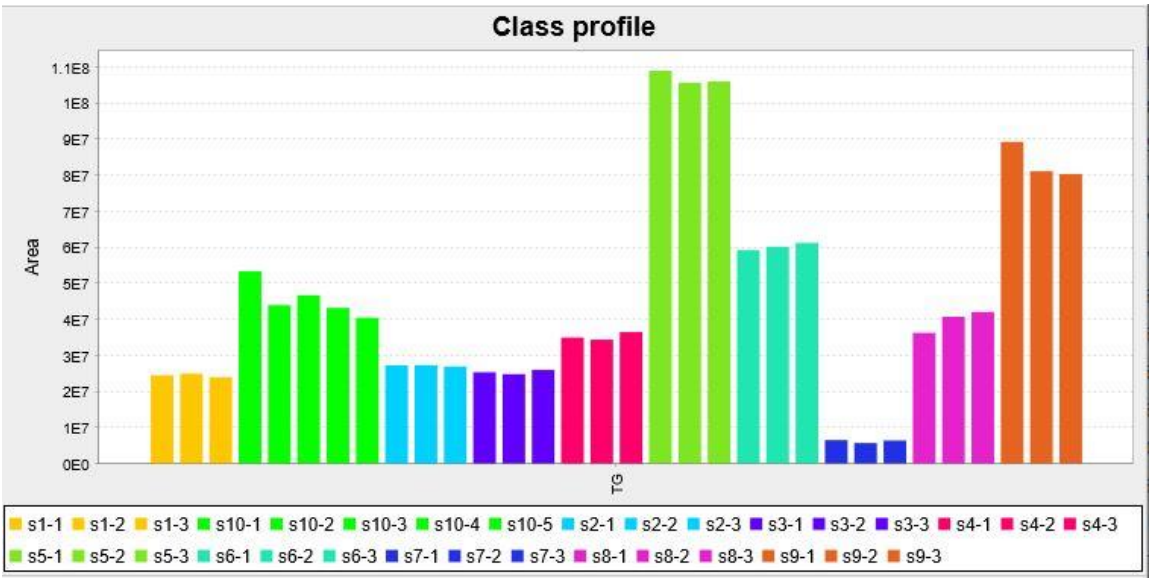

Figure 21: TGs identified with identification grade A or B in Sample 3.

4.3.4 Lipid content results after DCM:MeOH extraction

In this section, the results represent lipids in Sample 4 when comparing lipid extraction with DCM as extraction solvent and DCM:MeOH as extraction solvent. 1018 lipids were identified in Sample 4 using the data from AquireX in Lipidsearch. 260 lipids met the set criteria (see section 3.6.3) of identification grades and lipid classes. Among these 260 lipids, PCs, PEs, and TGs were identified, whereas the most significant portion was TGs. In Figure 22, s1 is the ID samples, and s4 is the blank samples. s3 represents the sample of extraction with DCM:MeOH 2:1, where PLs were identified. No PLs were identified using only DCM for extraction (s2 in Figure 22), only TGs. Even the number of TGs is seemingly lower than the DCM:MeOH 2:1 extraction. The amount might be lower due to slight differences in concentration, but this is not strongly considered in this thesis. Because of these results, it was determined to use a mixture of DCM and MeOH for lipid extraction to ensure a complete lipidome profile overview.

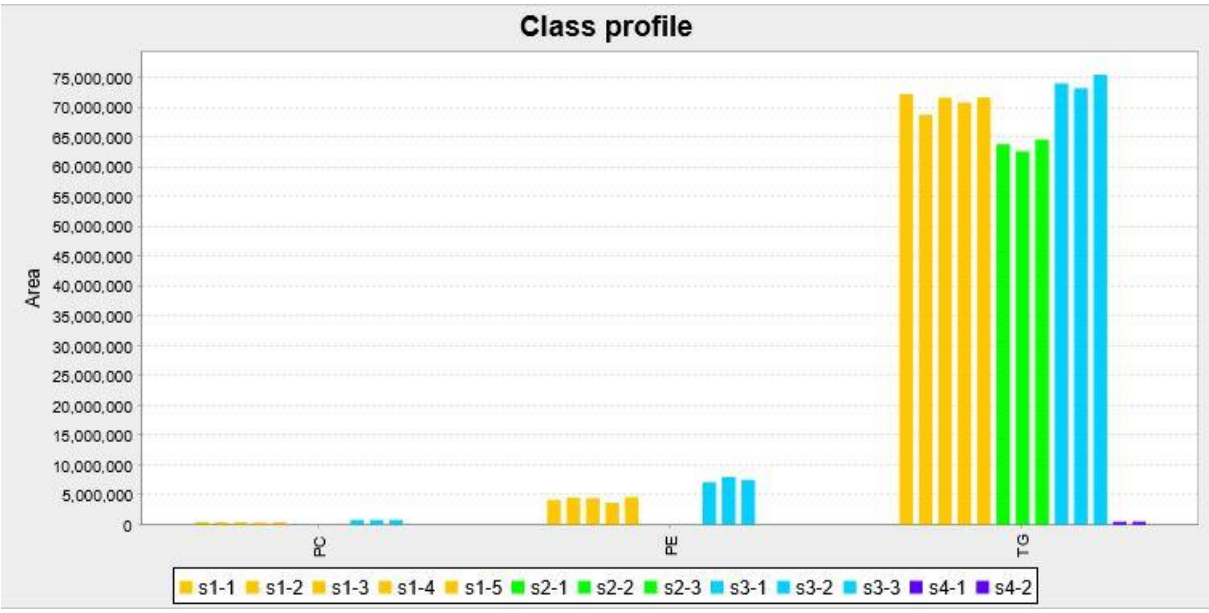

Figure 22 Sample 4. Lipid classes from the left: PC, PE, and TG.

4.3.5 Concentrations

In all analyses except for Sample 4 and 5, 1 mg/mL concentration was used when solving the lipids in isopropanol. As described in section 3.5.3.1, isopropanol could not dissolve several lipids as some lipids stayed in a solid state. Given the known weight of the lipids in the flask

before solving it, the solid lipids pose a significant amount of the total lipids. This is problematic since the actual concentration in isopropanol is hard to predict, and the UHPLC-MS results could be improved if the concentration were as high as attempted. For further investigation, it is recommended to solve the lipids in isopropanol before filtering off the solid lipids and see how much weight was lost. Most lipid samples contained 100-200 mg of lipids, and about 50-200 mL of isopropanol was added before analysis. It would be best to evaporate off the isopropanol for an accurate weight. Still, evaporation of this amount of isopropanol in a rotavapor is quite time-consuming, and it could be challenging to obtain complete dryness. Since the solid lipids were at the bottom of the container, some isopropanol could be removed to decrease the evaporation time. Though most likely, some of the less visible solid lipids may be lost if some of the isopropanol is removed before evaporation. Other methods for evaporation, such as methods using heat and nitrogen gas, could be performed. The solid lipids could be weighed too, but it might also be challenging to dry properly.

4.4 PHA precipitation

It is possible that there was little to no PHA present in the lipid extracts as it has yet to be known how the MMC's complete lipid profile looks. The bacteria might produce mostly TGs and little to no PHA, but this might change fast depending on the environment in the SBR. If there is no PHA present, it would not be necessary to perform any PHA precipitation, which would make the process of analyzing the lipidome much easier and less time-consuming. When attempting to dissolve the lipids in isopropanol before analysis, some lipids stayed solid, as described earlier in section 3.5.3.1. Some of these lipids may be PHA, in which case we do not need to precipitate PHA before this step. This depends on the results from the analysis of the solid lipids that Maria Wilhelmsen Hoff will carry out.

Using isopropanol and methanol is about equally effective as anti-solvents if there is PHA present in the extract due to similarities in structural properties. Heptane did not precipitate anything as expected since it is non-polar. A polar anti-solvent would eventually precipitate the most non-polar lipids, such as PHA if added in sufficient amounts. Heptane is non-polar and would further facilitate the solubility of PHA in the extract. Because of this, no further sample analysis was seen as necessary, and it was not included in Sample 3. If there is PHA in the extract, one might think that the precipitation is easier to perform if the lipids are solved in a more hydrophobic solvent than DCM, for example, hexane or heptane. Though,

amphiphilic lipids such as PLs would not be soluble in heptane or hexane since they are non-polar solvents.

Shortly before this thesis was handed in, preliminary GC-MS analysis of the precipitant indicated that very small amounts of PHA were present in the sample, analyzed as the methyl ester of 3-hydroxy butyric acid.

4.5 Lipid analysis

Results from lipid sample analysis are presented in this section. Five lipid samples were analyzed using LC-MS, and the numbers and graphs presented are from lipid identification performed in LipidSearch with data collected using AcquireX.

Table 7 Lipids identified with set criteria (section 3.6.3). [A]ID sample containing a sample of herring roe, the exact number of lipids from SBR biomass is unavailable (section 4.5.1). [B]First analysis with the new protocol for precipitating PHA (section 3.5.3). [C]Comparison of extraction methods, including a sample of single strains produced by Maria Wilhelmsen Hoff (section 4.5.1). The exact number of lipids from SBR biomass is not available [D]Comparison of lipid extraction in DCM and DCM+MeOH (2:1).

LIPID SAMPLE	TOTAL LIPIDS IDENTIFIED	LIPIDS IDENTIFIED WITH SET CRITERIA	NUMBER OF EPA OR DHA
SAMPLE 1	1125[A]	430[A]	196[A]
SAMPLE 2[B]	971	265	52
SAMPLE 3	332[C]	181[C]	46[C]
SAMPLE 4[D]	1018	260	61
SAMPLE 5	1054	272	83

4.5.1 Limitations concerning ID samples

The ID sample is a pooled sample that consists of lipids from all samples within the given run. The ID sample of Sample 1 contained lipids from a sample of herring roe. The Herring roe sample was added because no PLs were found in the bacterial samples, which was suspicious, and the Herring roe sample was used to confirm that the analytical method worked as intended. The ID sample of Sample 3 contained lipids from single strains produced by Maria Wilhelmsen Hoff. LipidSearch depends on the ID samples to identify the lipids in the

samples for this thesis. It is impossible to create alignments in LipidSearch based on only the lipids from this project for Sample 1 and 3 without including the herring roe lipids and single-strain lipids. Because of this, ID samples from Sample 1 and 3 will still be included but will not be representative of lipids from the SBR.

4.5.2 Lipid class profiles

The following figures show lipid class profiles of lipids extracted from the biomass from the SBR. All lipids displayed and discussed in section 4.5 follow the criteria described in section 3.6.3 of identification grade and lipid class.

4.5.2.1 Sample 1

In Figure 23, Sample 1 is presented. s2 represents the ID samples containing lipids from herring roe, and s3 is the lipids from the SBR. s1 is the blank samples. The lipid classes from left to right are PC, PE, and TG. Few lipids were obtained since the extraction method was sub-optimal, and most lipids were precipitated by adding lipid extract to ice-cold methanol. This fault of the method was yet to be discovered before lipid analysis. No PLs were identified in Sample 1. Only some TGs were identified.

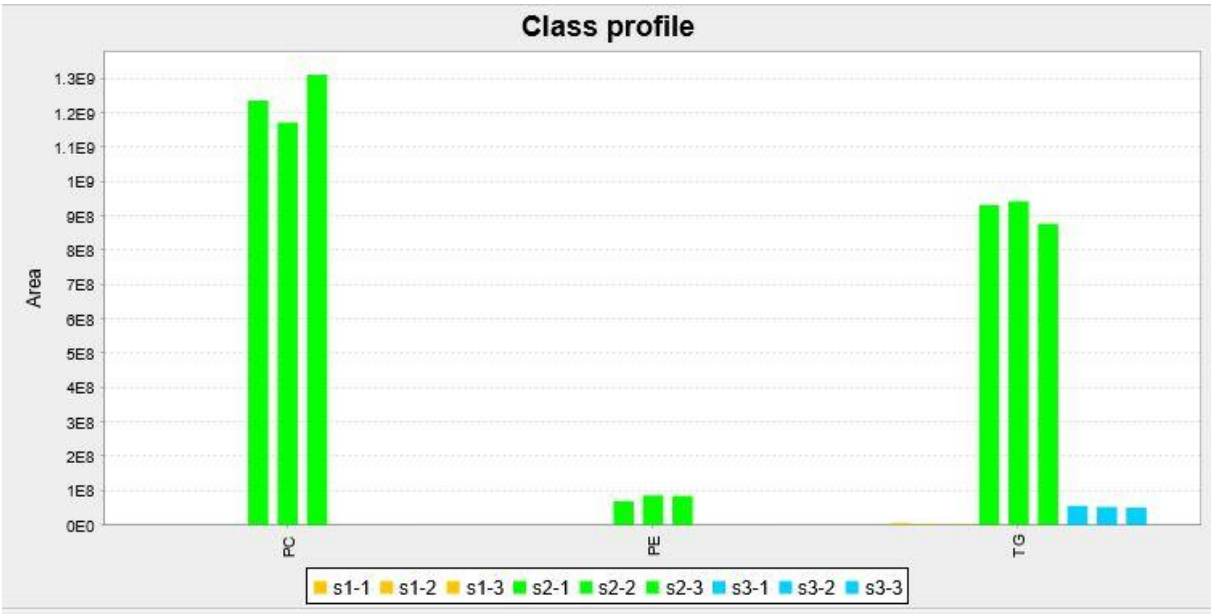

Figure 23 Sample 1. From left to right, PC, PE, and TG.

4.5.2.2 Sample 2

In Figure 24, Sample 2 is presented where s1 is the blank samples, s2 is the ID samples, and s3 and s4 are the lipid samples from Sample 2. For this sample, it was attempted to precipitate PHA by adding MeOH dropwise to the extract until precipitation occurred before removing the precipitant from the extract. A total of 971 lipids were identified, and 265 identified lipids are by the set criteria (section 3.6.3). 38 PLs of these lipids were and 227 lipids were TGs. The intensity of the signals was higher than for Sample 1, which confirms that most lipids most likely precipitated while performing the lipid extraction for Sample 1. Lipid classes from left to right in Figure 24 are PC, PE, and TG. Some lipids were identified in the blank sample, which might be because there was lipid residue in the UHPLC column after the lipid samples were analyzed.

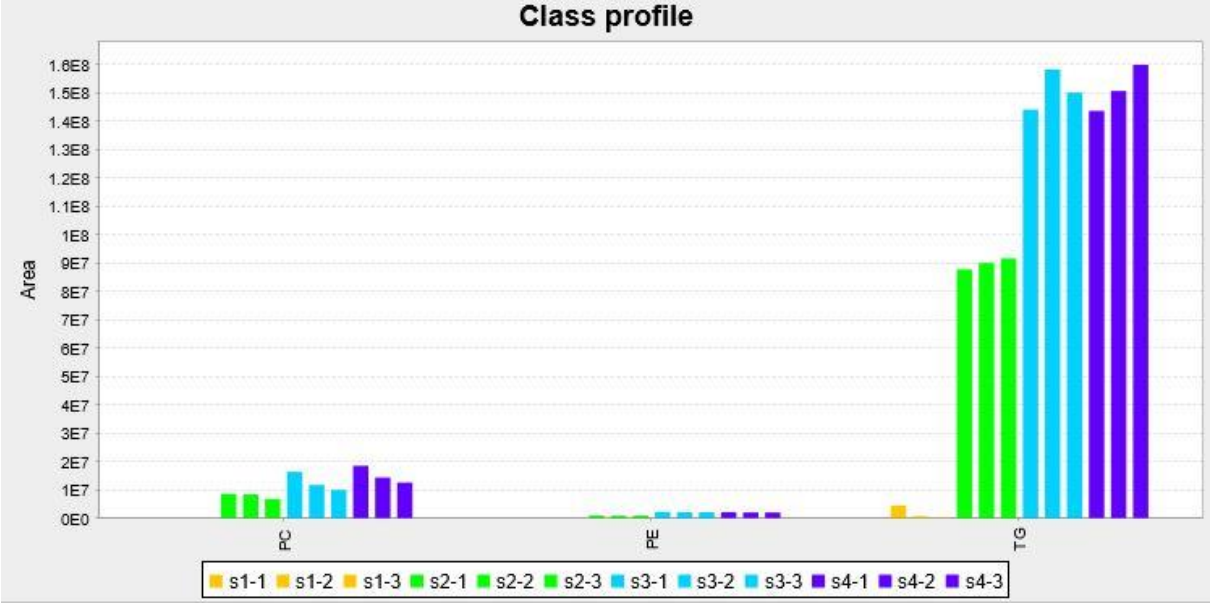

Figure 24 Sample 2. From left to right, PC, PE, and TG.

4.5.2.3 Sample 3

In Figure 25, Sample 3 is presented. 332 lipids were identified, where 181 lipids were identified with the set criteria (section 3.6.3). s1, s2, s3, and s4 are lipids from sonication followed by direct extraction for 30 minutes, 1 hour, 2 hours, and 4 hours, respectively. s6 is lipids from direct extraction, and s9 is from Soxhlet extraction. s7 and s8 are lipids from attempted PHA precipitation in isopropanol and methanol, respectively. S10 is from the ID samples. The ID samples contain lipids from a sample conducted by Maria Wilhelmsen Hoff, who works with single-strained bacteria (section 4.5.1). s5 consists of these lipids as well. The lipid class presented is TG. No PLs were obtained in the analysis of Sample 3. If this was the case only for the sonicated samples, it could be concluded that the PLs were lost in the sonication medium since PLs are partially soluble in water. However, this was not the case here since no PLs were present in the non-sonicated samples either. It was also discussed whether the bacteria in this batch of accumulated biomass contained any PLs. Still, it was later discovered for Sample 4 that the bacteria did contain PLs (section 4.5.2.4). The occurrence of PLs is further discussed in section 4.5.4. The number of TGs obtained was higher than that of Sample 1, which is likely because the lipid extract was not added to ice-cold methanol, which caused precipitation of most lipids.

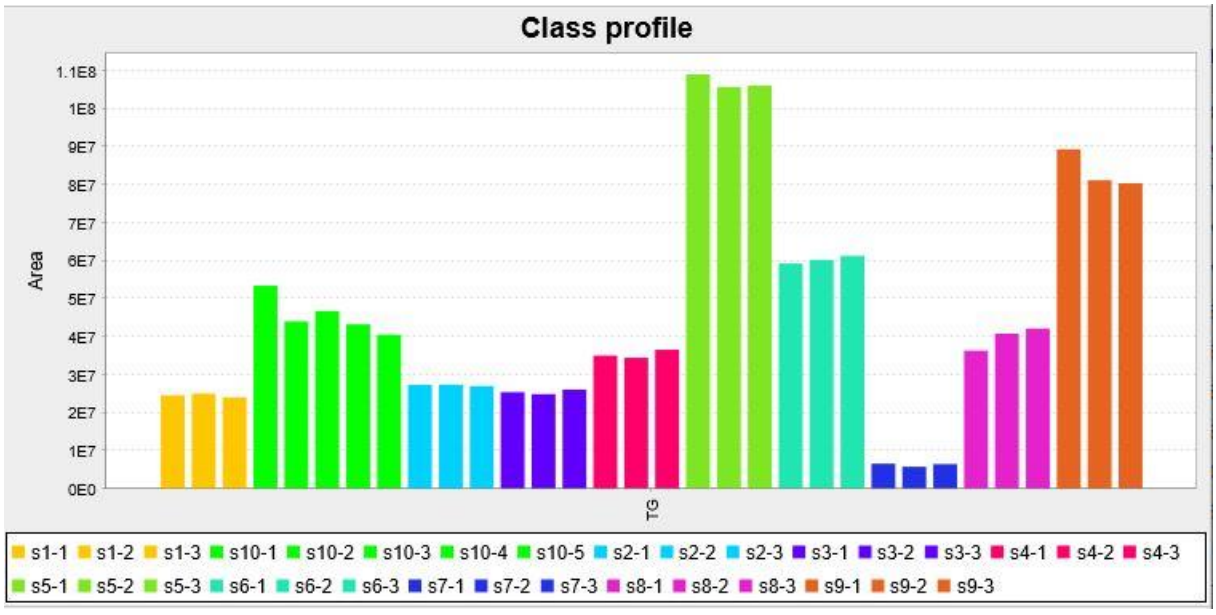

Figure 25 Sample 3. The only lipid class identified is TG.

4.5.2.4 Sample 4

In Figure 26, Sample 4 is presented. 1018 lipids were identified, whereas 260 lipids were identified with the set criteria (section 3.6.3). This is about three times as many lipids in total as in Sample 3, which was 332, but the concentration during analysis is also doubled for Sample 4. The SBR biomass was identical for Sample 3 and 4. Sample 4 include a high number of lipids with identification grade C or D, but the low grading could be due to low concentrations. s1 is the ID samples, s2 is the lipids extracted using only DCM as extraction solvent, and s3 is the lipids extracted using a mixture of DCM and MeOH. s4 is the blank samples. The lipid classes from left to right are PC, PE, and TG. No PLs were identified using only DCM as extraction solvent in Sample 3, while some were identified using DCM:MeOH as extraction solvent. In addition, more TGs were identified using DCM:MeOH than with pure DCM, and as a consequence, it was decided to continue all work with DCM:MeOH 2:1 as extraction solvent. Some lipids were identified in the blank sample, which might be because there was lipid residue in the UHPLC column after the lipid samples were analyzed.

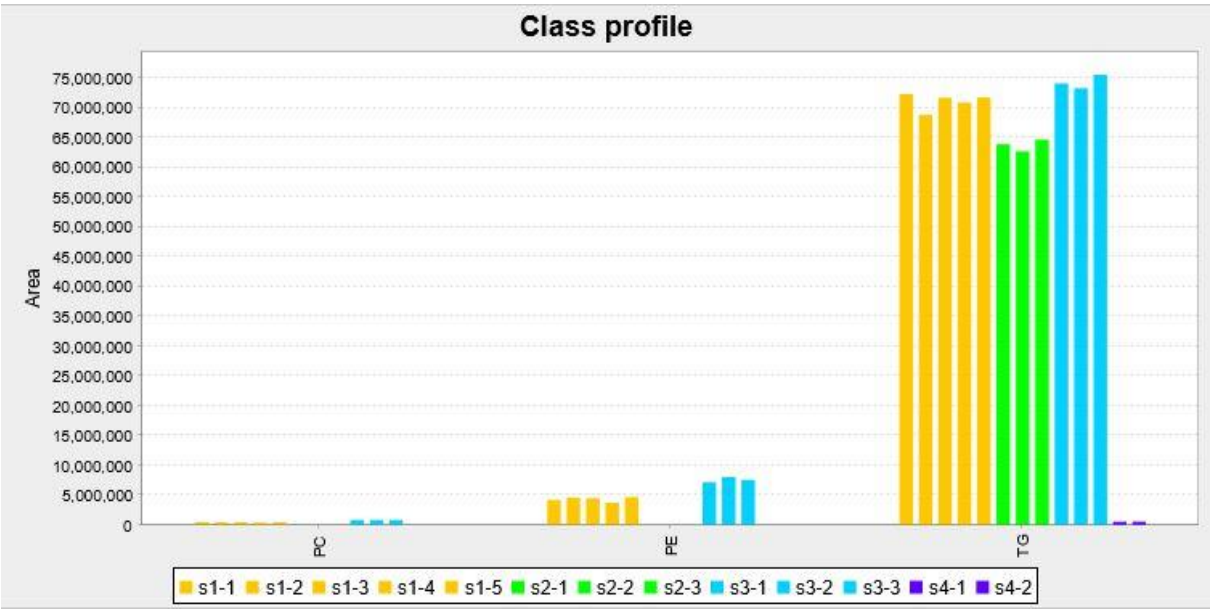

Figure 26 Sample 4. From left to right, PC, PE, and TG.

4.5.2.5 Sample 5

In Figure 27, Sample 5 is presented. 1054 lipids were identified, where 272 identified lipids met the set criteria (section 3.6.3). s1 is the ID samples, s2 is the lipid samples, and s3 is a blank sample. Lipid classes from left to right are PC, PE, and TG. The final method of sonication followed by direct extraction in DCM:MeOH was used for Sample 5. Some lipids were identified in the blank sample, which might be because there was lipid residue in the UHPLC column after the lipid samples were analyzed.

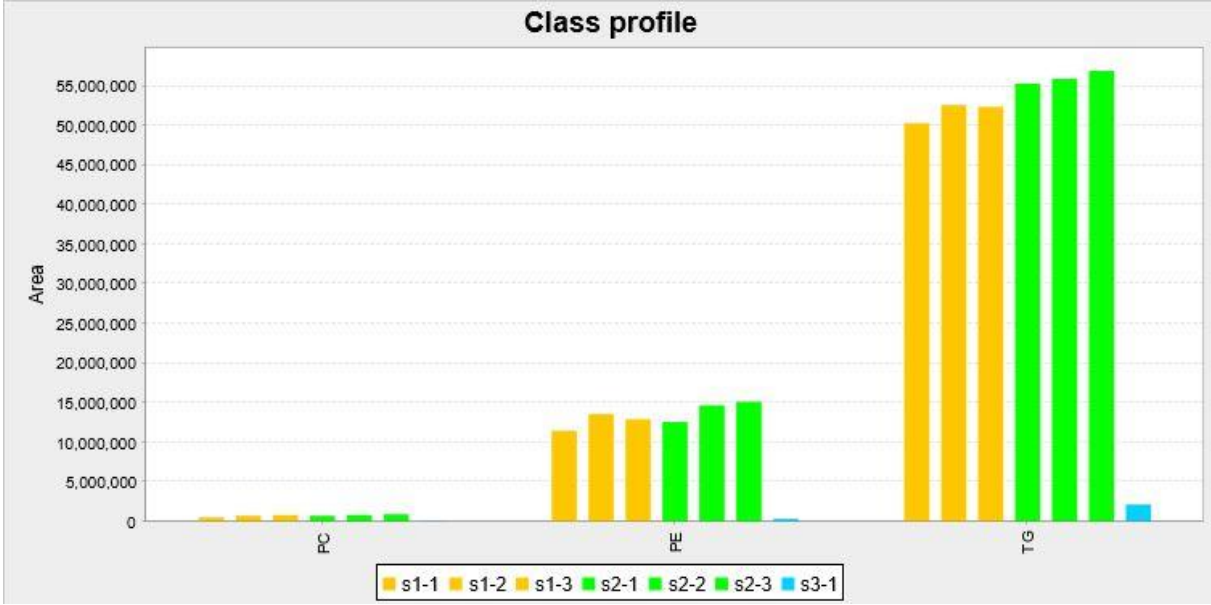

Figure 27 Sample 5. From left to right, PC, PE, and TG.

4.5.3 Triacylglycerols

TGs were identified in all lipid samples and is the lipid class of highest occurrence in the lipid analyses performed in this project. About 40-50% of lipids in each sample were TGs when looking at lipids with identification grade A-D. TGs are used as energy storage in the bacteria and are accumulated in the bacteria under certain conditions, which are controlled and monitored in the SBR. The results from the lipid extraction confirm that the MMC in the SBR does accumulate TGs, and it would be interesting to look further into which TGs are accumulated in the future. Many of the TGs also contained one or two substituents of DHA or EPA, which is further discussed in section 4.6.

4.5.4 Phospholipids

Other PLs in the lipid samples were PG, PS, PI, LPC, LPE, LPS, LPI, and LPG. About 75% of the PLs identified were PC and PE. PG was the third most common PL and accounted for about 8% of the total number of PLs. No PLs with identification grade A or B were identified in Sample 3, and it is not certain why. It could be because DCM cannot extract some of the polar lipids, but there were PLs detected in Sample 2 (Figure 24), where DCM was used as the extraction solvent. PLs have been present in the lipid profiles after changing the extraction solvent from DCM to DCM+MeOH in Sample 4 and 5. It is expected to find some PLs in the lipid samples since there are PLs in the cell membranes of many bacteria, but this is only certain once the complete bacteria profile in the MMC is identified. It is possible that the sonicator probe and extraction solvent are not able to break down the cell wall properly, but theoretically, this should not be an issue. Other microorganisms have been detected in the SBR before, such as microalgae, which often contain PLs. The detected PLs in Sample 1, 2, 4, and 5 may come from potential existing microalgae in the SBR. Microalgae could explain variations in the lipid profile for the biomass from the SBR. This is, however, not certain and should be further examined. Figure 28 presents examples of chromatograms and suggested structures from LipidSearch for a detected PC and a detected LPC.

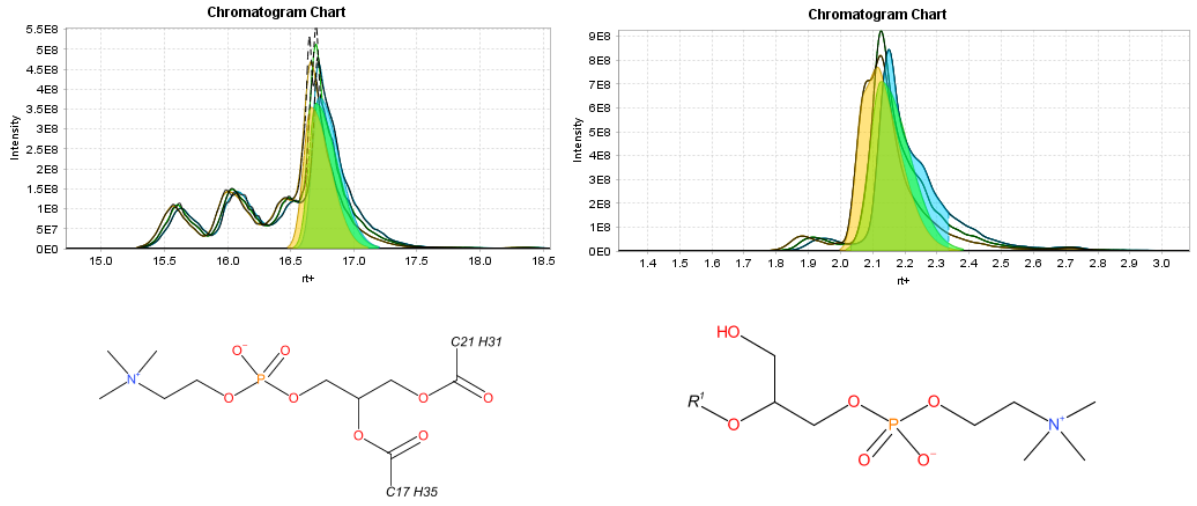

Figure 28 Chromatograms from grade A-identified PC(18:0_22:6) to the left and grade A-identified LPC(16:0) to the right and their structures.

4.5.5 Wax esters

With the set criteria of identification grade (section 3.6.3), only a few WEs were identified in Sample 1-5. Two WEs were identified in Sample 1, but they both were of identification grade C. One WE was identified with grade C in Sample 2, and one with grade C in Sample 5. It is unlikely that the bacteria in the SBR produce any WEs based on these results. LipidSearch could not suggest the complete structure of the identified WEs, only a molecular formula. Figure 29 presents the chromatogram of a WE from Sample 2. The molecular formula suggested by LipidSearch is WE (O-24:5_18:1)+NH4 and no more details are given by LipidSearch concerning which of the carbon chains belong to the FA and the fatty alcohol. As briefly discussed in section 4.5.5, there might have been microalgae present in the SBR whilst performing analysis. The microalgae might be producers of WEs. It is common in plants that the fatty alcohol is long-chained, and microalgae and plants share many characteristics. Because of this, in the molecular formula from Figure 29, it is likely that the fatty alcohol contains 24 carbons, and the FA contains 18 carbons.

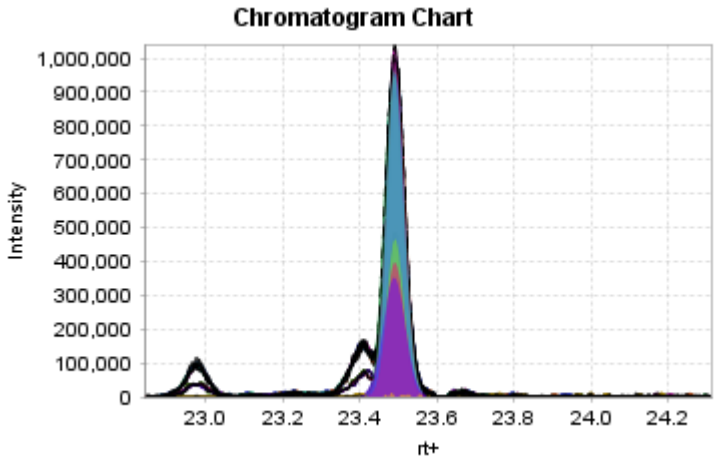

Figure 29 Chromatogram of grade C-identified WE (O-24:5_18:1)+NH4 from Sample 2.

4.5.6 ID sample profiles

It was attempted to create an alignment for all lipids combined from Sample 1-5 to compare the lipid classes of all samples. The LipidSearch software could not handle that many samples in one alignment. For instance, Sample 2 consists of 12 separate samples, which would construct a total of about 50-60 samples in an alignment if Sample 1-5 were aligned. It was not necessary to align all lipid samples within Sample 1-5 to create a comparison since the respective ID samples contain all lipids identified within Sample 1-5. Because of this, all ID

samples from Sample 1-5 were aligned to compare lipid classes and the set criteria of identification grade and lipid classes (section 3.6.3) were applied. The ID sample alignment aimed to compare the presence of PLs, TGs, and WEs, not to compare the number of lipids present in the separate ID samples. The concentrations of the samples had some variation and will not be considered in this section. Figure 30 present lipid classes in ID sample alignment 1 where s1, s2, s3, s4, and s5 are the ID samples from Sample 1, 2, 3, 4, 5, respectively. A total of 2814 lipids were identified and 906 of the lipids met the set criteria of identification grade and lipid classes (section 3.6.3). 272 of the 906 lipids contained DHA, EPA, or both in their molecular structure.

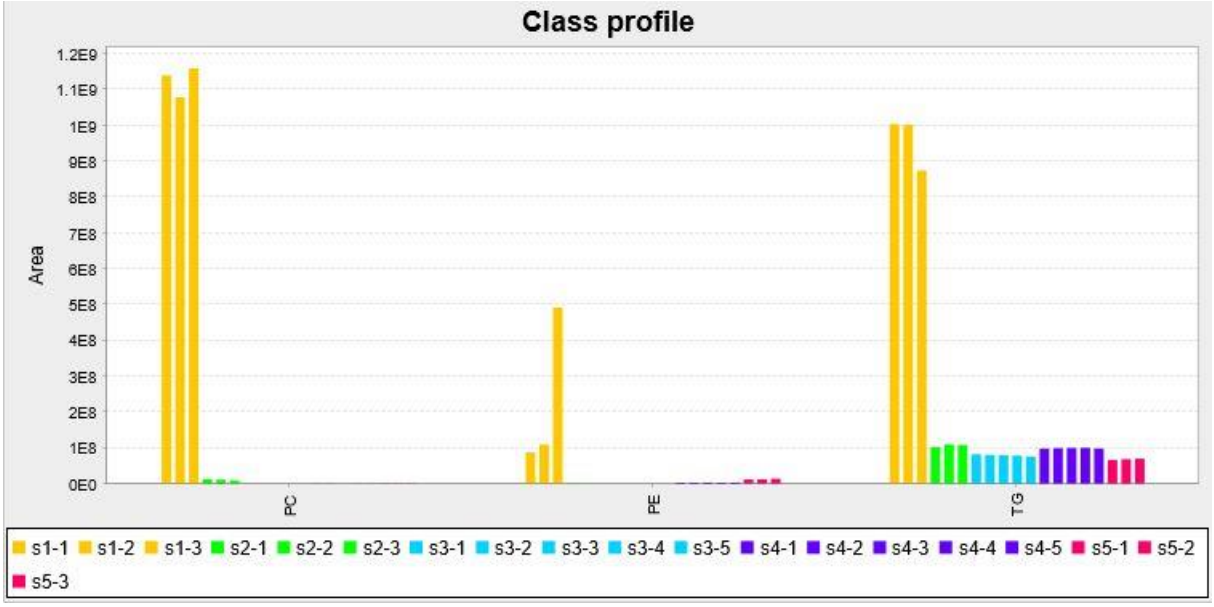

Figure 30 ID sample alignment 1. Lipid class profiles for ID samples with set criteria. From left to right, PC, PE, and TG.

The ID sample from Sample 1 contained, as explained in section 4.5.1, lipids from both Sample 1 and a sample of herring roe lipids. Because of this, s1 in Figure 30 do not represent lipids from the SBR. The basis for comparison of lipid classes from Sample 1 is absent because of this, and it was decided to exclude Sample 1 from the alignment. The ID sample of Sample 3 contained lipids from single strains produced by Maria Wilhelmsen Hoff and is neither representative of the SBR lipid class profile. Without statistical analysis, the deviation of Sample 3 compared to Sample 2, 4, and 5 does not seem as great as for sample 1. A new alignment was performed, ID sample alignment 2, excluding Sample 1, but keeping Sample 3 for further comparison (Figure 31). The set criteria of identification grade and lipid classes

(section 3.6.3) were applied. In Figure 31, s1, s2, s3, and s4 represent the ID samples from Sample 2, 3, 4 and 5, respectively. When excluding Sample 1 in ID sample alignment 2, the number of lipids identified changed from 2814 to 2117, meaning up to 700 identified lipids came from the Herring roe sample alone.

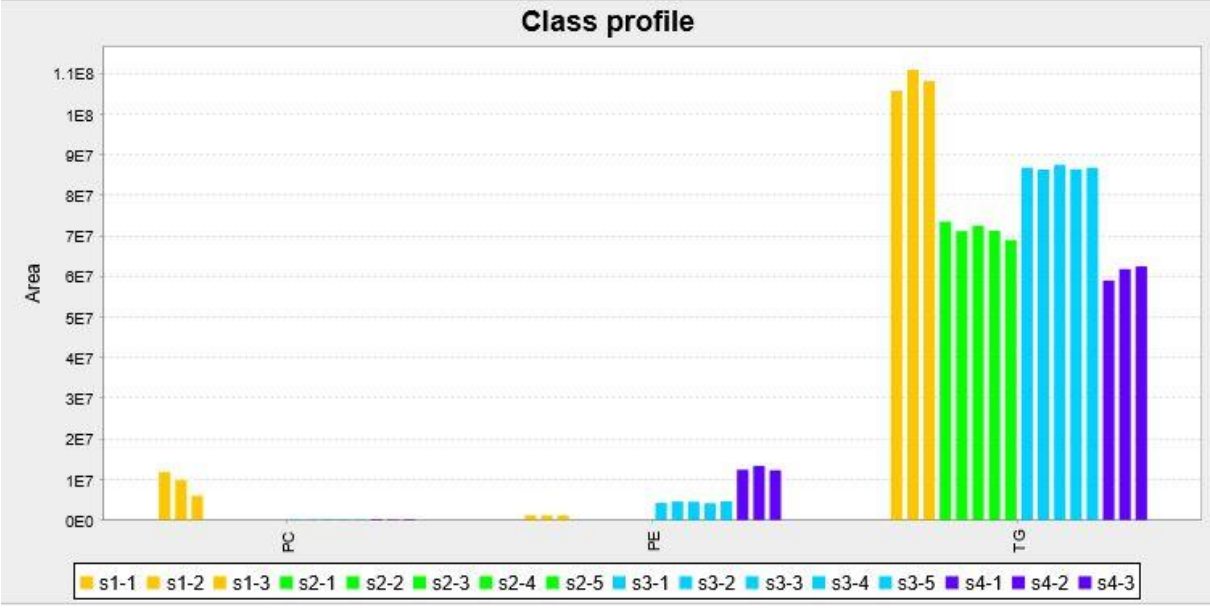

Figure 31 ID sample alignment 2. Lipid class profiles for ID samples with set criteria. From left to right, PC, PE, and TG.

4.5.7 Other lipid classes

4.5.7.1 Ceramides

Several ceramides were found in all lipid samples. In ID sample alignment 2, 348 ceramides were identified, where 124 were identified with identification grade A or B. When performing alignment for Sample 2 and 3, no ceramides were identified. It is not certain why they were identified when ID sample alignments 1 and 2 were made. Examples of peak area for ceramides in each sample are presented in figure 32, which was seen up to 1 500 000, compared to TGs with areas up to 3 000 000. In Figure 32, s1, s2, s3, and s4 represent ID sample 2, 3, 4, and 5, respectively. Remember that the concentrations in samples 4 and 5 are 2 mg/mL, while Sample 2 and 3 are of 1 mg/mL. Several ceramides had a larger area in Sample 4 and 5 compared to Sample 2 and 3. This might be because the lipids in Sample 4 and 5 were extracted using DCM:MeOH instead of only DCM as extraction solvent. Theoretically, it

does not add up since ceramides are hydrophobic, and their extraction would not benefit from adding MeOH to the extraction solvent. This is especially for long-chained ceramides, as they are very hydrophobic. Ceramides are not commonly found in bacteria, but some bacterial species may produce some ceramides to survive in extreme environments (26). The bacteria in the SBR are arctic, and they might possess a mechanism for survival that includes the production of ceramides. Ceramides will not be further discussed in this thesis as ceramides were not a lipid of interest. It might be interesting for further projects to look more deeply into ceramide production in bacteria.

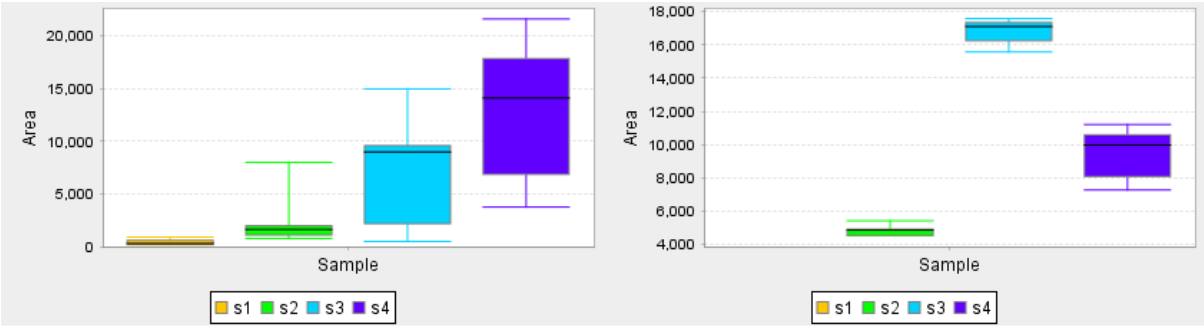

Figure 32 Box plot for the measured area for two long-chained ceramides. Cer(d18:2_42:0) to the left and Cer(d18:2_42:3) to the right.

4.5.7.2 Monoacylglycerols and diacylglycerols

Several MGs and DGs were identified in all lipid samples. In ID sample alignment 2, 341 MGs and DGs were identified, where 58 were identified with identification grade A or B. MGs and DGs are closely related to TGs, one of the lipid classes of interest. MGs and DGs could also be interesting as they can also function as energy storage for bacteria. However, they do have other functions in the bacterial cells, such as precursors involved in the biosynthesis of lipids and as signaling molecules. MGs and DGs could also be products of metabolism of TGs, but it has yet to be determined. Some of the DGs and MGs identified in this project did also contain DHA and EPA. Their direct relevance to the project has yet to be thoroughly discussed but might be interesting to look at in future projects.

4.6 DHA and EPA

Both docosahexaenoic acid (DHA, 22:6) and eicosapentaenoic acid (EPA, 20:5) were identified in most lipid samples in both TGs and PLs, and DGs and MGs. The amount of DHA and EPA which fit the set criteria are given in Table 7. Some TGs in all samples contained two DHA molecules as FAs. There is no reason to believe that the DHA and EPA

came from sources other than the bacteria since only PA is used as feed during fermentation. It would be of great interest to look further into the occurrence of DHA and EPA as they are useful as feed additives in the lipid-rich BM and other fish feed industries. In Figure 33, examples of TGs from Sample 5 containing two DHA molecules and their identified structure are presented.

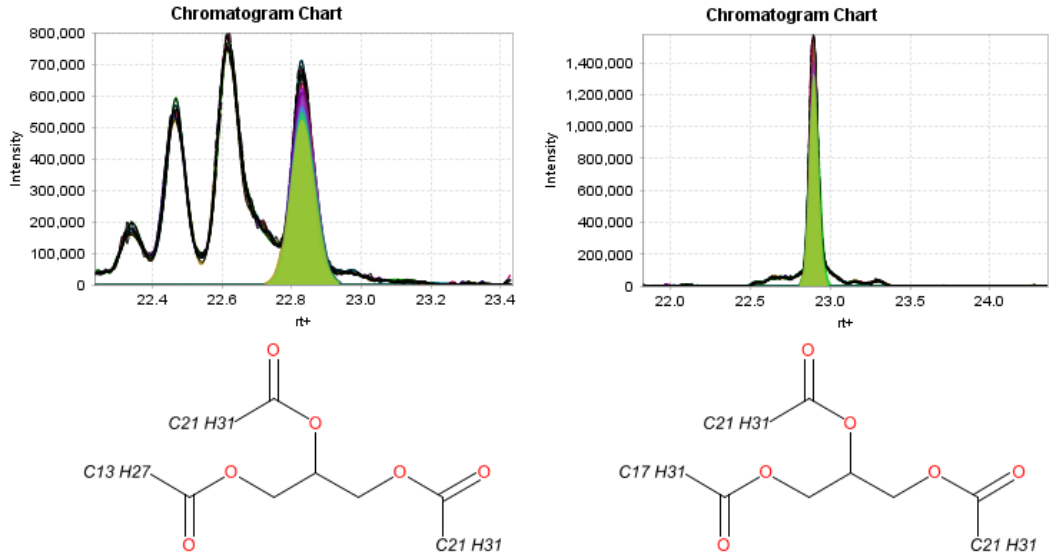

Figure 33 Examples of chromatograms from TGs from Sample 5 containing two DHA molecules. The belonging identified structure below. The left chromatogram and structure belong to TG(14:0_22:6_22:6), and the right to TG(18:2_22:6_22:6).

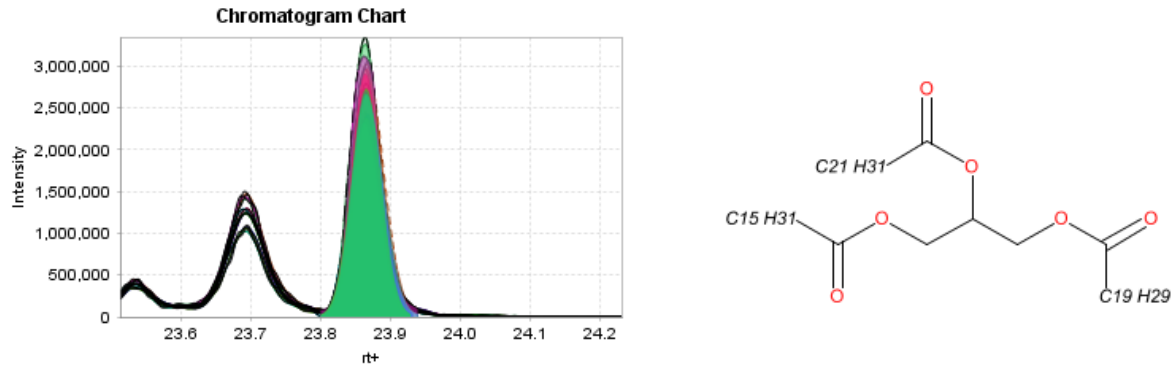

Figure 34 From Sample 4. Chromatogram of TG containing both DHA and EPA, TG(16:0_20:5_22:6), and its identified structure.

5 Conclusion and future perspectives

The thesis aimed to characterize the lipid profile/lipidome in lipid-rich bacterial biomass from a mixed microbial culturing process. Several lipid classes were identified, and the classes of main interest were TGs, PLs, and WEs. Several TGs were identified in all lipid samples, of which many contained FAs of interest, such as DHA and EPA. PLs were identified in Sample 2, 4, and 5, but not in Sample 1 and 3. None to a few WEs were identified in all lipid samples, and it was concluded that the MMC did not accumulate any WEs. Other lipids such as ceramides, MGs and DGs were also identified in the lipid samples, but their relevance for this project is still to be discussed.

The lipid profile probably varies depending on the SBR's state and how accumulation is performed. The variation in lipid content should be further examined in future projects.

The original lipid extraction method was not optimal, as fewer lipids than expected were obtained. Soxhlet extraction, direct extraction, and sonication followed by direct extraction in DCM were tested as lipid extraction methods. Lipid yield was used to compare the methods, and the lipid yield was highest for sonication followed by direct extraction in DCM. None to a few PLs were obtained when using only DCM as extraction solvent, and it was examined if using a mixture of DCM and MeOH would extract more PLs. The analysis showed PLs when using a mixture of DCM and MeOH, and the protocol was adjusted. The reproducibility of the final extraction protocol should be examined.

A method for properly separating PHA from the remaining lipids should be further investigated. A portion of the dried extracted lipids did not dissolve when attempting to dissolve it in isopropanol before analysis. It is not certain yet whether the solid lipids are PHA or if it contains other lipids of interest.

No quantification of lipids by the MS was performed in this thesis, but it would be interesting to examine quantities in future projects.

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Appendix

Appendix 1: MS Method

```
Thermo Scientific SII for Xcalibur Method

---- Overview ----
Name: New Instrument Method
Comment:
Run time: 32.500 [min]
Instrument: Vanquish_H on thermo-jr7hgt84
Description:
---- Script ----
initial      Instrument Setup
             ColumnComp.PrehtLeft.ReadyTempDelta: 1.00 [°C]
             ColumnComp.PrehtLeft.TempCtrl: On
             ColumnComp.PrehtLeft.Temperature.Nominal: 60.00 [°C]
             ColumnComp.PrehtLeft.EquilibrationTime: 1.0 [min]
             ColumnComp.CC.Mode: StillAir
             ColumnComp.CC.ReadyTempDelta: 0.50 [°C]
             ColumnComp.CC.TempCtrl: On
             ColumnComp.CC.Temperature.Nominal: 60.00 [°C]
             ColumnComp.CC.EquilibrationTime: 1.0 [min]
             ColumnComp.Column_B.ActiveColumn: No
             ColumnComp.Column_B.SystemPressure: "Pump"
             ColumnComp.Column_D.ActiveColumn: Yes
             SamplerModule.Sampler.PunctureOffset: 0 [µm]
             SamplerModule.Sampler.WashSpeed: 20.0 [µl/s]
             SamplerModule.Sampler.InjectWashMode: Both
             SamplerModule.Sampler.WashTime: 5.0 [s]
             SamplerModule.Sampler.DispenseSpeed: 5.000 [µl/s]
             SamplerModule.Sampler.DrawSpeed: 5.000 [µl/s]
             SamplerModule.Sampler.Pump: "Pump"
             SamplerModule.TempCtrl: On
             SamplerModule.Temperature.Nominal: 25.0 [°C]
             PumpModule.Pump.%B_Selector: %B1
             PumpModule.Pump.%A_Selector: %A3
             PumpModule.Pump.%A1_Equate: "Water 1 mM NH4FA 0.01 FA"
             PumpModule.Pump.%A2_Equate: "Water 0.1% FA"
             PumpModule.Pump.%A3_Equate: "50% ACN 1 mM NH4FA 0.01 FA"
             PumpModule.Pump.%B1_Equate: "50/50 IPA/MeCN 1 mM NH4FA 0.01 FA"
             PumpModule.Pump.%B2_Equate: "MeCN 0.1% FA"
             PumpModule.Pump.%B3_Equate: "95% ACN 1 mM NH4FA 0.01 FA"
             PumpModule.Pump.Pressure.LowerLimit: 0 [bar]
             PumpModule.Pump.Pressure.UpperLimit: 1517 [bar]
             PumpModule.Pump.MaximumFlowRampUp: 6.00 [ml/min²]
             PumpModule.Pump.MaximumFlowRampDown: 6.00 [ml/min²]
             ColumnComp.LowerValve.CurrentPosition: 6_1
             ColumnComp.UpperValve.CurrentPosition: 6_1
-2.500 [min] Equilibration
             PumpModule.Pump.Flow.Nominal: 0.600 [ml/min]
             PumpModule.Pump.%B.Value: 30.0 [%]
             PumpModule.Pump.Curve: 5
0.000 [min]
```

```
Thermo Scientific SII for Xcalibur Method

             PumpModule.Pump.Flow.Nominal: 0.600 [ml/min]
             PumpModule.Pump.%B.Value: 30.0 [%]
             PumpModule.Pump.Curve: 5
0.000 [min]  Inject Preparation
             Wait ColumnComp.Ready And SamplerModule.Sampler.Ready And PumpModule.Pump.Ready
0.000 [min]  Inject
             SamplerModule.Sampler.Inject
0.000 [min]  Start Run
             ColumnComp.CC_Temp.AcqOn
             ColumnComp.PrehtLeft_Temp.AcqOn
             PumpModule.Pump.Pump_Pressure.AcqOn
0.000 [min]  Run
             PumpModule.Pump.Flow.Nominal: 0.600 [ml/min]
             PumpModule.Pump.%B.Value: 30.0 [%]
             PumpModule.Pump.Curve: 5
20.000 [min]
             PumpModule.Pump.Flow.Nominal: 0.600 [ml/min]
             PumpModule.Pump.%B.Value: 75.0 [%]
             PumpModule.Pump.Curve: 5
25.000 [min]
             PumpModule.Pump.Flow.Nominal: 0.600 [ml/min]
             PumpModule.Pump.%B.Value: 95.0 [%]
             PumpModule.Pump.Curve: 5
30.000 [min] Stop Run
             ColumnComp.CC_Temp.AcqOff
             ColumnComp.PrehtLeft_Temp.AcqOff
             PumpModule.Pump.Pump_Pressure.AcqOff
```

Method Summary

Method Settings

Application Mode: **Small Molecule**
Method Duration (min): **30**

Global Parameters

Ion Source

Use Ion Source Settings from Tune: **True**
FAIMS Mode: **Not Installed**

MS Global Settings

Infusion Mode: **Liquid Chromatography**
Expected LC Peak Width (s): **3**
Advanced Peak Determination: **False**
Mild Trapping: **False**
Default Charge State: **1**
Enable Xcalibur AcquireX method modifications: **True**
Internal Mass Calibration: **EASY-IC™**

Divert Valve A

Time (min)	Position
0	1-2

Experiment#1 [AcquireX lipid characterization HCD-CID-MS3]

Start Time (min): **0**
End Time (min): **30**
Cycle Time (sec): **1.5**

Master Scan:

MS OT

Detector Type: **Orbitrap**
Orbitrap Resolution: **120000**
Use Quadrupole Isolation: **True**
Scan Range (m/z): **250-1500**
RF Lens (%): **40**
AGC Target: **Standard**
Maximum Injection Time Mode: **Custom**
Maximum Injection Time (ms): **50**
Microscans: **1**
Data Type: **Profile**
Polarity: **Positive**
Source Fragmentation: **Disabled**
Use EASY-IC™: **True**
Scan Description:

Filters:

Intensity

Filter Type: **Intensity Threshold**
Intensity Threshold: **1.0e5**

Dynamic Exclusion

Exclude after n times: **1**
Exclusion duration (s): **2**
Mass Tolerance: **ppm**
Low: **10**
High: **10**
Exclude Isotopes: **True**

Targeted Mass Exclusion

Mass List

Mass List Type: **m/z**
Time Mode: **Start/End Time**
Include Intensity Threshold: **True**
Add Mass List Targets Determined by Xcalibur AcquireX: **True**

Compound	m/z	t start (min)	t stop (min)	Intensity Threshold
	524.265	0	30	1E+20

Exclusion mass width: **ppm**
Low: **10**

High: **10**

Data Dependent

Data Dependent Mode: **Cycle Time**
Time between Master Scans (sec): **1.5**

Scan Event Type 1:

Targeted Mass

Mass List

Mass List Type: **m/z**
Time Mode: **Start/End Time**
Include Intensity Threshold: **True**
Add Mass List Targets Determined by Xcalibur AcquireX: **True**

Compound	m/z	t start (min)	t stop (min)	Intensity Threshold
	524.265	0	30	0

Mass Tolerance: **ppm**
Low: **10**
High: **10**
Set Collision Energy per Compound: **False**
Perform dependent scan on most intense ion if no targets are found: **True**
Use Group IDs: **False**

Scan:

ddMS^2 OT HCD

Isolation Mode: **Quadrupole**
Isolation Window (m/z): **1.5**
Isolation Offset: **Off**
Activation Type: **HCD**
Collision Energy Mode: **Stepped**
HCD Collision Energy Type: **Normalized**
HCD Collision Energies (%): **25,30,35**
Detector Type: **Orbitrap**
Orbitrap Resolution: **15000**
Scan Range Mode: **Define First Mass**
First Mass (m/z): **140**
AGC Target: **Standard**
Maximum Injection Time Mode: **Custom**

Maximum Injection Time (ms): **50**
Microscans: **1**
Data Type: **Profile**
Use EASY-IC™: **True**
Scan Description:

Data Dependent

Data Dependent Mode: **Scans Per Outcome**

Scan Event Type 1:

Targeted Mass Trigger

Mass List

Mass List Type: **m/z**

Compound	m/z
	184.0733

Mass Tolerance: **ppm**
Low: **10**
High: **10**
Use Group IDs: **False**
Trigger Only with Detection of at Least N Ions from the List: **False**
Only Ion(s) Within Top N Most Intense: **True**
n :: **3**
Only Ion(s) Above the Threshold (Relative Intensity, %): **False**
Trigger Type: **Continue Trigger**

Intensity

Filter Type: **Intensity Threshold**
Intensity Threshold: **5.0e4**

Scan:

ddMS^2 OT CID

MS^n Level: **2**
Scan Priority: **1**
Isolation Mode: **Quadrupole**
Isolation Window (m/z): **2**
Isolation Offset: **Off**

Activation Type: **CID**
Collision Energy Mode: **Fixed**
CID Collision Energy (%): **32**
CID Activation Time (ms): **10**
Activation Q: **0.25**
Multistage Activation: **False**
Detector Type: **Orbitrap**
Orbitrap Resolution: **15000**
Scan Range Mode: **Auto**
AGC Target: **Standard**
Maximum Injection Time Mode: **Custom**
Maximum Injection Time (ms): **50**
Microscans: **1**
Data Type: **Profile**
Use EASY-IC™: **False**
Scan Description:
Number of Dependent Scans: **1**

Scan Event Type 2:

Targeted Loss Trigger

Mass List

Mass List Type: **m/z**

Compound	m/z
	217.2042
	215.1885
	245.2355
	243.2198
	273.2668
	271.2511
	269.2355
	287.2824
	285.2668
	301.2981
	299.2824
	297.2668

	295.2511
	315.3137
	329.3294
	327.3137
	325.2981
	323.2824
	321.2668
	319.2511
	343.345
	357.3607
	355.345
	353.3294
	351.3137
	349.2981
	347.2824
	345.2668
	371.3763
	385.392
	383.3763
	413.4233

Mass Tolerance: **ppm**
Low: **10**
High: **10**
Trigger Only with Detection of at Least N Ions from the List: **False**
Only Ion(s) Within Top N Most Intense: **True**
n :: **3**
Only Ion(s) Above the Threshold (Relative Intensity, %): **False**
Trigger Only with Detection of Correct Charge State of the Product Ion: **False**
Ignore Charge State Requirement for Unassigned Ions: **False**
Trigger Type: **Continue Trigger**

Precursor Ion Exclusion

Exclusion mass width: **m/z**
Low: **0.5**
High: **4**

Intensity

Filter Type: **Intensity Threshold**
Intensity Threshold: **5.0e4**

Scan:

ddMS^3 OT CID

MS^n Level: **3**
Scan Priority: **1**
MS Isolation Window (m/z): **1.5**
MS2 Isolation Window (m/z): **2**
Isolation Offset: **Off**
Activation Type: **CID**
Collision Energy Mode: **Fixed**
CID Collision Energy (%): **35**
CID Activation Time (ms): **10**
Activation Q: **0.25**
Multistage Activation: **False**
Detector Type: **Orbitrap**
Orbitrap Resolution: **15000**
Scan Range Mode: **Auto**
AGC Target: **Standard**
Maximum Injection Time Mode: **Custom**
Maximum Injection Time (ms): **65**
Microscans: **1**
Data Type: **Profile**
Use EASY-IC™: **False**
Scan Description:
Number of Dependent Scans: **3**

Appendix 2: LipidSearch definitions

Item	Description
Rej	Rejection flag.
ID	An ID uniquely assigned to the combination of lipid molecule and BaseRt.
LipidMolec	Lipid molecule name.
ClassKey	This is a lipid class key.
SubClassKey	Lipid subclass key.
FaKey	The names of fatty acids.
FaGroupKey	Name of fatty acid chain being added up.
CalcMass	The molecular weight of the lipid molecule.
BaseRt	The representative retention time of the lipid molecule.
MolecFormula	Lipid molecular composition formula.
IonCnt	The number of Adduct Ions assigned to this lipid molecule.
IsoCnt	The number of molecules assigned as isomers of this lipid molecule.
PDBCnt	The number of links in the public database.
TotalSmpIDRate	The value (%) calculated by the following formula. [Number of samples that identified any ion in the molecule] / [Total number of samples] * 100
TotalGrade	This grade is given by the degree of product ions assigned to all lipid ions in this lipid molecule group. A: All of the class-specific ions and substituent-specific ions that specifies the structure are assigned. B: One of the class-specific ions and substituent-specific ions that specifies the structure. Partial assignment C: Either a class-specific ion or a substituent-specific ion that specifies the structure is assigned D: A product ion that specifies the structure is not assigned < / div>