Investigating the Potential of Microalgae *Chlamydomonas sp.* and *Stichococcus deasonii* as a Sustainable Feedstock for Biofuel Production: Effects of Fishwater and Synthetic Saltwater Growth Medium and Biomass Separation

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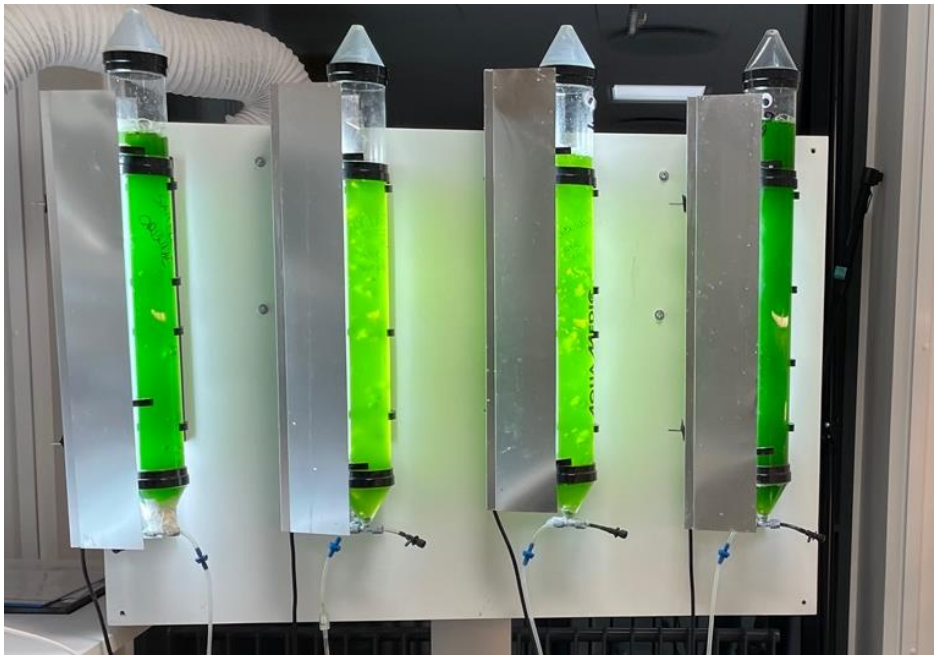

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STUDENT REPORT

Title Page

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Abstract

This thesis investigates the potential of two microalgae strains, *Chlamydomonas sp.* and *Stichococcus deasonii*, isolated from samples collected at the Esbjerg harbour in Denmark, as a sustainable feedstock for biofuel production. The study analyses the effects of two different growth medium - fishwater and synthetic saltwater supplemented with plant fertilizer - on the growth and lipid production of these microalgae. The flocculation properties of the microalgae, a critical aspect of biomass separation in microalgae-based biofuel production, are also evaluated.

Laboratory-scale experiments are conducted using 500 mL blue cap flasks continuously aerated with atmospheric air and artificial light from fluorescent lamps. Microalgal growth is assessed using optical density at 600 nm and a hemacytometer, while lipid extraction is carried out with sonication and organic solvents (methanol and chloroform) and analysed using FAME and GC-MS.

Microalgae growth was superior in artificial saltwater medium, averaging 16,678 cells/mL/h, compared to 4,250 cells/mL/h in fishwater. Across all cultures, the dry matter averaged 4% and the lipid content approximately 8%. Dominant fatty acids were identified as C18-3, C18-2, C16, and C18.

The results of this study have implications for industrial-scale production at a local aquaculture company called Alpha-Aqua, where a circular economy model will be employed to convert fishwater that would otherwise go to waste, into biofuel and/or high-value bioproducts including fishfeed. By assesing the growth and lipid production of microalgae strains in different growth medium, and by addressing the challenge of biomass separation through the study of flocculation properties, the thesis aims to contribute to the development of a sustainable and economically viable microalgae-based biofuel industry.

Keywords: Microalgae, biofuel, flocculation, circular economy, aquaculture, fishwater, aquaponics.

Preface

This report was completed by Sofia Vayas Pedersen (BIO9-4-E22) in the Bioengineering course and within the Energy department at Aalborg University Esbjerg, as a one year long master thesis during the fall semester 2022 and spring semester 2023. A special acknowledgement goes to my supervisors Professor Mette Hedegaard Thomsen and PhD fellow Malthe Fredsgaard for their advice and recommendations. To Jiwan Kumar Chettri for his support and work from AlphaAqua. To Jeppe Jensen from the Fiskeri- og Søfartsmuseet in Esbjerg for providing fishwater. Furthermore, a special thanks are owed to laboratory technicians Julaine Tania Enas, Lilian Melo Bondig, Dorte Spangsmark and Linda Birkebæk Madsen for their constant help with the laboratory equipment and for sharing their expertise. Finally, gratitude is given to Aalborg University Esbjerg for providing access to the laboratories, as well as the resources and materials needed to complete this project.

Illustrations in this report have been created using the softwares Draw.io®, BioRender® and Microsoft PowerPoint®.

Aalborg University, June 1st, 2023

Abbreviations

Abbreviation	Description
ATJ	Alcohol-to-jet
CN	Cetane number
DM	Dry matter
DW	Dry weight
ER	Endoplasmic reticulum
EROI	Energy return on energy invested
FA	Fatty acid
FAME	Fatty Acid Methyl Ester
FT	Ficher-Tropsch
FW	Fishwater medium
GC-MS	Gas Chromatography-Mass Spectrometry
HEFA	Hydro processed esters and fatty acids
HTL	Hydrothermal liquefaction
ID	Ignition delay
ISO	International organization for standardization
LCA	Life cycle assessment
MQ	Milli-Q
MUFA	Monounsaturated fatty acids
OD	Optical density
PUFA	Polyunsaturated fatty acids
RAS	Recirculating aquaculture systems
SD	Standard deviation
SDG	Sustainable development goals
SFA	Saturated fatty acids
SW	Saltwater medium supplemented with commercial nutrients
TES	Total energy supply
UAE	Ultrasound assisted extraction
VAP	Value added product

Table of Contents

Introduction

In today's world one of the most pressing issues is the global energy crisis [1]. The demand for energy is constantly increasing but in recent years it has reached an all-time high due to a combination of factors including a rapid growth of population and industrialization, the extraordinarily rapid recovery following the pandemic and of course the consequences felt from Russia's invasion of Ukraine in February 2022 [1].

During the winter of 2022-2023, the energy crisis was more tangible and present in everyone's mind than ever before [2]. In 2021, Russia was the world's largest fossil fuels exporter and before the conflict in Ukraine, Russia was a significant supplier of natural gas to the EU, accounting for around 40-50 % of all EU's natural gas imports [3], [4]. This has drastically changed since the start of the war as Russia decided to cut its gas exports by 80 % to the EU and this resulted in a significant increase in the gas and electricity costs in Europe. Precisely the gas went up by 144 % and the electricity went up by 78 % when compared to the averages observed between 2000 and 2019 [5].

As a society, we rely extremely much on energy. We need it to power our industrial sector, we need it for transportation, and we constantly use it in our everyday life's [6] up to 80 % of our total energy supply (TES) comes from fossil fuels [6] . Of these 80 %, 29 % comes from oil, 27 % comes from coal and 24 % comes from natural gas [6]. These forms of non-renewable energies all have a finite nature. This provokes an imbalance between the supply and demand of fuels and that in turn provokes an inevitable hike in the fuel prices.

Therefore, there is a real need to find sound and innovative solutions that can balance on the one hand the constant need for energy to continue our economic development and on the other hand the vital preservation of our environment [7]. These two aspects many times don't go hand in hand, nevertheless, researchers and scientists are constantly looking for solutions among other alternative energy sources. Such as wind-, solar-, nuclear- or bio-energy. One such solution could be the production of microalgae-biofuel, which is the focus of this report.

Over the last two decades, there has been a significant surge of interest in biofuels as a sustainable alternative to fossil fuels, with bioethanol and biodiesel being at the forefront of this trend. Bioethanol is commonly mixed with regular gasoline to reduce the carbon footprint of internal combustion engines [8]. The concept of liquid biofuels has been around for several decades and presents a practical way to mitigate CO_2 emissions by recycling carbon emissions into new biomass through photosynthesis, which can then be converted into usable fuel [9]. Therefore, different generations of biofuels have emerged. The first generation of biofuels which are produced from crops such as corn and sugarcane, compete with food production, cause deforestation, and require substantial freshwater resources [10]. The second generation of biofuels are produced from lignocellulosic biomass offering a more sustainable but are costly and energy-intensive alternative [10].

Given the limitation and ethical questions that are associated with first- and second-generation biofuels, a third and promising generation has risen, which uses aquatic organisms like microalgae as feedstock [11]. Algae-based biofuels require minimal land, microalgae proliferate rapidly, accumulate high lipid content, and have the added benefits of CO2 absorption and reduction in greenhouse gas emissions [12].

The process in a microalgae biorefinery consists of the steps illustrated in Figure 1. First the microalgae are cultivated by providing light, water, carbon dioxide and nutrients, especially nitrogen and phosphate in the form of water-soluble nutrients, this is usually carried out in an open or closed bioreactor. Once there is concentrated biomass, it is harvested, typically done by sedimentation, flocculation, centrifugation, or flotation. Then it can be dried. Figure 1 also shows a "wet route" illustrated by the dashed arrow, which, if employed would eliminate the drying process thus lowering the production cost [13]. Extraction is performed after cell disruption and the different microalgae fractions can subsequently be used to produce various products that can be classified into fuel and non-fuel groups [13], [14].

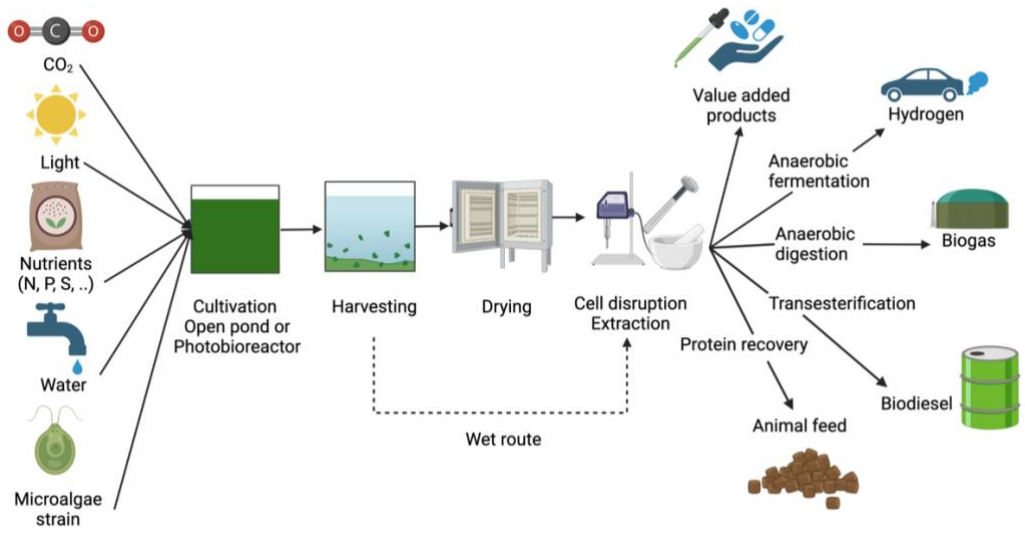


Figure 1 - A simplified pipeline model for a microalgae biorefinery. Created with BioRender.com.

To make any biorefinery economically viable, it is important to exploit the maximum amount of side streams available. In a case study by Khoo et al. (2019) [15], the carbohydrate fraction obtained from algal biomass can be utilized to produce bioethanol through fermentation, while the lipids can be trans-esterified to produce biodiesel [16]. The waste biomass generated can then be subjected to thermal treatments such as pyrolysis or gasification to produce bio syngas and hydrocar. Additionally, the nutrient and secondary metabolites such as pigments can be extracted and utilized as biofertilizers and bio nutrients [16]. Figure 2, illustrates a few examples of these products that can be obtained from algal biomass. Biodiesel, Bioethanol, biobutanol, biohydrogen and biogas are common fuel and energy products that can be obtained from microalgae biomass [14], [17]. Carbohydrates, pigments, proteins, sugars and fish feed are common non-fuel products that can be obtained from microalgae biomass [14].

Algal based biofuels	Value added products
• Biodisel • Bioethanol • Biobutanol • Biohydrogen • Biogas	• Carbohydrates • Pigments (β-carotenoids) • Proteins (Phycobiliproteins) • Vitamins (A, B1, B2, B6, B12, C, E) • Fish feed

Figure 2 - Fuel and non-fuel applications of algal biomass.

Figure 3 shows the wide range of fields where microalgae biomass can be applied. Moreover, the arrow demonstrates which sectors have higher or lower market prices [18].

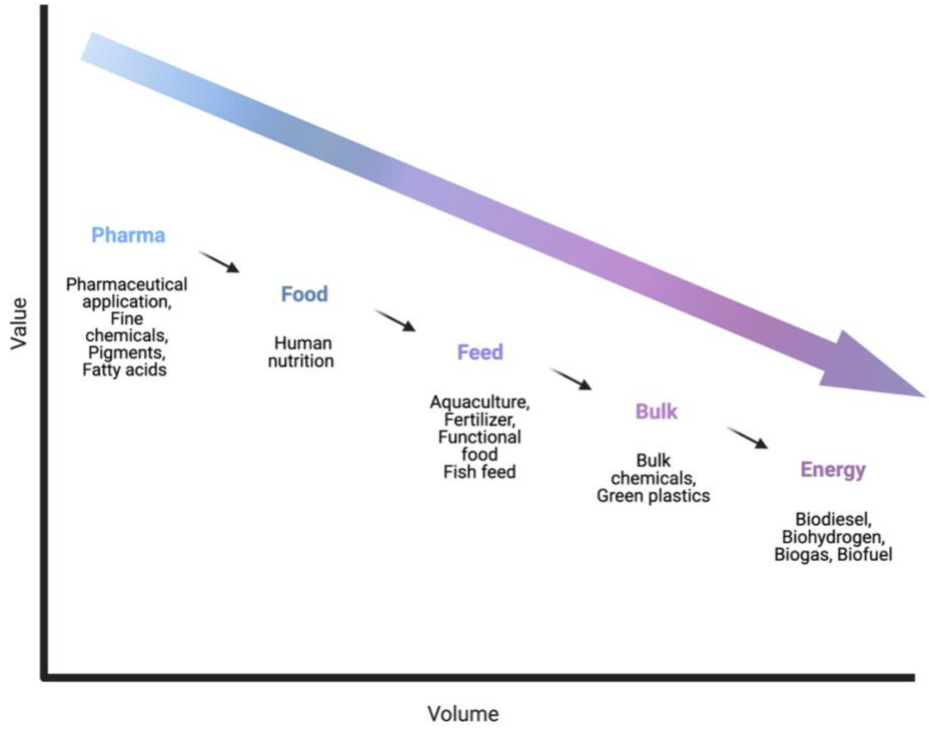


Figure 3 - Bioeconomy of value-added products obtained from microalgae. Created with BioRender.com.

Despite the numerous advantages of algae-based biofuels, there are still true challenges and bottlenecks within the process that need to be addressed and resolved before this technology can become a mainstream energy source [19]. These include the high cost of production at a large-scale cultivation and the high energy requirements mainly when needing to separate the cultivated biomass from the liquid culture medium and the extraction of the intracellular lipids as the cell wall of microalgae is quite rigid. However, if one were to find a suitable growth medium, a low energy harvesting method and an effective and cost-efficient lipid extraction method, this technology could be a game changer. This is why, research and developments efforts are ongoing and are still needed to continue making breakthroughs allowing this promising renewable energy form to truly kick off and unleash its full potential.

I Literature review

I.1 Microalgae

Microalgae are small aquatic organisms that can perform photosynthesis and therefore play a vital role in the global oxygen production and carbon fixation. These organisms can be found in many different environments, including freshwater and marine systems and they always have a curial role in the aquatic ecosystems.

As is often the case in nature, the diversity within microalgae is immense; it is estimated that there are between 200,000 and several million species of microalgae compare to around 250,000 species of higher plants [20]. This vast diversity of these microalgae represents an almost untapped resource [20].

These microorganisms can be used in many ways, including for instance as feedstock for biofuels, as feed for aquatic animals, as a way of purifying water and helping diminish pollution or for cosmetics, medicine or even as food for humans [20]. Indeed, in recent years there has been more and more research carried out, aiming to understand more about microalgae to tap into this vast array of possible products that can be extracted from them [20].

I.1.1 Microalgae classification

This section covers the world of microalgae classification. Like all living organisms, microalgae are classified using the system called taxonomy. This is a biological discipline that tackles the challenge of providing a reliable reference system that identifies, describes, classifies, and names all living organisms [21]. Taxonomy allows us to understand the diversity of life on Earth and to organize it into groups based on shared characteristics [21]. This would seem simple enough but the sheer number of species being discovered yearly, makes this a greater challenge than one would think. Furthermore, in the case of microalgae, the evolutionary origin of algal diversity makes it even more difficult to create a classification scheme that is phylogenetically meaningful [22] . This has made it so that multiple different classifications are currently in use.

Microalgae, like all living organisms, follow the same hierarchical classification system, which includes the following levels: Domain, Kingdom, Division or Phylum, Class, Order, Family, Genus, and Species. Each level represents a progressively smaller group of organisms that share increasingly specific characteristics. To establish links between different groups of microalgae researchers will examine different aspects, including but not limited to their fossil records, their phylogeny, their flagella and plastids or their ecology and distribution [23].

Microalgae can be classified into either the eukaryotic or prokaryotic domain, and then categorized further into either the Protista also called Plantae kingdom, based on their cellular structure and mode of nutrition [23].

Next, microalgae are grouped into various divisions also called phyla, examples include Chlorophyta (green algae), Rhodophyta (red algae), and Bacillariophyta [23].

These phyla can then be further classified into classes, each with unique characteristics such as the presence or absence of flagella or the type of photosynthetic pigments and the presence of a silica shell [23].

Moving up the taxonomy ladder, there is the order, family, genus, and ultimately the species, with each category containing members that share increasingly specific characteristics [24]. It is widely agreed upon within the scientific community that the species is the fundamental unit for taxonomy [24].

I.1.1.1 Procaryotic microalgae

I.1.1.1.1 Blue green microalgae: Cyanobacteria; chloroplast

Cyanobacteria, also known as blue-green algae, create a bit of debate within the scientific community because as its name might hint towards, there is some debate if they should be considered as bacteria or as algae. In the article written by Marter et al. 2021 they explain that cyanobacteria could be one of the worst taxonomies examples among the whole prokaryotic lineages [25]. They explain that the reason for this is that there was once a four-decade-long jurisdictional conflict between two nomenclature entities, on the one hand there was the International Code of Nomenclature of Prokaryotes (ICNP; "Prokaryotic Code") and on the other hand there was the International Code of Nomenclature for algae, fungi and plants (ICN; "Botanical Code") this debate ended in a nomenclatural deadlock [25]. Indeed, despite their common name, blue-green algae, are not technically algae because they lack many of the defining characteristics of algae, such as the presence of a true nucleus and other organelles. However, as cyanobacteria are microorganisms that are capable of photosynthesis, they are considered by many to be microalgae [25].

These microorganisms include around 2000 species and are considered to be one of the earliest forms of life on Earth [20]. Many times, cyanobacteria dominate the algal community, this is because they are extremely fast at up taking and storing nutrients. Polyphosphate granules serve a place for cyanobacteria to store phosphate, whereas nitrogen, carbon and energy is reserved in a polymer called cyanophycin, carbon and energy can also be stored in the form of cyanophycean starch [20].

Like all other microalgae, cyanobacteria produce various bioactive compounds, including hepatotoxins or neurotoxins which can cause serious health hazards for the public health when cyanobacterial blooms occur, but on the other hand can be used for commercial purposes [20]. The most used cyanobacteria in biotechnology are *Spirulina (Arthrospira) platensis* [20].

I.1.1.2 Eucaryotic microalgae

I.1.1.2.1 Green microalgae

Green microalgae are rich in chlorophyll a and b, this is what gives them a characteristic green colour. These microalgae will usually store their energy in the form of starch within small double membrane organelles called plastids, found inside the endoplasmic reticulum (ER) [23]. In the green microalgae, Chlorophyceae represent the largest group, they include about 2,500 species. The best-known species within this group are *Chlorella, Chlamydomonas, Dunaliella* and *Haematococcus* [23].

I.1.1.2.2 Euglenida

These microalgae exhibit both animal- and plant-like features. There is estimated to be around 800 species belonging to this group, they are all flagellated and have flexible cell walls, this allows some of them to show a crawling-like capability [23].
The Euglenida often lack the photosynthetic pigments and therefore need organic matter as nutrition. This means that they have several unique pathways and metabolites making them an interesting group for biotechnology investigation. However, this also means that they often require a complex growth medium [23].

I.1.1.2.3 Rhodophyta

The presence of beta-carotene gives these algae a red colour. Contrary to the green algae, Rhodophyta will store their energy in the cytosol and not in plastids, but also in the form of starch [23]. The pigment beta-carotene is commonly known as vitamin A. This pigment cannot be chemically synthesised which is why it is usually in high demand. Astaxanthin is another form of beta-carotene which is for example, commonly used to make salmon flesh more red. Due to these factors carotene from red algae is valued at approximately 700 euros per kilo [26].

I.1.1.2.4 Haptophyta or Prymnesiophyta

These are yellow to Brown algae that consist of approximately 500 species. The colour of these microalgae is mainly given through the presence of xanthophylls and is very similar to the colour of diatoms [23]. Some species belonging to this group are being used for feed of shellfish larvae and particularly oysters [27].

I.1.1.3 Extremophilic algae

It is the case of some microalgae that they not only can survive in extreme conditions but need these extreme conditions to grow, these algae are called extremophiles [20]. An example can be *Dunaliella salina* that require extremely high salt concentrations to grow or the cyanobacteria *spirulina* that exhibit optimal growth at high pH (between 9 and 11) [28] . This can be a true advantage in mass outdoor cultures where contamination is a persistent issue [20].

I.1.2 Microalgae present at Aalborg University Esbjerg

I.1.2.1 Chlamydomonas sp.

Chlamydomonas sp. belongs to the Chlorophyceae phylum [20]. It is a model system for research within the microalgae world [29]. They are typically smaller than 10 µm, unicellular and mobile due to its two flagella [29]. They reproduce asexually and can grow both heterotrophically and autotrophically [30]. Figure 4 shows an electron micrograph of *Chlamydomonas* sp., where different cellular compartments can be observed such as vacuoles (Va), the nucleus (Nc), the nucleolus (Ncl), mitochondrias (Mt), chloroplasts (Cp), and pyranoids (Py) [29].

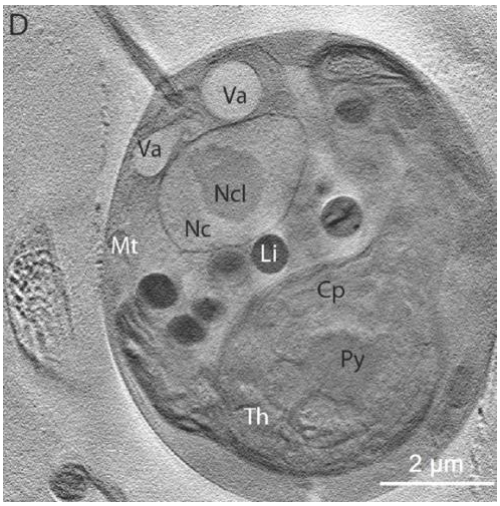


Figure 4 - Electron micrograph of *Chlamydomonas sp.*, showing vacuole (Va), nucleus (Nc), nucleolus (Ncl), mitochondria (Mt), chloroplast (Cp), and pyranoid (Py). Figure from source [29].

I.1.2.2 Stichococcus deasonii

Microalgae with a Stichococcus-like morphology are highly common and can be found in practically all types of habitats from freshwater, brackish, marine to hot acidic springs and snow [31]. *Stichococcus deasonii* are around 10 µm in size and have a rod shape. They propogate by asexual reproduction [32].

I.2 Biofuels

I.2.1 Classification of biofuels

I.2.1.1 First-generation biofuels

The first generation of biofuels utilized biomass from crops that were originally intended for human consumption, such as corn and sugarcane, which are commonly used in the USA and Brazil, respectively [10]. The principle consists of taking the sugars, starches and cellulosic biomass and converting them into bio-alcohols which can then be blended with conventional fossil fuels, this causes an increase in the octane number of the fuel thus lowering its volatility [11]. Anto et al. 2020, explain that first-generation biofuel date back to the 19th generation.

However, first generation biofuels present certain limitations. First, there is the big controversy of "food vs fuel", the cultivation of crops solely for energy purposes raises ethical concerns, particularly as the global population continues to grow rapidly [33]. Second, Doshi et al. 2016 state that there is a relatively low return on investment. Indeed, if one looks at the energy return on energy invested (EROI) for fossil fuels compared to biofuel from first generation feedstock, that of the first generation is a fair bit lower than that of fossil fuels, namely 1.1 - 5.8 EROI compared to 9 – 10 EROI respectively [34]. Third, first-generation biofuels compete with the food industry, driving up the prices of food crops, as well as meat and dairy products that rely on these crops as animal feed [35]. This increase in price of food crops downgrade the economic feasibility for biofuels from the first-generation feedstock [11]. Fourth, the cultivations of some of the first-generation feedstock such as sugar cane, greatly contributes to the deforestations in the Amazonian Forest and the devastating loss of biodiversity that goes with it [36]. And finally, biofuels require a large amount of freshwater, which is a scarce resource, with biomass crops having a water footprint that is 72 times higher than fossil fuels and 240 times higher than solar energy [36], [37]. These facts question the sustainability of first-generation biofuels.

I.2.1.2 Second-generation biofuels

Second-generation biofuels try to overcome some of the limitation from the first generation by using non-edible lignocellulosic biomass such as tree biomass, grass, jatropha, agriculture residues, straw, demolition wood etc. [11]. These crops are cheaply available and given the lower dependency on food the return on investment can be improved with an EROI of 11 [34]. Nevertheless, second-generation biofuels also present certain limitations, indeed the fermentation of this type of biomass requires a pre-treatment step to transform the cellulosic polymers into carbohydrates the microbes can digest. Because of this, second-generation biofuels tend to be more expensive and require more energy to manufacture than first-generation biofuels [38].

I.2.1.3 Third-generation biofuels

Third-generation biofuels come from photosynthetic organisms such as microalgae, cyanobacteria micro- or macro-algae [11]. The advantages of using algae as feedstock for biofuel are numerous. Starting with the facts that they have the ability to mitigate CO_2, that they require low aera for cultivation and that they can thrive in harsh conditions [11], [12], [39]. Indeed, Algae can grow in many different types of water, including freshwater, seawater, and even industrial wastewater [40], [41]. If one looks at the oil content and the growth rate of algae it is approximately 20 to 30 times faster in growth than the food yielding crops, and the oil content of algae is around 30 times higher than that of the first- and second-generation biofuels feedstocks [42].

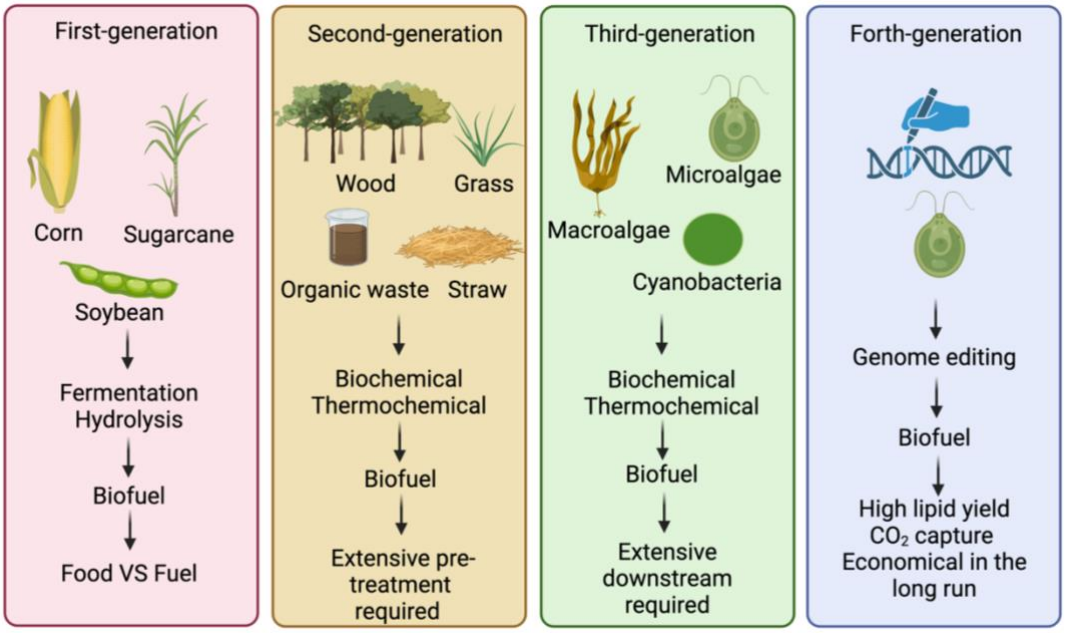


Figure 5 - Biofuel generations, figure inspired from Anto et al.[7] and created with BioRender.com.

Table 1, describes the advantages and limitations that can be found for each generation of biofuel feedstock. All the advantages from the first-generation feedstock are also true for the second- and third generation. Similarly, all the advantages seen in the second-generation are also true for the third-generation. The drawbacks that are seen in second-generation biofuels are overcome by algae-based biofuels, namely the controversial "food VS fuel" debate, the use of arable lands and the water use [11].

Table 1 - The advantages and disadvantages of biofuel feedstock generations [11].

Types of feedstocks	Advantages	Disadvantages	Ref.
First-generation (food crops: grains, sugarcane, vegetable oils etc.)	- Increases the financial assistance to agriculture rural communities. - Sustainable fuel limiting the greenhouse gas emissions.	- Food VS Fuel - Increase in food and feed prices. - High land requirements. - High energy requirements.	[43], [44]
Second-generation (lignocellulosic energy crops: non-edible crops, agricultural waste, forest residue, municipal biowaste)	- Doesn't compete with food and has less impact on food prices. - Can be cultivated on degraded and marginal lands. - Lower argi-chemical inputs compared to first generation.	- Pre-treatment of biomass is required. - Takes longer time for the full final feedstock to be produced. - Regulatory and consistency of the feedstock is complicated.	[45]–[47]
Third generation (photosynthetic organisms: microalgae, macroalgae, cyanobacteria)	- Easy to cultivate on barren lands. - Converts CO_2 into fuel. - Algal biomass per unit area is higher than any other feedstock. - Completely renewable feedstock. - Can grow on sewage- , salt-, industrial waste-water.	- Production cost of algae-based biofuels is slightly higher than that of other feedstock sources. - The presence of unsaturated oils can make biofuel from algae less stable than that from other sources.	[48], [49]

I.2.2 Types of Biofuels

All sorts of fuel that are produced from the combustion of biomass fall under the term of biofuels. The term "bio" specifies the organic nature of the feedstock, such as plants, animal matter or microorganisms. Given that plants and microalgae produce oil from sunlight and air and can do so year after year, their fats are renewable [50].

Microalgae biomass can be divided into 3 main fractions: the lipids and hydrocarbon fraction, the carbohydrates, and the bulk biomass. These three fractions can give different forms of fuels and energy depending on which process is used.

I.2.2.1 Transesterification

The lipids and hydrocarbons fraction can be transistorized to produce biodiesel. The transesterification process consists of converting triglycerides in the presence of alcohol, usually methanol or ethanol due to their low cost and availability [51], [52]. In other words, the manufacturing of biodiesel is done by converting oil and fats from renewable sources such as plants, recycled cooking fats, animal fats or oils from microorganisms into fatty acid methyl esters also known as FAME or biodiesel [50]. In the manufacturing process, the proportions are as follows: 50 g of oils or fats are reacted with about 5 g of short chain alcohols, usually methanol or ethanol and with the presence of a catalyst that will usually be sodium or potassium hydroxide this will produce 50 g of biodiesel and 5 g of glycerine as a coproduct of the biodiesel process [50].

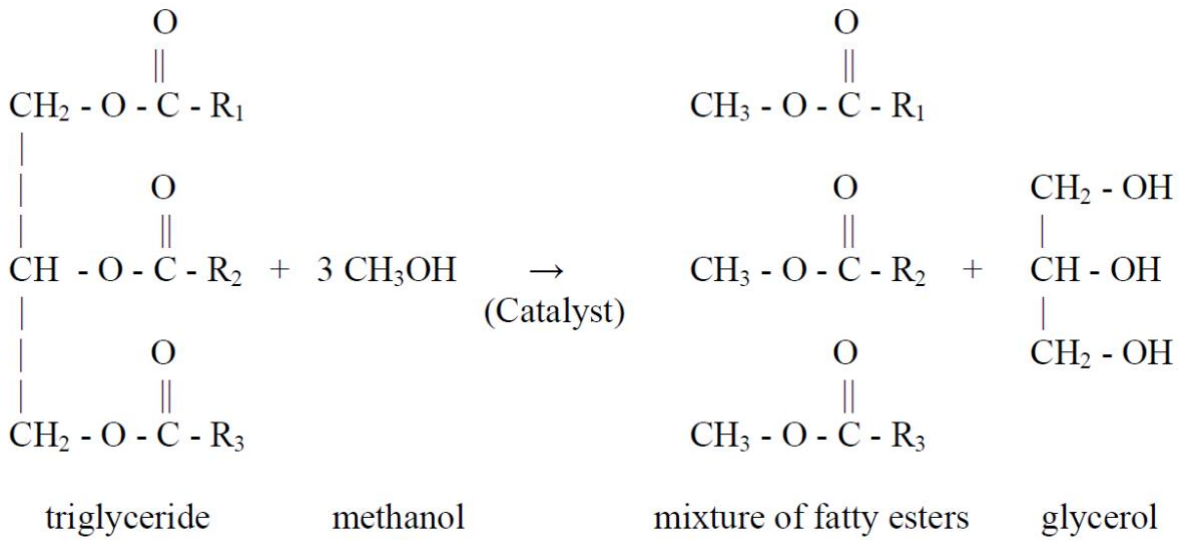


Figure 6 - Transesterification process to biodiesel from [53].

I.2.2.2 Hydrothermal liquefaction (HTL)

A process of hydrothermal liquefaction (HTL) can also be done, this is when biomass is converted into liquid under high pressure (4-22 Mpa) and high temperatures (~250 - ~375 °C) [54]. This process requires enough time for the molecules to break down [54]. The oil that emerges from this process is called bio-crude which, due to its high O_2 content and low hydrogen-to-carbon ration, it needs to undergo a hydrotreating process consisting of treating the biocrude with H_2 at high temperature and high pressure in the presence of a catalyst [55]. Through a distillation process, this upgraded biocrude can be converted into jet fuel or biodiesel [54].

I.2.2.3 Hydro processed esters and fatty acids (HEFA)

HEFA, or hydro processed esters and fatty acids, is the process where any type of oil including microalgae oil are hydrogenated and isomerized, this process results in the production of long chained hydrocarbons [54]. With an additional process of breaking down these larger hydrocarbon molecules into smaller ones (crackling), aviation fuel can be obtained [54]. Aviation fuel produced by HEFA process is high in energy content, is thermally stable and has low pollutant emissions [56]. As such, since 2008, HEFA fuels blended at a 50% limit with conventional jet fuels have been successfully tested by major airlines such as KLM and Luftansa [54], [57].

I.2.2.4 Fermentation

The carbohydrates fraction can be converted into bioethanol, biobutanol and biohydrogen through fermentation. Fermentation consists of using microorganisms to transform sugars into new products such as food, drinks, medicine, and fuels through chemical reactions [58].

I.2.2.5 Alcohol-to-jet (ATJ)

Alcohol-to-jet (ATJ) is a form of advanced fermentation where biomass of sugars is converted into long-chained hydrocarbons which have the same characteristics as conventional jet-A1 fuels [54].

I.2.2.6 Pyrolysis

Pyrolysis is another process which can also produce jet fuel, first the biomass needs to be dried, grinned, and chopped. Then it is heated at extremely high temperatures ranging from 400 to 600 °C [54]. The resulting bio-oil contains a vast amount of oxygen which is an unwanted compound in the final fuel, therefore this O_2 needs to be removed by hydrotreatment in order to obtain jet fuel [54].

I.2.2.7 Gasification also known as Ficher-Tropsch (FT)

Gasification also known as Ficher-Tropsch (FT) is a thermochemical conversion where the carbon-rich biomass is transformed into syngas [54]. Syngas consists primarily of carbon monoxide (CO) and hydrogen (H2) [54]. This is an endothermic reaction meaning it will consume heat, therefore these reactions are usually done in high temperatures and in the presence of a catalyst [59]. The syngas can then be converted into aviation fuel; however, it is important to keep in mind that the need for a catalyst makes FT an expensive option [54], [60].

I.2.2.8 Anaerobic digestion

Lastly, the bulk biomass can be converted into biogas by anaerobic digestion, as the name suggests this is the process where microorganisms break down organic matter in the absence of oxygen [61].

I.2.2.9 Microalgae biofuel

Many of these processes have already been employed to convert microalgae oil into biofuels. For example, gasification and Fischer-Tropsch and sugar-to-jet can become the future alternative process to convert microalgae to bio-jet fuel.

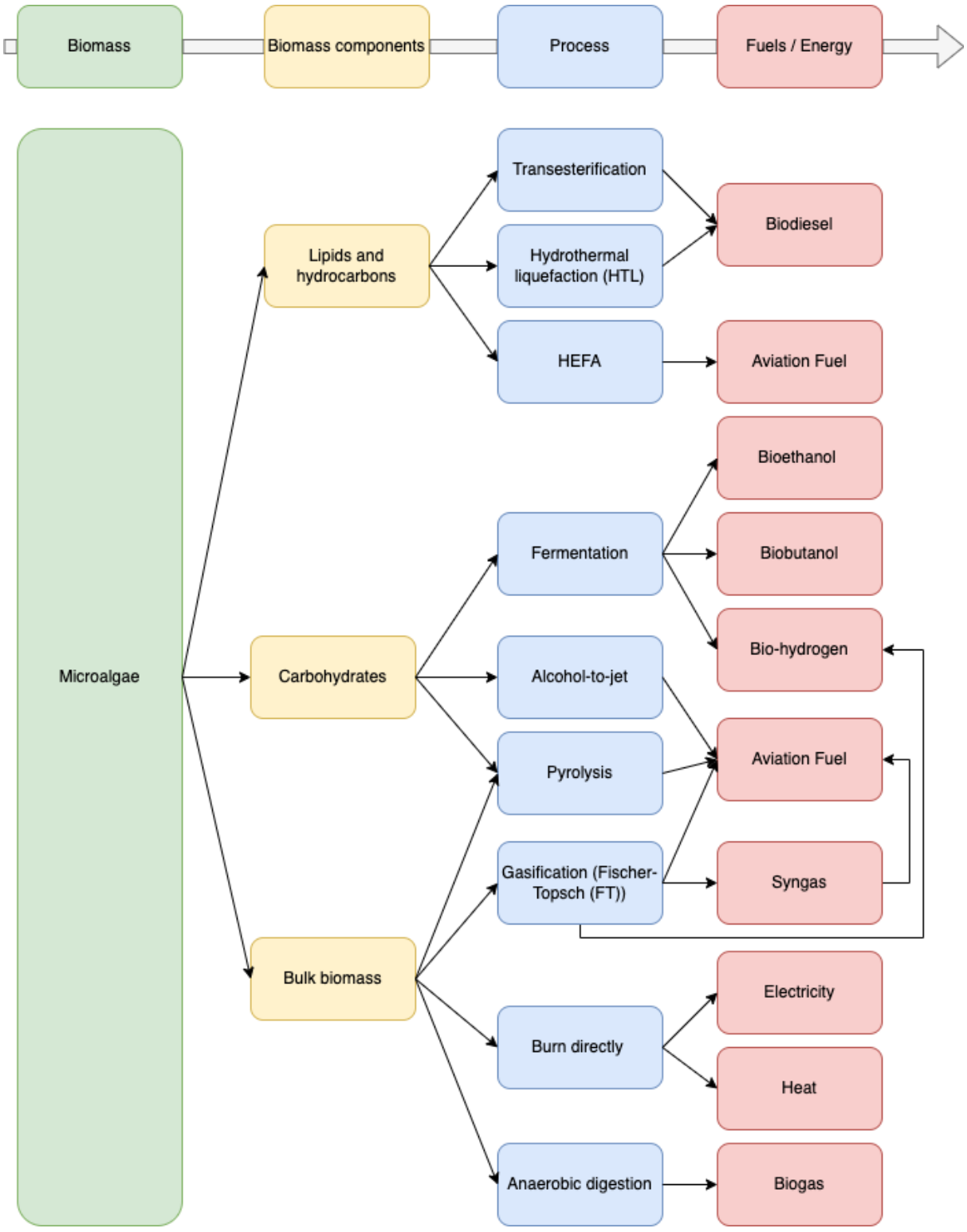


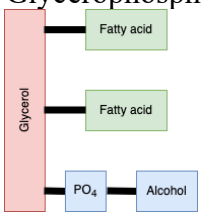
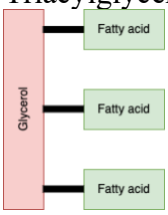
Figure 7 - Routes for microalgae production figure adapted from Hoang et al. 2022 [17], [54], [55].

I.3 Lipids and high value products

I.3.1 Lipids

Microalgae produce two main groups of lipids: structural lipids (polar lipids) and non-polar lipids.

Table 2 - Lipids in microalgae.

Lipids	Polar lipids	Non-polar lipids	Ref.
	Glycerophospholipids	Triacylglycerols (TAGs)	[62]
			[63]
	Amphipathic (hydrophilic and hydrophobic)	Hydrophobic	[63]
Main role	Cell structure	Energy storage	[62]

Many properties of biodiesel depend on the types of fatty acids that are used, these properties include the cetane number, the density, the viscosity, the flash point, the oxidative stability, and the cold filter plugging point [64]. The ideal biodiesel would be made only from monounsaturated fatty acids (MUFA) and fewer polyunsaturated and saturated fatty acids (PUFA and SFA) [50], [64]. The cetane number (CN) and the ignition delay (ID) are indicators of the fuel's quality in the diesel engine realm and are closely related to the amount of MUFA compared to PUFA and SFA [65]. The CN is usually between 15 and 100 and the higher it is, the better. The CN is closely correlated to the ID which is the time that it takes between the fuel being released into the engine and the onset ignition [65]. The shorter the ID the better [65]. These three values are related in the sense that the shorter the ID, the higher the CN and vice versa. And, to tie it all together, the more MUFA, the higher the CN and the shorter the ID [65].

Microalgae have a high lipid-productivity [19]. This can be seen in Table *3* where various types of crops and their average crop yield in litres of oil by a biomass per acre of land are described. Microalgae exhibit a remarkably high oil yield per acre. It ranges from 19,000 to 57,000 litres per acre, which is 279 to 838 times higher than corn, 105 to 314 times higher than soybean, 49 to 148 times higher than sunflower, 40 to 118 times higher than rapeseed, 38 to 114 times higher than jatropha, and 8 to 24 times higher than oil palm. This would make microalgae a highly attractive and sustainable alternative for oil production [18]. However, it is also important to look at the fatty acid profile, which are also shown in Table 3. Most crops have more unsaturated fatty acids than saturated ones, except for palm oil and microalgae, which have more saturated fatty acids.

Table 3 - Variations in oil production rates and fatty acid profiles among different biomasses.

Fatty Acid	Corn % w/w [18], [66]	Soybean % w/w [18], [67]	Sunflower % w/w [18], [67]	Rapeseed % w/w [18], [68]	Jatropha % w/w [18], [69]	Oil palm % w/w [18], [67]	Microalgae *Chlamydomonas reinhardtii* % w/w [18], [70]	Microalgae *Stichococcus sp* % w/w [18], [71]
Total oil yield								
Oil yield (L/acre)	68.13	181.68	386.07	480.69	495.83	2,403.47	19,000 - 57,000	
Saturated Fatty acids (SFA)								
C12:0	-	-	-	-	-	0.2	7.48	-
C14:0	-	-	-	0.05	0.1	1.1	2.46	2.12
C16:0	11.88	30.4	30.7	4.84	14.2	44.1	16.26	17.61
C17:0	-	-	-	0.14	0.1	-	-	-
C18:0	1.92	5.2	2.1	0.14	7	4.4	9.22	0.54
C20:0	0.28	-	-	0.5	0.2	-	-	-
C22:0	-	-	-	0.3	-	-	-	-
Total SFA	14.08	35.6	32.8	5.97	21.6	49.8	35.42	20.27
Monounsaturated Fatty Acids (MUFA)								
C16:1	-	-	7.6	0.06	0.7	0.2	7.7	-
C18:1	29.6	10.1	56	62.73	44.7	39	23.29	4.5
C20:1	-	-	-	1.25	-	-	-	0.15
Total MUFA	29.6	10.1	63.6	64.04	45.4	39.2	30.99	4.65
Polyunsaturated Fatty Acids (PUFA)								
C16:2	-	-	-	-	-	-	6.49	0.19
C16:3	-	-	-	-	-	-	5.21	-
C18:2	24.68	42.6	3.1	22.4	32.8	10.6	11.18	4.78
C18:3	0.86	11.7	-	7.5	0.2	0.3	10.31	5,71
Total PUFA	25.54	54.3	3.1	29.9	33	10.9	33.19	4.97

I.3.2 Value added Products (VAP)

As mentioned in the introduction, microalgae have emerged as a promising feedstock for various industries, including food, cosmetics, and biofuels. [72], [73]. In this section, we will explore the potential of microalgae for the production of value-added products such as antioxidants, pigments, vitamins, biomass and others, as shown in Figure *8*.

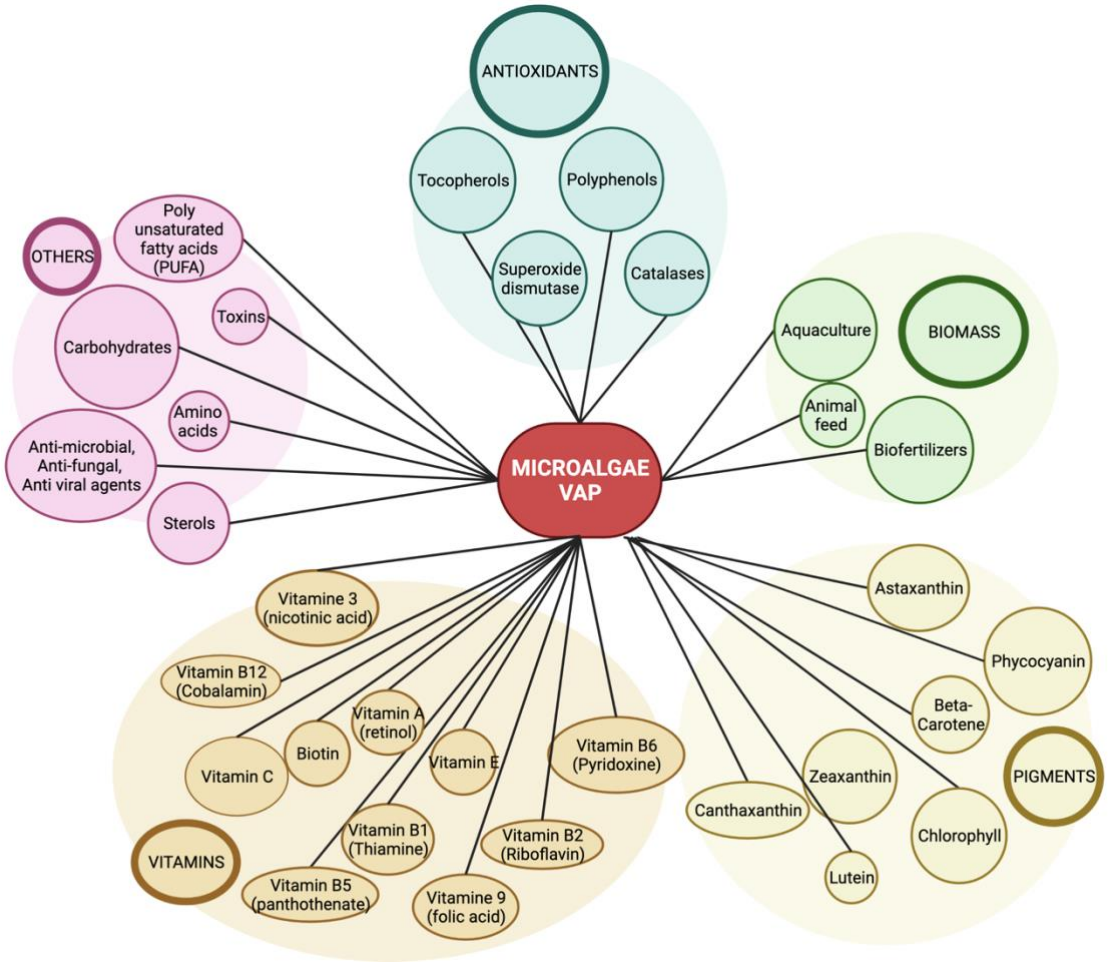


Figure 8 - Microalgae value added products (VAP) adapted from [74] and created with BioRender.com.

Table 4 outlines the dry biomass, the lipid yield, the dry weight of high value compounds and the biorefinery applications of different microalgae strains. This table shows that most microalgae produce roughly 3 g/L of dry biomass. Some species, however, exhibit more extreme variations, for example *Dunaliella salina* that can attain up to ~50 gDM/L in biomass. When focusing on the lipid yields shown in this table, it is possible to see a considerable variation even within the same strain. Literature suggests that some strains can accumulate more than 50% of their dry weight in lipids, however the actual range usually fall between 5 and 20 %. In this table the highest lipid content is attributed to the species *Chlamydomonas reinhardtii* with 15 to 57 % of lipids. The dry weight of high value compounds also varies

significantly from species to species however in some cases, there can be up to 70 % of the dry wight that can be used as high value compounds, such as proteins, sugars, or pigments.

Table 4 - Comparative evaluation of high value compounds and lipid content in different microalgal phyla for biorefinery applications.

Microalgae	Dry biomass (g/L)	Lipid yield (%)	Dry weight high value compounds content (%)	Biorefinery application	Ref.
Dunaliella tertiolecta	2.15	11.44 ± 1.8	36	Protein, sugar, pigments	[75]
Chlorella vulgaris	1.94	9.95 ± 2.1	58	Protein, sugar, pigments, b-1,3-glucan, nutrient source for humans	[17], [75]
Spirulina platensis and Arthrospira platensis Bacteria	2.18	11 ± 2.2	60-71	Protein, sugar, pigments, food sup-plement for humans, cattle, poultry, aquarium fish, ornamental birds and horses	[17], [75]
Botryococcus braunii	3.11	33 ± 2	58	Protein, sugar and pigments	[75]
Dunaliella salina	46.91 – 18.99	23	1.64 - 0.34	β-carotenoids	[76], [77]
Chlamydomonas reinhardtii	2.06 – 1.25 0.2 – 2.7	15 - 57	13 - 59	Protein, Sugar	[78], [79]
Stichococcus bacillaris	3.46 - 3.79	6.5 – 13.8			[80]

Table *5* shows the vast array of VAP that can be extracted from different microalgae.

Table 5 - Value added products that can be communalised from different microalgae strains. Adapted from [20] (OP: Open pond; PBRs: photobioreactors; P: phototropic cultivation; H: heterotrophic cultivation).

Microalgae	Product	Cultivation reactors	Cultivation mode	Application	ref
Aphanizomenon flosaquae	Vitamins, fatty acids, phycocyanin Glycoproteins, vitamins, lipids	OP, PBRs	P	Pharmaceuticals, nutrition, cosmetics	[81]
Chlamydomonas reinhardtii	Polysaccharides, lipids, functional proteins, pigments, hormones, vaccines, antibodies	OP, PBRs	P,H		[82]
Chlorella minutissima	Eicosapentaenoic acid	PBRs	P,H	Pharmaceuticals, nutrition	[81]
Chlorella protothecoides	Biomass, lipids, tocopherol	PBRs	P,H	Pharmaceuticals, nutrition	[81]
Chlorella vulgaris	Lipid, biomass	OP, PBRs	P,H	Health food, food supplement, feed	[20]
Crypthecodinium cohnii	Docosahexaenoic acid	PBRs	H	Pharmaceuticals, nutrition	[81]
Dunaliella salina	Carotenoids, β-carotene	OP, PBRs	P,H	Health food, food supplement, feed	[20]
Euglena gracilis	α-Tocopherol, biotin	PBRs	P,H	Pharmaceuticals, nutrition	[81]
Galdieria sulphuraria	C-phycocyanin	PBRs	P,H	Health food, cosmetics	[81]
Haematococcus pluvialis	Carotenoids, astaxanthin	OP, PBRs	P,H	Health food, pharmaceuticals, feed additives	[20]
Isochrysis galbana	Fatty acids	OP, PBRs	P,H	Animal nutrition	[20]
Lyngbya majuscule	Immune modulators	OP, PBRs	H	Pharmaceuticals, nutrition	[20]
Monodus subterraneus	Eicosapentaenoic acid	OP, PBRs	P	Pharmaceuticals, nutrition	[20]
Nannochloropsis oculata	Lipids	OP, PBRs	P,H	Pharmaceuticals, cosmetics, nutrition	[81], [83]
Nitzchia laevis	Eicosapentaenoic acid	PBRs	H	Pharmaceuticals, nutrition	[81]
Odontella aurita	Fucoxanthin, fatty acids	OP	P	Pharmaceuticals, cosmetics, baby food	[20]

Parietochloris incise	Arachidonic acid	PBRs	P	Pharmaceuticals, nutrition	[81]
Phaedactylum tricornutum	Lipids, fatty acids	OP, PBRs	P,H	Pharmaceuticals, nutrition	[20]
Porphyridium cruentum	Polysaccharides	PBRs	P,H	Pharmaceuticals, cosmetics, nutrition	[81]
Prototheca moriformis	Vitamin C	PBRs	H	Pharmaceuticals, nutrition	[81]
Shizochytrium sp.	Docosahexaenoic acid	PBRs	H	Pharmaceuticals, nutrition	[81]
Spirulina platensis	Phycocyanin, biomass	OP, PBRs	P	Health food, cosmetics	[20]
Tetraselmis suecica	Lipids, PUFA	OP, PBRs	P,H	Pharmaceuticals, nutrition	[81], [83]

I.4 Aquaponics a circular aquaculture-based industry

As stated by Khan et al. 2021, circular bioeconomy models are becoming a critical component of the green technology transition [84]. A circular economy model must balance the economic, environmental, and social objectives. These are the fundamental principles that connect bioeconomy, green economy, and circular economy [84]. The main idea of circular economy is to have a business model where the processes and the units demonstrate an economic feasibility while minimizing waste and environmental impacts [84]. Circular economy is based on three main and simple principals; the first principal is "no waste", this means that the products must be biodegradable and renewable. The second principal is that the consumed resources must be recovered, and this must be done without posing any danger to the ecosystems. And the third principal, is that the energy used along the process must come from sustainable and renewable energy sources [84].

Microalgae based circular bioeconomy can serve as a foundation for a circular aquaculture-based industry as part of a larger circular bioeconomy which can meet several of the UN Sustainable Development Goals (SDG) [84], [85]. A study conducted by Sutherland et al. 2021, explains the roles and or products of microalgae which can assist with the achievement of several SDG [86]. For example, SDG 2. "Zero Hunger", by providing high quality protein, and polyunsaturated fatty acid as an additive for human consumption or as feed for agriculture and aquaculture [86]. SDG 6. "Clean Water and Sanitation", where microalgae can play a vital role in wastewater treatment [86]. SDG-7 "Affordable and Clean Energy", here, microalgae can be used as feedstock for the third generation of biofuels [86]. SDG-12 "Responsible Consumption and Production", as there is the possibility of producing microalgal bioplastics [86]. SDG-14 "Life Below Water", microalgae can assimilate the diffused pollutants as nutrient and thus do bioremediation [86]. SDG-15 "Life on Land", through bio stimulants microalgae can help to improve crop plant growth and yield as well as strengthen the plant's tolerance to stress factors [86].

Microalgae farming can be done in non-arable land, it reduces the freshwater demand, the nutrients used in the process can be recycled and reintroduced as secondary raw materials, the microalgae biomass can have several commercial uses and finally, the microalgae convert atmospheric CO_2 into nutrient-rich sustainable feedstock [84].

Moreover, many marine species such as fish larvae and filter feeders like clams, krills, and whales rely on microalgae as their main source of nutrition [87]. Aquaculture companies therefore typically produce microalgae in-house, usually on a small scale of around 100 m^2, to ensure a constant supply of fresh feed for their hatcheries [87].

Aquaponics are integrated productions of fish cultivation and hydroponic crops. Hydropnic crops consists growing crops with or without soil as a physical support and where the fish waste is used as plant fertiliser [88], [89]. The state-of-the-art within aquaculture relies on Recirculating Aquaculture Systems (RAS). This solution is both eco-friendly and reduces the

waste output. Furthermore, such systems have a low water demand which is becoming a crucial factor as the freshwater supply in the world is gradually decreasing [90].

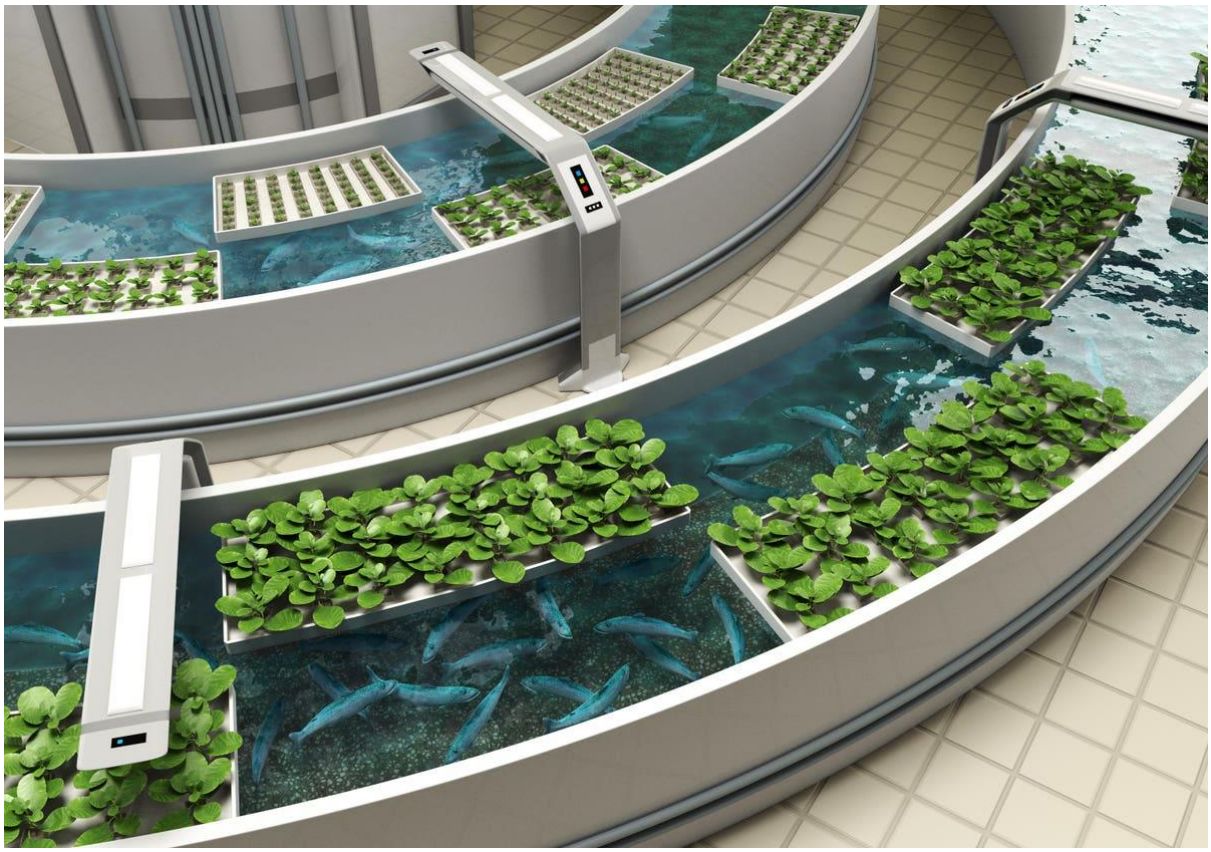


Figure 9 – Aquaponic system, picture taken from forbes magazine reference [91]. Aquaponics is the symbiosis of aquaculture with hydroponics.

It is estimated that about 0.133 kg of nitrogen is produced per kg of raised fish in aquaculture facilities [92]. Moreover, in order to meet the environmental standards, aquaculture facilities need to employ treatment strategies to remove the nitrogen and phosphorus from the effluent streams. It has been documented that over 99% of the total nitrogen and phosphorus resources can be removed from the municipal wastewater by microalgae [93].

Therefore, there are currently several examples where microalgae are already being used in circular business models. In a study by Gatto et al. 2021, a circular economy-based business model is described, where microalgae biomass is used for serval different products including, agriculture, nutrition, cosmetics, and aquaculture [84]. Another example is AlgaePro, a Norwegian company where technologies for growing microalgae are being investigated by recycling urban waste, CO_2 and waste heat from industrial sites [94]. A project called SaltGae conducted mainly in Italy and Slovenia aims to meet European initiatives by cultivating microalgae to absorb nutrients from agriculture wastewater and then once the water is cleaned, the algae are dried and used for cosmetics, animal feed and fertilisers [95]. In a study conducted by Tejido-Nuñez et al. 2020, two microalgae strains *Chlorella vulgaris* and *Tetradesmus obliquus* were co-cultivated in a RAS [89]. Similarly, Milhazes-Cunha et al. 2017, proposed to cultivate microalgae by using the excess ammonium in aquaculture effluent.

Fish feed can be produced alongside other valuable products from the microalgae and reintroduced into the aquaculture system [90]. This is the same principal that the local aquaculture industry Alpha Aqua in Esbjerg, Denmark is aiming for. This circular economy model using microalgae is presented in Figure 10.

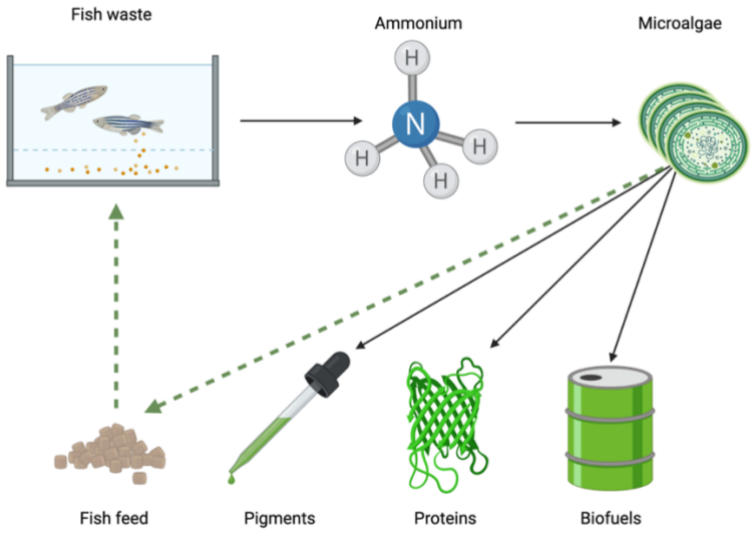


Figure 10 - Circular aquaculture-based industry. Created with BioRender.com.

I.5 Comparison between open and closed cultivation systems

The two most common methods for cultivating microalgae are either open systems such as open ponds, raceway ponds and tanks, or closed controlled systems that use bioreactors [96].

One of the first recorded attempt at scaling up microalgae cultivation, was carried out by Johnson et al. in 1988 by using open raceway ponds [97].

Both systems have different advantages and disadvantages. The main advantages seen when using open systems is the minimal capital and operation costs, as the maintenance and cleaning is easier and given that the primary energy input would be sunlight [98]. Additionally, the mixing of the culture is easier for example with a paddle wheel in the case of raceway ponds [98]. The biggest disadvantage, however, comes from the high risk of contamination for example from birds and the vulnerability to adverse climate conditions [96], [98]. Open systems also often require larger land areas to scale up [96], [98].

The main advantage of closed systems, also called photobioreactors (PBRs) is the control that the operators have over the growth conditions, such as the culture temperature, the evaporation, the pH, sufficient nutrients and CO_2 etc... [96], [98]. The PBRs generally require less space and have a great decrease in contamination issues [96]. On the downsides, the PBRs generally have higher designing and operation costs. They tend to be more complicated to maintain and clean and in addition, overheating and bio fouling are often observed [96].

Integrating the benefits and limitations of both open and closed systems, hybrid systems have recently gained attention. Hybrid systems, a combination of open and closed cultivation systems, are designed to maximize the advantages of both systems, aiming to mitigate the weaknesses of each [98]. In the initial phase, the microalgae are grown in a closed system to reduce contamination risk and control the growing conditions optimally. Following this, the microalgae are transferred to an open system for cost-effective mass cultivation [98]. This system seeks to balance the control and efficiency of photobioreactors with the economic feasibility of open ponds. Despite the promising potential, practical implementation of hybrid systems is still in early stages, with ongoing research aimed at addressing operational challenges such as system design and optimization, energy efficiency, and species-specific cultivation conditions [99].

All in all, however, the choice of the cultivation system depends on the purpose of the cultivation and the specifics needs that come with it [100]. For example, for wastewater treatment, circular open ponds can be used. Contrary to the production of pharmaceuticals products or high value nutritional products where a more controlled growth is required, and thus only a closed system would meet these needs [97].

In Table 6, the main advantages and disadvantage of both types of cultivations systems (open and closed) are listed.

Table 6 - Advantages and disadvantages of open vs closed operation systems. Adapted from [96], [98], [100].

Cultivation systems	Advantages	Disadvantages
Open	• Higher production volume possible. • Easier to clean. • Easy to scale up.	• Difficult to control the culture conditions. • High contamination risk • Larger land area requirements.
Closed	• Higher cell densities. • Can be sterilised, low contamination risk. • Better control of growth conditions	• Difficult to clean. • Higher designing and operation costs. • Difficult to scale up.

I.6 The bottlenecks in microalgae-based biofuels.

I.6.1 Microalgae harvesting

Microalgae size can range between 5 µm (*chlorella*) to more than 100 µm (*Spirulina)* [101]. As such, these small microorganisms can be challenging to harvest. Apart from their small sizes, other characteristics that make microalgae difficult to harvest is the fact that they usually have a low cell density, are mobile and their surface is negatively charged[102]. Therefore, different techniques have been investigated for the harvesting and dewatering of microalgae. The physical techniques include flotation, centrifugation, sedimentation, flocculation, ultrasonic aggregation, and filtration [102]. A combination of one or more of these techniques can also be employed [102]. However, these techniques usually require a high amount of energy and have high costs. As such when employing these techniques, the energy that would go into producing one barrel of algae-based biofuel would be larger than the energy that same barrel would provide [102].

Given these limitations, other harvesting methods have been investigated such as biological methods in order to make microalgae harvesting more affordable and eco-friendlier [103].

Flocculation refers to the process where individual microscopic organisms, like bacteria or algae, cluster together to form larger, aggregated masses or "flocs". This process enhances separation and removal efficiency in waste treatment or biofuel production.

Several bacterial strains, fungi, yeast and cyanobacteria have been investigated as bio-flocculants. In a study conducted by Prochazkova et al. 2015, the use of brewer's yeast was used to harvest *Chlorella vulgaris* grown in freshwater [104].

In a study conducted by An et al. 2016, the researchers experimentally investigated sludge bacteria and identified a large gene cluster that was required for floc formation [105]. In order to conclude that this gene cluster was indeed responsible for the floc formation, the authors of this study genetically modified the identified gene cluster of the gram-negative bacteria *Zoogloea resiniphila MMB*. Two in-frame deletion mutants were created, asnB and asnH, in these mutants, the genes asnB and asnH from the identified gene cluster were knocked out. The mutant asnH, exhibited a delay in the floc formation compared to the wild type which served as a control. The mutant asnB, completely lacked the ability to flocculate.

Table 7 shows some of the biological methods used for microalgae biomass recovery, adapted from [102], [106] .

Table 7 - Biological methods for microalgae biomass recovery: overview and comparison [102], [106]

Method	Microalgae	Biomass Recovery efficiency (%)	Settling Time (h)	ref
Bio-flocculation	*Pleurotus ostreatus*	64.86	2.5	[107]
Bio-flocculation	*Chlorella vulgaris* + eggshell	99	0.3	[108]
Bio-flocculation	*Aspergillus niger* for *Microalgaen scenedesmus* sp.	99.4	48	[109]
Bio-flocculation	*Botryococcus braunii*	97	12	[110]
Bio-flocculation (chiton)	*Chlorella* sp.	99	0.2	[111]
Bio-flocculation (Cationic guar gum polymer)	*Chlorella* sp. and *Chlamydomonas* sp	92-95%	0.15-0.5	[112]
Bio-flocculation (Cationic inulin polymer)	*Botryococcus sp.*	88	0.15	[113]
Flocculation by pre-cultivated fungus	*Chlorella pyrenoidosa* with *Aspergillus fumigatus*	99	3	[114]
Co-culture	*Penicillium* sp. spores or pellets	98.26	2.5	[115]
Co-culture	Non-filamentous microalgae cells with filamentous fungi	98.1	1	[116]
Co-culture	*Spirulina maxima* and *Synechococcus subsalsus*	98	48	[117]
Co-cultivation	Synechocystis PCC 6803 with Aspergillus fumigatus	100	48	[118]
Fugal pelletization	*Aspergillus* sp.	~100	24	[119]
Microalgal–Fungal Pelletization	*Aspergillus lentulus*	92	24	[120]
Magnetic separation	*Botryococcus braunii* and *Chlorella ellipsoidea*	55.9 and 5.83 mg-dry biomass/mg -particles respectively	0.01	[121]
Magnetic separation	*Nannochloropsis maritima*	95	0.06	[122]
Electrochemical	*Ankistrodesmus falcatus*	91	0.5	[123]

I.6.2 Lipid extraction

The production of biodiesel from microalgal cells is hindered by the high energy demand and high costs required for the recovery and purification of lipids [124]. Kumar et al. 2015, stated that among the difficulties seen in the commercial deployment of microalgal biofuel technology the cost-effective and efficient extraction of lipids was still one of the main bottlenecks [19].

As stated by to Ghasemi Naghdi et al. 2016, an ideal extraction method for producing biofuels should fulfil several criteria, including safety, cost-effectiveness, robustness, efficiency, selectivity, environmental friendliness, feasibility for large-scale production, and absence of product contamination [124].

Moreover, the available techniques for extracting lipids from microalgal cells typically involve concentrating and dewatering the microalgae before extraction, which is both energy-intensive and inefficient [124]. Furthermore, the challenges of overcoming the thick and sturdy cell walls in oleaginous microalgae must also be addressed [124]. To address these limitations, Ghasemi Naghdi et al. (2016) suggested employing wet lipid extraction techniques thus disrupting the algal cells and extracting the lipids while they are still in solution as well as using solvent-free extraction which is a promising alternative for industrial-scale production, though it is still in the early stages of development [124].

The methods used for extracting oil from microalgae can be divided into two main categories: mechanical and chemical methods [125].

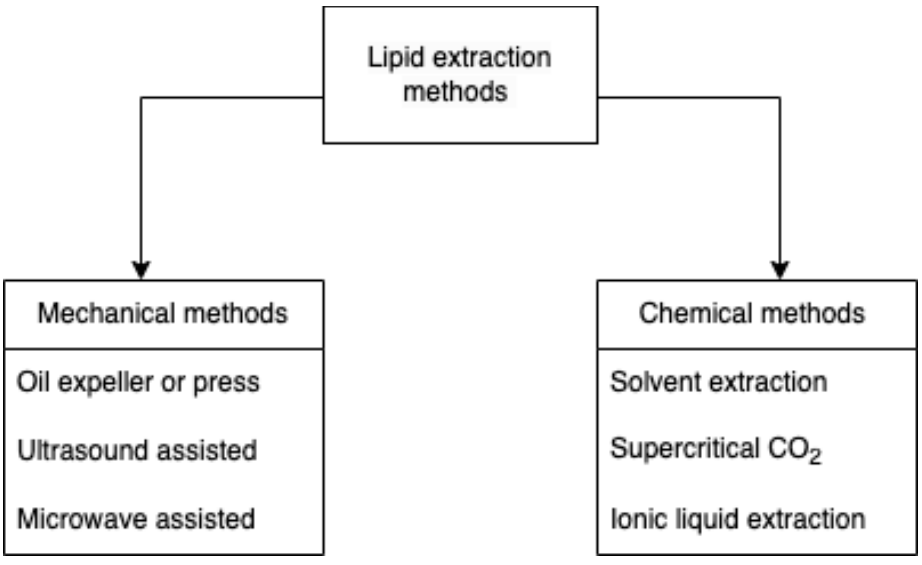


Figure 11- Diagram illustrating different techniques for extracting lipids from microalgae adapted from [126].

I.6.2.1 Extraction of lipids using mechanical techniques

I.6.2.1.1 Expeller or press

In this technique, a mechanical press is used in order to break the cells and press out the lipids from dried biomass [126]. The big drawback with this method is that it is slow compared to other techniques [127].

I.6.2.1.2 Ultrasound assisted extraction (UAE)

The principal behind UAE, is that when liquid is sonicated it generates sound waves which will propagate through the liquid medium and produce a cycle of alternating high- and low- pressure [128]. During the low-pressure part of the cycle "bubbles", "cavities" or "voids" are created and during the high pressure cycle they collapse violently thus damaging the cell wall allowing the extracts to be released as shown in Figure 12.

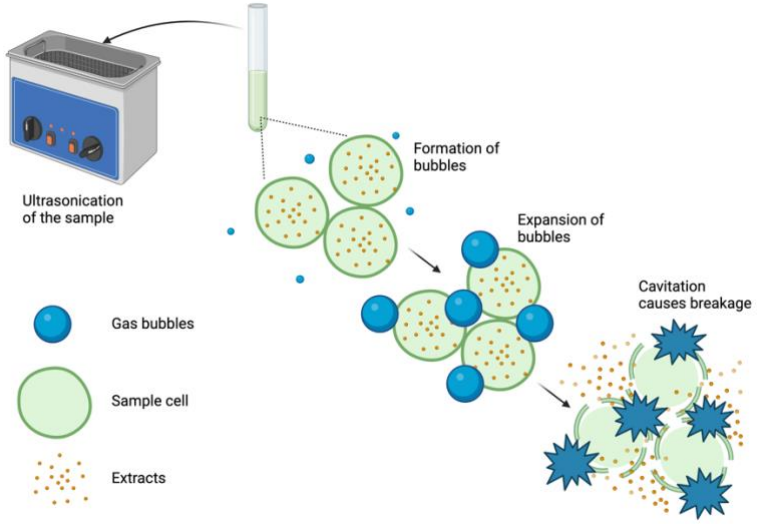


Figure 12 - Visual representation of the process of ultrasound-assisted extraction created with Created with BioRender.com and inspired from [129].

Mubarak et al. 2015, explain that due to the fact that microalgae have a thick cell wall, many of the intracellular lipids that are present, are not extracted when using methods such as solvent extraction and mechanical methods [126]. Therefore, it is suggested to use ultrasonic assisted extractions techniques.

I.6.2.1.3 Microwave assisted extraction

In this technique the use of microwaves which are electromagnetic radiations with a frequency from 0.3 to 300 Ghz, can penetrate the biomaterials and start to interact with polar molecules such as water within the biomass [126]. This does that the whole sample is uniformly heated and causes the lipids to be released from the cells [126]. In a study conducted by Chad et al. 2011 measured the lipids within 13 green algae strains using microwave assisted Nile red fluorescence [130]. By using 50 s in the microwave oven for pre-treatment of the algae biomass

and 60 s in the microwave oven for the staining process they could effectively quantify the lipid content of the algae [130].

I.6.2.2 Extraction of lipids using chemical solvents

Lipids are insoluble in water but soluble in organic solvents such as hexane, chloroform, methanol, acetone, ethanol and many more. Organic solvents have been used for many years to extract lipids from biological samples. For example, the Folch method was first described by Jordi Folch in 1957 and is still considered a standard method for the extraction of lipids from cellular material [131]. As such, the available extraction methods, such as Soxhlet extraction with n-hexane or Bligh and Dyer method with a chloroform/methanol solvent mixture are all derived from expensive conventional methods used for oil-bearing terrestrial crops and suffer from safety and environmental issues due to the use of organic solvents which makes the process toxic and harmful for the environment, rendering these methods inapplicable for industrial-scale production [124], [132], [133].

The major drawback of using toxic solvents like hexane and chloroform for the extraction of algae oil is that it can have an adverse effect on health and on the environment [134]. However, as the use of organic solvents for the extraction of lipids is one of the oldest techniques it is reliable and therefore is usually used at a laboratory scale for characterisation, but it is not fit for large scale production.

I.7 Life cycle assessment of biofuels (LCA)

This section aims at giving a quick overview of the LCA of biofuels, however it is of course possible to dive much further into this subject and it would be essential to do so in the case of a large-scale production.

The life cycle assessment is a mythology used all around the world which aims to analyse the impact generated by a product, process, or pathway throughout its existence “from cradle to grave”, this means that it is from the raw material cultivation, through production and includes the utilisation phases [135], [136].

Usually, when conducting a LCA “the impact of a product throughout its life cycle” refers to the environmental and human health impact. LCA aims to be an overall ecological assessment therefore, the economic and social factors are usually left aside [135].

LCA was initially used for industrial products and since there the International Organization for Standardization ISO 14040-series was created, showing how to conduct a standard LCA. Nevertheless, LCAs have since been applied in several different ways, thus often creating diverging results [136].

In a study by Kaltschmitt et al. 1997, the authors explained what parameters are included in the balance of a LCA of biofuels. They look at the energy sources used throughout the life cycle, and record them separately based on the final energy source (i.e., electricity, fuel oil, etc.) and based on the primary energy source (i.e. coal, mineral oil, uranium etc.). Furthermore, they explain that the other parameters that are taken into consideration are a wide range of airborne emissions such as carbon dioxide (CO2), methane (CH4), laughing gas (N20), nitrogen oxide (NO), sulphur dioxide (SO), non-methane hydrocarbons (NMHC), ammonia (NH3), dust, diesel particulates, hydrogen chloride (HC1), benzol, formaldehyde and benzo(a)pyrene, TCDD for dioxine and furane [135] .

Similarly, Gnansounou et al. 2009 evaluated the LCA of wheat-to-bioethanol [136]. Kolosz et al. 2020, made a study showing a generic aviation fuel path life cycle assessment, where they created a schematic diagram showing the different steps in the LCA of jet-fuel, from the biomass extraction until the combustion in the aircraft [137]. Each steps requires energy and resources and likewise each step is responsible for emissions and material waste. Figure 13, was inspired by this diagram.

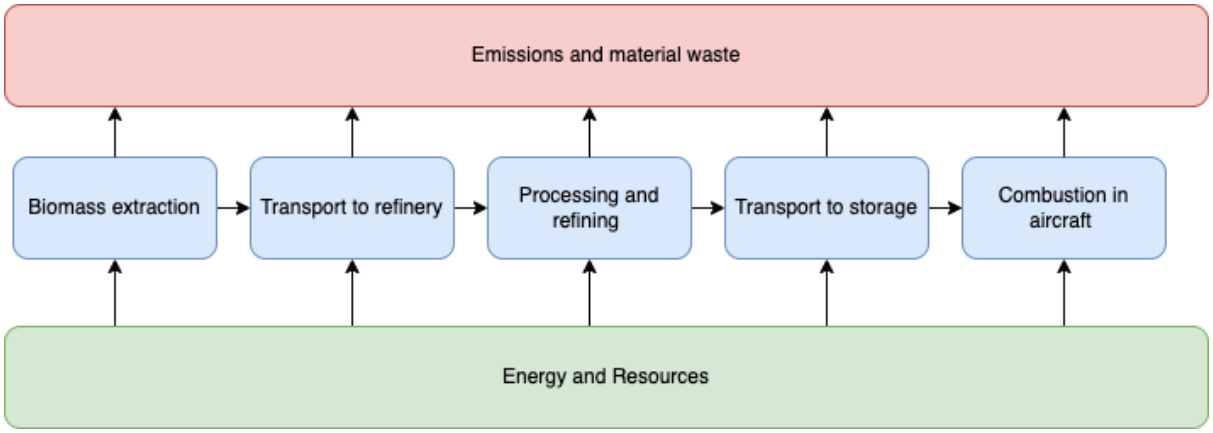


Figure 13 - Generic aviation fuel path life cycle assessment, figure inspired from [137].

The same kind of life cycle assessment can be made for a microalgae-based biofuel, this is shown in Figure 14.

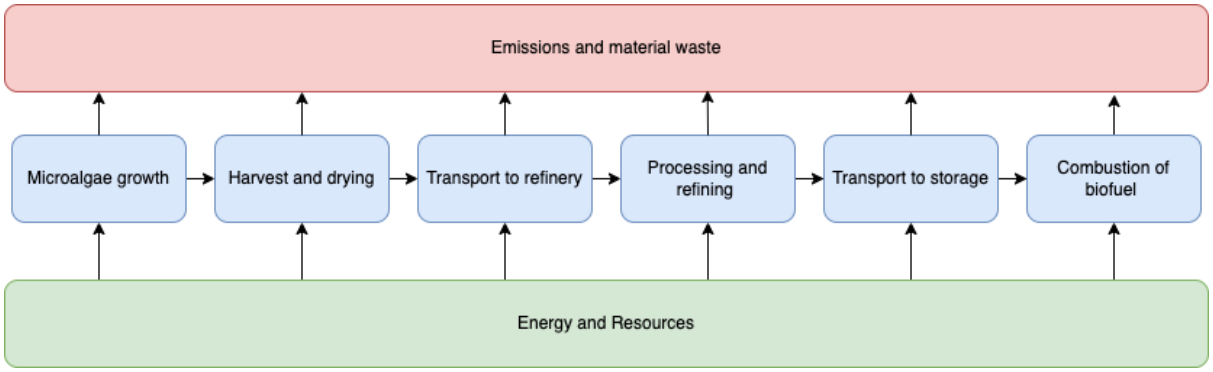


Figure 14- Generic microalgae based biofuel path life cycle assessment.

Adesanya et al. 2014, performed a LCA for the production of biodiesel from microalgae *Chlorella vulgaris*. Figure 15 shows the material flow in this cultivation system and downstream processes for the production of biodiesel. In the study, the authors use a hybrid cultivation system that coupled a tubular photobioreactor and a raceway pond in a two-way production, the former was used for the growth of microalgae and the later for the lipid accumulation [138]. Then the algal culture was allowed to sediment and flocculate in the presence of a flocculant. Centrifugation is then carried out the resulting concentrated algae slurry is then dried followed by a stage of cell disruption. The downstream process consists of a reactor where a solvent extraction is done with hexane. There are two outputs streams from this solvent extraction reactor, there is one stream containing the oil and hexane and one stream with the rest of the algal residue. The algal residue stream can be injected into an anaerobic digestor in order to produce biogas. The oil and hexane portion are run through a stripper in order to recover and recycle the hexane. The oil is further refined by Alkaline neutralisation, where the free fatty acids are saponified by an alkaline solution such as caustic soda (NaOH). The H^+ of the carboxyl group of the free fatty acids react with the OH^- group of the NaOH resulting in soap and water [139], [140]. Additionally, other non-glyceride materials are

removed, i.e. phospholipids, pigments and insoluble impurities [139], [140]. All these unwanted materials are combined into the anaerobic digestor and can also produce biogas. Whereas the refined oil is put into reaction with an alcohol to produce biodiesel and glycerol (co-product).

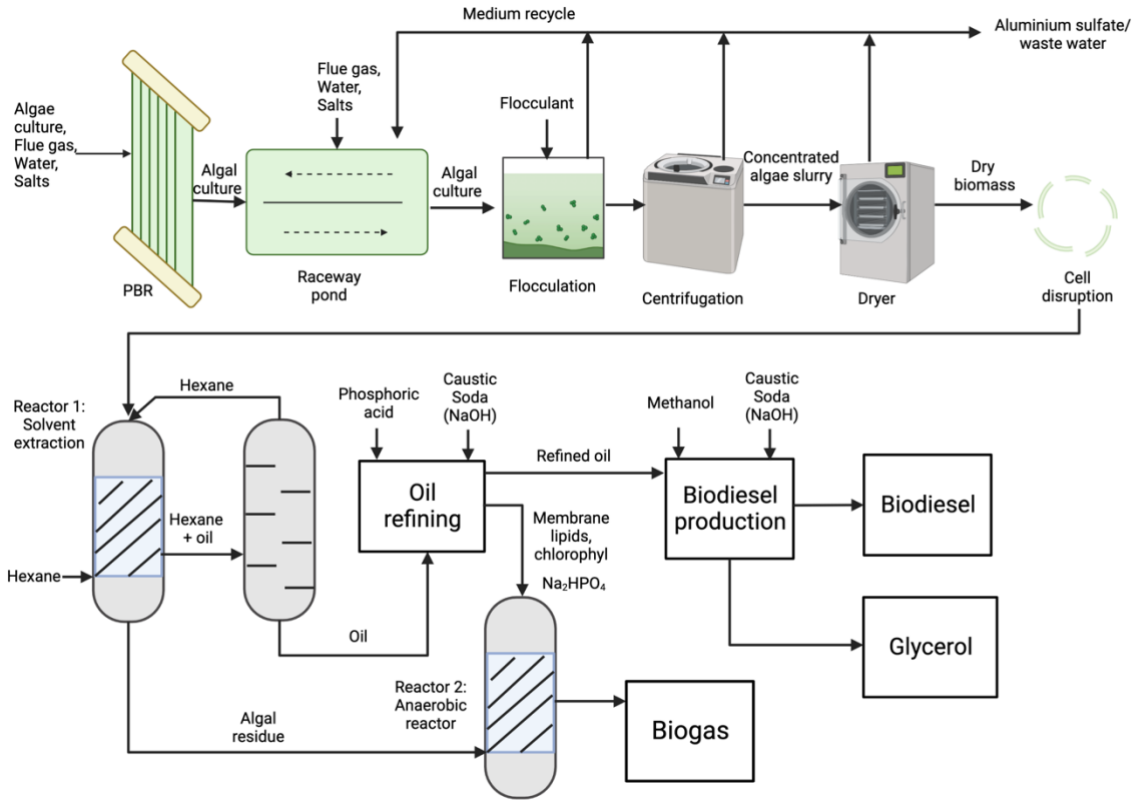


Figure 15- Process flow diagram of a hybrid cultivation system and the downstream processes for the production of biodiesel. This figure was adapted from the study conducted by Adesanya et al. 2014 [138] *and Created with BioRender.com.*

From this study, Adesanya et al. 2014, were able to conclude that the production of microalgal biodiesel with the above-described system, presents an overall significantly lower environmental impact than fossil-derived diesel [138]. More specifically microalgae-based biodiesel has 42% savings in global warming potential (GWP) and 38% savings in energy requirements (FER) when compared to fossil-derived diesel [138].

I.8 Literature review conclusion

In conclusion, microalgae cultivation offers numerous advantages and opportunities. Microalgae exhibit rapid growth and require minimal land and freshwater compared to traditional crops. They contain various bioactive compounds that can be extracted along with lipids and other biomass fractions, enabling the production of diverse value-added products and biofuels. While microalgae hold great potential in addressing energy crises and supporting UN sustainability goals, ongoing research and efforts are focused on overcoming bottlenecks in cultivation, harvesting, and product extraction. These advancements are crucial for making the microalgae industry economically viable in order to fully use its potential.

Problem statement and project objectives

The world's increasing energy demand and the need to reduce greenhouse gas emissions has led to a growing interest in biofuels as a sustainable alternative to fossil fuels. Microalgae-based biofuels are particularly promising due to their high lipid content and ability to grow in a variety of environments. However, the commercialization of microalgae-based biofuels faces several challenges, including the cost-effective production of biomass and the efficient separation of biomass from the growth medium. This project aims to address these challenges by investigating the potential of two microalgae strains, *Chlamydomonas sp.* and *Stichococcus deasonii*, isolated from water samples collected at the Esbjerg harbour in Denamrk, as feedstock for biofuel production. Their growth and lipid production in different growth medium are evaluated and the challenge of biomass separation through the study of flocculation properties is adressed. The findings of this study may contribute to the development of a sustainable and economically viable microalgae-based biofuel industry. In light of this, this the main hypothesis of this Master Thesis project is :

Chlamydomonas sp. and *Stichococcus deasonii* are two promising microalgae strains that can be used for lipid production. Fishwater can serve as a suitable alternative substrate replacing synthetic saltwater and commercial nutrients. Bioflocculation can assist in the formation of microalgae flocs to facilitate the separation of these microalgae strains from the water.

A set of project objectives is stated here below to investigate the main hypothesis:

1. Achieve a high-volume biomass production in Photobioreactors (PBRs) for comprehensive characterization of microalgae biomass using analytical methods.

2. Conduct laboratory-scale experiments to compare the growth and lipid production of microalgae strains in two distinct growth medium, namely fishwater and synthetic saltwater supplemented with plant fertilizer.

3. Evaluate the flocculation properties of the microalgae strains, which are a critical aspect of biomass separation in microalgae-based biofuel production.

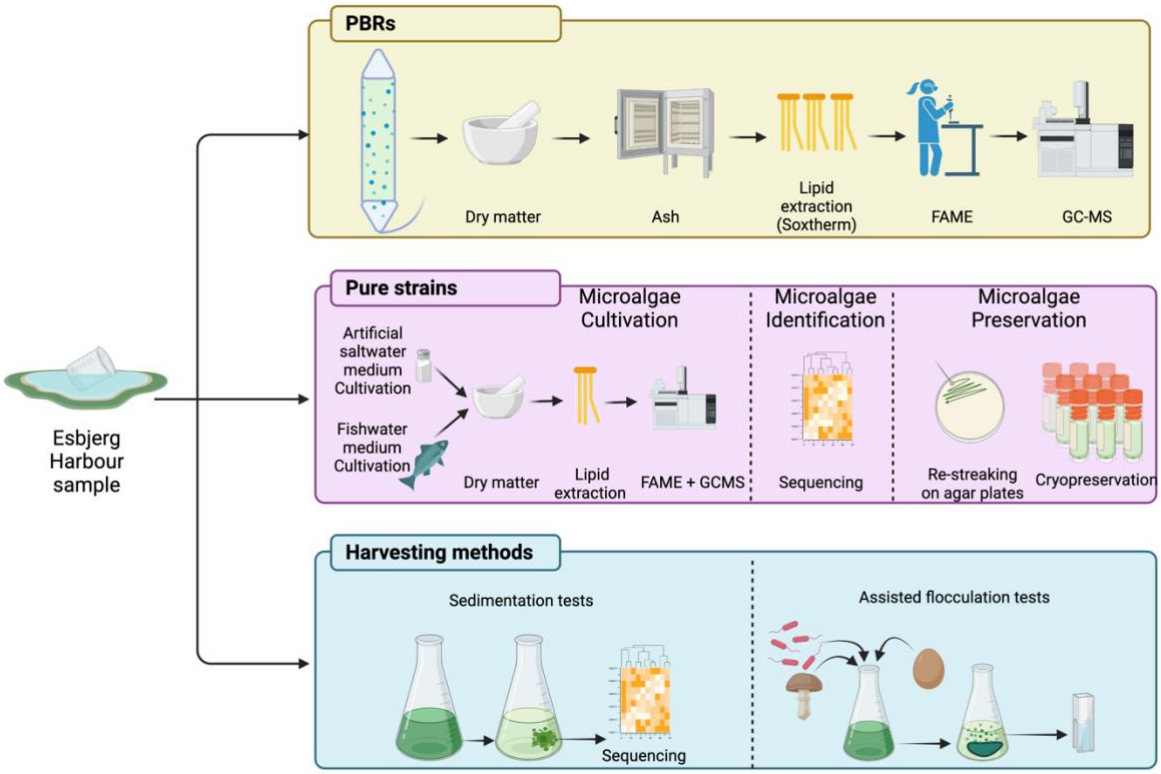
Overall, the project aims to provide insights into the potential of microalgae strains as a sustainable source of biofuel and contribute to the development of a circular economy model.

II Material and Methods

The following chapter sets out to describe the procedures adopted to study the microalgae samples present in Aalborg University Esbjerg and investigate their potential for microalgae-based biofuel.

II.1 Schematic Overview

Here below is a schematic overview of the procedures and experiments conducted to meet the project objectives.



The first phase of the laboratory work in this master thesis involved cultivating a large amount of biomass inside 2L photobioreactors. Gravimetric methods, Fatty Acid Methyl Ester (FAME) analysis, and Gas Chromatography-Mass Spectrometry (GC-MS) were used to characterize parameters such as dry matter, ash content, and lipid content.

The second phase, the isolated microalgae strains (*Chlamydomonas* sp. and *Stichococcus deasonii*), were cultivated for 30 days under the same experimental conditions, except for the variation in substrate. The first experiments used artificial saltwater supplemented with commercial nutrients, while the second case used fishwater. Microalgae growth was assessed using a combination of methods, including optical density (OD) measurements and microscopic analysis with a hemocytometer. Similarly, to the previous phase the dry matter and lipid content of the microalgae were determined using the same analytical techniques.

The third and last part of this project involved conducting sedimentation and assisted flocculation tests to characterize the aggregation behaviour of these microalgae strains.

II.2 Photobioreactors (PBRs)

II.2.1 Inoculation of Photobioreactors (PBRs)

The samples collected at Esbjerg harbour were cultured in 2L photobioreactors (PBRs) (Aqua Medic light reactors, Germany). To maintain the viability of the microalgae cultures, regular washing, re-inoculation, and feeding were performed. Every 2-3 weeks, 10 mL of fertilizer was added to each PBR for feeding. Re-inoculation was conducted once a month or every two months by unplugging the PBRs, emptying them, and retaining 100-200 mL of each culture. The PBRs were then washed with water and soap before the retained samples were reintroduced and filled up with artificial saltwater to the first rim. The artificial saltwater used had the same concentration as seawater (30-33 ‰), which was achieved by mixing 30-33 g of sea salts in 1 L of demineralized water. The sea salts were purchased from pet stores (Bonnie Dyrecenter, Esbjerg, Denmark) while the plant fertilizer was obtained from a common garden store (Esbjerg Planteskole, Esbjerg, Denmark).

Figure 16 - Picture of 2L photobioreactors containing samples collected at the Esbjerg harbour.

It is important to note that the PBRs used in this research have an opening at the top, which makes it a non-sterile cultivation system. However, since the microalgae cultures rely solely on the plant fertilizer and light for nutrition, only microalgae should be able to grow within the PBRs.

II.2.2 Dry matter and ash content in Photobioreactors

The dry matter of all four photobioreactors was determined by the gravimetric method as described in the section: II.6.1 Dry Matter and Ash Content.

II.2.3 Lipid content in photobioreactors

To determine the lipid content of PBR I and PBR III, a soxhterm extraction was performed to extract the lipids from the microalgae cultures, see section: II.6.2 Soxtherm lipid extraction.

Fatty acid methyl ester (FAME) was then prepared from the extracted lipids, followed by GC-MS analysis of the FAMEs, see section: II.6.4 Fatty Acid Methyl Ester (FAME) and Gas Chromatography-Mass Spectrometry (GC-MS).

This allowed for the identification and relative quantification of the fatty acid composition in the microalgae cultures, which is indicative of their lipid content.

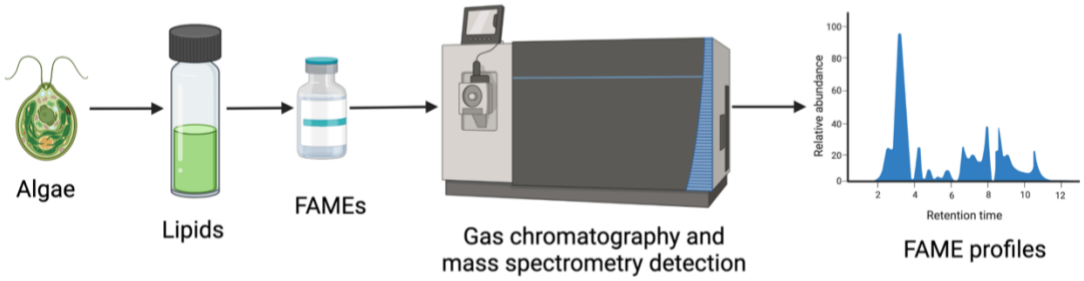


Figure 17 - Schematic representation of the analysis of fatty acid methyl ester (FAME) of microalgae by gas chromatography and mass spectroscopy (GC-MS).

II.3 Pure strains

As part of previous semester projects, conducted at Aalborg University, Esbjerg, Denmark two pure strains of microalgae have been isolated from the initial samples collected from the Esbjerg Harbour. As such, it was essential to find a suitable preservation technique, allowing proper viability and purity for the experiments conducted.

II.3.1 Preservation of the pure strains

II.3.1.1 Plating and re-streaking on agar

A short-term solution for preserving the pure strains was to re-streak them on solid medium. The recipe for the agar plates medium can be found in Annexe 1: Growth medium. This was done by scooping up a small amount of pure culture from a agar plate with the help of plastic inoculating loops and spreading it on new fresh plates. The re-streaked plates were incubated at room temperature under artificial fluorescent light for around 10 days. The plates can then be stored in the fridge.

II.3.1.2 Cryopreservation

As a long-term solution, cryopreservation using glycerol stocks proved to be a viable solution. The protocol used for was adapted from protocols.io and is described here below [141].

Microalgae cultures were placed at 4 °C for 3 days. The microalgae cultures were then diluted to an optimal concentration of $1x10^6$ cells/mL. This is verified by using a counting chamber. In the case of this study, serial dilutions were performed as follows:

- D0: 10 µL of the initial culture in 990 µL of saltwater (33 to 35 g of salts in 1L of water).
- D1: 10 µL of D0 in 990 µL of saltwater.
- D2: 100 µL of D0 in 900 µL of saltwater.

Under a laminar flow hood, 1 mL of filter sterilized 10 % glycerol and 1 mL of the diluted microalgae culture were added into cryopreservation tubes also called cryogenic tubes. The samples were then left at room temperature for 5 minutes. And progressively frozen down: first they were left in the fridge for 30 minutes, then in the normal freezer for another 30 minutes and finally, they were stored in the -80 °C freezer.

The culture regeneration was done with two different methods. For method 1, agar plates were prepared and for method 2, centrifuge tubes with artificial saltwater and plant fertiliser growth medium were prepared (see Annexe 1: Growth medium). The cryopreservation vials were allowed to warm up at room temperature for approximately 1 minute. They were then placed into 30 – 40 °C water bath until melted.

Figure 18 - cryopreservation or cryogenic tubes thawing in water bath.

Under a laminar flow, the outside of the tubes was quickly and thoroughly cleaned with 70% (v/v) ethanol.

For method 1, the cells were aseptically transferred into previously prepared agar plates and spread using Drigalski glass spatulas. They were left at room temperature and with constant light exposure.

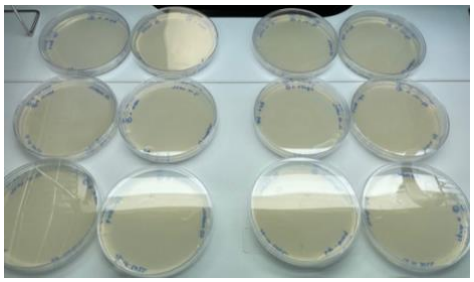


Figure 19 - Cryopreservation culture regeneration on agar plates (method 1).

For method 2, the cells were transferred into previously prepared centrifuge tubes. The lids were loosened up and a strip of tape was placed on the lids. They were also left at room temperature and with natural light exposure.

Figure 20 - Culture regeneration in liquid growth medium (method 2).

II.3.2 Sequencing of pure cultures

Sequencing of the pure cultures was done at the Department of Energy at Aalborg University in Aalborg, Denmark.

First, the samples are registered in the lab and DNA is extracted from all the organisms in the sample using DNeasy® PowerWater® Kit [142]. To validate the size and purity of the DNA extracts gel electrophoresis using Tapestation 2200 and Genomic DNA screentapes (Agilent, USA) was used. Qubit dsDNA HS/BR Assay kit (Thermo Fisher Scientific, USA) was used for measuring DNA concentration.

The extracted microbial community DNA is processed, and sequencing amplicon libraries are prepared using a custom protocol. The libraries are specifically focused on a particular region of the organisms' genetic material called the 16S/18S rRNA gene variable regions 4-8 (abeV48-A).

Next PCR (polymerase chain reaction) amplification is carried out. Each PCR reaction contains up to 25 ng of extracted DNA used as a template, 0.5 mM dNTP mix, 0.01 units of Platinum SuperFi DNA Polymerase (Thermo Fisher Scientific, USA), and 500 nM of each forward and reverse primer in the supplied SuperFI Buffer. The following steps were carried out during the PCR:

- First, an initial denaturation phase at a temperature of 98 °C for a duration of 3 minutes.
- Secondly, 25 cycles of amplification, where each cycle consisted of heating the mixture to 98 °C for 30 seconds, cooling it down to 62 °C for 20 seconds, and then raising it to 72 °C for 2 minutes.
- Finally, the reaction was completed by a final elongation step at 72 °C for 5 minutes.

The forward and reverse primers both include 24 nucleotide barcode sequences followed by the sequences targeting the archaea/bacteria/eukarya 16S/18S rRNA gene variable regions 4-8 (abeV48-A) shown here below:

[515FB] GTGYCAGCMGCCGCGGTAA
[1391R] GACGGGCGGTGWGTRCA

In the primer sequence here above, there are the usual 4 bases: cytosine (C), thymine (T), adenine (A) or guanine (G). But there are also degenerate bases such as R (A or G), Y (C or T), S (strong C or G), and W (weak A or T). The use of these degenerate bases allow primers to bind to a range of DNA sequences that contain slight variations in the sequence of nucleotides, this can improve the specificity and sensitivity of the PCR reaction.

The amplicon libraries that result from the PCR were purified following a standard protocol for CleanNGS SPRI beads (CleanNA, NL) with a bead to sample ratio of 3:5. DNA was eluted in 25 µL of nuclease free water (Qiagen, Germany). The SQK-LSK114 kit (Oxford Nanopore

Technologies, UK) was used on the purified amplicon libraries to prepare the sequencing libraries which could then be loaded onto a MinION R10.4.1 flowcell and sequenced using the MinKNOW v22.03.6 software (Oxford Nanopore Technologies, UK).

Bioinformatic processing was then done using the SILVA 16S/18S rRNA 138 SSURef NR99 full-length database in RESCRIPt format. The DNA from each microbe in the community contains specific taxonomic marker genes that allow for identification and abundance estimation. For instance, the 16S ribosomal RNA (16S rRNA) gene is used for bacteria and archaea, and the internal transcribed spacer (ITS) is used for eukaryotes. DNA sequencing is used to count the number of marker gene copies from each microbe in a sample, and this count is used to estimate the relative abundance of the microbe in the sample community. To obtain a high resolution of the community structure, at least 10,000 marker genes are DNA sequenced from each sample. The taxonomic marker genes are then compared to a reference database for identification of the microbes in the community. The sample preparation and DNA sequencing for the taxonomic marker genes were conducted according to the latest research standards. The organism abundances presented in the analysis are based on the count of each taxonomic marker gene in the sample, it is however noteworthy that these abundances may be influenced by factors such as DNA extraction, gene copy number, and primer biases and do not necessarily represent the absolute organism abundances in the sample.

II.4 Artificial saltwater versus fishwater cultivation medium

II.4.1 Concept

In literature, it is described that microalgae can thrive in fishwater, allowing for a circular economy model in fish farming industries, see section: I.4 Aquaponics a circular aquaculture-based industry. As such, the experiment was designed to compare the growth and lipid content of the two microalgal species grown in different culture medium, on the one hand artificial saltwater and plant fertiliser medium and on the other hand fishwater medium.

II.4.2 Experimental Design

II.4.2.1 Algal strains

Three replicates were prepared for each treatment, including *Chlamydomonas* sp., *Stichococcus deasonii*, a mixture of both and controls with no initial microalgae inoculum.

The initial concentrations of the cultures in both cultivations (with artificial saltwater medium and with fishwater medium is presented in Table 8.

Table 8 - Initial inoculation concentrations. a. is in saltwater and commercial nutrients medium, and b. is in fishwater medium. The OD_{600} was measured with a spectrophotometer and the cell densities were estimated with the help of a hemocytometer.

Culture	*Chlamydomonas* sp.	*Stichococcus deasonii*	Mix (*Chlamydomonas* sp.: *Stichococcus deasonii*) (1:1)	Controls
Initial OD_{600} of the purified microalgae stock	a. 0.5532 b. 0.5554	a. 0.6219 b. 0.6343	NA	NA
Initial cell density of the purified microalgae stock (cells/mL)	a. 215000 b. 222500	a. 182500 b. 260000	NA	NA
Initial concentration of the microalgae in blue cap flasks (cells/mL)	a. 7100 b. 7500	a. 6200 b. 9500	a. 6600 b. 8500	a. 0 b. 0

a. Cultivation in saltwater and commercial nutrients medium.
b. Cultivation in fishwater medium.

II.4.2.2 Culture conditions

The microalgae were cultured in 500 mL blue cap flasks containing 300 mL of saltwater and 1.5 mL of plant fertiliser or fishwater (salinity: 29 – 29 ‰, pH: 8, fish type: bristling) obtained from the Fiskeri- og Søfartsmuseet in Esbjerg, Denmark, with a constant air (Aquarium air pump, am-top cr-20, Denamark, 1.5 L/min, 50/60hz) and light supply (F18W/865 Luxline Plus, Sylvania, Germany). The cultures were grown at 25°C (room temperature) for 30 days. The air and water supply tubes were fitted with 0.45 µm filters to avoid contamination. The air supply tube was also fitted with a Marina air stone for more uniform aeration and circulation within the container.

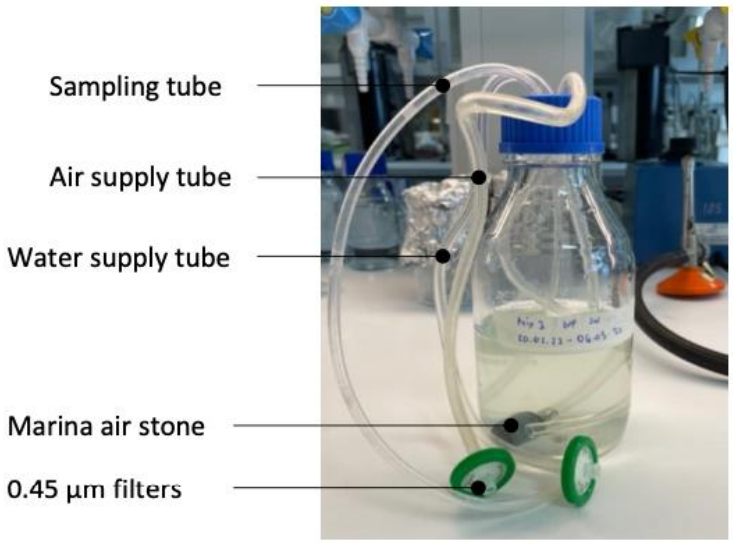


Figure 21 – Picture of the 500 mL blue cap flask used in the experiment. The flask contains a water supply tube, an air supply tube with an air stone for aeration, both tubes are fitted with 0.45 µm filters to prevent contamination, and a sampling tube for collecting microalgae samples.

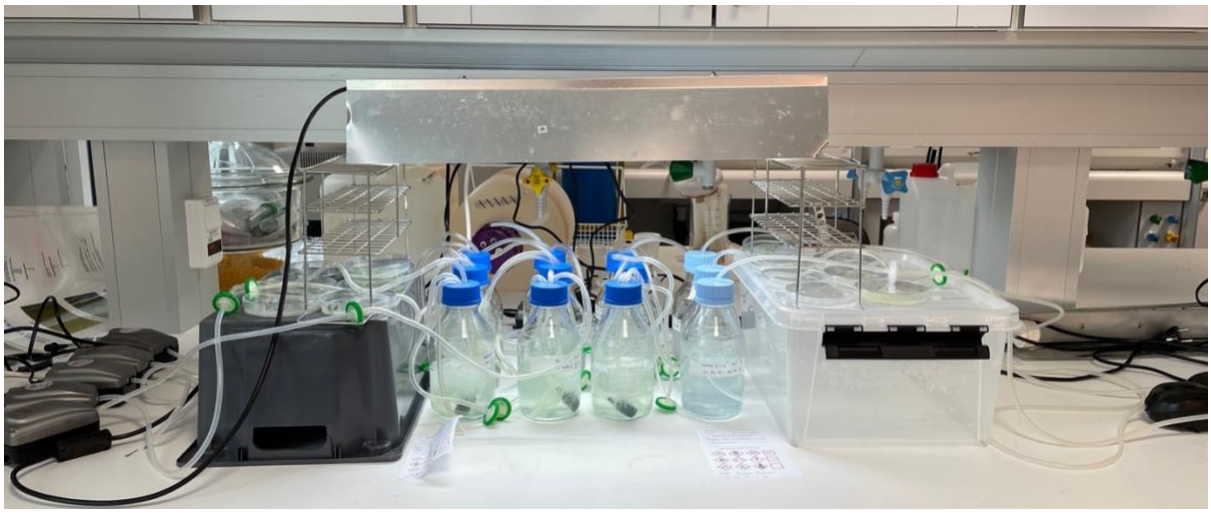


Figure 22 - Picture of the overall setup. The flasks were maintained under constant light and air supply, allowing for the growth of microalgae cultures.

II.4.2.3 Sampling and Monitoring:

Cell growth was monitored every second or third day by counting the cells in a Toma cell counting chamber and measuring the optical density at 600 nm as that is within the wavelenghth rage where the least number of pigments absorb as shown in Figure 23. Before sampling, the water level in the flasks was adjusted to 300 mL with MQ water (synergy UV water purification system).

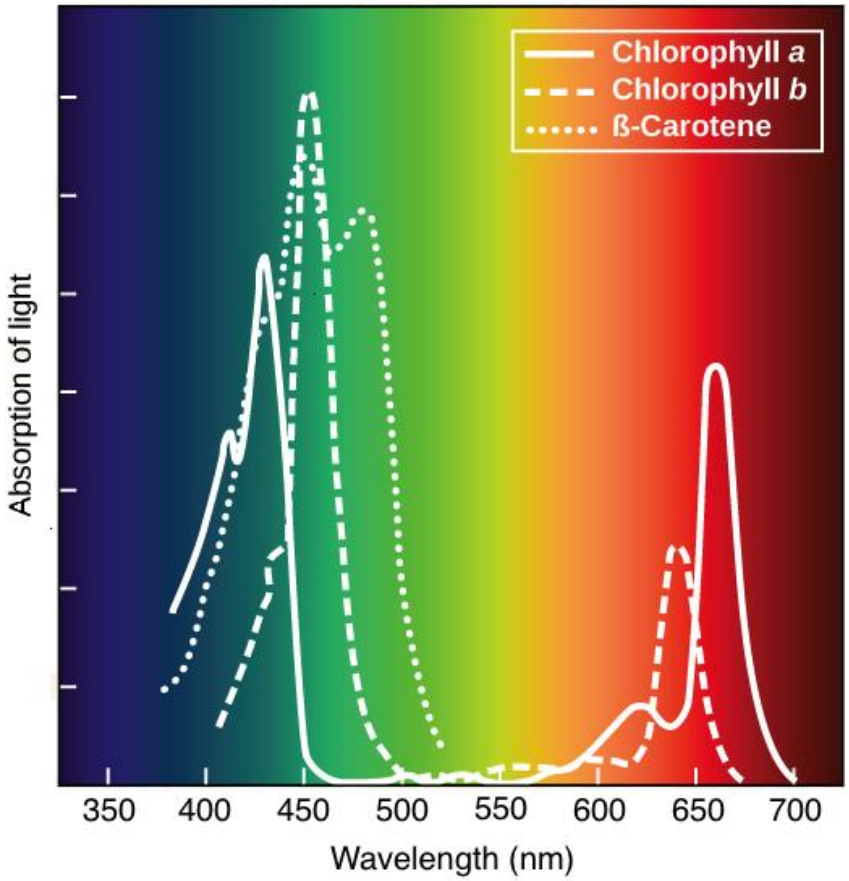


Figure 23 - Absorption spectra of pigments [143].

II.4.2.3.1 Optical density measurements:

The measurements of optical density were made with the help of a Cary 60 UV-Vis Spectrophotometer (Agilent, Denamrk). It is important to note that in order to obtain reliable optical density (OD) measurements, the samples were diluted sufficiently to ensure that the OD fell within the linear range of the spectrophotometer, typically between 0.05 and 0.8 [144]. Moreover, it is also necessary to keep in mind that OD measurements is a method that is quick and easy however it often yields inaccurate estimations due to several factor [145]. First the pigment contents that are present in microalgae can in some cases distort the OD measurements. This can however be somewhat overcome by choosing an appropriate wavelength. In this case, 600 nm was chosen as that is the wavelength where most pigments absorb a minimum of light. Second, the medium can also itself undergo changes in turbidity

throughout the experiment which can also affect the measurements. Finally, the size, shape, and aggregation of cells may also change throughout the cultivation [145]. Due to these factors, it is important to combine the OD measurements with another technique for calculating the cell growth. Therefore, the Toma cell counting chamber was used.

II.4.2.3.2 Hemacytometer

A Toma cell counting chamber was used. The frame of the counting chamber is composed of a large central square (1 mm^2) which can be fully seen with the 10X objective. This large square is further divided into 16 medium squares and those are in turn divided into 25 smaller squares. 10 µL are put under the coverslip. This does that the cell suspension reaches a height of 0.1 mm [146]. Taking these factors into consideration the volume of one large square will be:

$$1mm\ x\ 1mm\ x\ 0.1mm\ =\ 0{,}1\ mm^3\ =\ 10^{-4}mL\ =\ 0.1\ \mu L$$

Using the 10X objective the counting area is located and using the 40X all the cells within 4 of the 16 medium squares are counted. In Figure 24, here below, the four squares on the diagonal line (1, 7, 11 and 13) are counted. Furthermore, all the cells within the square and those that are on the top and right line of the square are included in the count, even if they are partially outside of the square. This is also illustrated in Figure 24, here below where the cells in green are counted but not those in red.

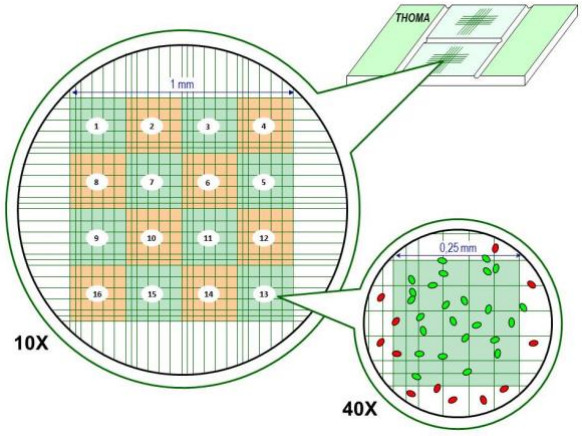


Figure 24 - Thoma cell counting chamber. Illustration from http://insilico.ehu.eus/ [146].

Once the number of cells is counted, the next step and final step is to calculate the microalgae cell concentration, to do this the following formula is used:

$$Cell\ concentration\ =\ \frac{Average\ number\ of\ cells\ counted\ *\ Dilution\ factor}{Volume\ of\ area\ counted}$$

$$Cell\ concentration\ (cell/ml) = \frac{Average\ number\ of\ cells\ counted\ *\ Dilution\ factor}{10^{-4}}$$

II.4.2.4 Harvesting and Analysis:

After 30 days, the cultures were harvested by washing in 0.9% saltwater and centrifugation at a speed of 5000g for 15 min to eliminate all the medium. 0.9% saltwater was used instead of MQ water in order to avoid osmotic shock. The pellet was resuspended in 30 mL of water and half of this sample was used to measure the dry matter by drying overnight at 105°C. The other half was placed overnight in a 60°C, and then the lipids were extracted using ultrasound and methanol and chloroform (1:2 v/v) see section: II.6.3 Lipid extraction using sonication and organic solvents (methanol and chloroform).

II.5 Flocculation and sedimentation experiments

The literature review explains that biofuel applications require a cost-effective harvesting method for microalgal biomass, which involves a two-stage process. Initially, a bio flocculation process is employed, followed by gravity sedimentation [147]. Additionally, a previous experiment conducted during a semester project also conducted at Aalborg University Esbjerg, observed that microalgae in a mixed culture agglomerated around a piece of cotton that accidentally fell into an Erlenmeyer flask (see Figure 25).
Similarly, in natural settings, it is occasionally observed that microalgae tend to accumulate around objects like wood or leaves. However, to the best of current knowledge, no specific studies or research have been conducted on physically agglomerating microalgae into solid materials like wood. Nevertheless, extensive research has explored the formation of microalgal flocs in the presence of bacteria and fungi, as well as the investigation of unconventional materials such as eggshells. Previous microscopic analysis has shown that these microalgae tend to agglomerate around fungi contamination as shown in Figure 26. These findings led to the selection of certain biological materials for further investigation into their potential role in microalgae flocculation.

Figure 25 – Picture of a microalgae culture with a piece of cotton that accidentally fell into an Erlenmeyer flask.

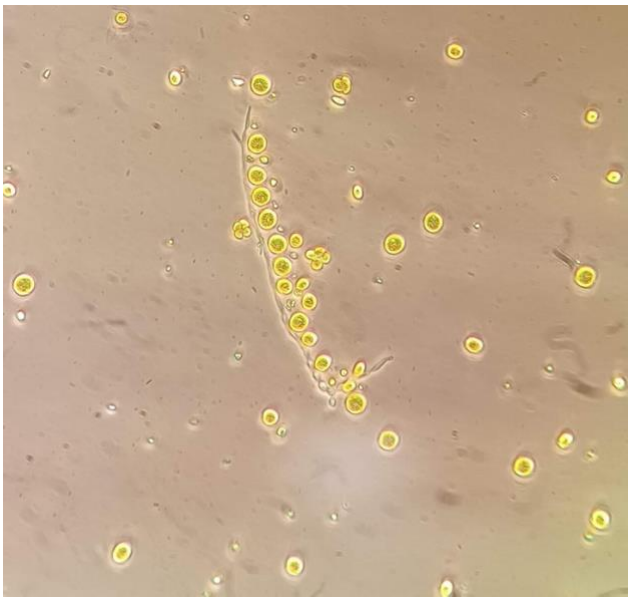


Figure 26 – Microscopic picture, of microalgae sp. agglomerating around fungi contamination X40.

II.5.1 Sedimentation tests

Samples from all four photobioreactors, were left unstirred on the counter at room temperature in 1 L Erlenmeyer flasks. This was done to see how fast the microalgae would sediment when left unstirred. Ideally, the optical density at 600 nm of the top supernatant would have been measured after 10, 20, 30, 40, 50, 60, 90 and 150 minutes [107]. However, for this experiment only visual observations were made.

II.5.2 Co-flocculation tests

Ten 500 mL Erlenmeyer flasks were filled with 300 mL of microalgae samples from a 2L photobioreactor. These flasks were then inoculated with different materials to test the flocculation / sedimentation properties of these microalgae in the presence of a co-flocculant. Table 9, lists the tested materials and the corresponding sample numbers and names. These materials were chosen due to their availability at the Aalborg University Esbjerg, and due to previous experiments and literature review. Indeed, during previous experiments conducted during semester projects here at Aalborg University Esbjerg, it was observed that when changing the salinity level an osmotic shock occurred causing these microalgae to sediment extremely quickly, this has also been observed in literature [148]. The use of fungi has been described in a study conducted by Luo et al. 2019, where *leurotus ostreatus*, an edible fungal strain was used as biofloculant [107]. Salicornia fibbers were chosen due to their abundant availability, due to ongoing research on Salicornia at Aalborg University Esbjerg, and finally, the thread was used to see if simple mechanical flocculation occurred, this would be a novel concept. A flask without any additional inoculum was used as a control. The flasks were left at room temperature and the flocculation or sedimentation of each sample was observed. Unfortunately, the ODs of the supernatants was not recorded and therefore, it would be advisable to redo these experiments.

Table 9 - List of tested materials and their corresponding sample numbers and sample names.

Sample number	Sample name	Tested material
1	Thread	A thread wrapped around a carboard.
2	Thread + fertiliser	A thread wrapped around a carboard and dipped in fertiliser.
3	Whole mushroom	A small whole mushroom.
4	Whole mushroom + fertiliser	A small whole mushroom dipped in fertiliser.
5	Chopped up mushroom	A big white mushroom chopped up (5g).
6	Flower	Flower
7	Fine cut salicornia fibbers	Fine cut salicornia fibbers (3g).

8	Roughly cut salicornia fibbers	Roughly cut salicornia fibbers (4g).
9	Osmose	High salt concentration (to test osmotic shock)
10	Control	Nothing

Figure 27- Materials to test any assisted flocculation. From left to right: A thread wrapped around a carboard and dipped in fertiliser. A small whole mushroom dipped in fertiliser. A small whole mushroom. A thread wrapped around a carboard. Fine cut fibbers (3g). Roughly cut fibbers (4g). A big white mushroom that will be chopped up (5g). A flower.

II.6 Analytical methods

II.6.1 Dry Matter and Ash Content

The Dry matter (DM) was determined by gravimetric methods. The procedure was performed in triplicates. First the crucibles were combusted in a muffle furnace for 2 to 3 hours at 575 °C, after which they were placed into a desiccator and cooled too room temperature. With the help of tongs, the crucibles were weighed, and the exact weight was recorded (W_a). With the help of a paster pipette approximately 10 g of the liquid samples were transferred to the pre-weighed crucibles, and the weight of the wet sample is recorded (W_s). The crucibles containing the wet samples were placed in the oven at 105 ± 3 °C and left to dry for at least 12 hours. The crucibles were then moved into the desiccator once more and left to cool down to room temperature. The crucibles were then weighed, and the exact weight was once more recorded (W_b). In order to determine the ash content, which includes all the inorganic residue, the crucibles were placed in the muffle furnace at 575 ± 25 °C for at least 12 hours. The crucibles were then place for a last time in the desiccator to cool to room temperature and then weighed (Wc).

II.6.2 Soxtherm lipid extraction

This procedure was conducted under a fume hood and gloves, glasses and lab coats were used. In order to extract the lipids from the microalgae samples by soxtherm, at least 5 g of dried biomass was grinded by hand with the help of a mortar until it had the consistency of dust.
Next the weight of the thimble was recorded. For this a labelled beaker was placed on the analytical scale and the weight was tired, with the help of tongs the thimbles were placed inside the beakers and the weight of the thimble was recorded. Next, with the help of a spoon rhe dried biomass was put into the thimble and once more the exact weight was recorded. Then with the help of tweezers the thimble was covered with cotton and the weight was recorded.

Figure 28 – Picture of thimbles inside labelled beakers and tongs.

Then the Soxtherm cup with the thimble holder and 3-5 small stones was weighed. Next the prepared thimbles with the biomass and the cotton were placed into the thimble holder by using tweezers. Then the soxtherm program (Soxtherm Manager) was stated and the fume hood, the water and the airflow (3.8 bars) were turned on and the solvent outlet was closed. Under the fume hood, 155 mL of Hexane was poured into the soxtherm cup, and the cup was placed into the soxtherm as shown in
Figure 29. The extraction was started by clicking on the start tag in the program. The soxtherm then operated automatically for the next 2 and a half hours. Once the extraction was completed, the soxtherm cups were left to cool down for 30 minutes and then left under the fume hood until the remaining hexane was evaporated. The cups were then weighed to calculate the lipid content and the lipids in the cups were further used for FAME.

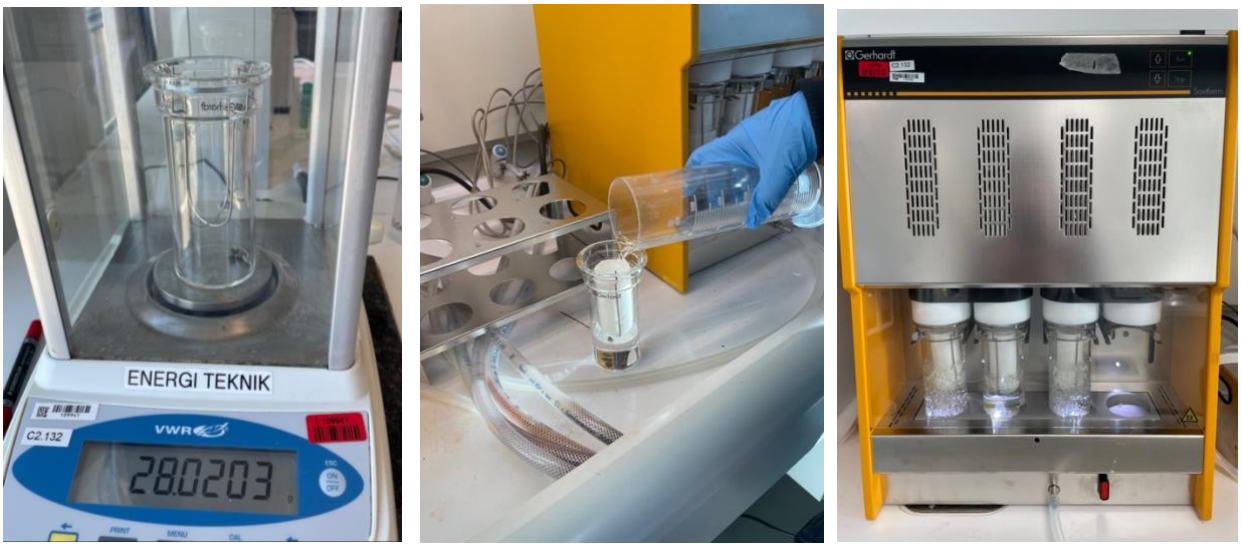


Figure 29 – From left to right: Picture1: weighing of the soxtherm cup, with the thimble holder and the 3-5 small stones. Picture 2: hexane being poured into the soxtherm cup. Picture 3: Soxtherm.

II.6.3 Lipid extraction using sonication and organic solvents (methanol and chloroform)

When the microalgae sample was too small for soxtherm lipid extraction the following procedure was done: the microalgae sample was transferred into a centrifuge tube. The tube was then centrifuged at 3000 rpm for 5 minutes. The supernatant was carefully removed without disrupting the pellet. Next, 40 mL of 0.9% autoclaved saltwater was added to the cell pellet, and the cells were resuspended by gently pipetting up and down or vertexing. The supernatant was carefully removed, and this process was repeated three times.

The resulting pellet was dried in a 60 °C conventional oven overnight for 8 hours. Then, approximately 0.2 g of dried biomass were weighed, and the exact weight was recorded. To these sample, 5 mL of methanol and chloroform (1:2 v/v) were added and vortexed for 30 seconds followed by sonication at 40 °C for 30 min using an ultrasonic bath. After sonication the top layer was filter through normal paper filter into a previously weighed glass vial and the filter was washed with a small amount (2-5 mL) of methanol and chloroform (1:2 v/v) to remove any remaining lipids. The bottom layer was then extracted again with another volume

of 5 mL of chloroform and methanol (1:2 v/v). This step was repeated to ensure complete lipid extraction. After the final extraction, the top lipid layer was once again filtered and washed as previously mentioned with a small amount of chloroform and methanol. The filtered lipid extract was evaporated using nitrogen gas until all the solvent has evaporated and the lipids were completely dry. Finally, the dried lipids were weighed, and the percentage of lipid content was calculated.

II.6.4 Fatty Acid Methyl Ester (FAME) and Gas Chromatography-Mass Spectrometry (GC-MS)

The extracted lipids were then analysed by Fatty Acid Methyl Ester (FAME) and Gas Chromatography-Mass Spectrometry (GC-MS). For this the dried microalgal samples were prepared and subjected to Fatty Acid Methyl Ester (FAME) extraction. The extraction process involved converting the fatty acids present in the samples into their corresponding methyl esters through the process of transesterification. The chemical reaction is shown in Figure 30.

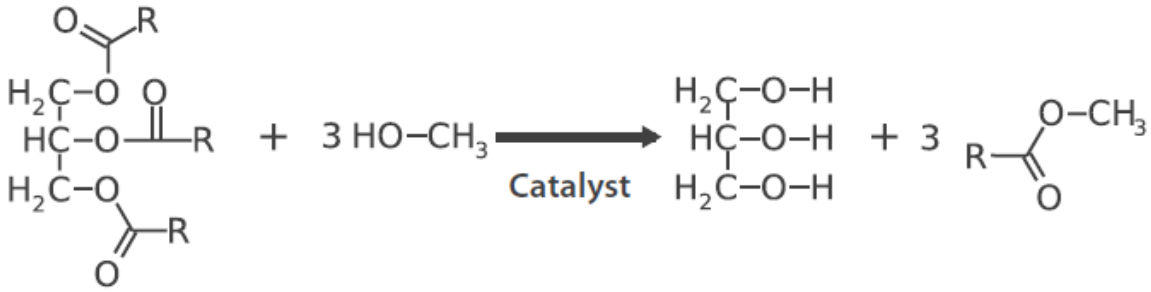


Figure 30 - Fatty acid methyl esters (FAME) reaction. Glycerol-bound fatty acids react with methanol in the presence of a catalyst to give mixture of glycerol and biodiesel (FAME).

The glycerol-bound fatty acids were converted through a transesterification process into their corresponding methyl esters, by using methanolic sodium hydroxide. Additionally, the hydrolytic free fatty acids and free fatty acids present in the sample were methylated through a catalytic reaction with boron trifluoride. Finally, the methyl esters were extracted from the reaction mixture using heptane. A schematic representation of the steps involved in the FAME protocol is shown in Figure 31.

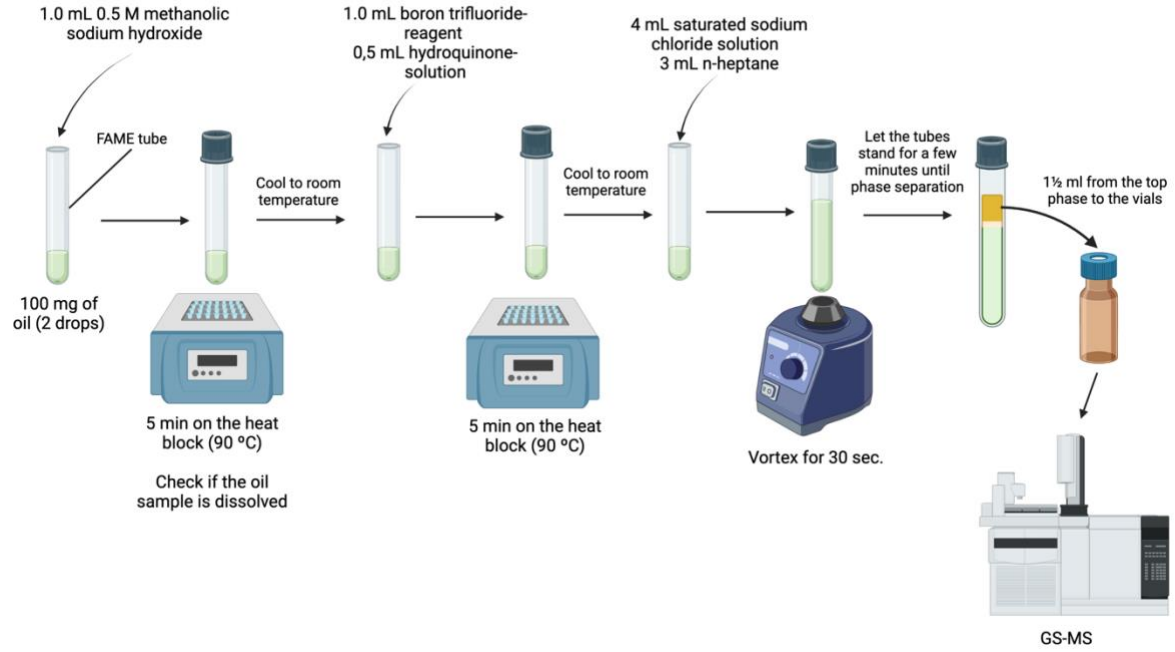


Figure 31-Schematic Representation of the FAME Protocol. Created with BioRender.com.

The FAME extracts were then analysed using Gas Chromatography Mass Spectrometry (Perkin Elmer instruments, Clarus 500 mass spectrometer, Denmark) for fatty acid profiling. The fatty acid methyl esters are eluted and separated on the GC capillary column according to their boiling point and their polarity. The GCMS data obtained from the analysis were processed and analysed using dedicated software. In the resulting chromatograms, the x-axis represents the retention time, which is a measure of how long it takes for each compound to travel through the chromatographic column. It indicates the time at which different compounds elute or separate from the column. The retention time allows to identify the fatty acid in question. The y-axis of the chromatogram represents the detector response or signal intensity. It reflects the relative abundance of the various fatty acid methyl esters present in the microalgae oil sample. The higher the signal, the greater the amount of that specific compound.

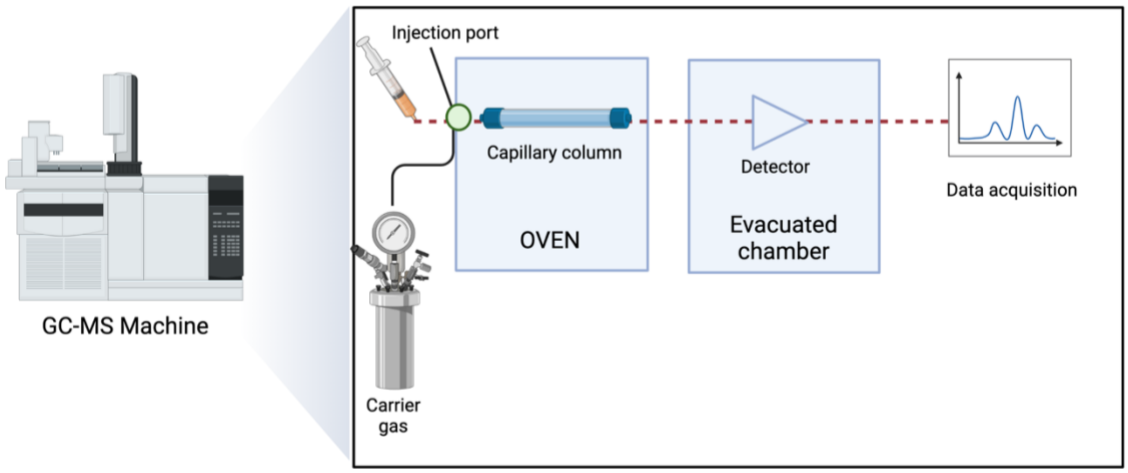


Figure 32 - Schematic representation of a GC-MS apparatus. Created with BioRender.com

II.7 Calculation methods

II.7.1 Standard deviation

All experiments were conducted in triplicates, as such the standard deviation could be calculated according to the following equation:

$$Standard\ deviation\ = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \mu)^2}{n-1}}$$

Where, n is the number data points, x_i is each of the values of the data points and μ is the sample mean.

II.7.2 Dry Matter

The dry matter of algae was calculated according to the following equation:

$$Dry\ matter\ \%\ = \frac{W_b - W_a}{W_s} * 100\ \%$$

Where W_a (g) is the weight of the crucible, W_b (g) is the weight of the crucible after the sample drying at 105 ± 3 °C for 12 h, W_s (g) is the weight of the wet sample.

II.7.3 Ash content

The ash content was calculated according to the following equation:

$$Ash\ \%\ = \frac{W_c - W_a}{W_b - W_a} * 100\ \%$$

Where W_a (g) is the weight of the crucible, W_b (g) is the weight of the crucible after the sample drying at 105 ± 3 °C for 12 h, W_c (g) is the weight of the crucible and the sample after drying at 575 ± 25 °C for 12 h.

II.7.4 Lipid content

$$Weight\ of\ the\ extracted\ lipids = Weight\ of\ the\ vial\ containing\ the\ dried\ lipid\ sample - Weight\ of\ the\ empty\ glass\ vial$$

$$Lipid\ content\ (\%\ wet\ weight)\ = \frac{Weight\ of\ the\ extracted\ lipids}{Weight\ of\ the\ microalgae\ sample} * 100$$

II.7.5 Relative abundance of each fatty acid

To determine the relative abundance of each fatty acid, percentages were calculated with the results obtained from the GC-MS. This involved dividing the peak area (or peak height) of each fatty acid by the total peak area (or peak height) of all detected fatty acids. The resulting values were then multiplied by 100, providing the relative abundance of each fatty acid as a percentage.

II.7.6 Growth kinetics

II.7.6.1 Specific growth rate

The growth rate is described by the following equation:

$$\frac{dBt}{dt} = \mu Bt \qquad (1)$$

Where,

- t represents the value of the dependent variable (B) that changes over time. In this case it is the biomass measured by the OD_{600} value at a specific time t.
- μ represents the specific growth rate. It will determine the rate at which the exponential growth occurs.
- t represents the independent variable; in this case it is time in days or hours.

The solution for the equation (1) is the well-known exponential growth equation:

$$Bt = B0 * e^{\mu * t} \qquad (2)$$

Where,

- B0 is the initial biomass value.

In order to obtain equation (2) the growth curve of each culture was plotted on a logarithmic scale and then trimmed so that only the log phase (exponential growth phase) was present. An exponential trendline was then fitted and its corresponding equation was displayed allowing to determine the specific growth rate parameter.

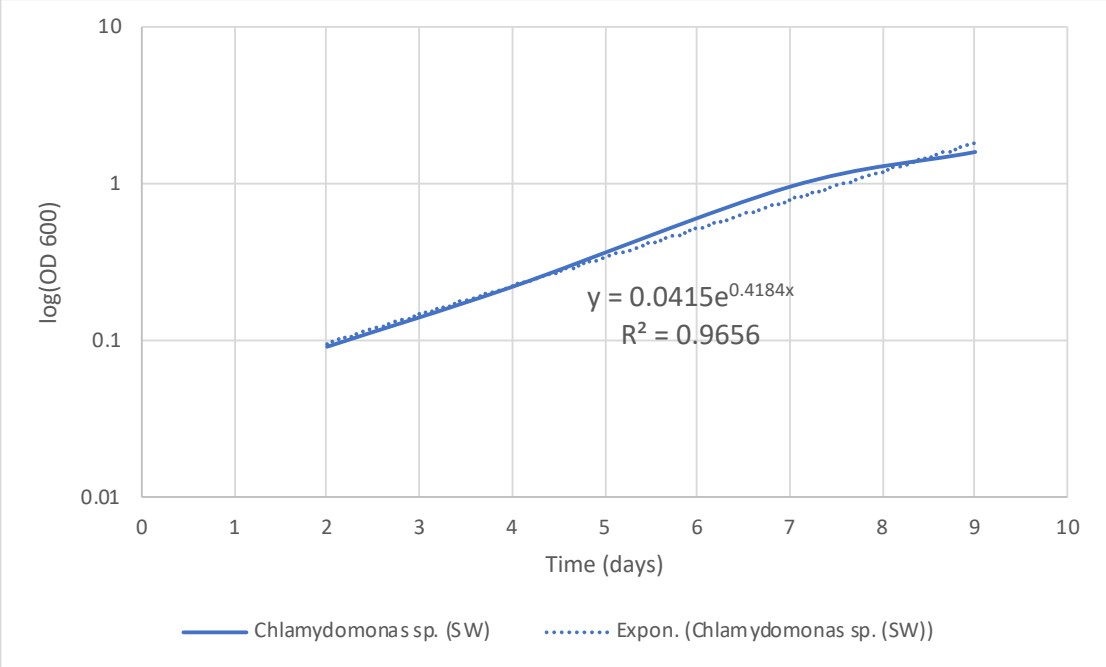


Figure 33- Growth curve of Chlamydomonas sp. Grown in saltwater supplemented with plant fertiliser was plotted on a logarithmic scale and trimmed to include only the log phase (exponential growth phase). An exponential trendline was fitted, and its corresponding equation was displayed, enabling the determination of the growth rate parameter.

In the example illustrated in the graph of Figure 33 the growth rate (k) is equal to 0.4184.

II.7.6.2 Doubling time

The doubling time (DT) represents the time it takes for the dependent variable to double in size. It is typically measured in units of time and can be determined with the following equation:

$$Doubling\ time\ (DT) = \frac{Ln(2)}{\mu} \qquad (3)$$

By plugging in the value of μ in equation (3), the doubling time could be determined.

II.7.6.3 Productivity

The productivity (P) is the increase in microbial biomass over time.

$$Pt = \frac{dBt}{dt} \qquad (4)$$

By combining equations (1) and (3), the following equation is obtained.

$$Pt = \mu Bt \qquad (5)$$

III Results and discussion

III.1 Cultivation of large quantities of microalgae biomass in photobioreactor for characterisation of dry matter, ash and lipid extraction and quantification.

The Aalborg University in Esbjerg houses four photobioreactors that contain microalgae samples isolated from the Esbjerg harbour. These photobioreactors are equipped with a continuous flow of air and light, they are non-sterile due to an opening on the top. Microscopic examination confirmed the presence of fungi and bacterial contamination, indicating the lack of sterility. However, since the medium lacks a carbon source, these heterotrophs are unable to thrive, unlike the microalgae that can derive their carbon from carbon dioxide, water and light. Additionally, when 1-2 mL samples from each PBR were plated on agar plates containing glucose, extensive growth of contaminants was observed, this can be seen in Figure 34.

Figure 34 - Growth of contaminants on agar plates with glucose. The figure displays the growth of contaminants on agar plates containing glucose. Each row corresponds to samples from a specific PBR, with PBR 1 in the first row, PBR 2 in the second row, PBR 3 in the third row, and PBR 4 in the fourth row. Within each row, the first column shows a 1/100 dilution, the second column depicts an inoculum of 1/10,000 dilution, and the third column represents a 1/100,000 dilution. The images provide visual evidence of the extent of contaminant growth.

To gain a deeper understanding of these microalgae sample and to get familiarized with the methods to be employed in future experiments, several analyses were conducted on the biomass obtained from these photobioreactors. These measurements included determination of dry matter and ash content. Subsequently, lipids were extracted using the soxtherm method, and the extracted lipids were further analysed using FAME and GCMS techniques. The following section presents the results obtained from these analyses.

III.1.1 Dry matter and ash content in photobioreactors

The experiment aimed to determine the dry matter (DM) and ash content in the four PBRs (PBR1, PBR2, PBR3, and PBR4). The results, depicted in bar graphs, revealed variations in DM among the PBRs.

The mean DM values for PBR1, PBR2, PBR3, and PBR4 were found to be 5.4 %, 9.8 %, 7.4 % and 9.6 % respectively. Corresponding to these means, the standard deviations for DM were 0.7 %, 1.2 %, 0.3 % and 1.5 % respectively.

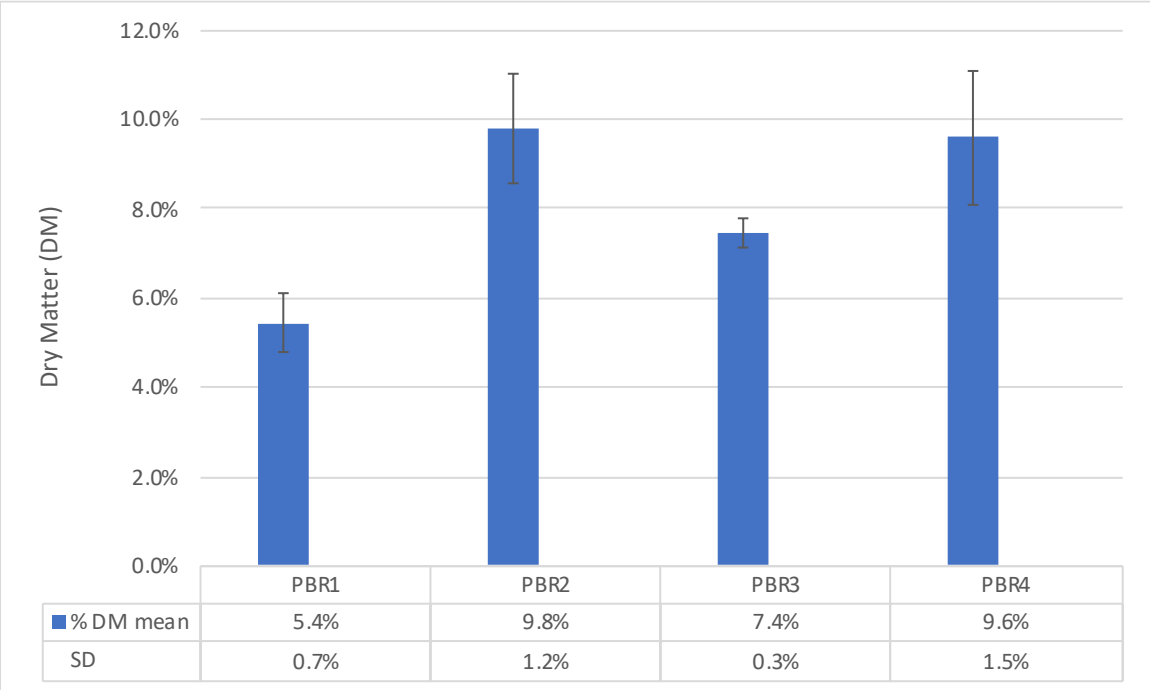


Figure 35 - The bar graph presents a comparison of DM among the four PBRS (PBR1, PBR2, PBR3, and PBR4). Each bar represents the mean DM value for a specific PBR, while the error bars depict the standard deviations.

The mean ash content percentages were 78 %, 75.4 %, 77.2 %, and 75.8 % for PBR1, PBR2, PBR3, and PBR4 respectively, with standard deviations of 0.2%, 1.4%, 0.2%, and 0.1%.

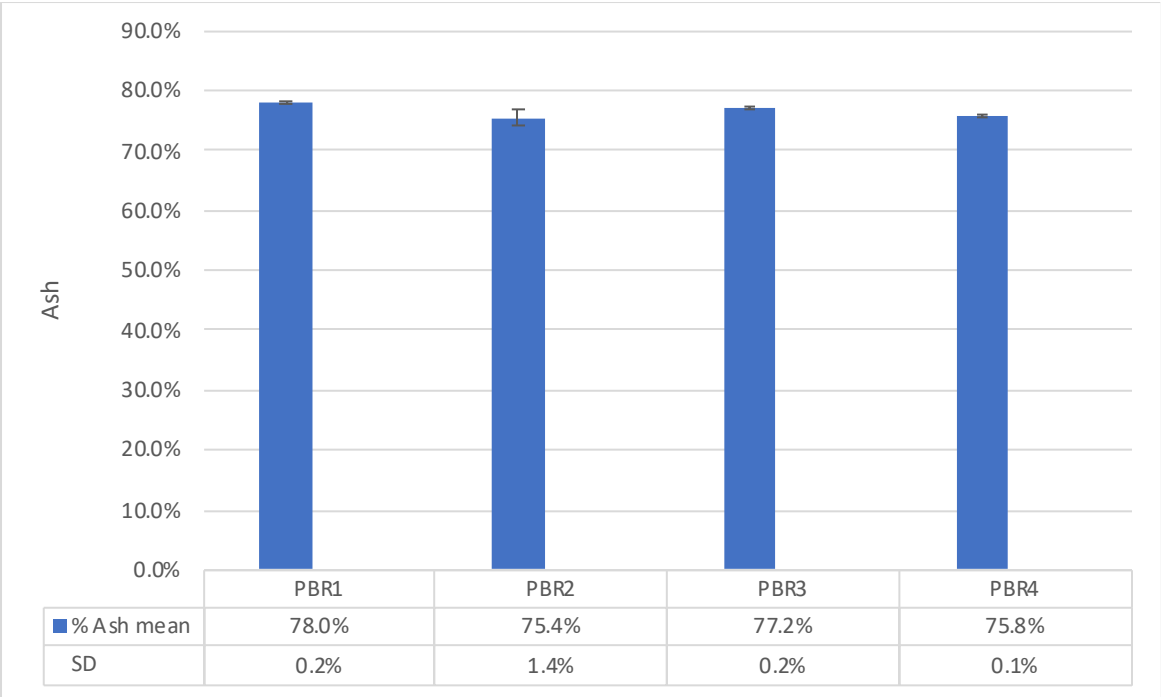


Figure 36 - The bar graph presents a comparison of the Ash content among the four PBRS (PBR1, PBR2, PBR3, and PBR4). Each bar represents the mean Ash value for a specific PBR, while the error bars depict the standard deviations.

In theory, since the PBRs were inoculated simultaneously with the same initial volume (100-200 mL) in 2L of medium, and exposed to similar conditions of air and light, it could be expected for them to exhibit similar DM and ash content. However, the observed differences among the PBRs, particularly in terms of DM, suggest two potential hypotheses.

First, it is possible that the different species present in the PBRs exhibit varying nutrient uptake patterns, resulting in differences in DM and ash content. Further investigation could be carried out in order to determine if certain species are more efficient at assimilating nutrients or if they have unique metabolic characteristics affecting their biomass composition.

Secondly, it is also possible that the conditions within the PBRS are not entirely uniform. Discrepancies in light intensity or variations in the strength of the pumps could impact nutrient distribution and uptake among the PBRs, contributing to the observed differences in DM and ash content.

To shed light on these hypotheses, further experiments should focus on analysing the growth dynamics of pure microalgae strains under sterile conditions and within more controlled environments.

III.1.2 Lipid content in photobioreactors

The composition of fatty acids was assessed in two distinct photobioreactors, designated as PBR1 and PBR3. This analysis was conducted by employing Fatty Acid Methyl Ester (FAME) analysis via Gas Chromatography-Mass Spectrometry (GC-MS).

Figure 37 shows the chromatogram obtained for PBR1.

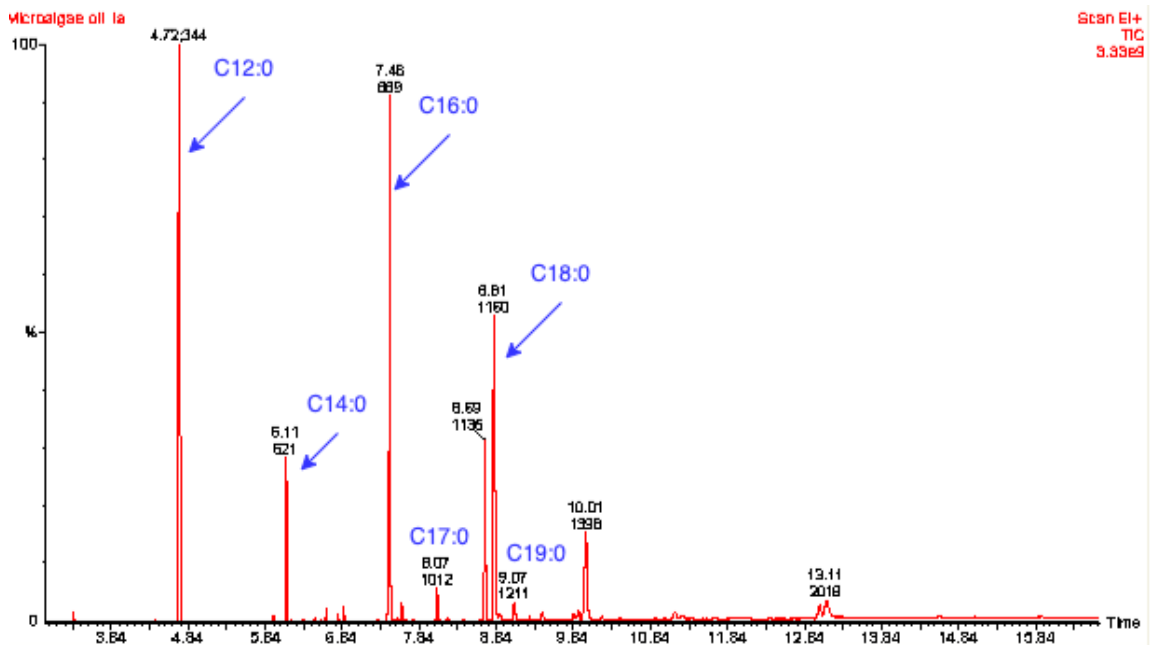
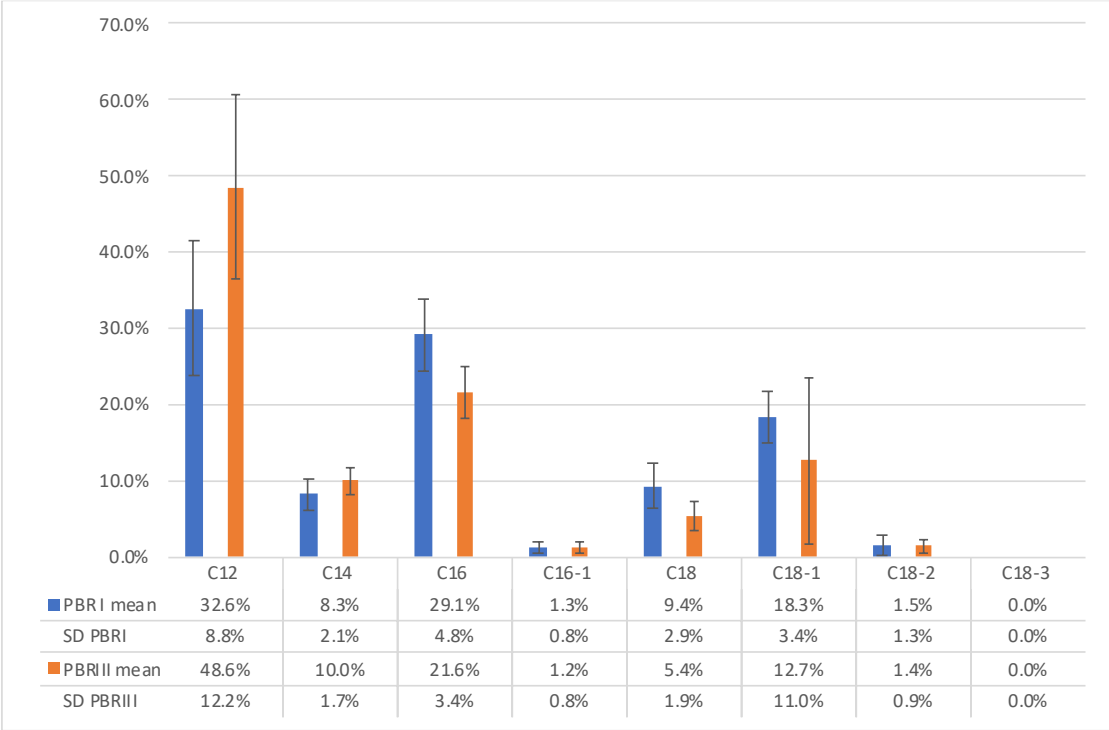


Figure 37- Chromatogram revealing the FAME composition of microalgae oil extracted from PBR1.

Figure 38, shows a bar graph of the FA composition in PBR1 and PBR3, with their standard deviations. Both PBRs have similar FA compositions, indicating comparable fatty acid ratios in the microalgae oil samples.



	C12	C14	C16	C16-1	C18	C18-1	C18-2	C18-3
PBRI mean	32.6%	8.3%	29.1%	1.3%	9.4%	18.3%	1.5%	0.0%
SD PBRI	8.8%	2.1%	4.8%	0.8%	2.9%	3.4%	1.3%	0.0%
PBRII mean	48.6%	10.0%	21.6%	1.2%	5.4%	12.7%	1.4%	0.0%
SD PBRIII	12.2%	1.7%	3.4%	0.8%	1.9%	11.0%	0.9%	0.0%

Figure 38 - Bar graph illustrating the relative proportions of different fatty acids in the microalgae oil obtained from PBR1 and PBR3.

In PBR1, the fatty acid C12 is dominant, constituting 32.6 %. This is followed by C16 at 29.1 % and C18-1 at 18.3 %. Other fatty acids include C18 at 9.4 %, C14 at 8.3 %, and C18-2 at 1.5 %. C16-1 C18-3 in PBR1 have values of 1.3 % and 'not detected', respectively.

In PBR3, C12 is again the most abundant fatty acid, representing 48.6 %. C16 at 21.6 % is the second most prevalent, with C18-1 following at 12.7%. C14, C18, C16-1 and C18-2 are also present in PBR3 at lower percentages and C18-3 is once again 'not detected'.

An examination of the compositions from the perspective of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs), reveals PBR1 contains 79 % SFAs, 20 % MUFAs, and 2 % PUFAs. PBR3 has 86 % SFAs, with MUFAs and PUFAs at 14 % and 1 %, respectively.

Literature indicates that the ideal biodiesel is primarily composed of monounsaturated fatty acids (MUFA), with fewer polyunsaturated and saturated fatty acids (PUFA and SFA) [50], [64]. In these PBRs, there is a larger amount of SFA than of MFA. However, all these fatty acids can be used in biofuel production, despite higher SFA percentages not being ideal.

III.2 Pure strains

III.2.1 Preservation of the pure strains

During this experiment, it was observed that reviving cryopreserved microalgae was faster when using agar plates (option 1) compared to liquid medium (option 2). In the agar plates some colonies are already visible, for both strains six days after the culture regeneration (see Annexe 2: Culture regeneration after cryopreservation pictures). Whereas in the liquid medium, 12 days after regeneration the Chlamydomonas strains are still not revived (see Annexe 2: Culture regeneration after cryopreservation pictures).
In both cases, in liquid or solid medium, *Stichococcus deasonii* is found to have a faster revival rate compared to *Chlamydomonas sp.*. This suggest that microalgae rates may vary depending on the microalgae species.

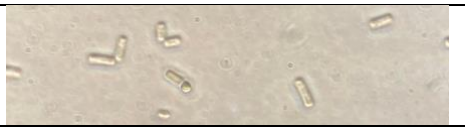
Alternatively, microalgae can be stored on agar plated in a normal refrigerator, but this technique has a maximum storage time of one to three months.

Restreaking has also proven to be a useful method for maintaining the purity and viability of isolated microalgae strains. However, in a recent experiment, it was observed that *Chlamydomonas sp.* failed to grow on agar plates despite repeated restreaking attempts. The cause of this unexpected result remains unclear and warrants further investigation.

III.2.2 Sequencing of pure strains

Through sequencing it was determined that the two strains isolated from samples collected at the Wadden sea in Esbjerg, Denmark belong to the species *Chlamydomonas sp.* and *Stichococcus deasonii.*

Table 10 - Sequencing-based taxonomic classification of the two microalgae isolated from natural samples, with microscopic images.

Microscopic picture at X40		
Kigdom	Eukaryota	Eukaryota
Phylum	Chlorophyta	Chlorophyta
Genus	Chlorophyceae	Trebouxiophyceae
Species	Chlamydomonas_sp	Stichococcus_deasonii

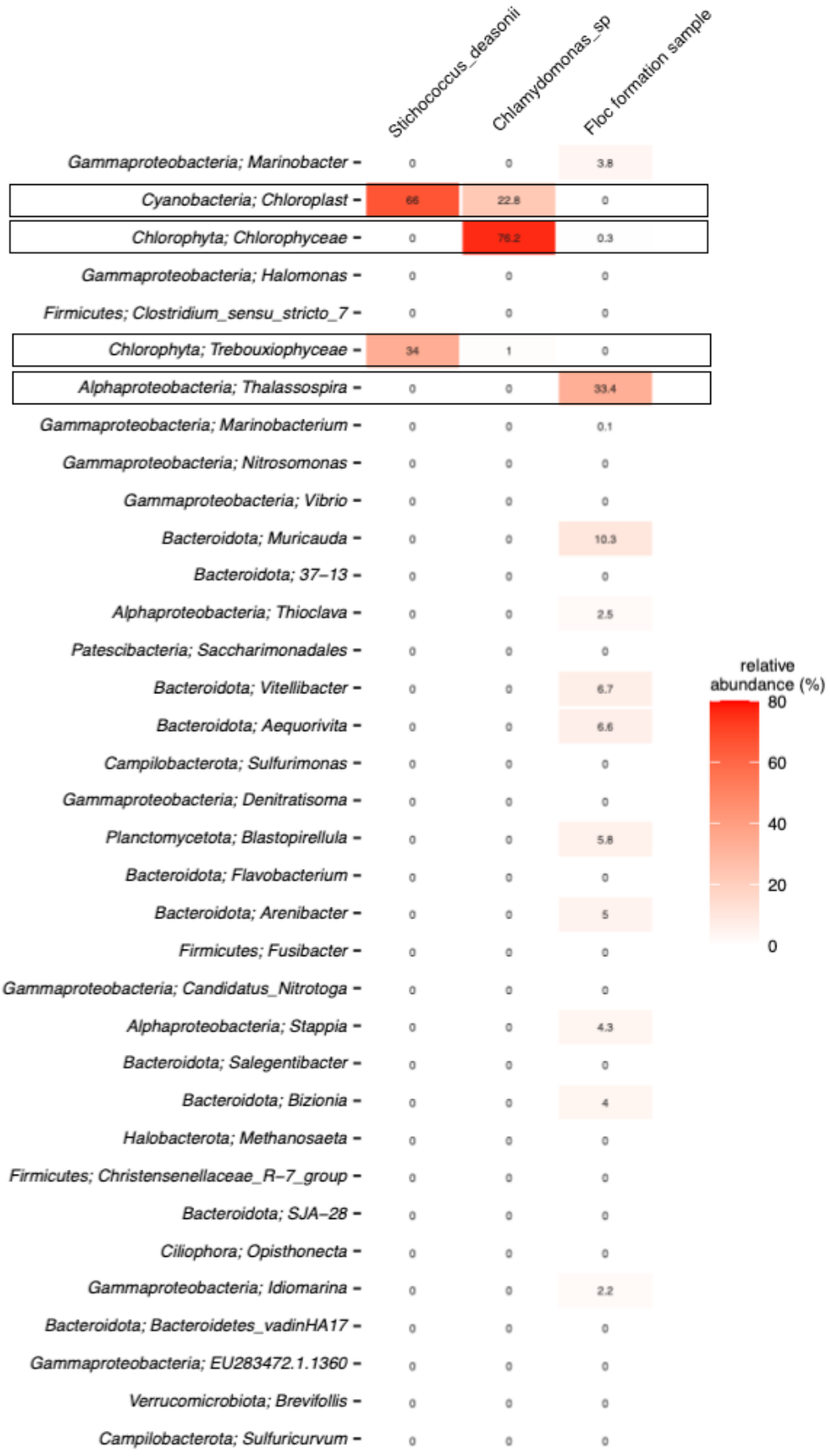
A heatmap was generated to visualize the abundance of different organisms in each of the three samples that were sequenced: two purified microalgae cultures and one unknown mixed culture that formed a floc. In both microalgae samples, a high abundance of cyanobacteria/chloroplasts

was observed, although it should be noted that this is expected as all microalgae contain chloroplasts where photosynthesis occurs.

Apart from the chloroplasts, the first microalgae sample showed the highest abundance of 34% for chlorophyta; Trebouxiophyceae, specifically the species *Stichococcus deasonii*. The second sample had an abundance of 76.2% for chlorophyta; Chlorophyceae, specifically the species *Chlamydomonas sp*. Notably, all other species in the heatmap had an abundance of 0, indicating that the cultures were pure and free of contaminants. The third sample, collected from a floc formation, showed a different pattern. This sample had a higher abundance of bacteria compared to the microalgae samples, with a notable presence of the Alphaproteobacteria phylum of 33.4 %. Further details on this sample can be found in the section III.4 Flocculation and sedimentation experiments.

Overall, the heatmap allowed for a clear visualization of the abundance of different organisms or organelles in each sample, highlighting the purity of the microalgae cultures and the presence of different organisms in the floc sample.

Table 11 - Samples composition heatmap (abeV48−A, Genus level)



III.3 Artificial saltwater versus fishwater cultivation medium

III.3.1 Growth curves

In the following section, the aim is to examine the growth of various cultures. This includes *Chlamydomonas* sp., *Stichococcus deasonii*, a mixture of both species in a 1:1 ratio, and controls. These cultures were cultivated in two different types of medium namely a saltwater medium supplemented with commercial nutrients (SW), and a fishwater medium (FW).

Figure 39 displays the growth trajectory of the microalgae when cultured in either artificial saltwater supplemented with plant fertiliser (SW) or in fishwater (FW) medium. The analysis is based on the mean optical density values of triplicates. The evaluated cultures include the mean OD of *Chlamydomonas* sp., the mean OD of *Stichococcus deasonii*, the mean OD of a mixture of both species (1:1), and the mean OD of the control samples that initially did not contain a microalgae inoculum.

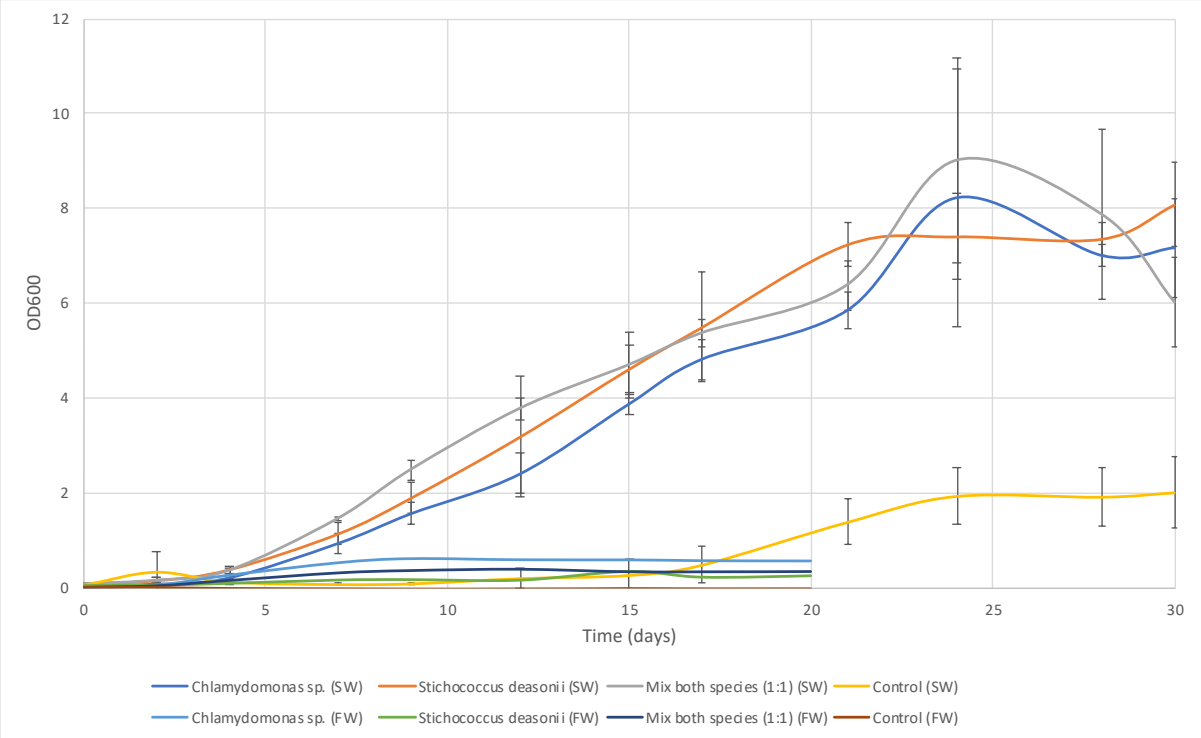


Figure 39 - Growth curves of four microalgae cultures cultivated in artificial saltwater medium supplemented with commercial nutrients (SW) and fishwater (FW). The y-axis represents the mean optical density (OD) values, while the x-axis represents time (in days). The saltwater cultures were grown for a duration of 30 days, whereas the fishwater cultures were grown for 17 days. No additional nutrient supplementation was provided to the cultures throughout the experiment.

From an initial examination, several general observations can be made. To begin with, the standard deviation error bars are notably large, indicating considerable variation from the mean within the triplicates.

The controls cultured in SW (yellow line in Figure *39*), which were not inoculated with any microalgae, exhibited contamination, as indicated by their green coloration (see Figure 40).

The growth of the contaminating organisms, predominantly *Stichococcus deasonii* according to microscopic observation (see Figure 40), in the controls, initiated on the ninth day of culture. This contamination indicates that the cultures were not kept under aseptic conditions, which could affect the reliability of the results. Contrarily, the controls in the fishwater medium showed no signs of contamination.

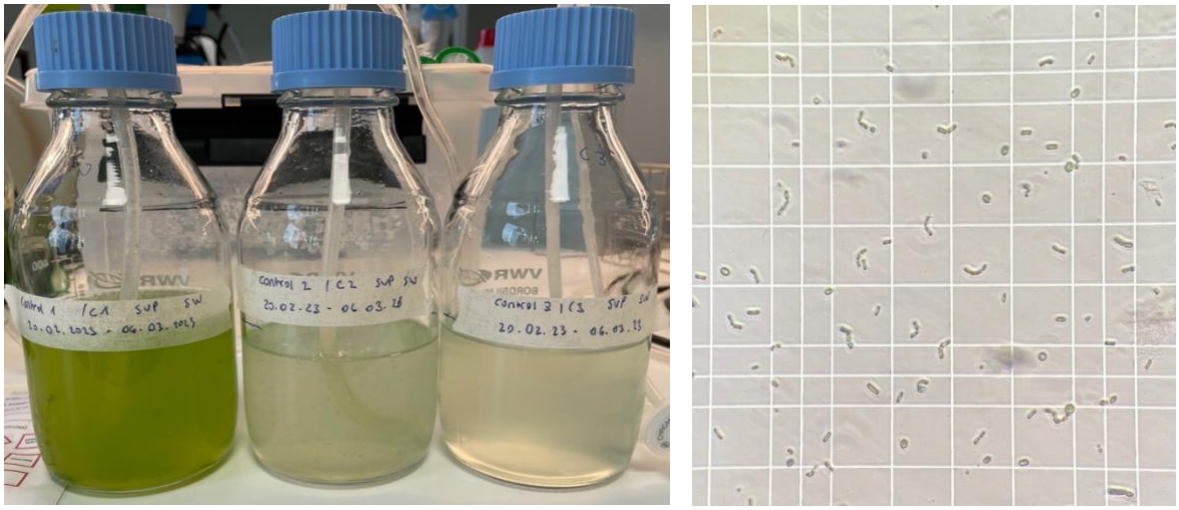


Figure 40 - Left: Picture of the green color observed in the control cultures (at day 15). Right: Microscopic observation (X40) of a sample from control 1.

Looking at the cultures cultivated in the saltwater supplemented with plant fertiliser (SW), it was noticed that the growth patterns of the three cultures, excluding the controls, were quite similar and displayed the typical growth pattern seen in microorganisms. This involved an initial lag phase lasting approximately 2 days, succeeded by an exponential phase that continued up until around day 20 for all three cultures. This was followed by a stationary phase for all cultures which lasted until day 28, after which a death phase was observed in the mixed culture from day 28 to day 30.

In contrast, the cultures grown in the fishwater medium revealed a different pattern. Indeed, no clear exponential phase was discernible for any of the cultures.

The data indicates that, under the conditions tested, the microalgae cultures have a lower performance in fishwater medium compared to artificial saltwater medium supplemented with commercial nutrients. A hypothesis for this observation is that the fishwater lacks sufficient nutrients for the microalgae. During the experiments, local aquaculture company Alpha Aqua was undergoing renovations, and no fish were present in the tanks, impacting the availability of nutrient-rich fishwater. As a workaround, fishwater was sourced from an Aquarium and Museum in Esbjerg (Fiskeri-& Søfartsmuseet). A potential explanation for the observed lack of growth using the museum-sourced fishwater could be the result of the clean conditions maintained in their tanks, leading to a deficiency of fish waste, which serves as nutrients for microalgae.

III.3.2 Growth kinetics

To further analyse the growth of these microalgae the growth kinetics were obtained by plotting the growth curves on a logarithmic scale (see Figure *41*) for all the microalgae cultures including the cultures of *Chlamydomonas sp.*, *Stichococcus deasonii*, mixture of both species (1:1) and controls, both in the medium containing the artificial saltwater supplemented with commercial plant fertiliser (SW) and in the medium containing the fishwater (FW).

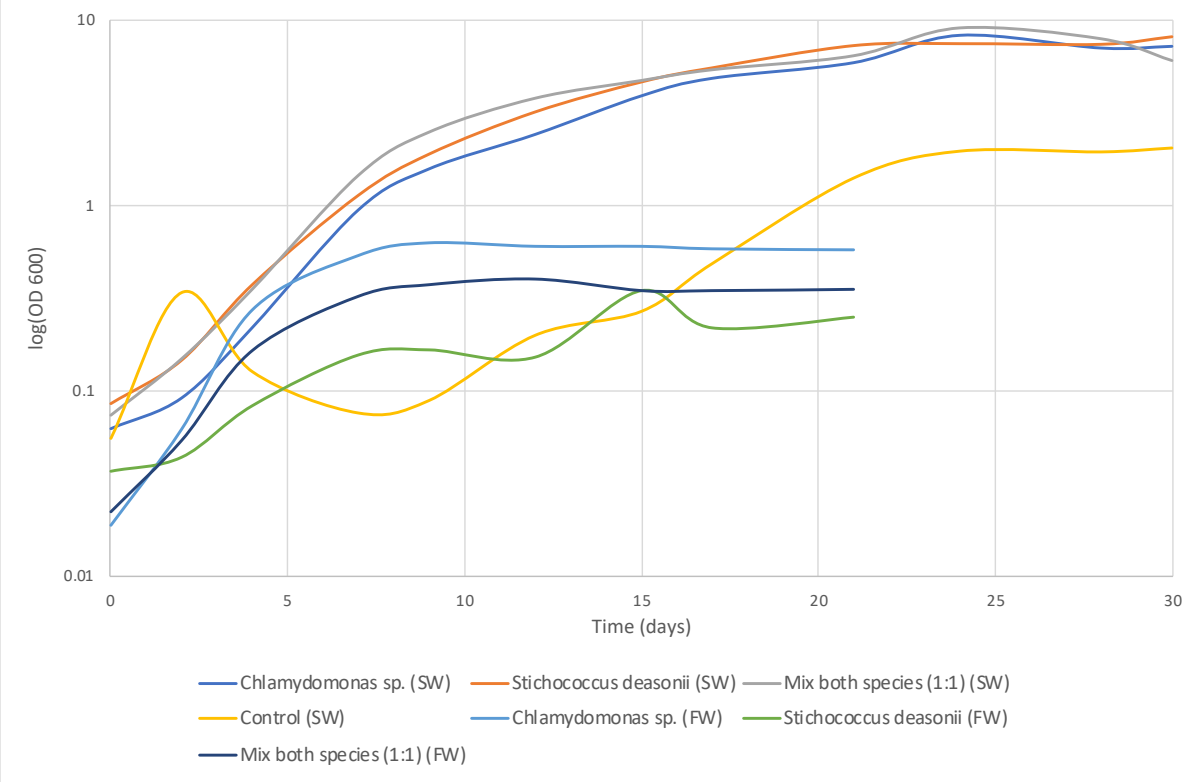


Figure 41 - Growth curves of four microalgae cultures cultivated in artificial saltwater medium supplemented with commercial nutrients (SW) and fishwater (FW), with the y-axis representing the mean optical density (OD) values on a logarithmic scale, and the x-axis representing time (in days). The saltwater cultures were grown for a duration of 30 days, whereas the fishwater cultures were grown for 17 days. No additional nutrient supplementation was provided to the cultures throughout the experiment.

From these results the growth kinetic values were estimated and calculated as described in section II.7.6 Growth kinetics. The obtained values are shown in Table 12.

Table 12 - Growth kinetics from the experiments run to compare the growth of 4 microalgae cultures grown in artificial saltwater with commercial nutrient medium vs fishwater medium.

Cultures	Medium	*Chlamydomonas* sp.	*Stichococcus deasonii*	Mix both species (1:1)	Control
Period of the exponential growth phase (t0,t) (days)	SW	2,9	2,15	2,9	15,24
	FW	2,7	2,7	2,7	NA
Exponential growth equation	SW	y = 0.0415e0.4184x	y = 0.0999e0.3094x	y = 0.0688e0.4116x	y = 0.0101e0.2251x
	FW	y = 0.0346e0.4135x	y = 0.0279e0.2499x	y = 0.0318e0.3469x	NA
Specific growth rate (μ) (d^{-1})	SW	0.42	0.31	0.41	1.41
	FW	0.41	0.25	0.35	NA
Biomass at time t (Bt) (cells/mL)	SW	956,667 ± 303,987	2,758,333 ± 38,188	1,033,333 ± 289,756	840,000 ± 588,791
	FW	246,667 ± 102,021	108,333 ± 62,567	112,500 ± 45,000	NA
Doubling time (d^{-1})	SW	1.66	2.24	1.68	0.49
	FW	1.68	2.77	2.00	NA
Doubling time (h^{-1})	SW	40	54	40	12
	FW	40	67	48	NA
Productivity (cells/mL/day)	SW	400,269	853,428	425,320	1,185,744
	FW	101,997	27,073	39,026	NA
Productivity (cells/mL/h)	SW	16,678	35,560	17,722	49,406
	FW	4,250	1,128	1,626	NA

Legend:

- SW = Cultivation in saltwater and commercial nutrients medium.
- FW = Cultivation in fishwater medium.
- NA = Not applicable (no growth).

The first row of Table 12 displays the duration of the exponential growth phase in days. The exponential growth phase for the *Chlamydomonas* sp. culture and the mixed culture in SW medium extended from day 2 to day 9. For *Stichococcus deasonii* in SW medium, this phase spanned from day 2 to day 15. The contamination in the controls occurred later in the experiment, resulting in an exponential growth phase from day 15 to day 24. For all cultures grown in fishwater, the exponential growth phase was observed from day 2 to day 7.

The second row presents the exponential growth equation, determined experimentally as outlined in the section II.7.6 Growth kinetics. This equation enabled the determination of the specific growth rates of each culture. Both in SW and FW, the *Chlamydomonas* sp. exhibited the highest specific growth rate of 0.42 (SW) and 0.41 (FW). The mixed cultures of both species followed with 0.41 (SW) and 0.35 (FW), and lastly, the *Stichococcus deasonii* cultures

registered growth rates of 0.31 (SW) and 0.25 (FW). The contaminated controls in SW registered the highest growth rate of all at 1.41.

The third row shows the biomass in cells/mL at the end of the exponential growth phase (t), these values were experimentally determined with the help of a hemacytometer. It is important to consider the large standard deviations, which make some comparisons difficult. However, some conclusions can be drawn, for example, it can be seen that the values are substantially lower when the cultures were grown in fishwater compared to artificial saltwater medium with commercial plant fertiliser. Moreover, the culture obtaining the highest biomass value at time t, is the *Stichococcus deasonii* culture grown in artificial saltwater medium.

The fourth and fifth rows, depict the doubling times of each microalgae culture. In the case of *Chlamydomonas sp.* grown in artificial saltwater medium (SW) and in fishwater (FW) the doubling time is approximately 40 hours. This finding indicates that, under the specific conditions of these experiment, it takes approximately 40 hours for the population of *Chlamydomonas sp.* to double in size.

In existing literature, the doubling time for *Chlamydomonas noctigama* has been documented as 9.5 hours [149]. Consequently, a discrepancy emerges between the reported doubling time for *Chlamydomonas* sp. and the experimental finding in this study. This difference in doubling time might be attributable to various factors. Environmental conditions such as light intensity, temperature, and nutrient availability can significantly impact the growth rate of microalgae. Genetic variability in different strains or isolates of *Chlamydomonas* sp. might also result in differing doubling times. Discrepancies may also stem from differences in experimental methods, including culture maintenance, sampling methods, and growth measurement procedures. As this experiment is the initial attempt at cultivating these microalgae, the observed doubling time is within reasonable bounds but could potentially be reduced with further optimisation experiments.

The doubling time of *Stichococcus deasonii* was approximately 54 hours and 67 hours for the cultures grown in SW and FW respectively.

Doubling time of the mixed culture *Chlamydomonas* sp. and *Stichococcus deasonii* (1:1) in artificial saltwater medium was approximately 40 and 48 hours for the cultures grown in SW and FW respectively.

The doubling time of the controls grown in artificial saltwater medium was a lot smaller than any of the other cultures with approximately 12 hours.

Once again, it is important to keep in mind that due to the large variation observed within the triplicates it is difficult to compare these values in a significant manner.

Finally, the last two rows of Table 12, show the productivity of each microalgae culture. It is possible to see a big difference in the productivity from the cultures grown in artificial saltwater

with commercial nutrients (SW) which is much larger than those of the cultures grown in FW. These range from 16,678 cells/mL/hour (SW) to 4,250 cells/mL/hour (FW) in the case of *Chlamydomonas sp.*. And the difference is even greater in the case of *Stichococcus deasonii* where is goes from 35,560 cells/mL/hour (SW) to 1,128 cells/mL/hour (FW). In the case of the mixed culture, the values are more similar to the cultures of *Chlamydomonas sp.* with 17,722 cells/mL/hour (SW) to 1,626 cells/mL/hour (FW). And lastly, the culture with the greatest productivity of all is that of the controls in grown in SW, with 49,406 cells/mL/hour.

III.3.3 Correlation analysis of optical density (OD600) and cell density in microalgae growth

Figure 42 and Figure 43, illustrate the correlation between the optical density measured at a wavelength of 600nm (OD_{600}) and the cell density (cells/mL) in the growth of microalgae in an artificial saltwater medium with plant fertiliser and a fishwater medium.

The x-axis represents the OD_{600} values, which serve as a measure of the microbial biomass concentration, while the y-axis displays the corresponding cell densities in cells/mL. A linear trendline was fitted to the data points, with the regression line forced through the origin to set the y-intercept to zero. The trendline equations and the correlation coefficient (R^2) are displayed alongside. The closer R^2 is to 1, the stronger the relationship between the OD_{600} measurements and cell densities. The R^2 value reveals the variance in cell density that can be explained by this linear relationship with OD_{600}.

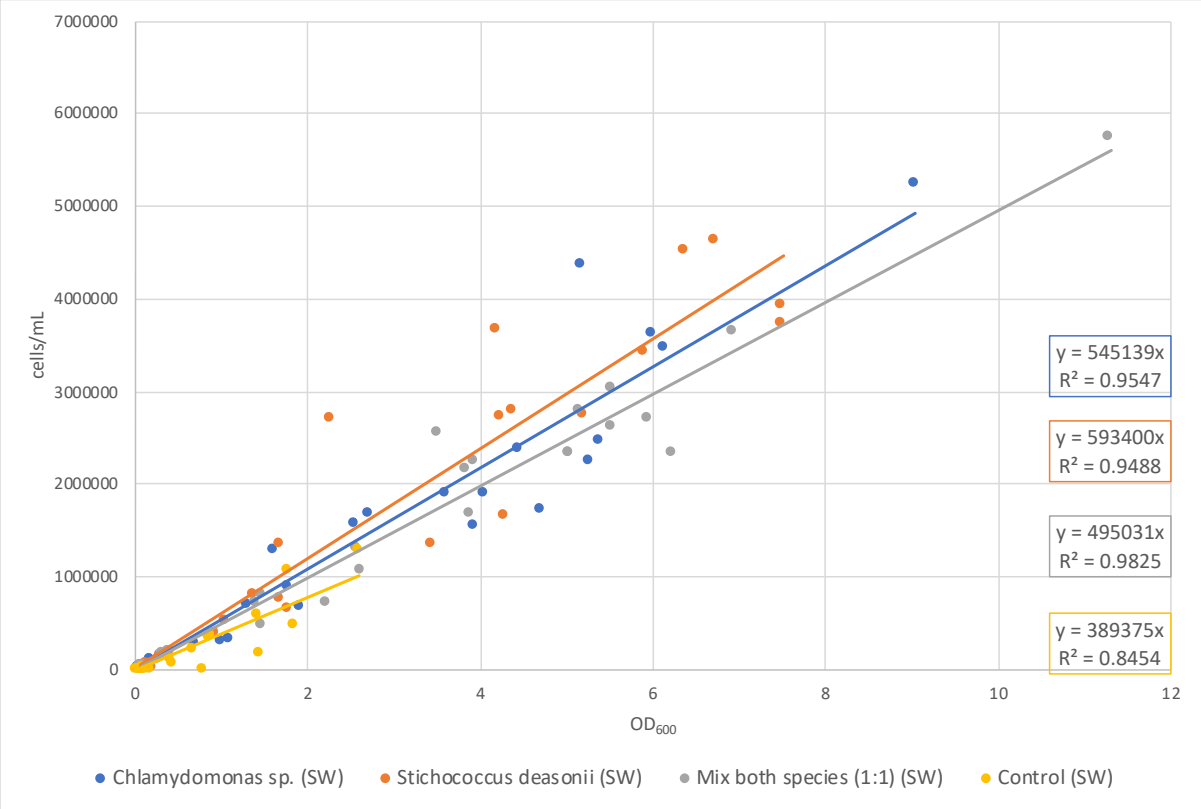


Figure 42- Relationship between optical density (OD600) and cell density (cells/mL) in four microalgae cultures grown in artificial saltwater and commercial nutrients medium. Linear trendlines were fitted and the corresponding trendline equations and correlation coefficient (R^2) was displayed.

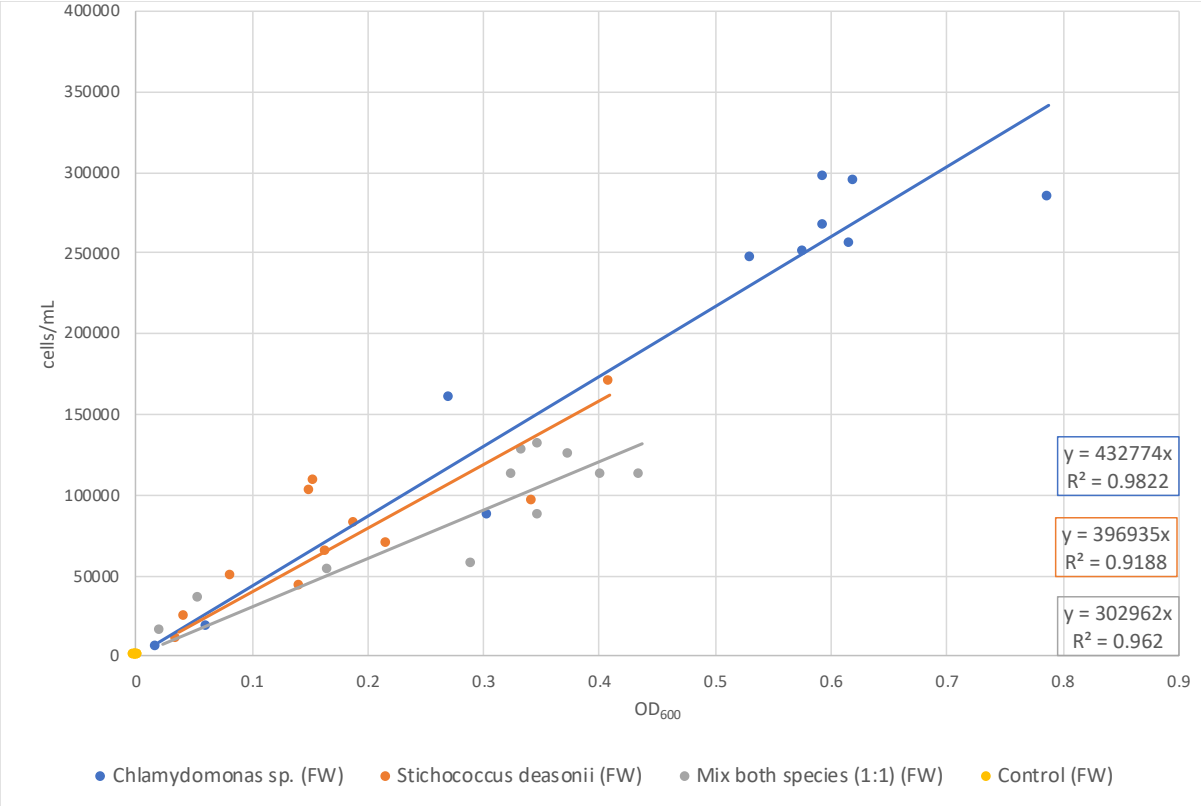


Figure 43 - Relationship between optical density (OD600) and cell density (cells/mL) in four microalgae cultures grown in fishwater medium. Linear trendlines were fitted and the corresponding trendline equations and correlation coefficient (R^2) was displayed.

In all microalgae cultures, an increase in optical density corresponds to an increase in cell density, following a linear pattern. The slope of the equation represents the average increase in cell density per unit increase in OD_{600}. Furthermore, all the regression coefficients, excluding the controls in the saltwater medium, boast R^2 values higher than 90%. This suggests that over 90% of the variance in cell density can be explained by the linear relationship with OD_{600}. These high R^2 values indicate a strong correlation between the two variables (OD_{600} and cell density) in the microalgae cultures.

The results obtained suggest that OD_{600} measurements can serve as reliable estimations for cell density in microalgae cultures, proving convenient for assessing biomass concentration. These results demonstrate the potential use of OD_{600} as a reliable proxy for estimating overall cell density. This approach could prove beneficial for optimising growth conditions, monitoring cell proliferation, and evaluating cultivation system efficiency in microalgae-based industries. However, it's essential to remember that while OD_{600} provides a solid estimate of cell density, it doesn't account for certain aspects such as the physiology or health of the microalgae.

Table 13 compares the linear relationships and correlation coefficients across all the microalgae cultures.

Table 13 - Comparison of regression parameters in microalgae cultures with Chlamydomonas sp., Stichococcus deasonii, and mixed culture.

Culture	Linear relationship	Explained varience
Chlamydomonas sp. (SW)	(cells/mL) = 545139 (OD600)	95%
Stichococcus deasonii (SW)	(cells/mL) = 593400 (OD600)	95%
Mix both species (1:1) (SW)	(cells/mL) = 495031 (OD600)	98%
Control (SW)	(cells/mL) = 389375 (OD600)	85%
Chlamydomonas sp. (FW)	(cells/mL) = 432774 (OD600)	98%
Stichococcus deasonii (FW)	(cells/mL) = 396935 (OD600)	92%
Mix both species (1:1) (FW)	(cells/mL) = 302962 (OD600)	96%
Control (FW)	NA	NA

The observed differences in trendline equations and R^2 values underscore the necessity of species-specific and experimental-conditions-specific analyses when studying microalgae growth dynamics. Each microalgae species exhibits unique characteristics and growth patterns, leading to variations in the OD_{600} and cell density relationship. However, once the regression is established, the OD_{600} can estimate the cell density, and vice versa, in these experiments.

III.3.4 Dry matter and lipid content

After the 30-day experiment conducted to investigate the growth of the four different microalgae cultures in artificial saltwater medium, the dry matter and the lipid content was experimentally calculated for each culture: *Chlamydomonas sp., Stichococcus deasonii*, a mixed culture consisting of a 1:1 ratio of *Chlamydomonas sp. and Stichococcus deasonii,* and the controls. Triplicates were performed for each culture to ensure statistical robustness, and the standard deviations of the dry matter and lipid content were calculated.

Histograms presenting the mean values of the dry matter (DM) , the mean lipid content, and the lipid composition along with the standard deviations for each microalgae culture, were generated to visualize the results (

Figure 44 and Figure *45*).

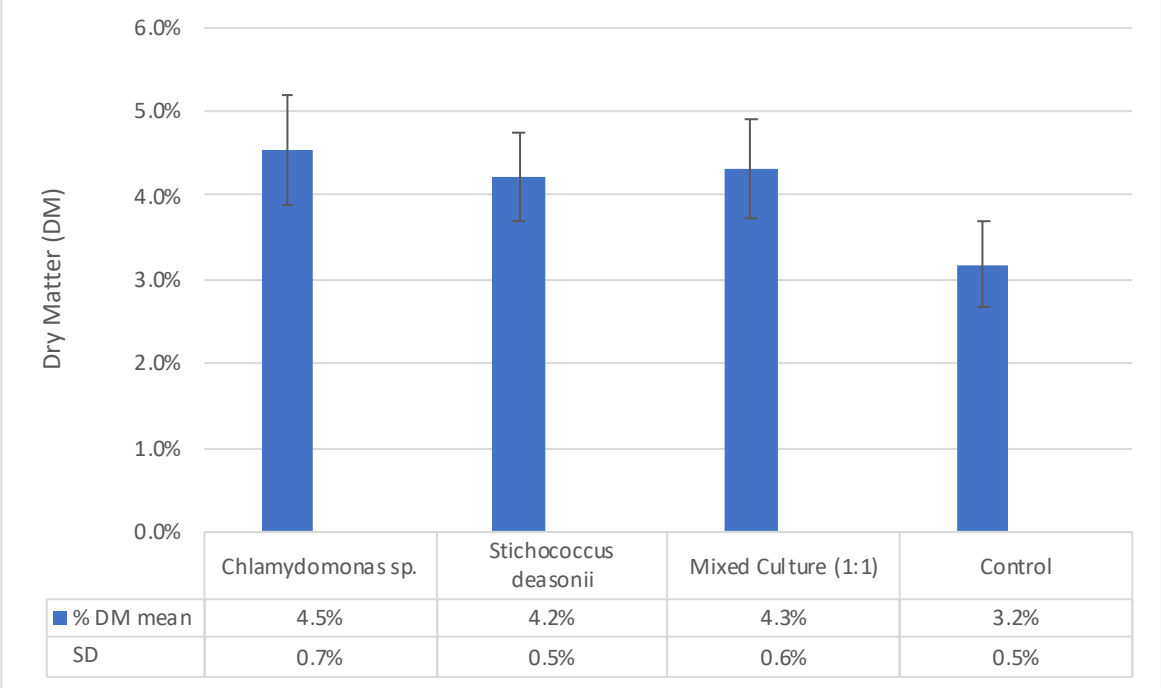
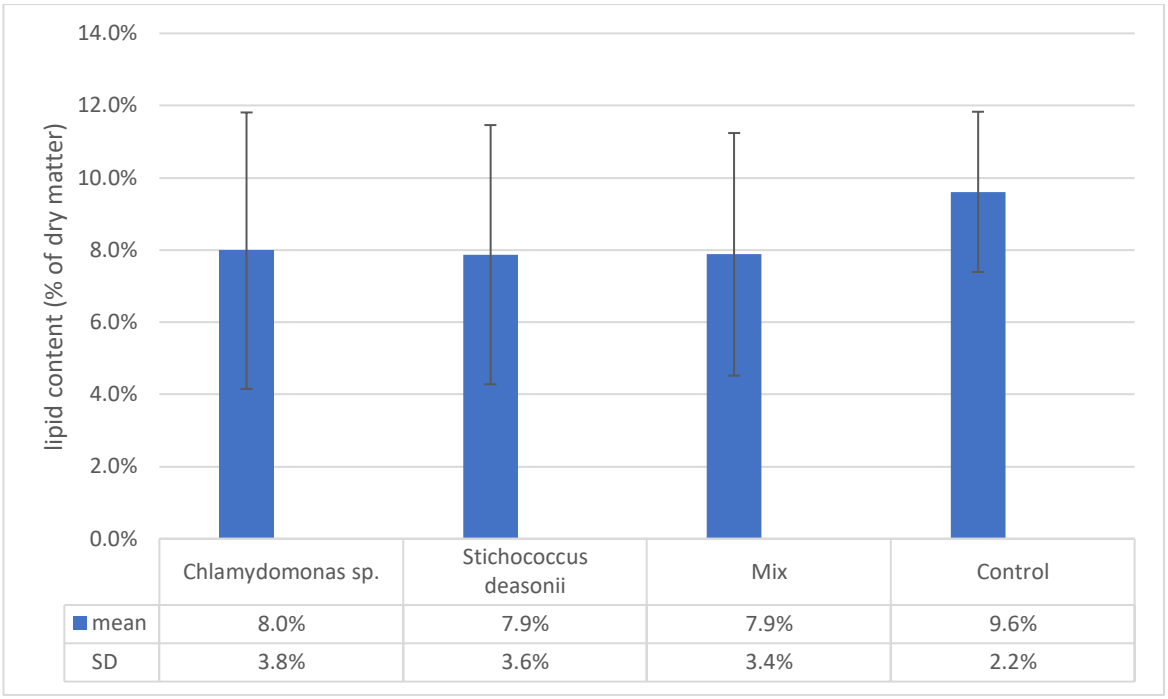
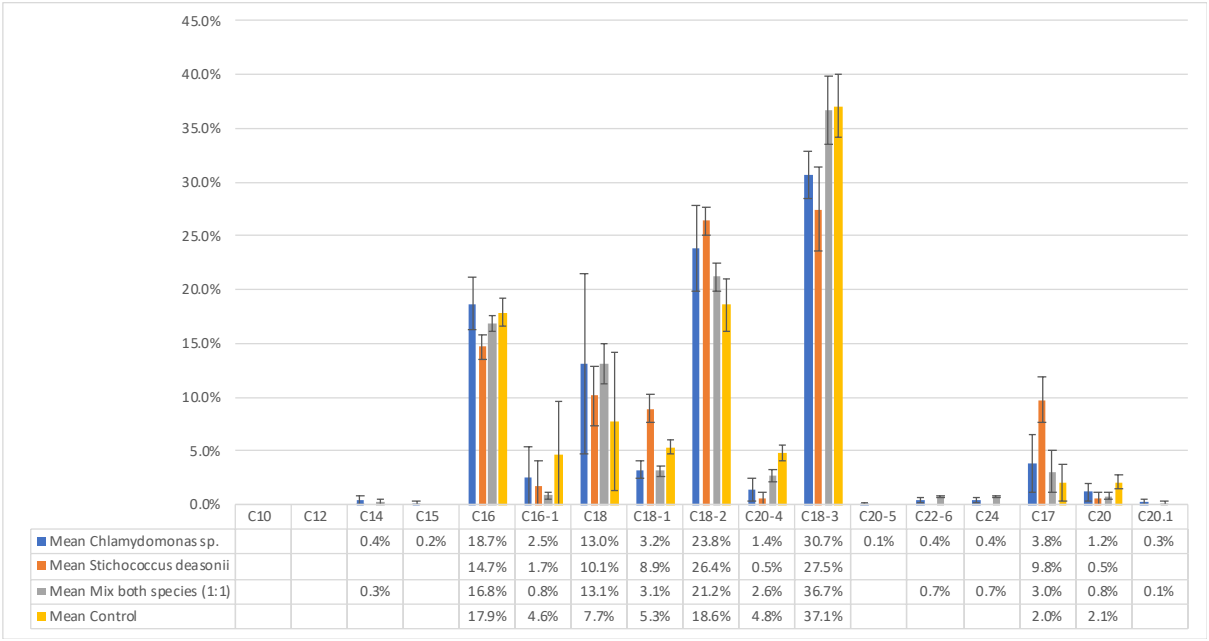


Figure 44 - Bar graph showing the mean dry matter and the corresponding standard deviations of four microalgae cultures after 30 days of cultivation in artificial saltwater: *Chlamydomonas* sp., *Stichococcus deasonii*, a mixture of those two species (Mix), and controls.



	Chlamydomonas sp.	Stichococcus deasonii	Mix	Control
mean	8.0%	7.9%	7.9%	9.6%
SD	3.8%	3.6%	3.4%	2.2%

Figure 45 - Bar graph showing the mean lipid content as a percentage of the DM and the corresponding standard deviations of four microalgae cultures after 30 days of cultivation in artificial saltwater: *Chlamydomonas* sp., *Stichococcus deasonii*, a mixture of those two species (Mix), and controls.



	C10	C12	C14	C15	C16	C16-1	C18	C18-1	C18-2	C20-4	C18-3	C20-5	C22-6	C24	C17	C20	C20.1
Mean Chlamydomonas sp.			0.4%	0.2%	18.7%	2.5%	13.0%	3.2%	23.8%	1.4%	30.7%	0.1%	0.4%	0.4%	3.8%	1.2%	0.3%
Mean Stichococcus deasonii					14.7%	1.7%	10.1%	8.9%	26.4%	0.5%	27.5%				9.8%	0.5%	
Mean Mix both species (1:1)			0.3%		16.8%	0.8%	13.1%	3.1%	21.2%	2.6%	36.7%		0.7%	0.7%	3.0%	0.8%	0.1%
Mean Control					17.9%	4.6%	7.7%	5.3%	18.6%	4.8%	37.1%				2.0%	2.1%	

Figure 46 - Gas chromatography mass spectrometry (GCMS) chromatograms of fatty acid methyl esters (FAME) extracted from four microalgae cultures: *Chlamydomonas* sp., *Stichococcus deasonii*, a mixed culture, and control samples.

The histograms revealed little variations both dry mass (DM) and lipid content and composition across the various microalgae cultures.

Indeed, from *Figure 44*, it can be seen that *Chlamydomonas sp.* exhibited the highest DM mean (4.5%), followed closely by the mixed culture (4.3%) and *Stichococcus deasonii (*4.2%). The controls had the lowest DM mean (3.2%). When considering the standard deviations, no substantial difference can be deduced.

From Figure *45*, it can be seen that the mean lipid content for *Chlamydomonas sp.*, *Stichococcus deasonii*, Mix, and controls was even more similar amongst themselves with 8%, 7.9%, 7.9%, and 9.6%, respectively.

Figure 46 reveals that the primary fatty acids present in all the microalgae samples in a decreasing order are C18-3, C18-2, C16, and C18.

From these results, three primary observations can be noted. Firstly, by referring to Table *3*, the existing literature reports that the microalgae *Chlamydomonas reinhardtii* predominantly contains the following fatty acids, listed in descending order: C18-1, C16, C18-2, C18-3. Similarly, *Stichococcus* sp. is reported to principally consist of these fatty acids, also listed in descending order: C16, C18-3, C18-2, C18-1.

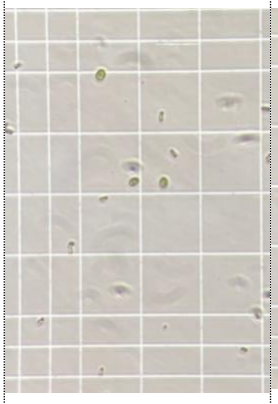
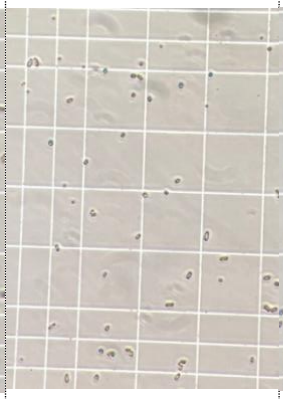
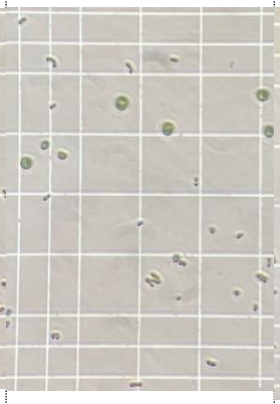
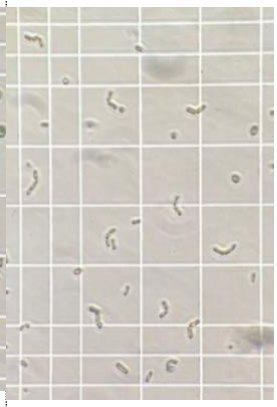
As such, when comparing these experimental findings with what is reported in the literature, it's clear that while the order and amounts may vary, the fatty acid patterns are quite similar.

Secondly, it is noteworthy that the control groups demonstrated the highest lipid yield of all the cultures. The main distinguishing factor between the controls and the other cultures lies in the initial inoculum. As previously mentioned, the control groups did not receive any initial microalgae inoculation, therefore the presence of microalgae can be attributed to contamination. As such, this suggests that the initial inoculum concentration may represent a potential variable for optimization.

Thirdly, as demonstrated by the contamination in the controls, it is possible to say that the conditions in this experiment were not kept sterile. Microscopic observation of microalgae cultures confirmed the omnipresence of *Stichococcus deasonii* across all samples (see

Table 14). Therefore, it is hypothesised that *Stichococcus deasonii* contaminated all the cultures. This likely accounted for the lack of significant differentiation among the cultures, as most observed outcomes could primarily be traced back to *Stichococcus deasonii.*

Table 14 - Microscopic pictures (X40) taken on day 15 of the artificial saltwater cultivation. *Stichococcus deasonii* is present in all three cultures.

Culture	*Chlamydomonas sp.*	*Stichococcus deasonii*	Mixed culture	Controls
Microscopic picture (X40) on day 15.				
Description	Some rod-shaped microalgae assumed to be *Stichococcus deasonii* and some round microalgae assumed to be *Chlamydomonas sp.*	Only rod-shaped microalgae assumed to be *Stichococcus deasonii.*	Same as column 1, some rod-shaped microalgae assumed to be *Stichococcus deasonii* and some round microalgae assumed to be *Chlamydomonas sp.*	Mostly rod-shaped microalgae assumed to be *Stichococcus deasonii.* Very few round microalgae assumed to be *Chlamydomonas sp*

Overall, the results suggest that *Stichococcus deasonii* is a strong competitor in microalgae cultures and may have potential for lipid production. This could prove useful in open systems cultivation where the chosen microalgae strain needs to be a robust strain in order to withstand environmental stress and contamination.

III.3.5 Conclusions based on the comparison of microalgae growth in artificial saltwater medium versus fishwater medium.

The observations drawn from this experimental comparison indicate superior microalgae growth in artificial saltwater as opposed to fishwater. This was reflected in a notably higher productivity, with the artificial saltwater medium averaging 16,678 cells/mL/h, compared to 4,250 cells/mL/h in the fishwater medium. It is however noteworthy that at the time of writing this report, the fishwater experiments were ongoing. Therefore, parameters such as dry matter, lipid content and lipid composition have thus far only been conducted for the artificial saltwater experiments. It would be highly recommended to do the same analysis for the fishwater experiments.

Nevertheless, a hypothesis was considered, stating that nutrient deficiency, potentially due to the absence of fish waste, might exist in the museum-sourced fishwater, hence influencing the growth. To investigate this hypothesis, future work could include making a nutrient profile of the fishwater, specifically focusing on key nutrients such as the primary macronutrients (Nitrogen (N), Phosphorus (P), Potassium (K)). The secondary macronutrients (i.e., Calcium (Ca), Magnesium (Mg), Sulfur (S)) and the micronutrients (i.e., Copper (Cu), Iron (Fe) and Zinc (Zn)). By employing methods such as inductively coupled plasma (ICP) the quantity of many of these nutrients could be detected.

Another way of testing this hypothesis, would be to add these necessary nutrients to the fishwater microalgae cultures using common plant fertiliser. If the microalgae then proliferate more, it would support the idea that the fishwater was lacking in these nutrients.

III.4 Flocculation and sedimentation experiments

III.4.1 Sedimentation tests

When the microalgae cultures from the PBRs were left unstirred on the counter at room temperature, a floating clump was observed see Figure *47*. This clump could be extracted as it had a gelatinous texture. If it were possible to control the formation of this aggregation, it could have significant impacts for microalgae harvesting purposes.

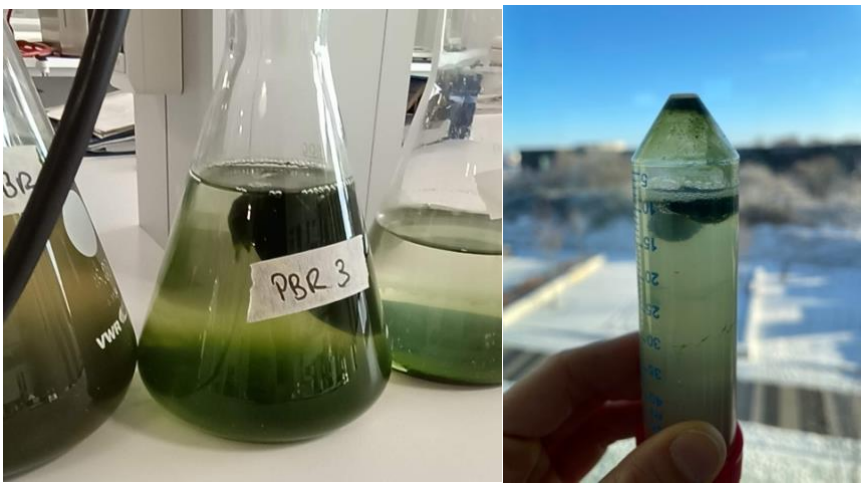


Figure 47 - An atypical aggregation observed in microalgae samples from the 2L photobioreactors left on the counter.

In order to control this phenomenon, it first needed to be understood. Therefore, microscopic observation was conducted to see if there was any visible bacteria or fungi contamination that would explain it as bio flocculation as described in literature.

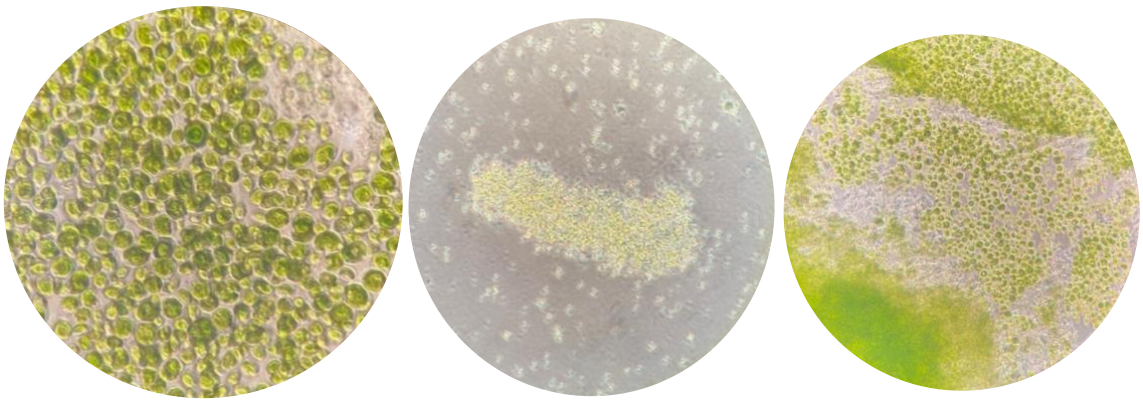


Figure 48 - Microscopic observation (from left to right: X100, X40, X40) of the floating clumps. No fungi or bacteria are apparent.

Under the microscope the culture seemed relatively free of contaminants see Figure *48*. On the other hand, when plated on agar plates containing glucose (see VI.1.2 Artificial saltwater medium with glucose) contamination was clearly visible see Figure *49*.

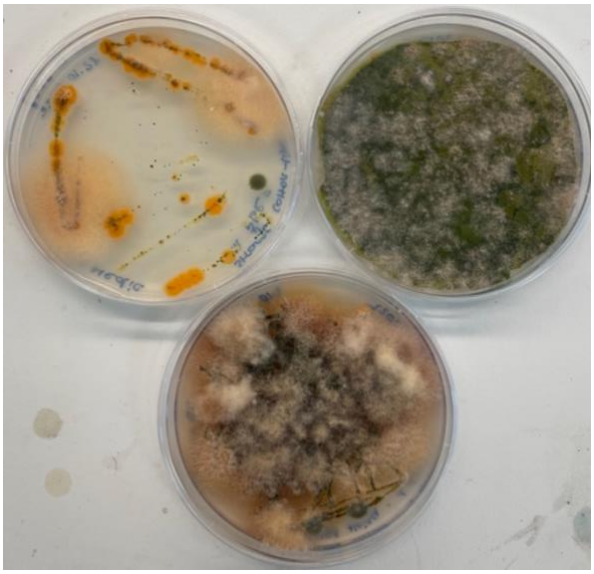
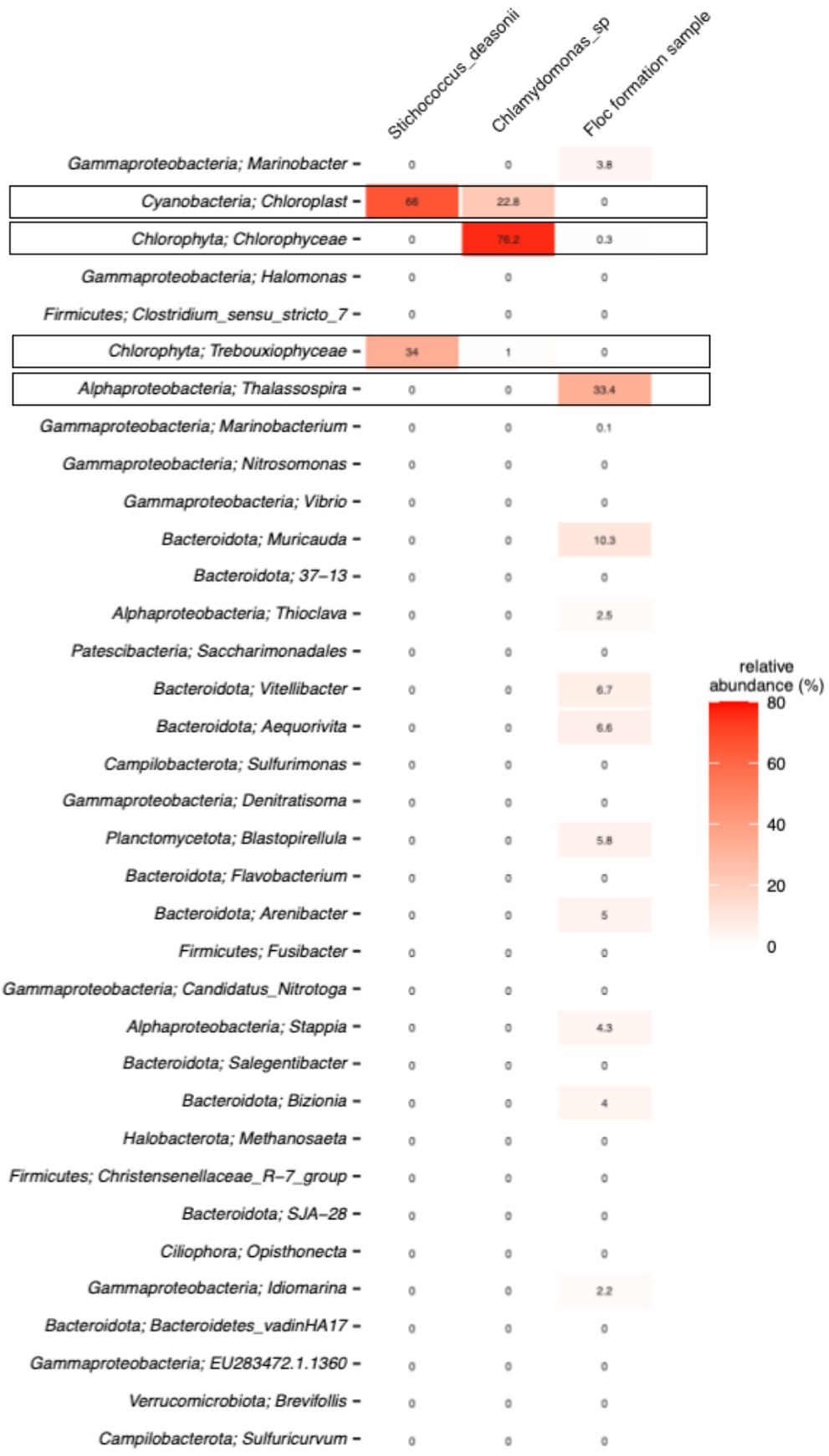


Figure 49 - Plating of the floating clump on agar plates containing glucose. Visible growth of bacteria and fungi.

A sample of the aggregated floc was sent for sequencing. The heatmap here below shows the results. In this heatmap, it can be seen that the most abundant species identified is *Alphaproteobacterial*, *Thalassospira*. Previous research has investigated similar bacteria in relation to their ability to induce bioflocculation in wastewater [105]. This flocculation phenomenon has been attributed to a specific gene cluster within these bacteria. Indeed, when this gene cluster was genetically knocked out in a study, the flocculation effect ceased to occur. Therefore, future projects involving genetic engineering of the bacteria found in the clump aggregation could be carried out to examine whether the microalgae still exhibit the same flocculation behaviour when exposed to these mutants and the wild-type strains of the bacteria.

Table 15 - Samples composition heatmap (abeV48−A, Genus level).



III.4.2 Flocculation tests

The experiment consisting of looking at the effects of different materials on the flocculation of microalgae is showed in Figure 50. From left to right the flask are inoculated with the following materials:

1. A thread wrapped around a carboard.
2. A thread wrapped around a carboard and dipped in fertiliser.
3. A small whole mushroom.
4. A small whole mushroom dipped in fertiliser.
5. A big white mushroom chopped up (5g).
6. Flower
7. Some fine cut fibbers (3 g).
8. Some roughly cut fibbers (4 g).
9. High salt concentration (to test osmotic shock) (5 g of salt).
10. Control

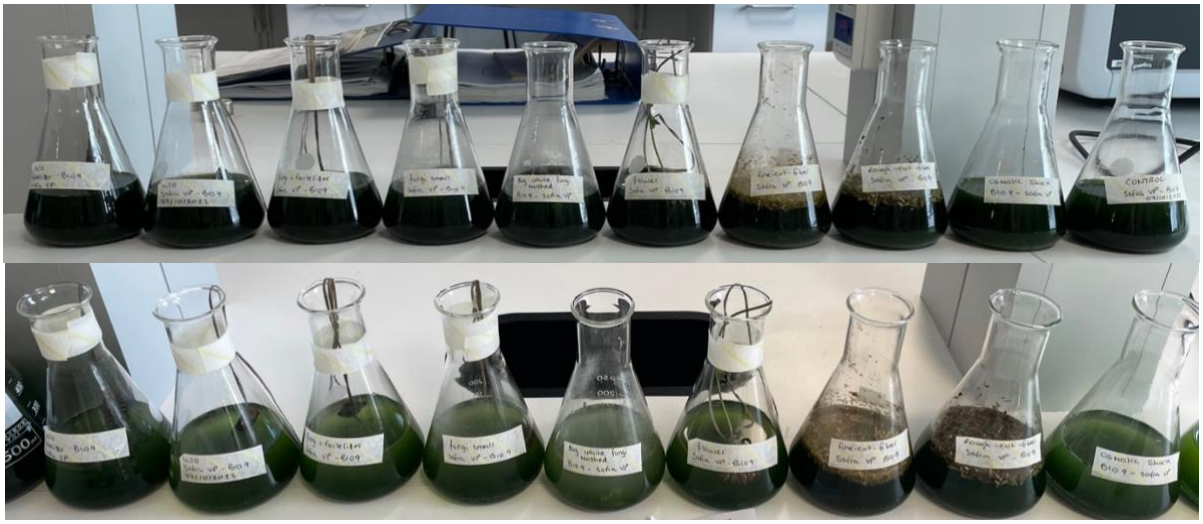


Figure 50 - Sedimentation, flocculation tests. Upper picture taken the day of inoculation; lower picture taken 3 days later.

The thread (1), the thread + fertiliser (2), the small whole mushroom (3), the small whole mushroom + fertiliser (4) and the flower (6) had no effect on whatsoever.

The following was observed in the flask with the big white chopped up mushroom (5): initially, the chopped-up fungi went to the bottom of the flask, after 3 hours the water at the top seemed to be more transparent indicating a quicker sedimentation compared to the control. However, after 3 days the fungi went to the top creating a lot of foam. Some microalgae attached themselves to the fungi but not at all, all the microalgae. Most microalgae just sedimented at the bottom just like the control.

In the case of both for the finely cut fibbers (7) and the roughly cut fibbers (8), the fibbers stayed at the top and with regards to the algae flocculation nothing happens.

In the case of the flask containing a large amount of salt (9), it seems pretty similar to the control after 3 days. The supernatant was removed and demineralised water was added to test the osmotic shock, but no effect was seen.

Overall, the algae flocculation has not seemed to be improved when inoculating them with any of the above-mentioned tests.

These preliminary experiments marked the onset of this project. Subsequent analysis revealed a number of related studies documented in scientific literature which yielded superior results. These promising experiments could potentially be reproduced to determine their impact on these specific microalgae (refer to section I.6.1 Microalgae harvesting). Additionally, it is advisable to systematically sample the supernatant at fixed time intervals, as suggested by various literature sources (commonly at 0, 5, 10, 20, 30, 40, 50, 60, 90 and 150 minutes), to quantify flocculation rather than relying on visual inspections alone.

IV Conclusion and perspectives

Microalgae cultivation has several advantages, these small photosynthesic microorganisms exhibit rapid growth and require minimal land and freshwater to grow. They are a source of various bioactive compounds and have a high lipid yield enabling the production of diverse value-added products and biofuels.

Microalage can help in addressing the omnipresent energy crises and supporting UN sustainability goals. However, research and efforts are needed to allow optimal cultivation, harvesting, and product extraction in order to fully use its potential.

Therefore, this master thesis sought to investigate the cultivation of two distinct microalgae strains isolated from samples collected at the Esbjerg harbour, Denmark, and identified by genomic sequencing as strains belonging to the species *Stichococcous deasonii* and *Chlamydomonas* sp. as feedstock for biofuel production.

Both strains were cultivated using an artificial saltwater medium enriched with commercial nutrients and fishwater supplied by the "Fiskeri- og Søfartsmuseet" located in Esbjerg, Denmark.

The experiment comprised three replicates of each culture, featuring *Chlamydomonas* sp., *Stichococcus deasonii*, a mixture of both species (1:1), and controls that did not incorporate an initial microalgae inoculum. Growth dynamics were monitored through optical density measurements and cell density estimated with the use of a hemacytometer. The tracking period spanned 30 days for the artificial saltwater medium supplemented with commercial nutrients (SW) and 20 days for the fishwater medium (FW).

In all microalgae cultures, a robust correlation between optical density (OD_{600}) and cell density was identified. The findings showed that the microalgae demonstrated superior growth rates in the artificial saltwater medium compared to the fishwater. This was reflected in a notably higher productivity, with the artificial saltwater medium averaging 16,678 cells/mL/h, compared to 4,250 cells/mL/h in the fishwater medium.

Moreover, it was observed that the artificial saltwater control cultures were predominantly contaminated by *Stichococcus deasonii*. Dry matter, lipid content, and lipid composition were assessed for the artificial saltwater medium experiments, as the fishwater experiments were in progress at the time of this report. Dry weight was determined via gravimetric methods, while lipids were extracted using sonication and organic solvents (methanol and chloroform). The lipids were then analysed using FAME + GC-MS.

Outcomes revealed minimal variation in the dry mass (DM), lipid content, and composition across the different microalgae cultures. The DM averaged around 4 % in all cultures, with the lipid content approximately 8 %. C18-3, C18-2, C16, and C18 were recognized as the dominant fatty acids in all microalgae samples.

Given these findings, two primary hypotheses emerged. The initial hypothesis states that there is a potential nutrient deficiency in the fishwater sourced from the museum. Validating this hypothesis would involve creating a nutrient profile for the fishwater or introducing necessary nutrients to the fishwater microalgae cultures in the form of commercial plant fertiliser. If the microalgae then proliferate more, it would support the idea that the fishwater was lacking nutrients.

The second hypothesis suggests that *Stichococcus deasonii*, due to its competitive nature and lipid production potential, could serve as a resilient strain for open system cultivation, demonstrating tolerance to environmental stress such as contamination.

Deducing explicit conclusions from the bioflocculation experiments proved difficult given the lack of precise measurements. Nonetheless, the emergence of a floating microalgae aggregation, which may be linked to bacterial bioflocculation as indicated by a literature review, was observed. Sequencing revealed a significant presence of *Alphaproteobacteria*, specifically *Thalassospira*, previously associated with bioflocculation in wastewater studies. These studies attributed flocculation to a specific gene cluster within these bacteria, which, when disrupted or terminated, ceases flocculation. Consequently, a potential future project could involve genetic engineering of these bacteria to evaluate possible changes in microalgae flocculation.

In conclusion, the cultivation of microalgae presents significant promise for energy solutions and sustainability objectives, especially when incorporated within a circular economy model using fishwater as a nutrient source. Nevertheless, the necessity for additional research to enhance this model's scalability becomes clear. Further investigations could explore variations in initial inoculum, light conditions, pH, and air flows or the induction of stress conditions like nitrogen starvation to optimize growth conditions in fishwater.

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VI Annexes

VI.1 Annexe 1: Growth medium

VI.1.1 Artificial saltwater medium

- 5 mL/L plant fertiliser
- 20g/L agar (if needed)
- Artificial saltwater to volume

VI.1.2 Artificial saltwater medium with glucose

- 5 mL/L plant fertiliser
- 20 g/L glucose (2 %)
- 20 g/L agar (if needed)
- Artificial saltwater to volume

VI.2 Annexe 2: Culture regeneration after cryopreservation pictures

In the figures below, the agar plates are positioned in the following order:

Stichococcus deasonii Dillution: D0	Stichococcus deasonii Dillution: D0	Chlamydomonas Dillution: D0	Chlamydomonas Dillution: D0
Stichococcus deasonii Dillution: D1	Stichococcus deasonii Dillution: D1	Chlamydomonas Dillution: D1	Chlamydomonas Dillution: D1
Stichococcus deasonii Dillution: D2	Stichococcus deasonii Dillution: D2	Chlamydomonas Dillution: D2	Chlamydomonas Dillution: D2

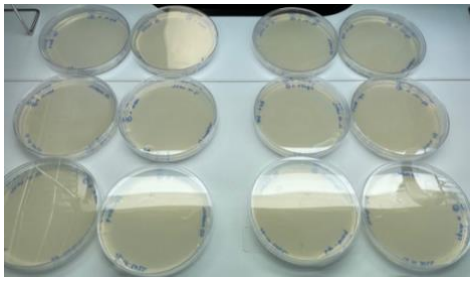

Figure 51- Cryopreservation culture regeneration on agar plates at T = t0

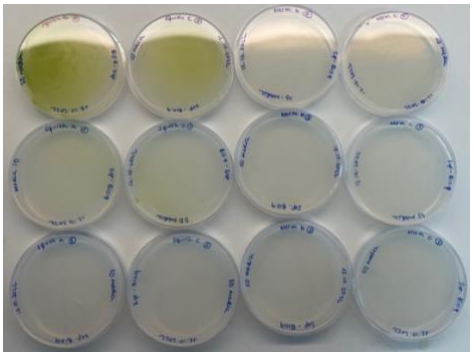

Figure 52- Cryopreservation culture regeneration on agar plates at T = t0 + 5 days

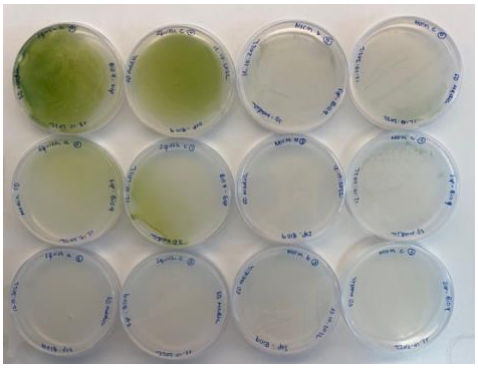

Figure 53- Cryopreservation culture regeneration on agar plates at T = t0 + 6 days

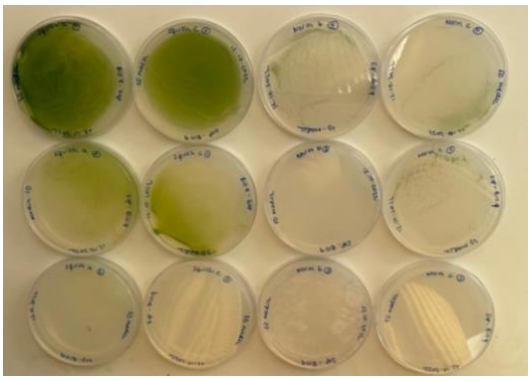

Figure 54- Cryopreservation culture regeneration on agar plates at T = t0 + 7 days

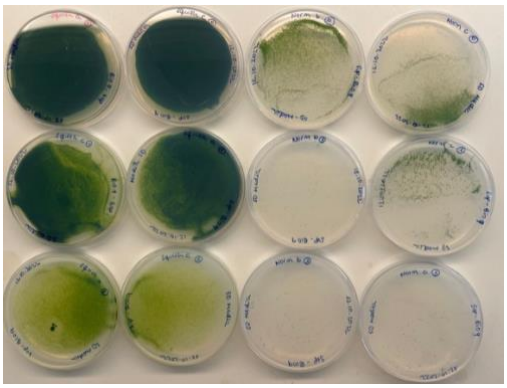

Figure 55- Cryopreservation culture regeneration on agar plates at T = t0 + 10 days

Figure 56 - 12 days after culture regeneration in liquid growth medium. To the left is *Chlamydomonas* and to the right is *Stichococcus deasonii*.