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Study of carbohydrates in *Euglena gracilis*

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The literature review deals with the basics of microalgae, microalgal cultivation and harvest, and the organism Euglena gracilis. The carbohydrates found in E. gracilis are discussed, with the focus on the storage carbohydrate paramylon. The review also deals with effects of cultivation conditions on composition of microalgae.

The aim of the experimental work was to investigate carbohydrate composition in *E. gracilis*, and in this way increase the knowledge of the microalgae. E. gracilis cultivated in five different environments was studied for content of the beta-glucan paramylon, as well as free sugars and oligosaccharides. As the method used for determination of paramylon content was a gravimetric method, a glucose measurement, protein determination and size-exclusion chromatography were performed on the paramylon isolated. In addition, the effect of supercritical fluid extraction (SFE) of the biomass to extract high value compounds on the overall carbohydrate composition and content was also investigated. In addition, the SFE samples were also analysed according to the AOAC method for dietary fibre

The paramylon content in the *E. gracilis* biomass was between 22 and 40 % of the dry biomass. SEC analysis of this paramylon isolated showed that it was of molecular weight around 150 kDa, but that it was not only paramylon that had been isolated, but the isolates also contained impurities. This was also confirmed by the analysis of glucose and protein in the isolates. Possible compounds that can have been isolated with the paramylon are leftover peptides bound to the tight paramylon structure, chlorophyll, or glycoproteins. The most abundant sugars found in *E. gracilis* biomass were mannitol, trehalose and glucose, with a total content of and the total content of the samples were from between 2.4 and 14.9 % of the *E. gracilis* of the total dry mass. There were also some other unquantified free sugars, such as lactose seen in the *E. gracilis* biomass. The oligosaccharide content was considered low and not further quantified.

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PREFACE

This master thesis project was carried out at the Department of Food and Environmental Sciences University of Helsinki from February to September 2017 as a part of the LEVARBIO project. The research was performed under supervision of Prof. Vieno Piironen, Minnamari Edelmann, Anna-Maija Lampi and Leena Pitkänen. I wish to sincerely thank all my supervisors for giving me the opportunity to take part in their research and pursue this thesis project. I would especially like to thank Minnamari Edelmann for her fantastic support, patience and help throughout the course of the project. Thank you.

I would also like to acknowledge the guidance and support I have received from all staff and students working simultaneously with me in the laboratories in the department. Finally, I want to express thanks to my dear family whom I hold dear for all support along the way, as well as friends and colleagues of whom I have had the joy to share this journey with.

Mathias Rudolf Amundsen

Parma May 2018

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1 INTRODUCTION

In 2014 the European Union adapted a strategy to develop bioeconomy in Europe, where the development and use of algae technologies was of key importance (Enzing et al. 2014). Microalgae constitute a diverse group of photosynthetic organisms growing in aquatic environments, and some species of microalgae were among the first organisms to inhabit planet earth. Today microalgae are among the most oxygen producing organisms on the planet (Darzin et al. 2010). One of the reasons why microalgae are regarded as interesting organisms to develop the bioeconomy in Europe is their many potential uses. They can improve the nutritional and functional quality of foods (Chacón-Lee & González-Mariño 2010), be used to produce biofuel (Darzin et al. 2010), and feed for animals (Kleivdal et al. 2013). or be used to extract compounds for a variety of uses.

There has long been interest in microalgae cultivation due to their rapid multiplication, efficient conversion of sunlight to energy and short growth cycles. In addition, microalgae can grow in traditionally non-arable lands, and using food industry waste or public wastewater as cultivation medium (Darzin et al. 2010). In fact human use of microalgae goes back hundreds of years, and microalgae are used in many regions of the world including Central America, Africa, and Asia (Borowitzka 2013). The term microalgae came in 1869 simultaneously as microalgae was first cultivated in simple inorganic media. As with many new technologies, the first attempts had limited success, and it was not until the success of Beijerinck in 1890 and Miquel in 1892 that modern microalgae cultivation had its first bloom (Borowitzka 2013). Besides as a food, the first products produced were food supplements and nutraceuticals and they still are the major commercial products on the market today (Darzin et al. 2010). Products of the spirulina and chlorella are considered as so called "superfood" and are today eaten by health concerned consumers all over the world (Chacón-Lee & González-Mariño 2010). Algal carbohydrates have long been used in commercial applications, and it is believed that microalgal polysaccharides can have varied industrial and commercial uses as well (Matos et al. 2017). Different microalgae produce different carbohydrates that can be utilized in unique ways (Varfolomeev & Wasserman 2011) not only as a healthy ingredient in foods (Matos et al. 2017) but also as structural ingredients (Wells et al. 2017)

Among the first organisms cultivated in a laboratory was *Euglena gracilis,* a single celled eukaryotic protozoon of the Euglena genus that grow in freshwater (Buetow 2001). *E. gracilis* has been widely studied as it is easy to handle and produces high value products. It can be cultivated on a variety of carbon and nutrient sources, and dependent on the cultivation can produce a variety of compounds that can be utilized by humans. These high value products include all 20 amino acids, paramylon, long chain polyunsaturated fatty acids and a variety of vitamins and minerals (Rodriguez-Zavala et al. 2010, O'Neill et al. 2015).

The most abundant carbohydrate found in *E. gracilis* is paramylon. The first mention of this β-1,3-glucan was in 1850, so it is a widely studied compound. Paramylon belongs to the dietary fibres and works as an energy and carbon storage molecule in *E. gracilis*. As it is a dietary fibre it is not digestible by humans but is considered to have several other health effects. Paramylon is a β-1,3-glucose chain folded in a right turning triple helix, which is very tightly packed. This is again packed in fibrillar form fold to membrane enclosed units of high crystallinity. Paramylon has molecular size of approximately 500 kDa (Barsanti et al. 2011). Thought the structure of paramylon is well described, the synthesis of the compound has not been studied in detail. A search of patents of paramylon revealed over 670 available patents, which shows that there has been industrial interest in the compound. Other carbohydrates in *E. gracilis* include the disaccharides trehalose and sucrose produced against osmotic pressure as well as glucose and mannitol (Dwyer 1986). However, the knowledge on the amounts of these sugars in *E. gracilis* is limited

Further knowledge on the carbohydrate content in *E. gracilis* is important in assessing its potential as a food ingredient. The literature review gives a theoretical background why and how microalgae generally and *E. gracilis* are cultivated and which carbohydrates are present in *E. gracilis*. The aim of this master thesis was to study the type and quantities of carbohydrates found in *E. gracilis* grown in chemical Hutner medium and on a dairy retentate. This was to study both how the organism can grow on an optimally created medium for its growth as well as to assess the potential of a dairy side-stream as its cultivation medium. It was also tested whether extracting lipids from the biomass affected the yield of carbohydrates in *E. gracilis*.

2 LITERATURE REVIEW

2.1 MICROALGAE

In this literature review microalgae are defined as single or colonial organisms with habitat in aquatic environments according to the definition of Chacón-Lee and González-Mariño (2010). There are more than 150.000 species of microalgae identified and among them there is diversity in both genetics and expressed characteristics (Guiry & Guiry 2017). Of the large number of species only a small proportion has ever been studied, only a few thousands are held in gene banks around the world, and even fewer are cultivated in large numbers (Darzin et al. 2010). To classify the microalgae several approaches for nomenclature and general classification are used. A typical approach is to classify the microalgae based on their phenotypic character. Typical microalgal classes include green algae (*Chlorophyceae*), diatoms (*Bacillariophyceae*), yellow-green algae (*Xanthophyceae*), golden algae (*Chrysophyceae*), red algae (*Rhodophyceae*), brown algae (*Phaeophyceae*) and picoplankton (*Prasinophyceae* and *Eustigmatophyceae*) (Darzin et al. 2010). Among these, the three most abundant classes are the eukaryotes; diatoms, green and golden algae (Enzing et al. 2014). Diatoms are the most dominant form of phytoplankton with an estimate of more than 100.000 species found. The diatoms are also the largest producer of biomass on earth. Green algae are abundant in fresh water, and the golden algae are known for their production of toxins (Enzing et al. 2014).

The most cultivated microalgae are clearly *Chlorella* and *Spirulina*, but also *Dunaliella, Haematococcus, Schizochtrium, Scenedesmus, Aphanizomenon, Scenedesmus, Aphanizomenon, Odontella and Porhyridium* are commonly cultivated (Chacón-Lee & González-Mariño 2010). There are many theoretical advantages of growing microalgae compared to traditional plants. Like traditional plants microalgae convert solar energy and different carbon sources into energy through photosynthesis. Microalgae can grow rapidly, with reports showing between 1 and 4 cell division per day (Darzin et al. 2010). As microalgae also are small in size, the harvesting cycles become short. Sometimes they can be harvested as often as every day, which is extremely rapid compared to traditional plants that can be harvested once or twice per year. This short growth cycle gives flexibility to meet fluctuations in the market demands on short notice and limits the damage of a bad growth season (Chen et al. 2013). Another feature largely attributed to the simple structure of microalgae, is the high theoretical conversion rate of radiation to biomass compared to traditional plants. Microalgae can convert roughly 6 % of the total incident radiation into new biomass. Whereas sugar cane, an effective

terrestrial plant, only can convert about 3.5-4 % (Darzin et al. 2010). It is important to note that for microalgae these figures are potential yields, and different growth factors will affect the conversion. These factors include weather conditions and influence of other organisms, which are rarely optimal, thus optimal growth is rarely reached (Darzin et al. 2010). Other benefits of microalgae compared to traditional plants include being able to grow in saline water or wastewater streams, and possibility of cultivation in lands unsuitable for the cultivation of other plants. These features are both beneficial as lack of freshwater and land for cultivation are regarded as limited resources to feed a growing world population (Chen et al. 2013).

For productive cultivation of microalgae, a key challenge is to find suitable algal species. The organism should be able to maintain high growth rates whilst growing in a relatively cheap medium and producing the right high value components (Darzin et al. 2010). Some believe that genetic modification of interesting species could be a solution to the problem (Snow & Smith 2012). But as current regulatory practices and societal pressure limit the use of many of the novel techniques in biotechnology, other techniques may be more feasible (Darzin et al. 2010, Snow & Smith 2012). Other challenges currently faced by microalgal production includes efficient recovery and preservation of the organisms, creation of sustainable systems for largescale systems, proving safety of the products as well as market entry and market approval (Chacón-Lee & González-Mariño 2010). Different techniques for cultivation will be explored in section 2.4, in relation to how the technique effects on the growth of the microalgae. The first organisms to be cultivated commercially and that reached market approval were *Chlorella* and *Spirulina* (Chacón-Lee & González-Mariño 2010). They still are among the species most produced and consumed. Different microalgae produce different carbohydrates that can be utilized in unique ways. Green algae contain starch, while red algae contain unique α-polyglucans called floridean starches more structurally similar to amylopectin than glycogen (Varfolomeev & Wasserman 2011). The majority of species of blue-green microalgae contain glycan like polysaccharides, whereas some species contain α-polyglucans (Varfolomeev & Wasserman 2011) and red microalgae are known to contain sulphate polysaccharides. The viscosity and charged groups in the polysaccharides are important features when applied by different industries. For instance, it is important for the food industry that the polysaccharides are stable across temperatures, pH, and ionic strength (Varfolomeev & Wasserman 2011). Though focusing on a small number of species, the bioactivity of microalgal carbohydrates have been proven over many years of experiments (Wells et al. 2016).

A large proportion (~75 %) of the 9000 tonnes of microalgal biomass produced in 2010 was low volume, high price products, including food supplements and nutraceuticals (Chacón-Lee & González-Mariño 2010, Darzin et al. 2010) . The microalgal biomass can also be used for a variety of other products, either "as such" or processed. Proposed uses of microalgae include use in food, supplements, feed, nutraceuticals, energy crops for bioenergy, and as raw material for several industries (Enzing et al. 2014). For instance, many microalgae produce eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), polyunsaturated fatty acids that have been proven to have improving qualities to human health (EFSA 2014). Microalgal EPA and DHA could be marketed as food supplements with an added health claim. Due to the structure of microalgae these fatty acids are often more easily extracted from microalgae than from traditional sources like fatty fish (Chacón-Lee & González-Mariño 2010). The protein quality of microalgae is also high, with favourable or equal biological value, efficiency ratio and true digestibility as compared to the conventional plant proteins (Chacón-Lee & González-Mariño 2010). There are already such protein products on the market in the USA (Figure 1) and there is a large market potential. Microalgae are also a good source of carbohydrates, as microalgal carbohydrates usually have few limitations on use and application. Carbohydrates found in microalgae are diverse and range from simple sugars to complex polysaccharides like starch and cellulose (Chacón-Lee & González-Mariño 2010). In addition to the more complex structures, microalgae often contain important minerals and can produce almost all vitamins (Chacón-Lee & González-Mariño 2010). All these components can be added to products or sold as supplements.

Figure 1 Products available containing microalgae. A smoothie by the Norwegian producer Bama containing spirulina, a protein powder by the Swiss producer Alver containing chlorella, skin creams from American Aloe Infusion and Sunchlorella, as well as fish feed from Ocean nutrition and a food supplement from BioNutrition

The beneficial composition of microalgae is the reason for the vision of incorporation into a range of products. Products where microalgae have already been included are beverages, health products, cosmetics, and food supplements (Figure 1). Challenges with addition of microalgae to foods as an ingredient are their strong colour and strong sea odour (Chacón-Lee & González-Mariño 2010). For Asian consumers, these smells and colours have been connected to high quality products, whereas in western societies they are often connected to poor quality. Therefore, adding microalgae in exotic products can be a more efficient way than adding microalgae like spirulina to shakes as has been tried with mixed success (Chacón-Lee & González-Mariño 2010). Microalgae have also been regarded as a potential source of biofuel (Darzin et al. 2010). For the fish farming industry microalgae would be a much longed source of marine ingredients especially regarding long-chained fatty acids (Kleivdal,et al. 2013). Microalgal polysaccharides have been suggested to have varied industrial and commercial uses as a high content can be obtained by controlling the culture conditions in cultivation (Matos et al. 2017). In foods microalgal carbohydrates can be added as an ingredient for energy or as dietary fibre, they can also be added as structural compounds to food and feed (Wells et al. 2017). Carbohydrates can also be used as fuel either by fermentation to ethanol or butanol, by anaerobic digestion to methane or by thermochemical processes making fuel intermediates (Darzin et al. 2010).

2.2 THE *EUGLENA* GENUS

The *Euglena* genus is a genus of microalgae with long history of research, as it was already observed and recorded in 1674 by the Dutch Anton van Leeuwenhoek. In a letter to the royal society he wrote about a green organism he had found in a lake in Ireland. Following him many further researchers studied the organism, but it was not before 1830, more than 150 years later the German Christian G. Ehrenberg first established the name Euglena (Buetow 2001). Organisms in the *Euglena* genus have a hybrid genome that contains Euglena-specific, Kinetoplastida-specific, and eukaryotic genes and genes acquired during secondary endosymbiosis. It can be hard to classify the evolutionary history of an organism that has degree of horizontal gene transfer (Ahmadinejad et al. 2007). Thus, *Euglena* has been regarded as *protista*, flagellates, *kinetoplastida* and photoautotrophs, but the current classification is as *protozoa* (Figure 2) (Ahmadinejad et al. 2007). There are more than 250 species of *Euglena* that have been recorded and described in the scientific literature (Buetow 2001). One of them, *E. gracilis* was among the first microalgae domesticated. This is because it is easy to grow on many different media on both small and large scale. In addition, *E. gracilis* is easily fractioned despite its rigid pellicle (Buetow 2001).
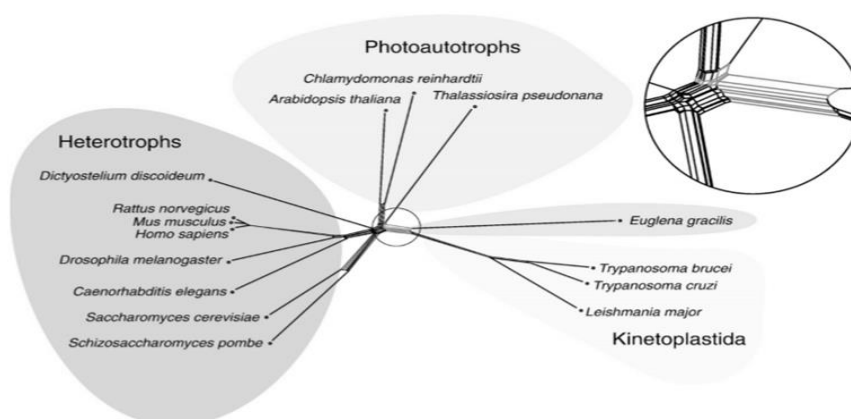

Fig. 4. A phylogenetic network reconstructed for the concatenated alignments of 259 globally distributed genes. The relevant splits are enlarged on the right.

Figure 2 The genentic placement of E. gracilis compared to other heterotrophs, photoauttrophs and knetoplastida (Ahmadinejad et al. 2007)

2.2.1   E. *gracilis*

E. gracilis is the most widely studied species of the Euglena genus. It is single celled, mostly spindle shaped, has a strong green colour, and has two flagella of which one is particularly long (Figure 3) (Buetow 2001). Like the other species of the genus, *E. gracilis* is naturally found in freshwater and it is approximately 50 μm long and 10 μm wide (Buetow 2001). Surrounding the *E. gracilis* cell is a rigid pellicle. Compared to other plants, in *E. gracilis* this pellicle is special, as the typical cell wall consists of polysaccharides or glycoproteins (O'Neill et al. 2015). The pellicle is, however, built up of 70-80 % protein, 6-17 % carbohydrates and 12-17 % lipids (Priesfield, Scholten-Beck & Ruppel 1997). The cell membrane complex (pellicle) is rigid with ridges and grooves and has four fibrils and a subpellicular tubule of the endoplasmic reticulum. It also has a layer of mucilage that is secreted through muciferous channels to the outside of the cell, which coats the pellicle (Buetow 2001). Inside the cell *E. gracilis has* centrally located chloroplasts that bundles chlorophyll a and b. These are the photosynthetic

Figure 3 The E. gracilis cell and important features of the organism. The cell colour is green, and it has a long distinctive tail (BiologyWise) .

centres of *E. gracilis*, and thus the storage carbohydrate paramylon is found in the centre of the chloroplast in pyrenoids covered with paramylon (Buetow 2001). The chloroplasts are surrounded by three-layered saucer-shaped membranes with a wavy margin. (Buetow 2001). Though *E. gracilis* mostly is spindle shaped, it can often also change its shape. Euglenoid movement is a feature of all organisms of Euglena, in which they continuously expand and extract in a way resembling movement (Buetow 2001). *E. gracilis* also changes its structure according to its environment. For instance, in highly saline medium it takes a globular form (Takenaka et al. 1997). The characteristic *E. gracilis* flagellum is between ½ to ⅔ of the length of the cell and is located close to a photoreceptor known as the stigma. By beating the flagellum *E. gracilis* creates movement important to optimise the photosynthesis. The stigma or the "red eye" gives signals the flagella when to beat and when to stop beating according to the amount of light it receives. Thus, optimising the light angle for photosynthesis(Buetow 2001). *E. gracilis* has a spherical 45-chromosome nucleus. The chromosomes are condensed at all phases of the cell cycle, making cell division occur through a special mitosis where the nuclear envelope and the endosome do not disintegrate (Buetow 2001). The cell mitochondrial
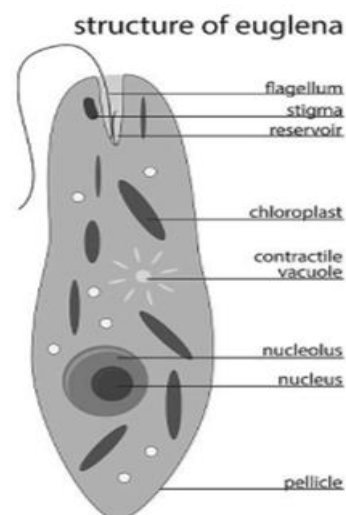
reticulum is large, and branches throughout the cell. Branching and thickness varies according to nutritional situation and growth phase of *E. gracilis* (Buetow 2001).

2.2.2 Nutritional requirements

One of the reasons *E. gracilis* was among the first microalgae cultivated was its adaptability to many different environments. However, it is worth noting that the adaptability of the metabolism is dependent on the growth conditions in particularly considering the light conditions (Matsuda, Hayashi & Kondo 2011). *E. gracilis* can grow both aerobically and anaerobically, both producing sugar (autotrophy) and using sugars (heterotrophy) in their metabolism or a combination of the two (Matsuda, Hayashi & Kondo 2011). There are several essential nutrients for *E. gracilis* including, thiamine (vitamin B_1), cobalamin (vitamin B_{12}), phosphorus, sulphur, nitrogen, and some minerals (Buetow 2001). *E. gracilis* is, however, very adaptable with regards to the carbon source, as it can utilize a variety of different compounds e.g. glucose, glutamate, malate, pyruvate, lactate, and ethanol (Rodriguez-Zavala et al. 2010). *E. gracilis* can also tolerate extremely stressful environments, such as environments with high concentrations of heavy metals or having low pH (pH 2.5-3.5) (Rodriguez-Zavala et al. 2010). Therefore, one of the proposed media for *E. gracilis* cultivation have been wastewater and production side streams (Markou et al. 2012). In media with unsuitable composition or in other need *E. gracilis* can absorb nutrients and engulf other small organisms (phagocytosis) (O'Neill et al. 2015)

2.2.3 Compounds produced by *E. gracilis*

One of the reasons why *E. gracilis* is a very adaptable organism is the 30.000 genes coding for synthesis of different compounds *(O'Neill et al. 2015)*. Many of these compounds are often produced as responses to the different environmental stresses, at different growth stages of the microalgae and according to time of cultivation (Coleman et al. 1988, Schwarzhans et al. 2015, Zeng et al. 2016). The effect of changes in the culture conditions on composition of the cell will be explored in section 2.4. Among the compounds *E. gracilis* produces are all the 20 amino acids, the polysaccharide paramylon, and several polyunsaturated fatty acids (Rodriguez-Zavala et al. 2010). Other compounds of interest include vitamin A, vitamin C, vitamin E, flavonoids, carotenoids, and chlorophyll (O'Neill et al. 2015). In their research Shigeoka et al. (1986) showed that 97 % of all tocopherols produced by *E. gracilis* was α-tocopherol. When cultivated at 27°C under 2000 lux in Koren-Hunter medium the protein, lipid, and carbohydrate contents of E. *gracilis* pellicle were 68.7, 17.9, and 13.5 %, respectively (Nakano et al. 1987).

2.3 CARBOHYDRATES IN EUGLENA

Despite not having the traditional cell wall polysaccharides, *E. gracilis* contains a variety of carbohydrates including polyglucans and more simple sugars (O'Neill et al. 2015).

2.3.1 Paramylon

The most abundant carbohydrate in *E. gracilis* is the β-1,3-glucan paramylon with CAS Number: 9051-97-2(Sigma-Aldrich Co.). As already mentioned, paramylon is closely associated with the chloroplasts in *E. gracilis*. In the chloroplasts, paramylon folds to granules and is surrounded by a membrane in a similar way as starch is folded (Barsanti et al. 2011). Therefore, when in 1850 the German professor J. Gottlieb was to name his newly characterised compound, he combined the two Latin words par (by) and amylon (starch) to give the name paramylon. When Clarke and Stone (1960) studied paramylon, they noted an important difference compared to starch, paramylon did not dye with iodine. They found a structural difference in the compounds made the iodine stay in the amylose helix, in difference to paramylon where the iodine dye did not remain. Paramylon folds in unique granular morphology dependant on the species of *Euglena* they are synthesised in. The granules can have different shapes and be either few and large or abundant and small or a combination of the two (dimorphic) (Monfils et al. 2011). In *E. gracilis*, the paramylon granules are typically ellipses and short rods, but they can also have shallow cup-shaped granules that cap the pyrenoids in the chloroplast or are in vicinity of the chloroplasts (Monfils et al. 2011).

It is well known that paramylon is synthesised in the offshoots of the chorindome (Calvayrac et al. 1981). The paramylon synthesis is not fully described in scientific literature, but a general description was made by O'Neill et al. (2015). They describe the process as an enzymatic process where glucose-1-phosphate reacts with urine triphosphate to form uridine diphosphate (UDP)-glucose with the help of the enzyme UTP-glucose-1-phosphate uridylyltransferase (Figure 4). With the help of paramylon synthase, an enzyme composed of at least seven different proteins, the glucose units from this UDP-glucose binds together in β-1-3-bonds and form paramylon. The same enzyme also helps transfer the paramylon to form granules (O'Neill et al. 2015).
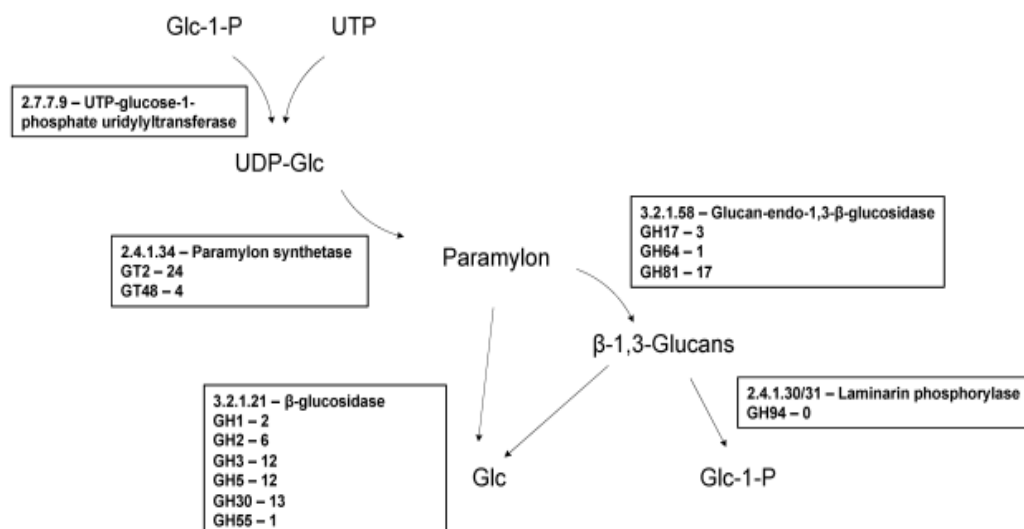
Figure 4 General pathway for synthesis and breakdown of paramylon from and to glucose-1-phosphate and to glucose. (O'Neill et al. 2015)

Paramylon is a linear β-1,3-glucan with a molecular size of approximately 500 kDa (Barsanti et al. 2011). Conformation of paramylon in solid and hydrous state is folding in a right turning triple helix where one 360° turn in the helix consists of six monosaccharide units (Barsanti et al. 2011). The paramylon triple helix is very stable, due to three types of hydrogen bonding; intermolecular bonding in the x-y plane (hexagonal hydrogen bonding), hydrogen bonding between adjacent O_2 molecules in the same chain, and intermolecular hydrogen bonding between different chains in a different x-y plane (left handed helical bonding) (Barsanti et al. 2011). To destabilize hydrogen bonds in the triple helix a strong base with pH >12, a solvent like dimethyl sulfoxide (DMSO) or a temperature above 135°C is needed.

The paramylon triple helixes are packed tight in higher order aggregates of microfibrils, sized approximately 4-10 nm (Figure 5). These microfibrils are in *E. gracilis* oval-shaped and surrounded by membrane, the purity of the paramylon molecule and its structural order makes the paramylon easy to isolate from *Euglenoid* species (Barsanti et al. 2011). The paramylon granules are highly stable storage molecules and are resistant against both extensive chemical and physical stress (Barsanti et al. 2011).

Also, other β-1,3-glucans besides paramylon are found in microalgae, e.g. curdlan, panhuman and callose. Though sharing several chemical characteristics with paramylon, such as forming triple helixes, they have different properties, largely attributed to the sidechains and how they fold 3-dimensionally (Barsanti et al. 2011). One clear example of different properties is the unique crystallinity of >90 % in paramylon compared to curdlan which has 30 % crystallinity. This high crystallinity is not often seen in carbohydrates of the size of paramylon (Barsanti et

al. 2011). This also affects the solubility of the compound. Paramylon is insoluble whereas curdlan is soluble in water (Barsanti et al. 2011).

To utilize paramylon for metabolic purposes *E. gracilis* synthesises an endo-1,3-β-glucanase to break down the polymer (Takeda et al. 2015). The human digestive system does not contain β-1,3-glucanase to hydrolyse paramylon and β-1,3-glucans, thus they are to dietary fibre. With paramylon it is worth noting that the effects of different dietary fibres are dependent on several factors such as water solubility (Kuda, Enomoto & Yano 2009). However, effect of paramylon as a dietary fibre has been studied in mice, and it showed comparable health effects as other similar β-1,3-glucans. Other health effects of paramylon that have been studied, include a protective effect on the liver against acute hepatic injury by carbon tetrachloride (CCl_4) poisoning, inhibition of atopic dermatitis-like symptoms and antitumor, cholesterol lowering and anti-HIV effects in mice (O'Neill et al. 2015)
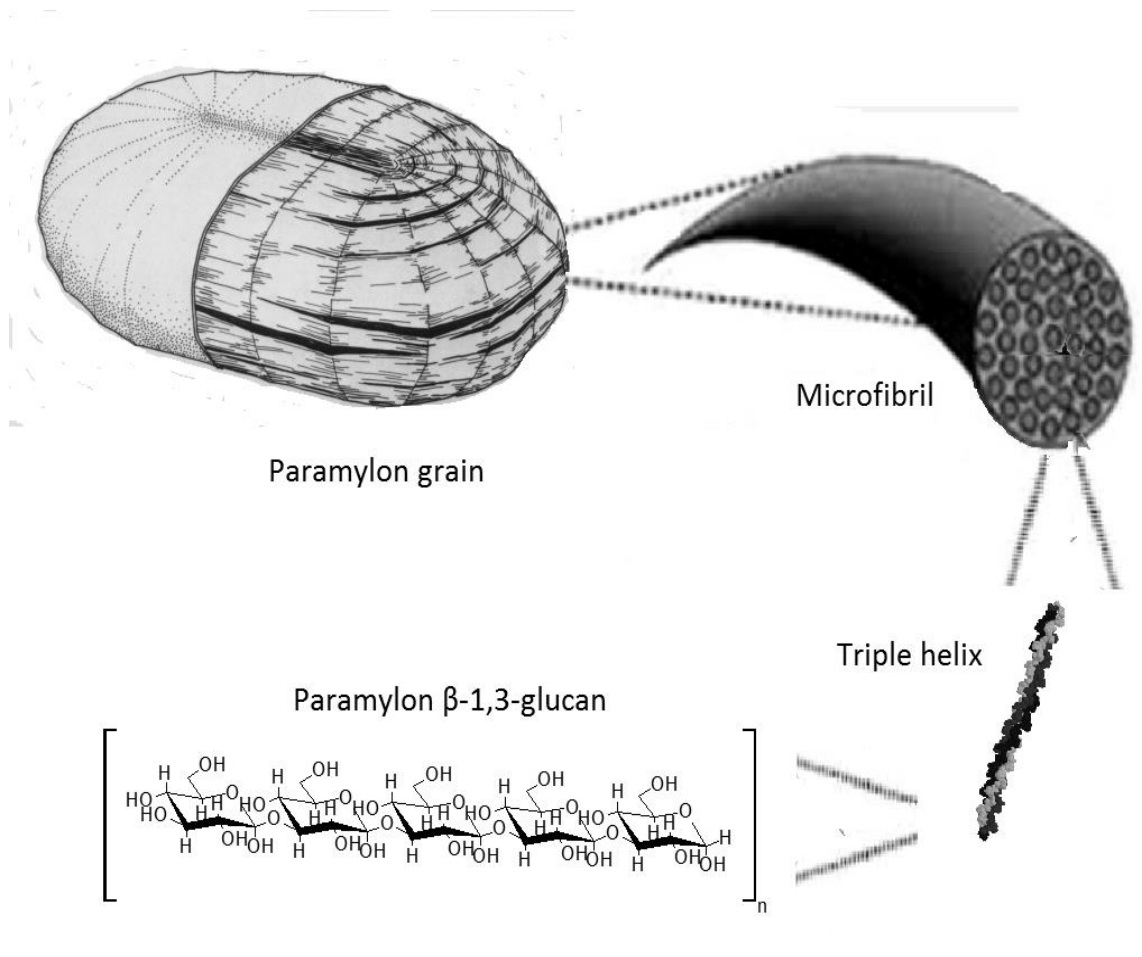

Figure 5 The basic chemical structure of paramylon, its three-dimensional structure, how it folds to fibrills and grains and that are differently dispersed in the cell.

2.3.2 Other carbohydrates found in *E. gracilis*

Amongst others the oligosaccharide raffinose, disaccharides trehalose (Porchia et al. 1999, Takenaka et al. 1997) and sucrose (Porchia, Fiol & Salerno 1999), the sugar alcohols mannitol (Dwyer 1986) and sorbitol (Takenaka et al. 1997) and the monosaccharide glucose (Takenaka et al. 1997) have been isolated from *E. gracilis.* Trehalose is a disaccharide that works as a storage and transport carbohydrate, but in addition functions as a stress protectant. Like paramylon trehalose only consists of glucose units but they are linked by a 1-1 alpha bond. In stressful conditions *E. gracilis* rapidly breaks down paramylon in a rapid process involving many enzymes and regulators (Takenaka et al. 1997). When *E. gracilis* is put under osmotic pressure this synthesis occurs to prevent high leakage of water from the cell, which in the worst case could lead to cell death (Takenaka et al. 1997) . Small amounts of glycoproteins linked to amongst others the flagella of *E. gracilis* have also been reported. Despite of their presence their structure has not been well characterised. There is, however, clear evidence that *E. gracilis* is able to synthesise this kind of glycoproteins (O'Neill et al. 2015).

2.4 CULTIVATION AND HARVEST OF *E. GRACILIS*

Cultivation of all organisms however large or complex they are, are based on the same principles, but the complexity of the challenges and the potential gains are largely divergent. To grow there must be availability of nutrients and other growth factors in amounts sufficient for the specific organism (Chacón-Lee & González-Mariño 2010). A good example of this is analysing traditionally grown plants and plants grown in novel soil less cultivations techniques as hydro ponds (FAO, n.a.). For the plant to grow the basic nutritional needs must be fulfilled in any case. Though being different species with different needs, it should be possible to grow microalgae on a large scale as is done with other microorganisms.

Success of any microalgal cultivation is dependent on several cultivation parameters (Figure 6), such as nutrient content, temperature, pH, mineral content, light exposure, and agitation (Chacón-Lee & González-Mariño 2010). The composition of nutrients that can be extracted from microalgae is not only highly variable between species but is also to a large extent affected by the cultivation conditions (Darzin et al. 2010). Thus, altering any of the previously mentioned parameters will eventually lead to a different product as the metabolism may change.
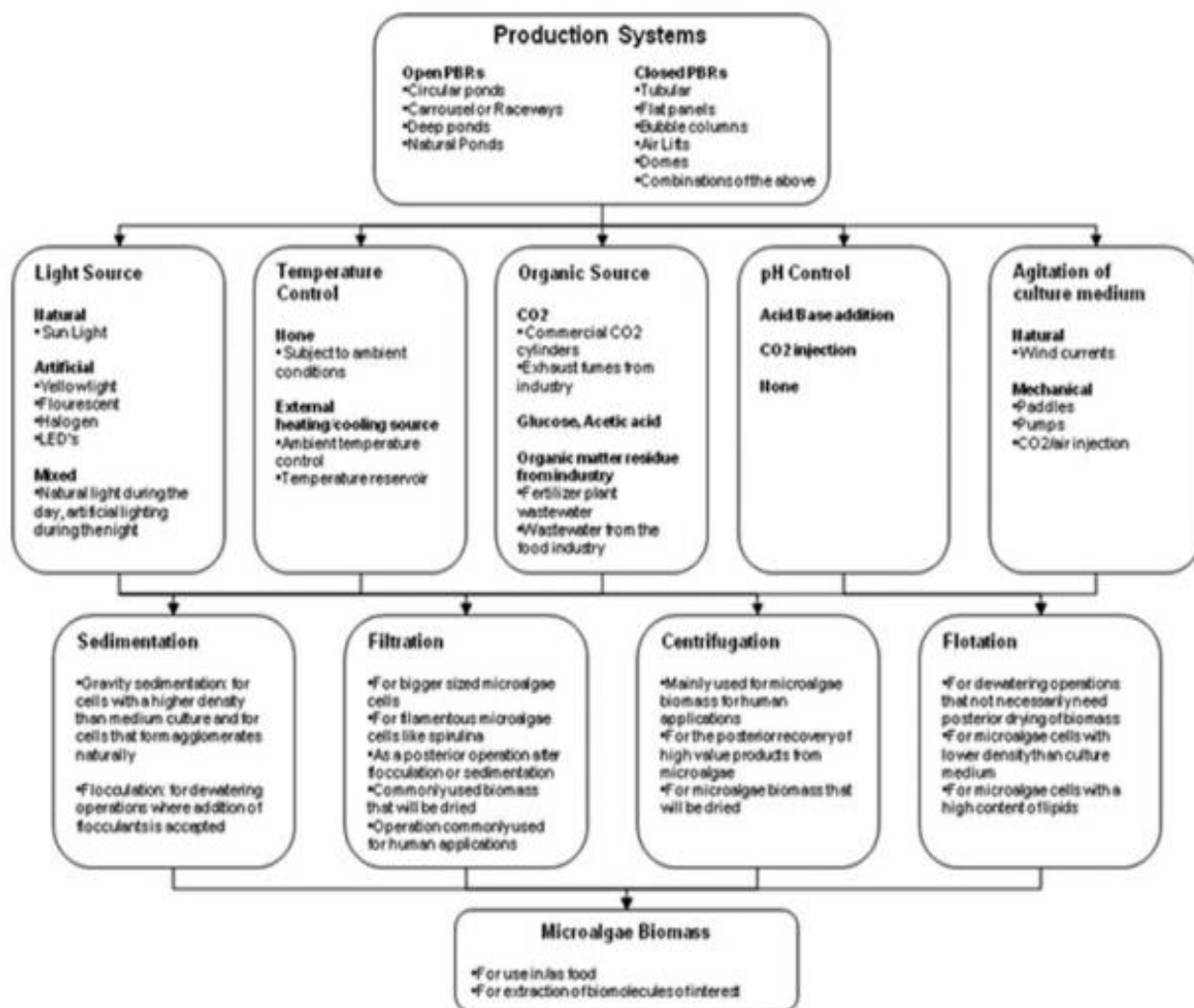
Figure 6 Parameters that can be changed to alter the cultivation of microalgae (Chacón-Lee & González-Mariño 2010)

Changing the nutrient composition is an effective way to initiate a change in metabolism. By changing the available carbon, nitrogen and light source, the metabolism can change from phototropic, heterotrophic, mixotrophic to photoheterotrophic. As metabolism and the input changes, the price of cultivation, cell density and the influence of competing microflora all change, which in the end can affect the success of the microalgal culture (Table 1). For an efficient and successful cultivation of *E. gracilis* a good medium of low price and that does not compete with already available food resources must be utilized (Darzin et al. 2010).

Table 1 Comparison of cultivating microalgae with different metabolisms affects cell density, cost and scale up (Chenet al. 2011)

Cultivation condition	Energy source	Carbon source	Cell density	Reactor scale-up	Cost	Issues associated with scale-up
Phototropic	Light	Inorganic	Low	Open pond or photobioreactor	Low	Low cell density High condensation cost
Heterotrophic	Organic	Organic	High	Conventional fermentor	Medium	Contamination High substrate cost
Mixotrophic	Light and organic	Inorganic and organic	Medium	Closed photobioreactor	High	Contamination High equipment cost High substrate cost
Photoheterotrophic	Light	Organic	Medium	Closed photobioreactor	High	Contamination High equipment cost High substrate cost

The climate in the region of growth has high influence in case *E. gracilis* is cultivated outdoors. To grow all microalgae have need for high amounts of radiation and adequate temperatures. However, unlike terrestrial plants microalgae are damaged by too direct solar radiation, which can cause cell death (Darzin et al. 2010). The minimum yearly average amount of radiation considered adequate for microalgal cultivation ($1500 \ kWh\text{-}m^2\text{-}yr^{-1}$) is found in most regions of the world (Darzin et al. 2010). However, the possibilities of cultivation are also affected by other factors, such as temperature adequate for year around production. This makes cultivation in large parts of northern Europe impossible outdoors, as the media and organisms would freeze. In such cases it is possible to use artificial light sources or systems where sunlight is captured from outside to indoor systems (Chen et al. 2011). These can sometimes be advantageous as stability of the light source can increase yields (Chen et al. 2011). It is also advisable to have production in areas with little extreme weather, to reduce the risk of damage to infrastructure. In ponds the temperature affects the level where the light inhibition occurs. An enhancement of light inhibition is seen at lower temperatures, low temperatures also decreases the uptake of nutrients and affects the cell-membranes reducing growth (Markou et al. 2012). The growth of algae biomass increases with temperature until its optimum growth temperature, above this optimum growth rates again decreases (Markou et al. 2012).

Benefits of growing some microalgal species compared to traditional terrestrial plants, is that microalgae can grow not competing for scarce resources. Several microalgae can grow in saline,

brackish or wastewater and are therefore not competing of clean drinking water (Darzin et al. 2010). Though *E. gracilis* is a freshwater organism it can grow in water with salt by converting paramylon to smaller carbohydrates to resist the osmotic pressure (Takenaka et al. 1997). By cultivating *E. gracilis* in an area not competing with traditional plants and with favourable topography, it will not occupy land that could be used for cultivation of other food sources (Darzin et al. 2010). To obtain optimal growth additional CO_2 must often be used to enrich the growth medium. Therefore, microalgae can be cultivated capturing CO_2 which otherwise would be released to the atmosphere. To have efficient cultivation, it is important to have the cultivation near a source of CO_2, as transportation of the gas may drastically increase the cost (Darzin et al. 2010) and environmental impact of cultivation. Among the available cultivation techniques there are four considered as having potential for mass cultivation of microalgae (Darzin et al. 2010). Open ponds and intensive ponds which are grown in open air, and photobioreactors and fermenters which are closed systems (Figure 7). Currently closed photobioreactors and open pond systems have the highest production volumes, but the different systems each have their own advantages and disadvantages.

Figure 7 A plated bioreactor, an open raceway pond and a traditional fermenter all considered to be potential ways to mass cultivation of microalgae

Harvesting microalgae is one of the key areas for development in microalgal processing, because of the technological challenges and the influence the step has on overall cost (Darzin et al. 2010). The aim of the harvest is to achieve microalgae with as high dry matter content as possible, using as little energy as possible in a timely manner. Current available systems for harvest include bulk harvest, centrifugation, flocculation (auto flocculation, chemical coagulation and electrolytic process), gravity sedimentation, filtration and screening, flotation, and electrophoresis (Chen et al. 2011). The difficulties in harvesting are due to the small size of the microalgae, difficulties separating the media and the organism, and the necessity of

handling large volumes (Chacón-Lee & González-Mariño 2010). According to the review by Chen et al. (2011) an optimal harvest method of algae for biofuel production should be species independent and use minimal energy and chemicals. However, another view to the problem is that an economically feasible system for a single organism or production process could be more efficient. Using gravity for harvesting is only feasible for large microalgae as their rate of sedimentation is high, whereas mass cultivation of algae needs high overflow rate favouring flotation (Chen et al. 2011). Micro strainers are possible for small algae but slime on the fabric may force continuous cleaning of the strainers. Improvement of harvesting technologies is a requirement to achieve economically feasible microalgae production (Chen et al. 2011).

2.4.1 The effect of cultivation on growth rate and biomass

Rapid cell growth and high final biomass are perhaps the most important factors when evaluating the success of the *E. gracilis* cultivation. Schwarzhans et al. (2015) found in their experiments that higher biomass yield was achieved cultivating *E. gracilis* in heterotrophically grown cultures compared to photoheterotrophically grown cultures. It was also shown that higher concentrations of protease peptone medium enhanced the growth rate but did not affect the dry cell weight (Schwarzhans et al. 2015). Another study in wastewater showed that the optimal way is to have a mixotrophic metabolism because this yields a higher growth rate than what is achieved with a phototropic metabolism (Markou et al. 2012) According to Schwarzhans et al. (2015) an increase in the nitrogen concentration in the growth medium also increases the growth rate of microalgae. Optimization of the cultivation conditions for higher concentrations of one molecule, e.g. by nitrogen starvation to increase carbohydrate content, can decrease the total available biomass (Markou et al. 2012). This is important to keep in mind when searching for the optimal growth conditions, as a higher percentage of lipids can be annulled by a lower biomass. A possible solution to prevent this kind of effect is to have a two-stage cultivation. A primary step with optimal conditions for growth, followed by a secondary cultivation step where the media or growth conditions is changed to optimise for production of a specific compound (Markou et al. 2012).

2.4.2 The effect on carbohydrate composition

The growth conditions can have an immense effect on the carbohydrate content in *E. gracilis*. A natural variation in normal conditions is from 6-17 %, but under extreme conditions O'Neill et al. (2015) cited a content of carbohydrates up to 85 %, whereas Barsanti et al. (2011) measured up to 95 %. Under ambient growth conditions higher amounts of paramylon have been found in cells with heterotrophic metabolism compared to cells with photoheterotrophic

metabolism (Schwarzhans et al. 2015). A study by Zeng et al. (2016) showed that the highest levels of paramylon in the cells are seen in the early stages after inoculation, after which the paramylon is probably broken down to synthesise other cell components. However, another study showed that paramylon is primarily produced during the exponential growth phase and that it is degraded when the cells transit to the stationary growth phase (Schwarzhan set al. 2015). According to Zeng et al. (2016) the light conditions effects the time of paramylon accumulation, but not the end concentrations. They showed that paramylon is accumulated at earlier stages of the *E. gracilis* development when cultivated in the dark compared to when a light source is available. Another study by Matsuda et al. (2011) showed that *E. gracilis* cultivated in the dark had reduced levels of paramylon. This is, however, contrary to the findings of Rodrıguez-Zavala et al. (2010). They found that cultivation in the dark was an efficient way to accumulate paramylon, tyrosine and a-tocopherol. However, when cultivated in the light it has been shown that higher degree of irradiation leads to carbohydrate accumulation (Matsuda et al. 2011).

Cultivation of *E. gracilis* in media with low levels of nitrogen or high content of glucose is shown to yield high amounts of paramylon (Rodriguez-Zavala et al. 2010). It is likely that when grown in these conditions more paramylon is accumulated due to the availability of glucose and due to heterotrophic metabolism. Nitrogen starvation has also been shown to increase carbohydrate and lipid accumulation in green algae. Though the effect often is stronger in lipids, some species accumulate more carbohydrates. Low level of iron also seems to increase the content of carbohydrates in combination with nitrogen starvation and further also high light intensity (Markou et al. 2012). There is evidence that the effects of nitrogen starvation are dependent and synergistic with limitation of other cultivational factors affecting carbohydrate content (Markou et al. 2012).Effects of phosphorous on carbohydrate accumulation has largely been determined by the ratio of 3-phosphoglycerate to inorganic phosphorous in some experiments. When there are low levels of intracellular phosphorous, there are higher contents of carbohydrates and high biomass production. Sulphur starvation leads to inhibition of cell division, which is due to lack of building blocks for proteins and cell components required for growth, which again leads to accumulation of carbohydrates. The most efficient strategy to yield high amount of carbohydrates when cultivating microalgae is sulphur starvation. (Markou et al. 2012). If E. *gracilis* is grown in high salt concentrations it converts paramylon to smaller trehalose compounds to give resistance to the increased osmotic pressure (Takenaka et al. 1997).

2.4.3 The effect on protein composition

Cultivation of microalgae in the light appears to yield higher content of protein compared to cultivation in the dark (Matsuda et al. 2011). Reducing the content of nitrogen in the cultivation media will reduce the accumulation of protein and lower the total biomass. Theoretically a reduction in iron content would consequently lead to a reduction in protein content, as iron is essential in nitrogen uptake for microalgae. However, despite this no indirect effect of iron reduction on the protein content has been observed (Markou et al. 2012).

2.4.4 The effect on lipid composition

Achieving high lipid concentrations is important in both biofuel and food applications. In light grown microalgae, the concentration of lipids was higher than in cells grown in the dark, and when cultivated without available oxygen *E. gracilis* accumulates wax esters (Schwarzhans et al. 2015). The total content of lipids is, however, unaltered as the organisms grown in the dark accumulated more of other compounds (Schwarzhans et al. 2015). If cultivated in the light it has been shown that higher degree of irradiation also leads to an accumulation of lipids (Markou et al. 2012). According to the results achieved by Schwarzhans et al. (2014) the amount of lipids in the cells could almost double.

In *E. gracilis* it is not only the content of lipids that changes with the growth environment, but also the fatty acid composition is altered. To achieve cultivations with high concentrations of the beneficial fatty acids DHA and EPA, photoheterotrophic cultivations have shown the best results (Schwarzhans et al. 2015). In both mixotrophic and heterotopic conditions more long chain unsaturated fatty acids are produced when cultivated in the light than when cultivated in the dark (Zeng et al. 2016). Schwarzhans et al. (2015) also found similar results which shows that photoheterotrophically grown cells have enhanced n-3/n-6 ratio because of the increased production of fatty acids like EPA, DHA, α-Linolenic acid (ALA) and C20:3n3. It is worth noting that the EPA and DHA concentrations accumulate in the early or late stationary phase (Schwarzhans et al. 2015). According to Schwarzhans et al. (2015) an increase in the nitrogen concentration in the growth medium increases the concentration of polyunsaturated fatty acids. When grown anaerobically and in the dark *E. gracilis* produces higher concentrations of wax esters (Matsuda, Hayashi & Kondo 2011). A study also showed that high level of iron in the cultivation media causes accumulation of lipids (Markou et al. 2012).

2.4.5 Toxins

Under the right conditions in nature microalgae can suddenly blossom widely and simultaneously occasionally produce large quantities of toxins. Some of the toxins produced by microalgae are lethal to other marine life. Even for humans these toxins can be harmful if consumed directly or through shellfish that accumulate the toxins in their flesh (Enzing et al. 2014). This kind of human intoxications from novel microalgal sources have been reported (Enzing et al. 2014). Recent examples include finding microcystin in a microalgal food supplement in Norway October 2017 (Søreide 2017). In microalgal blossoms there are also harmful volatiles near the surfaces, but the most harmful compounds produced by microalgae are the toxins (Delia et al. 2015). Microalgal toxins can be either hydrophilic or lipophilic, and depending on the route of ingestion, the solubility of the compounds and their mode of action their toxicity changes. The most common toxins are the water-soluble saxitoxins that can cause paralytic shellfish poisonings. These are mostly produced by the dinoflagellate species belonging to *Alexandrium* spp., *Gymnodinium catenatum* and *Pyrodinium bahamense* (Delia et al. 2015). Other toxic compounds produced include okadaic acid, synthesised by *Dinophysis* and *Prorocentrum*, which is one of the compounds responsible for diarrhoeic shellfish poisoning (Figure 8). Other toxins produced by microalgae are brevotoxins, domoic acids, spirolides, gymnodimines, palytoxin, aplysiatoxins, ciguatoxins and cyanotoxins (Figure 8) (Delia et al. 2015). These toxins cause neurotoxic, paralytic, amnesic, diarrhoeic shellfish poisoning, Ciguatera fish poisoning, and cyanobacterial neurotoxins (Enzing et al. 2014).
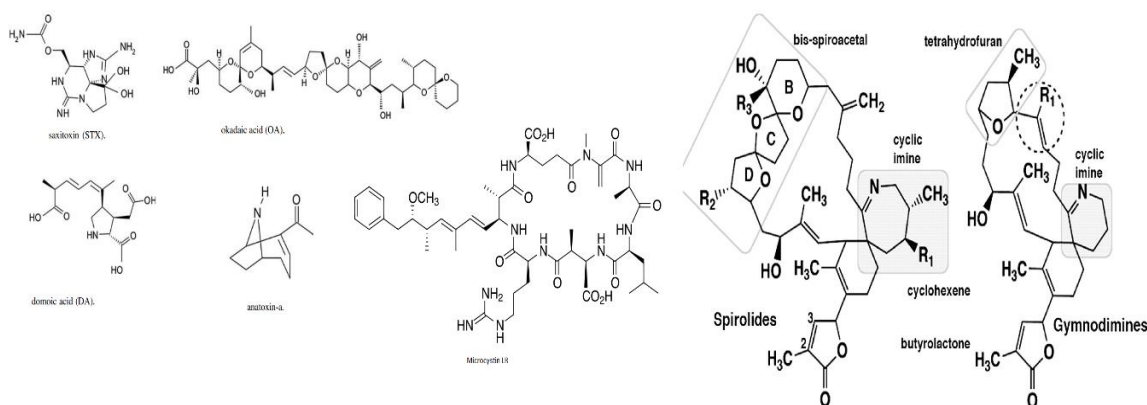
Figure 8 Saxitoxins, okadaic acid, domoic acid, anatoxin-a, microcystin LR, a spirolides and a gymnodimines are among the most common microalgal toxins

Dinoflagellates, diatoms, and cyanobacteria contain the largest number of toxin producing species of microalgae (Delia et al. 2015). Knowing toxicity on strain level is important, because studies have shown difference in toxicity among strains of the same species (Delia et al. 2015).

For instance, it is known that cyanobacteria generally are toxin producers, t, However, spirulina, that is classified as a cyanobacterium y produces toxins at very low levels. There are species in the *Euglena* genus known for producing toxins, particularly *E. sanguinea* is known to produce ichthyotoxin (Zimba et al. 2010). However, there have been no studies showing *E. gracilis* as a toxin producer. However, *E. gracilis* is known to produce some exogenous toxic free amino acids, but their effect is very limited (Oda et al. 1982).

3 EXPERIMENTAL RESEARCH

3.1 AIMS OF THE STUDY

E, gracilis produces a large variety of interesting compound that can be extracted. A research group from the University of Helsinki Department of Food and Environmental Sciences together with research groups from Department of Environmental Sciences and Häme University of Applied Sciences (HAMK) took part in the Levarbio and Algomeg projects. In those projects, the lipid and protein characteristics in algal biomasses have been studied, but the carbohydrate composition has not been characterized in detail. Therefore, the primary focus of this master thesis work was to study the carbohydrate composition of the *E. gracilis* biomass. To study the polysaccharide content in *E. gracilis,* a gravimetric method was used and the content of free sugars in the *E. gracilis* biomass was analysed using anion exchange chromatography. An effect of growth medium and conditions on the composition of carbohydrates in produced algal biomasses was investigated. In addition, the effect of supercritical fluid extraction (SFE) on the carbohydrates of biomasses was studied. The SFE samples were also analysed according to the AOAC method for dietary fibre.

3.2 MATERIALS AND METHODS

3.2.1 Test materials

The samples used in this study were freeze-dried biomasses of *E. gracilis* cultivated by the University of Helsinki, Department of Environmental Sciences, Lahti Campus (HYYL) and at Häme University of Applied Sciences (HAMK). The media used for cultivation was either a synthetic Hutner medium or a side stream from production of lactose free milk, so-called retentate (Table 2). The synthetic Hutner medium is a widely used medium for cultivation of *E. gracilis* (Appendix 1). Biomasses from three different cultivation batches in Hutner medium were studied. Euglena 2 biomasses (Table 2) were obtained from cultivation, where growth medium contained double amount of $(NH_4)_2HPO_4$ (0,533 g/l) compared with a basic Hutner medium (Appendix 1). Euglena 8 biomasses were from cultivation where source of phosphorous was KH_2PO_4 and its concentration was 2.6 g/l. Cultivation batches Euglena 2 and 8 included of three biological replicates, so-called photobioreactors R1, R2, R3 and volume of each was 200 l. For SFE *E. gracilis* was cultivated as a continuous cultivation in Hutner medium with KH_2PO_4 (2,6 g/l) in photobioreactors (200 l). Sample of the biomass from this

cultivation, before SFE (Pre SFE) and after two different SFE-extraction i.e., at temperature 30 °C (SFE30) and at 50 °C (SFE50) were studied for carbohydrate content. This was done to analyse the effect of the extraction.

In addition of biomasses cultivated in synthetic medium, carbohydrates were investigated in biomasses from cultivation in dairy retentate a side stream of lactose free milk production. E. gracilis was grown in five-litre bottles filled with the retentate. Two bottles were cultivated under dark (sample R-dark) and two were cultivated under normal light-dark period (R-light).

The biomasses were freeze-dried at the site of cultivation and stored at -20°C to preserve their original composition as well as possible. Shortly before analysis, the samples were kept in an exicator for 30 minutes and the biomass was milled to ensure homogeneity.

Table 2 The biomass samples analysed in this study the media used for cultivation, location, and eventual notes on changes in the cultivation medium.

Sample	Analysis code	Medium	Cultivation notes
Euglena 2	Euglena 2	Synthetic Hutner medium	$(NH_4)_2HPO$ 0.533 g/l Increased amounts of phosphate and nitrogen in cultivation
Euglena 8	Euglena 8	Synthetic Hutner medium	KH_2PO_4 2.6 g/l Increased amounts of phosphate
Supercritical fluid extraction (Aromtech)	PreSFE, SFE30 & SFE50	Chemical Hutner medium	Hutner 9-11 combination KH_2PO_4 - 2.6 g/l Increased amounts of phosphate
Retentate light	R-Light	Retentate	In luminated condition
Retentate dark	R-Dark	Retentate	Covered by a garbage bag.

For comparison, a paramylon standard (>99.9 % paramylon) (89862-1G-F Lot# BCBR1082V, Sigma, Merck KGaA, Darmstadt, Germany), also isolated from *E. gracilis,* was analysed using the same methods used in the analysis.

For quantification of free sugars in HPLC analysis, standards of D(+)-glucose (K34425937524, Merck, Darmstadt, Germany), D(-)-fructose (70942670, Merck, Darmstadt, Germany), D(+)-galactose (Lot:0001333964, Sigma, Merck, Darmstadt, Germany), D(+)-trehalose (No.T5251, Merck, Darmstadt, Germany), arabinose (2690, 1.01492.0100, Merck, Darmstadt, Germany), sucrose (S7903, Lot:44406512, Merck, Darmstadt, Germany) and mannitol (Cerestar, Lot.

BJ/0905, Cargill's R.L., Minneapolis, MN, USA) were used to quantify these sugars in the extracts.

3.2.2 Methods

Methods used in this study were from previously published papers or standard procedures at the University of Helsinki, Department of Food and Environmental Sciences. The experiments were constructed around the gravimetric method for determination of paramylon by Grimm et al. (2015) (Figure 9). Paramylon isolated by this method was characterized further by glucose determination (Dubois 1956), protein determination using the Kjeldahl method (AOAC, 1995) and size exclusion chromatography (SEC) (Pitkänen et al. 2017). The low molecule weight carbohydrates in the water and ethanol extracts were determined by high-performance liquid chromatography (HPLC) (Xu et al. 2017). In addition, dietary fibre was analysed by the AOAC method (AOAC International 1995).
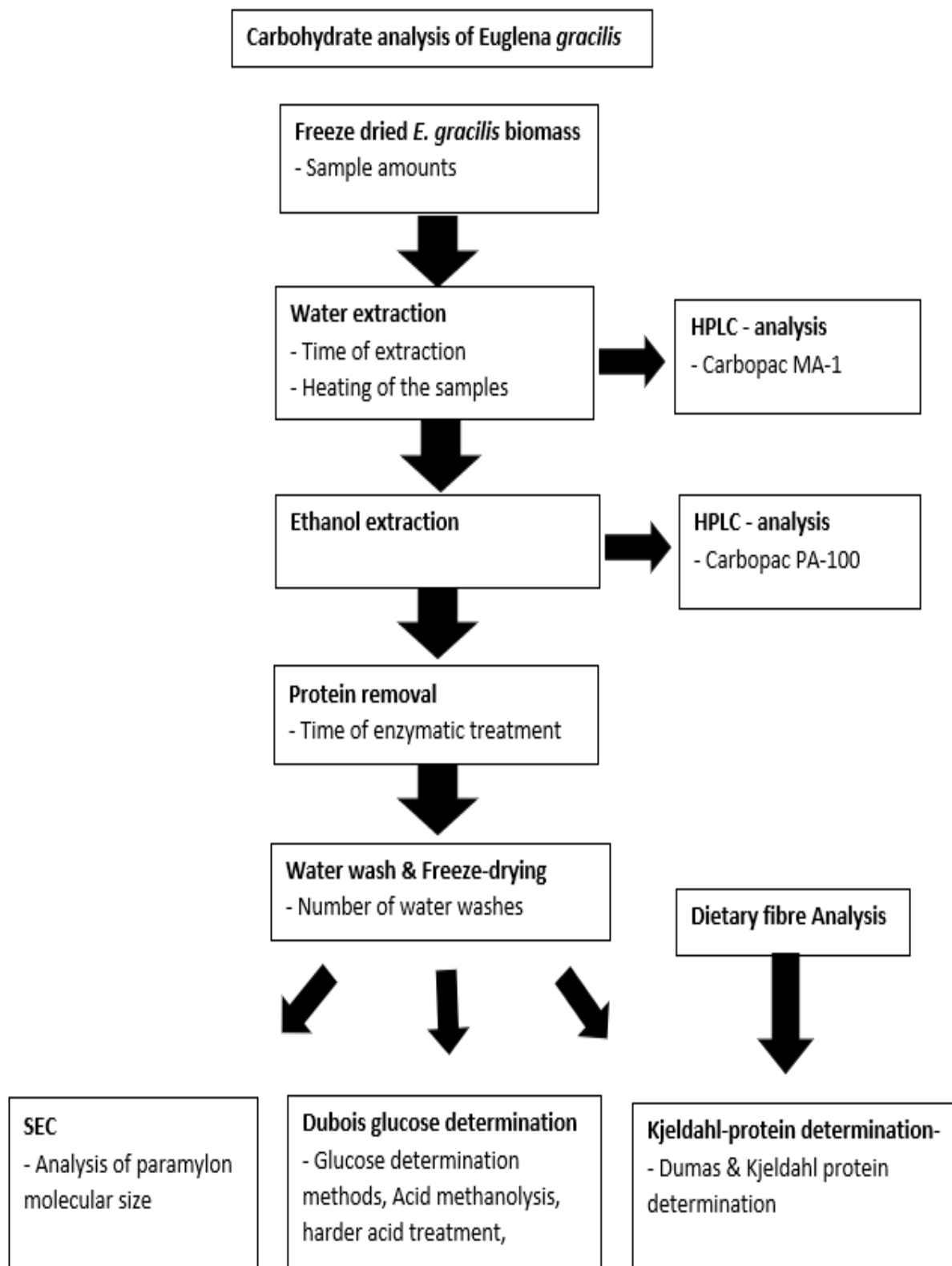
Figure 8 Overview of the experimental design of this experiment, in grey under each section is the parameters that were attempted to be changed to improve the analysis.

3.2.3 Analysis of paramylon content

The gravimetric determination of paramylon was performed based on the method by Grimm et al. (2015). Approximately 250 mg of *E. gracilis biomass* was weighed (150 mg for biomass cultivated on dairy retentate) to a centrifugation tube where it was mixed with 5 ml of MilliQ-water (MilliQ, Millipore, Merck KGaA, Darmstadt Germany). The analysis was performed in triplicates. The tube was placed in a water bath (95-100°C) for 10 minutes and was occasionally shaken. Thereafter the biomass water mixture was cooled down before it was placed in an ultrasound bath for 30 minutes. This was done to disrupt the *E. gracilis* pellicle and improve extraction of the free sugars. The mixture was then centrifuged at 8000 rpm for 10 minutes (settings used also later in the experiment) (Z323, HERMLE Labortechnik GmbH, Wehingen, Germany) to separate the water soluble and insoluble parts. The supernatant with the water-soluble compounds was transferred to an Eppendorf tube and stored at -20 for further analysis of free sugars. The sediment was then suspended in 5 ml of 95 % of ethanol (Etax A, Altia OY, 05200 Rajamäki, Finland) and centrifuged to extract ethanol soluble compounds such oligosaccharides and certain lipids. Therefore, after centrifugation the supernatant was transferred to a plastic bottle. The sediment was resuspended in 5 ml of 95 % of ethanol and again centrifuged. The supernatant containing ethanol was combined with the supernatant from the first ethanol extraction in the plastic bottle and stored at -20°C for further analysis of oligosaccharides.

The sediment was thereafter suspended in 5 ml trypsin (1 mg/ml) (T4799-5G, Sigma, USA, Lot#SLBR1752V) 100 mM sodium-di-hydroxide buffer (pH 7.6) ($Na_2H_2PO_4$ (H_2O), E. Merck, D6100, Darmstadt, Germany) and incubated at 37°C for 12h±4h to hydrolyse the proteins enzymatically. After incubation, 5 ml of 500 mg/ml urea solution (Urea-U5378-1006, Lot: BCBS3360, Sigma-Aldrich, Germany) was added and mixed before the solution was centrifuged. The supernatant containing the protein fragments was then discarded. The sediment was re-suspended in 5 ml urea and again centrifuged, and the supernatant was discarded. To remove leftover urea and peptide fragments the supernatant was suspended in 5 ml milliQ water, centrifuged and supernatant discarded. This was repeated three times. The remaining part was freeze-dried for 24h, and the remaining paramylon was then weighed. To attain the amount of paramylon in the biomass the weight of paramylon was divided by the initial biomass weighed. As the method used to isolate the paramylon was a gravimetric method, which has no positive control of the result, further analysis of the isolate was performed to test

the purity. The isolate was analysed for monosaccharide content, protein content and tested by SEC compared to a paramylon standard.

3.2.4 Analysis of glucose content of the paramylon isolates

The purity of paramylon isolates was studied by hydrolysing paramylon to glucose units, and a spectrophotometric analysis of the content based on the method of (DuBois et al. 1956). Two standard solutions of 0.6 mg/ml D-(+)-glucose (Merck, K34425937524) in MilliQ water were used to make a regression curve for quantification of the glucose content. According to the method procedure, five concentrations (20, 40, 60, 80 and 100 µl) of standard solution were mixed with 5 % phenol (puriss, p.a., Sigma-Aldrich, Steinhem, Germany) and H_2SO_4 (95-98 %). The acid is added to hydrolyse paramylon, whereas the phenol binds to the glucose to form an orange coloured complex. To measure the absorbance the samples were measured spectrophotometrically (UV-1800, Shimadzu, Kyoto, Japan) at 495 nm. Absorbance and concentration of D-(+)-glucose were plotted against each other, and by linear regression Microsoft Office Excel (Microsoft, USA) a formula for the calibration curve and the correlation factor were retrieved.

The monosaccharide analysis was performed on the isolates of two of the three parallels of each reactor, in duplicates. Paramylon isolate (20 mg) was dissolved in 25 ml 0.5 M NaOH. From this solution a 100 µl aliquot was mixed with 5 % phenol and H_2SO_4 (95-98 %) were added and let hydrolyse for at least 5minutes. The hydrolysates were analysed spectrophotometrically similar to the standards. The correlation between absorption and monosaccharide content obtained from the calibration curves were used to quantify the monosaccharide content in the isolates.

3.2.5 Analysis of protein content of the paramylon isolates

The protein content was also determined in paramylon isolates because it was one of the possible impurities in isolates. Protein determination of the paramylon isolates was performed based on the AOAC standard method for determination of protein content (AOAC International 1995). Paramylon isolate (40 mg) was weighed, and a Kjeltabs 1000 CK (Thompson & Capper LTD, United Kingdom) and 95-98 % H_2SO_4 were added. Thereafter, the samples were hydrolysed at 400°C for one hour in a digestor (Digestor 2010, Foss, Hilleroed, Denmark). Post hydrolysation the samples were left to cool for approximately two hours, before addition of milliQ water. The titration of the samples was performed the following day by an automatic titration (Kjeltec 2300 Analyzer unit, Foss Hilleroed, Denmark). Due to machine malfunction,

only a part of the titrations was performed according to the correct titration procedure. Only for8/26 of the samples a result was obtained, due to a malfunction in the supply of NaOH to the tubes. Therefore, the number of analysed isolates for protein content is limited. The protein content was calculated using the standard nitrogen conversion rate for food of 6.25.

3.2.6   SEC analysis of the paramylon isolates

SEC analysis was performed on 6 paramylon isolates, one from each of the cultivations and the paramylon standard. Approximately 4 mg paramylon isolate was suspended in 2 ml 0.1 M LiBr DMSO and left for two days to dissolve. After two days many of the samples still had not fully dissolved. However, as the results would not be used to quantify the content of paramylon but used as a qualitative analysis it was assumed that large enough content of paramylon had dissolved. This solution was then filtered through a 0.45 μm filter (Millipore, Billerica, MA, USA) and transferred to a chromatography vial. The SEC instrument consisted of an integrated auto sampler and pump module (Viscotek GPCmax, Malvern Instruments Ltd., Worcestershire, UK), a column oven (CROCO-CIL, Cluzeau Info Lab, Sainte-Foy-laGrande, France), and a combined static light scattering and viscometric detectors (Viscotek 270 Dual Detector, Malvern Instruments Ltd), and a DRI detector (Viscotek VE 3580, Malvern Instruments Ltd). The light scattering detector included two scattering angles: 7° and 90° and was working at wavelength of 670 nm. The separation columns were two Jordi (Jordi Labs, Mansfield, USA) columns; Xstream mixed bed which covers molar masses from 100 to 10.000.000 g/mole and X-stream 500. The dimensions of both columns were 250×10 mm, and the samples were analysed at 60 °C, with a flow rate of 0.8 ml/min. Injection volume of 100 μl was used. The results were interpreted using the chromatographic OmniSEC software (Malvern Panalytical, Almelo, Netherlands). The results of SEC analysis were compared to that of a paramylon standard, and the results of the refractive index (RI) and right-angle light scattering detectors were chosen to illustrate the interpretations as they give a good indication of similarities and differences between the standards and the samples.

3.2.7   HPLC analysis of extracts of the *E. gracilis* biomass

The extracts were first filtered through Amicon ultra centrifugal filter units (0.2μm) (Millipore, Billerica, MA, USA) to remove any larger fragments extracted. Thereafter the extracts were placed in three HPLC vials prepared for analysis, so that analysis of each extract was performed in triplicate. The analysis was based on a method described in Xu et al. (2017). Mono and disaccharides from the water extract, and oligosaccharides from the ethanol extract were quantified by an HPAEC-PAD system. This was equipped with a CarboPacMA-1 analytical

column (4 × 250 mm i.d., Dionex) for quantification of mono and disaccharides, and CarboPacPA-100 analytical column (4 × 250 mm i.d., Dionex) for quantification of oligosaccharides. As well as a DECADE electrochemical detector (Antec Leyden, The Netherlands) a Waters 2465 pulsed amperometric detector (Waters, USA), a Waters 717 Autosampler and two Waters 515 pumps. Injection volume of both the ethanol and water extracts was 10 µl. The elution was done with a mobile phase consisting of 1:1 milliQ water:1 M NaOH with the flow rate of 0.40 ml/min. The time set between each injection was 55 minutes. In addition to the primary analysis, the water extracts were also analysed with a gradient elution that started from 40 mM NaOH (25 min), then to 620 mM (10 min), and finally to 40 mM. The flow rate was 0.4 mL/min and the injection volume was 10 µl.

As a standard for quantification, a solution of milliQ water containing 1 mg/ml of D(+)-glucose, D(-)-fructose, D(+)-galactose, D(+)-trehalose, arabinose, sucrose and mannitol was also analysed to quantify these sugars in the water extract. These sugars were chosen based on the pre-tests and literature knowledge of sugars that had been previously been identified in E. gracilis. The standard solution was analysed as such injecting 10 µl and 5µl, in dilution 1:10 injecting 25 µl, 10 µl and 5µl, and in dilution 1:25 injecting 25 µl, 10 µl and 5µl to create a standard curve for quantification.

Creation of the standard curves and later quantification of the free sugars in the water extract was done by using the chromatographic software Empower 2 (Waters Corporation Milford, MA, USA). The same software was also used to illustrate the low content of oligosaccharides in the ethanol extracts. All calculations made including the average content, standard deviation and CV % of free sugars in the extract was calculated using Excel 2016 (Microsoft, Redmond, Washington, USA).

3.2.8 Dietary fibre analysis of the *E. gracilis* biomass

Analysis of dietary fibre of the biomass was performed based on the AOAC standard method for determination of dietary fibre in foods (AOAC International 1995). Approximately 400 mg of freeze dried *E. gracilis* biomass was weighed to a Erlenmeyer flask in four replicates. This was mixed with MES-Triz-buffer (MES hydrate (M8250-100g, Sigma, USA, Lot#SLBL4492V), Trizma® base (T1503-100g, Sigma, USA, Lot#SLBD2964V) and α-amylase (A3306-10 ml, Sigma, USA, Lot#SLBP3233V) were added to the mixture. The flask was placed in boiling water (95-100°C) in which it was kept for 35 minutes. After this, the sample was cooled to 60°C and milliQ water and amyloglucosidase (A9913-10 ml, Sigma,

USA, Lot#091M8701V) were added. The flask was then placed in another water bath (60°C) in which it was kept for 30 min. The mixture pH was thereafter adjusted to between 4.1 and 4.8 by adding 0.5M HCl. After the pH had been adjusted protease (P3910-500 mg, Sigma, USA, Lot#SLBP8132V) was added and the mixture was placed in a water bath (60°C) in which it was kept for 30 minutes. After this ethanol pre-heated to 60°C was added to the mixture, and the sample was left to precipitate overnight.

To remove any moisture a glass sinter and celatom® (C8656-1kg, Sigma, USA, Lot#SLBK4025V) were placed in a heating chamber (105°C) for 12hours. They were thereafter placed in the exicator for 30 minutes, before the weight of the sinter with and without celatom were noted and the cinters again placed in an exicator.

The precipitated mixture was thereafter poured over and filtered through the sinter containing celatom, by help of vacuum suction. To remove lipids and other impurities 95 % of ethanol (Etax A UN1170, 16381, Altia OY, 05200 Rajamäki, Finland), 78 % of ethanol and acetone were poured over and filtered through the sinter. When this was done, the sinter was again placed in a heating chamber (105°C) for 12hours. After 30 minutes in an exicator to reduce the possibility of the sample to reabsorb moisture the sinter weights were noted. To find the amount of dietary fibre in the *E. gracilis* the weight of the sinter with celatom before filtration was subtracted from the last weight. This result should be corrected for protein and mineral content, but no corrections were performed, as there was problem with the protein determinations better described in section 3.3.3.

3.3 RESULTS

3.3.1 Paramylon content of the *E. gracilis* biomass

The average content of paramylon isolated from the *E. gracilis* biomass on a dry weigh basis was 215-395 mg/g (Table 3). The highest content was measured in the SFE samples, whereas the lowest concentration measured was in *E. gracilis* cultivated in the dark (R-dark). The coefficient of variation of cultivational and analytical replicates for R-Dark was, however, 50.6 %. The variation was also above 10 % in *E. gracilis* cultivated under the conditions of Euglena 2, -8 and R-Light (Table 2). The variation among the analytical replicates was much lower than among the replicates cultivated in different reactors under same conditions. The coefficient of variation among the analytical replicates was only above 10 % in the biomass cultivated under conditions of Euglena 2 (Appendix 2).

Table 3 Average paramylon content in the E. gracilis biomass. Number of sample replicates from each cultivation (N), average content of crude paramylon (g/100g), the standard deviation of the replicates (g/100g), the variance of the results (CV %) in % of the gravimetric paramylon analysis.

Sample	N	Average (mg/g)	Std dev	CV %
Euglena 2	9	286	7.5	28.0
Euglena 8	9	340	3.9	11.0
Pre SFE	3	364	2.4	6.7
SFE30	3	395	2.8	7.0
SFE50	3	389	0.7	1.7
R-Light	6	263	3.3	12.6
R-Dark	6	215	10.9	50.6

3.3.2 Characteristics of the paramylon isolates

The paramylon isolates were hard pellets at the bottom of the centrifugal tubes. A visual inspection of the isolates suspended in a 0.5M NaOH solution intended for glucose analysis was performed. There were evident differences in colour and characteristics of the *E. gracilis* biomasses (Figure 10). The isolates of biomass cultivated on dairy retentate dissolved completely and were clear in colour; isolates of the Euglena 8 biomass were green and did not completely dissolve. Of the Euglena 2 biomass medium one parallel was light green and one had a strong fresh green colour, and the biomass cultivated on Hutner medium and that extracted by SFE were dark green to brown and clear. As the paramylon standard completely dissolved in the same solution it can be assumed that the paramylon isolated in the gravimetric analysis contained impurities.
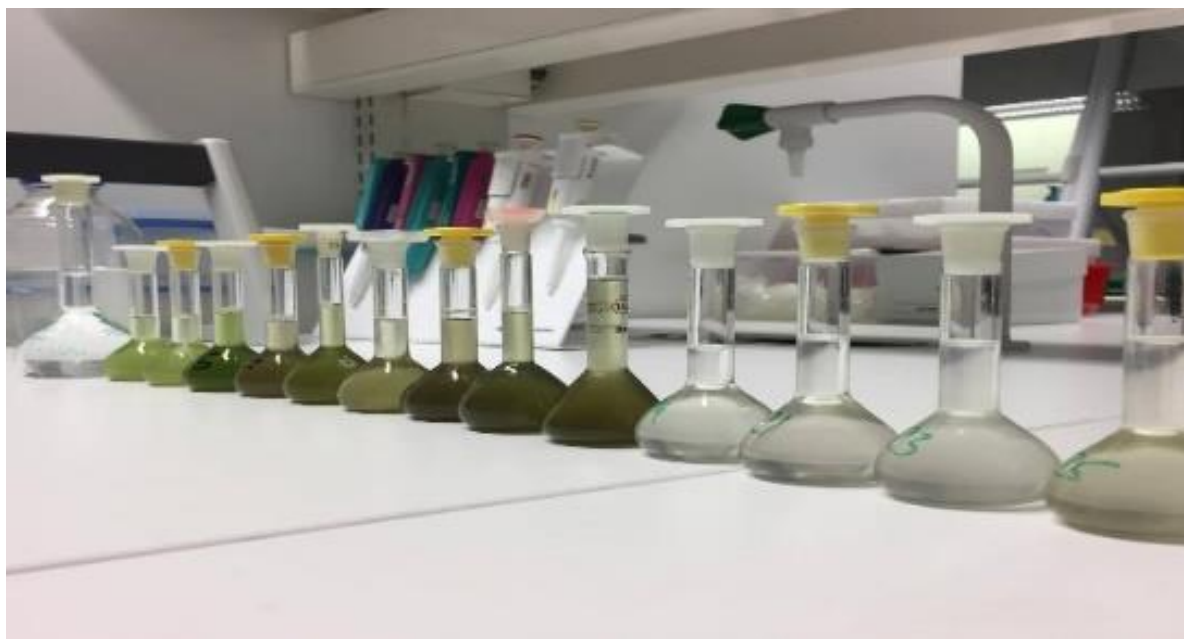

Figure 90 Differences in colour seen in the isolated paramylon from E. gracilis biomass. The isolates were dissolved in 0.5M NaOH. On the far left is the standard solution of glucose.

3.3.3 Glucose content of the paramylon isolates

The average concentration of glucose in the paramylon isolates was 16.6-35.5 % (Table 4). The lowest concentration was found in the isolates of the biomass after SFE at 50°C (SFE50), and the highest in the retentate cultivated in the light (R-light). The coefficient of variation of cultivation and analytical replicates was high for all samples and as high as 59 % in Euglena 2. Also, among the SFE samples the variability in the glucose content in the isolates was large, from 16.6-29.1 %. This variation was large considering the similarities in the performed SFE extractions. Performing the analysis on a standard paramylon sample gave an average glucose content of 85.6 %, but the results varied from 72-100.6 %.

Table 4 Average glucose concentration (%) of the paramylon isolates, the number of sample replicates from each cultivation (N), the standard deviation of the replicates (g/100g), the variance of the results (CV %).

Sample	N	Average glucose concentration (%)	Std dev	CV (%)
Euglena 2	5	25.9	15.3	58.8
Euglena 8	6	30.5	8.7	28.4
Pre SFE	2	21.6	-	-
SFE30	2	29.1	-	-
SFE50	2	16.6	-	-
R-light	4	35.3	6.3	17.8
R-dark	3	23.8	4.0	16.7

3.3.4 Protein content of the paramylon isolates

The measured protein content of the paramylon isolates was 11-44 % using Jones conversion factor (6.25) of nitrogen content to protein (Table 5). Due to the analytical problems described in section 3.3.3, number of isolates analysed for protein content was limited. The paramylon isolate from the biomass of R-light cultivation was the only with more than one isolate analysed for protein content (Table 5). For the Euglena 8 isolates the analysis was not performed at all due to the machine malfunction.

Table 5 Average protein concentration (%) of the paramylon isolates, the number of isolates from each cultivation (N) standard deviation (Std dev) and coefficient of variation (CV %)

Sample	N	Protein (%)	Std dev	CV (%)
Euglena 2	1	30.2	-	-
Euglena 8	0	-	-	-
Pre SFE	1	28.3	-	-
SFE30	1	28.3	-	-
SFE50	1	43.5	-	-
R-Light	3	10.5	2.9	27.5
R-Dark	1	26.0	.	-

3.3.5 SEC of the paramylon isolates

By comparing the SEC results of the paramylon standard and the paramylon isolates it was indicated that paramylon had been isolated but that there were also impurities in the isolates. The light scattering method gave an estimated molecular mass of paramylon standard and the paramylon in the isolates of 150kDa. The response of all the analysed samples on SEC with the RI- detector showed a similar trend. The largest response occurred at elution volume of 15 ml, for both the paramylon standard and the isolates (Figure 11). The response was, however, larger for the paramylon standard. There was also a response at retention volume 30 ml, most likely an analytical response as it was also seen in pure DMSO LiBr solution used to dissolve the isolates. After volume 41 ml responses of different magnitudes were also seen. The results showed that there thus was paramylon in the samples, but as the isolates dissolved less well than the standard it was not possible to compare the amounts.

Figure 10 Responses of the RI detector for the selected samples and the paramylon standard in the SEC analysis, the x-axis gives retention volume and the y- axis gives the RI detector response.

When the results from the right-angle light scattering (RALS) detector were analysed, a similar trend could be seen. There was a clear response in the light scattering at the retention volume 15 ml (Figure 12). Between the volumes 18 ml and 38 ml there was a large response that only to a small degree was seen in the analysis of the paramylon standard. This was yet another indication that the paramylon isolates were not pure paramylon but contained impurities that scattered light.

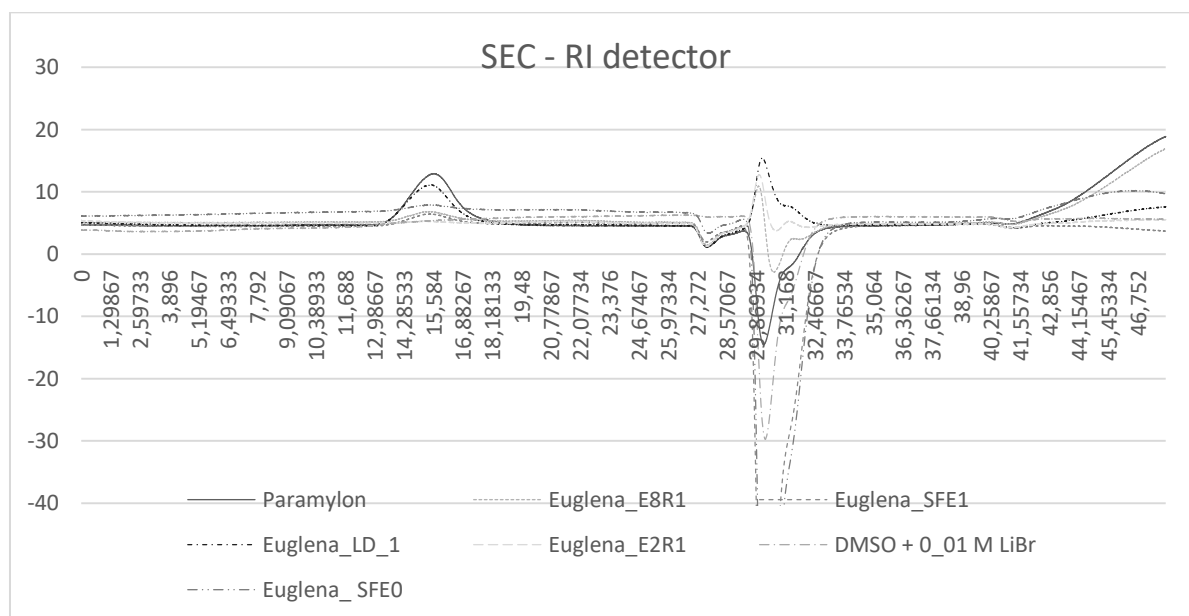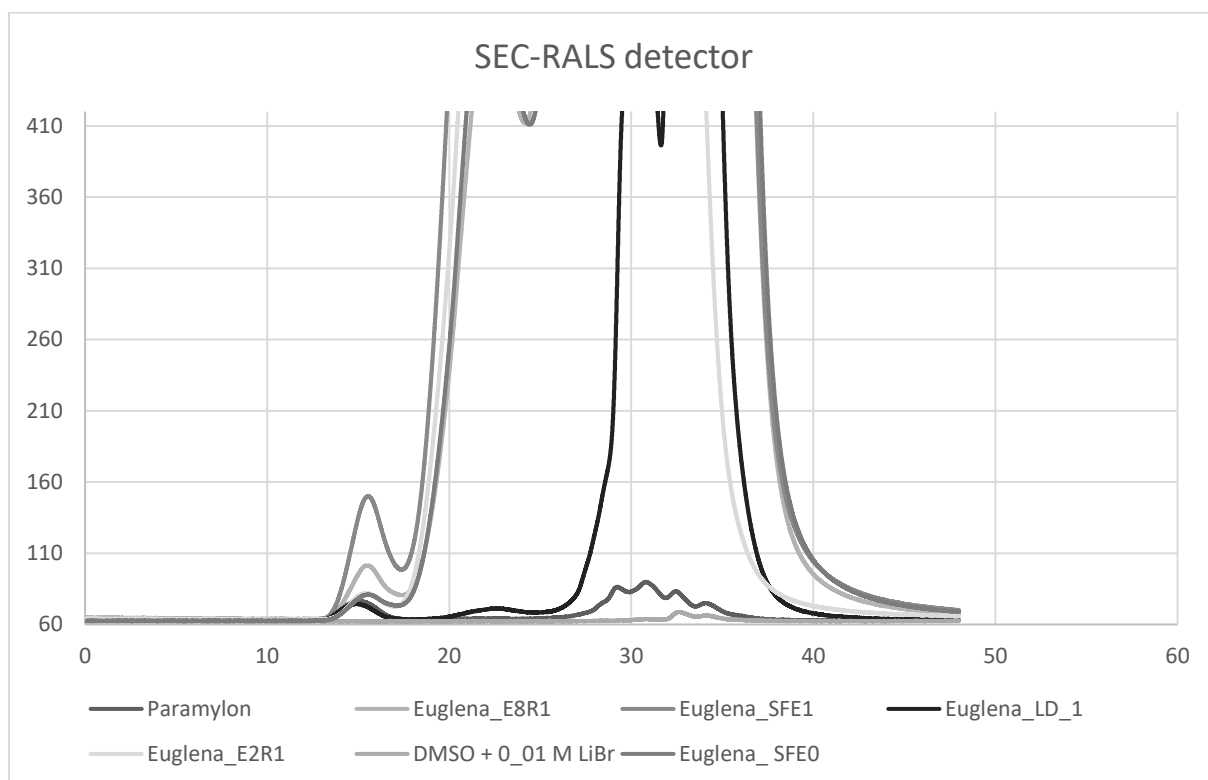*Figure 11 Responses of the RALS detector for the selected samples and the paramylon standard, the x-axis gives retention volume and the y- axis gives the RALS detector response.*

3.3.6 Total composition of the paramylon isolates

Through the SEC it was clear that the carbohydrate isolated was paramylon, but that there also were impurities in the isolates. According to the glucose determination, it was measured that approximately 30 % of the isolates were pure paramylon (Figure 13). No correction of glucose content was made based on the results of the paramylon standard due to large variation in the analysis. The protein content of the isolates was 10-40 %, and the remaining approximately 30 % of the paramylon isolates was not characterized to be either protein of carbohydrates in these analyses.

Figure 12 Composition of the paramylon isolates, in black the carbohydrate (paramylon) content, in light grey the content of protein measured,

3.3.7   Content of free sugars in the water extract of *E. gracilis* biomass

Identification of compounds

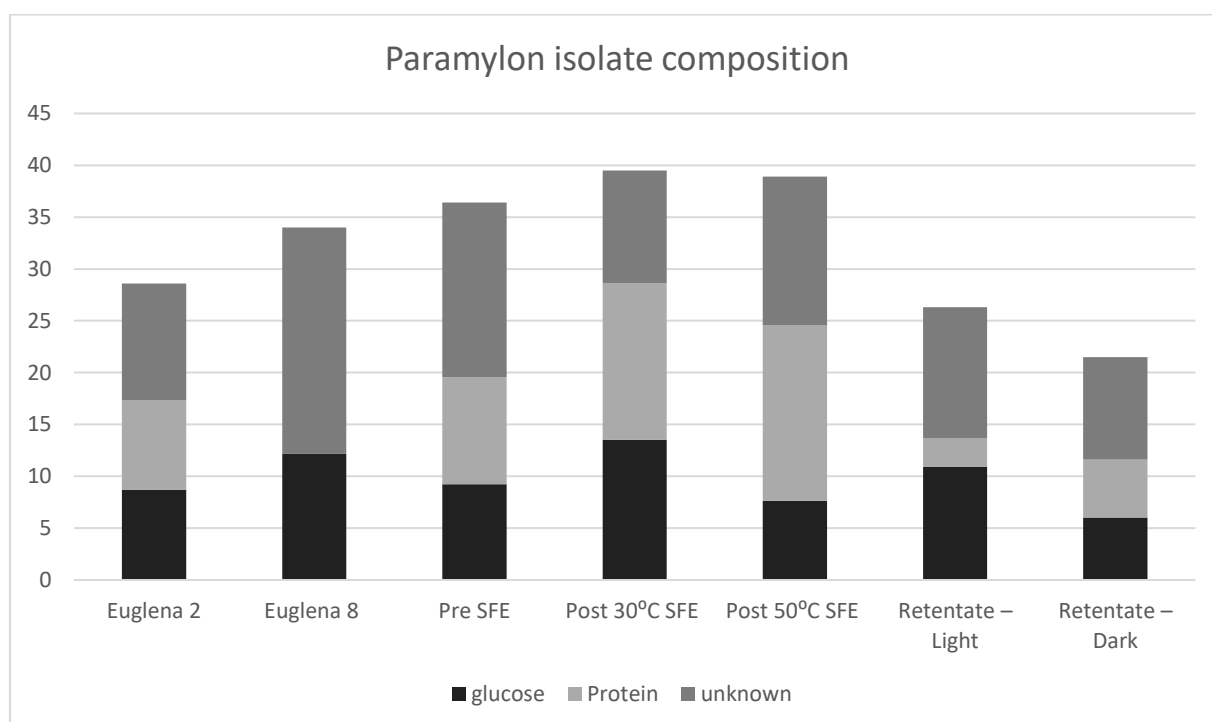In pre-tests of water extracts of the *E. gracilis* biomass on the CarboPac-MA1 column, compounds eluting at 9.3, 17.7, 19.1, 20.7, 22.3, 24.4, 25.8, 27.3 and 34.3 min were identified. Identification was done by comparing these retention times with scientific literature, trehalose (17.7 min), mannitol (20.7 min), arabinose (22.3 min), glucose (24.4 min), galactose (27.2 min) (Figure 14). The large response seen at approximately 9 minutes did not substantially decrease after a 50-fold dilution of the extracts (Appendix 4). Therefore, it was assumed that it was an system response.
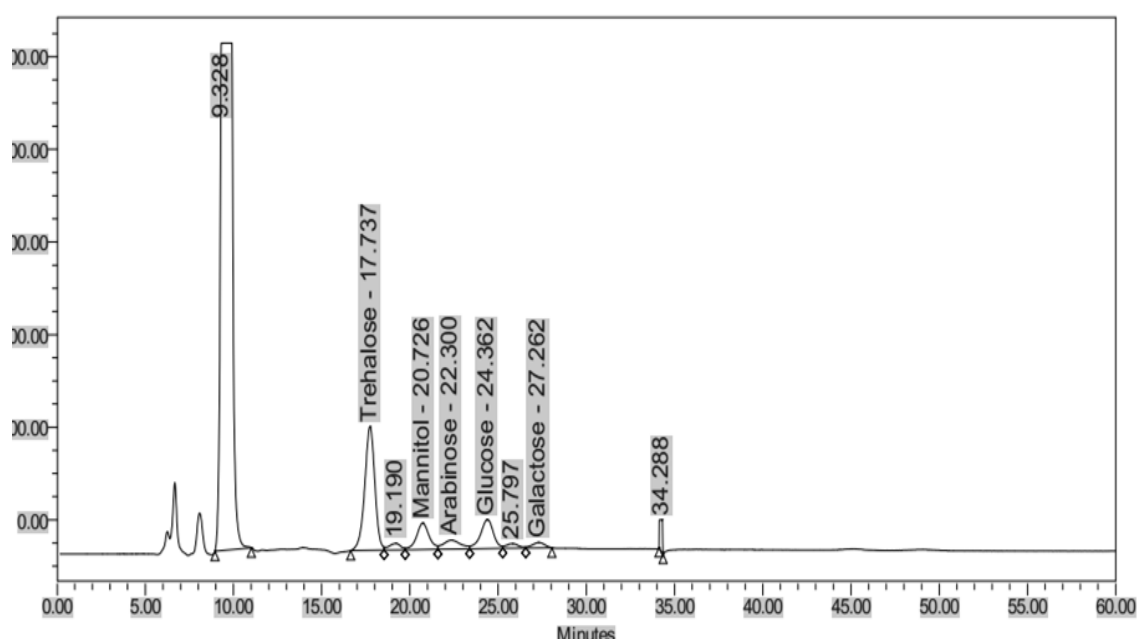
Figure 14 Chromatogram of a water extract of E. gracilis biomass Euglena 2 analysed on CarboPac MA-1 column, x-axis gives time of elution and the y-axis gives the response of the detector in mA.

In addition to the free sugars identified from the pretesting also other sugars were identified in the samples during full analysis. Particularly two compounds, one eluting at 27 and one at 30 minutes in the dark grown retentate samples were of significant magnitude (Figure 15). Because the R-dark, was cultivated on dairy retentate containing lactose, a standard solution of lactose was tested and compared with the results. The elution time of lactose was 30 minutes, an indication that the compound retained at 30 minutes in the isolate was lactose. However, as lactose was only seen retrospectively, it was not quantified.

Figure 135 Chromatogram of all water extracts of E. gracilis biomass, analysed on CarboPac MA-1 column, x-axis gives time of elution and the y-axis gives the response of the detector in mA.

The elution time of the compound quantified as sucrose eluting (44 minutes) in the different samples shifted quite substantially from 43-44,5 minutes (Figure 16). This could have been either due to differences in the amount of the compounds in the sample or because there were different compounds eluting at approximately this time in the extracts.

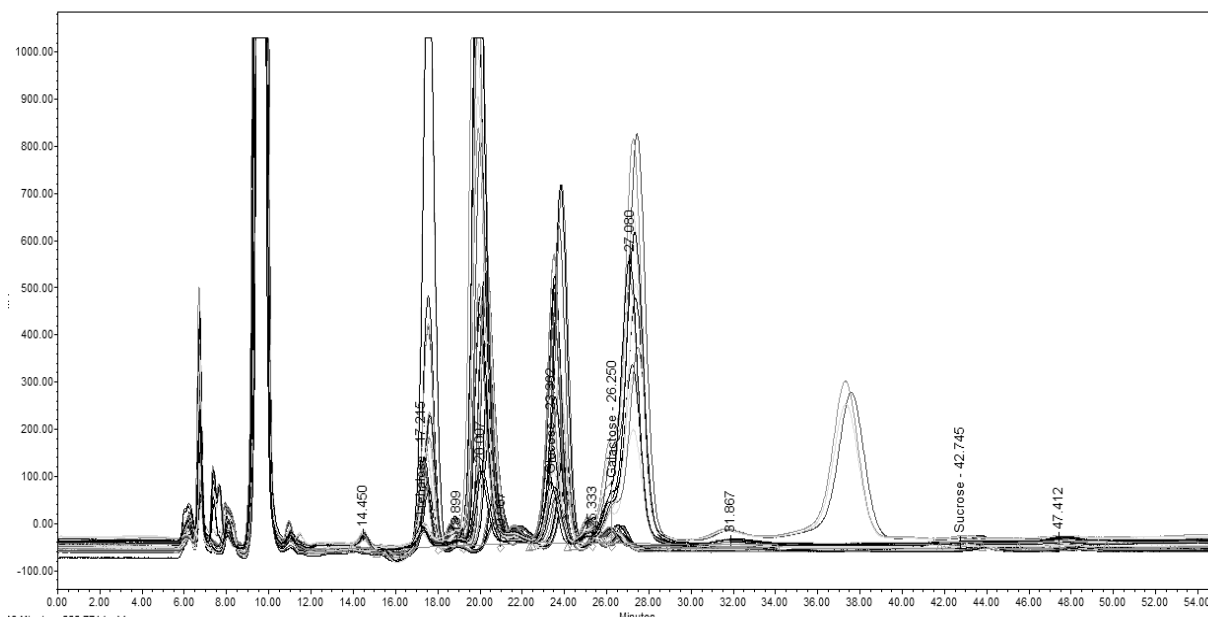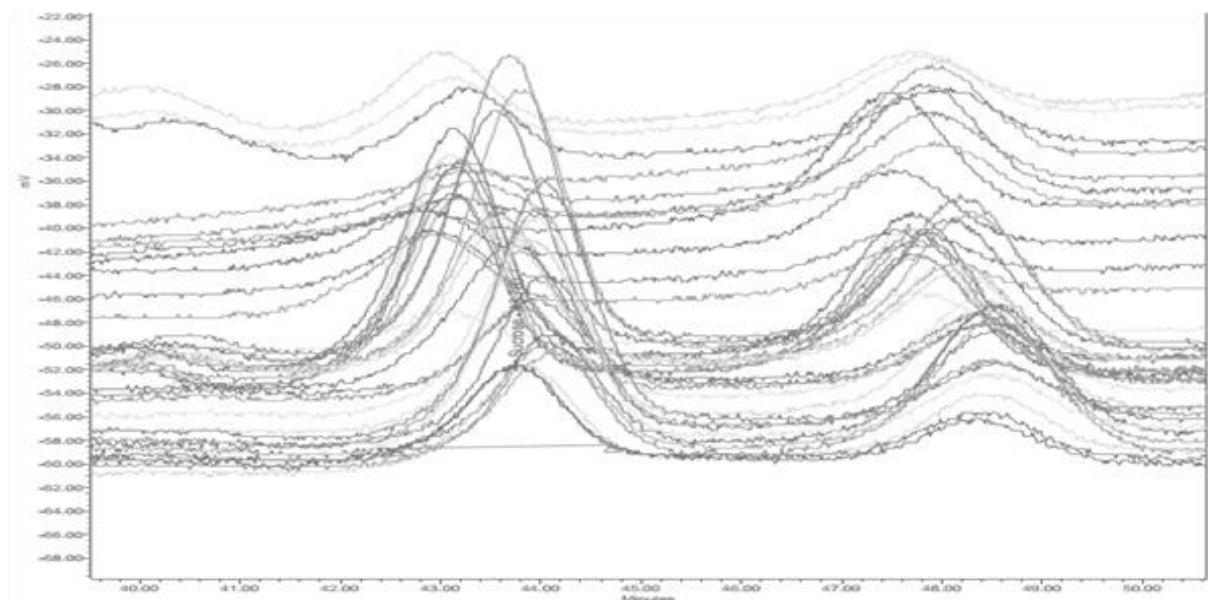*Figure 16 Shift in elution times of compound quantified as sucrose in the chromatogram of all water extracts of E. gracilis biomass, analysed on CarboPac MA-1 column, x-axis gives time of elution and the y-axis gives the response of the detector in mA.*

Calibration curves & limits of determination

The standard solution consisted in order of elution trehalose, mannitol, arabinose, glucose, galactose, fructose, and sucrose (Table 6). Despite mannitol and arabinose had good separation in pre-testing their separation was later not possible with the settings of analysis. Therefore, all samples were also analysed on the same column with a gradient in the flow rates described in section 3.4.2. This lead to sufficient separation of mannitol and arabinose. Thus, to quantify mannitol and arabinose the method with a gradient elution was used, for the remaining five sugars the method without gradient in the elution was used for quantification (Table 6). The elution of all compounds was generally faster using gradient elution, with a larger difference in compounds with long elution times. The difference in elution time for sucrose was 10 minutes, between the two methods (Appendix 4).

Table 6 Time of elution of the compounds in the standard solution (Time), formula used for calculation of the content of the sugar (Formula) fit of calibration curve and the coefficient of determination (R^2) without and using gradient elution (a)

Compound	Time	R^2	Formula
Trehalose	17.3	0.999593	$Y=5.53^{0.006x}+2.69^{0.004}$
Mannitol[a]	21.2	0.927671	$Y=1.23^{0.006x}+3.71^{0.005}$
Arabinose[a]	23.1	0.930276	$Y=9.71^{0.005x}+3.09^{0.005}$
Glucose	23.6	0.999359	$Y=1.13^{0.007x}+2.01^{0.004}$
Galactose	26.2	0.999585	$Y=1.38^{0.007x}+5.60^{0.004}$
Fructose	28.6	0.999440	$Y=2.36^{0.006x}+4.48^{0.004}$
Sucrose	42.6	0.999376	$Y=6.18^{0.006x}+1.17^{0.005}$

Content of free sugars in the water extract

The concentration of free sugars in the water extracts of *E. gracilis* were between 24 mg/g and 149 mg/g (Figure 17). Besides the biomass of R-light, all biomasses contained below 55 mg/g of free sugars. The most abundant free sugar in all samples was mannitol followed by trehalose and glucose. Only small amounts of arabinose and galactose were found in the samples. The determination of sucrose was uncertain, and no fructose was measured in the samples. The variation coefficient (CV %) for the total amount of sugars was generally as high as 54 % in Euglena 2 and above 45 % in the retentate samples (Appendix 3).
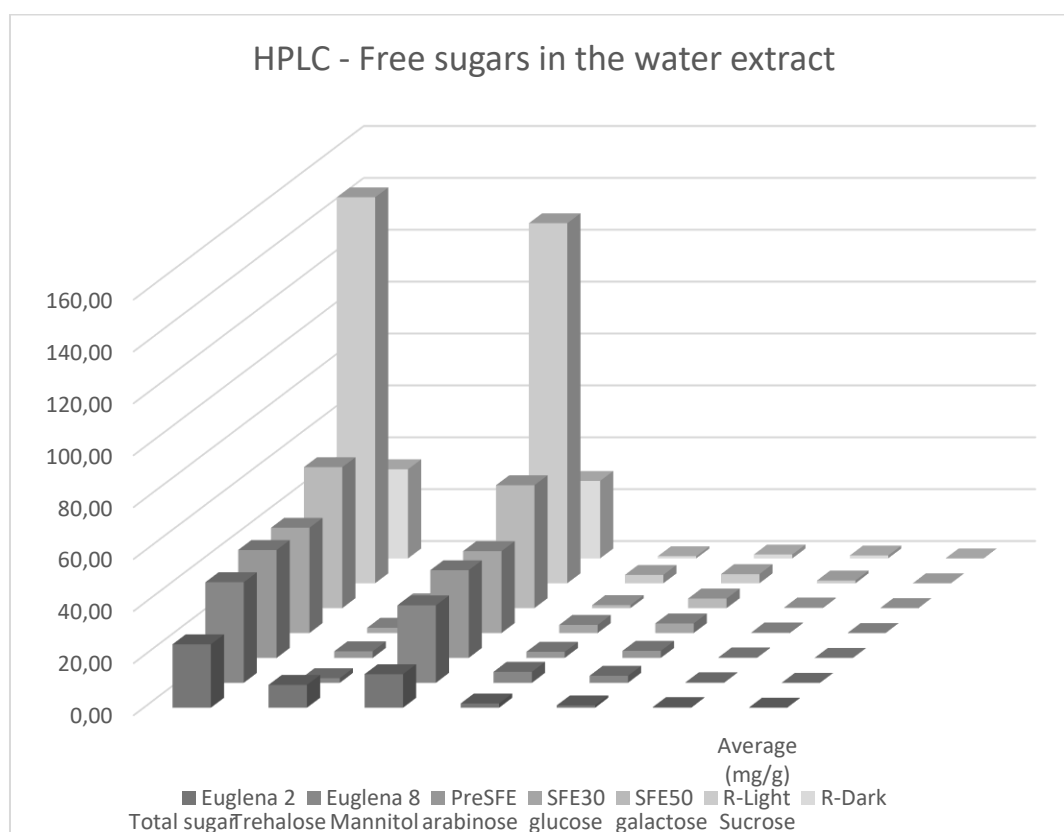
Figure 147 Average concentration of the quantifiable free sugars in the E. gracilis samples analysed (mg/g) in the E.gracilis biomasses (Table 2)

3.3.8 Content of free sugars in the ethanol extracts of *E. gracilis* biomass

The content of oligosaccharides in the ethanol extracts of the *E. gracilis* biomass was in low levels (Figure 18). In determination of free sugars from the ethanol fraction, only small amounts of sugars were found, and they were not characterized. As illustrated in figure 18 there were compounds eluting at 5, 10, 16, 33 and 34 minutes.
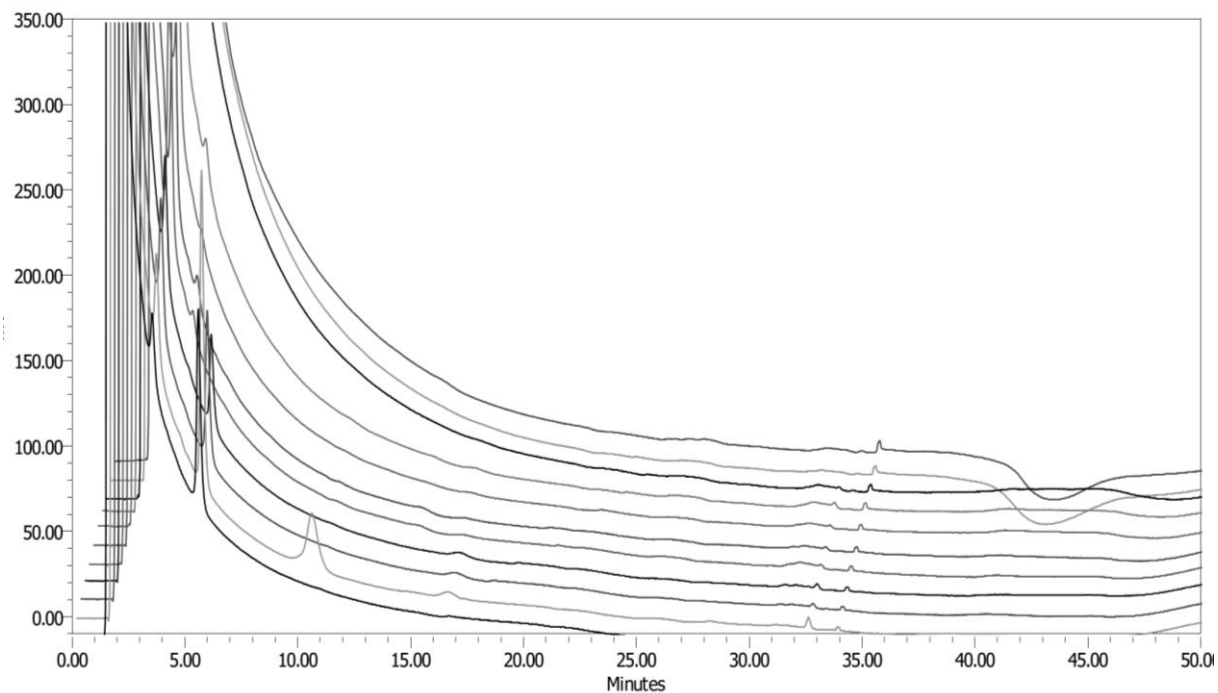
Figure 18 Chromatogram of ethanol extracts of E. gracilis biomass, of one of each of the cultivation replicates analysed on CarboPac PA-100 column, x-axis gives time of elution and the y-axis gives the response of the detector in mA

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3.3.9   Content of dietary fibre in the *E. gracilis* biomass

The average content of dietary fibre was 438-457 mg/g dry *E. gracilis* biomass (Table 7). There was no significant difference between the content of dietary fiber after SFE at the two temperatures. However, as no corrections for nitrogen content was made due to the analytical difficulties described in 3.3.3 the results have not been corrected for protein content. The mineral content had in previous experiments been shown as negligible and was not performed in this experiment.

Table 7 Content of dietary fibre (g/100g) in E. GRACILIS biomass that has undergone SFE number of analytical replicates (N)

Sample	N	Dietary fibre (mg/g)	Std. dev	CV %
PreSFE	4	457	10	2.1
SFE30	4	440	45	10.2
SFE50	4	438	16	3.8

3.4 DISCUSSION

3.4.1 Isolation of paramylon

The paramylon content of the *E. gracilis* biomass analysed in this experiment was between 22-40 % of the dry cell weight. This was higher than what has been found in other studies analysing *E. gracilis* cultivated in synthetic Hutner medium, such as in the study of Nakano et al. (1987) which reported a paramylon content of approximately 14 %. Grimm et al. (2015) measured a content of paramylon between 9.3 ± 0.1 g/L and 0.78 ± 0.01 g/L in samples that had dry cell weight between 12.3 ± 0.14 g/L and 3.4 ± 0.02 g/L (Grimm et al. 2015). It is hard to give an exact concentration of paramylon due to the way the results are presented in the previously mentioned study, but it is in the range of 23-76 % comparing the high and low values. It is, however, evident that there was no validation or correction done to the isolates to double check the results of the gravimetric method. Impurities are normal in this type of analysis and are the reason why also the AOAC method for determination for dietary fibre has corrections for protein and ash content. In this study it was chosen to perform a protein correction as this is done in the AOAC method for analysis of dietary fibre (AOAC, 1995), as well as a control of the content of monosaccharides and SEC analysis of the isolates. In the AOAC method a correction for ash content is usually performed. This was not done in this study as in previous analysis of the *E. gracilis* biomass cultivated under these conditions had been found to be negligible (Edelmann 2017).

3.4.2 Purity of the paramylon isolates

It was evident that the paramylon isolates obtained from the *E. gracilis* also contained other compounds than paramylon. The visual inspection of the isolates showed that there were clear differences between the paramylon standard and the isolates in colour and structure. The pure paramylon standard was a white powder, whereas the paramylon isolates obtained in this study were green-white hard pellets. It is well established that there is a high content of chlorophyll in the *E. gracilis* biomass (Buetow, 2001), and chlorophyll was most probably also the compound giving the isolates their green colour. This was also confirmed by the further analysis of the isolates.

The glucose determination of the paramylon isolates revealed that below half of the isolate content was glucose. This was not completely unexpected as similar results were recorded when analysing the dietary fibre content in the *E. gracilis* biomasses according to the AOAC method (Edelmann 2017). The method used to determine glucose content in the isolates, which was

based on the method by Dubois et al. (1956), appeared to produce results with high uncertainty. As paramylon is a polyglucan (Barsanti et al. 2011) its glucose content should theoretically be 100 %. As the analysis of the paramylon standard gave a glucose content between 72 and 100 %. This shows that it is difficult to consider whether the analysis gave a representative estimate of the glucose content in the isolates. The method should according to Hall (2007) be accurate for this kind of sample. As the glucose standard used correspond well to the paramylon, which only consists of glucose units. Due to the uncertainty also, other methods for glucose analysis were tested in the pre-testing phase. However, the method by Dubois et al. (1956) was chosen as the other methods tested gave even less reliable results. Acid methanolysis did not hydrolyse the paramylon and heated hydrolysis with sulphuric acid was a too powerful hydrolysis for the purpose.

The protein content of the paramylon isolates was substantial, as it was measured to between 10 and 44 % of the biomass content. Though other nitrogenous compounds should not be present due to the nature of the analysis, it was evident that there were impurities in the isolates. The content of chlorophyll in the isolates may have led to an overestimation of the protein in the paramylon isolates. To correct for these non-protein compounds, much lower conversion factors, for instance factors as low as 3, have been used in estimating nitrogen content in other microalgae (Lourenço et al. 2004). The conversion factor used to determine the protein content is of high importance and may affect the results significantly. In this experiment no such correction of the results was done, to keep in line with the correct correction factor used in previous analysis of the dietary fibre of *E. gracilis* at the university of Helsinki (Edelmann 2017). If the conversion factor of 3 would have been used in this experiment, even larger proportions of the isolates would be undetermined. However, it is worth noting that the overall reliability of the results of the Kjeldahl analysis was limited. This was due to the machine problems that occurred as described in section 3.3.2, where only 8 out of 26 isolates analysed had a partially successful result. Therefore, the overall conclusions that could be drawn based on the protein analysis was limited. Other nitrogen containing compounds that may affect the results in this experiments are glycosylated proteins, they are according to O'Neill et al. (2015) one of the hallmarks of Eukaryotic cells. To get a better overview of the protein content an amino acid analysis of the isolates would show exactly the amount of protein in the samples. This together with sequence analysis of the proteins in the nitrogen fraction, could give valuable information on what compounds are so closely related to the tight paramylon structure.

Based on comparison of the SEC analysis results of the paramylon isolates and the paramylon standard, a similarity in the results was evident. However, as also seen in other analysis other compounds were present as signals appeared when analysing the isolates that did not appear when analysing the standard sample. The analysis gave an indicative molecular weight of 150 kDa. This is substantially lower than the 500 kDa claimed by both the producer of the standard and the figures found in literature (Sigma-Aldrich Co. , Barsanti et al. 2011). As this indication was made in comparison to other β-glucans, the molecular mass in this experiment may have been underestimated due to the tight structure of paramylon. The high crystallinity may react differently to the light compared to other similar beta glucans. However, the SEC analysis gave valuable qualitative results, but the limited number of paramylon isolates tested limits the conclusions that can be drawn from the results. However, it is also possible that a correct molecular weight was obtained for paramylon in this study, as size and structure of paramylon change according to the growth conditions (Barsanti et al. 2001).

An improved isolation must be applied to increase the purity of the paramylon isolates if an industrial application is wanted. A benefit of using this method compared to the AOAC method for dietary fibre was the simultaneous water and ethanol extraction and possibility of further analysis of the isolates. It is however evident that like in the AOAC method the fraction of compounds containing nitrogen is high using the method by Grimm et al (2015). Several patents have been made to give improved isolation of paramylon, so an efficient extraction of the compound is feasible. By analysing the amino acid content and structure in the isolates, an better idea for improvements of the extraction could be achieved. It could give indication to what additional enzymatic hydrolysis could be utilized to remove remaining protein fraction, that later could be removed to a urea or water extraction. It is however also evident that compounds which should have been extracted with either the water or ethanol extraction, such as chlorophyll, were subject to several extractions but were not removed.

3.4.3   The effect of cultivation conditions on the paramylon content

To evaluate the effect of different cultivation conditions on the paramylon content in biomass, a given cultivation condition must yield repeatable amounts of paramylon. The variation of paramylon content among the *E. gracilis* biomasses cultivated in the same conditions but in different reactors was between 11 and 50 %. This large variation makes it difficult to interpret whether an effect seen was due to a change of cultivation medium or other conditions. . This is well highlighted when comparing the *E. gracilis* biomass grown in different light conditions. *E. gracilis* grown in the light had a paramylon content of approximately 200 mg/g, whereas the *E. gracilis* grown in the dark had a paramylon content varying between 100-300 mg/g. Due to this variation, no conclusion can be drawn on the effect of cultivation conditions on the content of paramylon in *E. gracilis*. In the experiments previously conducted in the university of Helsinki on the same *E. gracilis* biomasses, content of lipids were 7-10 % and the protein content 50-60 % (Edelmann 2017). If it is considered that carbohydrates, protein and lipids normally account for a large proportion of the cell weight it would be likely that the carbohydrate content would be around of 30 % in these experiments dependant on the cultivation conditions. The content of paramylon appears to be lower than this. There was a slightly lower content of isolated paramylon in the *E. gracilis* biomass prior to extraction (364 mg/g) than after SFE extraction at both 30^0C (395 mg/g) and at 50^0C (389 mg/g). The increase is approximately 7-8 %, which is comparable to the amount of lipids in the samples extracted by SFE (University of Helsinki unpublished, 2018). Therefore, the increase in measured paramylon content may be due to the extracted lipids. This shows that SFE does not influence the paramylon content and that paramylon can be utilized after SFE extraction.

3.4.4   Content of free sugars in the extracts of the *E. gracilis* biomass

The total concentration of free sugars in the *E. gracilis* biomass was from 24-149 mg/g. The total concentration of sugars in the sample may, however, be underestimated. A visual inspection of the chromatograms showed that some sugars, such as lactose, were found in the biomass but were not quantified. Mostly these free sugars would not affect the reported content of sugars in the biomass. Of the free sugars quantified the clearly most abundant compound was mannitol followed by trehalose, arabinose, glucose and galactose. Also, Dwyer (1986) found mannitol and trehalose to be the most abundant sugars in *E. gracilis* grown both in the light and in the dark. The levels of mannitol in *E. gracilis* grown on dairy retentate were especially high, particularly in the biomass grown in the light. If it is shown that *E. gracilis* can accumulate this amount of sugar over time when grown on dairy retentate, and the mannitol is

easily extracted by water extraction, it can be explored further for production. Mannitol could potentially be used as an artificial sweetener (E 421) and as a medicine as it is on the WHO list of essential medicines (WHO 2017). It is likely that *E. gracilis* accumulates mannitol in response to stress (Dwyer 1986).

It is natural to find glucose in *E. gracilis* as it is a natural part of the photosynthesis and is a precursor for paramylon (O'Neill et al. 2015). It is, however, also possible that the glucose in the cell is due to engulfment of nutrients from the cultivation media as this is a feature of *E. gracilis (O'Neill et al. 2015).* This would also account for why there appeared to be identified in large quantities in the *E. gracilis* grown on dairy retentate. However, it is outside of the scope of this experiment to evaluate why it only occurred in part of the samples grown on the retentate.

The ethanol extracts of the *E. gracilis* biomass were analysed to see whether the organism contained any oligosaccharides. However, analysis of the chromatograms of the ethanol extracts revealed that the oligosaccharide content was low. The exact content of oligosaccharides in the *E. gracilis* biomasses was not analysed. The quantities of sucrose found in the sample were also very small, in addition the retention time of the peak shifted rather much. Sucrose is according to Porchia et al. (1999) also used by *E. gracilis* as a stress response.

Comparing the content of free sugars in the biomass from before and after the SFE, there seems to be no significant difference. This was to be expected as the solubility of mono and disaccharides in hydrophobic liquids is low and the extraction conditions were much too mild to further break down the sugars. The conditions are also too weak to hydrolyse paramylon and thus release more free sugars, as paramylon is a highly resistant compound and needs temperatures above 135°C for such to happen (Darzin et al. 2010), There was no apparent differences between *E. gracilis* cultivated under different growth conditions on the accumulation of sugars.

3.4.5 Content of dietary fibre in the *E. gracilis* biomass

Analysis of dietary fibre content of the biomasses pre and post SFE revealed that SFE did not affect the unadjusted dietary fibre content. The content of dietary fibre in the *E. gracilis* biomass was approximately 25 % higher than the experimental results of paramylon obtained using the method of Grimm et al. (2015). Comparison between the gravimetric analysis of Grimm et al. (2015) and the AOAC dietary fibre (AOAC International 1995) is interesting as the methods are comparable. They are comparable as both hydrophilic and lipophilic compounds are extracted, and proteins hydrolysed enzymatically. The results obtained in this experiment are

not adjusted for protein and mineral content, but previous dietary fibre analysis of *E. gracilis* performed at the University of Helsinki (Edelmann 2017). showed a content of as high as 70 % protein in the isolated dietary fibre. This supports the notion that there also are impurities in the paramylon isolates obtained by the method of Grimm et al (2015). Gravimetric method like the ones used in this experiment has the weakness that the certainty of obtaining a pure isolate can always be questioned, as there is no real control of the compound pureness without any further testing (AOAC International 1995). As the protein correction could not be performed on the isolates due to the machine malfunction no corrections of protein content was performed to give an accurate estimation of the fibre content.

4 CONCLUSIONS

In this study, the main carbohydrates found in the biomasses of *E. gracilis* were paramylon, mannitol and trehalose. The crude contents of paramylon were between 220 and 400 mg/g which constitutes a substantial proportion of the dry cell weight. There appeared to be more paramylon in the biomasses cultivated on Hutner medium compared to when cultivated in dairy retentate. The effect of the light conditions on the paramylon content could not be analysed due to large variations in carbohydrate content among biomasses cultivated under same conditions of light. There was no effect of SFE on the content of paramylon or free sugars in the *E. gracilis* biomass. In this study the method did not yield high purity paramylon and corrections were needed to give a more accurate content for the paramylon content. Corrections had not been made in the original method. A large proportion of these impurities of the isolate include nitrogen containing compounds most likely chlorophyll and peptides that are associated with the paramylon. SEC analysis indicated that there was a peak eluting at the same volume for the paramylon standard and the isolates indicating the same molar mass. Further analysis of the paramylon isolates would be beneficial for further research as this would give a better overview of what measures needs to be taken to improve extraction of paramylon.

The content of free sugars quantified in this experiment was between 2.4 and 14.9 % of the E. gracilis of the total dry mass. The most abundant free sugars were mannitol, trehalose and glucose. Also, the possibility for *E. gracilis* to engulf nutrients was shown as lactose was found in high quantities in some of the samples grown in dairy retentate.

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Appendix 1 Composition of Hutner chemical cultivation media

$(NH4)_2\ HPO_4$	0.2
$MgSO_4\ 7H_2O$	0.5
$CaCl_2$	0.2
H_3BO_3	0.0144
Vitamin B_1	0.0025
Vitamin B_{12} ($\mu g\ L^{-1}$)	20
Trace element solution (in g 100 mL^{-1} MΩ water)	**1.0 ml L^{-1}**
$ZnSO_4\ 7H_2O$	4.4
$MnSO_4\ H_2O$	1.16
$Na\ MoO_4\ 2{*}H_2O$	0.3
$CuSO_4\ 5H_2O$	0.32
$CoCl_2\ 6H_2O$	0.28
Fe-solution (in g 100 mL^{-1} MΩ water)	**1.0 ml L^{-1}**
$(NH_4)_2SO_4\ Fe\ (SO_4)_2\ 6H_2O$	1.14
EDTA	1.0

Appendix 2 Results of gravimetric analysis of paramylon content

Sample	Reactor	parallel	g/100g	Average R	Std dev R	CV % R	min R	max R	Average S	Std dev S	CV % S	min S	max S
Euglena 2	1	13	21.8	20.3	3.51	17.2	16.3	22.9	28.6	8.0	28.0	16.3	40.2
		14	16.3										
		15	22.9										
	2	4	33.1	37.7	3.97	10.5	33.1	40.2					
		5	40.2										
		6	39.7										
	3	1	26.6	27.7	0.90	3.2	26.6	28.2					
		2	28.2										
		3	28.1										
Euglena 8	1	7	37.2	37.4	2.00	5.4	35.5	39.5	34.0	3.9	11.5	27.7	39.5
		8	39.5										
		9	35.5										
	2	1	27.7	29.6	1.84	6.2	27.7	31.4					
		2	29.6										
		3	31.4										
	3	1	33.5	34.9	2.46	7.1	33.4	37.7					
		2	37.7										
		3	33.4										
SFE	Pre	1	34.8	36.4	2.44	6.7	34.8	39.2	38.3	2.4	6.2	34.8	41.7
		2	39.2										
		3	35.1										
	30	1	41.7	39.5	2.78	7.0	36.4	41.7					
		2	36.4										
		3	40.4										
	50	1	39.6	38.9	0.68	1.7	38.2	39.6					
		2	38.9										
		3	38.2										
R- Light	1	1	29.6	28.8	1.34	4.7	27.3	29.6	26.3	3.3	12.6	21.1	29.6
		2	27.3										
		3	29.6										
	2	1	24.1	23.7	2.50	10.5	21.1	26.1					
		2	21.1										
		3	26.1										
R-dark	3	1	30.6	31.3	0.82	2.6	30.6	32.2	21.5	10.9	50.6	10.9	32.2
		2	31.3										
		3	32.2										
	4	1	10.9	11.6	0.62	5.4	10.9	12.2					
		2	11.6										
		3	12.2										

Appendix 3 Results of HPLC analysis of free sugars

Sample Name	sample weight	Trehalose	Mannitol	Arabinose	Glucose	Galactose	Fructose	Sucrose	To
E2R1-13	0.2628	7.23	11.47	0.00	0.59	0.21	0	0	
E2R1-14	0.2472	6.78	9.95	2.63	0.63	0.22	0	0	
E2R1-15	0.2674	6.30	9.24	2.60	0.64	0.24	0	0	
E2R2-4	0.3039	14.91	20.81	0.51	0.86	0.12	0	0	
E2R2-5	0.2567	17.53	24.68	0.74	1.25	0.14	0	0	
E2R2-6	0.2862	15.34	27.69	1.15	1.26	0.14	0	0	
E2R3-1	0.2771	3.68	4.35	2.45	0.88	0.36	0	0	
E2R3-2	0.2712	3.69	4.11	2.16	0.88	0.33	0	0	
E2R3-3	0.3069	3.24	3.52	2.17	0.85	0.31	0	0	
E8R1-7	0.2506	3.17	43.54	2.22	1.56	0.14	0	0	
E8R1-8	0.2797	2.90	40.19	1.64	1.61	0.14	0	0	
E8R1-9	0.2568	0.00	43.85	1.87	0.00	0.00	0	0	
E8R2-4	0.246	1.69	17.50	3.17	1.67	0.24	0	0	
E8R2-5	0.2506	1.72	16.78	3.17	1.68	0.24	0	0	
E8R2-6	0.2468	1.80	17.50	3.53	1.78	0.22	0	0	
E8R3-1	0.2826	1.61	34.06	2.76	5.45	0.28	0	0	
E8R3-2	0.2954	1.52	17.18	17.10	5.16	0.29	0	0	
E8R3-3	0.2591	1.56	36.82	2.61	5.23	0.29	0	0	
E-PreSFE-1	0.255	2.49	33.35	1.63	2.61	0.20	0	0	
E-PreSFE-2	0.2694	2.45	34.06	3.60	2.77	0.20	0	0	
E-PreSFE-3	0.2535	2.66	33.55	1.79	2.76	0.22	0	0	
E-SFE30-1	0.2956	2.08	34.91	2.88	3.72	0.24	0	0	
E-SFE30-2	0.2675	2.21	33.03	4.11	4.54	0.24	0	0	
E-SFE30-3	0.3371	1.71	26.48	2.34	2.77	0.16	0	0	
E-SFE50-1	0.2951	2.03	30.63	0.00	3.93	0.24	0	0	
E-SFE50-2	0.3469	1.60	45.20	1.23	2.81	0.19	0	0	
E-SFE50-3	0.2747	1.95	66.16	2.24	4.35	0.25	0	0	
E- Rlight-1	0.1319	0.68	154.81	4.81	3.56	0.87	0	0	
E- Rlight-2	0.135	0.78	180.00	4.30	3.67	1.07	0	0	
E- Rlight -3	0.147	0.71	149.66	1.87	2.65	1.12	0	0	
E- Rlight -1	0.1629	0.83	146.29	3.81	3.71	1.14	0	0	
E- Rlight -2	0.1546	9.61	98.03	2.33	3.48	1.00	0	0	
E-Rlight-3	0.1493	0.84	103.78	2.04	3.99	1.07	0	0	
E- RDark-1	0.1466	0.58	111.26	2.59	1.26	0.00	0	0	
E- RDark-2	0.1445	0.87	18.06	0.17	1.18	2.84	0	0	
E- RDark-3	0.1429	1.08	22.50	1.64	1.82	2.24	0	0	
E-RDark-1	0.1362	1.43	22.91	0.48	1.43	0.88	0	0	
E-RDark-2	0.1417	0.88	2.27	0.17	1.69	0.00	0	0	
E-RDark-3	0.1428	1.12	2.18	0.05	1.65	0.88	0	0	

Appendix 4 Comparison chromatograms of all E. gracilis water extracts analysed (method with and without gradient and of the extract diluted 1:50 analysed on CarbopacMA-1 colmun