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Cultivation of seven different species of marine microalgae using simulated flue gas mimicking effluents from paper mills as carbon source

Master of Science Thesis in the Master Degree Program Biotechnology

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Abstract

The use of fossil fuels is unsustainable, both due to limited supply and also due to large emissions of CO_2 with effects on global warming. Biofuels is a viable alternative but can, as produced today, only provide a limited amount of fuels needed. Biofuels are currently mainly derived from terrestrial plants, requiring large amounts of arable land. Biofuels from microalgae on the other hand do not require arable land and can, theoretically, replace fossil fuels completely. In addition, biofuels from microalgae could use flue gases from industry as carbon source and waste waters as growth medium for nutrient supply. In Sweden, the large paper industry is a particularly interesting potential provider, both due to its extensive carbon dioxide emissions and available waste waters containing nitrogen and phosphorous. In this thesis, a simulated flue gas mimicking that of paper mills with 15 % CO_2, 100 ppm NO and 10 ppm SO_2 was used to cultivate seven species of marine microalgae and to study its effect on growth and cellular composition. Of the species tested, *Nannochloropsis salina* was identified to be the most promising candidate due to its tolerance to the simulated flue gas; the specific growth rate obtained from optical density measurements was barely affected by the increasing CO_2 concentrations. The doubling time calculated from optical density was sustained at 79 hours throughout the experiment. However, the doubling time obtained from cell counts almost doubled from 42 to 87 hours when NO and SO_2 was switched on. For the other species a general decline in specific growth rate could be observed with increasing CO_2 concentrations. *Dunaliella salina* and *Dunaliella tertiolecta* were the species with the lowest doubling times, around 25 hours, but these were only sustained in the beginning of the experiments with CO_2 levels between 0 - 8 %. The cell composition analyses performed showed to be problematic, possibly due to the marine medium or too low cell content in the samples. The constituents of the simulated flue gas all contribute to a lower pH and the marine species used in this study was very sensitive to pH below 7 as seen by decreased biomass levels in the cultures at these pH levels. Hence, the pH must be controlled to avoid cultivation collapse. In this study, NaOH additions and Hepes buffer was used to maintain pH around 7. The results indicate that the simulated flue gas is stressful for the microalgae tested but by using an adaptation period the gas tolerance might be improved. The waste waters of paper mills in Sweden contain on average levels of nitrogen and phosphorous that roughly corresponds to the levels in f/2 medium which indicates that these could be used as growth medium. Two types of waste waters from Nordic Paper Bäckhammar were tested in a separate experiment but further studies is needed to establish whether marine algae can grow in it.

Acknowledgements

During the spring of 2011 Mathias Bark and I were looking for an interesting subject for a master thesis. We contacted Eva Albers, at Industrial biotechnology at Chalmers, after an intriguing lecture about microalgae and she contacted her cooperation partner Susanne Ekendahl at SP Technical Research Institute of Sweden. It was decided that we would perform two parallel studies regarding microalgae and flue gas at SP. Mathias and I had mostly studied bacteria and yeast at Chalmers and were therefore quite 'green' in the microalgae field.

I would like to thank Mathias for a successful cooperation and Eva and Susanne who have supported us throughout the study and were always there for helpful discussions.

I would also like to thank all the co-workers at SP Chemistry and Materials department for making us feel welcome and for plenty of help with equipment, analyzes and questions. Special thanks go to Catrin Lindblad for among other things practical help in the lab; to Rauno Pyykkö and Bo-Lennart Andersson for helping with the setup and testing of the gas distribution system; to Peter Söderberg and Lena Brive for helping us with the triglyceride analysis; and to Mathias Johansson for explaining and helping with the CHN-analysis.

Thank you all!

Niklas Engström

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1. Introduction

1.1. Background

The use of fossil fuels is unsustainable due to the limited availability and the resulting extensive green-house gas emissions (Chisti 2008; Verma, et al. 2010; Ekendahl, et al. 2011). The production of renewable biofuels is therefore highly interesting. Biofuels cannot directly reduce green-house gas emissions since the sequestered CO_2 is released again when the fuel is used, but the CO_2 is recycled and the fuel is as a result CO_2 neutral (Chisti 2008; Ekendahl, et al. 2011; Kumar, et al. 2010; Sheehan, et al. 1998).The replacement of fossil fuel, however, will reduce the emission of CO_2.

Biofuels such as ethanol and biodiesel are today mainly produced from terrestrial plants such as sugar cane, rapeseed, and palm that require vast amounts of arable land (John, et al. 2011; Chisti 2008; Griffiths and Harrison 2009; Ma and Hanna 1999; Xu, Miao and Wu 2006). The use of arable land for the production of biofuels is controversial since this competes with food production (Chisti 2008; Griffiths and Harrison 2009). Microalgae on the other hand, can be cultivated without the need of arable land and are also in general much more effective than terrestrial plants in capturing solar energy (Malcata 2011). According to Li et al (2008) switchgrass, which is the fastest growing terrestrial plant, can convert less than 0.5% of the total solar energy received in a typical midlatitude location whereas microalgae may convert up to 10% (Li, et al. 2008). The doubling time and water requirements are also factors that greatly favor microalgae. The typical doubling time for algae is around 24 hours (Chisti 2008; Malcata 2011). According to Kliphuis et al (2010) microalgae requires around 1.5 liters of water to produce 1 liter of biodiesel compared to 10 000 liters for land crops (Kliphuis, et al. 2010). Chisti (2008) has estimated that the USA would need to use over 60% of its agricultural cropping land to be able to cover its need for biodiesel using palm. This can be compared to around 3% for a microalga with 30% oil content (Chisti 2008).

Microalgae requires large amounts of CO_2, it takes approximately 1.8 ton CO_2 to produce 1 ton of microalgal biomass (Chisti 2008). To be able to obtain the high cell densities that are required to make algal cultivation profitable CO_2 needs to be supplied in concentrations higher than in the air. To further improve the prospects of microalgae cultivations industrial flue gas and waste water could be used. Industrial flue gas could be a rich source of CO_2 at little or no cost (Chisti 2008), which is important since CO_2 acquisition otherwise is a significant cost driver (Clarens, et al. 2010). Flue gas as CO_2 source has been successfully used in several other studies (Ekendahl, et al. 2011; Sheehan, et al. 1998; Borkenstein, et al. 2011; Negoro, et al. 1991; Sawayama, et al. 1995; Yun, et al. 1997). Many types of waste waters contain phosphorous, nitrogen and trace metals which are necessary for microalgal growth, and the use of this would reduce both costs and eutrophication effects (Chisti 2008; Kumar, et al. 2010; Huang, et al. 2010; Kong, et al. 2010; Patil, et al. 2008). Waste heat from the industry could be used to lower energy costs when concentrating the biomass by evaporation before harvesting and to control the temperature of the cultivations (Ekendahl, et al. 2011; Kumar, et al. 2010).

With soaring oil prices and increased environmental awareness microalgae seems very promising. The main challenge, however, is the economics of production (Ekendahl, et al. 2011; Patil, et al. 2008). Most of the data regarding e.g. lipid content and growth rates are from studies on bench-scale with controlled conditions (Kumar, et al. 2010). Less is known about the feasibility of scaling up to commercially viable production sizes or how competition from other microbial species, that are likely to contaminate, will affect the production (Ekendahl, et al. 2011; Kumar, et al. 2010). Because of the

small cell size and relatively low biomass concentrations of microalgae, harvesting, dewatering and lipid extraction are steps that consume large amounts of energy (Kumar, et al. 2010). To be able to cultivate with higher cell densities further research is needed with regard to among other things efficient light and gas transfer (Kumar, et al. 2010).

1.2. Problem formulation

The unsustainable use of limited fossil fuels and the enormous emissions of carbon dioxide into the atmosphere pose global challenges.

Many industries struggle with falling profitability and are looking for ways to increase it. Pulp and paper mills are emitting large amounts of carbon dioxide and have lots of waste water and excess heat that could be turned into something useful and profitable. By using the carbon dioxide, waste water and heat microalgae can be cultivated to produce biofuels. This has been tested for other types of industries but not for pulp and paper mills (Borkenstein, et al. 2011; Matsumoto, et al. 1995). It is of importance to find species that both can live and thrive in the flue gas and the wastewater and that are suitable for the production of biofuels.

1.3. Aims and target groups

The main aim with this thesis was to find suitable marine species for cultivation in flue gas from pulp and paper mills in Sweden. The goals were to find species that:

- have relative high specific growth rates, preferably with doubling times below 30 hours.
- tolerate high levels of CO_2 (~15%) in simulated flue gas and still maintain the cellular activity with at least 80% of the growth rate using low levels of CO_2 (~1%).
- tolerate NO and SO_2 impurities in simulated flue gas and still maintain the cellular activity with at least 80% of the growth rate using high levels of CO_2 (~15%).
- have a cell composition that is suitable for biofuel production, with high levels either of triglycerides or carbohydrates.

In order to reach these goals for the work within this study a broad range of marine species of microalgae was selected, representing several applications and properties. The average actual flue gas composition for the pulp and paper mills in Sweden was investigated to be able to simulate a typical flue gas in the experimental study. The average actual wastewater content of nitrogen and phosphorous was investigated for existing mills and a preliminary growth test of algae in waste water from one of these mills were made.

Fulfilling of these tasks will lead to an increased knowledge of the effects of flue gas on marine microalgae and prepare for further studies which in the end hopefully can lead to a commercialization of microalgae cultivation using waste streams from pulp and paper mills.

The target group of this thesis is primarily pulp and paper mills, but also other industries with suitable waste streams that wish to reduce their environmental impact and at the same time increase their profit. For the scientific community this study may lead to identification of research questions around effects of industrial conditions on cellular performance that is needed to be answered to aid the industrial cultivation of algae.

1.4. Method

Seven marine species were included in the main experiments. They were cultivated in 250 ml Erlenmeyer flasks (E-flasks) with marine laboratory medium (f/2). The simulated flue gas contained

15% of carbon dioxide, 100 ppm nitrogen monoxide and 10 ppm sulfur dioxide. Optical density, cell count and dry weight were used to measure growth. Cellular content of macromolecules was determined by analyzing the levels of carbohydrates, proteins and triglycerides. A CHN-content analysis was also performed. Two types of actual wastewater from Nordic Paper Bäckhammar were used to make a visual growth test on the species. The waste waters were mixed with autoclaved natural seawater to obtain a brackish salinity.

This study was limited to seven marine microalgae species in the main experimental runs and a further seven species were included in pre-cultures and side experiments. There was no temperature control, meaning that the temperature of the cultivations changed slightly during one day due to changes in room temperature from e.g. heat from the fluorescent lamps.

2. Theory

2.1. General information about microalgae

Algae are of extreme importance for life on earth. According to Day et al (1999) algae stand for approximately 50 % of the global photosynthetic activity and form the basis of the food chain for over 70 % of the world's biomass (Day, Benson and Fleck 1999). Algae can also be the cause of death. In response to eutrophication algal populations may become too large and thereby reduce the water transparency and cause the death of other photosynthetic organisms (Barsanti and Gualtieri 2005). The production of toxins by some algae can also cause massive fish and bird deaths (Barsanti and Gualtieri 2005). It has been estimated that there are about 200,000 – 800,000 different species of microalgae, of which 35,000 species has been described so far (Research on microalgae within Wageningen UR 2011). The general definition of an alga is an organism with chlorophyll *a* and a thallus that is not differentiated into roots, stem or leaves (Tomaselli 2007). This definition includes the prokaryotic cyanobacteria as an alga. Microalgae are microscopic algae that can be unicellular, colonial or filamentous. Microalgae can be found all around the world, mainly in waters but also on the surface of soil (Tomaselli 2007).

Most algae are considered photoautotrophs, and they are consequently depending on sunlight for energy and CO_2 as carbon source (Barsanti and Gualtieri 2005). However, most algal divisions also contain heterotrophic species that can acquire external organic carbon either by taking up dissolved substances (osmotrophy) or by phagocytosis (Barsanti and Gualtieri 2005). Algae that lack the ability to synthesize all essential components are called auxotrophic. On the other hand, it is thought that algae use a spectrum of nutritional strategies and therefore are in fact mixotrophic (Barsanti and Gualtieri 2005). The contribution of each strategy varies from species to species and depends on the general environmental conditions (Barsanti and Gualtieri 2005). Heterotrophy is more important when light is limiting and autotrophy when particularly food is in short supply (Barsanti and Gualtieri 2005).

2.1.1. Photosynthesis in microalgae

The process of photosynthesis converts light energy and inorganic compounds into organic material (Masojidek, Koblizek and Torzillo 2007). In eukaryotic microalgae the photosynthetic apparatus is organized in organelles called chloroplasts. The chloroplasts contain alternating layers of lipoprotein membranes, called thylakoids, and an aqueous phase that is called stroma. In prokaryotic cyanobacteria the photosynthetic membranes are located in the peripheral region, called chromoplast. The photosynthesis can be divided into two parts: the light reactions and the dark reactions. The light reactions occur at the photosynthetic membranes and convert the light energy to chemical energy, see eq. 1 for a summary of the light reactions.

$$2NADP + 3H_2O + 2ADP + 2P_i \quad light\ energy \quad 2NADPH_2 + 3ATP + O_2 \qquad eq.\ 1$$

The dark reactions occur in the stroma. Here, the $NADPH_2$ and ATP produced in the light reactions are used in the reduction of carbon dioxide to carbohydrates, which is summarized in eq. 2.

$$CO_2 + 4H^+ + 4e^- \quad 2NADPH_2, 3ATP \quad CH_2O \ + H_2O \qquad eq.\ 2$$

At low light irradiances the rate of photosynthesis is linearly dependent on light intensity. When the light intensity increases the photosynthesis becomes less and less efficient until it reaches a maximum where the enzymatic reactions becomes limiting. The cell is then light-saturated. When a cell is

exposed to high irradiances for a longer period the rate normally decreases below the light-saturated value which is called photoinhibition (Masojidek, Koblizek and Torzillo 2007).

The pigments that harvest light energy can be divided into three groups: chlorophylls, carotenoids and phycobilins. The chlorophylls (green) and the carotenoids (yellow or orange) are lipophilic while the phycobilins are hydrophilic. There are various types of chlorophyll molecules that are called *a*, *b*, *c* and *d*. All of them have two major absorption bands at 450-475 nm and 630-675 nm. Chlorophyll *a* is present in all oxygenic photoautotrophs. Carotenoids have several different roles in the photosynthetic apparatus. They are used as accessory light-harvesting pigments that transfer excitation to chlorophyll *a*, they have structural functions and they provide protection against excess irradiance and reactive oxygen species. The absorption range is between 400 and 550 nm. Phycobilins are found in cyanobacteria and red algae and are like carotenoids accessory pigments. Their absorption range is between 500 and 650 nm (Masojidek, Koblizek and Torzillo 2007).

Photosynthesis is affected negatively by sub-optimal temperatures and high oxygen concentrations. Osmotic stress also gives negative effects on photosynthesis (Vonshak and Torzillo 2007).

2.1.2. Species and biodiversity

There are several different kinds of algal divisions, or phylum, with their own characteristics. Among these divisions are Chlorophyta, Bacillariophyta, Cryptophyta, Heterokontophyta and Cyanobacteria which all are represented in this thesis. The classification is, however, under constant re-modeling and classes may change division and so on. The classification used here will be the one used by *Artdatabanken Dyntaxa* (Artdatabanken - Swedish Species Information Center 2012). Only divisions represented in the experiments will be presented here.

Chlorophyta, also called green algae, consists of a large variety of species which can be found all around the world (Tomaselli 2007). They range from microscopic to macroscopic and comprise four different classes: Micromonadophyceae, Charophyceae, Ulvophyceae and Chlorophyceae. Chlorophyta have both chlorophyll *a* and *b* and also several carotenoids. During stress, such as nitrogen deficiency, carotenoids can be synthesized and accumulated outside the chloroplasts giving the cells an orange or red color. The storage compound for Chlorophyta is mainly starch which is composed of amylose and amylopectin. The starch is formed within the chloroplasts, unlike other algae (Tomaselli 2007).

Bacillariophyta, also called diatoms, consists of three classes: Coscinodiscophyceae, Fragilariophyceae and Bacillariophyeae (Round, Crawford and Mann 1990). The Coscinodiscophyceae class is a centric diatom, which means it has radial symmetry, whereas the Fragilariophyceae and Bacillariophyeae are pennate diatoms, which mean they have bilateral symmetry (Tomaselli 2007; Round, Crawford and Mann 1990). Diatoms are mostly unicellular but can be attached to one another in chains or colonial aggregations. The color of diatoms are generally golden brown due to that the green color from chlorophylls *a*, c_1 and c_2 is masked by the brown and yellow color of fucoxanthin and β-carotene (Tomaselli 2007). The main storage compound is chrysolaminarin but also lipids are present (Tomaselli 2007). Diatoms have a siliceous cell wall, called frustule, which structure is highly varied between species and therefore used for classification (Tomaselli 2007). This cell wall means that diatoms need silicon. In most of the places diatoms live, the main form of silicon in solution is silicic acid, $Si(OH)_4$, and the uptake of this can sometimes be so efficient that its presence in water becomes undetectable by standard water analysis techniques (Round, Crawford and Mann 1990).

Cryptophyta consists of one class: Cryptophyceae. The Cryptophyceae are typically free-swimming, asymmetric cells with two flagella (Barsanti and Gualtieri 2005). They contain chlorophyll *a* and c_2 and they store polysaccharides as starch granules. Most are photosynthetic, but heterotrophic nutrition also occurs (Barsanti and Gualtieri 2005).

Heterokontophyta consists of several classes, among which Eustigmatophyceae is one. Most of the Heterokontophyta are marine, but they can also be found in freshwater and terrestrial environments (Barsanti and Gualtieri 2005). They contain chlorophyll *a*, c_1, c_2 and c_3, except for Eustigmatophyceae which only have chlorophyll *a*. The main accessory pigments are β-carotene, fucoxanthin, and vaucheriaxanthin which often outnumber the chlorophylls resulting in golden color. The primary storage compound is the polysaccharide chrysolaminarin, which is located in special vacuoles in the cytoplasm. The growth is generally photoautotrophic but can also be heterotrophic (Barsanti and Gualtieri 2005).

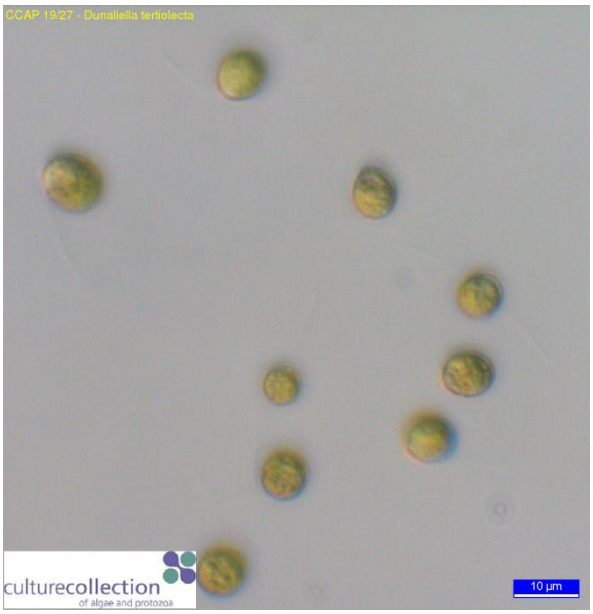
Cyanobacteria, also called blue-green algae, are prokaryotic algae with chlorophyll *a* that consists of two classes: Cyanophyceae and Prochlorophyceae (Tomaselli 2007). The name, Cyanophyceae, is due to that phycocyanin and phycoerythrin usually mask the color of the chlorophyll. The main storage compound is glycogen, but also cyanophycin is stored in large granules. Cyanobacteria have a cell wall composed of peptidoglycan with an external lipopolysaccharide layer and some species may also have a mucilaginous sheat. Cyanobacteria can grow singly or in filaments and the filaments can be branched or unbranched, with uniseriate or multiseriate arrangement. The filaments can contain cells that are differentiated into heterocysts, which are specialized nitrogen-fixing cells, and/or akinetes, which are cells that are dormant. Some species can float due to gas vacuoles. Prochlorophyceae have some major differences compared to Cyanophyceae. For example, they also have chlorophyll b, they have a starch-like polysaccharide as main storage compound and no cyanophycin or gas vacuoles (Tomaselli 2007).

2.1.2.1. *Dunaliella tertiolecta* and *Dunaliella salina* (Chlorophyta)

Dunaliella sp. is a biflagellate alga that is characterized by an ovoid shape often similar to that of a pear (Tomaselli 2007). *Dunaliella sp.* have a thin elastic plasma membrane that is covered by a mucous layer and lacks a rigid polysaccharide cell wall. This enables the cells to rapidly change their volume in response to changes in osmotic pressure. *Dunaliella sp*. occurs naturally in a wide variety of habitats but most often in waters with high salt content. It is the most halotolerant eukaryotic photosynthetic organism known but it does not require high salt concentrations. Besides the elastic membrane, *Dunaliella sp.* uses glycerol to handle osmotic pressure. The intracellular concentration of glycerol is directly proportional to the extracellular salt concentration. Some species of *Dunaliella*, e.g. *D. tertiolecta* and *D. salina*, contain strains that have the ability to accumulate large amounts of β-carotene. This occurs when the cells are exposed to high solar radiation but is also affected by other environmental stresses such as nitrogen deficiency or other nutrient deficiencies, elevated salt levels, extreme pH and low temperature. It is thought that the globules protect the cell against injury by high intensity radiation under growth limiting conditions. Since *Dunaliella sp.* thrives in high salt concentrations it is very suitable for mass cultivation in open ponds due to that the contamination risk is minimized. It also tolerates temperatures from around -5°C to above 40°C which makes cultivation easier. *Dunaliella sp.* is today widely distributed as β-carotene extracts, as nutritional supplement for humans and for feed use (Tomaselli 2007).

D. tertiolecta (Figure 1) can grow in the pH range of 6.0-9.3, but the optimum lies between pH 7-8 (Hinga 2002). The strain used in experiments in this thesis is called GUMACC5 and is originally

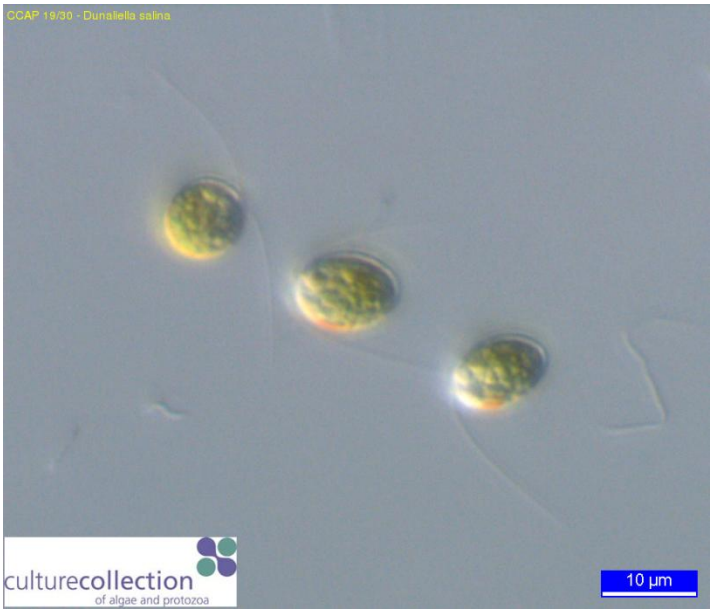
isolated in the sea close to the marine research station at Tjärnö, Strömstad (the Sven Lovén Centre for Marine Science Tjärnö).



Kingdom: Plantae
Division: Chlorophyta
Class: Chlorophyceae
Order: Volvocales
Family: Dunaliellaceae

Figure 1. *Dunaliella tertiolecta*; The scale in the bottom right corner shows 10 µm. Photo used with permission of CCAP – Culture Collection of Algae and Protozoa.

The optimum pH for growth for *D. salina* (Figure 2) is 9-11, but when cultivated the pH is often maintained at around 7.5 (Hosseini Tafreshi and Shariati 2008). The strain used in experiments in this thesis is called CCAP 19/30.



Kingdom: Plantae
Division: Chlorophyta
Class: Chlorophyceae
Order: Volvocales
Family: Dunaliellaceae

Figure 2. *Dunaliella salina*; The scale in the bottom right corner shows 10 µm. Photo used with permission of CCAP – Culture Collection of Algae and Protozoa.

2.1.2.2. *Phaeodactylum tricornutum* (Bacillariophyta)

P. tricornutum (Figure 3) belongs to the Bacillariophyceae class and is consequently a pennate diatom. It is special in many ways. It has the rather remarkable property of being pleiomorphic, which means it

can change shape and size (De Martino, et al. 2007). This is related to its atypical cell wall, which compared to other diatoms is less silicified and *P. tricornutum* appears in fact to be unique in that it does not require silicic acid. There are three major morphological types: fusiform, which can be seen in Figure 3, triradiate and oval. The three morphotypes are physiologically different and it appears as the oval type is induced as an adaptation to suboptimal conditions. *P. tricornutum* has been widely cultivated for both research purposes and as feed for the aquaculture industry due to its high oil content and ease of cultivation (De Martino, et al. 2007). It is not however often found in nature (Round, Crawford and Mann 1990; De Martino, et al. 2007). It is thought to be a coastal species that occurs in unstable environments, such as estuaries and rock pools (De Martino, et al. 2007).

The pH optimum for growth for *P. tricornutum* is 7.7 (Fredin 2009), but it can grow in the pH range of 6.1-10 (Hinga 2002). The temperature range lies from < 15 to 27°C, with optimum at 24°C (SERI Microalgal Technology Research group 1986).

The strain used in experiments in this thesis is called GUMACC2, which is the same as UTEX642.

Kingdom: Chromista
Division: Bacillariophyta
Class: Bacillariophyceae
Order: Naviculales
Family: Phaeodactylaceae

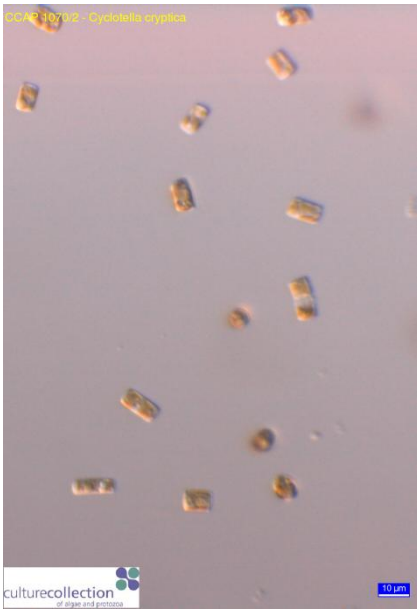
Figure 3. *Phaeodactylum tricornutum* with a fusiform morphology; The scale in the bottom right corner shows 10 µm. Photo used with permission of CCAP – Culture Collection of Algae and Protozoa.

2.1.2.3. *Cyclotella cryptica* (Bacillariophyta)

*C. cryptic*a (Figure 4) is a centric diatom which has been cultivated for aquaculture purposes, mainly because of its ability to produce eicosapentaenoic acid (AquaFUELs 2009). The cells of *C. cryptica* are short and drum-shaped, they live individually or in filaments in the form of chains or sometimes clusters that are covered by mucilage. The cell size is between 5-25 µm and the morphology depends highly on the salinity. It is a planktonic species that can be found in marine or brackish waters. *C. cryptica* can grow heterotrophically on glucose in the absence of light, which is thought to be used for survival at the bottom of waters or in muds. It can also grow photoheterotrophically (AquaFUELs 2009).

The pH range for growth for *C. cryptica* is 7-9, but the optimum is between pH 7 and 8 (SERI Microalgal Technology Research group 1986). The temperature range lies between 16 and 35°C with optimum at around 30°C depending on strain (SERI Microalgal Technology Research group 1986).

The strain used in experiments in this thesis is called CCMP33.



Kingdom: Chromista
Division: Bacillariophyta
Class: Coscinodiscophyceae
Order: Thalassiosirales
Family: Stephanodiscaceae

Figure 4. *Cyclotella cryptica*; The scale in the bottom right corner shows 10 µm. Photo used with permission of CCAP – Culture Collection of Algae and Protozoa.

2.1.2.4. *Arthrospira platensis* (Cyanobacteria)

Arthrospira is a genus of filamentous cyanobacteria (Hu 2007). The filaments are characterized by loosely spiral-shaped trichomes that are arranged in an open helix and covered in a mucilaginous sheet. Depending on the species and conditions, however, the geometry can vary from a coiled helix to a completely straight shape. The trichomes are generally a few millimeters long and consist of cells that are cylindrical and 3-12 µm in diameter. *Arthrospira* is as other cyanobacteria Gram-negative with a cell wall containing a major structural layer of peptidoglycan. *Arthrospira* can be found in a variety of habitats but mainly in alkaline, brackish and saline waters where they often become the predominant species and form large blooms. The optimal growth temperature is 35-38°C and the pH optimum is 9.5-9.8, which makes is an alkalophile. Generally it is considered photoautotrophic, but may use mixotrophic or even heterotrophic growth in the presence of organic carbon sources such as glucose or acetate. *Arthrospira* has been extensively cultivated as food supplement, as feed, for fine chemicals and as therapeutic agent (Hu 2007).

In experiments a strain of *A. platensis* (Figure 5), called CCMP1295, is used.

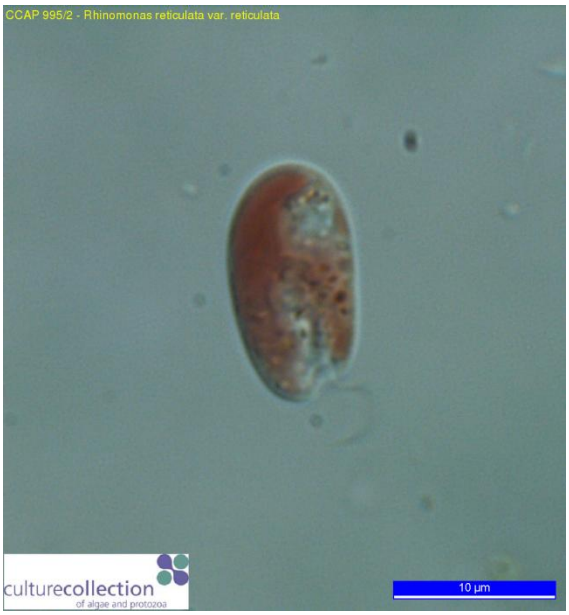
Kingdom: Bacteria
Division: Cyanobacteria
Class: Cyanophyceae
Order: Oscillatoriales
Family: Phormidiaceae

Figure 5. *Arthrospira platensis*; The scale in the bottom right corner shows 20 µm. Photo used with permission of NCMA – National Center for Marine Algae and Microbiota.

2.1.2.5. *Rhinomonas reticulata* (Cryptophyta)

R. reticulata (Figure 6) is like other Cryptophyta a free-swimming biflagellate. The cells are red-brown and the cell size is typically 8-12 µm in length and 3-5 µm in width (Cerino and Zingone 2006; Novarino 1991). Conditions suitable for optimum cultivation is poorly studied.

The strain used in experiments in this thesis is called GUMACC127, which is the same as CCAP 995/2.



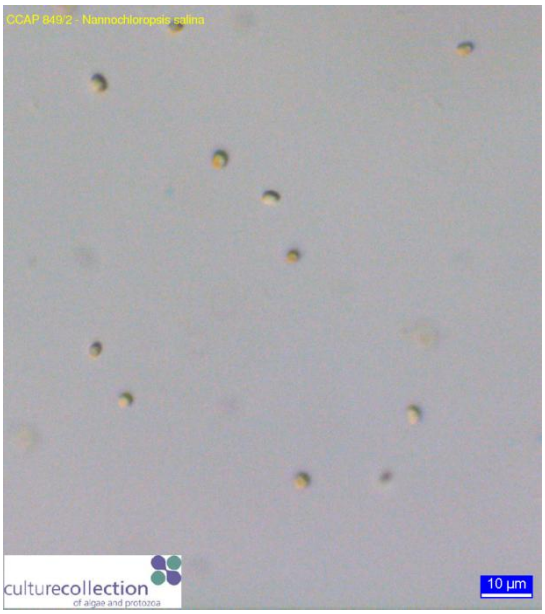
Kingdom: Chromista
Division: Cryptophyta
Class: Cryptophyceae
Order: Pyrenomonadales
Family: Pyrenomonadaceae

Figure 6. *Rhinomonas reticulata*; The scale in the bottom right corner shows 10 µm. Photo used with permission of CCAP – Culture Collection of Algae and Protozoa.

2.1.2.6. *Nannochloropsis salina* (Heterokontophyta)

Nannochloropsis is a genus with non-flagellate small cells, 2-4 µm in diameter (Tomaselli 2007). Like the other known species of Eustigmatophyceae the cells are green coccoid that are either single, in pairs or in colonies (Barsanti and Gualtieri 2005). *Nannochloropsis* has a cell wall of polysaccharides and do not accumulate starch (Tomaselli 2007). It has received a great deal of interest as a source of polyunsaturated fatty acids, since it can accumulate large amounts of eicosapentaenoic acid. *Nannochloropsis* is commonly cultivated in fish hatcheries as feed for rotifers and to enhance growth in larvae tanks (Zitelli, Rodolfi and Tredici 2007). The optimum pH for growth for *N. salina* (Figure 7) is 9, but it can grow in the range of pH 5-10.5 (SERI Microalgal Technology Research group 1986). The temperature range for growth lies between 17-32°C with optimum at around 28°C (SERI Microalgal Technology Research group 1986).

The strain used in experiments in this thesis is called CCMP1777.



Kingdom: Chromista
Division: Heterokontophyta
Class: Eustigmatophyceae
Order: Eustigmatales
Family: Monodopsidaceae

Figure 7. *Nannochloropsis salina*; The scale in the bottom right corner shows 10 µm. Photo used with permission of CCAP – Culture Collection of Algae and Protozoa.

2.2. Culturing of microalgae

Several factors influence the growth of microalgae. These can be divided into environmental factors, nutrients and type of cultivation system.

2.2.1. Environmental factors

Important environmental factors for cultivation of microalgae are light, being the primary energy source for many species of microalgae, temperature and pH. Mixing is of importance in order to obtain high cell densities and also the effects of using a flue gas as carbon source.

The amount of light required for cultivation depends on the culture depth and the algal density. A deeper and/or denser cultivation requires more light (Barsanti and Gualtieri 2005). The light can come either from the sun or from fluorescent lamps. Too high light intensities can cause photo inhibition and could also cause overheating. If using artificial light the most efficient is to use light from either the blue or red spectrum since these are the most active part of the light spectrum for photosynthesis. Normally the light intensity used is 10-15% of full daylight (100-200 µE sec^{-1} m^{-2}). The cells normally

need both short and longer cycles of light/dark. The short cycles can be achieved by mixing whereas the longer cycles are simply achieved by the natural day/night cycles or by turning of the artificial light. Some species can grow under constant illumination but most species prefer a dark period (Barsanti and Gualtieri 2005).

The temperature of the cultivation should generally be close to the temperature at which the algae were collected (Barsanti and Gualtieri 2005). Commonly cultivated species generally tolerate temperatures between 16°C to 27°C, but this vary depending on species and strain but also on the composition of the medium. Generally, cultivations are kept at a constant temperature around 18-20°C (Barsanti and Gualtieri 2005).

In natural seawater the pH is normally around 8 and the pH range for most cultivated marine microalgae is between pH 7 and 9, but this is species specific (Barsanti and Gualtieri 2005). To maintain a desirable pH the cultivation should be aerated and for high density cultures an inflow of carbon dioxide helps to maintain pH (Barsanti and Gualtieri 2005). Generally the bicarbonate buffer system helps to maintain a stable pH, but may be overwhelmed at high cell densities or when other components than carbon dioxide effect the pH. In laboratory cultivations the additions of buffers may be considered if needed, but for larger scale setups this would probably be too costly. Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer is a zwitterionic buffer with a pK_a at 7.55 at 20°C, which makes it an effective buffer at pH values around 6.8-8.2 (Good, et al. 1966). Hepes is generally considered a good all-around buffer for biological purposes.

Mixing is an important aspect when culturing algae, particularly in dense cultures. It is important to prevent cells from settling, to expose the cells to equal amounts of nutrients and light, to avoid thermal stratification, to improve gas exchange, and also to ensure that the cells experience alternating periods of light and darkness (Barsanti and Gualtieri 2005; Tredici 2007). Generally, algae are quite sensitive. Naturally, they do not experience much turbulence, so the mixing cannot be too strong. The amount of mixing possible to apply depends on the species. Filamentous cyanobacteria and dinoflagellates generally do not cope with being mixed by pumps. Air bubbling is a more gentle way of mixing but can also stress and damage cells (Barsanti and Gualtieri 2005; Tredici 2007).

Flue gases contain different components and concentrations depending on the fuel that is being burned and how efficiently the fuel is burnt. A typical flue gas contains mostly N_2, around 4-15% CO_2, around 5% O_2, 0-400 ppm SO_2 and 50-300 ppm NO (Oilgae 2009). Coal gives larger amounts of CO_2 than natural gas or syngas. Fossil fuels generally emit larger amounts of SO_2 (Sheehan, et al. 1998). CO may also be a major part of a flue gas, but it does not seem to pose a problem to microalgae cultivation due to its low solubility in water. Several studies, such as Negoro et al (1991) using 300 ppm NO and 0-400 ppm SO_2, have shown that NO_x and SO_x may inhibit growth. Other studies, such as Zeiler et al (1995) and Brown (1996), using 150 ppm NO and 200 ppm SO_2 have shown that these more moderate levels are not inhibitory to algal growth. Sheehan et al (1998) have concluded that NO_x and SO_x in flue gases generally do not pose a problem for microalgal culture and that the inhibitory effect found in previous studies such as Negoro et al (1991) is due to acidification of the medium and the resulting pH drop. Both NO and SO_2 contribute to a lowered pH since they when dissolved in water are converted to strong acids. NO is converted to nitrous acid (HNO_2) and nitric acid (HNO_3), while SO_2 is converted to sulfurous acid (H_2SO_3) and sulfuric acid (H_2SO_4). Flue gases may also contain soot, particles and heavy metals. Heavy metals are known to be toxic to most organisms and so also to microalgae (Kaplan 2007). Many species of algae have the ability to take up and bind heavy metals in complexes as a way of dealing with the toxicity. If the flue gas contains heavy metals these may be

accumulated in the algal cells, as was seen by Borkenstein et al (2010) who noticed that lead was accumulated in microalgal cultivations grown with flue gas from a cement plant burning coal and various surrogate fuels.

Power plants are estimated to stand for around 40% of the worldwide greenhouse gas emissions and are thus an abundant source of CO_2 (Oilgae 2009). In addition, petrochemical industries and steel manufacturers have large emissions and also paper industries emit significant levels of CO_2. An important aspect when looking at possible industries which could contribute the CO_2 source is the available land close to that industry. Cultivation of microalgae does require some space and this is something that is often lacking near large industries. It would of course be possible to build pipelines to transfer the flue gas to a nearby location but this increases costs. Another aspect is water. Since microalgae need water, preferably with nutrients in it, it would be beneficial if the industry is near water or has waste water that could be used. Waste waters from power plants do not generally support algal growth, both due to lack of nutrients and high levels of heavy metals (Oilgae 2009). Paper industries are a promising sector, especially in Sweden, since they both have high CO_2 emissions and waste waters with nutrients.

2.2.2. Nutrition

Several nutrients are necessary for microalgal growth. The most important are carbon dioxide, being the primary carbon source, nitrogen and phosphorous but trace metals and vitamins are also needed.

Carbon dioxide is highly soluble in water and when dissolved it combines with water and form bicarbonate and carbonate ions, as can be seen in equation 3:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2H^+ \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_2 + OH^- + H^+ \quad eq.\ 3$$

Sea water contains naturally on average 45 ml of total carbon dioxide per liter (at atmospheric pressure), but most of it is in the form of bicarbonate or carbonate ions. The amount of dissolved carbon dioxide is around 0.23 ml per liter (Barsanti and Gualtieri 2005). During photosynthesis, carbon dioxide fixation leads to accumulation of OH^- and an increase in pH (Grobbelaar 2007). The use of carbon dioxide shifts the equation and the bound ionic forms release more free carbon dioxide (Barsanti and Gualtieri 2005).

The most common way of distributing carbon dioxide to cultivations is through bubbling (Tredici 2007). Adsorption could however be a problem, since the bubbles generally have a short residence time in the cultivation. In open systems this is especially problematic since this leads to losses of carbon dioxide to the atmosphere. The most efficient way seems to be to bubble very small bubbles into a column with culture going the opposite direction (Tredici 2007).

The carbon dioxide could come directly from the surrounding air and bubbled through the cultivation, but for dense cultures the low amounts of carbon dioxide in the air (~0.03%) will be limiting (Barsanti and Gualtieri 2005). Pure carbon dioxide could also be used in mixture with air to desired concentrations but this will increase costs due to the purchase of pure carbon dioxide. Flue gases are a promising solution in supplying carbon dioxide and at the same time reduce the emission of this gas to the atmosphere.

Oxygen is produced in the photosynthesis. High levels of oxygen (<30 mg O_2 l^{-1}), however, are known to inhibit growth of microalgae (Vonshak and Torzillo 2007). Oxygen accumulation is one of the main problems when culturing algae in closed photobioreactors at high densities. Mixing of some sort is

needed to increase the gas exchange of both carbon dioxide and oxygen, but the cells are quite fragile which limits the amount of mixing possible.

Nitrogen is considered the second most important nutrient after carbon contributing to produced biomass (Grobbelaar 2007). In a cell the content of nitrogen can vary between 1% to more than 10%. The content varies between different groups of microalgae, for example diatoms are low in nitrogen, but it also varies depending on the supply and availability. Algae can use many forms of organic nitrogen compounds. Nitrate (NO_3^-), ammonia (NH_4^+) and urea are the compounds most often used (Grobbelaar 2007). Ammonia is often the preferred N-source for microalgae because it does not have to be reduced before amino acid synthesis (Barsanti and Gualtieri 2005). The use of both nitrate and ammonia affects the pH (Grobbelaar 2007). If ammonia is the sole N-source the pH can drop due to the release of H^+ ions and when using nitrate as sole N-source the pH increases. Some cyanobacteria also have the ability of fixating N_2 into ammonia. Generally, nitrogen should be provided in excess in cultivations to prevent it from being a limiting factor. However, nitrogen deficiency can be used to increase the cellular content of for example polysaccharides and lipids, often at the cost of decreased growth (Grobbelaar 2007). Microalgae can also use NO from flue gases as a nitrogen source but generally the amounts accessible are not enough to solely meet the nitrogen requirements of an algal cultivation (Yoshihara, et al. 1996; Sheehan, et al. 1998).

Phosphorous is an essential nutrient that often is one of the most important growth limiting factors, even though the cell contains less than 1% of phosphorous (Grobbelaar 2007). The preferred form of microalgae is orthophosphate (PO_4^{-2}). Phosphorous is important for many cellular processes, among these energy transfer and synthesis of nucleic acids. The reason phosphorous often becomes limiting is that it easily bounds to other ions, for example CO_3^{-2} and iron, which leads to precipitation which is inaccessible to cells (Grobbelaar 2007). Most microalgae can produce phosphatases on the cell surface which makes them capable of using organic phosphate as P-source (Barsanti and Gualtieri 2005). The ratio of N:P in the medium is of importance as it determines the potential productivity (Grobbelaar 2007). The so called Redfield ratio states that the ratio of carbon, nitrogen and phosphorous should be 106C:16N:1P (Grobbelaar 2007).

Trace metals are needed for some important organic molecules, such as coenzymes used in the photosynthesis (Barsanti and Gualtieri 2005) or for example Co which is essential for vitamin B_{12} production (Grobbelaar 2007). In natural waters, the availability of Fe, Mn, Zn, Cu, Co and sometimes Mo and Se can be growth limiting (Barsanti and Gualtieri 2005). In cultivations, metal chelators are often used to avoid precipitation, such as EDTA and disodium salts (Grobbelaar 2007).

Most microalgal species that have been investigated require vitamin B_{12}, around 20% require thiamine and less than 5% require biotin. These vitamins are therefore routinely added in cultivations (Barsanti and Gualtieri 2005).

2.2.3. Cultivation systems

There are basically two ways of cultivating microalgae. The easiest, cheapest, and historically mostly used are open systems. The alternative is a closed system, which is more expensive but enables better control and higher cell densities. In both types of systems illumination is needed, either directly from the sun or from lamps, generally some kind of mixing is also needed to provide a more efficient gas exchange.

Open systems can be further divided into lakes and natural ponds and different kinds of constructed ponds. These are all open to the surrounding, meaning that the risk of contamination by other

undesired species will happen sooner or later. To minimize this risk only some species can be cultivated in open systems, for example those that can live at high pH or salinity where other organisms normally do not live. Open systems offer little or no control over the growth conditions and the cell densities are generally pretty low, which makes harvesting more expensive. Evaporation can also be a major problem. The cost of building and maintaining open systems are, however, generally much cheaper than for closed systems (Tredici 2007).

Examples of natural ponds are around Lake Chad and in volcano craters in Myanmar where *Arthrospira* is cultivated (Tredici 2007). In Mexico *Arthrospira* has been cultivated commercially with a productivity of around 10 g m^{-2} d^{-1}. In addition, the largest natural ponds for commercial production are located in Australia in which *Dunaliella* are cultivated.

The constructed open ponds can be of different types, of which the so-called raceway ponds (Figure 8A), circular ponds and inclined systems are the most common (Tredici 2007). Large raceway ponds (1000-5000 m^2) are used by Cyanotech in Hawaii and Earthrise Farms in California which cultivates *Arthrospira*, (Tredici 2007; Barsanti and Gualtieri 2005). In Israel, Nature Beta Technologies Ltd. cultivates *D. salina* in raceway ponds. Long term productivity is typically around 12-13 g m^{-2} d^{-1} (Tredici 2007). Circular ponds are used in cultivation of *Chlorella* in for example Japan, Taiwan and Indonesia. Drawbacks with circular ponds are that they require a rather expensive concrete construction and more energy is needed for mixing. Inclined systems use the gravity to create mixing when the cultivation flows along a sloped surface. With inclined systems it is possible to obtain high mixing and high cell densities. The drawbacks are however among other things the cost of constantly pumping the culture to the top and high evaporation losses. Inclined systems are not in commercial use today (Tredici 2007).

Closed systems, or photobioreactors, are closed to the surrounding which greatly reduces the risk of contamination and thus enables the cultivation of practically any microalgae. Closed systems offer much more possibilities for control over the cultivation conditions and the cell densities can reach much higher values compared to open systems. Productivities often reach 30 g m^{-2} d^{-1} and in some cases even up to 130 g m^{-2} d^{-1} (Tredici 2007). Closed systems, however, often need some kind of cooling, gas exchange is much more problematic at the high cell densities obtained and fouling may be an issue. They are also much more expensive to build and operate compared to open systems. They have therefore mostly been used to produce high value products (Barsanti and Gualtieri 2005).

Photobioreactors can be divided both on their design and on their mode of operation (Tredici 2007). The design can for example be flat or tubular; horizontal, inclined, vertical or spiraled; manifold or serpentine. The mode of operation includes air or pumped mixing and single-phase or two-phase reactors. In single-phase reactors the gas exchange takes place in a separate gas exchanger while in a two-phase reactor the gas exchange takes place continuously in the reactor itself (Tredici 2007).

Tubular reactors (Figure 8B) are a commonly used type of photobioreactor in outdoor cultivations (Oilgae 2009). Tubular reactors are built of tubes with small enough diameters so that the light can penetrate to the center of the tubes and thereby maximize the surface area available for photosynthesis. Often the culture is circulated between the tubes and a tank to give the cells necessary time away from light. Some of the advantages with tubular reactors are that they can have a high productivity and a large surface exposed to light. It can, however, be difficult to control the temperature and photoinhibition is a common problem encountered. Scaled-up reactors also have problems with mass transfer of gases and distribution of light (Oilgae 2009).

Flat reactors, or flate-plate reactors (Figure 8C), uses as the name implies flat plates in which the algae is cultivated (Oilgae 2009). These reactors can yield high photosynthetic efficiencies and are claimed to be very suitable for mass cultures of algae. The oxygen accumulation is also said to be less than for tubular reactors. However, flat reactors also suffer from the problems with temperature control and scale-up (Oilgae 2009).

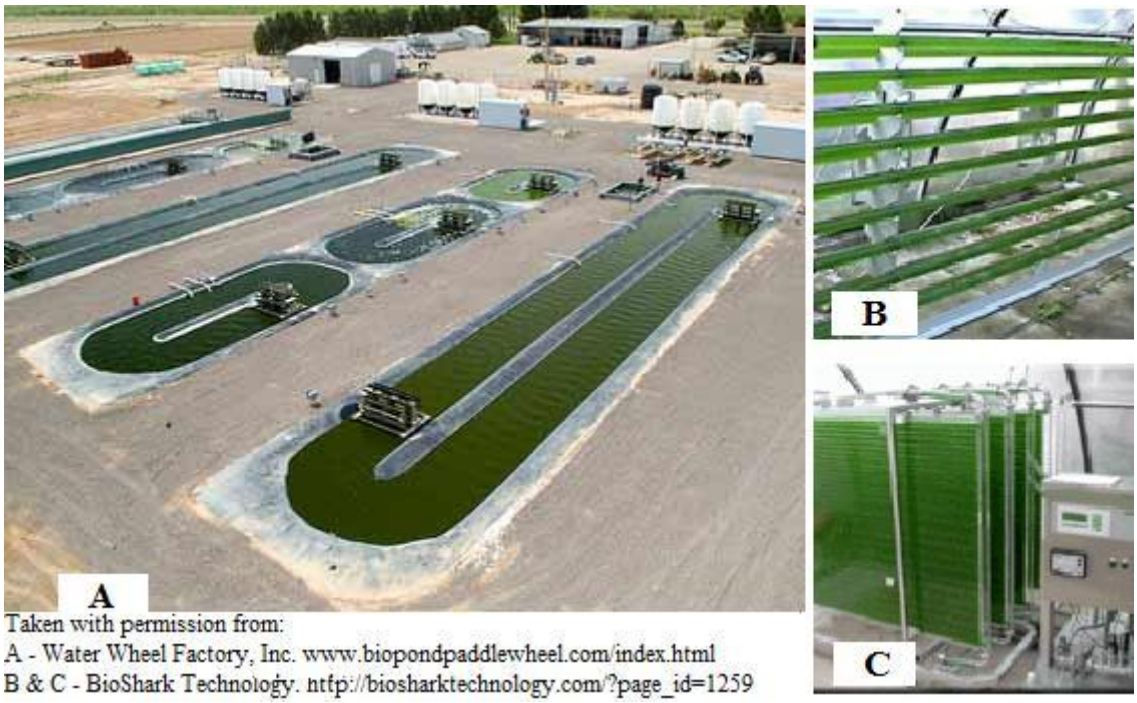


Figure 8. Different cultivation systems. Photo A shows several raceway ponds. Photo B shows a tubular reactor and photo C a flat-plate reactor.

The simplest way of cultivating algae in closed systems are probably to use vertical cylinders and sleeves (Tredici 2007). The mixing is provided solely by the injecting of compressed gas at the bottom. The reactors could, like other types of reactors, be produced by glass or plastic that can be cleaned and is used over and over again. It is also possible to create sleeves out of polyethylene bags of different sizes. Sleeve reactors have a short life due to biofouling, but are inexpensive and easy to replace (Tredici 2007). These simple reactors could be a promising alternative to the other, often quite expensive reactors.

A third alternative could be to combine an open and a closed system. A closed system, which enables more control and less risk of contamination, can be used for a cultivation during longer time to produce large amounts of biomass (Rodolfi, et al. 2008). This biomass is then transferred, preferably diluted to increase the amount of light on each cell, to an open system with N-limiting growth medium. N-limitation and light increase is a good way of increasing the lipid productivity. Since the N-limited growth in the open system only lasts for a few days there is less risk of any contaminating organisms to take over and the whole process would be cheaper than doing the same in two different closed systems (Rodolfi, et al. 2008). A combination with a closed and an open system has been done successfully by Huntley & Redalje (2007) in a commercial-scale (2 ha) production system. The average annual oil production was equivalent to >420 GJ ha^{-1} $year^{-1}$, which is more than the most optimistic estimates for biofuel production from terrestrial plants, and with a maximum production equivalent to more than 1000 GJ ha^{-1} $year^{-1}$ (Huntley and Redalje 2007). Huntley and Redalje are

convinced that a rate of 3200 GJ ha^{-1} $year^{-1}$ can be achieved using the same system with other species (they used *Haematococcus pluvialis*).

2.2.4. Harvesting biomass

When cultivating microalgae for the production of biofuels, or other products, there is a need to separate the biomass from the culture medium (Molina Grima, Acién Fernández and Robles Medina 2007). This is often a main cost contributor that can stand for 20-30% of the total cost of production. The reason for the high costs is that the microalgal cells are small and the concentrations are generally low, meaning that large volumes needs to be processed (Molina Grima, Acién Fernández and Robles Medina 2007). The main methods of harvesting are centrifugation, filtration, flocculation and flotation (Oilgae 2009).

Centrifugation uses increased gravitational forces to increase the sedimentation rate. Almost all microalgal types can be centrifuged and this has been the main method to harvest microalgal biomass. Centrifuges are generally easy to clean and sterilize and they are efficient (Molina Grima, Acién Fernández and Robles Medina 2007). However, centrifugation is energy-intensive and expensive, and is therefore more useful for high-value products. It may be too costly for lower-value products such as biofuels (Pienkos and Darzins 2009). Centrifugation can also damage the cells (Molina Grima, Acién Fernández and Robles Medina 2007).

Filtration can be used with pressure or with vacuum; it can be continuous or discontinuous (Ekendahl, et al. 2011). Filtration can be used to separate more fragile species, but the small size of many microalgal species may be a problem (Molina Grima, Acién Fernández and Robles Medina 2007). Filtration is like centrifugation expensive and generally more appropriate for high-value products.

By aggregating the cells in clumps the separation of them is made easier. Flocculation means that a polymer is added to impose aggregation whereas aggregation due to pH adjustments or electrolyte addition is called coagulation (Molina Grima, Acién Fernández and Robles Medina 2007). Large volumes can be handled by using flocculation or coagulation and has been commonly used in waste treatment ponds. The chemicals and processes used are, however, quite expensive and is only slightly less expensive than centrifugation. For high-value products it can be used as a pre-treatment to centrifugation or filtration (Molina Grima, Acién Fernández and Robles Medina 2007). The added chemicals are often difficult to remove from the separated algae (Oilgae 2009). By increasing the pH to around 12, flocculation can be induced without the addition of any other chemicals (Molina Grima, Acién Fernández and Robles Medina 2007; Rodolfi, et al. 2008). So-called autoflocculation, where the algal system flocculates on its own, can also be induced by interrupting the carbon dioxide supply (Oilgae 2009).

Froth Flotation uses an adjusted pH and air bubbling in a column to create a froth in which the algae accumulates and can be removed (Oilgae 2009). The pH required depends on the species. Dissolved Air Flotation uses a combination of froth flotation and flocculation and can remove 70-90% of the algae from a cultivation (Oilgae 2009).

The most promising low-cost alternative for biofuels production is according to Pienkos and Darzins (2009) to take advantage of natural sedimentation, possibly enhanced by autoflocculation or pH induced flocculation. Flotation using small amounts of chemical flocculants could also be cost-effective. However, more research is needed to bring down the costs of harvesting (Pienkos and Darzins 2009).

After harvesting of the biomass, there is still typically around 90% (w/w) of water in the microalgal slurry (Kumar, et al. 2010). Generally, the biomass needs to be further dried to avoid the slurry from being spoiled. Sun-drying is cheap but requires a large area. Spray-drying and freeze-drying are effective but expensive and more applicable for high-value products (Molina Grima, Acién Fernández and Robles Medina 2007). After the drying it may be of interest to disrupt the algal cells to release intracellular components. The method used to disrupt the cells depends on how the microalgal cell wall is constructed and the nature of the desired component. Common mechanical methods include homogenizers and bead mills. Chemical methods include alkaline lysis using hydroxide, which is an effective and inexpensive method, and organic solvents (Molina Grima, Acién Fernández and Robles Medina 2007).

2.3. Biofuels from microalgae

Biofuels can be defined as a solid, liquid or gas fuel consisting of, or derived from biomass (Patil, et al. 2008). According to Patil et al (2008) only biodiesel and bioethanol are today produced on an industrial scale. These so called first generation biofuels are obtained from food crops such as for example rapeseed or sugarcane. Biofuels produced from non-food products, such as microalgae, are generally called second generation biofuels (Patil, et al. 2008). Others consider fuels derived from non-food parts of food crops and lignocellulosic biomass to be second generation while fuels from algae are called third generation biofuels (John, et al. 2011) Apart from biodiesel and bioethanol, microalgae could also be used to produce biogas such as methane or hydrogen gas (Benemann 2007).

2.3.1. Biodiesel

The global biodiesel market increases with an average annual growth of 42% and is estimated to reach 37 billion gallons (~140 billion liters) by 2016 (Gouveia och Oliveira 2008). Food crops can be used to produce biodiesel but microalgae seems to be the only renewable source that has the potential to completely replace petroleum-derived transport fuels without competing with food production (Gouveia och Oliveira 2008).

A microalga contains various lipid types, such as polar phospholipids and neutral triglycerides. For biodiesel production the triglycerides are the main precursor (Huang, et al. 2010; Verma, et al. 2010). To convert the algae to biodiesel or a bio-oil there are different ways to go, *e.g.* transesterification, thermochemical conversion or hydroprocessing.

To obtain biodiesel the lipids in the algal cells are subjected to a transesterification process (Oilgae 2009; Verma, et al. 2010). Transesterification basically means that the lipids are chemically converted to its corresponding fatty esters (Oilgae 2009). This needs to be done to reduce the viscosity of the algal oils and to increase the fluidity (Huang, et al. 2010). Algal oils are generally much more viscous than petroleum-based diesel and cannot be used directly in engines. In the transesterification process, the triglycerides react with an alcohol, *e.g.* methanol, often with help from a catalyst to yield biodiesel. Alkali catalysts are most commonly used commercially due to higher reaction rates and conversion compared to acid catalysts. However, free fatty acids in the oil react with the alkali catalyst and forms glycerin and water, thereby removing the catalyst from further reactions (Huang, et al. 2010). If the concentrations of free fatty acids are higher than 4-5% the oil first is needed to be pretreated in an acid esterification process (Oilgae 2009; Huang, et al. 2010). After the transesterification process the biodiesel can be separated and the glycerin can be used to make for example soap (Oilgae 2009).

A thermochemical conversion process uses high temperatures in absence of oxygen and can be divided into pyrolysis and direct hydrothermal liquefaction (Carlsson, et al. 2007). Pyrolysis uses temperatures around 350-530°C in which different fractions are created where oils and gases can be recovered

(Carlsson, et al. 2007; Huang, et al. 2010). Before pyrolysis the biomass needs to be dewatered. In liquefaction, a lower temperature and high pressure is used (~300°C and 10 MPa) and it is possible to use wet biomass, which means that the costs can be reduced. The oils produced from either of these processes are derived from all organic compounds within the algal cells and not only lipids which mean a higher overall yield (Carlsson, et al. 2007).

Another alternative is to use catalytic hydroprocessing, which is a conventional petroleum refining process (Pienkos and Darzins 2009; Carlson, et al. 2010). The microalgal triglycerides are converted to n-alkanes by adding hydrogen gas under high temperature and pressure to produce biodiesel, gasoline or jet fuel (Carlson, et al. 2010).

In microalgae lipids function as membrane components, storage products, metabolites, and energy source (Becker 2007). The lipids can be divided into polar and non-polar, where polar lipids such as phospholipids and glycolipids are found in the membranes and where non-polar lipids mainly consists of triglycerides and free fatty acids (Becker 2007).

Griffiths and Harrison (2009) have summarized the literature values for lipid content and doubling time for several algal species. The highest lipid content for a certain species can be obtained by using different cultivation conditions such as nitrogen or silicon deficiencies. Griffiths and Harrison have listed the average values for laboratory cultivations from literature and in Table 1 are the highest average values for lipid content listed together with the doubling time in nutrient replete cultivations (Griffiths and Harrison 2009). Of these species, *C. cryptica* and *N. salina* are the ones with the highest lipid content found, which is obtained under deficient conditions, silicon and nitrogen respectively. High lipid content can improve the efficiency of biomass processing (Rodolfi, et al. 2008).

Table 1. Average laboratory lipid content and doubling time summarized from literature by Griffiths and Harrison, 2009. The highest lipid contents are obtained at three different conditions: a) Nutrient replete, b) Nitrogen deficient or c) Si deficient. The doubling time are obtained from nutrient replete cultivations.

Species	Highest lipid content (% dw)	Growth condition	Doubling time (h)
D. tertiolecta	18	Nitrogen deficient	11
P. tricornutum	26	Nitrogen deficient	25
C. cryptica	38	Silicon deficient	13
A. platensis	13	Nutrient replete	14
D. salina	19	Nutrient replete	11
R. reticulata	Not reported	Not reported	Not reported
N. salina	46	Nitrogen deficient	Not reported

The lipid productivity is another important aspect. An increase in lipid content is often counterbalanced by a decrease in growth (Griffiths and Harrison 2009). Of the species encountered in this thesis *Tetraselmis suecica* has the highest lipid productivity according to the summary by Griffiths and Harrison (2009). *N. salina* and *P. tricornutum* are two others that perform well in this respect (Griffiths and Harrison 2009). Also Rodolfi et al (2008) recommends *N. salina* due to its high lipid productivity, but also because it has the rather unusual ability to synthesize lipids not only at the expense of other cellular components but from newly fixed carbon. However, the small size makes harvesting more expensive and the tough cell wall and presence of polyunsaturated fatty acids could also be a problem for biodiesel production (Rodolfi, et al. 2008).

The algal oils used for biodiesels need to be compatible with the engines used (Rodolfi, et al. 2008). For example, there are restrictions on the level of total unsaturation (iodine value). In terms of fatty

acid profile for producing biodiesel, *Nannochloropsis* sp. and *D. tertiolecta* can be used if combined with other microalgal oils and/or vegetable oils (Gouveia och Oliveira 2008)

2.3.2. Bioethanol

Bioethanol is a supplement or replacement to ordinary gasoline and is the biofuel that is thought to be the most widely used (John, et al. 2011). Ethanol is generally not used on its own in engines but most often mixed with unleaded gasoline (Oilgae 2009). Ethanol is a high octane fuel and replaces lead as an octane enhancer in gasoline. It also reduces the price of the fuel and reduces the amount of harmful emissions. The most commonly used mixtures of ethanol and gasoline is E10 and E85. E10 consists of 10% ethanol and 90% of gasoline. This fuel can be used in all vehicles, without any modifications, that normally run on gasoline. E85 consists of 85% ethanol and 15% gasoline and only specially adapted vehicles can use it, so called Flexible Fuel Vehicles. These vehicles can normally use straight gasoline and mixtures up to 85% ethanol (Oilgae 2009).

The ethanol produced today is mainly derived from food crops that are fermented in similar processes as for beer and wine making (Oilgae 2009). The use of food crops to produce fuels is problematic since this could create rising food prices and shortages (John, et al. 2011; Oilgae 2009). It is also more or less impossible to produce enough ethanol from food crops to be able to replace gasoline since food crops requires large amounts of arable land. Chisti (2008) has estimated that if sugarcane would be grown to produce ethanol, around 60% of the total cropping area in the U.S. would be needed to cover the energy usage equivalent to the biodiesel used in the U.S. Algae can be used to produce ethanol without competing for arable land with food crops and without the need to remove rainforest on behalf of sugarcane production (John, et al. 2011).

Like in beer and wine fermentations the carbohydrates in algae is converted by yeast into ethanol. Carbohydrates in microalgae are in the form of starch, cellulose, sugars and other polysaccharides (Becker 2007). The amount of carbohydrates in algae depends on, as for all cellular components, on species. Typically the amount is between 10-60% in dry matter (Becker 2007; Harun, et al. 2010).

The carbohydrates needs to be hydrolyzed into simpler sugars, in a process called saccharification, before they can be fermented. This can be done by for example acid or enzymatic hydrolysis. If an amylase producing yeast is used the disrupted algal cells can fermented in a single step (John, et al. 2011). A possibility would be to separate the lipids from the algae to first produce biodiesel and that the carbohydrates are then used to produce ethanol (Oilgae 2009).

2.3.3. Biogas and hydrogen

Algae could also be used to produce methane, *i.e.* biogas, and hydrogen gas.

Methane gas, CH_4, is most often used in the form of the fossil fuel known as natural gas. It is burned in gas turbines or steam boilers to produce electricity, compressed it can be used as a vehicle fuel and in many countries it is used domestically for heating and cooking purposes (Oilgae 2009). Burning methane, which is the simplest hydrocarbon, produces more heat per mass unit and less carbon dioxide than any other hydrocarbon. Due to its excellent burning characteristics it is popular as cooking gas and it is claimed to be more environmental friendly as a vehicle fuel compared to gasoline and diesel. Natural gas is, however, a fossil fuel and thus unsustainable. Bio-methane on the other hand can be produced by for example anaerobic fermentation of biomass, *i.e.* anaerobic digestion. Producing methane from algae can be seen as the simplest way of turning algae into fuel and can potentially yield high amounts. Theoretically methane can be produced by anaerobic fermentation from all the

constituents of the alga – carbohydrates, lipids and proteins. Methane could also be produced from algae by pyrolysis or gasification (Oilgae 2009).

Hydrogen gas, H_2, has several favorable properties as a fuel but also several disadvantages. Among its favorable properties are that it is non-toxic and produces no CO_2 emissions when used. It contains large amounts of energy per unit weight and it is also easily converted to electricity in fuel cells (Oilgae 2009; Carlsson, et al. 2007). Among the disadvantages are that H_2 is difficult to store and transport and that it is extremely reactive and flammable. To be able to use H_2 as a fuel a new and expensive infrastructure needs to be constructed (Oilgae 2009; Carlsson, et al. 2007). Hydrogen gas could be produced by microalgae in three different ways. Green algae can under certain circumstances produce H_2 but this has, despite extensive research, not yet been proved to be practically feasible (Benemann 2007). The second way to produce H_2 from microalgae is to gasify the biomass into syngas, which is a mixture of CO and H_2 (Oilgae 2009). The gasifying of the biomass is done by applying heat and pressure to the biomass together with steam and controlled amounts of oxygen. The H_2 is then separated by absorbers or special membranes. Large plants are needed to make this process economically feasible. The third way is to ferment the algal biomass into methane gas and by steam reformation turn the methane into hydrogen gas instead. This is the most common way of producing H_2 and also the cheapest (Oilgae 2009).

3. Methods

3.1. Marine algal strains and pre-cultures

After a literature search fourteen species were selected and obtained for further investigation, see Table 2 below. They were chosen on the basis of success in previous studies regarding high lipid or carbohydrate content, tolerance to flue gas components and/or good growth. These species were all grown in flasks under similar conditions as those in the experiments, see chapter 0, but without any forced gas flow and with 16 hours light and 8 hours dark cycles. This was made to get a first idea of which species that seemed to thrive and grow fastest, but also to maintain and obtain enough volume of them for the later experiments. A standard f/2 medium with artificial seawater (Instant Ocean) were used, see Appendix A. Species nr 2, 3 and 12 in the table below also required an addition of silica. The salinity was set to approximately 2.5% to mimic the salinity of the Swedish west coast and this salinity was also used in the later experiments. All of these species were also grown in 1%, 0.25% salinity and in fresh water medium (3N-BBM +V recipe taken from CCAP (CCAP - Culture collection of algae and protozoa 2012), see Appendix A) to see how well they coped and if they might be interesting for further investigation in a parallel master thesis (Bark 2012) working with fresh water species. The salinity was measured with a conductivity meter (WTW LF318). Only visual growth was investigated. Seven species were then chosen for further experiments, no. 1-7 in Table 2 below.

Table 2. Species and strain name of the fourteen selected microalgae species. No. 1 - 7 were chosen for further experiments.

	Species	Strain name	Species reproduce in Sweden*
1	*Dunaliella tertiolecta*	GUMACC5	Yes
2	*Phaeodactylum tricornutum*	GUMACC2	Yes
3	*Cyclotella cryptica*	CCMP333	Yes
4	*Arthrospira platensis*	CCMP1295	Yes
5	*Dunaliella salina*	CCAP 19/30	Yes
6	*Rhinomonas reticulata*	GUMACC127	Yes
7	*Nannochloropsis salina*	CCMP1777	Yes
8	*Tetraselmis suecica*	CCAP 66/4	Yes
9	*Tetraselmis sp.*	Strain T_A, Isolate from Eko Agus Suyono, Gadjah Mada Unversity, Indonesia	Unknown
10	*Chlorella salina*	CCAP 211/25	Yes
11	*Chaetoceros muelleri*	CCAP 1010/3	Yes
12	*Isochrysis galbana*	CCAP 927/14	Yes
13	*Nannochloropsis limnetica*	CCMP505	Unknown
14	*Nannochloropsis oculata*	GUMACC104	Yes

*** Reproduction information is taken from Dyntaxa (Artdatabanken - Swedish Species Information Center 2012).**

3.2. Experimental setup of cultivation system

The setup is a modification from Valadi et al. (Valadi, et al. 2001). Twelve 250 ml Erlenmeyer flasks (E-flasks) were lined up in two rows, six in each row, see Figure 9 and Figure 10. Two fluorescent lamps (Osram L 18W/77 Fluora) were put on both outer sides of the flasks and two were put above them. The lamps were lit during 20 hours and off during 4 hours each day of cultivation. The irradiance on the flasks was approximately 15 W/m2. The irradiance was measured with a spectroradiometer (Optronic Laboratories, model 754-O-PMT) between 250 and 800 nm. The flasks

were sealed with a rubber stopper into which metal tubes were inserted. One tube directed the flue gas into the medium. This tube was prolonged with a rubber tube with a hole in it and lay on the bottom of the flask in order to distribute the gas more evenly and impose agitation of the cells. A second tube allowed for sampling and a third for outflow of gas. The components of the flue gas was mixed by coupling the different flows together and the gas was then led through filters (Acro 50, 0.2 µm PTFE filters) into a flask containing de-ionized water to humidify the gas in order to minimize evaporation from the culture flasks. The gas was then led to the culture flasks. The outflow was filtered using a potassium permanganate filter to remove SO_2. The experimental setup was shared with a parallel study using fresh water algae (Bark 2012).

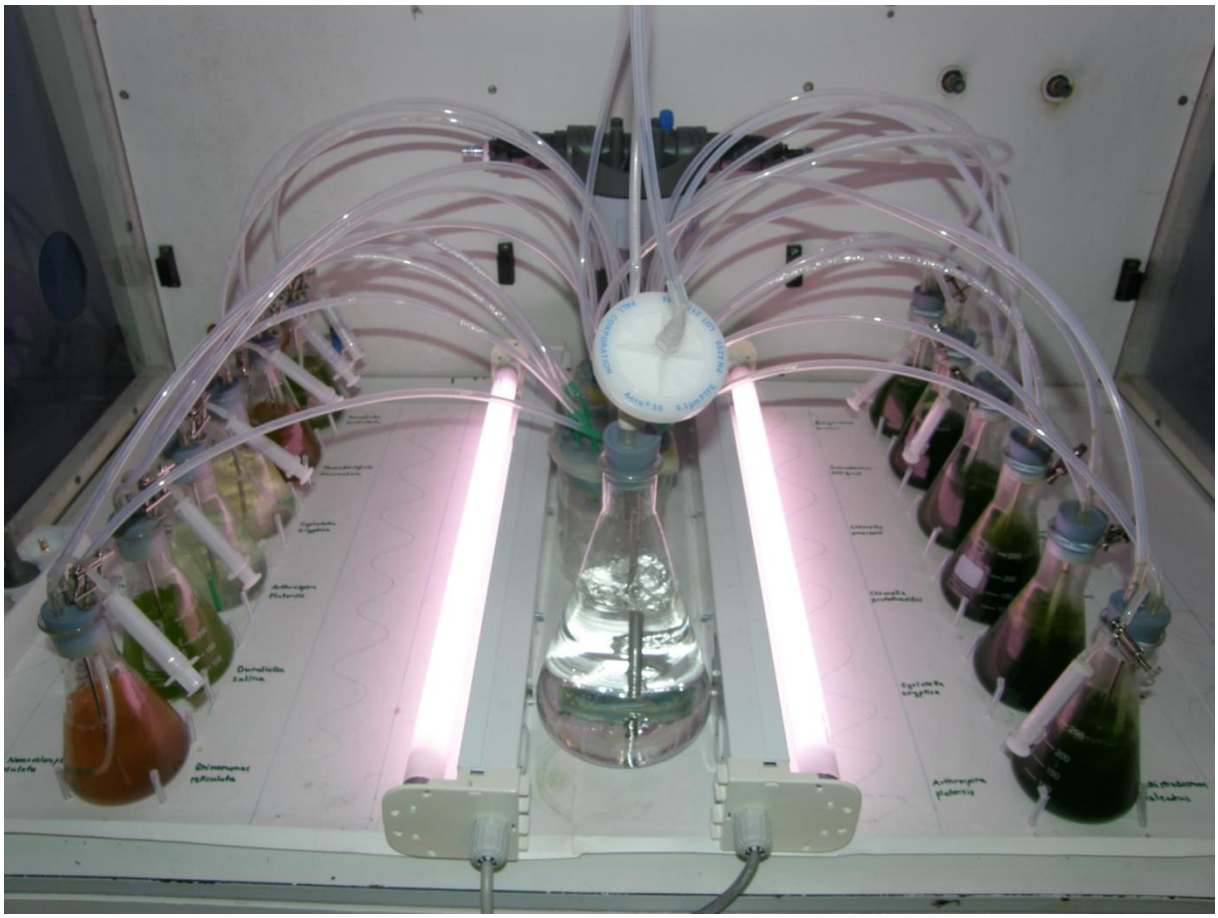


Figure 9. Experimental setup. The large E-flask is the gas humidifier. The gas first goes though a filter and then bubbles through de-ionized and autoclaved water and continues to the gas distributer, which is standing behind the gas humidifier. From the gas distributer the gas is distributed to the twelve E-flasks. The outflow from the twelve E-flasks is returned to a beaker, identical to the gas distributer, to bring together the outflows to one single outflow that is led to a sulfur dioxide filter and then released into the fume hood.

An artificial flue gas was used resembling actual flue gas from Swedish paper mills. The components of the artificial flue gas were limited to carbon dioxide, nitric oxide and sulfur dioxide and the levels was determined to be 15 %, 100 ppm and 10 ppm, respectively (see also Results section). The gases were delivered separately in three standard gas tubes (Air Liquide) with flow meters and diluted with compressed air to obtain the correct concentrations. The concentrations in the gas tubes were 99% CO_2, 1732 ppm NO and 979 ppm SO_2. The total gas flow was measured with a Gilian Gilibrator 2 Primary flow calibrator (Bubble Generator (Standard flow), Range: 20 cc – 6 LPM). The combined gas concentrations were measured with a Vaisala Carbocap Hand-held carbon dioxide meter GM70, an Eco Physics CLD 700 El-ht Chemiluminescence NO/NO_x Analyzer and a Thermo Environmental Instruments Inc. 43C SO_2 Analyzer.

Figure 10. Experimental setup; A close-up photo of the experimental setup. The long silver colored metal tube is the sampling tube which is connected to the syringe that can be seen in the top right corner. The rubber tube to the right of the sampling tube is leading the inflow of gas to the bottom of the E-flask where it is allowed to bubble up through the cultivation. To the right of the sampling tube the tube leading the outflow of gas can be seen.

3.3. Pre-tests for establishing cultivation system and gas inflow

1) Approximately a total of 3.5 l/min of gas flow, with around 15% CO_2, 100 ppm of NO and 10 ppm of SO_2 was used from start. To the E-flasks with 200 ml f/2 medium 20 ml algal suspension was added. For this test species no. 1 to 6 in Table 2 was used.

2) Approximately a total of 2 l/min of gas flow and approximately 7.5% of CO_2 were let to flow in the E-flasks containing only 180 ml of media for about 90 min. The pH were after this 5.5 – 5.6 in the flasks. Algal suspension was added, 40 ml to each flask, for species no. 1 to 6 in Table 2. The reason for this test was to test if a lower total gas flow, lower concentration of CO_2, a postponed start of NO and SO_2 and a larger part of added algal suspension give a more favorable pH than obtained in the previous pre-test (1).

3) A continuation of pre-test 2. The cultivations had all lost color and looked weak. Approximately 30 ml of suspension was removed from all of the flasks and a new 50 ml of algal suspension was added to each one to try and recover them. The reason for this test was to see if fresh algal suspension could be able to recover a poor cultivation.

3.4. Changes in pH by inflow of gas

1) A test of the buffering capacity of natural seawater versus f/2 media.

Two different types of seawater were used. The first was water taken from 40 m depth at Kristineberg 2010-03-12 and the second was surface water taken from Långedrag 2010-01-30. Kristineberg is a field station located at Fiskebäckskil, Lysekil, on the west coast of Sweden. Långedrag is located in Gothenburg, also on the west coast of Sweden. The outflow from the experimental setup was lead to

an E-flask containing seawater or f/2 media for one hour. The gas flow was less than 2 l/min and CO_2 around 8%.

2) A test of the pH effect of SO_2 in f/2 media.

This test was performed in one of the E-flasks of the experimental setup. The total gas flow was around 2 l/min with approximately 8% CO_2, around 100 ppm NO and 0-10 ppm of SO_2 for two hours. The pH was first measured with the amount of SO_2 set to zero after this the media was replaced and the amount of SO_2 set to 10 ppm.

3.5. Flue gas cultivation - Experimental run 1

In experimental run 1 six of the species were tested, no. 1-6 in Table 2 above. The cultivations started with 220 ml of medium and 30 ml of algal suspension in each E-flask. The total gas flow was initially set to zero and then after half an hour a flow of 0.8 l/min of compressed air was used. The total flow was after little more than one day increased to a total of 1.4 l/min and CO_2 was raised incrementally. NO and SO_2 was switched on at a later stage in the experiment, see Table 3. Two refills with fresh medium were made to ensure sufficient nutrient supply. The first refill was made at approximately 142 hours from start; around 50 ml fresh medium was added, giving a total of approximately 250 ml in the E-flasks. The second refill was made at approximately 284 hours from start; around 70 ml of the cell suspension was first removed and around 100 ml fresh medium added, giving a total of approximately 250 ml in the E-flasks. In experimental run 1 the pH dropping was counteracted by 1M NaOH. For information on when this happened during the experiment and at what times different samples were taken see Table 23 in Appendix DAppendix .

Table 3. Experimental run 1; Total gas flow and gas concentrations throughout the experiment. For CO_2 the concentration was measured twice, a first time when the new concentration was set and a second time before the concentration was raised again.

Time from start (h)	Total flow (l/min)	CO_2 (%)	NO (ppm)	SO_2 (ppm)
0	0	0	0	0
0.5	0.8	0	0	0
25	1.4	1.0–2.0	0	0
96	1.4	2.9–3.2	0	0
121	1.4	5.9-6.5	0	0
168	1.4	9.1–9.5	0	0
194	1.4	12.3–12.7	0	0
261	1.4	15	0	0
308	1.4	15	100	0
311	1.4	15	100	10
356	1.4	15	100	10

3.6. Flue gas cultivation - Experimental run 2

In experimental run 2 seven of the species were tested, no. 1-7 in Table 2 above. In experimental run 2 Hepes buffer was used to avoid a pH drop, like the one experienced in experimental run 1, see chapter 4.7 and Figure 13. The cultivations started with 220 ml of medium and 30 ml of algal suspension in each E-flask. The total gas flow was initially set to 1.4 l/min of compressed air. The concentrations of CO_2, NO and SO_2 were set according to Table 4.

Table 4. Experimental run 2; Total gas flow and gas concentrations throughout the experiment. For CO_2 the concentration was measured twice, a first time when the new concentration was set and a second time before the concentration was raised again.

Time from start (h)	Total flow (l/min)	CO_2 (%)	NO (ppm)	SO_2 (ppm)
0	1.4	0	0	0
1.5	1.4	0.95 – 1	0	0
23	1.4	3.3	0	0
44	1.4	6.7 – 7.5	0	0
69	1.4	15.6 – 15.9	0	0
141	1.4	15.6 – 15.9	100	10
362	1.4	15.6 – 15.9	100	10

3.7. pH measurements and analyses of cell concentration and biomass

pH measurements were taken regularly throughout the experiments with a SymPhony SP80PD pH-meter. A sample of approximately 2 ml was taken from each cultivation into a test tube 1-3 times a day except during weekends.

Optical density was measured, generally 1-2 times a day except during weekends, as the absorbance at 750 nm (Griffiths, Garcin, et al. 2011) in 1 ml cuvettes (VARIAN Cary 50 with software Advanced Reads or PERKIN-ELMER Lambda 9 UV/VIS/NIR Spectrophotometer with software Lambda 19) with sterile f/2 medium as blank. The average of two measurements (read twice each time) with mixing in between was done on the samples to obtain reliable data. In experimental run 2 the samples were directly frozen after sampling due to unavailability of the spectrophotometer.

Cells were counted with a Leitz Wetzlar Dialux 20 microscope with 40x magnification in a Bürker counting chamber, in which 15 squares were counted to give a mean value. This mean value was multiplied with $4x10^6$ to give total amount of cells per ml. The samples were first frozen and measured after both of the experimental runs were ended. For the first experimental run samples were measured from the second medium refill and to the end (from 260 to 356 hours from start). For the second experimental run samples were measured throughout the experiment.

Whatman GF/C filter were used for the dry weight analysis. The filters were first labeled and pre-conditioned in a room with constant temperature and humidity, 50 ± 5 % relative humidity and 23 ± 2 ºC, overnight and after this weighed. From each cultivation 20 ml of sample were filtered and then washed twice with 10 ml of NH_4HCO_3 to remove salt and nutrients from the medium (Zhu and Lee 1997). The filters were dried at 100°C for 2.5 h and then returned to the constant room with 50 ± 5 % relative humidity and 23 ± 2 ºC overnight and after this re-weighed. Duplicates were made for each sample. For the first experimental run the dry weight was measured at the end (356 hours from start). For the second experimental run the dry weight was measured in the middle (140 hours from start).

Dry weight was measured to determine the densities in the cultivations in g/l. The optical density at the same time point was also used to connect dry weight and optical density. Estimations on dry weight were then calculated from the optical density measurements at other time points. These estimations were used to calculate the amount of triglycerides, proteins and carbohydrates in g/gDW.

The time it takes for a cell population to double is called generation time or doubling time (Madigan and Martinko 2006). When a population doubles with a regular time interval the growth is exponential. By plotting the cell numbers, or optical density, on a logarithmic scale and time on an

arithmetic scale (a semilogarithmic graph) the specific growth rate can be calculated for sections of the growth curve that show exponential growth. The slope of a fitted line during exponential growth is equal to the specific growth rate. By dividing the natural logarithm of 2 by the specific growth rate the doubling time for that period is given.

In both experimental runs specific growth rates were calculated for both cell count and optical density for sections showing exponential growth.

3.8. Analyses of macromolecular composition

Samples were taken at two different time points during the cultivations, frozen directly, and all samples were analyzed after the experimental runs were finalized.

The quantification of triglycerides was done by hexane extraction from algal biomass followed by acid esterification with methanol and analysis of silated fatty acid esters with GC/MS (Agilent technologies, 5975 inert XL Mass selective detector) as described in (Söderberg, Utvinning och kvantifiering av triglycerider i algodlingar - Extraction and quantification of triglycerides from algacultures 2011). For estimating the total weight of triglycerides from the glycerol standard an estimated average weight of 870g/mol was used (Söderberg, Utvinning och kvantifiering av triglycerider i algodlingar - Extraction and quantification of triglycerides from algacultures 2011). See Appendix G for more information.

The carbohydrate analysis was performed according to the phenol-sulphuric acid method by Herbert et al (1971) with glucose as standard; see Appendix F for more information. Measurements were performed in microtiter plates and the absorbance measured at 488 nm in a FLUOstar Omega plate reader (BMG LABTECH).

The proteins were extracted by 1 % SDS and analyzed on the supernatant by the method of Lowry (Lowry, et al. 1951) using the Biorad Dc kit (BioRad) with Bovine Serum Albumin as standard. The absorbance was measured at 750 nm in a FLUOstar Omega plate reader (BMG LABTECH). For the cultures with HEPES buffered medium a precipitation step was done before the analysis with 13.6 mg/l deoxycholic acid followed by 5.8 % trichloroacetic acid. The kit was used directly on the protein pellet. For further details see Appendix H.

3.9. CHN-analysis

The CHN-analysis was performed at the end for both experimental runs. As much sample as possible were collected in 50 ml Falcon tubes which were centrifuged at 6000 rpm for 5 min. The supernatant was removed and the pellet re-suspended in distilled water up to approximately 50 ml again. This solution was then centrifuged again at 6000 rpm for 5 min and the supernatant removed. The pellets were then dried inside the tubes at 60°C until completely dry. The dried pellets were roughly grinded with a spoon, packed in metal foil and measured in the CHN-analyzer (Leco CHN 2000).

3.10. Test of growth in waste water

Two different kinds of waste water were obtained from the paper mill Nordic Paper Bäckhammar, Sweden and composition is given in Table 5. The first one, called “RIA”, is water that has gone through a treatment plant to remove nitrogen and phosphorous from the water using biological treatment and precipitation. This water is normally emitted to the recipient. The second one, called “Reactor 2”, is water taken after the biological treatment but before the precipitation step.

Table 5. Analysis of the actual wastewaters from Nordic paper Bäckhammar. Analysis performed by AK Lab AB (Borås, Sweden).

	RIA	Reactor 2
Total-N, mg/l	4.2 ±11%	13 ±11%
Total-P, mg/l	0.13 ±11%	1.8 ±11%
Nitrate-N (NO_3-N), mg/l	<500 ±14%	<500 ±14%
Ammonium-N(NH_4-N) mg/l	2.3 ±14%	2.3 ±14%
Phosphate-P (PO_4-P), mg/l	0.043 ±16%	0.48 ±16%
Sulfate (SO_4), mg/l	248 ±12%	169 ±12%
BOD7 , mg/l	9±14%	68 ±14%
pH (25°C)	4.7	6.7

The plus/minus percentage gives the uncertainty of measurement.

Both of these waste waters were mixed 50/50 with natural seawater, obtained from Kristineberg, 2010-03-12 at 40 meters depth. This solution was poured in small flasks and inoculated with all the different algae species from Table 2 to find out which species that seems most suitable for this kind of medium. The algal suspensions were centrifuged and re-suspended in the seawater solution to remove nutrients from the f/2 medium.

4. Results

4.1. Flue gas and waste water data from Swedish paper mills

The gas effluents from paper mills vary depending on what type of kiln that is used and what type of fuel that is burned. Data from several mills were requested (Appendix B) and the data received are presented in Table 6. The level of CO_2 vary between 0-24% with the highest levels coming from lime kilns. The mean value for the data is about 11% (median 12%). The levels for the simulated flue gas to be used in the experiments were selected based on the average calculated on the data received first, rounded off to be 15% CO_2, 100 ppm NO, and 10 ppm SO_2.

Table 6. Gas effluents from Swedish paper mills. Mean values for the components at the bottom of the table. The artificial flue gas in this study was based on first mean values. The second mean values consist of all values received, after the artificial flue gas concentrations were decided.

Pulp- and paper mills	Type of kiln	CO_2 (%)	CO (ppm)	NO_X (ppm)	NO (ppm)	NO_2 (ppm)	SO_2 (ppm)	O_2 (%)
Billerud Karlsborg	Bark	11.4	189		75.7		9.9	8.8
Billerud Gruvön	Bark	13.0		50.0			< 1	
Billerud Gruvön	Recovery boiler	18.0		70.0			< 1	
Billerud Gruvön	Lime kiln	24.0		140			< 1	
Billerud Gruvön	Gas destruction	6.0		80.0			5.0	
Arctic Paper Munkedal	Kiln	14.5	30.0	134	134			1.6
Arctic Paper Grycksbo	Oil			65.4	65.4			5.0
Stora Enso Skoghall	Kiln 11	15.0	82.0	50.0	47.0	2.3	7.0	5.4
Stora Enso Skoghall	Kiln 12	12.0	281	66.0	65.0		0.5	8.6
Stora Enso Skoghall	Lime kiln	20.4	104	20.6	21.0		1.0	5.1
Stora Enso Skoghall	Recovery boiler	13.8	20.0	75.7	75.4		0.6	5.7
Stora Enso Skoghall	Gas	6.0	1.9	22.3	22.0		0.1	10.5
Munksjö Aspa Bruk	Recovery boiler	14.3		68.0				
Nordic Paper Bäckhammar	Recovery boiler	13.1	64.5	68.7	64.2		28.3	6.1
Nordic Paper Bäckhammar	Lime kiln			180			3.0	
Nordic Paper Bäckhammar	Bark	9.9	159	86.0	71.1		11.4	10.0
Nordic Paper Bäckhammar	Flash dryer			15.0			12.0	
Holmen Paper Braviken	Solid fuel	10.0	200	87.0		87.0	5.6	
Holmen Paper Braviken	Oil	0.01		121		121	15.7	
Holmen Paper Hallsta	Kiln		680		52.7	3.4	9.8	
Waggeryd Cell	Flash dryer	0.3	18.0	3.3			2.0	20.4
Rottneros	Recovery boiler	15.1	7.2		70.7		25.4	
Rottneros	Kiln						1.5	
VIDA AB	Sulphite kiln	13.0	300		50.0		60.0	
VIDA AB	Solid fuel	12.0	250		100			
Munksjö Paper Billingsfors	Recovery boiler min.	7.9	118		65.1		9.9	
Munksjö Paper Billingsfors	Recovery boiler max.	9.3	272		73.4		33.7	
Munksjö Paper Billingsfors	Solid fuel min.	5.1	407		104		22.0	
Munksjö Paper Billingsfors	Solid fuel max.	10.8	1135		124		29.8	
Munksjö Paper Billingsfors	Mix min.	4.7			131		14.5	
Munksjö Paper Billingsfors	Mix max.	7.7			148		41.1	
Mean value (from first data received)		13.3	214	73.4	77.1		9.0	
Mean value (total after all data received)		11.1	227	73.8	77.0	53.4	12.5	

Blank boxes indicate that compound data either not were available or measured.

The wastewater streams from the investigated paper mills vary depending on where in the system it is taken. The levels also vary over time. The paper mill Waggeryd Cell had by far the highest values, both for nitrogen and phosphorous, which greatly affects the mean values at the bottom of Table 7. With all data the mean values are 9 mg/l for nitrogen and 1.4 mg/l for phosphorous, which is comparable with the levels in the f/2 medium used in the experiments (12.4 mg/l nitrogen and 1.1 mg/l phosphorous), see Appendix A. Without the data from Waggeryd Cell, the mean values are around 5 mg/l nitrogen and 0.6 mg/l phosphorous.

Table 7. Levels of nitrogen and phosphorous in wastewater streams from Swedish paper mills.

Pulp- and paper mill companies	Wastewater	Nitrogen (mg/l)	Phosphorous (mg/l)
Billerud Karlsborg	Total	3.0	0.4
Billerud Gruvön	Total	2.1	0.3
Billerud Gruvön	To biologic purification	4.6	1.6
Billerud Gruvön	From biologic purification	7.8	1.0
Stora Enso Skoghall	Total	3.5	0.3
Munksjö Aspa Bruk	Total	3.0	0.8
Munksjö Aspa Bruk	Total	4.0	1.0
Nordic Paper Bäckhammar	RIA	1.9	0.3
Nordic Paper Bäckhammar	Reactor 2	2.0	0.02
Holmen Paper Braviken	Total (to receiving waters)	6.0	0.3
Holmen Paper Hallsta	Total	8.0	0.9
Smurfit Kappa Kraftliner Piteå	Total	3.2	0.9
Waggeryd Cell	To sewage plant minimum	18.0	5.0
Waggeryd Cell	To sewage plant maximum	64.0	10.0
Rottneros	Sewage water	1.4	0.6
Munksjö Paper Billingsfors	Total minimum	6.9	0.1
Munksjö Paper Billingsfors	Total maximum	13.0	0.1
Mean value		9.0	1.4

4.2. Visual salinity test in pre-cultures

Waste waters generally have a low salinity which means that to be able to use marine species a mixture of seawater and waste water is needed. This would generate brackish water and therefore the selected strains were tested in marine medium with different salinities. The visual salinity test on all the fourteen species showed that most of them grow best in the higher salinity medium, that most can grow successfully at lower salinities, and that fresh water medium generally is not preferred, see Table 8. This means that many of these species probably could grow in different mixtures of seawater and possible waste waters. These are, however, just preliminary results. The sole parameter considered was visual growth. More thorough investigations are needed to ensure these results.

Table 8. Visual salinity test of growth in pre-cultures. The species were grown in fresh water medium, 3N-BBM+V, and three different salinities of f/2 medium.

Species	Growth performance			
	3N-BBM+V	f/2 2.5 ‰	f/2 10 ‰	f/2 25 ‰
Dunaliella tertiolecta	+	+++	++	+++
Phaeodactylum tricornutum	-	+	+	+++
Cyclotella cryptica	-	+	+++	+++
Arthrospira platensis	-	+++	+++	+++
Dunaliella salina	-	++	++	+++
Rhinomonas reticulata	-	-	-	+++
Nannochloropsis salina	++	+++	++	++
Tetraselmis suecica	-	+	+	+++
Tetraselmis sp.	-	+	+	+++
Chlorella salina	++	++	+	+
Chaeotoceros muelleri	-	-	Not determined	+++
Isochrysis galbana	-	-	-	++
Nannochloropsis limnetica	-	++	-	++
Nannochloropsis oculata	-	-	-	++

Scale: ”-” = ”poor” to ”+++ ” = ”very good”.

4.3. Test of waste water

The objective for this test was to get an idea whether the species could grow in waste water from a paper mill. A mix with seawater was used to get a brackish salinity that typically would be found in the Baltic Sea. This could be a possible way of cultivating marine species at larger scale using natural seawater to obtain salinity and also possibly nutrients, while the bulk of nitrogen and phosphorous comes from the waste waters.

The test of waste water was limited to visual examination of growth. In Figure 11A are the two types of waste water without the addition of seawater or algae seen. The waste water “RIA” seems to enable some growth based on the color of the flasks, see Figure 11B. For the waste water “Reaktor 2” it is harder to say whether growth has occurred or not due to that this waste water from start was more colorful and contained particles, see Figure 11C. The growth was slower than for the f/2 media used, which is reasonable since this media is developed to sustain growth of marine algae. It should be said that the algae did not have any time to adapt to the waste waters beforehand. A more promising result could perhaps be obtained if the algae were accustomed to the waters for a longer period. In Appendix B are photos from the first and the 16th day after inoculation that can be compared to the photos here. Further experiments need to be made to conclude whether the species can grow in it or not.

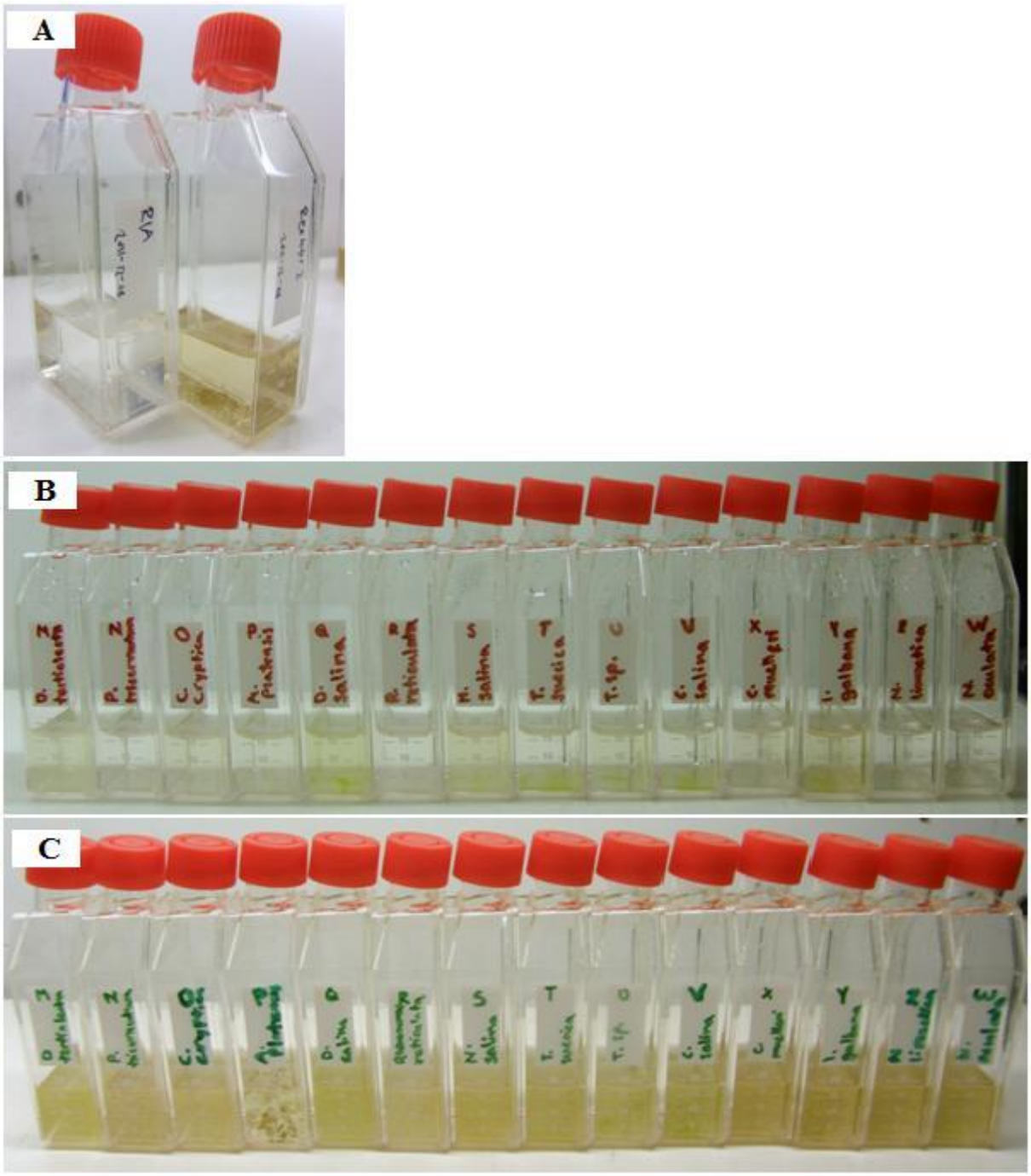


Figure 11. Test of waste water; A - Waste water "RIA" to the left and "Reaktor 2" to the right. This is unsterilized waste water without any seawater or added algae. B - Waste water "RIA" mixed with seawater and inoculated with algae. The photo is taken a little more than one week after inoculation. C - Waste water "Reaktor 2" mixed with seawater and inoculated with algae. The photo is taken about one week after inoculation. The species are placed in the same order, left to right, as in Table 2.

4.4. Pre-test 1 for establishment of cultivation system

To establish the performance of the constructed cultivation system a pre-test was done with six marine species (*D. tertiolecta*, *P. tricornutum*, *C. cryptica*, *A. platensis*, *D. salina* and *R. reticulata*) with a total gas flow of approximately 3.5 l/min distributed to twelve flasks (including six fresh water species from the parallel study) with 15% CO_2, 100 ppm of NO and 10 ppm of SO_2. The light intensity was approximately 45 W/m^2.

The starting OD was low, below 0.1 for all the six species tested in this stage, even if the intention was to have a starting OD at 0.2, but the algae was however visible to the naked eye in the E-flasks from start. After one day of cultivation the color had diminished in all flasks but the optical density was however similar or even a bit higher than from start. The pH was measured in the E-flasks after one day of cultivation and was found to be very low, in the range of 3.9-4.96, which could be compared to the pH of medium which is 8.53 for f/2 and 8.37 for f/2 +Si. After two days of cultivation the carbon dioxide tube was depleted by either a leakage or the gas tube was not as full as anticipated and the cultivation test was terminated. The diminished color of the algae may possibly be caused by death of algae as a result of the low pH.

4.5. Pre-test 2 and 3 for establishing regime of gas inflow

To avoid the large pH drop seen in the first pre-test, the total gas flow and the starting concentrations of CO_2, NO and SO_2 were lowered in pre-test 2 and 3. The total gas flow was 2 l/min, and from start the CO_2 was 7.5% and no NO and SO_2 was included. In addition, a larger volume of cell suspension was added to start the cultivations.

Two days from start the cultivations had lost most of its color, only the *D. tertiolecta* culture had a faint green color and pH in the range of 4.0-5.2. A 4 hour dark period was started, and after this the pH in the cultivations were further decreased to 3.7-4.9, of which the *D. salina* culture showed the lowest and the *R. reticulata* culture the highest pH value. To try and save the cultivations, 30 ml was removed and 50 ml of new algae suspension was added. The following day all cultivations again looked very pale. Over the weekend all cultivations had lost color and looked dead. pH was from 2.5 to 3.0. The lowered gas flow and levels of components in the gas was done to try and maintain a higher pH but it was found that this was not enough. The conclusion from this is that the gas concentrations should be increased step by step. A too large increase will rapidly lower the pH and inhibit the algae.

An additional problem discovered was the measurements for *C. cryptica*. This alga grows in a filamentous like way, which makes it difficult to obtain accurate samples. The samples content of cells can vary depending on how much of or how many filaments of *C. cryptica* that is extracted. Since the filaments are not evenly distributed in the flasks the samples will vary a lot and the results on biomass and cellular composition will not be reported.

4.6. Changes in pH by inflow of gas

Since the pre-tests indicated that the gaseous compounds in the inflow affect the pH of the media additional tests were done.

1) A test of the buffering capacity of natural seawater versus f/2 media.

A pH measurement was made before and one after the addition of gas, see Table 9. The pH decreased for all three tested solutions, but most for the f/2 media even though this had the highest starting pH. This should mean that f/2 based on artificial sea water has a lower buffering capacity than natural seawater.

Table 9. Additional pH-test; Test of the buffering capacity of two different types of natural seawater and f/2 media.

	Seawater 40 m	Seawater surface	f/2
pH before gas	7.86	7.63	8.50
pH after 1 h with gas	5.91	5.78	5.52
% pH of starting value	75%	76%	65%

2) A test of the pH effect of SO_2 in f/2 media.

The pH was measured twice with, and twice without, SO_2 in the gas flow, see Table 10. The results show that the addition of 10 ppm of SO_2 does not change the pH after two hours. This implicates that SO_2 does not have a major impact on pH, at least not after two hours. However, the effect of SO_2 might not be seen so soon. The CO_2 is thought to stand for the rapid pH drop to around 5.5, but we have seen in our experiments that the pH drops to around 2.5, which cannot be due to CO_2. Another possible contribution may be from NO, which forms HNO_3 in the media.

Table 10. Additional pH-test; Test of the effect of SO_2 on f/2 media after 2 h of bubbling in f/2 medium.

pH of f/2 media: 8.45		
pH after:	Without SO_2	With SO_2
1h	5.45	5.48
2h	5.47	5.48

4.7. Effects of flue gas on pH and growth, experimental run 1

Based on the experiences from the pre-tests the influence of the simulated flue gas on growth and cellular composition was studied in two experimental runs with increasing CO_2 levels and NO and SO_2 included at the end of the runs. The results on the effects of the simulated flue gas on pH, cell count and optical density will first be presented for experimental run 1, then for experimental run 2. A comparison of the growth measured as cell count and optical density is made at the end of each section. The components of the simulated flue gas was not turned on at full concentrations from start; how the CO_2 was increased and when the NO and SO_2 was switched on for experimental run 1 can be seen in Figure 12.

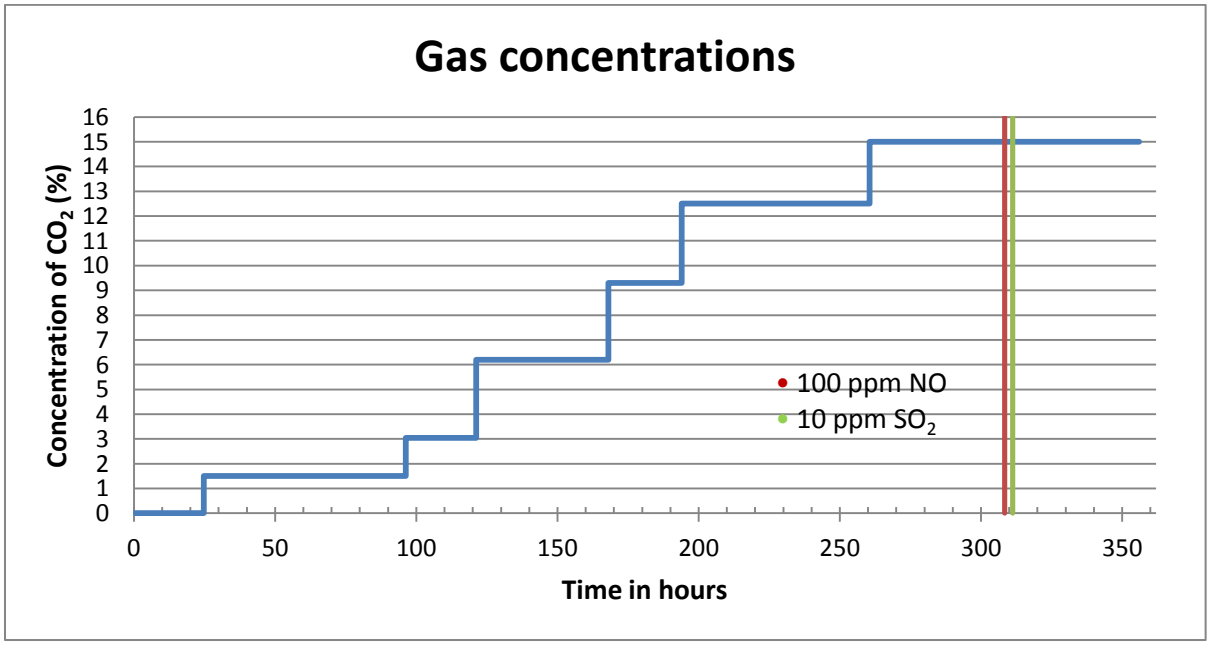


Figure 12. Gas concentrations used in experimental run 1; The blue line shows the incrementally increasing CO_2 concentration. The red line shows the time point in which 100 ppm NO was switched on and the green line where 10 ppm SO_2 was switched on.

4.7.1. pH-measurements

For experimental run 1 the pH started at a high value and decreased rapidly at similar levels for all strains, see Figure 13. Unfortunately, the measurements between 23 and 145 h were lost. At 168 h the pH was increased by addition of 2 ml of 1M NaOH. The pH was after this fairly stable around pH 6.5.

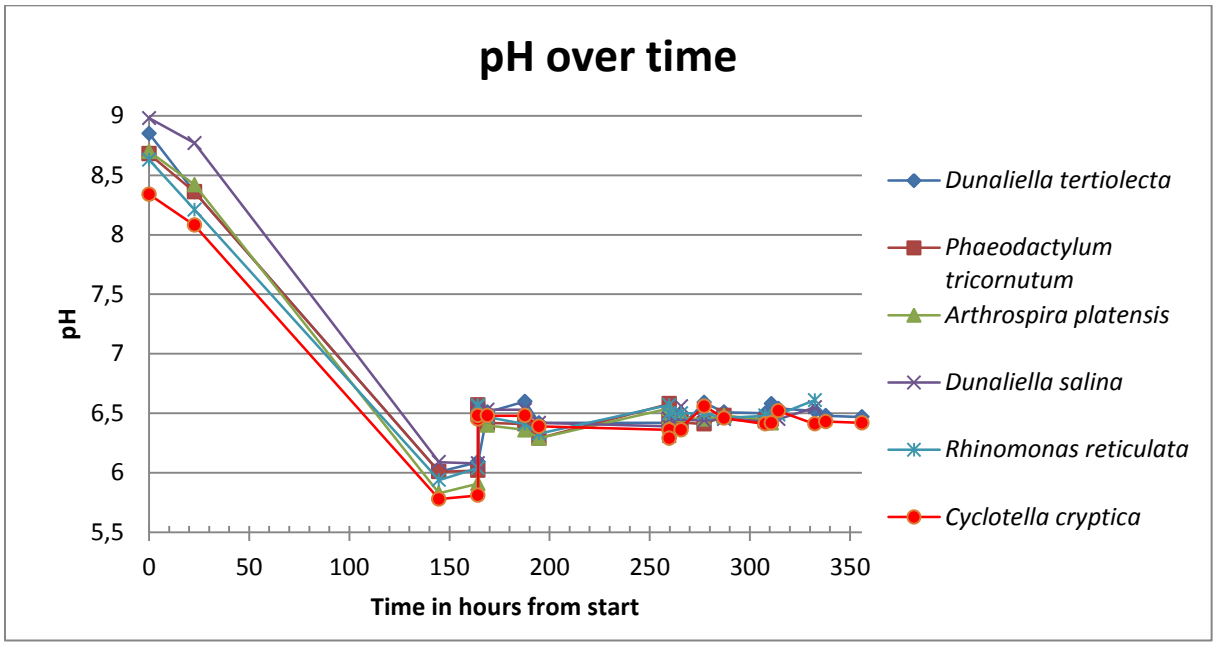


Figure 13. Experimental run 1; pH measured over time in hours from start. The pH drops are during the period of increasing the CO_2 concentration. The sudden raise in pH at 169 h is due to the addition of 1M NaOH.

4.7.2. Growth performance evaluated by cell count measurements

The cells were counted starting at approximately 260 hours from start (Figure 14 and Appendix I). The cultivations were after this refilled with fresh medium to ensure that the growth would not be limited, thereof the initial large decrease in cell count. From the second point, at 284 hours from start, no further medium refills were made. It can be seen that *P. tricornutum* had the highest cell count throughout the cultivation and that this species is the one with the longest sustained increase in cell count. This is also one out of two species, together with *R. reticulata*, that did not show decreasing cell count in the last measurement.

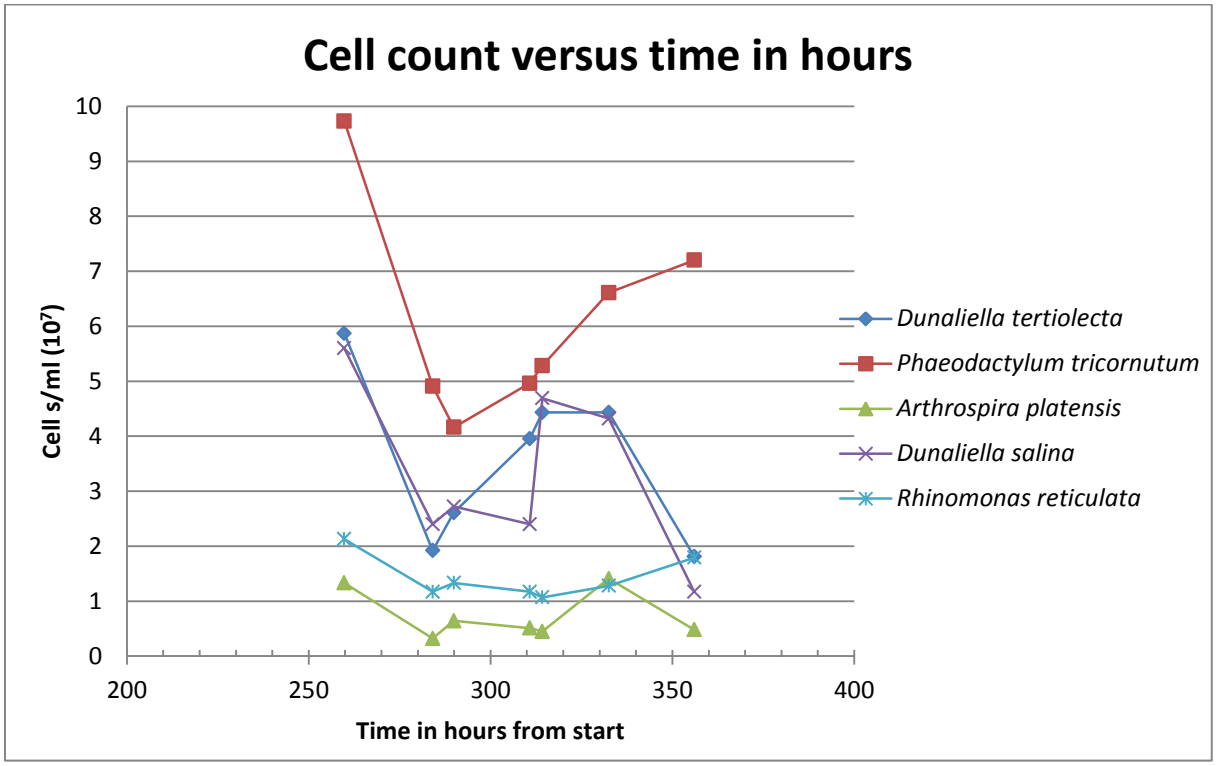


Figure 14. Experimental run 1; Cell count versus time in hours from start. The cell count started at 260 h from start and the next sample is at 284 h which is after the second refill with fresh medium.

The specific growth rates and doubling times were calculated and can be seen in Table 11. The exponential growth phase for *D. tertiolecta* has the largest slope and gives therefore the highest specific growth rate and lowest doubling time, but this applies only for a little more than 30 hours. For *D. salina* linearity could be found if the deviating data point at 311 h was removed. The corrected exponential growth phase for *D. salina* is similar to that of *D. tertiolecta*, with a slightly higher doubling time. For *P. tricornutum* two exponential growth phases have been identified. The first stretches from time point 290 – 356 and is the longest fitted trend line for experimental run 1. The second corresponds to a shorter time period (311 – 333 hours) within the first were the specific growth rate is higher.

It can be seen in Figure 24 in Appendix I that the growth for *P. tricornutum* slowed down towards the end. The decrease in growth seems to start approximately 24 h after the switching on of NO and SO_2, at around 333 hours from start. The same decrease in growth can be seen for both the species of *Dunaliella*, but the decrease starts earlier than for *P. tricornutum*. The decrease start somewhere after 314 h where the exponential growth phases for both species of *Dunaliella* ends. This is about 6 h and 3 h after NO and SO_2 respectively were switched on. After about 333 hours from start the cell count declines for the *Dunaliella* species which does not occur for *P. tricornutum*. This indicates that both of the *Dunaliella* species, at these conditions, cannot cope with the concentrations of NO and SO_2 used. However, they both seem to tolerate the CO_2 concentration and have the highest doubling rates in this experiment. *D. tertiolecta*, *D. salina* and *A. platensis* all showed decreased cell counts for the last measurement. This indicates that these cultivations were in poor condition and that something in the growth conditions are inadequate or too stressful.

The results for *R. reticulata* were quite different. First towards the end a sustained increase could be seen, before this the growth was irregular and the cell count increased and declined alternately. The

doubling time at the end was approximately 56 h, close to the best doubling time for *P. tricornutum*. This indicates that *R. reticulata* could cope with the concentrations used but need a longer period to adjust. It would have been interesting to run the experiment for a longer period of time to see if the late increase continues.

The cell count for *A. platensis* was very irregular, with values going constantly up and down. This can be interpreted as the conditions were not favorable for this species. However, an error in measurement could have occurred due to that *A. platensis* can form gas vacuoles and thereby float, which could be seen in the cultivations. This could mean samples taken were not representative for the culture.

Table 11. Growth in experimental run 1; Data from the trend lines from fig. "Ln cell count versus time in hours" in Appendix I.

1st trend line	*D. tertiolecta*	*P. tricornutum*	*A. platensis*	*D. salina**	*R. reticulata*
Time points (h)	284 - 314	290 - 356	-	284 – 314	314 - 356
Spec. growth rate, μ (h^{-1})	0.025	0.009	-	0.022	0.012
Doubling time (h)	27	80	-	31	56
R^2 value	0.970	0.957	-	1	0.990
2nd trend line	*D. tertiolecta*	*P. tricornutum*	*A. platensis*	*D. salina*	*R. reticulata*
Time points (h)	-	311 - 333	-	-	-
Spec. growth rate, μ (h^{-1})	-	0.013	-	-	-
Doubling time (h)	-	54	-	-	-
R^2 value	-	0.997	-	-	-

***With the deviating data point at 311 h removed.**

4.7.3. Growth performance evaluated by optical density

In Figure 15 an increase in optical density indicating an initial growth can be seen for all species, except for *A. platensis*. At 145 hours from start is the first data point after the first refilling of media, which explains the sudden decrease in optical density. The second large decrease can be seen at time 284 hours which is the first data point after the second refilling of media. This is also the first point after the amount of carbon dioxide has been raised to 15%. From here on the measurements has also been made with another spectrophotometer which may affect the results. At 311 hours from start was the first point after switching on NO and at 314 hours is the first point after switching on SO_2. The data for optical density can be found in Appendix J.

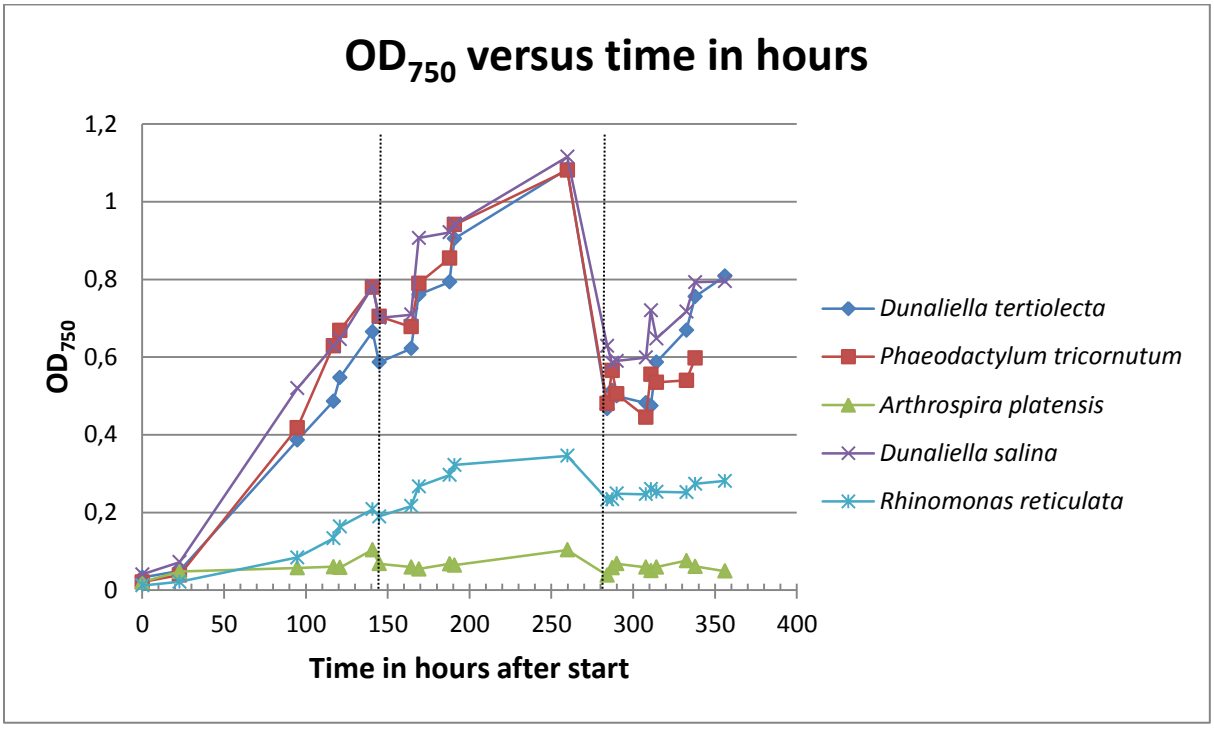


Figure 15. Cell concentration in experimental run 1; Optical density at 750 nm versus time in hours from start. The black, vertical dotted lines shows were the additions of fresh medium were made.

After the first refilling of medium the specific growth rate decreased for all species (*A. platensis* has no calculated growth rate before this), as can be seen in Table 12. After the second refilling of medium the specific growth rates for *P. tricornutum*, *A. platensis* and *R. reticulata* seemed to have decreased even further. *D. tertiolecta* and *D. salina* have slightly higher specific growth rates after the second refilling.

P. tricornutum has the highest specific growth rate, giving a doubling time of about 23 h, while *R. reticulata* has the slowest (apart from *A. platensis*) with a doubling time of 34 h. These high specific growth rates in the beginning could be quite expected since the CO_2 was increased incrementally, resulting in higher pH from start and that NO and SO_2 not yet have been switched on.

From the first measurement after the first medium refill, at 145 h, the specific growth rates quite dramatically decreases for *D. tertiolecta*, *P. tricornutum*, *D. salina* and *R. reticulata*. *R. reticulata* increased its doubling time to 61 h while the others all go over 130 h which almost can be considered stationary. The concentration of CO_2 was still quite low when this decrease started, at 145 hours the concentration was approximately 6.2%. NO and SO_2 was still not on. A possible reason could be a low pH. The pH at 145 h was around 6 for all species. Since several of the pH values from the start are missing it is not clear at what time the pH reached this level. At 168 h the pH was raised to 6.5 but this is still quite low. If the pH dropped to this low level close to 145 h this is a probable reason for the decreased specific growth rate.

Table 12. Growth in experimental run 1; Data from the trend lines from fig. "Ln OD_{750} versus time in hours" in Appendix J.

1st trend line	*D. tertiolecta*	*P. tricornutum*	*A. platensis*	*D. salina*	*R. reticulata*
Time points (h)	23 - 141	0 - 121	169 - 260	0 - 117	0 - 141
Spec. growth rate, μ (h^{-1})	0.023	0.030	0.007	0.025	0.020
Doubling time (h)	30	23	102	28	34
R^2 value	0.968	0.995	0.981	0.989	0.997
2nd trend line	***D. tertiolecta***	***P. tricornutum***	***A. platensis***	***D. salina***	***R. reticulata***
Time points (h)	145 - 260	164 - 260	284 - 290	145 - 260	145 - 191
Spec. growth rate, μ (h^{-1})	0.005	0.004	0.098	0.004	0.011
Doubling time (h)	131	169	7	173	61
R^2 value	0.849	0.788	0.954	0.773	0.931
3rd trend line	***D. tertiolecta***	***P. tricornutum***	***A. platensis***	***D. salina***	***R. reticulata***
Time points (h)	314 - 356	-	311 - 333	314 - 356	287 - 356
Spec. growth rate, μ (h^{-1})	0.008	-	0.017	0.005	0.002
Doubling time (h)	88	-	41	136	315
R^2 value	0.930	-	0.934	0.821	0.769

After the second refill of media both the species of *Dunaliella* had low specific growth rates, but higher than after the first refill. The optical density values for *P. tricornutum* at this phase went up and down but the general feeling is that the culture is in a more or less stationary phase. The same can be said for *R. reticulata*, but indications for a low growth rate could be seen. The optical density measurements for *A. platensis* display varying values; the short exponential growth phases that can be identified seem more or less random. Both species of *Dunaliella* are arguably the most promising when looking at optical density, since they show the highest specific growth rates at the end of the experiment when all gases were at their highest concentrations.

In the middle of experimental run 1 the spectrophotometer that had been used became unavailable. This led to that a different spectrophotometer had to be used for the remaining measurements. This is not preferable since different machines can differ. One sample was measured with both machines to estimate the difference; this can be seen in Table 33. The value for optical density is lower with the second spectrophotometer for all species except *A. platensis* where the value is more or less the same. This means that the samples measured with the second spectrophotometer are underestimated compared to if measured with the first spectrophotometer.

4.7.4. Comparison of growth characteristics determined by different analytical methods

When comparing the results regarding specific growth rate and doubling time between cell count and optical density at 750 nm clear differences were seen, see Table 13. Since cell count only was performed towards the end of the experiment no comparisons can be made before 284 h. The time

spans in which linearity of logarithmic cell concentration data could be found did not correspond well between cell count and optical density.

Table 13. Experimental run 1; Comparison of the doubling times found for both cell count and optical density at 750 nm.

Species	Cell count		OD_{750}	
	Trend line (h from start)	Doubling time (h)	Trend line (h from start)	Doubling time (h)
D. tertiolecta	284 - 314	27	314 - 356	88
P. tricornutum	290 - 356 311 - 333	80 54	- -	- -
A. platensis	- -	- -	284 - 290 311 - 333	7 41
D. salina	284 – 314*	31	315 - 356	136
R. reticulata	314 - 356	56	287 - 356	315

***With the deviating data point at 311 h removed.**

4.8. Effects of flue gas on pH and growth, experimental run 2

Below are the results on the effects of the simulated flue gas on pH, cell count and optical density for experimental run 2. In contrast to experimental run 1, cell count was measured throughout experimental run 2, enabling more comparison between the two types of growth measurement. The components of the simulated flue gas was not turned on at full concentrations from start; how the CO_2 was increased and when the NO and SO_2 was switched in can be seen in Figure 16.

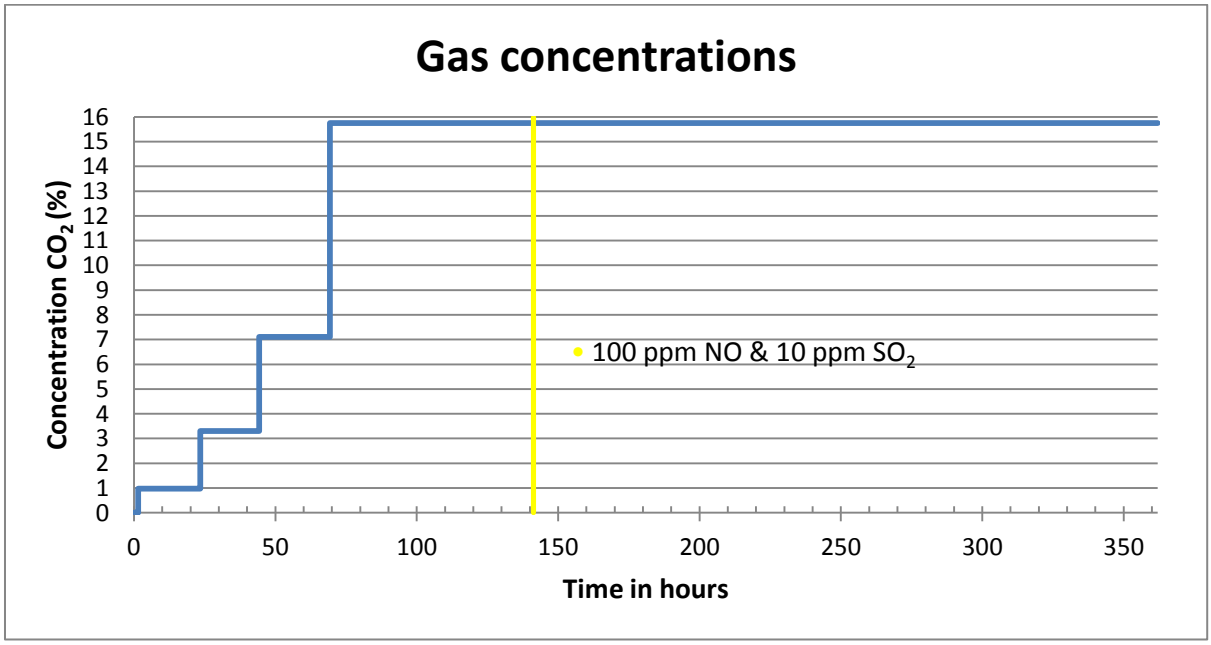


Figure 16. Gas concentrations used in experimental run 2; The blue line shows the incrementally increasing CO_2 concentration. The yellow line shows the time point in which 100 ppm NO and 10 ppm SO_2 was switched on.

4.8.1. pH-measurements

For experimental run 2 the pH starts at a lower value compared to the first run, see Figure 17. Hepes buffer was added in this run which gave less decrease in pH with increased gas concentrations. The pH landed on around pH 7 when all gases were at their final concentrations. For more detailed information see Appendix E.

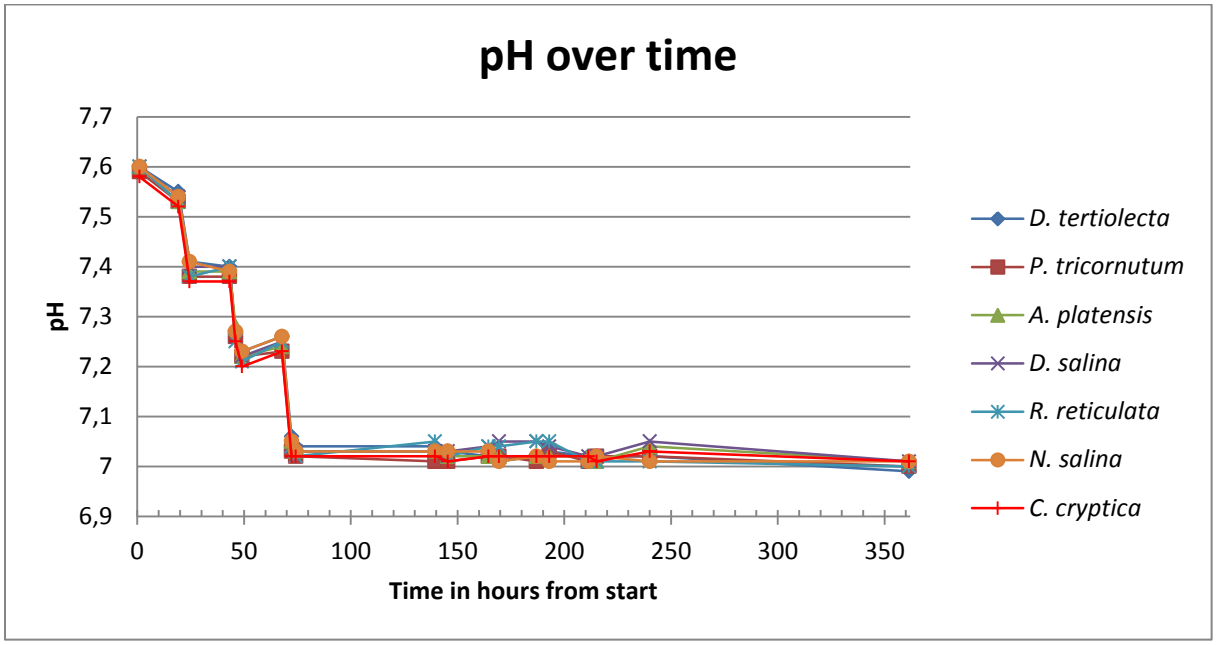


Figure 17. Experimental run 2; pH measured over time in hours from start. The pH drops as the CO_2 concentration increases and lands close to pH 7.

4.8.2. Growth performance evaluated by cell count measurements

In Figure 18 it can be seen that *N. salina* had the highest cell count throughout the measurements and that this was the only one with sustained increase in cell count. All of the other species showed at least one decrease in cell count during the experiment. For all species, except *N. salina* and *P. tricornutum*, a decrease in cell count in the last measurement was seen. This indicates that these cultivations were in poor condition and that something in the growth conditions were inadequate or too stressful. The data regarding cell count can be found in Appendix I.

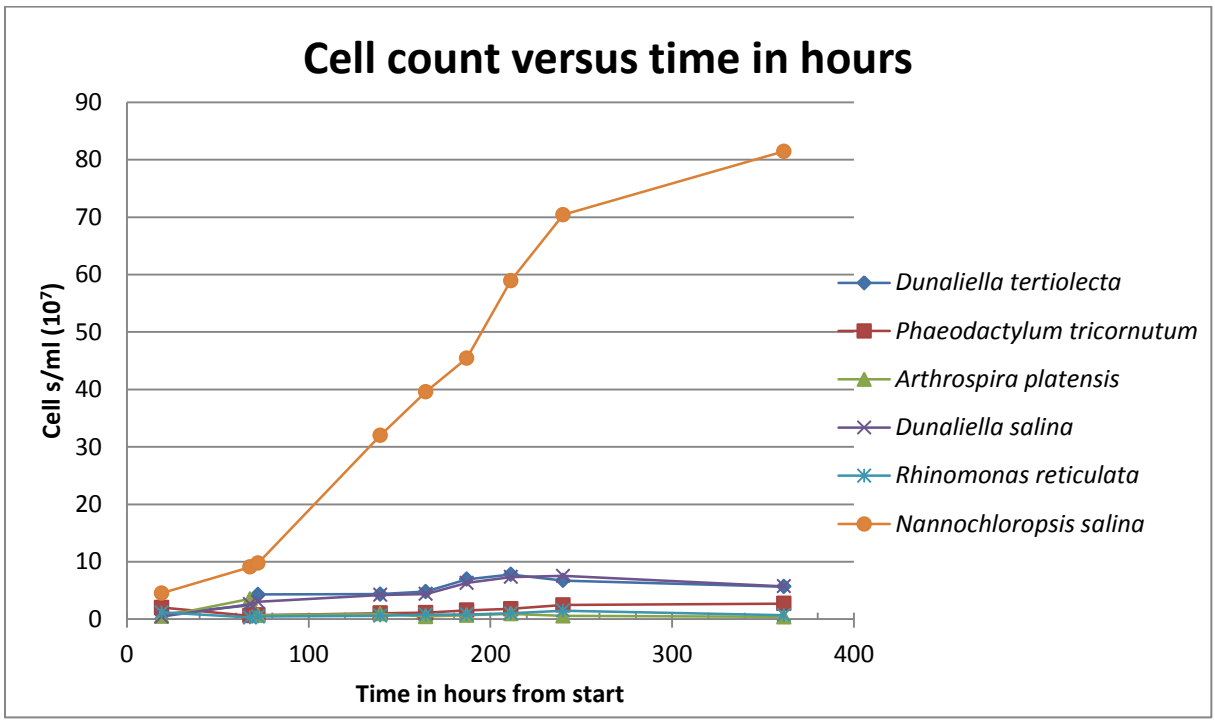


Figure 18. Experimental run 2; Cell count versus time in hours from start.

All species have at least one identified exponential growth phase, which can be found in Table 14 where the calculated specific growth rate and doubling time is presented.

D. tertiolecta, *D. salina* and *N. salina* all three showed similar growth characteristics. An initial growth increase that was relatively high, followed by a lag phase (no obvious lag phase for *N. salina*) and thereafter a lower growth increase. *P. tricornutum* and *R. reticulata* showed the opposite by first having a decline in cell count then a relatively low increase followed by a higher increase.

At 140 hours, when *N. salina* started to decrease in growth, was when NO and SO_2 was switched on. At 73 hours, when *D. tertiolecta* and *D. salina* started to decline in growth, was right after the carbon dioxide was raised to 15.6 – 15.9%.This indicates that for *N. salina* the addition of SO_2 and NO imposes a stress which lowers the specific growth rate. For both of the *Dunaliella* species, the high amount of CO_2 seemed here to be the reason for lower specific growth rate. The new growth increases for the both *Dunaliella* species started at approximately 24 h after the addition of NO and SO_2 which suggests that these gases do not affect them that much. The later decrease in growth could, however, be partly due to these gases.

Table 14. Growth in experimental run 2; Data from the trend lines from fig. "Ln cell count versus time in hours" in Appendix I.

1st trend line	***D. tertiolecta***	***P. tricornutum***	***A. platensis***	***D. salina***	***R. reticulata***	***N. salina***
Time points (h)	19 – 72	68 - 140	165 - 211	19 - 72	72 - 165	19 - 140
Spec. growth rate, μ (h^{-1})	0.027	0.007	0.015	0.036	0.005	0.016
Doubling time (h)	26	98	46	19	128	42
R^2 value	0.925	0.955	0.991	1	0.937	0.996
2nd trend line	***D. tertiolecta***	***P. tricornutum***	***A. platensis***	***D. salina***	***R. reticulata***	***N. salina***
Time points (h)	165 - 211	165 - 240	-	72 - 165	187 - 240	140 - 240
Spec. growth rate, μ (h^{-1})	0.010	0.010	-	0.004	0.012	0.008
Doubling time (h)	67	70	-	165	59	87
R^2 value	0.906	0.979	-	0.970	0.991	0.993
3rd trend line	***D. tertiolecta***	***P. tricornutum***	***A. platensis***	***D. salina***	***R. reticulata***	***N. salina***
Time points (h)	-	68 - 240	-	165 - 211	-	-
Spec. growth rate, μ (h^{-1})	-	0.008	-	0.011	-	-
Doubling time (h)	-	90	-	63	-	-
R^2 value	-	0.979	-	0.937	-	-

P. tricornutum and *R. reticulata* showed the opposite by first having a decline in cell count then a relatively low increase followed by a higher increase. This could be interpreted as the cells need a longer period to adjust to the conditions. But the conditions are altered continuously. It could also be so that the altered conditions are more favorable for them. At around 73 hours the highest amount of carbon dioxide was just put on, this was when these two start their first increase. The NO and SO_2 was

put on at 141 hours, their specific growth rates increased at 165 hours for *P. tricornutum* and 187 hours for *R. reticulata*.

After 240 h the growth for *N. salina*, *P. tricornutum* and *R. reticulata* slowed down a great deal. For *R. reticulata* the next measurement was even lower than the one for 240 h. The same could be seen for the both *Dunaliella* species, but at one data point earlier, at 211 h.

As in experimental run 1, the both *Dunaliella* species showed the fastest doubling time. A curious result is that the doubling time generally is the same or even higher in experimental run 2. One could expect that the specific growth rate and doubling time would be improved in experimental run 2 since the pH was both higher and more stable. For *N. salina* no such comparison was possible since it was not included in experimental run 1.

The fact that the samples were frozen before measurement could have affected the results. Different species may cope with freezing differently, some might be more resistant and the cell count is not affected while others might be sensitive and thereby render lower than actual cell counts. In Appendix I the effect of freezing can be seen on optical density. This makes comparisons between species difficult. One could expect, however, that since all samples have been treated the same way that the differences are the same between samples within a species. The actual number of cells might be lower, but trends and differences between samples should still be the same.

4.8.3. Growth performance evaluated by optical density

N. salina was the only species that showed consecutive increasing optical density values, see Figure 19 and Table 15. *N. salina* also reached by far the highest value; it did however start with the highest optical density. All species, except *N. salina* and *P. tricornutum*, showed a decrease in optical density in the last measurement. The decrease in optical density for all others indicates that these cultivations were in poor condition and that something in the growth conditions was inadequate or too stressful. The data for optical density can be found in Appendix J.

D. tertiolecta and *D. salina* both showed a high specific growth rate in the beginning of this experiment, as in experimental run 1, with a doubling time of about 24 hours. The doubling time was lower here than in experimental run1, which may be due to a more stable pH. After about 140 hours the specific growth rate for these was greatly reduced and the optical density started declining steadily after 193 hours. At 142 h were the NO and SO_2 switched on which indicates that these gases were stressful for both the species of *Dunaliella*.

N. salina did not have as high doubling time, having instead about 79 hours, but the specific growth rate was quite stable and long instead. No clear stationary or death phase could be seen for *N. salina* which indicates that it tolerates and is able to grow in these conditions.

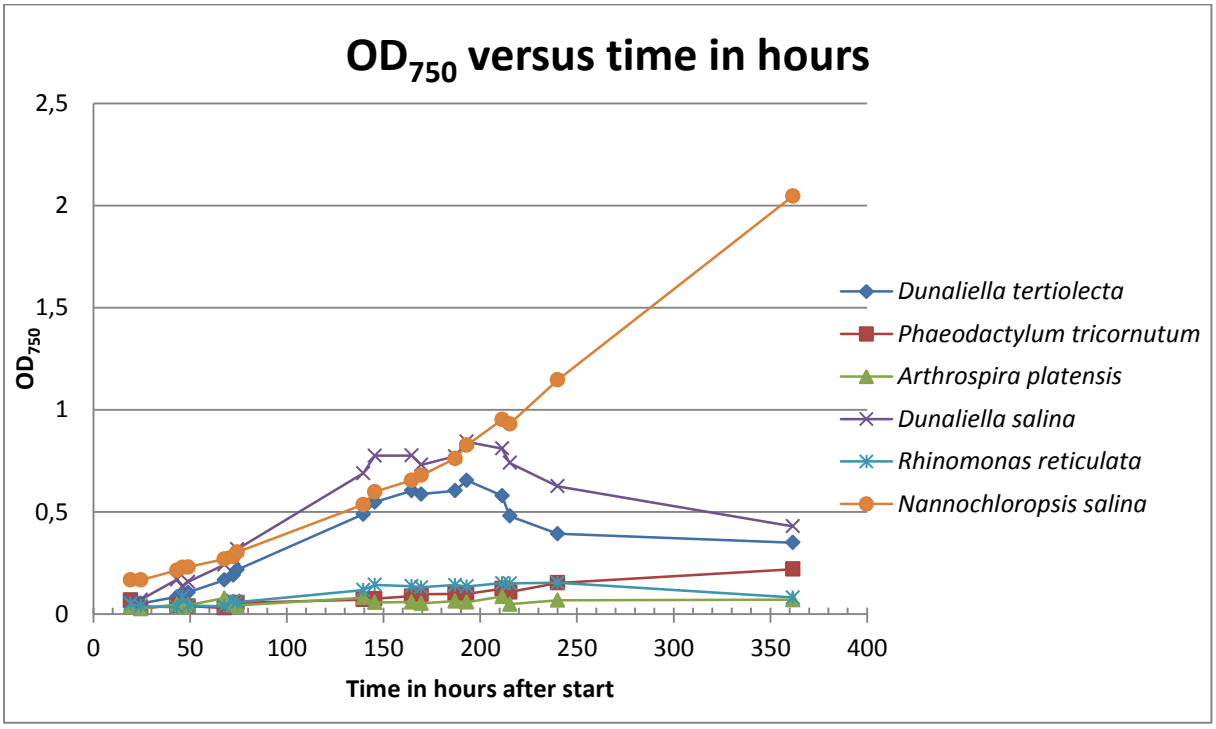


Figure 19. Cell concentration in experimental run 2; Optical density at 750 nm versus time in hours from start.

P. tricornutum had a jagged start in optical density. But at time 68 – 74 hours it had an extremely high, although short, specific growth rate. The specific growth rate successively decreases but remain positive. This is quite strange, since *P. tricornutum* had the shortest doubling time at the start of experimental run 1. Changed from that run was the addition of Hepes and a more stable pH, which was thought to be more favorable for all species. It may be so that *P. tricornutum* needed some time to adjust to these changes.

R. reticulata experienced a similar jagged start as *P. tricornutum*, followed by a relatively high specific growth rate, giving a doubling time of about 59 hours. But at time 146 hours it entered a more or less stationary phase and decreases quite a lot to the last measurement. At 142 h were the NO and SO_2 switched on which indicates that these gases were stressful also for *R. reticulata*.

The optical density for *A. platensis* did not vary a lot in numbers, but the specific growth rate goes constantly up and down. No exponential growth phase could be identified in its data. Either *A. platensis* did not cope well with the conditions or there has been some problem with sampling and/or measurement. It has been shown that *A. platensis* was the species used here which was most sensitive to freezing, see Table 34 in Appendix J. A theory is that the freezing has more or less destroyed the samples thereof the more or less same result throughout the experiment.

Table 15. Growth in experimental run 2; Data from the trend lines from fig. "Ln OD750 versus time in hours" in Appendix J.

1st trend line	*D. tertiolecta*	*P. tricornutum*	*A. platensis*	*D. salina*	*R. reticulata*	*N. salina*
Time points (h)	25 - 74	68 - 74	-	19 - 74*	74 - 146	25 - 240
Spec. growth rate, μ (h^{-1})	0.028	0.095	-	0.029	0.012	0.009
Doubling time (h)	25	7	-	24	59	79
R^2 value	0.993	0.978	-	0.995	0.984	0.993
2nd trend line	***D. tertiolecta***	***P. tricornutum***	***A. platensis***	***D. salina***	***R. reticulata***	***N. salina***
Time points (h)	140 - 193	140 - 170	-	-	-	-
Spec. growth rate, μ (h^{-1})	0.004	0.010	-	-	-	-
Doubling time (h)	161	71	-	-	-	-
R^2 value	0.824	0.978	-	-	-	-
3rd trend line	***D. tertiolecta***	***P. tricornutum***	***A. platensis***	***D. salina***	***R. reticulata***	***N. salina***
Time points (h)	-	215 - 362	-	-	-	-
Spec. growth rate, μ (h^{-1})	-	0.004	-	-	-	-
Doubling time (h)	-	161	-	-	-	-
R^2 value	-	0.880	-	-	-	-

***The value for sample no. 5 at 43 hours is not included in the trend line for *D. salina* due to that this value deviates too much and therefore is regarded as unreliable.**

In experimental run 2 the first spectrophotometer was still unavailable and it was decided that the samples should be frozen and measured when the first spectrophotometer became available again. As seen for the cell count, freezing of samples is not optimal. Cells may be destroyed and hence give a lower impact on optical density. The effect of freezing is species specific as can be seen in Table 34, where re-measurements of samples from experimental run 1 are listed, freezing lowers the optical density. Sample 16 has been measured both before and after freezing with the first spectrophotometer and the value after freezing is between 46 – 96 % of the initial value, where *D. salina* has the highest value, closely followed by *D. tertiolecta*. *A. platensis* has the lowest value and seems to be most affected by freezing. The values for optical density are therefore underestimated for experimental run 2.

This means that comparisons between experimental runs are troublesome. Comparisons within experimental run 2 are also difficult since the species are affected differently by the freezing. However, trends and specific growth rates should be preserved within a species since all samples are treated the same way.

4.8.4. Comparison of growth characteristics determined by different analytical methods

In contrast to experimental run 1, cell count was performed throughout the experimental run. As in experimental run 1, the doubling times derived from cell count and optical density and their time spans

did not generally match, see Table 16. However, for both of the species of *Dunaliella* the initial doubling times and time spans correspond fairly well.

Table 16. Experimental run 2; Comparison of the doubling times found for both cell count and optical density at 750 nm.

Species	Cell count		OD_{750}	
	Time span	Doubling time (h)	Time span	Doubling time (h)
D. tertiolecta	19 – 72 165 - 211	26 67	25 - 74 140 - 193	25 161
P. tricornutum	68 - 240 165 - 240	90 70	140 - 170 215 - 362	71 161
A. platensis	165 - 211	46	-	-
D. salina	19 - 72 72 - 165 165 - 211	19 165 63	19 - 74* - -	24 - -
R. reticulata	72 - 165 187 - 240	128 59	74 - 146 -	59 -
N. salina	19 - 140 140 - 240	42 87	25 - 240	79

***The value for sample no. 5 at 43 hours is not included.**

The reason for that the time spans and doubling times do not correspond can be that there are different types of growth. Cell count only measures the amount of cells whereas optical density measures the amount of absorbed/refracted light and therefore is affected not only by cell number but also size and amounts of cellular components. It could be that in the time ranges were cell count is increased but not optical density, the increase in number is counterbalanced by a decrease in size and/or density of the cells (or less light capturing components). When the optical density increases but not cell numbers, it could be due to an increased cell size and/or higher cellular density (or more light capturing components).

4.9. Dry weight for experimental run 1 and 2

For experimental run 1 the dry weight was measured only at 356 hours from start, see Table 36 in Appendix K. This dry weight was then used together with optical density to calculate an estimation of the dry weight at 117 and 311 hours from start, since these samples were used in other analytical measurements that need a dry weight coupled to them, see Table 17 below.

Table 17. Experimental run 1; Dry weight coupled to OD_{750}, which is used to estimate the dry weight for other samples in the experimental run.

Species	Mean dry weight at 356 h (g/l)	OD_{750} at 356 h	Correlation between dry weight and OD_{750} ((g/l) /OD)	Estimated dry weight at 117 h (g/l)	Estimated dry weight at 311 h (g/l)
D. tertiolecta	0.48	0.848	0.56	0.27	0.27
P. tricornutum	0.28	0.635	0.43	0.27	0.24
C. cryptica*	0.28	-	-	-	-
A. platensis	0.10	0.040	2.50	0.15	0.13
D. salina	0.58	0.976	0.59	0.37	0.42
R. reticulata	0.28	0.327	0.84	0.11	0.22

***No estimation is done for *C. cryptica* due to lack of reliable data.**

For experimental run 2 dry weight was determined at 140 hours from start, see Table 37 in Appendix K. This dry weight was then used together with optical density to calculate an estimation of the dry weight at 68 hours from start, since this sample, together with the sample at 140 hours, were used in other analytical measurements that was needed to be correlated to the dry weight see Table 18 below.

Table 18. Experimental run 2; Dry weight coupled to OD_{750}, which is used to estimate the dry weight for other samples in the experimental run. No estimation is done for *C. cryptica* since the OD values differ greatly between measurements and no reliable data can be obtained.

Species	Mean dry weight at 140 h (g/l)	OD_{750} at 140 h	Correlation between dry weight and OD_{750} ((g/l) /OD)	Estimated dry weight at 68 h (g/l)
D. tertiolecta	0.60	0.489	1.23	0.21
P. tricornutum	0.23	0.071	3.17	0.10
C. cryptica*	0.48	-	-	-
A. platensis	0.35	0.081	4.34	0.34
D. salina	0.60	0.689	0.87	0.21
R. reticulata	0.35	0.119	2.96	0.11
N. salina	0.63	0.536	1.17	0.31

***No estimation is done for *C. cryptica* due to lack of reliable data.**

The dry weight data were used to determine the percentage of carbohydrates, proteins and triglycerides. For experimental run 1 both the cellular composition samples used needed an estimated dry weight. In experimental run 2 only one sample needed an estimate since dry weight measurement was performed at 140 hours from start. A concern with using estimated dry weights is the accuracy. Peter Söderberg has tried to correlate optical density and dry weight for *Botryococcus braunii* without any success (Söderberg, Discussion regarding coupling of OD and DW of microalgal cultures 2012). This means that the estimated dry weights are uncertain and therefore also the calculated percentages for carbohydrates, proteins and triglycerides. To give more accurate values a dry weight should be established for every sample that is analyzed.

4.10. Cellular composition, experimental run 1 and 2

In experimental run 1 the percentage of protein was generally quite high, see Table 19, the amount of protein in algae is typically around 40-60% of dry matter (Becker 2007). This gave a high total percentage for most of the species. A few protein samples were re-measured and these showed differing values compared to the first measurement and the variation between duplicates was also here large. A possible reason for the high values could be that something in the medium was affecting the results. Some of the carbohydrate samples also show high variability but not as much as the protein samples. The levels of carbohydrates and triglycerides generally show increasing values from the first to the second measurement (except carbohydrates for *A. platensis* and triglycerides for *R. reticulata*). A reason for the increasing values could be stress, which could induce accumulation of storage compounds, since at 311 h from start the CO_2 into the cultivations was at the highest level, NO has just been switched on, but not the SO_2.

Table 19. Experimental run 1; Summarized results for content of carbohydrates, proteins and triglycerides at 68 and 311 h from start. Where re-measurements were available the average is used here.

	Carbohydrates (%)		Proteins (%)		Triglycerides (%)		Total (%)	
Species	**117 h**	**311 h**	**117 h**	**311 h**	**117 h**	**311 h**	**117 h**	**311 h**
D. tertiolecta	9	24	77	62	3	5	89	91
P. tricornutum	11	16	74	72	7	16	92	104
A. platensis	12*	10	88	96	2	2	102	108
D. salina	22	23	54	44	4	8	80	75
R. reticulata	7	14	147	78	10	5	164	97

***The first measurement showing 90% is disregarded here.**

In experimental run 2 the protein percentage was dramatically lower, see Table 20. The protein samples were treated to remove the effect of the medium. Many of the samples give values close to or even under zero. The reason for this could be that there was too little cell content in the samples; the values generally increase from the first to the second measurement.

Table 20. Experimental run 2; Summarized results for content of carbohydrates, proteins and triglycerides at 68 h and 140 h from start.

	Carbohydrates (%)		Proteins (%)		Triglycerides (%)		Total (%)	
Species	**68 h**	**140 h**	**68 h**	**140 h**	**68 h**	**140 h**	**68 h**	**140 h**
D. tertiolecta	n. a.	11	n. a.	3	~0	1	0	15
P. tricornutum	~0	8	6	9	1	2	7	19
A. platensis	n. a.	10	3	10	~0	~0	3	20
D. salina	n. a.	26	13	3	2	2	15	31
R. reticulata	n. a.	n. a.	1	7	n. a.	1	1	8
N. salina	n. a.	1	1	4	2	2	3	7

n. a. measurement with values below zero.

The weight of ash in a microalgal cell typically is around 5-20 % of the dry weight, depending on species (Zhu and Lee 1997)(Ekendahl, et al. 2011). The ash-free weight generally consists of around 90% or more of carbohydrates, proteins and lipids (Zhu and Lee 1997). For more data regarding the carbohydrates see Appendix L, for protein see Appendix M and for triglycerides see Appendix N.

4.11. CHN-analysis, experimental run 1 and 2

The CHN-analysis was made at the very end of each experimental run. All samples, in both experimental runs, showed very low values, see Table 21 and Table 22. The total percentage was highest for *N. salina* with 75 weight% accounted for by carbon, hydrogen and nitrogen.

Table 21. Experimental run 1; CHN-analysis.

Species	Carbon (weight %)	Hydrogen (weight %)	Nitrogen (weight %)	Total (weight %)	Weight of dry sample (mg)
D. tertiolecta	33	5	3	41	54.9
P. tricornutum	24	3	2	29	51.6
C. cryptica	14	2	1	17	58.9
A. platensis	11	2	1	14	30.5
D. salina	32	5	2	39	77.9
R. reticulata	21	3	2	26	53.7

Table 22. Experimental run 2; CHN-analysis.

Species	Carbon (weight %)	Hydrogen (weight %)	Nitrogen (weight %)	Total (weight %)	Weight of sample (mg)
D. tertiolecta	40	6	8	54	8.6
P. tricornutum	42	8	3	53	2.1
C. cryptica	24	4	6	34	80.5
A. platensis	20	4	5	29	5.7
D. salina	47	7	7	61	9.5
R. reticulata*					
N. salina	61	9	5	75	31.9

***Samples too small to analyze.**

The low levels of the CHN data indicate that the analysis did not give fully correct values. A probable reason is that there were too much salt left from the medium when the samples were dried which increases the ash weight. Thus, to possibly obtain more correct data the samples probably need a more thorough washing to remove salt from the medium and more material of each sample should be used in the analysis (approx. 150 mg dry weight and absolutely more than 10 mg) and if possible be done in replicates (Johansson 2011). The amount of dry sample was in both experimental runs were low, especially in experimental run 2 were the amount was lower than 10 mg in several cases.

5. Discussion

The main aim for this study was to find marine microalgae species that are suitable for cultivation in flue gas from Swedish pulp and paper mills; with focus on tolerance to the gas and obtaining good growth. Seven species were selected for the main experiments using a simulated flue gas containing 15% CO_2, 100 ppm NO and 10 ppm SO_2. In addition, 14 species (including the seven from the main experiments) were cultivated in two types of actual waste water from Nordic paper Bäckhammar in a preliminary study to test the possibility to use these waste waters as growth medium.

5.1. The most promising species

None of the species tested could maintain a doubling time below 30 hours when the highest flue gas concentrations were used. Only *N. salina* could maintain over 80% of its growth rate at low CO_2 concentrations when exposed to the highest CO_2 concentration. When NO and SO_2 were switched on, *N. salina* could maintain its growth rate based on optical density, but not on cell count which was roughly halved.

This makes *N. salina* the species tested in this study that seems to be the most tolerant to the gas concentrations used. The specific growth rate obtained from optical density at 750 nm is barely affected during the experiment for this species. The doubling time based on optical density is about 79 hours, which is high compared to other species in this study; *D. tertiolecta* and *D. salina* had a doubling time of 25 hours in the beginning of the second run. However, the growth phase of *N. salina* lasted almost throughout the whole experiment whereas the specific growth rates for the *Dunaliella* species (and the others) were unstable and generally decreased with time. Also when looking at cell counts *N. salina* showed the most promising results. A clear decrease in specific growth rate, based on cell counts, can be seen after the addition of NO and SO_2. The doubling time goes from 42 hours to 87 hours after the addition of NO and SO_2. This tells us that the gases do pose some kind of stress on the cells. Since the optical density increase is stable, this indicates that when NO and SO_2 is added the cells grow more in size and/or in density than they do in numbers. An increased cell size and/or accumulation of some cellular component would however probably be beneficial from a biofuel production point of view. *N. salina* has also been recommended in literature for among other things its high lipid productivity (Rodolfi, et al. 2008; Griffiths and Harrison 2009). The doubling times observed in this experiment are high compared to literature, see Table 1. The reason for this is probably a combination of sub-optimal conditions regarding for example light, temperature and pH and the stress imposed by the flue gas. From the visual salinity test *N. salina* also showed to thrive in many different salinities, from fresh water medium to f/2 medium with 2.5% salinity. This adds to the benefits of *N. salina* since if using waste waters mixed with seawater the salinity may be quite low.

5.2. pH considerations

From the pre-tests it was shown that a control of pH is necessary. As has been seen in the theory section the optimal pH for these marine algae are in the range of pH 7-11, many with an optimum above pH 8 (SERI Microalgal Technology Research group 1986; Hosseini Tafreshi and Shariati 2008; Hu 2007; Hinga 2002; Fredin 2009). Despite the attempts to avoid pH dropping in the experimental runs, the pH was at the end around 6.5 in run 1 and around 7 in run 2. Thus, the pH in the experimental runs in this study was not optimal. A higher and more stable pH would most likely result in better growth rates and perhaps better tolerance to the simulated flue gas due to the loss of pH stress. A good way of controlling the pH would be to use a pH-controlled gas inflow. This has been used successfully in other studies (Borkenstein, et al. 2011; Zeiler, et al. 1995). This means that the

concentration and/or flow of CO_2 are regulated automatically by continuous pH measurements. By doing this the pH can be maintained at a desired pH with low variations. However, this was not possible in these experiments due to that equipment needed was not available.

In laboratory scale it is possible to use, as was done in this study, a buffer or NaOH/HCl to adjust pH, this has also been done in several other studies (Negoro, et al. 1991; Ben-Amotz 1995; Burkhardt, Riebesell and Zondervan 1999) and this should be done to keep the pH above 8 throughout the whole cultivation to elucidate the effects of the flue gas. This works fine on a small scale, but for larger scales and for commercial purposes the addition of buffers and/or NaOH/HCl increase costs and is therefore not desirable.

Another possible solution would be to add flue gas or CO_2 at regular intervals instead of a constant flow or by pH-regulation. This has been done previously by for example (Brown 1996). This is a simpler way of controlling pH but it will most likely result in a wider pH range and increase the risk of reaching unwanted pH levels.

5.3. Flue gas components and concentrations

The actual average flue gas from Swedish pulp and paper mills is not very different than other types of flue gases. Since a large amount of biomass is being burned in the paper mills the levels of SO_2 is quite low compared to flue gases derived from the burning of fossil fuels. This flue gas should therefore be gentler to microalgae than fossil fuel derived ones. The simulated flue gas used in this study seemed to impose a stress on all the tested marine algae. *N. salina* appears to be the species tested here that is most tolerant to the gas concentrations used. However, since the pH was fairly low this might have affected the tolerance to the gas for many of the species. With a more optimal pH the gas tolerance might have been higher for some of the species. Several studies on both simulated and actual flue gases have been performed. Matsumoto et al (1995) successfully cultivated strains of *N. salina*, *P. tricornutum* and *Tetraselmis* sp. using an actual flue gas from a power plant containing around 14% CO_2, 185 ppm SO_x and 125 ppm NO_x. Nagase et al (1998) successfully cultivated *D. tertiolecta* in a simulated flue gas containing 15% CO_2 and 100 ppm NO (pre-cultures cultivated with 15% CO_2). Sheehan et al (1998) concludes that flue gas, in general, should not present a major problem in algae cultivations. The gas concentrations used in this study (15% CO_2, 100 ppm NO and 10 ppm SO_2) might not have been the problem, but rather the adaptation of the algae to the gas. The pre-cultures used did not have any active inflow of gas, which led to high pH values and low levels of dissolved CO_2. The addition of CO_2 in the experiments was made in a stepwise manner, but this only gave the algae hours to adapt to higher CO_2 concentrations. If the pre-cultures for a longer period of time had been adapted to a high CO_2 concentration, as Nagase et al (1998) did, the results may have been different and corresponded better to literature.

5.4. Cell content analyses

The analyses for cell content were problematic for these marine species. The values show large variability between duplicate measurements and generally give low values which do not seem reasonable. For the first experimental run for example, the average carbohydrate percentage is 19% with an average of 7 percentage point difference between duplicate measurements. For the protein analysis in experimental run 1 the average protein content is 75% with an average of 15 percentage point difference between duplicate measurements. The reason for the large variability could not be established in this study but it is believed that the marine medium has affected the results or that the cell content was too low in many of the samples. A parallel study using the same methods but for fresh water microalgae showed less variability and more reliable results (Bark 2012). Those samples

generally contained more cells so it remains unclear whether the poor results were due to the marine medium or the low cell content. For further studies using marine algae it is recommended to obtain higher cell densities, or if the problems remain use alternative methods. For the CHN-analysis a more thorough washing of the cells would probably be enough.

5.5. Wastewater

The visual growth tests of the two types of wastewater showed similar results. Some growth could be seen but there was no big difference for any of the species. This could mean that these types of wastewaters cannot support growth of the tested marine species. However, the species were not adapted beforehand to this kind of medium. With an adaptation period we might have seen more promising results. More thorough experiments are needed to establish whether these species can live in these kinds of waste waters.

An initial concern was that the levels of nitrogen and phosphorous would be too low in the wastewaters. This generally does not seem to be the case. The average values obtained from several pulp and paper mills for these compounds are roughly 9 mg/l of nitrogen and 1.4 mg/l of phosphorous, see Table 7. In the waste water "RIA" the levels are lower than the average and in the waste water "Reactor 2" the levels are a bit higher than the average values, see Table 5. For the f/2 medium used the levels are roughly 12.4 mg/l of nitrogen and 1.1 mg/l of phosphorous, see Appendix A. This means that the average values and the values for "Reactor 2" are more or less the same as for the cultivation medium. In the medium there are also trace metals and vitamins, if these are adequate in the wastewaters have not been investigated in this study.

Several studies have investigated the use of microalgae to reduce the amounts of nutrients in mainly municipal sewage and different types of industrial waste waters. Sheehan et al. (1998) have listed studies from the 1970's and forward and Oilgae (2009) have listed some companies that uses waste waters to produces biofuels.

6. Conclusions

From the results in this thesis the species most suitable for cultivation in flue gas from Swedish pulp and paper mills seems to be *N. salina*. If a more dilute flue gas were to be used *D. tertiolecta* and *D. salina* are also of interest due to their high specific growth rates.

In the experiments performed in this thesis increased concentrations of CO_2, NO and SO_2 lowers the specific growth rates of the microalgae tested. The tolerance to these gases could perhaps be increased by adapting the algae to them for a longer period of time, which was not done in these experiments. A dilution of the flue gas could also be an alternative. A possible problem with using flue gas containing CO_2, NO and SO_2 is they lower the pH. The gas flow cannot be on constantly since this most likely would lead to a pH drop and cell death. A pH-controlled gas inflow seems to be the most straightforward way of dealing with this.

The average actual waste waters from Swedish pulp and paper mills contain levels of nitrogen and phosphorous close to the levels in f/2 medium used in these experiments. The average nitrogen content is slightly lower and the average phosphorous content is slightly higher than the f/2 medium. Based on this, waste waters from paper mills could be used as growth medium for microalgae. However, marine microalgae generally prefer a salinity that is at least close to 2.5%, which means that salt or seawater needs to be mixed with the waste water. Adding salt also adds on the costs and adding seawater dilutes the waste water nutrient levels. Two waste water types from Nordic Paper Bäckhammar were tested in this thesis. Waste water "Reactor 2" contained both more nitrogen and more phosphorous than f/2 medium. Waste water "RIA" contained roughly half the level of nitrogen and close to 10% of the phosphorous compared to f/2 medium. Both were mixed 50/50 with seawater with unknown composition. None of these waste water mixes showed visual growth comparable to that seen in f/2 medium. A reason could be lack of adaptation – with an adaptation period the results may be more promising. Further studies are needed.

The cell composition analyses showed to be problematic for these marine species. A possible reason could be the salt in the medium. A parallel study using fresh water microalgal species were analyzed at the same time, with the same procedures and did not show the same irregularities, see Bark (2012). Either the methods used in this thesis need to be adjusted to work better with a marine medium or other methods should be considered for future experiments.

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Appendix A

Recipe and information regarding f/2 medium

Solution 1

75 g $NaNO_3$
5 g $NaH_2PO_4 \cdot H_2O$
Dilute to 1 liter, autoclave, store in fridge

Trace metal solution

3.15 g $FeCl_3 \cdot 6H_2O$
4.36 g $Na_2EDTA \cdot 2H_2O$
0.18 g $MnCl_2 \cdot 4H_2O$
0.022 g $ZnSO_4 \cdot 7H_2O$
0.010 g $CoCl_2 \cdot 6H_2O$
0.0098 g $CuSO_4 \cdot 5H_2O$
0.0063 g $Na_2MoO_4 \cdot 2H_2O$
Dilute to 1 liter, autoclave, store in fridge

Vitamins

Vitamin 1
0.1 g Thiamine·HCl (vit B1)
Dilute to 100 ml, sterile filter, store in freezer, short-term in fridge

Vitamin 2
0.01 g Biotine (vit H)
0.01 g Cyanocobalamin (vit B12)
Dilute to 100 ml, sterile filter, store in freezer, short-term in fridge

Silica stock

2.2 g $Na_2SiO_3 \cdot 5H_2O$
Dilute to 100 ml, autoclave, store in fridge

Cultivation medium

To roughly 1 liter filtered and autoclaved/filter sterilised sea water/ artificial sea water add:
1 ml solution 1
1 ml trace metal solution
200 µl vitamin 1
10 µl vitamin 2

If needed, add 1 ml silica stock

HEPES buffer

$C_8H_{18}N_2O_4S$ – 238.304 g/mol

The concentration should be 0.1-0.2 M in the medium. For 0.2 M solution, 47.6608 g is needed. This is added to roughly 1 liter of filtered artificial seawater, pH-adjusted with 10M NaOH and then autoclaved.

Amount of Instant Ocean needed:

Conductivity experiment. 100ml of 24°C f/2 medium (unfiltered, no vitamins – particles are allowed to settle before measurement).

Conductivity (mS/cm)	Amount Instant Ocean (g)	Salinity (%)*
9.4	0.66	0.53
13.8	1.00	0.80
20.2	1.50	1.20
31.8	2.50	1.98
37.7	3.00	2.39
39.9	3.20	2.55

*converted from: http://www.fivecreeks.org/monitor/sal.html

This means that around 32 g/l of Instant Ocean is needed to obtain a salinity of around 2.5%.

Actual medium used: 40.2 mS/cm giving a salinity of 2.57%.

Nitrogen content in f/2 medium.

Mole fraction of nitrogen:

$$\frac{M_N}{M_{NaNO_3}} = \frac{14.01}{(22.99 + 14.01 + 3 \times 15.99)} = 0.165$$

Solution 1 contains 75 g/l $NaNO_3$ which gives a concentration of nitrogen:

$$C_{N_{sol.1}} = \frac{M_N}{M_{NaNO_3}} \times C_{NaNO_3} = 0.165 \times 75000 = 12360\, mg/l$$

For 1 liter of medium, 1 ml of solution 1 is used:

$$C_{N_{f/2}} = \frac{C_{N_{sol.1}} \times V_{sol.1}}{V_{f/2}} = \frac{12360 \times 0.001}{1} = 12.36\, mg/l$$

Phosphorous content in f/2 medium.

Mole fraction of phosphorous:

$$\frac{M_P}{M_{NaH_2PO_4 \cdot H_2O}} = \frac{30.97}{(22.99 + 4 \times 1.01 + 30.97 + 5 \times 15.99)} = 0.225$$

Solution 1 contains 5 g/l $NaNO_3$ which gives a concentration of phosphorous:

$$C_{P_{sol.1}} = \frac{M_P}{M_{NaH_2PO_4 \cdot H_2O}} \times C_{NaH_2PO_4 \cdot H_2O} = 0.225 \times 5000 = 1120\, mg/l$$

For 1 liter of medium, 1 ml of solution 1 is used:

$$C_{P_{f/2}} = \frac{C_{P_{sol.1}} \times V_{sol.1}}{V_{f/2}} = \frac{1120 \times 0.001}{1} = 1.12\ mg/l$$

The ratio N/P is close to 11:1 (g/g) in this medium.

P: n = m / M = 1122.518 / 30.973762 = 36.24093

N: n = 12361.985 / 14.0067 = 882.57655

N/P = 24.353 mol/mol

Silica content in f/2 modified medium.

Mole fraction of silicon:

$$\frac{M_{Si}}{M_{Na_2SiO_3 \cdot 5H_2O}} = \frac{28.09}{(2 \times 22.99 + 28.09 + 8 \times 15.99 + 10 \times 1.01)} = 0.132$$

The silica stock contains 2.2 g/ml Na_2SiO_3 which gives a concentration of silicon:

$$C_{Si_{stock}} = \frac{M_{Si}}{M_{Na_2SiO_3}} \times C_{Na_2SiO_3} = 0.132 \times 22000 = 2910\ mg/l$$

For 1 liter of medium, 1 ml of the silica stock is used:

$$C_{Si_{f/2}} = \frac{C_{Si_{stock}} \times V_{stock}}{V_{f/2}} = \frac{2910\ \times 0.001}{1} = 2.91\ mg/l$$

Recipe and information regarding 3N-BBM+V medium

3N-BBM+V (Bold Basal Medium with 3fold Nitrogen and Vitamins; modified)

Stock solutions in g / 1000 ml water	**for 1 liter final medium**
(1) 25.0 g $NaNO_3$	30.0 ml
(2) 2.5 g $CaCl_2 \cdot 2\ H_2O$	10.0 ml
(3) 7.5 g $MgSO_4 \cdot 7\ H_2O$	10.0 ml
(4) 7.5 g $K_2HPO_4 \cdot 3\ H_2O$	10.0 ml
(5) 17.5 g KH_2PO_4	10.0 ml
(6) 2.5 g NaCl	10.0 ml
(7) Trace element solution (see below)	6.0 ml
(8) Vitamin B1 (see below)	1.0 ml
(9) Vitamin B12 (see below)	1.0 ml

Make up to 1 litre with distilled water. Autoclave at 15 psi for 15 minutes.

Trace metal solution

Add to 1000 ml of distilled water 0.75 g Na2EDTA and the minerals in exactly the following sequence:

$FeCl_3 \cdot 6\ H_2O$	97.0 mg
$MnCl_2 \cdot 4\ H_2O$	41.0 mg
$ZnCl_2$	5.0 mg
$CoCl_2 \cdot 6\ H_2O$	2.0 mg
$Na_2MoO_4 \cdot 2\ H_2O$	4.0 mg

Vitamin B1 (8) 0.12 g Thiaminhydrochloride in 100 ml distilled water. Filter sterile.
Vitamin B12 (9) 0.1 g Cyanocobalamin in 100 ml distilled water, take 1 ml of this solution and add 99 ml distilled water. Filter sterile.

The only source of N is the $NaNO_3$.

Mole fraction of N: $\frac{M_N}{M_{NaNO_3}} = \frac{14.01}{22.99 + 14.01 + 3 \cdot 15.99} = 0.165$

The concentration of $NaNO_3$ in the stock solution is C_{NaNO_3}= 25000 mg/l. The concentration of N is:

$$C_{N\ (Stock\ solution)} = \frac{M_N}{M_{NaNO_3}} \cdot C_{NaNO_3} = 0.165 \cdot 25000 = 4120\ mg/l$$

By diluting to final medium with 30 ml stock solution to 1 liter of H_2O the final concentration of N is:

$$C_{N\ (Final)} = \frac{C_N \cdot V_{Stock}}{V_{Final}} = \frac{4120 \cdot 0.03}{1} = \mathbf{123.6}\ mg/l$$

For P there are two sources, by the compounds K_2HPO_4 and KH_2PO_4. The concentrations of the P-compounds in the stock solution are $C_{K_2HPO_4}$= 5.713 g/l = 5713 mg/l and $C_{KH_2PO_4}$ = 17.5 g/l = 17500 mg/l. The concentration of P is divided in C_{P_1} and C_{P_2} due to the two compounds:

$$C_{P_1\ \ Stock\ solution} = \frac{M_P}{M_{K_2HPO_4}} \cdot C_{K_2HPO_4} = \frac{30.97}{2 \cdot 39.10 + 1.01 + 30.97 + 4 \cdot 16.00} \cdot 5713 = 1020\ mg/l$$

And: $C_{P_2\ \ Stock\ solution} = \frac{M_P}{M_{KH_2PO_4}} \cdot C_{KH_2PO_4} = 3980\ mg/l$

Diluting to final medium 10 ml from stock solution up to 1 liter, the concentration is:

$$C_{P\ \ Final} = \frac{C_{P1\ \ Stock\ solution} \cdot V_{Stock}}{V_{Final}} + \frac{C_{P2\ \ Stock\ solution} \cdot V_{Stock}}{V_{Final}} = \frac{1020 \cdot 0.01}{1} + \frac{3980 \cdot 0.01}{1} = \mathbf{50.0\ mg/l}$$

Hence, the N/P quotient is ~ 5:2 (g/g).

Appendix B

Data acquisition from Swedish paper mills

Swedish paper mills were contacted by telephone and/or email and requested to declare the components and concentrations of their different flue gases and waste waters. Thirteen paper mills supplied data for flue gases, often for several different kilns at each mill and/or maximum and minimum values. Eleven paper mills supplied data for waste waters, often from different purification steps or maximum and minimum values. The data were compiled and mean values calculated that formed the basis for the decision of what concentrations would be used in the artificial flue gas in the experiments.

Appendix C

Test of waste waters

Photos of the two types of actual waste water tested. Photos were taken each day after inoculation and the ones shown here are after one day and after 16 days for both “RIA” and “Reactor 2”.

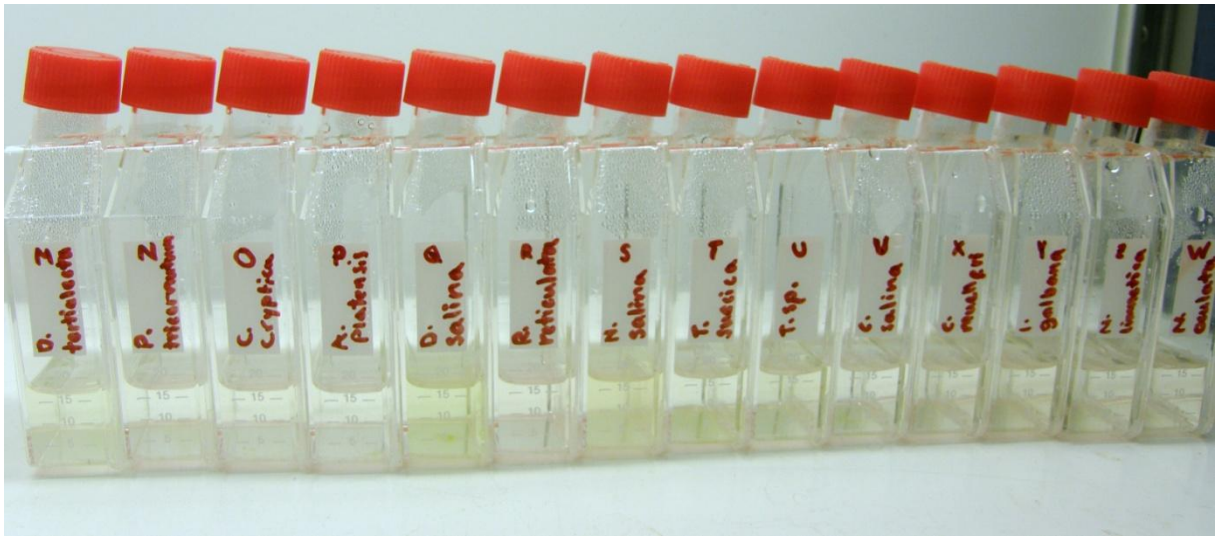

Figure 20. Test of waste water; Waste water "RIA" mixed with seawater and inoculated with algae. The photo is taken a little more than one day after inoculation. The species are placed in the same order, left to right, as in Table 2.

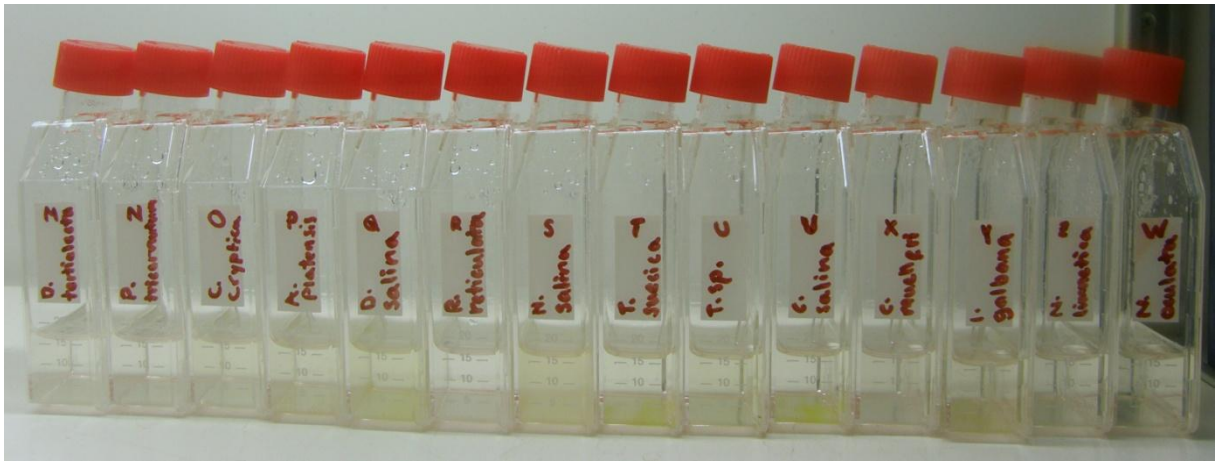

Figure 21. Test of waste water; Waste water "RIA" mixed with seawater and inoculated with algae. The photo is taken a little more than 16 days after inoculation. The species are placed in the same order, left to right, as in Table 2.

Figure 22. Test of waste water; Waste water "Reaktor 2" mixed with seawater and inoculated with algae. The photo is taken a little more than one day after inoculation. The species are placed in the same order, left to right, as in Table 2.

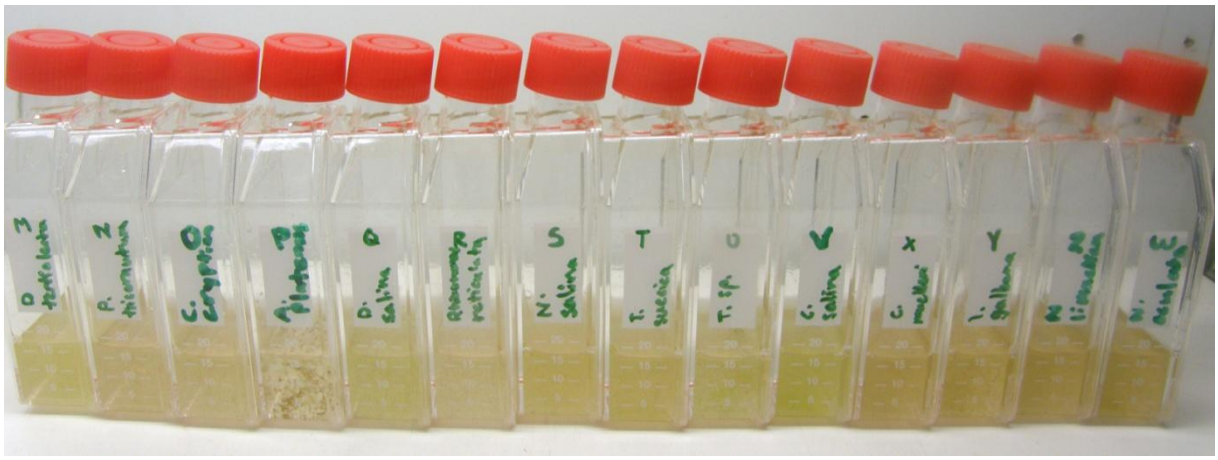

Figure 23. Test of waste water; Waste water "Reaktor 2" mixed with seawater and inoculated with algae. The photo is taken a little more than 16 days after inoculation. The species are placed in the same order, left to right, as in Table 2.

Appendix D

Experimental implementation

Experimental run 1

Table 23. Experimental run 1; Table over time, measurements, gas changes and other actions during the experimental run.

Sample no.	Time from start (h)	Action	Total flow (l/min)	CO_2 (%)	NO (ppm)	SO_2 (ppm)
1	-22	pH	0	0	0	0
2	0	pH, OD	0	0	0	0
	0.5	Gas change	0.8	0	0	0
3	23	pH, OD	0.8	0	0	0
	25	Gas change	1.4	1.0–2.0	0	0
4	95	pH, OD	1.4	1.0–2.0	0	0
	96	Gas change	1.4	2.9–3.2	0	0
5	99	pH	1.4	2.9–3.2	0	0
6	117	pH, OD, Carb., Prot., Triglyceride	1.4	2.9–3.2	0	0
7	121	OD	1.4	2.9–3.2	0	0
	121	Gas change	1.4	5.9-6.5	0	0
8	122	pH	1.4	5.9-6.5	0	0
9	141	pH, OD	1.4	5.9-6.5	0	0
	142	Refilling of media to all, approx. 50 ml of extra media giving a total of approx. 250 ml				
10	145	pH, OD	1.4	5.9-6.5	0	0
11	164	pH, OD	1.4	5.9-6.5	0	0
	165	Removes 100 ml of suspension and adds 100 ml new cell suspension to *Cyclotella cryptica*				
	168	Adds 2 ml of 1M NaOH to all cultivations				
	168	Gas change	1.4	9.1–9.5	0	0
12	169	pH, OD	1.4	9.1–9.5	0	0
13	188	pH, OD	1.4	9.1–9.5	0	0
14	191	OD	1.4	9.1–9.5	0	0
	194	Gas change	1.4	12.3–12.7	0	0
15	195	pH	1.4	12.3–12.7	0	0
16	260	pH, OD	1.4	12.3–12.7	0	0
	261	Gas change	1.4	15	0	0
17	266	pH	1.4	15	0	0
	277	Adds 1 ml 1M NaOH to all cultivations				
18	277	pH	1.4	15	0	0
	284	Removes approximately 70 ml of cell suspension from all cultivations, down to the 150 ml line and adds medium up to the 250 ml line. Adds 1 ml 1M NaOH				
		New spectrophotometer used from here on				
19	284	OD	1.4	15	0	0
20	287	pH, OD	1.4	15	0	0
21	290	OD	1.4	15	0	0
22	308	pH, OD	1.4	15	0	0
	308	Gas change	1.4	15	100	0
23	311	pH, OD, Carb., Prot., Triglyceride	1.4	15	100	0
	311	Gas change	1.4	15	100	10

24	314	pH, OD	1.4	15	100	10
25	333	pH, OD	1.4	15	100	10
26	338	pH, OD	1.4	15	100	10
27	356	pH, OD, CHN, DW	1.4	15	100	10

Experimental run 2

Table 24. Experimental run 2; Table over time, measurements, gas changes and other actions during the experimental run.

Sample no.	Time from start (h)	Action	Total flow (l/min)	CO_2 flow (%)	NO flow (ppm)	SO_2 flow (ppm)
0	0	Start	1.4	0	0	0
1	1	pH	1.4	0	0	0
2	-	Sample lost	1.4	0	0	0
	1.5	Gas change	1.4	0.95 – 1	0	0
3	19	pH, OD	1.4	0.95 – 1	0	0
	23	Gas change	1.4	3.3	0	0
4	25	pH, OD	1.4	3.3	0	0
5	43	pH, OD	1.4	3.3	0	0
	44	Gas change	1.4	6.7 – 7.5	0	0
6	46	pH, OD	1.4	6.7 – 7.5	0	0
7	49	pH, OD	1.4	6.7 – 7.5	0	0
8	68	pH, OD, Carb., Prot., Triglyceride	1.4	6.7 – 7.5	0	0
	69	Gas change	1.4	15.6 – 15.9	0	0
9	72	pH, OD	1.4	15.6 – 15.9	0	0
10	74	pH, OD	1.4	15.6 – 15.9	0	0
11	140	pH, OD, Carb., Prot., Triglyceride, DW	1.4	15.6 – 15.9	0	0
	141	Gas change	1.4	15.6 – 15.9	100	10
12	146	pH, OD	1.4	15.6 – 15.9	100	10
13	165	pH, OD	1.4	15.6 – 15.9	100	10
14	170	pH, OD	1.4	15.6 – 15.9	100	10
15	187	pH, OD	1.4	15.6 – 15.9	100	10
16	193	pH, OD	1.4	15.6 – 15.9	100	10
17	211	pH, OD	1.4	15.6 – 15.9	100	10
18	215	pH, OD	1.4	15.6 – 15.9	100	10
19	240	pH, OD	1.4	15.6 – 15.9	100	10
20	362	pH, OD	1.4	15.6 – 15.9	100	10
21	362	CHN	1.4	15.6 – 15.9	100	10

Appendix E

pH-measurements

Experimental run 1

See Table 25 for pH data. The volume taken from the pre-cultures had been diluted with fresh media, but the pH for samples no. 1 were still quite high at around pH 8.5. After about 22 hours almost all of the cultivations had increased even further in pH. An half an hour after the set starting point a gas flow composed of compressed air was switched on with about 0.8 l/min flowing though the twelve E-flasks. This gave a decrease in pH for all cultivations due to the increased dissolved carbon dioxide. Between sample no. 4 and 9 there was a problem with the pH electrode, so there are no reliable data from these samples. After almost 25 hours the gas flow was changed to a total of 1.4 l/min and the carbon dioxide was switched on and put to 1-2%. The percentage of carbon dioxide was after this increased bit by bit. At sample no. 10 the pH electrode was back in service and the carbon dioxide had reached a level of 5.9-6.5%. This led to a plunge in pH down to around pH 6. Almost twenty hours after, at samples no. 11, the pH values were the same or even higher, indicating that the cultivations coped with the low pH. However, a pH around 6 was considered too low, especially since the amount of carbon dioxide was planned to reach 15%, therefore 1M NaOH was added to all of the cultivations. With the addition of NaOH the pH was quite stable at around pH 6.5, even with a maximum of 15% carbon dioxide, 100 ppm nitrogen monoxide and 10 ppm of sulfur dioxide. Even though the pH was kept fairly stable in the later part of the experiment, a pH of around 6.5 is lower than the optimum for most marine algae. This experiment shows that the pH needs to be closely monitored. Either a buffer is needed in the media or the gas flow must be coupled and regulated with continuous pH measurements to control and keep the pH stable.

Table 25. Experimental run 1; Table over pH-measurements.

Sample no.	Time from start (h)	*Dunaliella tertiolecta*	*Phaeodactylum tricornutum*	*Cyclotella cryptica*	*Arthrospira platensis*	*Dunaliella salina*	*Rhinomonas reticulata*
1	-22	**8.65**	**8.36**	**8.35**	**8.62**	**8.65**	**8.56**
2	0	**8.85**	**8.68**	**8.34**	**8.70**	**8.98**	**8.63**
		0.5h: Compressed air at 0.8 l/min					
3	23	**8.36**	**8.36**	**8.08**	**8.42**	**8.77**	**8.21**
4-9	95 – 141	Problem with the measurements, no reliable data available. 25h: CO_2 is raised to 1.0–2.0 %, total gas flow of 1.4 l/min 96h: CO_2 is raised to 2.9–3.2 % 121h: CO_2 is raised to 5.9–6.5 %					
10	145	**6.01**	**6.01**	**5.78**	**5.83**	**6.09**	**5.94**
11	164	**6.09**	**6.02**	**5.81**	**5.91**	**6.08**	**6.04**
		165h: Removes 100 ml of suspension and adds 100 ml new cell suspension to “O”. 168h: Adds 2 ml of 1M NaOH to all cultivations. 168h: CO_2 is raised to 9.1–9.5 %					
12	169	**6.51**	**6.52**	**6.45**	**6.49**	**6.52**	**6.57**
13	188	**6.60**	**6.57**	**6.48**	**6.53**	**6.53**	**6.56**
		194h: CO_2 is raised to 12.3–2.7%					
15	195	**6.42**	**6.42**	**6.39**	**6.40**	**6.42**	**6.47**
16	260	**6.51**	**6.41**	**6.36**	**6.36**	**6.39**	**6.41**
		260.5: CO_2 is raised to 15%					

17	266	**6.29**	**6.29**	**6.29**	**6.29**	**6.31**	**6.33**
		277h: Adds 1 ml 1M NaOH to all cultivations.					
18	277	**6.59**	**6.58**	**6.56**	**6.54**	**6.56**	**6.57**
		284h: Removes approximately 70 ml of cell suspension from all cultivations, down to the 150 ml line and adds medium up to the 250 ml line. Adds 1 ml 1M NaOH.					
20	287	**6.51**	**6.45**	**6.46**	**6.44**	**6.45**	**6.50**
22	308	**6.50**	**6.43**	**6.41**	**6.49**	**6.44**	**6.50**
		308h: NO is raised to 100 ppm					
23	311	**6.58**	**6.43**	**6.42**	**6.47**	**6.45**	**6.46**
		311h: SO_2 raised to 10 ppm					
24	314	**6.54**	**6.41**	**6.52**	**6.45**	**6.43**	**6.48**
25	333	**6.52**	**6.48**	**6.41**	**6.48**	**6.48**	**6.47**
26	338	**6.48**	**6.44**	**6.43**	**6.45**	**6.45**	**6.49**
27	356	**6.47**	**6.45**	**6.42**	**6.42**	**6.55**	**6.61**

Experimental run 2

See Table 26 for pH data. In experimental run 2 Hepes buffer was used to maintain a stable pH since the experimental setup did not allow for a gas-controlled pH system. The pH of the media containing the buffer Hepes was very low, pH 5.42. To raise the pH before start a total of 15 ml 10M NaOH was added to give a pH of about 7.60.

Table 26. Experimental run 2; Table over pH-measurements.

Sample no.	Time from start (h)	*D. tertiolecta*	*P. tricornutum*	*C. cryptica*	*A. platensis*	*D. salina*	*R. reticulata*	*N. salina*
		0: Gas flow of 1.4 l/min, only compressed air from start						
1	1	**7.60**	**7.59**	**7.58**	**7.60**	**7.60**	**7.60**	**7.60**
		1.5h: CO_2 is raised to 0.95 – 1%						
3	19	**7.55**	**7.53**	**7.52**	**7.53**	**7.54**	**7.53**	**7.54**
		23h: CO_2 is raised to 3.3%						
4	25	**7.41**	**7.38**	**7.37**	**7.39**	**7.40**	**7.38**	**7.41**
5	43	**7.40**	**7.38**	**7.37**	**7.39**	**7.40**	**7.40**	**7.39**
		44h: CO_2 is raised to 6.7 – 7.5%						
6	46	**7.26**	**7.26**	**7.25**	**7.28**	**7.26**	**7.25**	**7.27**
7	49	**7.23**	**7.22**	**7.20**	**7.22**	**7.22**	**7.21**	**7.23**
8	68	**7.26**	**7.23**	**7.23**	**7.24**	**7.25**	**7.25**	**7.26**
		69h: CO_2 is raised to 15.6 – 15.9%						
9	72	**7.06**	**7.03**	**7.02**	**7.05**	**7.04**	**7.04**	**7.05**
10	74	**7.04**	**7.02**	**7.02**	**7.03**	**7.03**	**7.02**	**7.03**
11	140	**7.04**	**7.01**	**7.02**	**7.03**	**7.03**	**7.05**	**7.03**
		141h: NO raised to approx. 100 ppm, SO_2 raised to approx. 10 ppm						
12	146	**7.03**	**7.01**	**7.01**	**7.02**	**7.03**	**7.02**	**7.03**
13	165	**7.02**	**7.02**	**7.02**	**7.02**	**7.04**	**7.04**	**7.03**
14	170	**7.01**	**7.02**	**7.02**	**7.02**	**7.05**	**7.04**	**7.01**
15	187	**7.02**	**7.01**	**7.02**	**7.02**	**7.05**	**7.05**	**7.02**
16	193	**7.03**	**7.03**	**7.02**	**7.02**	**7.04**	**7.05**	**7.01**
17	211	**7.02**	**7.01**	**7.02**	**7.02**	**7.02**	**7.01**	**7.01**
18	215	**7.02**	**7.02**	**7.01**	**7.01**	**7.02**	**7.01**	**7.02**
19	240	**7.02**	**7.02**	**7.03**	**7.04**	**7.05**	**7.01**	**7.01**
20	362	**6.99**	**7.00**	**7.01**	**7.01**	**7.01**	**7.00**	**7.01**

With increasing amount of carbon dioxide in the gas flow the pH decreased in a similar fashion for all the cultivations. When all the gases where at their maximum levels the pH was stable at around pH 7 for all cultivations. This is a much better range than in experimental run 1, but still not optimal. With

an increased concentration of Hepes or having a higher starting value of the medium the pH might have been even more stable around 7.5. Still, a gas-controlled pH system would probably be better and easier to take to a larger scale.

Appendix F

Carbohydrate analysis

Ref: Herbert D, Phipps PJ & Strange RE, 1971, Chemical analysis of microbial cells. In Methods in microbiology, vol. 5B, pp. 209-344, Eds. JR Norris & DW Ribons. Academic Press, London.

Solutions:

5 % phenol (5.555 ml 90% phenol up to 100 ml)
10 mM glucose standard stock (0.1g/50 ml)
alt. use standard in glc kit =0.5 g/l=2.78 mM (Roche/r-Biopharm)

Hydrolysis and furfural formation, do each sample, standard, blank in duplicate:

Measurement in microtiter plates:

Method in Flourstar: Total carbohydrates (measuring absorbance at 488 nm three times),

Make standard curves from a glucose stock at 0.5 g/l. Use a standard curve with standards diluted between 20x and undiluted (0.025-0.5 g/l).

- Pipette 30 µl of duplicate mQ water, standards, and appropriate diluted samples.
- Add 30 µl of phenol solution with a multipipette and mixing accordingly.
- Add 150 µl of sulphuric acid with a multipipette and mixing accordingly.
- Incubate 30 minutes
- Measure absorbance

Appendix G

Triglyceride analysis

Master thesis work by Peter Söderberg; ”Utvinning och kvantifiering av triglycerider i algodlingar *Metod för att kvantifiera triglycerider i alger”*.

Summary of the method for quantification of triglycerides, translated from Peter Söderbergs master thesis (Söderberg, Utvinning och kvantifiering av triglycerider i algodlingar - Extraction and quantification of triglycerides from algacultures 2011):

1) A 1 ml sample was taken from each cultivation at two different time points during the cultivations and centrifuged for 20 min in 5000 rpm.
2) The supernatant was removed and the pellet was frozen until later analysis.
3) When the final sample had been taken, all samples were thawed and the pellets re-suspended in 1 ml of distilled water. The samples were then subjected to ultrasound treatment for 1 min with an amplitude of 11 µm. This was done to lyze the cells.
4) 400 µl of hexane were added to the lyzed samples and then centrifuged to obtain a phase separation. 50 µl of the hexane phase were then transferred to a vial in which the hexane is left to evaporate.
5) 200 µl of acid methanol (4 M HCl in methanol with glucose as an internal standard) were added to the vials, which were then put in an ultrasound bath for 10 min. The vials were left overnight at 60°C to evaporate the acid methanol.
6) To remove residual HCl, 100 µl of methanol were added to the vials which were then let to evaporate again.
7) 100 µl of Tri-Sil TP (mixture of trimethylsilylimidazole (TMSI) in dry pyridine) solution were added to the vials, which were then put in 60°C for 1h.
8) The samples were diluted 1:10 with dichloromethane.
9) A GC/MS were used to analyze the samples which were compared with a standard curve for glycerol to be able to calculate the amount of glycerol in the samples.

For estimating the total weight of triglycerides from the glycerol standard an estimated average weight of 870g/mol is used (Söderberg, Utvinning och kvantifiering av triglycerider i algodlingar - Extraction and quantification of triglycerides from algacultures 2011).

Appendix H

Protein analysis

Standard curve concentrations for experimental run 1.

Table 27. Experimental run 1; Protein standard curve concentrations.

Standard curve	Protein concentration (g/l)
1 % SDS Blank	0
16x dilution	0.0788
14x dilution	0.0900
12x dilution	0.1050
10x dilution	0.1260
8x dilution	0.1575
4x dilution	0.3150
2x dilution	0.6300

Standard curve concentrations for experimental run 2.

Table 28. Experimental run 2; Protein standard curve concentrations.

Standard curve	Protein concentration (g/l)
Blank	0
50x dilution	0.028
25x dilution	0.056
12.5x dilution	0.112
6.25x dilution	0.224
5x dilution	0.280

Appendix I

Cell count

Experimental run 1

Table 29. Experimental run 1; Cell count was performed in the later part of the experiment. The amount of cells has been counted in fifteen squares of the counting chamber. Given in the table is also the total amount of cells, dilution and mean value for the counted squares.

Counted squares →	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Total cells	Dilution	Mean value for squares, with dilution
Dunaliella tertiolecta																		
Sample no. 16	1	1	3	0	1	1	0	1	1	3	4	2	2	1	1	22	10	14.7
19	5	1	2	2	3	3	3	2	1	4	1	3	1	0	5	36	2	4.8
21	5	3	2	2	2	4	3	2	7	4	2	4	6	0	3	49	2	6.5
23	10	8	6	6	6	1	2	5	4	4	7	2	5	5	3	74	2	9.9
24	5	3	4	8	9	4	3	4	7	6	7	6	2	8	7	83	2	11.1
25	5	8	9	6	4	1	7	4	6	6	3	5	4	6	9	83	2	11.1
27	4	17	0	1	5	3	0	0	0	2	0	0	2	0	0	34	2	4.5
Phaeodactylum tricornutum																		
Sample no. 16	4	5	7	2	1	4	5	1	5	5	8	6	5	6	9	73	5	24.3
19	4	8	5	5	12	1	8	1	5	8	5	6	6	11	7	92	2	12.3
21	5	6	0	3	5	7	5	5	8	5	4	4	5	8	8	78	2	10.4
23	10	4	7	4	4	6	7	4	8	8	7	7	3	4	10	93	2	12.4
24	7	6	6	6	4	2	6	9	7	5	11	10	6	5	9	99	2	13.2
25	7	6	9	9	12	5	8	3	7	8	11	13	7	9	10	124	2	16.5
27	10	6	7	8	7	8	12	8	14	9	7	6	14	12	7	135	2	18.0
Arthrospira platensis																		
Sample no. 16	8	7	0	0	0	0	2	24	3	2	0	0	0	4	0	50	1	3.3
19	1	1	1	0	1	0	0	2	2	0	1	1	1	1	0	12	1	0.8
21	2	0	0	0	1	0	0	1	2	0	0	15	0	2	1	24	1	1.6
23	1	0	1	0	1	0	1	2	1	1	3	2	2	3	1	19	1	1.3
24	2	1	0	0	0	4	0	0	6	3	0	1	0	0	0	17	1	1.1
25	1	10	7	0	1	0	12	0	5	0	0	0	0	17	0	53	1	3.5
27	3	2	2	1	1	1	0	1	2	0	2	0	0	1	2	18	1	1.2
Dunaliella salina																		
Sample no. 16	3	4	1	3	2	3	6	1	3	1	4	1	4	5	1	42	5	14.0
19	5	4	1	2	3	3	4	2	3	3	5	5	2	1	2	45	2	6.0
21	3	3	7	6	0	5	4	2	3	3	3	3	4	1	4	51	2	6.8
23	3	4	1	4	4	4	3	4	4	2	3	4	1	1	3	45	2	6.0
24	4	9	5	7	4	3	4	8	6	5	9	6	5	7	6	88	2	11.7
25	5	8	7	1	4	8	5	4	4	9	5	12	1	5	3	81	2	10.8
27	4	1	1	3	1	2	1	2	1	1	0	1	1	3	0	22	2	2.9
Rhinomonas reticulata																		
Sample	1	4	3	2	2	2	3	3	2	1	1	3	4	7	2	40	2	5.3

no. 16																		
19	4	4	2	2	1	2	3	3	3	4	2	4	5	5	0	44	1	2.9
21	4	0	3	3	3	4	2	3	5	6	5	3	2	3	4	50	1	3.3
23	4	1	2	2	4	3	1	5	2	2	2	1	7	6	2	44	1	2.9
24	5	1	4	1	5	1	4	5	0	4	4	1	2	1	2	40	1	2.7
25	6	7	2	1	1	4	5	5	2	5	3	2	2	1	2	48	1	3.2
27	3	5	5	5	5	7	5	4	8	4	1	3	5	6	1	67	1	4.5

Table 30. Experimental run 1; Cell count. The mean value is used to calculate amount of cells per milliliter. A standard deviation is given in terms of both mean value of cells per square and cells per milliliter.

	Mean value for squares with dilution	Cells / ml (10^7)	Std deviation for squares	Std deviation in cells/ml (10^7)
Dunaliella tertiolecta				
Sample no. 16	14.7	5.9	11.3	4.5
19	4.8	1.9	3.0	1.2
21	6.5	2.6	3.6	1.4
23	9.9	4.0	4.8	1.9
24	11.1	4.4	4.3	1.7
25	11.1	4.4	4.4	1.8
27	4.5	1.8	8.8	3.5
Phaeodactylum tricornutum				
Sample no. 16	24.3	9.7	11.5	4.6
19	12.3	4.9	6.1	2.5
21	10.4	4.2	4.2	1.7
23	12.4	5.0	4.6	1.8
24	13.2	5.3	4.7	1.9
25	16.5	6.6	5.3	2.1
27	18.0	7.2	5.5	2.2
Arthrospira platensis				
Sample no. 16	3.3	1.3	6.3	2.5
19	0.8	0.3	0.7	0.3
21	1.6	0.6	3.8	1.5
23	1.3	0.5	1.0	0.4
24	1.1	0.5	1.9	0.7
25	3.5	1.4	5.5	2.2
27	1.2	0.5	0.9	0.4
Dunaliella salina				
Sample no. 16	14.0	5.6	8.1	3.2
19	6.0	2.4	2.7	1.1
21	6.8	2.7	3.5	1.4
23	6.0	2.4	2.4	1.0
24	11.7	4.7	3.7	1.5
25	10.8	4.3	5.9	2.4
27	2.9	1.2	2.3	0.9
Rhinomonas reticulata				

Sample no. 16	5.3	2.1	3.1	1.2
19	2.9	1.2	1.4	0.6
21	3.3	1.3	1.5	0.6
23	2.9	1.2	1.9	0.8
24	2.7	1.1	1.8	0.7
25	3.2	1.3	2.0	0.8
27	4.5	1.8	1.9	0.8

In Figure 24 the natural logarithm of the cell count versus time in hours from start is presented. By identifying linear parts with at least three data points for each species it is possible to calculate the specific growth rate and doubling time for that part.

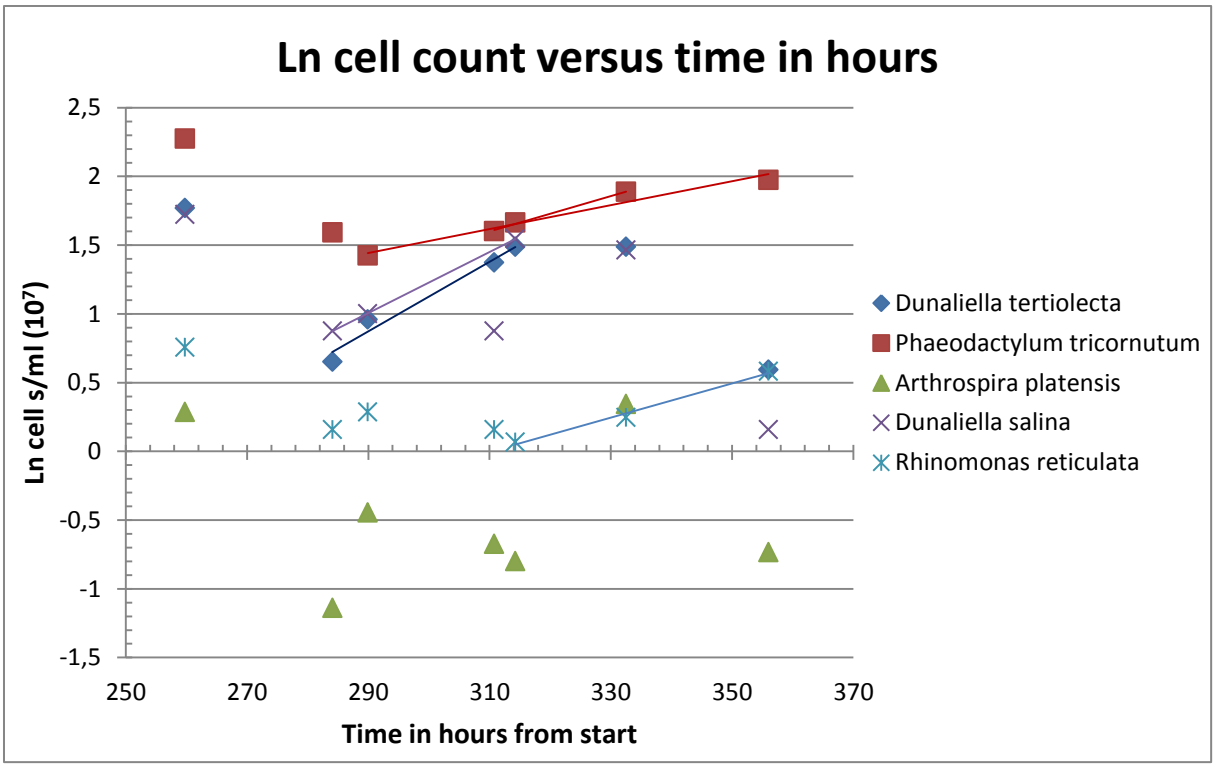

Figure 24. Experimental run 1; Ln cell count versus time in hours from start. Trend lines have been fitted to appropriate points to calculate the specific growth rate and doubling time. Trend lines have been fitted when three or more consecutive data points are aligned on linearity. For *D. salina* the deviating data point at 311 h has been removed to be able to fit a trend line. *A. platensis* could not have any trend lines fitted to its data.

Experimental run 2

Table 31. Experimental run 2; Cell count was performed throughout the experimental run. The samples has be frozen and thawed before counting. The amount of cells has been counted in fifteen squares of the counting chamber. Given in the table is also the total amount of cells, dilution and mean value for the counted squares.

Counted squares → / **Sample no. ↓**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**Total cells**	**Dilution**	**Mean value for squares, with dilution**
Dunaliella tertiolecta																		
3	2	1	2	0	0	2	3	3	4	2	6	1	3	1	2	32	1	2.1
8	6	8	4	8	6	6	8	9	4	4	8	5	5	7	3	91	1	6.1
9	8	5	6	5	11	7	7	13	8	14	18	31	14	8	6	161	1	10.7

11	8	9	13	9	12	10	10	12	7	14	11	14	13	8	13	163	1	10.9
13	7	7	4	8	9	3	7	1	4	5	4	4	9	10	8	90	2	12.0
15	2	3	3	4	1	5	3	2	2	4	8	7	10	5	6	65	4	17.3
17	6	9	3	6	5	5	2	6	3	4	6	6	5	4	3	73	4	19.5
19	5	3	2	5	3	7	5	5	1	5	5	3	3	6	5	63	4	16.8
20	3	2	4	4	3	5	3	4	5	3	5	5	1	3	3	53	4	14.1
Phaeodactylum tricornutum																		
3	4	3	7	3	6	5	6	5	5	6	4	10	3	5	3	75	1	5.0
8	1	2	2	2	1	2	2	0	2	0	3	2	1	0	2	22	1	1.5
9	4	1	0	1	3	0	2	1	1	3	1	2	4	0	3	26	1	1.7
11	1	2	4	4	6	3	3	1	1	1	2	2	3	2	4	39	1	2.6
13	3	2	2	3	3	2	5	3	4	4	3	3	2	2	1	42	1	2.8
15	2	2	0	2	3	7	6	4	4	2	3	6	6	5	6	58	1	3.9
17	3	5	1	5	6	5	5	6	9	2	5	4	3	4	4	67	1	4.5
19	8	6	6	8	10	7	1	6	8	4	8	3	6	3	8	92	1	6.1
20	8	8	9	10	9	7	3	4	4	5	10	6	5	10	4	102	1	6.8
Arthrospira platensis																		
3	0	2	2	1	2	2	1	1	1	0	3	0	1	1	1	18	1	1.2
8	16	12	5	5	4	7	10	13	17	7	10	8	6	4	8	132	1	8.8
9	2	2	1	0	2	1	1	2	3	3	2	1	0	1	4	25	1	1.7
11	5	2	0	0	1	9	2	1	0	0	7	0	1	0	6	34	1	2.3
13	1	3	2	0	0	2	2	2	1	0	1	0	1	1	1	17	1	1.1
15	6	0	2	1	2	2	2	1	0	1	2	3	1	1	1	25	1	1.7
17	2	2	1	1	2	2	1	3	2	1	3	0	7	3	4	34	1	2.3
19	2	1	3	2	1	1	0	2	2	0	1	2	3	1	0	21	1	1.4
20	1	1	1	1	0	1	1	1	2	1	1	0	1	2	1	15	1	1.0
Dunaliella salina																		
3	1	1	0	2	3	0	0	2	2	0	2	3	0	0	1	17	1	1.1
8	6	6	12	4	4	4	10	5	10	8	7	1	6	7	7	97	1	6.5
9	9	6	7	8	8	6	4	8	7	10	5	8	12	8	7	113	1	7.5
11	5	4	3	6	6	7	4	1	8	7	5	4	8	7	4	79	2	10.5
13	7	2	6	8	10	3	5	5	4	8	6	4	6	6	2	82	2	10.9
15	7	5	4	4	6	5	2	4	3	4	2	3	1	3	6	59	4	15.7
17	3	4	3	7	4	3	2	5	1	4	4	1	5	4	5	55	5	18.3
19	3	4	1	4	2	1	3	6	3	3	4	5	3	3	2	47	6	18.8
20	2	1	5	2	1	4	4	3	2	3	3	4	3	3	3	43	5	14.3
Rhinomonas reticulata																		
3	2	3	2	1	3	4	3	5	5	3	2	3	2	2	4	44	1	2.9
8	2	0	1	1	1	0	0	1	0	0	1	1	1	1	0	10	1	0.7
9	1	1	0	1	2	1	0	2	3	1	1	0	1	1	2	17	1	1.1
11	2	1	0	2	0	1	1	2	2	2	3	2	2	0	2	22	1	1.5
13	1	3	1	0	4	2	3	3	2	2	1	2	3	0	2	29	1	1.9
15	1	2	1	3	2	2	3	3	4	2	2	0	1	1	3	30	1	2.0
17	3	3	3	0	3	4	0	3	3	3	1	4	1	5	2	38	1	2.5
19	3	0	1	3	1	4	0	1	5	2	1	2	2	2	1	28	2	3.7
20	2	2	3	1	2	3	2	4	1	0	1	0	0	3	2	26	1	1.7
Nannochloropsis salina																		
3	14	12	11	10	8	14	8	12	12	13	13	8	9	12	13	169	1	11.3
8	17	23	27	26	20	25	22	15	18	23	22	20	32	25	25	340	1	22.7
9	29	30	29	24	28	24	23	23	20	25	20	25	26	25	15	366	1	24.4
11	32	34	24	30	45	39	41	37	56	39	44	42	44	46	47	600	2	80.0
13	45	43	42	41	44	52	41	58	55	50	48	60	53	57	53	742	2	98.9
15	25	27	27	33	25	27	22	17	29	29	26	36	40	27	36	426	4	113.6
17	30	28	26	25	31	34	25	31	25	26	28	28	30	33	42	442	5	147.3
19	24	25	27	25	28	34	31	26	28	28	31	29	35	32	37	440	6	176.0

20	33	30	30	38	42	34	33	30	33	35	36	32	34	36	33	509	6	203.6

Table 32. Experimental run 2; Cell count. The mean value is used to calculate amount of cells per milliliter. A standard deviation is given in terms of both mean value of cells per square and cells per milliliter. *C. cryptica* has not been counted.

Sample no.	Mean value for squares with dilution	Cells / ml (10^7)	Std deviation for squares	Std deviation in cells/ml (10^7)
Dunaliella tertiolecta				
3	2.1	0.9	1.6	0.6
8	6.1	2.4	1.9	0.8
9	10.7	4.3	6.8	2.7
11	10.9	4.4	2.3	0.9
13	12.0	4.8	5.2	2.1
15	17.3	6.9	10.1	4.0
17	19.5	7.8	7.1	2.8
19	16.8	6.7	6.5	2.6
20	14.1	5.7	4.8	1.9
Phaeodactylum tricornutum				
3	5.0	2.0	1.9	0.8
8	1.5	0.6	0.9	0.4
9	1.7	0.7	1.4	0.6
11	2.6	1.0	1.5	0.6
13	2.8	1.1	1.0	0.4
15	3.9	1.6	2.1	0.8
17	4.5	1.8	1.9	0.8
19	6.1	2.5	2.5	1.0
20	6.8	2.7	2.5	1.0
Arthrospira platensis				
3	1.2	0.5	0.9	0.3
8	8.8	3.5	4.1	1.7
9	1.7	0.7	1.1	0.5
11	2.3	0.9	3.0	1.2
13	1.1	0.5	0.9	0.4
15	1.7	0.7	1.5	0.6
17	2.3	0.9	1.7	0.7
19	1.4	0.6	1.0	0.4
20	1.0	0.4	0.5	0.2
Dunaliella salina				
3	1.1	0.5	1.1	0.5
8	6.5	2.6	2.8	1.1
9	7.5	3.0	2.0	0.8
11	10.5	4.2	4.0	1.6
13	10.9	4.4	4.5	1.8
15	15.7	6.3	6.7	2.7
17	18.3	7.3	7.9	3.2
19	18.8	7.5	8.1	3.3
20	14.3	5.7	5.6	2.3
Rhinomonas reticulata				
3	2.9	1.2	1.2	0.5
8	0.7	0.3	0.6	0.3
9	1.1	0.5	0.8	0.3
11	1.5	0.6	0.9	0.4
13	1.9	0.8	1.2	0.5

15	2.0	0.8	1.1	0.4
17	2.5	1.0	1.5	0.6
19	3.7	1.5	2.8	1.1
20	1.7	0.7	1.2	0.5
Nannochloropsis salina				
3	11.3	4.5	2.2	0.9
8	22.7	9.1	4.3	1.7
9	24.4	9.8	4.0	1.6
11	80.0	32.0	15.8	6.3
13	98.9	39.6	13.1	5.2
15	113.6	45.4	23.4	9.4
17	147.3	58.9	22.6	9.0
19	176.0	70.4	23.4	9.4
20	203.6	81.4	19.3	7.7

In Figure 25 the natural logarithm of the cell count versus time in hours from start is presented. By identifying linear parts with at least three data points for each species it is possible to calculate the specific growth rate and doubling time for that part.

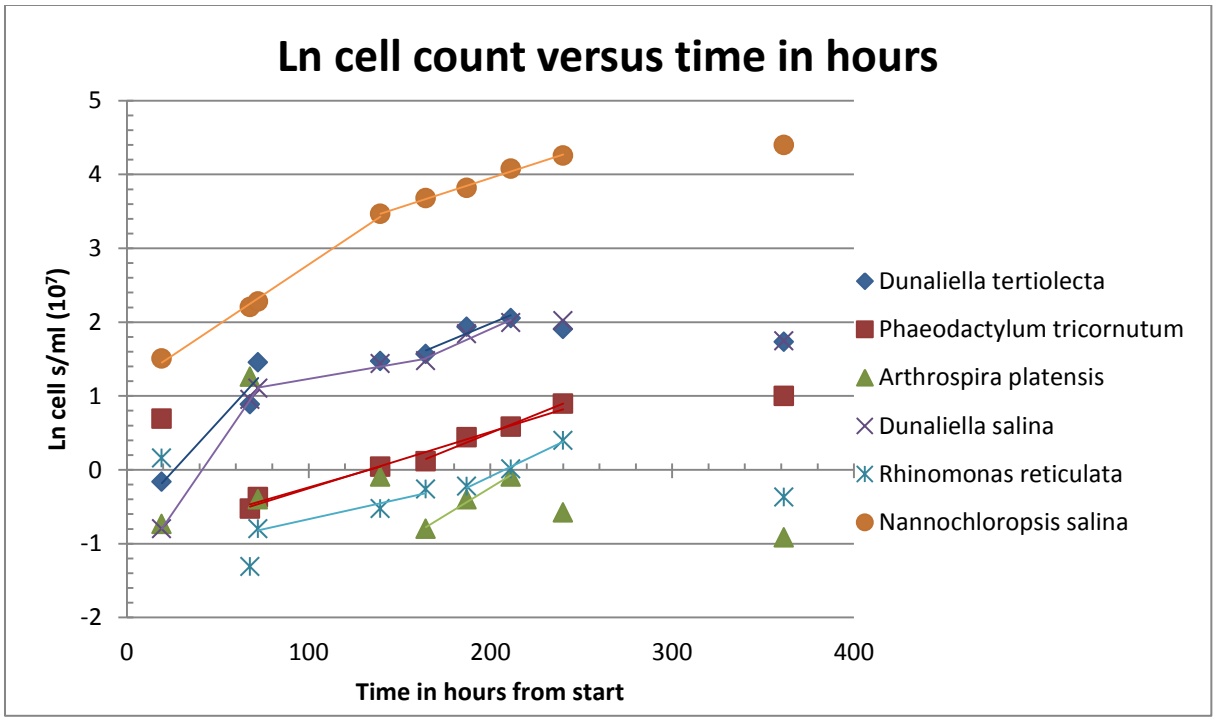

Figure 25. Experimental run 2; Ln cell count versus time in hours from start. Trend lines have been fitted to appropriate points to calculate the specific growth rate and doubling time. Trend lines have been fitted when three or more consecutive data points are aligned on linearity. All species have at least one trend line fitted to its data.

Appendix J

Optical density

Experimental run 1

Table 33. Experimental run 1; Table over optical density at 750 nm.

Sample no.	Time from start (h)	*Dunaliella tertiolecta*	*Phaeodactylum tricornutum*	*Cyclotella cryptica*	*Arthrospira platensis*	*Dunaliella salina*	*Rhinomonas reticulata*
2	0	0.033	0.021	0.011	0.021	0.041	0.012
3	23	0.050	0.039	0.012	0.048	0.072	0.021
4	95	0.386	0.418	0.099	0.057	0.520	0.084
6	117	0.486	0.629	0.019	0.060	0.628	0.133
7	121	0.547	0.669	0.048	0.058	0.646	0.164
9	141	0.665	0.780	0.033	0.104	0.782	0.208
10	145	0.586	0.705	0.017	0.068	0.701	0.190
11	164	0.622	0.678	0.028	0.060	0.710	0.217
12	169	0.761	0.790	0.492	0.054	0.907	0.270
13	188	0.793	0.854	0.108	0.068	0.921	0.296
14	191	0.905	0.941	0.069	0.064	0.942	0.322
16	260	1.084	1.081	0.173	0.104	1.116	0.346
		New spectrophotometer used from here on					
19	284	0.467	0.480	0.118	0.039	0.629	0.234
20	287	0.514	0.565	0.099	0.058	0.585	0.234
21	290	0.500	0.505	0.101	0.068	0.590	0.249
22	308	0.482	0.445	0.099	0.059	0.599	0.247
23	311	0.475	0.555	0.089	0.051	0.720	0.260
24	314	0.587	0.535	0.381	0.060	0.648	0.253
25	333	0.669	0.540	0.182	0.076	0.717	0.252
26	338	0.756	0.597	0.153	0.061	0.793	0.274
27	356	0.808	0.546	0.119	0.049	0.795	0.281
		Sample 27 re-measured with old spectrophotometer					
27	356	0.848	0.635	0.135	0.040	0.976	0.327

Table 34. Experimental run 1; Re-measurements of optical density at 750 nm after samples has been frozen. First is the additional measured OD and beneath is the percentage of this OD compared to the original OD. At the bottom there is an average percentage for each species and a total average percentage for all species combined. Sample no. 16 has been measured both times with the same spectrophotometer while the other ones have been measured with different spectrophotometers before and after freezing.

	Dunaliella tertiolecta	*Phaeodactylum tricornutum*	*Cyclotella cryptica*	*Arthrospira platensis*	*Dunaliella salina*	*Rhinomonas reticulata*
Sample no. 16	0.982	0.776	-	0.048	1.072	0.291
Percentage of previous measurement	91	72	-	46	96	84
Sample no. 19	0.467	0.412	-	0.012	0.542	0.205
Percentage of previous measurement	93	86	-	32	86	88

Sample no. 21	0.439	0.469	-	0.074	0.607	0.249
Percentage of previous measurement	88	93	-	108	103	100
Sample no. 23	0.427	0.444	-	0.015	0.459	0.170
Percentage of previous measurement	90	80	-	31	64	65
Sample no. 24	0.565	0.424	-	0.032	0.646	0.234
Percentage of previous measurement	96	79	-	53	100	92
Sample no. 25	0.650	0.469	-	0.031	0.718	0.211
Percentage of previous measurement	97	87	-	40	100	84
Average percentage of previous measurement	92	83	-	52	91	86
Total average percentage of previous measurement: 81						

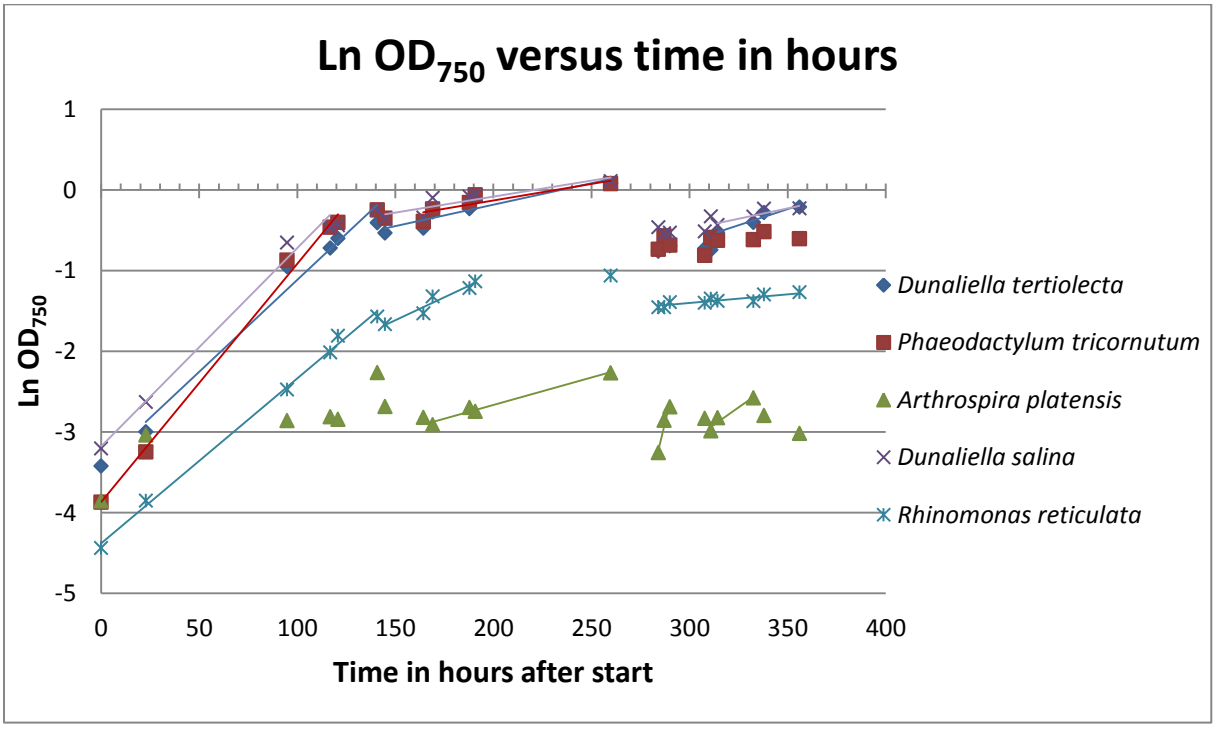

Figure 26. Experimental run 1; Ln OD_{750} versus time in hours from start. Trend lines have been fitted to appropriate points to calculate the specific growth rate and doubling time. Trend lines have been fitted when three or more consecutive data points are aligned on linearity.

Experimental run 2

Table 35. Experimental run 2; Table over optical density at 750 nm. No measurements were made for *C. cryptica* due to too large variance between measurements. Samples have been frozen and tawed once before measuring.

Sample no.	Time from start (h)	*Dunaliella tertiolecta*	*Phaeodactylum tricornutum*	*Arthrospira platensis*	*Dunaliella salina*	*Rhinomonas reticulata*	*Nannochloropsis salina*
3	19	0.053	0.069	0.038	0.063	0.051	0.167
4	25	0.053	0.029	0.027	0.070	0.040	0.167
5	43	0.085	0.042	0.047	0.169	0.034	0.215
6	46	0.088	0.043	0.034	0.136	0.084	0.228
7	49	0.106	0.039	0.045	0.158	0.042	0.230
8	68	0.167	0.031	0.078	0.242	0.038	0.270
9	72	0.191	0.051	0.045	0.274	0.066	0.282
10	74	0.217	0.056	0.042	0.317	0.059	0.303
11	140	0.489	0.071	0.081	0.689	0.119	0.536
12	146	0.549	0.075	0.057	0.775	0.143	0.600
13	165	0.604	0.088	0.059	0.777	0.137	0.655
14	170	0.588	0.097	0.052	0.731	0.132	0.679
15	187	0.604	0.099	0.064	0.772	0.142	0.762
16	193	0.655	0.099	0.059	0.84	0.135	0.827
17	211	0.579	0.124	0.086	0.811	0.152	0.952
18	215	0.480	0.108	0.048	0.740	0.150	0.931
19	240	0.393	0.153	0.068	0.626	0.153	1.147
20	362	0.350	0.220	0.070	0.430	0.082	2.046

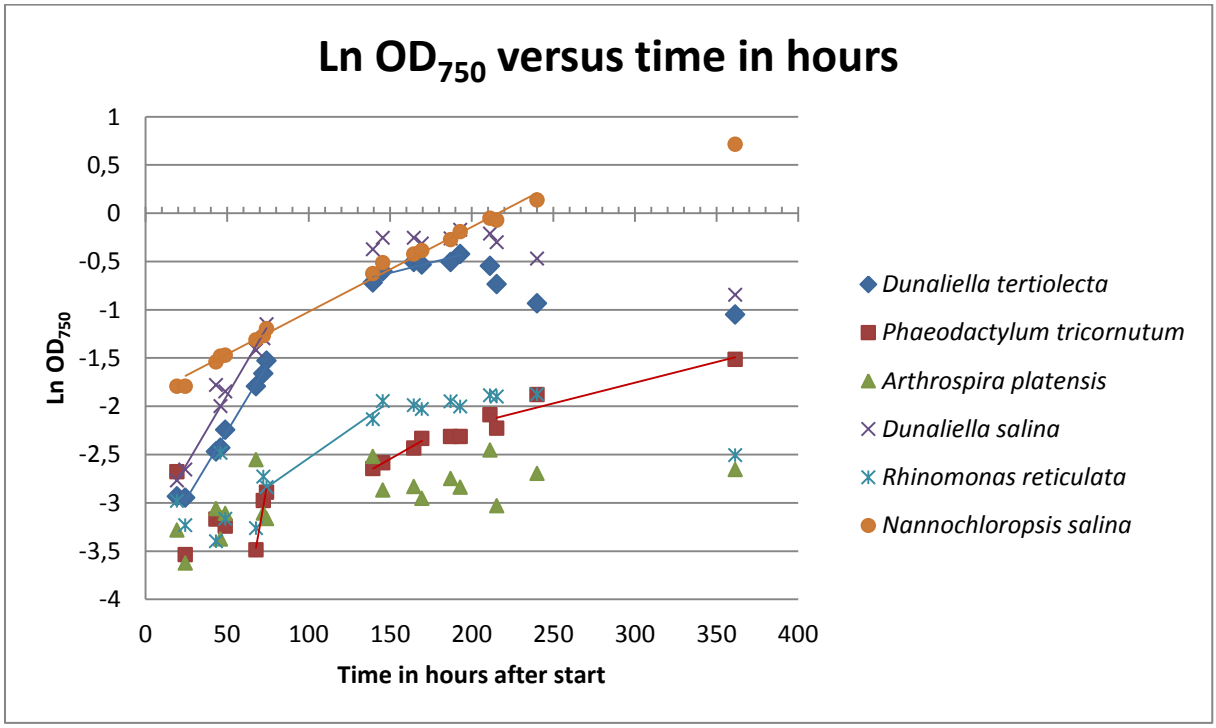

Figure 27. Experimental run 2; Ln OD750 versus time in hours from start. Trend lines have been fitted to appropriate points to calculate the specific growth rate and doubling time. Trend lines have been fitted when three or more consecutive data points are aligned.

Appendix K

Dry weight

The standard deviation for *A. platensis* is high relative to its values. This may be due to that *A. platensis* contains gas vacuoles which can obstruct sampling. The amounts of cells were also low for *A. platensis* which makes the measurement more sensitive. Both the *Dunaliella* species showed by far the densest cultures.

Table 36. Experimental run 1; Dry weight performed at the last sample in experimental run 1, no. 27.

	Filter A (g)	Filter B (g)	Sample A (g)	Sample B (g)	Dry weight of sample A (g)	Dry weight of sample B (g)	Mean dry weight (g)	Std. dev. between samples	Mean dry weight (g/l)
D. tertiolecta	0.122	0.124	0.133	0.132	0.011	0.008	0.0095	0.0021	0.48
P. tricornutum	0.125	0.124	0.130	0.130	0.005	0.006	0.0055	0.0007	0.28
C. cryptica	0.122	0.125	0.127	0.131	0.005	0.006	0.0055	0.0007	0.28
A. platensis	0.125	0.124	0.128	0.125	0.003	0.001	0.0020	0.0014	0.10
D. salina	0.124	0.125	0.135	0.137	0.011	0.012	0.0115	0.0007	0.58
R. reticulata	0.124	0.124	0.129	0.130	0.005	0.006	0.0055	0.0007	0.28

The standard deviations in experimental run 2 are generally much higher than in experimental run 1. The reason for this is unclear. The method used was the same in both experimental runs. *N. salina* showed the lowest deviation and the highest value. Both the *Dunaliella* species gave the second densest cultures.

Table 37. Experimental run 2; Dry weight performed at 140 hours from start.

	Filter A (g)	Filter B (g)	Sample A (g)	Sample B (g)	Dry weight of sample A (g)	Dry weight of sample B (g)	Mean dry weight (g)	Std. dev. between samples	Mean dry weight (g/l)
D. tertiolecta	0.124	0.125	0.142	0.131	0.018	0.006	0.0120	0.0085	0.60
P. tricornutum	0.125	0.125	0.132	0.127	0.007	0.002	0.0045	0.0035	0.23
C. cryptica	0.126	0.127	0.140	0.132	0.014	0.005	0.0095	0.0064	0.48
A. platensis	0.126	0.124	0.130	0.134	0.004	0.010	0.0070	0.0042	0.35
D. salina	0.123	0.121	0.131	0.137	0.008	0.016	0.0120	0.0057	0.60
R. reticulata	0.121	0.121	0.127	0.129	0.006	0.008	0.0070	0.0014	0.35
N. salina	0.120	0.121	0.132	0.134	0.012	0.013	0.0125	0.0007	0.63

Appendix L

Carbohydrate data

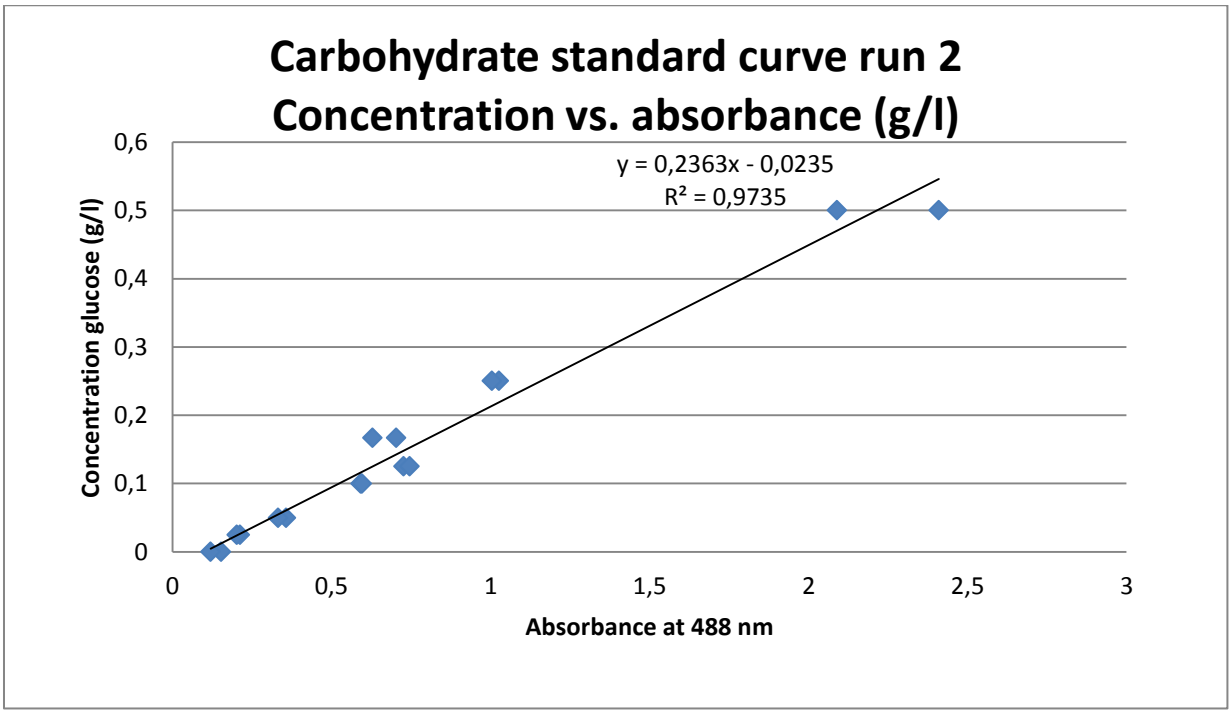

Figure 28. Experimental run 1 and 2; Standard curve for carbohydrates, using glucose.

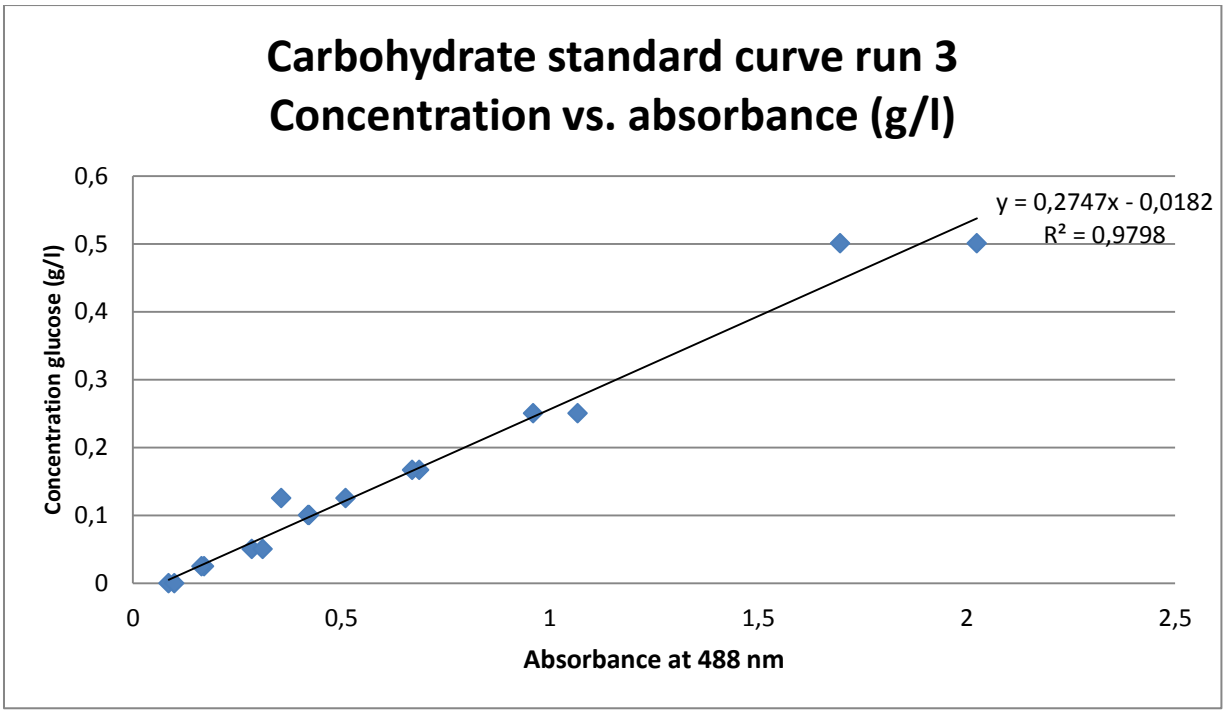

Figure 29. Experimental run 2; Standard curve for carbohydrates.

The amount of carbohydrates in g/l was calculated with the help of a glucose standard curve, Figure 28. To be able to determine the percentage of carbohydrates in the cells an estimated dry weight was used, see Table 17. The samples for *A. platensis* and *R. reticulata* showed high variability and were therefore re-measured, see Table 39. For *A. platensis* the first measurement at 117 hours was obviously incorrect, the re-measurement seems more probable. The values for *R. reticulata* are quite different between the first measurements and the re-measurements. Which values that are most correct is unclear. A trend can be seen for all species, except *A. platensis*, in that the carbohydrate amount increases towards the end of the experiment compared to the beginning, see Table 38.

Table 38. Experimental run 1; Carbohydrate analysis. The percentage content of carbohydrates is calculated with an estimated dry weight.

	Carbs. g/l	Estimated dw (g/l)	% carbs. (g/g)	Avg. % carbs. (g/g)	Std deviation between measured mean values (g/l)
***D. tertiolecta* 117h**	0.029	0.27	10	9	0.018
	0.023		8		
***D. tertiolecta* 311h**	0.066	0.27	25	24	0.016
	0.061		23		
***P. tricornutum* 117h**	0.032	0.27	12	11	0.017
	0.027		10		
***P. tricornutum* 311h**	0.044	0.24	18	16	0.041
	0.031		13		
***A. platensis* 117h**	-	0.15	-	90	0.837
	0.271		181		
***A. platensis* 311h**	0.017	0.13	13	10	0.026
	0.008		6		
***D. salina* 117h**	0.077	0.37	21	22	0.017
	0.083		22		
***D. salina* 311h**	0.086	0.42	20	23	0.060
	0.105		25		
***R. reticulata* 117h**	0.012	0.11	11	10	0.007
	0.010		9		
***R. reticulata* 311h**	0.025	0.22	11	23	0.154
	0.076		35		

- the sample was too small to give a value above zero. Considered to be zero.

Table 39. Experimental run 1; Carbohydrate analysis. Re-measured values for experimental run 1. The percentage content of carbohydrates is calculated with an estimated dry weight.

	Carbs. g/l	Estimated dw (g/l)	% carbs. (g/g)	Avg. % carbs. (g/g)	Std deviation between measured mean values (g/l)
***A. platensis* 117h**	0.032	0.15	21	12	0.084
	0.004		3		
A. platensis **311h**	0.021	0.13	17	10	0.053
	0.004		3		
***R. reticulata* 117h**	0.005	0.11	5	3	0.009
	0.002		2		
***R. reticulata* 311h**	0.011	0.22	5	6	0.005
	0.013		6		

Experimental run 2. Many samples contained too little to give a measureable value. This means that only a few species have values for both samples, both at 68 and at 140 hours from start. Values for the sample at 68 hours are the ones that are missing most of the time and the reason for this could be that the cell content in the samples was too small.

Table 40. Experimental run 2; Carbohydrate analysis. The percentage content of carbohydrates is calculated with an estimated dry weight for the sample at 68 hours from start. For the sample at 140 hours the actual dry weight is used.

	Carbs. g/l	Estimated dw (g/l)	% carbs. (g/g)	Avg. % carbs. (g/g)	Std deviation between measured mean values (g/l)
***D. tertiolecta* 68h**	-	0.21	-	-	0.009
	-		-		
***D. tertiolecta* 140h**	0.071	0.60	12	11	0.015
	0.066		11		
***P. tricornutum* 68h**	-	0.10	-	~0	0.026
	~0		~0		
***P. tricornutum* 140h**	0.026	0.23	11	8	0.042
	0.012		5		
***A. platensis* 68h**	-	0.34	-	-	0.011
	-		-		
***A. platensis* 140h**	0.038	0.35	11	10	0.012
	0.034		10		
***D. salina* 68h**	-	0.21	-	-	0.002
	-		-		
***D. salina* 140h**	0.161	0.60	27	26	0.040
	0.147		25		
***R. reticulata* 68h**	-	0.11	-	-	0.029
	-		-		
***R. reticulata* 140h**	-	0.35	-	-	0.008
	-		-		
***N. salina* 68h**	-	0.31	-	-	0.011
	-		-		
***N. salina* 140h**	0.006	0.63	1	1	0.012
	0.010		2		

- the sample was too small to give a value above zero. Considered to be zero.

The amount of carbohydrates in g/l was calculated with the help from a glucose standard curve, Figure 29, which can be found above. To be able to determine the percentage of carbohydrates in the cells an estimated dry weight was used for the sample at 68 hours, see Table 18. For the sample at 140 hours the actual dry weight could be used.

Appendix M

Protein data

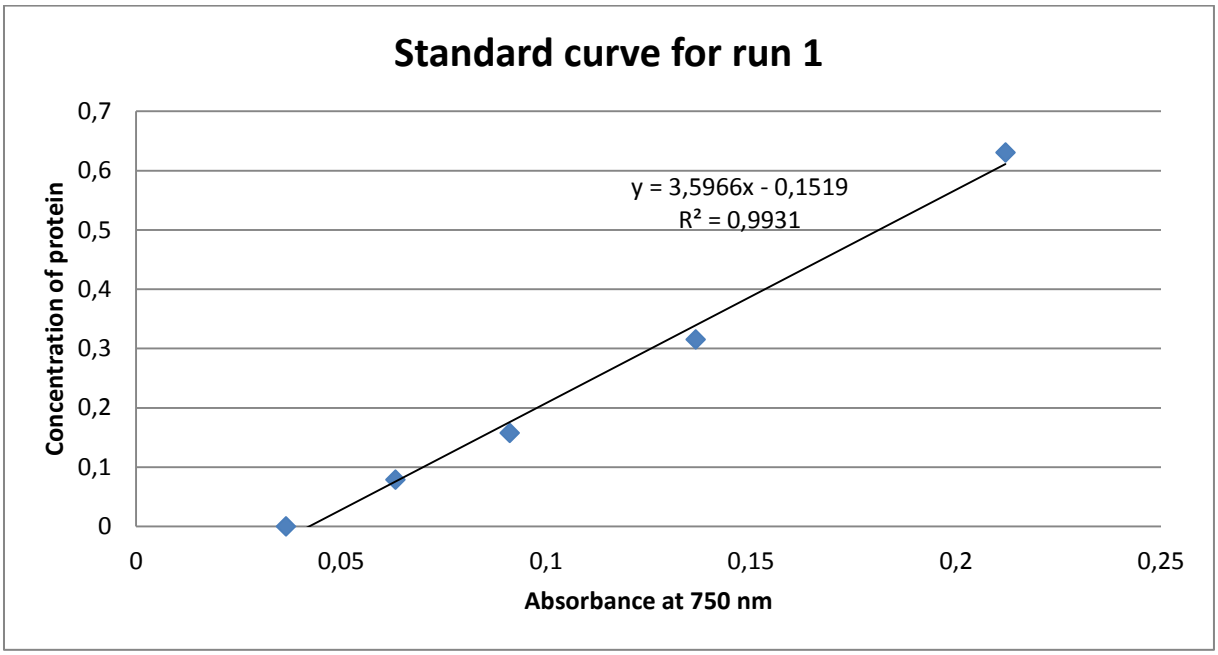

Figure 30. Experimental run 1; Standard curve for the first out of two runs in the protein analysis. This standard curve is used to calculate the amount of protein for the samples in experimental run 1.

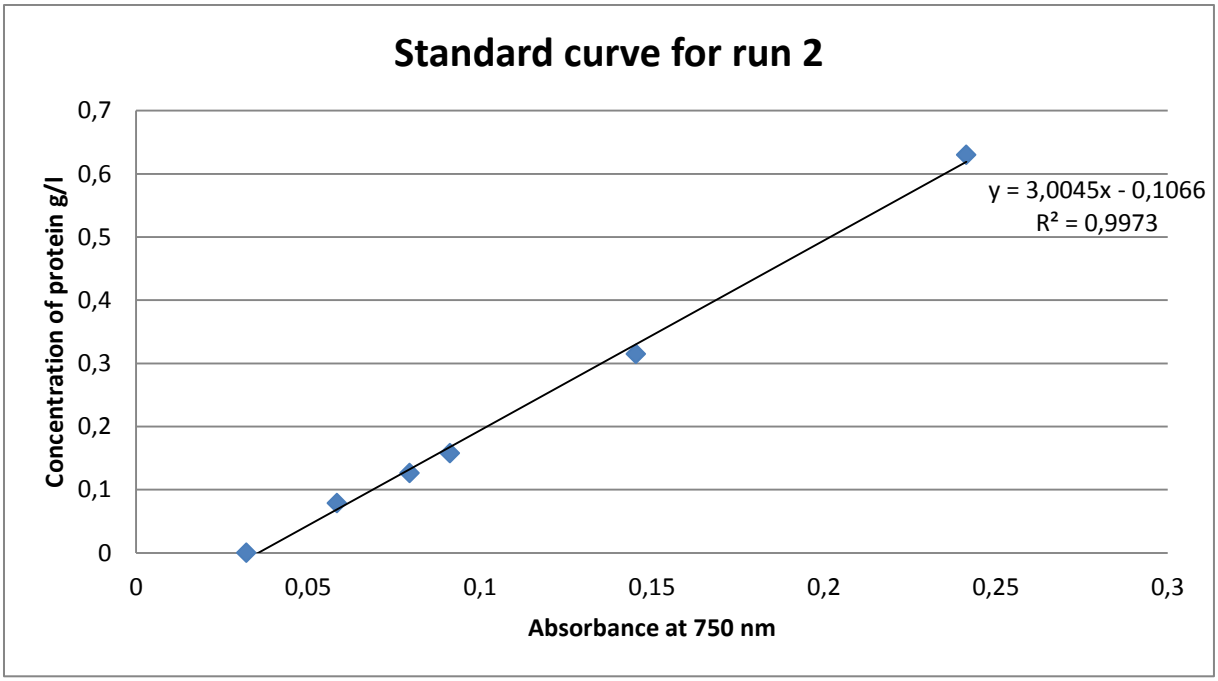

Figure 31. Experimental run 1; Standard curve for the second out of two runs in the protein analysis. This standard curve is used to calculate the amount of protein for the re-measured samples in experimental run 1.

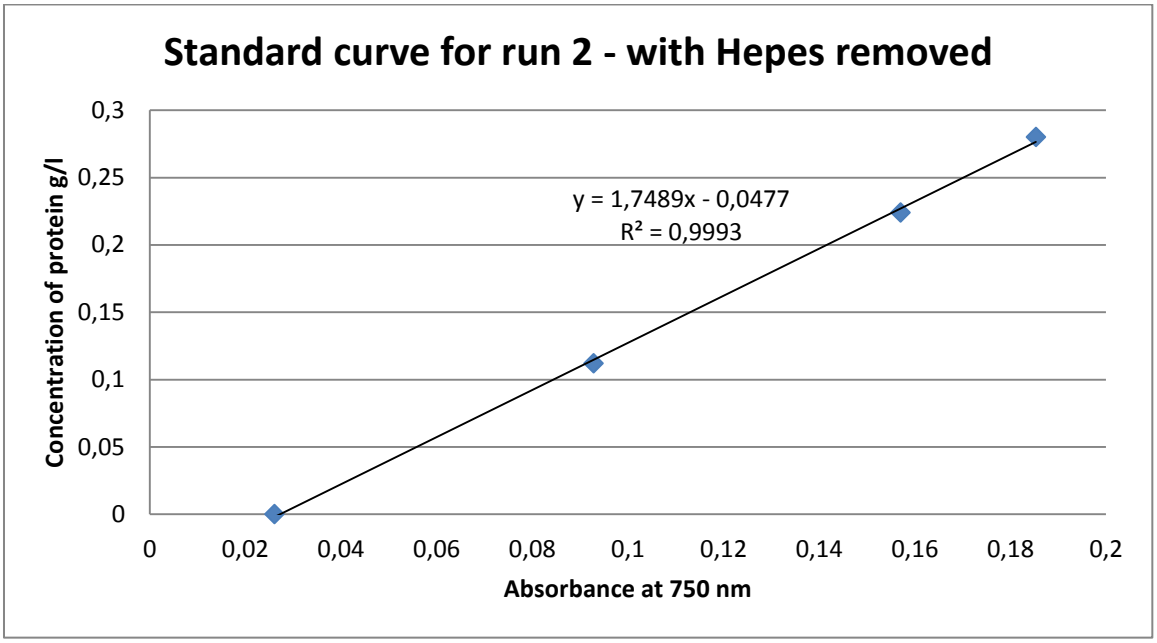

Figure 32. Experimental run 2; Standard curve for the samples in experimental run 2, with the effect of the media removed.

The amount of protein in g/l was calculated with the help from a protein standard curve, Figure 30. To be able to determine the percentage of protein in the cells an estimated dry weight was used, see Table 17. The re-measured samples use the standard curve in Figure 31. The results from the protein analysis for experimental run 1 show high values. The deviation between the duplicate measurements is in many cases large. A few samples were re-measured and these show differing values compared to the first measurement, the variation between duplicates is also here large. A possible reason for the high values could be that something in the medium is affecting the results.

Table 41. Experimental run 1; Protein analysis results from the first protein run. To be able to estimate the protein content of each species an estimated dry weight is used.

Species and hours from start	Measured mean value	Protein (g/l)	Estimated dry weight (g/l cells)	Protein % (g/g)	Protein % (g/g) Average	Std deviation between protein concentration (g/l)
***D. tertiolecta* 117h**	0.090	0.172	0.27	63	72	0.032
	0.103	0.218		80		
***D. tertiolecta* 311h**	0.082	0.142	0.27	53	62	0.032
	0.094	0.187		70		
***P. tricornutum* 117h**	0.086	0.157	0.27	58	78	0.077
	0.116	0.266		97		
***P. tricornutum* 311h**	0.080	0.134	0.24	56	64	0.028
	0.091	0.174		72		
***A. platensis* 117h**	0.076	0.120	0.15	80	88	0.018
	0.083	0.145		96		
***A. platensis* 311h**	0.073	0.111	0.13	88	96	0.014
	0.079	0.131		104		
***D. salina* 117h**	0.098	0.200	0.37	54	54	0.002
	0.099	0.202		55		
***D. salina* 311h**	0.115	0.263	0.42	62	50	0.074
	0.087	0.159		37		

***R. reticulata* 117h**	0.088	0.166	0.11	148	147	0.002
	0.088	0.163		145		
***R. reticulata* 311h**	0.087	0.159	0.22	73	78	0.015
	0.093	0.181		83		

Table 42. Experimental run 1; Protein analysis. Re-measured samples in run 2, using the standard curve for protein run 2.

Species and hours from start	Measured mean value	Protein (g/l)	Estimated dry weight (g/l cells)	Protein % (g/g)	Protein % (g/g) Average	Std deviation between protein concentration (g/l)
***D. tertiolecta* 117h**	0.098	0.189	0.27	69	82	0.025
	0.122	0.260		95		
***P. tricornutum* 117h**	0.093	0.174	0.27	64	69	0.006
	0.102	0.201		74		
***P. tricornutum* 311h**	0.099	0.191	0.24	79	79	0.001
	0.098	0.188		78		
***D. salina* 311h**	0.084	0.147	0.42	35	39	0.008
	0.095	0.180		42		

The amount of protein in g/l was calculated with the help from a protein standard curve, Figure 32, which can be found in aboveAppendix . To be able to determine the percentage of protein in the cells an estimated dry weight was used for the sample at 68 hours from start, see Table 18. The results from experimental run 2 show much lower values than from experimental run 1. The sample at 68 hours for *D. tertiolecta* showed negative values, possibly due to that the cell content was too small. The other species, except *D. salina*, show increased values in protein content from 68 hours to 140 hours from start. At 68 hours the CO_2 concentration was approximately 7.1% and at 140 hours approximately 15.8%.

Table 43. Experimental run 2; Protein analysis. Samples treated to remove the effect of the media. Using the standard curve for protein run 2 with Hepes removed. An estimated dry weight is used for the sample at 68 hours while the actual dry weight is used for the sample at 140 hours. The second sample for *R. reticulata* at 68 hours is calculated as zero.

Species and hours from start	Measured mean value	Protein (g/l)	Estimated dry weight (g/l cells)	Protein % (g/g)	Protein % (g/g) Average	Std deviation between protein content (g/l)
***D. tertiolecta* 68h**	0.024	-	0.21	-	-	0.004
	0.026	-		-		
***D. tertiolecta* 140h**	0.039	0.021	0.60	3	3	0.001
	0.039	0.020		3		
***P. tricornutum* 68h**	0.030	0.005	0.10	5	6	0.001
	0.031	0.007		7		
***P. tricornutum* 140h**	0.041	0.024	0.23	11	9	0.005
	0.037	0.017		7		
***A. platensis* 68h**	0.033	0.009	0.34	3	3	0.002
	0.034	0.012		3		
***A. platensis* 140h**	0.066	0.068	0.35	20	10	0.046
	0.029	0.003		1		
***D. salina* 68h**	0.044	0.029	0.21	14	13	0.002
	0.042	0.027		13		
***D. salina* 140h**	0.040	0.022	0.60	4	3	0.002
	0.038	0.019		3		
***R. reticulata* 68h**	0.028	0.002	0.11	2	1	0.003
	0.026	-		-		
***R. reticulata* 140h**	0.042	0.026	0.35	8	7	0.005
	0.038	0.020		6		
***N. salina* 68h**	0.028	0.001	0.31	~0	1	0.003
	0.030	0.005		2		
***N. salina* 140h**	0.040	0.022	0.63	3	4	0.006
	0.045	0.030		5		

Appendix N

Triglyceride data

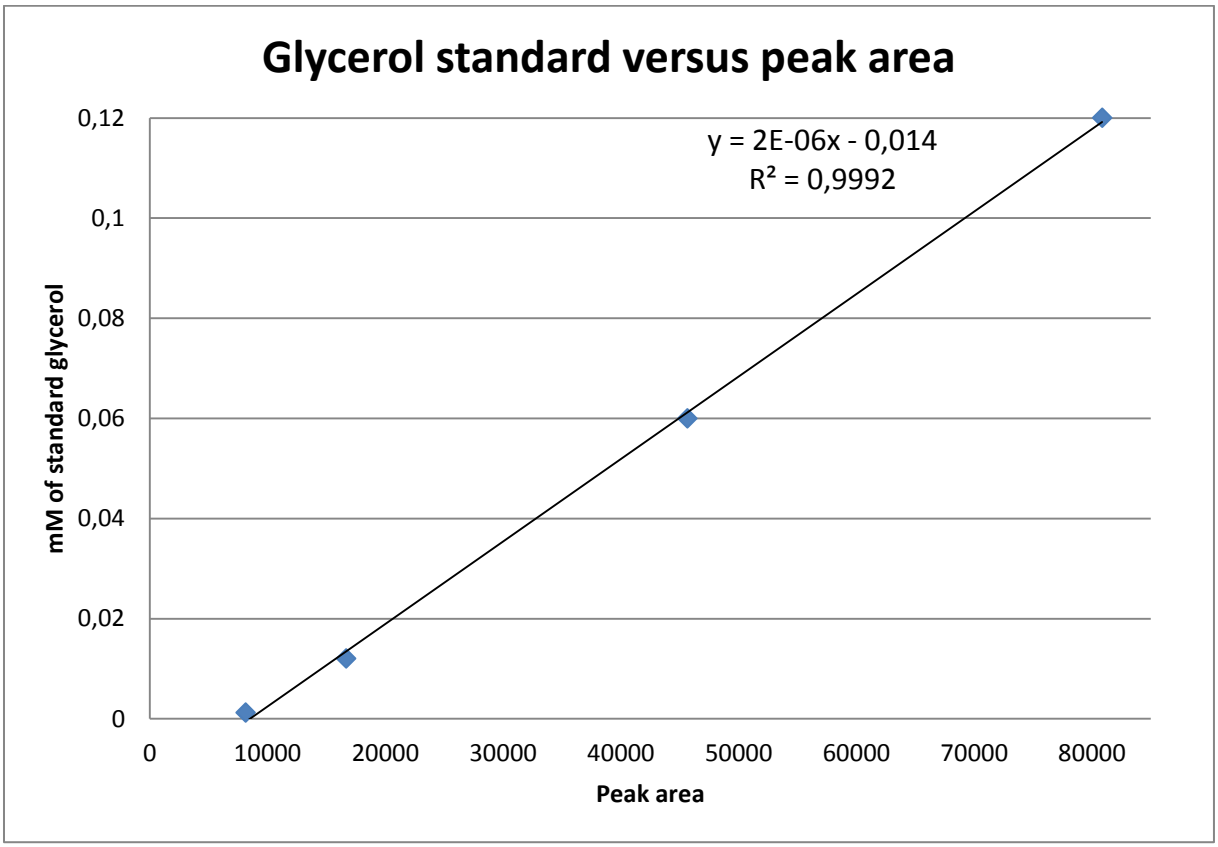

Figure 33. Experimental run 1 and 2; Glycerol standard curve used to calculate the amount of triglycerides in the cells. An estimated average of 870 g/mol for a triglyceride is used to calculate total triglycerides from this glycerol standard.

The amount of triglycerides in g/l was calculated with the help from a glycerol standard curve, Figure 33. To be able to determine the percentage of triglycerides in the cells an estimated dry weight was used, see Table 17.

All of the samples show quite low values for triglycerides. The amount of triglycerides increases between both sample points for all species except *R. reticulata*. An explanation for this could be an increased stress on the cells, as carbon dioxide increases – NO had been switched on not long before, at 308 hours, so it is less likely that the cells have had time to respond to the addition of NO. SO_2 had not yet been switched on.

Table 44. Experimental run 1; Triglyceride analysis. The total triglyceride content is coupled to the estimated dry weight. An estimated average of 870 g/mol for a triglyceride is used to calculate total triglycerides from the glycerol standard.

Species	Part	Peak area	Total triglycerides (g/l)	Estimated dw (g/l)	% triglycerides (g/g)
***D. tertiolecta* 117h**	Pellet	12995	0.008	0.27	3
	Supernatant	12374			
***D. tertiolecta* 311h**	Pellet	22408	0.013	0.27	5
	Supernatant	10720			
***P. tricornutum* 117h**	Pellet	35093	0.020	0.27	7
	Supernatant	7165			
***P. tricornutum* 311h**	Pellet	56618	0.037	0.24	16
	Supernatant	11117			
***A. platensis* 117h**	Pellet	-	0.003	0.15	2
	Supernatant	11031			
***A. platensis* 311h**	Pellet	7665	0.002	0.13	2
	Supernatant	9821			
***D. salina* 117h**	Pellet	16855	0.014	0.37	4
	Supernatant	16619			
***D. salina* 311h**	Pellet	39610	0.033	0.42	8
	Supernatant	22014			
***R. reticulata* 117h**	Pellet	21611	0.012	0.11	10
	Supernatant	9141			
***R. reticulata* 311h**	Pellet	18984	0.011	0.22	5
	Supernatant	10484			

- the sample was too small to give a value above zero. Considered to be zero.

The amount of triglycerides in g/l was calculated with the help from a glycerol standard curve, Figure 33. To be able to determine the percentage of triglycerides in the cells an estimated dry weight was used for the sample at 68 hours, see Table 18. For the sample 140 hours the actual dry weight could be used.

Experimental run 2 shows even lower values for triglycerides here than in experimental run 1. This could be explained by the reduced stress on the cells due to a higher and more stable pH. The values increase between the both samples, as in experimental run 1. The CO_2 was increased between the both samples and might account for the increased triglyceride content.

Table 45. Experimental run 2; Triglyceride analysis. The total triglyceride content is coupled to the estimated dry weight for the sample at 68 hours to give an estimate on the triglyceride percentage in the cells. For the sample at 140 hours the actual dry weight is used. An estimated average of 870 g/mol for a triglyceride is used to calculate total triglycerides from the glycerol standard.

Species	Part	Peak area	Total triglycerides (g/l)	Estimated dw (g/l)	% triglycerides (g/g)
***D. tertiolecta* 68h**	Pellet	7817	0.001	0.21	0.3
	Supernatant	-			
***D. tertiolecta* 140h**	Pellet	16059	0.007	0.60	1.2
	Supernatant	8294			
***P. tricornutum* 68h**	Pellet	7995	0.001	0.10	0.9
	Supernatant	7265			
***P. tricornutum* 140h**	Pellet	12646	0.004	0.23	1.8
	Supernatant	-			
***A. platensis* 68h**	Pellet	-	~0	0.34	~0
	Supernatant	7190			
***A. platensis* 140h**	Pellet	8444	0.001	0.35	0.3
	Supernatant	7079			
***D. salina* 68h**	Pellet	-	0.005	0.21	2.4
	Supernatant	14277			
***D. salina* 140h**	Pellet	18290	0.015	0.60	2.5
	Supernatant	16916			
***R. reticulata* 68h**	Pellet	-	-	-	-
	Supernatant	-	-		-
***R. reticulata* 140h**	Pellet	8258	0.002	0.35	0.5
	Supernatant	8439			
***N. salina* 68h**	Pellet	13770	0.006	0.31	1.9
	Supernatant	8825			
***N. salina* 140h**	Pellet	11230	0.013	0.63	2.0
	Supernatant	20808			

- the sample was too small to give a value above zero. Considered to be zero.