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

Master's Thesis in the Master Degree Programme, Biotechnology

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Abstract

Today a lot of carbon dioxide is released into the atmosphere due to human activities. A way of recycling the carbon and reuse waste for something beneficial is needed. Microalgae have the capacity to fixate inorganic CO_2 into organic matters and purify flue gases from this component. A desirable characteristic of many species of microalgae is the capability of producing and accumulating lipids suitable for biodiesel. Some algal species can also accumulate polysaccharides as *e.g.* starch for bioethanol purposes. A large and cheap carbon source, as CO_2 enriched air, is needed for a large scale production of algal biomass and exhaust gases from many industries could be possible alternatives. Potentially for future use, wastewater from an industry could be growth medium and provide nutrients, mainly nitrogen and phosphorous, needed for growth of microalgae. The risk of eutrophication would then be minimized.

This project involved screening and investigation of growth of eleven different freshwater microalgae species with simulated flue gas, mimicking flue gas from Swedish pulp- and paper mills, as carbon source. Batch cultures in Erlenmeyer flasks (250 ml) were used for cultivation and a gas distribution system was set up for providing artificially produced flue gas of 15 % CO_2, 100 ppm NO and 10 ppm SO_2 which was bubbled through the cell suspensions. Growth was followed by OD_{750} and cell count and higher specific growth rate means higher carbon fixation capacity. All freshwater species showed a quality of being able to grow in the high concentrations of the three constituents in the flue gas and can thereby work as a carbon source for microalgae. At the stated highest levels of the flue gas components the specific growth rates were generally decreased. *Scenedesmus* simris002, *Chlamydomonas reinhardtii* and *Nannochloropsis salina* showed high specific growth rates; they were pH tolerant and seemed to resist all components in the flue gas. The pH was a function of added flue gas constituents and concentrations (lowering the pH) and growth of algae (increasing the pH). The pH must be controlled when cultivating algae as both literature and experiments during this project indicate that too low pH kills the algae. Lipids, carbohydrates and protein content (% g/g) were investigated at late exponential phase, as potential oil or starch producer is of interest for biofuel purposes. The most promising species for carbohydrates were *Chlorella emersonii* (45 %) and *Chlorella sorokiniana* (34 %). The lipid content was estimated by a correlation where the values for proteins (g/g %), carbohydrates (g/g %) and assumed ash weight of 10 (g/g %) were subtracted from 100 % and the rest was assumed to be the proportion of lipids. The species with highest estimated lipid content were *Botryococcus braunii* (57 %) and *Chlorella vulgaris* (52 %). As for the protein content *N. salina* (66 %) and Nedsjön isolate (64 %) obtained the highest values.

Two types of wastewater from two different purification steps, obtained from *Nordic Paper Bäckhammar AB*, were tested as growth medium in small flasks with loose caps. The waters were the only source of nutrients for growth of the cells. The freshwater species cultivations were followed visually and *S.* simris002, *Scenedesmus obliquus* and *C. sorokiniana* showed the most promising results of being able to grow in the wastewater, even though growth seemed to be limiting due to low exchange of gas and CO_2 or too low nutrient concentration. The strain of *N. salina* showed qualities of growing in freshwater, brackish water and marine water which is a desirable characteristic if diluting the growth medium with a nearby lake or sea.

Abbreviations and explanations of important words

The different parts of the report contain words that need explanations. They are collected in this chapter to make it easier for the reader to follow the report.

3N-BBM+V	Bold's Basal medium with threefold of N (+ vitamins) [1]
Autospore	A spore of algae, non-motile, asexual, reproductive, with adult characteristics even before being released, hence a miniature of the cell that creates it [2].
Autotroph	Organism capable of using CO_2 as only carbon source [3].
CCAP	Culture collection – of algae and protozoa [1]
NCMA (previously CCMP)	The Provasoli-Guillard National Center for Culture of Marine Phytoplankton [4].
Chlorophyta	Green algae [5].
Chloroplast	Organelle where photosynthesis occurs in eukaryotes [3].
Chromophores	Chromophores are the portions of the light absorbing pigments and absorbs light at specific wavelengths. [6].
Chrysophyta	Golden algae, exists in orange, yellow or brown color [7].
Chrysolaminarin	Polymer of carbohydrates as carbon storage (diatoms) [8].
Chemical oxygen demand (COD)	An indirect measurement of how much organic compounds that exist in water, estimating water quality (mg/l). It refers to the amount of O_2 consumed per l of H_2O.
Coenobic form (coenobia)	Cells are in a colony with a fixed number of cells. The colony has no or little specialization [2].
Coenocytic	Multiple nuclei, single unit with multiple linked cells.
Cyanophyta	Blue-green algae, or cyanobacteria [8].
Eukaryote	An organism with a membrane-enclosed nucleus and most likely contain other organelles within the cell [3].
Exponential phase	A growth period of a population where the growth is increasing at an exponential rate [3].
Extremophile	An organism growing optimally at extreme conditions such as extreme temperature or pH [3].
Ferredoxin	Ferredoxins are iron-sulfur proteins and works as electron transfer complexes and facilitates this transport [6].
Filamentous	A kind of form of the microorganisms, very long rods where the length is much larger than the width [3].
Fucoxanthin	Pigment in diatoms, brown and golden algae. It masks the color of other carotenoids, giving a brownish color [2].
Inhibition	Reduction of microorganisms in number or in growth due to alterations in the environment [3].
Genus	Related species collected in a taxonomic group [3].
Heterotroph	An organism which gain their carbon and energy source from organic compounds created by other organisms [9].
Hydrogenase	H_2 is produced as hydrogenase combines e^- with H^+ [10].
Isogamous reproduction	A sexual reproduction of gametes with similar morphology and the fertilization happens as two different mating types fuse and forms a zygote [2].
Medium (plural media)	Nutrient solution where microorganisms are grown in [3].

Mixotroph	An organism which can use organic compounds as carbon source and inorganic compounds as electron donors [3]. Respiratory and photosynthetic metabolism co-works [9].
Morphology	How the shape is for an organism [3].
Nitrogenase	Enzyme used by organisms to fix atmospheric N [10].
Nucleolus	A region containing ribosomes where the ribosomal RNAs are transcribed and further processed [6].
Nucleoplasm	Similar to cytoplasm, but exist in nucleus.
Nutrient	Substrate taken from the environment of the cell and consumed for catabolic or anabolic reactions [3].
Organelle	A membrane-enclosed structure found in eukaryotes [3].
Phaeophyta	Brown algae [11].
Phospholipids	Lipids with a substituted phosphate group together with two fatty acids on the glycerol backbone [3].
Photoautotroph	A cell capable of using light as only energy source and CO_2 as the only carbon source [3].
Photoheterotroph	Organisms capable of using light as energy source and organic compounds as carbon source [3].
Photosystem	Structural units in cellular membranes where light is captured and converted to chemical energy. Plants, algae and cyanobacteria have two systems, *PSI* and *PSII* [6].
Phototroph	An organism where light is used as energy [3].
Phycocyanin	A pigment-protein complex. Is an accessory pigment to chlorophylls and absorbs orange and red light giving a blue pigment within cells indicating a blue color [12].
Phylogeny	Construction of evolutionary trees by relationships and ordering of organisms into higher taxa [3].
Phytoplankton	All photoautotrophic microorganisms in water.
Pyrenoid	Center of chloroplasts where CO_2-fixation occurs [2].
Rhodophyta	Red algae [13].
Sporangium	Enclosed space where distinct spores from meiosis are formed. Single celled or multicellular [2].
Strain	A population of cells within one species where all cells come from one single cell [3].
Syngas	Syngas is a mixture of CO and H_2 [10].
Thylakoid	Thylakoids are bound to membranes in chloroplasts that contain the photosystems [6].
Vegetative reproduction	Asexual reproduction in plants, no production of spores.
Wastewater	Liquid from domestic sewage or industry and it cannot be discarded into lakes or streams if it is not treated [3].

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1. Introduction

Microalgae have got a lot of attention worldwide as a common goal for the human population is to reach a sustainable society where biofuels are produced. Waste streams from different industries containing valuable carbon is released into the atmosphere and the goal of this research is to develop a sustainable CO_2 reduction process for flue gas from Swedish paper mills and use unexploited resources with a suitable alga for future purposes of producing biofuel from waste. The project can be seen as a screening where several freshwater microalgae were collected and tried out as possible CO_2 fixers. The report focuses on freshwater microalgae species and was made in parallel to a similar project treating marine microalgae species performed by Engström [14]. Batch cultures with freshwater medium and artificially produced flue gas bubbled through the cell suspensions were used to make the algae grow. The specific growth rates were examined to distinguish which species that was growing the best. Growth was followed by optical density and cell count measurements and a cell content analysis was also made to get a clue about the biofuel producing capacity of the species.

1.1. Background

Today's use of limited fossil fuels together with exhaust gas emissions are two of the main problems in the world. A steady increase in human population leads to an increase in global energy demands and thereby higher emission levels of CO_2 [15]. Global warming or not, the atmospheric CO_2 concentrations clearly increase due to emissions [16]. If governmental policies do not change an increase in energy demand will rise with 60% until the year of 2030 [15] and the predicted emission in year 2100 will be over 26 billion tons CO_2 per year [17]. To reach a sustainable society where CO_2 emissions are decreased and biofuels are produced, use of certain biotechnologies will be crucial. Industrial factories and power plants contribute mostly to the emissions of CO_2 where the flue gases contain levels of 15-20 % CO_2 [17]. A large industry in the Nordic countries, including Sweden, is the pulp and paper mills which release exhaust gas with high levels of CO_2 [18]. No functional cleaning technique exists today to decrease the CO_2 emissions. The possibility to recycle carbon and fix it into biomass and further convert it to valuable compounds such as lipids or carbohydrates is tempting. A quite unexplored area within biotechnology is the use of microalgae. Terrestrial plant species only contribute with 3-6 % of the fixation from the atmospheric CO_2 and microalgae fix CO_2 10-50 times more efficiently than these [17]. Microalgae have rapid biomass formation compared to plants, mostly due to better access of nutrients, minerals and water surrounding the cells [19].

As microalgae are found almost everywhere in different ecosystems it is possible to find a broad spectrum of different wanted characteristics and abilities, certainly among extremophiles [20]. In theory, microalgae are miniature factories and use photosynthesis to live. Sunlight is the energy source and inorganic CO_2 is mainly the carbon source [15]. Studies have shown that microalgae can be grown in flue gas and fix CO_2 with high efficiency if the concentrations of sulfur and heavy metals are low enough [18]. Borkenstein et al. [16] have made a similar study as the previous mentioned but with exhaust gas from a cement plant. *Chlorella emersonii* was used to study whether or not the doubling time was affected by flue gas compared to pure CO_2. The doubling time did not differ significantly which shows that flue gas can be used as carbon source, one step towards closing the carbon cycle. Cleaning of the gas and growth of microalgae is a win-win situation. The industries would not need to pay the high CO_2 taxes if the method would work.

Obtained biomass can be used for many purposes, but producing biofuels is probably most important. Methane gas, hydrogen gas, biodiesel and bioethanol are all alternatives possible with microalgae. Algae can also be used as animal fodder or simply combust it to use the energy [20]. Primarily, lipids and carbohydrates are wanted to have in the cells, lipids for biodiesel and carbohydrates for ethanol production [10]. The oil production in algae is far superior to *e.g.* soybean, palm, rapeseed and corn. Some species can accumulate lipids up to 80 % of dry weight [15]. Microalgae can replace fossil fuels completely [21]. Lipids would be produced from renewable resources and the production would be environmental friendly [22]. Oil production will not compete with use of arable land or with feedstock for food industry [15]. All microalgae in this project are possible species for oil production [23]. Another quality of microalgae is that nitrogen and phosphorous levels can be reduced in *e.g.* waste water from the industry since they are incorporated into biomass and can thus be removed [19]. This ability can be used to reduce biomass production cost. Use of microalgae at industrial scale has not yet come far in Sweden. However, Gentili [24] has developed a technique with *Avfall Sverige AB* and *Swedish Energy Agency* as financiers to purify municipal wastewater from N and P and keep these from being released in nature. The project was performed together with *Umeå Energi AB* and *UMEVA*.

Many challenges remain to be investigated before large scale cultivations of microalgae and biofuel production can be cost-effective. Fast growing species of algae generally contain lower amounts of lipids (<20 % of DW), whereas cells accumulating lipids (40 % to 50 % of DW) grow slower [21]. High lipid productivity is connected to cells being under stress, such as nutrient starvation [22]. Li et al. [22] claims that few species produce large quantities of lipids under favorable conditions. Therefore it is important to investigate species with an ability to produce lipids and find one with fast growth. Other significant challenges are to distribute light within the culture and to extract the oil through the tough cell wall without too high costs [10] and to avoid fouling, photoinhibition and mechanical stress due to *e.g.* agitation [25]. O_2 produced by algae can be toxic as O_2 get trapped and lead to local high concentrations [26]. Regarding flue gas as carbon source a challenge would be to achieve an injection system with optimum contact time between CO_2 and the algae [10]. The contribution of CO_2 can be hard to reach the required amounts necessary for highest growth [25]. High concentrations of CO_2, NO or SO_2 could lead to toxicity and low pH due to acid formation when dissolved in water [27].

The significance of this project is to investigate useful species and see whether or not they could be used in a first step to clean CO_2 from exhaust gases from paper mills. It could be possible to use the process water from industry and/or use water coming from a nearby lake or the sea as part of the growth medium. In Sweden there are many industries along the coast and also inland. The investigation of different species must be investigated how they thrive in the actual environment in which they will be cultured, *e.g.* waste water as growth medium and flue gas as carbon source.

1.2. Problem formulation

An effective way of cleaning CO_2 emissions from exhaust gas is presently missing. Use of fossil fuels is not sustainable and a sustainable solution where recycling of CO_2 is included must be researched. Besides the emissions and greenhouse effect, the industries need to pay CO_2-fees. Money can be saved for the industries with a successful technique and a cheap carbon source can be obtained for cultivation purposes. In theory algae are the optimal solution, but there are practical problems needed to be solved to obtain suitable microalgal processes which could be used at industrial scale. The best suitable microalga with high CO_2-fixation capacity needs to be found and the first step is to decide which species that can be used for further studies. It would be of great importance to investigate cell content and see whether or not the microalgae have abilities such as producing lipids or carbohydrates for biofuel purposes.

1.3. Aims and target groups

All kinds of power plants emitting large quantities of CO_2 could be potential carbon sources for algae. However, this project has Swedish paper mills as target group as Sweden has many mills around the country. The pulp- and paper industry is changing where less paper pulp is needed and alternative solutions for higher profitability is in their interest. The mills release large quantities of CO_2 and their exhaust gas will be mimicked in this study. The first part of the project is to find out the composition of the exhaust gases and decide the composition of the simulated flue gas. Furthermore, the study is a screening of eleven different species of microalgae in freshwater cultivations with the simulated flue gas bubbled through the cell suspensions as carbon source with a purpose to select a few species for further studies. The study aims to investigate the specific growth rate and thereby CO_2-fixation capacity. The goal is to find fast growing and robust species capable of cleaning flue gas from CO_2 to be used as a sustainable cleaning technique and to investigate the effect of flue gas on cell content, *i.e.* of lipids, carbohydrates and proteins. Slower growth can be compensated by high lipid/carbohydrate content and vice versa, which means that these qualities need to be considered when choosing the most suitable microalga for an overall good productivity. Growth in wastewater from *Nordic Paper Bäckhammar AB* at two different purification steps should be investigated as growth medium in a visual study. The best species should be selected for further scaling-up experiments and tested with real exhaust gas in future studies.

1.4. Restrictions and limitations

Because of a limit in the cultivation set up, eleven species were investigated, six the first run and five the second run. Only one strain of each species was studied. There are of course differences within one species so it is possible that different strains would have been better than the chosen ones [17]. However, it was more interesting to study eleven different species than eleven different strains within one species.

Artificially produced gas mimicked the real exhaust gas from paper mills with three constituents; CO_2, NO and SO_2. There would be chemical compounds in the real gas which cannot be mimicked artificially in the lab. However, the next step would be to investigate real exhaust gas from Swedish paper mills.

No artificial agitation was done. Agitation occurred naturally by the bubbles flowing out at the bottom and was relatively equal between the batch cultivations. Light was added artificially from fluorescence lamps. The light intensity did not vary as in a normal night/day cycle and a lower intensity was used compared to real sunlight. The temperature and pressure was the same for all cultivations, approximately room temperature and atmospheric pressure. All species have different optimal temperature for growth, but it was not possible to cultivate each of them at different specific temperatures.

2. Theory

2.1. Microalgae

Photosynthetic capacity exists among a diverse group of organisms including plants, algae and some photosynthetic bacteria [8]. The term algae were by phycologists previously referred to as any organism with *chlorophyll a* and lack of roots, stems or leaves and formerly included species of cyanobacteria which are nowadays regarded as prokaryotes [12]. Microalgae are microscopic algae where photosynthesis is used as an energy process to produce complex and energy rich compounds by fixating CO_2 and using surrounding building blocks [28]. This is the key for taking care of the sun's energy and to provide all organic life on earth with organic energy. Microalgae are regarded as the primary producers in the ocean. Microalgae are found almost everywhere and can live in marine, brackish and fresh water. Scientists have estimated the total number of algal species to over 50,000 in the world and only 30,000 species are identified and examined [20]. Even less species are really tried out biotechnological for industrial purposes. Biodiversity of microalgae is explained in *Appendix A* but is not essential for understanding the report.

2.1.1. Physical properties of photosynthetic eukaryotes

Microalgae have different morphology and physical properties. The nucleus of eukaryotes contains a set of chromosomes which contain most of the genome [29]. Three major cell organizations occur for microalgal species, namely *unicellular*, *colonial* and *filamentous* [29] but also *coenocytic* or *macrophytes* exist in *e.g.* green microalgae [2]. Microalgae can be motile and non-motile [2]. Motility is dependent on the existence of flagella and if lacking these cells are non-motile [29].

Biomass from microalgae mainly consists of carbohydrates, proteins and oils [8]. The microalgal cell wall consists of a microfibrillar layer of cellulose which can sometimes be enclosed with an amorphous layer, a protective layer, and both comprise the cell wall. The cell wall may be fortified with *plates* and *scales*. Sometimes there may be laminated polysaccharides outside of the outer amorphous coating. The plasma membrane is very thin and surrounds the cytoplasm containing organelles. Microalgae have different cell compartments where Golgi, mitochondria, ER, plastids, chloroplasts, ribosomes, lipid droplets, centrioles and vacuoles are common [8]. Flagella and microtubules may also occur [8]. A double nuclear membrane encloses the nucleus and contains nucleolus, genetic information as DNA molecules in chromosomes. Most microalgae are uninucleate, but multinucleate species exist [29].

A very important organelle in algae is the chloroplast, a flattened vesicle, with chlorophyll and other pigments, which is the site where the photosynthesis occurs [2]. Plants have 20 to 50 chloroplasts in each cell [6], whereas microalgae can have one or more chloroplasts within one cell and often they have only one big [2]. Furthermore, the chloroplasts have their own DNA coding for their own proteins [6]. Nowadays there is evidence showing that chloroplasts have evolved from symbiosis between eukaryotes and unicellular photosynthetic organisms (comparable to cyanobacteria). The internal structure of chloroplasts involves an inner membrane which embeds the stroma, similar to the mitochondrial matrix [6]. There exists alternating layers of thylakoids containing the chlorophyll, lipoprotein membranes and stroma, the aqueous phase [28]. Thylakoids are flat and saclike structures and all absorption of light occurs on or within the thylakoid membranes [6]. All NADPH and ATP produced are released into the stroma where the dark reaction occurs (further explained later) [6]. In cyanobacteria the thylakoids exist free in the cytoplasm, hence the photosynthesis occurs here [29]. Important polyunsaturated fatty acids exist in lipids in the cytoplasm and in the chloroplasts [29].

2.1.2. Reproduction and growth in a batch system

Vegetative, asexual, reproduction through cell division is common for most of the microalgal species. Other asexual reproduction occurs through fragmentation and production of spores. Sexual reproduction occurs for most species in a life-time but is not a universal feature [29]. In general, small microorganisms grow faster than big ones [3].

A culture growing in a closed system where nothing is added or taken away is called a batch culture [3]. An inoculum of cells is added and the batch culture is kept in an environment, which favors growth [30]. The growth is divided into three phases; the *lag-*, the *log-* and the *stationary phase* [3].

The *lag phase* varies in time depending on the growth conditions [3]. If an exponentially growing culture is transferred to a similar environment the exponential growth starts without delay, according to Madigan & Martinko [3]. If the cell sample is transferred from a stationary phase to a new fresh medium an adaptation occurs, *i.e.* a *lag phase*. If cells are coming from a rich medium to a poor medium the cells need to produce enzymes for production of essential metabolites missing, hence a long *lag phase* occurs.

Cells growing by division in *log phase*, synonymously *exponential phase*, are often in their healthiest state where one cell divides into two new cells which each divide into two new cells and so on. If the number of cells is plotted in a *semi logarithmic* way a straight line will be seen. Exponential growth cannot grow indefinitely in a batch culture and only occurs in the log phase.

The amount of absorbed energy most often depends on the amount of cells in a cell suspension rather than photon flux density [30]. However, a *linear phase* may, according to Lee & Shen [30], in some cases interrupt the growth cycle. If a stream of photons with low flux density passes through a cell suspension with low cell concentration some photons slip through. As the cell concentration increases there could be a point where all the photons are captured. The increase in cell density will be exponential until this limiting point and hereafter the biomass production occur at a linear rate as a function of added light.

Either log phase or the linear phase will last until any other limiting factor occurs. Finally, the nutrients start to deplete in a batch system as no new medium is added [3]. Madigan & Martinko [3] mean that either the essential nutrient is depleted or waste and toxic products accumulates and limit growth. As a result the exponential phase starts to decline and the *stationary phase* starts. At the end a *death phase* can occur as nutrients are depleted or toxic substances are released as there are too many cells in the solution [10]. The quality of the harvested cells in a batch culture varies with time and phase and is thus less predictable compared to continuous systems [10].

2.1.3. Factors influencing microalgal growth

To be able to thrive and grow, algae have several requirements on their environment and many important parameters affect the growth. When cultivating algae for production purposes, very important parameters to consider would be nutrients, light, pH, temperature and mixing.

2.1.3.1. Nutrients

Microalgae have two major possible ways for nourishment, namely *autotrophy* using light and/or *heterotrophy* using a chemical compound as energy source, respectively [31]. Algae growing in a heterotrophic way grow slower compared to autotrophy. During photoautotrophic growth, algae only need absorption of light and inorganic CO_2 to fulfill the energy demands. Other substrates, primarily water, get oxidized and O_2 is produced [31]. Water is crucial for algae to grow, but algae consume less water that higher land living plants [32]. When some organic compound, such as *e.g.* acetate, fructose

and glucose, additionally can be utilized as carbon sources (*mixotrophy*), the productivity of biomass would increase. *C. sorokiniana* was possible to grow *mixotrophically* during the light hours using both glucose and CO_2 and during the dark hours it was only growing on glucose [31]. In this way, it was possible to obtain higher cell concentration during a shorter time, since the specific growth rate is roughly the sum of the rates from algae living in photoautotrophic and heterotrophic ways, respectively [9]. In this study, only *photoautotrophy* as metabolic mode has been considered since the purpose is to clean exhaust gas from CO_2 [31].

There are some major elements needed for the formation of organic compounds within algae. These are carbon (C), hydrogen (H), oxygen (O), nitrogen (N), sulfur (S), phosphorous (P), potassium (K), sodium (Na), calcium (Ca), magnesium (Mg) and chlorine (Cl) [33]. Geider & MacIntyre [33] describes the phytoplankton stoichiometry as rather consistent and defined by the Redfield ratio, as C:N:P = 106:16:1. The trace elements needed for growth are iron (Fe), manganese (Mn), copper (Cu), zinc (Zn), cobalt (Co) and molybdenum (Mo). Some vitamins are required for some phytoplankton as they are not capable of synthesizing these themselves. Nitrogen and phosphorous are the two bulk elements which normally could be depleted during growth and can limit the biomass production.

Riebesell & Wolf-Gladrow [34] explain that the nutrient uptake occurs primarily by molecular diffusion. The rate of diffusion transport depend on three parameters, namely the concentration gradient from bulk medium to cell surface, the diffusion coefficient of the nutrient and the thickness of the diffusive boundary layer. These parameters can be affected by the microalga itself. The cells can control the nutrient concentrations at the cell surface and thereby controlling the concentration gradient. The diffusive boundary layer is also controllable to some extent by the organism. High concentrations of nutrients seem to inhibit growth at start of cultivations [19].

Carbon dioxide can be taken up and utilized by microalgae in two forms, namely HCO_3^- and CO_2. CO_2 is the only form of carbon with electric neutrality that can cross the membrane passively. When HCO_3^- is utilized as carbon source it occurs via active uptake or by extracellular conversion from HCO_3^- to CO_2 by enzymatic activity [34]. The ion CO_3^{2-} cannot be taken up and utilized.

A challenge to obtain an efficient bioprocess is to optimize the growth and find the best concentration of CO_2 so the cultivation time is reduced [10]. In addition, for production purposes a high final biomass concentration in the culture is needed for efficient down-stream processing which can be achieved by increasing the amount of CO_2 in the incoming air [30]. Nitrogen can be supplied as nitrate, ammonia or urea and phosphorous as inorganic $H_2PO_4^-$ or $H_2PO_4^{2-}$ [10]. Sulfur can be provided by inorganic sulfate and is crucial for formation of the essential amino acids, methionine and cysteine. Calcium is needed for preservation of the cytoplasmic membranes. Potassium is crucial for all algae and a lack of this element results in slow photosynthesis and hence slow growth. Potassium has several functions such as a co-factor for a number of enzymes and it participates in the protein synthesis. It also has a function in osmotic regulation. Magnesium is the central atom in the light harvesting chlorophyll molecule necessary for all photosynthetic algal species. Deficiency leads to disturbed cell division and the cells become peculiarly large. Iron is involved in the metabolism and is needed for nitrogen assimilation and it affects chlorophyll and phycocyanin synthesis. Trace elements are in the range of micro- or nanograms and affect the protein synthesis and growth.

2.1.3.2. Light and photosynthesis

The photosynthetic reaction is a redox reaction driven by light where chlorophylls harvest the light [28]. Light can be applied naturally by the sun or artificially by lamps and is essential for growth of photosynthetic organisms [30]. Photoautotrophs convert energy in sunlight and inorganic compounds, such as CO_2, into energy-rich organic compounds [28]. Masijodek et al. [28] mean that all living organisms on earth are dependent on photosynthesis.

Light energy is often measured as irradiance per unit area, *i.e.* in W/m^2 or $J/(m^2s)$ [6]. Terrestrial plants cannot convert the solar energy received efficiently, since around 1 W/m^2, *i.e.* less than 0.5 % of the total solar energy received can be converted in a mid-latitude location with a light irradiance of 200-300 W/m^2 [32]. In theory, microalgae can reach a 10 % total light energy conversion to primary products [35] or maybe even up to 20 % [32], but this is true only for low light intensities. When full sunlight intensity exposes the algae a light energy conversion of only 3 % can be achieved [35]. The light irradiance for short wavelength global light at Borås, Sweden, was measured by SMHI in a model giving an indication of how light irradiance changes by time (see *Figure 1*). Mean values were given for grid squares of 2500 m^2 each month from 2000-2012 [36]. The light irradiance was highest during the summer part of the year at almost ten times above the lowest level at the winter part of the year.

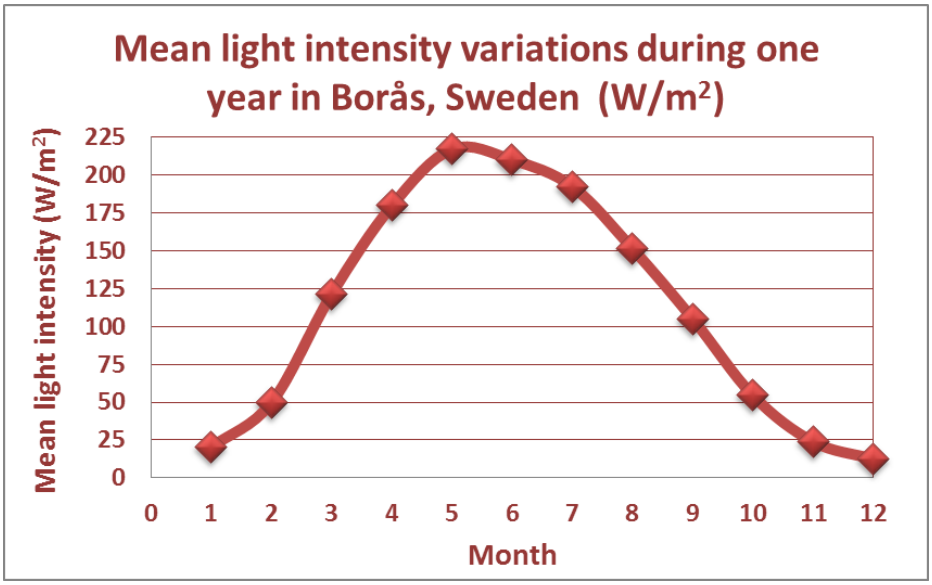

Figure 1. By permission of SMHI at 2012-02-29 [36]. Light irradiance measured each month during the period 2000-2012. Measurements were made in Borås, Sweden, where SP – Technical Research Institute of Sweden is located.

The visible light spectrum reaches from purple of 380 nm to red at 750 nm, which are the range of possible wavelengths for photosynthesis [6]. The quantum theory describes light as a photon stream. The photon energy is dependent on its frequency and wavelength. Blue light photons (~400 nm) have higher energy than red light photons (~700 nm). The photon must have the critical energy possible to excite one electron from the pigment and create a charge separation of the thylakoid membranes in the chloroplasts [6]. The summary of photosynthesis can be written as *Reaction 1* [33]:

Reaction 1 – Summary of the photosynthesis

$$CO_2 + 4\,H_2O + 8\ \text{photons} \xrightarrow{\text{Light energy, chlorophyll and enzymes}} O_2 + \frac{1}{6}\,C_6H_{12}O_6$$

The reaction is divided in two separate processes, the *light reaction* and the *dark reaction* [28]. The light energy is used in the light reaction to produce reducing power as NADPH, and the energy-rich compound ATP for further use in the dark reaction [6]. Two complementary photosystems are linked in series in the light reaction and both must be active to get maximum photosynthetic efficiency [33]. *Photosystem I* (*PSI*) absorbs light up to 700 nm and *Photosystem II* (*PSII*) absorbs wavelengths around 680 nm [6]. *PSI* and *PSII* are bound to photosynthetic thylakoid membranes. Both are multisubunits of transmembrane protein complexes where the center contain chlorophyll and electron transport agents [6]. The photosystem reactions are seen in *Reaction 2* [33]:

Reaction 2 – Photosystem II and photosystem I

$$\mathbf{PSII:}\ 2\,H_2O \xrightarrow{4\,hv} 4H^+ + 4e^- + O_2$$

$$\mathbf{PSI:}\ 2H^+ + 4e^- + 2\,NADP^+ \xrightarrow{4\,hv} 2\,NADPH$$

Electrons get excited by the photons of corresponding wavelength in one of the two reaction centers, *PSI* or *PSII*. The source of electrons comes ultimately from H_2O molecules and the final destination is $NADP^+$, which gets reduced to NADPH. A pH gradient is created in the thylakoid lumen as water molecules are broken down yielding protons and additionally protons translocated over the membrane. This gradient drives the ATP production, which leads to production of ATP and NADPH in the light reaction seen in *Reaction 3* [33]:

Reaction 3 – The light reaction

$$8\,h\nu + 2\,NADP^+ + 2\,H_2O + 3\,ADP + 3\,P_i \xrightarrow[+\ \text{chlorophyll}]{\text{Light energy}} 2\,NADPH + 3\,ATP + O_2 + 2H^+$$

The dark reactions occur in the stroma and NADPH and ATP are used in sequential biochemical reduction of CO_2 and fixating the carbon into organic compounds [6]. The dark reaction is the photosynthetic carbon reduction cycle, called the *Calvin cycle* [33], in which CO_2 enters the cycle and is converted to sugar phosphates. The biochemical energy is used for producing organic compounds aided by the *Calvin cycle enzymes*. RubisCO, *ribulose bisphosphate carboxylase*, is an enzyme which is vital in the Calvin cycle and catalyses carboxylations, the first step of CO_2 reduction to later be able to form glucose [3]. RubisCO activity is regulated to preserve a balance between obtained NADPH and ATP, versus biosynthesis of cell material and storage products [33]. The enzyme exists in many places in nature, also in microalgae, cyanobacteria and plants [3]. In summary, it takes 12 NADPH, 18 ATP and 6 CO_2 to produce one hexose in the dark reaction, *Reaction 4* [3] [33]. It can be converted to storage polymers, mainly starch and glycogen, and used to build cell material.

Reaction 4 – The dark reaction

$$CO_2 + 2\,NADPH + 2\,H^+ + 3\,ATP \xrightarrow{\text{Enzymes}} (CH_2O) + H_2O + 2\,NADP^+ + 3\,ADP + 3\,P_i$$

2.1.3.3. Light harvesting pigments

Organisms capable of utilizing photosynthesis all contain organic light harvesting pigments [28]. Masojidek et al. [28] describes three major classes of pigments, *chlorophylls*, *carotenoids* and *phycobilins*. Chlorophylls are green pigments and carotenoids are yellow or orange. These are both lipophilic. Phycobilins on the other hand are hydrophilic. The chlorophylls contain a tetrapyrrole ring where the central atom is Mg^{2+}. The chlorophyll is similar to hemoglobin and myoglobin, but in these molecules there is a Fe^{2+} instead of Mg^{2+} [6].

Chlorophyll a, b, c and *d* exist of which *chlorophyll c* lacks a long chain terpenoid alcohol [28]. The chlorophyll and partner pigments are kept in the membranes of the thylakoids attached to several proteins [6]. *Chlorophyll a* exists within all photoautotrophs and functions as the main light harvesting pigment. It is a primary part of the protein pigment complexes [37]. C*hlorophyll a* and *b* interact with proteins and membrane lipids by the hydrophobic phytol tail [6]. All chlorophyll molecules have two major absorption wavelengths, at blue-green (450-475 nm) and at red (630-675 nm) light. The *chlorophyll b, c* and *d* extend the absorption range of *chlorophyll a* thus making the absorbance spectrum unique for different species and depending on the ratio between chlorophyll types [6]. The total pigment content varies from 0.1 – 9.7 % of wet biomass and *chlorophyll a* can vary a 30-fold depending on changes within the species, temperature, light or level and type of nutrients [38].

Carotenoids are biological *chromophores* and exist in different variants within algae [6]. The carotenoids play different roles in the cells, but all functions are connected to light harvesting [6]. The absorption spectrum lies within the interval of 400 to 550 nm [28]. Carotenoids such as *carotenes* (*e.g.* α-carotene and β-carotene) and *xanthophylls* (*e.g.* lutein and zeaxanthin) could be present in algae. They facilitate photosynthesis by aiding excitation of *chlorophyll a*, function as a structural part in the pigment-protein complexes and protect and get produced as a defense mechanism to excess of irradiance, *i.e.* carotenoids are produced by cells under irradiance stress [28].

2.1.3.4. Light phenomenon and photosynthetic rate

At a low light intensity the rate of photosynthesis is proportional to light irradiance [26]. Richmond [26] explains that as the light intensity increases the photosynthesis becomes less efficient and finally comes to a maximum, the light saturated value. At this stage other cellular and enzymatic reactions utilizing energy compounds limits the photosynthesis. The light/dark (L-D) cycle also affects microalgal growth making previous fact about irradiance a bit more complex. Two important parameters are affected, the ratio of light and dark phases and the frequency of the cycle. A higher frequency of the L-D cycle makes it possible for stronger light to be more efficient for photosynthesis. Also, other parameters affect the photosynthetic productivity such as length of the light path, cell density and agitation.

Photoacclimation is a phenomenon as a response to changes in the light intensity [39]. Vonshak & Torzillo [39] report a balance between the light reactions in the chloroplasts and the energy demand for CO_2 fixation and other metabolic reactions. There are changes in light intensity during 24 hours of the natural light delivered. Photoacclimation exists to keep a high photosynthetic efficiency under a variety of light intensities by adapting (*i.e.* acclimating) the ability to harvest and use light. The process is complex and it changes cellular activities. There exists short-term and long-term photoacclimation. The short-term variant lasts for some seconds or minutes while the long-term can last for hours or days. The length required to acclimate and adjust pigmentation differ between species. Short-term photoacclimation use carotenoids to dissipate excess light and/or state transitions that can change the energy distribution between *PSI* and *PSII*. Long-term photoacclimation starts when the short-term variant is not enough to fulfill the adjustments. The long-term variant includes changes in

enzymatic activity and gene expression. This leads to changes in concentrations of photosynthetic complexes and photosystem stoichiometry.

At low light intensities, when the absorption is not enough, an increase in the *chlorophyll a* concentration occurs [39]. Vonshak & Torzillo [39] explain that a doubling in the *chlorophyll a* content does not lead to a doubling of light absorption rate. At high light intensities a stress response occurs. Carotenoids are produced by the cells and become higher relative to *chlorophyll a*. Two examples are stress responses where β-carotene production occurs in *Dunaliella salina* and astaxanthin in *Haematococcus pluvialis* [39]. They accumulate in lipid globules outside the chloroplasts and do not transfer energy to chlorophylls but act as protection for the reaction center.

Artificially produced light can be varied in light intensity by changing the distance from the culture to the light source [40]. Only high light intensities favor the storage lipids of primarily triglycerides which are favorable for biodiesel production [23]

2.1.3.5. Temperature

Temperature has a strong correlation to biochemical reactions and therefore affects microalgal growth [41]. Microalgae have different temperature optima for growth, usually somewhere between 20 to 30 °C [10]. Maximal productivity is obtained when the nutritional needs are fulfilled and the cultivation temperature is optimal [26]. Temperatures lower than 16 °C decrease normally the growth rate and over 35 °C many microalgae die [10]. There is a relationship between temperature and light intensity since lamps and sunlight emits heat [26]. The temperature of the flue gas, bubbled through the cell suspension, also affects the culture temperature and in addition, evaporation of water requires heat, which is taken from the surrounding air and medium [42].

A decrease in temperature often increases the amount of polyunsaturated fatty acids within the membranes [41]. According to Hu [41], an increased fluidity of the membrane system is achieved at low temperatures, essential for protecting *e.g.* the thylakoids and the photosynthetic machinery from photoinhibition. As temperature increases, the saturated fatty acids increases as these are more stable at higher temperatures [23]. Lipid classes and composition is affected by temperature rather than the total lipid content [41]. At temperatures beneath the optimal range, an increased enzyme production occurs as adaptation to preserve the photosynthetic efficiency [41].

2.1.3.6. Agitation

Some kind of agitation is necessary when culturing algal cells to assist nutrient and gas exchange at the contact site of cell surface and water [30]. Agitation minimizes the diffusion gradients at the cell surface and is important for reaching high biomass yields [31]. Turbulent flow leads to an increased exchange rate between the cells and the medium, important to have in mind when designing a photobioreactor. The cells are kept dispersed and thus settling and sedimentation of cells and growth on the walls of the cultivation equipment is avoided [31]. High cell densities with high photosynthetic activity produce high concentrations of dissolved oxygen which are poisonous for algae and can thus be avoided by efficient mixing [26].

Availability of light is small for cells in a high cell density suspension and can be increased by mixing, thereby creating minor photic zones still sufficient for photosynthesis. Enhancing the light/dark frequency leads to increased photosynthetic effectiveness and increased biomass production [25]. The mixing is crucial when the cell density is high to be able to reach even higher cell densities [26].

On the other hand, mixing can cause shear stress on photosynthetic organisms [25]. A challenge is to achieve an injection system of the gas with optimum contact time between CO_2 and algae without

damaging the cells [10]. Oilgae [10] mention that the problem can be fairly overcome by bubbling the CO_2 into the liquid and making a circulation and thus the absorption of the gas can become easier. Bubbling may cause stress at bubble development and bubble destruction, but is gentler to the cells than other mixing types [25]. A study of *C. reinhardtii* reported that gas bubbling of air through the cell suspension increased the growth rate due to a mixing effect [19].

2.1.3.7. Flue gas – effects of CO_2, NO_x, SO_2 and O_2

A suitable way to obtain cheap CO_2 enriched air is to use flue gases from different types of industrial activities as it is regarded as waste. Microalgae can be grown in flue gas and fix CO_2 with high efficiency if the concentrations of sulfur and heavy metals are low enough [18]. These properties have made algae an interesting and environmental friendly future alternative for cleaning flue gases from CO_2 and eventually produce biofuels. Flue gas derived from a power plant has several constituents, with somewhat different levels. The largest fraction consists of CO_2, most often varying between 10-15 %, and sometimes up to 20 % [10]. Microalgae exposed to CO_2 enriched air compared to only air show that biomass production increases as CO_2 levels increases. Too much CO_2 showed, in some reports, to have inhibitory effects due to a significant decrease in pH [19]. Flue gases also contain compounds such as O_2, NO, NO_2, CO and SO_2. Nitric oxide (NO) is rapidly oxidized to NO_2 in contact with O_2 through *Reaction 5* [27]:

Reaction 5 – Oxidation of nitric oxide to nitrogen dioxide

$$\mathbf{2\,NO\,(g) + \,O_2(g) \rightarrow 2\,NO_2(g) + NO\,(g)}$$

The gas coming in contact with the algae is a mixture of NO and NO_2 and some of the NO oxidize to NO_2 in the medium. There are studies showing that some species of microalgae can tolerate high levels of NO_x and SO_x [43]. Flue gases may have low values of O_2 as combustion consumes it to develop CO_2 which can limit oxidation of NO to NO_2. The presence of CO in the flue gas indicates limiting O_2 due to incomplete combustion, but the effect of CO on algae is unclear.

NO can be dissolved as the ion nitrite (NO_2^-) which increases in the culture medium when flue gas is added. This form can work as a nitrogen source for microalgae and therefore flue gas is an excellent choice as substrate for algae with both CO_2 and NO_2 [43]. NO_2 in flue gas can also serve as a nitrogen source for microalgae and growth is enhanced with higher levels of NO_2 [10]. SO_2 is also used in small amounts by algae but if concentrations are too high, growth is inhibited and the culture typically dies due to toxic effects or low pH [44]. Some species of *Chlorella* have been shown to tolerate high levels of NO and SO_2, up to 120 ppm and 50 ppm respectively [43]. Though, it was not the same species that could handle both.

2.1.3.8. Waste water cleaner from nitrogen and phosphorous

Algae thrive in wastewater as the impurities nitrogen and phosphorous are nutrients for algae. Algae can be used to reduce the levels of N and P as they are incorporated into biomass and can thus be removed [19]. The ability to reduce N and P can be coupled to reducing biomass production cost. Gentili [24] has developed a technique with *Umeå Energi AB* and *Swedish Energy Agency* to purify municipal wastewater from N and P. Gentli [24] describes how a combined power and heating plant can be coupled to the wastewater containing microalgae and successfully remove CO_2 from the inlet

gas and also reduce the N and P levels in the wastewater. Another interesting fact is that microalgae that thrive in these environments generally tend to contain a lot of oil [10].

An article on *Botryococcus braunii CCAP* 807/1 treats the possibility of using piggery wastewater as growth medium [45]. The wastewater was not pH adjusted and neither was it sterilized nor diluted. An et al. [45] concluded that *B. braunii* can reduce the N and P levels efficiently in piggery wastewater as a high cell density of 7 g/L was obtained. Other studies have shown that microalgae have the possibility to remove heavy metals from wastewater, but this ability is not wanted if producing biofuels [23]. Another study was made with *C. reinhardtii* to produce oil-rich biomass from wastewater by combining three different wastewaters, with different amounts of nutrients, and with added flue gas of CO_2 [19]. Kong et al. [19] found out that moderate amounts of added CO_2 enhanced algal growth but too much inhibited growth due to low pH. The study reached biomass concentrations of 2.0 gDW/l and 55.8 mg/l N and 17.4 mg/l P could be removed each day.

2.1.3.9. pH and salinity

The pH affects growth of microalgae, and different species and strains have different optima at which the fastest growth is achieved [19]. The pH optimum for *C. reinhardtii* is around 7.5 and this species was growing fastest around this pH [19]. The general pH optimum for most freshwater species is roughly between 7-9 [46]. Failure to keep the correct pH can lead to slow growth or culture collapse. The pH can affect the availability and solubility of CO_2 and minerals in the medium [10].

The pH is tricky as several factors affects pH from start to end in an algae culture with added flue gas. Formation of acids occurs by the dissolved gas components and lowers the pH [10] and growth of microalgae increases the pH [31]. As the cultivation of microalgae is progressing a gradual increase in pH will befall as a result of growth if no flue gas is added [31]. When HCO_3^- and CO_2 are used as carbon source for algae, both is leading to an increase in pH [34]. As CO_2 is taken up by the cell, the spontaneous conversion of HCO_3^- to CO_2 occurs in the medium, and thereby increasing the OH^- concentration and correspondingly the pH, *Reaction 6 (2)* [31]. As HCO_3^- is utilized as carbon source the uptake results in a charge difference which has to be compensated for [34]. It occurs via uptake of H^+ or by release of OH^-, hence also increasing the pH [34]. Cell suspensions with high cell densities and no added flue gas may have pH-values as high as 11 [31].

In freshwaters, there is a bicarbonate-carbonate buffer system (see *Reaction 6*) and preserves the pH optimal for cultivations of freshwater species, around pH 6.5 [31]. H_3PO_4 is also present in the medium [1] and as the pK_a for H_3PO_4 and H_2CO_3 are roughly in the same range [27] the phosphate buffer system and bicarbonate-carbonate buffer system would make the buffer capacity strong around pH = 6-8 [27].

Reaction 6 – Bicarbonate-carbonate buffer system

$$\mathbf{1:}\ 2\,HCO_3^- \Leftrightarrow CO_3^{2-} + H_2O + CO_2$$

$$\mathbf{2:}\ HCO_3^- \Leftrightarrow CO_2 + OH^-$$

$$\mathbf{3:}\ CO_3^{2-} + H_2O \Leftrightarrow CO_2 + 2OH^-$$

A buffered system may be used in more than one way. A pH adjuster, such as NaOH, may be added to the growth medium to adjust and maintain the pH without affecting growth [10] [47].

NO, SO_2 and CO_2 all form acids when dissolved in water (see *Reaction 7*); hence the pH decreases due to added flue gas [10]. If pH is too high CO_2 can be bubbled through to lower it [19]. Air with 33.33 % CO_2 increase growth, but higher concentrations inhibit growth due to pH decrease [19]. A pH-static control connected to the supply of CO_2 in the inlet gas can be added to the system directly to keep pH at an acceptable level [31]. Adding flue gas with 400 ppm SO_2, the pH can decrease to a value below 4 in less than 24 hours [19]. Levels of 150 ppm NO have been shown in reports to have no inhibitory effects on microalgae with pH adjustments [10]. The rapid oxidation of NO to NO_2 in air (see *Reaction 5*) indicates in *Reaction 7* that NO_2 also could contribute with too low pH by formation of nitrous acid [27].

Reaction 7 – Formation of the acids HNO_3, HNO_2, H_2SO_3 and H_2SO_2

Nitric acid: $3\ NO_2(g) + H_2O\ (l) \rightarrow 2\ HNO_3(aq) + NO\ (g)$

Nitrous acid: $NO_2(g) + NO\ (g) + H_2O\ (l) \rightarrow 2\ HNO_2\ (aq)$

Sulfurous acid: $SO_2(g) + H_2O\ (l) \rightarrow H_2SO_3\ (aq)$

Sulfuric acid: $SO_2(g) + O_2\ (g) \rightarrow 2\ SO_3\ (aq) \rightarrow 2\ SO_3\ (aq) + H_2O\ (l) \rightarrow H_2SO_4\ (aq)$

Larger pK_a-values indicate weaker acids and lower pK_a indicate stronger acids. If pH is higher than the pK_a-value, then the acid is dissociated [27]. The acids are collected in *Table 1* with pK_a-values [27].

Table 1. Acids formed from dissolved CO_2, NO, NO_2, SO_2 and SO_3. The components exist in flue gas as NO_2 and SO_3 are formed from NO and SO_2 respectively. Low pK_a-values indicate strong acids.

Gas component		pK_{a1}	pK_{a2}
Carbonic acid	H_2CO_3	6.4	10.5
Nitrous acid	HNO_2	3.4	-
Nitric acid	HNO_3	< 0	-
Sulfurous acid	H_2SO_3	1.9	7.2
Sulfuric acid	H_2SO_4	- 3.0	2.0

The gas constituents have different solubility in water and is an important parameter affecting the pH [10]. *Table 2* shows solubility of three flue gas constituents at 20 °C. SO_2 has highest solubility of the three and indicate that it could result in a large pH problem when using flue gas with this component. Little research has been made on solubility of the poisonous gas carbon monoxide (CO) [48], present in all exhaust gases according to *Table 5*. However, the solubility in water is low according to Jáuregue-Haza [48] and is higher in organic solvents. The solubility increases with increasing temperature and vice versa at lower temperatures [10].

Table 2. Solubility of three components, CO_2, SO_2 and NO, in flue gas at 20 °C [10].

Gas component	Solubility cc/ml H_2O
CO_2	0.88
SO_2	39.4
NO	0.05

The salinity affects how the microalgae grow. Conductivity can be used to measure salinity. Osmotic stress occurs due to wrong salinity for specific algae, resulting in that the photosynthetic activity is inhibited [39].

2.2. Microalgae species of the project

The species and strains used in the project were chosen from their abilities and qualities, such as fast growth and lipid production. A summary of all species is shown in *Table 3* with occurrence in Sweden and maximum % lipids of dry weight obtained in previous studies.

Table 3. The freshwater microalgae species used in this study are listed together with occurrence in Sweden [49] and maximum % lipids of dry weight obtained in previous studies [23].

Species	Occurrence	% Lipids in DW
Ankistrodesmus falcatus	Reproducing	28-40
Botryococcus braunii	Reproducing	25-86
Chlamydomonas reinhardtii	*Not found*	~ 23
Chlorella emersonii	*Not found*	63
Chlorella protothecoides	Reproducing	15-55
Chlorella sorokiniana	*Not found*	22
Chlorella vulgaris	Reproducing	14-56
Nannochloropsis salina	Reproducing	Unknown
Nedsjön isolate	Reproducing	Unknown
Scenedesmus obliquus	Reproducing	12-14
***Scenedesmus* simris002**	Reproducing	12-40

2.2.1. *Ankistrodesmus falcatus*

Ankistrodesmus falcatus (see *Figure 2*) belongs to green microalgae (Chlorophyta) and can form colonies with 2, 4, 8 or more cells, occasionally in bundles enclosed within a mucilaginous cover [5]. John [5] describes the cells as 1.5-7 µm wide and 20-165 µm long with a shape reminding of needles, very often curved with sharp edges. The species is well known for being tolerant to toxic waste and pollution and the cells are found plentifully in sewage and in fish-farm ponds, hence the species thrive in nutrient-rich environments. High pH and high bicarbonate concentrations show that *A. falcatus* have become dominant. *A. falcatus* has a quality to remove orthophosphate from the growth medium and could be a possible alternative as a phosphorous remover in sewage plants.

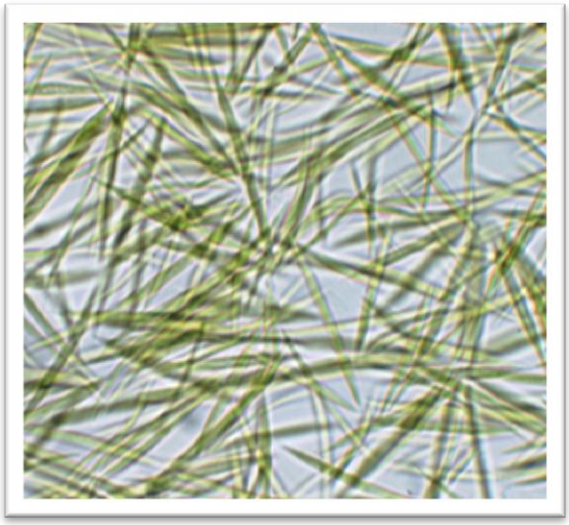

Figure 2. Ankistrodesmus falcatus
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http://www.ccap.ac.uk/strain_info.php?Strain_No=202/5C

2.2.2. *Botryococcus braunii*

Botryococcus braunii (see *Figure 3*) is a green microalga [5]. The cells are oval or egg-shaped and 3-6 µm wide and 6-11 µm long. All cells have an apical cap. The species is widely spread in different habitats [5]. *B. braunii* is one of the most promising species when it comes to hydrocarbon production, up to 86 % hydrocarbons per dry weight has been reported [10]. This species differ from other species as they do not produce triglycerides [50]. Metzger & Largeau [50] report production of hydrocarbons such as n-alkadienes and trienes, triterpenoid botryococcenes, tetraterpenoid, lycopadienes and numerous of ether lipids. Unfortunately, *B. braunii* grow slowly compared to other microalgae, with a doubling time often about 72 hours. With optimal growth the doubling time was reduced to 48 hours [10]. *B. braunii* is suggested to only become dominant if minimal competition occurs; hence the species is sensitive of being outrivaled. As already mentioned *B. braunii* (CCAP 807/1) was used to clean piggery waste water from N and P with successful results and a high obtained cell density [45].

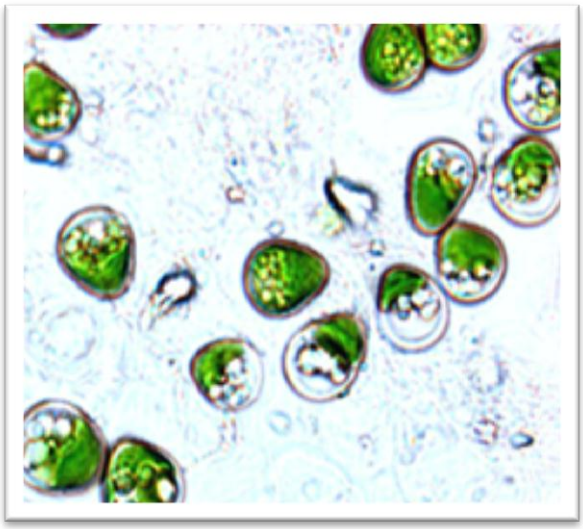

Figure 3. Botryococcus braunii
By permission of UTEX at 2011-12-14
http://www.sbs.utexas.edu/utex/algaeDetail.aspx?algaeID=3051

2.2.3. *Chlamydomonas reinhardtii*

Chlamydomonas reinhardtii (see *Figure 4*) is a green microalga [10]. Cells are spherical, or close to spherical, with a width of 8-22 µm and a length of 10-22 µm [5]. A large basal pyrenoid exists within the cell. Asexual reproduction by 4 zoospores occurs and isogamous sexual reproduction [5]. *C. reinhardtii* is a commonly used model organism and is one of three algae where the genome has been resolved [51]. Malcata [51] continues by pronouncing that refined metabolic engineering only has been possible with *C. reinhardtii*. This species has the potential to grow in a heterotrophic way with an organic carbon source such as acetate [9]. *C. reinhardtii* is also interesting due to a certain ability to switch from producing O_2 to H_2 [52]. It is known that *C. reinhardtii* can photoproduce H_2 when *PSII* is blocked. Yet no one knows the reason for this [10].

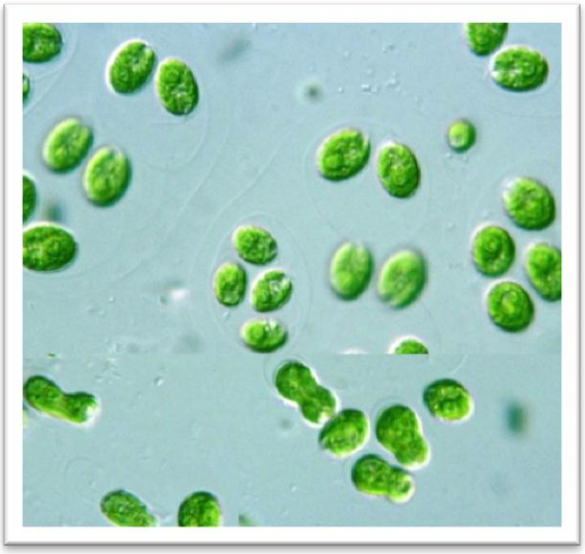

Figure 4. Chlamydomonas reinhardtii
By permission of Yuuji Tsukii at the Protist Image Galleries at 2011-12-15
http://protist.i.hosei.ac.jp/pdb/images/chlorophyta/chlamydomonas/Euchlamydomonas/reinhardtii/sp_10.html

2.2.4. *Chlorella emersonii*

Chlorella emersonii (see *Figure 5*) is a green microalga and the cells are close to spherical, with a width of 3-17 µm [5], living solitary or in groups. The pyrenoid is large and is surrounded by 2-4 starch grains. The autospores are ellipsoidal with 2, 4, 8 or sometimes even 16 in each sporangium [5]. A similar project to this study was performed on flue gas from cement plant [16]. Borkenstein et al. [16] suggests a method for cultivating *C. emersonii* with the purpose of cleaning 15 % CO_2 derived from the power plant. The cultivations were pH-controlled by controlling the inflow of the gas. Four 5.5 l airlift photobioreactors were tested, two with exhaust gas and two with pure CO_2 as references. The exhaust gas was treated with a flue gas scrubber and a dust separator. The study gave promising results where the doubling time was similar between the controls and the exhaust gas cultivations. No adverse effects could be distinguished for 30 days of cultivation. Cultivations with flue gas derived 2.0 g DW/l and the control derived 2.1 g DW/l.

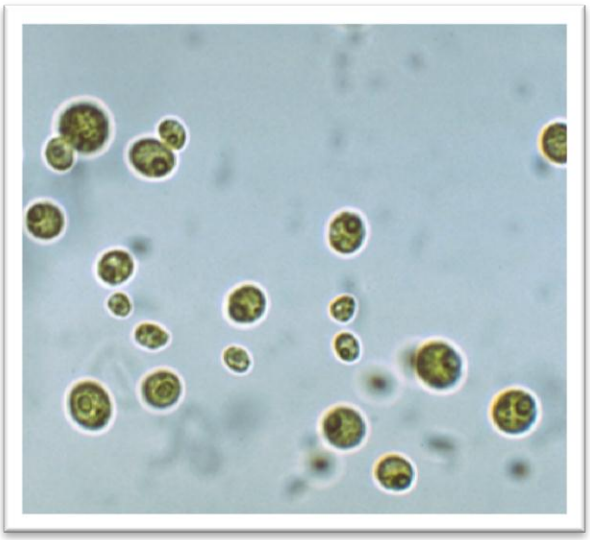

Figure 5. Chlorella emersonii
By permission of CCAP at 2011-12-14
http://www.ccap.ac.uk/strain_info.php?Strain_No=211/8C

2.2.5. *Chlorella protothecoides*

Chlorella protothecoides (see *Figure 6*) is a green microalga and the cells are spherical [5]. There are reports claiming that heterotrophic growth of *C. protothecoides* is possible [9]. *C. protothecoides* can generate 55 % lipids (g/g) during 144 hours of cultivation [53].

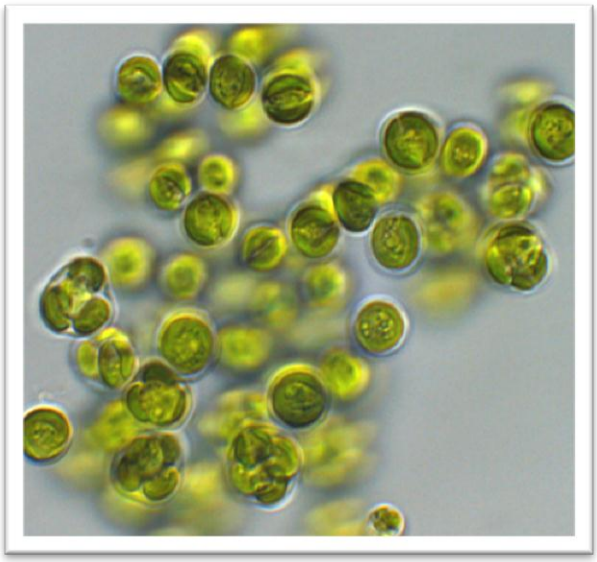

Figure 6. Chlorella protothecoides
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http://www.ccap.ac.uk/strain_info.php?Strain_No=211/54

2.2.6. *Chlorella sorokiniana*

Chlorella sorokiniana (see *Figure 7*) belongs to the division Chlorophyta and reproduces at a very high growth rate, and during 17-24 hours one cell has become four new cells [10]. *C. sorokiniana* is an algal species which is very tolerant to high irradiance, high temperatures and high CO_2 concentrations [54]. *C. sorokiniana* has a very high concentration of chlorophyll and have a fish-like odor [10]. This species has the potential to grow in heterotrophic and mixotrophic ways [9]. *C. sorokiniana*, contains lots of carbohydrates with potential use for bioethanol production.

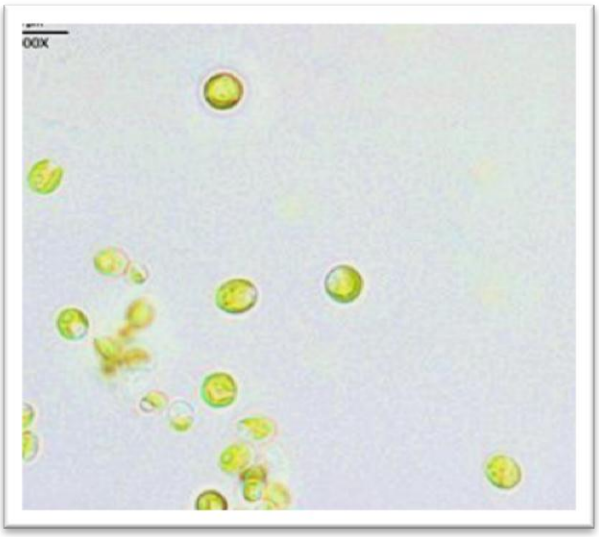

Figure 7. Chlorella sorokiniana
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http://web.biosci.utexas.edu/utex/algaeDetail.aspx?algaeID=2775

2.2.7. *Chlorella vulgaris*

Chlorella vulgaris, or synonymously called *Chlorella pyrenoidosa*, (see *Figure 8*) belongs to the division Chlorophyta and the cells are close to spherical [5]. The width of the cells is 1.5-10 µm. The pyrenoid is ellipsoidal or spherical and has 2-4 starch grains surrounding it. The autospores are spherical and each sporangium contains 2, 4, 8 or even 16 cells [5]. The same species and strain has been used for another study with flue gas conditions similar to this study, however with a bit lower concentrations of CO_2 of 10 %, NO of 57 ppm and SO_2 of 0.8 ppm [18]. A high carbon fixation capacity exists for this species. Some strains of *C. vulgaris* have shown a capability of producing a starch content of 37 % DW [10]. The species has the potential to grow in a heterotrophic way with an organic carbon source [9]. The strain of *C. vulgaris (CCAP 211/11B)* (same as in this study) is mentioned as unchanged, no genotypic or phenotypic changes, even after being cultivated under different environmental conditions for many years [55].

2.2.8. *Nannochloropsis salina*

Nannochloropsis salina (see *Figure 9*) is a marine species according to the homepage of NCMA [4], but was tried out in this study as a potential CO_2 cleaner in both freshwater 3N-BBM+V and f/2 medium and marine water medium by Engström [14]. The cells are round and small, 2-5 µm long and 2-3 µm wide [4].

2.2.9. Nedsjön isolate

An algal bloom occurred in the Swedish lake Västra Nedsjön close to Hindås, hence the name "Nedsjön isolate", and the species was isolated at 2011-06-27 by Susanne Ekendahl. The alga was sampled and the pre-culture was grown in E-flasks with the freshwater medium 3N-BBM+V. Microscopic studies show that cells are big and often grow in large clusters with many large cells and without obvious arrangements.

2.2.10. *Scenedesmus obliquus*

Scenedesmus obliquus (see *Figure 11*) is a green microalga and is a taxonomic synonym to *Acutodesmus obliquus* [5]. The cells are coenobia of 2, 4 or even 8 numbers of cells linearly arranged but can be unicellular which is the more common form when cultivating the algae. The size is 2-10 µm wide and 6-15 µm long. The cells are straight with sharp edges [5]. Two strains of *S. obliquus* in a study showed high growth rates and good ability to remove CO_2 in added gas [17]. The CO_2 consumption rates reached the top values 390 mg L^{-1} d^{-1} and 290 mg L^{-1} d^{-1} respectively which are outstandingly high. However, the study also showed that the productivity differed a lot between strains as many strains were investigated [17]. Other studies have shown that *S. obliquus* has a suitable fatty acid profile for diesel with mainly polyunsaturated fatty acids such as linolenic acid [56].

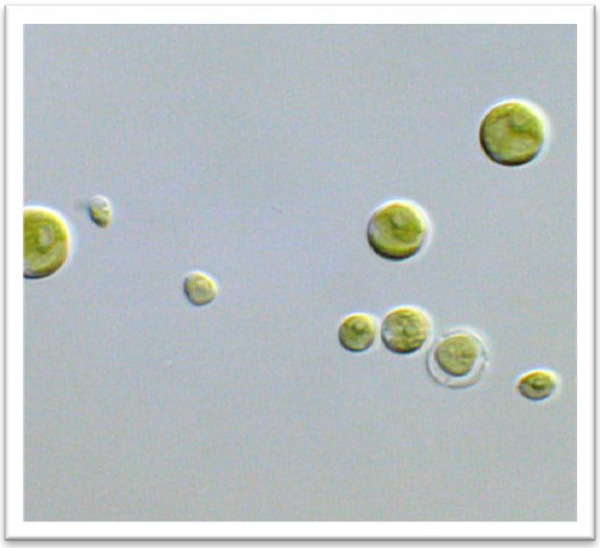

Figure 8. Chlorella vulgaris
By permission of CCAP at 2011-12-14
http://www.ccap.ac.uk/strain_info.php?Strain_No=211/11B

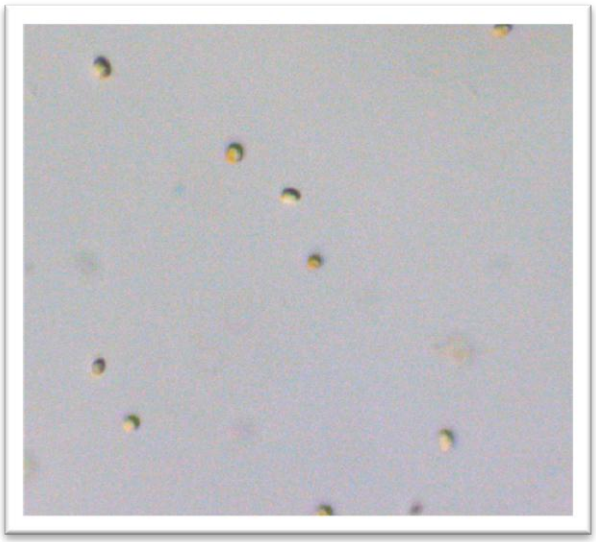

Figure 9. Nannochloropsis salina
By permission of CCAP at 2011-12-14
http://www.ccap.ac.uk/strain_info.php?Strain_No=849/2

2.2.11. *Scenedesmus* simris002

The genus *Scenedesmus* is the most taxonomically diverse group of the Chlorophyta division as there exists over 200 species. Species within *Scenedesmus* may form coenobic forms, organized and non-motile, where the number of cells is fixed within the colony [29].

The *Scenedesmus* simris002 strain (see *Figure 10*) was sent to *SP – Technical Research Institute of Sweden* from *Simrisalg AB* where previous studies have been made on this strain, hence the name "simris002" (see *Figure 10*).

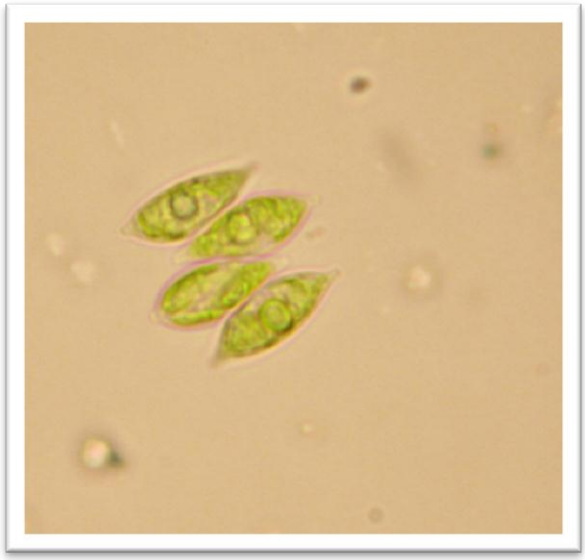

Figure 10. Scenedesmus simris002
By permission of Simrisalg AB and received at 2012-01-31

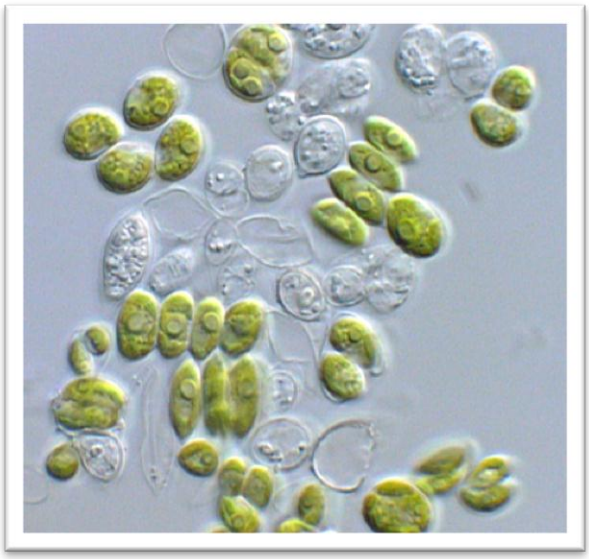

Figure 11. Scenedesmus obliquus
By permission of CCAP at 2011-12-15
http://www.ccap.ac.uk/strain_info.php?Strain_No=276/3A

2.3. Culturing of microalgae

There are different ways of culturing algae and several external parameters affect the growth. However, species and strains showing well-developed properties at lab scale may be worthless at large scale. Different environmental stress factors exist at large scale that cannot be mimicked at lab scale.

2.3.1. Challenges of scaling up

It is generally known when working with microorganisms that lab scale and industrial (large) scale have different environments. Lab scale has a controlled and evenly distributed environment while the large scale can have locally different conditions. The species and strains need to be robust and tolerant to changes within the environment. Plentiful of projects and reports show successful results at laboratory scale, but it is unclear how many of these reports that have resulted in successful pilot plants trials with "in-real-life-circumstances" [10].

Flue gas from *e.g.* steel industry or other power plants can be used as carbon source, but selecting and screening of tolerant algal species and strains need to be made to make the process economically feasible [23]. All power plants releasing high concentrations of CO_2 would be possible carbon sources and it will only be a matter of launching projects to develop a method, equipment and a suitable strain.

In this project three components (CO_2, NO and SO_2) are the only constituents in the artificial flue gas. Other compounds will be present in real life and there could be toxic compounds in the flue gas or in the wastewater affecting the algal growth [23]. High concentrations of CO_2, NO or SO_2 could lead to toxicity and lead to acid formation when dissolved in water and affect the pH [27]. Another challenge is to introduce nutrients such as CO_2 without disturbing and hindering the growth [10] and to achieve an injection system with optimum contact time between CO_2 and algae for highest growth [25]. The problem can be fairly overcome by bubbling the CO_2 into the liquid and making a circulation and thus the absorption can become easier [10].

Produced O_2 by algae can be toxic, but is mainly a problem for colonizing species with several cells attached to each other where O_2 get trapped and lead to local high concentrations [26]. Fouling, photoinhibition and mechanical stress due to *e.g.* agitation are other important challenges to overcome [25]. Light distribution in sufficient amounts and extraction methods of the oil through the tough cell wall is needed and still must be cost-effective [10]. It is difficult to illuminate at large scale as the cells are self-shading, and too much light lead to photoinhibition. A big challenge in Sweden is the varying night and day cycle during one year and the cultivations probably need added light during the dark months [18]. The temperature is neither constant and heat need to be added in some way during winter. It also costs a lot of money to harvest the cells as a lot of water has to be removed [10].

The CO_2 capture using microalgae will need space and land area that sometimes is not possible to have close to the power plants [10]. Oilgae [10] claims that pipelines are possible for the transport of CO_2 to the area where the algae are cultivated. This will of course cost some money, but quite a low cost within the range of 2-10 $ per ton of CO_2. Pipelines enable the idea of having one location where all the cleaning and production occur, a gathering point for many industries.

2.3.2. Cultivation systems for algae

Photobioreactors (PBRs) are designed reactors developed for cultivation of phototrophs such as microalgae [25]. The word photobioreactor is only used for closed systems and so far all designed PBRs have pros and cons. However, PBR:s and open systems allow the utilization of all known species around the world and enables the possibility of producing many high value products [25].

2.3.2.1. Open pond systems

Today, most of the used systems for cultivating microalgae are open pond systems [25]. The open pond systems are durable, sustainable, easier to handle, easier to clean and cheaper to build and control compared to closed systems [57]. However, there are drawbacks with open systems [57]; the contamination risk is high, space required is high, water and CO_2 losses are high, they are weather dependent and the biomass concentration reaches low values (0.1-0.2 g/l). Rainfall dilutes the culture and extreme summer sunlight leads to death [10]. Three developed systems exist; *inclined systems*, *circular pond*s and *raceway ponds*, the two last mentioned are the ones used at large scale [25].

Inclined systems use pumping and gravity as mixing, creating a turbulent flow as the suspension is flowing from the top to the bottom of a leaning surface [25]. Turbulent flow makes it possible to have thin tubes, less than 1 cm in diameter, and to a high surface to volume ratio compared to other systems. The system has problem with CO_2 desorption, evaporation losses, and some energy costs to pump the cell suspension to the top of the surface. Tredici [25] claims that in the long run there is not a considerably higher productivity than the ones obtained from raceway ponds.

Raceway ponds have a paddle wheel and paddle the suspension around in a “raceway”. It is cheap, but has drawbacks. It cannot have a depth more than 15 cm; otherwise the turbulent flow is disturbed. A large area is needed for the construction. Low cell concentration is achieved which obstruct the harvesting process and can lead to contamination [25].

Circular ponds have a rotating arm for mixing. The process is expensive due to the mixing required and an expensive construction [25].

2.3.2.2. Closed systems

Photobioreactors (PBRs) are defined photobioreactors used for phototrophs and are closed systems where the light does not fall directly on the cell suspension surface [10]. The light has to pass through some kind of transparent wall before it reaches the cells [25]. PBRs were developed to increase the productivity and maintain a monoculture [10], the risk for contamination is therefore low [25].

There are different PBRs. Some of them are tubular bioreactors, plate reactors or bubble column reactors [23]. They are expensive to run, hard to clean and fouling can occur [25]. But there are major advances by using PBRs instead of open ponds. The contamination risk is low, the space required is low, and the water and the CO_2 losses are minimized [57]. A process control is possible, no weather dependency exists and a high biomass production can be achieved. Oxygen poisoning is a larger problem in closed systems than open systems, hence it is essential to remove produced O_2 by sufficient mixing [10]. *Table 4* compares open and closed systems.

*Table 4. Modified table from Pulz [57] and Oilgae [10] with a comparison between open pond systems and closed systems. **Green color** indicates what is desirable for an algal culturing system and **red color** indicates the opposite.*

What is compared	Open pond systems	Closed PBR systems
Biomass concentration	Low	High
Contamination risk	Very high	Very low
Productivity	Low	High
Carbon dioxide losses	High	Low
Water losses	Very high	Very low
Oxygen inhibition	Low	High
Control	Low	High
Operating costs ponds	Very low	High
Required space	High	Low
Weather dependent	High	Low

2.4. Analysis of biomass and cell content

The cell content of algal cells is important to determine and consider when choosing species and strains. The whole idea of biofuel production depends on what cell composition the actual strain has. The growth and biomass formation is also important to know.

2.4.1. Determining cell concentration

There are different methods of estimating biomass and growth where optical density (OD), dry weight (DW) and cell count are commonly used methods. Dry weight and cell counting are time consuming methods whilst OD measurements are faster. For unicellular microorganisms optical density is proportional to cell count and dry weight, within certain limits [3]. However, Madigan & Martinko [3] explain that work has to be made beforehand. A standard curve has to be made to couple direct measurements, cell count or dry weight, to indirect measurements as OD.

2.4.1.1. Optical density (OD)

To follow growth of microorganisms optical density, or turbidity, is of great importance. Absorbance (or scattering) of light through a cell suspension can be directly coupled to biomass, furthermore coupled to DW or cell count on the same sample [38]. The method assumes that the cells are unicellular and that the scattered light is linear to the number of cells [3]. Madigan & Martinko [3] explain that appropriate dilutions have to be made to obtain a linear relationship between scattered light and biomass.

However, there are insecurities by OD-measurements for microalgae. Pigments in the cells affect the optical properties by absorbing and harvesting of light at certain wavelengths [38]. Harrison et al. [38] mean that it affects the correlation between OD and DW. Even though a standard curve has been made there can be errors in estimating biomass as pigment content varies depending on growth cycle phase and environmental circumstances. Harrison et al. [38] have several advices to minimize the errors. One of them is to use wavelengths outside the absorbance range of the light harvesting pigments, *i.e.* use wavelength with reported absorbance minimums. Two suggested wavelengths are 550 nm and 750 nm.

2.4.1.2. Direct microscopic count in a counting chamber

Cell count can be either viable count, where only live cells are of interest, or it can be a total count where all cells are counted, including dead cells [3]. Madigan & Martinko [3] mean that there are two ways of performing a direct microscopic count. The samples can be dried on slides and counted, or a counting chamber can be used if the cells are in a suspension. In the counting chamber a grid is engraved on the surface of the counting chamber. All squares have the same area (known) and by multiplying with a conversion factor a number of cells per milliliter is obtained. Drawbacks are that cell count includes both dead cells and living cells, small cells are hard to distinguish, and precision is hard to obtain. Dilutions could be necessary to get an acceptable number of cells to count [3].

2.4.1.3. Dry weight

Dry weight (DW) is a method for estimating biomass and is important to know for many aspects, among other things productivity and cell composition [58]. The DW is measured by taking a sample with known volume, drying it and expresses the obtained cell weight as a matter of suspension volume, *i.e.* the biomass concentration [58]. There are two main estimations, the dry weight (DW) and ash free dry weight (AFDW). The difference is that AFDW subtracts the weight of the salts after the sample has been ashed.

The filters must be conditioned and pre-weighed to be able to subtract the filter weights from the sample weights. The samples should be dried in an oven and then weighed together with the filter of known weight. Lee & Zhu [58] report that salts, outside of the cells, could be washed away before the drying step, especially important for marine species as the salt concentration is higher in the medium. They found that washing with 0.5 M ammonium bicarbonate (CH_4HCO_3) or 0.5 M ammonium formate (NH_4HCO_2) got lower weights compared to samples washed with only H_2O and non-washed samples. Therefore the authors recommend the two mentioned compounds for washing when performing DW-estimations.

2.4.1.4. Maximum specific growth rate

Madigan & Martinko [3] describe how exponential growth can be estimated with optical density or cell count by making a plot where the growth is plotted on a logarithmic scale and time arithmetically; hence a *semilogarithmic* plot is obtained. The linear correlation indicates that the cells are growing in their exponential phase and the specific growth rate can be read in the graph. The maximum specific growth rate in a batch culture is calculated by the slope of the straight line of the semilogarithmic plot, *Equation 1* [59]. There are fast growing species, where doubling time can be as low as 3.5 hours [15].

Equation 1 – Specific growth rate determination

$$\ln(X) = \ln(X_0) + \mu_{max} t$$

μ_{max} = Maximum specific growth rate (h^{-1})
X and X_0 = Relative measurements of the biomass concentration (*e.g.* OD)
t = Time

Note that the maximum specific growth rate is found in the log phase of the batch culture!

2.4.2. Cell content of algal cells

The cell content differs between species and it is of great importance to know the cell composition for industrial production purposes especially for biofuels.

2.4.2.1. General CHN Elemental Analysis

CHN analysis is an elemental analysis which makes it possible to conclude the elemental composition of a sample [60]. According to Boylan [60], the CHN analysis stands for Carbon-Hydrogen-Nitrogen analysis. The sample is combusted in excess of O_2 to break down compounds into simpler molecules. The elements oxidizes and will form CO_2, NO and H_2O. These compounds are detected and analyzed and by weighing them an empirical formula in weight percent of the sample can be made. However, it is very important that the samples are dried as H_2O otherwise would be left in the samples and a wrong weight would be obtained as well as too high levels of H.

2.4.2.2. Proteins

Proteins are an important part of the microalgae cells and the levels differ between algal species. Some species can contain large amounts of proteins, up to 47 % of DW [61]. Proteins are polymers of amino acids and are bonded together by peptide bonds [6]. The sequence of a protein is defined by the sequence of the corresponding gene. The proteins have many roles within cells and take part in basically all biochemical reactions within the cells [6]. From a biofuel perspective the proteins are not as essential as the carbohydrate and lipid content and will therefore not be treated further.

2.4.2.3. Carbohydrates

Carbohydrates exist in the forms of monosaccharides, oligosaccharides, and polysaccharides [6]. Hexoses are produced as a result of photosynthesis and the molecule can further be converted to storage polymers, polysaccharides, such as starch and glycogen and used to build cell material [3]. As relevant for this study, there has sometimes been found a relationship between high specific growth rate and starch content within cells and the starch is wanted as a potential source for bioethanol production [10].

2.4.2.4. Lipids

Lipids exist within algal cells and lipid productivity is thought as a key desirable characteristic when choosing algal species due to the possibility of producing biodiesel [62]. The lipids and fatty acids exist in the membrane, as storage products, or metabolites [6]. The largest portion of lipids often exists as a part within membranes [6]. Primarily lipid droplets exist in the cytoplasm, not in other organelles within the cell, and can therefore be extracted quite easily from the cells [10].

The most important structure among lipids is fatty acids which have a long hydrophobic hydrocarbon tail attached to a hydrophilic carboxyl group and are built in other types of lipids [6]. The components and amounts of lipids differ between species and classes [53] and the growth phase and environmental conditions affect the composition and productivity [59]. The fatty acids can be saturated and unsaturated with one or more double bonds [6]. Fatty acids are weak fatty acids with pK_a values around 4.5 [6].

Lipids within algal cells are divided into neutral lipids (triglycerides and cholesterols) and polar lipids (phospholipids and galactolipids) [53]. Triglycerides are the most desirable product for biodiesel production [23]. Triglycerides are often the form of which fatty acids are stored within organisms [6]. Mathews et al. [6] explain that triglycerides are triesters of fatty acids attached to one glycerol, and mean that mixtures of fatty acids often occur. All species do not produce lipids as triglycerides; *B.*

braunii is one example with no triglycerides, but containing *e.g.* terpenoids instead [50]. Triglycerides are produced by three steps in algal cells as seen in *Reaction 8* [53].

Reaction 8 – Triglyceride formation

Formation of acetyl coenzyme A in the cytoplasm

↓

Elongation and desaturation of carbon chain fatty acids

↓

Biosynthesis of triglycerides

It is generally known that microalgae can use both inorganic carbon (CO_2) and organic carbon sources (*e.g.* acetate or glucose) for growth and production of lipids [53]. Lowest yield of lipid formation resulting in a low content is achieved in the logarithmic growth phase, increasing formation at the end of logarithmic growth and highest in the stationary phase where growth is limited [59]. During logarithmic growth the lipid content mainly consists of polar membrane lipids, not suitable for biodiesel production [59]. Generally fast growing cells produce lower amounts of lipids (<20 % of DW) whereas cells accumulating high amounts of lipids (40 % to 50 % of DW) grow slower and more carbon is then partitioned into lipids [18]. Li et al. [22] report that few species produce large quantities of lipids under favorable conditions and the ones produced are primary polar lipids enriching the membranes and chloroplasts. It is not unusual that species accumulates between 20 – 50 % oil of dry weight if some kind of stress is applied on the cells, as nitrogen starvation [53], and some cells can accumulate even up to 86 % of DW as *B. braunii* [10].

Many microalgae have a desirable lipid profile suitable for biodiesel production and with high oxidation stability [23]. *Chlorella* species have short chain fatty acids mainly (C_{14} – C_{18}) which are suitable for biodiesel production [53], hence many *Chlorella* species were selected in this project. Huang et al. [53] mean that many algal species have elongated fatty acids (C_{20} – C_{22}) which are not suitable for biodiesel production; thereby the choice of algal species is important.

2.5. Biofuel production from microalgae

Biofuels are produced from biomass, such as plants or microorganisms [20]. Microalgae are miniature factories and can be used for production of biofuels. A second generation biofuel production would be a sustainable production as it will not collide with food or feed production and the idea is to use biomass that is not used for food purposes [10]. For biofuel production from algal biomass to be as efficient and economical as possible it is of great importance that all biomass is used as efficiently as possible and that all biomass is utilized [8]. Sheenan et al. [8] mean that there are many alternative biofuels from the biomass, mainly biodiesel and bioethanol but hydrogen gas, methane or combustion are other alternatives.

2.5.1. Biomass combustion

Flue gases can contribute with valuable CO_2 that can be fixed by algae at a high efficiency and can convert it to energy-rich biomass. The biomass can further be used to simply combust it [20]. The burning of biomass will lead to re-release of CO_2. On the other hand the corresponding amount of fossil fuel would not be burned and here is where the CO_2 release is saved [8]. In theory, the released CO_2 could again be led to a new culture of microalgae and get fixed once again, thereby recycling it and fix new light energy into new energy rich biomass for combustion.

2.5.2. Biodiesel

Biodiesel as biofuel has got a lot of attention the recent years due to environmental benefits compared to other fuels. Biodiesel is produced primarily from vegetable oils [53]. Biodiesel is a desirable product from many aspects and have significant environmental and economic impact [62]. It is made from renewable and non-toxic resources [56]. Biodiesel is biodegradable and is free from sulfur, and by using biodiesel no harmful chemicals would be released, not even aromatic compounds [53].

Large parts of the fatty acids in triglycerides are the desired kinds of lipids and therefore plant oils containing large amounts of these triglycerides have been used for biodiesel production so far [53]. Oil to biodiesel is mainly coming from terrestrial plants such as rapeseed, sunflower, soybean and palm and the production conquers with the food industry [56]. Oil production from microalgae may not conquer with land areas for food production or with feedstock for food industry [15]. Microalgae are a suitable choice for production of biodiesel compared to other oil crops due to similar fatty acids as plants, faster biomass formation, 10-50 times faster [32], and higher oil production [53]. Cultivations compared to production of other oleaginous crops are known for having bioremediation possibilities [23].

Biodiesel is regarded as an environmental friendly alternative to petro diesel and is a sustainable alternative, also beneficial from an economic aspect [62]. Lipid quality is important to investigate of the chosen microalgae as all lipids are not suitable for converting to biodiesel. Plant oil very often has carbon length of 14-18 which is feasible as biodiesel has about 15 carbons [53]. Many species have suitable lipid profiles with oxidation stability [23].

Lipids can be converted to biodiesel by transesterification with methanol, seen in *Reaction 9* [23]. Producing biodiesel from microalgae is technically, but not so far economically, achievable [62]. The production cost for biodiesel is too high and the main costs are the raw material (60-70 % of total cost) and the processing, as dewatering and extraction of the oil through the tough cell wall of microalgae [53]. Low algal productivity and high harvesting costs are two other challenges. Optimizing the algal growth and earning biodiesel needs CO_2 in high concentrations, possibly from flue gas [23] from paper mills.

Reaction 9 – Biodiesel formation

$$\text{Triglyceride (oil or fat)} + \text{Methanol} \rightarrow \text{Methyl Esters (Biodiesel)} + \text{Glycerol}$$

2.5.3. Bioethanol

Ethanol as fuel stands for ~ 80 % of the total ethanol production [10]. Ethanol is a high-octane fuel, used as an octane enhancer in gasoline, and is produced from renewable resources. Oilgae [10] contend that in the year of 2014 the demand for ethanol will be 100 billion liters of gasoline equivalents.

Ethanol can be produced by pure chemistry as a by-product from a reaction of ethylene and petroleum products [10]. However, these sources are not regarded as renewable and sustainable according to Oilgae [10]. Bioethanol is ethanol produced by fermentation of *e.g.* plant starch. Presently, the first generation bioethanol is produced by fermenting sugars or starch from agricultural plants to ethanol, a similar process as the one used for beer and winemaking. This method requires large amounts of food crops and is not a sustainable choice, and the food prices would also raise a lot. An alternative solution with bioethanol as a second generation biofuel not using food crops is desired. Carbohydrates such as starch can be converted to ethanol; hence algae are a well suited choice for biofuel production as some species store starch. There is a high possibility that algae can be a feedstock for ethanol as it contains polysaccharides and have thin cellulose walls [10]. Oilgae [10] report that cellulose (part of the cell wall) and starch (storage molecule) can be converted to ethanol by certain yeast. Three possible alternatives exist for ethanol production and all include a fermentation step. The three alternatives are fermentation of biomass, fermentation of residues after harvesting of oil or fermentation of syngas.

2.5.4. Methane gas

Methane is probably the simplest way of producing a biofuel from algal biomass as the biological and thermal processes necessary are not affected much of what constituents the biomass has [8]. Methane could be produced by anaerobic digestion of biomass or by methanation of syngas created from biomass [10]. Gasification could be used and it means involvement of breakdown of organic matter into methane [8].

2.5.5. Hydrogen gas

There are separate opinions about H_2 as a biofuel and many scientists mean that it will only be a green dream [10]. Other scientist's contend that H_2 has many advantages as a biofuel. It is renewable, it develops much energy per unit weight and it does not emit CO_2 when combusted [10]. The question still stands if microalgae can be used to generate H_2 for commercial purposes [52]. Today H_2 is produced from natural gas, a fossil fuel [10]. Photosynthetic microorganisms could make it possible to produce H_2 at large scale and thereby a renewable biofuel could be produced by a low energy requiring process [52]. H_2 is not only used for biofuel purposes even if the future goal is to achieve an alternative fuel [10]. There is a market for H_2 involving several areas. H_2 is important for producing NH_3 for fertilizers, for processing petroleum products and also for hydrogenating polyunsaturated fatty acids for margarine production [10]. However, there are problems needed to be solved before it can be used in large scale as fuel. Problems involve the storage, as it needs to be stored at low temperatures and high pressures, and it is very reactive and combustible. There are three major ways of producing H_2 from algae; a biochemical process, gasification and producing H_2 from methane [10] and these methods are further described in detail in *Appendix B.*

2.6. Content of gaseous compounds in flue gases from pulp- and paper mills

Gathering of information about gas and process water constituents from different pulp- and paper mills in Sweden was a part of the project. The pulp- and paper mills were contacted by e-mail and asked to report the content in their emissions, see *Table 5* for summary of data. The concentrations are approximate mean values from the earned values in the emission reports and these were used for deciding the most important levels in the simulated flue gas. From the first earned values the gas was decided to have 15 % CO_2, 100 ppm NO and 10 ppm SO_2. *Table 5* indicates that a broad spectrum of levels can be obtained by the exhaust gases from different pulp- and paper mills in Sweden.

Table 5. Constituents in the exhaust gases from different pulp- and paper mills in Sweden. The values are calculated mean values from emission reports and a mean value of the mean values is shown. The chosen flue gas constituents of CO_2 (15 %), NO (100 ppm) and SO_2 (10 ppm) was based on the levels in this table.

Pulp- and paper mills	Panna [1]	CO_2 (%)	CO (ppm)	NO_x (ppm)	NO (ppm)	NO_2 (ppm)	SO_2 (ppm)	O_2 (%)
Billerud Karlsborg	Barkpanna	11.4	189		75.7		9.9	8.8
Billerud Gruvön	Barkpanna	13.0		50.0			< 1	
Billerud Gruvön	Sodapanna	18.0		70.0			< 1	
Billerud Gruvön	Mesaugn	24.0		140			< 1	
Billerud Gruvön	Gasdestruktionsugn	6.00		80.0			5.00	
Arctic Paper Munkedal	Panna	14.5	30.0	134	134			1.60
Arctic Paper Grycksbo	Oljepanna			65.4	65.4			5.00
Stora Enso Skoghall	Panna 11	15.0	82.0	50.0	47.0	2.30	7.00	5.40
Stora Enso Skoghall	Panna 12	12.0	281	66.0	65.0		0.50	8.60
Stora Enso Skoghall	Mesaugn	20.4	104	20.6	21.0		1.00	5.10
Stora Enso Skoghall	Sodapanna	13.8	20.0	75.7	75.4		0.60	5.70
Stora Enso Skoghall	Gaspanna	6.00	1.90	22.3	22.0		0.10	10.5
Munksjö Aspa Bruk	Sodapanna	14.3		68.0				
Nordic Paper Bäckhammar	Sodapanna	13.1	64.5	68.7	64.2		28.3	6.10
Nordic Paper Bäckhammar	Mesaugn			180			3.00	
Nordic Paper Bäckhammar	Barkpanna	9.90	159	86.0	71.1		11.4	10.0
Nordic Paper Bäckhammar	Flingtork			15.0			12.0	
Holmen Paper Braviken	Fastbränsle	10.0	200	87.0		87.0	5.60	
Holmen Paper Braviken	Oljepanna	0.01		121		121	15.7	
Holmen Paper Hallsta	Panna		680		52.7	3.40	9.800	
Waggeryd Cell	Flingtork	0.30	18.0	3.30			2.00	20.4
Rottneros	Sodapanna	15.1	7.20		70.7		25.4	
Rottneros	Panna						1.50	
VIDA AB	Sulfitpanna	13.0	300		50.0		60.0	
VIDA AB	Fastbränsle	12.0	250		100			
Munksjö Paper Billingsfors	Sodapanna min.	7.90	118		65.1		9.90	
Munksjö Paper Billingsfors	Sodapanna max.	9.30	272		73.4		33.7	
Munksjö Paper Billingsfors	Fastbränslemin.	5.10	407		104		22.0	
Munksjö Paper Billingsfors	Fastbränsle max.	10.8	1140		124		29.8	
Munksjö Paper Billingsfors	Mixeri min.	4.70			131		14.5	
Munksjö Paper Billingsfors	Mixeri max.	7.70			148		41.1	
Värmeforsk-rapport	Använda värden	10.0			57.0		0.80	
Mean values (selected from first data recieved)		13.3	214	73.4	77.1		9.00	
Mean values (total after all data recieved)		11.1	227	73.8	77.0	53.4	12.5	

[1] No suitable translation was found for the naming of the equipment and hence the Swedish name is shown.

2.7. Content of nitrogen and phosphorous in waste water from pulp- and paper mills

Data about nitrogen and phosphorous levels in wastewater from the industries were also collected. Some mills contributed with gas constituents while others had information about the process water. The levels of N and P were different in different purification steps performed at the industry. *Table 6* summarizes mean values earned from each company. For nitrogen the values ranges from the lowest of 1.4 mg/l to the highest of 64 mg/l and for phosphorous the values ranges from the lowest of 0.02 mg/l to the highest of 10 mg/l. As can be understood from the table the values are somewhat different between different paper mills and different purification steps.

Table 6. Earned process water nitrogen and phosphorous values from different pulp- and paper mills in Sweden. The values were either mean values from emission reports or given mean values from the company.

Pulp- and paper mill companies	Wastewater	N (mg/l)	P (mg/l)
Billerud Karlsborg	Total	3.00	0.40
Billerud Gruvön	Total	2.10	0.25
Billerud Gruvön	To biologic purification	4.63	1.62
Billerud Gruvön	From biologic purification	7.75	1.04
Stora Enso Skoghall	Total	3.48	0.25
Munksjö Aspa Bruk	Total	3.00	0.75
Munksjö Aspa Bruk	Total	4.00	0.95
Nordic Paper Bäckhammar	RIA	1.89	0.33
Nordic Paper Bäckhammar	Reactor 2	2.04	0.02
Holmen Paper Braviken	Total	6.00	0.30
Holmen Paper Hallsta	Total	8.00	0.90
Smurfit Kappa Kraftliner Piteå	Total	3.15	0.88
Waggeryd Cell	To sewage plant minimum	18.0	5.00
Waggeryd Cell	To sewage plant maximum	64.0	10.0
Rottneros	Sewage water	1.40	0.63
Munksjö Paper Billingsfors	Total minimum	6.90	0.11
Munksjö Paper Billingsfors	Total maximum	13.0	0.11
Total mean value		8.96	1.38

3. Materials and method

The project was a screening of eleven different species of microalgae grown in freshwater medium to investigate which ones were growing fastest with added flue gas mimicking effluents from paper mills. The gas was distributed by a system connected to the algal cultures done in E-flasks and growth was followed by OD_{750}-measurements and cell count.

3.1. Strains of freshwater and marine algal species

A total of ten freshwater and one marine species of microalgae (see *Table 7*) were used in the experiments and obtained from CCAP [1] (Culture Collection of Algae and Protozoa, Scotland), NCMA [4] (National Center for Marine Algae and microbiota, U.S.A.) or isolated from natural habitats. In addition, some marine species were used for a growth test at different salinities.

Table 7. The studied species with strain name, culture collection origin and main habitat. The species and strains embrace the salinity test, the test runs and the flue gas experiments.

Species	Strain	Culture collection	Main habitat
Ankistrodesmus falcatus	*CCAP 202/15A*	CCAP	Freshwater
Arthrospira Platensis	*CCMP1295*	NCMA	Marine water
Botryococcus braunii	*CCAP 807/1 or UTEX 572*	CCAP	Freshwater
Chaeotoceros muelleri	*CCAP 1010/3*	CCAP	Marine water
Chlamydomonas reinhardtii	*CCAP 11/32C*	CCAP	Freshwater
Chlorella emersonii	*CCAP 211/11N*	CCAP	Freshwater
Chlorella protothecoides	*CCAP 211/54*	CCAP	Freshwater
Chlorella salina	*CCAP 211/25*	CCAP	Marine water
Chlorella sorokiniana	*CCAP 211/8K*	CCAP	Freshwater
Chlorella vulgaris	*CCAP 211/11B*	CCAP	Freshwater
Cyclotella cryptica	*CCMP 333*	NCMA	Marine water
Dunaliella salina	*CCAP 19/30*	CCAP	Marine water
Dunaliella Tertiolecta	*GUMACC5*		Marine water
Isochrysis galbana	*CCAP 927/14*	CCAP	Marine water
Nannochloropsis limnetica	*CCMP 505*	NCMA	Marine water
Nannochloropsis oculata	*GUMACC104*		Marine water
Nannochloropsis salina	*CCMP 1777*	NCMA	Marine water
Nedsjön isolate		Isolated by S. Ekendahl 2011-06-27 [(1)]	Freshwater
Phaeodactylum tricornutum	*GUMACC2*		Marine water
Rhinomonas reticulata	*GUMACC127*		Marine water
Scenedesmus obliquus	*CCAP 276/50*	CCAP	Freshwater
Scenedesmus sp.	simris002	Simrisalg AB	Freshwater
Tetraselmis sp.	T_A	Isolated by Eko Agus Suyono [(2)]	Marine water
Tetraselmis suecica	*CCAP 66/4*	CCAP	Marine water

[(1)] Isolated from the Västra Nedsjön close to Göteborg, Sweden.
[(2)] Isolated by the University of Gadjah Mada, Yogyakarta in Indonesia

3.2. Growth media for fresh water (3N-BBM+V) and marine (f/2) species and their storage

Two media rich in nutrients were used, for freshwater cultures *Bold's Basal Medium with 3-fold Nitrogen and Vitamins* (3N-BBM+V) [1] and for marine species f/2 medium [63] (for full recipes see *Appendix C*). The nitrogen and phosphorous content were calculated; 124 mg N/l and 50 mg P/l in the 3N-BBM+V medium and 12 mg N/l and 1 mg P/l in the f/2 medium (see calculations in *Appendix D*). The f/2 medium was prepared in artificial sea water (*Instant Ocean sea salt*), with salinities of 2.5‰, 10‰ and 25‰ representing brackish and marine waters.

For storage each algal strain was kept in a separate flask containing the appropriate type of medium and maintained with a light/dark cycle of 16/8, a temperature of 23 ± 2°C and loose caps on the bottles to obtain slow gas exchange and hence slow down growth (see *Figure 12*). Many of the storage cultivations were probably in stationary phase.

When handling non-reproducing algae in Sweden it is important to not spread the algae in Swedish waters and to minimize this risk all cell suspensions were autoclaved before discarding.

Figure 12. All the species were kept in storage flasks with loose caps containing the medium 3N-BBM+V for freshwater species and f/2 for marine species. The night/day cycle was 16/8 hours and the temperature was 23 ± 2°C.

3.3. Cultivation system and surrounding equipment

The cultivation system was constructed based on the anaerobic screening system for several cultures of the yeast *Saccharomyces cerevisiae* [64] (see *Figure 13* and *Figure 14*). Valadi et al. [64] demonstrate how E-flasks can be coupled to a gas distribution system which allows a screening technique of several strains simultaneously, in this experiment 12 batches. The equipment was homemade to a high extent. A pre-made racket with nails covered in silicone, four and four, kept each E-flask still on a line. The E-flasks could get detached.

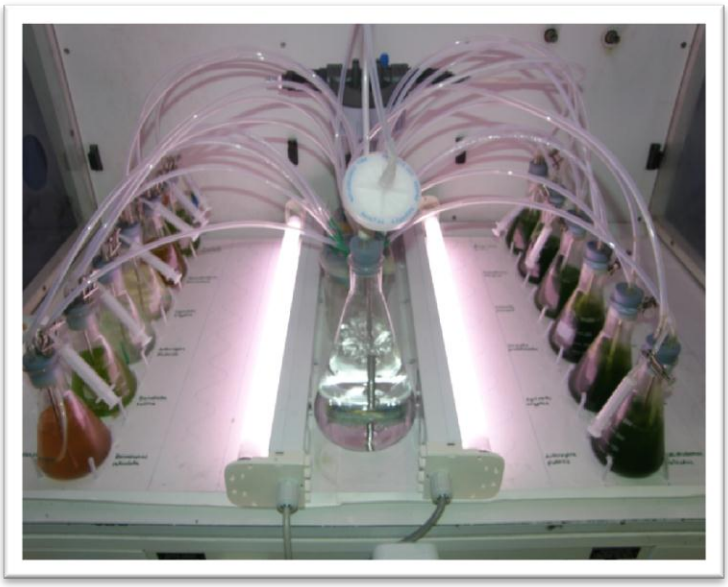

Figure 13. The equipment setup with distribution system according to Valadi et al. [64].

The E-flasks had rubber stoppers to seal the cultures. Three holes were drilled through each stopper. Stainless steel pipes were put through the holes, one for inlet gas, one for outlet gas and one attached to a syringe for sample-taking. Gas tight silicone tubes were attached to lead the gas flow. At the bottom of each flask a short plastic end was attached to each tube muzzle with a small hole which led the gas into the cell suspension by bubbling. The plastic ends leading the inlet gas could handle low flow rates and the differences between flasks were minimized. They were cut evenly, 5 cm each, and the end was melted to plug them. One small hole was made on each end by a thin needle. At the test runs plastic circles were attached instead of the short plastic ends, seen in *Figure 14*. All circles had 16 holes. However, at the low flow rate of 1400 ml/min distributed evenly on twelve flasks only one hole per end worked. The holes were placed downwards and the bubbles were forced down before heading to the surface. Agitation and contact time were enhanced, and different flow rates gave different agitations.

Illumination was applied by four oblong fluorescence lamps, *Osram L 18W/77 Fluora*, two were placed in the ceiling of the fume-hood cabinet and two along the E-flasks, six cultures for each lamp (see *Figure 13*). A timer was coupled to obtain an installed light/dark cycle.

Three gas tubes, from the company *Air Liquide*, containing CO_2 (100%), NO (1732 ± 17 mol-ppm distributed in N_2) and SO_2 (979 ± 20 mol-ppm distributed in N_2) were used (see *Figure 15*). The CO_2 tube had liquid content in equilibrium with the gas phase and could rapidly drop to zero as it was consumed. The gas flow was adjusted to desired concentrations by flow regulators. A precision regulator was coupled to the airflow to minimize the variations of the compressed air. A flow meter was coupled after all added gas flows to make the approximate total gas flow visible at all times by an airlifted metallic bullet (see *Figure 15*). The gas was bubbled through a distilled and autoclaved water bath to saturate with H_2O and minimize evaporation of water from the medium in the cultivation flasks. The gas outflow was led to an *AirSorb chemical filter* containing potassium permanganate cleaning SO_2 from the outlet gas. As much equipment as possible was autoclaved before use.

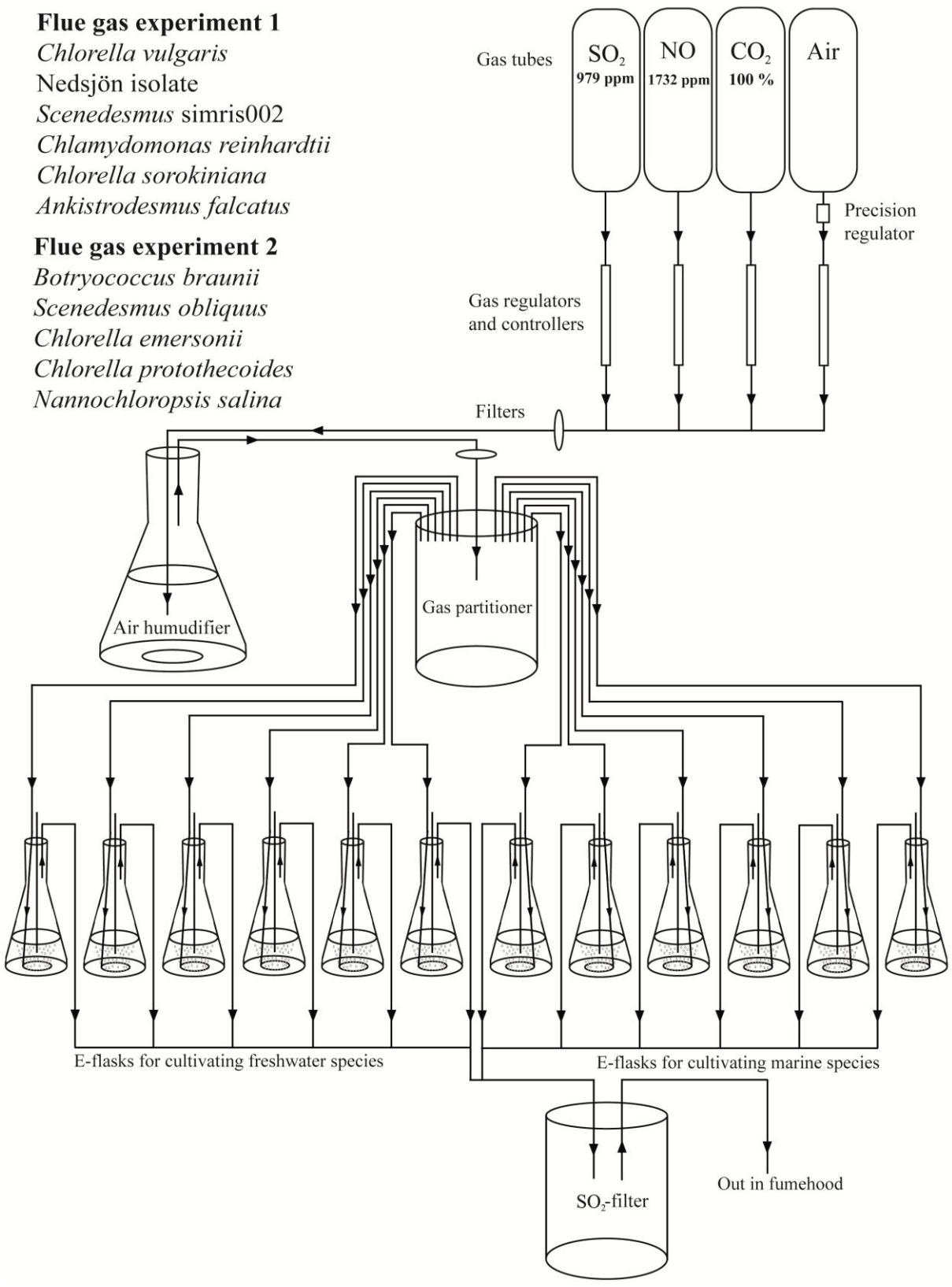

Figure 14. The setup of the equipment, constructed from Valadi et al. [64]. The middle line going down in each E-flask symbolizes the syringes where samplings were taken from. Twelve cultures could be investigated at the same time but only six were available for this project the others were used for a parallel project. The cultures of the eleven species investigated were done in two sets, Flue gas experiment 1 and 2.

3.4. Cultivations of microalgae and additional tests

The main experiments in the report were the flue gas experiments, but several additional tests were made during the project, regarding pH, salinity and absorbance.

3.4.1. Influence of flue gas on pH of the cultivation medium

Some pH tests were made as pH had impact on the growth of the algae. The first test was made by bubbling air through the medium 3N-BBM+V (150 ml) to see if pH was affected by only air. 2000 ml/min of air was divided in the system of flasks for three hours.

The second test was made to study the pH effect of having CO_2 (7.5 %) bubbled through only medium (3N-BBM+V) with a total gas flow of 3500 ml/min partitioned to twelve flasks. For each test, 200 ml of medium was added per E-flask without cells. Separate tests were made including either NO (100 ppm) or SO_2 (10 ppm).

Figure 15. Gas tubes of CO_2, NO and SO_2, flow regulators and a flow controller.

The third pH test was made to examine if pH measurements differed if performing them directly inside the E-flask compared to outside a cell culture. A gas inflow of 1400 ml/min with 5.9-6.5 % CO_2 was distributed to twelve flasks. The pH meter was put directly into the E-flask with parafilm wired around to avoid CO_2 from leaking out. The suspension inside was agitated by bubbles and was therefore mobile, but the test outside the E-flask in the test tube was still. The pH meter was kept in the test tube for approximately 5 minutes to see whether or not the pH changed over time outside the E-flask.

3.4.2. Salinity tolerance test of marine and freshwater microalgae

Brackish water as growth medium could be an alternative and thus a salinity test was made where the freshwater and marine species were grown in marine and brackish water medium f/2 and freshwater medium 3N-BBM+V. The f/2 medium was prepared in artificial sea water (*Instant Ocean sea salt*), with salinities of 2.5‰, 10‰ and 25‰ representing brackish and marine waters. Silicon (Si) was added to the diatom cultures as it is crucial for growth [68] [63]. The cultivations were performed in glass bottles (100 ml), with loose caps. 30 ml medium was added to sterile bottles together with 5 ml of pre-culture to start the cultivation. The cells were allowed to grow for 1-2 weeks at a temperature of 23 ± 2°C with 16/8 light/dark cycle. Growth was determined visually after shaken the bottles.

3.4.3. Absorbance test and comparison between OD_{550} and OD_{750}

An absorbance test concerning the two wavelengths 550 nm and 750 nm was made, since it was reported that there is absorbance minimums at these wavelengths [38]. Dilution series were made for the six species in *Flue gas experiment 1*. Pre-cultures were diluted six times; 5/5, 4/5, 3/5, 2/5, 1/5 and 0/5 (only medium). OD_{550} and OD_{750} were then measured for all species at all stated dilutions and the OD-values were plotted against the dilutions. The aim was to see whether or not a linear relationship occurred and if there was a difference in slopes between the two wavelengths, as different wavelengths can be absorbed by the pigments [38].

3.4.4. Flue gas test runs for improvements of methods and equipment

Three test runs (*Test run 1-3*) in freshwater 3N-BBM+V and algae with simulated flue gas added to the cultivations were performed and useful knowledge was earned from each test run and improvements of methods and equipment were made. The test runs are further described in *Appendices E-G* with performance, results and improvements. Each test run lasted for different times depending on the growth (if they were growing at all) or if other problems occurred.

3.4.5. Flue gas experiments

Two main experiments with simulated flue gas bubbled through the medium 3N-BBM+V and cells were started after the test runs; *Flue gas experiment 1* and *Flue gas experiment 2*. The species and strains investigated are visualized in *Figure 14* and *Table 7*. All experimental runs were made with twelve batches at the time divided on the freshwater and marine species. A night/day cycle was kept at 20/4 where the dark hours were between 6.00 am to 10.00 am. Light irradiance in the middle pointing towards the lamp was 13 W/m^2 and at the ends 8.5 W/m^2 and 2.7 W/m^2 in the middle upwards against the roof. The temperature was approximately room temperature. All E-flasks were shaken before sample-taking to distribute the cells. All samples were obtained through the syringe attached to each E-flask and 1 ml was always discarded before the samples were taken.

For all cultures the pH was measured simultaneously and controlled. Growth was followed with OD_{750} and the samples were frozen for further total cell count analysis. A total sample volume of 3 ml was taken regularly for OD_{750}, pH and cell count, 1 ml to OD_{750}/cell count and 2 ml to pH. At some occasion sampling for a full set of analyses was taken. Then a volume of 6 ml was needed of which additionally 1 ml was for lipid analysis and 2 ml for carbohydrate and protein analyses. The full sampling was taken twice in both runs according to *Table 8* and *Table* 9, the first closer to start and the second closer to the end. The lipid samples were centrifuged at 5000 rpm for 20 minutes and the supernatant was separated from the pellet into separate eppendorf tubes. The sample volume for carbohydrate and protein analysis was split evenly to four eppendorf tubes containing 0.5 ml each to ease the thawing and freezing process. The lipid-, carbohydrate-, and protein samples were frozen down with liquid nitrogen and put in a freezer for further use. The cell suspension volumes decreased by time as samples were taken. All remaining cell suspension was used for CHN analysis (100-150 ml). Biomass concentration was measured and coupled to OD_{750}.

3.4.5.1. Flue gas experiment 1

As pH reached values below 3 and the specific growth rate had decreased in all E-flasks in *Test run 3* (see *Figure 30* and *Table 29*), *Flue gas experiment 1* was remade from scratch. The purpose was to turn on the components one by one to investigate where the problem occurred and to avoid extreme pH-drops. First the cells were adapted to the new medium with light, then bubbling by air was added and hereafter the CO_2-levels were stepwise increased and finally NO and SO_2 were added. No NO or SO_2 were added until the final concentration of CO_2 (15 %) was reached. The experimental procedure was made according to the detailed protocol in *Table 8* and the gases were added according to *Figure 16*. The final concentration of CO_2 was not reached before the cell density was assumed as too high and two dilutions were performed to lower the cell density according to *Table 8*. At the dilution steps the volume was set to the same value for the twelve batches to keep them equal. At the end of the cultivation the tendency of growing on surfaces was examined visually.

3.4.5.2. Flue gas experiment 2

As *Flue gas experiment 1* was completed the results were evaluated for improvements. The marine species tested in parallel to the freshwater species had problem with slow growth and low pH in *Flue gas experiment 1* [14]. The fast growing species had to wait for the ones with slower growth to recover in pH after the CO_2 increase for the next increasing step of CO_2. HEPES buffer was used for the marine cultivations [14] and the CO_2 concentration could be stepwise increased faster with fewer steps. Mixing combined with light clearly enhanced growth in *Flue gas experiment 1* and therefore the bubbling by air was added directly at start, hence no acclimation or adaptation to the new medium were done. The detailed protocol of how the procedure was made is shown in *Table 9* and the gases were added according to *Figure 16*. No dilution was made as the stationary phase was not reached before highest concentration of CO_2 or even when SO_2 and NO were added.

Table 8. Flue gas experiment 1 was performed from start to end according to this protocol with different events of the experiment indicated. A stepwise increase of CO_2 from 0 % to the final 15 % was made and as the highest level of CO_2 was reached the NO (100 ppm) and SO_2 (10 ppm) were also added. Two dilution steps were performed to lower the cell densities.

Time (h)	Performance of *Flue gas experiment 1*
-	30 ml cell suspension was added to 220 ml sterile medium for startup. Only cells, medium and light were added the first 24 hours to make the microalgae to acclimate.
-	After 24 hours a total airflow of 800 ml/min, without extra added CO_2, NO or SO_2, was added and distributed to achieve mixing by bubbling.
0	OD_{750}-measurements started one hour after the added airflow. The cultivation start was set here as time 0 hours even though pH measurements were made earlier.
25	Adjustments to 1.35 % CO_2 with an increased total flow rate of 1400 ml/min.
97	Adjustments to 2.9-3.2 % CO_2.
117	Samples taken for lipid and carbohydrates/protein analysis.
121	Adjustments to 5.9-6.5 % CO_2.
141	Harvesting of 50 ml cells (discarded) from each batch to the remaining volume of 150 ml cell suspension in the E-flasks. Hereafter 100 ml fresh medium was added to the final volume of 250 ml. The cell suspensions were thereby diluted.
168	Adjustments to 9.1-9.5 % CO_2.
193	Adjustments to 12.3-12.7 % CO_2.
260	Adjustments to 14.9-15.1 % CO_2.
284	Harvesting of 100 ml cells (discarded) from each batch to the remaining volume of 100 ml cell suspension in the E-flasks. Hereafter 150 ml fresh medium was added to the final volume of 250 ml. The cell suspensions were thereby diluted.
309	NO was added by approximately 80.8 ml/min to the final total flow rate of 1400 ml/min making the concentration of 1732 ppm get diluted to approximately 100 ppm.
311	Samples taken for lipid and carbohydrates/protein analysis.
311	SO_2 was added by approximately 14.3 ml/min to the final total flow rate of 1400 ml/min making the concentration of 979 ppm get diluted to approximately 10 ppm.

Table 9. Flue gas experiment 2 was performed from start to end according to the protocol. The stepwise increase of CO_2 to final concentration of 15 % was performed in a higher rate compared to Flue gas experiment 1. NO (100 ppm) and SO_2 (10 ppm) were added earlier in the cultivation.

Time (h)	Performance of *Flue gas experiment 2*
0	30 ml cell suspension added to 220 ml sterile medium for startup. A total airflow of 1400 ml/min was added directly at start without extra added CO_2.
2	Adjustments to 0.95-1.09 % CO_2.
20	Adjustments to 3.3 % CO_2.
44	Adjustments to 6.7-7.5 % CO_2.
44	Cultivation of *C. emersonii* was pH adjusted as the cultivation changed in color from green to light yellow. 2 ml of 1 M NaOH was added.
68	Samples taken for lipid and carbohydrates/protein analysis.
69	Adjustments to 15.6-15.9 % CO_2.
140	Samples taken for lipid and carbohydrates/protein analysis.
140	Interruption for controlling CO_2 concentration.
143	NO and SO_2 was added as in *Flue gas experiment 1* to approximately 100 ppm and 10 ppm.

The three gas constituents, CO_2, NO and SO_2, were added at different times and concentrations during the flue gas experiments (see *Figure 16*). Note that CO_2 is given in % and NO and SO_2 in ppm.

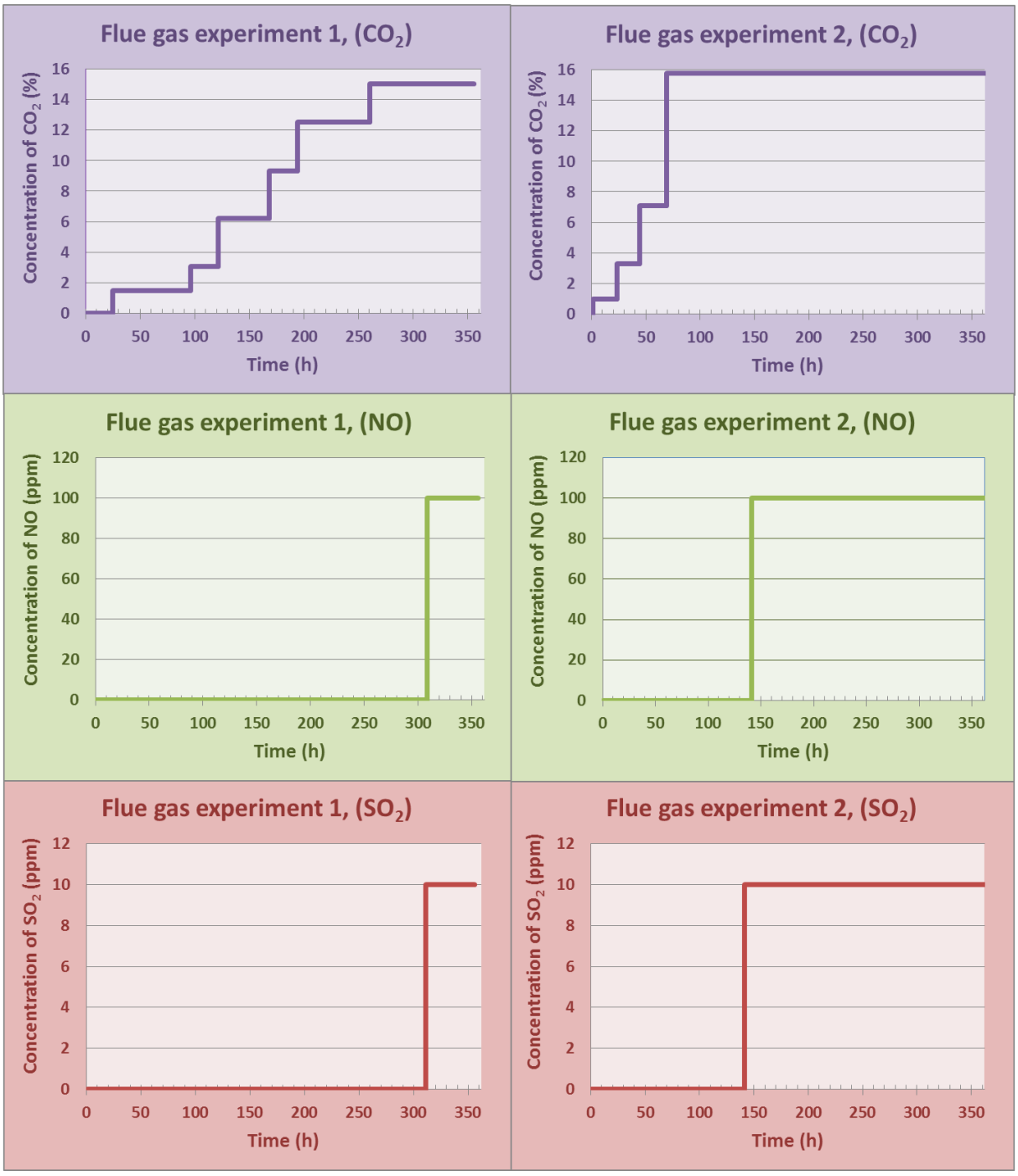

Figure 16. The order and time in which the gas constituent and their levels were added and then increased. In Flue gas experiment 2 the CO_2 (%) was increased faster and in fewer steps compared to Flue gas experiment 1. NO (ppm) and SO_2 (ppm) were added earlier in the second run.

3.4.6. Test of growth in two types of wastewater from *Nordic Paper Bäckhammar AB*

Parallel to the flue gas experiments another experiment was made by testing wastewater from the Swedish paper mill *Nordic Paper Bäckhammar AB* as growth medium. The wastewater could have a future purpose of working as growth medium in an algal cultivation contributing with nitrogen and phosphorous as building blocks for algae. The ten freshwater species (excluding the marine species *N. salina*) were tried out and were studied visually how growth was developing.

Two types of process water were tested, taken at different purification steps and were referred to as *RIA* and *Reactor 2* which refer to the purification steps, for composition see *Table 10*. *RIA* was purified water by biological purification and chemical mud separation. *Reactor 2* had only been purified by biological purification. The *RIA* water was adjusted by 1.0 M NaOH to pH 7.23 to be able to compare the two media.

Cells in 3 ml samples were taken from the storage flasks and divided into two 1.5 ml eppendorf tubes. The samples were centrifuged at approximately 4000 rpm for 15 min. The supernatant was discarded and the pellet was resolved in 200 µl autoclaved medium, *i.e.* the wastewater, thereby minimizing the amounts of nutrients and vitamins from the full medium 3N-BBM+V that could support growth. The resolved pellets were transferred to tissue culture flasks with loose caps containing 20 ml autoclaved wastewater. The light/dark cycle was 16/8. The flasks were shaken at least once per day to mix the culture, medium and air. The only CO_2 available was the amount dissolved in the media at start and the small exchange with the air. The cultivations were observed visually and photographed regularly to visualize how growth was developing. An additional test was made with only the two growth media in tissue culture flasks to see if some algal species came with the process water. These media were not autoclaved.

Table 10. Levels of phosphorous, nitrogen, pH, and other water quality parameters in the wastewaters, RIA *and Reactor 2, as analyzed by the ak LAB at Borås, Sweden.*

Subject matter	*RIA*	*Reactor 2*	Subject matter	*RIA*	*Reactor 2*
Total-N, mg/l	4.2 ±11%	13 ±11%	**Sulfate (SO_4), mg/l**	248 ±12%	169 ±12%
Nitrate-N (NO_3-N), mg/l	<0.50 ±14%	<0.50 ±14%	**Total-P, mg/l**	0.13 ±11%	1.8 ±11%
Ammonium-N(NH_4-N) mg/l	2.3 ±14%	2.3 ±14%	**pH (25°C)**	4.7[1]	6.7
Phosphate-P (PO_4-P), mg/l	0.04 ±16%	0.48 ±16%	**BOD7**	9.0	68 ±14%
Suspended substances, mg/l	<20 ±14%	160 ±14%	**COD(Cr)**	73	460

(1) Adjusted to pH 7.23 before culture start

3.5. Analytical instruments and sampling methods

Several analyses were performed during the project to investigate *e.g.* the growth and cell content. The analytical instruments, and how to use these, are important to know as well as sampling methods and performance.

3.5.1. Flow analysis calibrations

The total gas flow was measured by a *Gilian Gilibrator 2, Primary flow calibrator*, a bubble generator using a soap solution to determine the flow. The individual gas flow rates were calibrated on the individual flow regulators and were added to the total gas flow rate. Control measurements were made with each instrument on the total gas flow. For CO_2 a hand-held CO_2-meter, a *Vaisala Carbocap GM70*, was used. NO was analyzed with an *Eco Physics, CLD 700 El-ht, Chemiluminescence NO/NOx Analyzer* (see *Figure 17*). SO_2 was analyzed with a *Thermo Environmental Instruments Inc, USA, 43C SO_2 Analyzer*.

3.5.2. Salinity preparations in the marine medium f/2

Salinity was estimated by conductivity measurements done with a *WTW LF318 Conductivity meter* and converted to salinity at the actual temperature, room temperature. The conductivity meter was rinsed off with distilled water before measuring the salinity

3.5.3. Light intensity control

The light intensity was measured with a spectroradiometer of the kind *Optronic Laboratories 754-O-PMT*. The instrument was put in the fume cupboard at different directions where the algae were cultivated to estimate the light intensity. The instrument measured the light irradiance between 250-800 nm in different directions and gave values of W/m^2.

3.5.4. pH samplings and methods

The pH was measured by a pH-meter, *VWR International Sym**Ph**ony SP80PD*. The pH-meter was always rinsed off with distilled water before measuring. Bubble formation under the needle was also avoided. To avoid CO_2 from escaping, and thereby change the pH, the time between sample-taking and measurements was short. As the project went on pH got more important to check. The pH samplings were therefore made differently in the different experiments. In *Test run 1-3* the pH was not measured as often as in *Flue gas experiment 1* and *2*; hence the sample volume were larger. 10 ml sample was taken and put in a plastic cups in the test runs. In *Flue gas experiment 1* and *2* the plastic beaker was exchanged for a thinner test tube in glass where only 2 ml cell suspension was needed and made it possible to check pH more often.

Figure 17. The NO-meter used for measuring the NO and NO_x in the artificially produced flue gas.

3.5.5. Optical density (OD) analysis

OD_{750} was used to follow growth in a spectrophotometer, *VARIAN Cary 50*. The time intervals from sampling to OD_{750}-measurements were approximately the same at all samplings. The samples were diluted with medium appropriately, to result in OD_{750} of 0.2-0.5, if not already lower than this. The samples were put in eppendorf tubes before measurements and were not kept on ice. The ice was only used for *Test run 1-3* before realizing that condensation occurred on the cuvettes. The spectrophotometer gave an average of three measurements on each sample. The samples were analyzed twice, with mixing in between, and the mean values of these two OD_{750}-values were calculated. The samples were frozen down for further cell count analysis to minimize the total sample volume. Before running the OD_{750}-measurements with cells the spectrophotometer was zeroed with only medium. These were exchanged every day to avoid growth. The same cuvette was used for all measurements and errors due to differences between cuvettes were minimized.

3.5.6. Cell count analysis

As a complement to OD_{750}-measurements total cell counts were performed on selected samples in a counting chamber, Bürker with 0.1 mm depth, in a microscope, *Leitz Wetzlar Dialux 20*, with 40 times enlargement. Cell suspensions from OD_{750}-samplings were thawed quickly and counted. Damaged cells due to thawing were assumed to be countable. The chamber was cleaned with ethanol before each counting. A cover glass was put on and 200 µl samples were injected. Same dilution as OD_{750} was used to get an adequate number to count, 20-100 cells per square. The inner square of the possible squares was counted and 15 squares were counted for all samples. Cells on the left line and on the top line were also counted. To get cells/ml the counted mean value per square was multiplied with $4 \cdot 10^6$.

3.5.7. Dry weight (DW) and biomass concentration analysis

Whatman GF/C glass microfiber filters with diameter 55 mm and pore size 1.2 µm were used. The filters were marked with a pen and stored in a pre-conditioned room for 24 hours. The temperature was 23 ± 2°C and the relative humidity was RH = 50 ± 5%. All filters were weighed after the pre-conditioning. Solutions containing 0.5 M ammonium bicarbonate (NH_4HCO_3) was prepared and used for rinsing the samples [58]. 20 ml cell suspension was filtrated and all samples were rinsed two times with 10 ml ammonium bicarbonate. Duplicate samples were performed. The filters containing cells were dried in oven (100 °C) for 2.5 hours and then put back in the pre-conditioned room. The samples were weighed after a few hours and the pre-weighed filter values were subtracted. A mean value for both weights was calculated and the biomass concentration was calculated from the known sample volume. OD_{750}-measurements were made at the same sampling time and coupled to the actual biomass concentration.

3.5.8. CHN Elemental Analysis

All remaining cell culture, after the flue gas experiments, was used to earn as much dry biomass as possible. A minimum of 50 mg was needed for the analysis. The cell culture was put in plastic tubes and centrifuged for 5 min, 5000 rpm. The pellet was washed twice in distilled water (centrifuged 5 min, 5000 rpm). The tubes were put in oven at 80 °C until all liquid had evaporized. Tin foil cups were pre-weighed and the dry matter samples were put in these. The tin will not give rise to development of CO_2, NO or H_2O when burned. The cup was sealed and the air nitrogen was pressed out and a small metallic bullet was obtained. The bullets containing dry matter were put in the *LECO, CHN Elemental Analyzer 2000* model *601-800-900* to get C, H and N in weight percent.

3.5.9. Lipid analysis

The lipid analysis measured the quantity of triglycerides within the microalgae and was done by hexane extraction from algal biomass followed by acid esterification with methanol. Hereafter the analysis of fatty acid esters was made with GC/MS (Agilent technologies, 5975 inert XL Mass selective detector) and an estimated average weight of 870 g/mol [65] was used for estimating the total weight of triglycerides from the glycerol standard.

The experimental performance is described in *Appendix H* according to the attached protocol from Söderberg [65]. The analysis was started by thawing the deep frozen algae and the pellet was re-suspended in distilled water. The samples containing re-suspended pellets were lysed by ultrasound and re-frozen until the time for experiments. Same procedure was made on pellet and supernatant hereafter.

3.5.10. Carbohydrate analysis

The total carbohydrates were measured on the full cell suspension of the cultures using the sulfuric acid phenol method by Herbert et al. [66] with glucose as standard (see *Appendix I*). Microtiter plates were used for the analysis. In each analysis the medium was measured and used for background correction. No correction for carbohydrates in RNA and DNA was made.

3.5.11. Protein analysis

Total protein content in cells were performed by extracting with 1 % SDS and measured on the supernatant with the Protein Dc kit (BioRad) which is based on the Lowry et al. method [67], *Appendix J*. Microtiter plates were used for the analysis.

4. Results

To be able to use flue gas as carbon source and other waste streams as source for nutritional requirements in efficient cultivation of microalgae the influence of flue gas composition, physical factors like pH and salinity, and different types of process water on the algal growth and cellular composition are needed to be understood.

4.1. Influence of flue gas on pH of the cultivation medium

Since, CO_2 is readily dissolved in water and in equilibrium with carbonic acid an effect on pH of the medium by bubbling air could be expected. Thus, a pH test by bubbling air through the medium was done but only a small decrease in pH was observed after 2 hours and 45 min, seen in *Table 11*.

Table 11. The pH of 3N-BBM+V medium changed by only airflow added during 165 minutes.

Time from start (min)	pH
0	6.34
70	6.32
165	6.22

The second pH-test was made first with only medium and added air containing 7.5 % CO_2, secondly including NO (100 ppm) and thirdly also with both NO and SO_2 (10 ppm). By including NO and SO_2 at the given concentrations the pH seems to decrease faster compared to only having CO_2 at 7.5 % in the inlet gas, see *Table 12*. No large difference could be seen after two hours with or without SO_2.

Table 12. A pH test was made to see how the pH was affected by only CO_2 (7.5 %) added and compared to when all components were added with NO (100 ppm) and SO_2 (10 ppm).

Time (min)	Air, CO_2 (7.5 %)	Air, CO_2 (7.5 %), NO (100ppm)	Air, CO_2 (7.5 %), NO (100 ppm), SO_2 (10 ppm)
0	6.34	6.34	6.34
60	5.39	5.18	5.19
120	-	5.17	5.25

To examine whether or not pH measurements differed if performing them directly inside the E-flask compared to outside a pH test was made on the culture of *A. falcatus* in one of the twelve flasks of *Flue gas experiment 1* at 144.50 h from start with CO_2 of 5.9-6.5 %. A clearly increased pH was seen when measuring outside the E-flask compared to interiorly, see *Table 13*. The pH measured outside the E-flask slowly increased by time and the obtained value measured directly outside was most similar to the value measured interiorly. The pH increase was probably due to CO_2 released from the solution to the open air poor in CO_2, leading to a gradual increase in pH due to less acid formation.

Table 13. The pH measured directly inside the E-flasks of the culture with 5.9-6.5 % CO_2 compared to measurements outside performed immediately and after 5 min.

Place of pH meter	pH
Inside E-flask	6.19
Outside in test tube	6.27
Outside in test tube after 5 min	6.37

4.2. Salinity tolerance test of marine and freshwater microalgae

Since the water needed for algae cultivation possibly can be taken either as freshwater from lakes, brackish water from the Baltic Sea, or marine water from the sea it is important to know whether or not the microalgae can grow at different salinities. Some species could live in several environments and cope with both fresh and saline water conditions, suitable for cultivations of brackish water. The freshwater species thrived in lower salinities closer to freshwater and marine species thrived in higher salinities closer to marine water. As seen from the results presented in *Table 14*. Some species showed an ability to grow in a broad spectrum of salinities, *e.g. C. vulgaris*, *C. sorokiniana*, *A. falcatus* and *N. salina*. Some marine species grew well in low salinities (~ 2.5 ‰), but most freshwater species could not grow in higher salinities (~ 10 ‰). A reason could be that the surface water of the sea is diluted by rainfall and the marine species need to be tolerant to lower salinities. The marine species *N. salina* showed best performance and was thus able to grow in marine, brackish and freshwater systems; hence it was selected to the flue gas experiment for freshwater cultivations.

Table 14. Growth efficiency of different microalgae species grown in glass bottle cultures for ~ 1 week with different media: freshwater (3N-BBM+V) and with increased salinity in f/2 medium.

Species	Growth efficiency in different media [1]			
	3N-BBM+V	f/2 2.5 ‰	f/2 10 ‰	f/2 25 ‰
***Botryococcus braunii* (FS)** [2]	Good	Okay	Poor	- [3]
***Chlorella vulgaris* (FS)**	Very Good	Very good	Good	-
Nedsjön isolate (FS)	Very Good	Good	Good	-
***Scenedesmus* simris002 (FS)**	Very Good	Okay	Poor	-
***Scenedesmus obliquus* (FS)**	Very Good	Okay	Poor	-
***Chlamydomonas reinhardtii* (FS)**	Very Good	Good	Poor	-
***Chlorella sorokiniana* (FS)**	Very Good	Very Good	Good	-
***Chlorella emersonii* (FS)**	Very Good	Very Good	Poor	-
***Ankistrodesmus falcatus* (FS)**	Very Good	Very Good	Good	-
***Chlorella protothecoides* (FS)**	Very Good	Poor	Okay	Okay
***Phaeodactylum tricornutum* (MS)** [2]	Poor	Okay	Okay	Very Good
***Nannochloropsis salina* (MS)**	Good	Very Good	Good	Good
***Tetraselmis suecica* (MS)**	Poor	Okay	Okay	Very Good
***Tetraselmis sp.* (MS)**	Poor	Okay	Okay	Very Good
***Dunaliella tertiolecta* (MS)**	Okay	Very Good	Good	Very Good
***Chlorella salina* (MS)**	Good	Good	Okay	Okay
***Rhinomonas reticulata* (MS)**	Poor	Poor	Poor	Very Good
***Chaeotoceros muelleri* (MS)**	Poor	Poor	Okay	Very Good
***Isochrysis galbana* (MS)**	Poor	Poor	Poor	Good
***Nannochloropsis limnetica* (MS)**	Poor	Good	Poor	Good
***Cyclotella cryptica* (MS)**	Poor	Okay	Very good	Very Good
***Arthrospira platensis* (MS)**	Poor	Very Good	Very good	Very Good
***Nannochloropsis oculata* (MS)**	Poor	Poor	Poor	Good
***Dunaliella salina* (MS)**	Poor	Good	Good	Very Good

*[1] Classification of growth by visual inspection: **Very good**, **Good**, **Okay** or **Poor growth**.*
[2] FS – freshwater species, MS – marine species.
[3] The sign (-) signify "not tested". The freshwater species indicated reduced growth at f/2 (10 ‰) and hence; they were not tried at 25 ‰.

4.3. Absorbance test and comparison of OD_{550} and OD_{750}

Running algae in spectrophotometers are difficult compared to *e.g.* yeast in many aspects. A growth curve for yeast is predictable and the generation time is substantially smaller, hours instead of days. OD-measurements are also tricky when handling microalgae compared to other microorganisms as they contain light harvesting pigments absorbing the light, *e.g.* carotenoids and chlorophylls, and the pigment composition and content can vary in amounts depending on species, environmental factors and growth phase. This may give misleading results and hence an absorbance minimum is wanted when selecting the wavelength for OD measurements [38].

An absorbance test was made to minimize the error in OD-measurements due to absorption by the pigments and find a suitable wavelength with small absorption. 550 nm and 750 nm were recommended according to Harrison et al. [38] and were tried out. The plot for *C. vulgaris* where OD_{550} and OD_{750} were plotted against different dilutions is shown in *Figure 18*. The other five dilution series for five other species are presented in *Appendix K*. The line showed for both wavelengths a linear relationship between OD and the dilutions. However, a larger slope and also a higher absorbance could be seen for 550 nm compared to 750 nm for all freshwater green microalgae tested with dilution series and hence, a higher absorbance was seen for OD_{550} compared to OD_{750} for all species. Therefore OD_{750} was used to follow growth in the flue gas experiments to lower the absorbance disturbances. The R^2-values indicate confidential results.

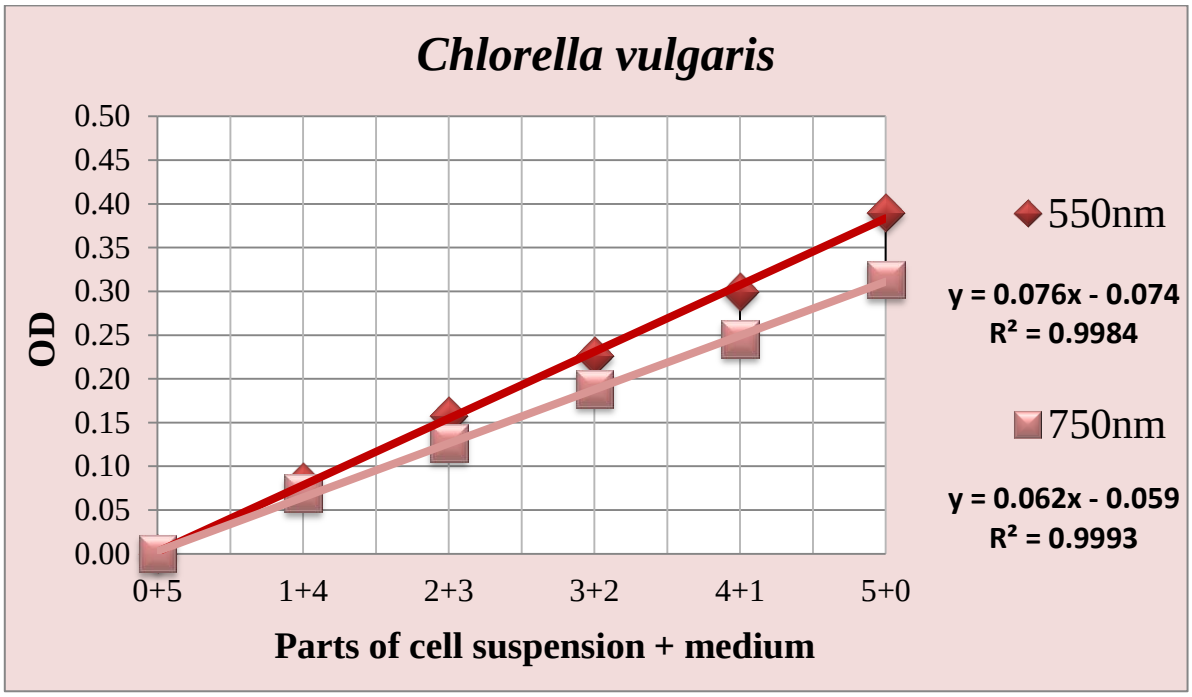

Figure 18. OD variations depending on dilutions of cell suspension. A linear relationship was obtained for two wavelengths but with two different slopes.

4.4. Flue gas experiment 1

The components of a flue gas may affect the algae growth and cellular components. Thus, flue gas experiments were done in which simulated gas containing CO_2 and additionally NO and SO_2 was added. Each species have one corresponding color in the plots and is the same through the rest of this chapter.

4.4.1. Influence of cultivations conditions on pH of the cultivation medium

The pH was affected by algal growth and the added flue gas. The pH was lowered as the components in the flue gas were added or increased (CO_2, NO and SO_2) and growth of the microalgae increased the pH by the use of CO_2 or HCO_3^-. As pH also has impact on the growth rate of the microalgae the pH was followed regularly and CO_2 was added as a function of pH (see *Figure* 19 and *Table 30* in *Appendix L*). The cultivations with highest pH from the pre-cultures reached the highest starting pH and the difference seemed to be maintained to some extent throughout the whole cultivation. Harvesting of cells and addition of medium was performed two times to get a lower cell concentration. Addition of new medium and increasing of CO_2 levels led to a decrease in pH.

The trends in pH changes and levels followed the same pattern for all strains (see *Figure* 19). All six freshwater species in *Flue gas experiment 1* survived until the end when performing the stepwise increase of CO_2 and all six species could recover the pH drops occurring as a result of the increased flue gas constituent levels, even though the pH became close to 6 for most species.

The cultivations of three species generally had higher pH and better pH recovery from pH-drops throughout the whole cultivation; *S.* simris002 (green line), N. isolate (red line) and *C. reinhardtii* (purple line). *C. reinhardtii* were the culture with lowest pH at start in the culture medium, but ended among the highest at the end at 356 h. The cultivation of *S.* simris002 was the one resulting in the highest pH (6.40) at the end whereas in the culture of *C. vulgaris* the pH showed the lowest values throughout the whole experiment. No buffered system was needed in the freshwater system to avoid low pH-values if performing a stepwise increase of the flue gas constituents. An initiation of a pH drop can be seen at the end (at 356 h) for all species.

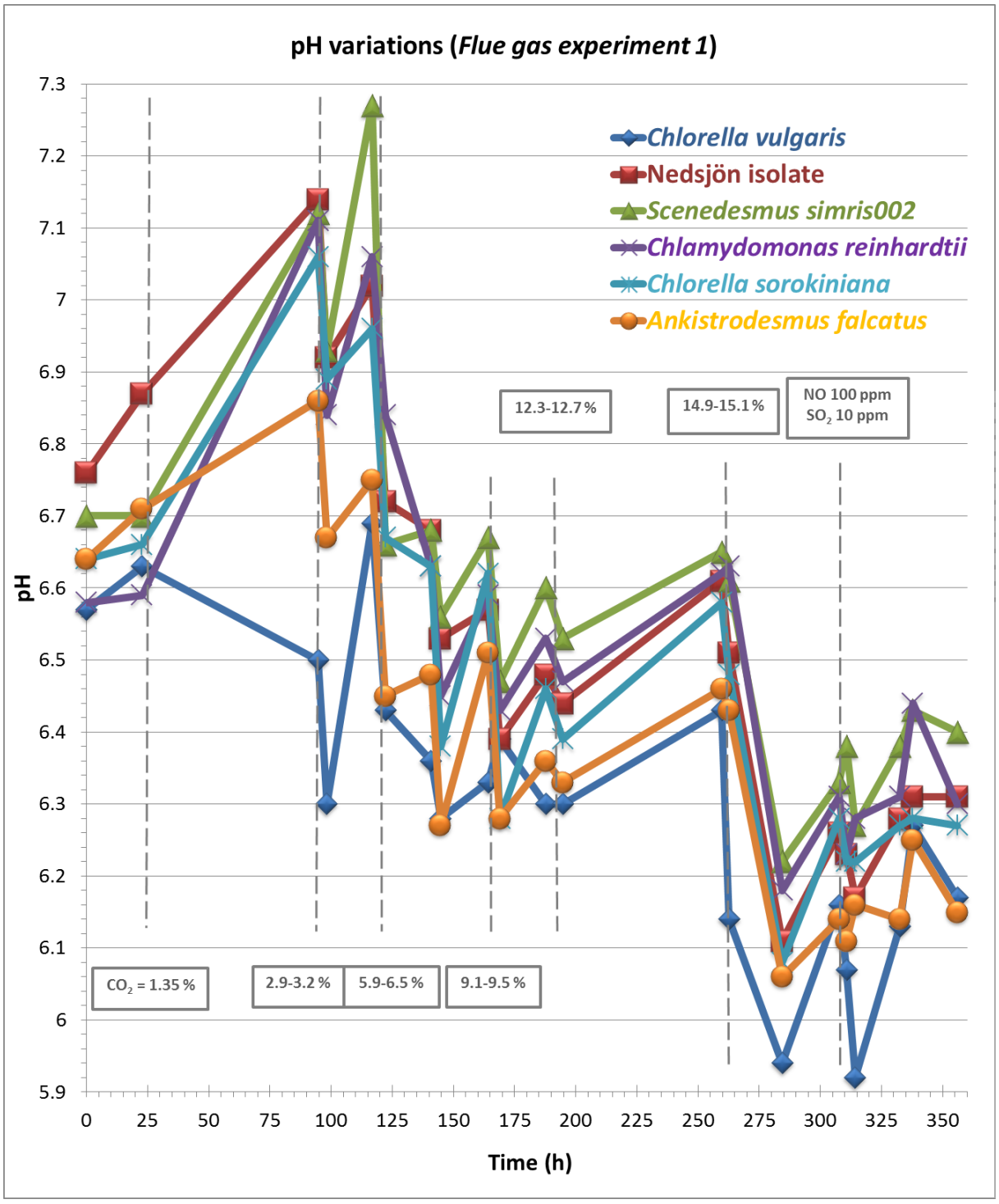

Figure 19. The pH variations in Flue gas experiment 1 for cultivations of the six investigated freshwater species. The CO_2 concentration was stepwise increased from approximately 0 % to 15 % and hereafter the NO (100 ppm) and SO_2 (10 ppm) were added according to the dotted lines in the figure. The total gas flow was increased from 800 to 1400 ml/min at 25 h. Dilution of cells were done at 141 and 284 h. The dotted lines illustrates when the gas constituents were increased or added and the colored lines corresponds to one species each.

4.4.2. Influence of cultivation conditions on the biomass formation and growth properties

Growth was followed by OD_{750} measurements (see *Figure 20*) for *Flue gas experiment 1*. Growth occurred in all E-flasks, the growth curves for each species followed the same pattern for all strains, and none of the species got inhibited by the flue gas. All species had increased the OD_{750} during 22 h of cultivation and thus the drop in pH by only airflow was counteracted and hence, only light and agitation by air-bubbling enhanced the growth which could be expected. At an increased gas flow rate of 1400 ml/min and CO_2 at 1.35 %, a good growth was achieved as seen from the large increases in cell concentration (OD_{750}) and pH (due to growth) at 95 hours from start (see *Figure 19* and *Figure 20*). Thus, all species had enhanced growth by a small amount of CO_2 added. Visual observations were made and thick and dark green cell suspensions were obtained after 95 hours of cultivation. N. isolate was the culture with the darkest green color. The species with highest OD_{750} at any time were *S.* simris002 (green line) and *C. reinhardtii* (purple line), indicated the fastest growth for these strains. N. isolate (red line) was generally the species with lowest OD_{750}. After 168 hours a thick sediment layer of cells was seen for all cultivations and aggregates formed as large clumps were seen for N. isolate, *C. sorokiniana* and *C. reinhardtii*.

The two additions of fresh medium at time 141 h and 284 h gave a dilution seen as a drop in OD_{750}. The second dilution was probably not enough for the fast growing species *S.* simris002 and *C. reinhardtii* to achieve the fastest possible growth at CO_2 (15 %), NO (100 ppm) and SO_2 (10 ppm) as they had OD_{750}-values close to 2 even after the dilution. The growth phase closer to the end of *Flue gas experiment 1*, where highest levels of CO_2 (15 %), NO (100 ppm) and SO_2 (10 ppm) were added, cell count was performed as a complement to the OD_{750}-measurements. *Figure 21* shows the cell number plotted against time. The culture with highest number of cells formed was *C. reinhardtii* (purple line) and the lowest one was N. isolate (red line).

The maximum specific growth rates and the generation times were estimated for each species and the growth curve was divided into *start phase* (0-1.35 % CO_2), *middle phase* (9.5-15 % CO_2), and *end phase* (15 % CO_2, 100 ppm NO and 10 ppm SO_2) as two dilutions were performed. *S.* simris002 (see *Figure 22*) was chosen to represent the general tendency of the species in *Flue gas experiment 1* as the growth curves were similar to each other. The specific growth rate from the cell count measured in the end was also determined, performed in the same way as the example with *S.* simris002 in *Figure 22*. For visualizing how the exponential phases for all species were determined in *Flue gas experiment 1* and *2* two plots (*Figure 38* and *Figure 39*) are shown in *Appendix N*, one illustrating logarithmic cell number and the other logarithmic OD_{750}. Both are from the end phases, but one from each experimental run.

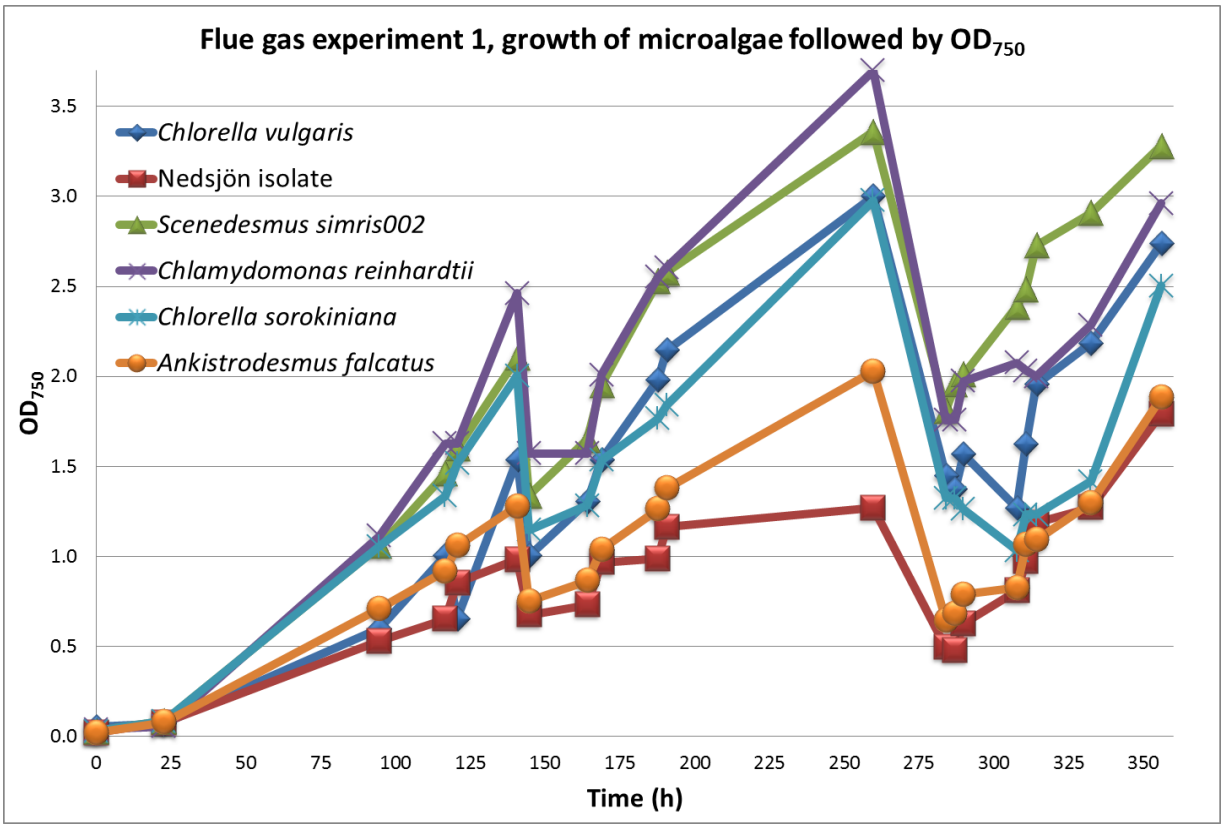

Figure 20. Cell concentration as OD_{750} in Flue gas experiment 1, the two drops in OD_{750} at time 141 h and 284 h symbolize the two dilutions performed. The second dilution step shows that S. simris002 and C. reinhardtii had OD_{750}-values close to 2 even after the dilution. Each colored line corresponds to one species each.

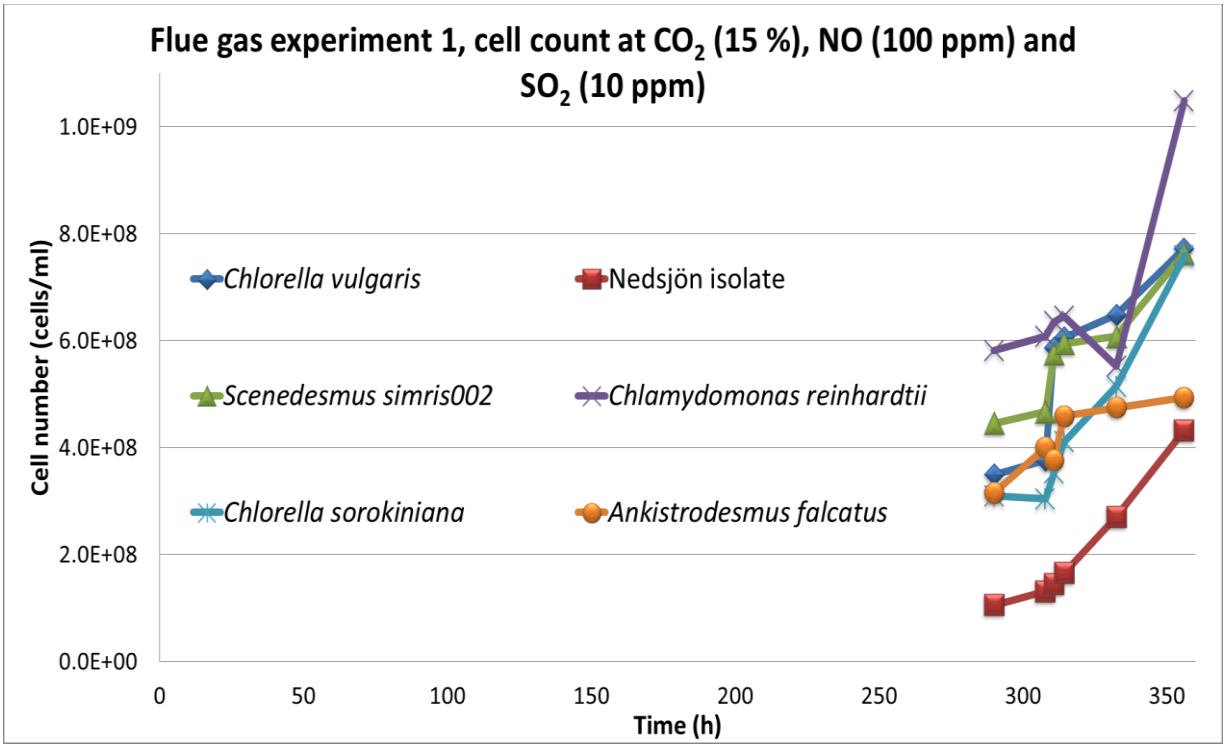

Figure 21. Concentration of cells as cell count (cells/ml) for Flue gas experiment 1 during the period when the highest levels of CO_2 (15 %), NO (100 ppm) and SO_2 (10 ppm) were added.

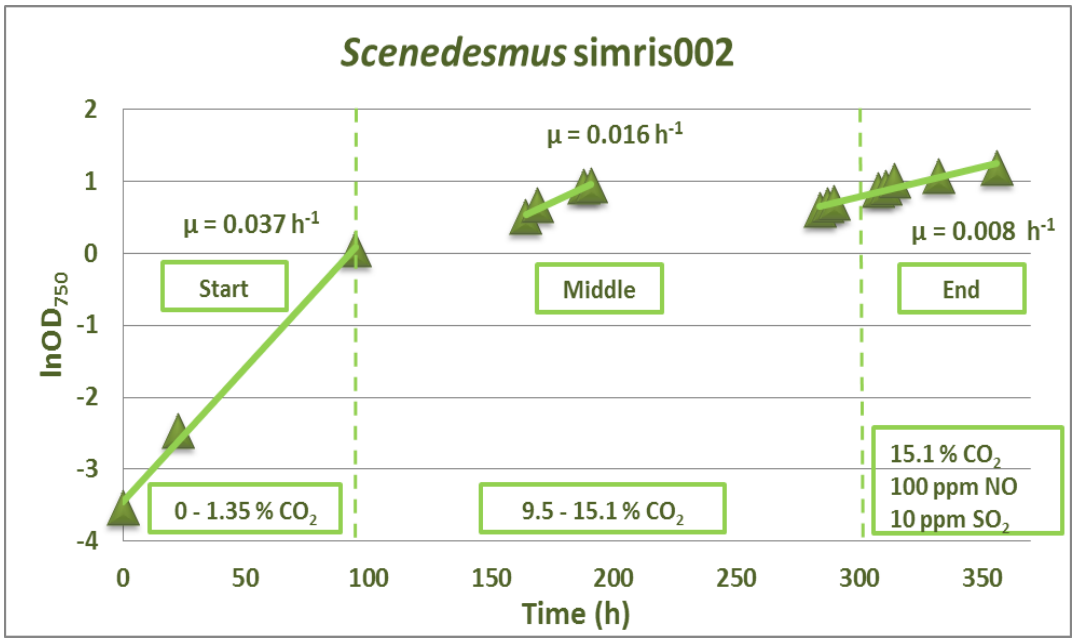

Figure 22. The logarithmic OD_{750} versus time for Flue gas experiment 1 for S. simris002 in the start phase (0-1.35 % CO_2), middle phase (9.5-15 % CO_2) and end phase where highest levels of CO_2 (15 %), NO (100 ppm) and SO_2 (10 ppm) were added. The slope corresponds to the specific growth rates.

Specific growth rates and generation times are summarized in *Table 15* from both OD_{750} measurements and cell count. In the start phase, higher specific growth rates were found as compared to in the middle or at the end of the cultivations for all species when determined from the OD_{750} measurements. It should be noted that the middle phase and the latest phase were seen after a dilution step and the OD_{750}-values were still high even after the dilutions. In *Figure 20* the OD_{750} values for *S.* simris002 and *C. reinhardtii* were close to 2 even after the second dilution. A fast growth at start makes the cultures reach high cell densities and the stationary phase faster and could slow down growth in the middle and the end phase. These later phases are therefore not comparable as the already high cell densities could have limited growth due to *e.g.* light limitations. Other reasons could of course be due to lower pH (~ 6-7) than the pH optimum for the species or inhibition by the components in the flue gas. At least the specific growth rates were lower at the end with CO_2 (15 %), NO (100 ppm) and SO_2 (10 ppm) added in the flue gas.

Four species of six showed higher specific growth rates from OD_{750} measurements at start (0-1.35 % CO_2), and thereby lower generation times, compared to the other two, namely *S.* simris002, *C. reinhardtii*, N. isolate and *C. sorokiniana*. *C. reinhardtii* had the lowest generation time of 17 h closely followed by *S.* simris002 with a generation time of 20 h. N. isolate was the species growing fastest, as determined both by cell count and OD_{750}, at the end of the cultivations when CO_2 (15 %), NO_2 (100 ppm) and SO_2 (10 ppm) added. The generation time was 41 h determined by OD_{750}-measurements and 40 h by cell count measurements.

Large differences between generation times for OD_{750}-measurements and cell count was seen for several species. At the end phase, *A. falcatus* had a generation time of 50 h as determined by the OD_{750}-measurements and 139 h by the cell count measurements. The reason could be that some kind of stress was applied on the cells and that they started to accumulate *e.g.* lipids and increased in size. OD_{750} would then have been affected while the number of cells was unchanged. However, the microscopic study could not strengthen this theory.

Table 15. The specific growth rates and generation times for Flue gas experiment 1 calculated from OD_{750}-measurements and cell count determined at the three different growth phases illustrated in Figure 22*;* start phase *(at 0-1.35 % CO_2),* middle phase *(at 9.5-15.1 % CO_2) and* end phase *(at 15.1 % CO_2, 10 ppm SO_2 and 100 ppm NO).*

Species	*C. vulgaris*	N. isolate	*S.* simris002	*C. reinhardtii*	*C. sorokiniana*	*A. falcatus*
$\mu_{OD\ start}$ (h^{-1})	0.030	0.027	0.037	0.042	0.035	0.031
$\mu_{OD\ middle}$ (h^{-1})	0.017	0.012	0.016	0.017	0.012	0.015
$\mu_{OD\ end}$ (h^{-1})	0.009	0.017	0.008	0.007	0.016	0.014
$\mu_{cell\ count\ end}$ (h^{-1})	0.006	0.025	0.010	0.011	0.017	0.005
$g_{OD\ start}$ (h)	23	26	19	17	20	22
$g_{OD\ middle}$ (h)	41	58	43	41	58	46
$g_{OD\ end}$ (h)	77	41	87	99	43	50
$g_{cell\ count\ end}$ (h)	116	28	69	63	41	139

For most of the regressions an R^2 coefficient above 0.91 was obtained.

4.4.3. Correlation of biomass concentration to OD_{750}

To be able to have a clue about dry weights at different OD_{750}-values the OD_{750} was coupled to the biomass concentration with a correlation factor that could be used to calculate biomass concentrations directly from the OD_{750}-measurements. Values of corresponding DW were calculated for the sampling times where lipid, carbohydrate, and protein analyses were performed for both flue gas experiments. A corresponding biomass concentration value (g DW/l) to OD_{750} at the sample occasion is shown. The samples for the correlations were taken at late log phase for both runs and these can be seen in the summary of the correlations in *Table 16*.

A high value of the correlation factor (g DW/l)/OD_{750} implies that the biomass concentration is large compared to OD_{750}. To calculate the biomass concentration from a large correlation factor means that a lower OD_{750}-value is required to reach large g DW/l. An OD_{750} of 1.8 corresponded to 0.83 g DW/l for N. isolate and if comparing to *e.g. C. reinhardtii* with an OD_{750} of 3.0 for 0.70 g DW/l one can understand that a larger correlation factor was obtained for N. isolate. Hence, OD_{750}-values are impossible to compare between species if not coupled to direct biomass as *e.g.* DW. N. isolate was a species growing in large colonies as seen in the microscope (see *Table 18*) and the cells probably shaded each other in the spectrophotometer by the large cluster formation, giving relatively low values compared to the biomass concentration. Neither were the large colonies identical and different numbers of cells were seen. OD_{750}-measurements are only working if the cells are evenly distributed and single celled within the suspension [3], otherwise the diffraction of light would show misleading results. As previously discussed N. isolate had high pH-recovery from pH-drops, but low OD_{750}-values (see *Figure 20*), and the high biomass formation indicate good growth and a healthy state.

Table 16. Correlation between biomass concentration (g DW/l) and OD_{750} determined from samples taken at late log phase. Values of biomass concentrations were calculated for the sampling times 117 h and 311 h where lipid-, carbohydrate-, and protein analysis was performed for both experimental runs.

Flue gas experiment 1	Biomass conc. (g DW/l)	OD_{750}	Correlation factor (g DW/l)/OD_{750}	OD_{750} (117 h)	Biomass Calc. g DW/l (117 h)	OD_{750} (311 h)	Biomass Calc. g DW/l (311 h)
C. vulgaris	0.60 ± 0.07	2.74 ± 0.001	0.22	1.01	0.22	1.62	0.36
N. isolate	0.83 ± 0.04	1.79 ± 0.023	0.46	0.65	0.30	0.97	0.45
***S.* simris002**	0.98 ± 0.04	3.28 ± 0.004	0.30	1.46	0.43	2.49	0.74
C. reinhardtii	0.70 ± 0.00	2.96 ± 0.001	0.24	1.63	0.39	2.03	0.48
C. sorokiniana	0.60 ± 0.00	2.50 ± 0.000	0.24	1.34	0.32	1.23	0.30
A. falcatus	0.48 ± 0.04	1.88 ± 0.000	0.25	0.92	0.23	1.06	0.27

From the correlation between OD_{750} and g DW/l the maximum g DW/l at any time for all six cultivations were calculated as higher OD_{750}-values were reached earlier in the cultivation process before the dilutions (see *Table 17*). The values are theoretical, but give an indication of the true values. Two dilutions were made and the highest possible OD_{750}-values were not reached which indicate that even higher theoretical biomass concentrations would be possible. *S.* simris002 had the highest theoretical cell concentration with 1.0 g DW/l closely followed by *C. reinhardtii* with 0.9 g DW/l.

Table 17. Highest obtained g DW/l at any time during Flue gas experiment 1. The values were estimated from the correlation between OD_{750} and g DW/l using the highest OD_{750} obtained. S. simris002 had the highest value of 1.0 g DW/l, closely followed by C. reinhardtii of 0.9 g DW/l.

Species	Correlation factor (g DW/l)/OD_{750}	Highest OD_{750} at any time	Biomass concentration g DW/l highest at any time
C. vulgaris **(260 h)**	0.22	3.0	0.7
N. isolate (356 h)	0.46	1.8	0.8
S. **simris002 (260 h)**	0.30	3.4	1.0
C. reinhardtii **(260 h)**	0.24	3.7	0.9
C. sorokiniana **(260 h)**	0.24	3.0	0.7
A. falcatus **(260 h)**	0.25	2.0	0.5

4.4.4. Microscopic study (during cell count)

As cell count was performed the cultures were studied under a microscope. Some cultures were contaminated whereas others were not. Several species could be seen in some cell cultures. *Table 18* describes the visual observations performed during the cell count.

Table 18. Visual observations were made during cell count. Some cultures were monocultures and others were consortia with several strains. Number of different types of algal cells seen and a description of each is given.

Species	Number of different types of cells observed	Description
C. vulgaris	1	Cells were small, round and green. Clusters present, but mostly single-celled.
N. isolate	3	Some *Scenedesmus* like cells, some small round and green and many large clusters of large green cells. Largest cells of all investigated species.
***S.* simris002**	1	Only *Scenedesmus* cells, single-celled or four and four.
C. reinhardtii	1	Small, round and green cells. Single-celled but also large clusters were common with 20-40 cells in each cluster.
C. sorokiniana	1	Small, round and green cells. Many single-celled. Extremely large clusters existed with over 100 cells per cluster.
A. falcatus	2	Some *Ankistrodesmus* cells were seen, more common at start. Small, round and green cells were taken over both in clusters and single-celled, similar to *Chlorella* species. The cultivation was used for a pH test at 144.5 h from start measured directly inside the E-flask and was most likely contaminated at that time.

More information about the shapes is described in the Theory chapter.

4.4.5. Cell content measurements of lipids, carbohydrates and proteins

The lipid, carbohydrate, and protein contents were investigated. The lipid analysis was unsuccessful as wrong caps were used in GC/MS and the samples had evaporated. The content at 117 h from start is summarized in *Table 19* and 311 h from start in *Figure 23*. Standard curves are presented in the *Appendix I* and *Appendix J*. If some points were completely dissociated from the linear relationship these were skipped, leading to better R^2-value.

The carbohydrate contents were similar between the species at 117 h (around 10-18 % g/g). As for the protein content, the N. isolate had at least a threefold higher content compared to the other species investigated (60 % g/g). Higher cell densities seemed to give the most realistic results and samples at 117 h were not used for lipid estimations.

Table 19. Composition (% g/g) of carbohydrates and proteins in the algae sampled at 117 h for Flue gas experiment 1. The added concentration of $CO_2$was at 117 h around 2.9-3.2 %.

Species, experiment 1 *(117 h from start)*	Carbohydrates (g/g %)	Protein (g/g %)
C. vulgaris	13.3	15.1
N. isolate	9.5	60.4
***S.* simris002**	15.6-16.2 [(1)]	19.9
C. reinhardtii	12.8-18.1 [(1)]	10.3
C. sorokiniana	12.1-14.1 [(1)]	13.4
A. falcatus	10.5	20.9

[(1)] Double values were obtained as two runs were made for these.
Total mean standard deviations on the duplicates: carbohydrates ± 1.7 (g/g %) and proteins ± 6.8 (g/g %)

The lipid content was estimated for the species at the time 311 h from start by using a correlation where the values for proteins (g/g %), carbohydrates (g/g %) and ash weight (approximate value) of 10 (g/g %) [18] were subtracted from 100 % and the rest was assumed to be the proportion of lipids (see *Figure 23*). *C. sorokiniana* had the highest carbohydrate content of 34 % (g/g), N. isolate had the highest protein content of 64 % (g/g) and *C. vulgaris* and *S.* simris002 had the highest estimated lipid content of 52 % (g/g) and 49 % (g/g) respectively. The values are approximate as the corresponding DW to each species and sampling time was calculated based on the correlation factors in *Table 16*.

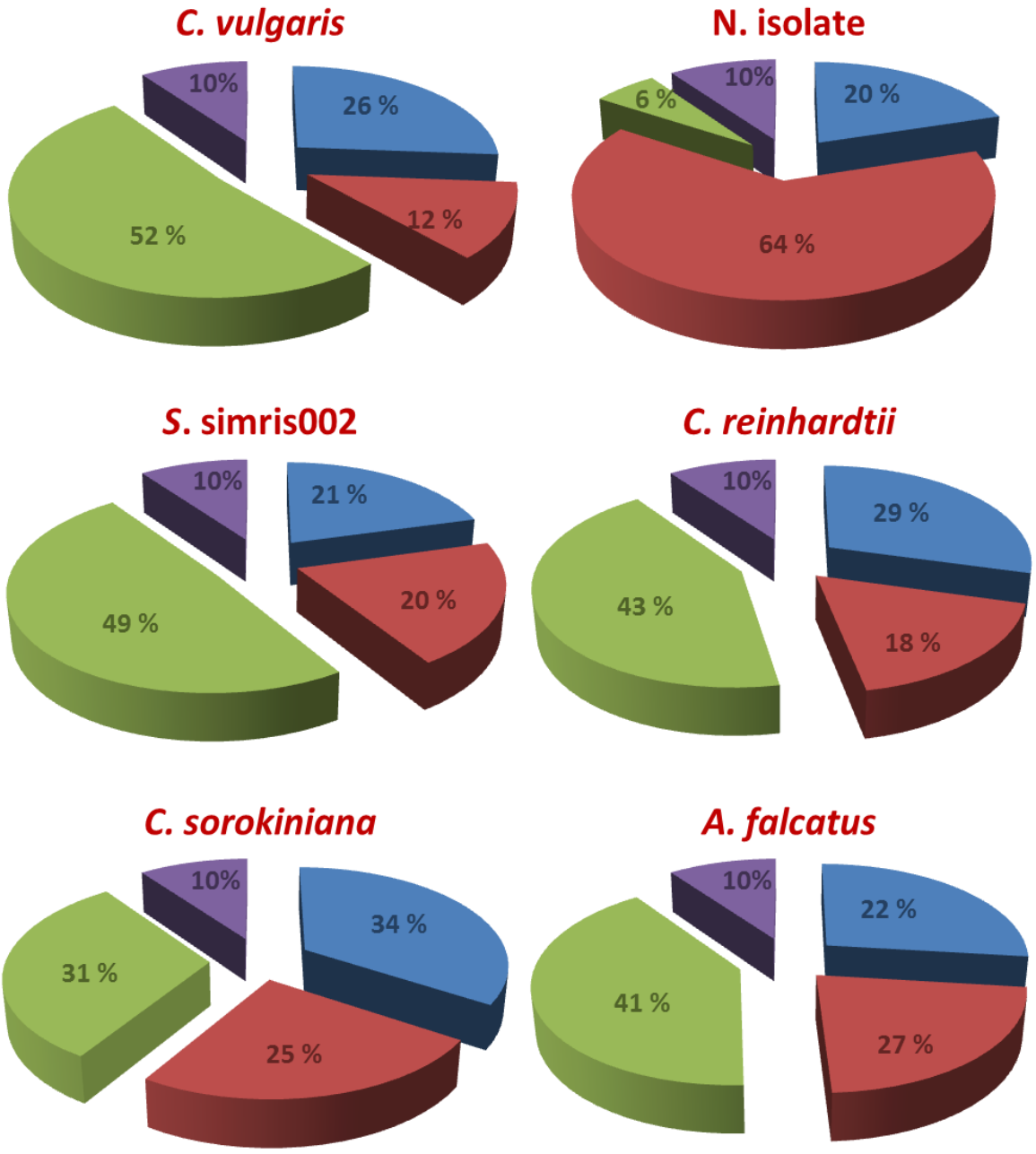

Figure 23. Cell composition of the species from Flue gas experiment 1 at time 311 h from start with ~ 15 % CO_2. Red = Total protein (g/g %), Blue = Total carbohydrates (g/g %), Purple = Assumed ash content (g/g %) and Green = Expected lipid content (g/g %). The lipid content was estimated for each species according to: 100 % - carbohydrates % - proteins % - 10 % assumed ash weight [18]. The total mean standard deviation on the duplicates was for carbohydrates ± 1.7 (g/g %) and proteins ± 6.8 (g/g %).

4.4.6. Growth on surfaces examined for the species in *Flue gas experiment 1*

Some species had a tendency to attach to the glass and grew at the bottom and at the walls of the E-flask, starting at approximately 100 h from start. This ability is not a wanted characteristic for a species for further experiments as less light reaches the algae. The polarity of the membranes can differ between species and growth conditions depending on the amount of produced polar membrane lipids [22]. One can suspect that different membrane polarities play a vital role in why some algae attached to the glass and some did not. Four of six species in *Flue gas experiment 1* showed these properties. *Figure 24* shows the emptied flasks and how they were looking approximately 15 days from start (356 h). *S.* simris002 and *C. vulgaris* were the only two microalgae not attaching to the glass. Several pictures of this phenomenon are shown in *Appendix O*.

Figure 24. Emptied culture flasks after cultivations for 356 h with C. vulgaris (A), N. isolate (B), S. simris002 (C), C. reinhardtii (D), C. sorokiniana (E) and A. falcatus (F).

4.5. Flue gas experiment 2

Flue gas experiment 2 was made similar to *Flue gas experiment 1* but the stepwise increase of the gas components was made faster and with larger increasing steps at the later part of the experiment. Each species has one corresponding color and is the same through this chapter.

4.5.1. Influence of cultivations conditions on pH of the cultivation medium

The pH variations are visualized in *Figure 25* (and the detailed *Table 31* in *Appendix M*). The pH was a function of added gas levels and algal growth and the stepwise increase of CO_2 levels were made depending on the pH. Five species were studied, according to *Figure 14*, and the cultivations with highest pH from the pre-cultures reached the highest starting pH. The difference in the starting pH of the cultivations was conserved throughout the experiment and the lines follow each other in a similar fashion (see *Figure 25*). All five freshwater species survived the stepwise increase of flue gas components and growth occurred for all cultures and they pH-increased after each addition of the components in the flue gas, even though the pH became as low as around 6 in the medium, sometimes even lower.

From *Figure 25* it can be seen that a small added volume of 1 M NaOH at time 44 h increased the pH for the culture *C. emersonii* (the green line), which was done since this culture did not look healthy, the color changed from bright green to yellow. After the base addition, the pH was higher compared to the rest of the strains until the end of the cultivation of this species. The green color was restored after a few days and hence, the pH has large impact on the culture health. The species with highest pH in the medium at the end, except the pH adjusted *C. emersonii*, was *C. protothecoides* (purple line). *B. braunii* was the one with lowest pH in the medium at most of the samplings and was often below pH 6 (blue line).

At the end of the experimental run, there occurred a pH-drop in the medium for all species, after NO (100 ppm) and SO_2 (10 ppm) had been added for approximately 9 days (213 h, from 143 h to 356 h). For *S. obliquus* (red line) this final decrease in pH was most pronounced for all the strains investigated However, the drop was not that large and none of the species had lower pH than 5.9 even after this time. No buffering system was needed to avoid low pH-values if performing a stepwise increase of the flue gas constituents.

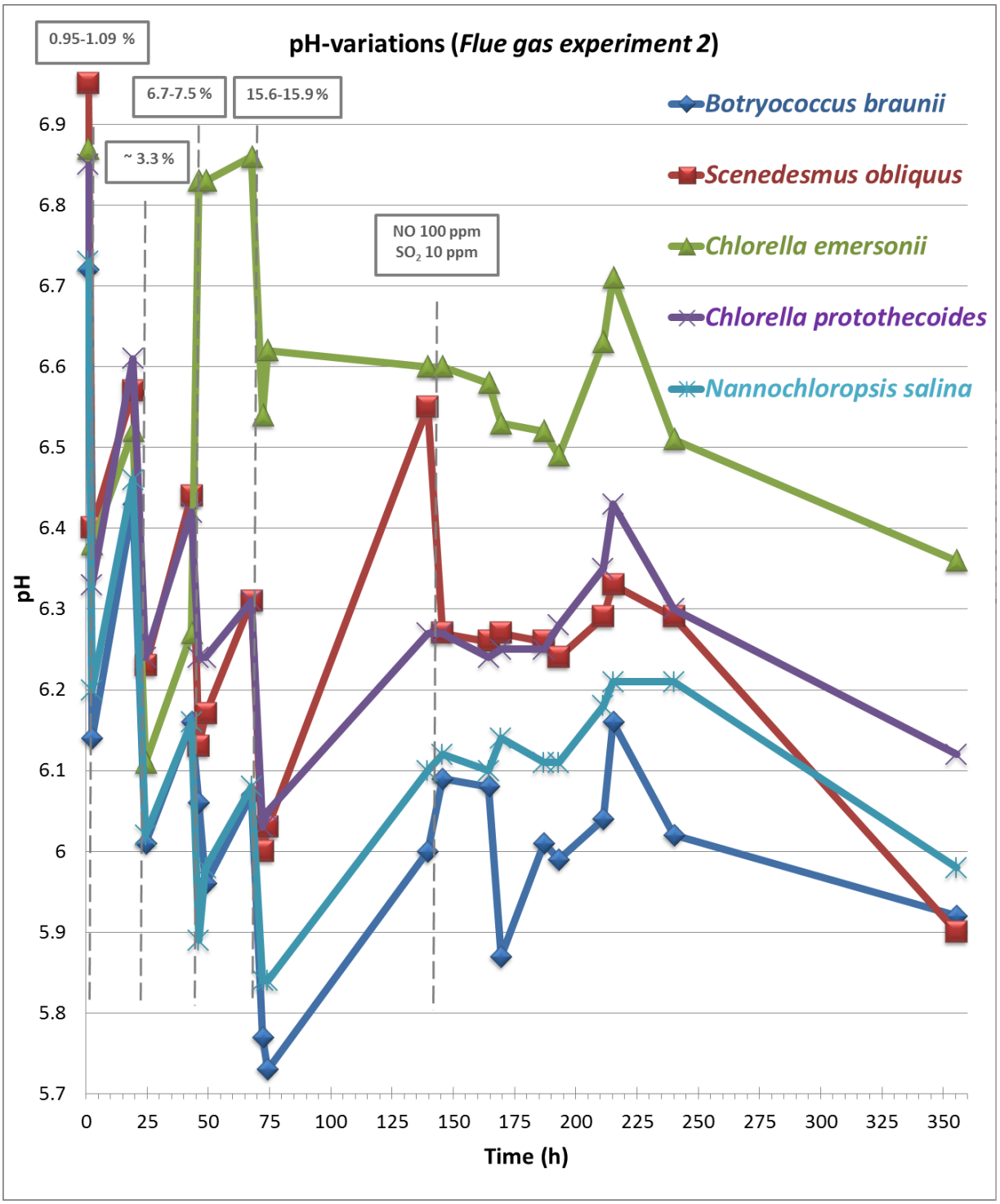

Figure 25. The pH variations in Flue gas experiment 2, in which five species were investigated. The time points for increase in gas components are indicated by dashed lines to the final level of NO (100 ppm) and SO_2 (10 ppm) together with 15 % of CO_2. The total gas flow was kept at 1400 ml/min from start to end of the cultivation. The colored pH-curves correspond to one species each.

4.5.2. Influence of cultivation conditions on the biomass formation and growth properties

Growth was followed by OD_{750} measurements (see *Figure 26*) for *Flue gas experiment 2*. Good growth occurred in all E-flasks and none of the species got inhibited by the flue gas despite the faster addition of the gas levels compared to *Flue gas experiment 1*. The species with highest OD_{750} at any time was *C. protothecoides* (purple line) with a value of 5. The species *N. salina* (light blue line) had low starting OD_{750} but was the one with second highest OD_{750}-value at the end, around 4.25. The starting OD_{750} values were not exactly the same between the species and the difference of the starting concentration affected the continuation of the growth curve. No dilution was performed as the highest gas levels were reached before the stationary phase. The growth curves closer to the end indicate a linear phase and the growth seemed to be limited.

Cell count was performed to follow growth as a complement to the OD_{750}-measurements and in *Flue gas experiment 2* the cultivations were followed by these measurements from start to the end of the run (see *Figure 27*). The numbers of cells were in the same order of magnitude and no large differences between the species could be seen in the growth curves. Larger variations in the data were seen for the cell counts compared to the OD_{750}-measurements. The species with highest reached number of cells per ml was *C. protothecoides* (purple line), closely followed by *N. salina* (light blue line). In both of the plots, OD_{750} and cell count, the cultivation of *S. obliquus* indicate smallest growth at the end and as previously mentioned the pH dropped at the end of the cultivation (see *Figure 25*) for this species. It started to grow slowly and the CO_2 then lowered the pH.

The growth curves in both *Figure 26* and *Figure 27* were similar to each other and *B. braunii* (dark blue line) was selected to visualize how the specific growth rates and generation times were obtained (see *Figure 28*). The OD_{750}-measurements and cell count numbers were plotted with logarithmic values against time to identify phases with exponential growth. Two phases were observed, one *start phase* (at 3.3-15 % CO_2) and one *end phase* (at 15 % CO_2, 100 ppm NO and 10 ppm SO_2). *Flue gas experiment 2* did not have a dilution step and harvesting of cells, *i.e.* no fresh medium was added during the run before the end phase and is not comparable with run 1. The end phase had higher biomass concentration compared to the start phase which could have limited the growth for this phase. The end phase in *Figure 26* indicated linear phases and that limited growth occurred in the later part of the experiment, thus the start had higher specific growth rates compared to the end of the cultivations. The start phase in *Flue gas experiment 2* is not comparable to *Flue gas experiment 1* as it refers to the increase of CO_2 from 3.3-15% (compared to 0-1.35 %) To see the logarithmic growth of OD_{750} for all species at the end phase see *Figure 39* in *Appendix N*.

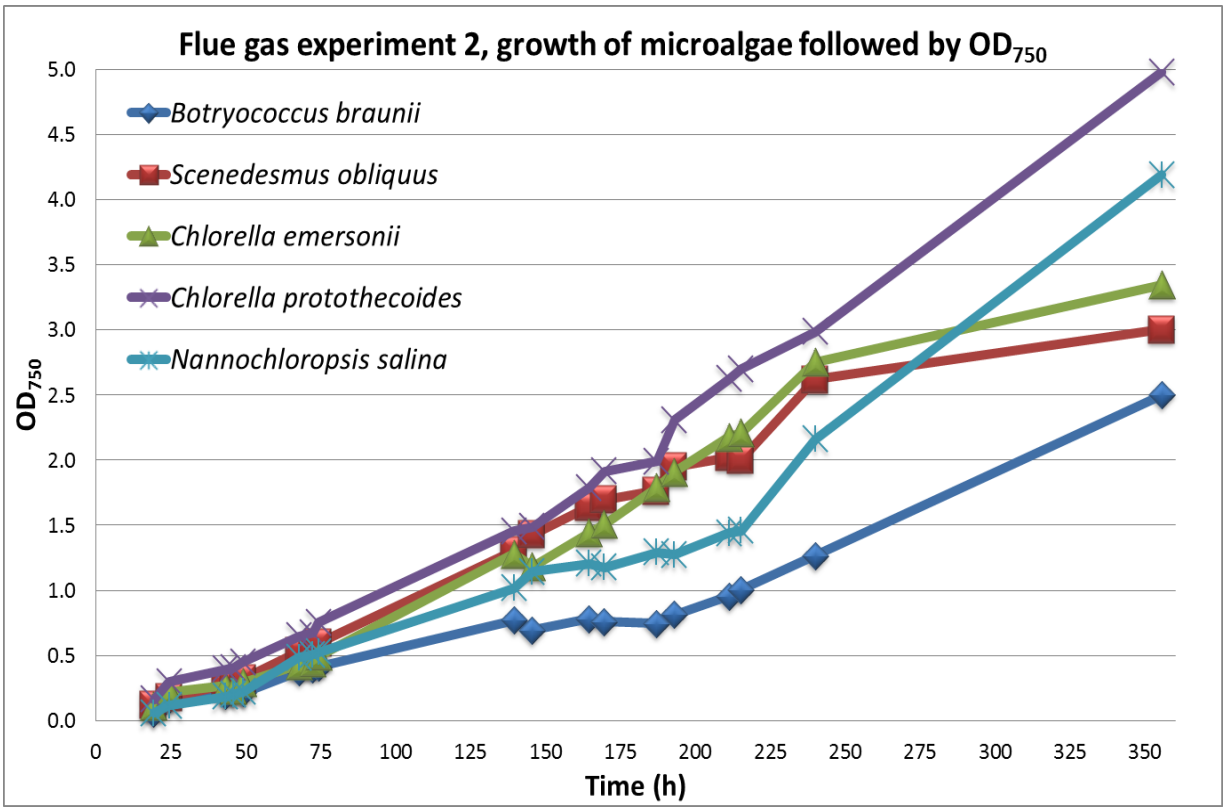

Figure 26. Cell concentration as OD_{750} in Flue gas experiment 2 versus time. Each colored line corresponds to one species each. No dilutions were performed during the experiment. Linear phases can be indicated in the second part of the cultivation.

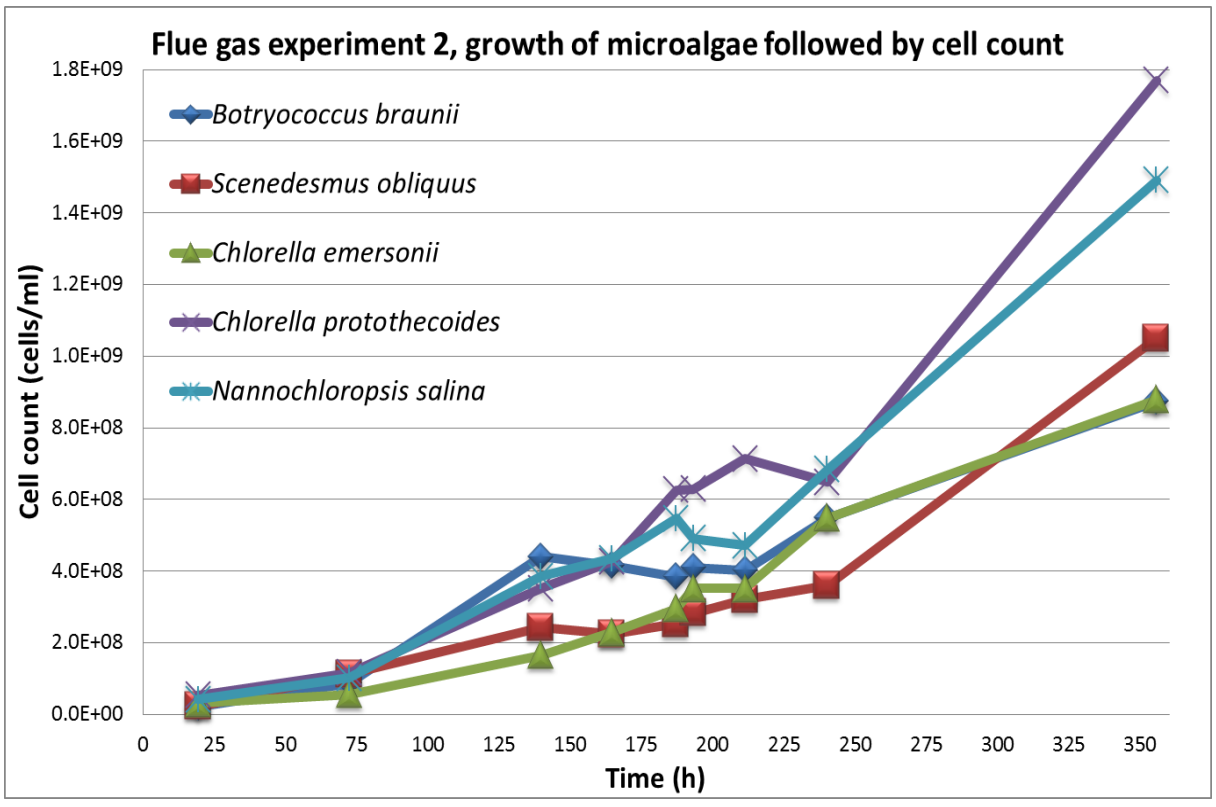

Figure 27. Concentration of cells as cell count (cells/ml) for Flue gas experiment 2 versus time. Each colored line corresponds to one species each. No dilutions were performed during the experiment.

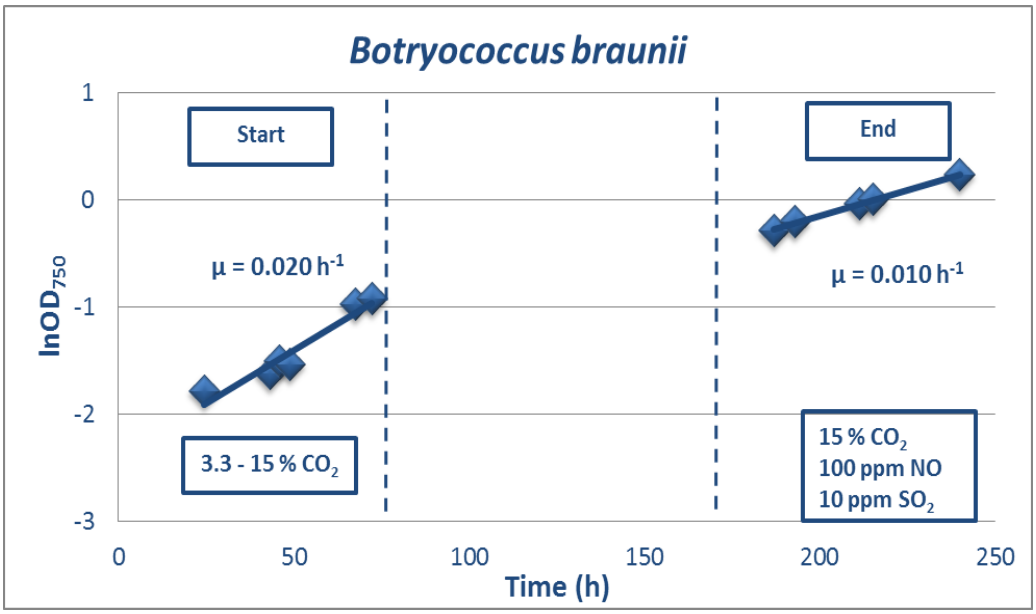

Figure 28. Logarithmic OD_{750}-values versus time for Flue gas experiment 2 at start where CO_2 was increased from 3.3 % to 15 % and at the end where CO_2 (15 %), NO (100 ppm) and SO_2 (10 ppm) was added. The specific growth rate was determined for each species in the same way as in this example both for OD_{750} and cell count measurements.

The growth rates and generation times are presented in *Table 20*. Two specific growth rates were calculated at two different times as previously explained, one closer to the start and one closer to the end. All species had faster growth at start compared to the end of the run. The slower growth could be due to higher biomass concentrations at the end which give *e.g.* light limitations, low pH or simply inhibition by the components in the flue gas.

N. salina was the species with fastest growth obtained from the OD_{750}-measurements and lowest generation time at start, g_{start} = 21 h, where CO_2 was increased from 3.3 % to 15 %. As the start phase in *Flue gas experiment 2* was harsher due to this faster increase of CO_2 compared to *Flue gas experiment* 1 the investigated strain of *N. salina* is a candidate for further experiments. The good quality of being able to live in a broad spectrum of salinities is also desirable. As for cell count *B. braunii* was among the fastest at start with a generation time of 28 h.

Table 20. The specific growth rates and generation times for Flue gas experiment 2 from OD_{750}-measurements and cell count determined for the two illustrated growth phases in Figure 28; start phase (at 3.3-15 % CO_2) and end phase (at 15% CO_2, 10 ppm SO_2 and 100 ppm NO).

Species	*B. braunii*	*S. obliquus*	*C. emersonii*	*C. protothecoides*	*N. salina*
$\mu_{OD\ start}$ (h^{-1})	0.020	0.025	0.016	0.018	0.033
$\mu_{OD\ end}$ (h^{-1})	0.010	0.006	0.008	0.007	0.008
$\mu_{cell\ count\ start}$ (h^{-1})	0.025	0.019	0.014	0.016	0.019
$\mu_{cell\ count\ end}$ (h^{-1})	0.004	0.007	0.008	0.007	0.006
$g_{OD\ start}$ (h)	35	28	43	39	21
$g_{OD\ end}$ (h)	69	116	87	99	87
$g_{cell\ count\ start}$ (h)	28	36	50	43	36
$g_{cell\ count\ end}$ (h)	173	99	87	99	116

For most of the regressions an R^2 coefficient above 0.91 was obtained. No harvesting of cells and no fresh medium was added during the run.

4.5.3. Correlation of biomass concentration to OD_{750}

The OD_{750} was correlated to the biomass concentration for the five species in *Flue gas experiment 2* to be used for estimation of biomass concentrations from OD_{750}-measurements. Values of corresponding DW were calculated for the sampling times where lipid-, carbohydrate-, and protein analyses were performed. A corresponding biomass concentration value (g DW/l) to OD_{750} at the sample occasion was calculated (see *Table 21*) and the samples were taken in log phase (at 68 h) and late log phase (at 140 h). Some species had large correlation factors and *S. obliquus* had as high correlation factor as N. isolate of the previous run. As explained later in *Table 23* the cultivation was contaminated and large clusters were seen, suspiciously similar to the cells seen in the N. isolate cultivation.

Table 21. Correlation between biomass concentration (g DW/l) and OD_{750} determined from samples taken at late log phase. Values of biomass concentrations were calculated for the sampling times 68 h and 140 h where lipid-, carbohydrate-, and protein analysis was performed for both experimental runs.

Flue gas experiment 2	Biomass conc. (g DW/l)	OD_{750}	Correlation factor (g DW/l)/OD_{750}	OD_{750} (68 h)	Biomass Calc. g DW/l (68 h)	OD_{750} (140 h)	Biomass Calc. g DW/l (140 h)
B. braunii	0.33 ± 0.04	0.77 ± 0.020	0.42	0.38	0.16	0.77	0.33
S. obliquus	0.60 ± 0.00	1.31 ± 0.004	0.46	0.53	0.24	1.31	0.60
C. emersonii	0.33 ± 0.04	1.28 ± 0.011	0.25	0.43	0.11	1.28	0.33
C. protothecoides	0.55 ± 0.07	1.45 ± 0.027	0.38	0.65	0.25	1.45	0.55
N. salina	0.43 ± 0.04	1.02 ± 0.012	0.42	0.48	0.20	1.02	0.43

From the correlation between OD_{750} and DW, the maximum g DW/l at any time for all five cultivations were calculated (see *Table 22*) from the higher OD_{750}-values reached later in the cultivation after the correlation was performed. The contaminated mixture (see the microscopic study in *Table 23*) of *C. protothecoides* was the one with highest value of 1.9 g DW/l closely followed by *N. salina* with 1.8 g DW/l. The values were generally higher compared to the values obtained in *Flue gas experiment 1* (compare with *Table 17*), but it should be noted that no dilution was performed in this run and the cultures were allowed to reach even higher OD_{750}-values and also higher biomass concentrations.

Table 22. Estimated highest levels of biomass concentrations (g DW/l) reached at any time during the cultivations of Flue gas experiment 2. The values were estimated from the correlation between OD_{750} and g DW/l from highest found OD_{750} at the end of the cultivations at 356 h.

Species	Correlation factor (g DW/l)/OD_{750}	Highest OD_{750} at any time	Biomass concentration g DW/l highest at any time
B. braunii	0.42	2.5	1.1
S. obliquus	0.46	3.0	1.4
C. emersonii	0.25	3.4	0.9
C. protothecoides	0.38	5.0	1.9
N. salina	0.42	4.2	1.8

4.5.4. Microscopic study (during cell count)

As cell count was performed the cultures were studied under a microscope. Some cultures, as the fast growing *C. protothecoides*, were contaminated and several species were seen, whereas others were not. *Table 23* describes the visual observations in *Flue gas experiment 2*.

Table 23. A microscopic study was performed during cell count and the cultures were examined whether or not they were monocultures or if contamination had led to mixtures. Number of different types of algal cells seen are given and a description of each.

Species	Number of different types of cells observed	Description
B. braunii	1	Very small, round and green cells, both in clusters and single-celled. Clusters with up to 60 cells were common.
S. obliquus	3	Mixture already at start. *Scenedesmus* cells, small round and green cells and large green cells were seen. Small cells similar to *Chlorella* species and large similar to N. isolate.
C. emersonii	1	Small, round and green cells. Clusters with 20 cells were common.
C. protothecoides	2	Mixture already at start with few *Scenedesmus* cells, later on more common. Small, round and green cells forming large clusters but also single-celled.
N. salina	1	Small, round and green. Similar to *B. braunii*. Mostly living in clusters with varying size, from 20-100 cells per cluster.

More information about the shapes is described in the Theory chapter.

4.5.5. Cell content measurements of lipids, carbohydrates and proteins

The lipid-, carbohydrate-, and protein content were investigated for the species in *Flue gas experiment 2*. The lipid analysis was unsuccessful as wrong caps were used in GC/MS and the samples had evaporated. The content at 69 h from start is summarized in *Table 24* and 140 h from start in *Figure* 29. The values are approximate as the corresponding DW to each species and sampling time was calculated based on the correlation factors in *Table 21*. Standard curves used for estimating the values are presented in *Appendix I* and *Appendix J*. If some points were completely dissociated from the linear relationship these were skipped, leading to better R^2-value. Higher cell densities seemed to give the most realistic results and a lipid estimation was not done for samples at 68 h.

Table 24. Composition (% g/g) of carbohydrates and proteins in the algae sampled at 68 h from start for Flue gas experiment 2*. Highest level of CO_2 (15 %) was added at time 69 h from start.*

Species, experiment 2 *(68 h from start)*	Carbohydrates (g/g %)	Protein (g/g %)
B. braunii	9.0	29.0
S. obliquus	17.5	55.1
C. emersonii	69.9	46.5
C. protothecoides	16.6	29.3
N. salina	7.5	25.0

Total mean standard deviations on the duplicates: carbohydrates ± 1.7 (g/g %) and proteins ± 6.8 (g/g %)

The lipid content was estimated for the species at the time 140 h from start by using the correlation where the values for proteins (g/g %), carbohydrates (g/g %) and ash weight of 10 (g/g %) [18] were subtracted from 100 % and the rest was assumed to be the proportion of lipids (see *Figure* 29). *C. emersonii* had the highest carbohydrate content of 45 % (g/g), *N. salina* had the highest protein content of 66 % (g/g) and *B. braunii* and *S. obliquus* had the highest estimated lipid content of 57 % (g/g) and 43 % (g/g) respectively.

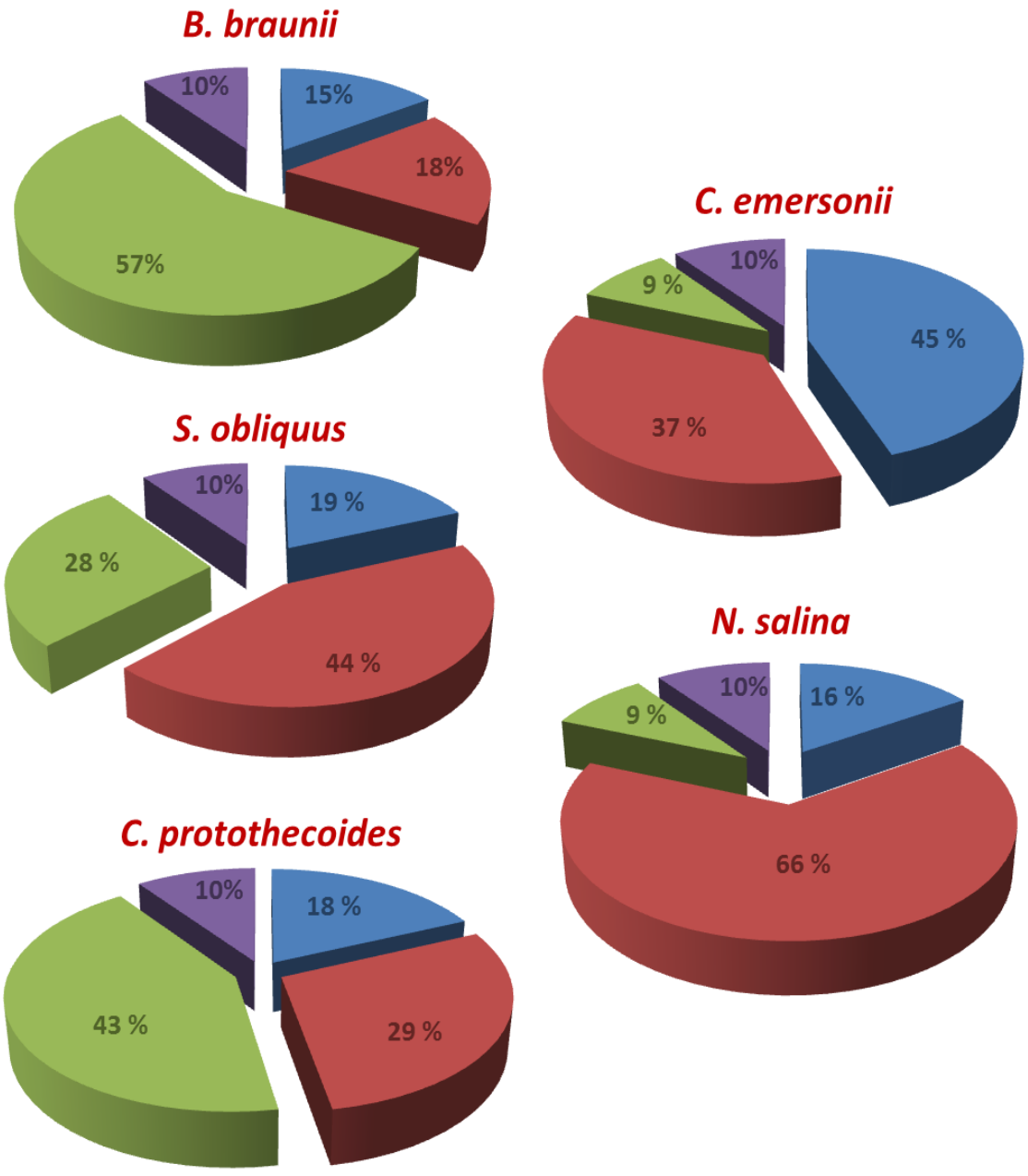

*Figure 29. Cell composition of the species from Flue gas experiment 2 at time 140 h from start with ~ 15 % CO_2. **Red = Total protein (g/g %)**, **Blue = Total carbohydrates (g/g %)**, **Purple = Assumed ash content (g/g %)** and **Green = Expected lipid content (g/g %)**. The lipid content was estimated for each species according to: 100 % - carbohydrates % - proteins % - 10 % assumed ash weight [18]. The total mean standard deviation on the duplicates was for carbohydrates ± 1.7 (g/g %) and proteins ± 6.8 (g/g %).*

4.6. Test of growth in two types of wastewater from *Nordic Paper Bäckhammar AB*

For evaluation of the possbibility to use wastewater from pulp and paper mills as a growth medium growth was followed in a visual study for added algal cells in wastewater *RIA* and *Reactor 2* from *Nordic Paper Bäckhammar AB* (see *Appendix P*). The names refer to two purification steps.

The freshwater medium used in the flue gas experiments was *Bold's Basal medium with threefold of nitrogen and added vitamins*. Calculations show that levels of N and P are high, 124 mg/l and 50.0 mg/l respectively. The N/P quotient is thereby 5:2 (mg/mg). The marine f/2 medium used in the salinity test had 12.4 mg/l of N and 1.1 mg/l of P giving an N/P quotient of ~ 11:1 (mg/mg). According to most of the data about their waste water handed in from the pulp and paper mills the concentrations of N and P are lower than the levels in the freshwater medium and approximately the same as in the marine medium (see *Table 6*). For the actual process water tested the total N concentration in *RIA* and *Reactor 2* were 4.2 mg/l and 13 mg/l respectively, and the total P the concentrations were 0.13 mg/l for *RIA* and 1.8 mg/l for *Reactor 2* (see *Table 10*)

For *RIA* water as growth medium it was found that growth occurred in several flasks within two days. After this time period the growth decreased due to limitations, nutritional or low CO_2 availability in the wastewater. The two *Scenedesmus* species, *S. obliquus* and *S.* simris002, showed highest reached cell densities together with *C. sorokiniana*. *C. reinhardtii*, *A. falcatus*, *C. protothecoides* and *B. braunii* also grew acceptable. The growth of *N. salina* in the wastewaters was not tested.

For water obtained from *Reactor 2* when used as growth medium, growth was difficult to distinguish because of the dark yellow color of the medium as compared to the green of the cells. After several days the green color was seen, and after 390 hours of cultivation some clear results could be seen. *B. braunii*, *S.* simris002, *S. obliquus* and *C. sorokiniana* were the ones with darkest green color. Growth also occurred for *C. reinhardtii*, *A. falcatus*, *C. protothecoides* and N. isolate.

S. obliquus, *S.* simris002 and *C. sorokiniana* indicated good growth for both *RIA* and *Reactor 2* as growth medium and could be alternatives for coupling wastewater and flue gas.

After one week, no growth could be seen in the flasks with only *RIA* and *Reactor 2* without added cells and without autoclaving them. After one month growth could be seen in both flasks as a green color and indicate indigenous species accompanied with the wastewater *RIA* and *Reactor 2*. Pictures of these are shown in *Appendix Q*.

4.7. CHN analysis

The CHN-analysis was performed for all eleven freshwater cultivated species on the remaining cell suspension at the end of the runs. As can be seen in *Table 25* the composition of the eleven freshwater species is similar to each other. For all species the carbon was approximately half of the weight 42-52 % (g/g), the hydrogen was 6.8-7.3 % (g/g) and the nitrogen was 7.1-8.8 % (g/g). *B. braunii* and *S.* simris002 were the species with highest percentage of C, 52 % (g/g). No correlation was found between high N % (g/g) and the high protein content for *N. salina* and N. isolate in the cellular content analysis.

Table 25. CHN-analysis was performed on remaining cultures after Flue gas experiment 1 and Flue gas experiment 2. Different weights of the samples were used due to different obtained dry weights. The cells were washed twice to remove excess salts.

Species	C (g/g %)	H (g/g %)	N (g/g %)	Sample weight (mg)
C. vulgaris	51	7.1	8.8	33
N. isolate	51	7.1	8.4	39
***S.* simris002**	52	7.4	8.6	84
C. reinhardtii	42	7.4	7.1	62
C. sorokiniana	50	6.9	8.6	44
A. falcatus	45	6.8	7.7	14
B. braunii	52	7.3	8.3	53
S. obliquus	47	7.2	7.7	110
C. emersonii	49	7.0	7.5	82
C. protothecoides	48	7.0	7.9	130
N. salina	50	7.0	8.0	92

5. Discussion

The main aim of the study was to investigate growth of microalgae with added artificially produced gas as carbon source mimicking effluents from Swedish paper mills. The wastewater from the Swedish paper mill *Nordic Paper Bäckhammar AB* was used as growth medium in an additional test. Several cultivations of the same species and more samples would have been needed to be able to have statistical safety for the lipids-, protein-, carbohydrate-, pH-, OD_{750}- and CHN-analyses. It is time consuming work and more samples would not have been possible in this project. The screening is anyhow an indication of which species should be used for further experiments with flue gas.

5.1. Flue gas as carbon source and specific growth rates

The primary challenge in this study was to reach the highest levels of CO_2, NO and SO_2, as they were stepwise added or increased to avoid pH drops, before high cell densities and the stationary phase were reached. Determination of the maximum specific growth rates in the exponential phase with highest levels of the gas constituents added was therefore hard and none of the two experiments succeeded to visualize the highest possible growth at highest levels of the gas constituents added. The microalgae grew too fast at start, a positive problem. The end phase is not comparable between the species as different biomass concentrations were reached at different times and fast growing species reached high concentrations earlier than the others. The specific growth rates were lower for all species closer to the end of the cultivation compared to the start; hence some factor or factors were inhibiting or limiting growth, *Table 15* and *Table 20*. If the reason was due to toxicity of the flue gas, low pH, or if other parameters were limiting such as too high reached cell densities or low light availability is hard to know. The specific growth rates were also generally higher at the end of experiment 1 compared to experiment 2 and a probable reason was the dilutions performed in the first run to lower cell densities and thus the cells could grow faster.

Too high cell densities slowed down growth, which means that faster growth were possible at higher concentrations. A probable reason for slow growth could be light limitation at higher cell densities. The precaution from the test runs to decrease the light intensity was unnecessary as it rather affected the growth negatively (see *Appendices E-G*). At start with low cell densities the light was probably enough to satisfy all cells [30] and the photosynthetic efficiency was proportional to the light intensity [26]. At higher cell densities the cells started to shade each other, the penetration depth decreased and the same number of photons was shared by more cells giving a *linear growth phase* proportional to the added light [30]. In *Figure 20* and *Figure 26* there are indications of linear phases and according to Lee & Shen [30] the light could be the limiting factor. The light intensity during one year in Borås [36] (see *Figure 1*) was ~ 10-225 W/m^2 and except during winter the light intensity is higher compared to the level of artificial light used in this study, 3-13 W/m^2 depending on the direction. *Flue gas experiment 1* showed that the cultures standing at each end of the row of the six cultivations, *C. vulgaris* and *A. falcatus*, grew the slowest (see *Table 15*). The light intensity was lower at the ends of the row of flasks, 8.5 W/m^2 compared to 13 W/m^2 in the middle pointing towards the lamp, and this fact strengthens the hypothesis that the light became limiting. Regarding the L-D frequency only a short time in the light would be enough to satisfy the energy demand for the cells [26]. Mixing enhances the L-D frequency and biomass formation [25]. But the mixing was probably sufficient due to the vigorously bubbling of gas and thus the limiting light could possibly not be compensated for by enhancing the L-D frequency, only by increasing the light intensity.

Kong et al. [19] reported in their study a specific growth rate of 0.024 h^{-1} for *C. reinhardtii* cultivated using flue gas with 33 % CO_2 in a biocoil. This indicates that fast growth can be achieved at the highest levels of the constituents in the flue gas and the growth rate reported is in the same range as

obtained in this project for *C. reinhardtii*, 0.007 - 0.042 h^{-1}. A similar study by Borkenstein et al. [16] with *C. emersonii* and real flue gas burned from stone coal at a cement plant (15 % CO_2) reached a specific growth rate of 0.005 h^{-1}, compared to 0.008-0.016 h^{-1} for the strain in this study. Another study claims that growth rates of 0.29 h^{-1} can be achieved by microalgae at optimal conditions (a generation time of 2.4 h), and indicates that higher specific growth rates are achievable also with flue gas as carbon source [15]. As almost all of the species had high reported lipid content in the literature [23] and lipid accumulation is connected to slow growth [21] there would definitely be species growing faster compared to the ones investigated in this study, but without containing the desirable lipids.

The cultivations of *C. reinhardtii*, *S.* simris002, *S. obliquus*, *C. protothecoides* and *N. salina* resulted in high levels of estimated biomass concentrations up to 1.9 g DW/l (see *Table 17*). Borkenstein et al. [16] reached biomass concentrations of 2-2.1 g DW/l with *C. emersonii* which is similar to the obtained biomass concentrations in this study. However another study with *C. reinhardtii* by Kong et al. [19] reached 2.0 g DW l^{-1} day^{-1} in a biocoil and indicates that even higher productivity is possible. Cultures of *S. obliquus* and *C. protothecoides* were mixtures of several species and good growth with added flue gas was seen for these. It could be so that a consortia of species make it possible to reach even higher cell densities than for monocultures and thus cultivation of consortia could be attractive alternatives for capturing CO_2 in the flue gas. The drawback would be that all species need to have the wanted characteristics as *e.g.* the correct lipid profile [53].

However, despite the slower growth rates at higher levels of the gas constituents, high biomass concentrations were obtained for all cultivations done in this project, *Table 17* and *Table 22*, thus, using cultivation of freshwater species as a mean to clean flue gas from CO_2 would be a possible alternative. From our experiment, it is suggested that *S.* simris002, *C. reinhardtii* or *N. salina* could be used for such purpose due to high obtained biomass concentration, pH-tolerance and high specific growth rate.

At large scale, the algae would not be able to utilize a satisfyingly large part of the high concentrations of CO_2 to make it possible to release the gas directly and re-circulation would be needed. To dilute the flue gas with air could be a suitable alternative as the specific growth rates were higher for all species at low concentrations of CO_2 and the algae could then fix a higher fraction of the added CO_2. A small increase of CO_2 (~ 1.35 %) made the algae grow fast with a generation time for *C. reinhardtii* as low as 17 h without optimizing other parameters important for growth [10]. Too high concentrations of the gas constituents led to pH problems, seen in *Test run1-3*, and could be avoided by diluting the gas to lower concentrations. It could also be motivated to increase the contact time between CO_2 and the algae, achieved in *e.g.* a long vertical column [10], and adapting the cells [3] to high gas concentrations by transferring cells from an already started cultivation with flue gas to a new one leading to a better growth and shorter lag phase. The E-flasks were not suitable for the cultivations as they are broadest at the bottom and the height of the cell suspension liquid was small; hence a small exposure and contact time between the gas and the algae occurred. It is important to enhance the injection system [10] and by a high column with increased contact time and with diluted gas the cleaned gas could possibly be released directly after leaving the reactor.

Real exhaust gas from the industry contains more compounds than the artificially produced gas and *e.g.* carbon monoxide (CO) was constantly appearing in the reported data of emissions obtained from the pulp and paper mills (see *Table 5*). It was not possible to investigate the effect of CO due to restrictions in handling this gas because its toxicity. Low solubility in water has been reported for CO and would thus not disturb algal growth [48]. This theory was strengthened by contact with Professor

Kirsten Heimann [44] who verified that CO would not be harmful to the algae [48]. The study with *C. emersonii* by Borkenstein et al. [16] concluded that real exhaust gas from a cement plant with burned stone coal had no adverse effects on growth compared to the control grown in pure CO_2.

The next step would be to combine two waste streams; flue gas as carbon source and wastewater as growth medium. When testing the wastewaters, *RIA* and *Reactor 2*, as medium growth of the algae stopped after only one-two days for *RIA* and after a few days more for *Reactor 2*, but growth was clearly limited in both wastewaters. The reason was probably low CO_2 availability and low nutrient levels, especially the phosphorous levels of 0.13 mg/l (*RIA*) and 1.8 mg/l (*Reactor 2*). Too low nutrient levels could be a problem and it could be motivated to add the lacking components. The NO in the flue gas can be dissolved to yield NO_2 which could be used as nitrogen source for the algae [10]. The critical parameter would therefore be the phosphorous content and as *Reactor 2* contained a tenfold higher total phosphorous levels compared to *RIA* it would be interesting to try this water coupled with flue gas. The summary of the wastewaters from different purification steps and different Swedish paper mills (see *Table 6*) also indicates that higher levels of both nitrogen and phosphorous is achievable.

5.2. pH in microalgal cultivations with flue gas

The simulated flue gas constituted of CO_2, NO, and SO_2 and all form acids when dissolved in water. The two last mentioned form strong acids, and are potential problems when cultivating algae with flue gas [27]. Oilgae [10] indicates that SO_2 could be a long-term problem due to higher solubility compared to CO_2 and NO [10] (see *Table 2*). The low concentration of 10 ppm of SO_2 added at the end indicates that it could take days or even weeks until the full saturation value would be reached. In our experiment, the pH decreased at the end as also NO (100 ppm) and SO_2 (10 ppm) had been included in the inflow of gas (see *Figure 19* and *Figure 25*). However, *Flue gas experiment 2* showed that by stepwise increasing CO_2 during 6 days and include NO and SO_2 and then after further 9 days of cultivation none of the five freshwater cultivations had pH below 5.9 and hence SO_2 would therefore not give pH-problems in the long run. The flue gas could rather be used to lower the pH by bubbling it through as too high pH very often is a problem in algal cultivations [19].

A connection between fast growth and recovery from pH drops were seen and most freshwater species recovered quickly after each increase of the inlet gas, *Figure 19* and *Figure 25*. *Test run 2-3* indicate fast growth at start with CO_2 (7.5 %), NO (100 ppm) and SO_2 (10 ppm) added, but stopped or decreased within 2 days for all species as pH became too low (pH < 3). The rapid acid formation was probably too harsh as fewer cells could fix CO_2 or HCO_3^- and increase the pH [34]. *S.* simris002 was the only species of the six surviving the low pH and still indications of growth were seen, *Figure 30* and *Table 29*.

There are alternatives to avoid extreme pH values at large scale cultivations. A pH regulation mechanisms can work, such as coupling inlet gas to a pH control and add gas when pH become too high [19], as Borkenstein et al. [16]. Buffered systems would be expensive as buffers need to be added in large amounts to the system at large scale, but if used, higher concentrations of flue gas could probably be added since too low pH is prevented by the buffering capacity. Low pH could be avoided by stepwise adding the flue gas, as successfully shown in this project. However, at lower pH the microalgae grew slowly, if anything at all. Faster growth could generally be achieved at the optimum pH [10], which is for freshwater green microalgae often somewhat higher at 7-9 [46] as compared to the levels seen in this project at 6.0-7.0. Optimum pH for *e.g. C. reinhardtii* is at 7.5 [19] and the cultivation was mostly around pH 6.5. Optimum growth could compensate for the cost of a buffered system, but there exist definitely suitable freshwater species with optimum growth around pH 6.

An interesting parameter affecting the solubility of the gas and thereby the pH would be the temperature, the solubility decreases with decreasing temperature [10]. A colder growth medium could thereby be a possibility to avoid low pH problems, but a colder environment would probably slow down growth [10] for most of the selected species. However, the Swedish climate would fit the lower temperature during a large part of the year.

5.3. Lipids, carbohydrates and proteins

The carbohydrate analyses implied that two species contained the highest levels; *C. emersonii* (~ 44 g/g %) and *C. sorokiniana* (~ 31-36 g/g %), and the type of carbohydrates is needed to be determined in future studies [10]. These two species are interesting as they contain rather high levels of lipids, 22 % (g/g) and 63 % (g/g) respectively, according to literature values [23] and as well high levels of carbohydrates shown in this study. Of course it is desirable to be able to utilize all parts of the biomass for highest profitability, the lipids could be extracted for biodiesel and the carbohydrates could be used for bioethanol fermentation [10]. Therefore the bioethanol and biodiesel possibilities must be taken in consideration when choosing the algal species for the purpose of cleaning flue gas from CO_2 as the biomass must be used for something beneficial and profitable. These characteristics should primarily be tested thoroughly for the previously selected species for further studies; *S.* simris002, *C. reinhardtii* and *N. salina*. However, to make the technique profitable there are challenges remaining to investigate, as dewatering and extraction of the oil through the tough cell wall still costs too much energy and money [53].

The levels of expected lipids showed that *B. braunii* together with *C. vulgaris*, *S.* simris002 and *C. protothecoides* would have approximately 50 % (g/g) lipids, which corresponded to the high literature values [23], in the phase closer to the end of cultivation. However, the total lipid content could definitely be higher if some kind of stress was applied on the cells, *e.g.* nitrogen starvation [22] or high light intensities [23], which start the lipid accumulation for storage. At such conditions of lipids and some species can accumulate up to 80 % lipids of the dry weight [15]. The lipid estimation values are approximate and are based on the values for carbohydrates and proteins in % (g/g) and the assumption that the dry weight matter only contained lipids, proteins, carbohydrates and an assumed ash content % (g/g) [18]. The lipid estimations are very uncertain due to these assumptions and estimations. More experiments and more samples would be needed for statistical safety and no conclusions can be drawn from these experiments, they are indications. The lipid composition is also of interest to investigate to know whether or not the lipids are suitable for biodiesel as many microalgae have desirable lipid profiles suitable for biodiesel production [53] with high oxidation stability [23].

6. Conclusions

Many species, both marine and freshwater species, can live and thrive in different salinities ranging from freshwater (~ 0‰) to marine water (~ 25‰) and in this study it was seen that the marine *N. salina* could live in fresh-, brackish-, and marine medium.

The absorbance is generally lower when performing OD_{750}-measurements of freshwater green microalgae compared to OD_{550}-measurements. OD_{750}-measurements can be used as an appropriate indirect cell concentration measurement of suspensions containing microalgae if coupled to a direct biomass as *e.g.* dry weight. However, OD_{750}-values are not comparable between species and strains and not even between growth phases. The large colonial forming species gave high biomass concentrations and low OD_{750}-values compared to other investigated species. More dry weight measurements are needed to be performed at different times in log phase to get a biomass/OD correlation.

Flue gas from paper mills could possibly provide the carbon source for several freshwater microalgae. All freshwater species investigated in this project could live in these environments with high levels of CO_2 (15 %), NO (100 ppm) and SO_2 (10 ppm) in the flue gas, but slower growth was seen at these high levels for all species. The pH is strongly dependent on concentrations of CO_2, NO and SO_2 and algal growth. The gas constituents lower the pH through acid formation and growth increases the pH through CO_2 and HCO_3^- utilization. Flue gas can thereby be used to prevent too high pH in the cultivations. Low pH (~ < 5.5-6) slow down growth and too low pH kill the microalgae (pH <3). By stepwise increasing the concentration of the gas constituents, and thereby avoiding pH drops, the microalgae can grow and thrive in combination with flue gas from paper mills. The process needs to be optimized by several factors such as optimal light, agitation, temperature, pH and contact time between gas and liquid.

For further experiments *S.* simris002, *C. reinhardtii* and *N. salina* are obvious choices due to fast growth, good tolerance to pH-changes and recovery from pH-drops, and high cell density obtained. *S.* simris002 showed an ability to tolerate low pH and still be able to grow at which others could not. *N. salina* showed the highest theoretical biomass concentration and a quality of growing at several salinities. N. isolate and *C. sorokiniana* are also possible alternatives due to fast growth and good pH-recovery at pH drops and the last mentioned could be interesting from a bioethanol perspective as it had high carbohydrate content. *C. protothecoides* and *S. obliquus* were contaminated with other microalgae giving a consortia and impressed with high cell densities and using such consortia in the cultivation could be alternatives as a CO_2 capture process. To further be able to produce chemical compounds or energy from the biomass of these species a more detailed cell composition analysis need to be made as *e.g.* investigating the lipid profiles and kind of carbohydrates existing.

Wastewaters from paper mills could be possible growth media for microalgae. The nitrogen and phosphorous levels are critical parameters and needed as nutrient for growth. However, the levels of these elements were low in *RIA* and *Reactor 2* water tested, but other types of wastewaters from other paper mills or purification steps contain higher levels of these nutrients. Next step would be to combine wastewater as growth medium with nitrogen and phosphorous and flue gas as carbon source. Possible candidates of algae with desirable characteristics in both flue gas and wastewater were *S.* simris002, *S. obliquus, C. protothecoides, C. sorokiniana* and *C. reinhardtii* and these could be used for further coupled experiments.

7. Outlook

The future for using microalgae to fixate CO_2 from waste streams looks bright, but some problems need to be solved and more research is needed on this area. Further studies should cultivate the algae at lower temperatures to see if the pH problem becomes smaller since at lower temperatures the solubility of the added flue gas is lower [10]. Real exhaust gas as carbon source with potential inhibitors such as carbon monoxide should be examined. Dry weights should be performed regularly, each day, to calculate the carbon dioxide fixation rate per day due to biomass formation.

A whole project should focus on the total lipids per dry weight that could possibly be produced for the eleven freshwater species in this study. A nitrogen starvation could be applied on the cells to enhance the lipid accumulation [22]. The lipid composition would also be of interest to investigate to see if the algae have suitable lipid profiles for biodiesel production [53].

The capacity of reducing nitrogen and phosphorous in different wastewaters, with known levels, should be followed each day in algae cultivations to investigate how fast the levels decrease. A comparison between the species should be done to distinguish the cultivations with highest N and P reduction rates and thus the best suitable species. The wastewaters could be possible growth media and could be coupled to flue gas in an algae cultivation system. A relatively simple experiment for further studies at *SP – Technical Research Institute of Sweden* could be to replace Bold's Basal medium with wastewaters obtained from Swedish paper mills and investigate the biomass formation capacity.

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Appendix A – Species and biodiversity

The biodiversity of photosynthetic microorganisms need to be clarified. Microalgae are usually separated due to their different light-harvesting pigments: *Rhodophyta* [3] are red algae, *Bacillariophyta* [68] are diatoms, *Chrysophyta* [7] are golden algae, *Phaeophyta* [11] are brown algae, *Chlorophyta* or *Streptophyta* [5] are green algae and *Phaeophyceae* [3] are brown algae. *Cyanophyta* are referred to as blue-green algae, or cyanobacteria, and are strictly referred to as prokaryotes due to the lack of membrane-enclosed structures such as nucleus, mitochondria and chloroplasts [12]. Cyanobacteria contain *chlorophyll a* and indicate green color, but more or less all species of *cyanophyta* can form the blue pigment phycocyanin and the red pigment phycoerythrin. However, the cyanobacteria are the main nitrogen-fixating organisms and this role is crucial for life on earth [8]. *Rhodophyta* are red algae and almost all are marine species, only 3 % of the species live in freshwater habitats [13]. Sheats & Sherwood [13] claim that almost all freshwater red algae are macroscopic and benthic. The freshwater variants also exist in marine habitats and compared to these the size ranges of freshwater species are smaller, 80 % of the length range of marine equivalent. Diatoms, *Bacillariophyta*, have golden-brownish chloroplasts due to various pigments, mainly fucoxanthin, and require silica for growth [68]. Silica is crucial for diatoms as it is part of the cell membrane in a polymerized form, hence without silica no new cells can be formed [8]. The *Chrysophyta*, golden algae, are similar to diatoms but have a complex set of pigment, mainly fucoxanthin, in the chloroplasts [7]. Kristiansen & Preisig [7] explain that fucoxanthin is golden-brownish and masks the green chlorophyll color. There exist around 1000 species of golden algae. Golden algae can produce oils and carbohydrates for storage purposes [8]. The brown algae, *Phaeophyta,* are characterized by brownish chloroplasts, one or more per cells, also due to fucoxanthin [11]. Wehr [11] claims that there exist few numbers of brown algae in freshwater habitats and the ones that do exist are smaller in size. The green microalgae are divided in two evolutionary groups, namely *Chlorophyta* and *Streptophyta* [5]. These are the evolutionary progenitors of the terrestrial flora [8] and the *Streptophyta* include the terrestrial plants together with some green algae [5]. John [5] explains that all remaining classes of green microalgae, *Chlorophyceae*, *Trebouxiophyceae* and *Ulvophyceae*, belongs to *Chlorophyta*. They are mostly found in freshwater systems as colonies or single-celled [8]. Green algae store mainly starch compounds and lipids at certain circumstances [8].

Appendix B – Hydrogen gas production

There are three major ways of producing H_2 from algae; a biochemical process, gasification and producing H_2 from methane [10].

Biochemical process

A biochemical process is possible where algae produce H_2 by enzymatic activity and enzymes are catalysts for cleavage of H_2O molecules [10]. The photosynthesis is divided into the *light reaction* and the *dark reaction*, previously described in *Reaction 3* [33] and *Reaction 4* [33]. During specific conditions some algae produce H_2 by the help of the enzyme *hydrogenase* [52], involving metal-containing active sites [35]. Green algae use hydrogenases in a light driven reaction to produce H_2 [35]. CO_2 is not reduced and instead H_2 is produced as the hydrogenase combines electrons from the photosynthetic electron transport chain with protons [10]. Hydrogenase is encoded by a gene existing in the nucleus but the actual protein functions and works in the stroma in the chloroplasts [52].

O_2 inhibits the hydrogenases, hence anaerobic conditions are strictly necessary [52]. The hydrogenase is a defense mechanism to make the algae survive in anaerobic conditions [10]. O_2 production can be switched off when culturing algae if depleting the sulfur [52] as sulfur depletion prevents the internal oxygen flow, hence the hydrogenase can function unhindered [10]. Sulfur absence works as a shift from normal photosynthesis where oxidation of H_2O produces O_2 and biomass to a metabolic change where O_2 is not evolved [52] and enables the possibility to produce large amounts of H_2 [52].

The *Reaction 10* illustrates formation of H_2 where X is the electron carrier ferredoxin [10]. Ferredoxin functions as electron carrier and donor to Fe-hydrogenase and connects the hydrogenase to the electron transport chain existing in the chloroplasts [52]. Water is the electron donor from the photochemical reaction and hence microalgae are water-splitting microorganisms. Oilgae [10] describes several methods of optimizing the H_2 production, one of them includes addition of Cu. Briefly described Cu turns off a piece of a chloroplast gene needed for *PSII* where O_2 is produced [33]. An anaerobic state starts hydrogenase production and thereby H_2.

Reaction 10 – Formation of hydrogen gas by enzymatic activity

$$\mathbf{2H^+ + 2X_{reduced} \rightarrow 6H_2 + 2X_{oxidized}}$$

Gasification

Another way of producing H_2 is through gasification [10]. Gasifying algal biomass by using steam, heat, high pressure and specific quantities of O_2 the biomass is broken down chemically to syngas, a mixture of CO, CO_2 and H_2. CO is further reacting with H_2O to form more H_2 and CO_2. As syngas is produced one of the key challenges is to separate the H_2 from the syngas and many techniques are being investigated for this purpose. This way of producing H_2 is most likely the most cost-effective way at large scale. Another challenge is to earn the large quantities of biomass required [10].

Reaction 11 – Formation of hydrogen gas by gasifying biomass

$$\mathbf{CO + H_2O \rightarrow CO_2 + H_2}$$

Hydrogen gas from methane

If fermenting biomass from algae methane is produced [10]. Traditional steam reformation methods could be an alternative way of producing H_2 from methane. High temperatures are needed and a metal based catalyst, *e.g.* nickel, and water vapor reacts with CH_4 to produce H_2 and CO, illustrated in *Reaction 12* [10]. The formed CO can react with H_2O as *Reaction 11* illustrates [10]:

Reaction 12 – Formation of hydrogen gas by fermenting biomass

$$\mathbf{CH_4 + H_2O \rightarrow CO + 3H_2}$$

Appendix C – Freshwater and brackish water media recipes

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 liter with distilled water, autoclave, and store in the fridge.

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.

f/2 medium

Solution 1 (Dilute to 1 liter, autoclave, and store in the fridge)

75 g	$NaNO_3$
5 g	$NaH_2PO_4 \cdot H_2O$

Trace metal solution (Dilute to 1 liter, autoclave, and store in the fridge)

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	$Na_2MoO_4 \cdot 2H_2O$

Vitamins (Dilute to 100 ml each, sterile filter and store in freezer)
0.1 g Thiamine·HCl (vit B_1).
0.01 g Biotine (vit H) + 0.01 g Cyanocobalamin (vit B_{12}).

To roughly 1 liter filtered and autoclaved/filter sterilized sea water/ artificial sea water add:
1 ml solution 1 + 1 ml trace metal solution + 200 µl vit B_1+ 10 µl vit H + vit B_{12}

Appendix D – Nitrogen and phosphorous content in 3N-BBM+V and f/2

The nitrogen and phosphorous content and N/P quotient were calculated for the two culture media.

Freshwater medium (3N-BBM+V)

The only source of N is the $NaNO_3$.

Mole fraction of N: $\frac{M_N}{M_{NaNO_3}} = \frac{14.01}{(22.99 + 14.01 + 3 \cdot 15.99)} = 0.1648$

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

$$C_{N\ (Stock\ solution)} = \frac{M_N}{M_{NaNO_3}} \cdot C_{NaNO_3} = 0.1648 \cdot 25000 = 4121 \text{ mg/l}$$

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

$$C_{N\ (Final)} = \frac{C_N \cdot V_{Stock}}{V_{Final}} = \frac{4121 \cdot 0.03}{1} \approx \mathbf{124\ mg/l}$$

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:

$$C_{P_1\ (Stock\ solution)} = \frac{M_P}{M_{K_2HPO_4}} \cdot C_{K_2HPO_4} = \frac{30.97}{(2 \cdot 39.10 + 1.008 + 30.97 + 4 \cdot 16.00)} \cdot 5713 = 1016 \text{ mg/l}$$

$$\text{And:} \quad C_{P_2\ (Stock\ solution)} = \frac{M_P}{M_{KH_2PO_4}} \cdot C_{KH_2PO_4} = 3983 \text{ mg/l}$$

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

$$C_{P\ (Final)} = \frac{C_{P1\ (Stock\ solution)} \cdot V_{Stock}}{V_{Final}} + \frac{C_{P2\ (Stock\ solution)} \cdot V_{Stock}}{V_{Final}} = \frac{1016 \cdot 0.01}{1} + \frac{3983 \cdot 0.01}{1} \approx \mathbf{50.0\ mg/l}$$

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

Marine water medium (f/2)

Mole fraction of N: $\frac{M_N}{M_{NaNO_3}} = \frac{14.01}{(22.99 + 14.01 + 3 \times 15.99)} = \frac{14.01}{84.98} = 0.1648$

Solution 1 has 75 g/l $NaNO_3$), hence N: $C_{N_{sol.1}} = \frac{M_N}{M_{NaNO_3}} \cdot C_{NaNO_3} = 0.1648 \cdot 75000 = 12360$ mg/l

1 ml of solution up to 1 liter medium: $C_{N_{f/2}} = \frac{C_{N_{sol.1}} \cdot V_{sol.1}}{V_{f/2}} = \frac{12360 \cdot 0.001}{1} \approx \mathbf{12.4\ mg/l}$

Mole fraction of P: $\frac{M_P}{M_{NaH_2PO_4 \cdot H_2O}} = \frac{30.97}{(22.99 + 4 \cdot 1.008 + 30.97 + 5 \cdot 15.99)} = 0.225$

5 g/l $NaH_2PO_4H_2O$, hence P: $C_{P_{sol.1}} = \frac{M_P}{M_{NaH_2PO_4 \cdot H_2O}} \times C_{NaH_2PO_4 \cdot H_2O} = 0.225 \times 5000 = 1120$ mg/l

1 liter of medium, 1 ml of solution 1 is used: $C_{P_{f/2}} = \frac{C_{P_{sol.1}} \times V_{sol.1}}{V_{f/2}} = \frac{1120 \cdot 0.001}{1} \approx \mathbf{1.12\ mg/l}$

Hence, the N/P quotient is ~ 11:1 (g/g)

Appendix E – Test run 1

Performance

The first test run was performed with everything added at start; light, agitation by bubbling, highest concentrations of all gases and high flow rate. Six species and equipment setup according to *Figure 14* were tried out. The distance between the lamps was only 20 cm and the distance from one lamp to the cultivation was approximately 7 cm. Premeasured light intensity was 20 W per fluorescence lamp and a small increase occurred when the light was lit in the roof by only a few W. The light intensity was measured to 40-45 W/m^2 for the exposed E-flasks.

The cultivations were made in E-flasks (250 ml). Sterile medium was filled to 200 ml. Then 20 ml of pre-cultured cell suspensions were added to each E-flask leading to a starting volume of 220 ml. Simulated flue gas with a flow rate of 3500 ml/min was more or less evenly distributed between twelve batches by bubbling it through the cell suspensions. The gas was mixed by three-way couplings with the most important constituents; CO_2 (100 %), NO (100 ± 17 ppm) and SO_2 (10 ± 20 ppm). The flow from each tube was set so the final concentrations became close to 15.0 % of CO_2, 100 ppm of NO and 10 ppm of SO_2, visualized in *Table 26*. A natural circulation occurred as the gas was added through the pipe at the bottom. The flow regulator and flow rate controller were read from the lower edge of all metallic bullets.

OD-measurements followed growth. As CO_2 was dissolved in the water suspension the pH was lowered, therefore after one day from start with algae and added gas, a sample of pH was measured to see if and how much the pH had changed. *Test run 1* and *Test run 2* had 16 light hours and 8 dark hours. The gases were added in following order after calculating the flow rates from each. The actual concentrations were measured and adjusted by the NO-meter, SO_2-meter and CO_2-meter.

Table 26. The calculated and added flow rates of the three gases for Test run 1. The concentrations of the tubes were read on a label on the specific tube.

Components	Total	Air	CO_2	NO	SO_2
Concentrations in tubes	-	-	100 %	1732 ± 17 ppm	979 ± 20 ppm
Flow rates (ml/min)	3500	2738	525	202	35

Results

One could see that one by one of the cultivations lost the green color. The OD was low for all cultivations already at start and did not increase during the three days of cultivations. The pH in only medium was 6.41 before running the cultivations. The pH was tested in all cultivations after 24 hours of cultivation (see *Table 27*) and a drop in pH was obtained for all cultivations.

Table 27. The pH measurements after 24 h of cultivation with all components added during this time period.

Day	Time (h)	*C. vulgaris*	N. isolate	*S.* simris002
2	**24**	3.74	4.18	3.86
		C. reinhardtii	***C. sorokiniana***	***A. falcatus***
		4.16	3.43	3.42

Appendix F – Test run 2

Performance

Extra precautions were made from knowledge earned from *Test run 1*. *Test run 2* was made with the same species as in *Test run 1*. Higher start concentration of cells was used compared to *Test run 1*. 180 ml of medium and 40 ml of cell suspension was mixed to a starting volume of 220 ml. A large problem in *Test run 1* was the low pH. As CO_2, NO and SO_2 give dissolved acids and the microalgae did not survive too high concentrations at start. Therefore the total gas flow was lowered to 2000 ml/min and new individual flow rates from the tubes were calculated, *Table 28*. The CO_2 concentration was lowered to 7.5 %, hence half of the wanted 15 %. The lower concentration was used to avoid large pH-drop at start and make the cells start growing before increasing to highest level of CO_2. The flows of NO and SO_2 were calibrated on the flow regulators to match the total flow rate of 2000 ml/min. Air was supposed to compensate the lower flow rate of CO_2 which made the total flow rate constant. The medium was first added to the E-flasks with gas flow added. The calculated flow rates and flow regulator values can be seen in the table below. One of the two lamps was put to a distance of 16 cm from the E-flasks. The other lamp was moved to the roof. The precaution was made to avoid photoinhibition or too long photoacclimation. Light irradiance in the middle pointing towards the lamp was 13 W/m^2, 2.7 W/m^2 in the middle upwards against the roof and at the end towards the lamp was 8.5 W/m^2. Some differences between the E-flasks was obtained but was not thought to be limiting and the cultivations are regarded as possible to compare. The total outflow was investigated to see if the outgoing gas flow was similar to ingoing gas flow.

Table 28. The added flow rates of the three gases for Test run 2. The concentrations of the tubes were read on a label on the specific tube.

Components	Total	Air	CO_2	NO	SO_2
Concentrations in tubes	-	-	100 %	1732 ± 17 ppm	979 ± 20 ppm
Flow rates (ml/min)	2000	1634	150	115.5	20.4

Results

Test run 2 was started by only having medium with added flue gas of 7.5 % CO_2. In an hour the pH changed from pH = 6.41 (only medium) to pH = 5.39. The pH was also measured in the wetting chamber with 7.5 % gas components and the pH had decreased to 2.92 within 24 h. This shows that pH depends much of the flue gas. Improvements from *Test run 1* made it possible to see visual differences after three days of cultivation. *C. vulgaris*, N. isolate and *S.* simris002, were green and grew while the other three, *C. reinhardtii*, *A. falcatus* and *C. sorokiniana*, had lost their color. The OD measurements strengthens the observations as the mentioned green algae had increased in OD while the other ones were approximately the same as at start. The gas outflow was 1600 ml/min and hence 400 ml/min loss existed in the system.

The project continued in *Test run 3* with improvements as growth decreased in *Test run 2*.

Appendix G – Test run 3

Performance

Test run 3 was a continuation of *Test run 2* with some improvements. The same medium and the previous cells were re-used, but with new added cells from the pre-cultures. The start OD was thought to get higher and the living cells could continue to grow. For the three well growing cultures of *C. vulgaris*, N. isolate and *S.* simris002 only 20 ml new pre-culture was added. For *C. reinhardtii*, *A. falcatus* and *C. sorokiniana* 30 ml culture was discarded from the batches and 50 ml pre-culture was added. The volume was approximately the same in all batches after the additions of fresh cells. Another problem to minimize is the risk of getting an extremely low pH during the dark hours of the night and day cycle. This was tried to be counteracted by increasing the number of light hours and thus avoid pH drop during the night. The light/dark cycle was henceforth 20 light hours and 4 dark hours and was applied in *Test run 3*, *Flue gas experiment 1* and *Flue gas experiment 2*. The 4 dark hours were put from 06.00 to 10.00 in the morning. Four days after adding new cells in *Test run 3* and six days from start of *Test run 2* a pH test was made for all species.

Results

The pH was below 3 for all cultivations after the experiment (see *Table 29*). All cultivations had lost the green color after six days of *Test run 2* and after four days of *Test run 3*. The growth decreased or stopped for all species, except for *S.* simris002 indicating growth at extremely low pH (see *Figure 30*).

Table 29. The low pH values obtained after four days of cultivation in Test run 3. Extremely low values were seen and all microalgae had lost the green color.

Species	pH	Species	pH
C. vulgaris	2.49	***C. reinhardtii***	2.59
N. isolate	2.56	***C. sorokiniana***	2.61
***S.* simris 002**	2.73	***A. falcatus***	2.62

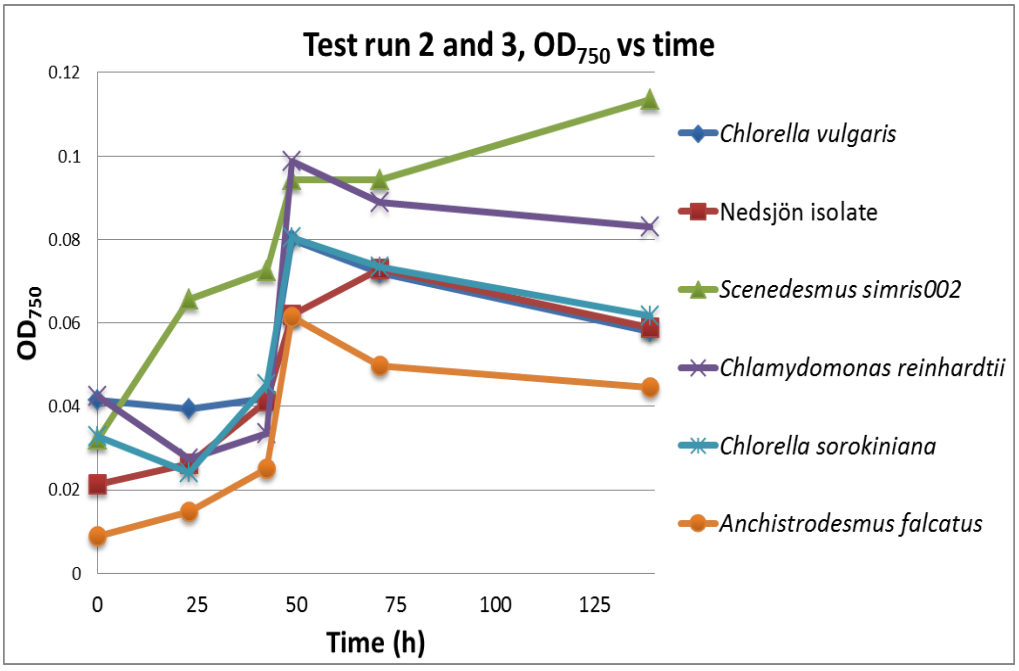

Figure 30. Growth curve of the six species in Test run 2 and Test run 3 measured by OD_{750}. One can see the added pre-culture at 48 h from start where Test run 3 started.

Appendix H – Protocol for quantifying triglycerides in microalgal cells

The protocol was modified from Söderberg [65].

Performance

- Sample a volume of 1 ml from each algal cultivation.
- Centrifuge for 20 min in 5000 rpm and discard the supernatant.
- Prepare solutions; 0.12 M glycerol standard, dilute 20 µl of 87 % glycerol with 2 ml MeOH.
- Also prepare a 120 ml reaction solution of approximately 4 M HCl in methanol together with glucose as internal standard. Solve 1.8 g glucose with 10 ml water, take 120 µl of this solution and mix with 40 ml concentrated HCl and 80 ml methanol.
- Re-suspend cells in water and lysate them with ultrasonic sound for 2x1 min with amplitude of 10 µm.
- Add 400 µl of hexane to all samples. Centrifuge for phase separation and transfer 50 µl of the hexane phase to a vial for reaction.
- Let the samples stand in ultrasonic bath for 10 min.
- Evaporate the samples.
- Make a standard curve from glycerol; 1.2 mM, 0.12 mM, 60 µM, 12 µM and 1.2 µM. Prepare a blank of an empty tube. Further instructions are shown at the end of the protocol.
- Prepare reference oils; vegetable oil diluted 1:1 with dichloromethane. Evaporate 4 µl before adding acid methanol.
- Add 200 µl of the acid methanol to all samples, including the standard curve and the blank. Run all the samples in an ultrasonic bath for 10 min. Let all samples stand 16h in 60 °C. Evaporate the methanol.
- Add 100 µl MetOH, evaporate once again to get rid of HCl.
- Add 100 µl Tri-Sil TP solution and let the samples stand for 1 h in 60 °C.
- Dilute samples 1:10 with dichloromethane.
- Analyze with GC/MS and calculate the amount of glycerol within the samples.

Arrangement of standard curve

- Take 100 µl from the glycerol solution. Dilute with 900 µl to 12 mM, mark as 1.
- Take 100 µl from tube 1 and dilute with 900 µl MetOH to 1.2 mM. Mark tube as 2.
- Take 100 µl from tube 2 and dilute with 900 µl MetOH to 0.12 mM. Mark tube as 3.
- Take 200 µl from tube 3 and dilute with 200 µl MetOH to 60 µM. Mark tube as 4.
- Take 40 µl from tube 3 and dilute with 360 µl MetOH to 12 µM. Mark tube as 5.
- Take 40 µl from tube 5 and dilute with 360 µl MetOH to 1.2 µM. Mark tube as 6.
- Take 200 µl from each tube and transfer to marked vials. Let them evaporate.

Appendix I – Protocol for total cellular carbohydrates

The test was performed according to a protocol taken from Herbert et al. [66].

Solutions and Samplings

- 5 % phenol (mixture of 5.56 ml of 90 % phenol filled to 100 ml)
- 10 mM of glucose standard stock (0.1 g/ 50 ml)
- Take samples at the time for investigation and freeze samples with liquid nitrogen.

Measurements in microtiter plates

Method in Flourstar Omega plate reader (BMG LABTECH): Total carbohydrates (measuring absorbance at 488 nm three times at each sample in duplicate samples). Make standard curves with the glucose standard of 0.5 g/l diluted from the glucose stock. Use a standard curve with standards diluted between 20x and undiluted (0.025-0.5 g/l).

- Pipette 30 µl of duplicate milli-Q water, standards, and appropriate diluted samples on a microtiter plate.
- Make a background of only medium in a few wells to be able to subtract this value from the earned values of the cell samples.
- Add 30 µl of phenol solution with a multipipette and mixing accordingly.
- Add 150 µl of sulfuric acid with a multipipette and mixing accordingly.
- Incubate for 30 min and hereafter measure the absorbance at 488 nm.

Absorbance was measured to estimate the carbohydrate content and two standard curves with glucose concentrations were used for the analysis, see *Figure 31*.

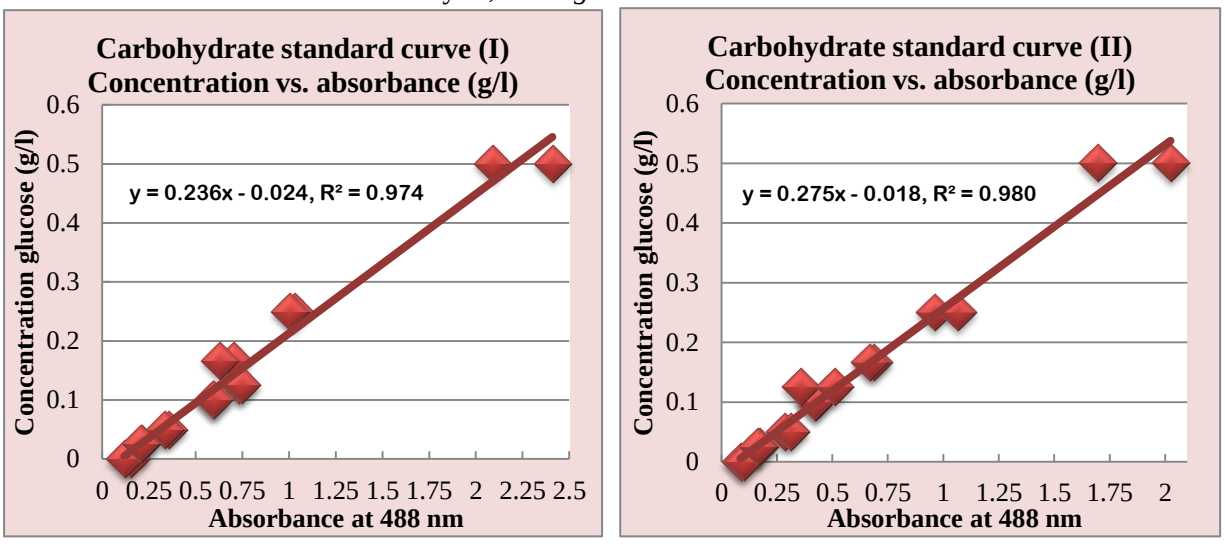

Figure 31. Two carbohydrate standards were made, hence two plots. The y-value corresponds to the concentration and was calculated by each equation given in the plots.

Appendix J – Protocol for total protein content in Biomass

The protein content experiments were made according to Lowry [67] by the Protein Dc kit (BioRad).

Solutions and Samplings

- Take a volume of 90 µl cell suspension and mix with 10 µl of 10 % SDS solution. Heat the samples in 5 min at 95 °C. Vortex and centrifuge the samples at 15000 rpm for 5 min. The supernatant should be used for further analysis.

Standard construction on Microtiter plates

- The standard (BSA) delivered with the kit should be used (approximately 1.3 to 1.5 g/l, check on the bottle).
- A dilution series should be prepared with 3-5 dilutions where the standard protein solution should be between 0.2 g/l to 1.5 g/l.
- Dilute the standards with 1 % SDS solution as the cell suspension is mixed to the final concentration of 1 % SDS.
- Make a standard curve with absorbance at the wavelength of 750 nm.
- Make the standard curve at each assay.

Analysis with BioRad Dc kit on Microtiter plates

- Take a suitable volume of sample, 10 µl, and add to dry microtiter plates.
- Add a volume of 25 µl reagent A from the kit into each well containing sample or standard with a multipipette.
- Hereafter add 200 µl of reagent B into all wells with a multipipette. Agitate the whole microtiter plate gently and avoid samples from getting mixed. If bubbles are formed a pipette tip can be used to pop them.
- Incubate the samples for 15 minutes.
- Measure the absorbance at the wavelength of 750 nm.

The protein content was measured from two standard curves, see *Figure 32*.

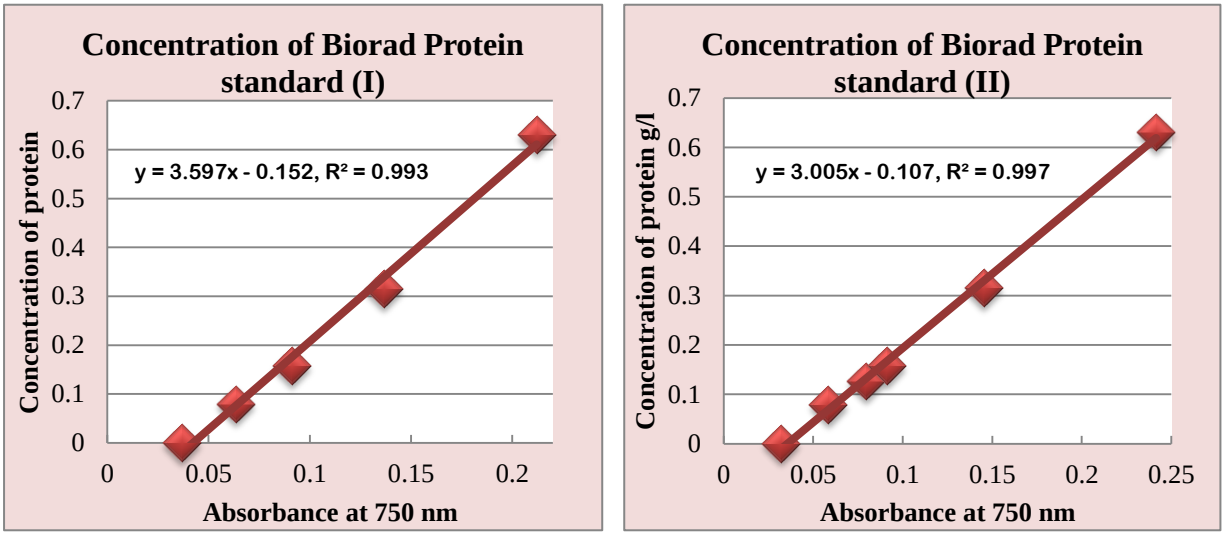

Figure 32. The protein content was measured by two plots. The y-value corresponds to the wanted concentration.

Appendix K – Absorbance test and comparison of OD_{550} and OD_{750}

An absorbance test was performed on; *C. vulgaris*, N. isolate, *S.* simris002, *C. reinhardtii*, *C. sorokiniana* and *A. falcatus*. The figure for *C. vulgaris* is shown in *Figure 18* in the *Results* chapter. The six green microalgae species were the ones tried out in *Flue gas experiment 1*. Pre-cultures were diluted to five different dilutions and OD_{750} and OD_{550} were performed on each species and each dilution. *Figure 33* to *Figure 37* together with *Figure 18* all indicate that OD_{550} got higher values compared to OD_{750} at all dilutions for all species.

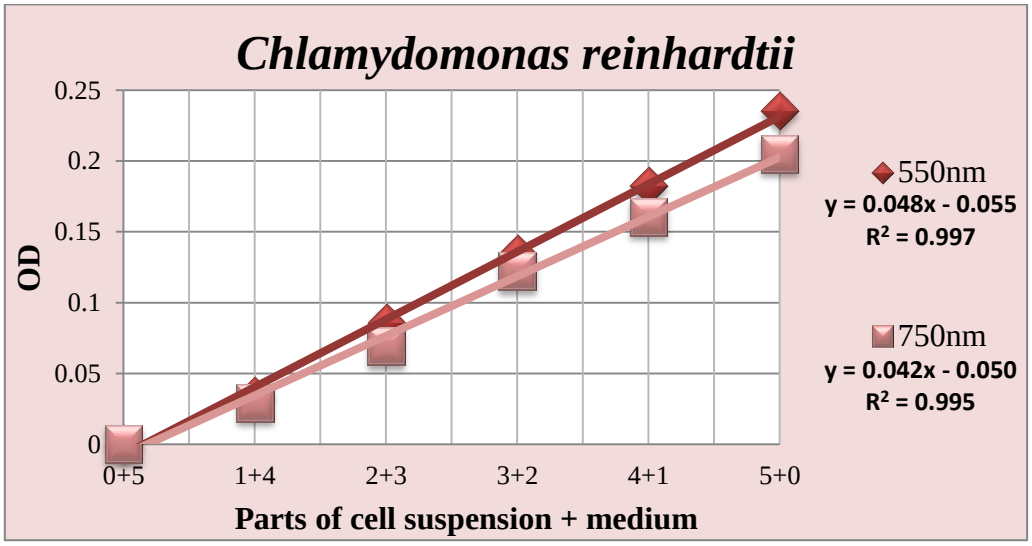

Figure 33. OD-measurements at 550 nm and 750 nm for six dilutions of for C. reinhardtii.

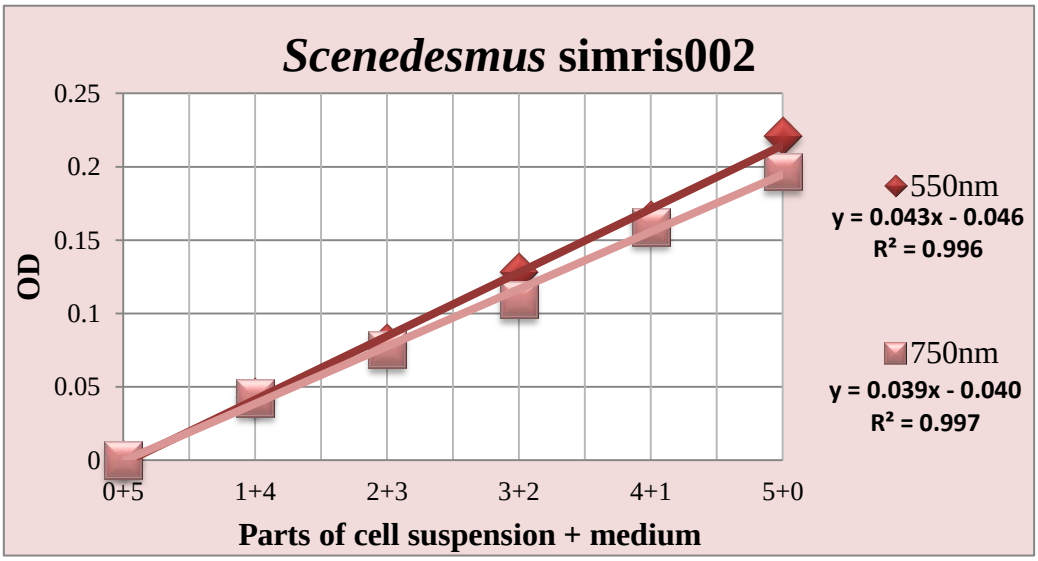

Figure 34. OD-measurements at 550 nm and 750 nm for six dilutions of for S. simris002.

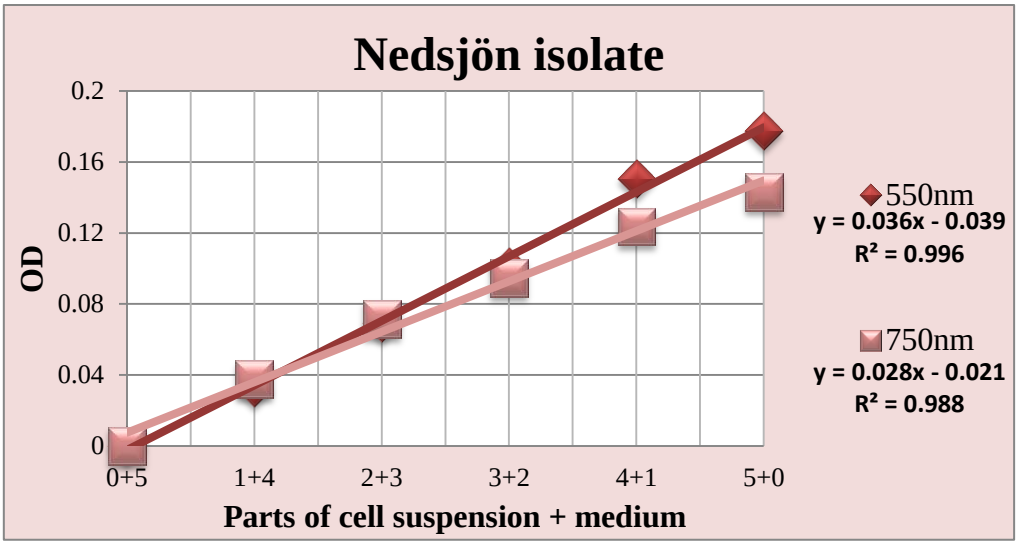

Figure 35. OD-measurements at 550 nm and 750 nm for six dilutions of for N. isolate.

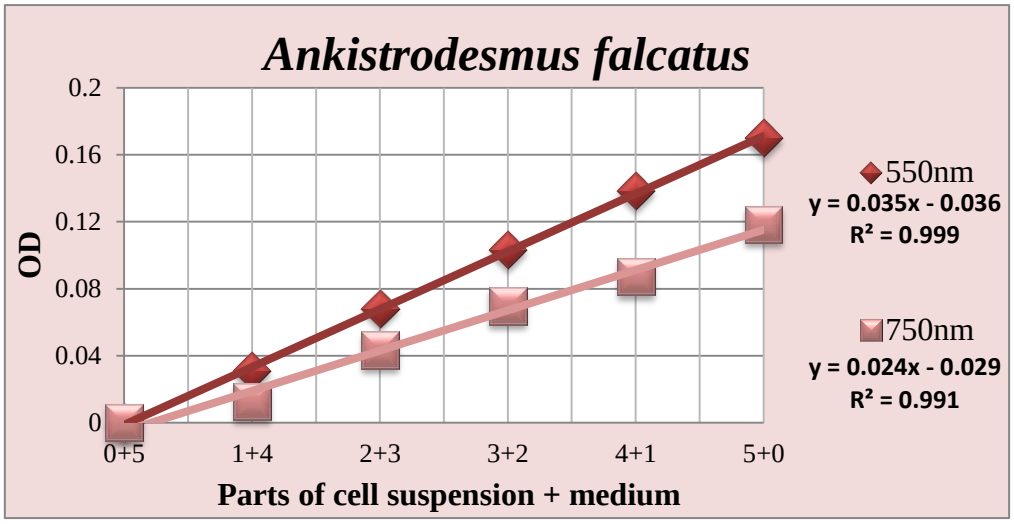

Figure 36. OD-measurements at 550 nm and 750 nm for six dilutions of for A. falcatus.

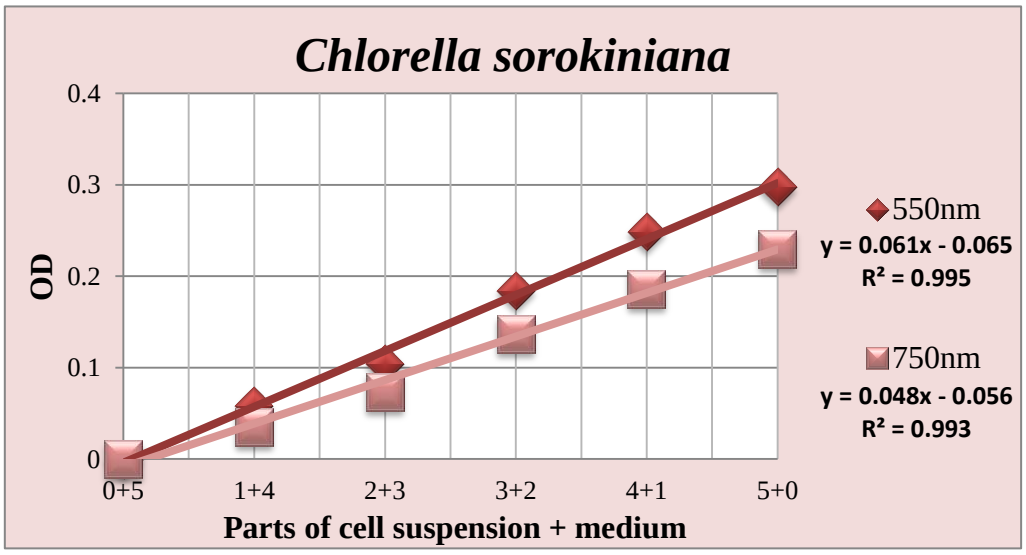

Figure 37. OD-measurements at 550 nm and 750 nm for six dilutions of for C. sorokiniana.

Appendix L – pH variations for *Flue gas experiment 1*

As a complement for the illustration of the pH variations in *Figure 19* for *Flue gas experiment* 1 a *Table 30* illustrates a detailed description and the exact order in which the pH measurements and when the flue gas constituents were added or increased.

Table 30. pH of the culture medium during Flue gas experiment 1 in which the CO_2 concentration was stepwise increased from approximately 0 % to 15 %. The other gas components NO (100 ppm) and SO_2 (10 ppm) were included in the gas mixture at 308 h and 311 h from start NO (100 ppm) and SO_2 (10 ppm) respectively.

Time	CO_2	Gas flow	pH in culture medium					
(h)	(vol. %)	(ml/min)	*C. vulg-aris*	N. isol-ate	*S.* simris-002	*C. reinh-ardtii*	*C. sorok-iniana*	*A. falc-atus*
-	0	0	6.51 [(1)]	6.68 [(1)]	6.70 [(1)]	6.51 [(1)]	6.62 [(1)]	6.54 [(1)]
0	0	0	6.57	6.76	6.70	6.58	6.64	6.64
0	A total airflow of 800 ml/min was started.							
22	0	800	6.63	6.87	6.70	6.59	6.66	6.71
25	CO_2 added to approximately 1.35 %. The total gas flow was increased to 1400 ml/min.							
95	~ 1.35	1400	6.50	7.14	7.12	7.11	7.06	6.86
97	CO_2 was increased to 2.9-3.2 %.							
98	2.9-3.2	1400	6.30	6.92	6.93	6.84	6.89	6.67
117	2.9-3.2	1400	6.69	7.02	7.27	7.06	6.96	6.75
121	CO_2 was increased to 5.9-6.5 %.							
122	5.9-6.5	1400	6.43	6.72	6.66	6.84	6.67	6.45
141	5.9-6.5	1400	6.36	6.68	6.68	6.63	6.63	6.48
141	Dilution of cells.							
145	5.9-6.5	1400	6.28	6.53	6.56	6.45	6.38	6.27
164	5.9-6.5	1400	6.33	6.57	6.67	6.59	6.62	6.51
168	CO_2 was increased to 9.1-9.5%.							
169	9.1-9.5	1400	6.39	6.39	6.47	6.43	6.28	6.28
188	9.1-9.5	1400	6.30	6.48	6.60	6.53	6.46	6.36
193	CO_2 was increased to 12.3-12.7 %.							
195	12.3-12.7	1400	6.30	6.44	6.53	6.47	6.39	6.33
260	12.3-12.7	1400	6.43	6.61	6.65	6.62	6.58	6.46
260	CO_2 was increased to 14.9-15.1 %.							
263	14.9-15.1	1400	6.14	6.51	6.61	6.63	6.48	6.43
284	Dilution of cells.							
284	14.9-15.1	1400	5.94	6.11	6.22	6.18	6.08	6.06
307	14.9-15.1	1400	6.16	6.26	6.33	6.31	6.28	6.14
308	NO added by 80.8 ml/min to 100 ppm.							
311	14.9-15.1	1400	6.07	6.23	6.38	6.23	6.22	6.11
311	SO_2 added by 14.3 ml/min to 10 ppm.							
314	14.9-15.1	1400	5.92	6.17	6.27	6.28	6.22	6.16
333	14.9-15.1	1400	6.13	6.28	6.38	6.31	6.27	6.14
338	14.9-15.1	1400	6.27	6.31	6.43	6.44	6.28	6.25
356	14.9-15.1	1400	6.17	6.31	6.40	6.30	6.27	6.15

[(1)] ***The different values of pH at start were depending on the pH in the pre-cultures.***

Appendix M – pH variations for *Flue gas experiment 2*

As a complement for the illustration of the pH variations in *Figure 25* for *Flue gas experiment 2* a *Table 31* illustrates a detailed description and the exact order in which the pH measurements and when the flue gas constituents were added or increased. As can be seen the stepwise increase of CO_2 was performed faster compared to *Flue gas experiment* 1 and NO (100 ppm) and SO_2 (10 ppm) were added earlier in the cultivation time period.

Table 31. The pH varying through time during Flue gas experiment 2 as CO_2 concentration was stepwise increased from approximately 0 % to 15 %, more rapidly performed compared to Flue gas experiment 1. At 143 h from start NO (100 ppm) and SO_2 (10 ppm) were included in the gas mixture.

Time	CO_2	pH in culture medium				
(h)	(vol. %)	*B. braunii*	*S. obliquus*	*C. emersonii*	*C. prototheco-ides*	*N. salina*
0	All cultivations were started with a total flow rate of 1400 ml/min. No CO_2 included.					
1	0	6.72 [(1)]	6.95 [(1)]	6.87 [(1)]	6.85 [(1)]	6.73 [(1)]
1.5	CO_2 increased to 0.95-1.09 %.					
2.5	0.95-1.09	6.14	6.40	6.38	6.33	6.20
19	0.95-1.09	6.43	6.57	6.52	6.61	6.46
20	CO_2 increased to approximately 3.3 %.					
25	3.3	6.01	6.23	6.11	6.24	6.02
43	3.3	6.16	6.44	6.27	6.42	6.16
44	CO_2 increased to 6.7-7.5 %.					
44	*C. emersonii* adjusted with 2 ml of 1 M NaOH as the color was light yellow.					
46	6.7-7.5	6.06	6.13	6.83	6.24	5.89
49	6.7-7.5	5.96	6.17	6.83	6.24	5.98
68	6.7-7.5	6.07	6.31	6.86	6.31	6.08
69	CO_2 increased to 15.6-15.9 %.					
72	15.6-15.9	5.77	6.00	6.54	6.03	5.84
74	15.6-15.9	5.73	6.03	6.62	6.05	5.84
140	15.6-15.9	6.00	6.55	6.60	6.27	6.10
143	SO_2 and NO added to approximately 10 ppm and 100 ppm respectively.					
193	*C. emersonii* was again green in color and seemed to thrive.					
146	15.6-15.9	6.09	6.27	6.60	6.27	6.12
165	15.6-15.9	6.08	6.26	6.58	6.24	6.10
170	15.6-15.9	5.87	6.27	6.53	6.25	6.14
187	15.6-15.9	6.01	6.26	6.52	6.25	6.11
193	15.6-15.9	5.99	6.24	6.49	6.28	6.11
211	15.6-15.9	6.04	6.29	6.63	6.35	6.18
215	15.6-15.9	6.16	6.33	6.71	6.43	6.21
240	15.6-15.9	6.02	6.29	6.51	6.30	6.21
356	15.6-15.9	5.92	5.90	6.36	6.12	5.98

[(1)] The different values of pH at start were depending on the pH in the pre-cultures. The gas flow rate was 1400 ml/min during the whole experiment.

Appendix N – Exponential phases examples for *Flue gas experiment 1-2*

Two examples of how the specific growth rates were calculated are visualized in *Figure 38* and *Figure 39*. The values are collected in *Table 15* and *Table 20*.

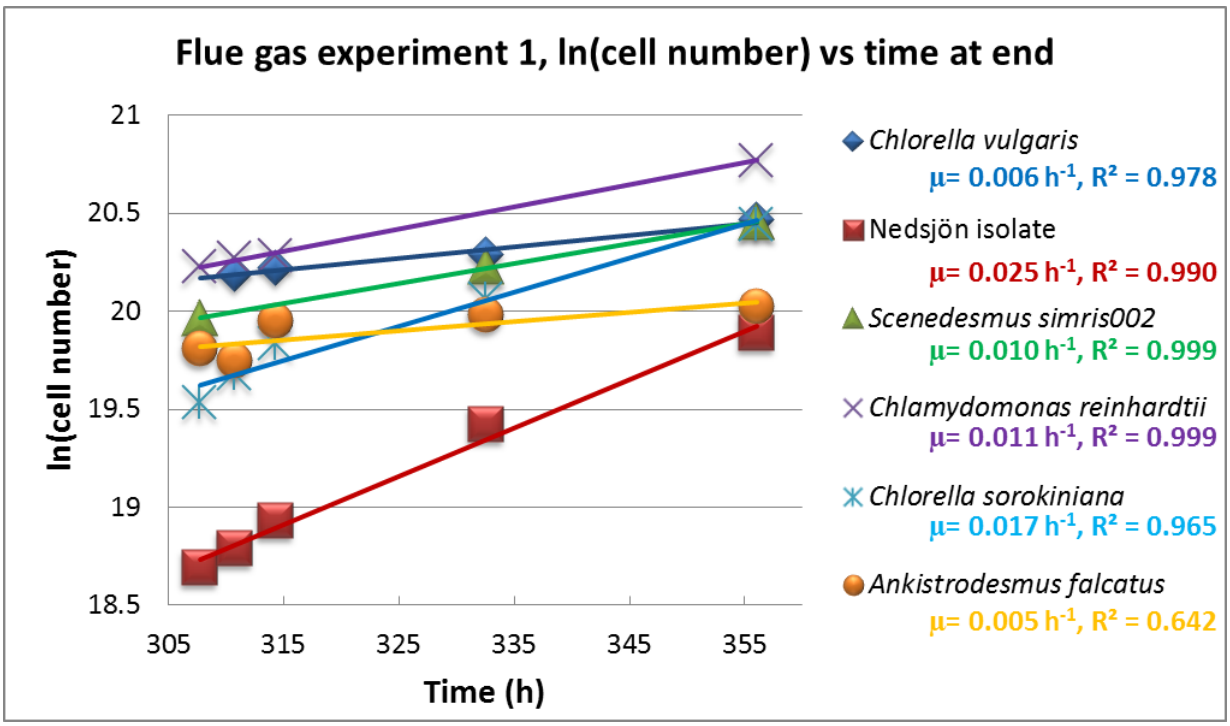

Figure 38. The logarithmic cell counts versus time for Flue gas experiment 1 in the latest phase where highest levels of CO_2 (15 %), NO (100 ppm) and SO_2 (10 ppm) were added.

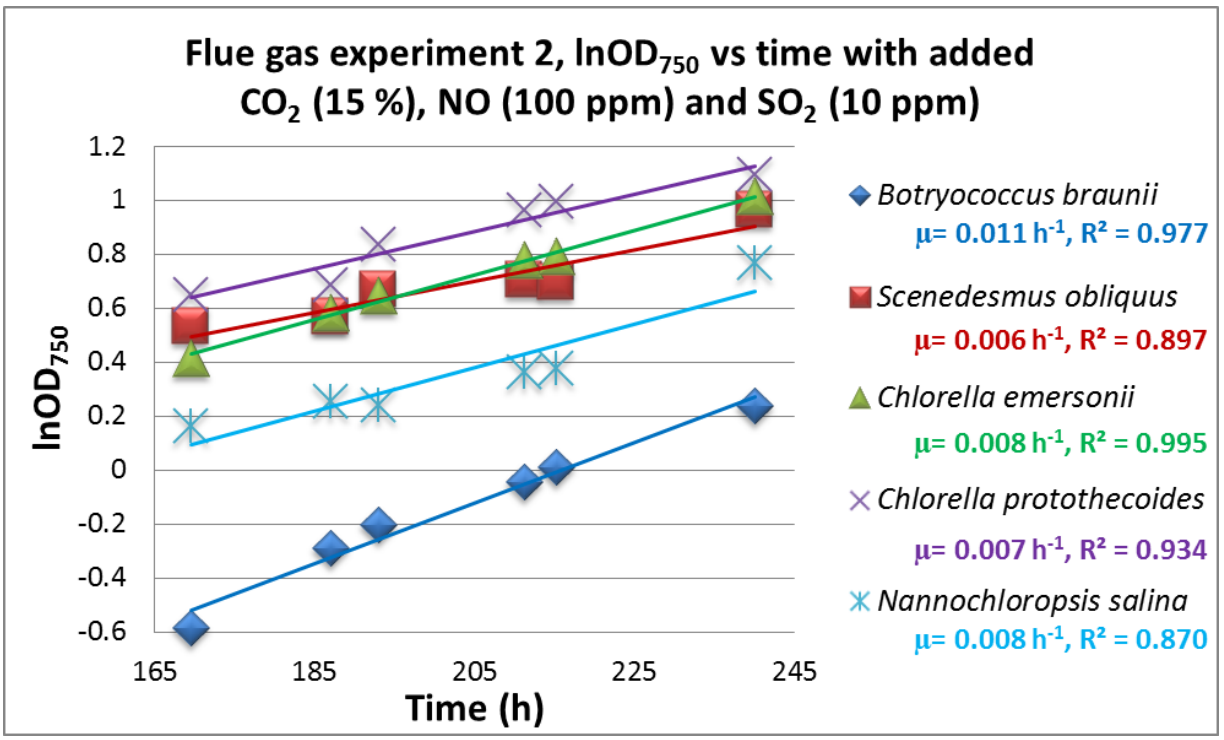

Figure 39. Logarithmic OD_{750} versus time for Flue gas experiment 2 at the end where CO_2 was approximately 15 %, NO (100 ppm) and SO_2 (10 ppm). Specific growth rate is given for each species. All specific growth rates are lower compared to the start of the cultivation.

Appendix O – Growth on surfaces examined for the species in *Flue gas experiment 1*

At the end of *Flue gas experiment 1* growth on the walls could be seen for four of the tested species. Following pictures show the similarities and differences. *C. vulgaris* and *S.* simris002 showed no tendencies of growing on the walls, *Figure 40*. On the next page it is also possible to see the E-flasks with no medium in them, probably giving a better insight of the growth on the walls.

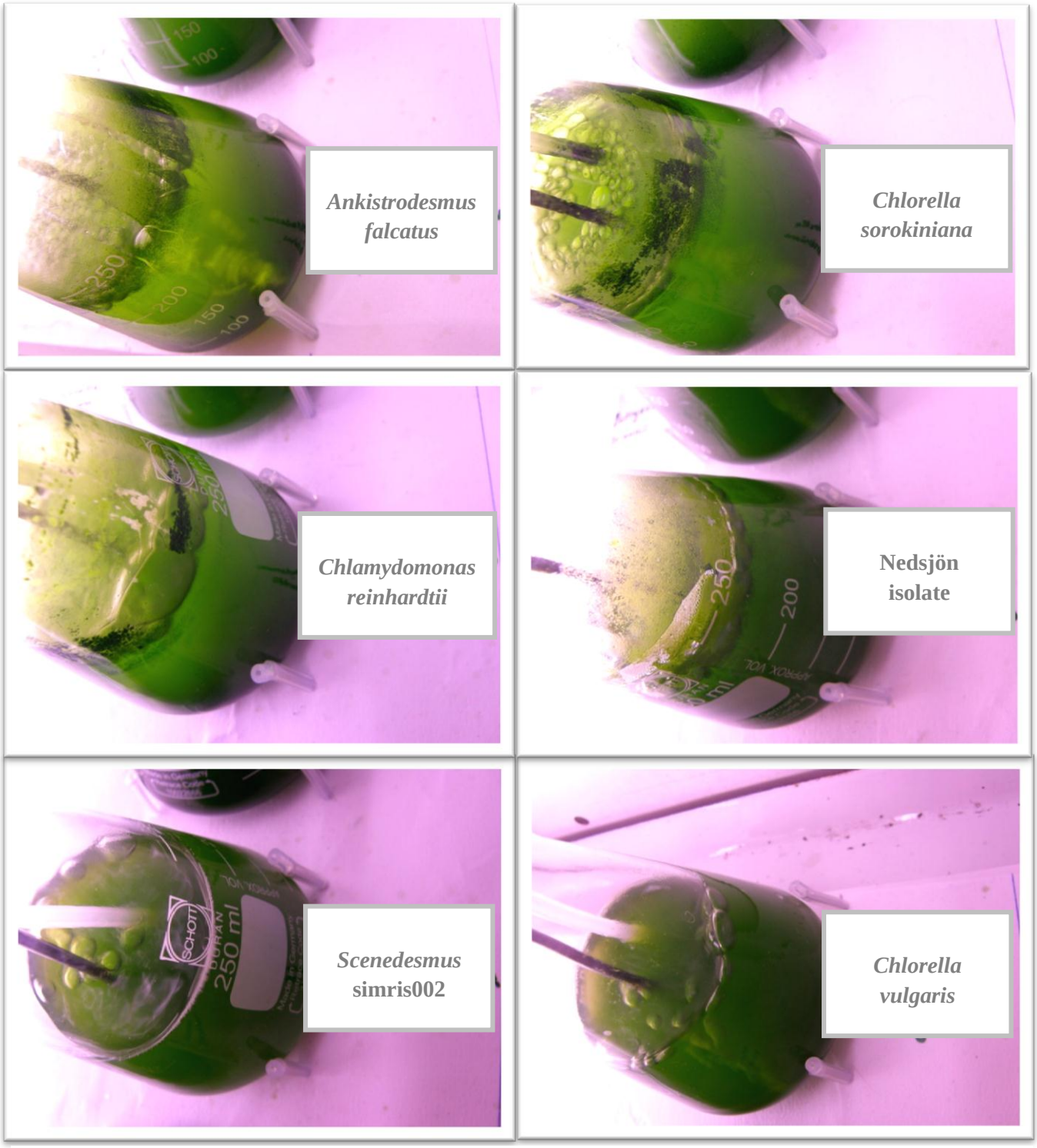

Figure 40. Growths on walls for the six species in Flue gas experiment 1. The picture was taken after the run was finished and before the cell suspension was taken for CHN-analysis.

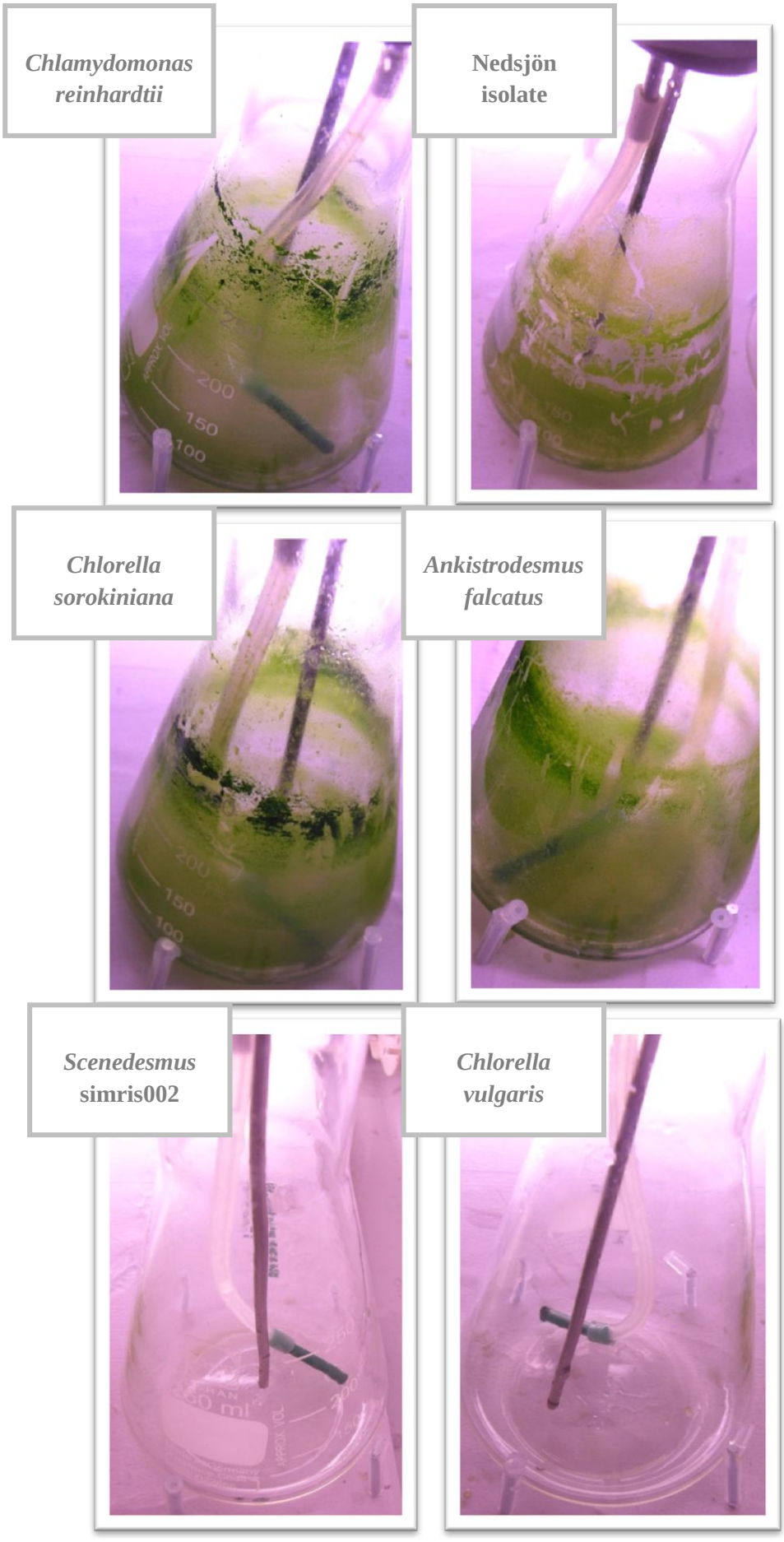

Figure 41. Growth on walls shown after cell suspensions taken for CHN analysis in Flue gas experiment 1. The E-flasks were rinsed with water before the pictures were taken.

Appendix P – Wastewater growth test for *RIA* and *Reactor 2*

Figure 42. Wastewater from Nordic Paper Bäckhammar AB as growth medium. Two cleaning steps gave rise to two different media, namely RIA and Reactor 2. The media were autoclaved before usage to avoid algal species brought from the media to grow.

Appendix Q – Growth check on *RIA* and *Reactor 2* without added cells

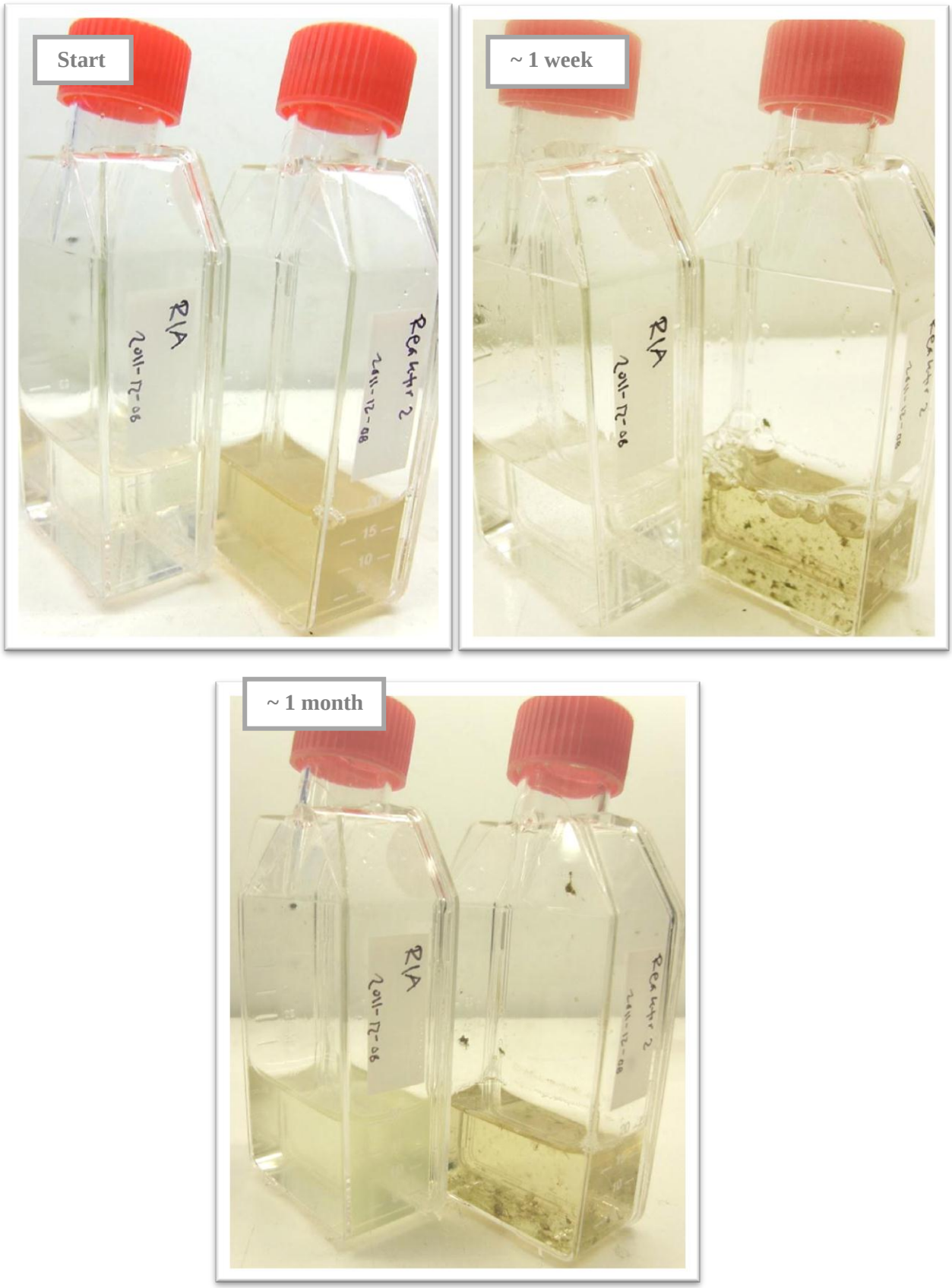

Figure 43. Flasks of only wastewater RIA and Reactor 2 from Nordic Paper Bäckhammar AB. They were not autoclaved as the potential growth of already existing species was of interest. The visual observation indicated obvious growth in the flasks stored for approximately 1 month.