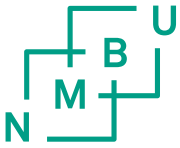
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Preface and acknowledgments

This thesis was written at the Norwegian University of Life Sciences (NMBU), Faculty of Veterinary Medicine and Biosciences, Department of Plant Sciences (IPV). The laboratory work was conducted in the Plant Cell Laboratory and in the greenhouses at Senter for Klimaregulert Planteforskning (SKP), during the period May 2013-February 2014. The thesis was done in context with project 203281: Use of Solar Energy for CO_2 capture, algae cultivation and hydrogen production – BioH_2.

With this, I give my greatest thanks to

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Ås, 14.5.2014

Marit Elisabeth Evjen

Abstract

The effect of high light intensities in form of daylight and simulated daylight conditions on the growth and quality of the green microalga *Chlamydomonas reinhardtii* was investigated. In the daylight experiments, cultures of *C. reinhardtii* were grown in a greenhouse with natural light conditions only, with a control of natural daylight plus a background light of 200 μmol m^{-2} s^{-1}. The cultures were analysed for dry weight and starch content. In addition, different initial cell densities were used in the two treatments (10 000 cells mL^{-1} and 50 000 cells mL^{-1}) to evaluate the effect on growth. The results showed that growth increased proportionally with natural light intensity during the days. During nights, growth (dry weight) slowed down or decreased. Starch accumulated during days, and decreased during nights. In the control treatments with continuous light in addition to daylight, dry weight and starch increased evenly throughout the experiment, before the growth stagnated and decreased 5-6 days into the experiment. The effect of the initial cell densities was not significant between the treatments. In the simulated light experiments, cultures were treated with 6 hours light periods of 500 and 1000 μmol m^{-2} s^{-1}, both with continuous backlight of 200 μmol m^{-2} s^{-1}, in addition to a control of continuous 200 μmol m^{-2} s^{-1}. The cultures were analysed for dry weight, starch, chlorophyll and photoinhibition. Results showed that the cultures in high light treatments suffered dynamic photoinhibition. Cells in the medium light treatment were also photoinhibited, but on a lower level. However, dry weight and starch did not differ significantly between the three treatments. Chlorophyll decreased severely during the light periods. As dry weight of all experiments was lower than expected, the effects of air bubbling (CO_2) velocity and content of calcium, magnesium or tap water in the growth medium were also tested. We concluded that a higher air bubbling velocity had a positive effect on algal growth, and that the High Salt Sueoka-medium should be enriched in calcium, magnesium, and sulphur.

Sammendrag

Grønnalgen *Chlamydomonas reinhardtii* ble testet for virkningen av sterkt lys på vekst og kvalitet. Det ble gjort to hovedforsøk: ett med kun dagslys, og ett med simulerte dagslysforhold. I det første forsøket ble mikroalgen dyrket i veksthus med kun naturlig innstråling, mot en kontroll med kontinuerlig bakgrunnslys på 200 μmol m^{-2} s^{-1}. Kulturene ble analysert for tørrvekt og stivelse. Her ble det også brukt forskjellig konsentrasjon i startkultur (10 000 celler mL^{-1} og 50 000 celler mL^{-1}), for å studere virkningen på vekst (tørrvekt). Resultatene viste at tørrvekt økte proporsjonelt med lysmengde på dagtid, og minsket eller sank i løpet av natten. Stivelse akkumulerte i løpet av dagen, men sank i løpet av natten. For kontrollen steg tørrvekt og stivelse jevnt (ekstra ved solfylte dager), før veksten stagnerte etter 5-6 dager og deretter sank. De forskjellige konsentrasjonene i startkultur ga ingen tydelig forskjell i vekst. I det andre forsøket ble den kraftige økningen av lysintensitet simulert ved å dyrke kulturer med lysperioder på 500 μmol m^{-2} s^{-1} og 1000 μmol m^{-2} s^{-1} i seks timer, samt kontinuerlig belysning på 200 μmol m^{-2} s^{-1}, mot en kontroll på kun kontinuerlig belysning på 200 μmol m^{-2} s^{-1}. Kulturene ble analysert for tørrvekt, stivelse og klorofyll. I tillegg ble fotoinhibering registrert. Resultatene viste at kulturene med sterkt lys (1000 μmol m^{-2} s^{-1}) ble kraftig fotoinhibert, men ikke kronisk. Kulturene i medium sterkt lys (500 μmol m^{-2} s^{-1}) ble også fotoinhibert, men i mindre grad. Tørrvekt og stivelse viste ingen tydelig forskjell i de tre behandlingene. Klorofyllmengden ble kraftig redusert i de sterkeste lysbehandlingene. Da tørrvekten hos samtlige forsøk var lavere enn forventet, ble hastighet på luftbobling med CO_2, samt mengde kalsium og magnesium og bruk av kranvann i mediet prøvd ut. Konklusjonen på disse forsøkene ble at mengde luftbobling med hell kan økes, samt at mengde kalsium, magnesium og svovel burde økes i High Salt Sueoka-mediet.

Abbreviations

ATP, ADP	Adenosine Triphosphate, Adenosine diphosphate
Bpm	Beats per minute
Con.	Continuously
DL	Daylight
DMSO	Dimetylsulfoxide
DW	Dry Weight
ETC	Electron Transport Chain
GOPOD	Glucose oxidase (GO) and Peroxidase (POD)
Fm	Maximum fluorescence
Fo	Yield fluorescence without PFD
Fv/Fm	Quantum efficiency (yield) / maximum efficiency of PSII
Fv	The difference between Fm and Fo
Ft-c	Foot-candle
g	G-force
HL	High light (continuous 200 μmol m^{-2} s^{-1} + 6h 1000 μmol m^{-2} s^{-1})
HS-medium	Suoeka's High Salt Medium (growth medium)
I_c	Light Compensation Point
I_h	Point where photosystems are saturated
I_m	Point where photosystems are inhibited
ML	Medium light (continuous 200 μmol m^{-2} s^{-1} + 6h 500 μmol m^{-2} s^{-1})
$NADP^+$ (NADPH*)	Nicotinamide Adenine Dinucleotide Phosphate, *reduced form
NIVA	Norwegian Institute of Water Research (Norsk Institutt for Vannforskning)
OCD	Optimal Cell Density

P680	Pigment 680. Photosystem II primary donor. Absorbing light by 680 nm
PAR	Photosynthetic Active Radiation
PFD	Photon Flux Density
PSI, II	Photosystem I, II
ROS	Reactive Oxygen Species
Rpm	Revolutions per minute
SKP	Centre for Climate Regulated Plant Research (Senter for Klimaregulert Planteforskning)

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Introduction

Sunlight is the most plentiful source of energy, and is the main driving force for the most vital biochemical reaction on this planet, namely the photosynthesis. In the photosynthesis, water molecules are split helped by sunlight energy. Not only is this reaction the source of oxygen, but it also generates electrons, which in a chain of reaction results in energy carriers. The energy carriers are then used in carbohydrate production, where atmospheric carbon is converted to carbohydrates. Another utility of the photosynthesis is to make clean energy directly from sunlight, such as hydrogen gas (H_2) for biofuel. Several phototrophic organisms such as bacteria and microalgae have the ability to produce hydrogen from sunlight and water (Asada & Miyake 1999; Ghirardi et al. 2000; Kosourov et al. 2002).

Chlamydomonas reinhardtii is a much used model organism due to its undemanding nature, simple life cycle and to several similarities to higher plant physiology on a cellular level (Sueoka 1960). The ability of microalgae to produce hydrogen gas was discovered in the 1940s (Gaffron & Rubin 1942), an ability *C. reinhardtii* also possess. The reaction is catalysed by the enzyme hydrogenase, which only functions in anaerobic conditions. However, this potential has not been much developed since (Kosourov et al. 2002; Melis & Happe 2001). One of the challenges of producing *C. reinhardtii* for hydrogen production is to increase the growth to sufficient amounts to make the production profitable. In particular, the amount of starch in the cells is important, as starch is the main electron donor when the cells start producing hydrogen gas.

The aspect of producing microalgae in natural daylight instead of, or in addition to artificial light is of high interest, as it is more efficient in terms of both general energy and economy. To this day, there are few studies done on the effects of producing microalgae in daylight, particularly in Norwegian conditions. The challenge with growing cultures in daylight is the highly variation of light intensities. In broad daylight, light irradiation can be as high as 2000 μmol m^{-2} s^{-1}. As vital as the sunlight is to growth and development of phototrophic organisms, such high levels may also damage plant tissues and thus have a negative impact on growth. High light intensities damage the D1-proteins connected to photosystem II (PSII). This phenomenon is named photoinhibition.

The aim of the thesis is to study the effects of high light exposure on growth and quality (in particular starch production) in the green microalga *C. reinhardtii*. This is to investigate

possibilities of benefiting from sunlight energy to produce *C. reinhardtii*, particularly in Norwegian conditions during the summer months. The experiments was done with both natural daylight, and simulated daylight conditions.

1 Literature review

1.1 Organism: *Chlamydomonas reinhardtii*

Chlamydomonas reinhardtii is a unicellular eukaryote green alga of the class Chlorophyceae. Its shape is ellipsoid and its flagella clearly define its front and backside. The full-grown volume of a *C. reinhardtii* cell is about 56.03 μm^3. It moves rapidly using its two antenna shaped flagella (Figure 1).

The nucleus is located in the centre of the cell. Inside the nucleus is the nucleolus, containing DNA. The Golgi vesicle is situated in the proximity of the nucleus. Surrounding the nucleus, the U-shaped chloroplast is causing the clear green colour of *C. reinhardtii* cells. Inside the chloroplast, thylakoid membranes are arranged either as single disks or stacked. Starch granules are located throughout the chloroplast. By the cell wall, the mitochondria and the eyespots are located, which consist of layers of orange granules. Each layer is covered with thylakoid membrane. The eyespots work as photoreceptors and are connected to the flagella. This way, the alga can orient itself and move toward or away from the light and different environments (Harris 2009).

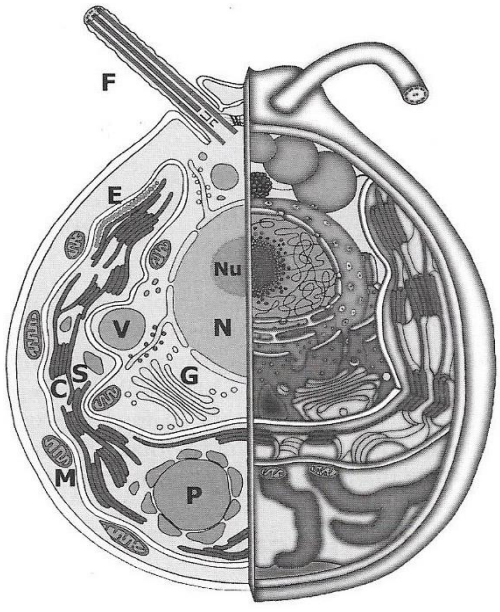

Figure 1: Illustration of the cell structure of *C. reinhardtii*. N: Central nucleus, Nu: Nucleolus, F: Flagella, C: Chloroplast, E: Eyespot, P: Starch-containing pyrenoid, M: Mitochondria, G: Golgi vesicle, S: Starch Grains, V: Vacuoles. From Harris (2009).

Chlamydomonas cells divide in a rapid tempo by multiple fission (Bisova et al. 2005) where each nucleus parts in two. The dividing nuclei often form clusters of up to 16 nuclei in one enveloped cell before the cytoplasm separate (Figure 2 – 16h). Grown in 12 hours light and 12 hours night, exponential growing cultures would undergo two or three divisions in 24 hours, where most divisions occur during the night-period (Bernstein 1964; Harris 2009).

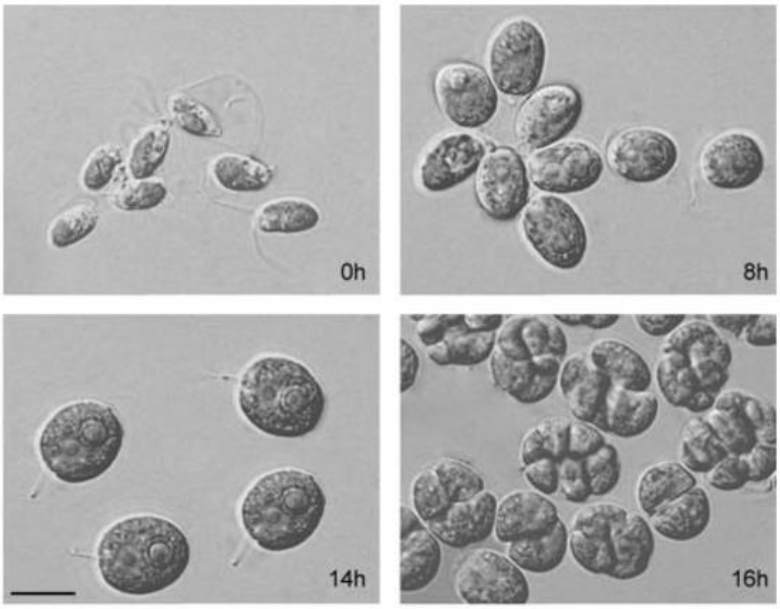

Figure 2: Cell division cycle of a synchronous culture of *C. reinhardtii*, grown on a 12:12 hour light-dark cycle. Pictures are taken during a 16-hour cycle (light period starts at 0), and show the rapid tempo in cell division and size increase of cells. Bar = 10µm. From Bisova et al. (2005).

1.2 Photosynthesis

Photosynthesis is a set of reactions that enables plants the solitary task to produce carbohydrates and oxygen from water, CO_2 and sunlight. The photosynthesis occurs in the thylakoid membrane in the chloroplast (Figure 3). It can be divided into three steps: water oxidation and O_2-evolution, the electron transport chain (ETC) and biomass formation (Calvin-Benson cycle). In short, photons are absorbed by light harvesting antennas in the photosystems I and II (PSI and PSII), which gain the energy to split water molecules. This reaction releases electrons and protons, which get transported from the lumen (the thylakoid inner space) through the electron transport chain (ETC), generating NADPH and ATP later used in the carbon-reactions (Taiz & Zeiger 2010). The three steps proceed as follows.

Water oxidation and O_2-evolution

Water oxidation takes place in the photosystem II-complex (PSII). The purpose of photosystem II (PSII) reaction centre is to split water molecules and gain sunlight energy to excite electrons. The core of PSII is made of membrane proteins, including the proteins D1 and D2. Connected to these proteins are the main chlorophyll donor P680 (pigment absorbing light at 680 nm), other chlorophylls, carotenoids, pheophytin and plastoquionine (Taiz & Zeiger 2010).

The splitting of water molecules occur due to a strong oxidation, making PSII the strongest oxidation centre known. The water molecules are oxidized and split by the following chemical reaction:

$$H_2O \rightarrow \frac{1}{2} O_2 + 2 H^+ + 2 e^-$$

One water molecule releases half an oxygen molecule, two protons and two electrons. The protons are released into the lumen, and sent through the ATP synthase system to the stroma. The electrons are excited by sunlight, and sent through the electron transport chain (Taiz & Zeiger 2010).

Electron Transport Chain (ETC)

In the electron transport chain (ETC), the electrons from the water splitting are transferred due to sunlight excitation through a series of redox-reactions and ends up producing the energy

carriers ATP and NADPH. ATP and NADPH are molecules with high-energy bonds, which release energy when breaking the phosphate bonds (Taiz & Zeiger 2010).

First, electrons reduce the chlorophyll centre P680 in PSII. Next, P680 is excited by sunlight, and then oxidized by sending the electrons to pheophytin. Then electrons are transferred to the plastoquinones PQ_A and PQ_B. The plastoquinones gain protons from stroma, before being reduced by electrons and then releasing protons to lumen. Next, electrons are transferred to cytochrome b_6f, oxidizing plastoquinones before the plastoquinones collect more protons from stroma. Electrons are transferred to plastocyanin and then reduce the chlorophyll centre in PS700 in PSI. P700 is then excited by sunlight, before it sends electrons to A0, A1 and a series of membrane-bound iron-sulphur proteins, before the electrons end up in soluble ferredoxin. Then, the chain ends with the enzyme ferredoxin-$NADP^+$ reductase reducing $NADP^+$ to NADPH. NADPH is then used in the Calvin cycle to produce carbohydrates.

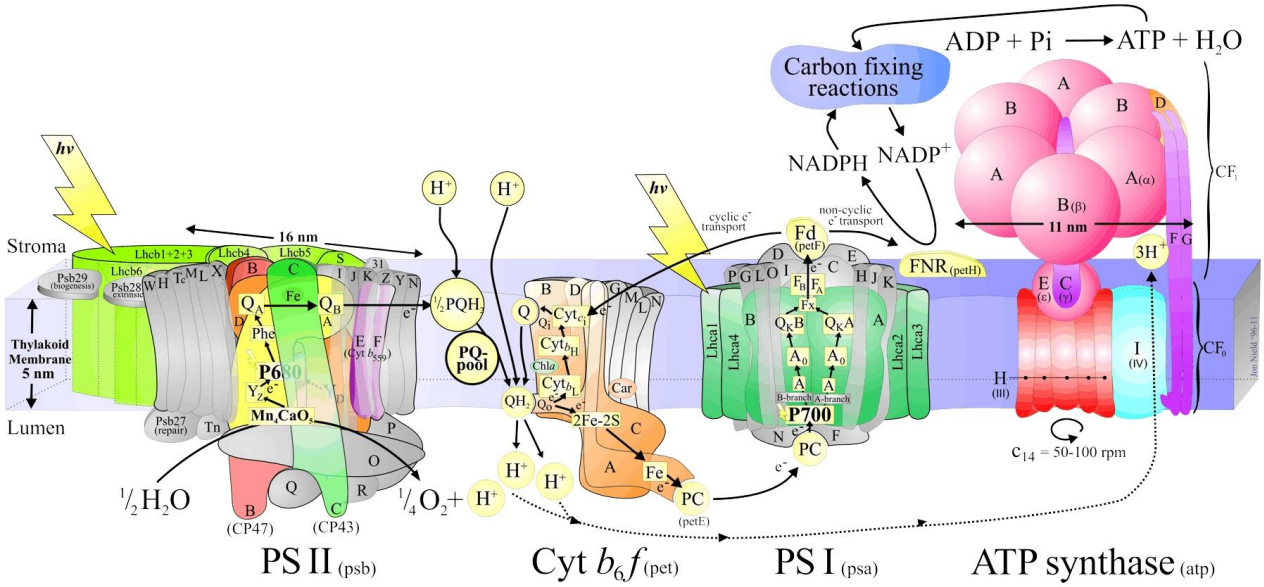

Figure 3: Illustration of the thylakoid membrane, showing PSII, Cyt b_6f, PSI and the ATP synthase system. Through water splitting, electrons are reducing PSII and protons are released into lumen. Electrons are excited by sunlight in PSII, and then are transported through several redox-reactions in the electron transport chain, generating NADPH. Ultimately, protons from lumen are sent through the ATP-synthase system, gaining ATP. ATP and NADPH are then used in the Calvin cycle. Illustration from QueenMaryUniversityLondon (2014).

The next step is the ATP synthase (Taiz & Zeiger 2010). Protons, both from the water splitting and the plastoquinone system, are highly concentrated in the lumen. Due to electrochemical potential gradient, the protons are pushed through the thylakoid membrane to stroma. This process synthesizes ATP, used in the Calvin cycle.

Biomass formation / The Calvin Cycle

The Calvin cycle captures inorganic carbon (CO_2) from the atmosphere and transforms it into simple carbohydrates. This happens in three steps. First, ribulose-1.5-bisphosphate (rubisco) is carboxylated by CO_2 and H_2O, which forms 3-phosphoglycerate. Second, 3-phosphoglycerate is reduced to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. Dihydroxyacetone leaves the cycle to form carbohydrates. This reaction demands one ATP and one NADPH from the light reactions. Third, ribulose-1,5-biphosphate is regenerated , using glyceraldehyde 3-phosphate and another ATP (Taiz & Zeiger 2010). This way, the cycle is complete, ready to accept more carbon.

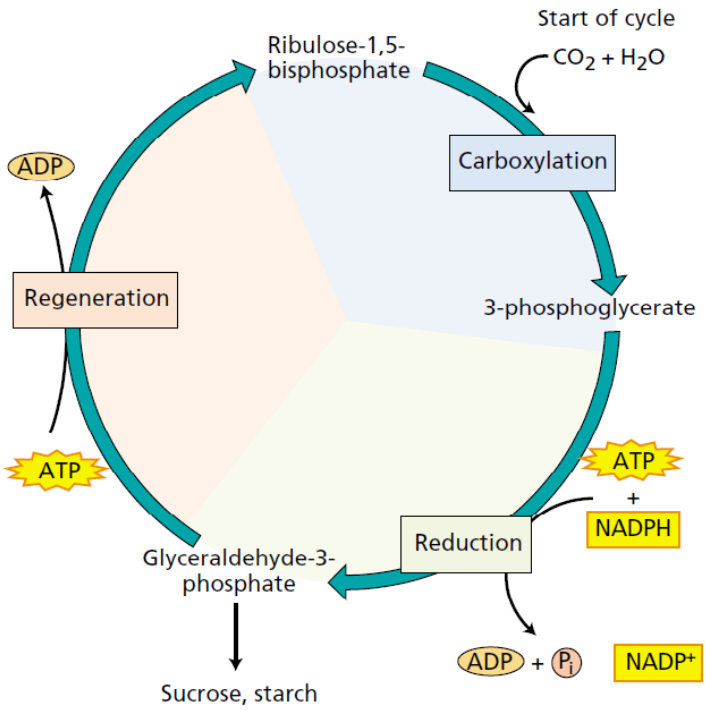

Figure 4: The Calvin cycle. The illustration shows the three steps carboxylation, reduction and regeneration of ribulose-1.5-phosphate. The cycle consumes CO2, H2O, ATP and NADPH, and gains NADP+, ADP and carbohydrates. From Taiz and Zeiger (2010).

The Calvin cycle is said to be light independent. Nevertheless, certain reactions are still regulated by light. This includes the enzyme rubisco, the ferredoxin-thioredoxin system (from ETC), and other enzyme-reactions (Taiz & Zeiger 2010).

Starch is one of the main products of photosynthetic carbon fixation (Klein 1987; Taiz & Zeiger 2010). In *Chlamydomonas*, high accumulation of starch is of particular interest, as the cell breaks down starch and produces hydrogen under anaerobic conditions (Melis 2002). Starch is made of amylose and amylopectin, and is synthesized during the day in the chloroplasts. During the night, linkages of the starch molecule may be cut to release maltose and glucose to support growth and other structures (Taiz & Zeiger 2010).

1.2.1 Hydrogen production

The first discovery of the ability to microalgae to produce hydrogen, was done by Gaffron and Rubin (1942). Later, a method called 2-stage photosynthesis and H_2-production has been presented (Ghirardi et al. 2000; Melis et al. 2000). This method consists of removing sulphur from the growth medium, which slows down the repair of D1 protein of the PSII, gradually making the culture anaerobic. In anaerobic conditions, starch or acetate will be the main electron donors. In addition, ferredoxin transfers its electrons to hydrogenase instead of ferrodoxin-$NAPD^+$ reductase. The electrons are then donated to protons and hydrogen gas is produced (Ghirardi et al. 2000; Melis & Happe 2001; Pyo Kim et al. 2006).

1.3 Influence of light on photosynthesis and growth

1.3.1 Light-response curve

Light is the most important factor in growth of photoautotrophic organisms. Richmond (2008) stated that if all other growth conditions are optimal, light intensity is the sole factor to limit growth in a thin culture.

The light-response curve (Figure 5) illustrates the influence of light intensity on photosynthetic rate. Light-response curves are made by measuring CO_2 fixation rates at increasing photon flux densities (PFD) (Taiz & Zeiger 2010). The curve starts below zero as measurements starts in darkness. In the dark, the respiration releases CO_2, which translates into negative CO_2 fixation rate. While increasing the light, the CO_2 fixation will at one point match CO_2 release. This is the light compensation point (I_c). When increasing the light further, the photosynthetic activity increase until the photosystems are saturated (I_s). From here on, increasing the light will not increase photosynthetic activity any further. At a certain level of PFD, the strong light will inhibit the photosystems (I_h). After this point, the photosynthetic activity decreases. The value of light intensity that leads to saturation and inhibition, depends on other growth factors, such as temperature, CO_2 and nutrients (Sorokin & Krauss 1958).

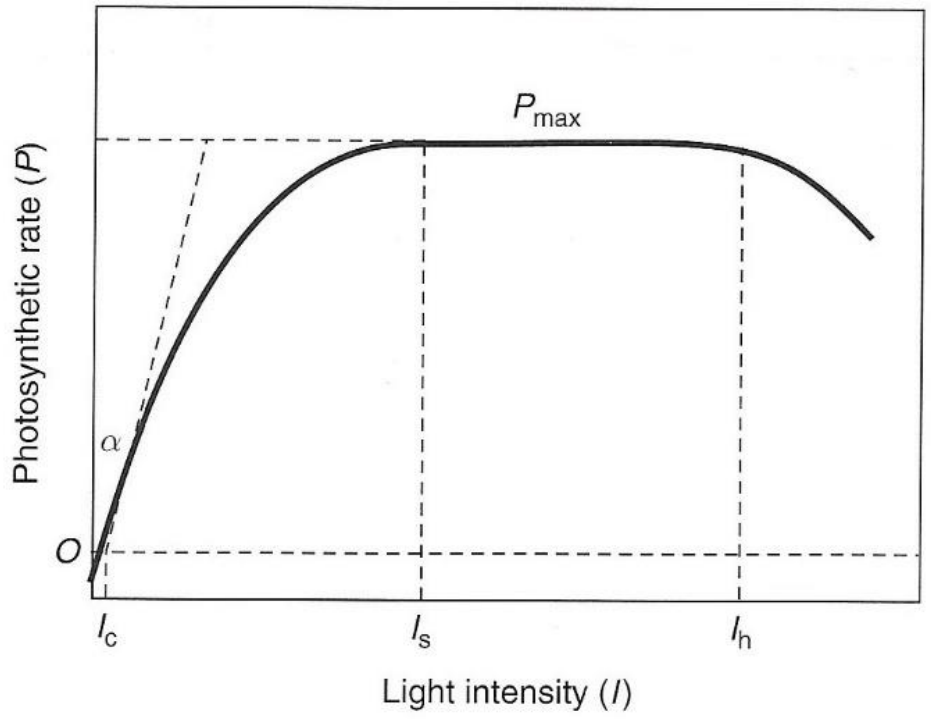

Figure 5: Light-response curve for photosynthetic organisms. The curve shows the relationship between photosynthetic rate and absorbed light (photon irradiance). I_c: light compensation point; I_s: Light saturation intensity; I_h: point of photoinhibition. From Richmond (2008).

Plants or microalga-cultures grown in high light intensities over time, may adapt to the environment, often by decreasing chlorophyll content and chlorophyll antenna size (light harvesting complexes) (Bonente et al. 2012; Polle et al. 2002; Taiz & Zeiger 2010).

1.3.2 Optimal cell density and self-shading

As stated by Richmond (2008), light energy received by photoautotrophic cells is a function of the photon flux density (PFD) reaching culture surface. Further into a culture, the cell density may affect photosynthetic productivity by decreasing amount of photons emitting through (Myers & Graham 1959). For a given light source an optimal cell density (OCD) exists (Myers & Graham 1959). OCD represents the highest net yield of cell mass per illuminated area. If the culture is dense, shading will occur. In shading cultures, the cells in the outer part of the growth system will absorb a greater part of the photons irradiated on the system. The light cannot reach further into the culture, making the inner cells less irradiated. A shaded culture can be divided into two areas: outer zone (photic zone) and the dark volume (Richmond 2008). In the photic zone, the photosynthetic system is saturated by light, but in the dark volume the light is below the compensation point (Figure 5: I_c) and photosynthetic activity is low.

1.3.3 Light/dark (L-D) cycles

In production systems, the culture is often in movement and cells change place between the photic zone near the light source and darker volume further into the culture. This leads to periodic illumination, named light/dark (L-D) cycles (Richmond 2008). Periodic illumination helps photoautotrophic cells utilize strong light without being damaged by chronic photoinhibition, especially in dense cultures (Burlew 1953; Grobbelaar 1991; Ogbonna et al. 1995; Richmond 2008). Periodic illumination can be used intentionally with a light system that provides light periodically, or by letting the culture be moved from a dark zone to a strongly illuminated zone, so that illuminated and dark cells changes places continuously.

1.3.4 Photoinhibition: Damage to photosystem II caused by strong light

Periods of high light intensity easily damage the photosystems. The phenomenon of photo-induced damage is called photoinhibition (Taiz & Zeiger 2010), and is a result of more light

energy absorbed by the PSII than it can utilize. Photo-induced damage appears as a decrease in DW and breakdown of chlorophyll.

Photoinhibition mainly affects PSII. Risk for photoinhibition depends on the redox state of the primary quinone acceptor (Q_A) in PSII. When Q_A is oxidized and ready to accept electrons, light absorption leads to electron transport from water to plastoquinone. The electrons go through the electron transport chain as usual, and the energy is quenched. If Q_A is reduced, the electron transfer chain will be altered, and this might lead to damage (Melis 1999), and the excess light excitation may damage the D1 protein directly (Taiz & Zeiger 2010). Another possibility is that excess electrons from PSI may lead to formation of excited oxygen molecules (reactive oxygen species (ROS)), if the $NADP^+$-sinks are outnumbered (Taiz & Zeiger 2010). ROS is extremely reactive, and may damage cell structures and lead to cell death. To prevent damage from the excess light excitation, the photosystems have protection systems. Carotenoids work as quenchers, and overtake the energy and dissipate it as heat through the xanthophyll cycle (Taiz & Zeiger 2010).

There are two types of photoinhibition: dynamic (reversible) or chronic, depending on the amount of excess light. Dynamic photoinhibition occurs in moderate amount of excess light. It can be detected as a decrease in quantum efficiency, and is caused by the turnover from light energy to emission of heat by the xanthophyll cycle. In dynamic photoinhibition, the maximum photosynthetic rate is not altered. Even stronger light and extreme conditions leads to chronic photoinhibition. If the photoinhibition is chronic, both quantum efficiency and maximum photosynthetic yield decrease. In this case, the D1 protein in the PSII centre is damaged and needs to be replaced by a newly synthesized protein. Damaged reaction centres are moved from the grana to the stromal lamellae, and damaged D1 is degraded. Then, new D1 proteins are synthesized. The D1 protein damage and repair occurs in dynamic photoinhibition as well, but in a pace that prevents decrease in maximum yield (Adir et al. 2003). In chronic photoinhibition, the process from chronic damage to complete recovery may take weeks or months (Taiz & Zeiger 2010).

Photoinhibition may occur more easily in certain conditions, such as low CO_2 or unfavourable temperature, or the presence of acetate in the medium (Fischer et al. 2006).

Chlorophyll fluorescence

Measurement of chlorophyll fluorescence is an often-used method of determining the activity of the photosystems. Its main advantage is that the method does not damage plant materials, and is thus suitable for on-going experiments.

As mentioned, absorbed light (photons) may excite electrons in the photosynthesis or be dispersed as heat. A third possibility is to re-emit the photons as light with higher wavelengths, - a phenomenon called chlorophyll fluorescence. Re-emitted light has a longer wavelength than the absorbed light. Illuminating plant material with a certain wavelength enables registration of the re-emitted chlorophyll fluorescence (Maxwell & Johnson 2000).

Prior to measuring fluorescence, the plant cells must be dark adapted to close the PSII reaction centres. This process takes about 15-20 minutes. When exposing plant cells to light again, the yield of chlorophyll fluorescence will quickly increase, before decreasing during the next minutes. This is fluorescence quenching. Two pathways can explain this reaction: photochemical quenching or non-photochemical quenching. In photochemical quenching, the rate of electrons transported away from PSII increases, due to light-induced activation of enzymes involved in carbon metabolism. In non-photochemical quenching, there is an increase in efficiency with energy converted to heat (Maxwell & Johnson 2000).

Prior to analysing fluorescent signals, one of the two pathways of non-photochemical and photochemical quenching needs to be turned off. Often a light doubling technique shuts the photochemical pathway, where an intense rapid light pulse saturates and closes the PSII reaction centres. The fluorescence yield will then reach a value equal to the value with no photochemical quenching (maximum fluorescence, F_m) (Maxwell & Johnson 2000).

To test the efficiency of PSII, one can measure Fv/Fm (quantum efficiency (yield), or maximum efficiency of PSII) (Maxwell & Johnson 2000) (see Equation 1, Appendix 1). Quantum efficiency reflects the amount of the absorbed photons that is used for generating chemical compounds, and is calculated by dividing the number of photochemical products by the total number of quanta absorbed (Taiz & Zeiger 2010).

Under optimal conditions, the Fv/Fm value should be around 0.83 (Maxwell & Johnson 2000). This means PSII reaction centres are open and reflects the maximum efficiency of light utilization. If the Fv/Fm value is below 0.83, the photosystems have been exposed for stress and are photoinhibited.

1.4 Nutrition

As higher plants, green algae have a demand of certain essential elements. For an element to be essential, the plant cannot fulfil its lifecycle without it, deficiency can only be prevented or treated with that particular element, and the element must be directly involved with nutrition (Arnon & Stout 1939). Essential elements includes the macro nutrients nitrogen, potassium, calcium, magnesium, phosphorus, sulphur and silicon, the micronutrients chlorine, iron, boron, manganese, sodium, zinc, copper, nickel and molybdenum; and obtained from air or water: hydrogen, carbon and oxygen (Richmond 2008; Taiz & Zeiger 2010). The content of nutrients in a medium depends on autotrophic or photoautotrophic growth (Richmond 2008). In addition to macronutrients and trace elements, chelates (EDTA) also are needed. The area close to a plant cell has often a low pH. Chelates wrap pH-sensitive trace elements and transfer them from the media and to the cells (Marschner & Marschner 2012).

The composition of nutrients in a medium depends on autotrophic or photoautotrophic growth (Richmond 2008). *Chlamydomonas* can be grown photoautotrophically, retrieving carbon in the form of CO_2 from the environment, requiring light for growth; heterotrophically on added organic carbon, often acetate, in the growth media, grown with no light; and mixotrophically, grown in light with acetate in the medium (Harris 2009).

Carbon, nitrogen and phosphorus are vital due to their roles as electron acceptors and energy precursors in the photosynthesis. For photoautotrophic growth, carbon is retrieved as CO_2 from the atmosphere. For heterotrophic growth in darkness, carbon must be added in the growth medium, often in the form of acetate (Harris 2009). Nitrogen is vital as an NADPH (nicotinamide adenine dinucleotide phosphate)-precursor and in biomass production (Richmond 2008). Phosphorus is a precursor for the energy compounds NADPH and ATP (adenosine triphosphate). Phosphorus is often supplied as phosphate (PO_4^{3-}).

Calcium and magnesium are essential for maintaining permeability of the cell membrane (Taiz & Zeiger 2010), and thus affect uptake of other nutrients positively. Calcium is particularly important in cell division (Sanders et al. 1999; Taiz & Zeiger 2010). Magnesium is vital for photosynthesis (formation of chlorophyll), respiration and synthesis of DNA and RNA (Taiz & Zeiger 2010).

As uptake of nutrients is dependent on an optimal pH (Bævre & Gislerød 1999; Marschner & Marschner 2012), it is useful to add a buffer to the growth medium. The optimal pH is 7.6, but is highly affected by certain physiological reactions. In photosynthetic CO_2-fixation, OH^- will

accumulate in the growth solution and pH will increase. Meanwhile, CO_2 release during respiration triggers an opposite reaction: pH decrease (Richmond 2008). In addition, the source of nitrogen affects the pH in the medium. Uptake of ammonium chloride releases H^+ ions. A buffer keeps the amount of protons in the solution stable.

Competition between ions with similar electronic charge often occurs. Therefore, a balanced amount of nutrients is needed to provide for certain elements. Common examples are nitrogen and chlorine; and potassium and nitrogen (Bævre & Gislerød 1999).

1.4.1 Growth medium

A wide range of growth media can be used for *C. reinhardtii* (Harris 2009). Choice of medium depends on many factors, such as sources of carbon and nitrogen, original habitat, cell composition, demand of trace elements, pH and vitamins, and purpose of algae production (Grobbelaar 2004; Harris 2009; Merchant et al. 2006; Vonshak 1986). The fact that in nature *C. reinhardtii* can be found in various environments suggests that it thrives in a variety of different media (Merchant et al. 2006). The most common medium is the Tris Acetate Phosphate (TAP) medium (Harris 2009). However, in this study, Sueoka's High Salt medium (HS) was used. This medium contains no acetate, which makes it suitable for photoautotrophic growth with CO_2 as the sole carbon source (Harris 2009).

In a culture containing acetate, acetate will be the main substrate for respiration (Melis et al. 2000). Media containing acetate will sooner enter an anaerobic phase, as respiration uses oxygen. Without acetate or atmospheric carbon, the carbon source will be starch, which can be broken down and utilized.

To see nutrients in a *C. reinhardtii*-medium in perspective with nutrient solutions of the other greenhouse grown crops of roses and tomatoes, content of nutrients in their respective growth solutions are listed in Table 1. The most obvious differences is the large amounts of phosphorus and potassium in the microalgae nutrient solution. Phosphorus has a high risk of being growth limiting for algae grown in liquid solution as it easily precipitates, which demands for high amounts (Grobbelaar 2004). In addition, K_2HPO_4/KH_2PO_4-compounds work as a buffer in the medium. Associated with this, the high amount of potassium may be explained by it being added as K_2HPO_4/KH_2PO_4-compounds (Harris et al. 1989). K_2HPO_4/KH_2PO_4 also improve the buffer capacity (Harris 2009). Moreover, the amount of calcium, magnesium and sulphur is rather low in the microalgae nutrients solution. These

nutrients are involved in cell division, chlorophyll formation and enzyme activity (Bævre & Gislerød 1999; Taiz & Zeiger 2010).

Table 1: Content of nutrients in ionic form (mg L^{-1}) in the final solution of Sueoka's high salt (HS) medium, a nutrient solution for greenhouse roses and a nutrient solution for greenhouse tomatoes. *Nutrient solution for greenhouse roses is from Bævre and Gislerød (1999). **Nutrient solution for greenhouse tomatoes: TotalGro 3-13-29 plus calcium nitrate. Source: TotalGro, P.O. Box 805, Winnsboro, LA 71295 (1- 800-433-3055) (Snyder 2001).

	Content of nutrients (ionic form) in mg L^{-1}		
Macro nutrients	HS-medium	Nutrient solution roses *	Nutrient solution tomatoes **
N	131.0	195.0	110.0
P	420.0	40.0	49.0
K^+	853.4	208.0	240.0
Ca^{2+}	2.7	150.0	100.0
Mg^{2+}	2.0	33.0	54.0
S	2.6	45.0	110.0
Micro nutrients			
Zn^{2+}	3.7	0.1	0.5
B	2.5	0.3	1.0
Mn^{2+}	0.9	0.9	1.0
Co	9.4	-	-
Cu^{2+}	0.3	0.1	1.0
Mo	0.8	0.1	0.1
Fe^{2+}	0.7	1.6	3.4

2 General Materials and Methods

2.1 Organism

Chlamydomonas reinhardtii, strain 137c(+) was obtained from NIVAs culture collection, where it was named CHL153. This strain belongs to one of three strains derived from G. M. Smith collection of 1945 (Harris 2009). This particular strain has a mutation and lacks the nitrate reductase enzyme, which makes it unable to grow on nitrate as a nitrogen source (Harris 2009).

2.2 Nutritional medium

C. *reinhardtii* was kept in two different growth stages: as a stock culture on agar and as a growth culture in a liquid medium. TAP-agar was used for the stock culture, while a liquid Sueoka's High Salt (HS) medium was used for the growing algae.

Both the TAP- and HS-medium were made in the Plant Cell Laboratory, SKP. pH was adjusted to 7.6 by adding 1M HCl. Daylight experiments number one and two and light period experiment number one were carried out without buffer. In all of the other experiments, 10-20 mL of 1 M $NaHCO_3$ were added to the medium to buffer the cultures.

TAP-agar

Stock cultures of *C. reinhardtii* were grown on TAP-agar (see Table 19 – Appendix 2). The stock cultures were transferred to fresh TAP-agar every three months.

pH was adjusted to 7.0 by adding 1 M HCl. The mixture was heated to 95 °C before adding agar. Then, agar was poured into Petri dishes, before autoclaved. The recipe gives a total of 250 mL TAP-agar, which corresponds to 10 Petri dishes.

Sueoka's High Salt-medium (HS)

The liquid HS-medium was used for all experiments. Stock solutions of salts, phosphorus and trace elements are shown in

Table 20 (appendix 2). Stock solutions were prepared in advance and stored at 4 °C until the medium was made. For the recipe of the HS-medium, see

Table 20 and Table 21 (listed in order of addition). pH was adjusted to 7.6 before autoclaving the medium. After autoclaving, the medium was cooled and stored at 4 °C until the start of

experiment. Prior to inoculating or starting experiments, the medium was room tempered. Buffer was added in some of the experiments to maintain a stable pH.

Inoculating culture

Some weeks prior to experiments, the algae stored on a TAP-agar were transferred to a freshly made liquid HS-medium. The liquid culture was then grown at the same conditions as the control in the experiments (normally PFD 200 μmol m^{-2} s^{-1}, 25 °C and 3% CO_2). The culture was inoculated every 3-4 days to be kept exponentially growing. When inoculating, about 2-3 mL from the mother culture were added to 320 mL fresh and room tempered HS-medium.

2.3 Photobioreactors (PBRs)

The photobioreactors used in the experiments were special made 380 mL glass tubes placed on a plastic rack in an aquarium with fluorescent lighting tubes on the backside (Figure 6, Figure 7). The aquarium was filled with water to control temperature. The PBRs were closed with a rubber cork. Through the rubber cork, glass tubes of inner diameter of 3.0 mm were providing gas exchange between the surrounding air and the inside of the PBRs. Air containing 3% CO_2 was added via a slim rubber hose.

The temperature of the water was kept at 25±1 °C using aquarium heating cobs (Eheim Jäger 3619 Aquarium Heater, 300 W, 220-240 V, Germany).

In the daylight-, light pulse- and CO_2-experiments, the temperature was registered manually, and deviations were noted. If the temperature was too high, a censor reacted and started to cool the water. In addition, the water pipes in the aquaria used in an experiment were connected with each other and an aquarium pumping system. The water was always in movement, providing a stable and homogenous temperature. To keep the temperature within wanted range, one could speed up or slow down the water supply to the given aquarium.

In the nutritional salt-experiments, the temperature was controlled using the computer software PLW PicoLog Recorder (UK).

The CO_2 level in the air bubbling was kept at 3.0% for all experiments. The bubbling in the tubes also ensured the mixing of the culture.

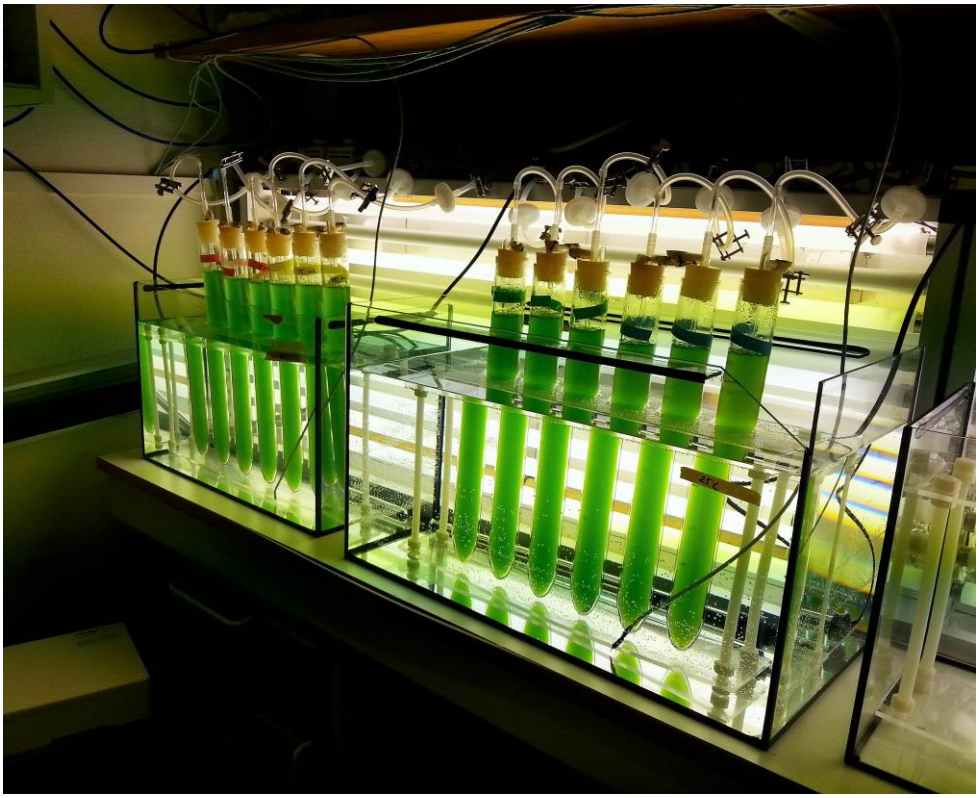

Figure 6: Illustration of photobioreactor-systems used in all the experiments. Note that the water exchange system (temperature control) is absent, unlike the systems used in the daylight- and high light exposure experiments. In illustration above, temperature was controlled by keeping a stable room temperature and heating cobs. M. Evjen, 2014.

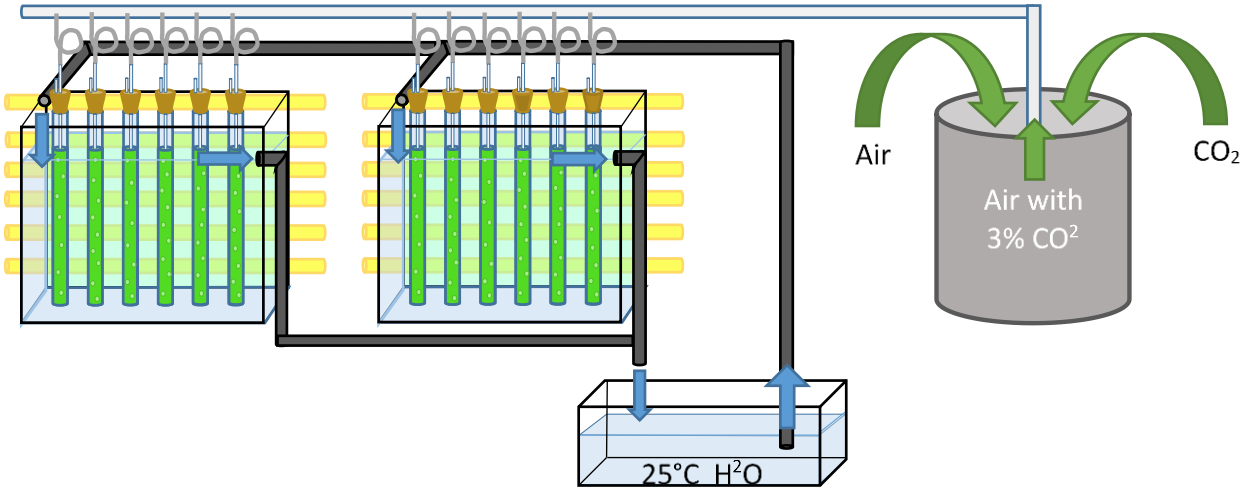

Figure 7: Illustration of photobioreactors used in the daylight and light period experiments. Aquaria were connected with water pipes to control temperature. Water was transported through the system using a pump placed in the water tank (bottom of the illustration). The water tank also contained a temperature control system. Blue arrows indicate flow direction of water. Green arrows indicate the flow direction of the air and CO_2 mix. In the light period experiments, three aquaria were connected in this way. In front of two of the aquaria, a high pressure sodium light system was placed (not shown in the illustration).
M. Evjen, 2014.

Greenhouse system

The concentration of CO_2 in the gas was controlled by mixing pure CO_2 with air in a large container. The desired amount of CO_2 was plotted in percentage into a connected computer using the computer software PC200W 4.0 data logger. Then, the diluted CO_2 was distributed to the algal tubes through a pipe system. The amount of CO_2 in the gas was monitored in real time using a CO_2-logger and adjusted as needed.

Bioreactor-room system

The CO_2-system in the photobioreactor-room was slightly different from the greenhouse. The amount of CO_2 was controlled by pressure using a capillary tube in a water container.

Pure CO_2-gas was transported from a CO_2-tank by tubes and through a valve before mixed with air in a container. The air-container was connected to the PBRs. A capillary tube connected to the CO_2-transport tube, was placed vertically in a water container. Thus, excess CO_2, was blown through the capillary tube, and then bubbled out through the water. Raising the capillary tube would decrease the pressure through the valve (seen by more CO_2 disappearing as bubbles), thus lower the amount of CO_2 added to the air and vice versa.

The background light of the bioreactors consisted of up to eight cool white fluorescent tubes. In total, the fluorescent tubes would give a photon flux density (PFD) of 200 μmol m^{-2} s^{-1}. The light intensity could be decreased by disconnecting one or more of the tubes. The light intensity was measured using a light meter (SKP 200/217/140, Skye Instruments, UK) in an empty algae tube in the aquarium. The light intensity value was determined by averaging measurements evenly distributed through the aquarium.

2.4 Cell counting

Prior to each experiment, cell number of start culture was determined using a haemocytometer (0.200 mm, 0.0625 mm^2, Fuchs-Rosenthal, Tiefe, DK). Then, the amount of algae needed for a start culture of 10 000 or 50 000 cells mL^{-1}, was calculated using Equation 2, Appendix 1.

2.5 Sampling

All samples were taken using an electronic pipette (Falcon Express Becton Dickinson Labware, S/N98154) with 10 mL pipette tips (VWR Serological pipettes). Approximately 6

mL from every tube were collected directly from the PBRs and poured into glass test tubes. The procedure was done as clean as possible, by working quickly using rubber gloves and sterile pipette tips. In experiments where photoinhibition was measured, 5 mL extra algal culture was collected in an additional test tube. The photoinhibition samples were immediately placed in a dark place for dark adaption.

2.6 pH measurements

pH measurements were done using a pH-meter (Orion 420A+). The pH-meter was calibrated on a daily basis. pH was measured in one sample at the time, directly from the test tubes. Test tubes were not in any way mixed prior to pH measurements. pH-values were noted, and the average of the three treatment parallels was calculated.

2.7 Absorbance

Absorbance (optical density (OD)) was used as an indicator of the growing stage of the cultures. The measurements were done with a spectrophotometer (Helios α Unicam UVA-054710) at 750 nm. The spectrophotometer was calibrated using diH_2O.

The absorbance levels were kept between 0.4 and 0.7 absorbance units (AU), as this was within the range within which the method of measurements was correct. (See testing the method of optical density, chapter 2.12.) If the absorbance exceeded 0.7 AU, the sample was diluted with diH_2O.

2.8 Fluorescence

Photoinhibition (Fv/Fm) was recorded with a fluorometer (Fluorpen FP100 SN-FP-244). Samples for photoinhibition analysis were taken separately. About 5 mL of liquid algae culture were needed for this procedure to be certain the fluorometer was able to reach the liquid inside the 13 mL glass test tube. After sample taking, the test tubes were placed in a dark place (cupboard or similar) for 15 minutes to achieve dark adaption. Then, in a dim lighted room, the Fv/Fm value was measured in one test tube at the time. The content of the tube was carefully mixed by inversion. The tube was then placed on a separate test tube rail, to prevent the remaining tubes to be affected by the measuring light from the fluorometer.

Then, Fv/Fm was measured. After all tubes were measured, the remains from the sample were used for starch and chlorophyll analysis.

2.9 Dry weight

Dry weight (DW) was determined using GF/F glass microfiber filters (0.7 µm pore size, 25 mm diameter, GE Healthcare (Whatman)). Prior to measuring, the filters were rinsed in diH_2O to remove dust, which could disturb the DW results, and dried in an oven (Heraeus Function Line) for 4 hours at 95 °C. The pre-weight of each filter was registered using a 3-decimal weight in mg (Mettler Toledo XP6).

DW was determined with three measurements per sample. Each filter was placed on a filter holder device (Cat No: XX2702550, Millipore Corporation Bedford, USA) connected to a vacuum pump (KNF) and rinsed with 10 mL of diH_2O. Algae samples were added, maximum 1 mL to each filter. The volume of algae sample used depended on the density of the culture. The vacuum pump was activated and the algae culture was drawn through the filter. Leaving the pump on, 2x10 mL of diH_2O was added to each filter, to make sure that all of the growing medium was rinsed off. When all of the liquid was drawn through the filter, the filters were dried in the oven for 4 hours at 95 °C. The filters were put in a vacuum desiccator containing silica gel to extract the remaining humidity of the filters. The filters were cooled down to room temperature before the post-weight was registered. The weight of the sample was determined by subtracting the pre-weight of the empty filter from the post-weight of the filter containing biomass.

2.10 Starch and chlorophyll analysis

Analysis for starch and chlorophyll were done using the same sample. 2 mL of each culture were transferred into Eppendorf-tubes and centrifuged (Eppendorf Centrifuge 5417 R, USA) for 20 min at 20 800 x g at 4 °C. The supernatant was removed by pipetting, and the pellet was stored at -20 °C until analysis.

Chlorophyll extraction

Procedure of extraction of chlorophyll was obtained from The Chlamydomonas Sourcebook (Harris et al. 1989).

The frozen samples were thawed in room temperature. To each sample, 200 mL of 95% ethanol was added, then mixed by vortexing to make sure that all chlorophyll was extracted. The tubes were centrifuged at 20°C, 20 800 x g for 15 min. When centrifuged, 0.25 mL of the supernatant containing the extracted chlorophyll were diluted 1:4 in 95% ethanol in the Eppendorf-tubes and mixed by pipetting. The rest of the supernatant was disposed.

Chlorophyll assay

Absorbance of the extracted chlorophyll samples was measured using a spectrophotometer with 1 mL 95% ethanol as a blank. Extracted chlorophyll was poured from the Eppendorf-tubes into cuvettes. The absorbance of each sample was measured at two different wavelengths: 649 nm and 665 nm. In between, the machine was re-calibrated with 95% ethanol.

Chlorophyll content was calculated using Equation 4 (see Appendix 1).

Starch extraction

The following method of starch analysis was based on an enzymic amyloglucosidase/α-amylase method from Megazyme Total Starch Kit (K-TSTA, Ireland), modified to fit microalgae samples by Daria Markina, PhD student at IPV/NMBU. The principle of the assay is to dissolve the starch from chlorophyll-emptied cells, turn it into a gel using DMSO and 100 °C heat, then hydrolyse the gel using α-amylase, before separating the glucose molecules apart using amyloglucosidase. Adding GOPOD will turn the solution pink, and the intensity of the colour is correlated with the amount of starch in the solution.

The cell pellet remaining after chlorophyll extraction was used for the starch assay. The Eppendorf tube containing the cell pellet was left open for some minutes to let the rest of the ethanol evaporate. Then, 20 µL of 80% ethanol were added to each pellet. Samples were mixed by vortexing to dissolve the pellet. Next, 0.2 mL of the solvent DMSO were added to the each pellet, helping to cell disruption and starch solubilisation. Then, about 0.5 mL of 0.5 mm glass beads (Cell Disruption Media, Scientific Industries, 888-850-6208) were added to the solution, completely covering it. Samples were then mixed by vortexing before being placed in a mixer mill (Retsch MM301), at 30 Hz frequency for 10 min. Afterwards, samples were quickly centrifuged in a micro-centrifuge (Labnet, Cat.No.: C1301T-230V) before being placed into heating box at 100^{o}C, 600 rpm for 5 min, solubilizing the starch. Then, 0.3 mL of properly diluted α-amylase were added, to cut the branches in the starch molecules. Samples were then mixed by vortexing. Next, samples were placed into a heating box, at 100 °C for 12

min. During this time, samples were mixed by vortex after 4, 8 and 12 min. Samples were then placed into heating box (Biosan, TS-100 Thermo Shaker), at 50 °C. Next, 0.4 mL Na-acetate buffer were added. Then 10µL of the enzyme amyloglucosidase were added, separating the glucose molecules apart. The samples were mixed, first by pipetting; then by vortexing. Then the samples were left in the heating box at 50 °C for 30 min. 70 µL diH_2O were added to each sample providing a total of 1 mL of each sample. Samples were mixed by vortexing. Then, the samples were centrifuged for 10 min. at 20 °C, 20 800 x g. While centrifuging, twice as many Eppendorf-tubes as there are samples, plus six extra, were marked and set to be ready.1 mL of GOPOD reactant was pipetted into each of the Eppendorf-tubes. In two of the tubes, 33.4 µL of diH_2O was added. This was used as a reagent blank in the measurements. In four other tubes, 33.4 µL of glucose standard from the starch kit was added. This was the glucose blank for the measurements. When the samples were centrifuged, 33.4 µL of the supernatant was added to every Eppendorf-tube of GOPOD, two replica of each. Then, the samples were put in a heating box to incubate at 50 °C for 20 min.

Starch assay

Absorbance was measured using a spectrophotometer at 510 nm. All of the samples, including reagent blanks and glucose blanks were poured into cuvettes. First, the spectrophotometer was calibrated with the reagent blanks. Then, the glucose blanks and all of the samples were measured. If there were many samples, the machine was re-calibrated regularly.

Amount of starch was calculated using Equation 5 (Appendix 1), given in % w/w (weight starch/DW algae in %).

2.11 Statistical analysis

The statistical software Minitab was used in statistical analysis, with the commando One-way Analysis of Variance (ANOVA).

The analysis was conducted with the difference in productivity (g L^{-1} d^{-1}) calculated for the different treatments. The productivity was calculated by dividing the maximum amount of DW during the experiment by the time (d) required to attain the maximum DW or starch. This was done with all three parallels (three PBRs in each experiment) and all repeats done within the parallels (three samples from each PBR) (n=9). The ANOVA-test was used to test if there

was a difference between the treatments on a 95% level of significance. If the p-value was under 0.05, a simple t-test was conducted to compare two treatments.

Statistical significance is presented in the tables next to the productivity-values. Statistical significance is indicated by letters (a, b or c), where same letters means no significant difference between treatments, and different letters indicates a significant difference on a 95 % level of certainty.

2.12 Validating the method: Absorbance

In absorbance measurements of the algae cultures in all experiments in this thesis, the algal culture was diluted when it reached a certain density. To make sure the method of measuring absorbance was accurate, a basic test of the method was conducted.

The absorbance of a sample of liquid culture of *C. reinhardtii* was measured in the spectrophotometer using four different dilutions: 1:1; 1:2; 1:4 and 1:8. Samples were diluted with diH_2O in 1 mL plastic cuvettes. After absorbance registrations, the measured value was divided by the dilution rate and plotted in a graph. The R^2-value was used to determine the validity of the method.

The results of the test are presented in Figure 8 A and B. The measurements had linear distribution, particularly in Figure 8B, where the undiluted value was omitted. This indicates that the method is linear when absorbance values are in the range of 0.2 and 0.7 AU. When the absorbance values exceed 0.7, the sample should be diluted to attain an absorbance between 0.2 and 0.7.

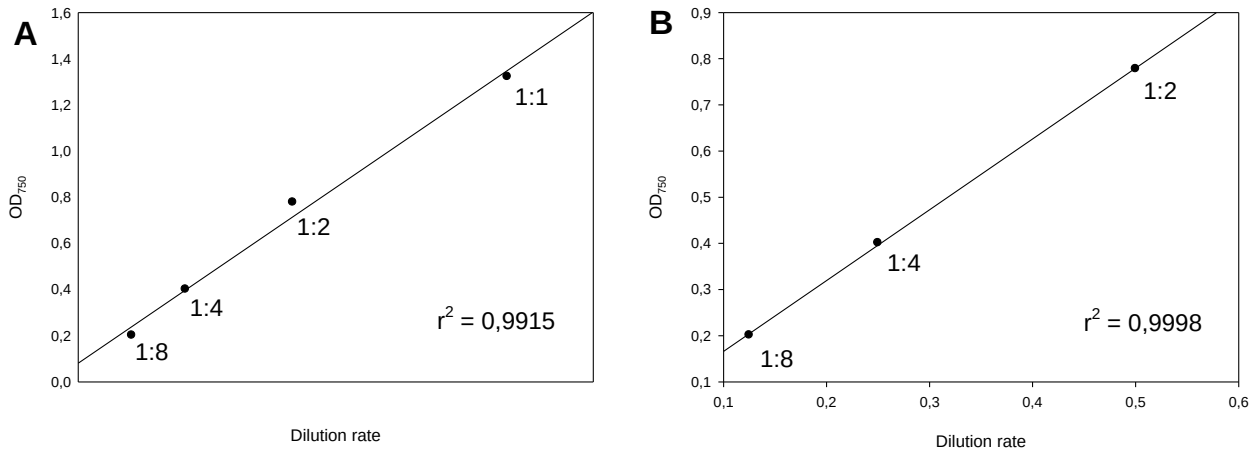

Figure 8: Validating the method of absorbance (OD) measurement: Absorbance of a culture of *C. reinhardtii* in different dilutions. A) Dataset includes undiluted sample (1:1). B) Undiluted sample (1:1) is omitted from the dataset.

3 Specific Materials and Methods

3.1 Effects of daylight (DL) and initial cell densities on growth and starch

The effect of daylight on growth, starch and chlorophyll content of *C. reinhardtii* was tested in the following experiments. The experiments were conducted in an acrylic greenhouse. The natural light in a greenhouse is app. 60% of the outdoor illumination (Bævre & Gislerød 1999).

The experiments were done with three repeats. The first experiment was conducted directly after summer solstice, then the two next experiments with monthly intervals. Due to the different weather conditions during these weeks, the results are presented separately for each experiment.

Materials and Methods

The daylight experiments were carried out with two different light treatments: first: a control treatment with continuous backlight of 200 μmol m^{-2} s^{-1} in addition to daylight (DL+200); and second: daylight only (DL). Within each of these two light treatments, there were two different start cultures concentrations of 10 000 (10c) and 50 000 cells mL^{-1} (50c). Each treatment was done with three parallels, making 12 tubes in total. See Table 2 for diagrammatic view. The microalgae were grown in HS-medium, with 3.0±0.2% CO_2 and 25±1 °C.

Table 2: Overview of the experimental setup for the daylight experiments. The treatments daylight plus 200 μmol m^{-2} s^{-1} with an initial cell density of 10 000 cells/ mL (DL+200 10c) and daylight plus 200 μmol m^{-2} s^{-1}, with an initial cell density of 50 000 cells/ mL (DL+200 50c) had a continuous 200 μmol m^{-2} s^{-1} background light during the whole experiment, in addition to natural daylight. The treatments daylight with an initial cell density of 10 000 cells/mL (DL10c) and daylight with an initial cell density of 10 000 cells/mL (DL50c) were grown with natural daylight only.

	DL+200 10c	**DL+200 50c**	**DL 10c**	**DL 50c**
Initial density	10 000 cells mL^{-1} (ca 0.0007 mg mL^{-1})	50 000 cells mL^{-1} (ca 0.003 mg mL^{-1})	10 000 cells mL^{-1} (ca 0.0007 mg mL^{-1})	50 000 cells mL^{-1} (ca 0.003 mg mL^{-1})
PFD	Daylight + continuous 200 μmol m^{-2} s^{-1}	Daylight + continuous 200 μmol m^{-2} s^{-1}	Daylight only	Daylight only

Samples were collected twice every day, at 08:00 am and 08:00 pm for 7-9 days, recording pH, absorbance and DW. For the two latter daylight experiments, samples for starch and chlorophyll analysis also were collected. The experiments were ended when the cultures had stopped growing according to absorbance measurements.

PFD was measured inside the greenhouse, just in front of the aquaria. The experiments were done in Ås, Akershus, 59 ^{o}N. The photobioreactors were placed in aquaria in a double acrylic greenhouse faced east west, providing sunlight all day. Inside the greenhouse, the aquaria were placed on a table facing south. A wall provided shadow on the backside (north) of the photobioreactors. In this way, there was not much light to consider before and after the samples were taken (08:00 am and 08:00 pm).

First repeat – June/July 2013

The first repeat of the daylight experiment was conducted during the weeks 26-27, 2013 (June 24th – July 2nd). This was shortly after summer solstice (June 21st), and the days were at their longest.

June 24th: Sunrise: 03:57 am
Sunset: 10:41 pm
= 18 h 43 min light hours.

July 2nd: Sunrise: 04:04 am
Sunset: 10:38 pm
= 18 h 34 min light hours.

Second repeat – July/August 2013

The second repeat of the daylight experiment was conducted during the weeks 31-32, 2013 (July 29th – August 5th).

July 29th: Sunrise: 04:53 am
Sunset: 10:03 pm
= 17 h 10 min light hours.

August 5th: Sunrise: 05:09 am
Sunset: 09:35 pm
= 16 h 24 min light hours.

Third repeat – August/September 2013

The third repeat of the daylight experiment was conducted during the weeks 36-37, 2013 (September 2nd – September 10th).

September 2nd:	Sunrise:	06:15 am
	Sunset:	08:16 pm
	= 14 h 01 min light hours.	
September 10th:	Sunrise:	06:34 am
	Sunset:	07:52 pm
	= 13 h 18 min light hours.	

3.2 Effects of high light (HL) exposure on growth, starch and chlorophyll

The high light exposure experiments were conducted as a simulated version of daylight experiments, but by more controlled terms. Cultures were illuminated for six hours every day with high light intensity, with a continuous backlight of 200 μmol m^{-2} s^{-1}. The scientific question was to investigate the effect of high light exposure on growth and quality.

Materials and Methods

The light period experiment was conducted in the same green house as used in the daylight experiments. For this experiment, a tent of light-reflective dark curtains was set up to prevent daylight disturbance. The tent was not 100% lightproof, as there was gaps by the top and the bottom to ensure air circulation, to prevent heated temperatures. With all artificial lights off, the light measured inside the tent was approximately 9 μmol m^{-2} s^{-1}. All start cultures had a cell density of 10 000 cells mL^{-1}.

Light treatments

In the high light exposure experiments there were three different treatments (

Table 3). First, 200μmol m^{-2} s^{-1} continuous backlight plus 6 hours light period of 500μmol m^{-2} s^{-1} (medium light: ML); second, 200μmol m^{-2} s^{-1} continuous backlight plus 6 hours light pulse of 1000μmol m^{-2} s^{-1} (high light: HL); and third, a control with continuous backlight of 200μmol m^{-2} s^{-1} only (control), with no light periods.

Inside the tent, three aquaria were set up in the same manner described in chapter 3.1.5. A high-pressure sodium (HPS) light system (GAN 4-550 AL 230 V, Superagro Norway; Philips, 400 W light bulbs) was set up in front of the aquaria with ML and HL treated cultures. The number of lamps and distance from the lamps to the algal tubes determines the light intensity.

A timer clock set for 6 hours controlled the length of the light periods: from 09:00 am to 3:00 pm. Fluorescent cool white tubes provided continuous backlight.

Table 3: Overview of the experimental setup for effect of light period on microalgae *C. reinhardtii*. All treatments had a continuous backlight of 200 μmol m^{-2} s^{-1}. The ML treatment was illuminated with a 6 h light period of 500 μmol m^{-2} s^{-1}. The HL treatment was illuminated with a 6 h light period of 1000 μmol m^{-2} s^{-1}. All treatments had three parallels.

	ML	**HL**	**Control**
Backlight	Continuous fluorescent backlight: 200 μmol m^{-2} s^{-1}	Continuous fluorescent backlight: 200 μmol m^{-2} s^{-1}	Continuous fluorescent backlight: 200 μmol m^{-2} s^{-1}
Light Period	6 h light period: 500 μmol m^{-2} s^{-1}, HPS	6 h light period: 1000 μmol m^{-2} s^{-1}, HPS	-

Sampling was done twice a day: before and after the light period (8:45 am and 3:00 pm). pH, absorbance, photoinhibition, DW, starch and chlorophyll were recorded with every sampling. Experiments were done with three repetitions: week 33, 35 and 41, 2013. Each experiment was ended when the culture stopped growing according to the absorbance measurements (about six days).

3.3 Additional experiments

During the daylight- and high light exposure experiments (chapter 4.1 and 4.2), maximum DW never exceeded 1.5 mg mL^{-1}. In similar experiments conducted by Leiv Mortensen (2013), maximum DW have reached over 3.0 mg mL^{-1} (personal communication). It was therefore of interest to investigate the difference between the methods leading to this difference in the results.

The main differences between the materials and methods between the experiments are:

a) The amount of air bubbling containing 3% CO_2
b) The water quality used in the nutritional medium: diH_2O or tap water

3.3.1 Effects of air bubbling velocity

In experiments conducted by Mortensen (unpublished), the amount of air bubbling containing CO_2 was given in a higher rate. In this experiment, differences in growth due to amount of air bubbling containing 3% CO_2 were investigated.

Materials and Methods

Three different gas bubbling regimes were used: low, medium and high bubbling. Low bubbling was similar to the level used in previous experiments, high bubbling was as high as possible with the available equipment, and medium bubbling was at an intermediate level between high and low bubbling (Table 4). For all of the treatments, CO_2 was mixed with air at 3.0±0.2%. Each treatment was carried out with three parallels of PBRs in addition to one plastic bottle of one litre. Concentration of start culture was 10 000 cells mL^{-1}, PBRs were illuminated continuously at a PFD of 200 μmol m^{-2} s^{-1} by cool white fluorescent tubes, and the cells were grown at 25 °C. Gas bubbling rate was measured with a flow meter for the high bubbling treatment. For the low and medium bubbling treatments, the bubble speed was too low to be registered with a flow meter. Instead, it was measured roughly by registering the time needed to fill a plastic bag of 1 litre. Thus it was hard to differentiate between the low and medium bubbling using this method.

Table 4: Overview of the experimental setup for the gas bubbling experiment on growth of microalgae *C. reinhardtii* cultures. There were three different gas bubbling treatments of 3% CO_2: low, medium and high bubbling, where previous cultures were grown with something between low and medium bubbling. All treatments had a background light of 200 µmol m^{-2} s^{-1} and a temperature of 25±1°C.

	Low bubbling	**Medium bubbling**	**High bubbling**
Gas bubbling rate (L h^{-1})	Ca 3.3	(not measured)	Ca 150.0
Bubble size (cm)	0.7	1.5	Large
Bubble release speed (bpm)	220	220	High

The experiment started November 4th, 2013. Aquaria with PBRs and plastic bottles were placed in a shading tent put up in a greenhouse. For the algae grown in PBRs, sampling was done once a day by collecting about 8.0 mL culture with an electronic pipette. During the experiment, pH, optical density, DW, starch and chlorophyll was registered in mentioned order. For the cultures grown in plastic flasks, samples were collected at the end of the experiment due to technical issues.

3.3.2 Use of extra calcium and magnesium or tap water

The low growth in previous experiments could be due to lack of magnesium and calcium in the growth medium. Studies have shown that in a medium for optimized growth, the demand of calcium and magnesium may be higher than what the HS-medium supplies (Kliphuis et al. 2012). In addition, high levels of these nutrients are likely to be found in Norwegian tap water. In his experiments, Mortensen (unpublished) prepared the HS-medium with tap water, instead of deionized water, and reached a DW of 3-4 g L^{-1} (personal communication).

Materials and Methods

The recipe of HS-medium was modified by splitting Beijerincks Solution in two: solution 1a: nitrogen and sulphate; and solution 1b: magnesium and calcium (Table 5). In this way, the amount of calcium and magnesium could be altered without affecting the amount of nitrogen, sulphate, phosphate and trace elements.

The experiment was conducted with different concentrations of calcium and magnesium in the medium (Table 6). The growth media of treatment 1-3 contained various amounts of the magnesium and calcium-solution, prepared with deionized water. The medium in treatment 4 was prepared using tap water.

1 L of each medium contained 5 mL of stock solution 1a, 5 mL of stock solution 2, 1 mL of Hutner's trace element solution and 20 mL 1 M $NaHCO_3$. Other conditions were start culture of 10 000 cells mL^{-1}, parallels of 3 tubes á 320 mL, 3.0±0.2 % CO_2, 200 μmol m^{-2} s^{-1} and temperature of 25±1 °C.

Table 5: Beijerinck's solution divided in two: stock solution 1a (nitrogen and sulphate) and stock solution 1b (magnesium and calcium).

Stock solution 1a: Nitrogen and sulphate	
NH_4Cl	100 g
K_2SO_4	2.88 g
Deionized water	To total of 1000 mL
Stock solution 1b: Magnesium and calcium	
$MgCl_2$ * $6H_2O$	3.25 g
$CaCl_2$ * $2H_2O$	2.0 g
Deionized water	To total of 1000 mL

Table 6: Experimental design of the nutrient experiment. The treatments consisted of modified HS-medium with different contents of solution 1b ($MgCl_2$*$6H_2O$ and $CaCl_2$*$2H_2O$) prepared with diH_2O or tap water. The rest of the nutrients in the media was kept as in the HS-medium.

	Control	**15 mL Sol1b**	**30 mL Sol1b**	**Tap water**
Volume Sol1b (mL L^{-1})	5.0	15.0	60.0	5.0
Water quality	diH_2O	diH_2O	diH_2O	Tap water

Samples were taken once a day using an electronic pipette. 8 mL algal culture were collected into glass test tubes. Growth factors were registered in the following order: pH, optical density and DW. Sampling for DW was started after 48 hours.

The experiment was conducted in the bioreactor-lab, Plant Cell Laboratory, SKP. The bioreactor system used was similar to the ones described in chapter 0, except from the water, which was kept still in the aquaria, the temperature only controlled by heating cobs and sensors. External light and temperature were controlled by keeping out natural daylight and keeping room temperature stable using air conditioning.

4 Results

4.1 Effects of daylight (DL) and initial cell densities on growth and starch

First repeat – June/July 2013

Dry weight/productivity

In general, the growth of *C. reinhardtii* increased with the amount of PFD during the day (Figure 9, a). After 7 days, the controls (DL+200) and the daylight (DL)-cultures reached about the same DW. However, the DW of the DL-cultures used more time to reach its peak. As seen in Table 7, the productivity in mg L^{-1} d^{-1} of the DL+200 is double of the productivity of the DL-treatments.

Despite the initial cell densities, the DL+200 10c-treatment quickly reached the same amount of DW as DL+200 50c. As for the daylight treatments, DL 10c did not reach the DW of DL 50c until 7 days into the experiment.

As for productivity, the DL+200-treatments were significantly higher than the DL-treatments, while the DW/PFD-rate was higher in the DL-treatments (Table 7).

Content of starch was not analysed in this experiment.

Table 7*:* Daylight experiment nr 1: Effect of daylight and initial cell density on growth of *C. reinhardtii*. Treatments: 1) Daylight + 200 μmol m^{-2} s^{-1}, initial cell concentration 10 000 cells/mL (DL+200 10c); 2) Daylight + 200 μmol m^{-2} s^{-1}, initial cell concentration 50 000 cells/mL (DL+200 50c); 3) Daylight, initial cell concentration 10 000 cells/mL (DL10c); 4) Daylight, initial cell concentration 50 000 cells/mL (DL50c). Column two: maximum DW attained during the experiment. Column three: total amount of PFD required to attain maximum DW. Column four: time (d) required to attain maximum DW. Calculations shown in Appendix 1: *Equation* 6. Unequal, raised letters indicate statistical significance at $\alpha = 5\%$.

	Max. DW attained (g L^{-1})	**Total amount PFD (mol m^{-2} d^{-1}) to attain max. DW**	**Days to attain max. DW**	**DW/PFD (mg L^{-1} / mol m^{-2} d^{-1})**	**Productivity (g L^{-1} d^{-1})**
1) DL+200 10c	1.1	138.6	4.5	0.008	0.24^{a}
2) DL+200 50c	0.9	120.5	3.5	0.007	0.26^{a}
3) DL10c	1.0	82.0	5.5	0.012	0.18^{b}
4) DL50c	0.9	66.8	5.0	0.013	0.18^{b}

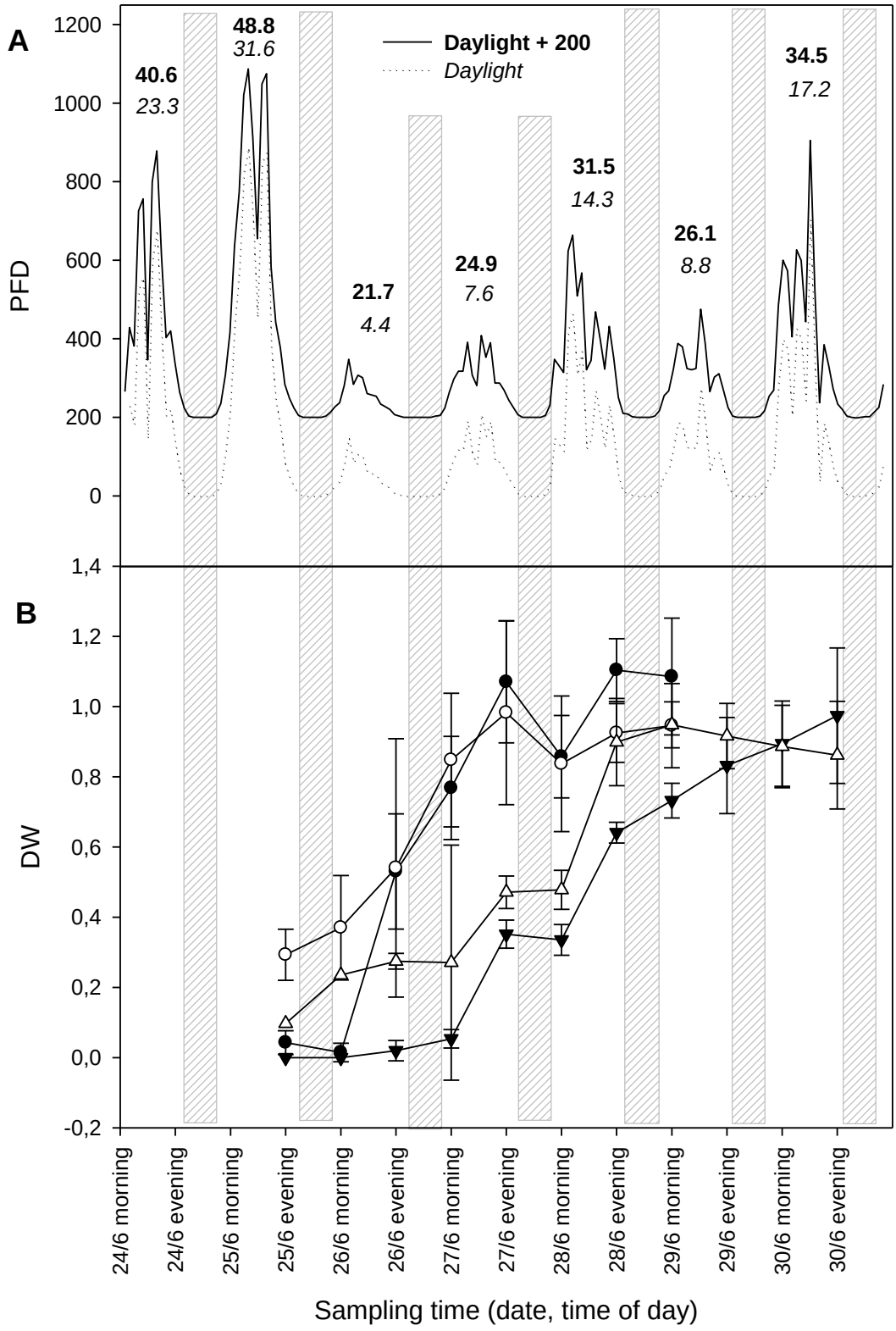

Figure 9: Daylight experiment nr 1: Effect of daylight and initial cell density on the growth of *C. reinhardtii*. A) PFD measured in front of the PBRs in the greenhouse, week 26-27, 2013. Top and bottom numbers indicate mol m^{-2} d^{-1} respectively in the control treatment and the daylight only treatment. B) DW in g L^{-1} measured twice a day. ● Daylight + 200 μmol m^{-2} s^{-1}, initial cell concentration 10 000 cells/mL. ○ Daylight + 200 μmol m^{-2} s^{-1}, initial cell concentration 50 000 cells/mL. ▼Daylight, initial cell concentration 10 000 cells/mL. Δ Daylight, initial cell concentration 50 000 cells/mL. Grey areas lines represent nights with low PFD.

Second repeat – July/August 2013

Dry weight and productivity

Similar to the first daylight experiment, the DW of the DL-treatments increased with increased PFD (Figure 10). In addition, DW-growth slowed down or decreased during nights. The DL+200-treatments had a clear and even exponential growth phase, before the stationary- and death phase.

Despite the initial cell density in the DL-treatments, the DL 10c and DL 50c-treatments quickly seemed to reach the same amount of DW (Figure 10B). In fact, DL10c exceeded DL50c after 3 days (Figure 10B). Apart from that, DL 10c and DL50c followed the same growth rhythm.

The growth rates of the DL+200-treatments were much higher. Here, DL+200 50c remained the densest culture. The growth rate of DW per day was significantly higher than for the treatments with daylight only (Table 8).

The DL+200 50c-treatment had the higher productivity rate. It was app. twice as high as the productivity of the DL-treatments. The productivity of the DL+200 10c-treatment was in between. The DW/PFD-rate was slightly higher in the DL-treatments (Table 8).

Starch

The contents of starch varied diurnally with the rhythm of the PFD-levels throughout the day. In the morning, starch content was low, and in the evening, starch content was high.

In the second repeat, the amount of starch was very similar between DL 10c and DL 50c. So was the DL+200 10c and DL+200 50c.

Table 9 shows that the starch produced in percentage mg d^{-1} is higher for the DL+200 than in the DL-treatments. However, as the amount of starch varies with the time of the day (Figure 10 C), it was difficult to choose time point for the maximum amount of starch for the DL-treatments.

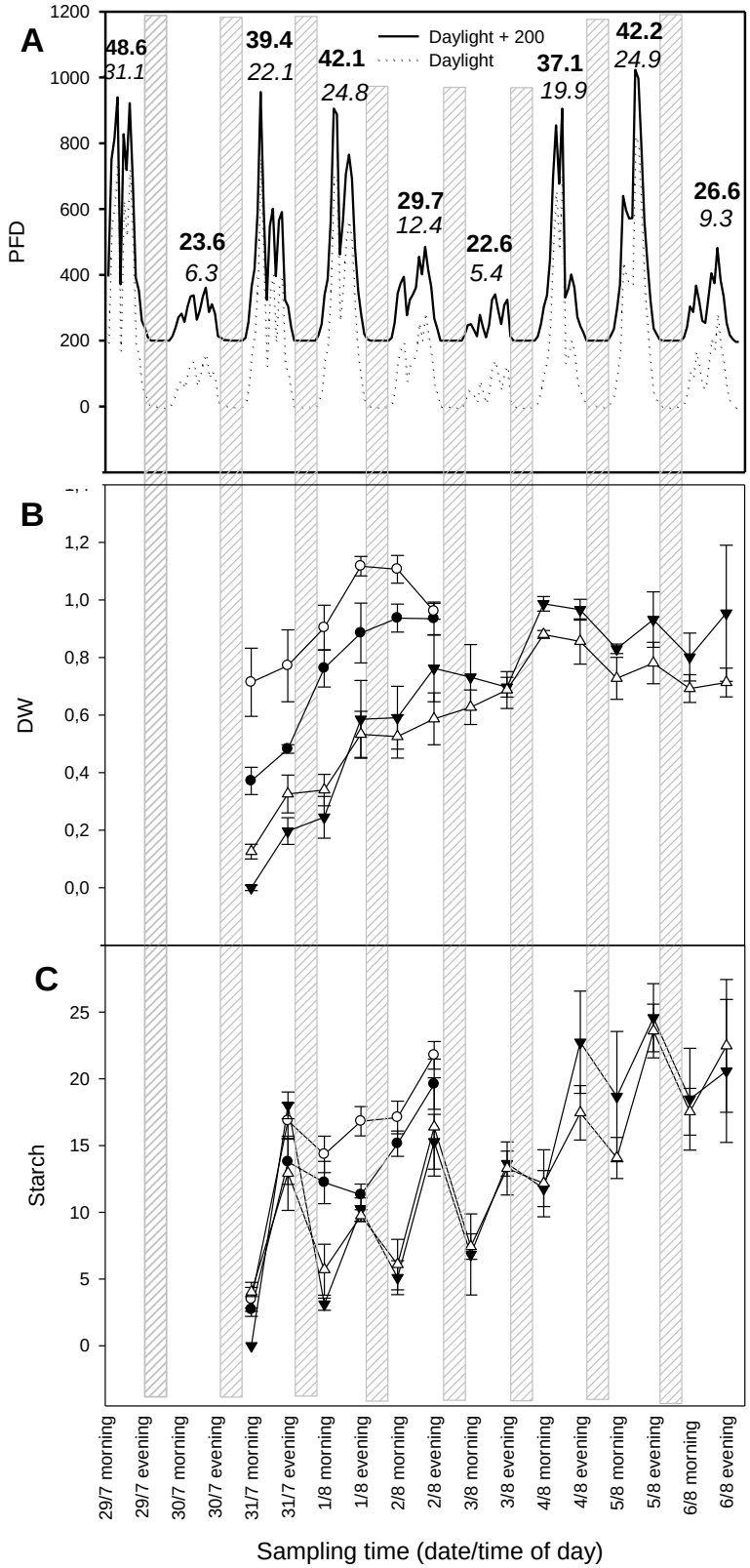

Figure 10: Daylight experiment nr 2: Effect of daylight and initial cell density on growth and starch content of *C. reinhardtii*. A) PFD measured in front of the PBRs in the greenhouse, week 26-27, 2013. Top and bottom numbers indicate mol m^{-2} d^{-1} respectively in the control treatment and the daylight only treatment. B) DW in g L^{-1} measured twice a day. C) Content of starch in % w/w. ● Daylight + 200 µmol m^{-2} s^{-1}, initial cell concentration 10 000 cells/mL. ○ Daylight + 200 µmol m^{-2} s^{-1}, initial cell concentration 50 000 cells/mL. ▼Daylight, initial cell concentration 10 000 cells/mL. Δ Daylight, initial cell concentration 50 000 cells/mL. Grey, tilted lines represent nights with low PFD.

Table 8: Daylight experiment nr 2: Effect of daylight and initial cell density on growth of *C. reinhardtii*. Treatments: 1) Daylight + 200 μmol m^{-2} s^{-1}, initial cell concentration 10 000 cells/mL (DL+200 10c); 2) Daylight + 200 μmol m^{-2} s^{-1}, initial cell concentration 50 000 cells/mL (DL+200 50c); 3) Daylight, initial cell concentration 10 000 cells/mL (DL10c); 4) Daylight, initial cell concentration 50 000 cells/mL (DL50c). Column two: maximum amount of DW attained during the experiment. Column three: total amount of PFD required to attain maximum DW. Column four: time (d) required to attain maximum DW. Calculations shown in Appendix 1: Equation 6. Unequal, raised letters indicate statistical significance at $\alpha = 5\%$.

	Max. DW attained (g L^{-1})	**Total amount PFD (mol m^{-2} d^{-1}) to attain max. DW**	**Days to attain max. DW**	**DW/PFD (mg L^{-1} / mol m^{-2} d^{-1})**	**Productivity (g L^{-1} d^{-1})**
DL+200 10c	1.0	149.9	4.5	0.007	0.22^{a}
DL+200 50c	1.1	124.3	3.5	0.009	0.31^{b}
DL 10c	0.7	67.7	6.0	0.010	0.12^{c}
DL 50c	0.7	67.7	6.0	0.010	0.12^{c}

Table 9: Daylight experiment nr 2: Effect of daylight and initial cell density on starch production of *C. reinhardtii*. Treatments: 1) Daylight + 200 μmol m^{-2} s^{-1}, initial cell concentration 10 000 cells/mL (DL+200 10c); 2) Daylight + 200 μmol m^{-2} s^{-1}, initial cell concentration 50 000 cells/mL (DL+200 50c); 3) Daylight, initial cell concentration 10 000 cells/mL (DL10c); 4) Daylight, initial cell concentration 50 000 cells/mL (DL50c). Column two: maximum amount of starch during the experiment. Column three: total amount of PFD required to attain maximum starch. Column four: time (d) required to attain maximum amount of starch. Calculations shown in Appendix 1: Equation 6. Unequal, raised letters indicate statistical significance at $\alpha = 5\%$.

	Max. starch attained (% w/w)	**Total amount PFD (mol m^{-2} d^{-1}) to attain max. DW**	**Days until max. starch**	**Starch accumulation rate (% w/w d^{-1})**
1) DL+200 10c	19.6	149.9	4.5	4.4^{a}
2) DL+200 50c	21.8	149.9	4.5	4.8^{a}
3) DL10c	24.6	98.1	7.5	3.3^{b}
4) DL50c	23.6	98.1	7.5	3.1^{b}

Third repeat – August/September 2013

Dry weight and productivity

Similar with the first and second daylight experiment, the DW of the DL-treatments increased with increased PFD. During nights, DW was slowed down or decreased. The DL+200-treatments had a clear and even exponential growth phase, before the stationary- and death phase.

Despite the initial cell density in the DL+200-treatments, the DL+200 10c and DL+200 50c-treatments reached the same amount of DW after app. 4 days. For the DL-treatments, DL 50c remained more dense than DL 10c, but the difference evened out 5-6 days into the experiment. Apart from that, DL 10c and DL50c followed the same growth rhythm.

The DL+200-treatment had a significantly ($\alpha = 5\%$) higher productivity rate than the DL-treatments. However, the DW/PFD-rate was slightly higher in the DL-treatments.

Starch

The content of starch in the DL-treatments varied diurnally with the rhythm of the PFD-levels throughout the day. In the morning, starch content was low, and in the evening, starch content was high. For the DL+200 treatments, the growth rate was slightly slowed down during nights, but still increasing.

The initial cell density-difference remained in the DL-treatments, until 7 days into the experiment, where DL 10c has a higher starch content than DL 50c. The DL+200-treatmens are quite similar to each other.

Table 11 shows that the starch produced in percentage mg d^{-1} is higher for the DL+200 than in the DL-treatments, and DL+200 10c is the highest. However, as the amount of starch varies with the time of the day (Figure 10 C), it was difficult to choose time point for the maximum amount of starch for the DL-treatments.

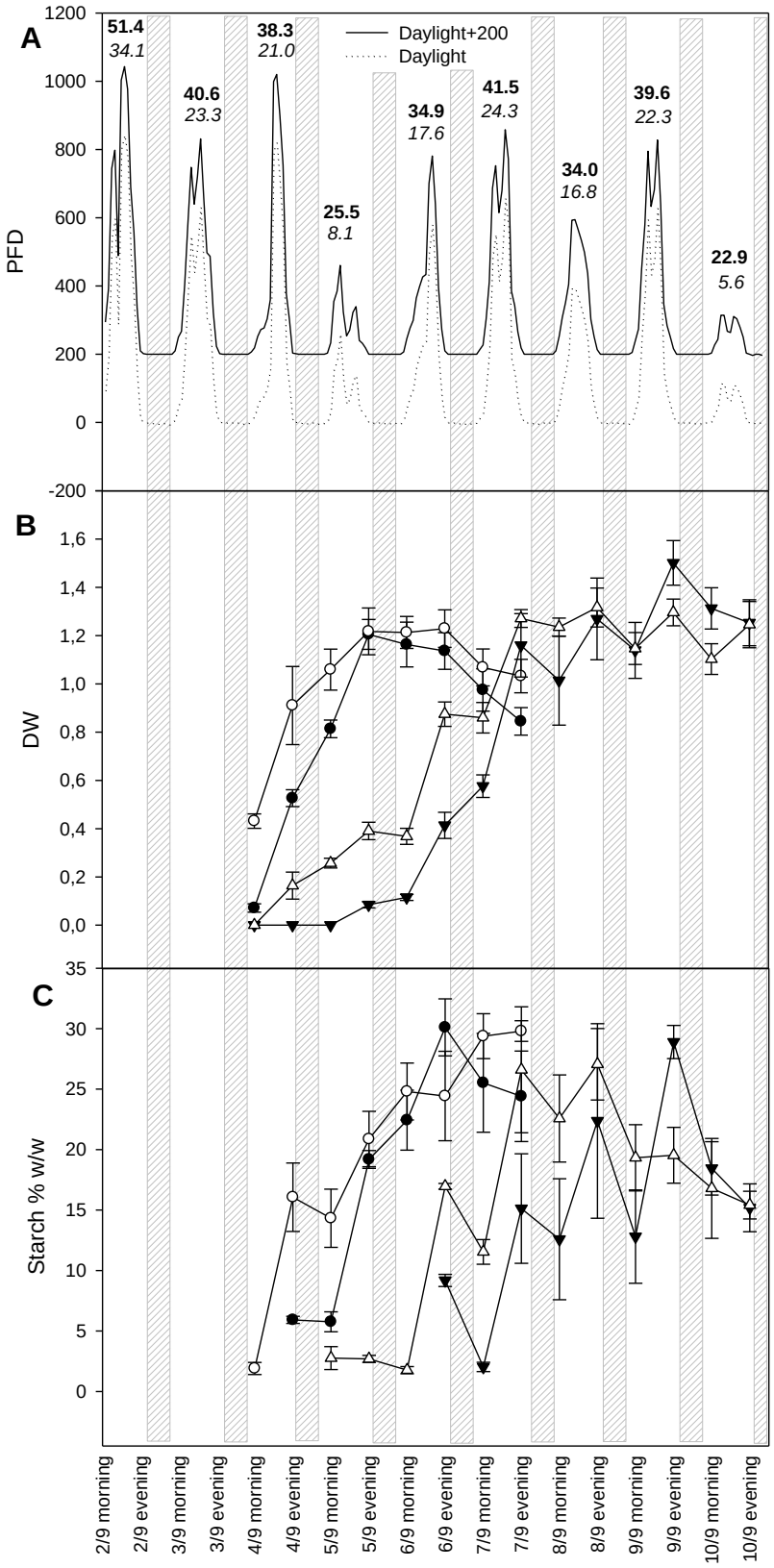

Figure 11: Daylight experiment nr 3: Effect of daylight and initial cell density on DW and starch of *C. reinhardtii.* A) PFD measured in front of the PBRs in the greenhouse, week 26-27, 2013. Top and bottom numbers indicate mol m^{-2} d^{-1} respectively in the control treatment and the daylight only treatment. B) DW in g L^{-1} measured twice a day. C) Content of starch in % w/w. ● Daylight + 200 μmol m^{-2} s^{-1}, initial cell concentration 10 000 cells/mL. ○ Daylight + 200 μmol m^{-2} s^{-1}, initial cell concentration 50 000 cells/mL. ▼Daylight, initial cell concentration 10 000 cells/mL. Δ Daylight, initial cell concentration 50 000 cells/mL. Grey, tilted lines represent nights with low PFD.

Table 10: Daylight experiment nr 3: Effect of daylight and initial cell density on growth of *C. reinhardtii*. Treatments: 1) Daylight + 200 μmol m^{-2} s^{-1}, initial cell concentration 10 000 cells/mL (DL+200 10c); 2) Daylight + 200 μmol m^{-2} s^{-1}, initial cell concentration 50 000 cells/mL (DL+200 50c); 3) Daylight, initial cell concentration 10 000 cells/mL (DL10c); 4) Daylight, initial cell concentration 50 000 cells/mL (DL50c). Column two: maximum amount of DW attained during the experiment. Column three: total amount of PFD required to attain maximum DW. Column four: time (d) required to attain maximum DW. Calculations shown in Appendix 1: Equation 6. Unequal, raised letters indicate statistical significance at $\alpha = 5\%$.

	Max. DW attained (g L^{-1})	**Total amount PFD (mol m^{-2} d^{-1}) to attain max. DW**	**Days to attain max. DW**	**DW/PFD (mg L^{-1}/mol m^{-2} d^{-1})**	**Productivity (g L^{-1} d^{-1})**
1) DL+200 10c	1.2	119.8	3.5	0.010	0.34^{a}
2) DL+200 50c	1.2	147.4	4.5	0.008	0.27^{a}
3) DL10c	1.5	97.9	7.5	0.015	0.2^{b}
4) DL50c	1.3	84.9	6.5	0.015	0.2^{b}

Table 11: Daylight experiment nr 3: Effect of daylight and initial cell density on starch production in *C. reinhardtii*. Treatments: 1) Daylight + 200 μmol m^{-2} s^{-1}, initial cell concentration 10 000 cells/mL (DL+200 10c); 2) Daylight + 200 μmol m^{-2} s^{-1}, initial cell concentration 50 000 cells/mL (DL+200 50c); 3) Daylight, initial cell concentration 10 000 cells/mL (DL10c); 4) Daylight, initial cell concentration 50 000 cells/mL (DL50c). Column two: maximum amount of starch during the experiment. Column three: total amount of PFD required to attain maximum starch. Column four: time (d) required to attain maximum amount of starch. Calculations shown in Appendix 1: Equation 6. Unequal, raised letters indicate statistical significance at $\alpha = 5\%$.

	Max. starch (% w/w)	**Total amount PFD (mol m^{-2} d^{-1}) to attain max. starch**	**Days to attain max. starch**	**Starch accumulation rate (% w/w d^{-1})**
1) DL+200 10c	31.1	147.4	4.5	6.91^{a}
2) DL+200 50c	29.8	178.8	5.5	5.42^{b}
3) DL10c	28.9	97.8	7.5	3.85^{c}
4) DL50c	27.1	84.9	6.5	4.16^{c}

4.2 Effects of high light (HL) exposure on growth, starch and chlorophyll

Presented results are average values of three repeats of the experiment. Results from each repeat are presented in Appendix 2. Thus, the standard deviation levels are quite high, but the results of every single repeat mainly follow the same shape as in the figure with average values.

Photoinhibition

The cultures of the HL treatment were strongly photoinhibited by the strong light (Figure 12A). However, comparing photoinhibition-values with DW results, this level of photoinhibition did not seem to have a significant effect on the DW (Figure 12B).

In the results from the individual experiments, the growth of the algae in the HL treatment was lower than both the control and the algae in the ML treatment in the first two experiments, but not in the third.

Dry weight and productivity

In general, the light periods had a positive effect on the DW, especially in ML treatment (Figure 12B). As seen in Table 12, the growth (DW per day) was higher in the ML treatment than in the other two treatments (however, not significantly).

Looking at the three repetitions of the experiment separately (Appendix 2: Figure 18), the effect of light periods was more diverse than presented in the average values. In the first and second repeat, the ML treatment displayed the highest growth (DW per day) (significantly only in the second exp.), but in the last experiment the HL treatment significantly displayed the highest growth (Table 13).

Starch

The strong light periods affected the content of starch positively. During the light periods, starch content increased in the cultures both in the ML and in the HL treatment. However, for the HL treatment, the growth rate was not as high as in the ML treatment (Figure 12C).

The content of starch varied from the second to the third repeat. In the end of the second repeat, the content of starch was significantly lower in the cultures in the HL treatment than in the ML treatment and the control (Figure 18 2C). However, in the third repeat, the three treatments displayed the same starch content (Figure 18 3C). The significance was tested at maximal DW points (Exp.2: t=140 h, Exp.3: t=90 h).

Chlorophyll

The strong light had a negative effect on the chlorophyll content (Figure 12D). The chlorophyll content decreased evenly during the experiments for both the treatments and the control, but more rapidly during the light periods.

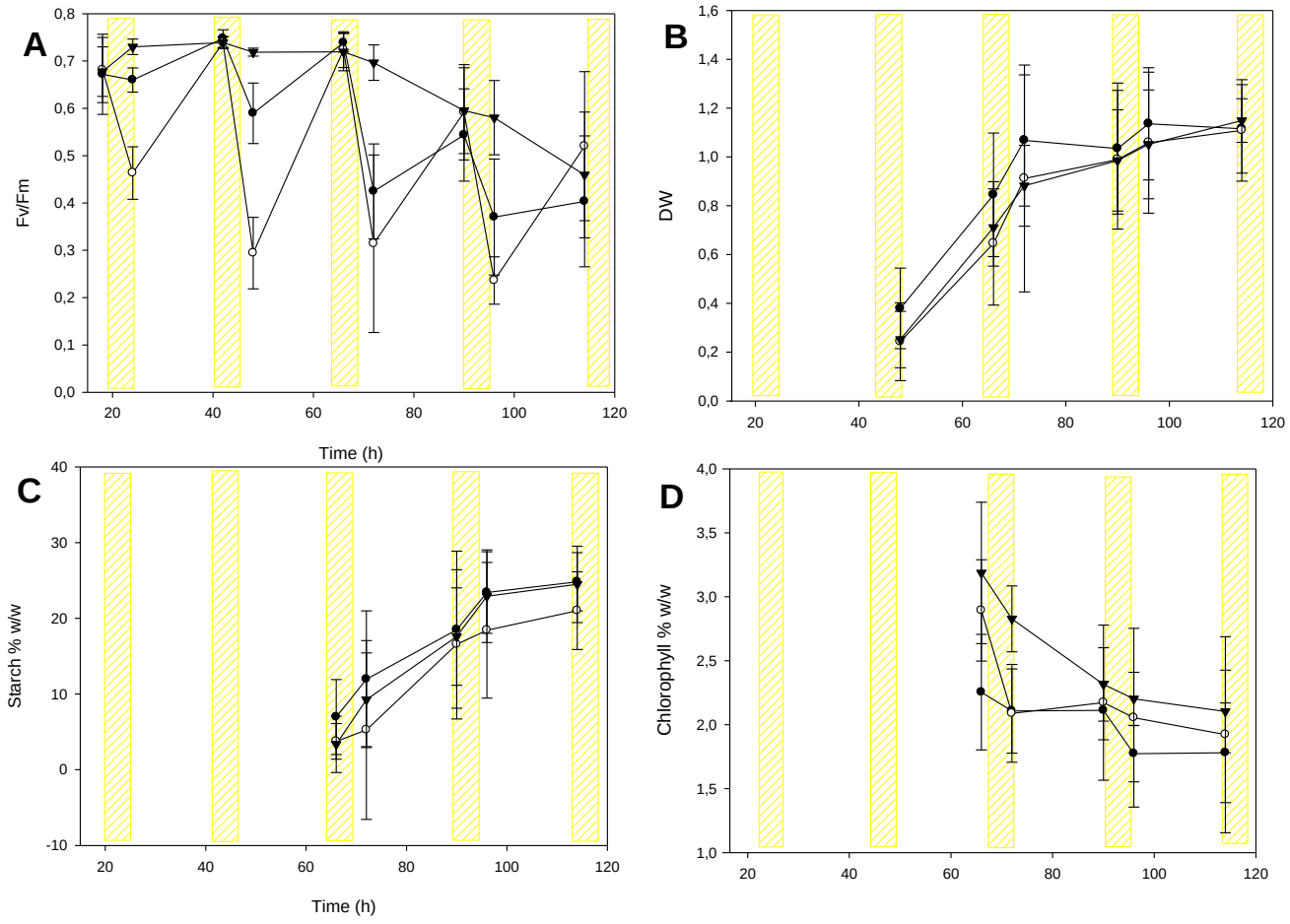

Figure 12: Effect of strong light periods on *C. reinhardtii*. Yellow areas represent light pulses of 6 hours. ● ML treatment. ○ HL treatment. ▼Control treatment. A: Effect on photoinhibition. B: Effect on DW. C: Effect on starch in % w/w. D: Effect on chlorophyll in % w/w.

Table 12: Effect of high light exposure on DW and productivity. Values are means of results from three repeats of the experiment. Calculations shown in Appendix 1: Equation .

	Max. DW attained (mg L^{-1})	**Total PFD required to attain max. DW (mol m^{-2} d^{-1})**	**Days required to attain max. DW**	**Biomass/PFD (mg L^{-1}/mol m^{-2} d^{-1})**	**Productivity (g L^{-1} d^{-1})**
Control	1.1	73.4	4.3	0.015	0.26
ML treatment	1.2	93.6	3.3	0.013	0.36
HL treatment	1.1	155.5	4.0	0.007	0.28

Table 13: Effect of high light exposure on starch accumulation rate. Values are means of results from three different repeats of the experiment. Calculations shown in Appendix 1: Equation 6.

	Max. starch attained (% w/w)	**Total PFD required to attain max. starch (mol m^{-2} d^{-1})**	**Days required to attain max. starch**	**Starch accumulation rate (% w/w d^{-1})**
Control	25.2	64.8	4.8	5.3
ML	26.4	118.8	4.4	6.0
HL	22.	181.4	4.9	4.6

Table 14: Effects of high light exposure on productivity and starch accumulation rate. The experiment was carried out in three repeats. Unequal, raised letters indicate statistical significance at α = 5%. Calculations shown in Appendix 1: Equation 6.

	1. Repeat	**2. Repeat**		**3. Repeat**	
	Productivity (g L^{-1} d-^{1})	Productivity (g L^{-1} d-^{1})	Starch accumulation rate (% w/w d^{-1})	Productivity (g L^{-1} d-^{1})	Starch accumulation rate (% w/w d^{-1})
Control	0.22^{a}	0.30^{a}	3.82^{a}	0.25^{a}	7.57^{a}
ML treatment	0.33^{a}	0.31^{a}	5.37^{a}	0.46^{b}	6.94^{a}
HL treatment	0.22^{a}	0.19^{b}	2.98^{a}	0.49^{b}	7.01^{a}

4.3 Additional experiments

4.3.1 Effects of air bubbling velocity

The regime of high air bubbling displayed an increased growth rate compared to the other two treatments. However, the DW still did not match the results from Mortensen (personal communication).

The pH of the low bubbling treatment had increased after two days. This effect was not observed in the other two treatments (Figure 13).

Comparing the growth of cultures in tubes of 320 mL and plastic bottles of 1000 mL, there was a difference only in the high and medium bubbling-treatments, where tubes displayed the highest DW. Comparing the results of the air bubbling velocity exeriment with the DW of the control in high light experiment nr 3, it is shown that both the medium and high bubbling treatments provide for better growth (Figure 14).

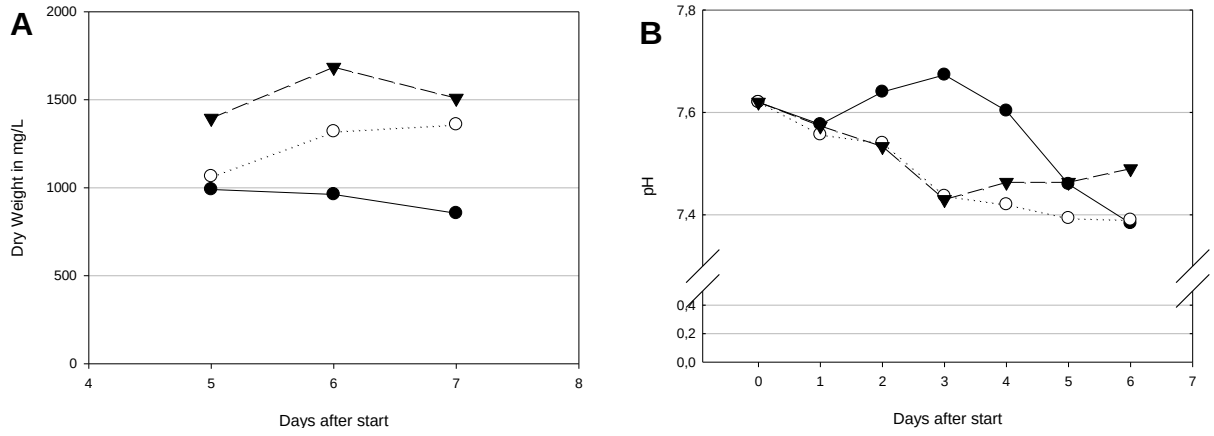

Figure 13: Effect of different gas bubbling treatments on a) DW; and b) pH, of the microalga *C. reinhardtii*. Gas bubbling treatments were low bubbling (●), medium bubbling (○) and high bubbling (▼).

Table 15: Effects of different air bubbling regimes on DW and productivity in *C. reinhardtii*. Calculations shown in Appendix 1: Equation 6. Unequal, raised letters indicate statistical significance.

Bubbling intensity	**Max. DW attained (g L^{-1})**	**Days required to attain max. DW**	**Productivity (g L^{-1} d^{-1})**
Low	1.0	5.0	0.20^{a}
Med.	1.4	6.0	0.23^{a}
High	1.7	6.0	0.28^{a}

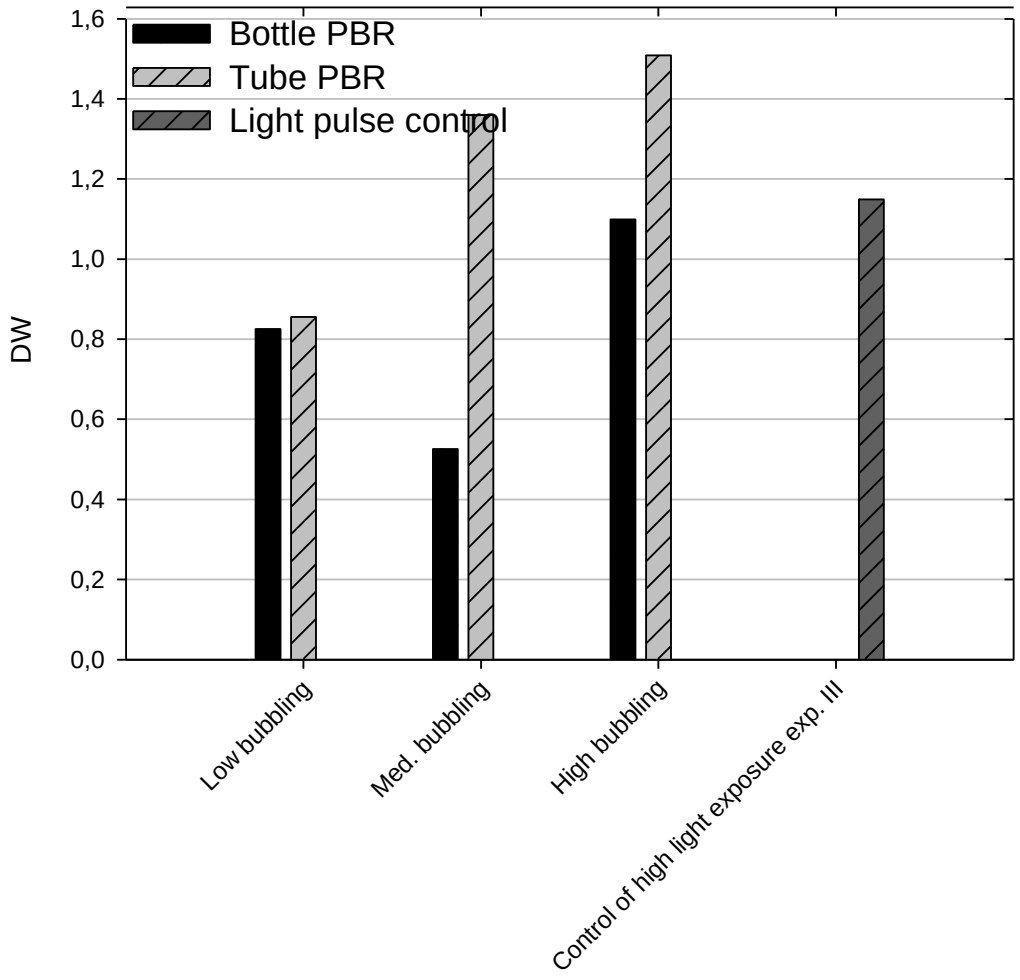

Figure 14: Comparison of DW between algae grown in glass PBRs or plastic PBRs after 7 days low, medium and high bubbling of air with 3% CO_2. Far right column represents the mean of maximum attained DW in the controls in high light exposure experiments, which had an air bubbling rate of something in between low and medium bubbling.

4.3.2 Use of extra calcium and magnesium or tap water

Overall, the results presented below indicate that a higher amount of calcium and magnesium is needed to ensure a high growth of the microalga *C. reinhardtii*, compared to the HS-medium. Kliphuis et al. (2012) also observe this.

DW was highest in the medium prepared with tap water. DW was also higher in treatments with higher amount of calcium and magnesium when compared to the growth in the HS-medium (Figure 15).

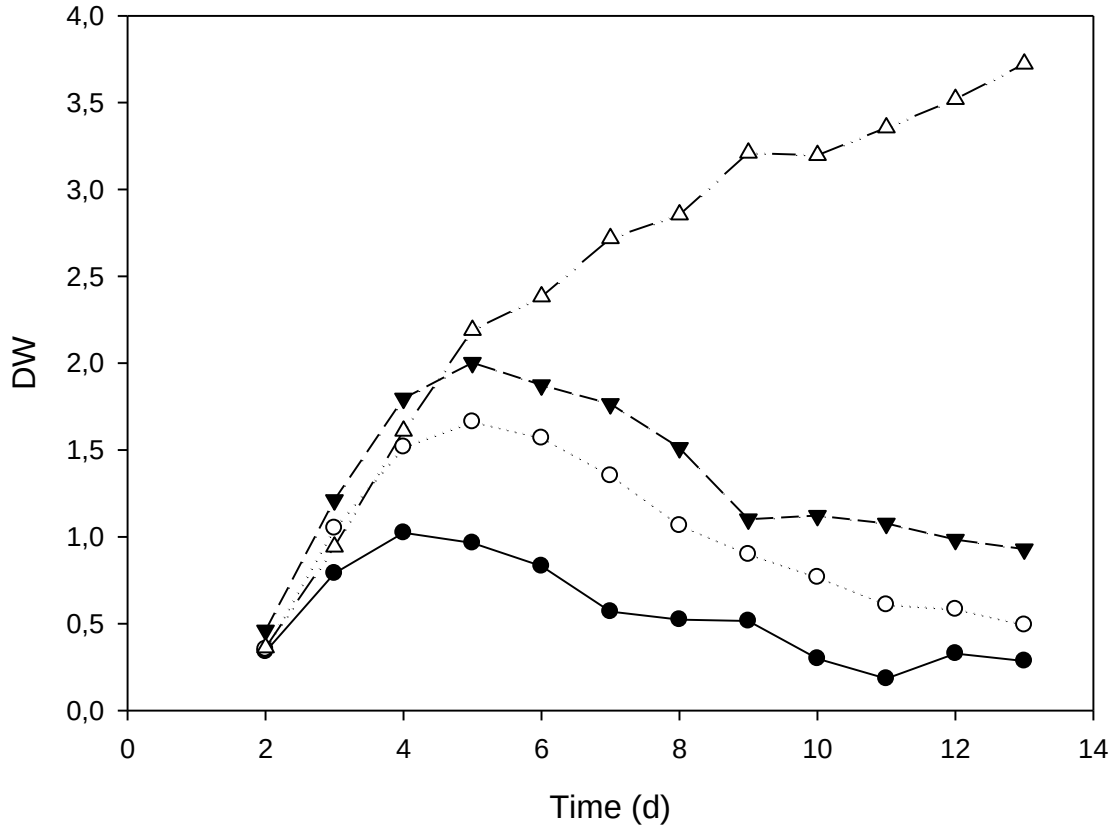

Figure 15: Effect of growing the microalgae *C. reinhardtii* in modified HS-media on DW in mg L^{-1}. The medium was varied by altering the amount of solution 1b (containing calcium and magnesium), and with diH_2O or tap water. Other nutrients were kept as original. ● Control: diH_2O with 5 mL solution 1b. ○ diH_2O with 15 mL solution 1b. ▼ diH_2O with 30 mL solution 1b. Δ Tap water with 5 mL solution 1b.

Table 16: Effects of concentrations of calcium and magnesium on DW and productivity of *C. reinhardtii*. The medium was varied by altering the amount of solution 1b (containing calcium and magnesium), and with diH_2O or tap water. The control treatment contained diH_2O with 5 mL solution 1b, the other treatments contained diH_2O with three and six times as much solution 1b, respectively. The tap water based medium contained 5 mL solution 1b. Other nutrients were kept as original. Calculations are shown in Appendix 1: Equation 6. Unequal, raised letters indicates statistical significance at $\alpha = 5\%$.

	Max. DW attained (g L^{-1})	**Days acquired to attain max. DW**	**Productivity (g L^{-1} d^{-1})**
Control	1.0	4.0	0.25^a
15 ml Sol1b	1.7	5.0	0.34^b
30 ml Sol1b	2.0	5.0	0.40^c
Tap water	3.7	13.0	0.28^d

5 Discussion

5.1 Effects of light on growth: DW and productivity

Altogether, the results from our experiments indicate that within the range of light intensities tested in this study, high light exposure (daylight or simulated) did not have a significant impact on growth or starch compared to the control treatments. Microalgae grown in daylight (diurnal light conditions) displayed better longevity than controls of continuous illumination and had a diurnal rhythm of starch accumulation.

Effect of high light intensities on the photosystems

Photoinhibition was registered before and after light periods in the high light exposure experiments. The quantum yield (Fv/Fm-values) in both ML- and HL-treatments was reduced after the light periods, by 20% and 60% respectively. However, the reduced quantum yield had little negative impact on the DW. The quantum yield of all treatments recovered completely (Fv/Fm = 0.7) after periods of low light. This indicates that the photoinhibition was dynamic (Maxwell & Johnson 2000). During dynamic photoinhibition, the quantum efficiency decreases, but the maximum photosynthetic rate remains unchanged (Taiz & Zeiger 2010). This means that the photosystems are not able to utilize all the photons absorbed, but still can function as normal and produce the same amount of photosynthetic compounds. The excess photons are dispersed as heat (quenched) through the zeaxanthin cycle (Taiz & Zeiger 2010). For chronically phtotoinhibited cultures, however, both quantum efficiency and maximum photosynthetic rate would decrease. The distinct pattern with reduced quantum yield after high light periods, and recovered quantum yield after low light periods is known as diurnal depression or diurnal photoinhibition (Ogren & Evans 1992; Vonshak & Torzillo 2004).

Photoinhibition was not registered in the daylight experiments. However, the PFD in the greenhouse rarely exceeded 1000 μmol m^{-2} s^{-1}. Considering the results from the high light exposure experiments with PFDs up to 1000 μmol m^{-2} $s^{-}1$ and only dynamic photoinhibition, photoinhibition was probably not chronic in the daylight experiments either.

Field studies of microalgae have shown that the quantum yield of cultures grown outdoors follow the same diurnal depression as observed in our high light-exposure experiments (Lu & Vonshak 1999; Torzillo et al. 1996). However, broad daylight has a PFD of up to 2500 μmol

m^{-2} s^{-1} and several studies have tested responses of *C. reinhardtii* with PFD up to such intensities (Falk et al. 1992; Falk & Samuelsson 1992; Fischer et al. 2006; Leverenz et al. 1990). In all these studies, cultures were chronically photoinhibited in light intensities in the range of 1600-2500 μmol m^{-2} s^{-1}, suffered damage to photosystems and had a decrease in growth. However, below this range, no chronic photoinhibition was detected. Thus, the light intensities used in our experiments probably were not high enough to induce severe damage. Inside a greenhouse, where an outside illumination of 2000 μmol m^{-2} s^{-1} would be decreased to app. 1200 μmol m^{-2} s^{-1}, broad daylight may be shaded just enough to prevent chronic photoinhibition.

Impact of high light intensities on DW and productivity

As the photoinhibition was shown to be dynamic, it did not have any impact on DW production in the high light exposure experiments. The three treatments displayed no significant difference in DW. Admittedly, there was a slight increase during the periods of high light for both the HL- and ML-treatments, but it was not proved to be significant (α = 5%). In addition, the ML-treatment had a slightly higher growth in total than HL and the control, but neither this was significant. Standard deviation was high, as there were some differences in DW between the three repeats. However, all three repeats followed the same pattern, with an exponential growth phase over 2-3 days, before a stationary growth phase.

Photoinhibition was not tested in the daylight experiments, but as the PFD rarely exceeded 1000 µmol m^{-2} s^{-1}, photoinhibition is assumed to be similar as to the high light exposure experiments. The effect of growing *C. reinhardtii* in daylight was an increase in DW proportionally with the PFD during the day. This was expected, as photosynthetic activity generally increases with light until photosystems are saturated (Richmond 2008; Taiz & Zeiger 2010).

An interesting result in the daylight experiments was a better utilization of light in the DL-cultures compared to the DL+200-cultures. This was reflected by a higher DW per PFD (μmol m^{-2} s^{-1}) in the DL-cultures versus the DL+200-cultures, even though productivity was lower. This was observed in all three repeats. Production of more biomass per photon in low light versus high light intensities was also observed by Bonente et al. (2012).

The daily illumination for the DL-cultures was app. 17 mol m^{-2} d^{-1} in all three experiments, which equals an average of app. 200 μmol m^{-2} s^{-1} during light hours. Thus, the average light

intensity of the DL+200-cultures was twice as high as in the DL-cultures during light hours. However, the daytime growth of the DL+200-cultures was similar to the daytime growth of DL-cultures, despite the double PFD, and the DW difference between the DL and DL+200 treatments probably is due to the night illumination solitary for the DL+200 treatments. This indicates that the photosystems were saturated at 200 μmol m^{-2} s^{-1}. This is also supported by the equal growth (DW) between the HL, ML and control-treatments. In other words, in light conditions above 200 μmol m^{-2} s^{-1}, the light intensity had surpassed the light saturation point (I_s) in the light response curve (Figure 5). This photosystem saturation threshold has been observed in other studies on *C. reinhardtii* (Pyo Kim et al. 2006), but as others have determined the photosystems to be saturated at 100 μmol m^{-2} s^{-1} (500 ft-c) (Sorokin & Krauss 1958), the light saturated point may also be even lower than 200 μmol m^{-2} s^{-1}. Moreover, the PFD of 200 μmol m^{-2} s^{-1} is quite established and has been used as a standard illumination in several experiments with *C. reinhardtii* (Kliphuis et al. 2012; Kosourov et al. 2002; Melis et al. 2000; Polle et al. 2002; Thyssen et al. 2001).

Growth curves

Figure 15 shows a typical growth curve for microalgae grown in daylight versus cultures grown with continuous illumination. During the exponential growth-phase, the growth was sufficiently high to prevent DW declining during the nights. As cultures entered the stationary growth phase, the DW decreased during the nights, but still increased during the days. This decrease in DW was probably due to accumulation and degradation of starch, and is also observed in similar experiments on green algae (Brányiková et al. 2011). Otherwise, the daylight and control-growth curves were quite similar, except that the DL-curve was delayed (in Figure 13, app. 3 days).

The daylight cultures showed increase in growth during the day, and stagnation or decrease during the night. This corresponds with the cell division of *C. reinhardtii* happening in the early hours of the morning, and not during night time (Harris 2009). Cultures grown in a continuous light environment, such as the DL+200-treatment and in the high light exposure experiments, were shown to express genes for cell division continuously (Bisova et al. 2005). Thus, the diurnal effect on growth was not displayed in continuously illuminated cultures.

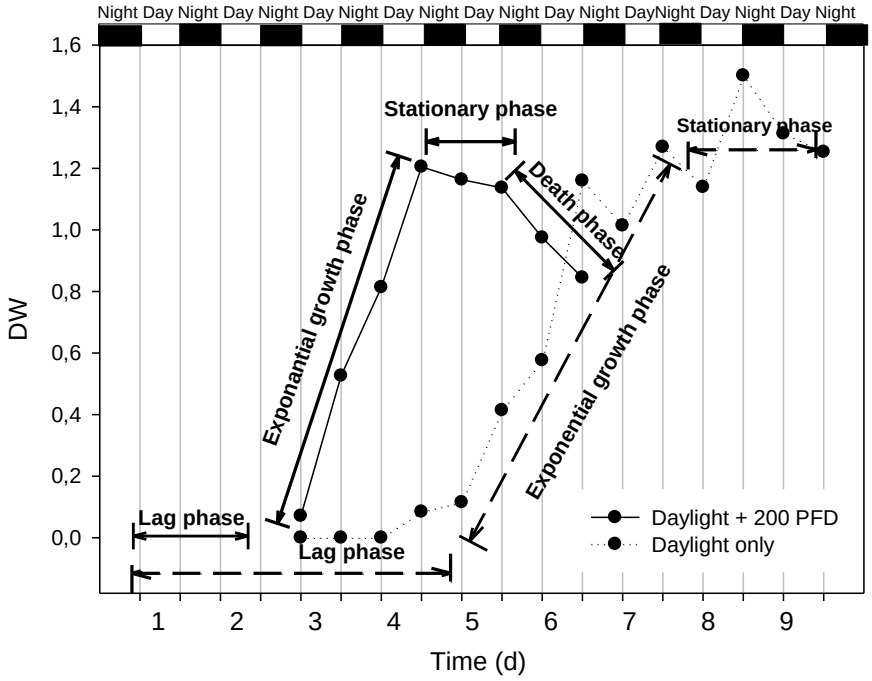

Figure 16: Typical growth curves for *C. reinhardtii* cultures grown in natural daylight, with backlight (full lines) and without backlight (dashed lines) of 200 μmol m^{-2} s^{-1} fluorescent cool white tubes. Cultures were started with a density of 10 000 cells mL^{-1}. The shape of the curve is dependent on the weather. This figure is from daylight experiment nr 3, which had sunny days with relatively high PFD 5-6 days into the experiment.

Effects of initial and later cell density on growth

The daylight cultures displayed no clear effects of the initial cell densities of 10 000 cells mL^{-1} and 50 000 cells mL^{-1}. In two of three repeats, we observed that the densest culture continued to be the densest, but this was not consistent. In dense cultures, microalgae may be self-shading, and hence have a lower efficiency on light utilization shading (Leverenz et al. 1990; Myers & Graham 1959; Richmond 2008). In our experiment, this would mean a lower growth in the cultures with an initial cell density of 50 000 cells mL^{-1}. From our results however, no clear difference in DW between the initial cell-treatments was observed. Perhaps, by measuring photon use efficiency we would have detected a difference between the cultures that could not be seen in DW. Possibly, the concentrations of the initial cultures were not different enough. With a higher difference, the effect of shading might have been clearer.

In a dense culture, it is important to have sufficient turbulence. Mixing the cultures with CO_2-bubbling creates a light/dark (LD)-cycle for the microalgae, as they are moved in rapid tempo from the edge of the culture where the light intensity is high to the inner parts of the culture,

where the light intensity is lower. This was shown to be positive for cells in strong light environments (Richmond 2008), as LD-cycles will decrease the time each cell is exposed to strong light. Turbulence in a culture does not only effect the photons reaching each cell, but also influences exchange rates of metabolites and nutrients, by enhancing the area around each cell (Grobbelaar 1991). With no turbulence, the microalgae will sediment, and both illumination and exchange rates of metabolites will be severely decreased.

5.2 Effects of high light intensities on quality: starch and chlorophyll

In the diurnal conditions of the daylight experiments, starch accumulates during the day and decreases during the night. The decrease is due to breakdown of starch to support growth structures (Taiz & Zeiger 2010). In other words, the accumulation (synthesis) and breakdown of starch follows a diurnal rhythm. This rhythm is stated to be determined by circadian control in cultures grown in diurnal conditions (Ral et al. 2006). Circadian control was not shown in the high light exposure experiments, probably because the microalgae were pre-grown in continuous light.

In the high light exposure experiments, the content of starch increased considerably during the ML and HL-periods. The high light intensity did not seem to damage the photosystems chronically, but had a positive impact on photosynthetic activity. The starch increase in the HL-treatment was not as prominent as in the ML-treatment. However, in the second repeat of the experiment, growth in the HL-treatment was slower than in the other treatments. In the third repeat, no difference was observed between the HL- and the ML-treatments. Therefore, we cannot claim that a lower starch production is a general tendency in light intensities of up to 1000 μmol m^{-2} s^{-1}.

Sampling time in starch analysis

Klein (1987) showed that starch accumulates in two periods during a day: in the beginning and in the end of light periods. In the middle of the light periods and in the dark, starch is degraded (Figure 17), to generate electrons. It is likely that this also applies for the cell cycles of our daylight experiments. Therefore, the real maximum amount of starch accumulated in the cells during the daylight experiment possibly was even higher than illustrated in figures 9-10. The morning sampling (08:00 am) was conducted at a time where the accumulated starch

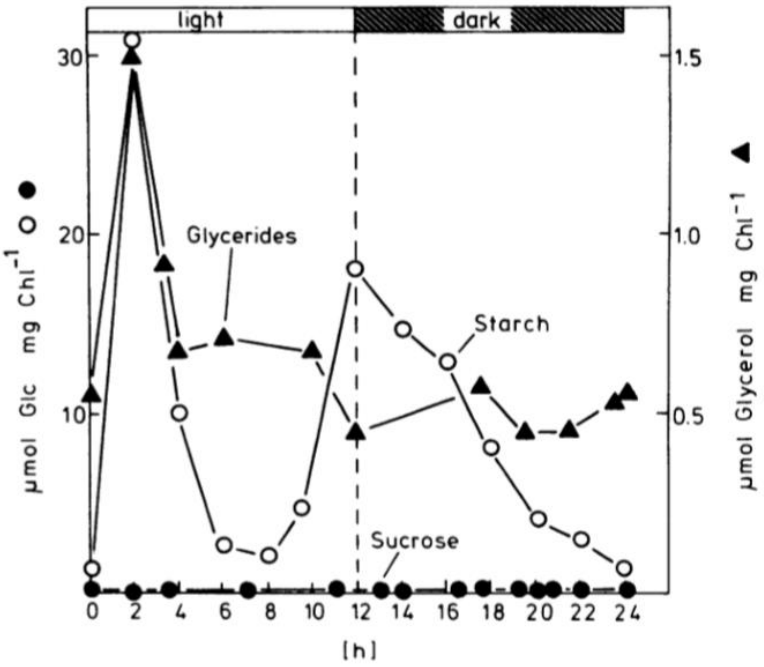

Figure 17: Intracellular concentration of starch, sucrose and glycerides in synchronously growing *C. reinhardtii* during a 24 h cell cycle with 12 h light and 12 h dark. From Klein (1987).

was low (as expected), and before the highest peak shown in Figure 17. In the evening sampling (08:00 pm) in the end of the light period, the starch accumulated to a higher degree. However, according to Klein's findings, the real peak of starch content could be as much as 1/3 higher in the beginning of the light period. It should be noted that the cultures of *C. reinhardtii* in Klein (1987) were grown synchronously with a diurnal rhythm of 12h light, and 12h dark.

Decline in growth and chlorophyll degradation

In all our treatments with continuous light conditions (the daylight control and the high light exposure experiments), the growth stagnated after app. 70 hours (about 3 days), and the cultures died after 5-6 days. This could be observed by a colour change from bright green to yellow, and then milky white. As for the culture grown in daylight only, the exponential growth phase was maintained about twice as long (about 6-7 days, depending on the sun conditions).

The longevity of the microalgae was better in the cultures with diurnal light rhythms compared to continuous light. In the daylight experiments, the bright green colour remained for a longer time in the DL-treatments than in the DL+200-treatments (data not shown). This

was probably due to the cell division rhythm. In diurnal light, the microalgae divide during night time/early morning, while in continuous light the microalgae divide continuously (Harris 2009; Rollins et al. 1983). Hence, microalgae grown in continuous light would have faster rate of cell division, nutrient spending and then decease sooner. Chlorosis is a typical symptom for deprivation of several nutrients, such as magnesium, calcium and sulphur (Bævre & Gislerød 1999; Taiz & Zeiger 2010).

A decrease in chlorophyll content per cell is also an adaption to higher light intensities (Bonente et al. 2012). Fischer et al. (2006) showed that high light intensities with PFD up to 2500 μmol m^{-2} s^{-1} significantly decreases the amount of chlorophyll and enhances cell death after a 4 hours exposure. The PFD used by Fischer et al. (2006) was over twice as high as the PFD used in our experiments, but the results of the high light-exposure experiments were similar: a rapid decrease of chlorophyll after high light exposure. The difference is that the HL-treatment evidently did not lead to cell death, while the 2500 μmol m^{-2} s^{-1} PFD treatment did. By the end of the experiment (140 hours), the recovered Fv/Fm value for all of the treatments had decreased to about 0.4. This can be seen in correlation with the decreasing chlorophyll levels. Chlorophyll bleaching is typical for cultures grown under high light intensities, both for algae cultures and higher plants (Fischer et al. 2006; Lambers et al. 1998).

It would also be interesting to measure the level of photon transmission through the increasingly denser culture, to further investigate self-shading and optimal cell density (Myers & Graham 1959). Possibly, a dense culture may explain growth stagnation, in terms of both nutrient deprivation and lack of photons throughout the culture.

5.3 Additional experiments

For both of our additional experiments, the initial cell density needs to be taken in perspective. The initial cell density of Mortensen's experiments was far higher than the initial cell densities in our experiments (personal communication), and this probably had an impact on the further growth. This tendency is also seen in the daylight experiments cultures with higher initial cell densities (50 000 cells mL^{-1}) had a better growth than cultures with lower initial cell densities (10 000 cells mL^{-1}).

5.3.1 Effects of air bubbling velocity

The general tendency in the air bubbling experiment was that higher air bubbling velocity gave higher productivity, and thus confirmed the hypothesis of this additional experiment.

The growth of cultures in 1 L plastic bottle-PBRs with low and medium bubble speeds was quite low. Probably, the low and medium bubbling speed were not able to stir up the culture properly. This resulted in microalgae sedimentation at the bottom of the PBRs. The cell division rate was probably affected negatively by the sedimentation in our experiment.

The maximum mean DW of control treatments in high light exposure-experiments was 1.15 g L^{-1}. This value was higher than the low bubbling-treatment, but lower than both the medium and high bubbling. This leads to the conclusion that our results indicate that a stronger bubbling regime does lead to higher growth, but still not as high as in experiments of Mortensen et al. (unpublished) and Kliphuis et al. (2012).

5.3.2 Use of extra calcium and magnesium or tap water in the HS-medium

Increasing the calcium and magnesium content in the medium positively influenced the growth. However, the growth was only slightly higher than the control – it never matched the DW of 3-4 g L^{-1} as in Kliphuis et al. (2012). More interestingly, a medium prepared with tap water instead of diH_2O lead to a prolonged exponential growth phase. The sampling of the cultures was ended after two weeks, but the cultures were left growing and visually examined regularly. 3-4 weeks after the start, the tap water culture was still green, while the others had died (data not shown). This indicates that the depletion of nutrients occurred 4-5 days into the experiments in the media prepared with deionized water.

The difference in growth between cultures grown in medium prepared with tap water and the other treatments may have occurred because the tap water contains other essential nutrients apart from calcium and magnesium. Sulphur is another nutrient of high content in the tap water-based medium and the optimized medium by Kliphuis et al. (2012) (Table 17). Sulphur is an essential macronutrient, and participates in chlorophyll formation, increases yield growth and activates enzymes (Taiz & Zeiger 2010). Deficiency of sulphur leads to chlorosis (Taiz & Zeiger 2010). This may also explain why the algae grown in tap water conserved their green colour up to a month after the experiment was started, as opposed to the algae grown in diH_2O, which lost chlorophyll after a week. Work done by Daria Markina on the growth medium composition of *C. reinhardtii*, concluded with increasing the content of sulphur in the HS-medium from 0.0058 g L^{-1} to 0.02 g L^{-1} (personal communication).

Chlorosis was observed in the diH_2O-based growth media quite soon compared with the tap based medium. Chlorosis is a deficiency symptom of both magnesium and sulphur, but also nitrogen (Taiz & Zeiger 2010). Nitrogen content was unfortunately not measured in the tap water due to testing method. However, in the medium used by Kliphuis et al. (2012), the nitrogen level was indeed higher than in the original HS-medium.

However, with an exception of the medium with a base of tap water, the algae grown in modified HS-medium still did not reach a density of 3-4 g L^{-1}, as in Kliphuis et al. (2012). The low growth was possibly due to precipitation of salts after autoclaving. The precipitation likely consisted of calcium carbonate or magnesium carbonate. Thus, it was hard to establish the actual amount of calcium and magnesium in the medium when the experiment was started. We can assume that the actual amount of calcium and magnesium was lower than planned. In experiments conducted by Kliphuis et al. (2012), the medium was sterile filtrated. Mortensen did not autoclave the HS-medium either in his similar experiments (personal communication). In addition, Kliphuis et al. (2012) used even more magnesium than what was added in our experiment. However, the reason for this could be that the source of magnesium was also the source of sulphate.

The HS-medium contains 1.9 mg/L magnesium and 2.7 mg/L calcium (Table 17). This is quite low compared to the enriched HS-medium used by Kliphuis et al. (2012), with a concentration of calcium and magnesium of 15.6 and 27.7, respectively. Compared to several other growth media suitable for *C. reinhardtii*, the HS-medium has a lower amount of calcium and magnesium, while the content of potassium and phosphorus is high (Harris 2009). However, different purposes of growing algae demand different contents of nutrients in the

media (Grobbelaar 2004). When comparing content of calcium and magnesium in the HS-medium with nutrient solution for the other greenhouse crops, such as roses and tomatoes (Table 1), the amount of calcium and magnesium is remarkably low in the HS-medium. The purpose of calcium and magnesium should not be more important to higher plants compared with microalgae, as their main contribution is not bound to higher-plant specific traits, but membrane production, nutrient uptake and chlorophyll formation (Taiz & Zeiger 2010). However, content of nutrients in DW of both roses, tomatoes and cultures of microalgae need to be taken into consideration. Nevertheless, further optimization of growth media for *C. reinhardtii* is needed.

Table 17: Overview of the nutrient contents in the control, 15 mL solution 1b (3 times original amount of Ca^{2+} and Mg^{2+}), 30 mL solution 1b (6 times original amount of Ca^{2+} and Mg^{2+}) and tap water treatment. Nutrients in the optimized HS-medium used in Kliphuis et al. (2012) are added in the last column. Calculations were done using Equation 3 (Appendix 1). Molecular weights are from Harris (2007). Phosphates and Hutner's trace elements are the same for all treatments. *Tap water column: Values are sums of nutrient content in the tap water plus nutrient content in the control. Amounts of micronutrients were negligible compared to Hutner's trace elements. **Tap water was not tested for this nutrient due to method of analysis

	Content of nutrients in mg/L (ppm)				
Nutrient (Ion)	Control: 5 mL Sol. 1b	15 mL Sol.1b	30 mL Sol.1b	Tap water* 5 mL Sol. 1b	Optimized HS-medium, Kliphuis et al. (2012)
N	131.0	131.0	131.0	131.0**	346.4
P	420.0	420.0	420.0	420.0	439.4
K^+	853.4	853.4	853.4	855.7	1429.2
S	2.6	2.6	2.6	13.6	36.6
Mg^{2+}	1.9	5.8	11.7	4.5	27.7
Ca^{2+}	2.7	8.2	16.4	20.7	15.6

5.4 Conclusions

Production of *C. reinhardtii* in daylight is a possible scenario in the conditions seen of the Norwegian summer. Little negative effects on DW and quality were observed by light intensities up to 1000 μmol m^{-2} s^{-1}. The photosystems were photoinhibited when exposed to light periods of 500 μmol m^{-2} s^{-1} or 1000 μmol m^{-2} s^{-1}, but not chronically. In higher light intensities, shading would be an option. However, better light conditions than a Norwegian summer would be beneficial for the yield.

In diurnal conditions, content of starch will increase during day and decrease during nights. Thus, if producing for *C. reinhardtii* for starch, harvest time is crucial and should be done towards the end of the day. Amount of PFD during the day also has a positive influence, which means that harvesting after a sunny day will provide for a better yield than after a clouded day. Diurnal growing conditions were also positive for longevity of the cultures.

We have also enlightened the need to optimize the growth medium for *C. reinhardtii* to achieve a better DW yield. In particular, calcium, magnesium and sulphur are needed in a higher level than what the HS-medium provides for. Medium prepared with tap water is shown to be beneficial.

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7 Appendix 1. Calculations

7.1 Equation 1

Fv/Fm = (Fm-Fo)/Fm = ϕ_{PSII}/qP

Where Fm = maximum fluorescence, Fo = yield fluorescence without PFD, Fv = the difference between Fm and Fo, qP = photochemical quenching (proportion of opened PSII reaction centres), ϕ_{PSII} = Quantum yield photochemistry (Proportion of absorbed energy used in photochemistry). Also, we have F_t = steady-state yield of fluorescence in light; F_o = yield of fluorescence in absence of PFD

7.2 Equation 2

When counting cells using a haemocytometer, the total number of cells were calculated using the following formula:

$$V_1 = \frac{C_2 V_2}{C_1}$$

Where

V_1 = Amount algae culture to add start culture in mL

V_2 = Volume of HS-medium in tubes (normally 320 mL)

C_1 = Cells counted using the haemocytometer

C_2 = Desired amount of cells/mL (10 000 or 50 000)

7.3 Equation 3

Calculating specific nutrient concentration in mg/mL

$$Specific\ nutrient\ concentration\ in\ mg/l = \frac{X}{Y} * Z$$

Where

X = Amount of nutritional molecules (salts, phosphates) added per litre final medium in mg

Y = Molecular weight of nutritional molecules in mg/mMol

Z = Molecular weight of specific nutrient in mg/mMol

7.4 Equation 4

Chlorophyll is calculated using the following formulas (Harris et al. 1989).

$$Total\ chlorophyll\ (in\ \mu l\ chl/ml\ culture) = \frac{(6.1 * OD665) + (20.04 * OD649)}{0.25}$$

$$Chlorophyll\ A\ (in\ \mu l\ chl/ml\ culture) = \frac{(13.7 * OD665) - (5.76 * OD649)}{0.25}$$

$$Chlorophyll\ B(in\ \mu l\ chl/ml\ culture) = \frac{(25.8 * OD649) - (7.6 * OD665)}{0.25}$$

, where OD649 is absorbance measured at 649 nm, OD665 is absorbance measured at 665 nm. The formula is divided with 0.25 due to the dilution of chlorophyll in ethanol (see chapter 3.1.13.1).

7.5 Equation 5

Starch was calculated by the following formula:

$$Starch\ in\ \%\ starch\ per\ mg\ DW = \frac{OD510}{ODGB * DW} * 45$$

Where DW is DW of the given sample, OD510 is the absorbance of the sample measured at 510 nm, ODGB is the mean value of the absorbance of the glucose blanks.

7.6 Equation 6

Equations used calculating productivity or growth or per PFD. See Table 18.

Table 18: Equations used calculating productivity or growth per PFD. Further explanations are below the table.

	Max. growth (mg DW L^{-1} or starch in %)	Total light PFD until max. growth (μmol photons m^{-2} s^{-1})*	Days until max. growth reached	Productivity	Growth/PFD	Increased growth in mg DW L^{-1} or % starch d^{-1}
Treatment	MG	PFD_t	D_m	$\frac{PAR_t}{D_m}$	$\frac{MG}{PAR_t}$	$\frac{MG}{D_m}$

*PFD_t is a summation of hourly registrations of PFD from the start of the experiment until maximum growth was reached.

Examples:

Daylight experiments:

$$\boldsymbol{PAR_t = SUM_{hourly\ PAR\ t1-Dm}}$$

Daylight experiments, control:

$$\boldsymbol{PAR_t = SUM_{hourly\ PAR\ t1-Dm} + ((200\mu mol * 24h) * Dm)}$$

Light pulse experiment:

$$\boldsymbol{PAR_t = \big((200\mu mol * 24h) * (Dm)\big) + ((1000\ or\ 500\mu mol * 6h) * LPm))}$$

Light pulse control-, Ca^{2+} and Mg^{2+}- and air bubbling experiments:

$$\boldsymbol{PAR_t = (200\mu mol * 24h) * Dm}$$

Where

t1 = beginning of the experiment

Dm = Days until maximum growth was reached

Lm = Number of light periods until maximum growth was reached

8 Appendix 2: Figures and tables

Table 19: Contents of TAP-agar (Harris, 1989), listed in order of addition.
The recipe gives a total of 250 mL.

TAP-agar nutrients	Amount
Tris Base	0.605 g
TAP Salts stock solution	6.25 mL
PO_4 Stock Solution	93.75 µL
Hutner's trace elements	0.25 mL
Acetate	0.25 mL
Deionized water	243 mL
Agar	3.75 g

Table 20: Stock solutions of Sueoka's High Salt-medium (HS). Overview of nutrients in salts solution, phosphate solution and Hutner's trace elements (Harris, 1989). The Hutner's trace elements salts are dissolved in H_2O (amount listed by their right), before mixed together.

Stock solutions		
Salt	**Content in g L^{-1}**	
Salts Solution (Beijerinck's solution)		
NH_4Cl	100.0	
$MgSO_4 * 7H_2O$	4.0	
$CaCl_2 * 2 H_2O$	2.0	
Deionized water to 1 L		
Phosphate Solution		
K_2HPO_4	288.0	
KH_2PO_4	144.0	
Deionized water to 1 litre		
Hutner's trace elements	**Content in g**	**mL**
diH_2O		
EDTA, disodium salt	50	250
$ZnSO_4 * 7H_2O$	22	100
H_3BO_3	11.4	200
$MnCl_2 * 4H_2O$	5.1	50
$CoCl_2 * 6H_2O$	1.6	50
$CuSO_4 * 5H_2O$	1.6	50
$(NH_4)_6Mo_7O_{24} * 4H_2O$	1.1	50
$FeSO_4 * 7H_2O$	5.0	50

Table 21: Sueoka's High Salt-medium. Overview of the contents of the salts solution, phosphate solution and Hutner's trace elements in the medium, and also content of the salts in g L^{-1} in the final medium.

Final HS-medium		
Stock solution	**Amount added to final medium**	**Content in g L^{-1} final medium**
Salts Solution (Beijerinck's solution)	5 mL	
NH_4Cl		0.5
$MgSO_4 * 7H_2O$		0.02
$CaCl_2 * 2 H_2O$		0.01
Phosphate Solution	5 mL	
K_2HPO_4		1.44
KH_2PO_4		0.72
Hutner's trace elements	1 mL	
EDTA, disodium salt		0.063
$ZnSO_4 * 7H_2O$		0.028
H_3BO_3		0.014
$MnCl_2 * 4H_2O$		0.006
$CoCl_2 * 6H_2O$		0.002
$CuSO_4 * 5H_2O$		0.002
$(NH_4)_6Mo_7O_{24} * 4H_2O$		0.001
$FeSO_4 * 7H_2O$		0.006
Deionized water	To total of 1 L	

High light exposure results individually.

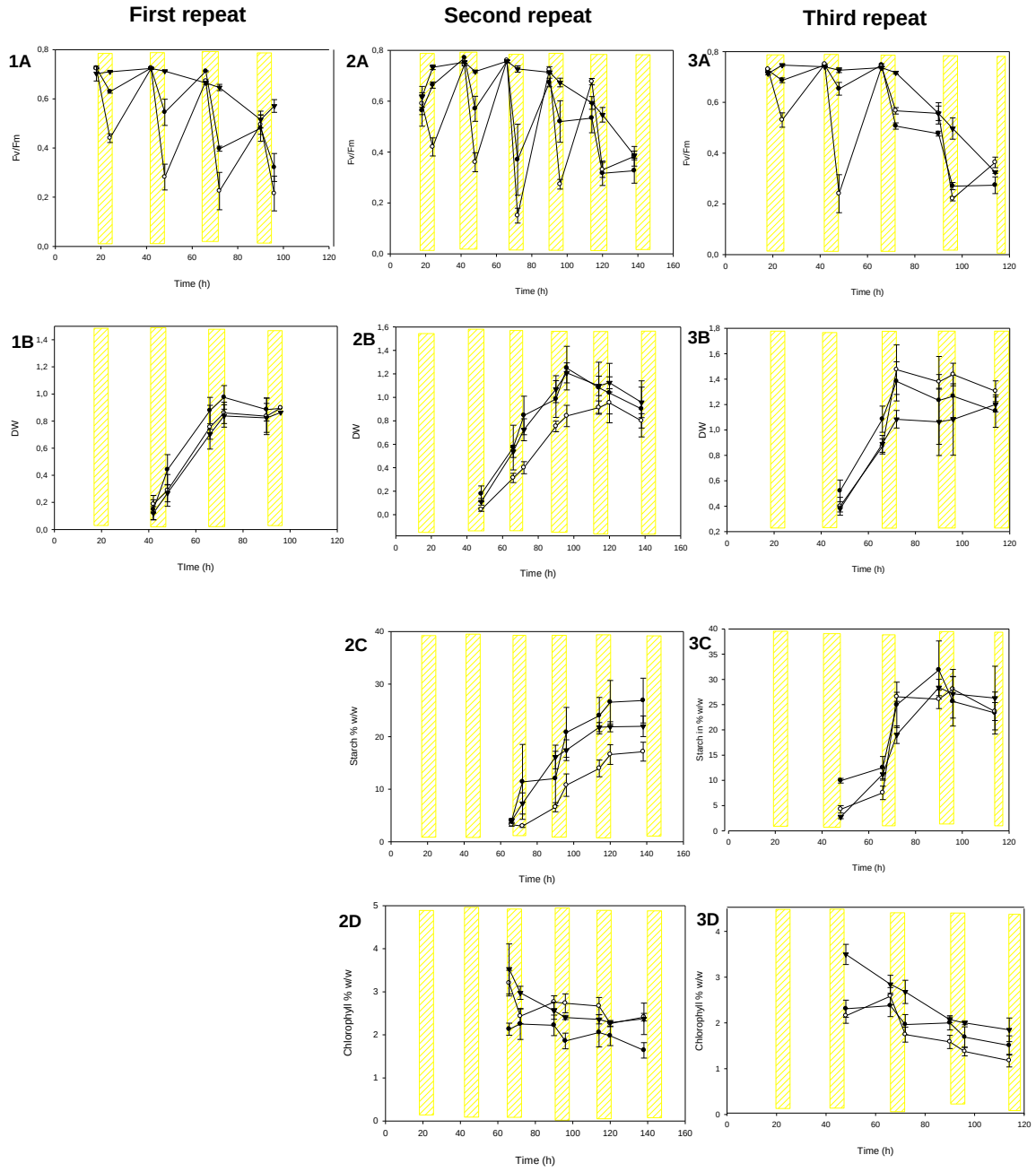
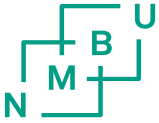


Figure 18: Effects of high light exposure on *C. reinhardtii*. Vertically is first, second and third repeat (1, 2 and 3). Horizontally is effects on A) Photoinhibition; B) DW; C) starch in % w/w and D) chlorophyll in % w/w. Yellow areas represent light pulses of 6 hours. ● = ML treatment, ○ = HL treatment, ▼= Control treatment.

Unequal results in the three repeats of high-light exposure experiments

The DW-results of the high light exposure experiments, varied significantly between the repeats (Table 18). This could be due to initial cell densities not being accurately 10 000 mL^{-1} in every repeat. Cell counting using a haemocytometer can be a challenge due to *Chlamydomonas*' cell division cycle, as they form clusters of up to 16 nuclei in one enveloped cell before the cytoplasm separate (Figure 2 – 16h). Clustering is also the reason why using an automatic cell counter is not suitable, as a cell counter would count the 16 cells in a cluster as one. An alternative method of determining start cell concentration is using DW. DW samples are done as the method described in chapter 2.9. After determining the post-weight, one can calculate how much inoculum to add a start culture. Meanwhile the filters are dried (four hours), cooled and weighed, the mother culture is placed in a refrigerator. The challenge of this method is that the culture might undergo changes while stored in the dark in a refrigerator (for instance starch breakdown), which will have an impact on the DW.

Cell counting with a haemocytometer would be easier and more accurate if the cell division was controlled. This can be done by growing the culture synchronously (a 12:12 hour light-dark cycle) as described by Harris (2009). This regime leads to cell division during dark period/early light period. In continuous illumination conditions, cells are shown to synthesize cell dividing components contentiously (Rollins et al. 1983). Therefore, growing the mother culture in a 12:12 hours light-dark cycle if possible, and counting cells at a time where cells are not in division could conceivably make cell counting easier.



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