Effects of culture conditions on the photoautotrophic growth and biochemical composition of *Chlamydomonas reinhardtii*, as a potential source for hydrogen production

Virkning av dyrkningsforholdene på fotoautotrof vekst og biokjemisk sammensetning av *Chlamydomonas reinhardtii*, som en potensiell kilde for bruk i produksjon av hydrogen

Philosophiae Doctor (PhD) Thesis

Daria Markina

Department of Plant Sciences
Faculty of Veterinary Medicine and Biosciences
Norwegian University of Life Sciences

Ås 2014

Thesis number 2014:85
ISSN 1894-6402
ISBN 978-82-575-1244-6

Supervisors

Main supervisor:

Professor Hans Ragnar Gislerød
Department of Plant Sciences
Norwegian University of Life Sciences
hans.gislerod@nmbu.no

Co-supervisors:

Dr. Leiv M. Mortensen
Department of Plant Sciences
Norwegian University of Life Sciences
leiv.mortensen@nmbu.no

Professor Jorunn Elisabeth Olsen
Department of Plant Sciences
Norwegian University of Life Sciences
jorunn.olsen@nmbu.no

Dr. Igor A. Yakovlev
Norwegian Forest and Landscape Institute
igor.yakovlev@skogoglandskap.no

Evaluation committee

Associate Professor Dr. Niels Thomas Eriksen
Aalborg University, Denmark
nte@bio.aau.dk

Assistant Professor Dr. ir. Packo P. Lamers
Wageningen University, the Netherlands
packo.lamers@wur.nl

Committee administrator:

Dr. Yeon Kyeong Lee
Department of Plant Sciences
Norwegian University of Life Sciences
yeonkyeong.lee@nmbu.no

Acknowledgments

The work presented in this thesis was carried out at the Department of Plant Sciences and at the Plant Cell Laboratory at the Norwegian University of Life Sciences during the period 2011 – 2015. The project was financed by the Norwegian Research Council (Project 20328: Use of solar energy for CO_2 capture, algae cultivation and hydrogen production – BioH_2) and the Norwegian University of Life Sciences.

First of all, I would like to sincerely thank my main supervisor, Hans Ragnar Gislerød, for his supervision, guidance, encouragement, and support throughout my PhD period. I would also like to thank Leiv Mortensen for the lively scientific discussions and his many ideas that undoubtedly maintained me aware of things I hadn't take into consideration and that contributed a good deal to the quality of my work. I am very grateful to Jorunn Olsen for her thorough reading and commenting on my papers and my thesis, which greatly improved the quality of this thesis. I would also to thank Yeon Kyeong for supporting me during the 're-submission' period.

A special thank to all the people from the Plant Cell Laboratory. You made my days in the lab enjoyable and made me feel at home there. I couldn't have wished for a warmer welcome when I first arrived, especially from Gry and Astrid. I also want to thank you all for your patience during the time I was learning Norwegian. Without those countless conversations (and cakes, sweets, and ice cream) at coffee breaks and lunches, I could have never progressed so rapidly. I value the ambience in the lab, how easy it is to talk to people there and how friendly everyone is. I will remember the great moments we had together with Gry, Astrid, Tone, Ida, Anne Guri, Yeon Kyeong, Linda, Dalia, Anette, Marit, Trina, Lars Harald, Zhibo, Shiori, Luz, Paul, Meseret, Amsalu. My years in the lab were a great life experience and more than colleagues, I consider you my friends. Thanks to Tone and Linda for always ordering materials for me. I am also very grateful to Dag for all his patience and technical help in the lab, without which I couldn't have done half the experiments I made.

I really don't have the words to express my gratitude to my best friend and my lab technician, my 'humble servant', my 'work wife', Silje. You know I could never have done this without you. I always dreamt about working with my best friend. But, I could never imagine how much fun it could be and how happy it would make me. On the serious side, thank you for all your patience with me, for helping me prepare the materials for my experiments and cleaning everything afterwards, for weighing endless amounts of filters, for taking samples for me, for being always so thorough when

taking the dry weight or doing anything else, for always willing to learn new stuff and foreseeing all my lab needs before I could even think about them myself. One couldn't really wish for a more perfect co-worker. Thank you for bringing in the radio to the lab. It was the best idea ever. Morgenklubben med Loven og Co at the radio Norge couldn't be a better start to a day of sample-taking and the disco-mood at top volume inside the algae room, in the media kitchen and the weighing room, as well as at my bench (although at lower volume) made the repetitive endless tasks so much more bearable. I don't think I can even start thanking you for all the other wonderful things you brought to my life, I would need a whole other thesis just for that. Thank you for always being there for me when I needed you, for fun stuff and less fun stuff like moving and driving me and my things, like Christmas trees, for introducing me to the knitting, for all the dinners and parties we've had together, for the best 17th of May week end in Bergen, for always listening to me, whether it was complaining or any little detail of my life I felt like sharing with you, for your wise advice, for teaching me Norwegian and correcting me, for all your perfect presents, for being so childish sometimes and always sitting beside me at coffee breaks and lunches. I dedicate this thesis to you, with all my love.

Huge thanks to all my friends in Ås that made my PhD years SO fun! Thanks to Rachel, Stephanie, Valeriya, Anja, and Bianca for the indescribable and unforgettable wine nights. To Davide, Paulo, and Sergio for all the dinners and parties we had at their houses. To Dimitris for our memorable birthday parties. To Emilio, because 'life is good'. Thanks to Felipe for bringing Latin America to Ås. To Michele for all the activities we did together and the conversation we had. And to all the others that I had the pleasure to meet and spend time with during these last four years. This PhD gave me a second opportunity to have a proper student life and I enjoyed it immensely.

I would especially like to thank Mauricio for your patience and for always listening to me, for being at my side when I needed you, for your advice and for all that you give me with your affection. Above all, thanks to my parents for believing in me, for listening and advising me during my PhD period, for always being there for me, no matter what. My mom has always been an inspiration to me, my role model, my everything. No words could express my gratitude and my love.

Ås, 2015.

Table of contents

1. Abstract

Hydrogen is a particularly attractive energy carrier, since its combustion produces water vapor only. The green microalga *Chlamydomonas reinhardtii* is able to produce hydrogen under sulfur-deficient anaerobic conditions and is used as a model organism for studying hydrogen metabolism in microalgae. Cultures with high biomass are required for an effective hydrogen production. Their biochemical composition, in particular, the contents of starch, protein, and chlorophyll are key factors affecting hydrogen production yield.

The aim of this thesis was to investigate the effects of culture conditions on the growth and biochemical composition of *C. reinhardtii* by use of photoautotrophic batch cultures. The studied variables included nutrient concentrations in the growth medium, concentration of CO_2 added to the cultures, light intensity, and temperature. We used factorial statistical designs to evaluate the individual and the interaction effects of these variables.

Three series of experiments were designed and response parameters, such as the pH of the cultures, their productivity and biomass yield, and their contents of starch, protein, and chlorophyll were measured. In the first series of experiments, a 2^4 full factorial design was first used to quantify the effects of ammonium (7.5 and 17.5 mM), phosphate (10.0 and 20.0 mM), sulfate (0.2 and 0.9 mM), and carbon dioxide (2.0 and 5.0% v/v) concentrations on the studied response parameters. Then, the number of treatments was extended to twenty-five (with 5.0 – 20.0 mM ammonium, 7.5 – 22.5 mM phosphate, 0.1 – 1.0 mM sulfate, and 1.0 – 6.0% carbon dioxide) in a central composite design and the responses were modeled using a second order equation. The obtained second-order surface responses were used for an optimization procedure that predicted maximum responses and the corresponding values of the studied variables, which were assessed by model validation experiments. The high phosphate concentrations were used to maintain a stable pH in the cultures. In the second series of experiments, the effects of light intensity (100 and 400 µmol m^{-2} s^{-1}), temperature (25 and 35 °C), and CO_2 concentration (3 and 9% v/v) on the growth and biochemical composition of *C. reinhardtii* cultures were quantified. Finally, the effects of increasing concentrations of calcium (0.068 – 0.68 mM) and magnesium (0.081 – 0.81 mM) on the growth and biochemical composition of microalgae were quantified in the third experiment.

The maximum productivity was predicted to be 0.87 g L^{-1} d^{-1} for 5 mM ammonium, 0.65 mM sulfate, and 6% CO_2 in the first series of experiments, and this result was confirmed by the model validation experiment. The productivity of the cultures was greatly influenced by increasing CO_2 concentrations. The productivity increased significantly (to 1.12 g L^{-1} d^{-1}) by increasing the light intensity to 400 µmol m^{-2} s^{-1} and the temperature to 35 ºC in the second series of experiments. A further increase in productivity to 1.97 g L^{-1} d^{-1} was achieved in the third experiment by increasing ten times the concentrations of calcium and magnesium in the medium in relation to the medium used in the two first series of experiments.

The biomass yield was positively influenced by the ammonium and sulfate concentrations, as well as by their interaction, but to a less extend by the CO_2 concentration. The maximum biomass yield measured in the first series of experiments was 1.57 g L^{-1} and it was 1.35 g L^{-1} in the second series of experiments. The concentrations of calcium (0.068 mM) and magnesium (0.081 mM) in the growth media used in these cultures were shown to be limiting for growth over a biomass of about 1 g L^{-1}, as we obtained a biomass of 4.83 g L^{-1} by increasing ten times the concentrations of calcium and magnesium.

The maximum predicted contents of starch and protein were quite high in the first series of experiments: 55 and 65% of dry weight, respectively. These results were confirmed by the model validation experiments. Starch and protein contents varied inversely to each other as response to varying growth medium composition. High concentrations of ammonium and sulfate enhanced protein accumulation, while cells grown in media with low concentrations of ammonium and sulfate accumulated starch as a general response to nutrient limitation, even if these two nutrients were not completely depleted from the medium. Both starch and protein contents increased with increasing light intensity in the second series of experiments, although starch did not exceed 11% of dry weight, due to the nutrient sufficiency of the growth medium. The chlorophyll content of the cultures increased with increasing concentrations of ammonium and sulfate in the media of the first series of experiments and with decreasing light intensity and increasing temperature in the second series of experiments.

For obtaining cultures with high biomass, high protein, and high chlorophyll contents, we recommend using media with increased concentrations of ammonium, sulfate, calcium, and magnesium. In such cultures, use of a good pH buffer is encouraged. Media with low ammonium

content are recommended for cultures with high productivity and starch content. By increasing light intensity (up to 400 µmol m^{-2} s^{-1}), temperature (up to 35 °C), CO_2 (up to 9% v/v), and calcium (to 0.68 mM or higher) and magnesium (to 0.81 mM or higher) concentrations, the productivity and starch content can be further increased.

Key-words

Biochemical composition, carbon dioxide, *Chlamydomonas reinhardtii*, light intensity, modeling, nutrient concentrations, photoautotrophic growth, temperature.

2. Sammendrag

Hydrogen er en spesiell attraktiv energikilde, siden utslippet ved forbrenning er vanndamp. Grønnalgen *Chlamydomonas reinhardtii* er i stand til å produsere hydrogen ved mangel på svovel under anaerobe forhold og brukes som modellorganisme for å studere hydrogenmetabolismen hos mikroalger. Det kreves kulturer med høy biomasse for en effektiv hydrogenproduksjon. Den biokjemiske sammensetningen av algene og spesielt innholdet av stivelse, protein og klorofyll er sentrale faktorer som påvirker effektiviteten i hydrogenproduksjonen.

Hensikten med dette arbeidet var å studere virkningen av dyrkingsbetingelsene på vekst og biokjemisk sammensetning hos *C. reinhardtii* dyrket fotoautotroft i batch kulturer. Innholdet av næringsstoffer i dyrkingsmediet, CO_2-konsentrasjon tilført algekulturen, belysningsstyrke og temperatur ble studert. Faktoriell design ble brukt som statistisk analyse for å studere hoved- og samspillseffekter av de ulike variablene.

Det ble lagt opp tre forsøksserier hvor en studerte pH i kulturene, produktivitet og biomassetetthet, og innholdet av stivelse, protein og klorofyll. I den første forsøksserien, som var et 2^4 faktorielt forsøk, ble virkningen av ammonium (7.5 og 17.5 mM), fosfat (10.0 og 20.0 mM), sulfat (0.2 og 0.9 mM) og karbondioksid (2 og 5 %) undersøkt. Forsøket ble så utvidet til 25 behandlinger (med 5.0 – 20.0 mM ammonium, 7.5 – 22.5 mM fosfat, 0.1 – 1.0 mM sulfat, og 1.0 – 6.0 % karbondioksid) i en «central composite design» og hvor virkningen av de ulike faktorene ble modellert ved bruk av andregradsligninger. De oppnådde resultatene ble brukt for å optimalisere virkningen på de studerte variablene, som igjen ble testet. Det høye fosfatinnholdet ble tilført for å holde en stabil pH i kulturene. I den andre forsøksserien ble virkningen av belysningsstyrke (100 og 400 µmol m^{-2} s^{-1}), temperatur (25 og 35 °C) og CO_2 (3 og 9 %) på vekst og biokjemisk sammensetning undersøkt. I den siste forsøksserien undersøkte en virkningen av kalsium (0.068 – 0.68 mM), og magnesium (0.081 – 0.81 mM), på vekst og biokjemisk innhold i *C. reinhardtii*.

Den maksimale veksthastigheten ble beregnet til 0.87 g L^{-1} d^{-1} ved bruk av 5 mM ammonium, 0.65 mM sulfat og 6% CO_2 i den første forsøksserien og resultatene ble bekreftet i testforsøk. Produktiviteten til *C. reinhardtii* økte ved økende tilførsel av CO_2. Produktiviteten

økte signifikant (til 1.12 g L^{-1} d^{-1}) ved å øke belysningsstyrken til 400 µmol m^{-2} s^{-1} og temperaturen til 35 ºC. En videre økning i produktiviteten til 1.97 g L^{-1} d^{-1} ble oppnådd i den tredje forsøksserien med å ti-doble kalsium og magnesium konsentrasjonen i forhold til innholdet i mediet brukt i de to første forsøksseriene.

Maksimal biomasse ble påvirket av konsentrasjonen av både ammonium og sulfat, så vel som samspillet mellom disse, men noe mindre av CO_2-tilførselen. Den maksimale biomassetettheten oppnådd i den første forsøksserien var 1.57 g L^{-1} og 1.35 g L^{-1} i forsøksserie to. Konsentrasjonen av kalsium (0.068 mM) og magnesium (0.081 mM) brukt i disse forsøksseriene viste seg å være for liten når tettheten i kulturene oversteg ca. 1 g L^{-1}, og det ble oppnådd en tetthet på 4.83 g L^{-1} ved å ti-doble disse konsentrasjonene.

Det maksimalt beregnede innholdet av stivelse og protein var meget høyt i den første forsøksserien, henholdsvis 55 og 65 % av tørrvekten. Disse resultatene ble bekreftet av testforsøkene. Innholdet av stivelse og protein i algene var negativt korrelert og reagerte forskjellig avhengig av sammensetningen av mediet. Høy konsentrasjon av ammonium og sulfat i dyrkingsmediet fremmet innholdet av protein, mens lav konsentrasjon av ammonium og sulfat førte til betydelig økning av stivelse, selv om det var en del igjen av disse stoffene i dyrkingsmediet. Både stivelse og proteininnholdet i algene økte med økende belysningsstyrke i forsøksserie to, men innholdet av stivelse oversteg ikke 11 % av tørrvekten, trolig fordi det var rikelig med ammonium og sulfat i dyrkingsmediet. Klorofyllinnholdet i algene økte med økende innhold av ammonium og sulfat i dyrkingsmediet og var høyest ved lav belysningsstyrke (100 µmol m^{-2} s^{-1}).

For å oppnå kulturer med høy biomassetetthet, høyt innhold av protein og klorofyll bør det anvendes høye konsentrasjoner av ammonium, sulfat, kalsium og magnesium. I slike opplegg er det viktig å bruke gode buffere for å holde en stabil pH i dyrkingsmediet. For å oppnå høy produktivitet og høyt innhold av stivelse, bør konsentrasjonen av ammonium i dyrkingsmediet være på et nivå slik at det oppstår en mangelsituasjon mot slutten av kulturene. Ved å bruke høye belysningsstyrker (opp til 400 µmol m^{-2} s^{-1}), temperaturer (opp til) 35 °C, konsentrasjon av CO_2 (opp til 9 %), kalsium (0.68 mM) og magnesium (0.81 mM) vil produktiviteten og stivelsesinnholdet kunne økes ytterligere.

Emneord

Biokjemisk sammensetning, *Chlamydomonas reinhardtii*, fotoautotrof vekst, karbondioksid, modellering, næringssammensetning av vekstmediet, temperatur, vekstlys.

3. List of papers

This thesis is based on the following articles:

Paper I:

Effects of ammonium, phosphate, sulfate, and carbon dioxide concentrations on starch, protein, and chlorophyll contents during photoautotrophic growth of *Chlamydomonas reinhardtii*

Daria Markina, Leiv M. Mortensen, Hans Ragnar Gislerød

Submitted to the Journal of Phycology

Paper II:

Modeling the effects of nutrient concentrations on the photoautotrophic growth and biochemical composition of *Chlamydomonas reinhardtii*

Daria Markina, Hans Ragnar Gislerød

Manuscript

Paper III:

Effects of light intensity, temperature, and carbon dioxide concentration on photoautotrophic growth and biochemical composition of *Chlamydomonas reinhardtii*

Daria Markina, Hans Ragnar Gislerød

Manuscript

Paper IV:

The importance of calcium and magnesium for the growth of *Chlamydomonas reinhardtii*

Daria Markina, Hans Ragnar Gislerød

Submitted to the Journal of Plant Physiology

4. Abbreviations

μ	Relative (or specific) growth rate
μ∞	Theoretical maximum growth rate
a	Gas-liquid interfacial area per unit of liquid volume
ADP	Adenosine diphosphate
ak_L	Volumetric mass transfer coefficient
ANOVA	Analysis of variance
APS	ATP-sulfurylase to form 5'-adenylyl sulfate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
C*	CO_2 solubility
C. reinhardtii	*Chlamydomonas reinhardtii*
CA	Carbonic anhydrase
cAMP	Cyclic adenosine monophosphate
Chl	Chlorophyll
DMSO	Dimethyl sulfoxide
DW	Dry weight
ETC	Electron transport chain
GOPOD	Glucose oxidase/peroxidase
GS/GOGAT	Glutamate synthase/glutamine:oxo-glutarate amino-transferase
H_2	Molecular hydrogen
HS	High-Salt Sueoka growth medium
Hyd1	Hydrogenase enzyme
I	Irradiance
k_L	Liquid mass transfer coefficient
LHC	Light-harvesting complex
mt	Mating type
NADPH	Nicotinamide adenine dinucleotide phosphate

nit	Nitrate reductase
NIVA	Norsk institutt for Vannforskning – Norwegian Institute for Water Research
OD	Optical density
P	Photosynthetic rate
p	p-value
PAPS	3'-phospho-5'-adenylyl sulfate
PAR	Photosynthetically active radiation
PBR(s)	Photobioreactor(s)
PFD	Photon flux density
PFOR	Pyruvate:ferredoxin oxidoreductase
Pmax	Maximum photosynthetic rate
PS	Photosystem
Psr	Phosphorus starvation response
Q	Intracellular nutrient quota
Q_{10}	Temperature coefficient
Q_{min}	Minimum intracellular cell quota
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rubisco	Ribulose-1,5-bisphosphate carboxylase-oxygenase
SAG	Sammlung von Algenkulturen – Culture collection of algae
TAG(s)	Triacylglycerol(s)
TAP	Tris-Acetate-Phosphate growth medium

5. Introduction

5.1. Bioenergy from microalgae

The current world energy supply is largely dependent on fossil fuels. Indeed, according to the International Energy Agency (2013), over 80% of the energy we use today derives from petroleum, coal, and natural gas. The intensive use of fossil fuels leads to major pollution problems. The major issue with the use of fossil fuels is the greenhouse gas emissions that drive climate change, increasing the atmospheric and oceans temperatures. On the other hand, the energy demand is rising in a world with growing population and increasing standard of living. Fossil fuels are, however, an exhaustible resource and their shortage is predicted to happen in the 21st century. To solve this environmental and energy crisis situation, we need to find alternative renewable and environment-friendly sources of energy. The development of renewable energies has enormous potential and some technologies are currently available, such as hydropower, wind energy with windmill parks, and solar energy with photovoltaics. Nevertheless, the transport fuels require liquid or gas fuels, which can be obtained from biomass, and are called biofuels.

Biofuels

Biofuels are produced from the sunlight energy and carbon dioxide through photosynthesis by plants, algae, and cyanobacteria. Solar energy is a major energy source, the total solar energy received at Earth's surface being over three orders of magnitude higher than the world's energy use (Larkum 2010). Biofuels are usually considered to be renewable and environment-friendly, and constitute therefore a sustainable alternative to fossil fuels. The main advantage of the biofuels over other renewable energy sources, such as wind or solar energy, is that the biofuels can be used in transportation, especially in air travel, and could eventually replace the fossil fuels in this area.

The term biofuel refers to solid, liquid, or gas fuels that are predominantly produced from biomass. The solid biofuels include wood, charcoal, plant residues, and animal dung, and are usually used for heating through burning. The liquid biofuels are bioalcohols (methanol, ethanol, propanol, and butanol), vegetable oils, and biodiesels and are usually used for transportation.

Biogas, biomethane, and biohydrogen are gas biofuels that can be used to produce electricity, for heating, or as transportation fuels.

The biofuels produced from sugar, starch, or vegetable oil derived from arable crops, such as cereals, sugarcane, and oil crops are commonly called first-generation biofuels (Juneja et al. 2013). The main limitations of producing biofuels in this way are the intensive agricultural input, the pollution due to the use of pesticides and fertilizers, the extensive land requirements, the intensive freshwater use, and, above all, the trade-off between the food crops and fuel crops production (Juneja et al. 2013). The use of food crops to produce biofuels is, according to the World Bank Group, the main factor driving up the food prices (Mitchell 2008). This affects countries in development the most.

The second-generation biofuels are fuels derived from lignocellulosic biomass and agricultural residues (Juneja et al. 2013). They circumvent several of the negative outcomes associated with the first-generation biofuels, but also require agricultural input, land, and freshwater that could be used for food crops (Juneja et al. 2013). The processing of this kind of biomass to convert it into fuels is also problematic.

The biofuels from algae are considered to be the third-generation biofuels (Juneja et al. 2013). Production of algal biofuels avoids most of the limitations of the first- and second-generation biofuels, including the food vs. fuel dilemma (Juneja et al. 2013). Algae have higher photosynthetic efficiency compared to the terrestrial plants (Chisti 2007). The maximum theoretical photosynthetic efficiency of conversion of solar energy into biomass is 4.6% for C3 plants and 6% for C4 plants (Zhu et al. 2008). Algae, however, can reach efficiencies up to 9% (Dismukes et al. 2008). Microalgae can reach higher biomass productivities due to their faster growth rates compared to terrestrial plants (Guschina and Harwood 2013; Marques et al. 2012). They also produce 10 – 20 times more oil (ha^{-1} $year^{-1}$) than any oil crops (Guschina and Harwood 2013).

Microalgae can grow in saline, brackish, and coastal seawater with little competition (Guschina and Harwood 2013). They are grown in liquid medium, which can be handled easily and the nutrients can be recycled (Juneja et al. 2013). Microalgae cultures use much less water than traditional crops, with the possibility of using non-potable water, including waste waters,

which can be treated in this way (Chisti 2007). Microalgae can also be cultivated in variable climates and non-arable lands, including marginal areas unsuitable for agricultural purposes, such as deserts and seashore lands (Marques et al. 2012). Production of microalgae can avoid certain environmental impacts, such as soil desertification and deforestation and it does not require use of pesticides (Chisti 2007). More importantly, cultivation of microalgae does not displace food crop cultures.

Biofuels from microalgae

- Brief history

The concept of using algae as a source of renewable fuels and energy dates back to 1931 (Borowitzka and Moheimani 2013) and it regained much attention during the 1990's oil crisis (Borowitzka 2013). It was first proposed in 1942 that microalgae might be suitable sources of lipids, which could be used to produce fuels (Harder and von Witsch 1942). Microalgae have been the subject of applied research for their commercial and industrial potential since the early 1950's when productivity and yield were first studied in mass culture by J. S. Burlew (1953). The commercial production of microalgae mainly for use as nutritional supplements and nutraceuticals started in the 1960's. The commercial farming of microalgae is, thus, less than 60 years old. The studies on microalgae mass culture in the 1970's made significant progress in understanding the nutrient requirements of microalgae and the effects of their limitations on growth, as well as the effects of temperature and light in outdoors cultures (Borowitzka 2013). Since then, several species of microalgae have been commercially produced, mainly in open pond systems, for use as nutritional supplements, pigments, and for aquaculture feed. Between 1980 and 2000, large research programs in Japan and United States focused on developing microalgal energy production systems (Barbosa and Wijffels 2013). In 1980, the United States Department of Energy began the 'Aquatic Species Program', which aimed at developing microalgae as a source of liquid oil fuels that would be able to compete with the fossil fuels (Ferrel and Sarisky-Reed 2010). Sheehan et al. (1998) noted in the conclusion to their report on the findings of this program that 'perhaps the most significant observation is that the conditions that promote high productivity and rapid growth (nutrient sufficiency) and the conditions that induce lipid

accumulation (nutrient limitation) are mutually exclusive. Further research will be needed to overcome this barrier, probably in the area of genetic manipulation of algal strains to increase photosynthetic efficiency or to increase constitutive levels of lipid synthesis in algal strains'. Since then, considerable research efforts have been made in this direction (for review, see Guschina and Harwood 2013).

- Liquid fuels from microalgae

When microalgal growth slows down due to nutrient limitation in the growth medium, carbon energy storage compounds, such as lipids and carbohydrates are synthesized and are accumulated in large amounts if the nutrient limitation situation is not reversed. Several species of microalgae accumulate substantial amounts (up to 50% of the dry biomass and more) of non-polar lipids, mainly in the form of triacylglycerols (TAGs) and hydrocarbons. Bioethanol also can be produced from microalgae, either via fermentation of the algal carbohydrates, or produced directly through microalgal photosynthesis (Chisti 2013). Then, the microalgae need to be harvested, dewatered, and the desired products extracted. Microalgal lipids can be converted to gasoline, kerosene (jet fuel), and diesel fuels that are indistinguishable from the equivalent fuels derived from petroleum (Chisti 2012). Liquid fuels derived from microalgae have performed outstandingly in tests (Haik et al. 2011). Microalgal transport fuels are therefore a proven technology.

- Hydrogen production from microalgae

Microalgae can also be used to produce gas biofuels such as biogas and biohydrogen. Hydrogen (H_2) is a particularly attractive energy carrier, since the only by-product of its combustion is water (Equation 1). H_2 also offers the highest mass-based chemical energy density and can be used in fuel cells or combustion engines (Lehr et al. 2012).

$$H_{2(g)} + \frac{1}{2} O_{2(g)} \rightarrow H_2O_{(g)} + 286\ kJ$$

However, the current technologies, by which H_2 is obtained are based on the fossil fuels cracking, which is accompanied by CO_2 emissions (Fouchard et al. 2005). One sustainable H_2 production process is the natural ability of certain green microalgae and cyanobacteria to produce H_2 in a

defined environment (Lehr et al. 2012). BioH_2 production using solar energy and electrons derived from water for the use as a renewable energy carrier is regarded as one of the most environmentally benign and sustainable energy solutions (Peters et al. 2013). The biggest advantage of producing H_2 using microalgae as compared to producing liquid biofuels is that H_2 is a gas and it is released from the cells into the medium and can be collected in the gas phase of the reactor. No elaborate down-stream processing is therefore required for its production. The green microalga *Chlamydomonas reinhardtii* produces the highest levels of H_2 reported to date and has been extensively used as a model organism for studying microalgal H_2 metabolism (Ghysels and Franck 2010). It is for this reason that this particular microalga was the microorganism of choice in the present study. Nevertheless, currently, the yields of H_2 production are far below those required for the production of H_2 from microalgae to be economically viable (Peters et al. 2013).

- Constrains and prospects to the commercialization of biofuels from microalgae

In spite of the immense potential of microalgae to produce liquid and gas biofuels, several constrains exist to the commercialization of this technology. The challenges are both technological and economical.

First of all, photosynthesis is an intrinsically inefficient process. Even if the microalgal photosynthesis is more efficient than the plants' one, the maximum theoretical efficiencies have not yet been attained in practice (Zhu et al. 2008). The low yields and photochemical conversion efficiencies are the main technological drawbacks (Akkerman et al. 2002). Other challenges include photoinhibition and other stresses, decline in biomass production yields due to competing microalgae, pathogens, and ageing, among others. Optimization of the light path length and of the photobioreactors (PBRs) design is necessary (Akkerman et al. 2002). The photosynthetic productivity and light use efficiencies could also be improved by reducing the size of the light-harvesting antenna complexes of the chosen microalgae strains (Beckmann et al. 2009; Borowitzka 2013; Melis et al. 1998; Polle et al. 2002).

On the other hand, production of microalgae is a rather energy-demanding process. A positive energy balance in the microalgal fuel production is imperative for it to make sense. The

large-scale, at which microalgae need to be produced to meet the requirements of bioenergy production, is challenging. Mixing of the cultures and sparging for both degassing and CO_2 supply require important mechanical energy input. Large amounts of water and nutrients are required for considerable amounts of microalgae to be produced. Sustainability of microalgal biofuels production requires the development of almost complete recycling of phosphorous and nitrogen in microalgal cultures (Chisti 2013). Sources of low-cost and concentrated CO_2 would also be necessary for large-scale production systems. The down-stream processes of harvesting, dewatering, and extraction of lipids from the microalgal biomass are also very energy-demanding and the current technologies are not suitable for the large-scale production of biofuels.

New microalgae strains need to be screened and improved through genetic engineering to increase the lipid accumulation or the H_2 production yields. Knowledge of how cultivation conditions affect productivity and biochemical composition of microalgae is important for choosing the optimal growth conditions for higher growth rates, biomass production, and level of lipids or carbohydrates accumulation (Guschina and Harwood 2013). More knowledge needs to be gained in different disciplines, integrated, applied, and demonstrated in an industrial context (Barbosa and Wijffels 2013).

The high cost of producing microalgae biomass postpones into the future the use of microalgal biofuels as an economical, renewable, and sustainable source of biofuels and bioenergy (Borowitzka 2013). Therefore it is essential to optimize all the factors influencing microalgae biomass production and reduce the costs. The costs of the PBRs and their operation and maintenance are the most critical to be reduced (Akkerman et al. 2002). On the other hand, it was early recognized that the economics of commercial utilization of microalgae largely depend on the cost of harvesting and dewatering (Soeder 1978). This is valid for liquid biofuels production. However, in the case of bioH_2, harvesting and dewatering are not necessary as H_2 is a gas and is released by the microalgae into the medium and can be collected directly from the overhead compartment of the PBRs.

A biorefinery approach (Figure 1) may be the only economically feasible way of using microalgae to produce bioenergy. A biorefinery is a facility that integrates biomass conversion processes and equipment to produce biofuels, power, and diverse chemicals from biomass (Demirbas 2009). The lipid content in microalgae can be as high as 50% of the dry biomass and

even higher percentages (as high as 60% of the dry biomass) are found as proteins and starch (Becker 2007; Singh and Gu 2010). These fractions can be used to produce biodiesel, bioethanol, biohydrogen, bioplastic, and other products (Chisti 2007; Hempel et al. 2011; Hirano et al. 1997; Hu et al. 2008; John et al. 2011; Melis et al. 2000; Nguyen et al. 2009; Ueno et al. 1998). The residual microalgae cake can be used for producing valuable co-products, such as carotenoids, astaxanthin, and other pigments, ω-3 fatty acids, vitamins, polysaccharides, and proteins, among others (Becker 2007; Borowitzka and Borowitzka 1990; Chen and Zhang 1997; Tripathi et al. 2002). These compounds can be used in a variety of commercial applications, such as aquaculture and animal feed, textile industry, nutraceuticals and dietary supplements, chemical and cosmetic industries, fertilizers industry, and others. The residual protein from the biomass also can be used for livestock, poultry, and fish feed additives (Singh and Gu 2010).

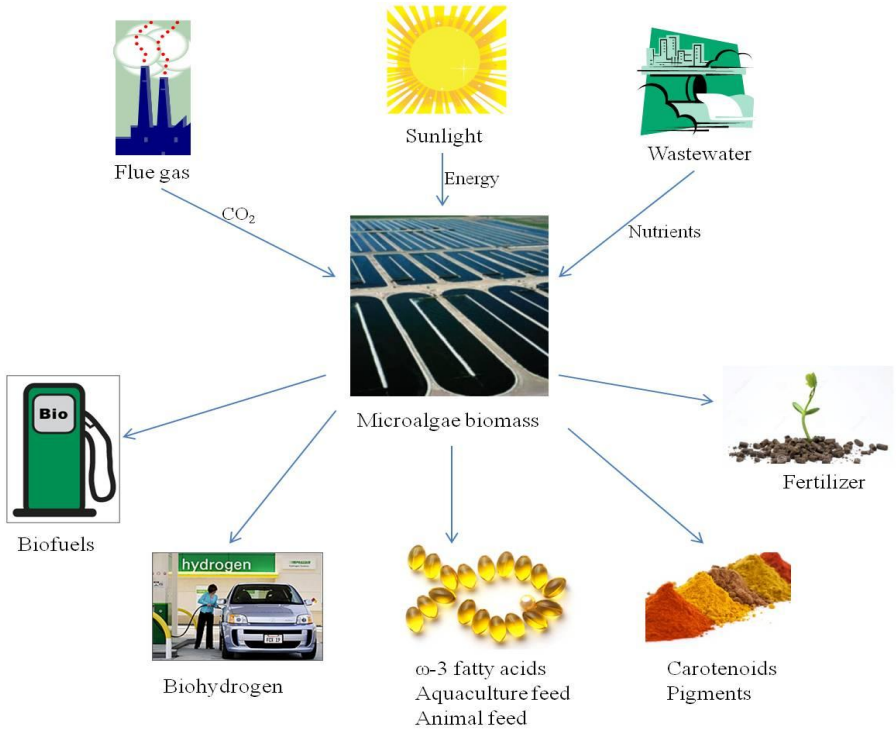

Figure 1. Schematic representation of the biorefinery concept, where sunlight energy, CO_2 from flue gases, and nutrients from wastewater are used to produce microalgae biomass for applications in biofuels, food, feed, pigments, fertilizers, and other commercial areas.

Another aspect of the biorefinery concept is the utilization of waste sources of nutrients (Figure 1). Use of CO_2 from flue gases and extraction of other nutrients from wastewaters would bring about environmental advantages of production of biofuels with microalgae, such as mitigation of green house gas emissions and wastewater treatment (Åkerström et al. 2014; Doucha et al. 2005; Jeong et al. 2003; Keffer and Kleinheinz 2002; Mortensen and Gislerød 2014; Shelef 1968; Wang et al. 2008). Integration of these various applications is complex and its feasibility yet needs to be demonstrated at large scale (Barbosa and Wijffels 2013).

In the short-term, the prospect of using microalgae to provide significant amounts of biofuels to sustain the human need for bioenergy is limited. However, several improvements to the process are foreseeable in the future and intensive research is being undertaken on these subjects. Biofuels from microalgae can only become a reality if the cost of production of microalgae is reduced, if the productivity of the desired products is maximized, and if other compounds are concomitantly produced for applications in food, feed, chemistry, and materials using microalgal biorefinery.

5.2. *Chlamydomonas reinhardtii*: morphology, physiology, and metabolism

Chlamydomonas reinhardtii is a green unicellular freshwater microalga, belonging to the phylum Chlorophyta, of the order Chlorophyceae, which is characterized by their flagella. *C. reinhardtii* was the first green microalga to have its genome sequenced (Merchant et al. 2007). However, genetic studies on this microorganism have been performed since as early as 1875 (Harris 2009). Due to this early genetic characterization, its fast growth and its metabolic adaptability, *C. reinhardtii* has been used as a model organism in various research aspects of cell and molecular biology of microalgae and higher plants (Ball 2005; Harris 2001; Hicks et al. 2001; Pröschold et al. 2005). The main areas of current investigations using this model system are chloroplast biogenesis, light perception, cell-cell recognition, cell cycle control, cell motility (including flagella structure and function and genetics of basal bodies) (Harris 2001), and, most recently, H_2 production (Melis et al. 2000).

Organism's origin

The species *Chlamydomonas reinhardtii* was first described in 1888 by P. A. Dangeard (Dangeard 1888), and named after Ludwig Reinhard(t), an Ukrainian botanist (Harris 1989). Most of the current laboratory strains of *C. reinhardtii* descend from a mating pair (of mating type mt^+ and mt^-) of clones derived from a zygospore isolated in a potato field in Amherst, Massachusetts, in 1945 and designated by G. M. Smith as isolate 137c (Harris 1989). In 1955, Ebersold received cultures from Smith, but these microalgae had two nuclear gene mutations, *nit-1* and *nit-2*, which prevented these cells from utilizing nitrate for growth (Harris 1989). The strain used in this study was the 137 c (mt^+) strain originally obtained from the SAG (Sammlung von Algenkulturen/Culture collection of algae) culture collection in Göttingen, Germany, where it is named SAG 34.89. This strain is equivalent to the Ebersold-Levine phenotype *nit-1, nit-2* Levine 137c(+), CC-125, and UTEX 2244 strains (SAG 2014). NIVA (Norsk institutt for vannforskning) culture collection in Norway purchased the SAG 34.89 strain in 2001, and it has been stored under the name CHL153 in the NIVA culture collection. We obtained the *Chlamydomonas reinhardtii* 137c (mt^+) NIVA strain personally on a Tris-Acetate-Phosphate (TAP) - agar solid medium Petri dish from Dr. Kari Skjånes, Bioforsk, Ås, Norway, in February 2011.

Cell morphology

Vegetative *C. reinhardtii* cells are of ellipsoid shape of approximately 10 µm in diameter and are characterized by two anterior flagella and a single cup-shaped basal chloroplast that surrounds the nucleus (Figure 2).

The cell nucleus and nucleolus are prominent in cross-sections of *C. reinhardtii*. The 120 mega bases nuclear genome is distributed on its seventeen chromosomes in the haploid state (Merchant et al. 2007). The nuclear membrane is continuous with the endoplasmic reticulum, and one to four Golgi bodies are situated nearby (Harris 2001).

The chloroplast occupies the basal two thirds of the cell. The chloroplast genome is of 195 kilo bases. Thylakoid membranes have the form of flat vesicles or disks and can be single or arranged in stacks inside the chloroplast (Harris 2009). This is the site for the light-dependent reactions of photosynthesis. One or more pyrenoids surrounded by starch bodies are found within the chloroplast (Ball et al. 1990). The pyrenoids are not membrane-bound organelles but specialized areas of the chloroplast that contain high levels of the Ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) enzyme (Kerby and Evans 1978). CO_2 fixation reactions of photosynthesis occur in pyrenoids.

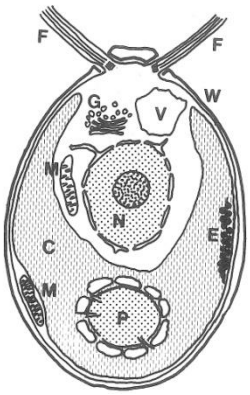

Figure 2. Schematic representation of *Chlamydomonas reinhardtii* cell (modified from Harris 1989). C, chloroplast; E, eyespot; F, flagella; G, Golgi apparatus; M, mitochondria; N, nucleus; P, pyrenoid; V, vacuole; W, cell wall.

The eyespot, or stigma, appears bright orange in light microscopy, due to high concentration of carotenoids (Harris 2001). It is located just inside the chloroplast membrane at the cell equator. The eyespot functions as a directional antenna that enables swimming cells to orient themselves with respect to unidirectional light (Harris 2001).

Mitochondria have elongated or branching structure and are dispersed throughout the cytosol (Harris 2001). The 15.8 kilo bases mitochondrial genome is linear and contains only a few genes (Harris 2001). Mitochondria are the site of cell respiration, which provides the energy (mainly in form of ATP) for the metabolic processes.

Two contractile vacuoles are located at the anterior end of the *C. reinhardtii* cell and are involved in osmoregulation (Harris 2001).

The two flagella are of 10 – 12 µm length and they arise from a pair of basal bodies located just beneath the apical end of the cell (Harris 2001). The flagella are responsible for cell motility and mating. The basal bodies play an essential role during mitosis, as they assume their role as centrioles.

Closely appressed to the plasma membrane, surrounding the cell, is the cell wall. The cell wall of *C. reinhardtii* consists primarily of carbohydrates and hydroxyproline-rich glycoproteins stacked in seven layers (Imam et al. 1985; Woessner and Goodenough 1994) and does not contain cellulose (Roberts 1974).

The life cycle of *Chlamydomonas reinhardtii*

- Sexual cycle

C. reinhardtii has a simple life cycle (Figure 3) (Harris 2001) and an easily cultivated haploid vegetative stage (marked 'haploid progeny' in Figure 3) (Pröschold et al. 2005). The haploid *C. reinhardtii* cells can be of two mating types: mt^+ and mt^- (Harris 2001), which, when deprived of nitrogen, differentiate into sexually competent gametes (Figure 3: gametogenesis). Plus and minus gametes start pairing along the length of their flagella (Figure 3: aggregation) initiating a cyclic adenosine monophosphate (cAMP)-mediated signal transduction cascade, which results in 'activation' that consists of a morphological change in the flagellar tips and

dissolution of the cell walls of the mating partners (Harris 2001). The mating partners fuse (Figure 3: plasmogamy) and the newly formed diploid zygote remains motile with four flagella. Formation of a hard, impermeable zygospore wall begins, chloroplasts disintegrate and lose their chlorophyll, and lipid bodies accumulate (Harris 2001). The zygospore wall gives protection against adverse environmental conditions: zygospores can remain viable in the soil for many years (Harris 2001). Zygospore maturates and germinates, meiosis occurs and four haploid vegetative cells are released (Harris 2001). Some of the mated pairs fail to initiate zygospore maturation pathways and begin to divide mitotically as stable vegetative diploids (Harris 2001).

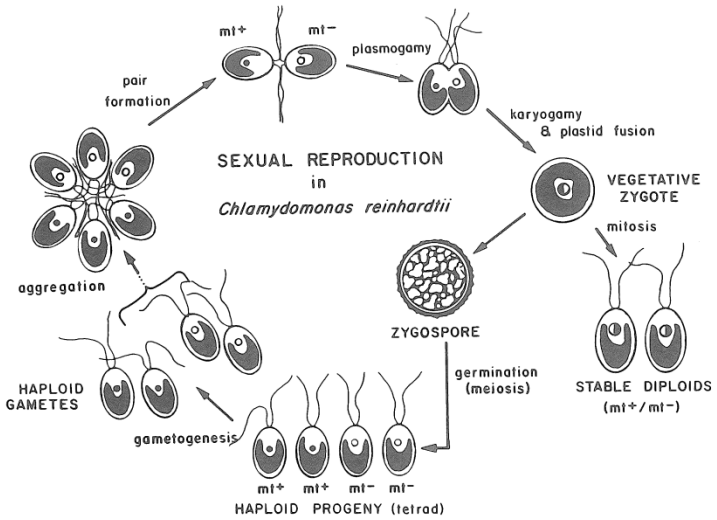

Figure 3. Schematic representation of the cell cycle of *Chlamydomonas reinhardtii* cells (Harris 1989).

- Vegetative cell growth

C. reinhardtii cells are able of three different modes of metabolism based on the energy source and model of utilization; these include: photoautotrophic, heterotrophic, and mixotrophic. Photoautotrophic cultures assimilate CO_2 through photosynthesis and, thus, require light as an energy source. Heterotrophic growth means culture in darkness with an organic carbon source, such as acetate (and acetate only (Sager and Granick 1953)) in the case of *C. reinhardtii* (Gfeller and Gibbs 1984; Gibbs et al. 1986; Heifetz et al. 2000). Heterotrophically grown cells metabolize the externally supplied acetate by O_2-consuming mitochondrial respiration, which, in this way, replaces chloroplast metabolism (Falkowski and Raven 2007). Mixotrophic cultures are capable

of using acetate as supplement or replacement for light and CO_2 as source of energy and carbon for growth (Falkowski and Raven 2007).

In the laboratory, wild type *C. reinhardtii* cells are easily grown in liquid or on solid agar media at pH in the range of 6.5 to 8.0, with no requirements for supplementary vitamins or other co-factors (Harris 1989). E. Harris (1989) recommends to use deionized water when preparing the culture medium for experimental work.

According to E. Harris (2001), optimal growth temperature is from 20 to 25 °C and adequate irradiance is in the range of 200 to 400 µmol m^{-2} s^{-1} photosynthetic active radiation (PAR). Photoautotrophic cultures of *C. reinhardtii* should be bubbled with 5% CO_2 for maximum growth (Harris 1989). Under these conditions, the average doubling time should be of 6 to 8 h.

Vegetative cells of *C. reinhardtii* divide by mitosis. If the cells are grown under light-dark illumination cycle, the cells remain in the G1 state of the mitotic cell cycle during the light phase and divide during the dark phase. Two to three mitotic divisions take place then in rapid succession, the daughter cells (4 to 8) are retained within a common mother cell wall (palmelloid cells, Figure 4) and then are released simultaneously (Harris 2001). The number of successive divisions that take place in a given cycle depends on the cell size reached during the G1 state. Many cellular processes are controlled by the circadian rhythm. If the cells are grown under continuous light, the mitotic divisions are asynchronous and can occur at any time point for each individual cell. In our experience, palmelloid cells from two to up to sixteen daughter cells can be observed in these conditions (Figure 4).

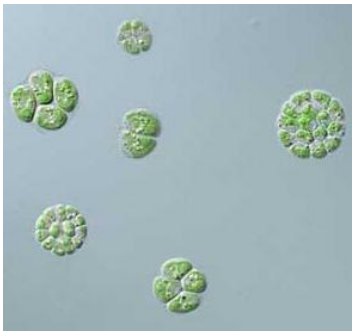

Figure 4. Palmelloid cells of *Chlamydomonas reinhardtii* (SIBS 2014). Up to sixteen daughter cells can be observed inside one single palmelloid cell surrounded by the mother cell wall.

Cell metabolism

- Photosynthesis

Photosynthesis is the main process responsible for the fixation of inorganic CO_2 into organic molecules with sunlight as energy source (Shastri and Morgan 2005). Organisms capable of photosynthesis obtain their energy by absorbing light energy for the reduction of CO_2 through oxidation of substrates, mainly water, and release O_2 (Field et al. 1998). Aquatic photosynthetic organisms fix almost half of the inorganic carbon from the atmosphere (Field et al. 1998).

The photosynthetic reactions occur in two steps. First, the light-dependent reactions are responsible for light absorption and oxidation of water molecules (Figure 5). These redox reactions produce chemical energy in form of adenosine triphosphate (ATP) and reductants in form of nicotinamide adenine dinucleotide phosphate (NADPH). The light-dependent reactions occur inside the chloroplast, across the thylakoid membranes (Raven 1980). The second step of photosynthesis consists of biochemical reactions that are responsible for CO_2 fixation.

In the first step of photosynthesis (Figure 5), light is intercepted by light-absorbing pigments, *i.e.* chlorophyll *a* and other photosynthetic pigments, which are organized in supra-molecular structures called antennae. The pigments are associated with specific proteins, forming the light-harvesting protein – chlorophyll *a* complexes (LHC), which are embedded in the thylakoid membranes (Dubinsky et al. 1995). By absorbing photons from the incident light beam, the pigments become excited. The excitation energy is then transferred to the reaction centers in the photosystems (PS), where it is trapped as chemical energy by oxidation of the excited state of primary electron donor by a primary electron acceptor (Ley 1980). The water molecule is oxidized at the PSII and charge separation occurs, one electron leaving the water molecule and O_2 being released (Dubinsky et al. 1995).

This electron is then transported by a cascade of redox reactions through what is called the electron transport chain (ETC). The ETC consists of several membrane-bound and membrane associated electron carriers. Two photosystems (PSII and PSI), each with a LHC, act in series through an intermediary complex containing cytochromes *b6* and *f*, among other redox molecules (Figure 5).

The net result is that the redox state of the electrons passing through the ETC is elevated from a level that brings about oxidation of water to a level capable of $NADP^+$ reduction (Harris 2009). The energy of the electrons excited by light is used to generate a proton motive force by the pumping of H^+ across the thylakoid membrane from the stroma into the lumen, against the pH gradient. A membrane-bound ATP synthase utilizes these protons to generate ATP. The ATP synthase is a complex machinery that utilizes the proton motive force by letting the protons flow along the pH gradient from the thylakoid lumen to the stroma to drive the phosphorylation of ADP into ATP. The ATP synthase couples in this way the reducing power generated by photosynthetic electron flow to the production of chemical energy storage compound, ATP (Harris 2009).

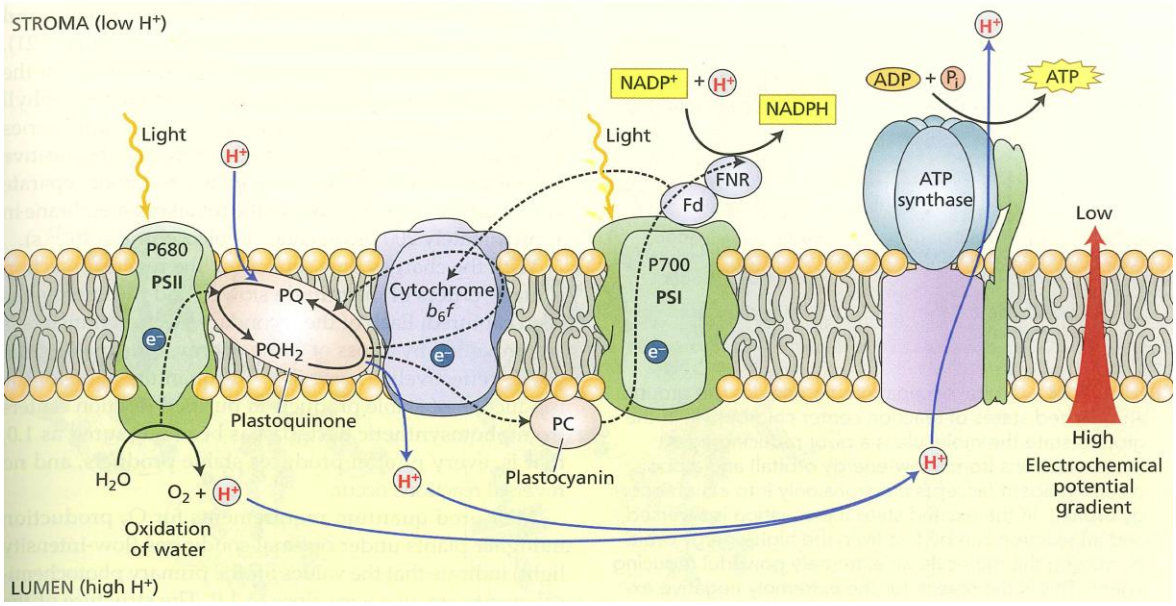

Figure 5. Schematic representation of the light-harvesting reactions of photosynthesis (Taiz and Zeiger 2010).

The ATP and NADPH generated by the light-dependent reactions are then used in the carbon fixing reactions of photosynthesis. The initial fixation of CO_2 is catalyzed by the Rubisco enzyme through the following reaction:

$$\text{Ribulose-1,5-bisphosphate} + CO_2 \rightarrow 2 * \text{3-phosphoglycerate}$$

This reaction is the first step of incorporation of the atmospheric carbon into carbon skeletons by the carbon reduction pathway, called Calvin-Benson cycle (Figure 6). This pathway decreases the carbon oxidation state from the highest value found in CO_2, to levels found in sugars. The enzymes of the Calvin-Benson cycle are not membrane-bound and are localized in the stroma of the chloroplast (Harris 2009).

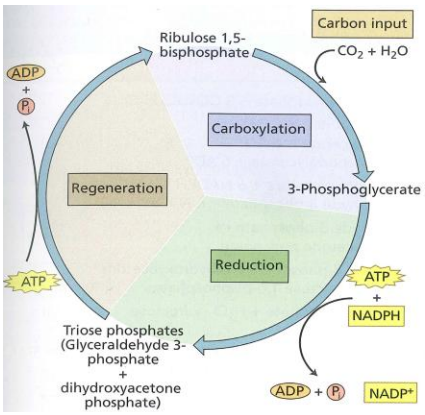

Figure 6. Schematic representation of the Calvin-Benson cycle (modified from Taiz and Zeiger 2010). The Calvin-Benson cycle consists of three phases: carboxylation, reduction, and regeneration. The first step is the incorporation of CO_2 into ribulose-1,5-bisphosphate by the Rubisco enzyme. Then, the product of this reaction, 3-phosphoglycerate, is reduced to 3C triose phosphates, which can then be used for the synthesis of starch and other carbon-containing components. Finally, ribulose-1,5-bisphosphate molecule is regenerated though a series of ten enzymatic reactions.

When the products of the Calvin-Benson cycle are formed in excess to be utilized by the growth metabolism of the cell, carbon is stored in high-energy carbon storage compounds, such as lipids and carbohydrates (Larkum et al. 2003). In case of such imbalance, *C. reinhardtii* is known to accumulate starch (Klein 1987). For this, intermediate metabolites of the Calvin-Benson cycle are converted into glucose through a set of enzymatic reactions, and glucose molecules are incorporated into the starch macro-molecules. The starch molecule consists of α-1,4 linked glucans branched through α-1,6 glycosidic linkages (Taiz and Zeiger 2010). In *C. reinhardtii*, starch is synthesized in the chloroplast (Levi and Gibbs 1984) by the ADP-glucose pyrophosphorylase, which adds glucose molecules to an already existing glucan chain (Taiz and Zeiger 2010).

- Photosynthetic rate is a function of irradiance

The fraction of solar radiation that is usable by photosynthetic systems is in the 400 to 700 nm range and is called photosynthetically active radiation (PAR). The amount of PAR incident per unit area is called photosynthetic photon flux density (PFD) and is expressed as micromoles of incident PAR photons per area per second (μmol m^{-2} s^{-1}).

The photosynthetic rate is a Poisson function of the irradiance. Indeed, the photosynthetic rate depends on the irradiance in a way that is usually graphically represented by the so called – 'photosynthesis versus irradiance' (P vs. I) curve (Figure 7).

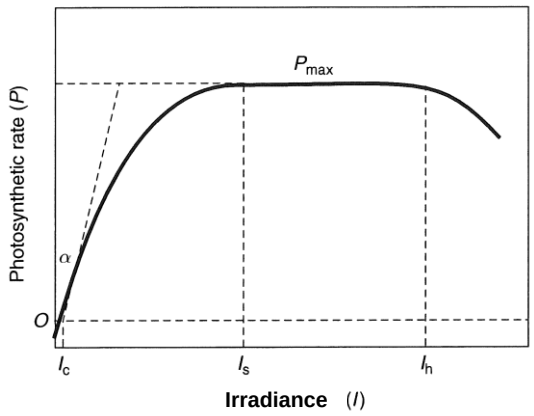

Figure 7. Irradiance-response curve of photosynthesis (modified from Richmond 2003). Photosynthetic rate is usually measured as O_2-evolution rate by a Clark-type oxygen electrode (Walker 1993). The intercept of the vertical axis, *O*, is the measure of O_2 uptake due to dark respiration. I_c is the light compensation point, where photosynthetic O_2 production rate is equivalent to the O_2 uptake rate by respiration. The initial slope, α, is the maximum efficiency of light harvesting and utilization. I_s is the irradiance level of onset of light saturation. P_{max} is the light-saturated rate of photosynthesis. It is the maximal attainable photosynthetic rate under saturating irradiance. I_h is the irradiance at which photoinhibition is set off.

The photosynthesis-irradiance curve can be divided into three distinct regions: a light-limited region, a light-saturated region, and a photoinhibited region. In the first region, the photosynthetic rate is linearly proportional to the irradiance. At low irradiance levels, the rate of photon absorption determines the rate of the steady-state electron transport from water to CO_2; that is why it is called the light-limited region. As the irradiance increases, the photosynthetic rate becomes increasingly non-linear and rises to the saturation level, P_{max}. Photosynthesis becomes less light-efficient. At light saturation, the rate of photon absorption exceeds the rate of steady-

state electron transport from water to CO_2. The enzymatic reactions utilizing fixed light energy become rate limiting. Further increases in irradiance beyond the light saturation can lead to a reduction in photosynthetic rate from the maximum saturation level (Baker and Bowyer 1994). This reduction, which is dependent on both, the irradiance and the duration of exposure, is called photoinhibition.

- Respiration

Aerobic (O_2-consuming) respiration is a set of catabolic reactions occurring in mitochondria, by which reduced organic carbon compounds generated during photosynthesis are oxidized. In this way, respiration generates the carbon precursors for biosynthesis of a full range of cellular components, such as nucleic acids, lipids, proteins, and structural polysaccharides. It results, thus, in cell growth, at the same time as it releases the energy stored in carbon compounds, generating ATP. Respiration also leads to production of reductants (NADH) and it releases CO_2. Respiration is therefore essential for growth and maintenance processes of the microalgal cell (Larkum et al. 2003).

5.3. Growing *Chlamydomonas reinhardtii*

Photobioreactors

In practice, microalgae are cultivated either in outdoors open ponds or in closed photobioreactors (Figure 8). The open ponds (Figure 8a) allow for large scale outside production of microalgae and are currently the most used in commercial production. They are relatively easy and inexpensive to build and operate. However, they present the inconvenience of easy contamination of the cultures and little control over growth conditions.

Closed PBRs, on the other hand, avoid these drawbacks, but they are more expensive and difficult to build and operate. They allow for more or less strict control over the different factors that affect microalgae growth, among others, light, temperature, mixing, pH, salinity, CO_2 supply and O_2 removal, nutrients concentrations, and culture mode. Many different designs of PBRs exist nowadays. Probably the most popular are the tubular PBRs that can be arranged vertically in racks (Figure 8b) or horizontally on the ground (Figure 8c). The flat panel PBRs can also be arranged at different angles (Figure 8d and e). A simpler version are the vertical plastic cylinders (Figure 8f) or sleeves (Figure 8g and h). Column PBRs are bubble columns or airlifts, which are placed vertically, aerated from below, and illuminated through transparent walls (Eriksen 2008). Column PBRs offer the most efficient mixing, the highest volumetric gas transfer rate, and the best controllable culture conditions (Eriksen 2008). Experimental PBRs are therefore often designs as bubble column, as was the case in this study.

Quite a lot of research is focusing on the optimal design of PBRs. The main factor to take into account is the light-use efficiency, a term that reflects how much biomass is produced with the available light (Eriksen 2008). For this, surface-to-volume ratio and light-path length, inclination and orientation, as well as mixing are important parameters to take into consideration (Richmond 2004).

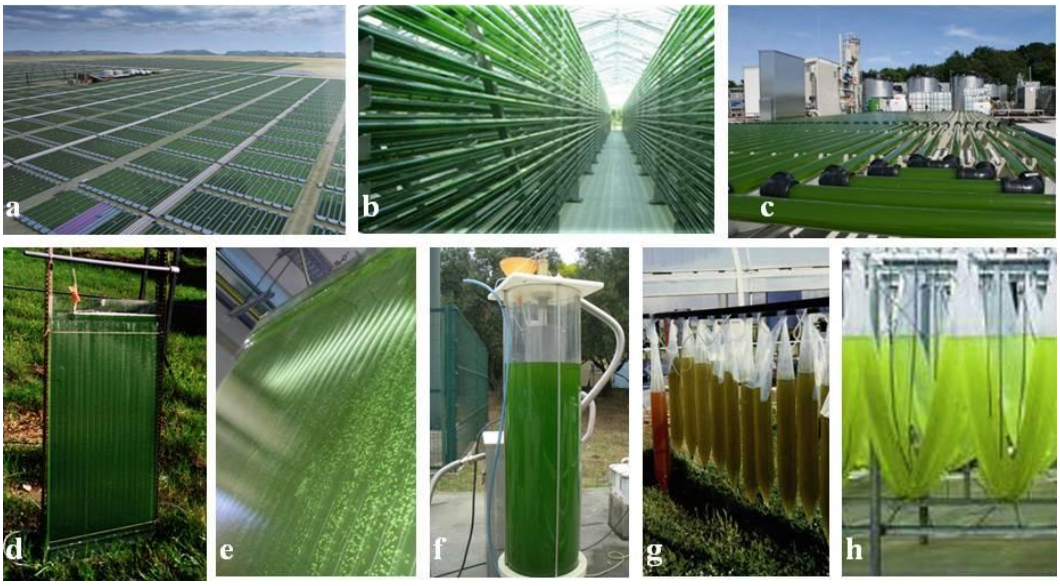

Figure 8. Different designs of photobioreactors (Gouveia 2011). (a) Race ponds in California, US. (b) Vertical tubular PBRs, Germany. (c) Horizontal tubular PBRs, at AlgaePARC, the Netherlands. (d) Vertical flat panel PBR, Portugal. (e) Inclined flat panel PBR, Portugal. (f) Vertical plastic cylinder PBR, Portugal. (g) Vertical plastic sleeves PBRs, Portugal. (h) Inclined plastic sleeves PBRs, Portugal.

Cultivation regimes

- Continuous cultures

Microalgae can be cultured in different regimes: continuous, batch, or even immobilized. In continuous regime, fresh growth medium is supplied and some of the culture is removed from the PBR continuously or intermittently (semi-continuous culture). In a continuous culture, the microalgae are maintained in a steady-state exponential growth. Growth can be defined as any form of accretion of the biomass of microalgae in a culture (Andersen 2005). The main factor controlling cell growth in a continuous system is the rate at which fresh medium is added (Becker 1994). In such cultures, when all the required nutrients are provided in excess, microalgae grow in a balanced way and display a uniform chemical and biochemical composition (Goldman 1980). Two kinds of control strategies of continuous systems are used in microalgae cultures:

- Chemostats, where the nutrient concentrations are maintained constant, or
- Turbidostats, where the microalgal concentration inside the PBR is constant.

- Batch cultures

In a batch culture, on the other hand, a limited amount of growth medium and microalgal inoculum are placed in a PBR and incubated in favorable conditions for growth (Richmond 2004). This is the most common method for cultivation of microalgae and is widely used in commercial cultivation systems (Richmond 2004). Batch cultures have some advantages in terms of expense, ease of manipulation, and required volume of medium (Andersen 2005). Batch culture mode was therefore chosen to be employed in this study.

The biomass concentration in a batch culture is a function of time and is usually graphically represented as a growth curve as in Figure 9 (Fogg and Thake 1987). The evolution of biomass concentration in a batch culture is commonly divided in five growth phases:

1) Frequently, just after fresh medium has been inoculated, a lag phase (zone 1 in Figure 9) occurs, where no apparent increase in cell number is observed. The conditions in the culture are different from the previous environment experienced by the microalgae cells (Becker 1994) and often, the cells are not adapted to the new environment and might be in an unhealthy condition (Becker 1994; Spencer 1954). Also, a portion of the newly inoculated cells might be not viable and does not contribute to the growth (Fogg and Thake 1987). On the other hand, the viable cells might not be in a condition to divide immediately, especially if the parent culture was an old one. Enzymes may have been inactivated and concentrations of metabolites may have decreased to a level insufficient for cell division (Hinshelwood 1946; Spencer 1954). During the lag phase, thus, the enzymes are restored and the substrate concentrations increase to the levels necessary for rapid growth. Some growth-enhancing factors might need to accumulate in the growth medium before the cells can start their division or, on the contrary, some toxic compounds that inhibit growth might be present in the medium (Fogg and Thake 1987; Huntsman and Barber 1975). The length of the lag growth phase is directly dependent on the age of the inoculum, diminishing as this entered the exponential phase of growth, being zero if the inoculum has been growing exponentially, then increasing according to the duration of the stationary phase (Fogg and Thake 1987). The length of the lag phase also varies inversely with the size of the inoculum (Fogg and Thake 1987). In this growth phase, there is high metabolic activity in the culture. The cells are

also much more sensitive to temperature and other environmental changes than cells in more mature stage of development (Becker 1994).

2) Once the cells have accumulated sufficient metabolites and the necessary enzymes are activated, the culture enters into the exponential growth phase (zone 2 in Figure 9), where the relative or specific growth rate (μ) accelerates continuously. The growth rate can be expressed in different measures of growth, such as cell numbers, volume of microalgal material, or cell nitrogen, which are approximately the same, but not necessarily identical, since the mean generation time may remain constant, while the mean cell volume or dry weight per cell alters (Fogg and Thake 1987). The increment in microalgal biomass (dN) in time (dt) is proportional (μ) to the biomass (N) in the population at any given moment:

$$\frac{dN}{dt} = \mu N$$

The cells divide at increasing rate, determined by the intrinsic nature of the organism and the culture conditions (Becker 1994). Measured under standard conditions, the relative growth rate is usually rather constant for a particular species (Fogg and Thake 1987). Cell size is the main determinant of the relative growth rate of a species, since it reflects the surface/volume ratio and, thus, the relative rate at which nutrients can be taken up (Fogg and Thake 1987). It is a general observation than small species grow faster than large ones (Banse 1976; Foy 1980).

For a given species, the relative growth rate is a function of temperature, light intensity, and other environmental factors. It increases with temperature, but optimal temperatures may vary with light intensity and concentration of nutrients (Eppley 1972; Eppley and Sloan 1966; Hutner et al. 1957; Maddux and Jones 1964). The relative growth rate has the same general relationship to light intensity as does the rate of photosynthesis, increasing proportionally to light intensity when light is the limiting factor and being independent of light intensity when saturating levels are reached (Fogg and Thake 1987; Sorokin and Krauss 1958). No self-shading effect occurs at the low cell densities in the exponential growth phase. However, the response to different light intensities alters quickly, while exponential growth is taking place (Steemann Nielsen et al. 1962). If nutrients are supplied in sufficient amounts, changes in nutrient concentrations caused by the uptake by the microalgae are so small that their effect on growth can be neglected (Becker 1994). The effect of supplying a low concentration of a particular

nutrient to a batch culture is observed on the shorter duration of the exponential phase rather than on the reduction of the relative growth rate (Fogg and Thake 1987).

In this growth phase, microalgae grow in a balanced way and display a uniform chemical and biochemical composition (Fogg and Thake 1987). The metabolic pattern characteristic of the exponential phase is of high photosynthetic capacity, which is devoted to production of protein and other cell constituents involved in growth, rather than storage products or cell wall constituents (Fogg and Thake 1987). Such actively growing cells have a high protein content, as much as 70% of dry weight, whatever the taxonomic position (Fogg and Thake 1987).

3) After some time, the cell concentration becomes high enough for the cells to begin to shade one another (phenomenon known as the self-shading effect) so that gradually a high absorption of incident light occurs. Only the cells at the surface will receive a light intensity saturating for photosynthesis, the bulk of the culture being light-limited, and if the culture is very dense, in virtual darkness. The culture transitions then into the linear growth phase (Melis et al. 1998; Sinetova et al. 2012). This transition is accompanied by a marked decrease in protein content (Fogg and Thake 1987).

In the linear growth phase (zone 3 in Figure 9), the growth rate (or productivity) is constant and the cell concentration increases linearly with time (Ogbonna et al. 1995b):

$$\frac{dN}{dt} = k$$

It is in the linear growth phase where the highest amount of biomass is produced in absolute terms. The linear growth phase lasts until exhaustion of a certain nutrient occurs (Sinetova et al. 2012) or the culture reaches a stage, where respiration begins to interfere. In nutrient-rich, well maintained cultures, this linear phase remains over a certain period of time (Becker 1994). The linear growth phase is relatively longer than the exponential growth phase, regardless of the irradiance (Ogbonna et al. 1995b). Ogbonna et al. (1995b) argued that during light-limited batch cultivation of photosynthetic cells, the linear growth rate is a better indicator of growth than the specific growth rate of the exponential growth phase. Ogbonna et al. (1995b) found a good correlation between the linear growth rates and the final cell concentrations for both *Chlorella* and *Spirulina* cells. Therefore in his study, we chose to study the productivity (g L^{-1} d^{-1}) of *C.*

reinhardtii cultures in the linear growth phase rather than the relative growth rate in the exponential growth phase.

4) As the culture enters the stationary growth phase, the overall growth rate decreases with increase in cell concentration (transition zone between linear and stationary growth phases, between zones 3 and 4 in Figure 9) (Ogbonna et al. 1995b) and the nutrients start to become limiting. The duration of this transition zone depends on the nature of the limiting factor (Fogg and Thake 1987). Nutrient limitation, experienced by the cells in the stationary growth phase, often leads to decrease in gross protein abundance in relation to carbohydrate and lipids and results in unbalanced growth (Falkowski and Raven 2007). Alteration of the pH of the medium results from a preferential uptake of particular constituents of the medium. Especially in the case of the use of ammonium as a nitrogen source, the pH decreases causing the medium to become too acidic to support growth (Fogg and Thake 1987). Oxidative breakdown of synthesized substances starts to reduce the constant increment of biomass and equilibrium is reached between the maximum microalgal biomass concentration and biomass loss due to degradation process. In the stationary growth phase (zone number 4 in Figure 9), the maximum attainable microalgal biomass concentration (or biomass yield) in a closed system is reached (Becker 1994). The biomass yield attained in the stationary phase depends on the nature of the limiting factor (Fogg and Thake 1987). If a nutrient is limiting, it is to be expected that the yield will be proportional to the amount supplied initially (Fogg and Thake 1987). Great changes in the pattern of metabolism and cell composition occur in microalgae during the exponential growth phase (Fogg and Thake 1987). For example, nitrogen-deficient *Monodus subterraneus* cells from an advanced stationary growth phase contain less than 10% of dry weight in protein, low amounts of chlorophyll and nucleic acids, but high contents of storage polysaccharides and lipids, accompanied by extremely low photosynthetic and respiratory activity (Fogg 1959). There may be some differences between microalgal classes in relation to their composition in the stationary growth phase, but these changes are small compared with the differences in cell composition, which a single species can experience in the course of growth in a culture (Fogg and Thake 1987).

5) Eventually, the unfavorable environmental conditions, the old age of the culture, the limited supply of light and nutrients, and the contamination by other microorganisms lead to the death of the culture (zone 5 in Figure 9). The reduced viability of the cell population is also due to the

release of growth-inhibiting organic substances into the medium (Jørgensen 1956). The time of the onset of the death phase varies enormously according to species and the conditions of culture. Often, the stationary phase may be maintained for several weeks, but sometimes the growth may decline catastrophically immediately at the end of the linear phase. Finally, the death rate becomes exponential, suggesting that death depends on some random event, and is followed by a complete collapse of the microalgal population (Becker 1994).

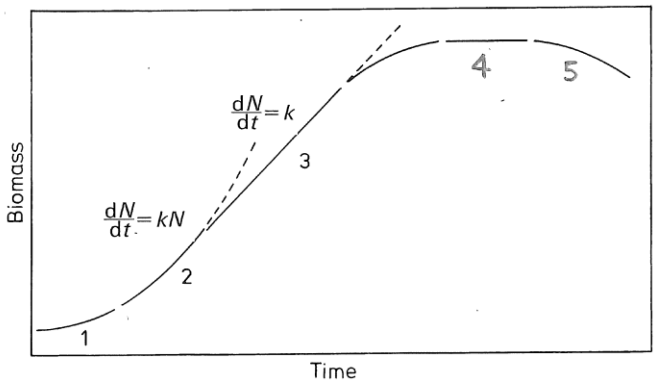

Figure 9. Evolution of microalgal biomass concentration with time in a batch culture (modified from Becker 1994). (1) Lag growth phase, (2) exponential growth phase, (3) linear growth phase, (4) stationary growth phase, and (5) death phase.

- Modeling of microalgal growth

Growth rate may be calculated directly from change of biomass with time, ir a single biomass determination can be used together with a rate measurement to give μ (Eppley and Strickland 1968). Growth modeling of organisms is important to understand their behavior under different environmental conditions, such as temperature, light intensity, pH, and nutrients (Carvalho and Malcata 2003; Çelekli et al. 2008; Çelekli and Yavuzatmaca 2009; Celekli et al. 2009; Dermoun et al. 1992; Ogbonna et al. 1995b; Tevatia et al. 2012; Xin et al. 2010; Zonneveld 1996; Zonneveld 1998b; Zwietering et al. 1990). Such models are used for prediction of microbial biomass production and optimization of growth conditions (Carvalho and Malcata 2005; Çelekli et al. 2008; Çelekli and Yavuzatmaca 2009; Jeong et al. 2008; Vieira Costa et al. 2002).

Several models exist on the dependency of microalgae steady-state growth in continuous culture on concentrations of nutrients dissolved in the growth medium. They describe the relative

growth rates as being related to a growth-limiting nutrient concentration in the medium. The growth limitation by a nutrient is defined by Lotka (1956) as follows: 'if one essential component is presented in limiting amounts, any moderate increase or decrease in the ample supply of the other components will have little or no observable influence on the rate of growth'. Some of these models (Geider et al. 1998; McCarthy 1980; Monod 1949; Morel 1987; Zonneveld 1998a; Zonneveld 1998b) use the Monod hyperbolic equation (Monod 1949). It describes the relative growth rate as a function of the concentration of the limiting nutrient that follows a Michaelis-Menten type of kinetics:

$$\mu = \mu_{max} \frac{S}{S + K_s}$$

where μ_{max} is the maximum specific growth rate of the organism, S is the concentration of the limiting nutrient, and K_s is the half-saturation constant, which corresponds to the concentration of nutrient at which $\mu = 0.5\ \mu_{max}$. However, this equation describes the growth rate as being dependent on the nutrient concentration in the medium and its uptake rate, while the relative growth rate depends more directly on the intracellular concentrations of the limiting nutrient rather than on its uptake rate (Droop 1968). The Droop curve (Figure 10) relates the growth rate to the intracellular quota (Q) of a nutrient, with zero growth at the minimum cell quota (Q_{min}) of a nutrient, which increases with additional nutrient supply, asymptotically approaching a theoretical maximum growth rate (μ_∞) at infinite quota. The minimum cell quota corresponds to the amount of a nutrient used in cell structure and machinery, and all quota above the minimum quota is nutrient stored for future growth

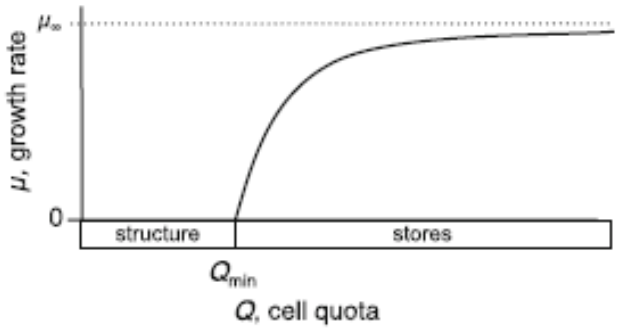

Figure 10. Droop function relating growth rate to cellular nutrient quota (Klausmeier et al. 2008).

Modeling of light-limited batch cultures of microalgae is, on the other hand, much more complex since there are continuous changes in both intensity and distribution of light within the reactor during the cultivation period. During high-cell density cultivation of photosynthetic cells, incident light is absorbed within the periphery of the illuminated surface. Thus, only the surface zone is illuminated, while the region away from the illumination surface may be completely dark. The proportion of the reactor volume, which receives enough light for cell growth decreases with increase in the cell concentration. Nutrient concentrations also vary over a wide range as the batch culture progresses through its different phases, as the microalgal cells consume the available nutrients for growth, in some cases until some nutrient becomes depleted. Many industrial processes are conducted using batch cultures and modeling for design and optimization of light-limited batch cultivation of microalgae is an important engineering challenge (Ogbonna et al. 1995b). Culture experiments are often used to help evaluate the effects of different environmental conditions on microalgal growth, physiology, or production of key metabolites (Andersen 2005). An efficient method to optimize culture parameters is to employ a statistically based factorial approach to experimental design (Anderson and Whitcomb 2000). This method uses statistics to identify the culture parameters and the interactions between them that are the most influential on achieving maximum biomass yield and productivity.

Culture conditions affecting growth and biochemical composition of *Chlamydomonas reinhardtii*

Growth factors such as nutrient concentrations, CO_2 levels, pH, irradiance, and temperature not only affect photosynthesis and productivity of the cell biomass of microalgae, but also influence the activity of cellular metabolism and, thus, the cell composition. For a single species, the variation in cell composition may vary manyfold, according to the culture conditions, under which it is grown. The biochemical composition of cells changes in response to signals received from the environment in a process known as acclimation. It is assumed that acclimation serves to increase growth rate under suboptimal conditions over the value that would be achieved if cellular biochemical composition was static (Geider et al. 1998). Acclimation may also serve to limit the damage that may occur as a consequence of exposure to adverse environmental conditions (Geider et al. 1998).

Nutritional factors

An essential element is an intrinsic component in the structure of the metabolism of an organism, whose absence causes severe abnormalities in growth, development or reproduction (Taiz and Zeiger 2010). If photoautotrophic microalgal cells are supplied with these essential elements, water, and light energy, they can synthesize all the compounds they need for normal growth.

Microalgal photosynthesis and growth are closely related to the availability of carbon, nitrogen, phosphorous, and sulfur, among other nutrients. When growth rates are plotted as a function of nutrient concentration, four zones are usually recognized (Figure 11):

1) A deficiency or limitation zone with low nutrient concentrations, in which growth increases rapidly when nutrients are supplied.
2) A transition zone, where the critical concentration is found. In this zone, growth is little affected by addition of more nutrients. This zone is known as the zone of the optimal concentration.
3) An adequate zone, where no increase in growth is found with an increase of supply of nutrients. Luxury storage takes place at these concentrations. This zone is fairly wide for macronutrients, but much narrower for micronutrients.
4) A toxic zone, where an increase in the concentration of nutrients leads to reduced growth (Richmond 2004).

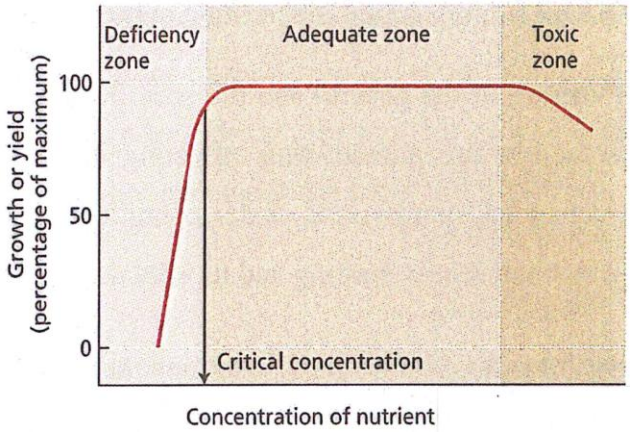

Figure 11. Relationship between microalgal growth and nutrient content in the growth medium (modified from Taiz and Zeiger 2010).

- Stress responses to nutrient limitations

When microalgal growth is limited by some nutrient, two kinds of responses are triggered in the cells: general responses and specific responses (Grossman 2000). The general responses occur at the cellular level and apply to limitation by any nutrient. The specific responses, on the other hand, are acclimation responses to a limitation in some specific nutrient.

The general responses entail various photosynthetic and metabolic changes that ultimately result in the temporary cessation of cell growth and cell division (Grossman 2000). The general responses to nutrient limitation include:

- cessation of cell division (Davies et al. 1996; Lien and Knutsen 1979),
- accumulation of carbohydrates (such as starch in the case of *C. reinhardtii*) or lipids, synthesis of which diverts energy and fixed carbon from cell growth and division (Ball et al. 1990; Ball et al. 1998),
- decline in photosynthetic rate and modification of photosynthetic activities (Davies et al. 1996; Peltier and Schmidt 1991; Wykoff et al. 1998),
- adaptation of the metabolism to reduced nutrient environment (Gauthier and Turpin 1994; Theodorou and Plaxton 1993).

According to Grossman (2000), some of these responses may be a consequence of changes in metabolism that occur because depriving a cell of nutrients blocks its growth and limits the utilization of reductant (NAD(P)H) and chemical bond energy (ATP) generated by photosynthetic electron transport. In this way, even if cells are grown under low to moderate light, the photosynthetic ETC will be completely reduced and, thus, the redox potential of the cell will be increased. This increase in the redox potential of the cell will have a global effect on cellular metabolism. On the other hand, with the decreased demand for reductant, the cell will tend to accumulate high potential electrons and excited chlorophyll molecules that can interact with oxygen, producing reactive oxygen species (ROS). These molecules cause extensive cellular damage, but they also function as regulatory signals that modulate metabolic activity.

Microalgae are also adapted to scavenge their environment for nutrients thanks to the specific responses, be it through structural changes, storage, or increase in nutrient utilization efficiency (Richmond 2004). The specific responses to nutrient limitation include:

- biosynthesis of transport systems that facilitate more efficient uptake of the limiting nutrient into the cell,
- induction of hydrolytic enzymes that enable cells to access alternative sources of the limiting nutrient. Some of these enzymes are exported to the surrounding medium or associated with the cell wall,
- changes in the cellular organization that help minimize the use of the limiting nutrient. Increase of specific intra-cellular enzymes that degrade macromolecules that contain the limiting nutrient, such as protein (nitrogen and sulfur source), polyphosphate, RNA (nitrogen and phosphorous source) and lipids (nitrogen and sulfur source),
- changes in components of the ETC, which are replaced by alternative molecules with analogous functions, but that do not use or contain the limiting nutrient (Grossman 2000).

Responses to nutrient limitation are highly regulated and are the consequence of sensing a specific deficiency and of alterations in cellular metabolism that result from the inability of the cell to assimilate nutrients, grow and divide (Grossman 2000).

As a result of the combination of general and specific acclimation responses to nutrient limitation, cell growth and secondary metabolism are altered, thus affecting cell composition. It is well established that considerable variation in the biochemical composition under conditions of nutrient limitation can be observed in microalgal cells, depending on which nutrient is limiting and to what degree (Juneja et al. 2013). Furthermore, environmental conditions, such as light and temperature, that are not strictly associated with nutrient limitation also integrate into acclimation processes (Grossman 2000).

- Carbon

Carbon constitutes about 50% of the microalgal cell dry biomass (Becker 1994) and it is the most essential of the non-mineral nutrients for microalgae growth. Carbon atoms are the building blocks of all the organic molecules and carbon skeletons are the core of all metabolites in a cell. Carbon sources vary between organic carbon for heterotrophic and mixotrophic microalgae growth, supplied in form of acetate in the case of *C. reinhardtii*, and inorganic carbon in form of CO_2, HCO_3^-, or CO_3^{2-} for photoautotrophic and mixotrophic microalgae growth.

CO_2 in varying concentrations is commonly supplied as bubbled gas into the microalgal cultures. This CO_2 needs to be transferred from a rising gas bubble into a liquid phase, in a process known as gas-liquid mass transfer, and, ultimately, to the site of carboxylation by the Rubisco enzyme inside the chloroplast of a microalgal cell, which can be considered as a solid particle. The transport of CO_2 from gas bubbles to the cells can be represented by a number of steps and resistances, as shown in Figure 12. These steps include:

- transfer from the interior of the bubble to the gas film,

- movement across the gas-liquid interface,

- diffusion through the relatively stagnant liquid film surrounding the bubble,

- the transport through the bulk liquid,

- diffusion through the relatively stagnant film surrounding the cell,

- movement across the liquid-cell interface, and finally

- intracellular diffusion through the cytoplasm, into the chloroplast to the site of the Rubisco enzyme.

The liquid film resistances around the bubbles usually control the overall transfer rate (Chisti 1989; Moo-Young and Blanch 1981). Thus, the easiest way to increase the availability of CO_2 to microalgal cells is by increasing the gas-liquid mass transfer (Kapic 2005).

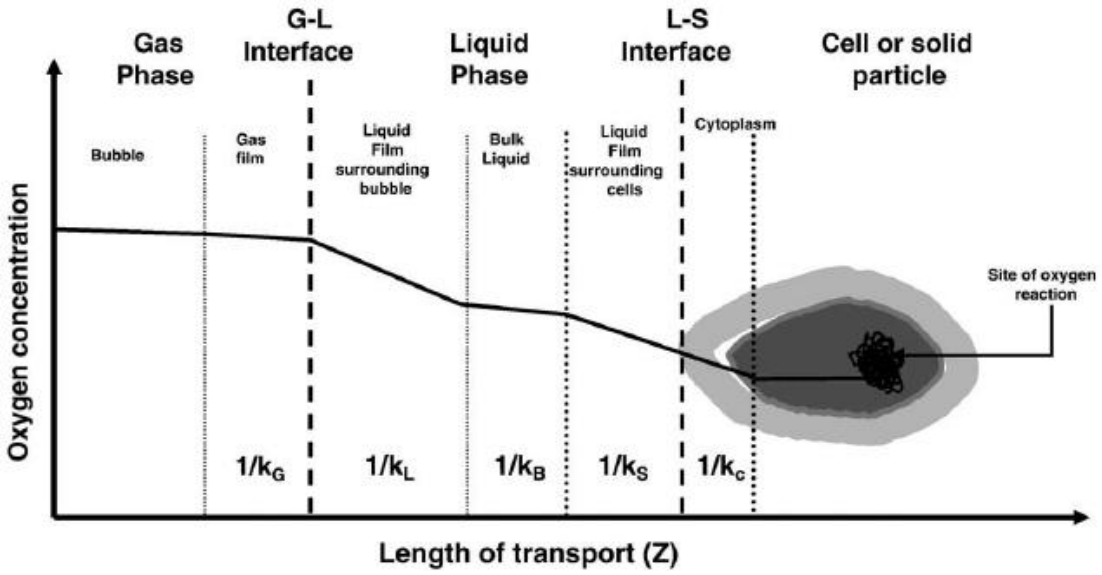

Figure 12. Steps and resistances for the gas transfer from a bubble into the cell. This figure represent the case of oxygen; however, the same principle is applied for CO_2. Source: Garcia-Ochoa and Gomez 2009.

The simplest theory on the gas-liquid mass transfer is the two-film model developed by Whitman (1923). Usually, the gas-liquid mass transfer rate is modeled according to this theory. It describes the flux through each film as the product of the driving force by the mass transfer coefficient, as follows:

$$J^0 = k_G\,(p_G - p_i) = k_L(C_i - C_L)$$

where J^0 is the molar flux of carbon dioxide (mol m^{-2} s^{-1}) through the gas-liquid interface; k_G and k_L are the gas and liquid mass transfer coefficients, respectively; p_G is the carbon dioxide partial pressure in the gas bubble; and C_L is the dissolved carbon dioxide concentration in the bulk liquid, with the index $_i$ referring to the values at the gas-liquid interface (Garcia-Ochoa and Gomez 2009). Since the interfacial concentrations cannot be measured directly, the overall mass transfer coefficients are usually considered. The equation can then be re-written as follows:

$$J^0 = K_G\,(p_G - p^*) = K_L\,(C^* - C_L)$$

where p* is the carbon dioxide pressure in equilibrium with liquid phase; C* is the carbon dioxide saturation concentration in the bulk liquid in equilibrium with the bulk gas phase; and K_G and K_L are the overall mass transfer coefficients (Garcia-Ochoa and Gomez 2009; Kadic 2010).

Taking into account that carbon dioxide is little soluble in water, it is commonly accepted that the greatest resistance for the mass transfer is on the liquid side of the interface and the gas phase resistance is negligible (Chisti 1989). The overall mass transfer coefficient is then equal to the local coefficient: $K_L = k_L$.

The carbon dioxide mass transfer rate per unit of PBR volume, N_{CO2}, is obtained by multiplying the overall gas flux by the gas-liquid interfacial area per unit of liquid volume (a):

$$N_{CO_2} = a J^0 = a k_L (C^* - C_L)$$

Due to the difficulty of measuring k_L and a separately, usually the product ak_L is measured and this parameter, called volumetric mass transfer coefficient, characterizes the transport from gas to liquid. To determine the CO_2 transfer capacity in practice, the CO_2 concentration in the gas phase and the concentration of the dissolved inorganic carbon in the liquid phase are measured.

The driving force of the gas-liquid mass transfer is the CO_2 concentration gradient between the gas phase and the liquid phase. Factors affecting this gradient include the solubility and the metabolic activity of the cells. The CO_2 solubility, C*, in electrolyte solution is usually smaller than the gas solubility in pure water, known as the salting-out effect. The CO_2 solubility depends on the pH (Figure 13) temperature, the pressure, concentration of nutrients, concentration and type of salts present and the chemical reactions (Hermann et al. 1995; Linek and Vacek 1981; Weissenborn and Pugh 1996).

It is the dissolved CO_2 concentration in the medium that determined the availability of CO_2 to the microalgal cells, and not the CO_2 concentration in the gas phase. To increase the dissolved CO_2 concentration in the growth medium, the concentration of CO_2 in the bubbled gas can be increased, the gas flow rate and the bubble-liquid interface surface can be increased, as well as the solubility of CO_2 in the liquid growth medium. By increasing the gas flow rate, the mixing of the cultures will also increase, facilitating the dissolution of CO_2 gas in the liquid and, thus, increasing the availability of CO_2 to the cells.

These three inorganic carbon species can be inter-converted through the following equilibrium reactions:

$$CO_2 + H_2O \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-}$$

When the pH of the growth medium decreases, the relative concentration of CO_3^{2-} decreases, while CO_2 and HCO_3^- levels increase (Figure 13). During photosynthetic CO_2 fixation, OH^- accumulates in the medium inducing a rise in pH (Azov 1982). However, when the cultures are bubbled with gas containing high concentration of CO_2, the pH of the medium decreases. This is due to the excess of the amount of supplied CO_2 in relation to the capacity of the microalgal culture to take up the dissolved CO_2. On the other hand, the affinity of microalgae for CO_2 increases at lower pH (Azov 1982; Rotatore and Colman 1991).

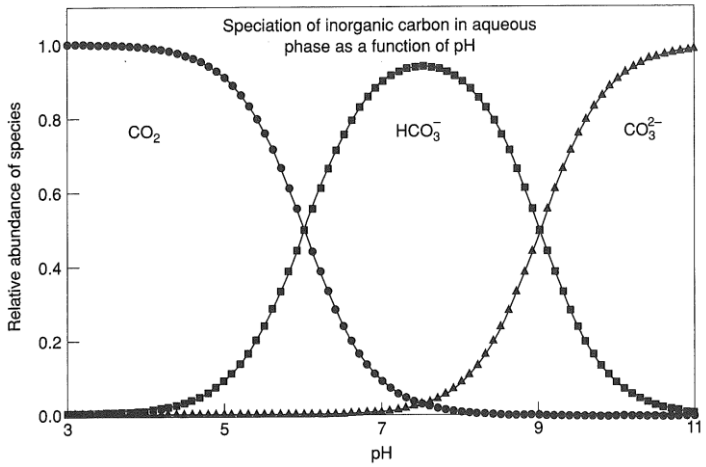

Figure 13 Distribution of the three inorganic carbon species, CO_2, HCO_3^-, and CO_3^{2-}, in aqueous solution as a function of the pH (Falkowski and Raven 2007).

Moroney and Tolbert (1985) have shown that *C. reinhardtii* cells grown under high CO_2 concentration (5% v/v) take up CO_2 from the medium, but not HCO_3^-, as do the cells grown at air-level (0.03%) CO_2 in a process known as carbon dioxide concentrating mechanism (Badger et al. 1977; Badger et al. 1980; Moroney and Ynalvez 2007; Yamano and Fukuzawa 2009). They postulated that the cells grown under high CO_2 concentration did not synthesize the inorganic carbon pump that facilitates HCO_3^- uptake (Moroney and Tolbert 1985). Microalgal cells take up CO_2 via passive diffusion (Berry et al. 1976).

The carbon assimilation pathway involves multiple forms of carbonic anhydrase (CA) enzyme, which converts CO_2 to H_2CO_3 and vice versa (Sultemeyer 1998). Expression of periplasmic CA is regulated by acetate and pH in *C. reinhardtii* (Van and Spalding 1999). Chloroplastic CA activity is required for photosynthesis of *C. reinhardtii* at ambient CO_2 concentrations (Amoroso et al. 1996; Funke et al. 1997) and the mitochondrial CA is also known to be induced at low CO_2 concentration (Eriksson et al. 1998).

As described above, CO_2 is incorporated into organic carbon skeletons by the Rubisco enzyme during the Calvin-Benson cycle reactions in the chloroplast. Carbon fixed by the cell can be utilized for respiration, as an energy source, and as raw material in the formation of new cells (Berman-Frank and Dubinsky 1999). In this way, in *C. reinhardtii*, starch and growth metabolism (use of carbon for biosynthesis) are the major sinks for photosynthetically fixed carbon. (Klein 1987). The level of starch is, however, controlled by other factors than the CO_2 fixation. Non-growing cells direct more than 50% of the fixed CO_2 into starch (Klein 1987).

Elevated CO_2 concentration leads to increased rates of carboxylation and decreased rates of oxygenation of Rubisco enzyme in C3 plants, resulting in a higher net rate of photosynthesis and increased synthesis of carbohydrates (Stitt and Krapp 1999). Elevated CO_2 concentrations have also been found to decrease the relative amount of proteins and pigments in *Spirulina platensis* cells, but increase the carbohydrate content (Gordillo et al. 1999). This change in the cell composition was accompanied by reduction in the maximum biomass yield (Gordillo et al. 1999).

- Nitrogen

Nitrogen is one of the main nutrients in microalgal metabolism. It is an essential constituent of all cellular proteins, nucleic acids, and chlorophylls. It constitutes between 1 and 20% of the cell dry biomass (Richmond 2004), depending on supply and availability. *C. reinhardtii* does not accumulate intracellular nitrogen storage compounds (Harris 2009). Therefore, nitrogen must be available in the medium for the growth of this microalga.

C. reinhardtii can assimilate a number of different nitrogen sources, such as nitrate, nitrite, urea, and ammonium, among others (Harris 1989). However, its preferred nitrogen source

is ammonium (NH_4^+) (Florencio and Vega 1983). Assimilation of ammonium is less energy demanding than that of other prevalent nitrogen sources, such as nitrate and nitrite (Grossman 2000; McCarthy 1980; Syrett 1981). Nitrate and nitrite need to be reduced to ammonium after entering the cell in order to be assimilated. The reduction steps are energy-demanding, and microalgae growth on nitrate is slower than on ammonium. Besides, the 137c strain of *C. reinhardtii* (used in this study) lacks the nitrate reductase activity (Nichols et al. 1978). It can not therefore utilize nitrate for growth and is usually grown on ammonium as nitrogen source.

The first step in the assimilation of extracellular ammonium is the uptake through transporters localized to the plasma membrane, chloroplast, and mitochondria (Stern 2009). *C. reinhardtii* has two ammonium transport systems (Franco et al. 1988). One is a low affinity ammonium transporter with high maximum uptake velocity, which is constitutively expressed. The other one is a high affinity ammonium transporter with a low maximum uptake velocity and is repressed by ammonium. The low affinity system functions under nitrogen-replete conditions while the high affinity system becomes predominant under ammonium limitation (Grossman 2000).

Once ammonium has entered the cell, its main flux is expected to be into the chloroplast, which is the main site of ammonium assimilation (Stern 2009). Here, ammonium is incorporated into carbon skeletons through enzymatic reactions of the glutamine synthetase/glutamine:oxo-glutarate amino-transferase (GS/GOGAT) (Lam et al. 1996). The amination of glutamate to form glutamine is catalyzed by GS, while the GOGAT reaction facilitates reductive amination of 2-oxoglutarate using the amide of glutamine as the N donor in the reaction, the product of the reaction being two glutamate molecule. Glutamate is a key molecule that couples the metabolism of amino acids to that of carbohydrates and lipids (Stern 2009).

The assimilation pathways for inorganic nitrogen are intrinsically dependent on organic carbon substrates, reductants (in case of nitrate and nitrite used as nitrogen sources), and ATP, that are supplied by both photosynthetic and respiratory pathways (Turpin 1991). This dependency is, however, a two-way street: when nitrogen is limiting, photosynthesis and respiration are affected, and growth is decreased (Morris et al. 1971; Turpin and Bruce 1990). Inorganic nitrogen taken up by the microalgal cell and assimilated into biochemically active compounds is recycled within the cell to meet changing physiological needs (Fujita et al. 1988;

Vergara et al. 1995). When their assimilatory capacity is exceeded, cells secrete ammonium, thus, avoiding any toxic effect of excessive intracellular amounts (Stern 2009).

Nitrogen-sufficient marine diatom *Skeletonema costatum* contains high concentration of protein (Dortch 1982). Protein-nitrogen can constitute as little as 10% (Conover 1975) and as much as 95% (Thomas and Krauss 1955) of the total cellular nitrogen. Non-limited cells of *Stephanodiscus minutulus* had a significantly higher chlorophyll *a* content as compared to nitrogen-limited cells in the study by Lynn et al. (2000).

Nitrogen deficiency, on the other hand, is known to reduce the protein content of microalgae (Dortch 1982; Fogg 1956; Gordillo et al. 1999; Holm-Hansen et al. 1959; Kilham et al. 1997; Lynn et al. 2000; Morris et al. 1974). This, in turn, results in a higher lipid/protein ratio (Converti et al. 2009; Falkowski and Raven 2007; Round 1984), at the expense of growth rate (Li et al. 2008). Microalgae grown in nitrogen-depleted cultures also tend to divert their photosynthetically fixed carbon to carbohydrate synthesis (Falkowski and Raven 2007; Gordillo et al. 1999; Lynn et al. 2000; Richmond 2004). In this way, nitrogen status, probably through its effect on the intracellular glutamate levels, acts as a metabolic switch between protein synthesis, under nitrogen-replete conditions, and carbohydrate or lipid synthesis, under nitrogen-limited conditions. Other effects of nitrogen limitation include decrease in O_2 evolution, CO_2 fixation, chlorophyll content, cell size and biomass production (Collier and Grossman 1992; Gordillo et al. 1999; Kolber et al. 1988; Lynn et al. 2000; Richardson et al. 1969; Round 1984). As nitrogen source is consumed from the medium in a batch culture, photosynthesis continues at a reduced rate, until cellular nitrogen falls below a particular species-specific threshold value (Q_{min}) (Gordillo et al. 1999). It is interesting to note that, for example, nitrogen-limited *Spriulina platensis* cells exhibit reduced CO_2 fixation capacity even under normal to high available CO_2 concentrations (Gordillo et al. 1999).

- Phosphorous

Although *C. reinhardtii* cells contain less than 1% of the dry biomass of phosphorous (Goldman 1980), it forms part of many structural and functional cellular components required for normal growth and development of microalgal cells. It is present in ubiquitous molecules such as

reductants (NADPH) and energy carriers (ATP). It is also present in nucleic acids, phospholipids, and intermediary metabolites, all essential for the cell.

The prevalent form of available phosphorous in the aquatic environment is inorganic phosphate, either as $H_2PO_4^-$ or as HPO_4^{2-} (Grossman and Takahashi 2001). Phosphate enters the cell by active transport across the plasma membrane powered directly or indirectly by ATP (Falkowski and Raven 2007). Phosphate is incorporated into organic compounds through various types of phosphorylation (Richmond 2004). In the cytosol, phosphate is available via reactions using ATP (or other nucleotide triphosphate) for direct incorporation into nucleic acid precursors and carbohydrate and hydrocarbon skeletons without electron transfer reactions (Falkowski and Raven 2007).

Under phosphate-replete conditions, microalgal cells are able to accumulate inorganic phosphate intracellularly in the form of polyphosphates, which are stored in granules (Healey 1982). This process is known as luxury uptake of phosphate (Healey 1982). However, once phosphate becomes deficient in the medium, the polyphosphate granules disappear (Healey 1982). It is probably due to this luxury uptake of phosphate and accumulation of polyphosphate granules inside the cells, that microalgae are able to divide up to four times after phosphate becomes deficient (Collier and Grossman 1992). Nitrogen and sulfur, in contrast, do not accumulate inside the cell and their limitation provokes immediate arrest of cell division (Collier and Grossman 1992).

The N:P ratio in the medium is important and determines the potential productivity of the culture (Richmond 2004). Immediate effects of phosphate limitation induce a decrease in the synthesis and regeneration of substrates of Calvin-Benson cycle and a consequential decrease in the rate of light utilization for carbon fixation (Barsanti and Gualtieri 2014). Phosphate deprivation also induces synthesis of several phosphatases and transporters (Chang et al. 2005; Dumont et al. 1990; Quisel et al. 1996; Rubio et al. 2001), and of Psr1 regulatory protein (Wykoff et al. 1999).

The supply of phosphate also determines the composition of the produced biomass (Richmond 2004). It is especially the cellular content of lipids and carbohydrates that is affected by the external and internal supplies of phosphate (Borowitzka 1988). Phosphorous starvation

also reduced chlorophyll *a* and protein content of microalgal cells, thereby increasing the relative carbohydrate content (Celekli et al. 2009; Collier and Grossman 1992; Healey 1982; Healey and Hendzel 1979; Kilham et al. 1997).

- Sulfur

Sulfur is an essential element incorporated into proteins, sulfolipids, polysaccharides, intermediary metabolites, molecules that function in photoprotection, electron carriers, and redox controllers (Ghysels and Franck 2010; Grossman 2000). Microalgal cells have very limited storage capacity for sulfur (Ghysels and Franck 2010), responding rapidly to the lack of sulfur in the growth medium. Sulfur must, therefore, be supplied in sufficient amounts in the medium for optimal growth of microalgae.

Sulfate anion (SO_4^{2-}) is the main form of sulfur taken up by the microalgae. The uptake of sulfate is facilitated by specific transport systems (Leustek and Saito 1999; Zhang et al. 2004) and is an ATP-dependent process, which involves secondary active transport facilitated by ion gradients (Matsuda and Colman 1995; Raven 1984; Weis et al. 2001). Following uptake, the sulfate anion must be activated by ATP-sulfurylase to form 5'-adenylyl sulfate or APS. The APS generated can either be phosphorylated into 3'-phospho-5'-adenylyl sulfate (PAPS) and used for sulfation of various metabolites by sulfotransferases (Varin et al. 1997), or it can serve as substrate for reduction of sulfate to sulfide (S^{2-}) and integrated into cysteine and methionine, which are then incorporated into proteins and other cellular metabolites (Bick and Leustek 1998; Leustek and Saito 1999; Saito 2000).

Sulfur deprivation induces several responses in *C. reinhardtii* cells, which is being thoroughly researched in relation to the H_2 production mechanism. These include induction of expression of high affinity transport systems (Yildiz et al. 1996) and of periplasmic arylsulfatase (Ohresser et al. 1997). Photosynthetic activity (O_2 evolution and CO_2 fixation) is reduced and protein synthesis is disrupted (Ghirardi et al. 2000; Wykoff et al. 1998). The chlorophyll content of the cells decreases (Melis et al. 2000; Zhang et al. 2002) and the composition of the thylakoid membrane changes upon sulfur deprivation (Melis et al. 2000). During the first day of sulfur

deprivation, cells stop dividing and start to accumulate large energy stores in the form of starch and lipids (Ghysels and Franck 2010; Zhang et al. 2002).

Sulfur deprivation is known to induce decrease in chlorophyll content of microalgae (Collier and Grossman 1992). Several species of microalgae are known to accumulate starch under sulfur-deprived conditions (Branyikova et al. 2010; Dragone et al. 2011; Ji et al. 2011; Yao et al. 2012).

- Calcium

The fraction of calcium in the elementary composition of microalgae is in the range of 0 - 80 mg Ca^{2+} g^{-1} dry biomass (0.0 – 8.0% of dry weight) (Healey and Stewart 1973). Calcium is required for cell membrane integrity and cell transport mechanisms (Fujii 1994). Calcium is also implicated as a second messenger in signal transduction of a wide variety of biotic and abiotic stimuli in both lower and higher plants (Rudd and Franklin-Tong 1999; Sanders et al. 1999). In *Chlamydomonas*, calcium signaling has been shown to be associated with motile responses (flagellar beat, phototaxis, chemotaxis), sensory responses (flagellar adhesion during mating), and the maintenance and removal of the flagella (flagellar length control, flagella excision) (Ermilova et al. 1998; Goodenough et al. 1993; Harz and Hegemann 1991; Kamiya and Witman 1984; Quarmby and Hartzell 1994; Tuxhorn et al. 1998). In addition to its role in signal transduction, calcium is an essential co-factor in photosystem II-driven oxygenic photosynthesis (Boussac et al. 1989; Krieger et al. 1993).

In *Dunaliella tertiolecta*, increased calcium concentrations (up to 0.3 mM) enhanced cell growth (Chow et al. 2015). Calcium starvation, on the other hand, is known to induce lipid accumulation in microalgae cultures (Deng et al. 2011; Gorain et al. 2013).

- Magnesium

The fraction of magnesium in the elementary composition of microalgae is in the range of 0.5 – 75 mg Mg^{2+} g^{-1} dry biomass (0.05 – 7.5 % of dry weight) (Healey and Stewart 1973). The magnesium content reported for *Chlorella* sp. ranges between 0.36 and 0.8% on a dry weight basis (Oh-Hama and Miyachi 1988). Chlorophyll molecules contain magnesium in their

structure, making the supply of magnesium indispensable for the photosynthetic activities of plants and algae. Furthermore, magnesium activates more enzymes than any other mineral nutrient (Epstein and Bloom 2004), some of which are ATPases, Rubisco, RNA polymerase and protein kinases (Marschner 1995; Shaul 2002).

Under increased concentration of magnesium (up to 6 mM), a significant rise in the biomass of *Chlorella vulgaris* and *Scenedesmus obliquus* was recorded in the study by Gorain et al. (2013). In *Dunaliella tertiolecta*, increased magnesium concentrations (up to 5.6 mM) also enhanced cell growth (Chow et al. 2015). The chlorophyll concentration in *Chlorella* cultures increased linearly about fifteen-fold when the initial magnesium concentration was increased from 8 µM to 115 µM (Finkle and Appleman 1953a). A further two-fold increase in the chlorophyll content was observed when the magnesium concentration was increased up to 2 mM (Finkle and Appleman 1953a). In these cultures, the chlorophyll content attained by the cells represented approximately 70 – 80% of the total magnesium that had been added to the growth media (Finkle and Appleman 1953a).

The absence of magnesium, on the other hand, is expected to prevent cell division and chlorophyll synthesis and, thus, to reduce growth yields (Finkle and Appleman 1953b). In plants, magnesium deficiency in leaves is associated with a massive accumulation of carbohydrates and elevated susceptibility to high light intensity (Cakmak and Kirkby 2008; Marschner and Cakmak 1989). In *C. reinhardtii*, magnesium deprivation decreases the protein content of the cells, but increases carbohydrates and lipids (Çakmak et al. 2014).

- Optimization of the growth medium composition for *Chlamydomonas reinhardtii*

Artificial media for growing fresh water microalgae have been developed empirically. The simple solutions of a few mineral salts used by pioneers such as Benecke and Beijerinck (1890) were modified by varying the proportions of the major solutes and trace elements were added, as these were discovered to be essential for healthy microalgae growth (Fogg and Thake 1987). Usually, researchers have tested a number of well-established recipes and used the most suitable one with a minimum of modifications (Fogg and Thake 1987). Only a few scientists (Krauss 1953; Miller and Fogg 1957; Rodhe 1948) have undertaken determinations of growth in

series of media, in which the concentrations and proportions of the constituents were systematically varied. Generally, the usefulness of a medium has been judged by the final yield of microalgal biomass (Fogg and Thake 1987). An optimal medium for microalgae cultivation is, on the other hand, strongly specific for each species and for each intended product (Mata et al. 2010).

Although *C. reinhardtii* has been grown in laboratories for several decades, there has been little development of a growth medium specific for this microalga. This microorganism acclimates rather easily to a variety of environmental conditions and media compositions (Dubini et al. 2009; Grossman 2000; Harris 2009). Therefore, several different media are used for its culturing (Harris 2009). Concentrations of nitrogen, phosphorus, and sulfur vary widely in the recipes of these media. Carbon sources also vary between organic carbon, supplied in the form of acetate, and inorganic carbon, in the form of HCO_3^- or CO_2 supplied at different concentrations in the bubbled gas. It is well documented that the nutrient status of the microalgae affects their photosynthesis and, thus, the productivity of the culture's biomass (Jo et al. 2006; Juneja et al. 2013; Richmond 2004). According to A. Richmond (2004), culture media should be formulated to supply nutrients in excess to ensure that they never become the rate-limiting factor. However, nitrogen, phosphorous, and carbon are often limiting. Oversupply of these nutrients is not a good strategy either, since it may lead to stress and reduced growth (Richmond 2004). Whereas there has been considerable interest in studying the responses of *C. reinhardtii* to conditions of nutrient limitation (see Grossman 2000 for review), so far, there has been little interest in determining the optimal growth conditions for this microalga (Jo et al. 2006; Lehr et al. 2012; Tamburic et al. 2011).

The culture conditions favoring specific product formation are usually not compatible with those required for maximal growth and biomass production (Richmond 2004). However, the cell composition of a microalga can be readily optimized by PBRs that control environmental conditions and through the use of multistage (or multiphase) cultivation strategy (Richmond 2004). The concept of multistage cultivation strategy is to ensure maximum production of biomass in one stage and maximum induction and accumulation of the desired products in the other (Richmond 2004). On the other hand, Klok et al. (2013) have argued that cells accumulate highly reduced carbon storage products, such as TAGs and starch, when the cultures are limited

for some nutrient as a result of energy imbalance between the anabolic processes and the photosynthesis. They suggest that it should be possible to create an energy imbalance by reducing the nutrient supply to the cells and, thus, force them to accumulate carbon storage compounds, while simultaneously allowing cell division to continue (Klok et al. 2013).

Environmental factors

- Light

Light is often considered to be the most important factor affecting microalgal growth and it is also the most difficult parameter to control in PBRs. The amount of incident light that enters the PBR is a function of light intensity and normal surface area (Hahn et al. 2004). At high cell density, microalgal productivity is limited by light attenuation inside the PBR due to shading effects of the layers of cells close to the surface of the PBR on the cells that are in the core of the PBR (Benemann 2000; Melis et al. 1998). The following PBR and process parameters determine the light distribution characteristics inside the PBR:

- the optical density (OD) of the culture, which depends on biomass concentration, pigmentation, and cell size and geometry,
- path length of light within the culture, which depends on the light source and the geometry of the PBR, and on latitude and season in outside cultures,
- mixing regime that affects light delivery to individual cells (Dubinsky et al. 1995).

In PBRs, microalgal cells are, therefore, exposed to varying irradiances (Grobbelaar 1991; Ogbonna et al. 1995a; Perner-Nochta and Posten 2007). Light absorption by *Chlorella* cultures approximately follows the Beer's law, the intensity of light decreasing exponentially as the path length through the algal suspension increases (Fogg and Thake 1987). The cells respond to this variation by modifying their metabolism and, thus, also their growth potential. The major physiological outcome of light-shade adaptation is the modification of growth rates (Falkowski and Owens 1980). Light affects growth of microalgae primarily through its impact on photosynthesis (Sorokin and Krauss 1958). The growth-irradiance curves are therefore similar to

photosynthesis-irradiance curves (Falkowski 1980). Accordingly, a typical irradiance curve for growth (Figure 14) presents three different portions:

1) A light-dependent portion, where the growth rate increases with increasing irradiance.
2) A light-independent portion or plateau, where the irradiance is saturating for growth.
3) A light-dependent portion, where the rate declines with increase in irradiance and where the cells are photoinhibited (Sorokin and Krauss 1958).

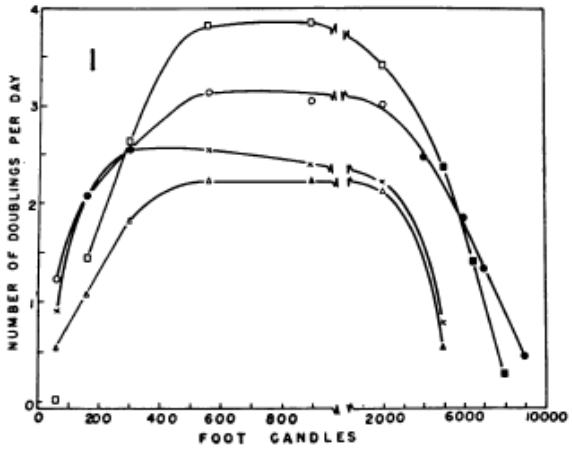

Figure 14. Irradiance curve for growth of four green microalgae species (Sorokin and Krauss 1958). The squares represent the response of *C. reinhardtii* growth to irradiance.

In addition to photosynthesis, light also drives photoacclimation. The photoacclimation process in microalgae leads to changes in the optical, biophysical, structural, biochemical, and physiological properties of the cell according to the availability of light (Dubinsky et al. 1995). These changes lead to optimization of light harvesting and utilization, increasing, thus, the photosynthetic efficiency (Dubinsky et al. 1995; Falkowski and Raven 2007). Photoacclimation is most often related to changes in abundance and composition of the photosynthetic pigments. The most obvious effect induced by acclimation to different light intensities is a strong reduction in chlorophyll content per cell in high light. This leads to a reduction of capacity for light absorption per volume of culture when the same cell density is considered, better penetration of light into the PBR and protects against excess absorbed light energy and, thus, photoinhibition (Bonente et al. 2012). In *C. reinhardtii*, acclimation to different light intensities also involves modulation of photosynthetic protein content per cell. High light induces a decrease in the

transcription and translation of LHC proteins (Durnford et al. 2003), suggesting modulation of antenna size.

Photoacclimation also affects carbon fixation, respiration rates, and biochemical composition of the organism, as well as cell volume (Falkowski and Raven 2007). Variation of growth rate under light limitation of photosynthesis has relatively little effect on the overall cellular composition (Falkowski and Owens 1980). However, there is often a tendency to accumulate more carbohydrates at higher light intensity levels (Falkowski and Owens 1980; Richmond 2004). This is due to the excess absorbed energy by the photosystems that the cells dissipate by directing it towards carbon fixation and synthesis of energy-rich carbohydrates (Hu 2004; Klok et al. 2013). *Chlorella* was found to accumulate high amounts of starch (from 8.5% up to 40% of DW) as the photon flux density increased from a mean of 215 to 330 μmol m^{-2} s^{-1} prior to cell division (Branyikova et al. 2010). *Dunaliella tertiolecta* showed a decrease in protein content and increase in lipid fraction with increased light intensities up to saturation (Cuhel et al. 1984). Carvalho et al. (2009), on the other hand, found that the cultures of *Pavlova lutheri* increased their cellular protein content as response to an increase in light intensity up to 195 μmol m^{-2} s^{-1}. *Phaeodactylum tricornutum* grown in low light also exhibits increased rate of protein synthesis (Morris et al. 1974).

- Temperature

Any microalgal specie's response to temperature is characterized by three 'cardinal temperatures' (Morita 1975): the lower and upper limits of temperature for growth, and the optimum temperature for growth at some point between the extremes. At the low temperature limit, there is loss of membrane function due to a phase change from liquid to gel or solid phase (Nedwell 1999). Below optimal temperature, growth rate increases with increasing temperature (Renaud et al. 2002). Growth at the optimum temperature results in maximum growth rate, but in minimal cell size (Harris 1986; Rhee 1982) and minimal cell contents of carbon and nitrogen (Rhee 1982). The efficiency of carbon and nitrogen utilization decreases at non-optimal temperatures (Darley 1982), while the cell volume increases (Goldman 1980). This means that it requires more carbon and nutrients to produce a cell at the same growth rate at non-optimal temperatures than it does at the optimal one (Darley 1982). The temperature range allowing

growth above optimum is usually quite narrow (Li 1980), the growth rate declining abruptly above the optimum temperature (Renaud et al. 2002). Increasing the temperature beyond the optimum reduces protein synthesis and, consequently, results in decreased growth rates (Konopka and Brock 1978). The upper limit of temperature is imposed by increasing rate of denaturation of key cellular components as temperature increases to the point that denaturation exceeds the rate at which these can be replaced, with consequent disruption of cell function (Nedwell 1999). The responses and the characteristics of a microalgal organism at a given temperature depend on the temperature experienced during growth (Li 1980). Harris (1989) recommends growing *C. reinhardtii* at a temperature range of 20 – 25 °C, and states that most strains tolerate temperatures in the range of 15 – 35 °C.

Temperature affects cell metabolism through several mechanisms. It affects the fluidity of the membranes, the diffusion of molecules, and mainly, the cellular biochemical reactions, through control of enzyme kinetics (Davison 1991; Raven and Geider 1988). Most biological reactions vary with temperature in such a way that with every 10 °C increase in temperature, the reaction rate approximately doubles (Vitova et al. 2011). This is expressed as a temperature coefficient Q_{10} of about 2 (Vitova et al. 2011). It has also been suggested that changes in citoplasmic viscosity under sub-optimal temperature conditions are responsible for less efficient carbon and nitrogen utilization under this condition (Hope and Walker 1975; Raven and Geider 1988).

Temperature affects cell processes to a large extent by control of photosynthetic rate (Setlík et al. 1972; Spudich and Sager 1980; Zachleder and van den Ende 1992). While the light-driven redox reactions of photosynthesis are influenced mainly by the amount of the available light, the carbon fixation reactions of photosynthesis are temperature-dependant enzymatic reactions. Analyses of biological rate processes as a function of temperature are often based on the Arrhenius equation that describes the influence of temperature on biochemical reactions rates (Geider et al. 1998; Li 1980):

$$k = A\, e^{-E\alpha/RT}$$

where k is the reaction rate constant, A is a constant (d^{-1}), E is the activation energy (J mol^{-1}), and R is the universal gas constant (J K^{-1} mol^{-1}), T being the temperature expressed in Kalvin scale.

The dependence of growth rate on temperature has been treated as an exponential function (Eppley 1972; Goldman 1980; Goldman and Carpenter 1974), as described by the Arrhenius equation, growth being the result of all the reactions occurring in the cell, or as a linear relation (Rhee and Gotham 1981). However, for each microalgal species, the Arrhenius equation is applicable only over a defined temperature range (Sorokin 1960). The optimum temperature for net photosynthesis is generally higher than the optimum temperature for growth (Li 1980).

Temperature effects on microalgal metabolism extend, however, beyond growth and photosynthesis. Temperature affects cell composition, nutrient uptake rates, and, in particular, nitrogen metabolism (Berges et al. 2002; Carvalho and Malcata 2003; Carvalho et al. 2009; Eppley 1972; Geider et al. 1998; Morris et al. 1974; Rhee and Gotham 1981; Sakamoto and Bryant 1999; Terry 1983; Thompson 1999; Thompson et al. 1992; Yoder 1979). Berges et al. (2002) observed that cellular carbon and nitrogen contents, protein, chlorophyll *a*, and C:N ratio increased with increasing temperature from 17 to 25°C in *Thalassiosira pseudonana* cultures. Rhee and Gotham (1981), on the other hand, observed an increase in protein concentration in *Scenedesmus* sp. with decreasing temperature. Shuter (1979) concluded that the increase in chlorophyll content with increasing temperature was a common response in microalgae. Thompson et al. (1992) studied the effect of temperature on the biochemical composition of eight species of marine phytoplankton and came to the same conclusion.

- pH

The pH of the growth medium can have a significant impact on microalgal metabolism (Chen and Durbin 1994; Goldman 1973; Goldman et al. 1982). It determines the solubility and the availability of CO_2 and of other essential nutrients (Andersen 2005). High pH limits the availability of carbon from CO_2 and lowers the affinity of microalgae to free CO_2, thus slowing down microalgal growth (Azov 1982; Rotatore and Colman 1991). Moroney and Tolbert (1985) determined that utilization of CO_2 for photosynthesis by *C. reinhardtii* was more efficient at a pH lower than neutral (< 6.95). Acidic conditions, on the other hand, can alter nutrients uptake (Gensemer et al. 1993) or induce metal toxicity (Anderson and Morel 1978; Sunda 1975) and, in this way, negatively affect microalgal growth. Maintenance of neutral intracellular pH in an acidic external environment would require an expenditure of energy to pump protons out of the

cell (Terry and Abadía 1986). In their modeling study on *Spirulina platensis*, Celekli et al. (2009), found that variations in pH significantly affected the amount of chlorophyll *a* and produced biomass.

C. reinhardtii grows photoautotrophically in simple mineral salts media, such as the High-Salt Sueoka (HS) medium (Sueoka 1960), over a pH range of 6.5 – 8.0 (Harris 1989). However, bubbling the cultures with gas containing high CO_2 concentrations, such as 5% v/v, uptake of ammonium by microalgae from the growth medium and, probably, also excretion of metabolites to the medium lead to a decrease in pH. A pH buffer is, therefore, usually used in the growth medium to maintain a stable pH. A pH buffer consists of a mixture of a weak acid and its conjugate base, or vice-versa, which inter-conversion tends to minimize changes in the concentration of H^+ in the solution, and, thus, to keep the pH stable. Phosphate buffer system, such as K_2HPO_4/KH_2PO_4, is present in the HS medium in quite high concentration (13.6 mM PO_4) (Sueoka 1960). On the other hand, the $CO_2/HCO_3^-/CO_3^{2-}$ system is the most important buffer present in freshwater and is the best means available to control and maintain specific pH levels that are optimal for the cultured microalga (Richmond 2004).

Interaction effects between the different growth factors on the growth and biochemical composition of microalgae

Liebig's minimum theory (1870) predicts that only one resource may limit growth at the time, but the validity of this postulate has been challenged over the past three decades (Andersen and Pedersen 2002). For many key resources, this theory inadequately describes the scenario, because the exploitation of one resource is not independent of the availability of others (Andersen and Pedersen 2002). There is evidence that some species may shift their acclimation strategies in response to combination of growth parameters in a different way that if they acted independently (Carvalho and Malcata 2003; Carvalho et al. 2009; Dermoun et al. 1992). The fact that the resource utilization is not independent on the availability of other resources also has important implications for the design of laboratory experiments (Andersen and Pedersen 2002).

When a microalgal cell is placed in a given environment that is adequate in terms of prevailing physicochemical conditions, it will grow exponentially until a growth parameter

becomes growth-limiting. The uptake of nutrients from the surroundings and the release of cell metabolites thereto occur at rates that depend on the internal cell control mechanisms, including adaptability to environmental conditions and genetic heritage (Richmond 2004). Even if the intrinsic characteristics of the cell population remain somewhat constant, the culture medium can be engineered in terms of several parameters, such as temperature, irradiance, and pH, whereas certain phenomena, such as acid-base equilibrium, ion strength, and gas-liquid equilibrium eliminate further degrees of freedom (Bailey and Ollis 1986). This metabolic complexity makes it rather difficult to describe the cell physiology as related to the combined effects of physical and chemical parameters (Carvalho and Malcata 2003).

Complex interactions exist between the chemical components, their availability, and the uptake by microalgae in cultures (Richmond 2004). Nutrient uptake depends on other environmental factors that influence microalgal growth, such as light, temperature, pH, and turbulence. The uptake and assimilation of one nutrient is also dependent on the availability and cellular content of other nutrients, as their metabolism is inter-dependent.

The assimilation of most macronutrients requires carbon skeletons (Falkowski and Raven 2007). Carbon and nitrogen metabolism are known to be interdependent (Fonseca et al. 1997; Huppe and Turpin 1994; Larsson et al. 1985; Stitt and Krapp 1999; Syrett 1981; Turpin 1991; Turpin and Bruce 1990). Under normal CO_2 (atmospheric level) and nitrogen sufficiency, the biomass yield of *Spirulina platensis* was the highest, while cultures under high CO_2 concentration (1%) and nitrogen limitation showed the lowest yield (Gordillo et al. 1999).

During light-dependent photosynthesis, nutrient uptake will depend on the available light energy, while at saturating irradiances nutrient uptake will be constant. Rhee and Gotham (1981) investigated the simultaneous limitations of light and nutrient on growth, and found that the combined effects were greater than the sum of individual effects and were not multiplicative. They concluded that within a certain range of growth rates, light and cellular nitrogen quota could compensate for each other in maintaining growth rate constant (Rhee and Gotham 1981). Under nutrient-sufficient conditions, cell quotas of carbon, nitrogen, and phosphorous, as well as cellular chlorophyll and protein concentrations were found to increase as irradiance decreased below saturation (Rhee and Gotham 1981). Both, carbon and nitrogen cell quotas, have been found to be positive exponential functions of irradiance (Thompson 1999). In *C. reinhardtii*, the

ferredoxin-dependent activity of the GOGAT enzyme in the ammonium assimilation pathway is known to increase in the light (Cullimore and Sims 1981).

Temperature influences the enzymatic reactions that mediate nutrient assimilation and utilization in the cell, and it also influences the rates of photosynthesis (Goldman and Carpenter 1974; Sorokin and Krauss 1962). This implies a synergism between temperature and light, which, again, will affect nutrient uptake (Richmond 2004). Non-optimal growth temperatures reduce the availability and utilization efficiency of nutrients (Sterner and Grover 1998).

Inversely, increased concentrations of some nutrients in the medium are known to increase the tolerance to higher temperature (Hutner et al. 1957; Maddux and Jones 1964). Eppley (1972) reported that both the temperature optimum and the maximum tolerated temperature by *Dunaliella tertiolecta* increasing with increasing salinity of the medium. Adaptations to higher or lower temperatures may also occur with varying light intensity (Hutner et al. 1957).

Many microalgae, such as *Chlorella pyrenoidosa*, show an increased saturation irradiance for growth at higher temperature compared to low temperature (Sorokin and Krauss 1962). High irradiance and warm temperature have been observed to promote carbon fixation since this pathway acts as a sink for excess energy (Morgan and Kalff 1979; Parker and Armbrust 2005). On the other hand, at high irradiance and low temperature cells are oversensitive to photoinhibition, in the sense that photoinhibition may be induced at relatively low irradiance due to suboptimal temperature conditions (Sakamoto and Bryant 1999; Vonshak et al. 2001).

There is a lack of knowledge on how to combine the different growth factors in order to optimize the biomass production and the desired chemical components of microalgae (Richmond 2004). Understanding the interactions between the various environmental variables is necessary to develop viable high-productivity microalgae systems, which produce the compound of interest at high rates. This is essential for successful scale-up of microalgal cultures in commercial systems for production of microalgal biofuels and other by-products (Juneja et al. 2013).

5.4. Hydrogen production by *Chlamydomonas reinhardtii*

Production of H_2 from solar energy using microalgae is an emerging field where significant development has been achieved in the last two decades.

Hydrogen production in green microalgae: historic overview of scientific discoveries

It was first discovered in the late 1930s that some species of unicellular green algae are able to metabolize H_2, consuming H_2 in the dark to drive cellular metabolism (Gaffron 1940) and producing H_2 in the light by oxidizing H_2O (Gaffron and Rubin 1942). The biochemical and genetic mechanisms behind H_2 production process weren't revealed until more than 50 years later, when the hydrogenase enzyme (Hyd1), responsible for transferring electrons to protons leading to molecular hydrogen, was purified and analyzed (Happe et al. 1994; Roessler and Lien 1984) and the *HydA* gene was isolated (Happe and Kaminski 2002). Hyd1 enzyme was shown to be localized to the chloroplast (Happe et al. 1994) and to accept electrons from the photosynthetic ferredoxin in the light (Happe and Naber 1993). H_2 metabolism in *Chlamydomonas* occurs only under anaerobic conditions, since the [FeFe]-hydrogenase is extremely sensitive to O_2 at the transcriptional and enzymatic activity levels (Ghirardi et al. 1997). H_2 metabolism in *C. reinhardtii* was considered to be of transient nature due to the need for anaerobic conditions, which are difficult to maintain for a photosynthetic microorganism. For this reason, H_2 production by *C. reinhardtii* was not regarded as having any biotechnological significance until the year 2000 (Philipps et al. 2012), when the team of A. Melis showed that sulfur deprived *C. reinhardtii* cultures were able to become anaerobic in gas-tight photobioreactors and produce significant amounts of H_2 for several days (Melis et al. 2000).

Why do microalgae produce hydrogen?

Production of H_2 under anaerobic sulfur-deprived conditions is a stress response in *C. reinhardtii* cells. Microalgae receive energy from light, but do not have access to sulfur or oxygen. The excess electrons generated by the ETC receiving light energy are released through the hydrogenase activity (Turner et al. 2008), H_2 being, thus, a sink for electrons. Under

anaerobic conditions, the oxidative phosphorylation, which normally produces ATP in the mitochondria is inactivated. Instead, electron transport via the hydrogenase pathway is coupled to photosynthetic phosphorylation in the tylakoid membrane (Arnon et al. 1961), generating ATP in the chloroplast, which is essential for the maintenance and repair function of the cell (Melis et al. 2000).

Biochemistry of hydrogen production in *Chlamydomonas reinhardtii*

Due to the extreme sensitivity to O_2 of the hydrogenase enzyme in *C. reinhardtii*, the protocol for H_2 production established by Melis et al. (2000) consists in two stages. First, cells are grown under normal aerobic conditions in order to accumulate biomass. Then, the culture is centrifuged, the cells are re-suspended in a sulfur-free medium and incubated under continuous illumination in gas-tight photobioreactors. The absence of sulfur in the medium blocks protein biosynthesis. This provokes partial and reversible inactivation of the PSII activity (Wykoff et al. 1998) by blocking the replacement of the D1 protein in the PSII reaction center, which is very rich in sulfur and has a rapid turnover. The photosynthetic activity of PSII declines gradually until it becomes inferior to the levels of mitochondrial respiration after about 24 – 30 h (Figure 15) of incubation in sulfur-deprived conditions. After this point, the cells consume all the oxygen they produce and the sealed culture becomes anaerobic in the light. Hydrogenase enzyme is then synthesized and activated, allowing for the H_2 formation.

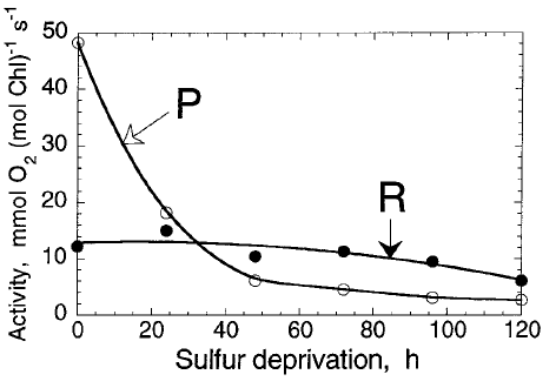

Figure 15. Response of photosynthesis (P) and respiration (R) rates to sulfur deprivation of *C. reinhardtii* cultures in gas-tight photobioreactors (Melis et al. 2000).

During the first 24 h of sulfur deprivation, the cells stop dividing and begin to accumulate large amounts of energy storage compounds, such as protein and, especially, starch (Figure 16) (Melis et al. 2000) as a general response to nutrient deprivation (Grossman 2000). On the other hand, the rapid degradation of the Rubisco enzyme (Zhang et al. 2002) suggests a decline in CO_2 fixation rates (Burgess et al. 2011).

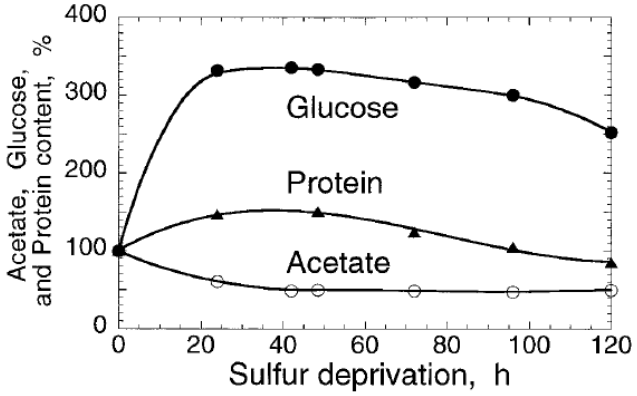

Figure 16. Sulfur-deprived cultures of *C. reinhardtii* accumulate large amounts of protein and, especially, of starch (measured as the amount of glucose) (Melis et al. 2000).

- Electron sources

H_2 production can result from three different electron transfer pathways (represented by the red numbers in Figure 17). The first is the PSII-dependent pathway, which involves residual water photolysis by PSII, the electrons being transferred through the photosynthetic ETC to PSI and then to ferredoxin. The ferredoxin donates the electrons to the hydrogenase enzyme (Gaffron and Rubin 1942) instead of transferring them to NADP, which is used in the carbon fixation pathway. The down-regulation of CO_2 assimilatory pathway in sulfur-deprived *C. reinhardtii* cells is an important pre-requisite for sustained H_2 production (Hemschemeier et al. 2008), so that the hydrogenase enzyme does not have to compete with alternative electron sinks (Cinco et al. 1993), like the carbon assimilation pathway.

The second pathway is PSII-independent, and it uses the photo-fermentation of endogenous organic compounds (such as starch, proteins, and lipids) as a source of reducing power, providing electrons to the photosynthetic ETC at the plastoquinone level through the chlororespiratory pathway. Externally added sources of organic carbon, such as acetate, increase

the electron flow through this pathway and thus, the H_2 production yields (Gfeller and Gibbs 1985).

The third pathway is light-independent and is a fermentative pathway, where pyruvate generated by starch catabolism may be oxidatively decarboxylated by pyruvate:ferredoxin oxidoreductase (PFOR) enzyme (Torzillo et al. 2014). This leads to a reduced state of the ferredoxin, which acts as an electron donor to the hydrogenase (Noth et al. 2013).

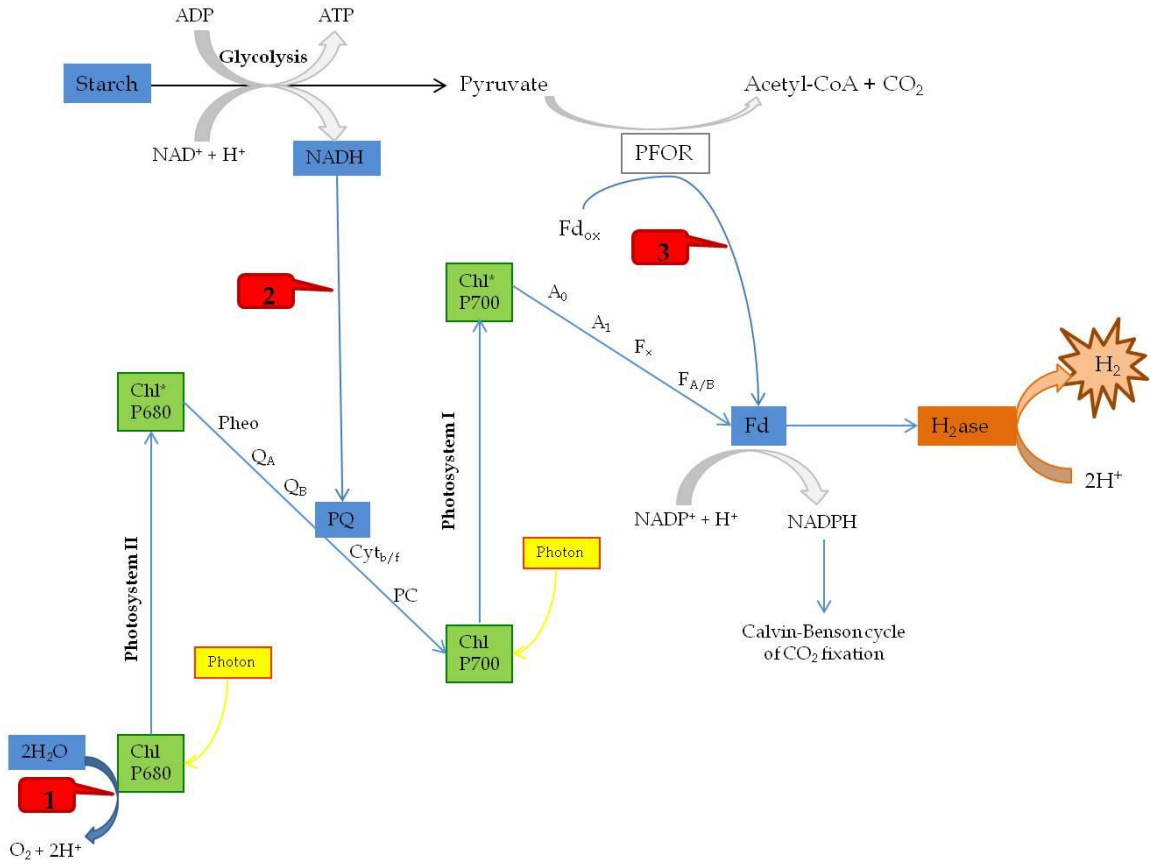

Figure 17. Schematic representation of the biochemistry of hydrogen production by sulfur-deprived *C. reinhardtii* cells.

- Required metabolites

Both water oxidation and endogenous catabolism of starch and protein contribute with electrons to H_2 production (Fouchard et al. 2005; Kosourov et al. 2003; Posewitz et al. 2004; Zhang et al. 2002). Organic substrate degradation also contributes to the respiratory consumption

of O_2, produced by the residual PSII activity of water splitting, during the H_2 production process and is, thus, responsible for maintaining the anaerobic state of the culture (Ghirardi et al. 2000; Kosourov et al. 2003; Melis 2002). Finally, substrate catabolism during H_2 production is necessary to maintain a proper intracellular redox potential that controls the expression of the hydrogenase gene in *C. reinhardtii* cells (Posewitz et al. 2004). The levels of accumulation of intracellular starch and protein are, therefore, essential for the H_2 production process.

Use of acetate for hydrogen production

Until now, the majority of laboratories studying H_2 production in *C. reinhardtii* have used the Tris-Acetate-Phosphate (TAP) medium (Gorman and Levine 1965), which contains acetate (Hahn et al. 2004; Jo et al. 2006; Kruse et al. 2005; Melis et al. 2000; Torzillo et al. 2009). Acetate is a relatively expensive compound, which is not available in nature and must be synthesized (Fouchard et al. 2005). The use of acetate in the culture medium increases, therefore, the cost of biomass and of H_2 production (Ghirardi and Amos 2004). It also serves as growth substrate to heterotrophic organisms, enhancing the risk of contamination by bacteria, fungi, and other microalgae (Ferrel and Sarisky-Reed 2010; Fouchard et al. 2005). Moreover, since H_2 combustion generates no carbon-based emissions, using an organic carbon source, such as acetate, for growing *C. reinhardtii* does not seem to make sense. From an energy conversion point of view, it appears more logical to use CO_2 as carbon source in microalgal cultures. By omitting acetate from the growth medium, *C. reinhardtii* is produced photoautotrophically with CO_2 and light as its only energy source. CO_2 is readily available in the atmosphere or from industrial sources, such as flue gas. Melis et al. (2000) argued that acetate is necessary as substrate in respiration for establishing and maintaining anaerobiosis in the H_2 production stage. However, it has been demonstrated that production of H_2 under photoautotrophic conditions is possible (Tolstygina et al. 2009; Tsygankov et al. 2006).

H_2 production under photoautotrophic conditions is, however, less efficient than under heterotrophic or mixotrophic conditions (Tolstygina et al. 2009; Tsygankov et al. 2006). One of the reasons for the low H_2 output may be the low level of starch accumulation during the aerobic phase of the sulfur deprivation stage (Tsygankov et al. 2006). Sulfur deprivation in itself is not sufficient to decrease PSII activity to such a level that anoxia is maintained in an acetate-free

medium (Fouchard et al. 2005). In this study, we aimed at obtaining high starch accumulation levels during the first stage of photoautotrophic production of biomass, prior to sulfur deprivation. In this way, the cells that are subjected to sulfur deprivation would already have accumulated enough starch before they are sulfur-deprived. Our working hypothesis was that high intracellular starch content would allow for establishing anoxic conditions in the beginning of the sulfur deprivation stage, with no need for acetate in the medium. Fouchard et al. (2005) showed that the aerobic growth phase could be conducted either mixotrophically or photoautotrophically without a major impact on the subsequent H_2 production.

6. Aims of the present study

The aim of this thesis was to investigate the effects of culture conditions on the growth and biochemical composition of *C. reinhardtii* photoautotrophic batch cultures, as a potential source for H_2 production. The individual and the interaction effects of nutrient concentrations in the growth medium, CO_2 concentration in the bubbled gas, light intensity, and temperature on the pH of the cultures, their productivity and biomass yield, as well as on the contents of starch, protein, and chlorophyll were examined.

Three series of experiments were designed:

1) The goal of the first one was to quantify (Paper I) and model (Paper II) the effects of ammonium, phosphate, sulfate, and carbon dioxide concentrations on the growth and biochemical composition of *C. reinhardtii* cultures.

2) The second series of experiments (Paper III) aimed at understanding how the combinations of different levels of light intensity, temperature, and CO_2 concentration affect the growth and biochemical composition of this microalga.

3) The third experiment (Paper IV) aimed at evaluating whether the calcium and magnesium concentrations used in previous experiments were limiting for the growth of *C. reinhardtii* batch cultures.

7. Materials and methods

The experimental work in this study was conducted in the Plant Cell Laboratory, at the Norwegian University of Life Sciences (NMBU), Ås, Norway. All the experiments were carried out in a special 'algae room' with no natural light and constant temperature, maintained at 15 ± 2 °C by an air conditioning system.

This PhD work was part of a collaboration project between the microalgae research group at the Plant Sciences Department, Norwegian University of Life Sciences, and the research institute Bioforsk (Norwegian Institute for Agricultural and Environmental Research), Ås: 'Use of solar energy for CO_2 capture, algae cultivation and hydrogen production – BioH_2'. The project consisted of two parts:

1) To gain knowledge about how the culture conditions affect the growth and biochemical composition of *C. reinhardtii* (NMBU), and
2) Testing the conditions for H_2 production by *C. reinhardtii* (Bioforsk).

7.1. Experimental setup

The details of the experimental setup used in each series of experiments are described in the Materials and methods sections in the corresponding Papers. Here, a brief general description is provided.

The experiments were performed in bubble column Pyrex glass tubular PBRs (Figure 18). The PBRs were placed in water bath aquaria and the temperature was set up manually by using aquarium heaters (Eheim Jager 3619, Eheim, Daizisau, Germany). Because it was not possible to regulate the temperature in the aquaria, the air temperature was kept at 15 ± 2 °C by air conditioning. The temperature in the aquaria was registered by a data logger connected to a computer, equipped with the PicoLog software (Picolog, Pico Technology, St Neots, UK). Air was mixed with pure CO_2 in a plastic box (50 L) and injected through latex and glass tubing into the bottom of the PBRs. The CO_2 concentration in the bubbled gas was set up manually by a capillary and water column system. The CO_2 concentration in the bubbled gas was registered by a data logger and a computer equipped with the PicoLog software. The variation in the CO_2 supply

was in the ± 10% range. The gas flow rate was regulated manually by valves set up on the inlet tubing of each individual PBR. The PBRs were continuously illuminated by cool white fluorescent tubes from one side. The light intensity was measured using a LI-190SA instrument (LI-COR, Lincoln, NE, USA) with a quantum sensor (400 – 700 nm) inside an empty PBR immersed in the water-bath aquarium, and the average PFD over the height of the PBR was estimated. The average light intensity incident on the PBRs was modified by turning on/off the necessary amount of lamps. The system was controlled several times a day over the duration of the experiments.

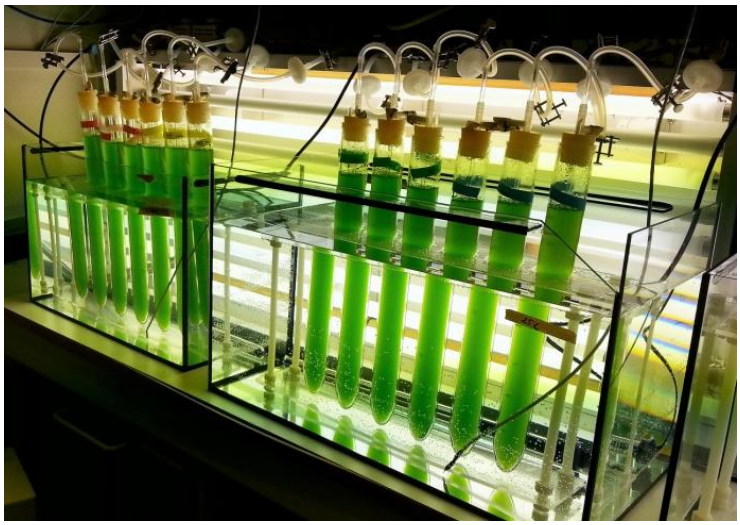

Figure 18. Photobioreactors used for batch cultures of *C. reinhardtii*. Photo: Marit E. Evjen.

7.2. First series of experiments: concentrations of ammonium, phosphate, sulfate, and carbon dioxide (Papers I and II)

The aim of the first series of experiments was to quantify (Paper I) and model (Paper II) the effects of ammonium, phosphate, sulfate, and carbon dioxide concentrations on the growth and biochemical composition of *C. reinhardtii* cultures, as a potential source for H_2 production.

For efficient H_2 production, cultures with high biomass are necessary (Tamburic et al. 2011). Starch and protein have been shown to be important electron sources for the hydrogenase enzyme (Melis et al. 2000; Zhang et al. 2002). Moreover, chlorophyll *a* concentration of 20 µg mL^{-1} has been shown to be optimum for maximum H_2 production yield (Hahn et al. 2004). Therefore, the productivity and the biomass yield of the cultures, as well as their contents of starch, protein, and chlorophyll were chosen as response parameters.

Preliminary experiments (data not shown) showed that varying concentrations of ammonium and phosphate in the HS medium, as well as CO_2 concentration bubbled into cultures had a significant effect on the growth of *C. reinhardtii*. Based on the results of these preliminary experiments, a range of nutrient concentrations was chosen, and the sampling times were decided. Sulfate was added to the experimental designs of the first series of experiments, due to its known effect on starch accumulation and its importance in the H_2 production process. The concentrations of ammonium in the first series of experiments varied between 5.0 and 20.0 mM, phosphate concentrations were between 7.5 and 22.5 mM, sulfate concentration varied between 0.1 and 1.0 mM, and CO_2 concentrations in the bubbled gas varied between 1.0 and 6.0 % v/v (Table 1 in Paper I). The phosphate concentrations used in this study were quite high (7.5 – 22.5 mM), due to the fact that K_2HPO_4/KH_2PO_4 was used as a pH buffer system, necessary to avoid acidification of the cultures as ammonium was consumed and metabolites excreted by the microalgae. The results of the preliminary experiments showed that when the concentration of phosphate was decreased below 7 mM, the pH of the cultures with high ammonium concentrations decreased below 4.0 and these cultures died. Additional pH buffers were, then, tested (data not shown) for their efficiency to stabilize the pH of the cultures. $NaHCO_3$ showed to be the most effective in maintaining stable pH and was, therefore, chosen to be used in the growth media at a concentration of 2 mM per percent CO_2 in the bubbled gas. The sampling

times were chosen to approximately correspond to the linear and the beginning of the stationary growth phases.

In Paper I, two concentrations of ammonium, phosphate, sulfate, and carbon dioxide were combined in a 2^4 full factorial design. One of the advantages of this experimental procedure is its ability to identify interaction effects between the studied variables. It also reduces the number of runs as compared to one-by-one factor experiments. The details of this statistical design are presented in Table 1 in Paper I. The experimental setup and the details of the various analyses are described in the Materials and methods section of Paper I. The individual and interaction effects of the concentrations of the four studied nutrients we quantified and the results of the analysis of variance (ANOVA) are presented in Table 2 in Paper I. The measured pH, dry weight (DW) of the cultures and their contents of starch, protein, and chlorophyll are presented in the figures in Paper I.

In Paper II, the number of treatments was extended from sixteen treatments in the 2^4 factorial design used in Paper I to twenty five treatments in a central composite design with additional eight axial points and one central point (Figure 19). The details of this statistical design are presented in Table 1 in Paper II and the experimental setup is described in the Materials and methods section of Paper II. The response parameters (productivity, biomass yield, and starch, protein, and chlorophyll contents) were modeled using a second order equation (Equation 2, Paper II). The details of the statistical analysis are presented in the Materials and methods section in Paper II. The obtained second-order response surfaces (Table 2 in Paper II) were then used for an optimization procedure that predicted maximum responses and the corresponding values of the studied variables. Model validation experiments were carried out.

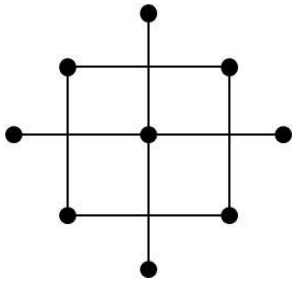

Figure 19. An example of a central composite design, based on a 2^2 full factorial design with a center point and four axial points.

The treatments in the first series of experiments were divided into blocks and carried out successively, according to the CO_2 concentration. The experiments were carried out over a seven-week period. Control treatments were not performed in this first series of experiments and the results could not been normalized. The vitality of the cultures could have varied over the experimental period.

The statistical models used in Paper II use the ANOVA method, which was also used for the analysis of the results in Papers I and III. For the ANOVA to be reliable, some basic assumptions must be satisfied. Namely, it assumes that the errors of the response data follows a normal distribution, that the observations are mutually independent and that the variances of the responses within the treatments are constant. The models used in Paper II were validated using the residuals analysis. The residuals are the differences between every individual observation and the corresponding model estimate of that observation. If the model is adequate, the residuals should present no structure; this means, they should not present any obvious distribution pattern (Montgomery 2009). The normality assumption can be checked by plotting a histogram of the residuals (Figure 20). If the normality assumption is satisfied, this histogram should look like a sample from a normal distribution centered at zero. A normal probability plot of the residuals is also usually constructed (Figure 20). It uses a t-test and if the error distribution is normal, this plot will look like a straight line, especially in the central values, more than on the extremes. By plotting the residuals vs. time (or observation order) (Figure 20), it is possible to detect correlations between the residuals and to test the independence assumption. Finally, a non-constant variance can be detected on the residuals vs. fitted values plot (Figure 20) if it presents any obvious structure or pattern.

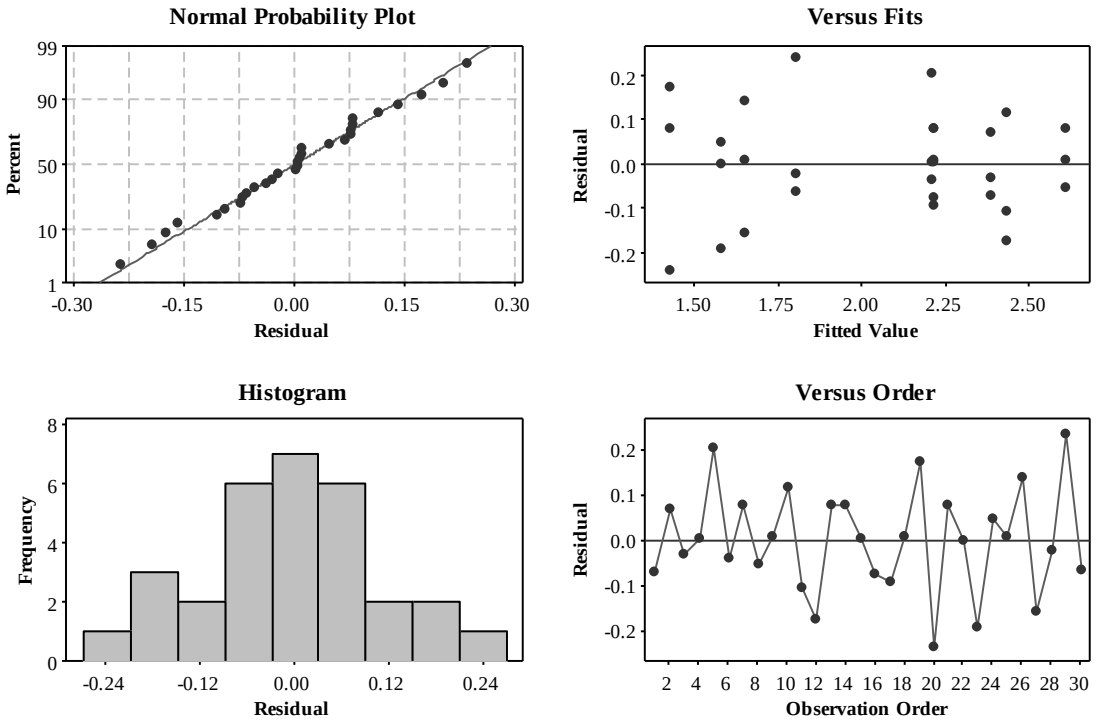

Figure 20. An example of the residuals distribution plots generated by Minitab of one of the models used in Paper II.

7.3. Second series of experiments: light intensity, temperature, and CO_2 concentration (Paper III)

The second series of experiments focused on the effects of the environmental factors: light intensity (100 and 400 µmol m^{-2} s^{-1}), temperature (25 and 35 °C), and CO_2 concentration (3 and 9% v/v) on the growth and biochemical composition of *C. reinhardtii*. A 2^3 full factorial design was used to study the effects of these three factors and their interactions. Table 2 in Paper III shows the details of this experimental design. The experimental setup is described in detail in the Materials and methods section of Paper III. The composition of the growth medium used in this series of experiments was determined by using the modeling results of the first series of experiments (Paper II) and optimizing the concentrations of ammonium, phosphate, and sulfate for maximum productivity and maximum biomass yield. The nutrient concentrations of this medium are detailed in Table 1 in Paper III.

In this series of experiments, the treatments were divided into blocks and carried out successively, at two different levels of light intensity. A control treatment (n = 3) with 100 µmol m^{-2} s^{-1}, 25 °C, and 3% CO_2 was carried out in parallel to the treatments at 400 µmol m^{-2} s^{-1}. A simple t-test showed that there was no significant ($p > 0.05$) differences between the measured dry weights of these control cultures compared to the cultures in treatment 1, with the same conditions.

7.4. Third experiment: calcium and magnesium concentrations (Paper IV)

The results, obtained by Leiv Mortensen (Mortensen and Gislerød 2014; 2015) and Marit Evjen (2014) from our research team, indicated a possible growth limitation by calcium and/or magnesium when *C. reinhardtii* is grown in the HS medium. Indeed, the HS medium (and the growth media used in the first two series of experiments) contains relatively low concentrations of calcium and magnesium (0.068 mM Ca and 0.081 mM Mg) compared to other culture media used for growing *C. reinhardtii* (Harris 2009). This experiment (Paper IV) was designed to investigate whether the concentrations of calcium and magnesium used in the first two series of experiments were limiting for growth. Four different concentrations of calcium and magnesium were used, ranging from the concentrations found in the HS medium (0.068 mM Ca and 0.081 mM Mg) to their tenfold (0.68 mM Ca and 0.81 mM Mg). The details of the experimental setup are described in the Materials and methods section of Paper IV.

7.5. Determination of the dissolved CO_2 concentration

The concentration of the dissolved CO_2 gas in the growth medium, and not the CO_2 concentration in the bubbled gas, determines the availability of CO_2 to the microalgae. A test was performed to determine the relationship between the concentration of CO_2 in the bubbled gas and the concentration of the dissolved CO_2 in the growth medium. The details of the experimental setup are described in the Materials and methods section of Paper I. The results showed a progressive increase in the dissolved CO_2 concentration from 100 to 430 mg L^{-1}, with an increasing CO_2 concentration in the bubbled gas, from 0.04% to 9.0% (Figure 21). In parallel to the increase in dissolved CO_2 concentration, the pH decreased from 7.52 ± 0.01 to 6.59 ± 0.01 (mean ± SD, n = 3) (Figure 21). The linear regression curve (order 2) between the CO_2 concentration in the bubbled gas and the concentration of dissolved CO_2 was highly correlated (R^2_{adj} = 0.955) and followed the function $y = -4.2\ x^2 + 73\ x + 108$, where y = dissolved CO_2 concentration in the medium (mg L^{-1}) and x = CO_2 concentration in the bubbled gas (% v/v).

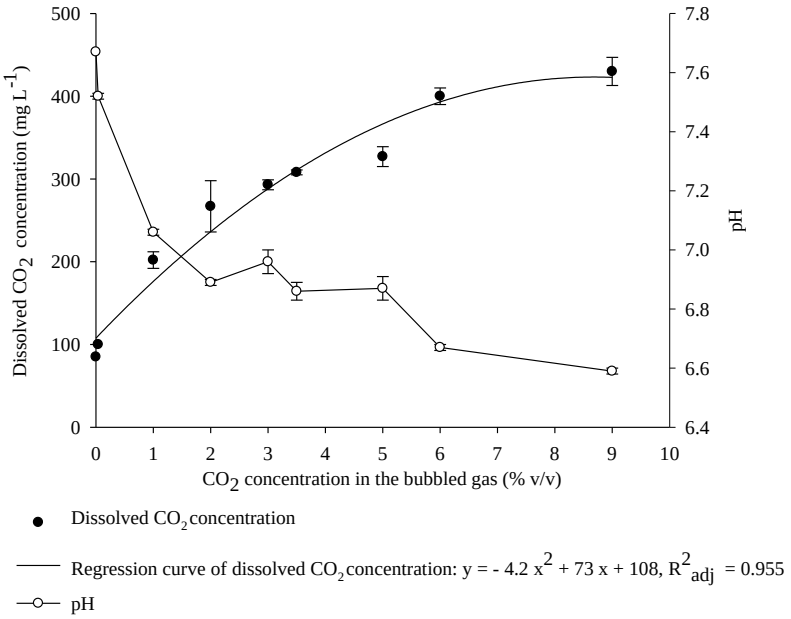

Figure 21. Effect of CO_2 concentration in the bubbled gas (% v/v) on the dissolved CO_2 concentration (mg L^{-1}) in the High-Salt Sueoka medium (means ± SD, n = 3) with the linear regression curve ($y = -4.2\ x^2 + 73\ x + 108$, R^2_{adj} = 0.955) fitted to the data and on the pH of the medium.

Additional measurements were performed to determine how different culture conditions could influence the dissolved CO_2 concentration in the PBRs. First, a test was carried out using much higher flow rate than the one used throughout our experiments with 3 and 9% CO_2 in HS medium. The results shown that there was no significant ($p > 0.1$) difference between the dissolved CO_2 concentrations at the two flow rates tested. A second test with 35 °C and 9% CO_2 showed that there was no significant ($p > 0.1$) difference between the dissolved CO_2 concentrations at 25 or 35 °C. Finally, a test was performed using the growth medium used in Paper III with 3 and 9% CO_2. The measured dissolved CO_2 concentrations in the growth medium used in Paper III were significantly ($p < 0.001$) lower than in the HS medium, with 130 ± 5 mg L^{-1} (mean ± SD, n = 3) instead of 293 ± 6 mg L^{-1} at 3% CO_2, and 187 ± 6 mg L^{-1} instead of 430 ± 17 mg L^{-1} at 9% CO_2. This difference in dissolved CO_2 concentrations between the HS medium and the medium used in Paper III was probably due to the different nutrient composition of the two media.

7.6. Determination of the biomass content in the cultures

The biomass content of a culture can be measured by different methods. These methods include cell counting, DW determination, optical density (OD) measurement, or assays of chlorophyll or protein content. These last two methods of determination of chlorophyll or protein content per unit volume of culture were not applicable for our experiments, since our study hypothesis was that the biochemical content of the cells should vary with the different culture conditions. Cell counting, especially using particle counters, such as Coulter counter, also presents a difficulty in the case of *C. reinhardtii* since this microalga forms palmelloid cells upon division. So, our methods of choice were measuring the DW of the cultures and their OD by spectrophotometry.

The DW of a culture was measured by filtering a small sample through a pre-weighed glass microfiber filter, which was then dried and weighed. The filters were pre-washed with deionized water in order to remove any residual material that otherwise might be lost in the procedure, thus affecting the measured DW of the sample, and dried. After a sample was filtered using a vacuum pump, the filters were washed with deionized water to remove all the remaining salts that are present in the growth medium. The filters were dried (4 h at 103.5 °C was enough to obtain constant weight) and then cooled down in a desiccator containing silica gel that extracts all the remaining water from the filter, and weighed again. We disposed of a microbalance (XP6, Mettler Toledo, Greifensee, Switzerland) with a very high degree of precision, it being able to measure the weight down to a µg. We were, therefore, able to use quite small filters (25 mm in diameter) and very small sample volume for each DW determination, which is very convenient in case of frequent sampling with an initial culture volume of 300 mL.

The various steps of this procedure had to be assayed and optimized before starting the experiments. Three filters were used for measuring the DW of each culture, to eliminate the errors associated with the method. The details of this procedure are described in the Materials and Methods section of Paper I.

The productivity of the cultures was measured approximately in the linear growth phase. The productivity (P) of the cultures ($g\ L^{-1}\ d^{-1}$) was calculated using the formula:

$$P = \frac{DW_2 - DW_1}{t_2 - t_1}$$

where $DW_{1\ (2)}$ is the DW determined at time $t_{1\ (2)}$ (in days). The biomass yield was defined as the maximum attained biomass measured during the experiments.

The measurement of the OD of a sample is much simpler and required little effort to set up. We needed, however, to determine the range of ODs, over which the method was linear. Outside that range, the cultures had to be diluted before the measurement was done. Theoretically, a linear relationship between the DW and the OD should exist. We plotted the DW vs. the corresponding OD of each one of our samples from all three series of experiments in Figure 22. The distribution was linear and could be approximated by the straight line of equation DW = 0.534 OD + 0.013, with R^2_{adj} = 95.5%. The Pearson correlation (Pearson and Hartley 1966) between the DW and the OD was 0.977 ($p < 0.001$). It would therefore be possible to measure OD and calculate the corresponding DW using this linear regression equation. Gordillo et al. (1999) also found a linear relationship between the OD at 750 nm and the DW of *Spirulina platensis* photoautotrophic batch cultures. The slope of their curve was 0.89, meaning that the conversion coefficient is species-specific and probably depends on the cell size and the pigment content. We observed, however, that the variance was not constant within the measured range. It was small for low values of OD and DW, and became larger as these increased.

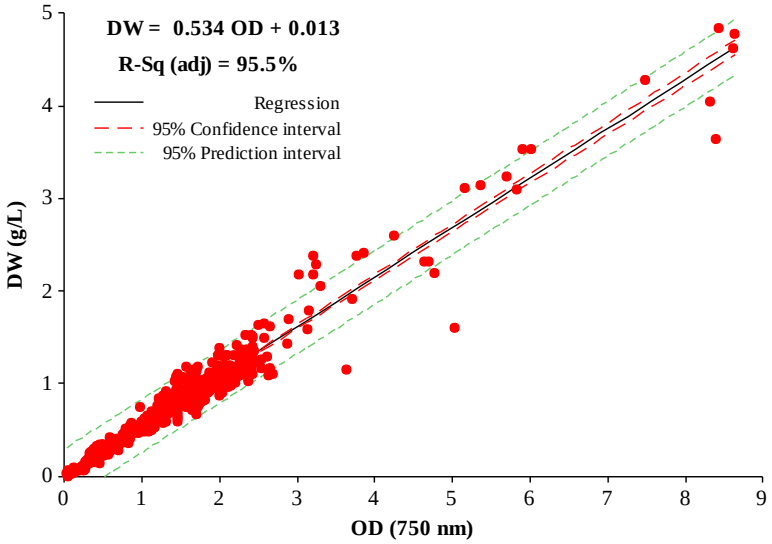

Figure 22. The linear relationship between the dry weight (DW) and the optical density (OD at 750 nm) of the samples of *C. reinhardtii* cultures in our experiments.

7.7. Setup of the biochemical assays

The assays for starch, protein, and chlorophyll also needed to be set up. The details of these biochemical assays are presented in the Materials and Methods sections of Paper I.

The major issue encountered was to disrupt the cells to extract the intracellular compounds. Several methods of cell disruption were tested: sonication, solubilization by detergents, freezing-thawing. The extent of cell breakage was evaluated by microscopic observation of the number of remaining intact cells. Finally, the cells were disrupted by mechanical force, by adding glass beads and shaking the samples on a mixer mill.

Different methods of starch assay were tested. First, the method of McCready et al. (1950), modified by Branyikova et al. (2010) was tested. This method relies on hydrolysis of starch by perchloric acid. Anthrone in 72% sulfuric acid is then used to colorimetrically determine the starch content in the solution against glucose standard curve. This method uses some quite 'nasty' solvents and it was difficult to establish its linearity. Sigma sa20 colorimetric starch assay kit (Sigma-Aldrich, St. Louis, MO, USA) based on autoclaving for starch solubilization followed by several enzymatic reactions was tested next. Due to some technical difficulty in dissolving the starch assay reagent supplied in the kit, this method wasn't used either. Finally, Megazyme starch assay (K-TSTA, Megazyme International, Bray, Ireland) was adapted to small volume samples (2 mL). Starch was solubilized by heat and dimethyl sulfoxide (DMSO) solvent, hydrolyzed by α-amylase and amyloglucosidase enzymatic reactions, and finally, the glucose concentration was assayed colorimetrically after the reaction with the glucose oxidase/peroxidase (GOPOD) reagent. A formula supplied in the Megazyme starch assay kit was used to calculate the starch content of the cultures as % of DW.

Proteins were solubilized by a buffer containing 1% Triton, after testing several buffers with different detergents at different concentrations, and different pH levels, so the whole cell protein extract was assayed. Bovine serum albumin (BSA) standard curve was prepared in the same buffer, making sure of its linearity. The colorimetric microplate assay kit from Bio-Rad (#500-0112, Bio-Rad, Hercules, CA, USA) based on the Lowry (1951) method was chosen over the Bradford method based Bio-Rad assay kit because of its compatibility with detergents used for protein solubilization. The protein concentrations in the samples were determined by

spectrophotometry measurements and using the BSA standard curve. The protein concentration was normalized to the DW and expressed as protein content in % of DW.

Chlorophyll was extracted directly from the microalgal cells without previous cell disruption. For this, several solvents were tested, and finally, 95% ethanol was chosen since it is more environment-friendly than other solvents, such as acetone or methanol, easier to use, and more efficient in extracting chlorophyll from *C. reinhardtii* cells. The absorbance of the chlorophyll extracts was measured using spectrophotometry and the formula supplied by Harris (1989) was applied to determine the total chlorophyll (*a* + *b*) concentration in the samples:

$$[Total\ chl](\mu g\ mL^{-1}) = 20.4 \times OD_{649} + 6.1 \times OD_{665}$$

When needed, the chlorophyll concentration was normalized to the DW of the sample and expressed as chlorophyll content in % of DW.

8. Main results and discussion

In this study, we quantified the effects of culture conditions, such as nutrient concentrations in the growth medium, CO_2 concentration in the bubbled gas, light intensity, and temperature on the growth and biochemical composition of *C. reinhardtii* photoautotrophic batch cultures. The first series of experiments (Papers I and II) aimed at evaluating the effects of concentrations of ammonium, phosphate, sulfate and carbon dioxide on the productivity, the biomass yield, and the contents of starch, protein, and chlorophyll for potential hydrogen production with *C. reinhardtii*. Paper I presents the growth curves and the variation in biochemical composition of a part of the data from the first series of experiments, while Paper II presents the statistical models using the complete dataset and the results of the validation experiments. Tables 1 and 2 (at the end of this section) summarize the main results of the first series of experiments. Next, we studied the effects of light intensity, temperature, and CO_2 concentrations on the same set of parameters. The results of the second series of experiments are presented in Paper III and summarized in Tables 3 and 4 at the end of this section. Finally, a question arose whether the concentrations of calcium and magnesium in the growth media used in the first two series of experiments could be limiting for growth. An experiment with four different concentrations of calcium and magnesium, ranging from those used in our media to their ten-fold, was carried out. The results of this experiment are presented in Paper IV and summarized in Table 5 at the end of this section.

8. 1. pH

The pH of the cultures decreased progressively with the time and the growth of microalgae (Table 2 and Figure 1 in Paper I; Figure 1 in Paper III; Figure 1 in Paper IV). Indeed, the pH of the cultures was negatively correlated to the measured dry weight (Tables 2 and 4). The optimum pH range for *C. reinhardtii* growth is 6.5 – 8.0 (Harris 1989). In some of the cultures in the different experiments carried out in this study, the pH decreased below 6.5. This was especially the case in media containing high concentrations of ammonium (17.5 – 20 mM) and relatively low concentrations of phosphate (7.5 – 10 mM) (Table 2 in Paper I). The pH at the end of the experiments also tended to be lower in the cultures grown at lower carbon dioxide concentrations (Table II in Paper I; Table 3 in Paper II), probably due to the fact that less bicarbonate was added to these cultures, as the amount of bicarbonate added to the growth media was proportional to the carbon dioxide concentration in the bubbled gas. This was a strategy that did not yield enough bicarbonate at the low CO_2 concentrations and culture conditions that yielded high biomass. The decrease in pH was particularly marked in the second series of experiments, in the cultures grown in 17.5 mM ammonium, 7.5 mM phosphate, at 3% carbon dioxide and 400 µmol m^{-2} s^{-1}, where the pH dropped significantly after only 48 h of growth and the cultures died (Figure 1 in Paper III). Consequently, for the experiments on calcium and magnesium (Paper IV), we increased the phosphate concentration to 15.0 mM in order to maintain a stable pH in a medium with high initial ammonium concentration (17.5 mM). This strategy was effective, and the pH in these experiments was stable and did not decrease below 6.25 (Figure 1 in Paper IV).

8. 2. Productivity

The productivity (g L^{-1} d^{-1}) of a culture reflects the speed of its growth. It is important to obtain high productivities of microalgal cultures because it reduces the operation time of the process, and therefore also reduces the costs. The productivities measured in this study correspond roughly to the productivity in the linear growth phase. It is in the linear growth phase where the biomass production is maximal in absolute terms. Ogbonna et al. (1995b) argued that during light-limited batch cultivation of photosynthetic cells, as was the case in this study, the linear growth rate (or productivity) is a better indicator of growth than the specific growth rate in the exponential growth phase.

The productivity of the cultures was greatly enhanced by increased CO_2 concentrations in the first series of experiments, with a 2.7-fold increase in productivity between 1 and 6% CO_2. It was also the case in the second series of experiments for the cultures grown at 400 µmol m^{-2} s^{-1}, but not at 100 µmol m^{-2} s^{-1}. The concentration of dissolved CO_2 in the PBRs at 6% CO_2 in the bubbled gas was twice as high as at 1% in the first series of experiments. There was also a 1.4-fold increase in the dissolved CO_2 concentration between 3 and 9% CO_2 in the second series of experiments. A perfect (R^2 = 1.00) quadratic relationship of equation $y = 1.14x^2 - 1.87x + 1.9$, where y is the increase in measured productivity and x is the increase in dissolved CO_2 concentration, was found using the data of Papers I, II, and III (Figure 23).

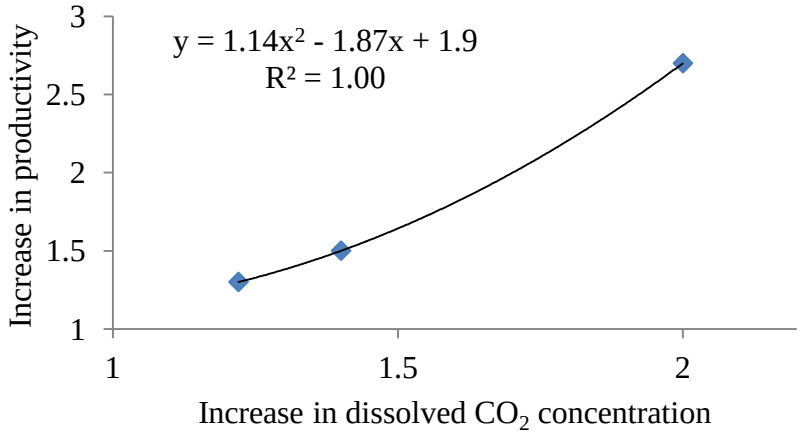

Figure 23. Quadratic relationship between the increase in productivity and the increase in the dissolved CO_2 concentration, with data taken from Papers I, II, and III.

In the first series of experiment, the concentration of ammonium had little influence on the productivity compared to the effect of CO_2 (Figure 1b in Paper II), probably due to the fact that it was not growth-limiting at the time points between which the productivity was measured. Increased concentrations of sulfate up to 0.55 mM did increase the productivity of the cultures (Figure 1b in Paper II), indicating that 0.1 and 0.2 mM sulfate were probably limiting for growth even at the early stage where productivity was measured. The maximum productivity predicted by the model in Paper II was 0.87 g L^{-1} d^{-1} for a medium containing 5.0 mM ammonium, 0.65 mM sulfate with 6.0% CO_2 (Figure 2a in Paper II; Table 1), which was confirmed by the model validation experiment (Figure 5a in Paper II). The maximum productivity corresponded to the medium with the lowest concentration of ammonium and the highest concentration of carbon dioxide tested in this study, showing for the negative interaction effect on the productivity between the concentrations of these two nutrients (Table 2 in Paper II). The optimum sulfate concentration for maximum productivity was 0.65 mM, which is much higher than the concentration of sulfate in the HS medium (0.182 mM) (Sueoka 1960), but is closer to the sulfate concentration in the TAP medium (0.51 mM) (Gorman and Levine 1965) (Table 1). We would therefore recommend increasing the sulfate concentration in these two growth media to 0.65 mM.

In the second series of experiments, high light intensity (400 µmol m^{-2} s^{-1}) and high temperature (35 °C) induced an earlier and faster growth of the cultures as compared to low light (100 µmol m^{-2} s^{-1}) and low temperature (25 °C) (Figure 2 in Paper III). The maximum productivity measured in these experiments was 1.12 g L^{-1} d^{-1} in the treatment with 400 µmol m^{-2} s^{-1}, 35 °C, and 9% CO_2, between 36 and 48 h of growth (Figure 2 in Paper III). The cultures clearly benefitted from the synergistic effect of high light intensity, high temperature, and high CO_2 concentration for their growth at early stage (Table 3 in Paper III). The productivity measured in the same conditions of light intensity and CO_2, but at 25 °C was not significantly ($p > 0.1$) different from 1.12 g L^{-1} d^{-1}, but it was measured 24 h later, between 60 and 72 h of growth, these cultures having a much longer lag phase than the cultures at 35 °C (Figure 2 in Paper III). The cells in these cultures probably received enough light energy and carbon dioxide available for fast growth. However, the temperature of 35 °C probably accelerated the accumulation of sufficient intracellular compounds for cell division, which allowed the cultures grown at 35 °C to start their fast growth earlier as compared to the cultures grown at 25 °C.

Mortensen and Gislerød (2014) also found that at 300 µmol m^{-2} s^{-1} there was no difference in productivity in the temperature range of 24 – 33 °C. It is worth mentioning that 35 °C approaches quite closely to the maximum temperature tolerated by *C. reinhardtii* cells. Indeed, Tanaka et al. (2000) showed that at 35 °C there was no synthesis of heat-shock proteins in *C. reinhardtii* cells, which are synthesized when cells are exposed to extreme high temperatures, however, they were present in cells exposed to temperatures over 37 °C.

The maximum productivity in the second series of experiments (1.12 g L^{-1} d^{-1}) was significantly ($p < 0.05$) higher than the maximum productivity measured in the first series of experiments (0.87 g L^{-1} d^{-1}). The growth medium used in the second series of experiments was designed using the results of the first series of experiments as to yield maximum productivity and biomass yield. This medium contained 17.5 mM ammonium, 7.5 mM phosphate, and 0.65 mM sulfate. The maximum productivity in the second series of experiments was measured at 400 µmol m^{-2} s^{-1}, 35 °C, and 9% CO_2. These combined conditions of nutrient-rich medium, high CO_2 concentration, and higher light intensity and temperature than the ones used in the first series of experiments acted synergistically to yield the higher productivity measured in the second series of experiments.

The productivity of the cultures was further increased by increasing the calcium and magnesium concentrations in the growth medium, in the third series of experiments (Paper IV). The maximum productivity measured in this experiment was 1.97 g L^{-1} d^{-1} in the treatment with ten times the concentrations of calcium and magnesium used in the first and second series of experiments and the HS medium. However, the difference in growth at different concentrations of calcium and magnesium was not significant ($p > 0.01$) before 2.5 days of growth, up to a biomass concentration of 0.95 – 1.18 g L^{-1} (Figure 2b in Paper IV). This indicates that the HS medium (Sueoka 1960) and the media used in the first two series of experiments has sufficient amount of calcium and magnesium to sustain microalgal growth up to about 1 g L^{-1}. The concentrations of calcium and magnesium used in the first two series of experiments did not therefore affect the growth of the microalgae up to this biomass.

8. 3. Biomass yield

Contrary to its effect on the productivity, increased CO_2 concentration did not increase the biomass yield of the cultures (Table 2 in Paper I; Table 3 in Paper III). The effect of CO_2 concentration on the DW of the cultures at 96 h of growth in Paper I was not significant (Table 2 in Paper I). However, by adding three more levels of CO_2 concentration in Paper II, we could observe an increase in biomass yield with increasing CO_2 concentration up to 3.5% (Figure 1c in Paper II) and the CO_2 concentration was included in the model for the biomass yield (Table 2 in Paper II). In the second series of experiments, the increased CO_2 concentration of 9% had a negative effect on the biomass yield at 100 µmol m^{-2} s^{-1} (Table 3 in Paper III). This is in accordance with the results obtained by Mortensen and Gislerød (2014; 2015), who also detected a negative effect of CO_2 concentrations higher than 9% on the growth of this strain of *C. reinhardtii*. The cultures might have benefited from higher CO_2 concentrations during their early growth stage, increasing their productivity with the increase in the available CO_2 concentration (Figure 23). However, the cultures in a later growth stage might have been less sensitive to the increase in CO_2 concentration, since their growth might have been limited by the availability of other nutrients in the growth medium (such as calcium and magnesium), hence no measured increase in biomass yield at higher CO_2 concentrations.

Both ammonium and sulfate concentrations, as well as their combination, influenced positively the biomass yield of the cultures in the first series of experiments (Table 2 in Paper I; Table 2 in Paper II). The growth of the cultures in low sulfate (0.1 – 0.2 mM) and low ammonium (5.0 – 7.5 mM) concentrations was therefore probably limited by these two nutrients. We calculated that 2.8 – 5.2 mM of nitrogen and 0.05 – 0.12 mM of sulfur was left in the medium at the time when the biomass yield was determined (Figure 11 in Paper I). Even if the cultures did not become depleted of these two nutrients, their growth might have been affected by their remaining low concentrations in relation to the availability of other nutrients, especially of carbon dioxide. The uptake and assimilation of ammonium, sulfate, and also phosphate are closely related to the carbon fixation. A balance must exist in the growth medium between the main macronutrients and carbon, so that these macronutrients are incorporated into carbon skeletons in an equilibrated manner (Grossman and Takahashi 2001). When all the required

nutrients are provided in excess, microalgae grow in a balanced way and display a uniform chemical and biochemical composition (Goldman 1980). This is rarely the case in batch cultures, where most frequently one of the nutrients becomes limiting for growth. The cells in the cultures grown at low ammonium and sulfate concentrations in the first series of experiments, probably experienced an imbalance of the available nutrients, with high available carbon dioxide and low ammonium and sulfate, which was reflected by their higher starch content as compared to the cultures grown in media containing high initial concentrations of ammonium and sulfate (Figure 4 in Paper I).

The maximum biomass yield measured in the first series of experiments was 1.57 g L^{-1} in the medium containing 12.5 mM ammonium, 7.5 mM phosphate, 0.55 mM sulfate, and 3.5% CO_2 (Table 1). The maximum predicted biomass yield by the model in Paper II was 1.66 g L^{-1} for a medium containing 20.0 mM ammonium, 7.5 mM phosphate, 0.66 mM sulfate, and 4.5% CO_2 (Figure 2b in Paper II; Table 2). However, the model validation experiment yielded a biomass of 1.26 g L^{-1} at 96 h of growth (Figure 5b in Paper II; Table 1), which was 24% lower than predicted. The predicted optimum sulfate concentration for the maximum biomass yield was the same as for the maximum predicted productivity. The predicted optimum ammonium concentration, however, corresponded to the opposite extreme (20.0 mM) as for the maximum predicted productivity (5.0 mM).

The medium composition used in the second series of experiments was similar to the one predicted for the maximum biomass in Paper II. It contained 17.5 mM ammonium, 7.5 mM phosphate, and 0.66 mM sulfate. The highest biomass measured in the second series of experiments was 1.35 g L^{-1} for the same growth conditions that yielded the maximum productivity, namely 400 µmol m^{-2} s^{-1}, 35 °C, and 9% CO_2 (Figure 2b in Paper III; Table 3). Surprisingly, it was lower than the maximum biomass measured in the first series of experiments (1.57 g L^{-1}), even if the growth medium was rich in nutrients, and higher light intensity, temperature, and CO_2 concentrations were used in the treatment that gave the highest biomass yield in the second series of experiments. We had expected that by increasing light intensity and temperature the biomass yield would be higher than in the first series of experiments. Knutsen et al. (2012) offer a somewhat speculative, but interesting, explanation for different growth yields

obtained in cultures grown during different seasons of the year. However, the lower biomass yield in the second series of experiments as compared to the first one could also be due to the negative effect of the CO_2 concentration, which was also observed by Mortensen and Gislerød (2014; 2015) in their studies with the same strain of *C. reinhardtii*. The results presented in Paper II (Figure 1c in Paper II) showed that 3.5% CO_2 yielded the highest biomass yield. The optimization procedure in Paper II indicated 4.5% of CO_2 as the optimal concentration for obtaining maximum biomass yield (Table 1). However, the model validation experiment (Figure 5b in Paper II) carried out with 4.5% CO_2 yielded a lower biomass (1.26 g L^{-1}) yield than the one measured at 3.5% CO_2 (1.57 g L^{-1}). The increased CO_2 concentration of 9% in the second series of experiments did not have a positive effect on the biomass yield, and a CO_2 concentration of about 3.5% could actually be the optimum one for obtaining high biomass yields with this microalga. Due to the fact that the two treatments with 400 µmol m^{-2} s^{-1} and 3% CO_2 showed a sharp decrease in pH and died after 72 h (at 35 °C) and 96 h (at 25 °C), it is difficult to evaluate the effects of the environmental factors studied in the second series of experiments on the biomass yield. In the other cultures, we observed an increase in biomass up to 96 h of growth, after which the cultures entered into a stationary growth phase. Light was limiting for growth at the level of 100 µmol m^{-2} s^{-1}, as the cultures grown at 400 µmol m^{-2} s^{-1} had higher productivities and higher biomass yields. A positive correlation was found between the biomass yield and the productivity of the cultures in both series of experiments (Tables 2 and 4). Ogbonna et al. (1995b) also found a good correlation between the linear growth rates and the final cell concentrations for both *Chlorella* and *Spirulina* cells.

The results obtained in the third experiment showed that the biomass yields of the cultures in the first and second series of experiment were limited by the concentrations of calcium and magnesium. Indeed, when the concentrations of calcium and magnesium were increased ten times (0.68 mM calcium and 0.81 mM magnesium) the biomass of the cultures attained 4.83 g L^{-1}, twice as much as in the treatment with calcium and magnesium concentrations used in first two series of experiments (0.068 mM calcium and 0.081 mM magnesium) (Figure 2b in Paper IV). The biomass concentration measured after 96 h of growth in the treatment with the lowest concentrations of calcium was 1.44 g L^{-1}, and was not significantly ($p > 0.1$) different from the

biomass measured at 96 h of growth in the cultures with similar concentrations of ammonium, sulfate, and carbon dioxide in the first series of experiments.

The cultures grown at the highest concentrations of calcium and magnesium entered into stationary growth phase after six days of growth, as did the other cultures in the third experiment (Figure 2b in Paper IV). The growth of these cultures might have been limited by nitrogen, and probably also by sulfur. As we calculated in Paper IV, the supplied amount of ammonium (17.5 mM) in the growth medium was sufficient to yield 2.6 g L^{-1} biomass containing 9.4% of DW nitrogen, which was the measured nitrogen content of the cells grown in media with non-limiting concentrations of ammonium at 5% CO_2 (as in Paper IV) in the first series of experiments (Figure 10 in Paper I). This would mean that the microalgae grown at the highest concentrations of calcium and magnesium could have started to experience nitrogen limitation at some point between days 3 and 4, as they reached a biomass higher than 2.6 g L^{-1} (Figure 2b in Paper IV). This was reflected by the diminished productivity between days 3 and 4 as compared to the productivity measured between days 2 and 3 in these cultures, with even further decrease in productivity between days 4 and 6 (Figure 2b in Paper IV). As explained in the introduction to Paper II, when the supply rate of one of the nutrient elements is lower than required for the maximum biosynthetic capacity of the cell, that element becomes limiting for growth (Bailey and Ollis 1986; Falkowski and Raven 2007). Growth of nutrient-limited populations slows down before total exhaustion of that nutrient (Bailey and Ollis 1986). The cultures experience, then a transitory growth phase between the linear and the stationary growth phases, where the growth slows down (as in the cultures grown at the highest concentrations of calcium and magnesium in the third experiment shown in Figure 2b in Paper IV). During this transitory growth phase, the cell content of the limiting nutrient probably decreases until it reaches its minimum cell quota (Droop 1973) and the cell division stops. The culture enters then into the stationary growth phase (as did the cultures grown at the highest concentrations of calcium and magnesium in the third experiment shown in Figure 2b in Paper IV), where the cell quota is maintained constant, at its minimum. The parameter that determined nutrient sufficiency or nutrient limitation status of a cell is the nutrient cell quota (Droop 1973), not the amount of nutrient in the growth medium, although a relationship between the two is expected.

8. 4. Starch content

During the nutrient-limited growth, several biochemical processes are affected. Photosynthesis and respiratory processes, as well as protein and pigments synthesis, are reduced (for references, check Paper II). Under these conditions, the cells experience an energy imbalance: the energy required for growth becomes lower than the energy supplied through photosynthesis (Hu 2004; Klok et al. 2013). Accumulation of highly reduced compounds, such as lipids and carbohydrates, that do not contain the limiting nutrient serves then as a sink for the assimilated carbon and as an alternative sink for the excess energy (for references, check Paper II). Klok et al. (2013) postulated that, if an energy imbalance is created by reducing the nutrient supply to the cells, cells division continues at the same time as lipids or carbohydrates accumulate, as they do in the classic nutrient depletion experiments (for references, check Paper II). This was observed in the cultures grown at high calcium and magnesium concentration in the third experiment, which accumulated starch (from 2 to 25% of DW) (Figure 3a in Paper IV) as their growth slowed down between days 3 and 4 as the ammonium in the medium became growth-limiting. The protein content of these cultures decreased accordingly from 30 to 14% of DW (Figure 3b in Paper IV), as did their chlorophyll content (Figure 4b in Paper IV). However, the starch content in these cultures decreased in the stationary growth phase after reaching its maximum at day 6, probably due to the accumulation of lipids, which were not measured, but their amount was that high as to be visible at the pipette tip when analyzing these samples for their starch content. Gardner et al. (2013) also observed a rapid accumulation of starch in their nitrogen-starved *C. reinhardtii* cultures, reaching its maximum and then gradually decreasing as TAG accumulated. Algal carbon reallocation from starch into lipid is a consequence of a switch in metabolic pathways to form lipid in preference to starch during nutrient limitation.

Similar responses to nutrient concentrations in the growth medium were observed in the first series of experiments. Indeed, cells grown in media with low content of ammonium and sulfate, especially at high CO_2 concentrations, accumulated quite high amounts of starch, up to 42% of DW in the medium containing 7.5 mM ammonium, 20 mM phosphate, 0.2 mM sulfate, with 5.0% CO_2 (Table 2 and Figure 4 in Paper I; Figure 3a in Paper II; Table 1). Negative interactions effects on the starch content of the cultures were observed between the

concentrations of ammonium and sulfate, ammonium and carbon dioxide, and between sulfate and carbon dioxide (Table 2 in Paper I; Table 2 in Paper II).

The maximum predicted starch content by the model in Paper II was 55% of DW for a medium containing 5 mM ammonium, 22.5 mM phosphate, 0.1 mM sulfate, with 6% CO_2 (Figure 4a in Paper II; Table 1), which is quite high value for microalgae (Dragone et al. 2011). The results of the model validation experiment confirmed the predicted response, with 52 ± 3% of DW starch for the predicted medium composition (Figure 5c in Paper II), which was not significantly ($p > 0.1$) different from the predicted value. The concentrations of ammonium and sulfate predicted to be optimal for maximum starch content corresponded to the lowest concentrations tested in the first series of experiments, while the concentrations of phosphate and sulfate were the highest tested. The fact that the predicted optimum concentrations are boundary values of the experimental design used in the first series of experiments leaves us with a question of how much starch could be accumulated if the ranges of the studied nutrient concentrations were expanded.

The goal of this study was to obtain high biomass cultures that contained high starch content. However, the predicted optimum media composition for the maximum biomass yield and for the maximum starch content were quite different (Table 1), with the medium for maximum predicted biomass yield containing high concentrations of ammonium and sulfate, conditions that are adverse for starch accumulation. Indeed, the starch content and the biomass yield were negatively correlated in the first series of experiment (Table 2b). We used the models obtained in Paper II for maximum biomass yield and for maximum starch content in an optimization procedure to obtain a medium that would give simultaneous high biomass yield and high starch content. This optimization procedure yielded the following medium composition: 5.0 mM ammonium, 7.5 mM phosphate, 0.65 mM sulfate, with 5.2% CO_2 and predicted a biomass yield of 1.14 g L^{-1} and a starch content of 44% of DW. The model validation experiment was carried out using this predicted medium composition and yielded a biomass yield of 1.31 ± 0.04 g L^{-1} (means ± SD, n = 3) and a starch content of 31.0 ± 1.9% of DW (means ± SD, n = 3). The measured biomass yield was significantly ($p < 0.001$) higher than the predicted biomass yield, but the measured starch content was significantly ($p < 0.001$) lower than the predicted. This predicted

medium composition was very similar to the medium predicted for the maximum productivity (Table 1). The model validation experiment yielded a productivity of 0.87 ± 0.01 g L^{-1} d^{-1}, which was the maximum productivity predicted by the model in Paper II (Table 1) and also measured in the model validation experiment for productivity (Figure 5a in Paper II).

The starch content in the second series of experiments did not exceed 11% of DW (Figure 3a in Paper III; Table 3), probably due to the nutrient sufficiency of the growth medium. The growth medium in the second series of experiments contained 17.5 mM ammonium and 0.65 mM sulfate (Table 1 in Paper III), which were the nutrients that had the largest effect on the starch content in the first series of experiments. The cultures in the first series of experiments grown in media containing similar concentrations of ammonium and sulfate had about 8% of DW of starch (Figure 4 in Paper I), which is on the same level as the starch content in the cultures in the second series of experiment. The highest starch content of 10.8% of DW was measured in the treatment with 400 µmol m^{-2} s^{-1}, 25 °C, and 3% CO_2 (Figure 3a in Paper III; Table 3), just before these cultures died.

8. 5. Protein content

The maximum protein content measured in the first series of experiment was quite high: 52% of DW in the medium containing 17.5 mM ammonium, 20.0 mM phosphate, 0.9 mM sulfate with 5.0% CO_2 (Figure 5b in Paper I; Table 1). The maximum predicted protein content by the model in Paper II was 65% of DW for a medium containing 20 mM ammonium, 22.5 mM phosphate, 1.0 mM sulfate, with 4.4% CO_2 (Figure 4b in Paper II; Table 1), which was confirmed by the model validation experiment (Figure 5d in Paper II, Table 1). The optimum predicted medium composition corresponded with the boundary values of the experimental design, which like in the case of predicted maximum starch content, leaves us with a question of how the protein content would have varied if the limits of the ranges of the concentrations of testes nutrients were expanded.

The protein content in the cultures in the first series of experiments was positively influenced by the concentrations of ammonium and sulfate (Figure 3b in Paper II; Figure 5 and Table 2 in Paper I). Positive interaction effects between the concentrations of carbon dioxide and ammonium, and of ammonium and sulfate were detected (Table 2 in Paper I; Table 2 in Paper II), opposite to the effects of these nutrients on the starch content of the cultures in the first series of experiments. Indeed, starch and protein contents of the cultures were negatively correlated in the first series of experiments, as response to varying nutrient concentrations in the growth medium (Table 2, Figure 24). A linear relationship between the starch and the protein contents was found (Figure 24). This was expected, as protein and starch make up for the major constituents of the cell. The nitrogen status of the cell acts as a switch in metabolic pathways from protein synthesis under nitrogen sufficiency to starch synthesis as nitrogen becomes limiting.

The protein content in the second series of experiments increased with increasing light intensity (Figure 3b and Table 3 in Paper III), while temperature and CO_2 concentration had no significant effect on the protein content of the cultures (Table 3 in Paper III). The maximum protein content was 43% of DW in the treatment with 400 µmol m^{-2} s^{-1}, 25 °C, and 9% CO_2, which was expected as the medium used in the second series of experiments contained high amounts of ammonium and sulfate (Table 1 in Paper III).

The cultures grown in the lowest concentrations of calcium and magnesium in the third experiments also contained quite high amount of protein (up to 42% of DW) up to day 5, as these cultures were not limited by ammonium or sulfate. On the contrary, and as mentioned previously, the cultures grown at higher concentrations of calcium and magnesium had a drop in their protein content from 30 – 37% of DW to 14 – 20% of DW between days 3 and 4, opposite to the simultaneous starch increase.

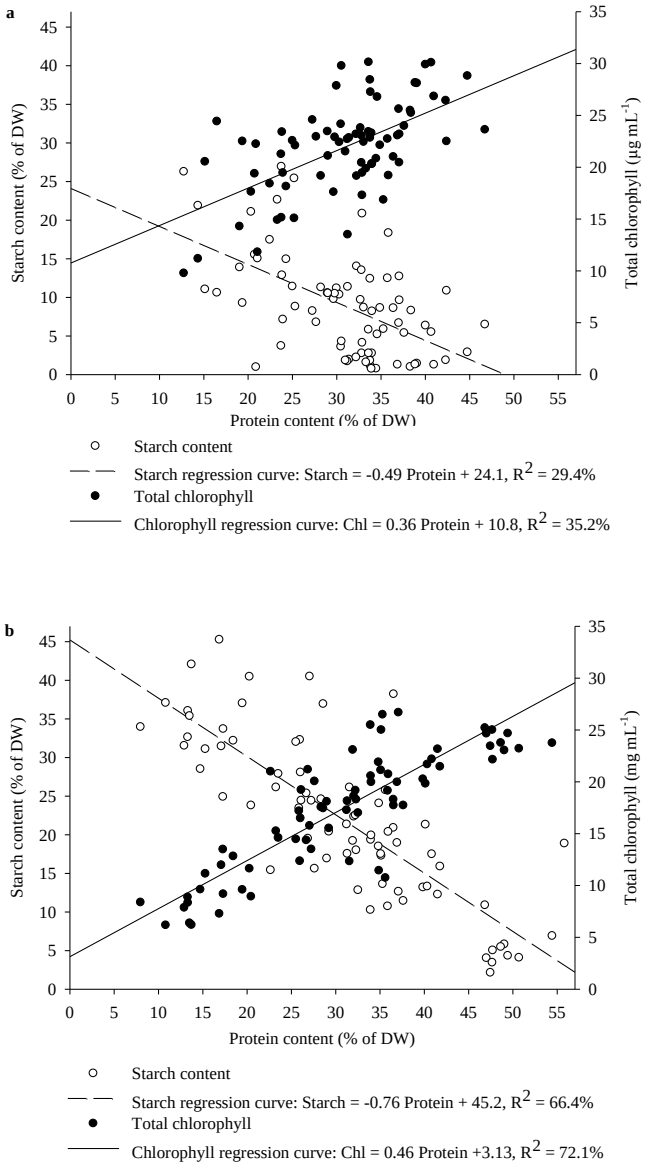

Figure 24. Linear relationships between the protein and starch contents and between the protein and chlorophyll contents in *C. reinhardtii* cultures at 67 h of growth (a), and 96 h of growth (b).

8. 6. Chlorophyll content

The chlorophyll content (as % of DW) increased with increasing concentrations of ammonium in the first series of experiments (Figure 7 and Table 2 in Paper I; Figure 3 in Paper II). This result was expected, as nitrogen is part of the structure of the chlorophyll molecule. Positive interaction effects were also detected between ammonium and carbon dioxide concentrations, and between ammonium and sulfate concentrations (Table 2 in Paper I; Table 2 in Paper II). The cultures grown in higher nutrient concentrations gave rise to higher biomass content (Table 2 in Paper I), and therefore higher chlorophyll content, due to photoacclimation to increased mutual shading. The chlorophyll content was influenced by the different nutrient concentrations as did the protein content of the cultures (Table 2 in Paper I). Indeed, a positive correlation was found between the protein content and the chlorophyll concentration (Table 2) and a linear relationship was established between these two parameters (Figure 24).

The model of Paper II predicted a chlorophyll concentration of 20 μg mL^{-1}, which was determined to be the optimum one for H_2 production by Hahn et al. (2004), for a medium containing 5.0 mM ammonium, 22.5 mM phosphate, 0.6 mM sulfate, with 4.0% CO_2 (Table 1). However, the results of the model validation experiment (Figure 5e in Paper II) yielded a chlorophyll content of only 5.5 μg mL^{-1} for that medium composition. Probably, the ammonium concentration in this medium was not high enough to yield 20 μg chl mL^{-1}, as its highest content was 13 μg mL^{-1} at 48 h after the start of the experiment and decreased thereafter.

Both the model for the chlorophyll content and the one for the biomass yield failed to predict correctly the optimum medium compositions for the desired chlorophyll content and for maximum biomass yield (Table 1). Both models had many significant terms in the equation (Table 2 in Paper II), which probably was excessive, making them little precise. When modeling a response, one should seek to establish a model with as little number of terms as possible. Both these model also contained significant terms of phosphate concentration (Table 2 in Paper II). The phosphate concentrations used in the first series of experiments were much higher than the phosphate requirement as a nutrient for the growth of *C. reinhardtii* cultures, as it was used as a pH buffer in the medium. Probably, if we would have eliminated those terms of phosphate

concentrations from these two models, their validity would have been improved and they would make better prediction of the optimal conditions for the desired chlorophyll content and maximum biomass yield of the cultures.

The chlorophyll concentration in the cultures (μg chl mL^{-1}) increased with the increasing concentrations of calcium and magnesium (Figure 4a in Paper IV), reflecting higher biomass concentration in the cultures grown at higher concentrations of calcium and magnesium. In their study on *Spirulina platensis*, Celekli et al. (2009) also observed a direct relationship between the biomass level and the amount of chlorophyll *a* throughout the culture period. However, when expressed as chlorophyll content per unit dry weight, little variation was observed between the treatments with different concentrations of calcium and magnesium (Figure 4b in Paper IV).

In the second series of experiment, the chlorophyll content of the cultures significantly decreased with increasing light intensity (Figure 3c and Table 3 in Paper III), showing for the photoacclimation of the microalgae to higher light intensity (Falkowski and Raven 2007). Temperature also had a positive effect on the chlorophyll content of the cultures, although less than the light intensity, as did the interactions of temperature with light intensity and CO_2 concentration (Table 3 in Paper III). Shuter (1979) and Thomspon et al. (1992) concluded in their studies that the increase in chlorophyll content with increasing temperature was a common response in microalgae.

The chlorophyll concentration in the first series of experiments was positively correlated to the biomass yield and to the protein content of the cultures (Table 2), as all three parameters benefitted from nutrient-rich (especially in ammonium) growth media. Media with low ammonium concentrations yielded higher pH, productivity, and starch contents, which were also positively correlated between each other. In the second series of experiments other kinds of correlations between the different parameter were found. Productivity, starch and protein contents were all positively correlated to the biomass yield, as all these parameters increased with increasing light intensity. Chlorophyll, on the other hand, correlated negatively with the biomass yield, protein and starch contents, as it decreased with increasing light intensity.

8.7. Tables

Table 1. Summary of the results of the first series of experiments (Paper II). Concentrations of the four main nutrients: ammonium, phosphate, sulfate, and carbon dioxide in the Tris-Acetate-Phosphate medium (Gorman and Levine 1965), the High-Salt Sueoka medium (Sueoka 1960), and the experimental media that yielded the maximum responses of productivity between 43 and 67 h of growth, biomass (dry weight) yield at 96 h of growth, as well as the maximum contents of starch, protein, and chlorophyll at 96 h of growth. The optimized media compositions are also given for the corresponding predicted responses and the results of the model validation experiments are indicated. NS, non significant.

Medium	TAP	HS	Max. productivity	Max. predicted productivity	Max. biomass	Max. predicted biomass	Max. starch	Max. predicted starch	Max. protein	Max. predicted protein	Max. chlorophyll	Predicted 20 µg chl mL^{-1}
NH_4 (mM)	7.48	9.35	12.5	5.0	12.5	20.0	7.5	5.0	17.5	20.0	17.5	5.0
PO_4 (mM)	1.0	13.6	15.0	NS	7.5	7.5	20.0	22.5	20.0	22.5	10.0	22.5
SO_4 (mM)	0.51	0.182	0.55	0.65	0.55	0.66	0.2	0.1	0.9	1.0	0.9	0.6
CO_2 (% v/v)			6.0	6.0	3.5	4.5	5.0	6.0	5.0	4.4	5.0	4.0
Predicted response				0.87 g L^{-1} d^{-1}		1.66 g L^{-1}		55% of DW	52% of DW	65% of DW	37 µg mL^{-1}	20 µg mL^{-1}
Measured response			0.87 g L^{-1} d^{-1}	0.86 ± 0.06 g L^{-1} d^{-1}	1.57 g L^{-1}	1.26 ± 0.01 g L^{-1}	42% of DW	52 ± 3 % of DW		64 ± 2 % of DW		5.5 ±0.5 µg mL^{-1}

Table 2. Significant ($p < 0.05$) Pearson correlation coefficients between the different parameters measured in the first series of experiments (of the complete data set with 25 treatments) at 67 h of growth (a) and at 96 h of growth (b). Significance levels: ^, $p < 0.05$; *, $p < 0.01$; **, $p < 0.001$.

a

	pH	Productivity ($g\ L^{-1}\ d^{-1}$)	DW ($g\ L^{-1}$)	Starch (% of DW)	Protein (% of DW)	Total chlorophyll ($\mu g\ mL^{-1}$)
pH		0.499**	-0.681**			
Productivity ($g\ L^{-1}\ d^{-1}$)	0.499**		0.966**			-0.301^
DW ($g\ L^{-1}$)	-0.681**	0.966**				-0.346*
Starch (% of DW)					-0.542**	-0.664**
Protein (% of DW)				-0.542**		0.594**
Total chlorophyll ($\mu g\ mL^{-1}$)		-0.301^	-0.346*	-0.664**	0.594**	

b

	pH	Productivity ($g\ L^{-1}\ d^{-1}$)	DW ($g\ L^{-1}$)	Starch (% of DW)	Protein (% of DW)	Total chlorophyll ($\mu g\ mL^{-1}$)
pH			-0.556**	0.612**	-0.556**	-0.575**
Productivity ($g\ L^{-1}\ d^{-1}$)			0.349*			
DW ($g\ L^{-1}$)	-0.556**	0.349*		-0.499**	0.513**	0.672**
Starch (% of DW)	0.612**		-0.499**		-0.899**	-0.852**
Protein (% of DW)	-0.556**		0.513**	-0.899**		0.813**
Total chlorophyll ($\mu g\ mL^{-1}$)	-0.575**		0.672**	-0.852**	0.813**	

Table 3. Summary of the results of the second series of experiments (Paper III). Levels of light intensity, temperature, and CO_2 concentration that yielded the maximum responses of productivity, biomass yield, as well as the maximum contents of starch, protein, and chlorophyll.

	Max. productivity	Max. biomass	Max. starch	Max. protein	Max. chlorophyll
Light intensity (µmol m^{-2} s^{-1})	400	400	400	400	100
Temperature (°C)	35	35	25	25	25
CO_2 concentration (% v/v)	9	9	3	9	9
Response	1.12 g L^{-1} d^{-1}	1.335 g L^{-1}	10.8% of DW	43% of DW	3.9% of DW

Table 4. Significant ($p < 0.05$) Pearson correlation coefficients between the different parameters measured in the second series of experiments at the first sampling time (a) and the second sampling time (b). Significance levels: ^, $p < 0.05$; *, $p < 0.01$; **, $p < 0.001$

a

	pH	Productivity (g L^{-1} d^{-1})	DW (g L^{-1})	Starch (% of DW)	Protein (% of DW)	Total chlorophyll (% of DW)
pH			-0.550*			
Productivity (g L^{-1} d^{-1})			0.499^	0.641*	0.643*	-0.793**
DW (g L^{-1})	-0.793**	0.499^		0.556*		
Starch (% of DW)		0.641*	0.556*			
Protein (% of DW)		0.643*				-0.564*
Total chlorophyll (% of DW)		-0.793**			-0.564*	

b

	pH	Productivity (g L^{-1} d^{-1})	DW (g L^{-1})	Starch (% of DW)	Protein (% of DW)	Total chlorophyll (% of DW)
pH						0.693**
Productivity (g L^{-1} d^{-1})			0.532^		0.743**	-0.692**
DW (g L^{-1})	-0.616*	0.532^			0.493^	
Starch (% of DW)						
Protein (% of DW)		0.743**	0.493^			-0.769**
Total chlorophyll (% of DW)	0.693**	-0.692**			-0.769**	

Table 5. Summary of the results of the third series of experiments (Paper IV). Maximum responses of productivity, biomass yield, and the maximum contents of starch, protein, and chlorophyll measured in treatments with different concentrations of calcium and magnesium.

Calcium - Magnesium concentrations (mM)	Maximum productivity ($g\ L^{-1}\ d^{-1}$)	Maximum biomass ($g\ L^{-1}$)	Maximum starch content (% of DW)	Maximum protein content (% of DW)	Maximum chlorophyll concentration ($\mu g\ mL^{-1}$)
0.068 Ca - 0.081 Mg	1.16	2.28	33.2	42.0	39.3
0.17 Ca - 0.203 Mg	1.34	2.59	23.5	38.2	55.4
0.34 Ca - 0.405 Mg	1.73	3.53	26.7	37.0	62.5
0.68 Ca - 0.81 Mg	1.97	4.83	32.3	40.5	78.6

9. Concluding remarks and future perspectives

This work presents the first step in the direction of obtaining an optimized medium for the growth of *C. reinhardtii*. We described how the different initial concentrations of nutrients in batch cultures, as well as light intensity, in the range of 100 – 400 μmol m^{-2} s^{-1}, and temperature, in the range of 25 – 35 °C, influence the biomass production and its composition. The statistical approach of factorial experimental designs was an efficient method to detect interaction between the different culture variables. This information is valuable when this microalga is to be cultivated for different purposes. It is possible to use the results of this study in the design of an appropriate growth medium and when defining culture conditions for obtaining fast growing and high biomass cultures or for producing starch, protein, or chlorophyll.

For obtaining *C. reinhardtii* cultures with high biomass, high protein, and high chlorophyll contents, we recommend using media with increased concentrations of ammonium, sulfate, calcium, and magnesium. In such cultures, use of a good pH buffer is encouraged. Media with low ammonium content are recommended for cultures with high productivity and starch content. By increasing light intensity (up to 400 μmol m^{-2} s^{-1}), temperature (up to 35 °C), CO_2 (up to 9% v/v), and calcium (0.68 mM or higher) and magnesium (0.81 mM or higher) concentrations, the productivity and starch content can be further increased. Usually, two-stage cultivation strategy is used to obtain cultures with high starch content, with biomass growth under nutrient-replete conditions and starch accumulation under nutrient deprivation. However, this study shows that high growth and accumulation of high levels of starch is possible in a single batch culture, if appropriate growth conditions are defined for obtaining high productivity in the linear growth phase in a medium that becomes nutrient-limited in the stationary growth phase for starch accumulation.

These conclusions were made for a certain combination of culture condition, such as type and geometry of PBR, culture regime, concentrations of nutrients not considered in this study, and light regime for the strain used in this study. The extrapolation of these recommendations to other culture conditions and systems is therefore difficult. The optimum culture conditions are particular for each species and production system. However, the general effects and the type of

interactions between the different culture variables detected in this study can be used as a start point when designing a production system.

The quality of the results obtained in this thesis could have been improved by using a more advanced PBR, with automatically controlled pH, temperature, light intensity, CO_2 concentration and the flow rate of the bubbled gas. However, by not controlling the pH, we could obtain information on how it is affected by the culture conditions (the pH being a measured parameter rather than a controlled study variable) and the growth kinetics of the microalgae. This information could be valuable for establishing a pH control strategy.

More frequent sampling could also improve the results of the thesis, giving the possibility for establishing models on the effects of culture conditions on the growth kinetics and detailed variation of the biochemical composition throughout the different growth phases of the cultures. More frequent analyses of the nutrient composition of the cells and of the growth medium could permit to determine the growth-limiting cell quotas and concentrations of the different nutrients in the medium.

It could be useful to extend the ranges of concentrations of the studied nutrients and increase the concentrations of calcium and magnesium, and probably also of iron and other elements, in the first two series of experiment to obtain a better overview of their effects and improved models. Phosphate concentration in the medium could be reduced, by using higher concentrations of bicarbonate in the medium or by establishing an automatic control of pH. In this case, the real effects of the phosphate concentration on the growth and biochemical composition of *C. reinhardtii* could be evaluated. It also would be interesting to repeat the third experiment with higher concentrations of calcium, magnesium, ammonium, and sulfate to determine if even higher biomass yield may be obtained with this microalga.

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Paper I

Effects of ammonium, phosphate, sulfate, and carbon dioxide concentrations on starch, protein, and chlorophyll contents during photoautotrophic growth of *Chlamydomonas reinhardtii*

Daria Markina*, Leiv M. Mortensen, Hans Ragnar Gislerød

Department of Plant Sciences, Norwegian University of Life Sciences, P.O. Box 5003, 1432 Ås, Norway.

*Corresponding author, e-mail address: daria.markina@nmbu.no, dmarkina@gmail.com, tel.: (+47) 64 96 61 38, fax: (+47) 64 96 56 01

Running title: *Chlamydomonas*: nutrients, growth and composition

Submitted to the Journal of Phycology

Abstract

The green microalga *Chlamydomonas reinhardtii* is able to produce hydrogen under sulfur-deficient anaerobic conditions. The efficiency of hydrogen production is known to depend on starch, protein, and chlorophyll contents of the culture. Microalgae are normally grown mixotrophically for the purpose of hydrogen production, while in this work they were grown under photoautotrophic conditions. We studied the effects of two ammonium (7.5 and 17.5 mM) and two sulfate (0.2 and 0.9 mM) concentrations at 2.0 and 5.0% CO_2 on the dry weight (DW in g L^{-1}) and the biochemical composition of *C. reinhardtii*. All treatments included two high-level phosphate concentrations (10 and 20 mM) to avoid acidification of the cultures. The biochemical composition of the cultures was much more affected by the different nutrient treatments than was their growth. The cells accumulated quite a high amount of starch (up to 40% of DW) in the low ammonium treatments, even though the cultures did not become depleted of nitrogen (3 – 5 mM N remaining in the medium) after 96 h of growth. Low sulfate treatments also resulted in significantly higher starch content than high sulfate treatments, especially in the high ammonium treatments. In the high ammonium and sulfate treatments, the protein content of the cells was 35 – 50% of DW. High ammonium concentration also had a significant, positive effect on the chlorophyll content of the cultures. The information obtained in this study makes it possible to produce microalgae with a well-defined biochemical composition of relevance for studying the process of hydrogen production.

Key-words

Chlamydomonas reinhardtii, chlorophyll, interaction effects, nutrients, photoautotrophic growth, protein, starch.

Introduction

Concern about global warming and environmental pollution due to greenhouse gases, and about energy supply and demand with projected shortage of fossil fuels in the 21st century, has led to considerable interest in developing renewable and environmentally friendly energy sources. Hydrogen (H_2) gas is an attractive alternative clean fuel and energy carrier, since its combustion only generates water vapor. The green microalga *Chlamydomonas reinhardtii* has been shown to produce the highest levels of H_2 reported to date, and it has been used extensively as a model organism for studying microalgal H_2 metabolism (Chochois et al. 2009; Ghysels and Franck 2010; Hemschemeier et al. 2008; Laurinavichene et al. 2004; Tsygankov et al. 2006; Zhang et al. 2002). Species such as *Chlorella sorokiniana* and *Chlorella salina* are also known to be capable of producing H_2 (Chader et al. 2009; Roy et al. 2014). Until now, the majority of laboratories studying H_2 production have supplied organic carbon substrate during the growth of *C. reinhardtii*. However, this has been shown not to be necessary (Fouchard et al. 2005; Kosourov et al. 2007; Tolstygina et al. 2009; Tsygankov et al. 2006).

Sustained H_2 production by *C. reinhardtii* is accomplished by means of a two-stage cycle: an aerobic biomass growth stage, followed by an anaerobic H_2 production stage under sulfur deprivation (Melis et al. 2000). Photofermentation of both protein and starch is recognized as contributing electrons for H_2 production (Chochois et al. 2009; Fouchard et al. 2005; Melis et al. 2000; Zhang et al. 2002). In addition, Hahn et al. (2004) found that the chlorophyll concentration in the culture played an important role, affecting the yield of H_2 production in *C. reinhardtii*. The optimal chlorophyll concentration has been suggested to be 20 – 25 μg mL^{-1} culture (Giannelli et al. 2009; Hahn et al. 2004). Since good light penetration into the culture is important for H_2 production, cultures with small antenna size would be preferred (Beckmann et al. 2009).

It is well known that the availability of nutrients in the growth medium greatly influences the biochemical composition of microalgal cells (Grossman 2000; Richmond 2004). In order to produce biomass with high starch content, it is necessary to suppress cell division through an environmental condition or by starvation of mineral nutrients (Markou et al. 2012). The freshwater microalga *Chlorella* was found to accumulate high amounts of starch (from 8.5% up to 40% of dry weight) as the photon flux density (PFD) increased from a mean of 215 to 330 μmol m^{-2} s^{-1} prior to cell division (Branyikova et al. 2010). In the marine microalga *Tetraselmis subcordiformis*, the starch content could reach 62% of dry

weight through sulfur depletion and 54% through nitrogen depletion (Yao et al. 2012). In the same species, nitrogen starvation increased the starch content more than four times and the hydrogen yield 5.5 times (Ji et al. 2011). Increasing the pH has also been found to increase the starch content of *C. reinhardtii* cultures (Tolstygina et al. 2009).

This study aimed to investigate how the medium composition in the biomass growth stage affects the growth and the biochemical composition of *C. reinhardtii* cultures. Eight different nutrient media were used, with two different concentrations of ammonium, sulfate, and phosphate under two concentrations of CO_2 in the bubbled gas. K_2HPO_4/KH_2PO_4 was used as a buffer system in order to reduce the acidification of the microalgae cultures as the ammonium in the medium is consumed. The two phosphate concentrations (10 and 20 mM) were therefore far above the requirement for this nutrient. The results were analyzed in order to characterize the microalgae with respect to the desirable composition of starch, protein, and chlorophyll before using them for H_2 production.

Materials and methods

Organism and culture conditions

The *Chlamydomonas reinhardtii* P. A. Dangeard 137c strain was obtained from the NIVA culture collection, Norway (CHL153). The microalgae were stored in sterile conditions in a 1.5% agar Tris-Acetate-Phosphate (TAP) (Gorman and Levine 1965) solid growth medium (Andersen 2005) on Petri dishes at room temperature and low light intensity. The microalgae were pre-cultured in liquid High-Salt Sueoka (HS) medium (Sueoka 1960) at 25 °C under continuous illumination from cool white fluorescent tubes (Philips Master TLD 36W/840 Reflex, Eindhoven, Netherlands) at a PFD of 200 µmol m^{-2} s^{-1}.

Experimental setup

Sterile batch cultures of *C. reinhardtii* cells were carried out in Pyrex glass tubular (coned at the bottom) photobioreactors (PBRs) with an inner diameter of 3.5 cm and a total volume capacity of 380 mL. The temperature was maintained at 25.0 ± 0.5 °C using water baths in aquaria. Cool white fluorescent tubes were used to provide continuous illumination at a PFD of 200 µmol m^{-2} s^{-1}. The light intensity was measured using a LI-COR model LI-190SA instrument (LI-COR, Lincoln, NE, USA) with a quantum sensor (400 – 700 nm) inside an empty PBR immersed in the water-bath aquarium, and the average PFD over the height of the PBR was estimated. Filtered (Acro 37 TF Vent Device, 0.2 µm PTFE membrane filters, PALL Life Sciences, Port Washington, NY, USA) air containing 2.0 ± 0.1% or 5.0 ± 0.1% CO_2, according to the experimental design (Table 1), was injected at the bottom of the PBRs (the bubble size was approximately 3 mm). The experimental medium was based on the HS medium (9.35 mM NH_4, 13.6 mM PO_4, and 0.182 mM SO_4) (Harris 2009), with varying concentrations of NH_4Cl, KH_2PO_4/K_2HPO_4 and K_2SO_4. Two CO_2 concentrations in the bubbled gas (2.0 and 5.0%), two concentrations of NH_4 (7.5 and 17.5 mM), two concentrations of PO_4 (10.0 and 20.0 mM), and two concentrations of SO_4 (0.2 and 0.9 mM) were combined in the experiments in a 2^4 full factorial design, as shown in Table 1, a total of sixteen treatments. Each treatment was carried out in three parallels. The treatments were divided into two blocks and carried out successively, at two different CO_2 concentrations. The initial pH of the media was adjusted to 7.50 with 1 M NaOH prior to sterilization by autoclaving. $NaHCO_3$ was used in the medium as a pH buffer, at a concentration of 2 mM per percent CO_2 in the bubbled gas. Microalgae pre-grown at the same CO_2 concentration as used in the experiments were inoculated into the experimental medium (300 mL) in PBRs to give a

concentration of 10^4 cells mL^{-1} at the start of the experiments. The cells were counted under a microscope, using a Fuchs-Rosenthal counting chamber.

Microalgae samples for pH and dry weight (DW) determination were collected at three time points, 43 h, 67 h, and 96 h after the start of the experiments. In addition, samples collected at 67 h and 96 h were analyzed for starch, protein, and chlorophyll contents.

Determination of dissolved CO_2 concentration

The concentration of dissolved CO_2 in the growth medium is important for microalgal growth, and not the concentration in the gas bubbled into the culture, although a close relationship is expected. A test was performed in order to document this relationship, where different concentrations (0.04 – 9.0%) of pure CO_2 mixed with air were bubbled through the PBRs filled with 300 mL HS medium, at 25.0 °C. The concentration of the dissolved CO_2 in three parallel PBRs was measured at each CO_2 concentration bubbled into the PBRs after a stabilizing period of three hours, using hand-held titration cells for titrimetric analysis (CHEMetrics Inc., Midland, VA, USA). The results showed a progressive increase in the dissolved CO_2 concentration from 100 to 430 mg L^{-1}, with an increasing CO_2 concentration in the bubbled gas, from 0.04% to 9.0% (Figure 1). In parallel to the increase in dissolved CO_2 concentration, the pH decreased from 7.52 ± 0.01 to 6.59 ± 0.01 (mean ± SD, n = 3). The linear regression curve (order 2) between the CO_2 concentration in the bubbled gas and the concentration of dissolved CO_2 was highly correlated (R^2_{adj} = 0.955) and followed the function $y = -4.2\ x^2 + 73\ x + 108$, where y = dissolved CO_2 concentration in the medium (mg L^{-1}) and x = CO_2 concentration in the bubbled gas (% v/v). By using this function, the concentrations of dissolved CO_2 were calculated to be 237 and 368 mg L^{-1} at 2.0 and 5.0% CO_2, respectively.

Biomass determination

The biomass of the cultures was determined by filtering 0.3 – 2.0 mL of the microalgal culture through pre-washed and dried (at 103.5 °C) GF/F glass microfiber filters (0.7 µm pore size, 25 mm diameter, GE Healthcare, Whatman, UK), using a vacuum pump. The filters containing biomass were then washed with 30 mL de-ionized water to remove adhering salts and dried for four hours at 103.5 °C. They were allowed to cool to room temperature inside vacuum desiccators containing silica gel (Silica gel, Merck KGaA, Darmstadt, Germany) prior to weighing (XP6, Mettler Toledo, Greifensee, Switzerland). The DW of the cultures

was calculated by subtracting the DW of the clean filter from the DW of the filter with biomass. Based on this value, the biomass concentration in the culture was calculated in g L^{-1}.

Chlorophyll assay

Chlorophyll was extracted from the cells with ethanol and assayed using the method described by Harris (1989). The amount of chlorophyll in the samples was normalized to the DW of the culture and calculated as % of DW.

Starch assay

Cell pellets of 2 mL samples left after chlorophyll assay were used for starch assay. A Megazyme total starch (amyloglucosidase/α-amylase method) assay kit (K-TSTA, Megazyme International, Bray, Ireland) procedure was adapted to the small sample volume. 20 µL of 80% ethanol was added to the cell pellet and the pellet was dispersed on a vortex mixer. Cells were disrupted by adding 0.2 mL DMSO (Sigma-Aldrich, St. Louis, MO, USA) and 0.5 mL 0.5 mm glass beads (Cell disruption media, SI-BG05, Scientific Industries Inc., New York, NY, USA) to the samples and shaking them on a mixer mill (MM301, Retsch, Haan, Germany) at 30 Hz frequency for 10 min. Samples were heated on thermo-shakers (TS-100, Biosan, Riga, Latvia) to 100 °C for 5 min. for starch solubilization. 0.3 mL of α-amylase (diluted 1/30 according to the Megazyme starch assay kit specifications) was added to the samples and the tubes were incubated at 100 °C for 12 min. The content of the tubes was mixed vigorously after 0, 4, 8, and 12 min. The tubes were placed at 50 °C, and 0.4 mL of sodium acetate buffer (200 mM, pH 4.5) was added to the samples, followed by 10 µL of amyloglucosidase. The samples were thoroughly mixed and incubated at 50 °C for 30 min. Then, 70 µL of distilled water was added to the samples in order to adjust the total sample volume to 1.0 mL. The samples were mixed thoroughly and centrifuged at 20800 x *g* (Eppendorf centrifuge 5417R, Eppendorf, Hamburg, Germany) for 10 min. at 20 °C. Then, 33.4 µL of the supernatant was added to 1 mL GOPOD reagent and incubated at 50 °C for 20 min. The same procedure was used for two reagent blanks (33.4 µL distilled water) and four glucose controls (33.4 µL D-glucose standard solution 1 mg mL^{-1}). The absorbance (optical density) of each sample and the glucose controls was measured at λ = 510 nm against the reagent blank. The starch content in the cultures was calculated as % of DW using the formula supplied in the Megazyme starch assay kit.

Protein assay

Cell pellets of 1 mL samples left after chlorophyll assay were used for protein assay. The cells were disrupted using 0.5 mL of 0.5 mm glass beads (Cell disruption media, SI-BG05, Scientific Industries Inc., New York, NY, USA) and shaking on a mixer mill (MM301, Retsch, Haan, Germany) for 10 min. at a frequency of 30 Hz. Tris-HCl solubilization buffer (50 mM, pH 8.3) containing 1% Triton X-100 was used to solubilize cell proteins. The Lowry method (Lowry et al. 1951) was applied using a Bio-Rad *DC* protein microplate assay kit II (#500-0112, Bio-Rad, Hercules, CA, USA) to determine the protein concentration in the samples. BSA standard curve (6 point in the 0.0 – 0.75 mg mL^{-1} range) was used to calculate the protein concentration in the samples. The protein concentration was normalized to the DW of the samples and calculated as % of DW.

Element analysis

To determine the total nitrogen, phosphorous, and sulfur content of the microalgae cultures, 15 mL samples were collected at 96 h after the start of the experiments and centrifuged for 10 min. at 3220 x *g*, and the supernatant was discarded. The cell pellet was washed twice with deionized water and frozen at -20 °C until analysis. The Norwegian standard method, NS 4743 (Standard Norge 1993), was used to measure the total nitrogen in the cells. The total phosphorous and sulfur content of the cells was analyzed using an Inductively Coupled Plasma Optical Emission Spectrometer (Optima 5300 DV, Perkin Elmer, Waltham, Massachusetts, USA), after the addition of HNO_3 to 10% v/v and decomposition by ultraclave (ultraClave III, Milestone, Sorisole, Italy) at 250 °C for 1.5 h.

Statistical analyses

The data were analyzed using the analysis of variance (ANOVA) procedure in the MINITAB software (V16, Minitab Inc., State College, PA, USA). The figures were created using SigmaPlot version 9.0 (Systat software Inc., San Jose, CA, USA).

Results

The pH of the cultures decreased progressively with time and the growth of the microalgae (Figure 2). The Pearson correlation coefficients between the pH and DW of the cultures were significant ($p < 0.001$) at 67 h and 96 h after the start of the experiments, they were -0.681 and -0.556, respectively. The increase in ammonium concentration from 7.5 to 17.5 mM significantly ($p < 0.01$) decreased the pH of the cultures, at both 67 h and 96 h after the start of the experiments, by 0.15 and 0.31, respectively. Increased sulfate concentration in the medium also significantly ($p < 0.05$) decreased the pH of the cultures by 0.26, but only at 96 h after the start of the experiments. As expected, the increase in phosphate concentration from 10 to 20 mM significantly ($p < 0.01$) increased the pH of the cultures, by 0.18 and 0.37 after 67 h and 96 h of growth, respectively (Table 2). Significant negative interaction effects on the pH were detected between carbon dioxide and phosphate concentrations ($p < 0.1$), and between ammonium and sulfate concentrations ($p < 0.05$) after 96 h of growth (Table 2).

The DW of the cultures increased throughout the growth period (Figure 3), except in treatments 10 (5.0% CO_2, 7.5 mM NH_4, 10 mM PO_4, and 0.9 mM SO_4) and 11 (5.0% CO_2, 7.5 mM NH_4, 20 mM PO_4, and 0.9 mM SO_4), where the DW didn't experience any change between 67 and 96 h of growth. The maximum DW (1.39 g L^{-1}) after 96 h of growth was registered for treatment 14 with high concentrations of ammonium, sulfate, and carbon dioxide. The increase in concentrations of ammonium and sulfate had a significant ($p < 0.001$) positive effect on the DW (about 0.15 g L^{-1} increase) of the cultures after 96 h of growth (Table 2). On the contrary, the increase in phosphate concentration in the medium had a significant negative effect on the DW of the cultures throughout the experimental period (Table 2). Significant positive interaction effects on the DW were detected between carbon dioxide and ammonium concentrations ($p < 0.001$), between ammonium and sulfate concentrations ($p < 0.01$), and between phosphate and sulfate concentrations ($p < 0.01$) after 96 h of growth (Table 2).

From 67 to 96 h after the start of the experiments, the starch content of the cultures strongly increased in the low ammonium (7.5 mM) treatments at both CO_2 concentrations, and it reached a higher content at 5.0% CO_2 (37% of DW in average) as compared to 2.0% CO_2 (28% of DW in average) (Figure 4). The highest starch content (41.4% of DW) was obtained at 7.5 mM ammonium and 20 mM phosphate concentrations after 96 h of growth. In the high ammonium (17.5 mM) treatments, the starch content did not reach more than 20% of DW after 96 h of growth. The cultures grown at 17.5 mM ammonium and 0.9 mM sulfate did

not accumulate starch at all, the maximum starch content being 12.5 and 5.0% of DW for 2.0 and 5.0% CO_2, respectively. The statistical analysis showed that the concentration of ammonium in the growth medium had a significant ($p < 0.001$) negative effect on the starch content, as did the combinations of ammonium and carbon dioxide concentrations, and of ammonium and sulfate concentrations (Table 2), both at 67 and 96 h of growth. After 96 h of growth, the sulfate concentration and the combination of carbon dioxide, ammonium, and sulfate concentrations had a negative effect on the starch content of the cultures (Table 2).

The protein content in the microalgae cultures decreased between 67 and 96 h of growth in all treatments with 7.5 mM ammonium (Figure 5). On the contrary, at 17.5 mM ammonium, the protein content of the cultures increased between 67 and 96 h of growth, especially in the treatments with 0.9 mM sulfate. The highest protein content (52% of DW) was measured in treatment 16 (5.0% CO_2, 17.5 mM NH_4, 20 mM PO_4, and 0.9 mM SO_4) after 96 h of growth. The statistical analysis showed that the same variables were affecting the protein content as those affecting the starch content of the cultures, but this time in a positive way (Table 2). Phosphate concentration also positively affected the protein content of the microalgae ($p < 0.05$) (Table 2).

The starch and protein contents of *C. reinhardtii* cultures were negatively correlated, with Pearson correlation coefficients of -0.544 ($p < 0.001$) and -0.867 ($p < 0.001$) after 67 h and 96 h of growth, respectively. The linear regression analysis yielded the following relationships between starch and protein contents: Starch (% of DW) = 31.1 – 0.64 * Protein (% of DW) ($R^2 = 0.42$) and Starch (% of DW) = 45.6 – 0.75 Protein (% of DW) ($R^2 = 0.63$) at 67 and 96 h of growth, respectively (Figure 6). The ANOVA showed significant negative effects of ammonium concentration ($p < 0.001$) and of the combinations of carbon dioxide and ammonium concentrations ($p < 0.001$); ammonium and sulfate concentrations ($p < 0.001$); and carbon dioxide, ammonium, and sulfate concentrations ($p < 0.05$) on the starch/protein ratio of the microalgae (Table 2).

The total chlorophyll (*a* and *b*) content in the cultures (as % of DW) was significantly higher when the microalgae were grown in the high ammonium ($p < 0.001$) treatments than in the low ammonium treatments (Figure 7, Table 2). The interaction of carbon dioxide and ammonium concentrations also had a significant ($p < 0.05$) positive effect on the total chlorophyll content of the microalgae, as did the interaction between ammonium and sulfate concentrations ($p < 0.001$) after 96 h of growth (Table 2). The chlorophyll content decreased toward the end of the experiments (from 67 to 96 h of growth) in all treatments, except in

264 treatment 7 (2.0% CO_2, 17.5 mM NH_4, 20 mM PO_4, and 0.2 mM SO_4). The chlorophyll *a*/*b*
265 ratio varied between 1.55 at 67 h in treatment 2 (2.0% CO_2, 7.5 mM NH_4, 10 mM PO_4, and
266 0.9 mM SO_4) and 2.10 at 96 h in treatment 16 (5.0% CO_2, 17.5 mM NH_4, 20 mM PO_4, and
267 0.9 mM SO_4) (Figure 8). Larger differences in the chlorophyll *a*/*b* ratio between the
268 treatments were found in microalgae grown at 5.0% CO_2 than in microalgae grown at 2.0%
269 CO_2. The concentrations of all four nutrients had a significant positive effect on the
270 chlorophyll *a*/*b* ratio, as did the interaction of carbon dioxide and ammonium concentrations
271 (Table 2).

272 The dry weight of microalgae and the corresponding weight of starch and protein were
273 calculated so that the cultures would contain 20 mg chl L^{-1} (Figure 9) after proper dilution of
274 the microalgae harvested after 96 h of growth for hydrogen production. This chlorophyll
275 concentration was shown to be the optimal one for maximal hydrogen output (Hahn et al.
276 2004). While a microalgae dry weight of 1.5 – 2.7 g L^{-1} would be necessary to contain this
277 chlorophyll concentration after 96 h of growth for the low ammonium treatments and 5.0%
278 CO_2, about 1 g L^{-1} would be sufficient for microalgae grown in high ammonium treatments
279 (Figure 9). High ammonium treatments would yield high protein content (32 – 52% of DW),
280 but low starch content (5 – 20% of DW). In the low ammonium treatment, on the other hand,
281 starch would contribute to a much greater part of the biomass (up to 42% of DW).

282 The N content in the microalgae after 96 h of growth varied between 6 and 11% of
283 DW in the high ammonium treatments, and between 3 and 6% of DW in the low ammonium
284 treatments (Figure 10). The P content varied between 0.8 and 2.4% of DW (Figure 10). The S
285 content in the microalgae varied between 0.27 and 0.73% of DW (Figure 10). The contents of
286 these three nutrients were negatively affected by the carbon dioxide concentration ($p < 0.05$
287 for N and $p < 0.01$ for P and S) (Table 2). A significant ($p < 0.001$ for N and S, $p < 0.01$ for P)
288 positive effect of the ammonium concentration was also observed (Table 2). The sulfate
289 concentration had a significant positive effect on the S content of the microalgae ($p < 0.01$),
290 but a negative effect on the P content ($p < 0.001$) (Table 2). We also observed significant ($p <$
291 0.05) negative interaction effect of carbon dioxide and phosphate concentrations on the N
292 content of the microalgae, and positive interaction effects of ammonium and phosphate
293 concentrations on the S content ($p < 0.05$), of ammonium and sulfate concentrations on all
294 three nutrients ($p < 0.05$), and, finally, of the combination of carbon dioxide, ammonium, and
295 sulfate concentrations on all three nutrients ($p < 0.05$ for P, $p < 0.01$ for N and S) (Table 2).

296 Calculations showed that 8.0 – 12.5 mM N was left in the medium after 96 h of
297 growth in high ammonium treatments, while 2.8 – 5.2 mM N was left in the medium after 96
298 h of growth in low ammonium treatments (Figure 11). The remaining S concentration after 96
299 h of growth was over 0.6 mM S in high sulfate treatments, while it varied between 0.05 and
300 0.12 mM S in the low sulfate treatments (Figure 11).

Discussion

It appears to be quite clear that the different nutrient treatments had a much greater effect on the biochemical composition of the microalgae than on their growth. This is in accordance with the results of Lee et al. (2012), who found that, while decreasing the ammonium concentration in the medium from 6.2 to 4.7 or 3.1 mM had no effect on the growth rate, a significant effect was measured on the primary metabolism and enzyme expression in *C. reinhardtii*. The starch content of our cultures showed most variation between the different treatments (up to ten times). Small differences in biomass were measured between the two CO_2 concentrations, as expected based on previous results with the same strain (Mortensen and Gislerød 2014; Mortensen and Gislerød 2015). However, the effect of the different nutrient treatments on the biochemical components was more marked at 5.0% than at 2.0% CO_2 in the present study.

The availability of nitrogen and sulfur might have been the reason for the different growth rates observed towards the end of the growth period at 5.0% CO_2. This was the case even when a quite high nitrogen concentration (over 2.8 mM) was left in the culture medium in the low ammonium treatments (7.5 mM) at the end of the experiments. Xin et al. (2010) found 0.86 mM N to be the half-saturation concentration for nitrogen uptake in *Scenedesmus* sp, which is much lower than the remaining nitrogen concentration in our cultures. The growth decrease in the low ammonium treatments was related to low sulfur concentrations remaining in the culture. Sulfur deficiency in the growth medium is known to stimulate starch accumulation in the microalgae cells (Yao et al. 2012). The high starch contents measured in the present study (30 – 40% of DW) could have caused a negative feedback effect on photosynthesis in spite of the high N concentration still remaining in the medium. It is difficult to find literature on direct feedback downregulation of photosynthesis by high starch contents in microalgae (when nutrients are not depleted). However, this is well described in C_3 plants, such as wheat and soybean, where starch accumulates in the chloroplasts and inhibits photosynthesis (Sawada et al. 2001; Sinha et al. 2011). Therefore, it should not be surprising if the high starch content, particularly occurring at high CO_2 concentration, could reduce photosynthesis and growth of the microalgae. Increasing the phosphate concentration slightly (but not significantly) increased the starch content probably due to increased pH of the culture, which is in line with previous results (Tolstygina et al. 2009). Though, in the present study, this effect was small compared to the effect of low ammonium and sulfate concentrations.

As expected, the protein content in the low ammonium and sulfate treatments decreased, probably due to the limitation of availability of nitrogen and sulfur for protein synthesis, at the same time as the starch content increased during the growth period (Guo et al. 2014; Ji et al. 2011; Markou et al. 2012). A relative decrease in the protein content took place since starch made up a greater part of the total biomass of the microalgae. Since nitrogen is also part of the chlorophyll molecule, the concentration of chlorophyll generally followed the same pattern as the protein content of the cultures. The chlorophyll *a*/*b* ratios (1.55 – 2.1) recorded in the present study are similar to those described by Wood (1979). A high chlorophyll *a*/*b* ratio is desirable, since this would mean more efficient photosynthesis, due to more reaction center pigments relative to antenna pigments (Beckmann et al. 2009). According to our results (Table 2), this would mean using high ammonium concentration to produce microalgae for the purpose of H_2 production, which would contradict the recommendation of using low ammonium concentration in order to obtain cultures with higher starch content.

For H_2 production, the content of starch, protein, and chlorophyll is important, as is the ability of the light to penetrate the microalgae culture. A chlorophyll concentration of 20 mg L^{-1} has been suggested to be optimal for H_2 production (Hahn et al. 2004). This means that, the higher the chlorophyll content in the microalgae cell, the lower the biomass of the culture needed, and the more light will penetrate into the culture. The combination of high ammonium and low sulfate concentrations in the present experiment produced microalgae that would give the desired chlorophyll concentration of 20 mg L^{-1} at a biomass concentration of about 1 g L^{-1}. The corresponding starch content would be about 15 – 20% of DW. High ammonium combined with high sulfate concentrations would yield about the same biomass concentration, but with a much lower starch content (5.0 – 12.5% of DW). If a high starch content of 30 – 40% of DW (obtained at low ammonium and low sulfate concentrations) is desired, this would require a biomass concentration of 1.5 – 2.7 g L^{-1} in order to establish a chlorophyll concentration of 20 mg L^{-1}. The light penetration in this case would be reduced by 2 – 3 times.

It can be concluded that different nutrient combinations that do not necessarily result in different growth rates could change the relative content of starch, protein, and total chlorophyll, as well as the chlorophyll *a*/*b* ratio. This information makes it possible to produce microalgae with a well-defined biochemical composition of relevance to studying the process of H_2 production.

Acknowledgments

This study was financially supported by the Research Council of Norway (Project 203281: Use of solar energy for CO_2 capture, algae cultivation and hydrogen production – BioH_2) and the Norwegian University of Life Sciences. We thank Dag Wenner and Silje Roksti for their technical assistance.

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List of tables

Table 1. Experimental design. Four variables (CO_2 concentration in the bubbled gas (% v/v), and the concentrations of NH_4, PO_4, and SO_4 in the growth medium) were combined in a 2^4 full factorial design in order to investigate their effects on the growth kinetics and the biochemical content of *C. reinhardtii* batch cultures. In total, sixteen treatments were applied. The treatments were divided into two blocks and carried out successively, at two different CO_2 concentrations. Each treatment was repeated in three photobioreactors.

Table 2. Results of the ANOVA. Average effects and significance levels of the effects of different nutrient treatments (C – CO_2 concentration in the bubbled gas (% v/v), N – NH_4 concentration in the growth medium (mM), P – PO_4 concentration in the growth medium (mM), S – SO_4 concentration in the growth medium (mM)) and their interactions on pH, dry weight (DW) per liter culture, the content of starch, protein, chlorophyll, nitrogen (N), phosphorous (P), and sulfur (S) of the microalgae after 43, 67, and 96 h of growth of *C. reinhardtii*. The experiments were carried out in two blocks at two different CO_2 concentrations. The effect of the CO_2 concentration and the effect of the block (time) were therefore confounded. Significance levels: ^, $p < 0.10$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Not significant, $p > 0.10$ (not indicated).

Tables

Table 1. Experimental design. Four variables (CO_2 concentration in the bubbled gas (% v/v), and the concentrations of NH_4, PO_4, and SO_4 in the growth medium) were combined in a 2^4 full factorial design in order to investigate their effects on the growth kinetics and the biochemical content of *C. reinhardtii* batch cultures. In total, sixteen treatments were applied. The treatments were divided into two blocks and carried out successively, at two different CO_2 concentrations. Each treatment was carried out in three parallels.

		Nutrient			
Block	Treatment	CO_2 (% v/v)	NH_4 (mM)	PO_4 (mM)	SO_4 (mM)
1	1	2	7.5	10	0.2
1	2	2	7.5	10	0.9
1	3	2	7.5	20	0.2
1	4	2	7.5	20	0.9
1	5	2	17.5	10	0.2
1	6	2	17.5	10	0.9
1	7	2	17.5	20	0.2
1	8	2	17.5	20	0.9
2	9	5	7.5	10	0.2
2	10	5	7.5	10	0.9
2	11	5	7.5	20	0.2
2	12	5	7.5	20	0.9
2	13	5	17.5	10	0.2
2	14	5	17.5	10	0.9
2	15	5	17.5	20	0.2
2	16	5	17.5	20	0.9

Table 2. Results of the ANOVA. Average effects and significance levels of the effect of different nutrient treatments (C – CO_2 concentration in the bubbled gas (% v/v), N – NH_4 concentration in the growth medium (mM), P – PO_4 concentration in the growth medium (mM), S – SO_4 concentration in the growth medium (mM)) and their interactions on pH, dry weight (DW) per liter culture, the content of starch, protein, chlorophyll, nitrogen (N), phosphorous (P), and sulfur (S) in the microalgae after 43, 67, and 96 h of growth of *C. reinhardtii*. The experiments were carried out in two blocks at two different CO_2 concentrations. The effect of the CO_2 concentration and the effect of the block (time) were, therefore, confounded. Significance levels: ^, $p < 0.10$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Not significant, $p > 0.10$ (not indicated).

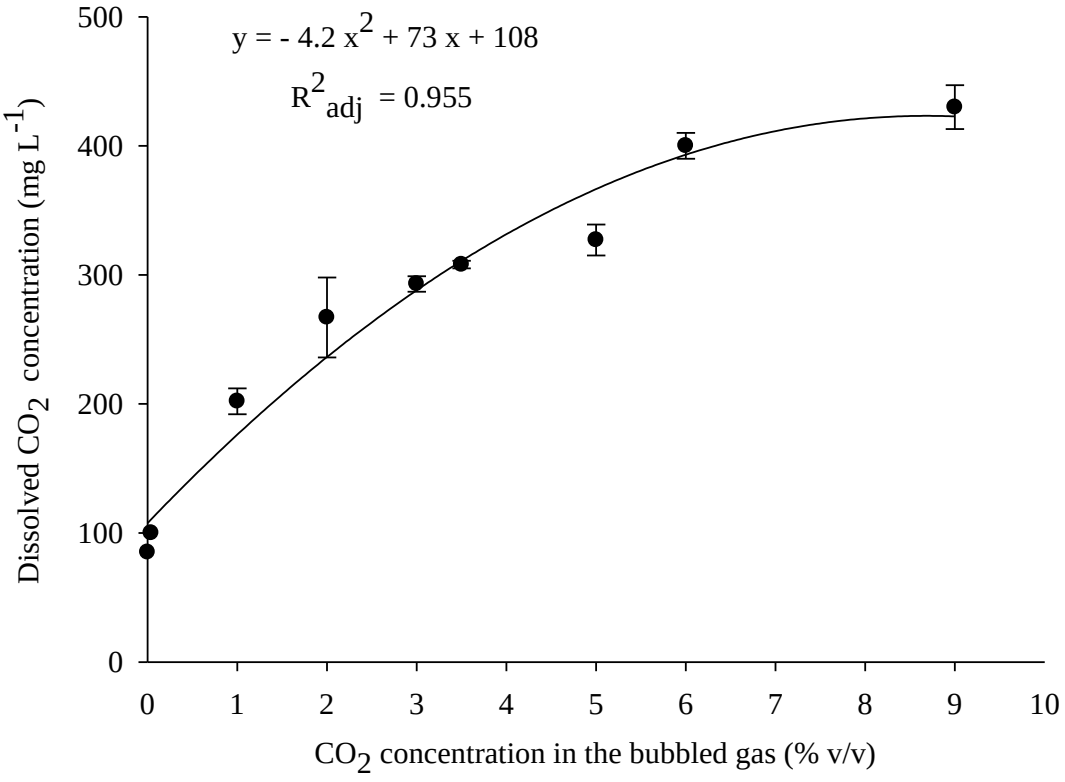
	pH			Dry weight ($g\ L^{-1}$)			Starch content (% of DW)		Protein content (% of DW)		Starch/protein		Total chlorophyll (% of DW)		Chlorophyll *a*/*b*		Nutrient content (% of DW)		
Nutrient	43 h	67 h	96 h	43 h	67 h	96 h	67 h	96 h	67 h	96 h	67 h	96 h	67 h	96 h	67h	96 h	N	P	S
C	-0.19***	-0.03	0.21^	0.009	0.157***	-0.028	0.3	4.0**	3.5*	-0.03	0.08^	0.30***	-0.003	-0.013^	0.10***	0.10***	-0.97*	-0.39**	-0.10**
N	-0.06	-0.15**	-0.31**	-0.004	-0.001	0.152***	-8.4***	-19.1***	3.7*	19.0***	-0.38***	-1.37***	0.052***	0.088***	0.14***	0.13***	3.38***	0.41**	0.16***
P	-0.05	0.18**	0.37**	-0.022*	-0.077^	-0.085**	1.3	0.5	4.1*	3.3*	-0.03	-0.16*	-0.010	0.009	0.04^	0.05*	0.62	0.05	0.01
S	0.02	-0.07	-0.26*	-0.003	0.070^	0.142***	-1.8	-6.0***	-0.3	3.0*	0.05	0.10	0.003	0.004	0.05*	0.12***	-0.03	-0.32**	0.12***
C x N	0.06	-0.06	0.10	0.014	-0.014	0.130***	-4.8***	-5.2***	3.4*	6.0***	-0.25***	-0.37***	0.020*	0.02*	0.11***	0.08**	0.46	-0.02	0.04
C x P	0.04	0.09^	-0.22^	0.013	-0.005	-0.034	2.1^	2.0^	-1.2	0.02	0.06	0.15^	-0.001	-0.008	-0.02	0.02	-1.12*	-0.09	-0.04
C x S	-0.04	0.04	0.10	0.013	-0.003	0.059	0.7	-0.8	-1.8	0.8	0.12**	0.05	-0.002	-0.008	0.07**	-0.002	0.66	-0.03	0.01
N x P	0.05	0.04	0.16	0.002	0.051	0.032	-0.6	-0.5	-3.2^	1.0	0.05	0.13^	-0.002	-0.010	0.01	0.11***	0.52	-0.02	0.06*
N x S	0.04	0.02	-0.24*	-0.003	0.004	0.102**	-2.9*	-4.1**	2.8^	7.0***	-0.20***	-0.44***	0.014	0.034***	0.02	0.07**	0.96*	-0.24*	0.08*
P x S	0.02	0.08	0.16	0.006	-0.032	0.105**	0.7	0.4	1.4	2.4^	0.004	-0.13^	0.001	0.006	0.04^	0.01	0.15	-0.06	0.001
C x N x P	-0.02	0.01	-0.13	-0.001	0.041	0042	-2.6*	-1.7	-0.6	-0.3	-0.07	-0.15^	0.002	0.010	0.02	-0.03	-0.33	-0.06	-0.08*
C x N x S	0.03	-0.06	0.09	-0.006	0.064	-0.038	-1.1	-4.2***	0.7	3.3*	-0.11*	-0.18*	-0.004	0.009	-0.001	0.07*	1.37**	0.26*	0.08**
C x P x S	0.03	-0.04	-0.1	-0.001	0.012	0.028	1.4	1.0	-1.3	-1.7	0.05	0.24**	0.002	-0.012	-0.01	-0.01	0.38	0.02	-0.01
N x P x S	-0.02	-0.05	0.10	-0.013	-0.007	-0.051	-0.2	-1.8^	-0.3	-1.9	0.01	0.12	-0.007	-0.010	-0.03	-0.001	-0.80^	-0.04	0.01
C x N x P x S	-0.04	0.06	-0.06	-0.016^	-0.097*	-0.100**	-1.9^	0.2	-0.03	0.8	-0.07	-0.19*	0.020*	0.021**	0.03	-0.03	0.21	0.05	-0.03

List of figures

Figure 10. Effect of the different nutrient treatments (NH_4/PO_4/SO_4 concentrations in mM) on the contents of nitrogen (N), phosphorous (P), and sulfur (S) in *C. reinhardtii* cells after 96 h of growth at 2.0% CO_2 (a) and 5.0% CO_2 (b).

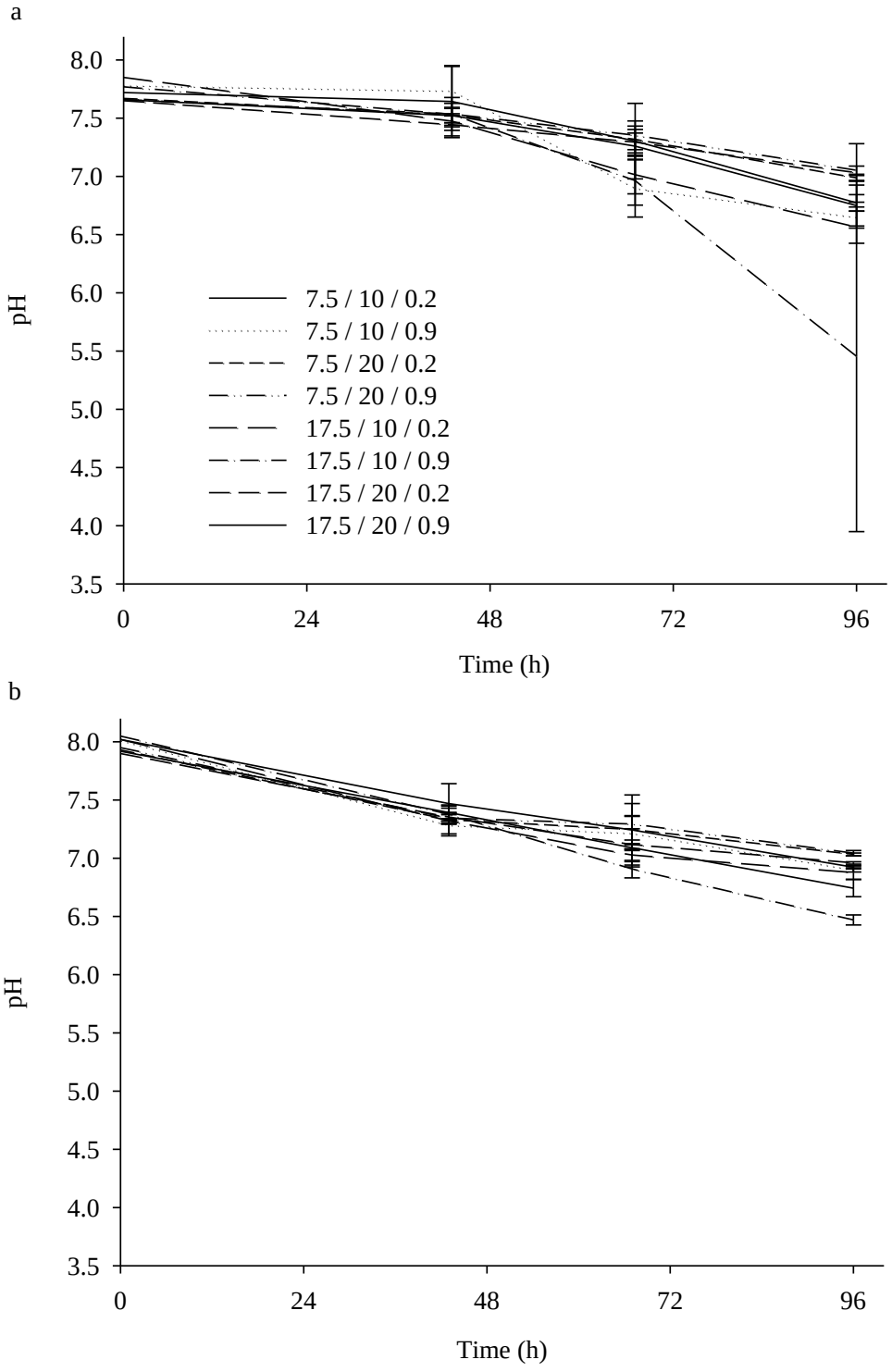
Figure 11. Effect of the different nutrient treatments (NH_4/PO_4/SO_4 concentrations in mM) on the residual nitrogen (a) and sulfur (b) concentrations (mM, mean ± SD, n = 3) in the medium after 96 h of growth of *C. reinhardtii* cultures at 2.0 and 5.0% CO_2.

538 **Figures**



539

540 Figure 1. Effect of the CO_2 concentration in the bubbled gas (% v/v) on the dissolved CO_2
541 concentration (mg L^{-1}) in the High-Salt Sueoka medium (means ± SD, n = 3) and the linear
542 regression curve ($y = - 4.2 x^2 + 73 x + 108$, $R^2_{adj} = 0.955$) fitted to the data.



543

544 Figure 2. Effect of the different nutrient treatments (NH_4/PO_4/SO_4 concentrations in mM) on
545 the pH (means ± SD, n = 3) of *C. reinhardtii* cultures grown at 2.0% CO_2 (a) and 5.0% CO_2
546 (b).

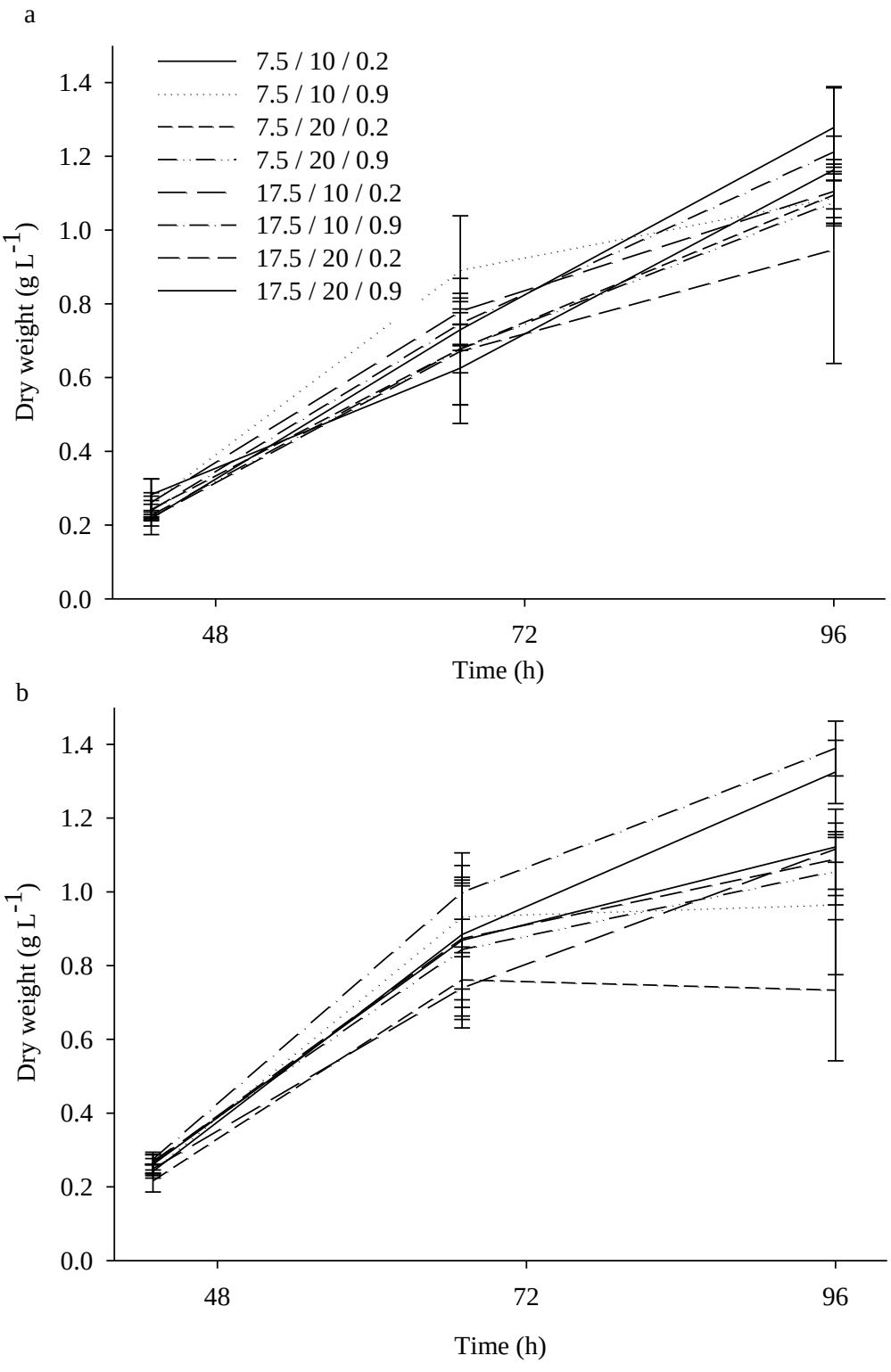
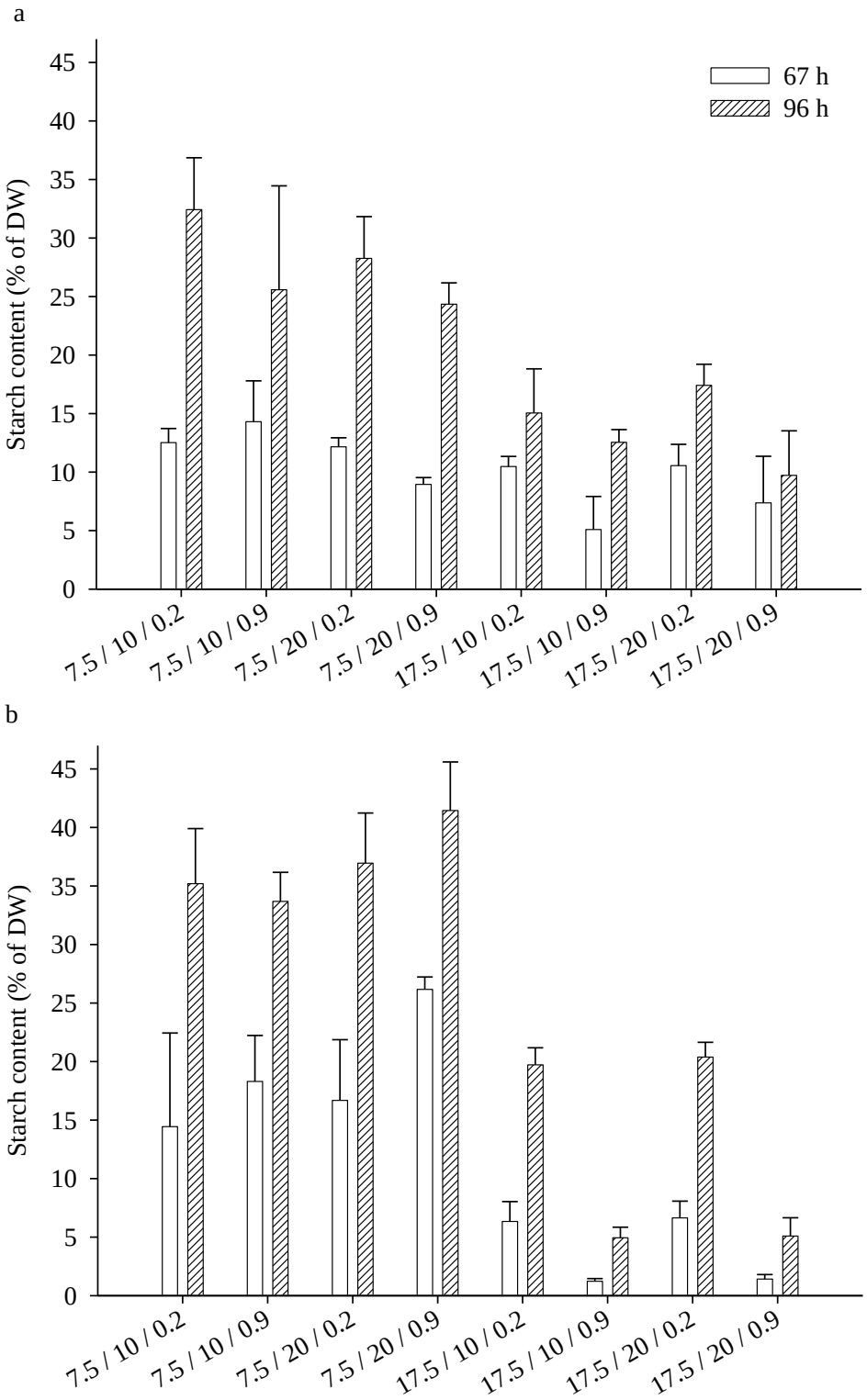
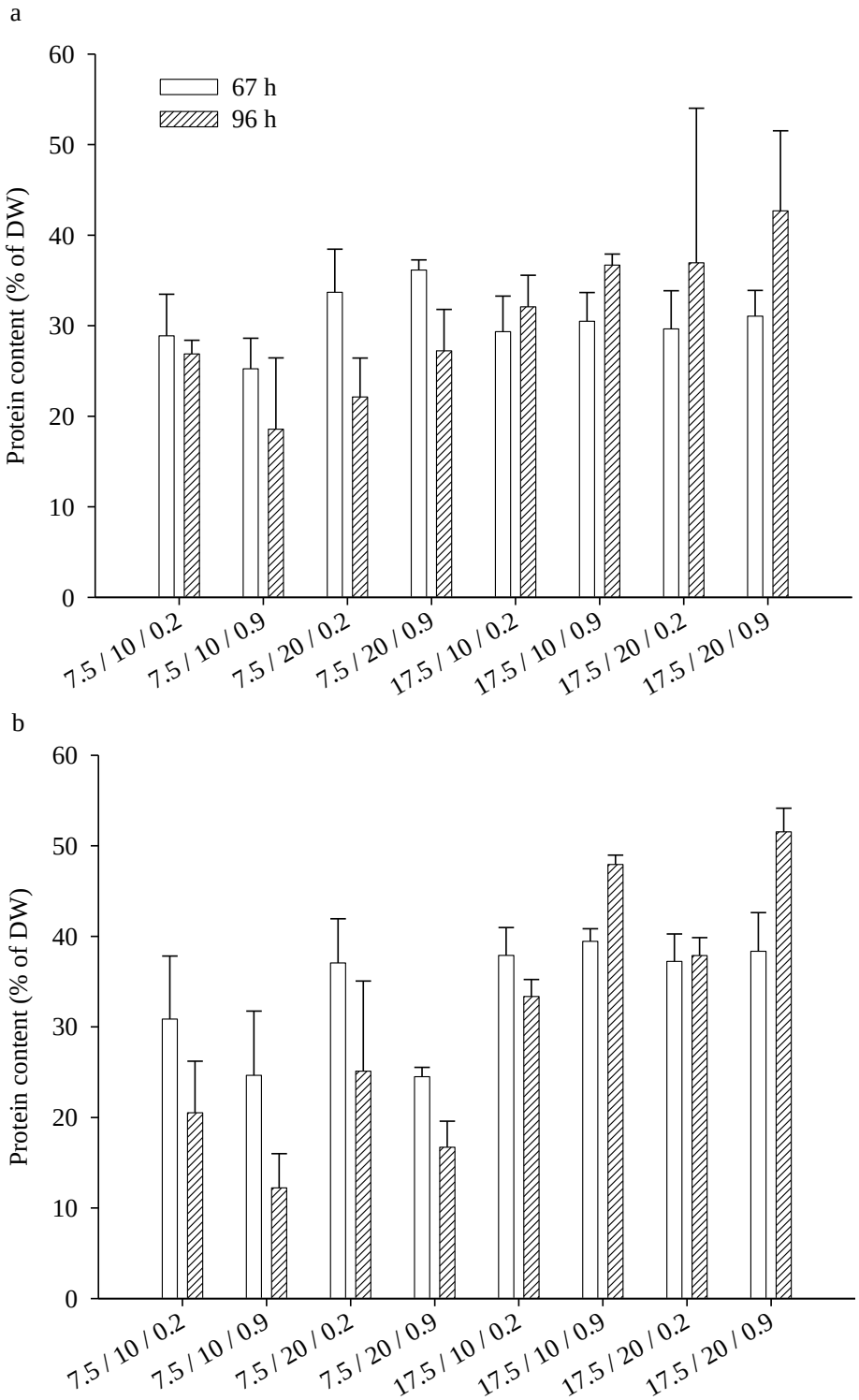


Figure 3. Effect of the different nutrient treatments ($NH_4/PO_4/SO_4$ concentrations in mM) on the dry weight (g L^{-1}, means ± SD, n = 3) of the *C. reinhardtii* cultures grown at 2.0% CO_2 (a) and 5.0% CO_2 (b).



551

552 Figure 4. Effect of the different nutrient treatments ($NH_4/PO_4/SO_4$ concentrations in mM) on
553 the starch content (% of dry weight (DW), means ± SD, n = 3) of the *C. reinhardtii* cultures
554 grown at 2.0% CO_2 (a) and 5.0% CO_2 (b).



555

556 Figure 5. Effect of the different nutrient treatments ($NH_4/PO_4/SO_4$ concentrations in mM) on
557 the protein content (% of dry weight (DW), means ± SD, n = 3) of the *C. reinhardtii* cultures
558 grown at 2.0% CO_2 (a) and 5.0% CO_2 (b).

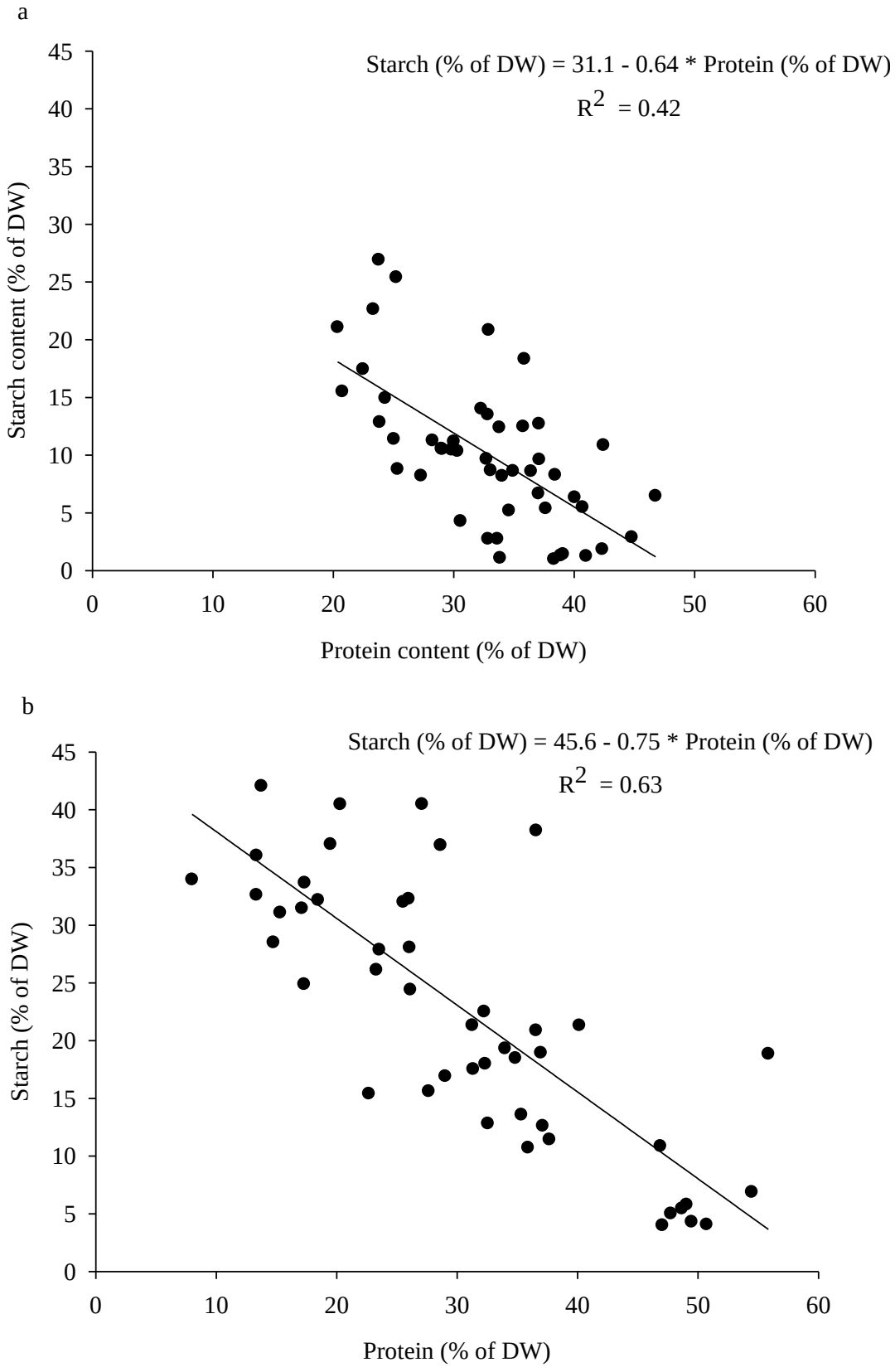
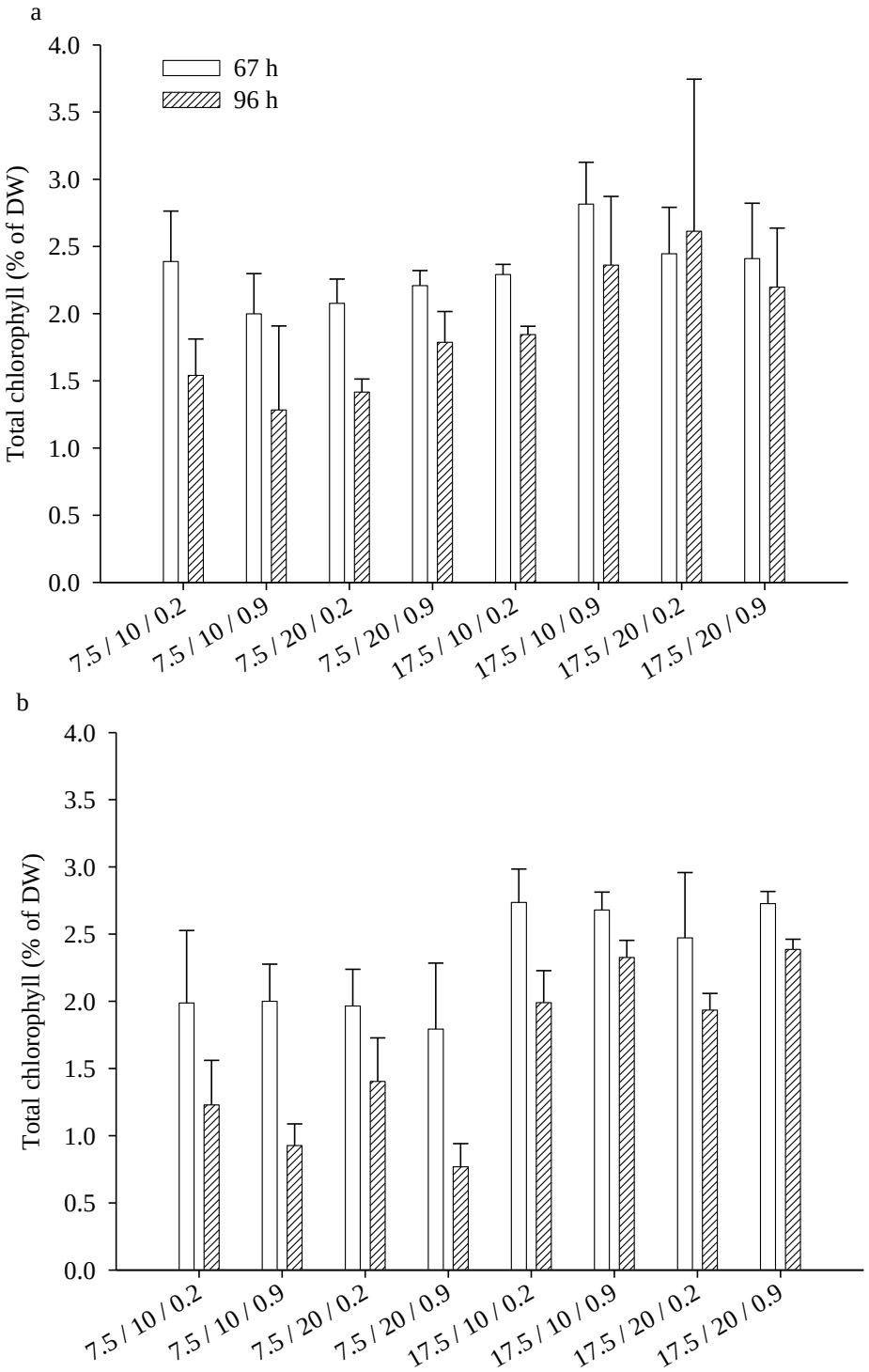


Figure 6. Relationship between starch and protein contents (% of dry weight (DW)) in C. *reinhardtii* cultures after 67 h (a) and 96 h (b) of growth.



562

563 Figure 7. Effect of the different nutrient treatments ($NH_4/PO_4/SO_4$ concentrations in mM) on
564 the total chlorophyll (*a* + *b*) content (% of dry weight (DW), means ± SD, n = 3) of the *C.*
565 *reinhardtii* cultures grown at 2.0% CO_2 (a) and 5.0% CO_2 (b).

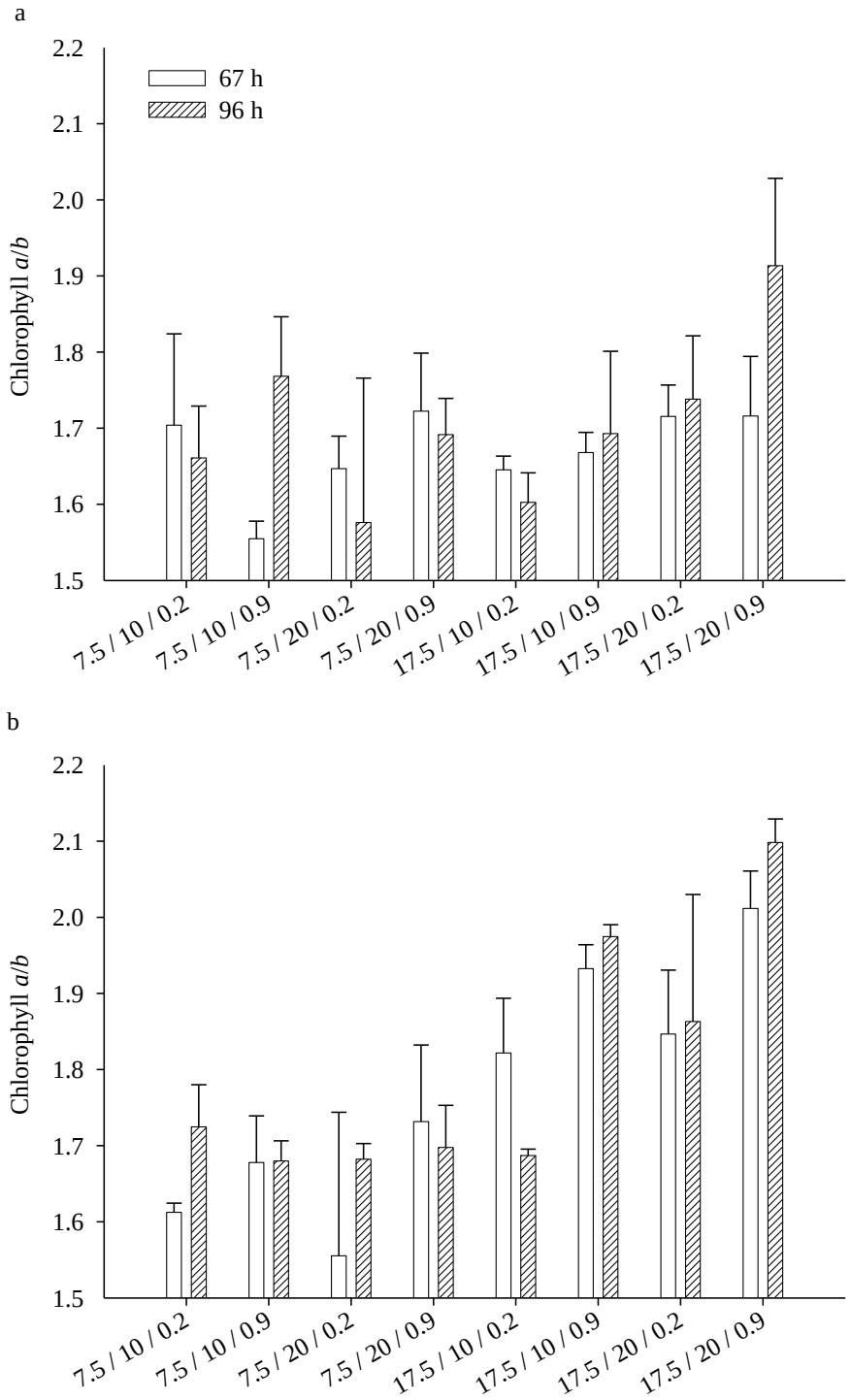
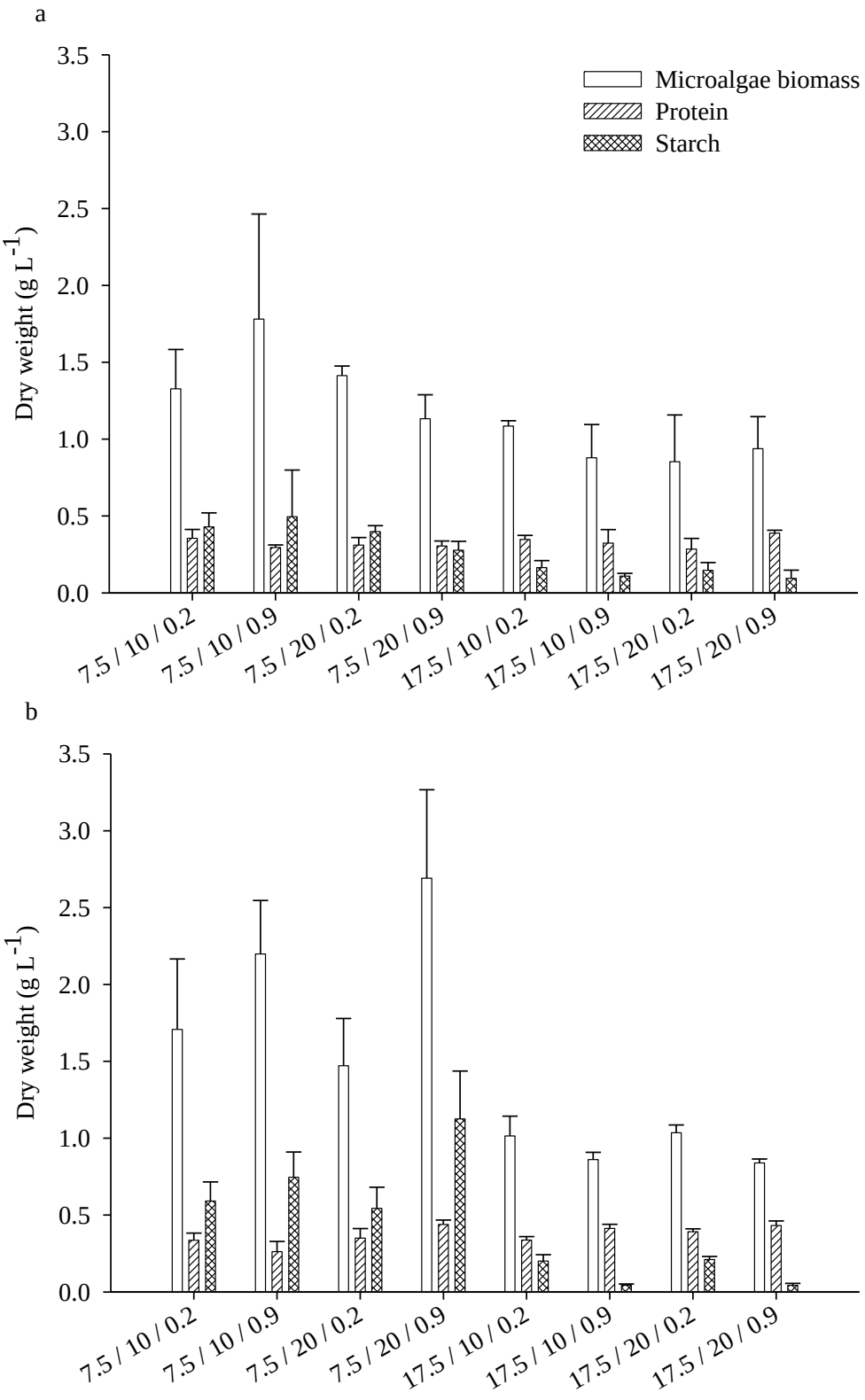
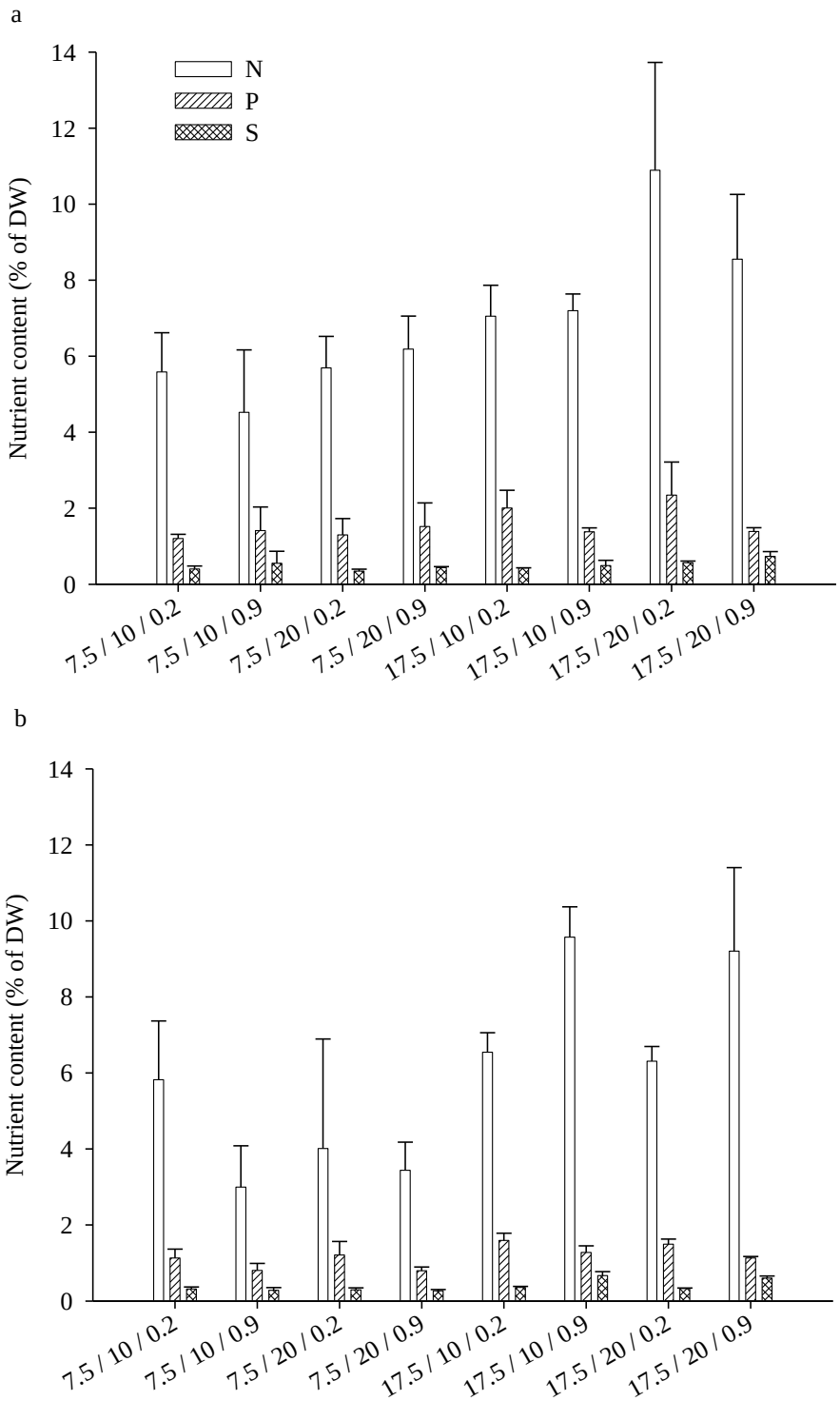


Figure 8. Effect of the different nutrient treatments (NH_4/PO_4/SO_4 concentrations in mM) on the chlorophyll *a*/*b* ratio (mean ± SD, n = 3) in the *C. reinhardtii* cultures grown at 2.0% CO_2 (a) and 5.0% CO_2 (b).



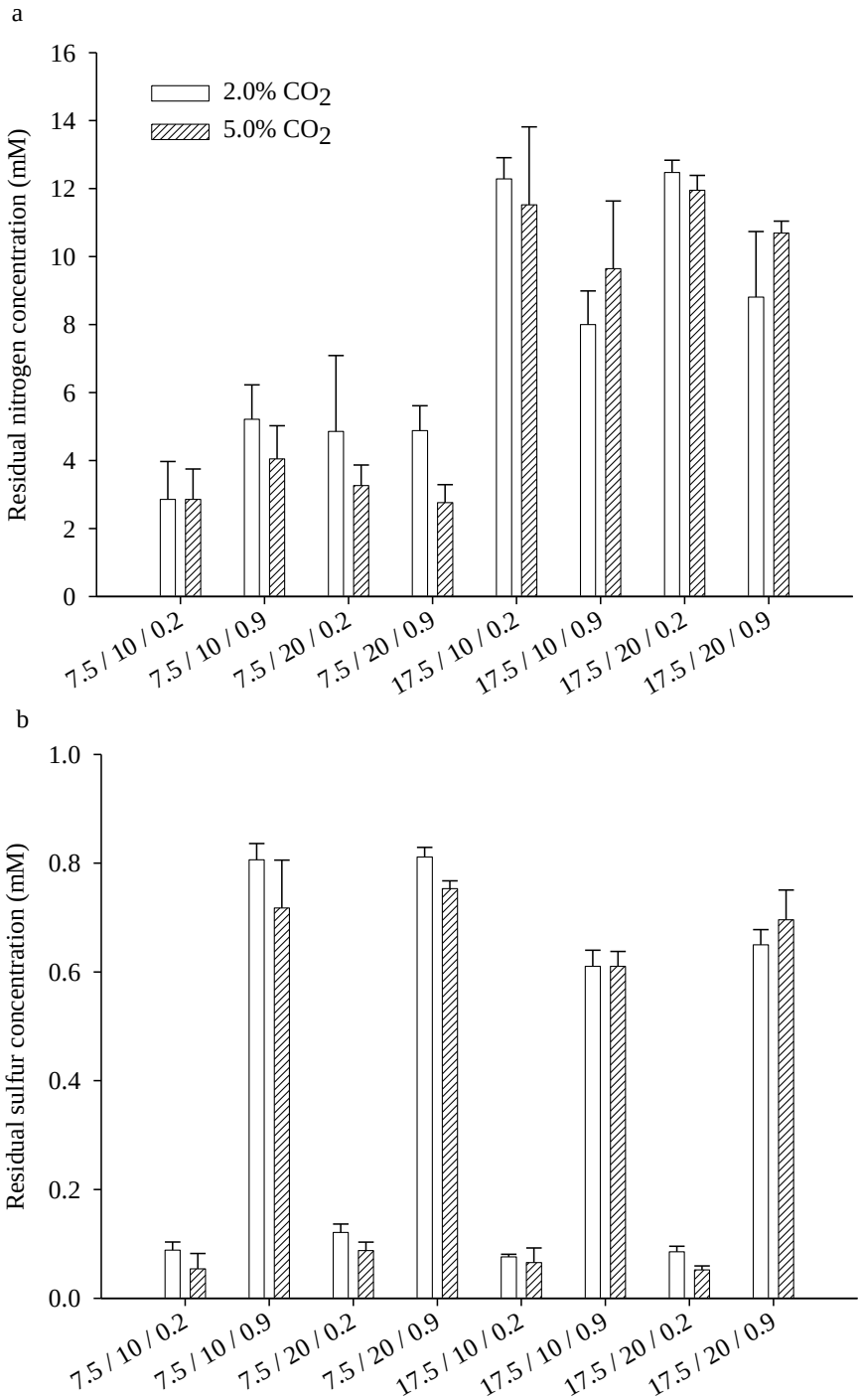
570

571 Figure 9. Dry weight of *C. reinhardtii* cultures, starch, and protein corresponding to 20 mg
572 chl L^{-1} calculated for the different nutrient treatments (NH_4/PO_4/SO_4 concentrations in mM) at
573 2.0% CO_2 (a) and 5.0% CO_2 (b) after 96 h of growth.



574

575 Figure 10. Effect of the different nutrient treatments (NH_4/PO_4/SO_4 concentrations in mM) on
576 the contents of nitrogen (N), phosphorous (P), and sulfur (S) in *C. reinhardtii* cells after 96 h
577 of growth at 2.0% CO_2 (a) and 5.0% CO_2 (b).



578

579 Figure 11. Effect of the different nutrient treatments (NH_4/PO_4/SO_4 concentrations in mM) on
580 the residual nitrogen (a) and sulfur (b) concentrations (mM, mean ± SD, n = 3) in the medium
581 after 96 h of growth of *C. reinhardtii* cultures at 2.0 and 5.0% CO_2.

Paper II

Modeling the effects of nutrient concentrations on the photoautotrophic growth and biochemical composition of *Chlamydomonas reinhardtii*

Daria Markina*, Hans Ragnar Gislerød

Department of Plant Sciences, Norwegian University of Life Sciences, P. O. Box 5003, 1432 Ås, Norway

*Corresponding author, e-mail: daria.markina@nmbu.no, dmarkina@gmail.com, tel.: (+47) 64 96 61 38, fax: (+47) 64 96 56 15

hans.gislerod@nmbu.no

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Abstract

The green microalga *Chlamydomonas reinhardtii* is able to produce hydrogen under anaerobic conditions. The production depends on the biomass concentration and starch, protein, and chlorophyll contents of the cultures. The aim of the present study was to model the effects of ammonium, phosphate, and sulfate in the growth medium, and of carbon dioxide in the gas bubbled into the culture, on the photoautotrophic growth and biochemical composition of *C. reinhardtii* batch cultures. A central composite design, with five levels of each variable, was used to combine the different concentrations of nutrients. Our models predicted maximum productivity of 0.87 g L^{-1} d^{-1} with 5.0 mM NH_4, 0.65 mM SO_4, and 6.0% CO_2, maximum starch content of 55% of dry weight with 5.0 mM NH_4, 22.5 mM PO_4, 0.1 mM SO_4, and 6.0% CO_2 and 65% of dry weight for maximum protein content with 20.0 mM NH_4, 22.5 mM PO_4, 1.0 mM SO_4, and 4.4% CO_2. These predictions were confirmed by the model validation experiments. The results of this modeling make it possible to produce microalgae with a well-defined biochemical composition for studying the process of hydrogen production.

Key-words

Chlorophyll, modeling, nutrients, photoautotrophic growth, protein, starch.

Introduction

Cultures of *Chlamydomonas reinhardtii* with high biomass are required for biohydrogen production (Tamburic et al. 2011). The starch and protein contents of the cultures play a central role in the hydrogen (H_2) production process, since the catabolism of these two substrates contributes electrons to the hydrogenase enzyme for H_2 formation (Chochois et al. 2009; Melis et al. 2000; Zhang et al. 2002). In addition, products of starch catabolism are substrates for mitochondrial respiration, and they therefore help to maintain the anaerobiosis of the culture that is necessary for the function of the hydrogenase enzyme (Kosourov et al. 2003; Zhang et al. 2002). Hahn et al. (2004) found that, at 20 µg chlorophyll mL^{-1}, H_2 production attained its maximum value and the yield was independent of increasing chlorophyll concentration. Usually, *C. reinhardtii* is grown mixotrophically on acetate as an organic carbon source for the purpose of H_2 production. However, this has been shown not to be necessary (Fouchard et al. 2005; Tolstygina et al. 2009; Tsygankov et al. 2006). We chose therefore to use photoautotrophic cultures in this study, since this makes more sense from the energy conversion point of view.

The nutrient status of the microalgae affects their photosynthesis (Falkowski and Raven 2007) and, consequently, their growth rate, biomass yield (Richmond 2004), and biochemical composition (Grossman 2000; Richmond 2004). The synthesis of many essential cellular components, such as proteins and pigments, requires other nutrients than carbon, hydrogen, and oxygen (Falkowski and Raven 2007). Nitrogen, phosphorous, and sulfur are structural constituents of a number of cellular components. The uptake and assimilation of these elements is closely associated with carbon fixation. A balance must exist between the main macronutrients and carbon, so that these macronutrients are incorporated into carbon skeletons in an equilibrated manner (Grossman and Takahashi 2001). When all the required nutrients are provided in excess, microalgae grow in a balanced way and display a uniform chemical and biochemical composition (Goldman 1980).

When the supply rate of one of the nutrient elements is lower than required for the maximum biosynthetic capacity of the cell, that element becomes limiting for growth (Bailey and Ollis 1986; Falkowski and Raven 2007). Growth of nutrient-limited populations slows down before total exhaustion of a nutrient (Bailey and Ollis 1986). Several biochemical processes are then affected. Photosynthetic and respiratory processes, as well as protein and

pigments synthesis, are reduced (Collier and Grossman 1992; Fogg 1956; Gordillo et al. 1999; Guo et al. 2014; Ji et al. 2011; Kilham et al. 1997; Klok et al. 2013; Kolber et al. 1988; Lee et al. 2012; Lynn et al. 2000; Morris et al. 1974; Richardson et al. 1969; Siaut et al. 2011; Turpin 1991; Wykoff et al. 1998). Under these conditions, the cells experience an energy imbalance: the energy required for growth becomes lower than the energy supplied through photosynthesis (Klok et al. 2013; Richmond 2004). This leads to over-reduction of the photosynthetic machinery and formation of oxygen-reactive species (Klok et al. 2013; Ledford and Niyogi 2005). Under these conditions, microalgae dissipate the excess energy as heat and fluorescence (Kolber et al. 1988). The accumulation of highly reduced compounds, such as lipids and carbohydrates, that do not contain the limiting nutrient serves as a sink for the assimilated carbon and as an alternative sink for the excess energy (Ball et al. 1990; Branyikova et al. 2010; Guo et al. 2014; Ji et al. 2011; Klok et al. 2013; Siaut et al. 2011; Wykoff et al. 1998). This allows the cell to continue to harvest light energy, while at the same time it reduces the formation of damaging reactive oxygen species (Klok et al. 2013). The accumulation of lipids and carbohydrates is therefore not necessarily coupled to a complete halt in growth as is often suggested (Ball et al. 1990; Branyikova et al. 2010; Guo et al. 2014; Ji et al. 2011; Siaut et al. 2011; Yao et al. 2012). Klok et al. (2013) postulated that, if an energy imbalance is created by reducing the nutrient supply to the cells, cell division continues at the same time as lipids or carbohydrates accumulate, as they do in the classic nutrient depletion experiments.

The development of an optimal medium for cultivation is specific to each species and to each intended product (Mata et al. 2010). Medium formulation studies are usually time-consuming and expensive. Central composite design is a powerful statistical tool used to model the relationships between a set of controllable experimental factors and observed responses. It has therefore been frequently applied to improve the productivity and yield of the desired product of various microalgal cultures (Burrows et al. 2008; Gong and Chen 1998; Hong and Lee 2008; Jeong et al. 2008; Nuutila et al. 1997; Wen and Chen 2001). The advantages of this experimental procedure include its ability to identify interaction effects between studied factors and that it reduces the number of experimental runs compared to ‘one factor at a time’ experiments. It produces second-order response surfaces that can be used in an optimization procedure.

The aim of this study was to model the responses of photoautotrophic batch cultures of *C. reinhardtii* to different combinations of nutrient concentrations in the growth medium and CO_2 in the bubbled gas. This was done with the goal of improving the growth conditions in order to obtain maximum biomass and desirable biochemical composition for further H_2 production. In a previous study, we investigated the effects of two concentrations of CO_2 (2.0 and 5.0%), NH_4 (7.5 and 17.5 mM), SO_4 (0.2 and 0.9 mM), and PO_4 (10.0 and 20.0 mM) on the contents of starch, protein, and chlorophyll in addition to the growth of photoautotrophic batch cultures of *C. reinhardtii* (Markina et al., submitted). Two concentrations of phosphate were used to avoid acidification of the cultures, and they were therefore much higher than the phosphate concentration required for growth of this microalga. A central composite design was used in the present study and the number of treatments was extended, with eight axial points and one central point. The experiments were divided into blocks and carried out successively over a seven-week period according to the CO_2 concentration. The possible effects of this blocking on the detection of interactions among the studied factors will be discussed. The productivity of the cultures, total biomass, starch, protein, and chlorophyll contents of the cultures were used as response parameters. The statistical models generated were used to optimize these parameters. Model validation experiments were carried out in order to confirm the predicted responses.

Materials and methods

Organism and culture conditions

The *Chlamydomonas reinhardtii* 137c strain was obtained from the NIVA culture collection, Norway (CHL153). The microalgae were stored in sterile conditions on a 1.5% agar Tris-Acetate-Phosphate (TAP) (Gorman and Levine 1965) solid growth medium (Andersen 2005) on Petri dishes at room temperature and low light intensity. The microalgae were pre-cultured in liquid High-Salt Sueoka (HS) medium (Sueoka 1960) at 25 °C and continuously illuminated by cool white fluorescent tubes (Philips Master TLD 36W/840 Reflex, Eindhoven, the Netherlands) at a photon flux density (PFD) of 200 µmol m^{-2} s^{-1}.

Experimental setup

This work was based on an extension of previously obtained results (Markina et al., submitted). In the present study, the number of treatments was extended from a 2^4 full factorial design to a central composite design with eight axial points and one central point (Table 1).

Sterile batch cultures of *C. reinhardtii* cells were carried out in bubble column Pyrex glass tubular (coned at the bottom) photobioreactors (PBRs) with an inner diameter of 3.5 cm and a total volume capacity of 380 mL. The temperature was maintained at 25.0 ± 0.5 °C using water baths in aquaria. Cool white fluorescent tubes were used to provide continuous illumination at a PFD of 200 µmol m^{-2} s^{-1}. The light was measures using a LI-COR model LI-190SA instrument (LI-COR, Lincoln, NE, USA) with a quantum sensor (400 – 700 nm) inside an empty PBR immersed in the water-bath aquarium, and the average PFD over the height of the PBR was estimated. Filtered (Acro 37 TF Vent Device, 0.2 µm PTFE membrane, PALL Life Sciences, Port Washington, NY, USA) air containing CO_2 at a concentration (% v/v) in accordance with the experimental design (Table 1) was injected into the bottom of the PBRs, allowing mixing by bubbling (the bubble size was approximately 3 mm). The concentration of the dissolved CO_2 in three parallel PBRs containing 300 mL of HS medium was measured for each CO_2 concentration bubbled into the PBRs (eight points in the 0.04 – 9% range) after a stabilizing period of three hours, using hand-held titration cells for titrimetric analysis (CHEMetrics Inc., Midland, VA, USA). The linear regression curve (order 2) between the CO_2 concentration in the bubbled gas and the concentration of dissolved CO_2 was highly correlated (R^2_{adj} = 0.955) and followed the function $y = -4.2\ x^2 +$

73 x + 108, where y = dissolved CO_2 concentration in the medium (mg L^{-1}), x = CO_2 concentration in the bubbled gas (% v/v) (Markina et al., submitted).

The experimental media were based on the HS medium (9.35 mM NH_4, 13.6 mM PO_4, and 0.182 mM SO_4), with varying concentrations of NH_4Cl, PO_4KH_2/PO_4K_2H, and K_2SO_4 as shown in Table 1. Each treatment was carried out in three parallels, and the experiments were carried out successively, divided into batches according to the concentration of CO_2 in the bubbled gas. PO_4KH_2/PO_4K_2H was used as a pH buffer to avoid acidification of the cultures. The concentration of PO_4 used in these experiments (7.5 – 22.5 mM) therefore greatly exceeded the microalgal requirement for this nutrient. The initial pH of the media was adjusted to 7.5 with 1 M NaOH prior to sterilization by autoclaving. $NaHCO_3$ was used in the medium as an additional pH buffer at a concentration of 2 mM per percent CO_2 in the bubbled gas. At the start of experiments (t = 0 h), cells from pre-grown cultures at the linear growth phase were inoculated into 300 mL of medium at a cell density of 10^4 cells mL^{-1}. The cells were counted under a microscope, using a Fuchs-Rosenthal counting chamber. The experiments were carried out over a seven-week period.

Model validation experiments were performed in three parallel PBRs with the same light and temperature conditions as previously. Nutrient concentrations in the model validation experiments corresponded to the optimum concentrations predicted by the different models.

Samples for dry weight determination and biochemical analyses were collected at three time points: t_1 = 43 h, t_2 = 67 h, and t_3 = 96 h (120 h for 1.0% CO_2 treatments because of slower growth). The pH of the cultures was monitored throughout the experiments.

Biomass determination

To collect the biomass, 0.3 – 2.0 mL of microalgal culture was filtered through pre-washed GF/F glass microfiber filters (0.7 µm pore size, 25 mm diameter, GE Healthcare, Whatman, UK) dried at 103.5 °C. The filters containing biomass were then washed with 30 mL deionized water to remove adhering salts, and thereafter dried for 4 h at 103.5 °C. The DW (g L^{-1}) of the sample was calculated by subtracting the DW of the empty filter from the DW of the filter with biomass. It was normalized to the culture volume filtered. The

productivity (P) of the cultures (g L^{-1} d^{-1}) was calculated using formula (1), where $DW_{1\ (2)}$ is the DW determined at time $t_{1\ (2)}$ (in days).

$$P = \frac{DW_2 - DW_1}{t_2 - t_1} \quad (1)$$

Chlorophyll assay

Chlorophyll was extracted from 1-2 mL samples using 95% ethanol and assayed using the described method (Harris 1989). The chlorophyll concentration was determined by optical density (OD) measurements (Heλios α, Thermo Fisher Scientific, Waltham, MA, USA) at λ = 649 nm and λ = 665 nm.

Starch assay

Cell pellets of 2 mL samples left after chlorophyll assay were used for starch assay. The Megazyme total starch (the amyloglucosidase/*α*-amylase method) assay kit (K-TSTA, Megazyme International, Bray, Ireland) procedure was adapted to a small sample volume. 20 µL of 80% ethanol was added to the cell pellet and the pellet was dispersed using a vortex mixer. Cells were disrupted by adding 0.2 mL of DMSO (Sigma-Aldrich, St. Louis, MO, USA) and 0.5 mL of 0.5 mm glass beads (Cell disruption media, SI-BG05, Scientific Industries Inc., New York, NY, USA) to the samples and shaking them on a mixer mill (MM301, Retsch, Haan, Germany) at 30 Hz frequency for 10 min. Samples were heated on thermos-shakers (TS-100, Biosan, Riga, Latvia) to 100 °C for 5 min for starch solubilization. Then, 0.3 mL of *α*-amylase (diluted 1:30 according to the Megazyme starch assay kit specifications) was added to the samples and the tubes incubated at 100 °C for 12 min. The contents of the tubes were mixed vigorously after 0, 4, 8, and 12 min. The tubes were placed at 50 °C and 0.4 mL of sodium acetate buffer (200 mM, pH 4.5) was added to the samples, followed by 10 µL of amyloglucosidase (20U). The samples were thoroughly mixed and incubated at 50 °C for 30 min. Then, 70 µL of distilled water was added to the samples in order to adjust the total sample volume to 1.0 mL. The contents of the tubes were mixed thoroughly and centrifuged at 20800 x *g* (Eppendorf centrifuge 5417R, Eppendorf, Hamburg, Germany) for 10 min at 20 °C. Then, 33.4 µL of the supernatant was added to 1 mL GOPOD reagent and incubated at 50 °C for 20 min. The same procedure was used for two reagent blanks (33.4 µL distilled water) and four glucose controls (33.4 µL D-glucose standard solution at 1 mg mL^{-1}). The OD of each sample and of the glucose controls was measured at

197 λ = 510 nm against the reagent blank. Starch concentration was calculated as the percentage
198 of DW of the samples according to the formula supplied in the Megazyme starch assay kit.

199 *Protein assay*

200 Cell pellets of 1 mL samples left after chlorophyll assay were used for protein assay.
201 The cells were disrupted using 0.5 mL of 0.5 mm glass beads (Cell disruption media, SI-
202 BG05, Scientific Industries Inc., New York, NY, USA) and shaken on a mixer mill (MM301,
203 Retsch, Haan, Germany) for 10 min at 30 Hz frequency. Then, Tris-HCl solubilization buffer
204 (50 mM, pH 8.3) containing 1% Triton was used to solubilize the proteins. The Lowry
205 method (1951) was applied using the Bio-Rad *DC* protein microplate assay kit II (#500-0112,
206 Bio-Rad, Hercules, CA, USA) to determine protein concentration in the samples. The OD (λ
207 = 750 nm) of the samples assayed on a microplate (CellBIND surface, Costar 3300, Corning
208 Incorporated, Corning, NY, USA) was measured using a microplate spectrophotometer (Asys
209 UVM340, Biochrom, Cambridge, UK) and Kim software (Kim32, 2004, Asys Hitech GmbH,
210 Cambridge, UK). A BSA standard curve (8 points in the 0.0 - 0.75 mg mL^{-1} range) was used
211 to calculate the protein concentration in the samples. The protein concentration was
212 normalized to the DW of the culture and calculated as the percentage of DW.

213 *Element analysis*

214 A 15 mL sample of *C. reinhardtii* was collected at the beginning of the stationary
215 growth phase (t_3) and centrifuged for 10 min at 3220 x *g* (Eppendorf centrifuge 5810,
216 Eppendorf, Hamburg, Germany), and the supernatant discarded. The cell pellet was washed
217 twice with deionized water and frozen at -20 °C until analysis. The Norwegian standard
218 method, NS 4743, was used to measure total nitrogen. Total phosphorous and sulfur were
219 analyzed using an Inductively Coupled Plasma Optical Emission Spectrometer, ICP (Optima
220 5300 DV, Perkin Elmer, Waltham, MA, USA), after the addition of HNO_3 to 10% v/v and
221 decomposition by ultraclave (ultraClave III, Milestone, Sorisole, Italy) at 250 °C for 1.5 h.

222 *Statistical analysis*

223 Three macronutrients were selected as studied variables: NH_4, PO_4, and SO_4 in
224 addition to the percentage of CO_2 bubbled into the cultures. Central composite design was
225 used to model the effects of the studied variables on the photoautotrophic growth and
226 biochemical composition of *C. reinhardtii*. The design matrix was a 2^4 full factorial design

with a central point and eight axial points. A total of 25 treatments, with each treatment carried out in three parallels, were performed, with five levels of each variable. The coded levels of the variables and their real values are shown in Table 1. The responses were modeled using the second-order equation:

$$\hat{y} = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum \sum_{i<j} \beta_{ij} x_i x_j + \varepsilon \quad (2)$$

where ŷ is the estimated response, *k* is the number of variables (k = 4, concentrations of NH_4, PO_4, SO_4, and percentage CO_2, in this study), β_0, β_i, β_{ii}, and β_{ij} are the intercept, linear, quadratic, and interaction coefficients, respectively, and ε is the random error.

The results were analyzed using the analysis of variance (ANOVA) method and regression analysis, which gives the regression equation of the estimated response as a function of the studied variables. First, a full quadratic model of equation 2 was fitted to the data. Then, the model was progressively reduced by backward elimination, discarding the terms with a p-value > 0.1. The residuals of the models, which compared the predicted values with the experimental response, were randomly distributed without any pattern, showing constant variance, normality and independence. The models were evaluated using the p-value of the regression coefficients (α = 10%) and the lack of fit test (α = 1%). The statistical analyses were performed using MINITAB software (V16, Minitab Inc., State College, PA, USA).

Results

pH

The pH of the cultures decreased throughout the experimental period (0 - 96 h) and with the growth of the microalgae (Markina et al., submitted). The pH at the end of the experiments (after 96 h of growth) ranged from 5.7 in treatment 20 (17.5 mM NH_4, 10.0 mM PO_4, 0.9 mM SO_4, and 2.0% CO_2) to 7.1 in treatment 25 (7.5 mM NH_4, 20.0 mM PO_4, 0.9 mM SO_4, and 2.0% CO_2). Phosphate concentration in the medium was the factor that had the largest influence on the pH after 96 h of growth. The pH increased with the increase in phosphate concentration (Figure 1a). The concentration of ammonium and sulfate had the opposite effect on the pH of the cultures (Figure 1a).

Productivity

The productivity of the cultures between 43 and 67 h of growth varied between 0.32 g L^{-1} d^{-1} in treatment 10 (12.5 mM NH_4, 15.0 mM PO_4, 0.55 mM SO_4, and 1.0% CO_2) and 0.87 g L^{-1} d^{-1} in treatment 9 (12.5 mM NH_4, 15.0 mM PO_4, 0.55 mM SO_4, and 6.0% CO_2). Carbon dioxide concentration had the largest positive effect on productivity compared to the other studied variables (Figure 1b).

The regression equation obtained after the analysis of variance (ANOVA) gave the estimated productivity as a function of the studied variables. First, a full quadratic model (equation 2) was fitted to the data. Then, the model was reduced by backward elimination, discarding the terms with a p-value > 0.1. Table 2 shows the retained terms after backward elimination was completed. The p-value of the variables of the model calculated by ANOVA gives the significance of each term in the model. The estimated regression coefficients of the variables, using data in uncoded units, correspond to the β-coefficients in the model of equation 2. The concentration of phosphate did not influence the productivity of the cultures in the studied range, since the p-value of all the terms including PO_4 was higher than 0.1. The quadratic terms of sulfate and carbon dioxide concentrations were significant ($p < 0.01$). A significant negative interaction effect ($p < 0.05$) on the biomass productivity was observed between ammonium and carbon dioxide concentrations. The productivity increased with increasing CO_2 concentration in the bubbled gas and with decreasing ammonium concentration in the growth medium (Figure 2a).

This model was used to determine the optimal values of the studied variables that would give the highest productivity. The solution of the optimization procedure gave the following optimal values for the studied variables: 5.0 mM NH_4, 0.65 mM SO_4, and 6.0% CO_2, and it predicted a productivity of 0.87 g L^{-1} d^{-1} for these values. Figure 2a shows the contour plot of the response surface of productivity using the fitted model and holding the sulfate concentration constant at its optimum value of 0.65 mM. The area of maximum productivity is located in the top-left corner of the plot, corresponding to high carbon dioxide and low ammonium concentrations (Figure 2a). To validate the results of the optimization procedure, microalgae were grown in the predicted optimal concentrations of the nutrients (5.0 mM NH_4, 0.65 mM SO_4, and 6.0% CO_2. 7.5 mM PO_4 were used for this experiment). The measured productivity was 0.86 ± 0.06 g L^{-1} d^{-1} (mean ± SD, n = 3) (Figure 5a), which did not differ significantly ($p > 0.1$, one sample t-test) from the predicted maximum productivity of 0.87 g L^{-1} d^{-1}.

Biomass yield

The biomass of the cultures after 96 h of growth varied between 0.74 g L^{-1} in treatment 5 (7.5 mM NH_4, 20.0 mM PO_4, 0.2 mM SO_4, and 5.0% CO_2) and 1.57 g L^{-1} in treatment 14 (12.5 mM NH_4, 7.5 mM PO_4, 0.55 mM SO_4, and 3.5% CO_2). Concentrations of carbon dioxide, phosphate, and sulfate had a significant effect on the total biomass of the cultures (Figure 1c).

Table 2 shows the terms retained in the final model for the estimated biomass attained after 96 h of growth as a function of concentrations of ammonium, phosphate, sulfate, and carbon dioxide. All the studied variables were significant ($p < 0.01$), as were their quadratic terms ($p < 0.1$ for x_1^2, $p < 0.001$ for x_2^2, x_3^2, and x_4^2) (Table 2). Significant positive interaction effects on the attained biomass were observed between ammonium and sulfate concentrations ($p < 0.001$), ammonium and carbon dioxide concentrations ($p < 0.01$), and also between phosphate and sulfate concentrations ($p < 0.01$) (Table 2).

This model was used to determine the optimal values of the studied variables that would yield the maximum biomass of the cultures at 96 h of growth. The optimization procedure gave the following values for the studied variables: 20.0 mM NH_4, 7.5 mM PO_4, 0.66 mM SO_4, and 4.5% CO_2, and it predicted a maximum biomass of 1.66 g L^{-1} for these values (Figure 2b). A model validation experiment was carried out with these nutrient

concentrations. The measured biomass after 96 h of growth was 1.26 ± 0.01 g L^{-1} (mean ± SD, n = 3) (Figure 5b). This was 24% lower than predicted by the model.

Starch content

The concentration of ammonium was the factor that mainly affected the starch content of the cultures after 96 h of growth, and the starch content increased with decreasing ammonium concentration (Figure 3a). The maximum measured starch content was 42% of DW in treatment 5 (7.5 mM NH_4, 20.0 mM PO_4, 0.2 mM SO_4, and 5.0% CO_2), which corresponds to low concentrations in ammonium and sulfate, but high amounts of phosphate and carbon dioxide. The productivity in this treatment was 0.55 g L^{-1} d^{-1}, and the total biomass after 96 h of growth, when the sample for starch was collected, was 0.74 g L^{-1}, the lowest measured in this study. The microalgae in treatment 13 (20.0 mM NH_4, 15.0 mM PO_4, 0.55 mM SO_4, and 3.5% CO_2) did not accumulate starch (3% of DW). This treatment corresponds to the highest ammonium concentration tested.

Table 2 shows the terms retained for the starch content model after completing the backward elimination procedure. The phosphate concentration term was not significant according to ANOVA, but the interaction effect between phosphate and carbon dioxide concentrations was significant ($p < 0.05$), so the phosphate concentration term was included in the model (Table 2). The quadratic term of the sulfate concentration was significant ($p < 0.001$) (Table 2). Significant negative interaction effects were detected between the concentrations of ammonium and sulfate ($p < 0.001$), ammonium and carbon dioxide ($p < 0.001$), and sulfate and carbon dioxide ($p < 0.05$) (Table 2).

The model used for optimization of the starch content after 96 h of growth yielded the following values: 5.0 mM NH_4, 22.5 mM PO_4, 0.1 mM SO_4, and 6.0% CO_2, predicting 55% of DW starch for these values (Figure 4a). This solution corresponds to the lowest concentrations of ammonium and sulfate, and to the highest concentrations of phosphate and carbon dioxide tested in this study (top-left corner in Figure 4a). A model validation experiment with these nutrient concentrations yielded a starch content of 52 ± 3% of DW (mean ± SD, n = 3) (Figure 5c). This was not significantly different from the value predicted by the model ($p > 0.1$, one sample t-test).

335 *Protein content*

336 Ammonium concentration was the most important factor affecting the protein content
337 of the cultures at 96 h of growth, with the protein content increasing as the ammonium
338 concentration increased (Figure 3b). The maximum protein content measured in our samples
339 was 52% of DW in treatment 7 (17.5 mM NH_4, 20.0 mM PO_4, 0.9 mM SO_4, and 5.0% CO_2),
340 closely followed by treatments 3 (17.5 mM NH_4, 10.0 mM PO_4, 0.9 mM SO_4, and 5.0% CO_2)
341 and 13 (20.0 mM NH_4, 15.0 mM PO_4, 0.55 mM SO_4, and 3.5% CO_2), where 48% of DW was
342 protein. These three media corresponded to a high ammonium concentration. The minimum
343 protein content was 12.4% of DW in treatment 12 (5.0 mM NH_4, 15.0 mM PO_4, 0.55 mM
344 SO_4, and 3.5% CO_2), corresponding to the lowest ammonium concentration tested.

345 Table 2 shows the terms retained in the final model of estimated protein content in the
346 cultures after 96 h of growth as a function of concentrations of NH_4, PO_4, SO_4, and CO_2. The
347 quadratic terms of ammonium and carbon dioxide concentrations were significant ($p <$
348 0.001). Significant positive interaction effects ($p < 0.001$) were observed between the
349 concentrations of ammonium and sulfate (Figure 4b), ammonium and carbon dioxide, as well
350 as between the concentrations of phosphate and sulfate. Phosphate concentration was not
351 significant ($p > 0.1$) as a main effect.

352 This model was used for optimization of the protein content in the cultures after 96 h
353 of growth and gave the following values: 20.0 mM NH_4, 22.5 mM PO_4, 1.0 mM SO_4, and 4.4
354 % CO_2 and it predicted the maximum protein content of 65% of DW. Figure 4b shows the
355 contour plot of the response surface using the fitted model (Table 2) and holding the levels of
356 the studied variables not displayed in the plot at their optimal values. The maximum response
357 can be seen in the top-right corner of Figure 4b. A model validation experiment with these
358 nutrient concentrations yielded a protein content of 64 ± 2% of the DW (mean ± SD, n = 3)
359 (Figure 5d). This was not significantly different from the value predicted by the model ($p >$
360 0.1, one sample t-test).

361 *Chlorophyll content*

362 Ammonium concentration was the most influential factor in relation to the chlorophyll
363 concentration at 96 h of growth (Figure 3c). The minimum chlorophyll concentration (9 µg
364 mL^{-1}) was measured at the lowest ammonium concentration in treatment 12 (5.0 mM NH_4,
365 15.0 mM PO_4, 0.55 mM SO_4, and 3.5% CO_2) and the maximum chlorophyll conentration (37

366 µg mL^{-1}) was measured at high ammonium concentration in treatment 3 (17.5 mM NH_4, 10.0
367 mM PO_4, 0.9 mM SO_4, and 5.0% CO_2). Phosphate and sulfate concentrations also strongly
368 influenced the chlorophyll concentration of the cultures (Figure 3c).

369 Table 2 shows the β-coefficients of the terms retained in the model for chlorophyll
370 concentration in the cultures after 96 h of growth as a function of concentrations of NH_4, PO_4,
371 SO_4, and CO_2. All the linear and quadratic terms were significant, as well as the interaction
372 terms of ammonium and sulfate concentrations ($p < 0.001$), ammonium and carbon dioxide (p
373 < 0.001), phosphate and sulfate ($p < 0.01$), and phosphate and carbon dioxide concentrations
374 ($p < 0.01$).

375 Hahn et al. (2004) showed that a chlorophyll concentration of 20 µg chl mL^{-1} was
376 optimal for H_2 production, giving the highest H_2 yields. We therefore applied the
377 optimization procedure to the chlorophyll concentration model in Table 2 to obtain 20 µg chl
378 mL^{-1}. The predicted nutrient concentrations for 20 µg chl mL^{-1} were 5.0 mM NH_4, 22.5 mM
379 PO_4, 0.6 mM SO_4, and 4.0% CO_2. A model validation experiment was carried out with these
380 nutrient concentrations, and the total chlorophyll concentration of the cultures after 96 h of
381 growth was 5.5 ± 0.5 µg mL^{-1} (mean ± SD, n = 3) (Figure 5e). This was almost four times
382 lower than predicted by the model.

Discussion

The present models indicate a strong effect of CO_2 concentration at an early growth stage (up to 67 h after the start of experiments) and a smaller, but still significant effect of CO_2 concentration on the biomass attained after 96 h of growth. This is in accordance with previous work on the same strain (Markina et al., submitted; Mortensen and Gislerød 2014; 2015). The strong effect of CO_2 concentration observed in this experiment at the early stage of growth could be related to the fact that the experiments were carried out successively over a period of seven weeks, with a possible difference in cell vitality and with only one treatment at 1.0 and 6.0% CO_2. However, it is also possible that the effect of a high CO_2 concentration could be very pronounced at an early stage of growth. Indeed, the concentration of dissolved CO_2 in our photobioreactors was twice as high at 6.0% CO_2 in the bubbled gas as it was at 1.0% (Markina et al., submitted). The difference in the availability of CO_2 between 1.0 and 6.0% was therefore much greater than the difference between 2.0 and 5.0% CO_2 in the bubbled gas. In their early growth stage, the cultures might be more sensitive to this difference in the availability of CO_2 than denser cultures (such as after 96 h of growth), which might have been limited by light or some of the nutrients in the growth medium.

Markina et al. (submitted) showed that the concentration of ammonium was the most influential on the starch content of the cultures grown at 7.5 and 17.5 mM NH_4. The present model confirmed this result, showing an almost linear curve, with the highest starch content in the cultures grown at 5.0 mM NH_4 and the lowest at 20.0 mM NH_4. This is in accordance with the results of Dragone et al. (2011), who found that the starch content (% of DW) was enhanced by decreasing the initial concentration of the nitrogen source in photoautotrophic batch cultures of *Chlorella vulgaris*. Similar results were obtained by Yao et al. (2012) in *Tetraselsmis subcordiformis* cultures and by Guo et al. (2014) in *Tribonema* sp. Ji et al. (2011) also found that *T. subcordiformis* cultures accumulated starch after two days of nitrogen starvation. *C. reinhardtii* cells are also known to accumulate starch under nitrogen starvation (Ball et al. 1990; Philipps et al. 2012; Siaut et al. 2011).

Sulfur deprivation is known to stimulate starch accumulation in several species of microalgae (Ball et al. 1990; Branyikova et al. 2010). Yao et al. (2012) found that decreasing sulfate concentration in the medium increased the starch content of *T. subcordiformis*

cultures. This was also the case in this study, although the effect of sulfate concentration on the starch content of the cultures was much less than the effect of ammonium.

The model predicted a significant negative interaction effect between the concentrations of ammonium and carbon dioxide on the starch content of the cultures, with the high starch content at low ammonium and high carbon dioxide concentrations. This could partly be related to the higher growth rate under high CO_2 and thus to a faster reduction of the nitrogen level in the growth medium. Markina et al. (submitted) showed that the level of nitrogen left in the medium after 96 h of growth was 2.8 mM or higher and, even at that nitrogen level, a high starch content (42% of DW) was observed in the cultures. This could be explained by an energy imbalance between carbon and nitrogen metabolism (Klok et al. 2013). A significant negative interaction effect on the starch content of the cultures was also predicted between the ammonium and sulfate concentrations, with the highest starch content predicted for low ammonium concentration, at low or high sulfate concentrations, and the lowest starch content predicted for high ammonium and sulfate concentrations (data not shown). The same negative interaction effects were observed by Markina et al. (submitted).

Protein and chlorophyll content varied in the opposite direction to the starch content under different growth conditions. Our models indicated that the content of both was positively affected by increasing concentrations of ammonium and sulfate. This is in accordance with the results by Lee et al. (2012), who found that increasing ammonium concentrations increased the cellular content of protein and chlorophyll in *C. reinhardtii* mixotrophic cultures. Protein content in *Tribonema* sp. displayed the same trend in the study by Guo et al. (2014). Ji et al. (2011) also found that *T. subcordiformis* cultures starved of sulfur or nitrogen showed a decreased protein content. Nitrogen starvation also reduced the cellular chlorophyll content in *C. reinhardtii* cells (Siaut et al. 2011). Moreover, Collier and Grossman (1992) showed that *Synechococcus* cells deprived of nitrogen, phosphorous, or sulfur were chlorotic in comparison to nutrient-replete cells.

The increased phosphate concentration increased the pH of the cultures. The low pH (5.9) observed at 7.5 mM PO_4 was probably due to high growth at that concentration, which was also reflected in the high chlorophyll concentration in the cultures.

This modeling provides mathematical tools for better understanding the physiology of *C. reinhardtii* photoautotrophic batch cultures. The present modeling confirms to a great extent the results of our previous study (Markina et al., submitted), namely that the different nutrient treatments had a much greater effect on the biochemical composition of the microalgae than on their growth. The maximum values of the response parameters reported in this study corresponded in some cases to boundary values of the experimental range and they may therefore not be the ultimate optimal values for these parameters. Moreover, the vitality of the microalgae may have been different over the seven-week period of the experiments. Furthermore, the maximum responses reported in this study are unique to a specific set of light and temperature conditions and there is thus room for further optimization.

Acknowledgements

This study was financially supported by the Research Council of Norway (Project 203281: Use of solar energy for CO_2 capture, algae cultivation and hydrogen production – BioH_2) and the Norwegian University of Life Sciences. We thank Leiv M. Mortensen and Jorunn E. Olsen for their valuable assistance during preparation of the manuscript and Dag Wenner and Silje E. Roksti for their technical assistance during the experiments.

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List of tables

Table 1. Experimental design. Four variables (x_1 – concentration of NH_4^+, x_2 – concentration of PO_4^{3-}, x_3 – concentration of SO_4^{2-} in the growth medium (mM), and x_4 – concentration of CO_2 in the bubbled gas (% v/v)) were combined in a central composite design, based on a 2^4 full factorial design with eight axial points and one central point, including five levels of each variable in 25 treatments. The treatments were divided into five blocks, and carried out successively, according to the CO_2 concentration. Experimental setup and coded levels of the studied variables for each treatment (A). Real values of the four studied variables (B).

Table 2. Estimated regression coefficients (β-coefficients in equation 2) of the model terms according to the ANOVA using data in uncoded units. x_1 – concentration of HN_4^+, x_2 – concentration of PO_4^{3-}, x_3 – concentration of SO_4^{2-} in the growth medium (mM), and x_4 – concentration of CO_2 in the bubbled gas (% v/v). The productivity (g L^{-1} d^{-1}) between 43 and 67 h of growth, the total biomass (dry weight (DW) in g L^{-1}) after 96 h of growth, starch and protein contents (% of DW), as well as total chlorophyll (a + b) concentration (µg mL^{-1}) after 96 h of growth of the *C. reinhardtii* cultures grown in the different media tested, were modeled. The experiments were divided into five blocks and carried out successively, according to the CO_2 concentration. The effect of the CO_2 concentration and the effect of the block (time) were therefore confounded. Significance levels: ^, $p < 0.10$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Not significant, $p > 0.10$ (not indicated).

Tables

Table 1. Experimental design. Four variables (x_1 – concentration of NH_4^+, x_2 – concentration of PO_4^{3-}, x_3 – concentration of SO_4^{2-} in the growth medium (mM), and x_4 – concentration of CO_2 in the bubbled gas (% v/v)) were combined in a central composite design, based on a 2^4 full factorial design with eight axial points and one central point, including five levels of each variable in 25 treatments. The treatments were divided into five blocks and carried out successively, according to the CO_2 concentration. Experimental setup and coded levels of the studied variables for each treatment (a). Real values of the four studied variables (b).

a

Block	Treatment	Coded levels			
		x_1	x_2	x_3	x_4
1	1	-1	-1	-1	1
1	2	1	-1	-1	1
1	3	1	-1	1	1
1	4	-1	-1	1	1
1	5	-1	1	-1	1
1	6	1	1	-1	1
1	7	1	1	1	1
1	8	-1	1	1	1
2	9	0	0	0	2
3	10	0	0	0	-2
4	11	0	0	0	0
4	12	-2	0	0	0
4	13	2	0	0	0
4	14	0	-2	0	0
4	15	0	2	0	0
4	16	0	0	-2	0
4	17	0	0	2	0
5	18	-1	-1	-1	-1
5	19	1	-1	-1	-1
5	20	1	-1	1	-1
5	21	-1	-1	1	-1
5	22	-1	1	-1	-1
5	23	1	1	-1	-1
5	24	1	1	1	-1
5	25	-1	1	1	-1

b

Variable	Compound	Real value				
		-2	-1	0	1	2
x_1	NH_4^+ (mM)	5.0	7.5	12.5	17.5	20.0
x_2	PO_4^{3-} (mM)	7.5	10.0	15.0	20.0	22.5
x_3	SO_4^{2-} (mM)	0.1	0.2	0.55	0.9	1.0
x_4	CO_2 (%)	1.0	2.0	3.5	5.0	6.0

623

Table 2. Estimated regression coefficients (β-coefficients in equation 2) of the model terms according to the ANOVA using data in uncoded units. x_1 – concentration of HN_4^+, x_2 – concentration of PO_4^{3-}, x_3 – concentration of SO_4^{2-} in the growth medium (mM), and x_4 – concentration of CO_2 in the bubbled gas (% v/v). The productivity (g L^{-1} d^{-1}) between 43 and 67 h of growth, the total biomass (dry weight (DW) in g L^{-1}) after 96 h of growth, starch and protein contents (% of DW), as well as total chlorophyll (a + b) concentration (µg mL^{-1}) after 96 h of growth of the *C. reinhardtii* cultures grown in the different media tested, were modeled. The experiments were divided into five blocks and carried out successively, according to the CO_2 concentration. The effect of the CO_2 concentration and the effect of the block (time) were therefore confounded. Significance levels: ^, p < 0.10; *, p < 0.05; **, p < 0.01; ***, p < 0.001. Not significant, p > 0.10 (not indicated).

Variable	Productivity (g L^{-1} d^{-1})	Biomass (DW in g L^{-1})	Starch (% DW)	Protein (% DW)	Chlorophyll (µg mL^{-1})
Constant	-0.273***	1.426***	28.016***	13.381***	31.087***
x_1	0.017	0.006***	0.356***	2.899***	1.358***
x_2		-1.12***	-0.689	-0.504	-4.814^
x_3	0.786**	1.13***	-12.099***	-45.905**	29.653***
x_4	0.271***	0.203**	4.207***	4.515^	2.474*
x_1^2		-0.001^		-0.14***	-0.072**
x_2^2		0.003***			0.173***
x_3^2	-0.604**	-1.56***	31.532***		-53.108***
x_4^2	-0.019**	-0.036***		-1.466***	-0.523*
x_1x_3		0.038***	-1.643***	2.567***	1.839***
x_1x_4	-0.005*	0.006**	-0.39***	0.414***	0.277***
x_2x_3		0.023**		1.216***	0.737**
x_2x_4			0.22**		-0.189**
x_3x_4			-2.41*		

List of figures

Figure 1. Main effects of the concentrations of NH_4, PO_4, SO_4, and CO_2 on the pH of *C. reinhardtii* cultures after 96 h of growth (a), on the productivity (g L^{-1} d^{-1}) of *C. reinhardtii* cultures between 43 and 67 h of growth (b), and on the attained biomass (dry weight in g L^{-1}) of *C. reinhardtii* cultures after 96 h of growth (c). The lines connect the average values of the measured parameter of the cultures for each level of the studied variables.

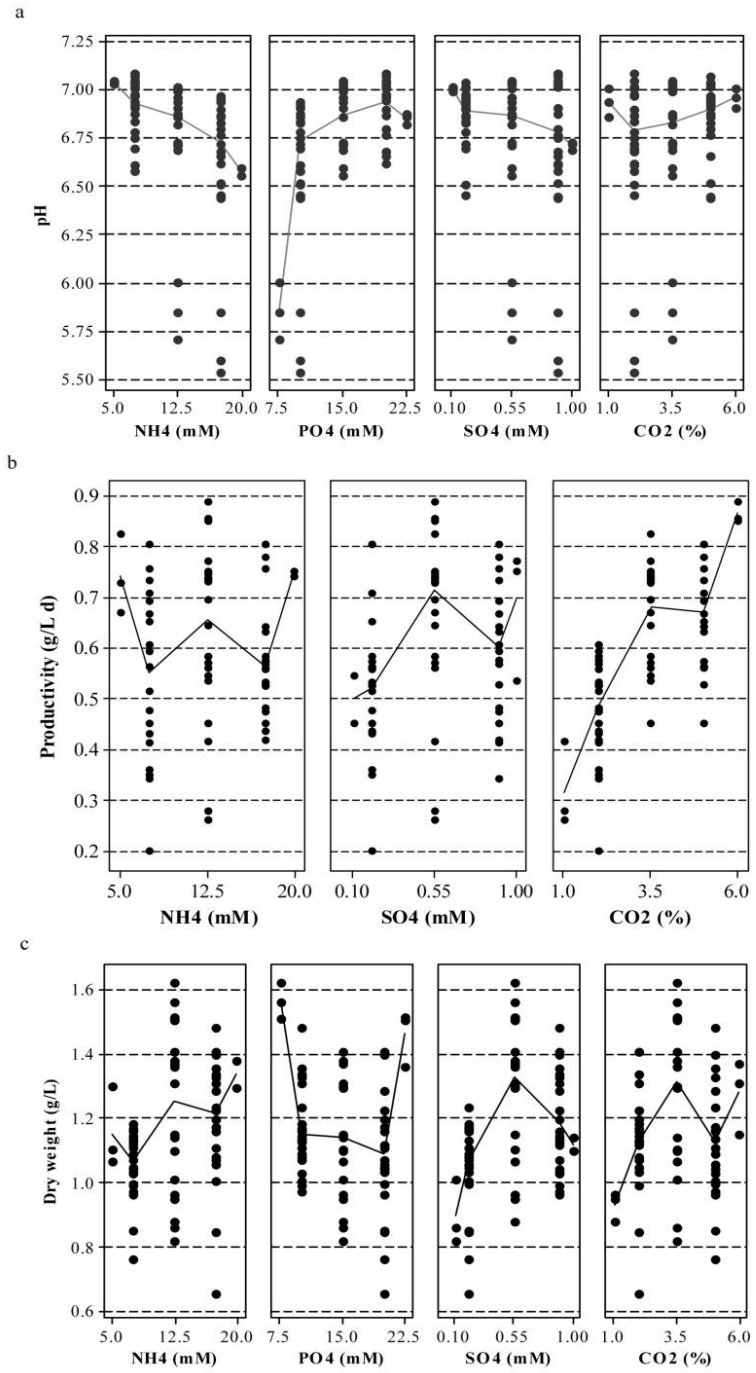
Figure 2. Contour plot of the response surface of productivity (P in g L^{-1} d^{-1}) of *C. reinhardtii* cultures between 43 and 67 h of growth vs. concentrations of NH_4 and CO_2, with the SO_4 concentration being held constant at its optimum (0.65 mM) (a), and of the biomass (dry weight (DW) in g L^{-1}) attained by the *C. reinhardtii* cultures after 96 h of growth vs. concentrations of CO_2 and SO_4, with the concentrations of NH_4 and PO_4 being held constant at their optimum (20.0 mM and 7.55 mM, respectively) (b). The optimization procedure predicted a maximum productivity of 0.87 g L^{-1} d^{-1} for the optimal values of the studied variables (5.0 mM NH_4, 0.65 mM SO_4, and 6.0% CO_2). The maximum predicted dry weight was 1.66 g L^{-1} for the optimal values of the four variables (20.0 mM NH_4, 7.5 mM PO_4, 0.66 mM SO_4, and 4.5% CO_2).

Figure 3. Main effects of the concentrations of NH_4, PO_4, SO_4, and CO_2 on the starch (% of DW) (a), protein (% of DW) (b), and total chlorophyll (µg mL^{-1}) (c) contents of *C. reinhardtii* cultures after 96 h of growth. The lines connect the average values of the measured parameter of the cultures for each level of the studied variables.

Figure 4. Contour plot of the response surface of starch content (% of DW) in the *C. reinhardtii* cultures after 96 h of growth vs. concentrations of CO_2 and NH_4, with the concentrations of PO_4 and SO_4 being held constant at their optimum (22.5 mM and 0.1 mM, respectively) (a), and of the protein content (% of DW) in the *C. reinhardtii* cultures after 96 h of growth vs. concentrations of SO_4 and NH_4, with the concentrations of PO_4 and CO_2 being held constant at their optimum (22.5 mM and 4.4%, respectively) (b). The optimization procedure predicted a maximum starch content of 55% of DW for the optimal values of the four nutrients (5.0 mM NH_4, 22.5 mM PO_4, 0.1 mM SO_4, and 6.0% CO_2). The maximum predicted protein content was 65% of DW for the optimal values of the four nutrients (20.0 mM NH_4, 22.5 mM PO_4, 1.0 mM SO_4, 4.4 % CO_2).

665 Figure 5. Growth curves accompanied by the evolution of pH; the starch, protein, and
666 chlorophyll contents in *C. reinhardtii* cultures grown in the optimum predicted media for
667 maximum productivity (a), maximum biomass (b), maximum starch (c), maximum protein
668 (d), and 20 μg mL^{-1} chlorophyll (e).

669 **Figures**



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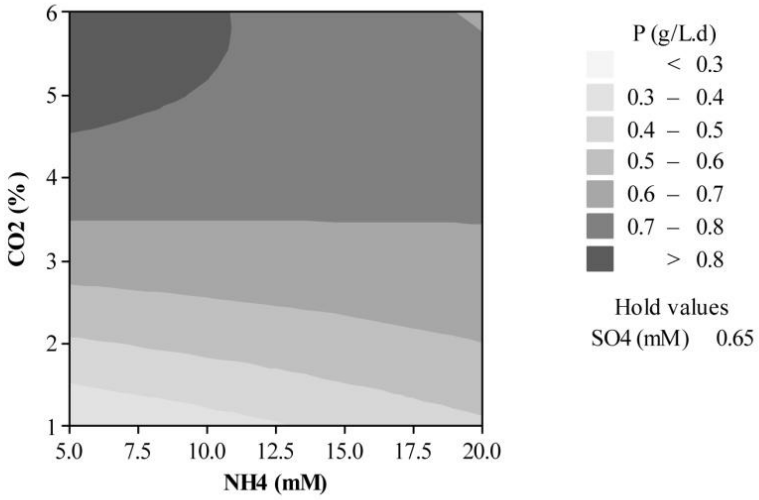
675 measured parameter of the cultures for each level of the studied variables.

a



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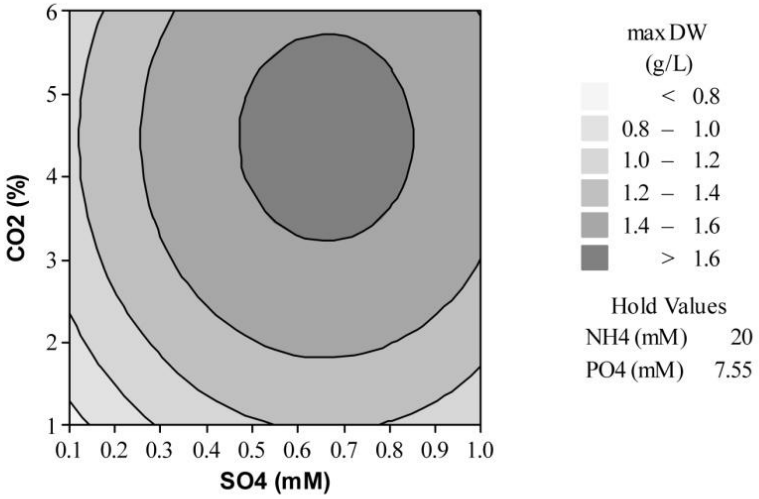

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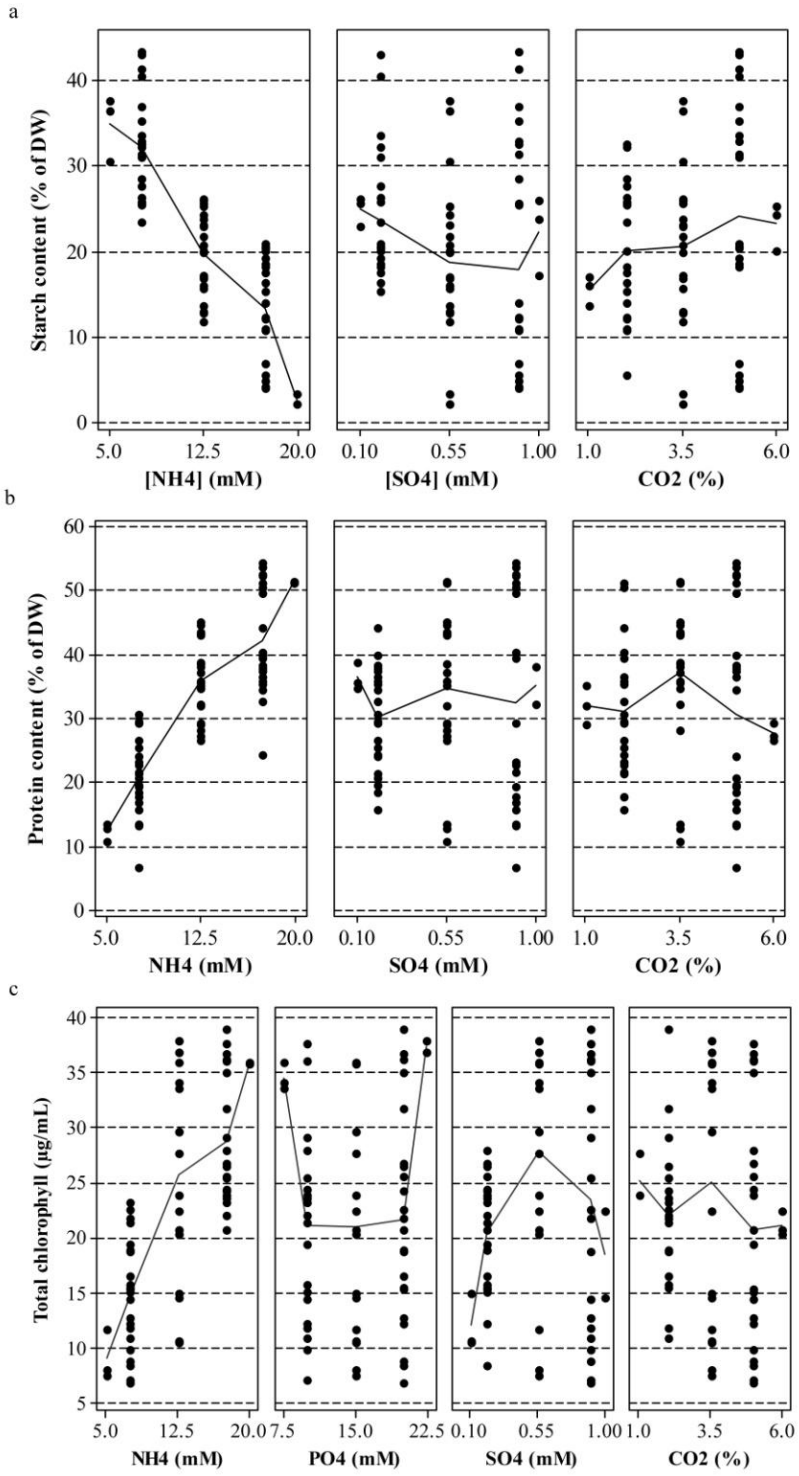

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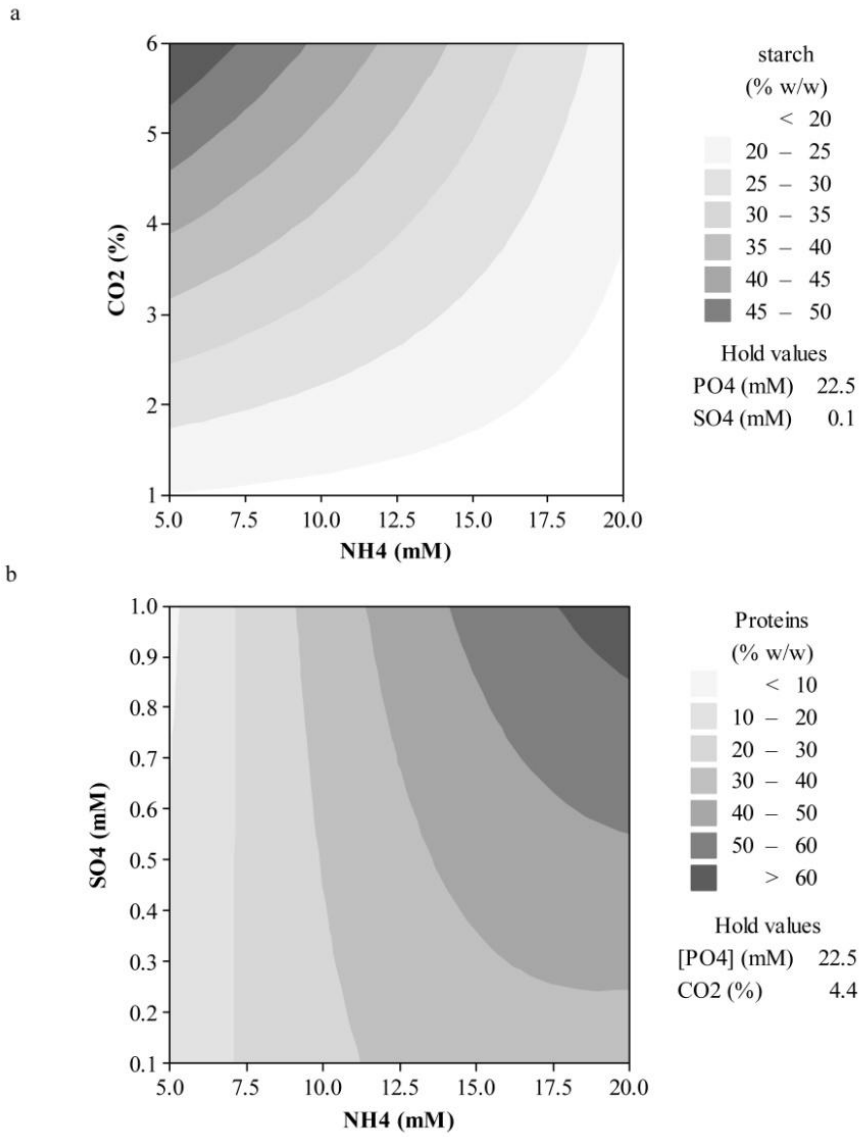

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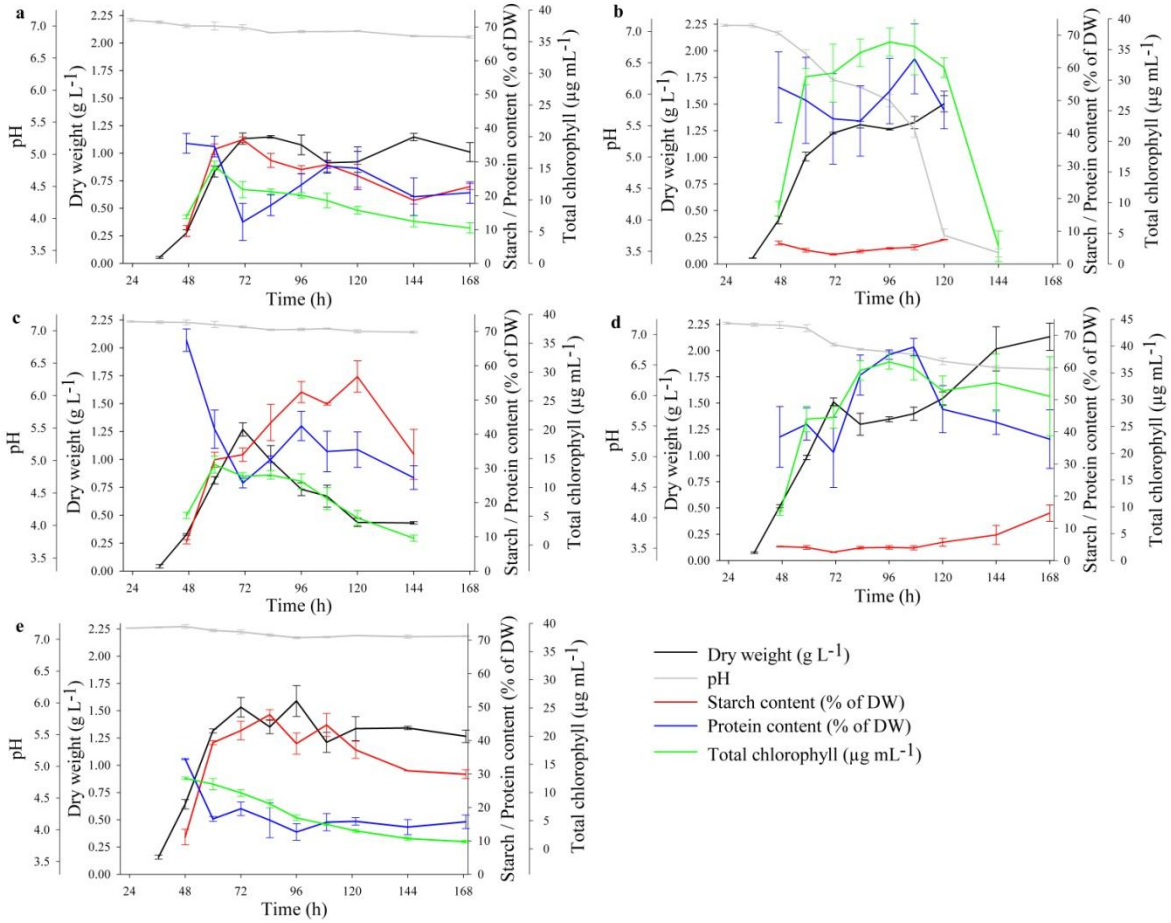

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705 chlorophyll contents of *C. reinhardtii* cultures grown in the optimum predicted media for
706 maximum productivity (a), maximum biomass (b), maximum starch (c), maximum protein
707 (d), and 20 µg mL^{-1} chlorophyll (e).

Paper III

Effects of light intensity, temperature, and carbon dioxide concentration on the photoautotrophic growth and biochemical composition of *Chlamydomonas reinhardtii*

Daria Markina*, Hans Ragnar Gislerød

Department of Plant Sciences, Norwegian University of Life Sciences, P.O. Box 5003, 1432, Ås, Norway

* Corresponding author, e-mail: daria.markina@nmbu.no, dmarkina@gmail.com, tel.: (+47) 64 96 61 38, fax: (+47) 64 96 56 15

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Abstract

The green microalga *Chlamydomonas reinhardtii* is able to produce hydrogen under sulfur-deficient anaerobic conditions. The efficiency of hydrogen production depends on the biomass concentration and starch, protein, and chlorophyll contents of the cultures. The aim of this study was to investigate the effects of the environmental factors on photoautotrophic growth and biochemical composition of *C. reinhardtii* batch cultures. Light intensity (100 and 400 µmol m^{-2} s^{-1}), temperature (25 and 35 °C), and CO_2 concentration (3 and 9% v/v) were combined in a 2^3 full factorial design. Both light intensity and temperature significantly enhanced the growth of the cultures: the maximum productivity (1.12 g L^{-1} d^{-1}) was measured in the treatments with 400 µmol m^{-2} s^{-1} and 9% CO_2. At 100 µmol m^{-2} s^{-1}, the biomass yield was higher at 3% CO_2. The chlorophyll content decreased with increasing light intensity. The starch content of the cultures did not exceed 11% of dry weight, probably due to nutrient sufficiency of the growth medium. The protein content increased from 30% of dry weight at 100 µmol m^{-2} s^{-1} to 41% of dry weight at 400 µmol m^{-2} s^{-1}, and reached 43% of dry weight in the treatment with 400 µmol m^{-2} s^{-1}, 25 °C, and 9% CO_2. The results of this study gave us a better understanding of the interaction effects between light intensity, temperature, and CO_2 concentration, which allows for better control of culture conditions.

Key-words

Carbon dioxide, *Chlamydomonas reinhardtii*, chlorophyll, light intensity, photoautotrophic growth, protein, starch, temperature.

Introduction

Chlamydomonas reinhardtii is a model organism used in biohydrogen production research (Antal et al. 2003; Fouchard et al. 2005; Hemschemeier et al. 2008; Kosourov et al. 2007; Kosourov et al. 2003; Kosourov et al. 2002; Laurinavichene et al. 2004; Laurinavichene et al. 2002; Melis et al. 2000; Posewitz et al. 2004; Ruhle et al. 2008; Tolstygina et al. 2009; Tsygankov et al. 2006; White and Melis 2006; Zhang et al. 2002). For hydrogen production, high cell density cultures are required (Tamburic et al. 2011). The biochemical content of the cultures also plays an essential role in this process (Burrows et al. 2008; Chochois et al. 2009; Doebbe et al. 2010; Giannelli et al. 2009; Hahn et al. 2004; Jo et al. 2006; Lehr et al. 2012; Melis et al. 2000; Morsy 2011; Philipps et al. 2012; Posewitz et al. 2004; Tamburic et al. 2011; White and Melis 2006; Zhang et al. 2002). Namely, high cell contents of starch and protein are desirable (Melis et al. 2000; Zhang et al. 2002). The chlorophyll concentration in the culture is as well highly influential on the hydrogen production yield (Giannelli et al. 2009; Hahn et al. 2004).

Environmental growth conditions are key factors that control both the growth and the biochemical composition of microalgae. Light is often considered as the most important factor affecting microalgal growth since it is the only energy source for photoautotrophic organisms. It is also the most difficult factor to control with high cell density cultures in photobioreactors due to the self-shading effect (Giannelli et al. 2009; Richmond et al. 2003). The microalgal cells are exposed to rapidly varying levels of light intensity due to the mixing of the cultures (Grobbelaar 1991; Ogbonna et al. 1995; Perner-Nochta and Posten 2007). The major physiological outcome of the light-shade adaptation is the modification of growth rates with variations in light intensity, due to the variations in the photosynthetic rates (Falkowski 1980; Sorokin and Krauss 1958). The growth-irradiance curves are therefore similar to photosynthesis-irradiance curves (Falkowski 1980). Variation of growth rate under light limitation of photosynthesis has relatively little effect on the overall cellular composition (Falkowski and Owens 1980). However, there is often a tendency to accumulate more carbohydrates at higher light intensity levels (Falkowski and Owens 1980; Richmond 2004). This is due to the excess absorbed energy by the photosystems that the cells dissipate by directing it towards carbon fixation and synthesis of energy-rich carbohydrates (Hu 2004; Klok et al. 2013). Another response to high light intensity levels is the decrease in the contents of photosynthetic pigments per cell, especially of chlorophylls *a* and *b* (Falkowski

1980; Falkowski and Owens 1980; Falkowski and Raven 2007; Geider et al. 1996; Geider et al. 1998). This response is a cell strategy to reduce the amount of absorbed energy and avoid photoinhibition when exposed to high light intensity (Baker and Bowyer 1994; Falkowski 1980; Falkowski and Owens 1980; Falkowski and Raven 2007; Melis et al. 1998). Conversely, when microalgae are exposed to low light intensity, the amount of pigments per cell increases so that the cells are able to harvest more of the available light energy from the environment (Eppley and Sloan 1966; Falkowski 1980; Falkowski and Owens 1980; Falkowski and Raven 2007). This ability of microalgae to adjust the amount of pigments in their photosystems to the amount of light they are exposed to is commonly known as photoacclimation (Falkowski and La Roche 1991). Photoacclimation also affects carbon fixation, respiration rates, biochemical composition of the organism, as well as cell volume (Falkowski and Raven 2007). Through these changes, photoacclimation leads to an increase in photosynthetic efficiency and, thus, to an optimization of light harvesting and utilization (Dubinsky et al. 1995; Falkowski and Raven 2007).

Temperature also influences the growth and the biochemical composition of microalgal cells (Carvalho and Malcata 2003; Carvalho et al. 2009; Eppley 1972; Goldman and Carpenter 1974; Goldman and Mann 1980; Li 1980; Raven and Geider 1988; Renaud et al. 2002; Thompson 1999; Thompson et al. 1992; Yoder 1979). Temperature affects cellular processes to a large extent through control of photosynthetic rate (Coles and Jones 2000; Davison 1991; Lindström 1984; Setlík et al. 1972; Spudich and Sager 1980; Zachleder and van den Ende 1992). While the light-driven redox reactions of photosynthesis are influenced mainly by the amount of available light, the carbon fixation reactions of photosynthesis are temperature-dependent enzymatic reactions. Growth at the optimum temperature results in maximal growth rate, but in minimal cell size, and carbon and nitrogen contents (Rhee 1982). Temperature also affects cell composition, short-term nutrient uptake, and, in particular, nitrogen metabolism (Eppley 1972; Morris et al. 1974; Sakamoto and Bryant 1999; Terry 1983; Thompson et al. 1992; Yoder 1979). Berges et al. (2002) observed that cellular carbon and nitrogen contents, protein, chlorophyll *a*, and C:N ratio increased with increasing temperature from 17 to 25°C in *Thalassiosira pseudonana* cultures.

Equilibration of CO_2 between aqueous solution and the gas phase is relatively slow. In addition, CO_2 diffusion in aqueous solution is about 10^4 times slower than in air (Raven 1977). Therefore microalgal cultures are often bubbled with gas containing relatively high concentration of CO_2, in the range of 1 to 5% v/v. It has been shown that *C. reinhardtii* cells

grown with high CO_2 (5% v/v) take up CO_2 from the medium, but not HCO_3^-, while cell grown with air take up both, as part of the carbon dioxide concentrating mechanism (Badger et al. 1977; Badger et al. 1980; Moroney and Tolbert 1985; Moroney and Ynalvez 2007). It was postulated that the cells grown at high CO_2 do not synthesize an inorganic carbon pump responsible for HCO_3^- uptake (Moroney and Tolbert 1985), while CO_2 is taken up via passive diffusion (Berry et al. 1976). Carbon fixed by the cell can be utilized for respiration, as an energy source, and as raw material in the formation of new cells (Berman-Frank and Dubinsky 1999). Elevated CO_2 concentrations have been found to decrease the relative concentrations of proteins and pigments in the cells, but increase carbohydrate content (Gordillo et al. 1999).

The effects of light intensity, temperature, and CO_2 concentration on microalgal growth and cell composition are known to be synergistic (Carvalho and Malcata 2003; Carvalho and Malcata 2005; Carvalho et al. 2009; Eppley and Sloan 1966; Sandnes et al. 2005; Sorokin and Krauss 1962; Suzuki et al. 1991). The levels of temperature and CO_2 are key factors for high light use efficiency related to growth (Mortensen 2004). The optimum light intensity for growth increases with increase in temperature (Shelef 1968; Sorokin and Krauss 1962). High light intensity and temperature promote carbon fixation since this pathway acts as a sink for excess energy (Parker and Armbrust 2005). On the other hand, at high light intensity and low temperature cells become more sensitive to photoinhibition (Coles and Jones 2000; Megard et al. 1984; Morgan and Kalff 1979; Tjahjono et al. 1994; Vonshak et al. 2001). On the other hand, the effect of CO_2 concentration on microalgal growth strongly depends on the light intensity (Carvalho and Malcata 2005). Limitation in the supply of light energy constrains the rate of transport and assimilation of inorganic carbon, so cells grown under light-limited condition exhibit reduced capacity to accumulate carbon (Beardall et al. 1998). In addition, the availability of CO_2 in aqueous solution and the uptake of CO_2 by the cell are temperature-dependent, since temperature is the main factor affecting diffusion.

Understanding the interaction effects between light intensity, temperature, and CO_2 concentration is essential for optimization of microalgal growth in controlled production systems. The use of factorial experimental designs permits to quantify the effect of each variable independently as well as their interactions. In this study, we combined two levels of light intensity (100 and 400 μmol m^{-2} s^{-1}), two CO_2 concentrations (3 and 9% v/v), and two

127 levels of temperature (25 and 35 °C) in a 2^3 full factorial design experiment. We quantified
128 their effects on the growth of *C. reinhardtii* batch cultures and their contents of starch,
129 protein, and total chlorophyll (% of DW).

Materials and methods

Organism and culture conditions

The *Chlamydomonas reinhardtii* 137c strain was obtained from the NIVA culture collection, Norway (CHL153). The microalgae were stored in sterile conditions on a 1.5% agar Tris-Acetate-Phosphate (TAP) (Gorman and Levine 1965) solid growth medium (Andersen 2005) on Petri dishes at room temperature and low light intensity. The microalgae were pre-cultured photoautotrophically in the experimental medium (Table 1) based on the High-Salt Sueoka (HS) medium (Sueoka 1960) at 25 °C and continuously illuminated by cool white fluorescent tubes (Philips Master TLD 36W/840 Reflex, Eindhoven, the Netherlands) at a photon flux density (PFD) of 100 µmol m^{-2} s^{-1}.

Experimental setup

Two levels of light intensity (100 and 400 µmol m^{-2} s^{-1}), two concentrations of CO_2 in the bubbled gas (3 and 9% v/v), and two levels of temperature (25 and 35 °C) were combined in a 2^3 full factorial design, as shown in Table 2, with a total of eight treatments. Each treatment was carried out in three parallels. The treatments were divided into two blocks and carried out successively, at two different levels of light intensity. A control treatment (n = 3) with 100 µmol m^{-2} s^{-1}, 25 °C, and 3% CO_2 was carried out in parallel to the treatments at 400 µmol m^{-2} s^{-1}. A simple t-test showed that there was no significant ($p > 0.05$) differences between the measured dry weights of these control cultures compared to the cultures in treatment 1, with the same conditions.

Sterile batch cultures of *C. reinhardtii* cells were carried out in Pyrex glass tubular (coned at the bottom) photobioreactors (PBRs) with an inner diameter of 3.5 cm and a total volume capacity of 380 mL. The experimental medium was a modified HS medium. The concentrations of ammonium, phosphate, and sulfate in this medium were determined using our previous results (Markina et al., submitted) and optimizing the medium composition to obtain maximum productivity as well as maximum biomass (dry weight) yield. Table 1 shows the detailed composition of the growth medium used. The initial pH of the medium was adjusted to 7.50 (pH-meter Orion 420A+, Thermo electron corporation, Waltham, MA, USA) with 1 M NaOH prior to sterilization by autoclaving. $NaHCO_3$ was used in the medium as a pH buffer, at a concentration of 2 mM per percent CO_2 in the bubbled gas. At the start of the experiments (t = 0 h), cells from pre-grown cultures at the linear growth phase were

inoculated into 300 mL of medium at a cell density of 10^4 cells mL^{-1}. The cells were counted under a microscope, using a Fuchs-Rosenthal counting chamber.

Cool white fluorescent tubes, placed at approximately 15 cm from the PBRs, were used to provide continuous illumination from one side at a PFD of 100 µmol m^{-2} s^{-1} and 400 µmol m^{-2} s^{-1}, according to the experimental design (Table 2). The light intensity was measured using a LI-COR model LI-190SA instrument (LI-COR, Lincoln, NE, USA) with a quantum sensor (400 – 700 nm) inside an empty PBR immersed in the water-bath aquarium, and the average PFD over the height of the PBR was estimated.

Filtered (Acro 37 TF Vent Device, 0.2 µm PTFE membrane filters, PALL Life Sciences, Port Washington, NY, USA) air containing 3 and 9% v/v CO_2, according to the experimental design, shown in Table 2, was injected at the bottom of the PBRs (the bubble size was approximately 3 mm). The CO_2 concentration in the gas mixture was recorded every five minutes by the PicoLog software (Picolog, Pico Technology, St Neots, UK). The concentration of dissolved CO_2 in three parallel PBRs filled with 300 mL of experimental growth medium at 25 °C was measured using hand-held titration cells for titrimetric analysis (CHEMetrics Inc., Midland, VA, USA). The dissolved CO_2 concentration was 130 ± 5 mg L^{-1} at 3% of CO_2 in the bubbled gas and 187 mg L^{-1} at 9% CO_2, at 25 °C. These two concentrations were significantly different at $p < 0.001$. Increase in temperature to 35 °C did not significantly ($p > 0.1$) influence the dissolved CO_2 concentration.

The temperature was maintained at a 25.0 ± 0.5 °C or 35.0 ± 0.5 °C, according to the experimental design (Table 2) using water baths in aquaria. The room temperature was maintained at 15 ± 2 °C and the water in the aquaria heated to the desired temperature by tubular aquarium heaters (Eheim Jager 3619, Eheim, Daizisau, Germany). The temperature in each aquarium was recorded every five minutes by the PicoLog software.

The pH, the optical density (OD at λ = 750 nm, Helios α, Thermo Fisher Scientific, Waltham, MA, USA), and the dry weight (DW in g L^{-1}) of the cultures were measured every 12 h after 24 h from the start of the experiment. Samples for chlorophyll, starch (2 mL), and protein (1 mL) assays were collected at 60 and 96 h of growth, except for the treatments 5, 6, and 8, where they were collected at 48 and 72 h, due to faster growth. These samples were centrifuged at 20800 x *g*, (Eppendorf centrifuge 5417R, Eppendorf, Hamburg, Germany), at 4 °C, for 20 min and the cell pellets stored at -20 °C until analysis.

Biomass determination

To collect the biomass, 0.3 – 2.0 mL of microalgal culture was filtered through pre-washed GF/F glass microfiber filters (0.7 µm pore size, 25 mm diameter, GE Healthcare, Whatman, UK) dried at 103.5 °C. The filters containing biomass were then washed with 30 mL deionized water to remove adhering salts, and thereafter dried for 4 h at 103.5 °C. The DW (g L^{-1}) of the sample was calculated by subtracting the DW of the empty filter from the DW of the filter with biomass. It was normalized to the culture volume filtered. The productivity (P) of the cultures (g L^{-1} d^{-1}) was calculated using formula (1), where $DW_{1\ (2)}$ is the DW determined at time $t_{1\ (2)}$ (in days).

$$P = \frac{DW_2 - DW_1}{t_2 - t_1}$$

Chlorophyll assay

Chlorophyll was extracted from 1-2 mL samples using 95% ethanol and assayed using the method described by Harris (1989). The chlorophyll concentration was determined by optical density (OD) measurements (Heλios α, Thermo Fisher Scientific, Waltham, MA, USA) at λ = 649 nm and λ = 665 nm.

Starch assay

Cell pellets of 2 mL samples left after chlorophyll assay were used for starch assay. The Megazyme total starch (the amyloglucosidase/*α*-amylase method) assay kit (K-TSTA, Megazyme International, Bray, Ireland) procedure was adapted to a small sample volume. 20 µL of 80% ethanol was added to the cell pellet and the pellet was dispersed using a vortex mixer. Cells were disrupted by adding 0.2 mL of DMSO (Sigma-Aldrich, St. Louis, MO, USA) and 0.5 mL of 0.5 mm glass beads (Cell disruption media, SI-BG05, Scientific Industries Inc., New York, NY, USA) to the samples and shaking them on a mixer mill (MM301, Retsch, Haan, Germany) at 30 Hz frequency for 10 min. Samples were heated on thermos-shakers (TS-100, Biosan, Riga, Latvia) to 100 °C for 5 min for starch solubilization. Then, 0.3 mL of *α*-amylase (diluted 1:30 according to the Megazyme starch assay kit specifications) was added to the samples and the tubes incubated at 100 °C for 12 min. The contents of the tubes were mixed vigorously after 0, 4, 8, and 12 min. The tubes were placed at 50 °C and 0.4 mL of sodium acetate buffer (200 mM, pH 4.5) was added to the samples, followed by 10 µL of amyloglucosidase (20U). The samples were thoroughly mixed and

incubated at 50 °C for 30 min. Then, 70 µL of distilled water was added to the samples in order to adjust the total sample volume to 1.0 mL. The contents of the tubes were mixed thoroughly and centrifuged at 20800 x *g* (Eppendorf centrifuge 5417R, Eppendorf, Hamburg, Germany) for 10 min at 20 °C. Then, 33.4 µL of the supernatant was added to 1 mL GOPOD reagent and incubated at 50 °C for 20 min. The same procedure was used for two reagent blanks (33.4 µL distilled water) and four glucose controls (33.4 µL D-glucose standard solution at 1 mg mL^{-1}). The OD of each sample and of the glucose controls was measured at λ = 510 nm against the reagent blank. Starch concentration was calculated as the percentage of DW of the samples according to the formula supplied in the Megazyme starch assay kit.

Protein assay

Cell pellets of 1 mL samples left after chlorophyll assay were used for protein assay. The cells were disrupted using 0.5 mL of 0.5 mm glass beads (Cell disruption media, SI-BG05, Scientific Industries Inc., New York, NY, USA) and shaken on a mixer mill (MM301, Retsch, Haan, Germany) for 10 min at 30 Hz frequency. Then, Tris-HCl solubilization buffer (50 mM, pH 8.3) containing 1% Triton was used to solubilize the proteins. The Lowry method (1951) was applied using the Bio-Rad *DC* protein microplate assay kit II (#500-0112, Bio-Rad, Hercules, CA, USA) to determine protein concentration in the samples. The OD (λ = 750 nm) of the samples assayed on a microplate (CellBIND surface, Costar 3300, Corning Incorporated, Corning, NY, USA) was measured using a microplate spectrophotometer (Asys UVM340, Biochrom, Cambridge, UK) and Kim software (Kim32, 2004, Asys Hitech GmbH, Cambridge, UK). A BSA standard curve (8 points in the 0.0 - 0.75 mg mL^{-1} range) was used to calculate the protein concentration in the samples. The protein concentration was normalized to the DW of the culture and calculated as the percentage of DW.

Statistical analysis

The data were analyzed using the analysis of variance (ANOVA) procedure in the MINITAB software (V16, Minitab Inc., State College, PA, USA). The figures were created using SigmaPlot version 9.0 (Systat software Inc., San Jose, CA, USA).

Results

The pH of the cultures decreased progressively with the time and the growth of the microalgae (Figure 1). The Pearson correlation coefficients between the pH and the dry weight of the cultures were negative and significant ($p < 0.05$) after 60 h of growth. A significant drop in pH (down to < 4.0) was observed after 72 h (at 35 °C) and 96 h (25 °C) in the treatments with 400 µmol m^{-2} s^{-1} and 3% CO_2 and these cultures died (Figure 1). The pH in the other treatments was in the range of 5.3 to 7.7 (Figure 1). Both light intensity and temperature, as well as their interaction, had a significant negative effect on the pH of the cultures, throughout the experiments (Table 3).

The cultures grown at 400 µmol m^{-2} s^{-1} and 35 °C started to grow earlier and grew faster than the cultures grown at 100 µmol m^{-2} s^{-1} (Figure 2), with the maximum productivity of 1.12 g L^{-1} d^{-1} measured in the treatment with the highest levels of all three factors between 36 and 48 h of growth. The highest productivity of the cultures in the treatment with 400 µmol m^{-2} s^{-1}, 9% CO_2, and 25 °C was not significantly ($p > 0.05$) different from 1.12 g L^{-1} d^{-1}, but it was measured 24 h later, between 60 and 72 h of growth. The minimum productivity of 0.45 g L^{-1} d^{-1} was measured in the treatment with 100 µmol m^{-2} s^{-1}, 9% CO_2, and 25 °C between 48 and 72 h of growth. The dry weight of the cultures increased during the first 96 h of growth, after which the cultures entered into a stationary growth phase (Figure 2). The maximum biomass (1.35 g L^{-1}) was measured in the same treatment as for the maximum productivity with 400 µmol m^{-2} s^{-1}, 9% CO_2, and 35 °C, with the highest levels of all three factors; and the minimum attained biomass (0.76 g L^{-1}) in the same treatment as for minimum productivity with 100 µmol m^{-2} s^{-1}, 9% CO_2, and 25 °C. The light intensity and the temperature, as well as their interaction had significant positive effects on the dry weight of the cultures, especially at the early stage of the cultures (Table 3). There was a significant ($p < 0.001$) positive interaction effect between CO_2 concentration and temperature on the dry weight in the early stage of the cultures (Table 3).

The starch content of the cultures was low (< 11% of DW) (Figure 3a). The starch content increased between the first and the second sampling at 25 °C, but decreased in the treatments with 35 °C, except for the treatment with 100 µmol m^{-2} s^{-1} and 3% CO_2 (Figure 3a). The lowest starch content (2.4% of DW) was recorded in the treatments with 100 µmol m^{-2} s^{-1} and 25 °C at 60 h of growth. The highest starch content (10.8% of DW) was measured in treatment 5 (400 µmol m^{-2} s^{-1}, 3% CO_2, 25 °C) at 72 h of growth, just before these cultures

died. Light intensity and temperature had a significant ($p < 0.001$) positive effect on the starch content in the first sampling, however, their interaction was negative (Table 3).

The protein content varied between 16% of DW in the treatment with 100 µmol m^{-2} s^{-1}, 9% CO_2, and 25 °C at 60 h of growth and 43% of DW in the treatment with 400 µmol m^{-2} s^{-1}, 9% CO_2, and 25 °C at 96 h of growth (Figure 3b). The protein content of the cultures increased between the first and the second sampling times (Figure 3b) and it was significantly ($p < 0.001$) higher at 400 µmol m^{-2} s^{-1} than at 100 µmol m^{-2} s^{-1}, at both sampling times (Table 3).

The total chlorophyll (*a* and *b*) content varied between 1.7 and 3.9% of DW (Figure 3c). The total chlorophyll content in the cultures was significantly ($p < 0.05$) higher at 100 µmol m^{-2} s^{-1} than at 400 µmol m^{-2} s^{-1} (Figure 3c, Table 3). CO_2 concentration also had a significant ($p < 0.001$) negative effect on the chlorophyll content at the first time of sampling, while the effects of the temperature and its interactions with light intensity and CO_2 concentration were significantly ($p < 0.05$) positive (Table 3).

Discussion

The growth of the cultures was positively influenced by the light intensity and the temperature, and their interaction. The maximum productivity (1.12 g L^{-1} d^{-1}) was measured in the treatment with the highest level of all three studied variables. This is in accordance with the results of Lien and Knutsen (1979), who concluded that 390 µmol m^{-2} s^{-1} and 35 °C were the optimal conditions for *C. reinhardtii* synchronous cultures. Mortensen and Gislerød (2014) compared the productivity of *C. reinhardtii* cultures at 75 and 300 µmol m^{-2} s^{-1} and found that 300 µmol m^{-2} s^{-1} yielded the highest productivity and that the productivity was the same at 24 and 33 °C.

The biomass yields measured in this study (0.76 – 1.35 g L^{-1}) were in the same range as measured previously for the same strain (Markina and Gislerød, unpublished; Markina et al., submitted). The growth of our cultures was limited by calcium and magnesium, with the use of a modified HS medium (Sueoka 1960) as indicated in Table 1, when the biomass increased over 1 g L^{-1}, as discussed by Markina and Gislerød (submitted). An increase in CO_2 concentration to 9%, at a light intensity of 100 µmol m^{-2} s^{-1}, led to a decrease in the dry weight of the cultures, in accordance with previous results obtained with the same strain (Mortensen and Gislerød 2014; 2015).

The cultures grown at 400 µmol m^{-2} s^{-1} and 3% CO_2 had a significant drop in pH that started after 48 h of growth, became chlorotic and died. These treatments had an intensive growth during the first days of the experiment, which needs a strong buffer for maintaining a stable pH. 2 mM $NaHCO_3$ per percent CO_2 was used in this experiment. This showed to not be enough buffer added for the fast growing cultures with 3% CO_2. However, cultures at 400 µmol m^{-2} s^{-1} and 9% CO_2 had a relatively stable pH and grew well until the end of the experiment, especially the ones at 35 °C, showing for the synergistic effect of light intensity, temperature, and CO_2 concentration.

The most obvious effect of the studied variables on the biochemical composition was on the chlorophyll content, which decreased with increasing light intensity. Andersen and Pedersen (2002) argued that the reduction in chlorophyll is not necessarily in contradiction with the observed increase in growth at high light intensity, because it is mainly the antennae pigments that are reduced, while the number of reaction centers remains constant or even increases (Falkowski and La Roche 1991; Fogg and Thake 1987). At 400 µmol m^{-2} s^{-1}, the chlorophyll content increased with increase in temperature. Shuter (1979) concluded that the

increase in chlorophyll content with increasing temperature was a common response in microalgae. Thompson et al. (1992) studied the effect of temperature on the biochemical composition of eight species of marine phytoplankton and came to the same conclusion. The maximum chlorophyll content (3.9% of DW) was observed in the treatments with 100 µmol m^{-2} s^{-1}, where it varied very little with variation in temperature and CO_2 concentration, corresponding to the treatments with low productivity and biomass. This value was higher than in the previously obtained results with the same strain grown at 200 µmol m^{-2} s^{-1} and in a medium with similar nutrient composition (Markina and Gislerød, submitted; Markina and Gislerød, unpublished; Markina et al., submitted), which confirms the negative effect of increase in light intensity on the chlorophyll content of the cultures.

The starch content of the cultures was low (< 11% of DW), probably due to the sufficiency of nutrients in the used medium. This is in accordance with the previous results obtained with the same strain grown in a medium with a similar composition (Markina and Gislerød, submitted; Markina and Gislerød, unpublished; Markina et al., submitted). Starch is known to accumulate in cells exposed to a nutrient limitation, nitrogen and sulfur limitations being the most effective (Ball et al. 1990; Branyikova et al. 2010; Dragone et al. 2011; Grossman 2000; Ji et al. 2011; Melis et al. 2000; Philipps et al. 2012; Yao et al. 2012; Zhang et al. 2002). Nonetheless, the starch content did increase with increasing light intensity. The freshwater microalga *Chlorella* was found to accumulate high amounts of starch (from 8.5% up to 40% of DW) as the photon flux density increased from a mean of 215 to 330 µmol m^{-2} s^{-1} prior to cell division (Branyikova et al. 2010). However, the environmental factors seem to have much less influence on the starch content than does the nutrient status of a culture (Markina and Gislerød, unpublished; Markina et al. 2015, submitted).

The protein content was positively influenced by the increase in light intensity. Carvalho et al. (2009) found that the cultures of *Pavlova lutheri* increased their cellular protein content from about 8 pg $cell^{-1}$ to 33.7 pg $cell^{-1}$ as response to an increase in light intensity up to 195 µmol m^{-2} s^{-1}. The protein range (16 – 43% of DW) measured in this study is in accordance with the previous results obtained with the same strain (Markina and Gislerød, submitted; Markina and Gislerød, unpublished; Markina et al., submitted).

It can be concluded that the increase in light intensity from 100 to 400 µmol m^{-2} s^{-1} and in temperature from 25 to 35 °C had a significant effect on the biomass of the cultures, considerably accelerating their growth. With the use of 100 µmol m^{-2} s^{-1}, the biomass yield

was higher at 3% CO_2 than at 9%. The contents of starch and protein increased with light intensity, contrary to the chlorophyll content. The results of this study gave us a better understanding of the interaction effects between light intensity, temperature, and CO_2 concentration, which allows for better control of culture conditions.

Acknowledgements

This study was financially supported by the Research Council of Norway (Project 203281: Use of solar energy for CO_2 capture, algae cultivation and hydrogen production – BioH_2) and the Norwegian University of Life Sciences. We thank Leiv M. Mortensen for his valuable assistance during preparation of the manuscript, and Dag Wenner and Silje E. Roksti for their technical assistance during the experiments.

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643 561

List of tables

Table 1. Experimental growth medium composition used in the present experiments. The growth medium was based on the High-Salt Sueoka medium (Sueoka 1960). The concentrations of ammonium, phosphate, and sulfate in the experimental medium were determined using our previous results (Markina and Gislerød, unpublished) and optimizing the medium composition to obtain maximum productivity as well as maximum biomass (dry weight) yield.

Table 2. Experimental design. Three variables (light intensity (µmol m^{-2} s^{-1}), CO_2 concentration (% v/v), and temperature (°C)) were combined in a 2^3 full factorial design in order to investigate their effects on the growth kinetics and the biochemical composition of *C. reinhardtii* photoautotrophic batch cultures. The eight treatments were divided into two blocks and carried out successively, at two different light intensity levels. Each treatment was carried out in three parallels.

Table 3. Results of the ANOVA. Average effects and significance levels of the effects of light intensity ('Light' in µmol m^{-2} s^{-1}), CO_2 concentration ('CO_2' in % v/v), and temperature ('T' in °C) and their interactions on the pH, the dry weight (DW) per liter culture, and on the contents of starch, protein, and chlorophyll of the cultures. The samples for starch, protein, and chlorophyll were collected at 60 and 96 h of growth, except for the treatments 5, 6, and 8, where they were collected at 48 and 72 h, due to faster growth. The experiments were carried out in two blocks at two different light intensities. The effect of the light intensity and the effect of the block (time) were therefore confounded. Significance levels: ^, $p < 0.10$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Not significant, $p > 0.10$ (not indicated).

Tables

Table 1. Experimental growth medium composition used in the present experiments. The growth medium was based on the High-Salt Sueoka medium (Sueoka 1960). The concentrations of ammonium, phosphate, and sulfate in the experimental medium were determined using our previous results (Markina and Gislerød, unpublished) and optimizing the medium composition to obtain maximum productivity as well as maximum biomass (dry weight) yield.

Nutrient	Concentration (mM)
NH_4	17.5
PO_4	7.5
SO_4	0.65
Ca	0.068
Mg	0.081
K	12.45
Cl	17.838
Na	2 mM / % CO_2
HCO_3	2 mM / % CO_2
Hutner trace elements solution	1 mL / L medium

674 Table 2. Experimental design. Three variables (light intensity (µmol m^{-2} s^{-1}), CO_2
675 concentration (% v/v), and temperature (°C)) were combined in a 2^3 full factorial design in
676 order to investigate their effects on the growth kinetics and the biochemical composition of
677 *C. reinhardtii* photoautotrophic batch cultures. The eight treatments were divided into two
678 blocks and carried out successively, at two different light intensity levels. Each treatment was
679 carried out in three parallels.

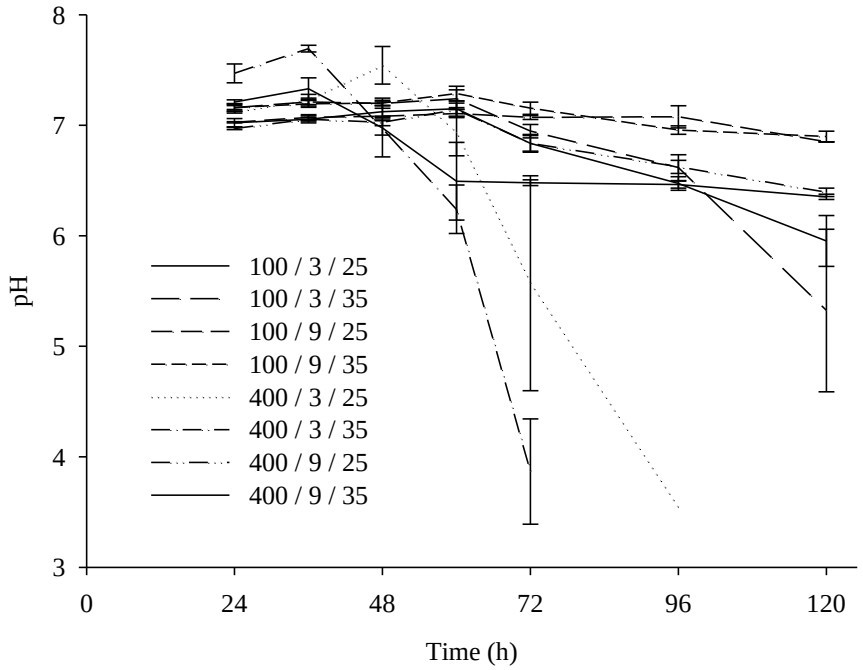
Treatment	Light intensity (µmol m^{-2} s^{-1})	CO_2 concentration (% v/v)	Temperature (°C)
1	100	3	25
2	100	3	35
3	100	9	25
4	100	9	35
5	400	3	25
6	400	3	35
7	400	9	25
8	400	9	35

680 Table 3. Results of the ANOVA. Average effects and significance levels of the effects of light intensity ('Light' in µmol m^{-2} s^{-1}), CO_2
681 concentration ('CO_2' in % v/v), and temperature ('T' in °C) and their interactions on the pH, the dry weight (DW) per liter culture, and on the
682 contents of starch, protein, and chlorophyll of the cultures. The samples for starch, protein, and chlorophyll were collected at 60 and 96 h of
683 growth, except for the treatments 5, 6, and 8, where they were collected at 48 and 72 h, due to faster growth. The experiments were carried out in
684 two blocks at two different light intensities. The effect of the light intensity and the effect of the block (time) were therefore confounded.
685 Significance levels: ^, $p < 0.10$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Not significant, $p > 0.10$ (not indicated).

	pH				Dry weight (g L^{-1})				Starch (% of DW)		Protein (% of DW)		Chlorophyll (% of DW)	
Factor	48 h	60 h	72 h	96 h	48 h	60 h	72 h	96 h	48/60 h	72/96 h	48/60 h	72/96 h	48/60 h	72/96 h
Light	-0.02	-0.49 ***	-1.31 ***	-1.71 ***	0.41 ***	0.43 ***	0.44 ***	0.39 ***	3.26 ***	1.95	13.45 ***	10.05 ***	-0.99 *	-1.39 ***
CO_2	-0.14 **	0.12	1.08 ***	1.71 ***	-0.03	-0.09 **	-0.01	-0.23 ***	0.30	-1.41	-2.9	2.79	-0.36 ***	0.28
T	-0.11 ^	-0.27 **	-0.47 **	-0.01	0.39 ***	0.33 ***	0.16 *	0.1 *	3.27 ***	-0.29	-1.96	-1.49	0.58 ***	-0.23
Light x CO_2	-0.12 *	0.11	0.86 ***	1.26 ***	0.01	-0.04	0.05	0.01	0.33	-0.02	-0.23	1.63	-0.15 ^	0.22
Light x T	-0.20 ***	-0.40 ***	-0.56 **	-0.04	0.27 ***	0.17 ***	0.01	0.08 *	-1.79 **	-2.79 *	-1.7	0.49	0.38 ***	0.04
CO_2 x T	0.14 **	0.03	0.33 ^	0.12 *	0.17 ***	0.17 ***	0.09	0.08 *	-0.31	-0.06	0.51	-1.14	0.20 *	0.26
Light x CO_2 x T	0.12 *	-0.01	0.34 *	-	0.13 **	0.10 **	0.001	-	-0.25	0.6	-6.46 *	0.08	-0.02	0.27

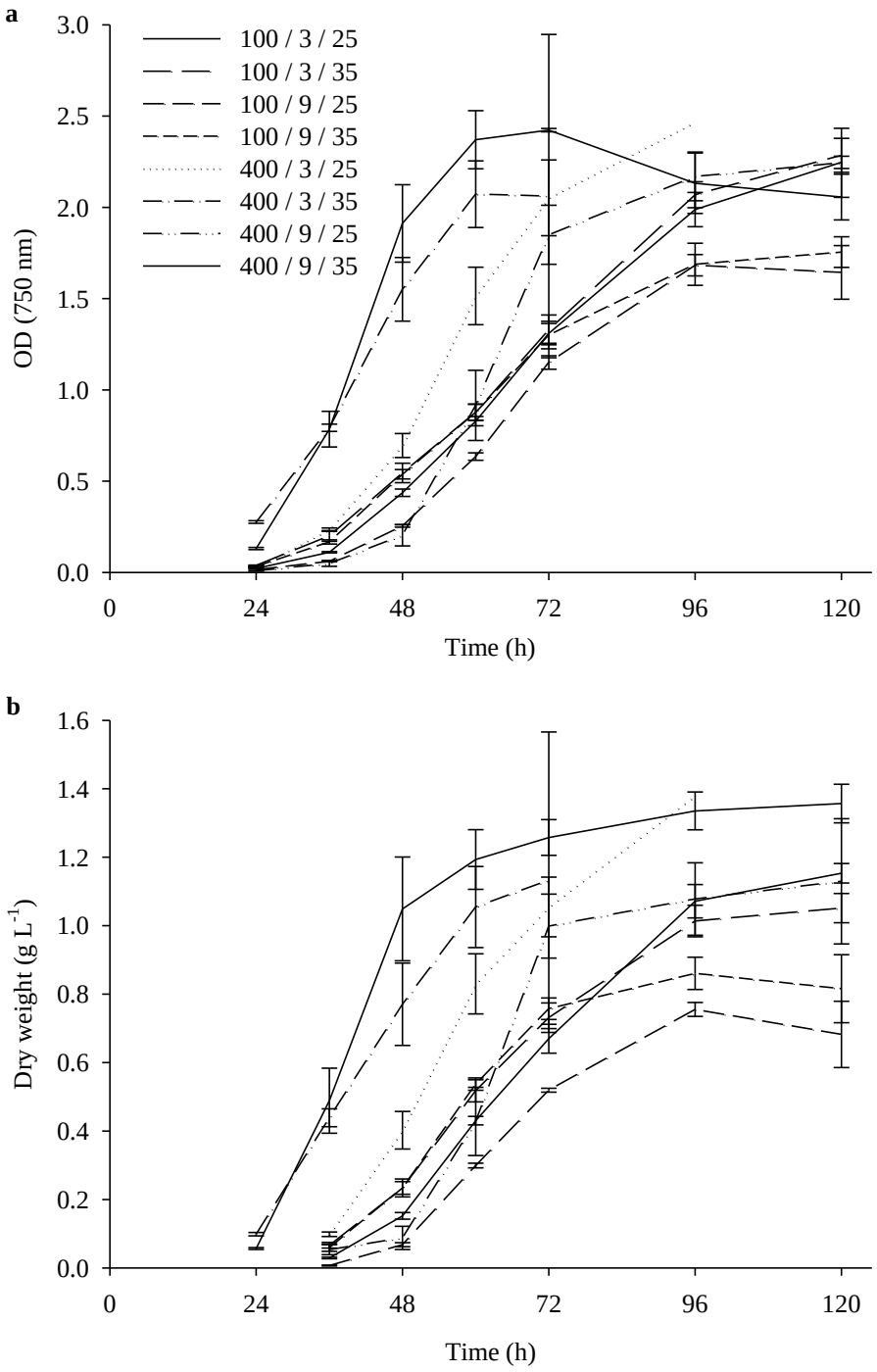
686 **List of figures**

695 **Figures**



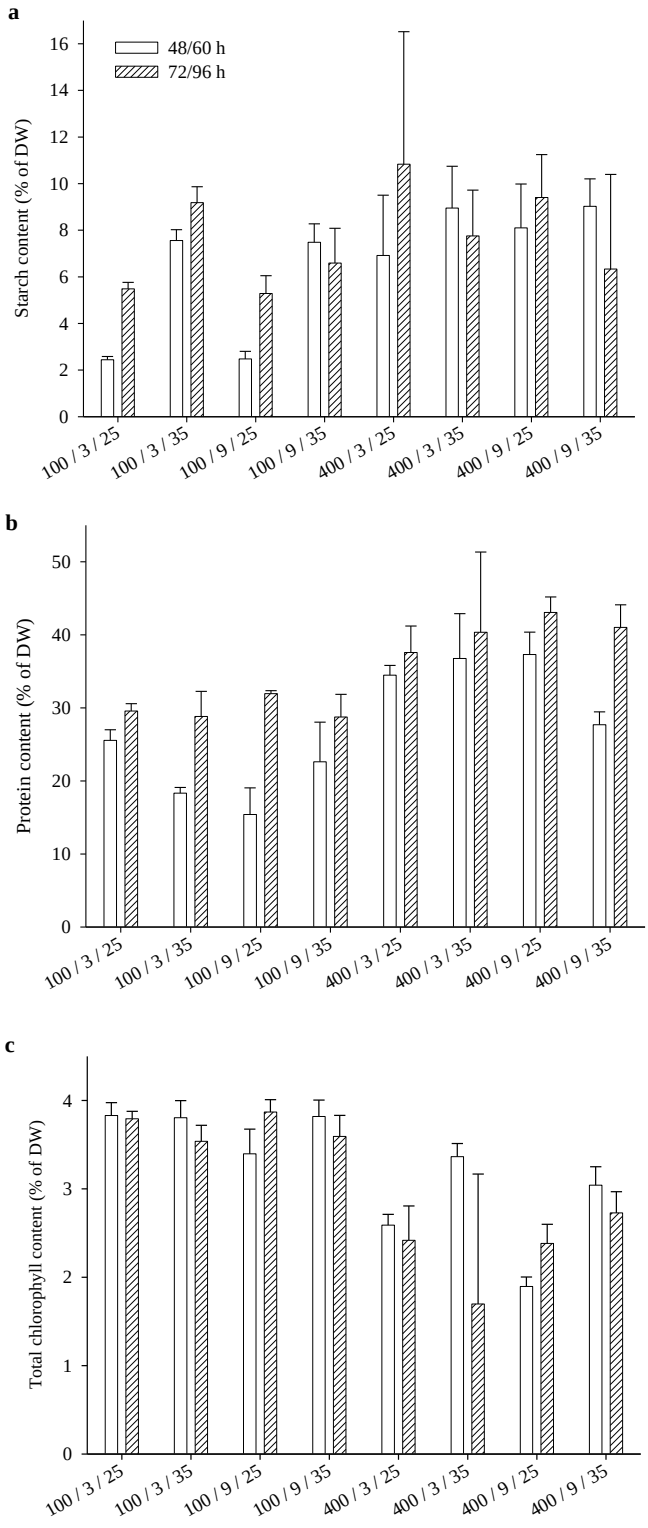
696

697 Figure 1. Effect of light intensity (µmol m^{-2} s^{-1}), CO_2 concentration (% v/v), and temperature
698 (°C) on the pH (means ± SD, n = 3) of *C. reinhardtii* photoautotrophic batch cultures.



699

700 Figure 2. Effect of light intensity (µmol m^{-2} s^{-1}), CO_2 concentration (% v/v), and temperature
701 (°C) on the optical density (OD, λ = 750 nm, means ± SD, n = 3) (a) and the dry weight (g L^{-1},
702 means ± SD, n = 3) of *C. reinhardtii* photoautotrophic batch cultures.



703

704 Figure 3. Effect of light intensity (µmol m^{-2} s^{-1}), CO_2 concentration (% v/v), and temperature
705 (°C) on the starch (a), protein (b), and total chlorophyll (c) contents (% of DW, means ± SD,
706 n = 3) of *C. reinhardtii* photoautotrophic batch cultures.

Paper IV

The importance of calcium and magnesium for the growth of *Chlamydomonas reinhardtii*

Daria Markina*, Hans Ragnar Gislerød

Department of Plant Sciences, Norwegian University of Life Sciences, P. O. Box 5003, 1432 Ås, Norway

*Corresponding author, e-mail: daria.markina@nmbu.no, dmarkina@gmail.com, tel.: (+47) 64 96 61 38, fax: (+47) 64 96 56 15

Submitted to the Journal of Plant Physiology

Abstract

The efficiency of hydrogen production by *Chlamydomonas reinhardtii* depends on the biomass concentration and starch, protein, and chlorophyll contents of the cultures. The growth media used for culturing *C. reinhardtii* vary widely in calcium and magnesium content, with the High-Salt Sueoka (HS) medium being particularly low in these two nutrients (0.068 mM calcium and 0.081 mM magnesium). The aim of this study was to examine whether these concentrations are limiting for the growth of *C. reinhardtii* batch cultures. Experiments were carried out with four different concentrations of calcium and magnesium, ranging from 0.068 to 0.68 mM calcium and from 0.081 to 0.81 mM magnesium. The results show that the calcium and magnesium concentrations of the HS medium do not affect the growth up to a biomass concentration of about 1 g L^{-1}, but inhibit further growth. By increasing the concentrations of calcium and magnesium tenfold, we obtained a biomass of 4.83 g L^{-1}. The starch content of the cultures also increased (from 17 to 32% of dry weight) with increasing concentrations of calcium and magnesium, probably due to nitrogen limitation. The chlorophyll concentration followed the same pattern. The protein content in the treatment with the lowest concentrations of calcium and magnesium was higher than in the other three treatments. It can be concluded that the concentrations of calcium and magnesium in the HS medium are not growth-limiting up to a biomass concentration of 1 g L^{-1}, but that they are so for higher biomass concentrations, and we recommend increasing them at least tenfold.

Key-words

Calcium, *Chlamydomonas reinhardtii*, chlorophyll, growth, magnesium, protein, starch.

Introduction

Cultures of *Chlamydomonas reinhardtii* with high biomass are required for biohydrogen production (Tamburic et al. 2011). The starch and protein contents of the cultures play a central role in the hydrogen (H_2) production process, since the catabolism of these two substrates contributes electrons to the hydrogenase enzyme for H_2 formation (Chochois et al. 2009; Melis et al. 2000; Zhang et al. 2002). The chlorophyll concentration in the culture is also highly influential on the H_2 production yield (Giannelli et al. 2009; Hahn et al. 2004).

The availability of the nutrients in the growth medium greatly influences the growth and the biochemical composition of microalgal cultures (Grossman 2000; Richmond 2004). Calcium and magnesium are both essential nutrients for microalgae. In earlier work by Markina et al. (submitted) and Markina and Gislerød (unpublished (b)), where we examined the effects of nitrogen, phosphorous, and sulfur on the growth of *C. reinhardtii* by using a modified High-Salt Sueoka (HS) medium (Sueoka 1960), the question rose of whether the low concentrations of calcium and magnesium could have a negative influence on the growth of the microalga. Knowledge about the effects of the concentrations of calcium and magnesium on the physiology of different species of microalgae is very limited as compared to other macronutrients, such as carbon, nitrogen, phosphorous, and sulfur.

The fraction of calcium in the elementary composition of microalgae is in the range of 0 - 80 mg Ca^{2+} g^{-1} dry biomass (0.0 – 8.0% of dry weight) (Healey and Stewart 1973). Calcium is required for cell membrane integrity and cell transport mechanisms (Fujii 1994). Calcium is also implicated as a second messenger in signal transduction of a wide variety of biotic and abiotic stimuli in both lower and higher plants (Rudd and Franklin-Tong 1999; Sanders et al. 1999). In *Chlamydomonas*, calcium signaling has been shown to be associated with motile responses (flagellar beat, phototaxis, chemotaxis), sensory responses (flagellar adhesion during mating), and the maintenance and removal of the flagella (flagellar length control, flagella excision) (Ermilova et al. 1998; Goodenough et al. 1993; Harz and Hegemann 1991; Kamiya and Witman 1984; Quarmby and Hartzell 1994; Tuxhorn et al. 1998). In addition to its role in signal transduction, calcium is an essential co-factor in photosystem II-driven oxygenic photosynthesis (Boussac et al. 1989; Krieger et al. 1993).

Calcium starvation induces lipid accumulation in microalgae cultures (Deng et al. 2011; Gorain et al. 2013).

The fraction of magnesium in the elementary composition of microalgae is in the range of 0.5 – 75 mg Mg^{2+} g^{-1} dry biomass (0.05 – 7.5 % of dry weight) (Healey and Stewart 1973). The magnesium content reported for *Chlorella* sp. ranges between 0.36 and 0.8% on a dry weight basis (Oh-Hama and Miyachi 1988). Chlorophyll molecules contain magnesium in their structure, making the supply of magnesium indispensable for the photosynthetic activities of plants and algae. Furthermore, magnesium activates more enzymes than any other mineral nutrient (Epstein and Bloom 2004), some of which are ATPases, RubisCO, RNA polymerase and protein kinases (Marschner 1995; Shaul 2002). The absence of magnesium, on the other hand, is expected to prevent cell division and chlorophyll synthesis and, thus, to reduce growth yields (Finkle and Appleman 1953b). In plants, magnesium deficiency in leaves is associated with a massive accumulation of carbohydrates and elevated susceptibility to high light intensity (Cakmak and Kirkby 2008; Marschner and Cakmak 1989). In *C. reinhardtii*, magnesium deprivation decreases the protein content of the cells, but increases carbohydrates and lipids (Çakmak et al. 2014).

The concentrations of calcium and magnesium vary widely in the different recipes for growth media of microalgae. Grobbelaar (2013) provided an overview of the variation of $CaCl_2$ in the growth media for microalgae from 0.22 mM to 0.56 mM and for $MgSO_4$ from 0.3 mM to 4.9 mM. The High-Salt Sueoka (HS) medium (Sueoka 1960) is particularly low in calcium (0.068 mM) and magnesium (0.081 mM) compared to other media used for *C. reinhardtii* cultures (Harris 2009). In a strategy for optimizing the growth and the biochemical composition of *C. reinhardtii* for the purpose of further H_2 production, it was essential to examine whether the concentrations of calcium and magnesium had an effect on these parameters. Experiments were carried out with four different concentrations of calcium and magnesium, with the concentrations ranging from the original concentration in the HS medium up to a concentration that was ten time higher. It was chosen to increase the concentrations of calcium and magnesium simultaneously, since their cellular fractions in microalgae are very similar (Healey and Stewart 1973; Ho et al. 2003). Their effects on the dry weight and on the contents of starch, protein, and chlorophyll of photoautotrophic batch cultures of *C. reinhardtii* were evaluated.

Materials and methods

Organism and culture conditions

The *Chlamydomonas reinhardtii* 137c strain was obtained from the NIVA culture collection, Norway (CHL153). The microalgae were stored in sterile conditions on a 1.5% agar Tris-Acetate-Phosphate (TAP) (Gorman and Levine 1965) solid growth medium (Andersen 2005) on Petri dishes at room temperature and low light intensity. The microalgae were pre-cultured in liquid High-Salt Sueoka (HS) medium (Sueoka 1960) at 25 °C and continuously illuminated by cool white fluorescent tubes (Philips Master TLD 36W/840 Reflex, Eindhoven, the Netherlands) at a photon flux density (PFD) of 200 µmol m^{-2} s^{-1}.

Experimental setup

Sterile batch cultures of *C. reinhardtii* cells were carried out in bubble column Pyrex glass tubular (coned at the bottom) photobioreactors (PBRs) with an inner diameter of 3.5 cm and a total volume capacity of 380 mL. The temperature was maintained at 25.0 ± 0.5 °C using water baths in aquaria. Cool white fluorescent tubes were used to provide continuous illumination at a PFD of 200 µmol m^{-2} s^{-1}. The light intensity was measured using a LI-COR model LI-190SA instrument (LI-COR, Lincoln, NE, USA) with a quantum sensor (400 – 700 nm) inside an empty PBR immersed in the water-bath aquarium, and the average PFD over the height of the PBR was estimated. Filtered (Acro 37 TF Vent Device, 0.2 µm PTFE membrane, PALL Life Sciences, Port Washington, NY, USA) air containing 5% v/v CO_2 was injected into the bottom of the PBRs, allowing mixing by bubbling (the bubble size was approximately 3 mm). The concentration of the dissolved CO_2 in the PBRs was determined to be 368 mg L^{-1} (Markina et al., submitted).

The experimental media were based on the HS medium, with 17.5 mM NH_4, 15.0 mM PO_4, 1.0 mM SO_4, and four different concentrations of $CaCl_2$ and $MgCl_2$. These concentrations corresponded to 1 x the concentrations in the HS medium (0.068 mM Ca, 0.081 mM Mg), 2.5 x (0.17 mM Ca, 0.203 mM Mg), 5 x (0.34 mM Ca, 0.405 mM Mg), and 10 x (0.68 mM Ca, 0.81 mM Mg), giving a wide range of tested concentrations. Each treatment was carried out in three parallels. The initial pH of the media was adjusted to 7.5 with 1 M NaOH prior to sterilization by autoclaving. $NaHCO_3$ was used in the medium as an additional pH buffer, at a concentration of 2 mM per percent CO_2 in the bubbled gas. At the

start of the experiments (t = 0 d), cells from pre-grown cultures at the linear growth phase were inoculated into 300 mL of medium at a cell density of 10^4 cells mL^{-1}. The cells were counted under a microscope, using a Fuchs-Rosenthal counting chamber.

Biomass determination

To collect the biomass, 0.1 – 1.0 mL of microalgal culture was filtered through pre-washed GF/F glass microfiber filters (0.7 µm pore size, 25 mm diameter, GE Healthcare, Whatman, UK) dried at 103.5 °C. The filters containing biomass were then washed with 30 mL deionized water to remove adhering salts and thereafter dried for 4 h at 103.5 °C. The dry weight (DW in g L^{-1}) of the sample was calculated by subtracting the DW of the empty filter from the DW of the filter with biomass. It was normalized to the filtered culture volume.

Chlorophyll assay

Chlorophyll was extracted from 1 – 2 mL samples using 95% ethanol and assayed using the method described by Harris (1989). The chlorophyll concentration was determined by optical density (OD) measurements (Helios α, Thermo Fisher Scientific, Waltham, MA, USA) at $\lambda = 649$ nm and $\lambda = 665$ nm.

Starch assay

Cell pellets of 2 mL samples left after chlorophyll assay were used for starch assay. A Megazyme total starch (amyloglucosidase/*α*-amylase method) assay kit (K-TSTA, Megazyme International, Bray, Ireland) procedure was adapted to a small sample volume. 20 µL of 80% ethanol was added to the cell pellet, which was dispersed using a vortex mixer. Cells were disrupted by adding 0.2 mL of DMSO (Sigma-Aldrich, St. Louis, MO, USA) and 0.5 mL of 0.5 mm glass beads (Cell disruption media, SI-BG05, Scientific Industries Inc., New York, NY, USA) to the samples and shaking them on a mixer mill (MM301, Retsch, Haan, Germany) at a frequency of 30 Hz for 10 min. Samples were heated on thermos-shakers (TS-100, Biosan, Riga, Latvia) to 100 °C for 5 min for starch solubilization purposes. Then, 0.3 mL of *α*-amylase (diluted 1:30 according to the Megazyme starch assay kit specifications) was added to the samples and the tubes incubated at 100 °C for 12 min. The contents of the tubes were mixed vigorously after 0, 4, 8, and 12 min. The tubes were placed at 50 °C, and 0.4 mL of sodium acetate buffer (200 mM, pH 4.5) was added to the samples, followed by 10 µL of amyloglucosidase (20U). The samples were thoroughly mixed and

incubated at 50 °C for 30 min. Then, 70 µL of distilled water was added to the samples in order to adjust the total sample volume to 1.0 mL. The contents of the tubes were mixed thoroughly and centrifuged at 20800 x *g* (Eppendorf centrifuge 5417R, Eppendorf, Hamburg, Germany) for 10 min at 20 °C. An appropriate volume (10 - 33.4 µL) of the supernatant was added to 1 mL GOPOD reagent and incubated at 50 °C for 20 min. The same procedure was used for two reagent blanks (10 - 33.4 µL distilled water) and four glucose controls (10 - 33.4 µL D-glucose standard solution at 1 mg mL^{-1}). The OD of each sample and of glucose controls was measured at λ = 510 nm against the reagent blank. The starch concentration in the cells was calculated as a percentage of DW of the samples according to the formula supplied in the Megazyme starch assay kit.

Protein assay

Cell pellets of 1 mL samples left after the chlorophyll assay were used for protein assay. The cells were disrupted using 0.5 mL of 0.5 mm glass beads (Cell disruption media, SI-BG05, Scientific Industries Inc., New York, NY, USA) and shaken on a mixer mill (MM301, Retsch, Haan, Germany) for at a frequency of 30 Hz for 10 min. Then, Tris-HCl solubilization buffer (50 mM, pH 8.3) containing 1% Triton was used to solubilize the proteins. The Lowry method (1951) was applied, using Bio-Rad DC protein microplate assay kit II (#500-0112, Bio-Rad, Hercules, CA, USA) to determine the protein concentration in the samples. The OD (λ = 750 nm) of the samples assayed on a microplate (CellBIND surface, Costar 3300, Corning Incorporated, Corning, NY, USA) was measured using a microplate spectrophotometer (Asys UVM340, Biochrom, Cambridge, UK) and Kim software (Kim32, 2004, Asys Hitech GmbH, Cambridge, UK). A BSA standard curve (8 points in the 0.0 – 1.75 mg mL-1 range) was used to calculate the protein concentration in the samples. The protein concentration was normalized to the DW of the culture and calculated as a percentage of DW.

Results

A significant drop in the pH of the cultures was observed between days 2 and 3 (Figure 1), which was parallel to the maximum increase in biomass (Figure 2). The pH of the cultures grown at the lowest concentrations of calcium and magnesium decreased more slowly than in the other cultures (Figure 1). The pH was stable at around 6.25 from day 6 and until the end of the experiments (Figure 1).

The cultures in all four treatments showed a rapid growth during the first days of the experiment, and the productivity increased from 1.16 to 1.97 g L^{-1} d^{-1} with increasing concentrations of calcium and magnesium (Figure 2b). The maximum dry weight was recorded on day 6. It was 4.83 g L^{-1} in the treatment with highest concentrations and 2.28 g L^{-1} in the treatment with the lowest concentrations (Figure 2b). A stationary phase was observed in all treatments from day 6 and until the end of the experiment (Figure 2), coinciding with the stabilized pH.

The starch content of the cultures increased sharply between days 3 and 4 of the three treatments with higher concentrations of calcium and magnesium (Figure 3a). In the treatment with the lowest concentrations of calcium and magnesium the highest increase was observed between days 4 and 5 (Figure 3a). After five days of growth, the starch content attained its maximum in all the cultures, except in the treatment with the lowest calcium and magnesium concentrations, where it continued to increase until the end of the experiment, while it decreased in the other treatments (Figure 3a). At the fifth day of growth, the starch content of the cultures increased with the increasing calcium and magnesium concentrations in the medium (Figure 3a). It varied from 17% of DW in the treatment with the lowest concentrations of calcium and magnesium to 32% of DW in the treatment with the highest concentrations of calcium and magnesium.

The protein content varied less than the starch content between the different treatments (Figure 3b). It decreased sharply between days 3 and 4, *i. e.* the opposite of the starch content, in the three treatments with higher concentrations of calcium and magnesium, and it increased from that point onwards, especially in the two middle treatments (Figure 3b). In the treatment with the lowest calcium and magnesium concentrations, the sharp decrease in protein content did not occur until day 6, reaching a minimum at day 10 (Figure 3b).

The chlorophyll concentration in the cultures increased with increasing concentrations of calcium and magnesium (Figure 4a). The treatment with the lowest calcium and magnesium concentrations had lower chlorophyll content (as % of DW) than the other treatments after eight days of growth (Figure 4b). The cultures in this treatment became almost completely chlorotic by day 15 of the experiment (data not shown).

Discussion

In the context of optimization of the growth and the biochemical composition of *C. reinhardtii* for the purpose of further H_2 production, we examined the effects of increasing calcium and magnesium concentrations on these parameters.

The biomass attained by the cultures increased with increasing concentrations of calcium (0.068 – 0.68 mM) and magnesium (0.081 – 0.81 mM) in the growth medium. Under increased concentration of magnesium (up to 6 mM), a significant rise in the biomass of *Chlorella vulgaris* and *Scenedesmus obliquus* was recorded in the study by Gorain et al. (2013). In *Dunaliella tertiolecta*, both calcium (up to 0.3 mM) and magnesium (up to 5.6 mM) enhanced cell growth (Chow et al. 2015).

At 2.5 days of growth, the biomass was in the range of 0.95 – 1.18 g L^{-1}, comparable to the dry weight measured for the same strain under similar growth conditions (Markina and Gislerød, unpublished (a, b); Markina et al., submitted). Up to this biomass concentration of 0.95 – 1.18 g L^{-1}, there was no significant ($p > 0.01$) difference in growth between the different calcium and magnesium concentrations tested, which indicates that the calcium and magnesium contents (0.068 mM Ca and 0.081 mM Mg) in the High-Salt Sueoka medium (Sueoka 1960) are sufficient up to this biomass concentration of *C. reinhardtii*.

The cultures in the treatment with the highest concentrations of calcium and magnesium, on the other hand, attained a biomass as high as 4.83 g L^{-1} after six days. The growth of these cultures might have been limited by nitrogen and/or sulfur. The supplied amounts of these two nutrients were sufficient to yield 2.6 g L^{-1} biomass with 9.4% of DW of nitrogen and 5.0 g L^{-1} at 0.64% of DW of sulfur, which were the nutrient contents of this strain grown in a medium with a similar composition to the one used in this study, not limited by nitrogen or sulfur (Markina et al., submitted). This means that the cultures with the three higher concentrations of calcium and magnesium would start to experience nitrogen limitation already at day 4. The cultures in the treatment with the highest concentration of calcium and magnesium might have become sulfur-limited at day 6 and would only contain about 5% of DW of nitrogen at that time point, which may explain the growth arrest of these cultures and the onset of a stationary growth phase. The onset of the stationary growth phase attained in the other treatments might be explained by the limitation of growth by calcium or

magnesium, since the attained biomass in these cultures increased with increasing concentrations of calcium and magnesium.

This possible nutrient limitation might also be what caused the accumulation of relatively high amounts of starch after three to five days of growth in the cultures with the three higher concentrations of calcium and magnesium. The starch content in these treatments increased with increasing biomass, probably due to an increase in the degree of nitrogen limitation as the biomass increased. Nutrient limitation by nitrogen, sulfur, or phosphorous has been reported to induce accumulation of carbohydrates, and particularly starch, in several strains of microalgae (Ball et al. 1990; Branyikova et al. 2010; Dragone et al. 2011; Grossman 2000; Hu 2004; Ji et al. 2011; Markou et al. 2012; Philipps et al. 2012; Siaut et al. 2011; Yao et al. 2012; Zhang et al. 2002). The starch content in the cultures grown at the lowest concentrations of calcium and magnesium was only 7% of DW after four days of growth. This is in line with previous results obtained with the same strain grown in media with a similar composition, with no limitation by nitrogen or sulfur (Markina and Gislerød, unpublished (a, b); Markina et al., submitted).

The protein content of the cultures grown in the three treatments with higher concentrations of calcium and magnesium was low after day 4 (14 – 20% of DW). This result would be expected for cultures suffering from nitrogen and/or sulfur limitation and with high starch content, since a negative correlation has previously been found between the amounts of these two cellular components in response to varying concentrations of ammonium and sulfate in the medium (Markina et al., submitted). The cultures growth at the lowest concentrations of calcium and magnesium had a protein content of 42% of DW after four days of growth. This is also in line with previous results obtained with the same strain grown un nutrient-replete conditions (Markina and Gislerød, unpublished (a, b); Markina et al., submitted).

The chlorophyll concentration increased with increasing concentrations of calcium and magnesium. The chlorophyll concentration in *Chlorella* cultures increased linearly about fifteen-fold when the initial magnesium concentration was increased from 8 µM to 115 µM (Finkle and Appleman 1953a). A further two-fold increase in the chlorophyll content was observed when the magnesium concentration was increased up to 2 mM (Finkle and Appleman 1953a). In these cultures, the chlorophyll content attained by the cells represented approximately 70-80% of the total magnesium that had been added to the growth media

(Finkle and Appleman 1953a). In a study carried out with tomato plants, the chlorophyll content in leaves also increased when the magnesium concentration in the nutrient solution was increased from 0.82 mM to 3.3 mM, but decreased when the calcium concentration was increased from 3.75 mM to 7.5 mM, probably due to the antagonistic effect of calcium on the uptake of magnesium (Hao and Papadopoulos 2004).

In this study, we chose to increase the concentrations of calcium and magnesium simultaneously, due to the similarity in their cellular fractions in microalgae (Healey and Stewart 1973; Ho et al. 2003), even if the metabolic roles of these two metals are quite different. In higher plants, their uptake is known to be antagonistic (Hao and Papadopoulos 2004; Ho et al. 1999; Paiva et al. 1998). Therefore, increasing the concentration of both calcium and magnesium simultaneously would probably be the correct strategy to avoid growth limitation by one of these two cations. On the other hand, transport antagonism between monovalent and divalent cations (Adams et al. 1992; Çakmak et al. 2014; Marcelle 1995; Marschner 1995) also needs to be considered when designing a growth medium.

It can be concluded that the increase in calcium and magnesium concentrations had a much larger effect on the growth than on the biochemical composition of *C. reinhardtii* photoautotrophic batch cultures. The concentrations of calcium and magnesium in the HS medium are not growth-limiting up to a biomass concentration of about 1 g L^{-1}, but that they are so for higher biomass concentrations, and we recommend increasing them at least tenfold for obtaining *C. reinhardtii* cultures with high biomass.

Acknowledgements

This study was financially supported by the Research Council of Norway (Project 203281: Use of solar energy for CO_2 capture, algae cultivation and hydrogen production – BioH_2) and the Norwegian University of Life Sciences. We thank Leiv M. Mortensen, Jorunn E. Olsen, and Sissel Torre for their valuable assistance during preparation of the manuscript, and Dag Wenner and Silje E. Roksti for their technical assistance during the experiments.

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442 **List of figures**

453

Figures

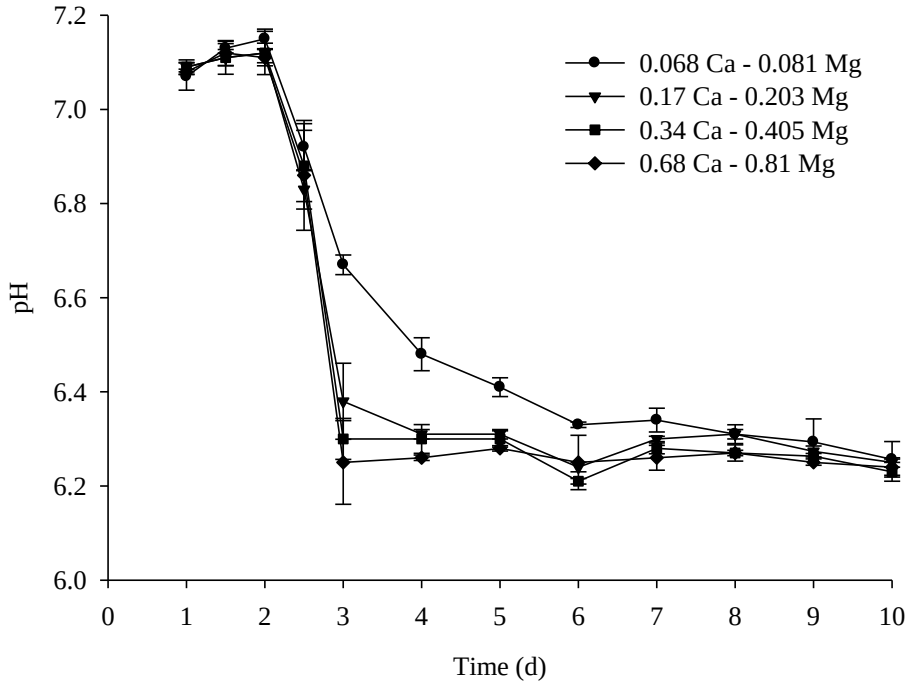
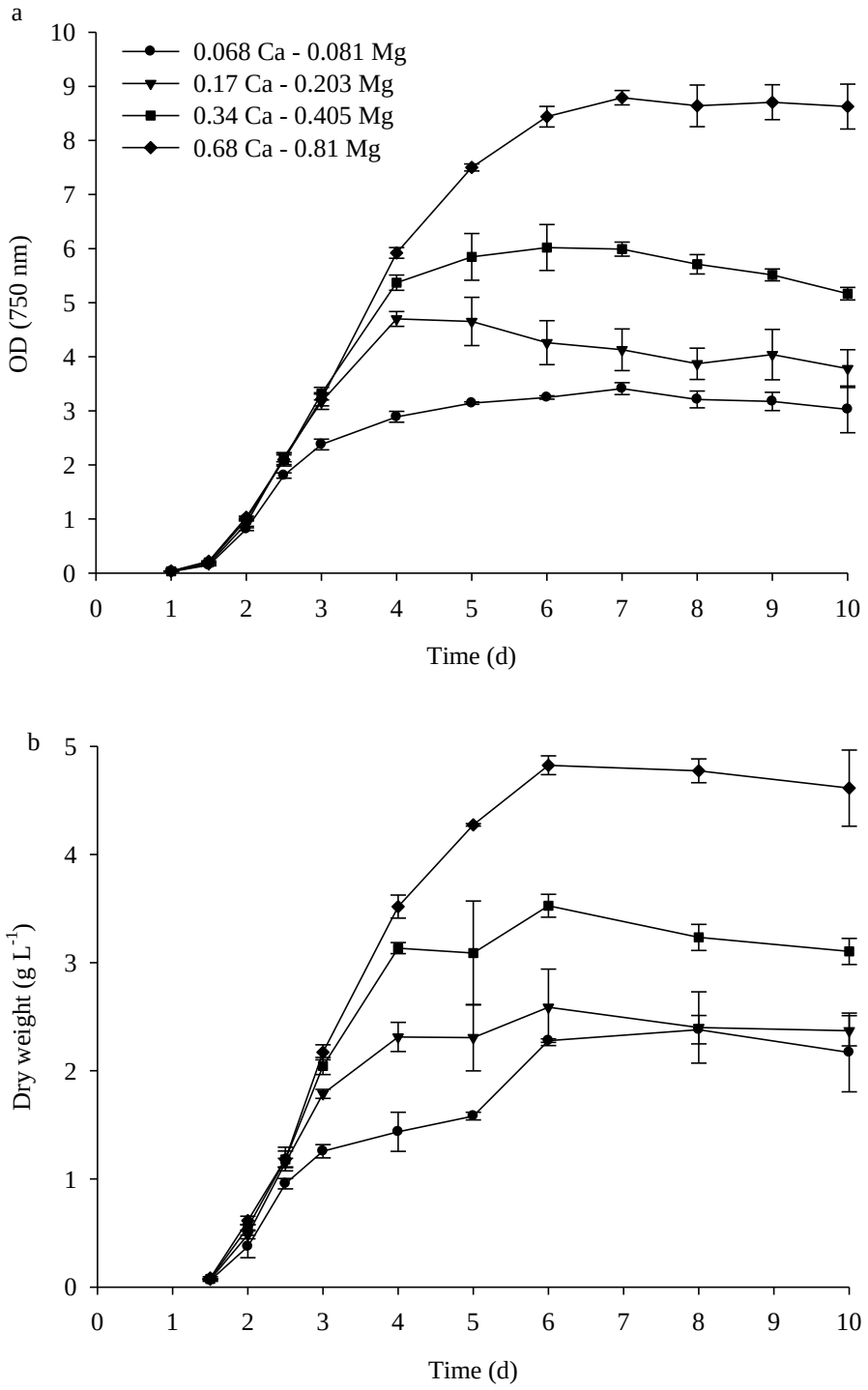


Figure 1. Effect of different concentrations of calcium and magnesium (mM) on the pH (means ± SD, n = 3) of the *C. reinhardtii* batch cultures.



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460 Figure 2. Effect of different concentrations of calcium and magnesium (mM) on the optical
461 density (OD, λ = 750 nm, means ± SD, n = 3) (a), and dry weight (g L^{-1}, means ± SD, n = 3)
462 (b) of the *C. reinhardtii* batch cultures.

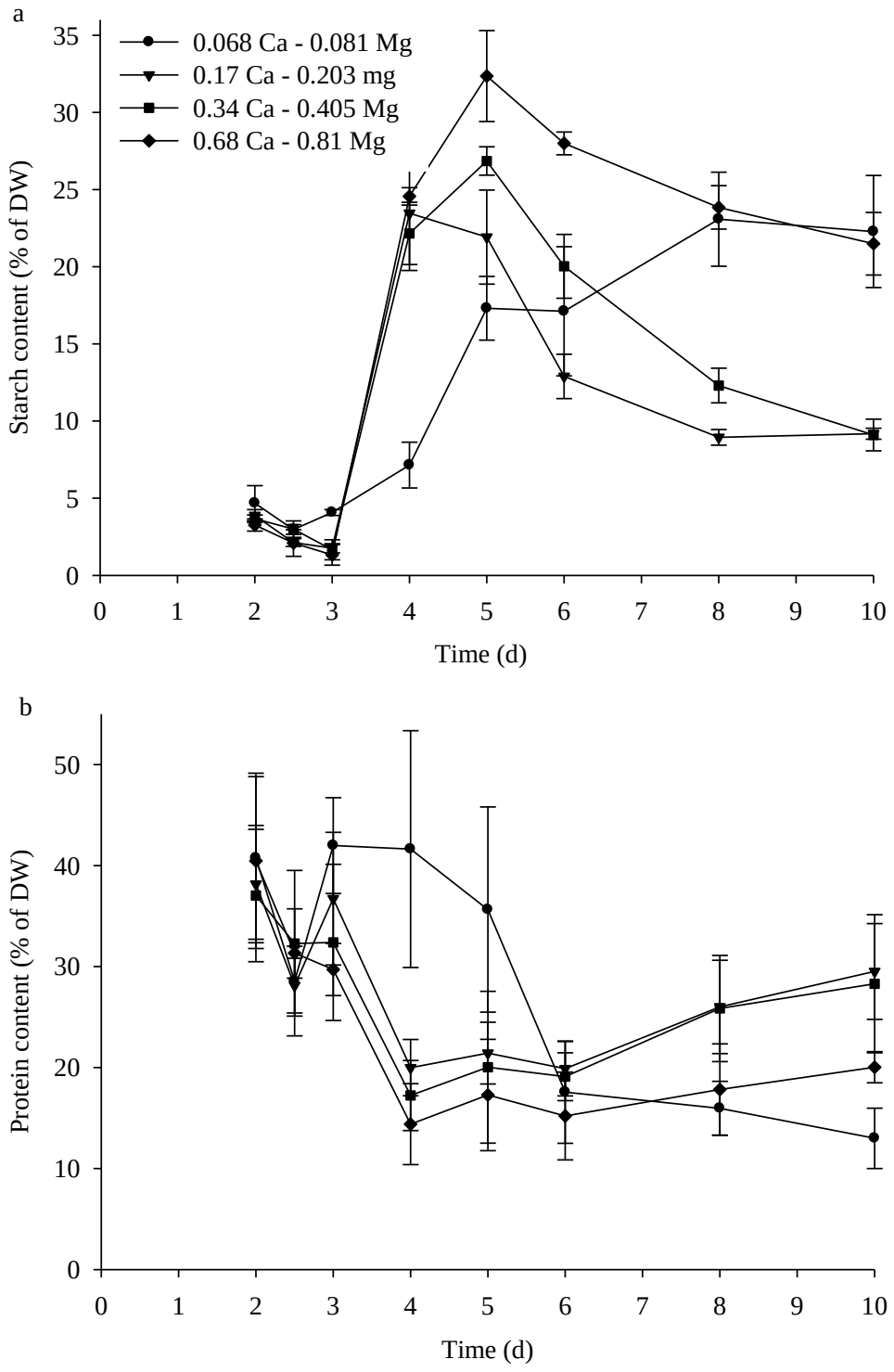

Figure 3. Effect of different concentrations of calcium and magnesium (mM) on the starch (a) and protein (b) contents (% of DW, means ± SD, n = 3) of the *C. reinhardtii* batch cultures.

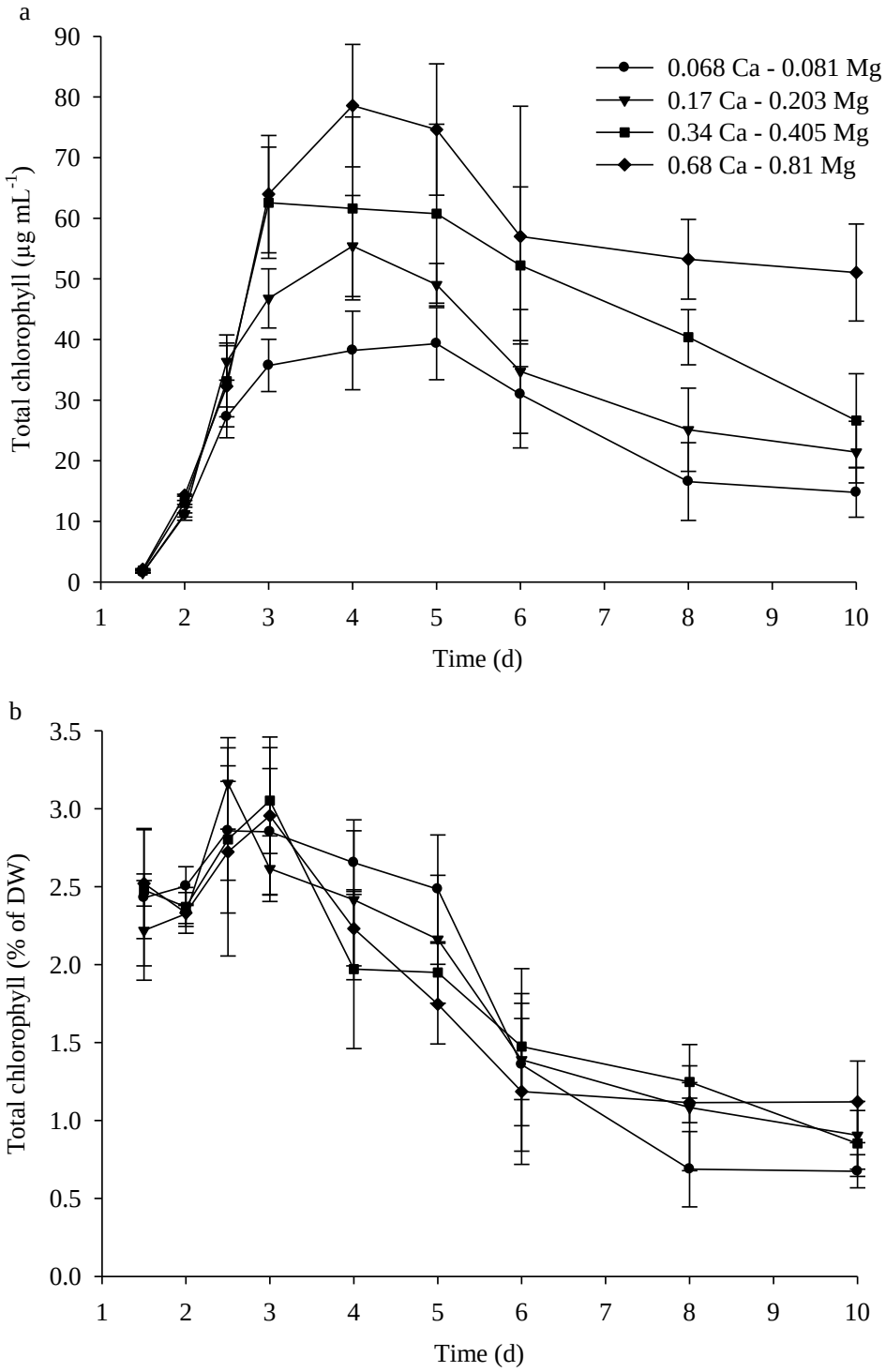

Figure 4. Effect of different concentrations of calcium and magnesium (mM) on the total chlorophyll concentration (µg mL^{-1}, means ± SD, n = 3) (a) and on the total chlorophyll content (% of DW, means ± SD, n = 3) of the *C. reinhardtii* batch cultures.