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Effect of nitrogen limitation on lipid and fatty acid composition of three marine microalgae

Hilde Karoline Fosse

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Supervisor: Kjell Inge Reitan, IBI
Co-supervisor: Matilde S. Chauton, SINTEF

Norwegian University of Science and Technology
Department of Biology

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Abstract

The feed used in the aquaculture industry today uses mainly fish oil as lipid source, but this fish oil will be a limiting resource if the industry shall expand and meet the future demand of food to a growing world population. Lipids from marine microalgae can be a potential replacement of fish oil in feed used in the aquaculture industry. Marine microalgae species are a natural source of lipids, and important sources of the fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). If lipids from marine microalgae shall be a realistic potential replacement of fish oil in feed, the microalgae production costs has to be reduced. One way to reduce the production costs is to increase the biological productivity of the marine microalgae. Different cultivation conditions affects the productivity and biochemical composition of the marine microalgae, and this can be utilized to get desired results. Nitrogen limitation in growth medium under microalgae cultivation has in many studies shown an increased lipid content in microalgae cells, but the effect is highly species specific. In this study three marine microalgae (*Phaeodactylum tricornutum, Rhodomonas baltica* and *Isochrysis* aff. *galbana* T-ISO) were cultivated under nitrogen limited growth conditions, by two different cultivation methods, batch culture and semicontinuous culture. The different cultivation methods gave different level of nitrogen limitation, and from lipid and fatty acid analysis the content of lipid and fatty acids were detected to see how the nitrogen concentration in growth medium effected the biochemical composition. The productivity of the three microalgae species were also examined. All three microalgae species accumulated lipids under strong nitrogen limitation. The content of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) had an overall increase, while the content of polyunsaturated fatty acids (PUFA) decreased with increased nitrogen limitation in the three microalgae species. The biomass, lipid and fatty acid productivity obtained in this experiment were generally lower than other studies have found. The productivity decreased in varying degree with extended nitrogen limitation.

KEYWORDS: *Phaeodactylum tricornutum* • *Isochrysis* aff. *galbana* T-ISO• *Rhodomonas baltica* • Lipids • Fatty acids • Nitrogen limitation • EPA • DHA

Sammendrag

I dagens akvakulturindustri er fiskeolje hovedkilden til omega-3 fettsyrer i fôret, men tilgang på fiskeolje vil være en begrensende faktor dersom industrien skal vokse, og for å kunne produsere mat til en voksende verdensbefolkning. Lipider fra marine mikroalger kan være en potensiell erstatter for fiskeoljen brukt i dagens fôr i akvakulturindustrien. Marine mikroalger er en naturlig kilde til lipider, og en viktig produsent av fettsyrene eikosapentaensyre (EPA) og dekosahexaensyre (DHA). Dersom lipider fra marine mikroalger skal kunne være en potensiell erstatter for fiskeolje i fôr, må kostnadene av mikroalgeproduksjon reduseres. En løsning som vil kunne redusere kostnadene, er å øke den biologiske produktiviteten hos de marine mikroalgene. Ulike miljøforhold under kultivering av marine mikroalger vil påvirke produktiviteten og den biokjemiske sammensetningen til mikroalgene, og dette kan utnyttes til å oppnå ønskede resultater. Nitrogenbegrensning i vekstmediet under kultivering av mikroalger har i flere studier vist å gi økt innhold av lipider i cellene hos mikroalger, men denne effekten avhenger av art. I denne studien ble tre marine mikroalger (*Phaeodactylum tricornutum, Rhodomonas baltica* and *Isochrysis* aff. *galbana* T-ISO) kultivert under nitrogenbegrensede vekstforhold, ved to ulike kultiveringsmetoder, batch kultur og semikontinuerlig kultur. De to kultiveringsmetodene gav ulikt nivå av nitrogenbegrensning, og fra lipid- og fettsyreanalysene ble innholdet av lipider og fettsyrer funnet for å se hvordan nitrogenkonsentrasjonen i vekstmediet påvirket den biokjemiske sammensetningen i mikroalgene. Produktiviteten til mikroalgene ble også funnet. Alle de tre mikroalgene akkumulerte lipider ved sterk nitrogenbegrensning. Innholdet av mettede og enumettede fettsyrer hadde en samlet økning, mens innholdet av flerumettede fettsyrer minket ved økende nitrogenbegrensning i mikroalgene. Biomasse-, lipid- og fettsyreproduktiviteten i dette studiet var generelt sett lavere sammenlignet med andre rapporter. Produktiviteten minsket i varierende grad ved sterkere nitrogenbegrensning.

NØKKELORD: *Phaeodactylum tricornutum* • *Rhodomonas baltica* • *Isochrysis* aff. *galbana* T-ISO • Lipider • Fettsyrer • Nitrogenbegrensning • EPA • DHA

Table of contents

ACKNOWLEDGMENTS ..... **III**

ABSTRACT ..... **V**

SAMMENDRAG ..... **VII**

TABLE OF CONTENTS ..... **IX**

ABBREVIATIONS ..... **XI**

1 INTRODUCTION **1**

1.1 LIPIDS FROM THE AQUATIC ENVIRONMENT 1

1.2 MARINE MICROALGAE 2

1.2.1 Influence of growth conditions on biochemical composition in microalgae *2*

1.2.2 Fatty acid accumulation in microalgae cells *3*

1.2.3 Rhodomonas baltica, Isochrysis aff. galbana and Phaeodactylum tricornutum *4*

1.3 MICROALGAE GROWTH AND METHODS FOR ALGAE CULTIVATION 4

1.3.1 Growth phases for microalgae cultivation *4*

1.3.2 Batch culture *5*

1.3.3 Semicontinuous culture *6*

1.4 AIM OF STUDY 6

2 MATERIALS AND METHODS **9**

2.1 EXPERIMENTAL SETUP 9

2.2 EXPERIMENTAL SYSTEM 9

2.2.1 Growth medium *10*

2.3 EXPERIMENTAL PROCEDURE 11

2.3.1 Batch culture *11*

2.3.2 Semicontinuous culture *12*

2.4 DETERMINATION OF GROWTH RATE AND DILUTION RATE OF MICROALGAE 12

2.5 CARBON ANALYSIS 13

2.6 LIPID AND FATTY ACID ANALYSIS 13

2.7 CALCULATION OF PRODUCTIVITY OF BIOMASS AND LIPIDS 14

2.8 STATISTICS 15

3 RESULTS **17**

3.1 GROWTH PARAMETERS 17

3.1.1 Batch culture *17*

3.1.2 Semicontinuous culture *20*

3.2 LIPID AND FATTY ACIDS CONTENT 23

3.2.1 Fatty acids composition *24*
3.2.2 Lipid and Fatty acid distribution compared with various nutrient limitation *25*
3.3 BIOMASS AND LIPID PRODUCTIVITY 29
4 DISCUSSION 31
4.1 CULTURE CONDITIONS - IRRADIATION, PH AND TEMPERATURE 31
4.2 GROWTH RATES AND PROGRESS IN THE DIFFERENT CULTIVATION METHODS 32
4.2.1 Batch culture *32*
4.2.2 Semicontinuous culture *32*
4.3 LIPID AND FATTY ACIDS CONTENT 33
4.3.1 Distribution of fatty acids with various nutrient limitation *34*
4.4 BIOMASS AND LIPID PRODUCTIVITY 36
4.5 CONCLUDING REMARKS 37
5 REFERENCES 39
APPENDIX 1 44
APPENDIX 2 45

Abbreviations

µm	Micrometer
µmol	Micromole
µ	Specific growth rate
μ_{max}	Maximum specific growth rate
mm	Millimeter
SFA	Saturated fatty acid
MUFA	Monounsaturated fatty acid
PUFA	Polyunsaturated fatty acid
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid
DW	Dry Weight
SD	Standard Deviation
SC	Semicontinuous culture
CO_2	Carbon dioxide
°C	Degree Celsius
$m^{-2}s^{-1}$	Square meter per second
ppm	Parts per million
rmp	Revolutions per minute
N_2	Nitrogen gas
PHA	*Phaeodactylum tricornutum*
RHO	*Rhodomonas baltica*
ISO	*Isochrysis* aff. *galbana* clone T-ISO

1 Introduction

According to the results of the *2015 Revision* of world's population prospect, the world population will be around 9.7 billion in 2050 and around 11.2 billion in 2100 (United Nations 2015). The rise in population will in turn increase the demand of food supply in the world. Today, the global terrestrial and marine primary food production are comparable in magnitude, but only 2% of the food supply is produced in the ocean (Duarte et al. 2009). A growing population is followed by the need for increased food production. The agriculture industry alone will most likely not be able to cover this need, due to limitation of farmland and access of freshwater (FAO 2011). To meet these challenges, the marine aquaculture food production have to increase the contribution of the overall food supply (FAO 2014).

If the aquaculture should be a part in meeting the demand of food to a growing population, the marine aquaculture must become less dependent on resources from fisheries. Fish from fisheries supplies the production of fishmeal and fish oil, used as important feed components in aquaculture (Chauton et al. 2015). The catches from fisheries have declined over the last twenty years and many fish stocks are at the time overexploited. This causes higher prices for fish oils and fishmeal and also unsustainable production (Duarte et al. 2009). If the aquaculture industry is going to sustain or increase the contribution of marine food, it has to reduce the input of wild fish in the aquaculture feed (Naylor et al. 2000).

1.1 Lipids from the aquatic environment

Foods from the aquatic environment have an important role in providing humans access to the health benefiting long-chain omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (FAO 2014). In the aquaculture industry, these essential fatty acids originate primarily from what the fish feed on, in other words, fish oil from their diet. For marine fish larvae, the lipids are important for development, by affecting the spawning and the egg quality for many fish species (Patil and Gislerod 2006). A shortage of polyunsaturated fatty acids (PUFA), as EPA and DHA, will have a negative effect the fertilization, fecundity and hatching rate. For humans the PUFA are particular important in new-born and infant development (Milledge 2011, Patil and Gislerod 2006, Patil et al. 2005). The PUFA have also shown great health effects regarding treatment of different diseases, like cancer, arthrosclerosis, psoriasis, reduced risk of heart disease and diseases of old age, such as Alzheimer's disease (Patil and Gislerod 2006). If the aquaculture industry, is going to ensure a healthy development

of marine fish larvae and also retain the important commercial application, saying that fish are a good source of omega-3 fatty acids, it's essential to find a good replacement for fish oil. These important omega-3 fatty acids will be the first limiting factor for further growth of the marine aquaculture (Carter et al. 2003). It is therefore crucial for the aquaculture industry to find alternative sources to these fatty acids, for use in farmed fish feed.

Marine microalgae are one of many possible alternatives that the feed production industry considers as a promising replacement in fish oil (Patil et al. 2005). Marine microalgae are the primary producers of EPA and DHA in marine food webs and they are highly productive (Barclay et al. 1994). The production of marine microalgae to use in feed for aquaculture is continuously progressing, and knowledge and experiences from the biofuel industry can to a great extend give useful information about large-scale production of microalgae. The overall biggest challenge for microalgae production for commercial utilization of microalgae biomass is the cost. The factor that has the greatest potential to reduce this cost is to increase the biological productivity (Chauton et al. 2015, Kleivdal et al. 2013).

1.2 Marine microalgae

Microalgae are unicellular eukaryote organisms, they are placed at the bottom of the entire aquatic food chain and contributing to the production of renewable resources by 100×10^6 tons of fish per year (Becker 2004). The size of microalgae can vary between a few micrometers (µm) to a few hundreds of µm, depending on the species. Phycologists defines algae as any organisms with chlorophyll *a* and a thallus, which are not differentiated into roots, stems and leaves (Tomaselli 2004). Compared with terrestrial plants, which are differentiated into roots, stems and leaves, the microalgae can have up to 10 times more efficient mass-transfer and growth than the terrestrial plants (Chauton et al. 2015). Marine microalgae are a natural source of many important nutrients, and are the primary producers of EPA and DHA, which accumulates in the marine food web. The productivity of microalgae and the favorable content of omega-3 fatty acids are major drivers to use them as a sustainable alternative to today's current use of fish oil in aquaculture feed.

1.2.1 Influence of growth conditions on biochemical composition in microalgae

According to Brown et al. (1997) microalgae contain on an average 30-40% protein, 10-20% lipids and 5-15% carbohydrates, but the biochemical composition are highly dependent on

growth conditions and species. Growth conditions influencing the biochemical composition are factors such as light, temperature, salinity and nutrient composition of the culture medium (Qiao et al. 2015, Reitan et al. 1994). The biochemical compositions in marine microalgae, particular the content of lipids, are important factors for considering microalgae for further use in aquafeed. In general, microalgae are metabolically very flexible and can be manipulated to produce more of desirable products by simply changing the cultivation conditions (Patil et al. 2005).

1.2.2 Fatty acid accumulation in microalgae cells

The potential of lipid production in microalga cells is, as mentioned earlier, species-specific and this can also be applied to the ability to produce PUFA (Chauton et al. 2015). In microalgae, the lipids serve as functional compounds and energy storage under limited growth conditions. High growth rates without any limitation of the growth will therefore generally not accumulate lipids. Nutrient limitation will generally reduce the growth and protein synthesis of the microalgae cells, which in turn will give an increased accumulation of photosynthetic products, such as lipids (Patil et al. 2005). A balance between microalgae biomass production and extended lipid production is essential for a cost-efficient microalgae production when they will be used as lipid-rich feed resource. Growth restrictions, especially extent of nutrients in growth medium, are essential factors regarding lipid accumulation in microalgae cells (Hu 2004). Nitrogen availability in the growth medium has a great effect on lipid metabolism in microalgae when the nutrient is limited. Limitation of nitrogen in growth medium is a well-known method for increasing the lipid content in microalgae, as it is cheap, easy to manipulate and also a reliable method for many algae species (Griffiths et al. 2012). In several microalgae species, the content of lipids have been up to 70% of the dry biomass under nitrogen limitation (Rodolfi et al. 2008). Although limited extend of nitrogen in growth medium may induce lipid accumulation in many microalgae species, it is not given that these lipids are suitable for fish oil replacement in aquafeed. Many studies, where nitrogen limited growth have been studied, show accumulation of saturated (SFA) and monounsaturated fatty acids (MUFA) as the main fractions of the lipid content in microalgae cells (Hu 2004, Rodolfi et al. 2008). Shall oil from microalgae replace the current use of fish oil in aquafeed, it has to contain PUFA, and particularly the fatty acids EPA and DHA. Nitrogen limited growth of microalgae must therefore not only focus on the induced content of lipids, but also which types of fatty acids it contains. A study done to examine the fatty acid profile for several microalgae species grown

under nitrogen limited conditions, reveal a lower content of PUFA compared with the same microalgae, grown under nitrogen replete conditions (Griffiths et al. 2012). In that research, *Isochrysis galbana* and *Phaeodactylum tricornutum* were examined, these will also be examined in this study.

1.2.3 *Rhodomonas baltica*, *Isochrysis* aff. *galbana* and *Phaeodactylum tricornutum*

The microalgae species that are examined and used here are all widely known marine species; *Rhodomonas baltica*, *Isochrysis* aff. *galbana* clone T-ISO and *Phaeodactylum tricornutum.* Several studies of these microalgae species have been done, regarding biomass production, the biochemical profile and the content of lipids (Chauton et al. 2013, Fernández-Reiriz et al. 1989, Griffiths et al. 2012, Patil et al. 2006, Qiao et al. 2015, Reitan et al. 1994, Rodolfi et al. 2008). *P. tricornutum* is a diatom belonging to the class Bacillariophyceae. Today, this microalgae species is widely used in China as aquaculture live food (Qiao et al. 2015). Since many diatoms are easy to cultivate, can reach a high biomass density and shows increased lipid content under nutrient limited growth conditions (Chauton et al. 2013), *P. tricornutum* is highly relevant as a source for oil in aquafeed. T-ISO belongs to the class Prymnesiophyceae and has for a long time been cultivated and used for aquaculture feed, due to the high content of PUFA (Liu and Lin 2001). The microalgae produces high levels of DHA, and is therefore of special interest for the aquaculture industry (Dunstan et al. 1993, Liu and Lin 2001). *Rhodomonas* sp. is a flagellate and a cryptophyte, found in the class of Cryptophyceae (Lafarga-De la Cruz et al. 2006). This class is important regarding primary production in both freshwater and in the marine environment (Patil et al. 2006). To this time, there is not much literature on *R. baltica*.

1.3 Microalgae growth and methods for algae cultivation

1.3.1 Growth phases for microalgae cultivation

Microalgae growth can be grouped into five different growth phases; lag phase, exponential phase, transition phase, stationary phase and mortality phase (fig. 1.1.)(Hoff and Snell 2007, Lee and Shen 2004). Lag phase is described as the time when there is little change in the cell density of the culture, due to adaptions to a new environment. After a period with acclimation to the new environment, the microalgae culture enters the exponential growth phase. First, the cells start to grow and multiply, until the cell division increases rapidly according to a logarithmic function. After the cell density has increased the cell division will be significant

reduced and the microalgae growth is in the transition phase. The microalgae cells have the highest nutritional value and density in this phase (Hoff and Snell 2007). Stationary phase occurs when the cell density remain relatively constant for a period. The time in stationary phase depends on the density of the culture, as it affects the access to nutrients. When the cell density decreases, the last phase of microalgae growth will occur, called the mortality phase. This will eventually happen due to exhaustion of nutrient, pH disturbance, oxygen deficiency or other important environmental growth factors.

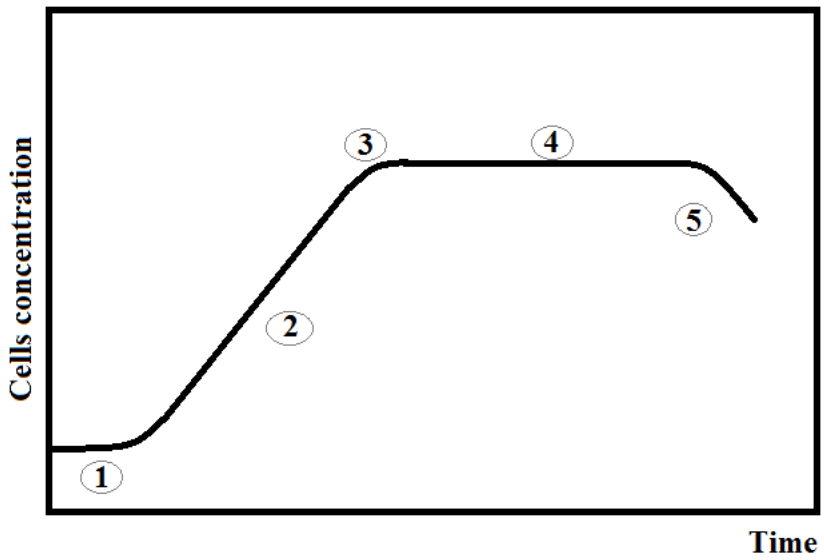

Fig. 1.1. Five typical growth phases of microalgae cultures. Lag phase (1), exponential phase (2), transition phase (3), stationary phase (4) and mortality phase (5).

1.3.2 Batch culture

Batch culture is the most widely used method for cultivation of microalgae cells (Lee and Shen 2004). This method has many advantages in terms of expense, the volume of media required and it's easily manipulated (Wood et al. 2005). The cultivation consists of prepared culture medium and algal inoculum, placed in a culture vessel, where the growth environment can be controlled and adjusted (Hoff and Snell 2007, Lee and Shen 2004). The growth medium is only supplied once, therefore the chemical environment will change continuously throughout the cultivation and this also applies to the biochemical composition of the microalgae cells. To ensure nutrient and gaseous exchange in the microalgae culture, some form of agitation has to be included in the culture (Lee and Shen 2004). If the cultivated microalgae is mixotroph or phototroph, CO_2 supply will be necessary. This will ensure excess of carbon used in photosynthesis and also regulate the pH in the culture (Lee and Shen 2004). Illumination of the culture can be done by either natural or artificial light sources.

1.3.3 Semicontinuous culture

In a continuous culture, fresh culture medium is supplied continuously or intermittently to the culture and a specific volume of the excess culture is removed (Lee and Shen 2004). Semicontinuous culture refers to a culture where the fresh culture medium is added and the excess culture is removed intermittently, at constant intervals. One type of continuous cultivation method is called chemostat cultivation. In this method the culture volume is constant, because the volume of added fresh medium and the volume of removed culture is the same (Lee and Shen 2004). With a constant cultivation volume the chemical environmental for the microalgae growth also remains constant and this in turn gives a stable biochemical composition within the microalgae cells. The definition of chemostat cultivation is not always defined as the same. Some define it as a cultivation method where new growth medium is added continuously to the culture and not intermittently, which also applies to the harvesting of the culture (Thomas and Dodson 1972). Regardless of which definition used, the essential in chemostat cultivation is that this method gives a stable chemical environment for microalgae growth. In a chemostat, the microalgae cells can grow with a constant rate of dilution and added new medium, which after a few days gives a steady state of the culture (Thomas and Dodson 1972). The culture growth will be constant from day to day, thus growth rate equals dilution rate. It is therefore easy to manipulate the growth rate of the culture by changing the dilution rate, in chemostat cultivation.

1.4 Aim of study

This present study is a part of a research project “MICRO-feed”, a project that explores the potential of cultured marine microorganism to become a new and sustainable feed source for the marine aquaculture industry. Marine microalgae produces essential fatty acids, important components in feed for the aquaculture industry.

The main objective in this study is to explore the content of lipids and fatty acids when microalgae are cultivated under nitrogen limited conditions. It is generally known that nitrogen concentrations in growth medium leads to increased content of lipids and fatty acids in microalgae species, but the production varies greatly between species. The production of EPA and DHA also varies between species and with different culture conditions.

Three marine microalgae explored in this study were cultivated by two different methods, batch culture and semicontinuous culture. The growth medium used was composed to give nitrogen

limitation. By use of the two different cultivation methods the microalgae cultures got different specific growth rate.

The semicontinuous cultures were selected in order to give a growth rate that correspond to 50% of the maximum specific growth rate, and the batch cultures were grown to stationary phase where the growth rate was close to zero. The aim of these strategies were to create a gradient in nitrogen limitation in the three microalgae cells. The effect of the various nitrogen concentrations was evaluated on the lipid and fatty acid profiles. Since EPA and DHA are the two fatty acids with highest value when considering microalgae as a replacement of fish oil in feed, the production of these fatty acids was the main focus.

2 Materials and methods

2.1 Experimental setup

All experiments were conducted at the laboratories of Norwegian University of Science and Technology (NTNU), Centre of Fisheries and Aquaculture (Sealab), and at SINTEF Fisheries and Aquaculture in Trondheim, between January and March 2016. The work was a part of the ERA-NET research project "MICRO-Feed" (ERANET-COFASP: Project No.: 248355/E40), a project that explores the potential of cultured marine microorganisms to become a new sustainable feed source for the marine aquaculture industry. Stock cultures of the marine microalgae *Rhodomonas baltica* (strain NIVA-5/91) and *Phaeodactylum tricornutum* (strain CCMP 2561) were obtained from SINTEF Fisheries and Aquaculture, Trondheim, while *Isochrysis* aff. *galbana* clone T-ISO (CCAP, strain 927/14) came from NTNU, Centre of Fisheries and Aquaculture. Two experiments were conducted, cultivation of microalgae in 1) batch culture, and 2) semicontinuous culture.

2.2 Experimental system

Seawater used in the cultures was filtered (0.22 µm) and autoclaved at 121 °C for 15 minutes (MLS 3020U Upright Autoclave, Sanyo, The Netherlands), and returned gradually to a pressure of 1 atm before it was used. The algae were grown in the pretreated seawater with f/2 medium (modified from Guillard (1975), see 2.2.1) in 1.2 L glass cylinders (LWS 05, Inst. Getreideverarbeitung GmbH, Germany, fig. 2.1), and each microalgae species was cultured in two parallels. Cultures were constant illuminated by artificial light (white LED, Evolys AS, Norway), with a light intensity of 150 µmol photon $m^{-2}s^{-1}$ at the culture surface. Temperature, controlled by double glass cylinders and water tubes, was kept constant at 22 °C. In the first two days of cultivation, in both batch and semicontinuous culture, filtered air was supplied to the cultures. From day three the filtered air was mixed with 650 ppm CO_2, due to increased pH. The supply of filtered air, together with magnetic stirring ensured constant mixing of the cultures.

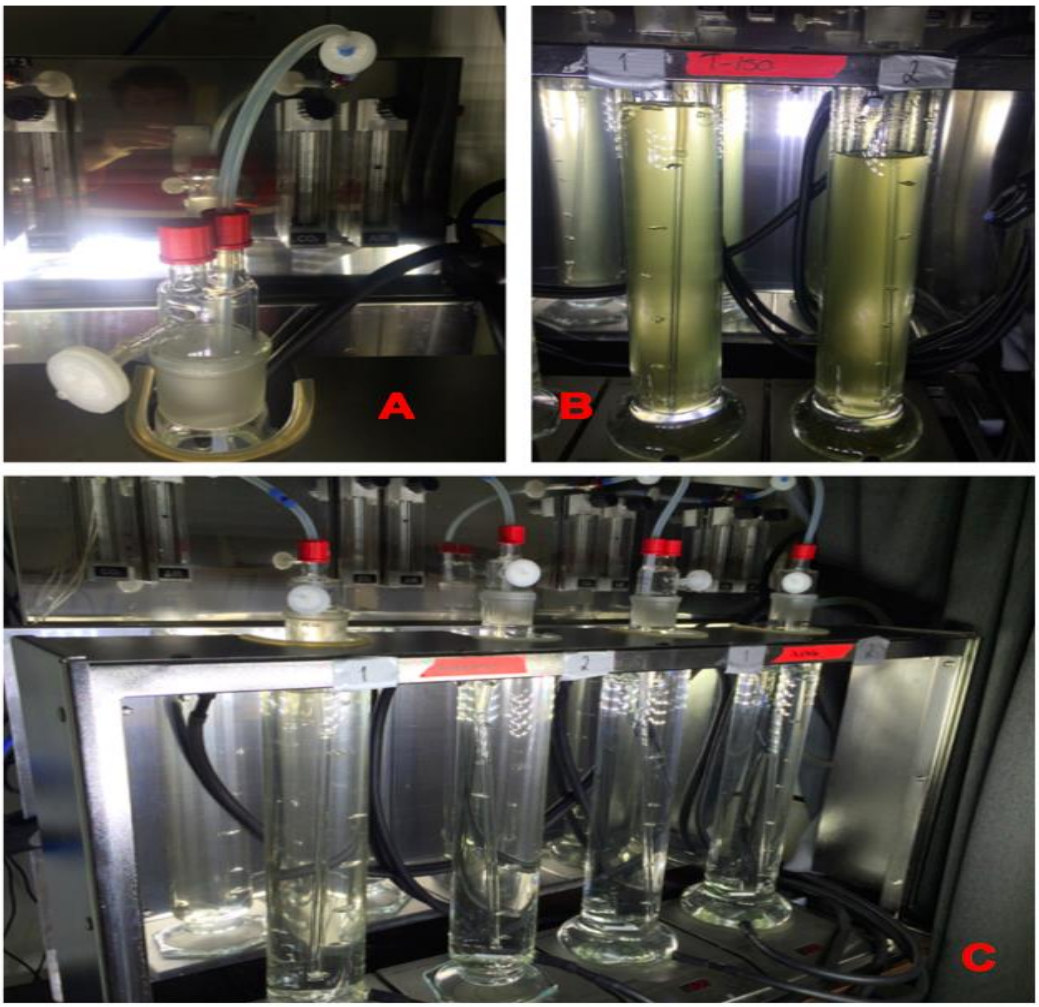

Fig. 2.1. Components of the experimental setup for cultivation of microalgae. Close-up picture of the filtered air supply to the glass cylinder (A). Two parallels of cultivated T-ISO (B) with air added, mixing the cultures. Overview of the system (C), a rack of four double glass cylinders placed on a magnetic field connected to water tubes, controlling the temperature.

2.2.1 Growth medium

The algae medium was modified from Guillard (1975), the f/2 medium. To ensure limitation of nitrate and sufficient amount of phosphate for the algae growth, 10% of the original amount of sodium nitrate and 25% more addition of sodium phosphate than the original recipe were used (table 2.1). The other components of the f/2 growth medium from Guillard (1975) had the same concentrations as the original recipe (appendix 1).

Table 2.1. Concentration of the nitrate and phosphate source in growth medium used in cultivations of the three species of microalgae.

Component	µmol L^{-1}
$NaNO_3$	88.24
$NaH_2PO_4 \bullet H_2O$	45.3

2.3 Experimental procedure

To ensure desirable and controlled growth of the algae, different measurements and registrations of the cultures were done every day during the cultivation:

- Registration of pH (PHM210 Standard pH meter, Radiometer Analytical, France), where the pH meter was calibrated every day with standard solution
- Photosynthetic condition (quantum yield, Q_y), using 10 mm square cuvettes (AquaPen-C AP-C 100, Photon System Instruments, Czech Republic)
- Instantaneous chlorophyll fluorescence, F_t, using 10 mm square cuvettes (AquaPen-C AP-C 100, Photon System Instruments, Czech Republic)
- Optical density (OD), using a spectrophotometer (UviLine 9100, Schott® Instruments, Germany) with absorption at 750 nm and with 10 mm square cuvettes. Seawater was used as a blank.
- Cell numbers with a coulter counter that count cells within the size range of 2-10 µm (Multisizer™ 3 Coulter Counter®, Beckman Coulter Inc., Miami, FL, USA)

2.3.1 Batch culture

The batch cultures where run in order to determine maximum specific growth rate (μ_{max}) under nitrogen limitation and cultivation of microalgae under strong nitrogen limitation. For the last purpose the batch cultures were harvested in late stationary phase of the batch cultures. Stock cultures of the algae were placed in glass cylinders, together with the pretreated seawater and modified f/2 medium (total volume of 1.2 L). After seven days of growth for *R. baltica* and T-ISO, and eight days of growth for *P. tricornutum*, when the algae cultures had been in the stationary phase for three to four days, the cultures were harvested by centrifugation at 4200 rpm for 7 minutes at 18 °C (Centrifuge 5804 R, Eppendorf™, Germany). The parallels of each

microalgae culture were pooled together during the centrifugation. Calculations of specific growth rate of batch culture are shown in 2.4.

2.3.2 Semicontinuous culture

The semicontinuous cultures were started with an inoculum from the batch cultures, with cell number between 24 000 – 70 000 per mL (table 3.4). The semicontinuous cultures were grown at a relative growth rate of 50% of μ_{max}, that was obtained from the batch cultures. Dilution rates (D) of the semicontinuous cultures were calculated in order to get a relative growth rate of 50% of μ_{max}. Calculations of D is shown in 2.4.

When the semicontinuous cultures reached a density of approximately 70% of maximum density (day 4, table 3.4.), exact volumes of 45-55% of the total volume for the individual microalgae species were harvested at the same time daily. Right after harvesting of cultures, the cylinders were refilled with the same amount of prepared seawater and modified f/2 medium. This was continued for twenty days to collect biomass material for analysis of lipids.

2.4 Determination of growth rate and dilution rate of microalgae

The specific growth rates of the algae (μ) were estimated in batch culture and can be expressed as

$$\mu = \ln\left(\frac{N_t}{N_0}\right)/t, \qquad [1]$$

where N_0 is the biomass at time t_0, and N_t is the biomass at time t. The maximum specific growth rate μ_{max} (table 3.1.) was calculated in the exponential initial growth phase (equation 1) when $\ln N$ versus time was linear and had the highest slope. After that, limitation of the growth resulted in lower growth rates.

Dilution rates (D) of semicontinuous cultures correlate directly to specific growth rates of the cultures, and D can be calculated from the specific growth rate corresponding to the 50% of μ_{max} of the cultures. D can also be expressed as

$$D = \Delta V/V_0 \qquad [2]$$

The daily replaced volume in the different cultures were estimated by equation 3. Since the growth rate was set to correspond to 50% of μ_{max}, $\mu - 50\% = 0.5\,\mu_{max}$ and the true growth rate ($\mu_{50\%}$) of the algae was thereafter estimated by equation 4.

$$\Delta V = V_0(e^{\mu t} - 1) \quad [3]$$

$$\mu_{-50\%} = \ln \frac{\left[\left(\frac{\Delta V}{V_0}\right)+1\right]}{t} + ln\left(\frac{N}{N_0}\right)/t, \quad [4]$$

Where ΔV is the daily (t = 1 day) replaced volume, V_0 is the rest volume after harvest, N and N_0 is the biomass at day_1 and day_0, respectively. By harvesting the exact volume found from the dilution rate every day, the algae cultures had a constant growth rate and a constant biochemical composition, which where independent of time in the established phase of steady state growth.

2.5 Carbon analysis

For carbon analysis, exact volumes of the cultures were filtered through 1.2 µm, 25 mm diameter GF/C glass microfiber filters (Whatman International Ltd., England) until saturation of the filters. The filters were ignited (480 °C, 2 h) before use. The filtered volume was registered, and the filters were stored at –20 °C. For removal of inorganic carbon, the filters were exposed to vapor of concentrated hydrochloric acid for 15 min. The outer circle of the filters were cut off, and the inner circle with the algal samples were transferred to tin capsules (Säntis Analytical AG, Switzerland) and placed inside a 96 well plate. Packed filters were dried in a heating cabinet at 60 °C for 48 h. Filtration of culture were done at day 3, 4, 5 and 7 in batch culture, and at the last day of the semicontinuous culture. To determine the content of carbon, the filters packed in tin capsules were analyzed using an Elemental Combustion System CHNS-O (Costech ECS, model 4010, Costech International, Firenze, Italy). SINTEF Fisheries and Aquaculture conducted the analysis.

2.6 Lipid and fatty acid analysis

The algae were harvested by centrifugation at 4200 rpm and 18 °C for 7 min (Centrifuge 5804 R, Eppendorf™, Germany) in 250 ml containers to remove most of the water. After transfer of the pellet to 15 ml tubes, they were rinsed with distilled water and centrifuged at 4200 rmp and 18 °C for 5 min. The concentrated algae were freeze-dried and stored under N_2 gas in a deep freezer holding –80 °C before lipid and fatty acid analysis.

A modified version of the Bligh and Dyer (1959) method were used to extract the lipids of *R. baltica* and T-ISO (fig. 2.2.). Extractions of lipids from *P. tricornutum* were more difficult than

for the two others microalgae species, due to the thick cell wall, therefor another method was used. For *P. tricornutum* the extractions of lipids were done by homogenizing in chloroform/methanol (2:1, v/v) and the content were determined gravimetrically (Folch et al. 1957). For the total lipid analysis, each sample has two technical replicates. After the samples were extracted and concentrated, they were placed in a desiccator for 24h and weighed on analyticalbalance. For the fatty acid methyl esters (FAMEs) preparation (fig. 2.2), samples from total lipid analysis were used and prepared by acid-catalyzed transesterification for 16h at 50 °C (Christie 2003). FAMEs were separated and quantified by gas-liquid chromatography (AutoSystem XL, Perkin Elmer, Waltham, MA). Keshuai Li, Postdoctoral researcher at NTNU, conducted the lipid and fatty acid analysis.

2.7 Calculation of productivity of biomass and lipids

The carbon analysis gave the carbon content per sample (µg/sample), and together with measurement of volume per sample, the content of carbon per mL was calculated (µgC/mL). The numbers of cells per mL culture for each microalgae species were counted every day and used to determine the content of carbon per cell (gC/cell). The average of the carbon content per cell from the two parallels in each microalgae were further used together with the average cell numbers in culture to get the content of carbon in culture. To find dry weight (DW) of the microalgae species, a constant (2.45, Kjell Inge Reitan pers.com) were multiplied with the number of carbon content in culture (equation 5).

$$DW = 2.45 * \frac{mg}{L} \quad [5]$$

Further on, the value of DW in each microalgae species and parallel were used as biomass value (N), to determine the biomass productivity (P) for each culture, expressed as

$$P = \frac{N * \Delta V}{culture\ volume} \quad [6]$$

where N is the biomass value, ΔV is the daily diluted volume and *culture volume* is the total volume of culture. The productivity value can be further used to find lipid productivity.

Productivity in the batch culture was calculated as the biomass at harvest divided by the number of culture days.

2.8 Statistics

Generation of graphs and calculation of standard deviation (SD) of the samples were performed with Microsoft *Excel 2013*. A t-test was used to analyze significant differences in cell numbers between the parallels of the different microalgae cultures, using the software *SigmaPlot 13.0*. For the t-test, the level of significance was set to 0.05

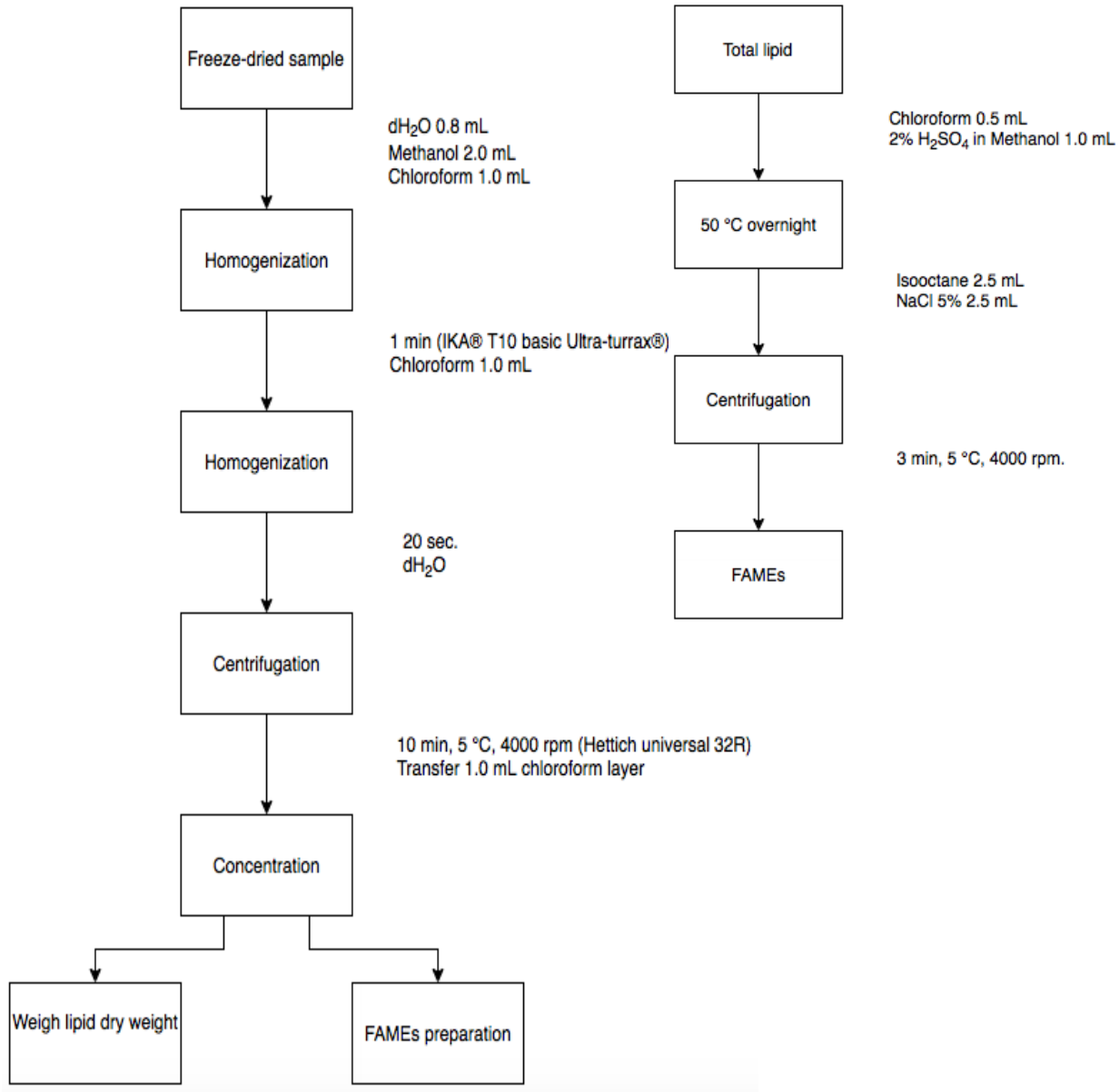

Fig. 2.2. Example of the procedure done for lipid extraction (left) and fatty acid methyl esters (FAMEs) preparation (right). The extractions of lipids in *R. baltica* and T-ISO were done by this procedure, and the procedure of FAMEs preparation were done to all three microalgae species.

3 Results

3.1 Growth parameters

3.1.1 Batch culture

The batch cultivations were conducted over seven days for T-ISO and *R. baltica*, and eight days for *P. tricornutum*, where all of the three microalgae species had an exponential growth and entered stationary phase before the harvest (fig. 3.1). Optical density were measured during the cultivation (fig. 3.2), and the pattern of measured values corresponds to the growth in cell numbers of the cultures.

The highest maximum specific growth rate (μ_{max} d^{-1}) in batch culture was obtained in *P. tricornutum* and the lowest in T-ISO (table 3.1). For *R. baltica* and T-ISO the μ_{max} were found between day two and day three, and for *P. tricornutum* between day three and day four. The specific growth rates of the three different microalgae species, were between −4.5 - 2.6 % of μ_{max} the last day in batch culture (table 3.2).

Table 3.1. Maximum specific growth rate (μ_{max} d^{-1}) for the three microalgae species obtained in batch culture, expressed as mean±SD.

	P. tricornutum	T-ISO	*R. baltica*
μ_{max}	1.63±0.01	1.19±0.02	1.40±0.10

Table 3.2. Number of cells (cells mL^{-1}), percentage of maximum specific growth rate (% $of\ \mu_{max}$) and biomass (mg DW L^{-1}) in the three microalgae species the last day in batch culture (mean, two parallels: 1, 2).

	Cells mL^{-1}		% of μ_{max}	mg DW L^{-1}	
	1	2		1	2
P. tricornutum	2.08×10^6	2.03×10^6	−4.54	34.7	33.9
T-ISO	1.78×10^6	1.72×10^6	1.50	46.0	44.4
R. baltica	2.51×10^5	2.51×10^5	2.62	35.1	35.1

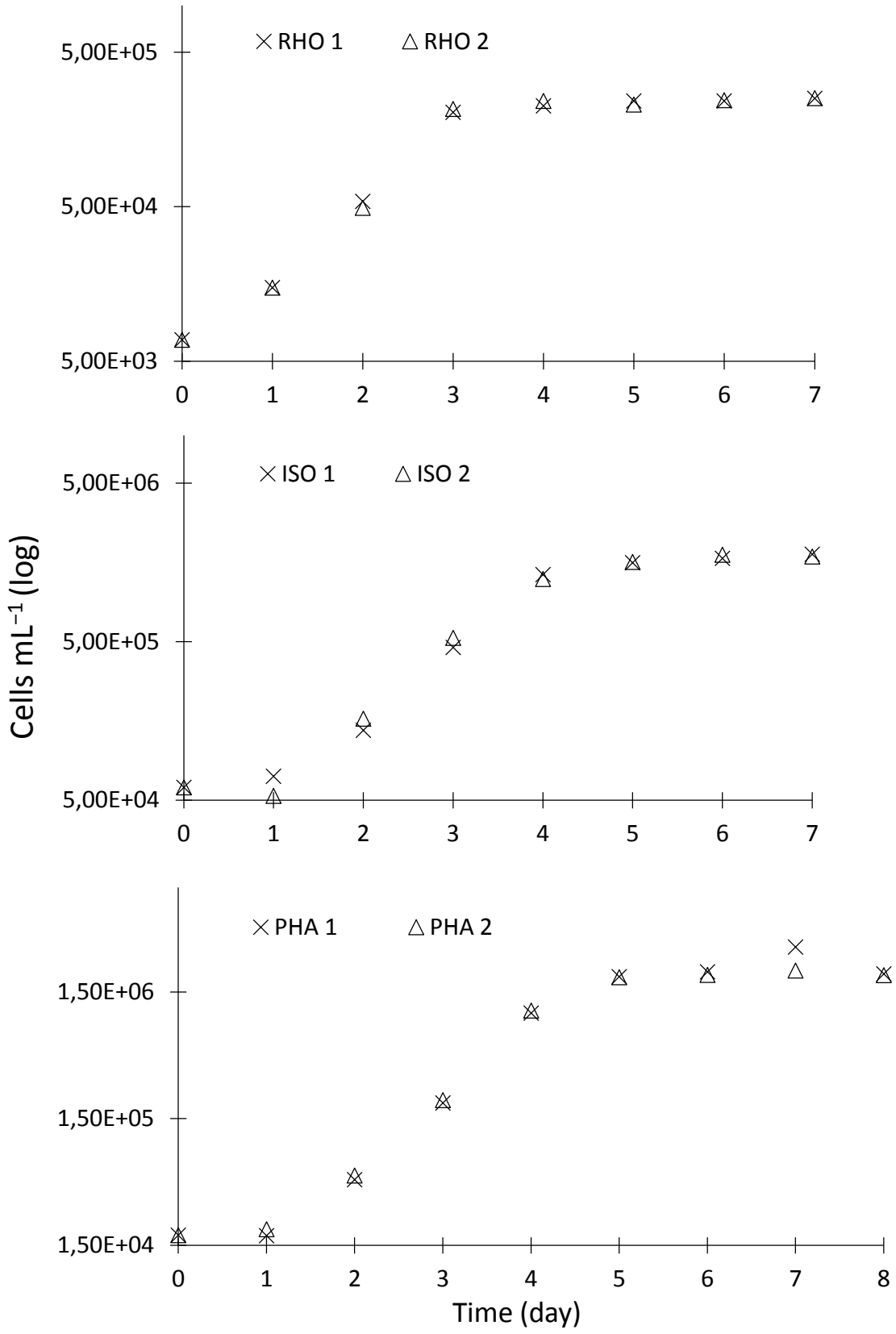

Fig. 3.1. Numbers of microalgae cells (cells mL^{-1}) over time (day), during the batch cultivation of the three microalgae species with two parallels (1, 2). *P. tricornutum* (PHA), *R. baltica* (RHO) and T-ISO (ISO)

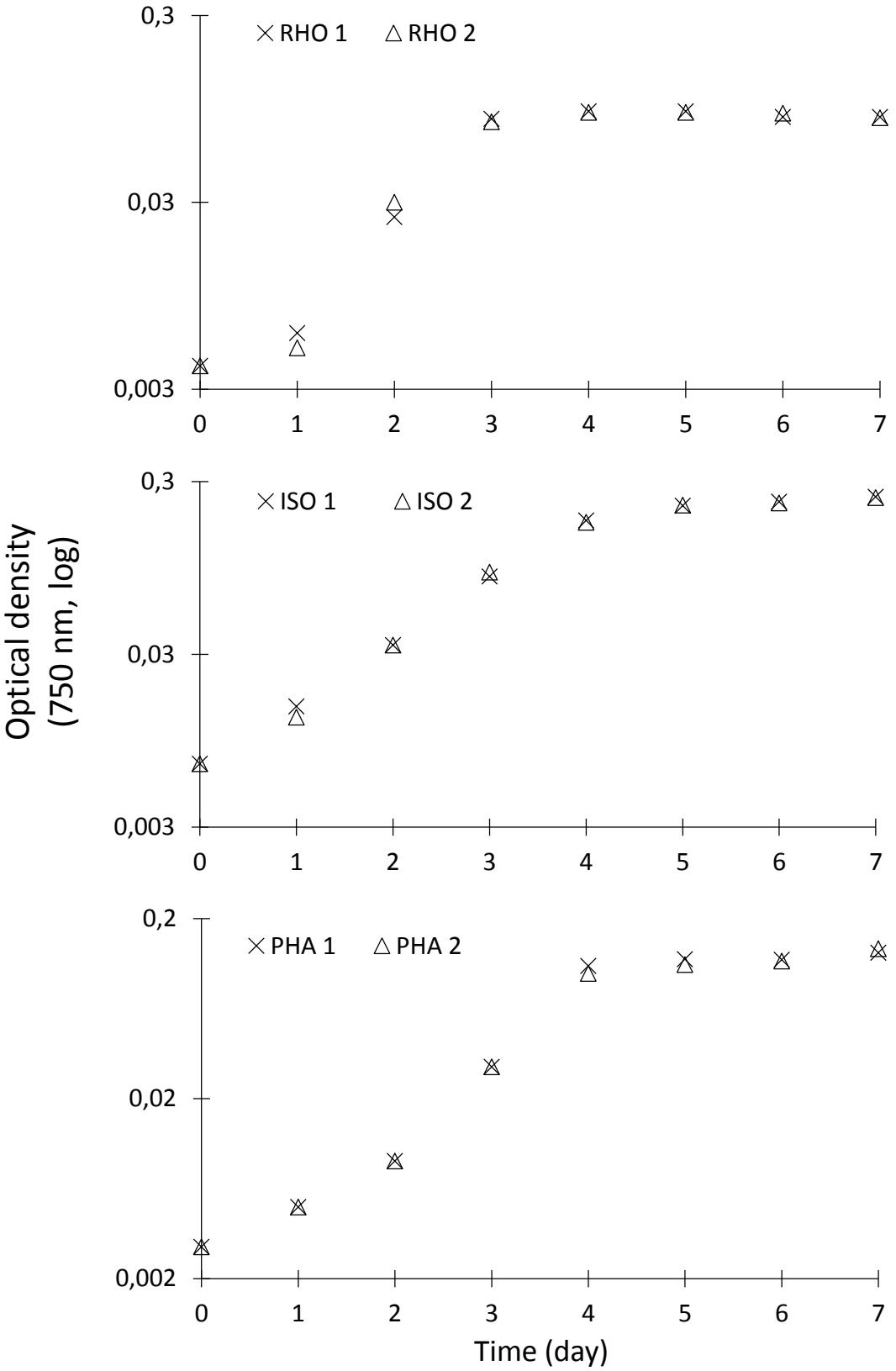

Fig. 3.2. Measured optical density with absorption at 750 nm over time (day), during the batch cultivation of the three microalgae species with two parallels (1, 2). Abbreviations are explained in fig. 3.1.

Table 3.3. Mean pH registered in the three microalgae cultures during batch and semicontinuous (SC) cultivation. Numbers in parenthesis represent the range.

pH	*P. tricornutum*	T-ISO	*R. baltica*
Batch	8.3 (8.1-8.6)	8.4 (8.1-8.6)	8.2 (7.8-8.8)
SC	8.7 (8.3-9.1)	8.7 (8.3-8.8)	8.5 (8.3-8.6)

During the two experiments, the pH in the cultures was on average between 8.2 - 8.7, with a range from 7.8 - 9.1 (table 3.3). From day three, in both cultivation methods, CO_2 was added to the cultures. CO_2 was supplied to ensure excess of carbon used in photosynthesis, this also contributes to regulate the pH in the cultures.

3.1.2 Semicontinuous culture

The semicontinuous (SC) cultivations were conducted over twenty days, and the dilution of the cultures started at day four of cultivation. Collections of the harvested culture were done from day six, when the cultures were in steady state, giving a stable chemical composition within the algae cells (fig. 3.3).

From day four, harvesting of culture were done daily in SC cultures and the cell density were nearly the same from day to day (fig. 3.3, table 3.4). This shows a steady growth, which indicates a dilution rate (D) that result in equal growth rate (μ) from day to day. The measurement of photosystem II efficiency (Q_y) gives also an indication of steady growth in the three microalgae species. During the stationary phase of cultivation the average numbers of cells were between 1.6×10^5 and 1.4×10^6 (table 3.5B). There were no significant differences (t-test, p = > 0.05) of the daily microalgae cell counts between the two parallels within the three microalgae species. Numbers of microalgae cells in the SC cultures were between 1.7×10^5 and 1.4×10^6 in late stationary phase, when the last harvest of culture were done (table 3.4, fig. 3.3).

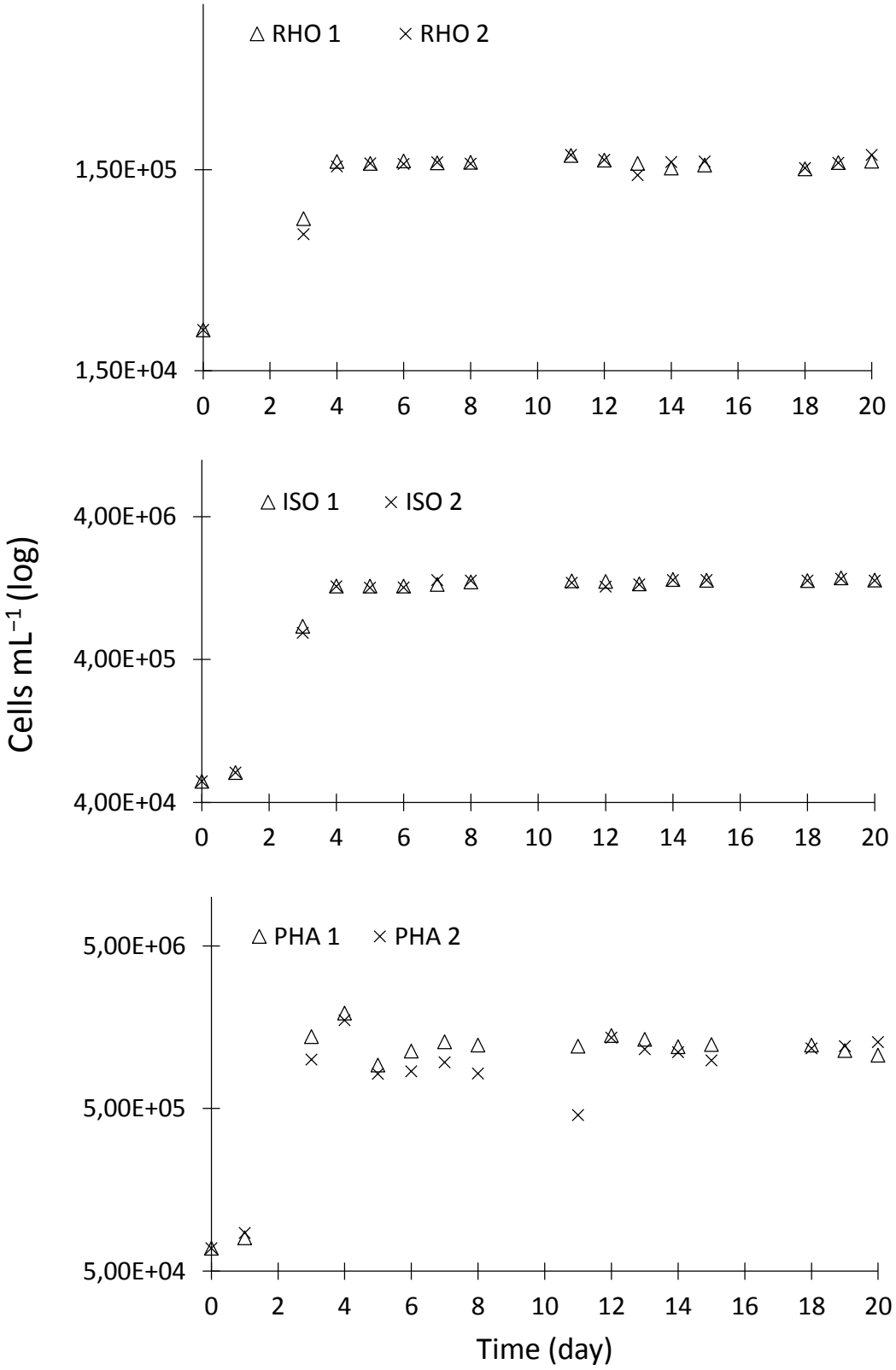

Fig. 3.3. Number of microalgae cells (cells mL^{-1}) over time (day), during the SC cultivation of the three microalgae species with two parallels (1, 2). The cultures were diluted daily, from day four until day twenty, when the cultivations were terminated. Collections of the harvested cultures were done from day six, when the microalgae were in steady state, giving a stable chemical composition within the algae cells. Abbreviations are explained

Table 3.4. Number of microalgae cells mL^{-1} culture at the first day (day 0, t_0), the first day of harvesting (day 4, t_4), day 8 (t_8), day 12 (t_{12}) and last day of the culture (day 20, t_{20}) in SC cultivation of microalgae. The numbers of microalgae cells are an average of the two parallels of each species cultured. From day 8, the numbers of microalgae cells in the culture are approximate equal from day to day, which indicates steady state of the cultures.

Day(t)	*P. tricornutum*	T-ISO	*R. Baltica*
t_0	6.94×10^4	5.61×10^4	2.39×10^4
t_4	1.84×10^6	1.30×10^6	1.60×10^5
t_8	1.03×10^6	1.41×10^6	1.62×10^5
t_{12}	1.39×10^6	1.35×10^6	1.67×10^5
T_{20}	1.18×10^6	1.43×10^6	1.71×10^5

Under the SC cultivation, the temperature for parallel two of *P. tricornutum* increased over two days (day 10 and 11), due to obstructed circulation of the water-cooling system. The increase in temperature lead to reduction in cell numbers in the culture (fig. 3.3) and reduced photosystem II efficiency (Q_y) (fig. 3.4). The culture was harvested as normal during these days, but the harvested culture was not collected for analysis. After two days, the culture reached a steady growth again, and the harvested medium was collected for analysis.

Table 3.5. A; Maximum specific growth rate (μ_{max} d^{-1}) and 50% of μ_{max} (μ_A, d^{-1}) of the algae culture, daily dilution rate (D) set to 50% of μ_{max} and daily volume diluted ($\Delta V, L$) in the three microalgae species in SC cultures. **B**; Cell numbers (cells mL^{-1}) and biomass (mg DW L^{-1}) during the steady state in the SC cultures in the three microalgae species (mean±SD, two parallels: 1, 2)

A	μ_{max}	μ_A	D	ΔV (L)
P. tricornutum	1.63±0.01	0.81±0.00	1.26	0.67
T-ISO	1.19±0.02	0.59±0.01	0.81	0.53
R. baltica	1.40±0.10	0.70±0.05	1.01	0.60

B	Cells mL^{-1}		mg DW L^{-1}	
	1	2	1	2
P. tricornutum	$1.2\times10^6\pm1.3\times10^5$	$1.0\times10^6\pm2.5\times10^5$	26.9±2.8	22.8±5.7
T-ISO	$1.4\times10^6\pm6.0\times10^4$	$1.4\times10^6\pm7.2\times10^4$	35.4±1.5	35.1±1.8
R. baltica	$1.6\times10^5\pm6.6\times10^3$	$1.6\times10^5\pm9.8\times10^3$	30.1±1.2	30.3±1.8

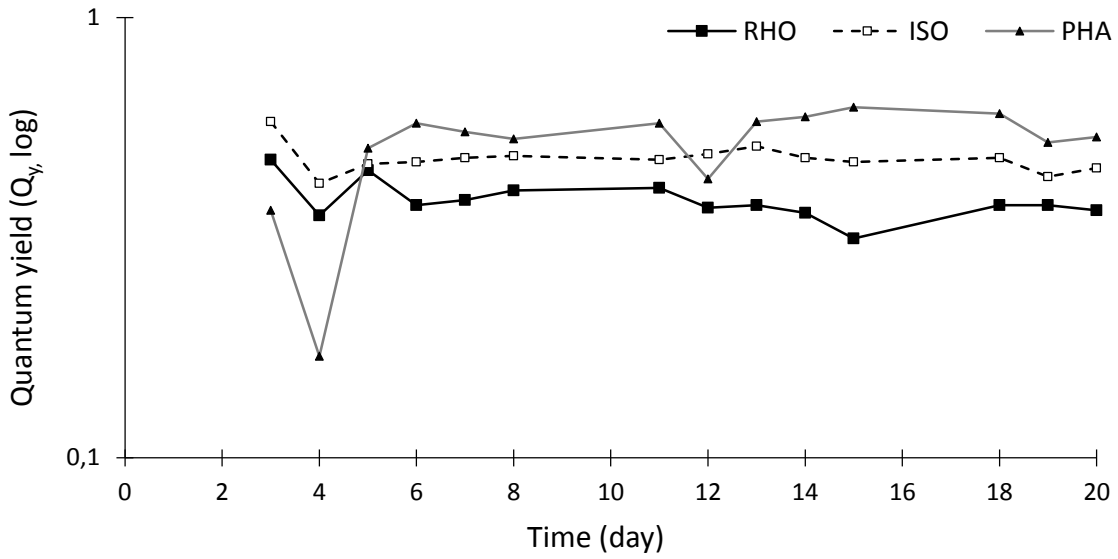

Fig. 3.4. Daily registrations of the photosystem II efficiency (Q_y) during semicontinuous cultivation. The graph shows an average between the two parallels of each microalgae species. Abbreviations are explained in fig. 3.1.

3.2 Lipid and fatty acids content

The content of total lipid of the three different microalgae species varied between 137 and 390 mg g^{-1} dry weight, and the total fatty acids constituted with 25-75% of the total lipids in the algae cells (fig. 3.5, table 3.6). The highest lipid content was found in T-ISO in batch culture, while *P. tricornutum* in semicontinuous culture had the lowest content of lipids. All three species of microalgae had a higher content of lipid with increased nutrient limitation, and for *P. tricornutum* the fraction of total fatty acids also increased. In T-ISO and *R. baltica* the fraction of fatty acids from total lipids decreased with increased nutrient limitation. From the variation in lipids and fatty acids content, we can see a pattern indicating that all algae species accumulate lipid when the access of nutrient is limited. The amounts of lipids, which remained unidentified in the microalgae cells from the analysis, varied between 2.3-7.7%.

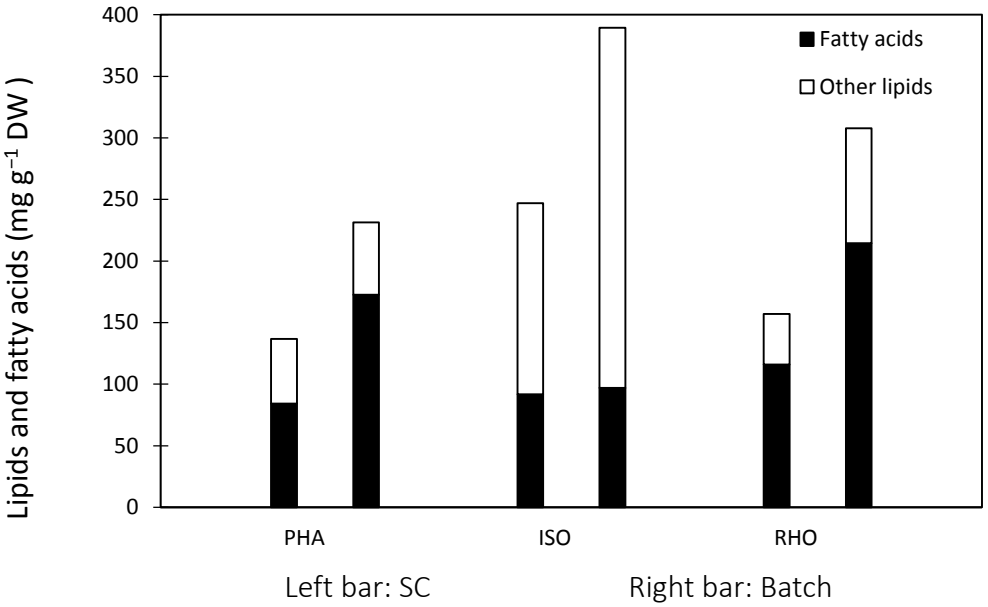

Fig. 3.5. Content of fatty acids and other lipids (mg g^{-1} DW) in microalgae cells grown semicontinuous (SC, 50% of μ_{max}) and in stationary phase in batch culture, with modified f/2 medium. Abbreviations are explained in fig. 3.1.

3.2.1 Fatty acids composition

All three species showed highest content of the saturated fatty acid (SFA) 16:0. The fatty acid profile for *P. tricornutum* is dominating of 14:0, 16:0, 16:1n-7 and 20:5n-3, while 18:1n-7, 18:1n-9, 18:2n-6, 18:4n-3 are found in lower quantities compared to the two other microalgae species (table 3.6, table 3.7). T-ISO and *R. baltica* had similar fatty acid profiles, with 14:0, 16:0, 18:1n-9, 18:2n-6 and 18:4n-3 as dominating fatty acids. For *R. baltica*, the fatty acid 18:3n-3 was found in high amounts, a fatty acid that is almost nonexistence in *P. tricornutum* and only in low amounts in T-ISO. The fatty acid 22:6n-3 (DHA) is only found in high amounts in T-ISO, while 20:5n-3 (EPA) fatty acid is nearly nonexistence compared to the content in the two others microalgae species. Results from the fatty acid profiles shows that the major SFA is 16:0 in all three microalgae species. The fatty acid 16:1n-7 in *P. tricornutum* and 18:1n-9 in T-ISO and *R. baltica* are the dominating monounsaturated fatty acid (MUFA). The presence of different polyunsaturated fatty acids (PUFA) varied in the different species of microalgae, but *P. tricornutum* showed the lowest amounts of PUFA compared to the two other microalgae species.

3.2.2 Lipid and Fatty acid distribution compared with various nutrient limitation

The content of total lipid in all three microalgae species were higher in batch culture compared to semicontinuous culture. Lipid content increased with increased nutrient limitation, with variations between 37-49% compared to the amounts of total lipids in microalgae species with lower nutrient limitation (table 3.6). For *R. baltica* the amounts of total lipid were nearly doubled, while the percentage of total fatty acids of the lipids remained almost constant. In *P. tricornutum* the fraction of total fatty acids increased, while the content in T-ISO decreased with extended nutrient limitation.

The fraction of SFA decreased in T-ISO when the nutrient limitation increased (fig. 3.6, table 3.7), contrary to *R. baltica* where the fraction of SFA increased. For *P. tricornutum* the amounts of SFA remained nearly constant independent of the extent of nutrient limitation. The content of MUFAs increased in *P. tricornutum* and *R. baltica*, and remained constant for T-ISO with stronger limitation of nutrient. The fatty acids 16:1n-7 in *P. tricornutum* and 18:1n-9 in *R. baltica* were the monounsaturated fatty acid that showed highest difference in fraction to variating nutrient supply, where both increased with stronger nutrient limitation. T-ISO was the only microalgae where the content of PUFA increased with increased nutrient limitation, which also applies to the total fraction of EPA and DHA. In *P. tricornutum* the amounts of PUFA decreased with over 40% and the polyunsaturated fatty acid EPA decreased with nearly 50% when the nutrient limitation was strongest.

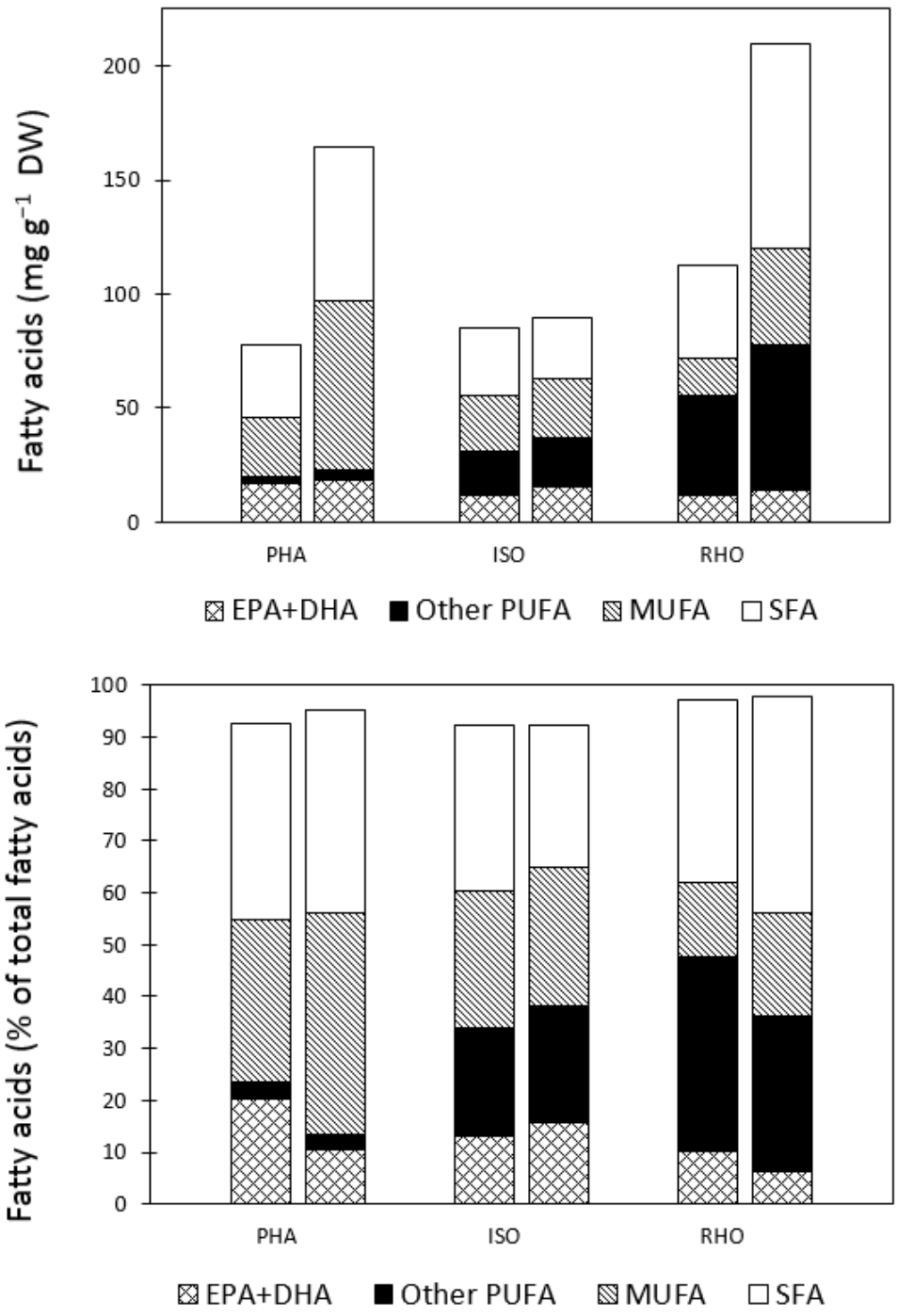

Fig. 3.6. Distribution of fatty acids; saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) grown in SC culture (50% of μ_{max}) and in stationary phase in batch culture, with modified f/2 medium. The upper panel shows quantitative content mg g^{-1} dry weight (DW), and the lower panel shows relative fatty acid composition in the three microalgae species. The bars to left represent SC culture (50% of μ_{max}) and the bars to right represent batch culture in stationary phase. Abbreviations are explained in fig. 3.1.

Table 3.6. Total lipid (mg g^{-1} DW), total fatty acids (mg g^{-1} DW), % fatty acids (FA) of total lipid and content of different types of fatty acids (mg g^{-1} DW) in semicontinuous (SC, 50% of μ_{max}) and batch culture, grown in modified f/2 medium expressed as mean ± SD. Technical duplicate samples (n=2). Numbers without SD had only one sample where the fatty acid was detected. — = not detected or < 0.2 mg g^{-1} DW.

	P. tricornutum		T-ISO		*R. baltica*	
	SC	Batch	SC	Batch	SC	Batch
Total Lipid	136.69±1.07	231.5±3.68	247.0±19.17	389.5±7.06	157.1±2.02	307.7±1.52
Total fatty acids	84.15±1.33	172.5±4.57	91.84±2.88	96.87±1.54	116.03±8.84	214.42±6.29
% FA of tot. lipid	61.6±1.46	74.6±3.16	37.3±1.72	24.9±0.06	73.8±4.68	69.7±2.39
Saturated						
C14:0	5.29±0.00	6.7±0.13	13.44±0.44	9.95±0.17	17.24±1.28	34.51±1.26
C15:0	0.28±0.00	0.39±0.01	0.13	0.2±0.01	—	0.59±0.02
C16:0	22.22±0.4	54.49±1.4	14.29±0.70	14.03±0.37	22.56±1.52	50.71±1.81
C17:0	—	—	—	—	—	0.49
C18:0	1.61±0.05	3.47±0.08	1.07±0.21	0.93±0.04	0.85±0.00	3.28±0.25
C20:0	—	—	—	0.35	—	—
C22:0	0.34±0.00	—	0.59±0.02	1.13±0.02	—	—
C24:0	2.06±0.01	2.55±0.07	—	—	—	—
∑SFA	**31.8±0.44**	**67.6±1.67**	**29.5±1.28**	**26.4±0.36**	**40.8±2.82**	**89.3±2.99**
Monounsaturated						
C16:1n7	25.19±0.47	69.94±1.84	1.17±0.09	1.83±0.20	3.02±0.32	3.63±0.16
C17:1	—	—	—	—	—	0.37
C18:1n7	0.42±0.07	1.52±0.02	0.72±0.02	0.8±0.02	3.85±0.29	4.45±0.15
C18:1n9	0.59±0.02	2.08±0.01	22.27±0.64	23.37±0.26	9.55±0.51	34.37±1.08
∑MUFA	**26.2±0.56**	**73.5±1.87**	**24.2±0.75**	**26.00±0.49**	**16.4±1.12**	**42.6±1.12**
Polyunsaturated						
C18:2n6	0.95±0.01	1.73±0.01	6.58±0.17	6.03±0.08	8.96±0.58	24.79±0.89
C20:3n6	0.89±0.03	—	—	—	—	—
C20:4n6	—	1.76±0.56	—	—	0.69±0.11	1.52±0.03
C22:5n6	—	—	2.16±0.08	2.32±0.03	—	—
∑n-6	1.8±0.03	3.5±0.58	8.7±0.25	8.3±0.10	9.7±0.69	26.3±0.92
C18:3n3	—	0.37	2.41±0.09	2.88±0.03	18.17±1.37	21.69±0.78
C18:4n3	1.11±0.00	1.31±0.02	8.1±0.31	10.54±0.13	15.01±1.14	15.78±0.56
C20:4n3	—	—	—	—	0.63±0.06	—
C20:5n3	14.89±0.08	16.49±0.35	—	0.55±0.09	7.32±0.53	9.04±0.20
C22:6n3	2.00±0.03	1.8±0.06	11.98±0.21	14.72±0.26	4.44±0.29	4.75±0.07
∑n-3	18.0±0.11	19.8±0.12	22.5±0.61	28.7±0.51	45.6±3.39	51.3±1.60
∑PUFA	**19.8±0.08**	**23.3±0.7**	**31.2±0.86**	**37±0.62**	**55.2±4.09**	**77.6±2.52**
EPA+DHA	**16.9±0.11**	**18.3±0.41**	**12±0.21**	**15.3±0.35**	**11.8±0.82**	**13.8±0.27**
UNKNOWN	6.30±0.25	8.10±0.30	6.99±0.01	7.43±0.07	3.58±0.81	4.89±0.36

Table 3.7. Distribution of fatty acids (% of total fatty acids) in semicontinuous (SC, 50% of μ_{max}) and batch culture, grown in modified f/2 medium expressed as mean ± SD. Technical duplicate samples (n=2). Numbers without SD had only one sample where the fatty acid was detected. — = not detected or < 0.2 mg g^{-1} DW.

	P. tricornutum		T-ISO		*R. baltica*	
% of total fatty acids	SC	Batch	SC	Batch	SC	Batch
Saturated						
C14:0	6.28±0.09	3.88±0.03	14.63±0.02	10.27±0.01	14.86±0.03	16.09±0.12
C15:0	0.33±0.01	0.23±0.00	0.14	0.20±0.01	0.15±0.00	0.27±0.00
C16:0	26.41±0.06	31.59±0.02	15.56±0.27	14.48±0.15	19.45±0.17	23.65±0.15
C17:0	—	—	—	—	—	0.23
C18:0	1.91±0.03	2.01±0.01	1.16±0.20	0.96±0.03	0.73±0.06	1.53±0.07
C20:0	—	—	—	0.36	—	—
C22:0	0.41±0.01	—	0.65±0.00	1.16±0.00	—	—
C24:0	2.45±0.06	1.48±0.00	—	—	—	—
∑SFA	**37.8±0.07**	**39.2±0.06**	**32.1±0.39**	**27.3±0.06**	**35.2±0.25**	**41.7±0.18**
Monounsaturated						
C16:1n7	29.94±0.09	40.54±0.00	1.27±0.06	1.89±0.18	2.60±0.08	1.69±0.03
C17:1	—	—	—	—	—	0.18
C18:1n7	0.49±0.07	0.88±0.01	0.79±0.01	0.82±0.01	3.32±0.01	2.08±0.01
C18:1n9	0.70±0.01	1.21±0.03	24.25±0.06	24.12±0.11	8.23±0.19	16.03±0.03
∑MUFA	**31.1±0.17**	**42.6±0.04**	**26.3±0.01**	**26.8±0.08**	**14.2±0.011**	**19.9±0.06**
Polyunsaturated						
C18:2n6	1.13±0.03	1.01±0.02	7.16±0.04	6.22±0.02	7.73±0.09	11.56±0.07
C20:3n6	1.05±0.05	—	—	—	—	—
C20:4n6	—	1.02±0.30	—	—	0.59±0.05	0.71±0.00
C22:5n6	—	—	2.36±0.02	2.39±0.01	—	—
∑n-6	2.2±0.07	2.0±0.29	9.5±0.02	8.6±0.03	8.3±0.04	12.3±0.07
C18:3n3	—	0.22	2.63±0.02	2.97±0.02	15.66±0.01	10.12±0.07
C18:4n3	1.32±0.02	0.76±0.03	8.81±0.06	10.88±0.04	12.94±0.00	7.36±0.04
C20:4n3	—	—	—	—	0.54±0.01	—
C20:5n3	17.70±0.19	9.56±0.05	—	0.57±0.08	6.31±0.02	4.21±0.03
C22:6n3	2.38±0.00	1.05±0.01	13.05±0.18	15.20±0.03	3.83±0.04	2.21±0.03
∑n-3	21.4±0.21	11.5±0.23	24.5±0.12	29.6±0.06	39.3±0.07	23.9±0.05
∑PUFA	**23.6±0.28**	**13.5±0.05**	**34.0±0.13**	**38.2±0.03**	**47.6±0.10**	**36.2±0.12**
EPA+DHA	**20.1±0.19**	**10.6±0.04**	**13.1±0.18**	**15.8±0.11**	**10.1±0.07**	**6.4±0.06**
UNKNOWN	7.5±0.18	4.7±0.05	7.6±0.25	7.7±0.05	3.1±0.47	2.3±0.23

3.3 Biomass and lipid productivity

The biomass and lipid productivity were found by calculations explained in section 2.7 and values obtained from carbon analysis. The microalgae species that had the highest biomass productivity was T-ISO and the lowest value was found in *P. tricornutum*, 15.6 mg $L^{-1}d^{-1}$ and 4.9 mg L^{-1} d^{-1} respectively (table 3.8). All three microalgae species had the lowest biomass productivity in batch culture. The biomass productivity from semicontinuous (SC) culture to batch culture was reduced by 60-67%, and the biggest difference was found in *R. baltica*.

The result of lipid and fatty acid content in the three microalgae species (section 3.2) were used to determine the productivity of lipid, and most interesting the productivity of EPA and DHA production (table 3.8). Highest lipid production was obtained in T-ISO and lowest in *P. tricornutum*, 3.8 mg $L^{-1}d^{-1}$ and 1.1 mg $L^{-1}d^{-1}$ respectively, same trend as for the biomass productivity. The lipid productivity were reduced in all microalgae species from SC culture to batch culture, by 35-42%.

The productivity of EPA and DHA production in the three microalgae species reflects the results found in section 3.2, reduced by 48-62% from SC culture to batch culture. This is a higher reduction than for the overall reduction in lipid productivity. The microalgae with highest EPA productivity was *P. tricornutum* and T-ISO had the highest DHA productivity, both found in SC culture. The microalgae with the greatest difference from SC to batch culture was *P. tricornutum* for EPA productivity and in *R. baltica* for DHA productivity, with a reduction of 62% and 72% respectively.

Table 3.8. Biomass productivity (mg $L^{-1}d^{-1}$) and productivity of total lipid, EPA, DHA and EPA+DHA (mg $L^{-1}d^{-1}$) per day of the three different microalgae species in batch and semicontinuous (SC) culture, grown in modified f/2 medium. – = not detected or < 0.01 mg $L^{-1}d^{-1}$.

	P. tricornutum		T-ISO		*R. baltica*	
	SC	Batch	SC	Batch	SC	Batch
Biomass Productivity	13.9	4.9	15.6	6.5	15.1	5.0
Lipid	1.9	1.1	3.8	2.5	2.4	1.5
EPA	0.21	0.08	–	–	0.11	0.05
DHA	0.03	0.01	0.19	0.10	0.07	0.02
EPA + DHA	0.24	0.09	0.19	0.10	0.18	0.07

4 Discussion

The main objective for this study was to investigate the possible accumulation of lipid and fatty acid in three different microalgae, grown at low nitrogen concentrations. In order to ensure nitrogen limitation in both batch and semicontinuous (SC) culture, the nitrogen concentration was set to only 10% of the original concentration and phosphate was 25% more added to the f/2 growth medium (Guillard 1975). The environmental conditions of the cultures will be discussed first. Thereafter, the progress in the two cultivation methods will be described, before evaluations of the production of lipid and fatty acids in the three microalgae species.

4.1 Culture conditions - Irradiation, pH and temperature

The cultures of the three microalgae had an irradiation of 150 µmol photon $m^{-2}s^{-1}$ at the culture surface during the experiment in both cultivation methods. For *Rhodomonas* sp. and *P. tricornutum* the growth is reported to be highest with a light intensity of 50-60 µmol photon $m^{-2}s^{-1}$, while T-ISO have an increased growth from 50 – 150 µmol photon $m^{-2}s^{-1}$ (Chaloub et al. 2015, Hammer et al. 2002, Kaplan et al. 1986, Qiao et al. 2015, Vu et al. 2015). This means that light intensity was initially sufficient for cellular growth and that most probably the cell growth of the microalgae cultures was limited by a nutrient. By use of the modified f/2 media the growth was believed to be nitrogen limited.

The pH in the cultures was regulated by supply of air mixed with CO_2 and was between 8.2 - 8.4 for the batch culture and between 8.5 - 8.7 for the SC culture (table 3.3). Very low or high pH in the culture will depress microalgae growth due to disruption of different cellular processes (Hoff and Snell 2007). Since the pH in the cultures of this experiment was kept relative constant, we can assume that the concentration of CO_2 mixed together with air supplying the cultures was adequate and supported a good culture environment for microalgae growth.

Cultivation temperature in batch culture and SC culture in this experiment was constant at 22 °C, controlled by a water tube system. The temperature will influence the biomass production, as well as the lipid quantity and quality of microalgae (Hoff and Snell 2007, Qiao et al. 2015, Renaud et al. 2002). All three microalgae species have an optimal growth with temperatures between 14 °C - 20 °C and the content of PUFA tend to increase with decreasing temperatures (Chaloub et al. 2015, Hoff and Snell 2007, Renaud et al. 2002, Zhu et al. 1997). Since the

culture temperature for this experiment was 22 °C, it can be assumed that the temperature was suitable for the growth of the three algae species.

4.2 Growth rates and progress in the different cultivation methods

4.2.1 Batch culture

All the three microalgae cultures had an expected growth curve, where the exponential growth phase was obtained after a few days in log phase. After five days of cultivation, all three microalgae species had entered the stationary phase (fig. 3.1). The pattern of growth can be compared with the fig. 1.1, which shows a typical picture of microalgae growth. The three microalgae were cultivated to stationary phase, which gave a strong limitation of nitrogen in the growth medium. When the cultures were harvested in stationary phase the biochemical composition of the microalgae cells reflected a growth under strong nitrogen limitation. The highest μ_{max} was obtained in *P. tricornutum* (1.63 d^{-1}) and the lowest in T-ISO (1.19 d^{-1}), this result is opposite found in earlier reports (Griffiths et al. 2012, Reitan et al. 1994). In those reports the *P. tricornutum* had a much lower μ_{max} (0.68 - 1.06 d^{-1}), while the value of μ_{max} for T-ISO were nearly the same as found here. The differences in μ_{max} indicates that it can be difficult to compare studies done where microalgae are cultivated, due to highly different environmental and physical cultivation conditions, which influences the growth and biochemical composition of the microalgae. When the batch cultures were harvested, the growth rate of all three microalgae species was under 3% (table 3.2) of the μ_{max}. *P. tricornutum* had the lowest growth rate (−4.54%) and *R. baltica* had the highest (2.62%). Considering the low growth rates, it is reasonable to assume that all three microalgae species were strongly nitrogen limited when they were harvested.

4.2.2 Semicontinuous culture

The dilution rate (D) in SC culture was set to correspond 50% of the μ_{max} (table 3.5A), which theoretically shall give a maximum production (Chauton et al. 2013). Ideally, a dilution rate in a SC cultivation in steady stat should be equal to the specific growth rate of the cultures (Lee and Shen 2004). With correct dilution rate, the cell numbers from day to day should be approximately equal. The progress in cultivation of the three microalgae cultures are shown in fig. 3.3 and table 3.4, and indicates cultures in steady state, due to the nearly constant cell numbers from day to day. Therefore, it can be assumed that the daily harvested microalgae

cultures have a constant biochemical composition. From table 3.4 the average cell numbers of the two parallels of each microalgae species are given of random days in the cultivation. Some variation in cell numbers can be seen, particular between day eight and twelve in the culture of *P. tricornutum.* A reasonable explanation to this is the increase in the culture temperature at day ten and eleven, which gave a disruption in growth conditions and reduced microalgae growth. This can also be seen in measurement of the photosystem II efficiency (Q_y) at day twelve (fig. 3.4). The dilution was performed as normal, but the harvested culture was not collected before the growth was in steady state again. From day thirteen the microalgae culture reached steady state, confirmed by the cell number counts and measurement of photosystem II efficiency (fig. 3.3, fig. 3.4). The registrations of cell numbers during the cultivations of the three microalgae species were generally constant. Small fluctuations in cell number can be explained by the method used to count cells. The coulter counter counts all particles within a size range, which means that other particles than microalgae cells will be counted, and this can give fluctuating cell numbers (Marie et al. 2005).

4.3 Lipid and fatty acids content

The overall lipid content in the three marine microalgae species varied a lot between the species and also between the two cultivation methods in each microalgae species, but all species had the highest content of lipids when they were cultivated in batch culture (table 3.6, fig. 3.5). This indicates that the algae species accumulates lipids when nitrogen excess is restricted, which are in agreement of previous reports (Breuer et al. 2012, Griffiths et al. 2012) The highest amount of lipids was found in batch culture for T-ISO (389.5 mg g^{-1} DW) and the lowest was found in SC culture in *P. tricornutum* (136.7 mg g^{-1} DW). *R. baltica* showed the greatest difference of lipid content between the two methods of cultivation, where the content increased with almost 50% from SC culture to batch culture. This indicates that *R. baltica* responds strongest to nitrogen limited growth medium by accumulate lipids when comparing the three microalgae species The percentage of fatty acid content of total lipid within the microalgae cells did not have the same reaction to stronger nutrient limitation as the content of total lipid. T-ISO and *R. baltica* had lower percentage content of fatty acids from total lipid from SC to batch culture, contrary to *P. tricornutum* that increased from 61% to 74%. If marine microalgae is going to be used as an economic efficiency replacement of fish oil, it is important to know about the quality of lipids and fatty acids. Specially, the quality of fatty acids in the microalgae used is important.

4.3.1 Distribution of fatty acids with various nutrient limitation

The fatty acid composition of P. tricornutum

The fraction of SFA was about the same for batch culture and SC culture (fig. 3.6, table. 3.6), a little higher fraction compared with other studies (Breuer et al. 2012, Patil et al. 2006, Reitan et al. 1994). The highest fraction of MUFA was obtained in batch culture and was dominated by the high fraction of the fatty acid 16:1n-7 in both cultivation methods, in agreement with other studies (Breuer et al. 2012, Patil et al. 2006, Reitan et al. 1994). Clearly, nitrogen limitation enhances the production of MUFA. The fraction of PUFA decreased with almost the half from SC culture to batch culture. The highest fraction of PUFA (23% of total fatty acids) was lower than other studies have found (Breuer et al. 2012, Qiao et al. 2015), but in those studies the growth medium had replete nitrogen concentrations. Compared with the results here, the PUFA production is influenced by the nitrogen concentration in the growth medium, by a reduced production. The fraction of EPA in this microalgae species is previous reported as high, and this is why this species is regarded as an important microalgae for the aquaculture industry (Hoff and Snell 2007). The nitrogen concentrations in both cultivation methods did not result in a higher content of EPA than previous studies have obtained. The content of EPA was generally lower than the content found in same microalgae species, with phosphorus limitation (Reitan et al. 1994) and also in other studies with nitrogen limitation (Breuer et al. 2012, Qiao et al. 2015). The microalgae species show a very similar biochemical composition as other researches have obtained under nitrogen limitation, where the main shift happens in the content of SFA, MUFA and the total amount of lipids, increasing with stronger nitrogen limitation (Breuer et al. 2012, Griffiths et al. 2012, Qiao et al. 2015).

The fatty acid composition of T-ISO

T-ISO had a great differences in the content of total lipids from SC culture to batch culture, coming from the increase in other lipids with increased nitrogen limitation (fig. 3.5). The content of fatty acid did not change much with increased nitrogen limitation, which gave a much lower percentage of fatty acids of the total lipid in batch culture compared with SC culture (table 3.6). The fraction of SFA decreases with increased nitrogen limitation (table 3.7), which is opposite compared to results obtained in other studies (Breuer et al. 2012, Griffiths et al. 2012, Reitan et al. 1994). Variations in results can be explained of the various environmental growth conditions since the mentioned studies all have some differences in cultivation conditions. The percentage fraction of MUFA was nearly constant between the two cultivation methods, which indicate that the MUFA production in this microalgae is not affected by various

nutrient concentrations, in agreement with Griffiths et al. (2012). T-ISO was the only microalgae species where the fraction of PUFA from total fatty acid increased with increased nitrogen limitation, and 18:4n-3 and DHA were the fatty acids that contributed to the increase. DHA fraction increased with nutrient limitation in T-ISO and the highest fraction was obtained in this microalgae compared with the two other microalgae species. The amount of DHA is similar as reported in Breuer et al. (2012) and a little lower than the value reported when the microalgae was phosphorus limited (Reitan et al. 1994). The biochemical composition of T-ISO with stronger nitrogen limitation gives a desired increase in PUFA, specially the content of DHA, but the values obtained here are generally the same or lower than found in other studies (Breuer et al. 2012, Harrison et al. 1990, Patil et al. 2006, Reitan et al. 1994).

The fatty acid composition in R. baltica

The difference between SC culture and batch culture regarding lipid content was greatest in *R. baltica* compared with the two other microalgae species, the amount was almost doubled (table 3.6). The fraction of fatty acids of total lipids was reduced from SC culture to batch culture, same trend found in T-ISO. *R. baltica* had the greatest increase of SFA content from SC culture to batch culture, compared with the two other species. In a report where the fatty acid composition of *Rhodomonas* sp. was detected under various cultivation temperatures, the amount of SFA were generally much lower than found here (Renaud et al. 2002). The exception was the culture grown under the highest temperature (33 °C) which obtained the same content found here. This may be an indication that high cultivation temperature has comparable effect on SFA production as nitrogen limitation in this microalgae species. The fraction of MUFA increased with decreased nitrogen concentration, and the fraction of the fatty acid 18:1n-9 got doubled, an effect only found in *R. baltica*. This fatty acid was the dominating MUFA in *R. baltica*, in accordance to the report by Fernández-Reiriz et al. (1989) and Patil et al. (2006). *R. baltica* had same effect in PUFA production as *P. tricornutum*, it decreased with increased nitrogen limitation. The two fatty acids which dominate the PUFA content were 18:3n-3 and 18:3n-4, a pattern also found in previous studies of *Rhodomonas* sp.(Fernández-Reiriz et al. 1989) (Patil et al. 2006). EPA and DHA fraction in *R. baltica* decreased when nitrogen limitation increased. The overall content of these two fatty acid were in general low compared with the two other microalgae species, which also may explain the relative low interest of this species as a promising source of fish oil replacement.

4.4 Biomass and lipid productivity

The variation of biomass productivity between the three microalgae species was low, between 13.9 and 15.6 mg L^{-1} d^{-1} in SC culture and between 4.9 and 6.5 mg L^{-1} d^{-1} in batch culture (table 3.8). Biomass productivity decreased in all three microalgae species in batch culture compared to the values in SC culture, an expected observation since the nutrient content in the culture decreased over time. The biggest difference in biomass productivity was found in *R. baltica*, with a decrease of nearly 70% from SC culture to batch culture. As mentioned earlier in this thesis, it can be difficult and maybe incorrect to compare studies against each other, since microalgae growth, productivity and biochemical composition are highly dependent on cultivation conditions. None of the referred literatures have identical cultivation conditions compared with each other or to the experiment done in this thesis, and this will give divergent results in varying degree. Even though it can be difficult to compare studies, it can give a unifying trend. In previous reports the biomass productivity of *P. tricornutum* is higher compared with the biomass productivity to T-ISO (Mata et al. 2009, Rodolfi et al. 2008). The opposite found in this experiment, but both of the referred literatures had conducted the microalgae cultivation with replete nutrient concentrations, contrary to the cultivations done here. This can indicate that the productivity of the microalgae *P. tricornutum* is more influenced than the productivity of T-ISO when cultivated with nutrient limitation.

The reductions of lipid productivity from SC culture to batch culture were lower in all three microalgae species than the reductions of biomass productivity. This indicates that the microalgae accumulates lipid when nitrogen concentration in growth medium is limited, which is also found in the lipid analysis. The accumulation of lipids in the three microalgae species in batch culture follows the pattern of lipid productivity. The productivity of EPA and DHA in the three microalgae species follows the biomass productivity, a major reduction from SC culture to batch culture. *P. tricornutum* had the highest productivity of EPA and T-ISO had the highest productivity of DHA. Both microalgae species had very low productivity of the opposite fatty acid.

All the values of biomass, lipid and fatty acid productivity obtained in SC cultures and batch culture are generally very low compared to previous reports (Breuer et al. 2012, Griffiths et al. 2012, Mata et al. 2009, Rodolfi et al. 2008). The results are not unexpected considering the very low nitrogen concentration in the growth medium. As mentioned previous in this thesis, nitrogen limitation is generally found to increase the production of lipids in many microalgae

species, which potentially can lead to increased PUFA content. In this study, the overall content of PUFA in the three microalgae species in both cultivation methods were about the same or lower obtained by previous studies. The connection between the productivity and the content of the desirable PUFA in *P. tricornutum* and *R.baltica* do not favor such strong nitrogen limitation shown in the growth medium in this study. The percent fraction of EPA and DHA decreased together with the productivity in these microalgae species. T-ISO was the only microalgae that had a positive correlation of decreased productivity and increased percent fraction of PUFA and DHA, obtained under strong nitrogen limitation in the growth medium.

4.5 Concluding remarks

The lipid and fatty acid profiles in the three microalgae species were in accordance with previous reports, and *P. tricornutum* and T-ISO had an expected high content of EPA and DHA respectively. Strong nitrogen limitation lead to accumulation of lipids in varying degrees. The percent fraction of PUFA content decreased for the microalgae *P. tricornutum* and *R. baltica*. T-ISO was the only microalgae that had positive correlation between increased nitrogen limitation and percent content of PUFA, including the content of DHA.

The overall biomass, lipid and fatty acid productivity in the three microalgae species were generally low compared with other reports, which may reflect the influence of the low nitrogen concentration in both cultivation methods. This indicates that the nitrogen concentration in the growth medium gave a limited growth of the microalgae species, which lead to a low biomass concentration.

In this thesis the main object was to explore the potential content of lipids and fatty acids when the microalgae species were cultivated under nitrogen limited conditions. Obtaining a high content of EPA and DHA was the main focus, due to the importance of these fatty acids in considering microalgae as a lipid replacement of fish oil in feed. This thesis has shown that the nitrogen concentration used here lead to higher lipid content, but the overall production of EPA and DHA were low due to a major decrease in productivity. Evaluating microalgae as lipid replacement for fish oil in feed, the results show that the microalgae *P. tricornutum* and T-ISO contains high values of EPA and DHA respectively and have therefore potential as a lipid source in feed. In light of the results obtained in this study, it would be useful to reconsider the extent of nitrogen limitation in the microalgae due to the major reduction of productivity. The

balance between productivity and nitrogen limitation is essential to obtain a desired result, where both biomass production and the content of EPA and DHA are high.

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Appendix 1

F/2 medium

The algae medium is modified from (Guillard 1975). To ensure depletion of nitrate and sufficient amount of phosphate for the algae growth, the recipe have only 10% of the original amount of sodium nitrate and 25% more of sodium phosphate than the original recipe.

Into 950 ml of filtered seawater, add the following components. Bring the final volume to 1 liter with filtered seawater.

Component	Stock Solution ($g \bullet L^{-1} dH_2O$)	Quantity Used
$NaNO_3$	75.0	0.10 ml
$NaH_2PO_4 \bullet H_2O$	5.0	1.25 ml
$Na_2SiO_3 \bullet 9H_2O$	30.0	1.00 ml
Vitamins Solution *	--	0.50 ml
Trace Metals Solution **	--	1.00 ml

Trace Metals Solution**

Into 950 ml of dH_2O, add the following components. Bring the final volume to 1 liter with dH_2O.

Component	Stock Solutions ($g \bullet L^{-1} dH_2O$)	Quantity used
$FeCl_3 \bullet 6H_2O$	--	3.15 g
$Na_2EDTA \bullet 2H_2O$	--	4.36 g
$MnCl_2 \bullet 4H_2O$	180.0	1.0 ml
$ZnSO_4 \bullet 7H_2O$	22.0	1.0 ml
$CoCl_2 \bullet 6H_2O$	10.0	1.0 ml
$CuSo_4 \bullet 5H_2O$	9.8	1.0 ml
$Na_2MoO_4 \bullet 2H_2O$	6.3	1.0 ml

Vitamins Solution*

Into 950 ml of dH_2O dissolve the Thiamine•HCl and add the other components. Bring the final volume to 1 liter with dH_2O

Component	Stock Solutions ($g \bullet L^{-1} dH_2O$)	Quantity Used
Thiamine•HCl	--	200.0 mg
Biotin	1.0	1 ml
Cyanocobalamin	1.0	1 ml

Appendix 2

Analytical settings and standard curves for carbon and nitrogen analysis.

Elemental Analysis Software
www.costechanalytical.com

Calibration	: ntnu_HILDE_MAI_2016	By	: sd
Description	:		
Created	: 06.10.2000 14:41:57	Modified	: 23.05.2016 15:51:46

Calculation	: ESTD	Mode	: Calibrate
Calibrate	: Automatic	Recalibration Type	: Average
Change Response	: Enable	Weight	: 0,25
Update Reten. Time	: Enable	Search Criteria	: 0,00%

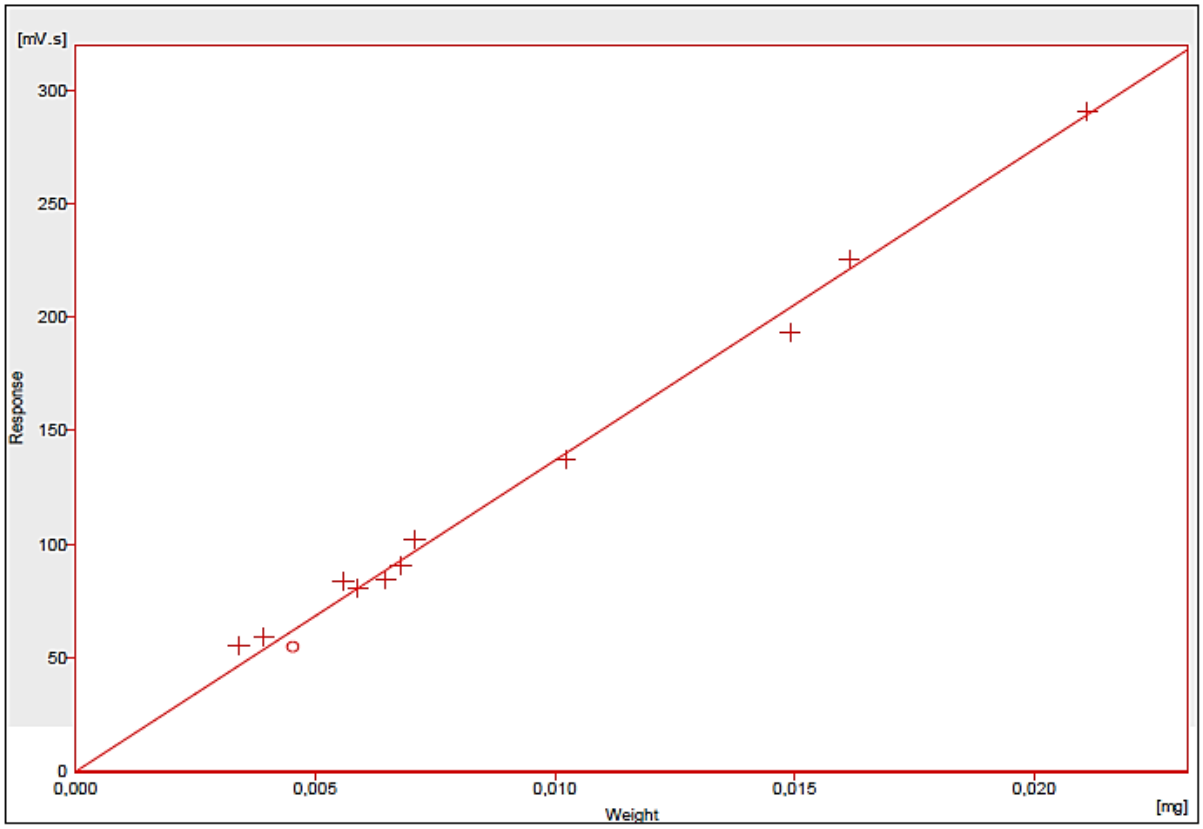
Calibration Summary Table (ESTD - ntnu_HILDE_MAI_2016 - Signal 1)

Used	Element Name	Reten.Time	Left Window	Right Window	Peak Type	Peak Color	LOD	LOQ	R B	Resp. Factor
☒	Nitrogen	1,735	0,500	0,500	Ordnr		0,000	0,000	A	0,0000
☒	Carbon	2,808	0,500	0,500	Ordnr		0,000	0,000	A	0,0000

Nitrogen - Signal 1 - 1,735 min.

Peak Type	: Ordnr
Left Window	: 0,5 min
Right Window	: 0,5 min
Response Base	: Area
Curve Fit Type	: Linear
Zero Type	: Curve from Zero
Weighting Method	: None
Subst. Equation	: Y = 13693,1019*X
Correlation Coef.	: 0,9974327
Residuum	: 5,4754 [mV.s]

	Response	Weight	Resp. Factor	Rec No.	Used	Deviation [%]
1	225,5481	0,01614	0,0001	1	☒	-2,0082
2	290,6397	0,02110	0,0001	1	☒	-0,5745
3	55,0836	0,00341	0,0001	1	☒	-15,2704
4	84,8420	0,00646	0,0001	1	☒	4,3363
5	102,3591	0,00708	0,0001	1	☒	-5,3423
6	137,2209	0,01023	0,0001	1	☒	2,0372
7	83,4855	0,00558	0,0001	1	☒	-8,4119
8	80,8141	0,00589	0,0001	1	☒	-0,1181
9	54,6850	0,00455	0,0000	1	☐	13,8828
10	59,4065	0,00393	0,0001	1	☒	-9,4961
11	192,8833	0,01491	0,0001	1	☒	5,8346
12	90,3407	0,00679	0,0001	1	☒	2,8536
13	0,0000	0,00000	0,0000	0	☒	-
14	0,0000	0,00000	0,0000	0	☒	-
15	0,0000	0,00000	0,0000	0	☒	-
16	0,0000	0,00000	0,0000	0	☒	-
17	0,0000	0,00000	0,0000	0	☒	-
18	0,0000	0,00000	0,0000	0	☒	-
19	0,0000	0,00000	0,0000	0	☒	-
20	0,0000	0,00000	0,0000	0	☒	-



Carbon - Signal 1 - 2,808 min.

Peak Type : Ordnr

Left Window : 0,5 min

Right Window : 0,5 min

Response Base : Area

Curve Fit Type : Linear

Zero Type : Curve from Zero

Weighting Method : None

Subst. Equation : Y = 35281,01142*X

Correlation Coef. : 0,9990881

Residuum : 60,05307 [mV.s]

	Response	Weight	Resp. Factor	Rec No.	Used	Deviation [%]
1	3996,7918	0,11076	0,0000	1	☒	-2,2300
2	5188,8901	0,14481	0,0000	1	☒	-1,5386
3	836,2073	0,02339	0,0000	1	☒	-1,3195
4	1458,8028	0,04436	0,0000	1	☒	7,2846
5	1718,4998	0,04855	0,0000	1	☒	-0,3171
6	2394,2214	0,07017	0,0000	1	☒	3,3957
7	1366,9457	0,03832	0,0000	1	☒	-1,1021
8	1414,3806	0,04045	0,0000	1	☒	0,9010
9	935,5416	0,03121	0,0000	1	☐	17,6931
10	934,5000	0,02694	0,0000	1	☒	1,7207
11	3510,6179	0,10230	0,0000	1	☒	2,8080
12	1619,6028	0,04656	0,0000	1	☒	1,4337
13	0,0000	0,00000	0,0000	0	☒	-
14	0,0000	0,00000	0,0000	0	☒	-
15	0,0000	0,00000	0,0000	0	☒	-
16	0,0000	0,00000	0,0000	0	☒	-
17	0,0000	0,00000	0,0000	0	☒	-
18	0,0000	0,00000	0,0000	0	☒	-
19	0,0000	0,00000	0,0000	0	☒	-
20	0,0000	0,00000	0,0000	0	☒	-