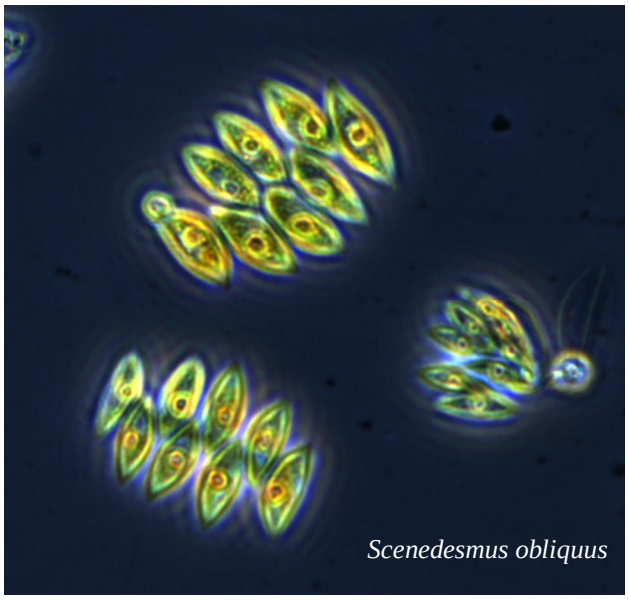
Optimizing lipid production by planktonic algae:

LIPIDO

2008-2010

End of project report, 1.6.2011

Edited by: Kristian Spilling



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Nordic Energy Research

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From the annual meeting held in Helsinki, March 2010. From left: Sigurbjörn Einarsson, Timo Tamminen, Jukka Seppälä, Heiko Rischer, Olav Vadstein, Herwig Stibor , Matilde Skogen Chauton, Anne-Kathrin Graber, Dagmar Enss, Maria Stockenreiter and Kristian Spilling.

Executive summary

The main topic of the LIPIDO project was to study how lipids accumulate in planktonic algae, and how this could be optimized for producing lipids for biofuel purposes. The main objectives were:

1) To screen for the most promising algal species for temperate environments

2) To optimize their growth and lipid yield as functions of growth conditions

3) To test the practical applicability of coupling algal culturing to CO_2 emission mitigation

4) To screen commercially interesting by-products from biomass of selected species

All of these topics have been addressed, and in the following report is a detailed description of results by the individual partners.

1
An extensive screening took place revealing several new strains that could be of interest as lipid producers. The main focus in this project was on temperate species; the Finnish partners focused on brackish water/Baltic Sea species (Ch 1 -2), the German partner on fresh water algae (Ch. 5) whereas the Norwegian and Icelandic partners focused on marine species of algae (Ch. 3, 4 and 5). Overall the results suggests that temperature is not a constrain for cultivating algae; it is just different species that thrive in different temperatures. Both the growth rate and lipid composition were comparable between the screened cold and warm water adapted algae, and availability of sunlight is the main constraint for algal cultivation in Northern Europe.

In addition to measure lipids using traditional methods (which are often time consuming and labor intensive) such as gas chromatography, we tested alternative methods for fast determination of lipid levels (Ch 2, Appendix I). Most notably, Nile Red staining was selected as a proxy for measuring lipid concentration. Reviewing the literature, we found that several approaches had been taken in terms of staining time and excitation /emission wavelengths. During the LIPIDO project the Nile Red staining method was compared between partners, and the method was standardized as far as possible considering the different instruments used for measuring the florescent properties of Nile Red stain. The literature review and protocol developed can be found in Appendix I. Additionally, different cultivation techniques and development of equipment for high throughput screening was also carried out in the LIPIDO project (Ch 3 and 4).

2
Various environmental parameters affect growth and the lipid composition and concentration in algal cells. In LIPIDO, the effect of light, temperature, salinity and different nutrient limitations on algal growth and lipid composition were studied for different species (Ch. 1-6). The results showed large species specific differences, with no general trend for all species. However, there is a good understanding of lipid synthesis theoretically, and work was undertaken to model the lipid synthesis (Ch. 4) and to follow the regulation of lipid metabolism on a genetic transcriptional level (Ch. 3).

Most of the previous work on utilizing algae for biofuel has concentrated on finding one optimum species that would be the best candidate for large scale cultivation. However, results from biodiversity experiments revealed that overall production of biomass, and also of lipids, may increase if more than one species are co-cultivated (Ch. 5). Different species may supplement each other and thereby increase the overall resource efficiency. In particular, complementary effects of light utilization enhanced the resource specific biomass and lipid production. Different algal classes provide different photosynthetic active pigments with different absorption spectra and diversity can therefore enhance the efficiency of light used for growth and lipid production.

In higher plants stress conditions can be induced by treating plants with one or several elicitors. In many cases the resulting reaction is an enhanced production of defence compounds i.e. secondary metabolites, but elicitation can also influence primary metabolites such as lipids. In a set of experiments chitosan was tested as an elicitor on *Chlorella* sp. and the results suggest that lipids may increase on a short term when using chitosan as an elicitor (Ch. 2).

3
Work on CO_2 uptake by algae was mainly undertaken by the Icelandic partner, Blue Lagoon, where they used flue gas from a geothermal power plant to feed the algal cultures. The results showed that algal growth was similar when grown in the flue gas compared with growth in a mixture of air and pure CO_2. In a joint project between Blue Lagoon,

SYKE and VTT the effect of different CO_2 concentration on lipid composition was studied. Preliminary results suggest that the CO_2 concentration influences the lipid concentration and composition. This is also supported by preliminary results from Univ. Oslo where a new experimental unit has been built to study the effect of CO_2 on algal growth (Ch. 4).

4
VTT was the main responsible partner for studying commercially interesting side products of algae and they concentrated on antimicrobial effects of algae. The effect of both *Chlorella pyrenoidosa* and *Scenedesmus obliquus* was tested on a series of known human pathogens and one non-pathogen bacterium. The results showed that *S. obliquus* had little or no effect on the survival and growth of the tested pathogens, but *C. pyrenoidosa* showed clear antimicrobial activity against all the pathogenic microbial strains tested. This effect was dose dependent and was also varying between the pathogens tested (Ch. 2).

Publications (as of May 2011)

Rischer, H (2009) Photosynthetic microorganisms as a future source of energy. In: K. Larjava (ed.), Energy Visions 2050, WS Bookwell Oy, Porvoo, 246-247.

Packer A, Li Y, Andersen T, Hu Q, Kuang Y, Sommerfeld M (2011) Growth and neutral lipid synthesis in green microalgae: A mathematical model Biores.Technol. 102: 111-117.

Spilling K, Seppälä J, Tamminen T (2011) Inducing auto-flocculation in the diatom *Phaeodactylum tricornutum* through CO_2 adjustment. J Appl Phycol. In press. DOI 10.1007/s10811-010-9616-5

Stockenreiter M., Graber A.-K., Haupt F. and Stibor H. (2011). The effect of species diversity on lipid production by micro-algal communities. J Appl Phycol. In press. DOI: 10.1007/s10811-010-9644-1.

In addition several papers originating from the LIPIDO project are in preparation and will be submitted soon.

1. Report from Finnish Environment Institute

By: Kristian Spilling, Jukka Seppälä and Timo Tamminen

Background

The need to reduce greenhouse gas emissions, increasing prices of fossil fuels, and related concerns about the future supply, all together have revived the interest in alternatives to fossil fuels. Bio-derived fuel is an interesting substitute for fossil fuels already being implemented into our energy portfolio. However, the common practice of using agricultural crops (e.g. palm oil) as feedstock for biofuel production has raised both societal and environmental concerns. There is therefore a need to develop new, sustainable biomass sources, if biomass-based fuel is to be utilized on a large scale. Microalgae offer a very promising source of biomass due to very high productivity, favorable biomass composition (e.g. high in lipids) and its potential of being incorporated with CO_2 mitigation and wastewater treatment. However, basic research on some key barriers is needed to bring the production cost of growing algae low enough for this technology to be implemented on a large scale. The main aim of this project was to investigate the potential of algae as feedstock for biodiesel production, where lipids, in particular triglycerides, were of main interest.

Results

All the results presented below were done in collaboration with VTT, which have determined all the fatty acid samples.

Screening

In order to identify high lipid yielding algae, with emphasis on local species (originating from Finnish coastal waters), we screened several phytoplankton species that were promising lipid producers (Table 1). In addition a few marine species that were known to be high lipid producers from the literature were also screened and used as benchmark species. In terms of environmental requirements the screened algae was divided into three different groups; cold water adapter Baltic Sea species (grown at 4°C, 6 PSU), warm water, Baltic Sea species (grown at 18°C, 6 PSU) and warm water, marine species (grown at 18°C, 35 PSU).

In total, twenty phytoplankton strains were screened for growth rate and lipid contents in both exponential and stationary growth phase (Figs 1 & 2). During the exponential growth phase, free fatty acids (FFA) and fatty acid methyl esters (FAME) comprised on average 9 % (range 2–27 %) of the dry weight in the studied species. During the stationary growth phase, under nitrogen limited conditions, the share of FFA and FAME in cells increased, and comprised on average 20% (range 11–27 %) of the dry weight in studied species.

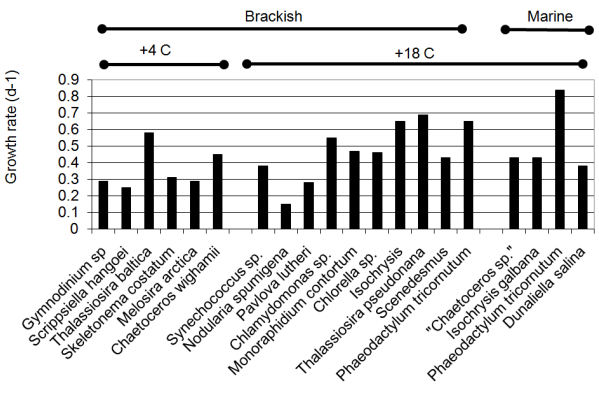

Fig 1. Exponential growth rate of the screened algae. The upper horizontal bars denotes the environmental conditions the cultures were grown under: Brackish at 6 PSU, Marine at 35 PSU and at 4°C and 18°C.

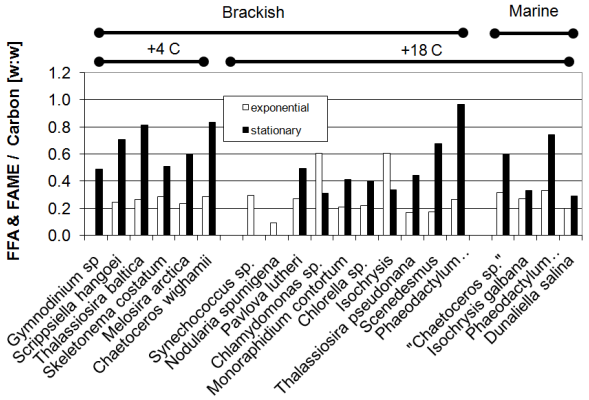

Fig 2. Total fatty acid content of the screened algae under exponential growth and under N-limitation. The upper horizontal bars denotes the environmental conditions the cultures were grown under: Brackish at 6 PSU, Marine at 35 PSU and at 4°C and 18°C.

Table 1: Species screened for lipid composition

Species	Class	Strain code	Growth rate [day^{-1}]
Baltic, cold water species (4° C, salinity 6)			
Gymnodinium sp.	Dinophyceae	GCTV-B4	0.29
Scrippsiella hangoei	Dinophyceae	SHTV-5	0.25
Thalassiosira baltica	Diatomophyceae	TVK-TBA-1	0.58
Skeletonema costatum	Diatomophyceae	TVK-SCO-1	0.31
Melosira arctica	Diatomophyceae	TVK-MAR-1	0.29
Chaetoceros wighamii	Diatomophyceae	TVK-CWI-1	0.45
Baltic, warm water species (18° C, salinity 6)			
Synechococcus sp.	Cyanophyceae	TV65	0.38
Nodularia spumigena	Cyanophyceae	TV-HEM	0.15
Pavlova lutheri	Prymnesiophyceae	TV3	0.28
Chlamydomonas sp.	Chlorophyceae	TV44	0.55
Monoraphidium contortum	Chlorophyceae	TV70	0.47
Chlorella pyrenoidosa	Chlorophyceae	TV216	0.46
Isochrysis sp.	Prymnesiophyceae	TV-ISOCHR	0.65
Thalassiosira pseudonana	Diatomophyceae	TV5	0.69
Scenedesmus obliquus	Chlorophyceae	TVK-SOB-1	0.61
Phaeodactylum tricornutum	Diatomophyceae	TV335	0.80
Marine, warm water species (18° C, salinity 35)			
Phaeodactylum tricornutum	Diatomophyceae	CCAP 1055/1	0.85
Isochrysis galbana	Prymnesiophyceae	CCAP 927/1	0.43
Dunaliella salina	Chlorophyceae	CCAP 19/18	0.41
Chaetoceros muelleri	Diatomophyceae	CCAP 1010/3?	0.43

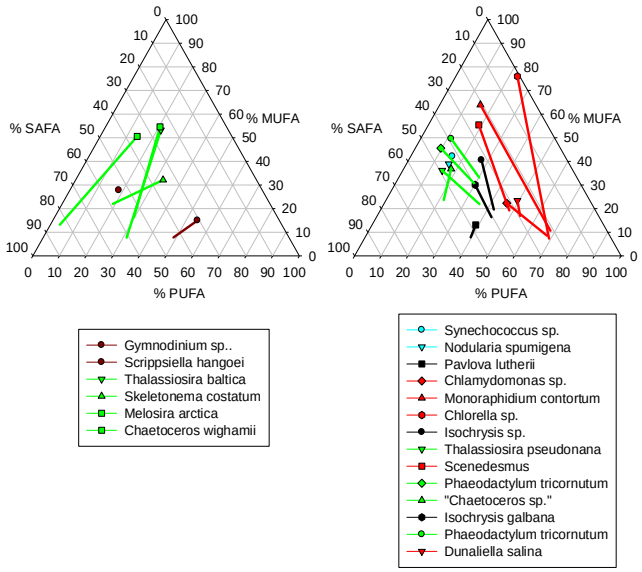

Fig 3. The ratio of SAFA:MUFA:PUFA, going from exponential (start of line) to stationary growth phase (point). Only one point of the stationary growth was determined. Different algal groups are denoted with different colors: diatoms – green, green algae – red, cyanobacterial – blue, dinoflagellates – brown and haptophytes - black.

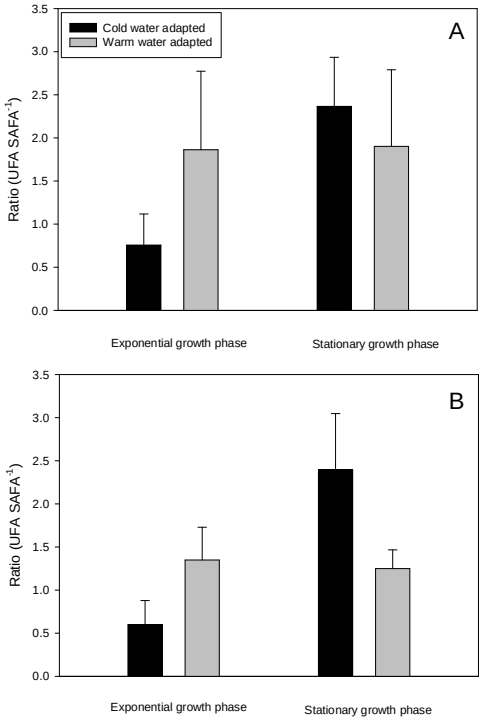

Fig 4. The ratio of unsaturated fatty acids (UFA) to saturated fatty acids (SAFA) for cold and warm water adapted species in exponential and stationary growth phase. The bars depicts the average values for the whole dataset (A; 6 cold water and 14 warm water species) and diatoms only (B; 4 cold water and 4 warm water diatoms). Error bars denotes standard deviation. Both free fatty acids (FFA) and fatty acid methyl esters (FAME) where included in the data.

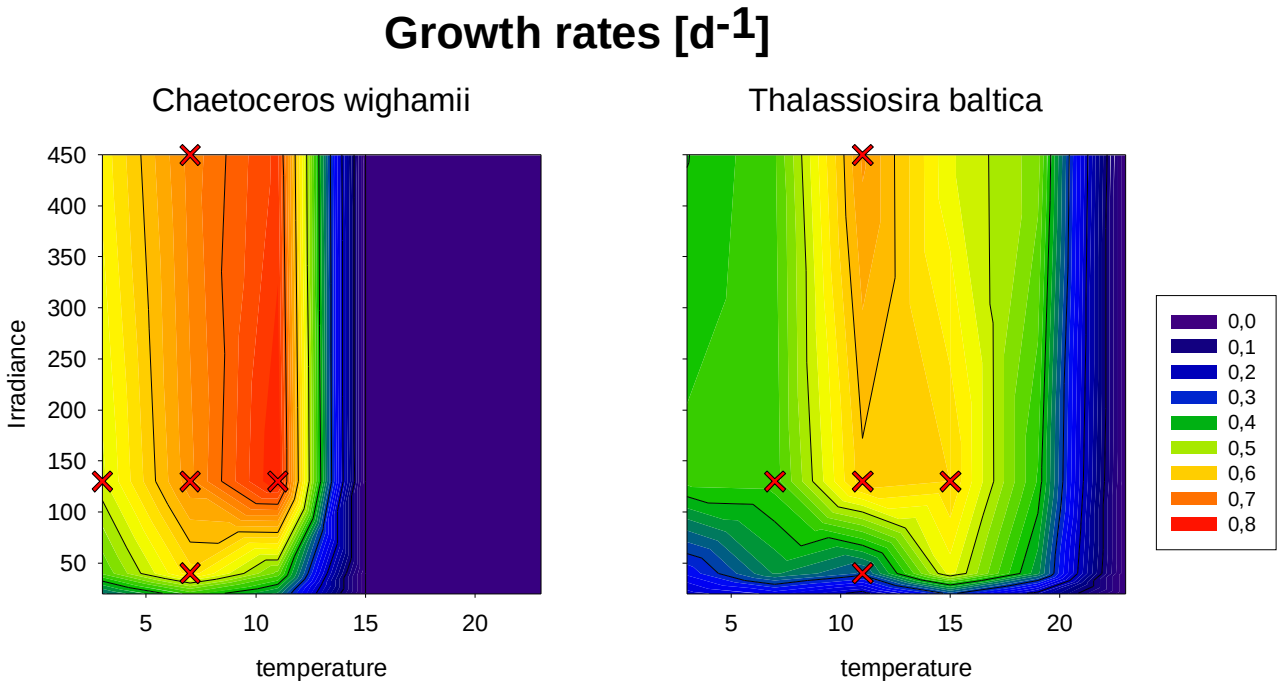

Fig 5. Growth rate (d^{-1}) of *C. wighamii* and *T. baltica* under different temperature and irradiance regimes. Crosses mark the combinations that were continued into stationary growth phase. Three limitations were used (P, N and Si limitation), which was induced by manipulation the nutrients in the medium.

In terms of growth rate and lipid content there was no large group specific differences, and the cold water adapted spices grew at almost similar rates as the warm water adapted species, perhaps with the exception of *Phaeodactulym tricornutum* which had clearly the highest growth rate of all tested species. There were furthermore no large difference between cold and warm water adapted species in terms of lipid content.

Fatty acid composition in algae adapted to different temperature

Temperature has been proposed to affect the amount of poly unsaturated fatty acids (PUFAs), and presence of PUFAs in the thylacoid membranes seem to be important for the photosynthetic machinery to work. Structural lipids are an integral part of the cells membranes, and the ratio between unsaturated fatty acids (UFAs) to saturated fatty acids (SAFAs) in the membranes affects the membrane fluidity. For this reason cold adapted plants generally contain more PUFAs than warm adapted plants. Most of the work on temperature effect on fatty acid composition has been done on higher plants and very little has been on this topic for algae.

Based from the results from the screening we analyzed the fatty acid composition in the algae presented in Table 1, and there seem to be some general differences between cold and warm adapted species (Fig 3). The cold adapted speces had unexpecidly a lower proportion of unsaturated fatty acids (UFA's) compared with the warm water adapted species in exponential growth phase (Figs 3 & 4), but the relative amount of UFA's increased in stationary growth phase. The warm water adapted algae had relatively stable proportion of total UFA's to SAFA's in exponential and stationary growth phase, but there was a general shift from PUFA's to MUFA's.

The relatively low amount of UFA's in the cold water adapted algae during exponential growth phase, when most of the fatty acids are bound to membranes is surprising as this is not found in higher plants. This suggests that there may be other mechanisms for controlling the membrane fluidity in cold water adapted algae compared with higher plants. This would however need further study as only a limited number of species were tested and most of the examined cold water species were diatoms.

Optimization

Based on the screening, two of the cold water algae with the best growth and lipid properties (*Thalassiosira baltica* and *Chaetoceros wighamii*) were picked for further tests of optimizing lipid production. This was done in a full factorial design experiment with temperature and irradiance as experimental variables. The growth and lipid concentration was determined in the exponential growth phase and the cultures were then split into three different nutrient manipulations. These manipulations were adjusted to give N, P and Si limited growth in stationary growth phase. Exponential growth is presented in Fig 5. The lipid content was mostly low (<5% of dry weight) during exponential growth, but

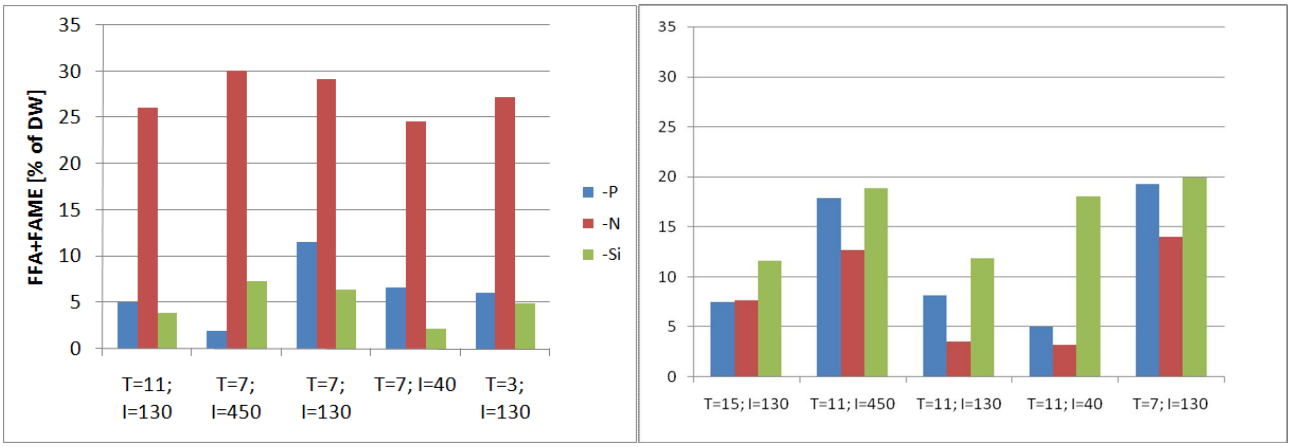

Fig 6. Fatty acid content of *C. wighamii (left panel)* and *T. baltica* (right panel) under different temperature (T, in °C) and irradiance (I, in µmol photons m^{-2} s^{-1}) regimes and different nutrient limitation (P, N and Si).

this increased during stationary growth phase to maximum of 20-30% lipids of total dry weight (DW) for *T. baltica* and *C. wighamii* respectively (Fig. 6). The nutrient limiting growth had a big impact on the accumulation of lipids; for *C. wighamii* N limitation was clearly increasing the lipid content whereas for *T. baltica* P and Si limitation produced the highest lipid content. The results clearly show that the affect of environmental parameters and nutrient limitation on the lipid metabolism is species specific.

Harvesting

Harvesting is a major cost of algal cultivation and finding a low cost way to dewater the algal suspension is critical for production of low cost commodities such as fuel. Aggregation due to polymers (flocculation) or electrolytes (coagulation) has been seen as a potential first step in algal harvesting, and several approaches have been taken. The most commonly used method has been to add a flocculating (or coagulating) agent, but this method has associated costs in handling and materials. Simply increasing the pH can also induce flocculation in some algae (Fig 7), and in a set of experiments it was demonstrated that *Phaeodactylum tricornutum* was able to increase the pH to self flocculating levels. The threshold for flocculation to start was at pH 10.5 (Fig 8). Algae raise pH when photosynthesizing, and the autoflocculation mechanism observed for *P. tricornutum* can be induced by turning off the CO_2 supply, potentially providing a simple, low-cost, initial dewatering step for this species (Spilling et al. 2011).

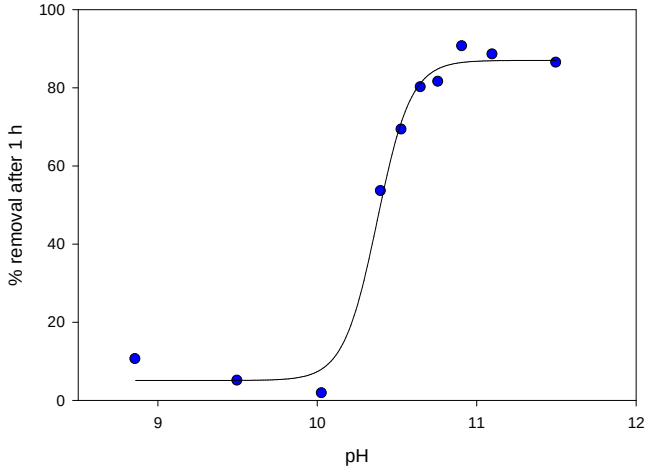

Fig 7. Removal of the biomass from the upper water layers in a cultivation vessel, 1 hour after adjusting the pH with NaOH. Flocculation starts to take place after a threshold of pH 10.5 has been passed.

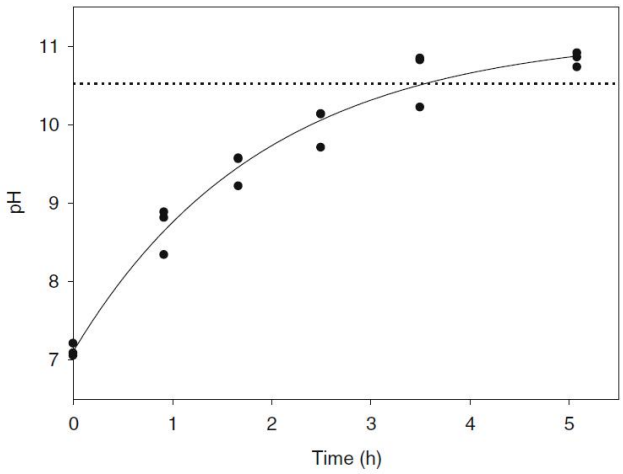

Fig 8. The increase of pH in a culture with concentration 1g dry weight / liter, in a few hours the pH is high enough to induce flocculation after aeration has been switched off. The culture here was kept in suspension with magnetic stirring.

2. Report from VTT Technical Research Centre Finland

By: Dagmar Enss, Heiko Richer and Kirsi-Marja Oksman-Caldentey

Methodical questions

Establishment of different methods for lipid analysis

Aims

In order to identify promising microalgae species for biodiesel production about 20 different species were cultivated and samples were taken in different growth phases. Besides lipid content also the lipid composition of a species is an important criterion for the biodiesel production. Therefore, two different methods focussing on lipid quality and quantity were established in our laboratory.

Both established methods were based on the same extraction protocol. The colorimetric test was meant as a high-throughput screening method for lipid quantity while the GC analysis gave more detailed information about lipid content and composition.

Material and methods

1) Lipid extraction

For cell disruption, 500 µL chilled (- 20 °C) methanol with 0.1 % butylated hydroxytoluene was added to 5 mg dry weight (DW) algae sample and incubated at -20 °C for 10 min in reaction vials. In pre-chilled racks, algae samples were disrupted with two 4 mm stainless steel balls in each vial in a mixer mill (3 min, 25 Hz, MM 301, Retsch, Haan, Germany). The following steps were performed at room temperature. To each sample 1000 µL chloroform and 150 µL internal standard (1549 mg/L triheptadecanoin, Sigma-Aldrich, 1029.6 mg/L heptadecanoic acid in chloroform:methanol, 2:1, Fluka, Sigma-Aldrich) was added and samples were mixed for 10 min. After centrifugation, supernatant was acidified with 300 µL 20 mM acetic acid, mixed for 5 to 10 min and centrifuged again. Organic phase was extracted two times with 500 µL chloroform, mixed 10 min and centrifuged. Organic phases were pooled and 750 µL were dried in a glass tube under nitrogen flow. The residue was dissolved in 300 µL isopropanol. Each sample was extracted twice and GC analyses were performed in duplicates, and the average of these measurements was used in data analysis. Isopropanol extracts were used for the colorimetric test.

For transmethylation and gas chromatography (GC) with flame ionisation detector (FID), fatty acid samples were dried again under nitrogen flow, taken up in 700 µL petroleum-ether and 125 µM sodium methoxide (97 %, dissolved in methanol) was added before boiling solution at 45 °C for 5 min. After cooling, 500 µL $NaHSO_4$ (15 % m/v) and 200 µL petroleum-ether was added and the samples were mixed. After separation of the two phases via centrifuging, the petroleum-ether phase was transferred to a GC vial, solvent was evaporated and residue was dissolved in 1000 µL hexane. For further GC analysis, 1 µL of this solution was used.

2) Colorimetric test with Serum Triglyceride Determination Kit as a high-throughput method

Test was performed with the Serum Triglyceride Determination Kit (#TR0100; Sigma-Aldrich) which is based on an enzymatic reaction and a coupled colour reaction. It was performed according to manufacturer`s manual but additionally samples were dissolved in isopropanol containing 5.1 % m/v Triton X-100 (Riedel-de Haën).

For a cheaper and convenient solution the test was also performed in microtiter scale. In comparison to manufacturer's manual the protocol was scaled down 1:4 and taken out in a 96-well microtiter plate (Nunc). Therefore the protocol was modified in the following way: Five µL sample dissolved in isopropanol with Triton X-100 (5.1 % m/v) was mixed on a plate shaker (30 s, step 5, Wallac) with 0.2 mL of Free Glycerol Reagent on a prechilled metal block and incubated with lid in oven (Venticell, MMM Medcenter) at 37 °C for 15 min on a prewarmed metal block. The absorbance at 540 nm was measured with microtiter scanner (Multiscan EX, Themo Labsystems) and used for calculating free glycerol content in samples.

The samples were replaced on prechilled metal block and 50 µL Triglyceride Reagent was added. Samples were mixed (30 s, step 5, Wallac) and incubated again in oven on a prewarmed metal block at 37 °C for 15 min. The absorbance at 540 nm was measured with microtiter scanner and used for calculating triglyceride content in samples.

For calibration glycerol and triolein (both Sigma) curves were generated.

3) Gas chromatography with flame ionisation detector (GC FID)

Fatty acids were separated and evaluated quantitatively by using a capillary gas chromatograph (7890A with sam-

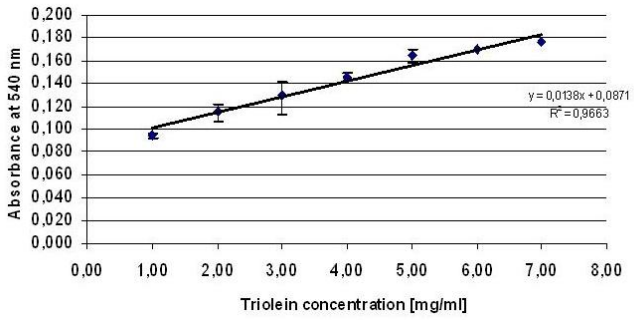

Fig. 1: Triolein calibration curve in colorimetric test

pler CTC ANALYTICS GC-PAL SYSTEM, Agilent Technologies) equipped with a BP-21 column (25 m × 0.2 mm × 0.3 mm, HP-FFAP Polyethylene Glycol TP, Agilent Technologies), and connected to a flame ionization detector. The injector temperature was 260 °C. For every sample, 1 µL was injected and transferred splitless to the column. The oven temperature was programmed to increase from 70 °C (1.5 min) to 240 °C at the rate of 7 °C/min. The carrier gas was helium with a pressure on 16.671 psi. Peaks were allocated to substances via multicapillary column gas chromatography and via comparison to reference substances (F.A.M.E. Mix, #1891, Supelco, Sigma-Aldrich). Altogether 30 fatty acids or fatty acid methyl esters were identified and quantified.

Results

Results indicated clearly that the lipid extraction method in combination with gas chromatography showed very high reproducibility concerning lipid quantity. All samples were analyzed at least twice; results varied only in negligible ranges. The additional information about the lipid composition led us to the conclusion that gas chromatography was the method of choice for lipid analysis.

During establishment of the colorimetric test several problems occurred: For a reproducible standard curve a triolein stocksolution (10 mg/mL) was prepared by emulsifying triolein in water with an ultrasonic bath and by vigorously mixing. Because of triolein's non-polar properties none of the methods led to a homogeneous, stable emulsion with reproducible results. Therefore, the emulator Triton X-100 (Fluka) was used (final concentration 0.1 % m/v) which led to acceptable results with the standard substance in concentrations between 1 and 7 mg/mL (Fig. 1).

Using the algae samples brought up another problem. The isopropanol extracts which were dried under nitrogen flow and taken up in the Triton X-isopropanol mixture were coloured in different green shades depending on the algae species. This colour interfered with the optical measurement and led to very inaccurate results. Therefore, we decided to analyze all samples via GC.

Establishment of axenic microalgae cultures and 16S rDNA analysis for selected algae

Aims

There are many examples that bacteria which are socialised with microalgae have a significant influence on those algae (Croft et al., 2005, Bruckner et al., 2008, Park et al., 2008). In order to work with algae cultures under controlled conditions we established several axenic cultures. Therefore a protocol for the cleaning procedure and two tests for approving the axenic status were established.

One of the tests on bacteria absence is the 16S ribosomal DNA (rDNA) analysis. 16S ribosomal DNA is partly highly conserved between different species of bacteria. Therefore PCR reactions amplifying and sequencing 16S rDNA is a method to check the occurrence and identity of bacteria in algae cultures. Because 16S rDNA is also part of the plastid DNA of microalgae the 16S DNA sequence of axenic algae helps also to identify the algae on species level as it was done for five of our axenic microalgae cultures.

Material and methods

1) Cleaning procedure

In order to study possible interactions between algae and associated bacteria first axenic cultures of *Scenedesmus* sp., *Chlorella* sp., *Nitzschia* sp., *Thalassiosira* sp., *Chaetoceros* sp. and *Melosira baltica* were established.

Therefore three parallel flasks with 1/10 volume algae in 9/10 volume T-2 medium, a modified f/2 medium (Guillard, 1975, Spilling et al., 2010) were cultured with the final volume of 100 mL under standard conditions (24 °C, 90 rpm with 12:12 light:dark cycle) for 1-2 weeks. Cells were harvested by filtration through 100 µm and 5 µm pore size filters (Millipore). The algal cells in filters were suspended into 50 mL of T-2 medium, centrifuged (10 min at 1 000 xg) and washed tree times. Resuspending cells in 50 mL T-2 medium containing 0.005 % Tween 20 (Fluka) and 0.1 M EDTA and incubating them at 20 °C for 1 h should destroy remaining bacteria. In the next step lysozyme (0.5 mg/L) was added, incubated at 20 °C for 10 min and then SDS (final concentration 0.25 % m/v) was added and incubated under the same conditions for 10 min. To remove SDS and lysozyme the cells were centrifuged and washed twice and then resuspended into 50 mL T-2 medium. According to literature (Demain and Elander, 1999; Nauerby et al., 1997; Ogawa and Mii, 2005; Sjahril and Mii, 2006) following antibiotics were added to algae cultures: Rifampicin (10 µg/ mL), ticarcillin (150 µg/mL), gentamycin (100 µg/mL), cefotaxime (250 µg/mL), ampicillin (250 µg/ mL) and meropenem (10 µg/mL). After 7 and 14 days cultivation under standard conditions with antibiotics 5 mL aliquots were

transferred into 25 mL T-2 medium. Axenic algal cultures were subcultured every week by transferring 20 mL culture of each flask into 80 mL fresh media. After three subculture cycles presence of bacteria was tested according to the two following protocols.

2) Approving axenic status

Culturing on medium plates..For the first test on bacteria absence 0.5-1 mL of each axenic algal cultures were streaked on a plate containing Marine Agar 2216 (BD Difco) and incubated for 3 weeks at 25 °C. Plates were observed for bacterial contaminations.

Analysis of 16S rDNA. DNA was extracted from cultures which were confirmed to be bacteria-free on plates according to a modified CTAB protocol (Doyle & Doyle, 1987). Ten mL of each algal suspension were collected into 2 mL tubes by sequentially centrifuging at 10 000 xg for 5 min (Biofuge primo R, Heraeus).

Optimization studies showed that homogenization of samples with an equal amount of small glass beads in a mixer mill (30 s, 30 Hz, MM301, Retsch) was most efficient. After homogenization 400 µL extraction buffer (50 mM Tris-HCl, 50 mM EDTA, 20 % sucrose, 2 % β-mercaptoethanol, pH 8,0) and additionally to literature a treatment with lysozyme (2,5 mg/mL) was added. In order to digest proteins samples were incubated for 1 h at 37 °C before proteinase K (0.2 mg/L in 1 % SDS solution) was added and the mixture was incubated at 55 °C for another 1 h. Finally 500 µL of a 1 % CTAB solution with 0.7 M NaCl was added and samples were incubated at 65 °C for 10 min. Nucleic acids were extracted twice with 600 µL phenol:chloroform:isopropanol (ratio 25:24:1) before it was precipitated with 670 µL ethanol (94 % m/v). Pellet was once washed with 70 % ethanol containing 30 µM ammonium acetate (Riedel-de Haën) and nucleic acid pellet was resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4).

For checking an approximately 1600 bp sequence part of 16S rDNA PCR experiments for each sample were performed with 1.25 U AmpiTaq® polymerase (Applied Biosystems) according to manufacture's instructions and with the following primers (200 nM each) from literature (Lane, 1991): Eubac27F: 5'-AGA GTT TGA TCC TGG CTC AG -3' and 1492R: 5'-GGT TAC CTT GTT ACG ACT T-3'. In contrast to manufacture's instructions precisely 40 ng genomic DNA and 2 mM $MgCl_2$ in combination with the following optimized temperature program lead to best results: Denaturation time 1.5 min at 95 °C was followed by 35 cycles at 95 °C for 30 s, 53 °C for 1 min, 72 °C for 1.50 min, and the final 72 °C for 7 min.

PCR products were analyzed electrophoretically on a 1 % (w/v) agarose gel containing 1 µg/mL ethidium bromide using TBE buffer. All samples presenting bands in the approximated size were cleaned up (High Pure PCR Product Purification Kit, Roche) and then used for sequencing reaction (Big Dye® Terminator v3.1 Cycle Sequencing RR-100, Applied Biosystems) both according to manufacture's manuals. Sequencing was done as a service.

Sequences were analyzed with BLAST, the Sequence Similarity Searching option in NCBI (National Centre for Biotechnology Information) database.

Results

With cleaning protocol we established axenic cultures of *Scenedesmus* sp., *Chlorella* sp., *Nitzschia* sp., *Thalassiosira* sp., *Chaetoceros* sp. and *Melosira baltica.* After the first antibiotic treatment all cultures were subcultured three times (once a week) before absence of bacteria in axenic algal cultures was confirmed on medium plates. Only in a very few culture lines bacteria were observed. These lines were discarded. However, for all clean lines a second antibiotic treatment was performed to ensure that cultures were definitely axenic.

16S rDNA from axenic algae cultures was partly amplified via PCR. PCR products were purified and sequenced with primer Eubac27F. Comparing the resulting approximately 500 bp sequence parts with published sequences in NCBI database showed that rDNA from axenic *Nitzschia* culture was 98 % identical with *Nitzschia thermalis* isolate C17 (FJ002224) and analyzed rDNA from axenic *Scenedesmus* culture even conformed to 100 % to *Scenedesmus obliquus* (strain UTEX 393, DQ396875). The analyzed 16S rDNA sequence part of axenic *Thalassiosira* culture showed 99 % identity with sequence of *Thalassiosira pseudonana* (isolate 11; FJ00221) while amplified DNA from axenic *Chlorella* culture was 98 % consistent with *Chlorella pyrenoidosa* (strain IAM C-101, AJ242752). Amplified rDNA of *Chaetoceros* was 100 % identical with *Chaetoceros muelleri* (isolate C12, FJ002219.1).

Biological questions

Elicitation of microalgae for enhancing lipid production

Aims. In plant cultures of higher plants stress conditions are induced by treating plants with one or several elicitors which are often chemical compounds. In many cases the resulting reaction is an enhanced production of defence compounds i.e. secondary metabolites (Dörnenburg & Knorr, 1995). It was also shown that elicitation has an influence on primary metabolites such as lipids. By adding yeast or fungal elicitors to alfalfa cell cultures acetyl-CoA carboxylase enzyme activity and transcripts were induced several fold (Shorrosh et al., 1994). Changes in lipid profile by elicitation was shown in *Nicotiana tabacum* (Tavernier et al., 1995).

In pre-tests we investigated the response of *Chlorella pyrenoidosa* and *Scenedesmus obliquus* upon the elicitors methyl jasmonate (MeJA) and chitosan in respect of lipid content. MeJA is a plant hormone which is produced in many plants in response to biotic and abiotic stress. Chitosan is a well-known elicitor in plant culturing of higher

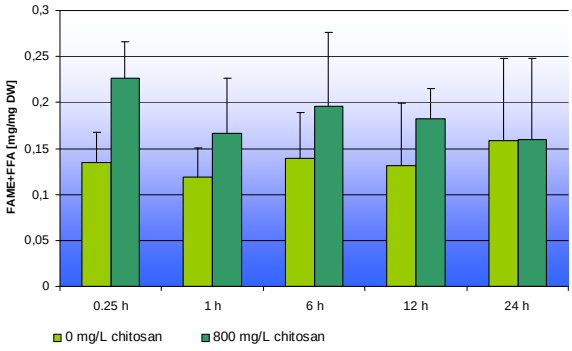

Fig. 2: Lipid content of chitosan-treated and non-treated *Chlorella* samples at different time points

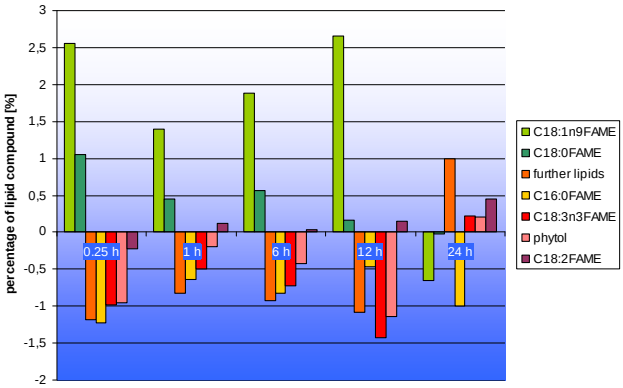

Fig. 3: Differences in lipid profiles of chitosan-treated and non-treated *Chlorella* samples at different time points

plants. It is a polysaccharide produced from shrimp carapace. Because shrimp carapace is a waste product in sea food industry it is a very cheap compound and therefore suitable for usage in high amounts that are needed for treating microalgae for biodiesel production in large scale.

Pre-tests

In the pre-tests 50 mL cultures of axenic *Chlorella pyrenoidosa* and *Scenedesmus obliquus* were cultivated for 14 day at 25 °C, 90 rpm shaking and under 12:12 light-dark cycle (illumination 200 µmol s^{-1} m^{-2}). Then either MeJA or chitosan was added at the following concentrations: MeJA: 0/25/50/100 mM; chitosan: 0/400/800 mg/L. Samples treated with MeJA were harvested after 0/18/24/48 h and those with chitosan after 0/24/48 h. Total lipids from all samples were extracted and analyzed via GC according to the protocol above (Methodical questions - Establishment of different methods for lipid analysis).

None of the *Chlorella* and *Scendesmus* samples treated with MeJA showed a significant higher lipid content than the control (non-treated samples). Also *Scenedesmus* samples which were treated with chitosan did not show an enhanced lipid content at any observed time point. However, *Chlorella* reacted on chitosan in high concentration with an initial increase in total lipids directly after elicitation. Because of the low amount of samples and varying results the main experiment with higher numbers of replicates and only one chitosan concentration was performed.

Main experiment - Material and methods

1) Purification of Chitosan

Chitosan [(poly(beta-(1,4)-D-glucosamine) from crab shells, Sigma] was prepared by dissolving in acetic acid (6 % v/v) by stirring over night. The insoluble fractions were discarded by centrifuging (23 000 xg, 30 min) chitosan was precipitated by adjustment of pH value to 6.0 with 2 M NaOH. Procedure was repeated once with only 3 h stirring and shorter centrifugation (23 000 xg, 10 min) Precipitation was washed three times with deionised water and lyophilized.

2) Elicitation procedure

Two weeks old axenic *Chlorella pyrenoidosa* and *Scenedesmus obliquus* cultures were used for elicitation experiment. Densities of algal cells were determinated with a Fuchs Rosenthal chamber (cell depth 0.2 mm, 0.0625 mm^2, Fortuna). Per algae species 34 samples, containing 50 mL culture in T-2 medium, a modified f/2 medium (Guillard, 1975, Spilling et al., 2010), were adjusted to density of 1.0 x 10^6 cells/mL and placed in a randomized order in the incubator (Multitron II, Infors) using a True Random Number Generator (http://www.random.org/lists). Two samples were used only for determination of growth at day 1, 3, 6, 8, 10, 13 and 14. Cultures were incubated at 25 °C, 90 rpm shaking and under 12:12 light-dark cycle until day 10 and then switched to continuous light (luminance 200 µmol s^{-1} m^{-2}).

Chitosan was added to cultures as a stock solution (6.6 mg/mL in 0.1 M acetic acid) with a final concentration of 690 mg/L culture. As a control, samples which were either non-treated or treated with equal amount of 0.1 M acetic acid were used. Samples were harvested by centrifugation 0.25 h, 1 h, 6 h, 12 h and 24 h after elicitation. Lipids were extracted and analyzed via GC as described above (Methodical questions - Establishment of different methods for lipid analytic).

Main experiment - Results

Chitosan-treated samples harvested after 0.25 h showed on average a total lipid content of 0.23 mg/mg DW. In comparison to untreated control group, which contained 0.13 mg/mg DW total lipids, the increase of fatty acid methyl esters (FAMEs) and free fatty acids (FFAs) accounts for 59.5 %. The Mann-Whitney test approved a significant ($p \leq 0.05$) increase in total lipid content of the chitosan treated samples.

During the following 24 h those additional FAME and FFA in chitosan-treated were stepwise reduced. While treated samples harvested after 1 h, 6 h and 12 h showed still an enhanced total lipid content in comparison to control samples, treated and untreated samples harvested after 24 h exhibit approximately the same total lipid content with 0.16 mg/mg total lipids on DW basis.

In addition to the total lipid amount also the lipid profiles of all samples were determined. Fig. 3 displays the differences in the six most abundant lipids and further lipids (sum of all other detected lipids) of chitosan-treated and untreated samples.

Oleic acid methyl ester (C18:1n9FAME) and stearic acid methyl ester (C18:0FAME) were increased in all treated samples harvested after 0.25 h, 1 h, 6 h and 12 h. Increase of those both compounds correlated with the increase in total lipids. The percentage of alpha-linoleic acid methyl ester (C18:3n3FAME), palmitoleic acid methyl ester (C16:0FAME), phytol and other lipids on the total lipids were decreased up to 12 h in the same ratio oleic acid methyl ester and stearic acid methyl ester were increased. Similarly to the lipid contents also the lipid profiles of treated and untreated samples harvested after 24 h did not show a significant difference any more. Obviously the adjustment of treated and untreated samples, observed for the total lipid content, was also visible at the lipid profile level.

Probably chitosan addition led to a stress situation in which *Chlorella* produced additional lipids. In order to confirm the results and to discover the underlying mechanism further studies have to be performed.

Screening for bioactive side-compounds: Tests on antimicrobial activity of Chlorella and Scenedesmus

Aims

In order to make economical use of algae biomass as a source for biodiesel it is necessary to utilize the biomass also for sidestream and downstream products. Therefore we tested two microalgae species for their antimicrobial activity. Lyophilized and homogenized axenic *Chlorella pyrenoidosa* and *Scenedesmus obliquus* cultures were tested for their antimicrobial activity against five pathogenic bacteria, one non-pathogenic bacterium and one fungus.

Material and methods

The microbes used in this study included both human pathogens and probiotic bacteria (Table 1). Bacterial strains *Staphylococcus aureus* VTT E-70045, *Escherichia coli* VTT E-94564T, *Escherichia coli* VTT E-093121, *Pseudomonas aeruginosa* VTT E-84219 and *Salmonella enterica* sv. Typhimurium VTT E-981151 used in the antimicrobial activity assay were cultured aerobically at 37 °C in Nutrient Broth (NB, Oxoid) with agitation (150 rpm) or on Nutrient Agar (NA, Oxoid). Probiotic bacterial strain *Lactobacillus rhamnosus* VTT E-96666 was grown in MRS (de Man Rogosa Sharpe) medium at 37 °C in anaerobic chamber. Yeast strain *Candida albicans* VTT C-85161 was grown at 37 °C on yeast mould agar (YMA) or with agitation of 150 rpm in yeast and mould broth (YMB).

Antimicrobial activity of the algal material on the selected microbial strains was measured in liquid cultures according to literature (Nohynek et al., 2006), modified to miniature size of 0.5 mL culture volume. Lyophilized and homogenized algae material was suspended in different concentrations (*Chlorella*: 50/25/5/1/0.25 mg/mL; *Scenedesmus*:

Table 1: Microbial strains used for activity tests

Strain and VTT catalogue numbers	**Source**	**Received from**
Candida albicans VTT C-85161	bronchomycosis	ATCC 10231
Escherichia coli T VTT E-94564^{T}	urine of cystitics patient	ATCC 11775
Escherichia coli uro VTT E-093121	clinical strain causing urinary tract infection	Benita Westerlund, University of Helsinki
Lactobacillus rhamnosus VTT E-96666	human faeces	ATCC 53103
Pseudomonas aeruginosa VTT E-84219	infected wound	ATCC 15692
Salmonella enterica sv. Typhimurium VTT E-981151	virulent human pathogen	National Public Health Institute
Staphylococcus aureus VTT E-70045 (ATCC 6538)	human lesion	Sanitized testing Laboratory, Switzerland

Table 2: Summarized results of activity tests

Compound tested	Conc. mg/ml	*Escherichia coli* T	*Escherichia coli* uro	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Candida albicans*	*Salmonella enterica* sv. Typhimurium	*Lactobacillus rhamnosus* GG
Chlorella	50			+++++				
	25	+++++	+++++		+++++	+++	+++++	+++
	5	+++++*	+++++	++++$_+$*	+	++	++++$_+$	+++
	1	±		+				
	0.5	-		-				
Scenedesmus	50			+				
	25	-	+		-	-	-	
	5	-	±	-	-	-	-	-
Controls	Conc. µg/ml							
Chloramphenicol	50	++++*	++++$_+$	++++ *	+++$_+$		+++	++++
	5	++++	++++	+++	++		+++	++
Hygromycin B	500					++++$_+$		
	50					++++		

-	no effect on growth
±, +, ++	delayed growth
+++, +++$_+$	inhibition of growth; static effect
++++, ++++$_+$	decrease of cell count; microbicidic effect
+++++	death of the culture (cfu below detection limit); microbicidic effect
*	tested twice

50/25/5 mg/mL) to 500 µL of microbial growth medium, which was inoculated with 1 % microbial culture grown over night. Microbial cultures without algae material were used as control. For comparing the antibiotic activity with commercial antibiotic compounds the same experiment was also performed with cultures containing chloramphenicol (for the bacterial strains) and hygromycin B (for the yeast strain).

The cultures were incubated in optimal growth conditions for each microbial strain. Microbial growth was followed by sampling the cultures altogether 4 times during the incubation period of 24 h. The samples were diluted in peptone saline, the proper dilutions were plated, and the microbial counts were recorded from the plates after incubation of 24 – 30 h. The inhibitory effects of algae extracts and antibiotics on the microbes were measured by comparing the control growth curve with those obtained from cultures with algae extracts.

Results

Chlorella pyrenoidosa showed clear antimicrobial activity against all the tested microbial strains but the effect was both dose and microbial species dependent (Table 2). *Pseudomonas aeruginosa* and *Candida albicans* were the most

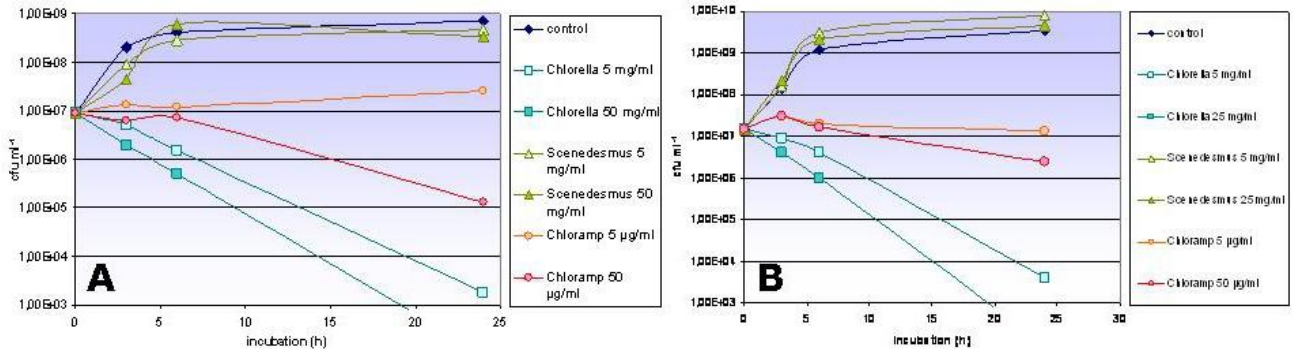

Fig. 4: Antimicrobial effect of disrupted *Chlorella pyrenoidosa* and *Scenedesmus obliquus* cells and chloramphenicol on A) *Staphylococcus aureus* E-70045 and *Salmonella enterica* sv. Typhimurium VTT E-981151

tolerant strains to disrupted *Chlorella* cells, whereas *E. coli* strains, *Salmonella* and *Staphylococcus aureus* were very sensitive to *Chlorella* in concentration of 5 mg/mL. The growth of probiotic *Lactobacillus rhamnosus* was inhibited with disrupted *Chlorella* cells but no bacteriocidic effect was detected. *Scenedesmus* cells caused only a weak antimicrobial effect against uropathogenic *E. coli* strain E-093121 (25 mg/mL) and *S. aureus* (50 mg/mL; Table 2).

To meet the requirements of an economically feasible downstream product we are performing the same bioactivity tests with residual algal biomass upon lipid extraction. Additionally we are also testing whether conditioned culture medium after algae harvest exhibits antimicrobial potential.

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3. Report from Norwegian Univ. Science & Technology (NTNU)

By: Matilde S. Chauton, Yngvar Olsen, Olav Vadstein

Background and aim

In the future we need sustainable alternatives to fossil fuels, and one place to search for new alternatives is among living plants or trees. Fuel production based on terrestrial crops such as lipid-rich plants or ligno-cellulose is undesirable due to excessive use of arable land and freshwater. Another emerging issue is that lipid rich plants from agriculture will be needed for human food. Instead marine microalgae are considered as a potential source for future biofuel production. Many species are lipid-rich as they store surplus carbon as lipids, and they can be cultivated in seawater systems placed in areas not suited for agriculture. Autotrophic algae have high photo efficiency when they use light energy and CO_2 to produce chemical energy in the cell, and algae cultivation can be considered as positive in terms of carbon budgets [1]. Many microalgae have short generation times and growing populations can be harvested almost continuously, and under the right circumstances as much as 50% of the dry weight may be lipids [2]. Resource limitation and stress, such as nutrient deprivation or unfavourable light conditions, is known to induce a shift from biomass synthesis and growth towards energy storage. Many microalgae store excess carbon/energy as lipids, and use some form of carbohydrate as a rapidly convertible energy and carbon source. The physiological mechanisms that lead to energy accumulation are, however, not fully understood. Nutrient limitation is one form of stress, but limitations in different nutrients lead to different effects: Nitrogen (N) limitation may hinder the protein synthesis and therefore the cell cycle progress, and silicate (Si) limitation is known to arrest the development through the cell cycle at a certain point before cell division occurs. Phosphorus (P) limitation affects the synthesis of ATP and reductants such as NADPH, membrane phospholipids and phosphorylation steps. At some point, cells switch from biomass synthesis/ growth to carbon accumulation and energy storage, but we do not fully understand the inducive factors at these points.

There are therefore questions that must be answered before microalgae production can be scaled up to commercially feasible levels, and the challenges are both on the biological/physiological side and the technological side [3]. In this project, the main objective has been to increase the understanding of the biological mechanisms behind lipid synthesis and accumulation in microalgae, and to evaluate the use of cultivated microalgae as a raw material in the production of biodiesel and other valuable products. Our work has been focused on experimental studies and evaluation of the ”added-value” in commercially interesting by-products.

The main goal was divided into the following sub goals (described in the following text):

- Optimizing algae growth and lipid yield as functions of growth condition:
- Growth yield and algal stoichiometry under varying degree of nutrient limitation
- Effect of day length variations (16/8 or 8/16 light/dark hours) and irradiance up-shift
- Microarray study of genetic transcription: what change in gene expression is induced by a shift from synthesis/cell growth to accumulation of excess energy/carbon?
- Reactor technology: development of data logging unit:
- Basic principles of measurement/functionality and system description
- Screening of commercially interesting by-products from algae biomass that is used for production of biodiesel:
- long-chained fatty acids, protein/carbohydrate-rich raw material, carotenoids
- Numerical modelling: parameter estimation and verification of numerical model developed together with Univ. of Oslo (UiO).

Progress report

The experimental work to study algae growth and lipid yield as a function of growth has been finished, and the collected data are being processed. One manuscript is *in prep.*, and the working title is ”Nutrient stress and carbon/energy storage in the microalgae *Phaeodactylum tricornutum*: characterisation of cells and lipid content in exponential fed-batch cultures”. Another subset of data will be incorporated into a manuscript on the effect of long vs. short photoperiod on carbon or energy storage. The experimental work to study gene expression in *Phaeodactylum tricornutum* is

also finished, and the collected data are under processing. This work forms the basis for at least one publication, and the working title of the first manuscript is ”Global analyses of carbon metabolism in a day/night cycle in the model diatom *Phaeodactylum tricornutum*”. Genetic analyses and manuscript elaboration is a collaboration with the research group of Prof. A. Bones (Dept. Biology/NTNU). A locally developed data logging system has been developed and implemented as a part of the project work. The first prototype has been in use in the cultivation work from the beginning, whereas the second version is being completed in the spring of 2011. An analysis of added-value compounds from the microalgae raw material will be performed as a part of the final compilation of project results, to be included with the work of the other colleagues of the Lipido project. Finally, an attempt to describe a numerical model to analyze lipid metabolism in microalgae will be made in collaboration with the Lipido partner at the Univ. of Oslo, Prof. T. Andersen in the spring/summer of 2011. For our part, the project will be terminated by the end of 2011.

Material and methods

Species selection

Based on relevant criteria such as ease of cultivation and high lipid yield, the following species were selected for further studies: the diatoms *Phaeodactylum tricornutum* and *Chaetoceros muelleri*, and the prasinophyte *Isochrysis* sp. These are marine microalgae, and e.g. *C. muelleri* and *Isochrysis* sp. are considered as efficient producers of lipids while *P. tricornutum* was chosen because the genome of this alga is sequenced and annotated, and whole genome microarrays are available. It is therefore a useful model alga for studies of gene expression. If subjected to the growth conditions that are optimal for lipid accumulation, *P. tricornutum* also produce enough lipids to be interesting for biodiesel production.

Growth conditions

Cultivation was performed in climate chambers, and the basic growth conditions were: constant temperature 20°C, irradiance 100-150 µmol photons m^{-2} s^{-1}, and a photoperiod of 16/8 light:dark hours (Figure 1). Growth medium was made from natural seawater of approximately 34 ‰ from the Trondheim fjord, and the water was filtered and autoclaved before addition of sterile-filtered nutrient solutions according to Guillard’s f/2-recipe [4]. The cultures were aerated with air supplemented with CO_2 (1-2% v:v) to prevent settling of cells on the bottom and CO_2 limitation. In our experimental work, the main experimental variables was limitation in different nutrients such as nitrogen (N), phosphorus (P) or silicate (Si), and different degrees of limitation. Furthermore, we have studied the effect of light resource availability, either through long or short photoperiod (*i.e.* day length) or through an up shift in ambient irradiance. The light:dark cycle was changed to 8/16 hours for the purpose of studying effects of photoperiod variations, and irradiance was increased to 500 µmol photons m^{-2} s^{-1} (continuous light) to study the effect of light stress.

As routine measurements, we used optical density (OD) at 750 nm as a proxy for changes in biomass or growth, and

Fig. 1. Cultivation of the diatom *Phaeodactylum tricornutum* in medium of different nutrient composition: N limited, unlimited, P limited.

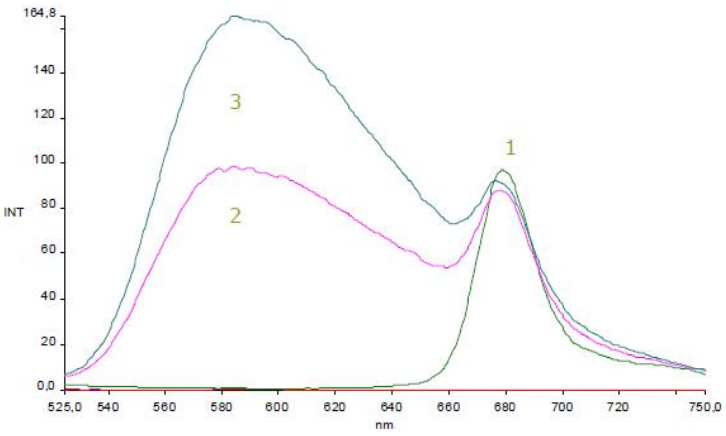

Fig. 2. Effect of Nile red staining on fluorescence emission: green curve (1) is autofluorescence from microalgae chlorophyll (peak around 680 nm) in unstained cells. Pink (2) and blue (3) curves shows the fluorescence after addition of Nile red (the pink curve is measured right after addition of the stain, the blue curve is measured after 15 min incubation in darkness): the peak around 585 nm is neutral lipid fluorescence. Exitation: 492 nm ±10.

1 http://www.merck-chemicals.se/spectroquant-tests/c_dtOb.s1O8EkAAAEdv_M1tkzg?back=true

Table 1. Fatty acid composition (as % of extracted lipids) of *Phaeodactylum tricornutum* under different growth conditions. Fatty acids that occur with less than 1% under all three conditions have been omitted from the table for the sake of simplicity.

Fatty acid	N limited	Si limited	Unlim
14:0 FA	6,9	6,2	6,0
16:0 FA	0,2	0,9	1,0
16:0 FA	20,7	15,0	14,5
16:1 n-7 FA	41,3	24,8	24,5
17:0 FA	0,9	2,3	2,5
17:0 FA	0,6	1,6	1,7
16:2 n-7 FA	1,5	4,3	4,0
16:3 n-4 FA	0,4	3,5	3,5
16:4 n-1 FA	2,9	8,1	7,8
18:1 n-9 FA	1,3	0,4	0,5
18:1 n-7 FA	1,2	3,4	5,4
18:2 n-6 FA	2,6	1,3	1,4
20:4 n-6 FA	1,2	0,6	0,6
20:5 n-3 FA	12,5	18,6	18,7
24:0 FA	1,4	2,9	2,5
Sum	**95,7**	**94,1**	**94,6**

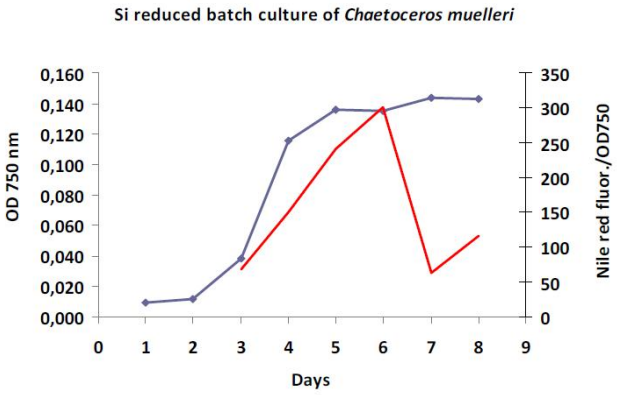

Fig. 3. Optical density (blue curve, proxy for biomass) and biomass-specific Nile red-fluorescence (red curve) in *Chaetoceros muelleri* grown in silicate limited batch culture over a period of 8 days.

Nile red-induced fluorescence as an indicator of neutral lipid contents based on the general protocol published by Chen et al. [5] and modifications based on collaboration with LIPIDO-colleagues (Figure 2). Cell counts were made by flow cytometry, and dissolved nutrients (NO_3^-, PO_4^+ or Si) were measured with auto analyzer and standard procedures [6] or Merck Spectroquant analysis kit. Elemental analysis of organic carbon and nitrogen was made by combustion and thermal conductivity detection, and chloroform extractable, neutral lipids were measured gravimetrically [7]. Water-soluble carbohydrates were measured by the phenol-sulphuric method [8].

Figure 3 shows an example of how neutral lipids (measured with Nile red-staining and fluorometry) accumulate in the cells of Si-limited *C. muelleri* when the exponential growth phase is over and the culture goes into stationary phase. After only one day, however, the Nile red fluorescence is low again: in terms of biomass production it is important to notice that the harvesting of lipid-rich cells has to be done at the right time since the lipid content seems to vary from day to day in such a cultivation system. Figure 4 shows an example of how the Nile red-stained cells can be analysed in a flow cytometer: The population of unstained cells have low autofluorescence in the wavelength window of the channel "FL2-H" (585±20 nm) and therefore appear to the left of the x-axis in the plot. When the same sample is stained with Nile red and incubated, the fluorescence from neutral lipids is seen as a shift along the axis.

Results and discussion

Optimizing algae growth and lipid yield as functions of growth condition

This work has been addressed through a series of experiments to study situations of nutrient stress or exposure to strong light, to learn more about the circumstances that leads to accumulation of lipids in stressed cells. The diatom *P. tricornutum* was studied in a fed-batch system under 5 different dilution rates and two different nutrient regimes, to induce either N or P limitation. Results in terms of growth, biomass production and cell chemistry was according to expectations, and under these circumstances this alga usually did not produce more than 10-15% (of dry weight) lipids, independent on growth rate or nutrient regime (Figure 5).

Detailed analysis of the fatty acid composition in batch cultures showed, however, that the lipid material can be highly suited for biodiesel production because of the high content of short chained, saturated fatty acids (Table 1). From this analysis it seems that growth under N limiting conditions led to accumulation of short-chained fatty acids with one or no double bonds, and that the content of EPA (20:5 n-3 FA) was slightly lower than in growth under Si limitation or no limitation. EPA is a long chained polyunsaturated fatty acid (PUFA) and as such, not involved in biodiesel production. However, EPA is an essential fatty acid that plays an important role in human nutrition and in feed for other organisms in aquaculture [9,10], and it has been shown to have antibacterial effects [11]. It may therefore be considered as a commercially interesting by-product, together with e.g. carotenoids.

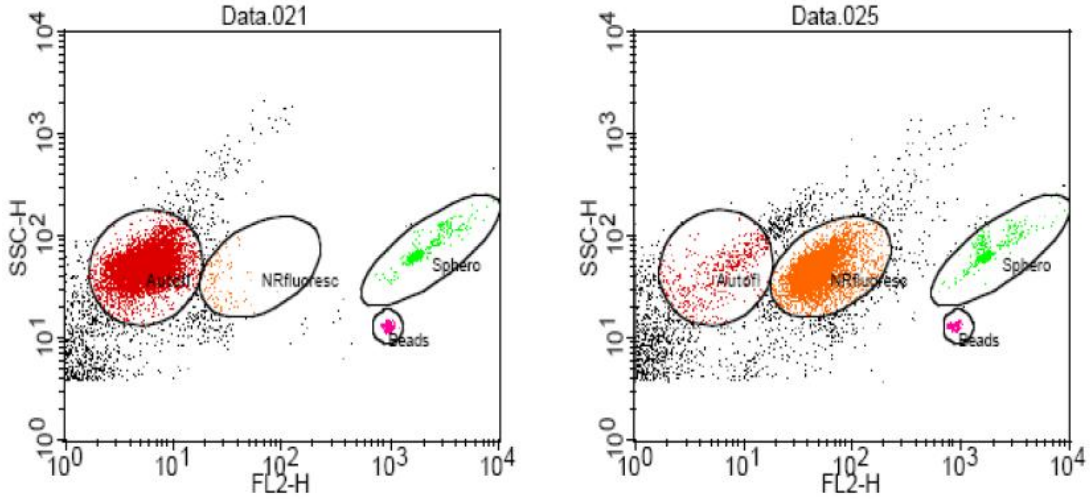

Fig. 4. Flow cytometry dot plot of unstained cells (left panel, 'Autofl') and same cells stained with Nile red (right panel, 'NR fluoresc'. Unstained cells have low autofluorescence while neutral lipids fluorescence strongly around 585±20 nm when excited with blue light (488 nm) and this is seen as a shift along the x-axis.

In another experimental setup, *P. tricornutum* and *Isochrysis* sp. were grown in N limited medium and studied during a light:dark cycle with focus on carbohydrate and lipid content (Figure 6). Storage carbohydrates were synthesized in the light period and as soon as the light energy was unavailable, the carbohydrates were broken down to provide energy and carbon for dark metabolism of other compounds. Measurements of Nile red-induced fluorescence showed, however, that the lipid content was high in the first half of the light period. Samples for analysis of total neutral lipids were collected at two points (when the Nile red-fluorescence indicated high and low lipid content) but the results did not show a clear correlation between the fluorescence and the measured lipid content. Instead, samples collected late in the light period had a higher content of neutral lipids (up to 20% of the dry weight in *Isochrysis* sp.). In both algae, the cellular lipid content was nearly doubled at the end of the light period, and this is important knowledge to determine optimal harvesting time in the production of biodiesel raw material.

Microarray study of genetic transcription

A microarray analysis provides data on the transcriptional level (where the DNA is read by RNA polymerase to produce a complementary copy) and shows up or down regulation of transcripts that may lead to synthesis of different enzymes. After studying some of the physiological characteristics of light or nutrient stress on *P. tricornutum*, we defined an experimental setup to study gene expression over the light/dark cycle and try to identify some factors that are involved in the metabolic shift from synthesis/cell growth to accumulation of excess energy/carbon.

It is necessary to avoid bacteria in the cultures, because bacterial RNA will be included in the analysis if present. Axenic cultures of the same strain of *P. tricornutum* was grown in sterile f/2-medium under the same growth conditions as described above, with photoperiod 16/8. Sampling for measurements of OD and NR-fluorescence, and analyses of cell numbers, C/N and carbohydrates was performed at selected time over a period of nearly 28 hours. Samples were also harvested for RNA extraction at the same times, and great care was taken to avoid any illumination of samples taken during the dark periods.

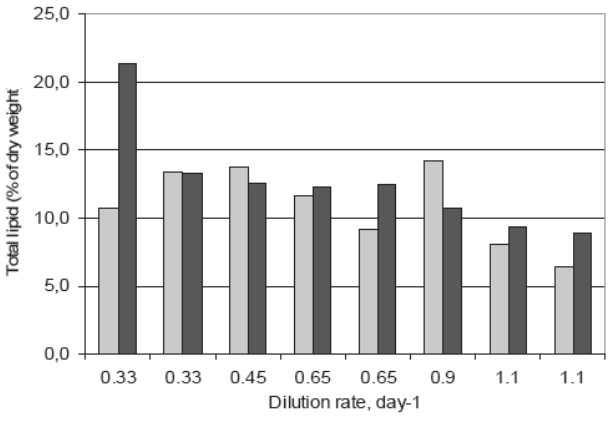

Fig. 5. Total neutral lipid content (% of dry weight) i *Phaeodactylum tricornutum* grown under 5 different dilution rates and two different nutrient regimes: N limitation (grey) and P limitation (black).

The data processing is not finished, but from some preliminary analyses it is clear that it is possible to follow activity on the transcriptional level through the metabolic pathways that are relevant for a better understanding of how microalga acquire and accumulate C into storage energy (lipids and carbohydrates). There was a correlation between the cyclic patterns of expression and the light/dark phases in many of the observed genes in our data from two replicate cultures grown under unlimited conditions (Figure 7), but the actual pattern of the cycles varied: some genes are up regulated in the light and quickly down regulated in the dark, or *vice versa*. Some genes are only moderately expressed throughout the light/dark phase, or highly up regulated throughout the cycle. It was possible to identify genetic activity in the majority

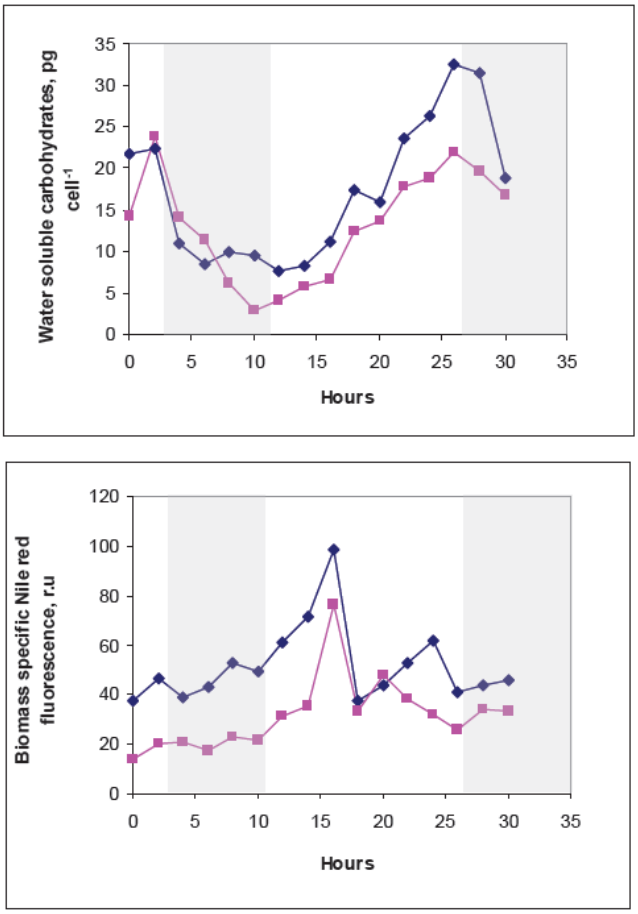

Fig. 6. Carbohydrates (upper panel) and Nile red fluorescence (lower panel) in *Phaeodactylum tricornutum* (pink curve/ squares) and *Isochrysis* sp. (blue curve/diamonds) grown in N limited medium under 16/8 light/dark periods. Grey part of panels are the dark period.

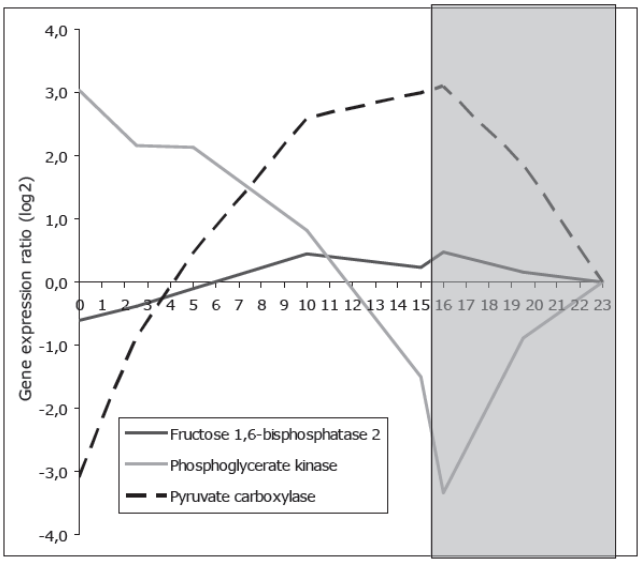

Fig. 7. Cyclic patterns of gene expression in *Phaeodactylum tricornutum* over a light:dark period (grey area is dark period), examples from the glycolysis (breakdown of glucose to provide pyruvate for other metabolic pathways). Gene expression ratio is log2 transformed and all samples were made relative to the last sampling point in the dark (23 h).

of the steps throughout the glycolysis (carbohydrate catabolism), synthesis of fatty acids (including elongation to longer hydrocarbon chains) and into the respiratory TCA cycle (Figure 8). From the data it is possible to learn more about the cellular processes that are involved in energy metabolism and production of fatty acids/lipids, and how these processes vary on a diurnal scale through light:dark phases. In this project we have seen that the cellular content of neutral lipids may vary with a factor 2 or more during the light:dark cycle, and this is important knowledge for production of algae biomass because it may ensure that harvesting is performed at the optimal time point.

Reactor technology: development of data logging and medium supply unit

As a part of the project work, we implemented a data logging unit developed especially for use in algae cultivation [12,13]. The system consists of a series of micropumps that can be used to add or remove medium/culture, light emitting diodes (LEDs, in this case infrared light that is not absorbed by photoactive pigments in the algae or other organic matter), and an electronic data logging and storing unit. The basic principles of functionality is that the LEDs are used to monitor the turbidity in a culture, and a custom made data program determines how much new medium should be added (or removed as superfluous culture volume). The calculation depends on the operational mode, and three different cultivation principles can be set up: chemostat, turbidostat, or exponential fed-batch. In our work, we have used the exponential fed-batch set up to cultivate various species of microalgae, and after a period of acclimation to growth conditions and biomass regulation to the actual nutrient availability, we saw that the population as a whole reached steady state in terms of chemical composition and biomass yield. The cultures can be filled and harvested several times, to produce cells of the same quality (Figure 9). Cell samples from different time points in the steady state-period showed great similarity in terms of cell numbers, chemical composition, lipid content and other characteristics throughout the period.

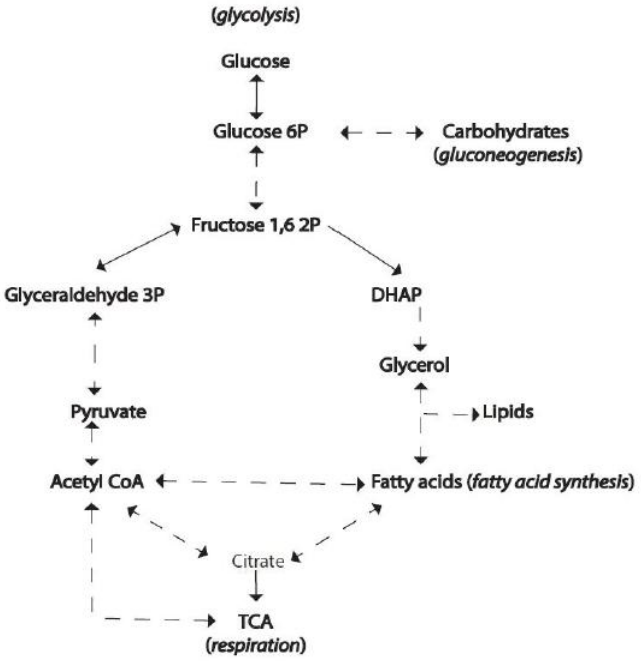

Fig. 8. Metabolic pathways that are relevant for the study of carbohydrate and lipid metabolism in the diatom *Phaeodactylum tricornutum*. Dashed lines indicate that there are several steps between the products, but only the main compounds are shown.

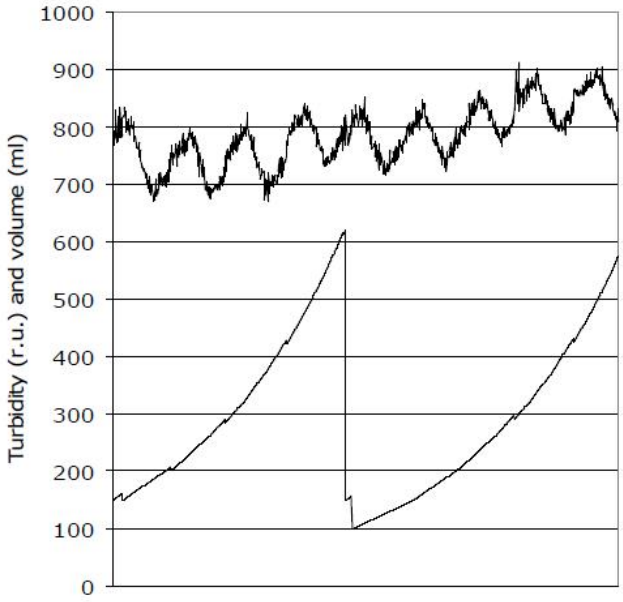

Fig. 9. Turbidity measurements (upper curve) and volume changes (lower curve) in an exponential fed-batch culture during a period of 9 days.

Screening of commercially interesting by-products

As mentioned, there are still challenges to be met before microalgae production can be scaled up to commercially feasible levels, and both physiological and technological aspects must be considered. Another important aspect to consider is the "added value" that lies in using other fractions of the algae biomass because the lipids that are used for biodiesel is only a small fraction of the total biomass. Other compounds of commercial interest are e.g. long-chained poly-unsaturated fatty acids and pigments (mainly carotenoids) that are used in aquaculture feed or health food production. If these components are removed the remaining matter is rich in proteins and carbohydrates, and it is possible to consider such raw material for fermentation and thereby increase the degree of exploitation of the microalgae biomass.

Numerical modelling:

The project plan included the idea of setting up a numerical model together with the project partner at UiO. When a proper model is defined, the following work will involve parameter estimation and verification of the model output. If possible, data from the previous experimental work will be used, but this work is scheduled to the summer of 2011 and can therefore not be reported in detail here.

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4. Report from Univ Oslo

By: Tom Andersen

I. Growth and neutral lipid synthesis in green microalgae: A mathematical model

Cultures suspended in growth media with low nitrogen (N) concentration yield biomass with significantly higher lipid content than those suspended in high-N media. On the other hand, cultures suspended under high-light tend to yield greater lipid content than those suspended under low-light (Hu et al., 2008; Rodolfi et al., 2009). The neutral lipid content can increase from zero to well over fifty percent of dry weight. This trend has been confirmed repeatedly by laboratory experiments (Hu et al. 2008, Scott et al. 2010, Scragg et al. 2002, Rodolfi et al. 2009).

Metabolic pathways downstream of the electron transport chain may serve as important defenses against oxygen radical production under nutrient limitation (Niyogi 2000). During N-stress, cell growth is halted, but carbon fixation may continue at rates exceeding the needs of the cell. It may be that the ‘‘uncoupling of photosynthesis and biomass production” as reviewed in Berman-Frank and Dubinsky (1999) is the mechanism that allows adequate electron sinks to function when cell growth is hindered as a result of nutrient limitation. Some species up-regulate nitrogen-free pigments or simply excrete excess photosynthate during stressful growth conditions – the many mechanisms with which phytoplankton handle excess carbon reduction is an important research area (Dubinsky and Berman-Frank 2001, Hessen and Anderson 2008).

Up-regulation of NL synthesis may be a means by which energy can be spent during stressed conditions, helping to maintain a safe turnover rate of the ATP and reductant pools sustained by the light reactions. Fatty acid production is expensive in terms of ATP and reducant requirements (Xiong et al., 2010). NLs store significantly more energy than carbohydrates do: 37 kJ/g versus 17 kJ/g, respectively; and, on a per-mass basis, NL synthesis requires twice the reducing energy (NADPH) than that of carbohydrate or protein synthesis (Hu et al. 2008). Thus, NL synthesis is an effective energy sink. It may be that certain species maintain a relatively high rate of photosynthesis during N-stress, but compensate by synthesizing NLs. Oleaginous species of algae use excess carbon and energy to synthesize storage lipids under N-stress, whereas non-oleaginous species synthesize carbohydrates or halt growth (Rodolfi et al. 2009).

Since N-limitation appears to be a key catalyst for excessive NL accumulation, an immediate question is whether or not ecological models of phytoplankton–nutrient interactions can be extended to this phenomenon. Ecological stoichiometry (Sterner and Elser 2002) in particular provides a useful foundation for mathematical models by considering the relationship between the elemental compositions of organisms and their environment. Given that NLs serve as C storage in N-limited environments, the N:C ratio of an algal suspension may provide the means of modeling TAG accumulation using plausible ecological models. The N:C ratio decreases with decreasing N-availability and increasing irradiance, both of which have been observed to increase NL synthesis. Can NL synthesis be simplified ecologically and mathematically as the cause (or effect) of a low N:C? This question is important, as ecological stoichiometry may be applicable to other bioengineering processes, see e.g. Mauzerall (2008).

Model description

Our model (Packer et al. 2011) assumes that algal mass is divided into two compartments: non-NL biomass A and neutral lipids L. Therefore the total algae density is the sum of the two compartments, A + L. The model is derived from four major assumptions:

- The specific growth rate of A is either N- or light limited. N-limited growth takes the form of the well-established (Droop) cell-quota model. An increase in A, the non-lipid dry weight, requires a fixed proportion of accumulated carbon.
- The net carbon fixation rate is governed by the standard single-hit Poisson model of photosynthesis, normalized to the chlorophyll content of A.
- Following Geider et al. (1998), chlorophyll a synthesis is coupled with nitrogen uptake. The proportion of nitrogen devoted to chlorophyll synthesis is regulated by the carbon utilization to carbon uptake ratio. Nitrogen uptake is regulated by the cellular N quota and by the ambient concentration of inorganic N.
- NL synthesis results from an excess of C-fixation relative to the C requirements for growth. Therefore, when all internal N stores are depleted, all increases in total biomass are due to *de novo* NL synthesis.

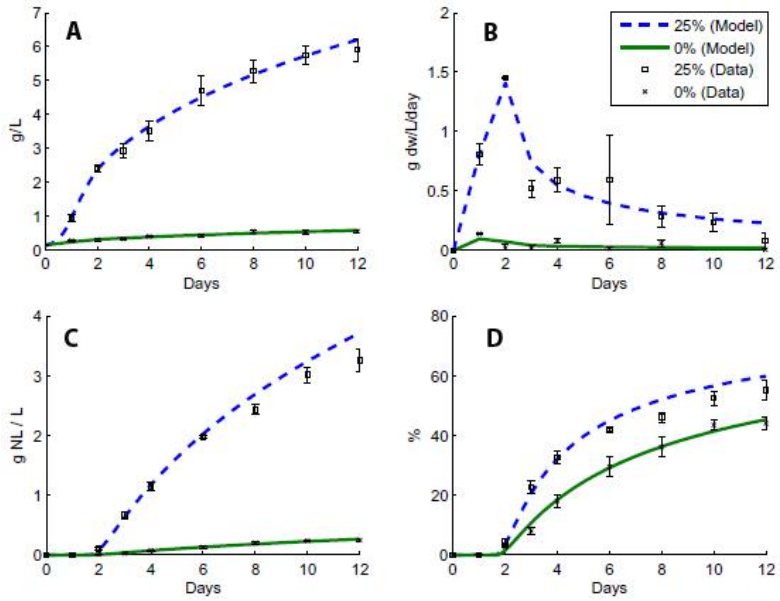

Figure I.1. Model simulations and observations for *Pseudochlorococcus* sp. growing in batch culture at low (0%) and high (25%) nitrogen supply. A) Biomass (dry weight), B) Biomass production rate, C) Neutral lipid concentration, D) Lipid content of dry biomass. Redrawn from Packer et al. (2011).

Experimental data

An experiment was designed to investigate the effect of nitrogen on the biomass and NL yield of the green microalgae *Pseudochlorococcum* sp. It was performed in batch culture using 60 ´ 10 ´ 3 cm cuboid-shape, flatpanel photobioreactors containing 1 L of growth medium with constant irradiance of 52 mol quanta m^{-2} d^{-1}. BG-11 medium was modified by reducing the $NaNO_3$ concentration to 0% or 25% of the original level (0 or 0.06 g N L^{-1}). All cultures were agitated by bubbling with 1.5% (v/v) CO_2 at a flow rate of 10 L min^{-1}. This CO_2 level has been optimized so that maximum biomass and lipid yield can be achieved without CO_2 becoming limiting. The initial biomass was such that inorganic N was depleted within the first 24 hours. Internal N stores are then probably exhausted in the next 24 hours after that, such that biomass production declines to zeros while a high lipid production rate is maintained for another 10 days (fig. I.1C,D).

Conclusions

The model demonstrates that NL production may be simplified within the framework of ecological stoichiometry. In addition, the decoupling of photosynthesis from cellular growth is a possible explanation for excessive NL synthesis in oleaginous green microalgae. Future experiments designed to measure information such as the N:C of biomass can help determine if there is a threshold N quota for excessive NL production.

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II. High-throughput niche screening of microalgae

The Huchinsonian (1957) niche is the hyper-volume within an N-dimensional space of environmental factors where a species can persist. Light, temperature, and salinity are the most important niche dimensions for selecting candidate organisms for mass culture in temperate coastal waters. The solar constant and the quantum yield of photosynthesis will ultimately determine the maximal attainable carbon fixation rate at given latitude, while the temperature and salinity will determine the net production yield through their effects on cellular maintenance costs and overheads for maintaining membrane potentials. Accurate mapping of algal growth response in a 3-dimensional niche space requires a work load proportional to the 3rd power of the number of grid points along each gradient axis: for example, $6^3 = 216$ experimental units are needed to explore a 6-step light-temperature-salinity (LTS) gradient for a single species. This means that there is need for efficient methods for running multidimensional growth experiments in limited space and with limited personnel resources. We here propose a method for achieving this based on disposable labware, standard lab instruments, and inexpensive, off-the shelf microcontrollers.

The basic experimental unit is standard 127 ´ 85 mm microtiterplates, usually in the 96 well format. The actual layout of gradients depends on the logistics: if no special incubation hardware is available then each plate can be arranged as a L ´ S gradient which can be incubated at different temperatures (Skjelbred et al., in prep.). If a L ´ T-gradient incubator is available (see below), then the individual plates usually represent different salinities. Algal growth is monitored daily by *in vivo* fluorescence, which can be measured non-destructively with a standard plate reader in less than 1 minute per plate. Specific growth rate (μ) is then estimated as the slope of a linear regression of log-transformed *in vivo* fluorescence against time.

The data can at that stage be considered as a collection of light response curves at different combinations of temperature and salinity. The second step of the data reduction is to fit a model curve to each light response and use the parameters of these models as aggregated data in the analysis. Light response models are in a sense arbitrary, albeit their parameters may have physical interpretations, such as in fig.II.1.

Growth rate – irradiance relationships are fitted to each T ´ S treatment combination using non-linear mixed effects models (R package nlme; Pinheiro and Bates 2000). The main advantage of this approach is that parameter estimates for badly constrained treatment combinations will be more similar to the overall average than with independent fits for each treatment combination (the so-called shrinkage effect). Fitted model parameters can then be visualized as functions of temperature and salinity by fitting generalized additive models (GAMs; Wood 2006). Figure II.2 shows that the particular strain in this experiment has the highest net specific growth rate in saturating light (ca. 0.6 d^{-1}) at 13 °C and a salinity of 22 psu, while it has the highest competitive ability for light (lowest E_0) at slightly lower temperature and slightly more saline water (12 °C and 24 psu). Comparisons across many species or strains can be visualized as the locations of their optima in T ´ S-space (figure II.3).

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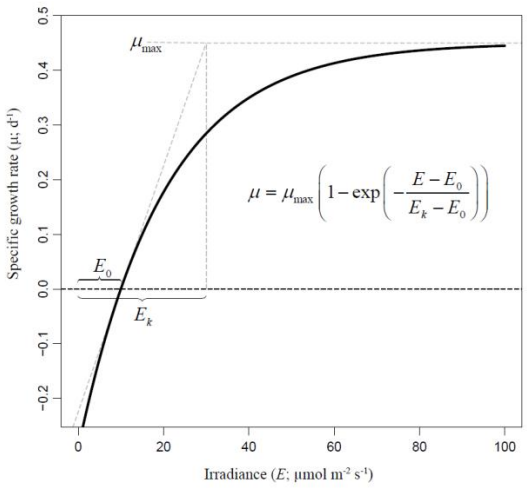

Figure II.1. 3-parameter model for net specific growth rate as function irradiance (Skjelbred et al., in prep.), with parameters maximal net specific growth rate (μ_{max}), compenstation irradiance (E_0), and saturation irradiance (E_k).

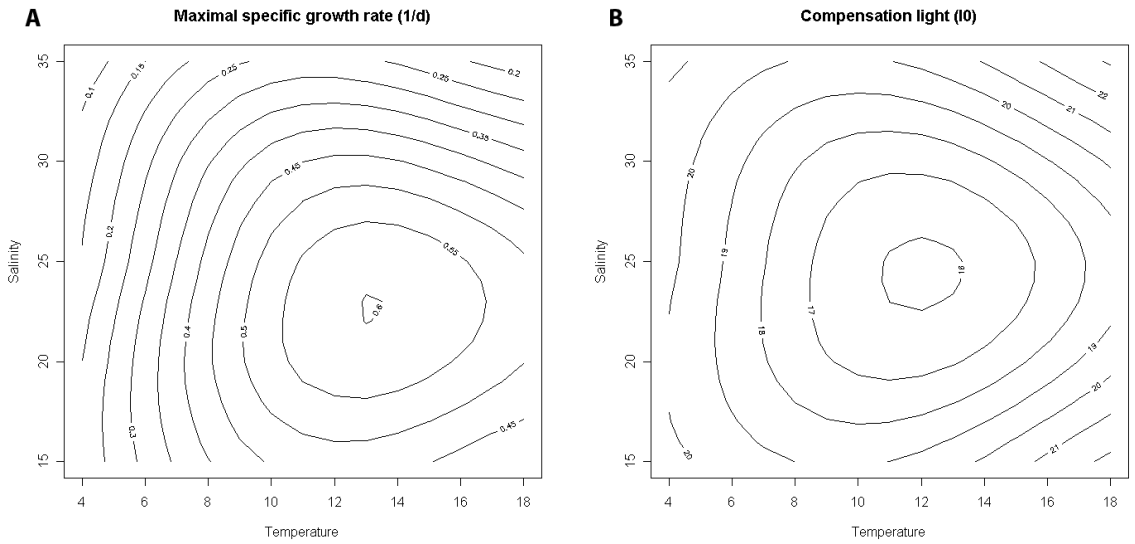

Figure II.2. Generalized additive models for maximal specific growth rate (A) and compensation irradiance (B) as function of temperature and salinity for strain UiO113 of the marine Dictyochophyte *Pseudochattonella farcimen* (Skjelbred et al., in prep.)

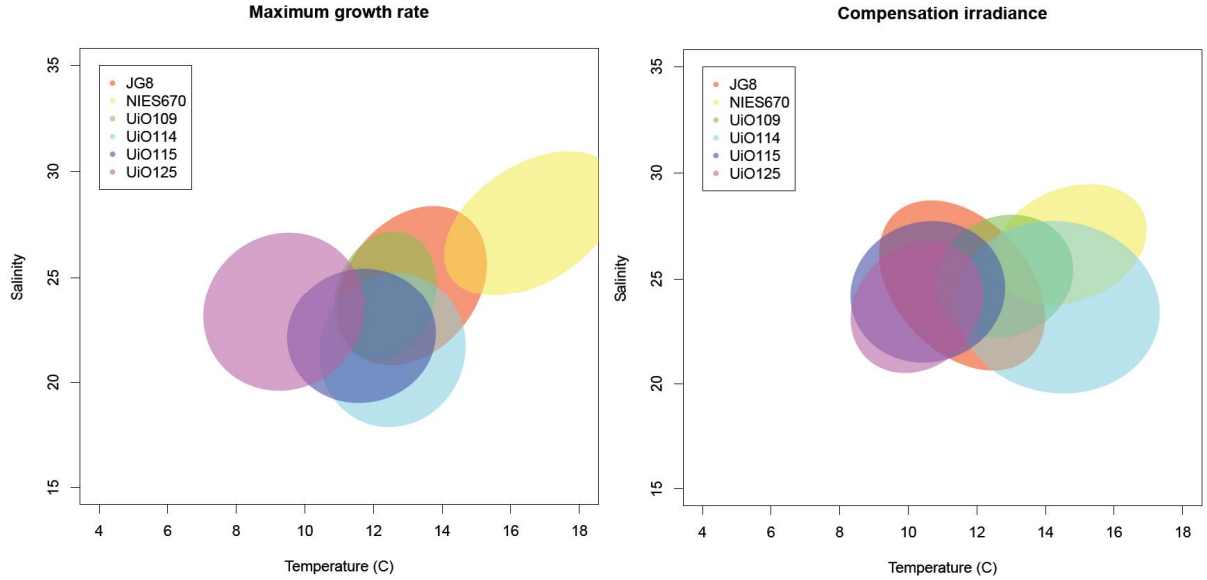

Figure II.3. Optimal temperature and salinity of 6 different strains of the marine dictyochophyte genus *Pseudochattonella* (Skjelbred et al., in prep.). 95% confidence ellipses for optima are estimated by 1000 bootstrap samples from each strain.

III. A compact light and temperature gradient incubator

High-throughput screening of light and temperature optima for growth and lipid yield is crucial for selecting suitable microalgal strains for biofuel production in temperate coastal environments. We have designed compact incubator based on standard electronic components which can estimate net, specific growth rate at 96 combinations of light and temperature in less than a week. The incubator consists of 2 independent modules for temperature and light gradients, with a 96-well microplate sandwiched between. This design allows the modules to also be used separately for experiments where only light or temperature gradients are needed. We use white microplates (for example Nunc 165306; white, optical bottom) intended for luminescence assays, to reduce crosstalk between wells while minimizing light loss by wall absorption.

Temperature gradient module

The temperature gradient module is based on the physical principle that a block of homogeneous heat-conducting material with ends clamped at fixed temperatures, will develop a linear temperature gradient between the hot and cold sides. In other words, the temperature at a given position in the block will be given by just its relative distance to the hot and cold ends. If a microplate is placed upon such a block with the rows aligned with the temperature gradient, the individual wells will have temperatures depending only on their row positions, but not their column positions.

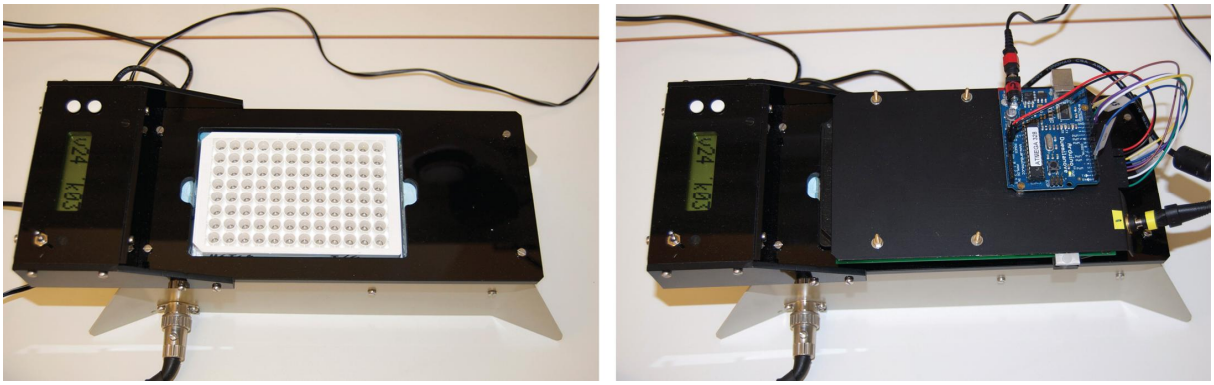

Figure III.1. Temperature gradient module, from the top with a 96-well plate in place (left), and with the light gradient module mounted on top (right).

The choice of material for the heat conducting block is crucial to the operation of the module. If the thermal conductivity is too high (e.g. aluminum), then the power consumption becomes prohibitively high. If it is too low (e.g. acryl) then the heat flow through the unit becomes too low to maintain a proper gradient. After substantial trial and error, we ended up with using stainless steel which has suitable heat capacity and conductivity, while also being inexpensive and having good machinability properties.

The stainless steel block is machined with groves to position the microplate on top and insulated on the sides. The ends are fitted with heating and cooling devices so that they can be clamped at constant temperatures. The cooling is done by thermoelectric Peltier elements while the heating is done by high-power resistors. End side temperatures are kept constant by a PIC microcontroller programmed to read temperatures by a pair of thermistors and adjust the heating/cooling power by pulse-width modulation. The unit is completely self-sustained with its own power source, and with microbuttons and a LCD display to set end-point temperatures. The unit is able to maintain a steady 2 to 24 °C gradient at room temperature.

Light gradient module

The light gradient module is based on white light-emitting diodes (LEDs), arranged in a 8 by 12 matrix such that each microplate well has one individually controllable LED positioned above it. The LEDs are controlled by Texas Instruments TLC5940 constant-current LED drivers. Since each TLC5940 can control 16 LEDs, we use 6 of these chips in a daisy-chain fashion to control the intensity of the 96 LEDs in the module. Each LED can then be set to 4096 different intensities by pulse-width modulation. The LED drivers are again controlled by an Arduino microcontroller, which is an open-source hardware project with an enthusiastic user community that has, among other things, developed a very suitable library for controlling TLC5940 LED drivers.

LEDs, even from the same production batch, do not give identical light output. By use of a miniature PAR sensor (Walz US-SQS/L), small enough to fit within a microplate well, one can establish calibration factors for individual LEDs such that light gradients can be programmed directly in PAR units. When used with the temperature gradient module, the light gradient is usually programmed as 8 steps perpendicularly to the temperature gradient, yielding 96 different light-temperature combinations. When used stand-alone the light gradient can also be programmed for other purposes, such as irradiance by photo period gradients.

The light gradient module is constructed on a single 2-layer printed circuit board. The layout was produced on a professional CAD system at the UiO Electronics lab, while the etching and silk-screening was done by a commercial printed circuit board production service. The circuit diagrams, print layouts, and software for the light gradient module will eventually be released to the open source hardware community. To save space, LED drivers and associated circuits were surface-mount components, while the LEDs were through-hole type – mainly because most surface mount LEDs have too wide illumination angle unless fitted with an appropriate lens. This may change if it is decided to produce larger series of the module.

IV. Increasing lipid yield in microalgal mass cultures by CO_2 control

Seawater contains only a few milli-molar of total inorganic carbon (TIC) and most freshwaters even less than that. This amount of TIC can be converted by photosynthesis into about 20-30 mg organic C per liter, which is enough biomass to give visible turbidity but not enough to capture more than a fraction of the available light energy unless the reactor is several meters deep. Since low biomass concentration will also entail extra harvesting costs, it is desirable to have as high biomass density as possible in commercial mass cultures. This means that practically any culture system designed for maximizing biomass production will become C-limited unless supplied with extra CO_2, usually by bubbling with air or CO_2-enriched air.

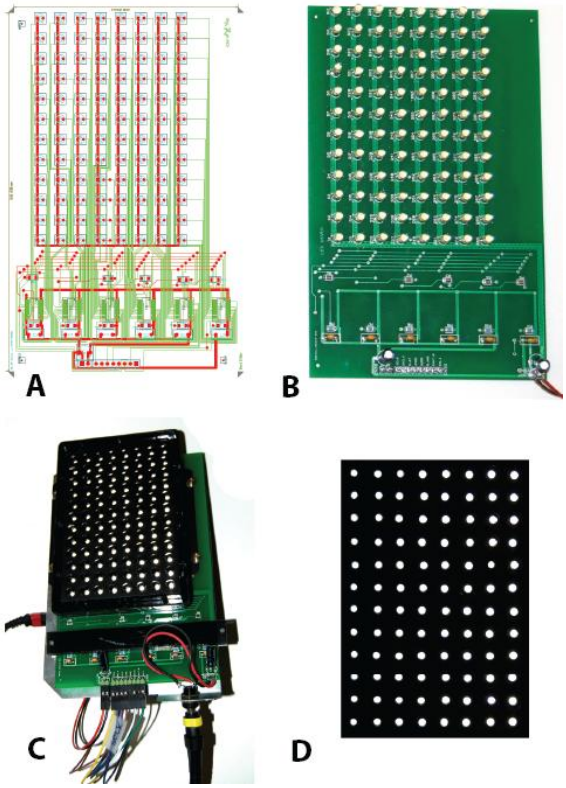

Figure III.2. Light gradient module: circuit board layout (A), printed circuit board populated with LEDs (B), assembled module with Arduino on bottom (C), calibrated LED matrix (D).

It is well-known that many algal species increase their lipid content under nutrient limitation. A simple and attractive explanation for this phenomenon is that the algae are diverting photo-synthetically produced reducing energy (NADPH) from biomass production to reducing carbo-hydrates to lipid, since lipids have higher energy density than other biochemical compounds. Most experiments where this has been demonstrated have used nitrogen as limiting factor, but there are reasons to believe that this mechanism can be induced by any limiting nutrient, including carbon. This raises particular interest because the carbon supply to a culture, as gaseous CO_2, is easier to regulate and modulate than the nitrogen supply though dissolved NO_3 or NH_4 ions. As CO_2 can be monitored by simple and well-proven technology such as pH electrodes or non-dispersive infrared spectroscopy (NDIR), it should also be easier to maintain precise control over C-supply than N-supply under mass-culture conditions.

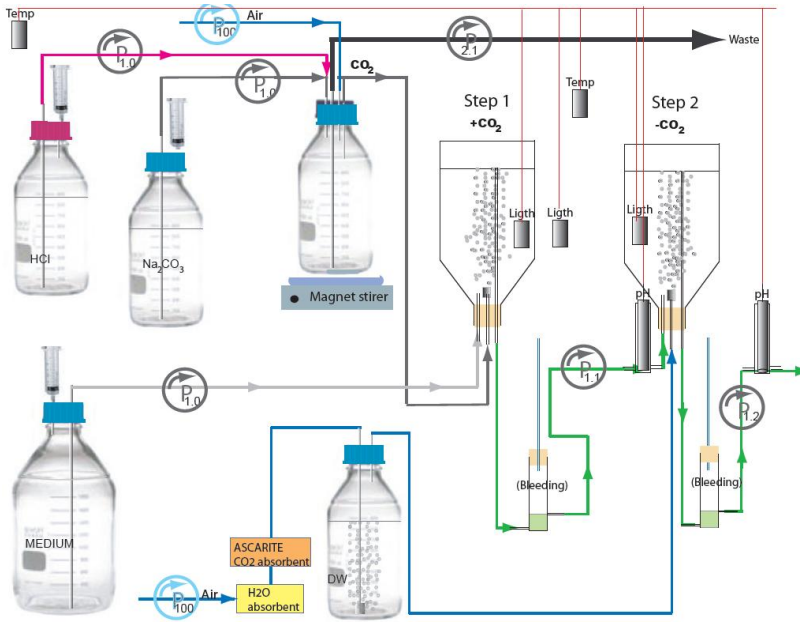

Figure IV.1. Flow scheme for the 2-stage chemostat system with CO_2 delivery module.

Experimental setup

We use a 2-stage chemostat system where both stages are receiving the same irradiance, but with the first stage being supplemented with surplus CO_2 while the second is forced to become CO_2-depleted. CO_2 for the first stage is generated by mixing continuous flows of $NaCO_3$ and HCl with known normalities. The generated CO_2 is mixed with CO_2-free air, and bubbled through the culture by way of a glass frit. Peristaltic pumps drive all gas and liquid flows of the CO_2 delivery system, such that exact CO_2 flows and partial pressures delivered to the culture can be calculated from pumping rates and simple stoichiometry. The second stage receives the same gas flow rate, but CO_2-free air made by running indoor air through a CO_2 trap (ascarite).

Figure IV.2. Incubator with CO_2 delivery system and data logging computer (left), internal rack with gas traps and pH electrodes (middle), chemostats with CO_2-repleted stage at top and CO_2-depleted stage at bottom (right).

The culture vessels are inspired by the design of Huisman et al. (2002), but using disposable tissue culture bottles (Nunc 159920 645 ml, non-treated) instead of custom-designed reactors. The planar geometry of the tissue culture bottles makes it easy to measure the irradiance entering and leaving the reactor. The amount of quanta absorbed within the reactor can then be easily calculated as the difference between irradiance in and out of the reactor. Combining this information with the carbon budget of the reactor gives a precise measure of the quantum yield; - the number of moles C fixed per mole of quanta absorbed. Each reactor has only one inlet and one outlet: a glass frit for entering air and medium, and a single glass tube as exit for air and reactor outflow. The outflow glass tube also maintains constant volume in the reactor, such that the dilution rate can be monitored as accumulated outflow divided by reactor volume. The flow between two stages passes through a gas trap such that CO_2-enriched air can be vented off before entering the CO_2-depleted stage. The whole system, except for medium reservoir and CO_2 delivery system, is housed within a commercial wine storage cabinet to maintain constant temperature. The light sources (standard cool-white fluorescent tubes) are mounted on the outside of the see-through door of the cabinet.

Each system is continuously monitored for turbidity, pH, and temperature by sensors and pH amplifiers connected to a Phidgets 8/8/8 I/O board. The Phidgets board has 8 analog inputs, of which we use 3 for light measurement (light out from each reactor, plus a common light in), 2 for pH measurement (1 pH electrode in the gas trap after each reactor), and 1 for temperature. The board is connected to a USB port on a PC, and controlled by a small Java program that writes the readings from all sensors to file every 10 seconds. The Java program also controls the duty cycle of the peristaltic pumps through one of the digital outputs on the Phidget board, connected to a solid-state relay.

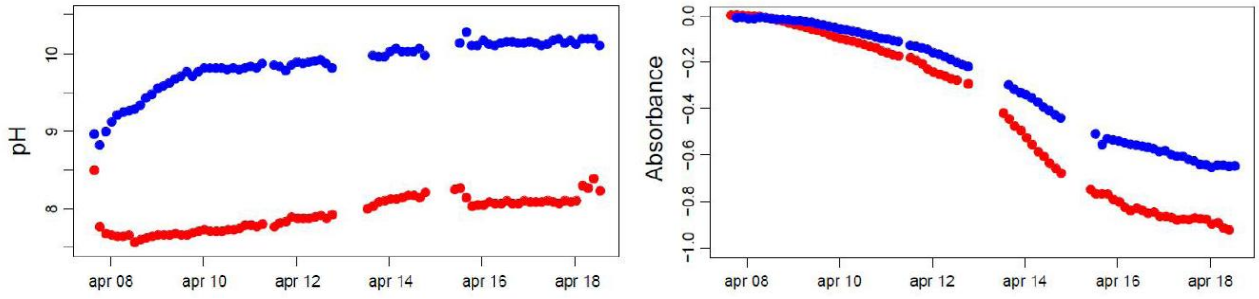

Figure IV.3. Test run of the 2-stage chemostat system with CO_2 control: pH in the 2 stages (upper), and light absorbance (lower). Red symbols are measurements from CO_2-repleted stage while blue symbols are from the CO_2-depleted stage.Gaps in series are due to data logging failure.

Preliminary results

Since Lipido-UiO received 1 year extra funding from the Norwegian Research Council, this part of the project is not yet completed. The following should therefore be taken as a proof of concept, rather than the final results. Figure IV.3 shows that the CO2 delivery system manages to maintain >2 pH unit difference between the 2 stages, even at very high biomass. As light absorbance (or log-transformed transmission) is expected to be proportional to biomass, the absorbance time series have the S-shaped appearance of logistic growth curves. It is interesting to notice that the CO_2-depleted stage has lower absorbance than the CO_2-repleted one, even though the latter is the source of the latter. This gives indications that the induced CO_2-limitation in stage 2 triggers substantial biochemical transformations, probably involving both pigments and storage products. Further analyses of elemental composition, lipid content, and photosynthetic pigments will reveal more about the nature of this transformation process.

5. Report from Ludwig Maximilians Universität (LMU)

By: Maria Stockenreiter, Florian Haupt, Herwig Stibor

Executive summary

Main objective and results
The following report includes the work and results of 36 project months. A number of laboratory experiments with laboratory and field algal strains were conducted to investigate the main subject, optimizing lipid production in planktonic algae.

I. Screening of peculiarly adapted algal strains
The first step of the project was to select productive algal strains suited for further investigations. For this purpose, different freshwater algal strains representing the major algal classes were cultivated at low and moderate temperature for more than one month. The experiments ran under highly controlled conditions with semi-batch cultures.
The main goal of this first step was to identify algal strains with high biomass and lipid production at low as well as moderate temperatures. Especially chlorophytes (*Chlamydomonas reinhardtii*, *Staurastrum tetracerum*) and cyanobacteria (*Anabaena cylindrica*) demonstrated highest biomass and lipid yields at both temperature regimes.

II. Effects of light intensity on high productive algal species
The second step was to cultivate the different algal strains used for screening under different light conditions. The aim was to determine the influence of low and high light irradiance on the total algal lipid content. In this experiment an additional algal strain (*Botryococcus braunii*) was cultivated, which is the most favoured algal strain in literature for biofuel production, because of its well known high lipid content. Higher light conditions resulted in higher biomass in almost all cultures, whereas the lipid content of the micro-algae was far less influenced by different light conditions.

III. Effects of nutrient limitation on algal lipid content
Previous studies have demonstrated that nutrient stress conditions, such as nitrogen starvation can induce higher lipid production in many algal species. The responsible mechanism is a stop of cell division under nitrogen limitation but an accumulation of photosynthetic products in form of lipids.
Four different algal strains were cultivated under nutrient stress conditions. Extreme nitrogen limitation resulted in significant higher algal lipid contents in all cultures. *Botryococcus braunii* was, as expected, the algal strain with the highest lipid yield. However algal growth rates decreased drastically with nitrogen shortage in all cultures, except for Anabaena cylindrica cultures. Some cyanobacteria such as Anabaena are able to compensate low dissolved nitrogen levels by fixing air borne nitrogen.

IV. Optimizing cultivation methods
To enhance both, biomass accumulation and lipid production at the same time we further investigated a two stage cultivation method to replace one stage semi-batch cultivation systems. In the first step of this cultivation method, algae were grown under optimal growth conditions in a chemostat resulting in high biomass. A daily rate was transferred into a second step (batch culture) where nutrients were limited.
In the first step of this cultivation method a full growth medium allows an enhancement of biomass accumulation. In the next step, the culture was transferred into nitrogen limited growth medium, where a further accumulation of photosynthetic products in the form of lipids occurred.
Staurastrum tetracerum and *Botryococcus braunii* showed in two stage cultivation higher population growth and cell specific lipid content as in semi-batch cultivation at comparable nutrient concentrations. Two stage cultivation cultures resulted in higher nutrient specific biomass production and lipid content of algae compared to 1-stage cultivation.

V. Diversity effects on algal lipid content (A+B)
Regarding the industrial cultivation methods for algal mass cultivation- which are either open pond systems or closed bioreactors- the experiments mentioned above were geared towards closed systems, which are the only possible sys-

tems to maintain monocultures of selected algal strains. In open systems a monoculture would not persist very long due to a constant biological input of microbes. The main goal of these experiments was to investigate how diverse multi species micro-algal communities perform in their growth and lipid production compared to highly selected strains of monocultures.

Algae from all major algal classes were grown in a large number of treatments differing in their diversity levels. Additionally, a comparison of the growth and lipid production of laboratory communities to natural lake and pond phytoplankton communities of different diversity extended these experiments.

The results show that lipid production increased with increasing diversity in both natural and laboratory micro-algal communities. The underlying reason for the observed 'diversity-productivity' relationship seems to be resource use complementarily.

More precise analyses of all experiments showed that complementary effects of light usage enhanced the resource specific biomass and lipid production. Different algal classes provide different photosynthetic active pigments with different absorption spectra and diversity can therefore enhance the efficiency of light use for growth.

Methods/Implementation

Two stage cultivation method cultures resulted in higher nutrient specific biomass production and lipid content of algae compared to single stage cultivation systems.

Up-scaling processes are currently under setup. The two stage cultivation method was taken as basis of a pilot medium - scale construction (1000 L) to grow diverse algal communities to high densities.

The cell specific lipid content was determined by fluorescence measurements of cells stained with Nile Red, which is a lipophilic vital dye with a shift of emission from red to yellow. It stains neutral lipids, which provide a differentiation between triglycerides, which are the most important lipid classes for biofuel production and membrane lipids. Additionally, there is only little overlap with chlorophyll-a auto fluorescence.

This method is a simple and well established for the rapid determination of algal lipids. There is a significant relationship between fluorescence and the lipid content of algae. It has been shown several times that the method of staining algal cells with Nile Red to estimate lipids is equally viable as the gravimetric method, which is commonly used for lipid determination. A major problem of Nile Red is that species vary in their uptake of this dye.

We combined Nile Red staining with modern imaging flow cytometer technologies. Analyses of the micro-algae lipid content with Nile Red fluorescence and using an imaging flow cytometer (FlowCAM®) has the unique advantage to estimate the lipid content of each algal cell in diverse communities without requiring the (practically impossible) physical separation of algal cells. This advantage also reduces the problem that species vary in their Nile Red uptake.

Conclusion

- Nitrogen reduction increased lipid production of different algal species.
- *Botryococcus braunii* was the algal strain with highest cell specific lipid content; however its growth rate was strongly influenced by nitrogen supply.
- A comparison of 1-stage and 2-stage cultures of *Staurastrum tetracerum* and *Botryococcus braunii* showed that 2-stage cultivation methods resulted in higher population growth and cell specific lipid content at comparable nutrient concentrations.
- Highly diverse communities produced higher yields of biomass in terms of carbon but also lipids compared to monocultures.
- Natural lake communities produced approximately the same amount of lipids than selected laboratory monocultures.
- Open pond systems with diverse algal communities may therefore represent an alternative to costly bioreactors using monocultures.
- A major underlying mechanism behind this observed diversity-productivity relationships was complementarity, which means that species complement one another in resource use efficiency or facilitate their growth.
- The dominance of a single highly productive species was not responsible for the observed positive effects of diversity on lipid production.
- The incorporation of the ecological advantages of diversity related resource use dynamics into algal biomass production may provide a powerful and cost effective way to improve biofuel production by enhancing resource use efficiency.

Report

Screening of algal strains

The production of biofuels from algae requires a comparison of several algal strains regarding growth and their lipid synthesis, to find the most suitable strain for optimizing its lipid content. For this purpose, investigations regarding their response to important ecological parameters and resources, such as temperature, nutrients and light leading to optimal growth are important. Temperature is one of the most important environmental factors influencing the reaction rate of biochemical and physiological processes (Lampert & Sommer 2007).

To accomplish the algal mass cultivation for biofuel production regions with low average temperatures, the variety of algal strains should cover algal strains whose growth is also ensured at low temperatures.

We used different freshwater algal strains representing the major algal classes to select the best algal strains growing at both, low and high temperatures for further optimization experiments. 12 different algal strains were cultivated with a modified phosphorus limited growth medium (Guillard & Lorenzen 1972) in cell flasks at 7°C and 20°C as semi batch cultures in a climate chamber. Light conditions were continuous 100 µmol photons m^{-2} s^{-1}; Experimental duration was 20 days (20°C) and 45 days (7°C).

Chlamydomonas reinhardtii, *Staurastrum tetracerum* (chlorophyta) and *Anabaena cylindrica* (cyanophyta) showed the highest total algal biovolume after 20 days of cultivation. At this time all cultures at 7°C showed lower total algal biovolume than at 20°C. However with time lag of 25 days, *Chlamydomonas reinhardtii* and *Anabaena cylindrica* showed nearly as high total algal biovolume as cultures at 20°C (Fig 1). For these reasons we used these three algal species for our following optimization experiments.

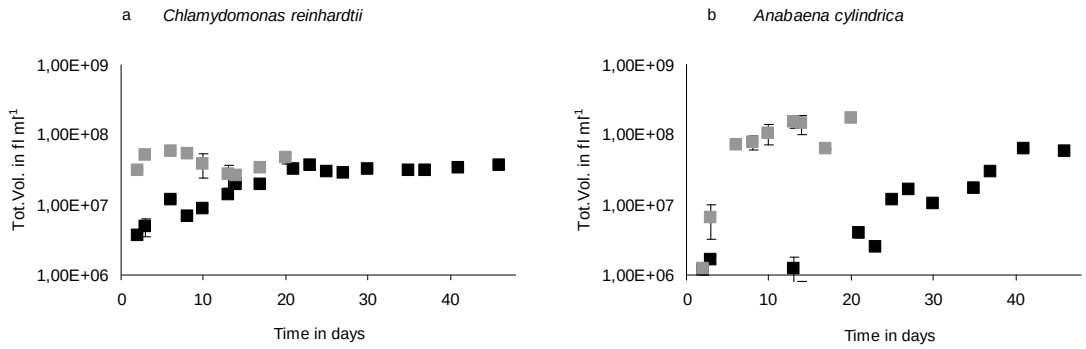

Figure 1: Total algal biovolume over time at 7°C (■) and 20°C (■) a) *Chlamydomonas reinhardtii* and b) *Anabaena cylindrica*. (error bars represent + 1SE).

Effects of light intensity and nutrient limitation on algal lipid content of selected species

Light and nutrients (e.g., phosphorus and nitrogen) are essential resources. Nitrogen (N) limitation can lead to decreasing cell division. However, the photosynthetic products in nitrogen limited cultures can be accumulated with almost the same rate than non- N limited cultures. Studies show that this can result in a fourfold increase of algal lipid content (Schenk et al. 2008), which tends to be inversely proportional to the rate of growth (Borowitzka 1988). Mainly the percentage of saturated fatty acids might be increasing during periods of nitrogen limitation (Ahlgren and Hyenstrand 2003; Xu N. et al. 2001; Piorreck et al. 1984).

We cultivated the above described selected algal strains under different light (30 µMol photons m-2 s-1; 100 µMol photons m-2 s-1) and nutrient (high and low nitrogen) conditions in semi batch cultures. Experimental duration was 38 days. Total lipids were estimated by measuring algal cultures stained with the vital dye Nile Red (9-diethylamino-5H-benzo[α]phenoxazine-5-one; HPLC grade; see appendix C) Nile Red) and a imaging flow cytometer (FlowCAM, see appendix B) FlowCAM®). Most treatments showed significant differences in their algal lipid content depended on the different nitrogen supply rates (Fig 2).

Cultures of *Botryococcus braunii*, *Chlamydomonas reinhardtii* and *Staurastrum tetracerum* showed significant increase of cell specific lipid contents at low nitrogen supply with *Botryococcus braunii* showing the strongest increase (Fig 2). The algal lipid content was marginally influenced by light supply. Lower light caused on average a very small increase in specific lipid content (Fig 2).

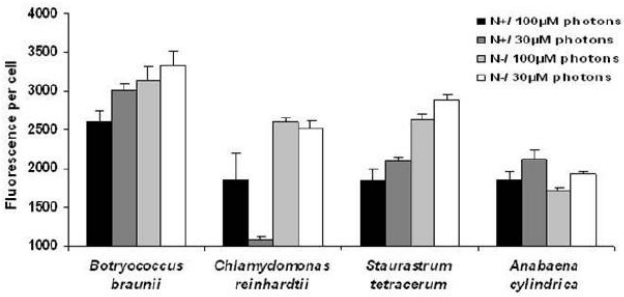

Figure 2: Mean values of fluorescence per cell of different algal species under different light conditions and nitrogen content at stationary phase. 1,0*E-11g lipid about 1000 fluorescence units (error bars represent + 1SE).

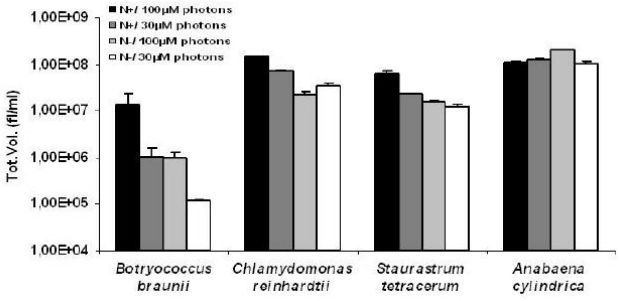

Figure 3: Mean values of total biovolume of different algal species under different light conditions and nitrogen content at stationary phase (error bars represent + 1 SE).

However, the lack of nitrogen in the nutrient solution caused a strong reduction in population growth in the semi batch cultures except for Anabaena cylindrica which is able to fix airborne nitrogen (Fig 3).

In summary, higher light intensities mainly influenced algal growth, while nitrogen limitation mainly influenced the algal lipid content positively. *Botryococcus braunii* was the algal species showing the largest response to nitrogen limitation with highest cell specific lipid content. However, its growth rate was strongly influenced by nitrogen supply resulting in very low biomass production at low nitrogen supply.

Optimizing cultivation methods

To optimize both, population growth and lipid production, we performed experiments to cultivate micro-algal species, *Botryococcus braunii* and *Staurastrum tetracerum*, in a two stage cultivation system.

In the first stage, we used a full WC- growth medium (Guillard and Lorenzen 1972) to ensure high population growth. In the second stage, which was inoculated continuously with algae grown in the first stage, was supplied with a growth medium with reduced nitrogen content (1.6 µgL-1). In this stage algae are still photosynthetic active, but due to the lack of nitrogen photosynthetic products were accumulated in form of lipids. The light conditions were 100 µMol photons m-2 s-1; Experimental duration was 30 days.

Staurastrum tetracerum and *Botryococcus braunii* showed in two stage cultivation higher population growth (Fig 4) and cell specific lipid content (Fig 5) as in 1-stage semi-batch cultivation at comparable nutrient concentrations.

As shown above, nitrogen reduction in 1-stage cultivation systems increased lipid production of different algal species. *Botryococcus braunii* was the algal species with the highest cell specific lipid content; however its growth rate was strongly influenced by nitrogen supply (less than 1% biomass established at high nitrogen supply was accumulated at low nitrogen supply however the cell specific lipid content increased substantially; Fig 4). 2-stage systems resulted in higher nutrient specific biomass production and lipid content of algae compared to 1-stage cultures. *Botryococcus* showed the same biomass in the second stage of the 2-stage system than in the nutrient rich first stage. However its cell specific lipid content increased substantially within the second stage (Fig 5).

In conclusion, the presented two stage system allowed a large enhancement of lipid production of micro algae. The first stage allowed high biomass production whereas the growth conditions within the second stage resulted in high lipid contents.

If a continuous cultivation of cultures with high biomass in stage one can be assured, an almost constant supply of huge amounts of algae with even high lipid content in the second step could be guaranteed.

Diversity effects on algal lipid content (A)

For the installation of infrastructures for the large-scale production of biofuel from micro-algae it is essential to establish cultivation methods that maximize lipid production, but that are still economically viable in terms of energy demand and resource supply. At the moment, the two most important methods for the mass cultivation of micro-algae are (1) monocultures growing in closed photo-bioreactors (PBRs), and (2) phytoplankton growing in open pond systems (Lehr and Posten 2009). The most noticeable difference, between these two micro-algal production systems, is

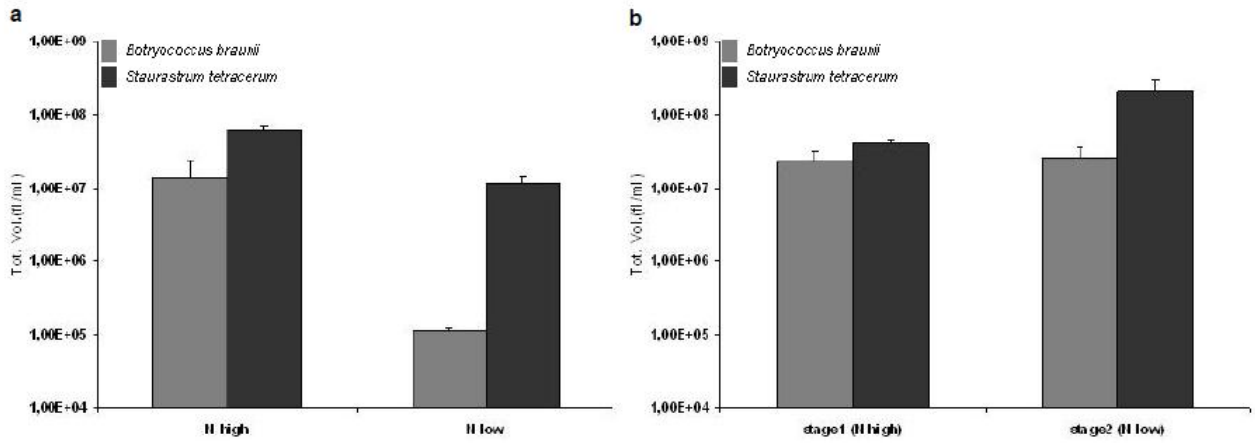

Figure 4: (a) Mean values of algal total biovolume in 1-stage cultures and (b) 2 stage systems at two different nitrogen supply rates (error bars represent + 1SE).

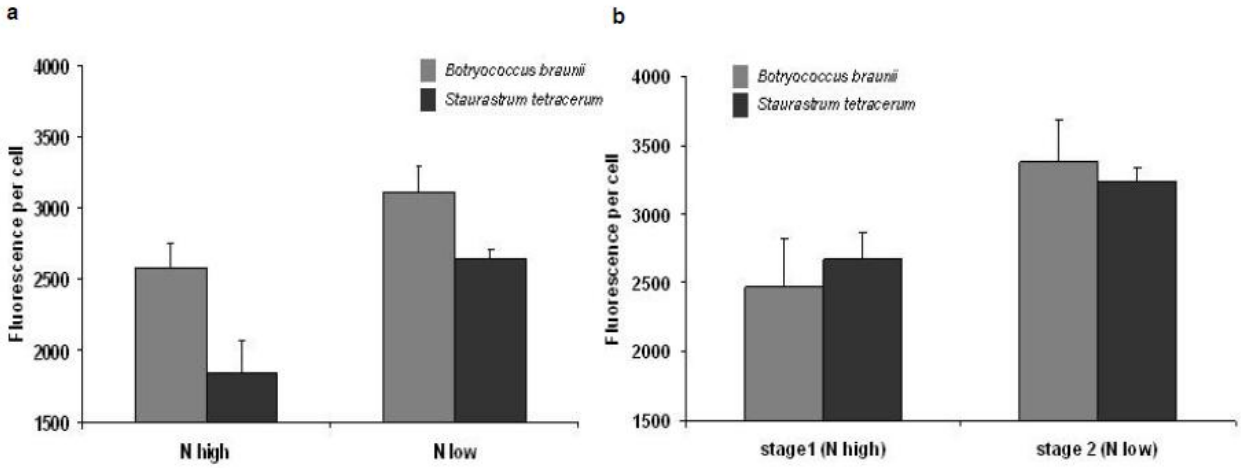

Figure 5: (a) Mean values of fluorescence per cell of Nile Red 1-stage cultures and (b) 2 stage system at two different nitrogen supply rates (error bars represent + 1SE).

the degree of exposure to the environment. For example, PBRs are usually technically sophisticated systems with a high control over environmental parameters and which must be closed to maintain selected micro-algal monocultures. In opposite, open pond systems offer less control about environmental conditions and are exposed to constant environmental input (Pulz 2001). This difference in production requirements result in higher maintenance of PBRs, which are therefore more costly in comparison to simple open pond systems (Borowitzka 1999). While it is possible cultivating monocultures of single 'lipid- rich' species or strains in open ponds, such monocultures would be unlikely to persist for long due to biological input from the environment (i.e. the addition of other micro-algae, protozoa and zooplankton). As a result, biomass production in open pond systems will necessarily include diverse phytoplankton communities.

The diversity of primary producer communities is currently not considered as an important parameter for the design of industrial pelagic food webs. However, evidence about a positive link between diversity and productivity within terrestrial and aquatic primary producer communities is increasing.

Based on the findings of recent studies dealing with diversity-productivity-relationships in aquatic primary producer communities we investigated experimentally whether diversity also can affect lipid production of micro-algae.

We investigated the growth and lipid production of micro-algae using species from all major freshwater algal groups. Algae were grown in a large number of treatments differing in their diversity level in fed-batch cultures. Additionally, we compared the growth and lipid production of laboratory communities to natural lake and pond phytoplankton communities of different diversity. The main goal of this experiment was to investigate how diverse multi species micro-algal communities perform in their growth and lipid production compared to highly selected strains of monocultures.

For this purpose we cultivated 22 algal strains with different diversity levels (1-4) and additionally samples from eight ponds and lakes located in Southern Bavaria in semi-continuous cultures at 20°C with light conditions of 90µMol Photons for seven days.

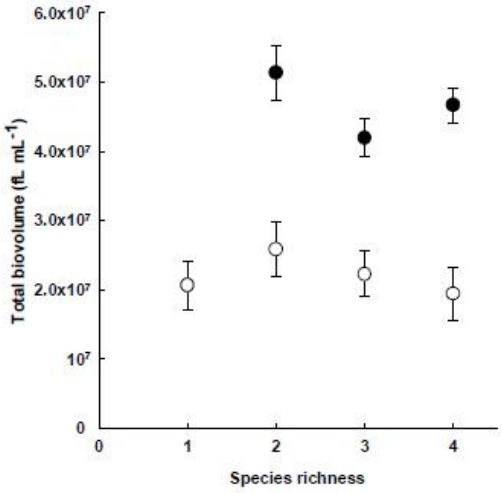

Figure 6: Biomass, determined as total biovolume (fL mL^{-1}) as a function of species richness. Mean values of measured (black circles) and expected (open circles) algal biomass are shown. Error bars represent ±1 SE (Stockenreiter et al. 2011).

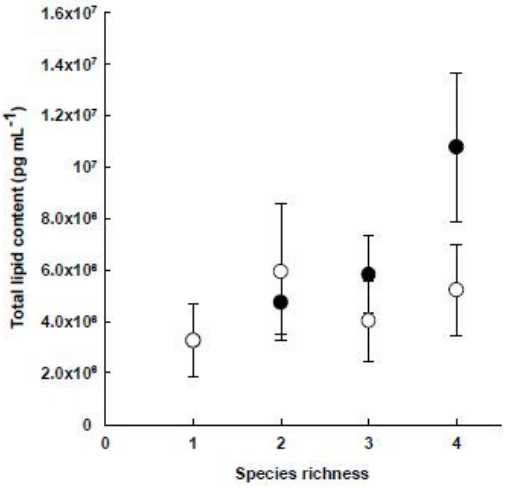

Figure 7: Total lipid content of algal cultures (pg mL^{-1}) as a function of species richness. Mean values of measured (black circles) and expected (open circles) lipid content are shown. Error bars represent ±1 SE (Stockenreiter et al 2011).

Our results show, that lipid production increased with increasing diversity in both, natural and laboratory micro-algal communities. The underlying reason for the observed diversity- productivity- relationship was obviously resource use complementarity (mathematical calculations see appendix D about calculations).

High diverse communities produced higher yields of biomass in terms of algal total biovolume (Fig 6) but also lipids (Fig 7) than compared to monocultures. Communities with mixtures of four different species showed twice as high mean algal lipid content as expected from the monocultures (Fig 7).

Diversity also influenced the cell specific algal lipid content as a quotient of the total algal lipid content and total algal biovolume. Mixtures of four different species showed a significant higher lipid content as expected from the monocultures (see appendix D) calculations). Species richness showed a significant influence on measured specific algal lipid content. Communities with four species showed significantly higher lipid content than communities with two and three different species (Fig 8).

The major underlying mechanism behind our observed diversity-productivity relationship was complementarity. Species complement one another in resource use efficiency or facilitate their growth. So, that the dominance of a single highly productive species was not responsible for the observed positive effects of diversity on lipid production.

In summary, we observed that (1) there is a link between biodiversity and lipid production in micro-algal communities. (2) The existing link is based on resource partitioning and facilitation among algal species and not on the dominance of a single highly productive algal species. (3) Diversity does not only influence positively algal biomass production and thereby increase lipid yields of more diverse communities; it does also influence the cell specific lipid content of micro-algae.

The comparison of eight natural lake phytoplankton communities with laboratory micro-algal communities showed that the lipid production of selected laboratory monocultures was not significantly higher than that of natural phytoplankton communities. This was an unexpected observation, as natural phytoplankton communities were not habituated to the nutrient-rich growth medium and the environmental conditions of the laboratory, unlike the long established laboratory cultures. Additionally, as species richness increased in the natural communities, lipid production also increased (Fig 9), which supported the diversity-lipid production relationship found in laboratory communities, as well as the diversity-productivity relationships found in natural phytoplankton communities in Scandinavian and Bavarian lakes (Ptacnik et al. 2008; Striebel et al. 2009).

However, our results demonstrate that highly diverse communities can produce higher yields of biomass in terms of both carbon and lipids when compared to monocultures. This enhancement of the yield efficiency of lipid production in diverse algal communities would be difficult to do only by technical means such as increasing resource supply. In addition, increasing the supply of resources is costly and usually correlated with high energy requirements. In contrast, an increase of the biological efficiency (resource use efficiency) of the system is usually self-financing. It is therefore important for biomass production systems to utilise all possible ecological options to increase the efficiency of the use of the supplied resources by integrating basic ecological principles into the cultivation systems.

Mechanistic aspects of diversity effects on algal lipid content (B)

Although we found that the underlying mechanism that micro-algal diversity can influence algal lipid production is

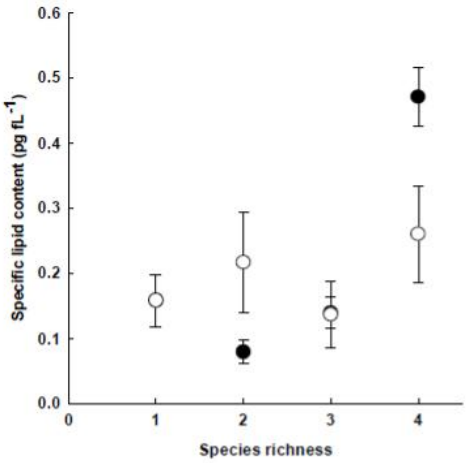

Figure 8: Specific lipid content, determined as lipid content per biovolume unit (pg fL-1) at the end of the experiment as a function of species richness. Mean values of measured specific lipid content (black circles) and of expected mean values of specific lipid content (open circles). Error bars represent ±1 SE (Stockenreiter et al 2011).

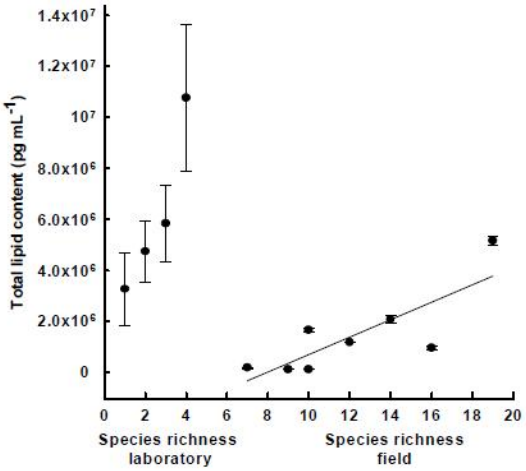

Figure 9: Total lipid content of laboratory and natural algal community cultures (pg mL-1) as a function of species richness. Mean values of total lipid content of laboratory algal communities (species richness one to four) and mean values of total lipid content of natural algal communities (species richness seven to 19; $y = 3.42 \cdot 1005 x - 2.72 \cdot 1006$; $r^2 = 0.658$; $p = 0.0146$). Error bars represent ±1 SE of different algal communities with identical diversity levels (laboratory treatments) or within lake/pond sample replicates (natural phytoplankton treatments) (Stockenreiter et al 2011).

related to complementarity (Stockenreiter et al. 2011) a more detailed analyses of the observed complementarity is however missing.

Recent studies point towards a complementarity in light use along the PAR spectrum (400-700 nm) as a main mechanism behind micro algal diversity – productivity relationships (Striebel et al 2009; Behl et al. 2011). A rich variety of photosynthetic pigments provided from diverse algal communities might exploit the existing light supply more efficiently, utilizing different wavelengths along the photosynthetically active radiation (PAR) spectrum (Falkowski et al 2004).

Diversity is often referred to as species richness neglecting other components of diversity (Diaz and Cabido 2001). However, species richness is only one part of a diverse community and its organization (Hillebrand and Matthiesen 2009). Diaz and Cabio (2001) could show that most of the recorded positive effects of species richness were the effect of functional richness and/or functional composition, where the term `functional group` stands for a class of species divided in their common biochemical/ecological functions (Hood et al. 2006). While the influence of species richness on community functioning within functional group is well studied (Cardinale et al. 2006; Duffy et al. 2007; Bruno and Cardinale 2008) the influence of species richness across functional groups remains limited (Scrosati et al 2011). Experiments with increasing algal functional group diversity in phytoplankton showed increased light-use complementarity with a strong increase in biomass-specific absorbance yield with increasing algal group diversity (Behl et al. 2011).

With regard to these arguments and our findings in our previous study, that species richness is positively linked to algal lipid-production we investigated the following hypotheses: (1) Diversity interact with the environmental parameter light. Light is exploited more efficiently in highly diverse communities, leading to higher lipid production in highly diverse communities. (2) Diversity – lipid productivity relationships are not only depending on species richness per se but also on the functional diversity of the micro-algal communities.

These hypotheses were tested via a series of laboratory growth experiments under highly controlled environmental conditions. We assessed the resource use efficiency in terms of light usage along the photosynthetically active radiation (PAR) spectrum and the lipid production of micro-algae communities using 23 species from all major algal groups. We created a gradient of functional group diversity using the four major freshwater algal classes (chlorophyta, diatoms, cyanophyta, and chrysophyta). Most micro-algal species have also other photosynthetic pigments beside chlorophyll a, their composition is taxon specific (Hager and Stransky 1970; Scheer 1999). The classification of algae is partly based on occurring pigments (van den Hoek 1995), thereby linking algal phylogenetic diversity to functional diversity in terms of light use. Communities assembled from species coming from various algal classes show usually larger differences between pigments than communities assembled from species coming from a single algal class. (Schlüter et al. 2006; Behl et al. 2011).

After data analyzing we found a statistically significant increase in the carbon specific average PAR absorbance with increasing species richness within single functional groups in mixtures of chlorophytes (Fig 10a). Whereas

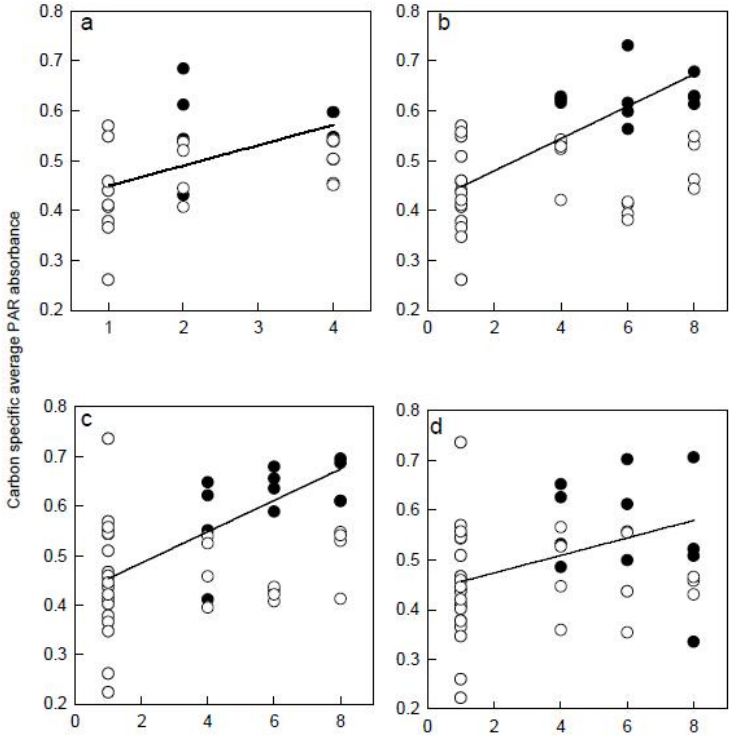

Figure 10: carbon specific average PAR absorbance within one (a) (chlorophyta), two (b), three (c) and four (d) functional group as a function of species richness for expected values (open circles) and measured values (filled circles). Lines for measured values (solid line) represent linear regression. Linear regression statistics are: (a) expected: $y = 0.02x – 0.41$; $r^2 = 0.11$; $P = 0.20$; measured: $y = 0.04x – 0.41$; $r^2 = 0.23$; $P = 0.05$. (b) Expected: $y = 0.004x – 0.44$; $r^2 = 0.03$; $P = 0.40$; measured: $y = 0.032x – 0.41$; $r^2 = 0.59$; $P < 0.0001$. (c) Expected: $y = 0.004x – 0.45$; $r^2 = 0.01$; $P = 0.51$; measured: $y = 0.031x – 0.42$; $r^2 = 0.42$; $P < 0.0001$. (d) expected: $y = 0.001x – 0.45$; $r^2 = 0.001$; $P = 0.90$; measured: $y = 0.012x – 0.44$; $r^2 = 0.15$; $P = 0.02$.

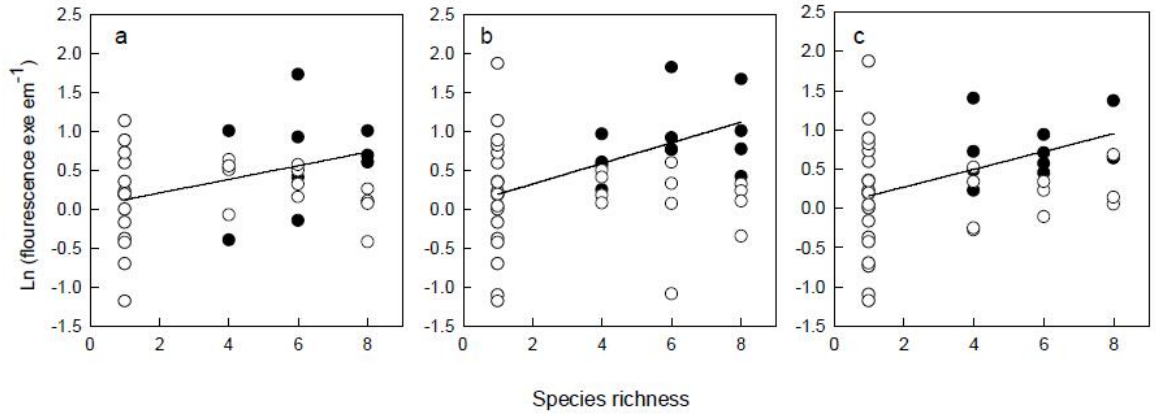

Figure 11: algal lipid content shown as ln (fluorescence ratio of Nile Red) within two (a), three (b) and four (c) functional group as a function of species richness for expected values (open circles) and measured values (filled circles). Lines for measured values (solid line) represent linear regression. Linear regression statistics are: (a) expected: $y = 0.0107x - 0.1450$; $r^2 = 0.0034$; $P = 0.77$; measured: $y = 0.0872x – 0.0379$; $r^2 = 0.14$; $P = 0.05$. (b) expected: $y = -0.0184x - 0.2119$; $r^2 = 0.0058$; $P = 0.68$; measured: $y = 0.1323x – 0.0629$; $r^2 = 0.23$; $P = 0.006$. (c) expected: $y = 0.0132x - 0.1117$; $r^2 = 0.0026$; $P = 0.78$; measured: $y = 0.1132x – 0.0434$; $r^2 = 0.16$; $P = 0.02$.

among functional algal groups we found a statistical significant increase in carbon specific average PAR absorbance coefficient with increasing species richness in mixtures with two, three and four different functional groups (Fig. 10b-d).

We didn't find a significant relationship between lipid content (shown as ln (fluorescence of cell stained with Nile Red)) and species richness within a single functional group, but there were clear effects in communities with different functional groups. We found a significant increase of lipid content with species richness in communities with two to four functional groups (Fig.11a-c).

Like in the previously described experiment the underlying mechanism of the observed over yielding in carbon specific average PAR absorbance and the micro algal lipid content was related to complementarity. The dominance of

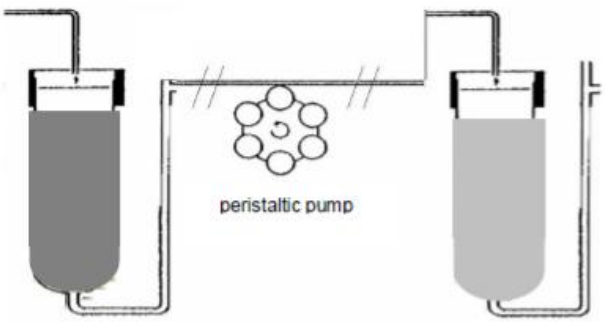

Figure: 12 Simplified sketch of flow batch system and peristaltic pump.

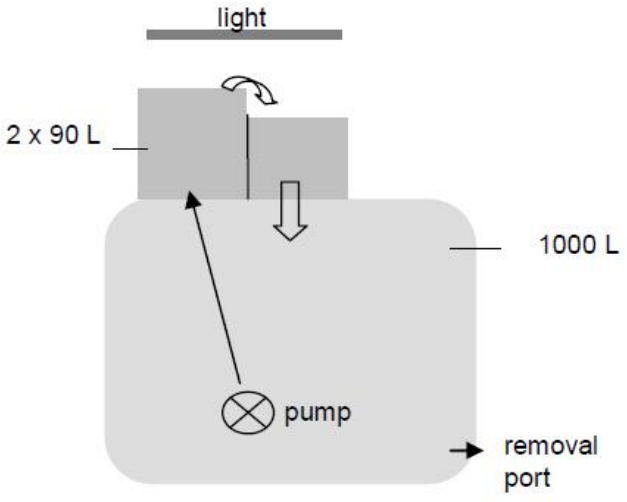

Figure 13: Up scaled two stage cultivation system for biomass and lipid enhancement.

a single highly productive species was not responsible for the observed positive effects of diversity on carbon specific average PAR absorbance and algal lipid production, which is supported by other studies dealing with diversity-productivity relationships (Behl et al 2011; Stockenreiter et al. 2011).

Our results demonstrate that there is a diversity-light-lipid relationship in algal communities. Results of carbon specific average PAR absorbance and Nile red fluorescence as a measure of lipid content showed a similar pattern with increasing species richness and functional group richness.

In accordance to the diversity-light-lipid production relationship, observed in our experiments, diversity parameters should be given more consideration with respect to biomass production, as a number of additional positive aspects of diversity on biomass production may be identified. Furthermore, highly diverse communities are also considered to produce more temporally stable ecosystem services, due to complementary effects among species that perform similar ecosystem functions (Ptacnik et al. 2008; Tilman 1996). This so called diversity-stability hypothesis states that species diversity mediates the functional stability of a community by compensating interactions to environmental fluctuations among the systems of concurrent performing species (McNaughton 1977). In summary, our results support the arguments of Smith et al. (2010) that it is important to base algal biomass production on modern ecological concepts of ecosystem dynamics, a point which is often not getting enough attention within the progress of technical optimizations of algal biomass production systems.

Appendix

A) Cultivation of algae

Two stage (Fig 12) production systems resulted in higher nutrient specific biomass production and lipid content of algae compared to single stage cultures.

Up scaling of laboratory two stage systems are currently under investigation (Fig 13). The 2 stage cultivation method was taken as basis of a medium scale (1000 litre) construction.

B) FlowCAM®

Our analyses of the micro-algae lipid content and using a imaging flow cytometer (FlowCAM®) at an excitation peak wavelength of 532 nm and an emission peak wavelength of 645 nm (green laser) has the unique advantage to estimate the lipid content of each algal cell in diverse communities without requiring the (practically impossible) physical separation of algal cells. It is possible to distinguish between the fluorescence of several species in communities by imaging and thereby to quantify the cell specific fluorescence of each micro-algal species in diverse communities. A fluorescence – lipid content calibration curve was fitted along a linear gradient of cell density of Saccharomyces cerevisiae var. carlsbergensis suspended in WC-medium, with a cell specific lipid content of 3.07·10-11g and a cell specific fluorescence range of 2750 ± 82 units.

C) Nile Red

Cell specific lipid content was estimated by staining neutral lipids with Nile Red, and measuring fluorescence with a FlowCAM® imaging flow cytometer. For staining micro-algae, 1 mg of fine-grained Nile Red (9-diethylamino-5H-benzo[α]phenoxazine-5-one; HPLC grade, Sigma Aldrich) was dissolved in 4 mL acetone (HPLC grade; Lee et al. 1998). Subsequently, 20 µL Nile Red solution were added to 5 mL of algal solution and incubated for 30 min in a darkened container. This method is a simple and well established method for the rapid determination of algal lipids (Eltgroth et al. 2005; Elsey et al. 2007; McGinnis et al. 1997). Lee et al. (1998) found a significant relationship between fluorescence and the lipid content of algae, and suggested that the method of staining algal cells with Nile Red to be equally viable as the gravimetric method, which is commonly used for lipid determination. According to, Sheehan et al. (1998) a major problem of Nile Red is that species vary in their uptake of this dye. However, in our analyses we compared multi species mixtures with their respective monocultures; so this effect should not have biased our results on lipid production as a function of diversity. Additionally, our analyses of the micro-algae lipid content with Nile Red fluorescence and using a imaging flow cytometer (FlowCAM®) has the unique advantage to estimate the lipid content of each algal cell in diverse communities without requiring the (practically impossible) physical separation of algal cells.

D) Calculations

The diversity related yield of algal biovolume, total algal lipid content and specific lipid content was calculated as the ratio of observed and expected values of these parameters at the end of the experiment. Expected values were based on a weighted average of the monoculture yields of the enclosed species in every community. For this purpose we determined the proportion of each species within the experimental communities by microscopy counting. The proportion of each species was multiplied by its species specific yield (biovolume) when growing in monoculture. These biomass values for each species in a mixture where added up to calculate the expected yield for each multi species treatment.

In addition, we calculated the contribution of complementarity and species identity (selection effect) to the observed yield according to Fox (2005):

$$\Delta Y = S * \overline{M} * \overline{RY} + S * Cov\left(M_i, \frac{RY_i}{RYT} - RY_{E,i}\right) + S * Cov\left(M_i, RY_i - \frac{RY_i}{RYT}\right) \qquad (I)$$

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6. Report from Blue Lagoon

By: Ása Brynjólfsdóttir, Halldór G. Svavarsson

Abstract:

A method to sequester the CO_2 from geothermal power plant's flue gas by means of photosynthetic microalgae is described. Some fundamental parameters of microalgae growth (pH level, CO_2 feed rate, temperature and illumination conditions) were analyzed in the process of optimizing its growth. Two unique microalgae species, one that thrives in the geothermal seawater of the Blue Lagoon and the other at Reykjanes peninsula were investigated. The results published here provide additional alternatives for managing CO_2 greenhouse gas emission from geothermal power plant and also provide additional value for the microalgae biomass production. It is demonstrated that geothermal flue gas can be efficiently used as a feedstock for microalgae cultivation.

Main objectives:

The main objective of the project was to provide a proof-of-concept level evaluation of algae as a potential raw material for biodiesel.
Our role was to explore the possibility of using flue gas from geothermal powerplant as a feedstock:

- Compare to commercially available CO_2.
- Testing - lab scale
- Upscaling – pilot scale

The work items listed above have all been executed.

Method implementation:

Experimental steps

- Capturing carbon dioxide from Svartsengi Geothermal Power Plant (Fig. 1)
- A comparison of microalgae cultivation from two different sources of CO_2: pure (commercial) and from geothermal flue gas.
- Two types of algae were cultivated: Blue Green Algae and Diatom Algae
- Lab scale experiments were carried out in 2.5 liters photo-bioreactor shown in Fig. 2
- Pilot scale growth of algae performed in a tubular reactor with total volume of 1000 Ltrs, shown in Figs. 3 and 4.

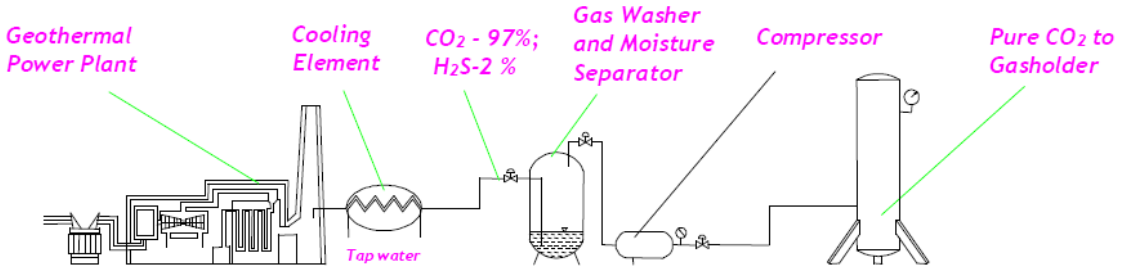

Fig. 1. Block diagram of capturing carbon dioxide from geothermal power plant.

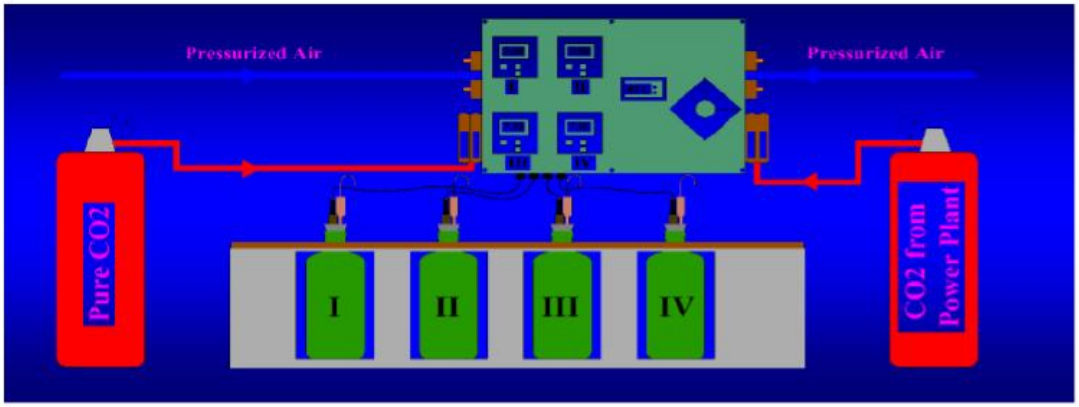

Fig. 2. Diagram of laboratory scale reactor for growth comparison.

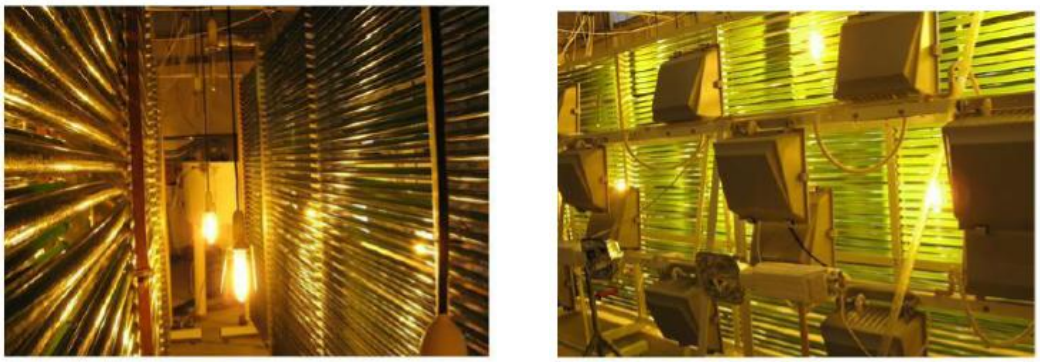

Fig. 3. Pilot scale reactor of total volume 1000 Ltrs.

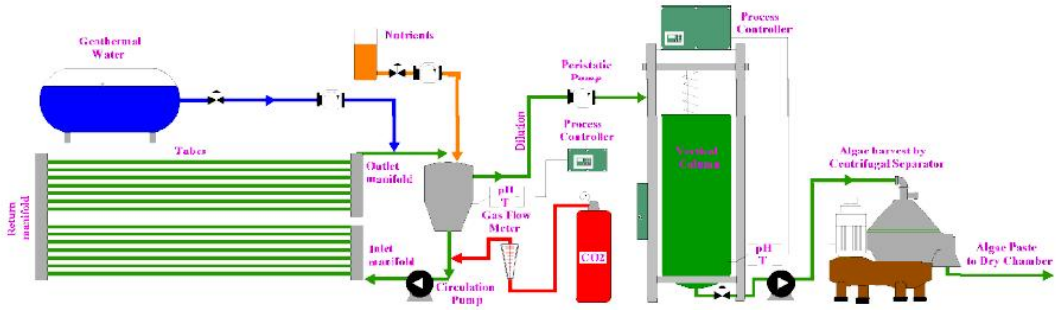

Fig.4 . Diagram of continuous system for algal biomass production.

Results:

Comparison of algae growth supplied by two types of CO_2 (Figure 5a and 5b):

- For blue green algae the average different of growth algae supplied by pure gas and from Geothermal Power plant was 2.41wt% and standard deviation σ =1.15%
- For diatoms the difference was higher and estimated by 7.48wt% and standard deviation σ =3.57%

A more detailed descrition of the results can be found in Appendix II

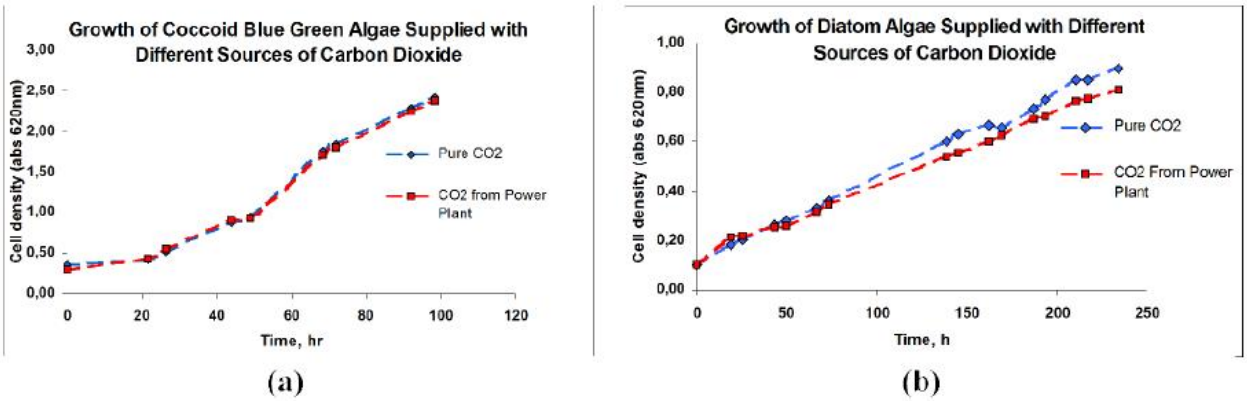

Fig. 5. Growth curves of blue green algae supplied with two types of gases (a); growth of diatoms supplied with two types of gases (b).

Conclusion:

It can be concluded from these preliminary results that supplying algae by CO_2 from Geothermal Power Plant does not lower its growth efficiency as compared to the commercially available gas.

Recommendations:

The informations obtained here are valuable to all industrial companies emitting CO_2 and to the algae industry in general and should be explored further. A Nordic collaboration on this topic is desireable and should be easily accomplished. Our results are currently being used in Icelandic research project focusing on lipid-producing algae fed on geothermal flue gases. A project executed by the Blue Lagoon research team and supported by the Icelandic Technology Development Fund. The outcoming of this will aim for production of valuable cosmetics, nutriciants (such as omega-3 fatty acids), fish feed and even biofuel. Due to the abundancy of geothermal sources in Iceland and especially on Reykjane peninsula, we see many industrial opportunities in this area.

APPENDIX I

NILE RED for measurement of lipids in phytoplankton: Summary of the internal methodological review for LIPIDO project

Contributions from SYKE, NTNU, LMU and VTT

Rapid and reliable measurements of neutral storage lipids are required while assessing phytoplankton as lipid producer for biofuel applications. Dye Nile Red has been widely used as a proxy for lipid content of cells. However, the method has several drawbacks and requires further optimization. In LIPIDO, various partners were using Nile Red method, and a working document was created, collecting experience and notes from the users and also reviewing the literature. This working document was used to discuss and agree some methodological aspects of Nile Red analysis, and it forms a common starting point for future work. In this annex, main recommendations of the working document are reviewed.

Key papers reviewed

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Nile Red concentrations and quality

Summary: Stock solutions 100-250 mg L^{-1} are generally used. Final concentrations are typically from 0.83 to 1.25 µg mL^{-1}, though Chen et al (2009) found 0.5 µg mL^{-1} to be the optimal concentration (but not much different from 0.5-2 µg mL^{-1}). No paper refers to different Nile Red qualities in phytoplankton lipid studies.

Recommendations:

Stock 250 mg L^{-1} (785µM) in acetone, stored in the dark.

Final concentration 1 µg mL^{-1} (3.14µM).

Technical grade Nile Red (Sigma N-3013) should not be used as it gives higher background values.

Cell concentrations

Summary: The aim of adjusting cell concentrations is to have measurements in the linear range of instrument's response. What matters in the analysis is the light attenuation at Nile Red wavelengths. The relationship between this and the cell numbers or optical densities at other wavelengths has not been studied. Therefore the given ranges should be considered as indicative only.

Recommendations:

For quantitative fluorescence measurements the cell and dye density should be low enough to avoid reabsorption effects. Optimally, optical density should be below 0.05 at excitation/emission wavebands.

Instead of doing time consuming absorption measurements (using stained samples!) daily for all samples, one should determine linear range for a fluorometer setup using serial dilutions with a Nile Red stained sample.

Staining time & temperature

Summary: Many studies use fixed time, without notice if it has been optimized. Sheehan et al (1998) "*The kinetics of fluorescence in stained cells varied in different species, presumably due to differences in the permeability of cell walls to the stain, and differences in how the lipid is stored in the cells, i.e., as large or small droplets. Fixing the stained cells with formaldehyde or ethanol preserved the Nile Red fluorescence for 2 hours, but cells that were chemically fixed before Nile Red staining did not exhibit the characteristic yellow fluorescence*." Some comments that fluorescence fades away quickly (in minutes) are related to microscopy and maybe strong illumination photobleach the stain.

Recommendations:

Nile Red fluorescence varies in time, and the reasons for these kinetics are unknown at time. As the kinetics vary between species (and maybe even between physiological stages) a kinetic check must be done for each species separately, and a time point for Nile Red measurements must be selected accordingly.

Wavelengths and instrumentation

Summary: Variations in the excitation and emission wavelengths in Nile Red measurements derive from the use of instruments with fixed optical setups. With spectral instruments the optimal wavelengths should be used (sometimes these do not coincide with maximum intensities, if spectra are not corrected for instrument optics).

Recommendations:

With spectral devices, the optimum wavelengths are close to ex 530nm/ em 575nm. However, the analysis shown below show that slight deviations (at least to lower wavelengths) do not really make any difference.

With instruments using fixed wavelengths the simulations of spectral window using spectral instruments is recommended, with subsequent validation of results using optimal wavelengths (though it may be difficult due to com-

plex optical configurations).

Corrections, standardizations etc. applied

Summary: Daily calibration of fluorometers (at least those with instabilities) should be performed. Fluorescence due to cells and Nile Red alone should be subtracted (though many times negligible when lipids have been accumulated). For some difficult (green algae) species Nile Red should not be routinely used without thorough study of Nile Red permeability.

Recommendations:

It would be beneficial to apply a solid secondary standard as a mean of correcting day-to-day variations of fluorometer.

Using a spike of Nile Red in water or in acetone may serve the same purpose, but is more vulnerable to errors due to high dilution required (e.g. spiking 10µl into 3 mL).

Nile Red fluorescence should be related to Triolein fluorescence, whenever possible.

APPENDIX II

Part of Indra Suryata's MSc thesis at Univ. Reykjavik, presenting work conducted at Blue Lagoon

By: Indra Suryata

PART II

2. CARBON EMISSION REDUCTION by means of microalgae cultivation

There is a growing scientific consensus that rising concentrations of carbon dioxide (CO_2) and other greenhouse gases are gradually warming the Earth's climate. The amount of damage associated with that warming remains uncertain, but there is some risk that it could be large and perhaps even catastrophic. Reducing that risk would require restraining the growth of CO_2 emissions — and ultimately limiting those emissions to a level that would stabilize atmospheric concentrations — which would involve costs that are also uncertain but could be substantial.

2.1 Carbon fixation process

As previously mentioned, there are several methods of CO_2 mitigation which have been studied; they are generally classified under two categories: (1) chemical reaction-based approaches and (2) biological CO_2 mitigation. Biological CO_2 mitigation itself has attracted much attention as an alternative strategy mainly due to its production of biomass as the byproduct during the photosynthesis process. This biological CO_2 mitigation can be done by plants and other photosynthetic microorganisms. Nevertheless, the potential for increased CO_2 capture in agriculture by plants has been estimated to contribute only 3–6% of fossil fuel emissions. This low value is largely because of the slow growth rates of conventional terrestrial plants.

In this project, microalgae are used to observe the feasibility of fixing the carbon gasses from the nearby geothermal power plant. Microalgae have the capability to fix carbon dioxide while capturing solar energy with efficiency up to 50 times greater than that of agricultural plants, with the same coverage/growing area. [Wang et. al., 2008].

2.2 Microalgae

Microalgae are a unicellular species which exist individually in nature or in chains or groups. Different species has different size which can range from just a few micrometers to a few hundreds of micrometers. Compared with higher plants, microalgae do not have roots, stems and leaves. Just like other plants, microalgae are capable to perform photosynthesis process. This process by microalgae is important for life on earth as they produce approximately half of the atmospheric oxygen and use simultaneously the greenhouse gas carbon dioxide to grow photoautotrophically [Benemann, 1997].

Microalgae have enormous biodiversity with approximately 200,000 – 800,000 species exist, of which about 35,000 species are described. Over 15,000 novel compounds originating from algae biomass have been chemically described. Most of these microalgae species produce unique products like carotenoids, antioxidants, fatty acids, enzymes, polymers, peptides, toxins and sterols. The chemical composition of microalgae is not intrinsic constant factor but varies over a wide range, depending on species and on cultivation conditions. It is possible to accumulate the desired products in microalgae to a large degree by changing environmental factors like temperature, illumination, pH, CO_2 amount, salinity and nutrients.

In view of CO_2 mitigation by microalgae, the strategy offers numerous advantages. First, microalgae have much higher growth rates and CO_2 fixation abilities as compared to conventional forestry, agricultural and aquatic plants. Second, it has the potential to completely recycle carbon dioxide because carbon is converted to chemical energy through photosynthesis process, which can be converted to fuels using existing technologies, such as transesterification process [Wang et. al., 2008].

The chemical reaction-based carbon mitigation strategy, as discussed previously, has disposal problems because both the captured CO_2 and the wasted absorbents need to be disposed of. Third advantage is that CO_2 biomitigation using microalgae could be made profitable from the production of biofuels and other novel bioproducts, as compared to the

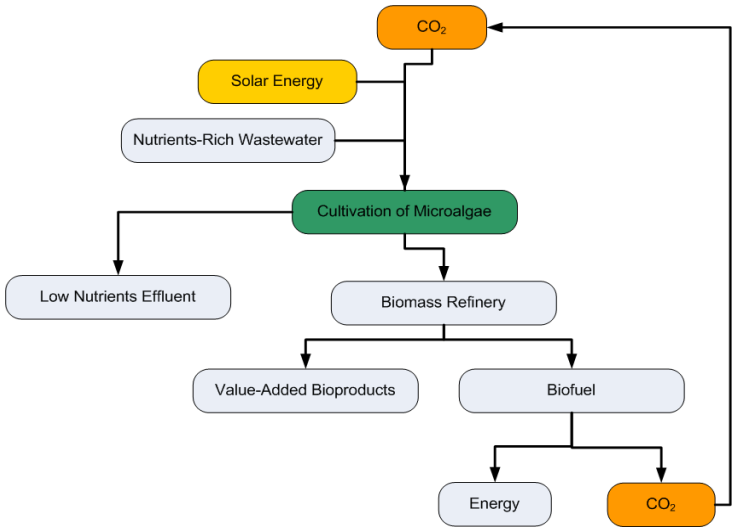


Figure 17. A conceptual flowchart for the complete "recycling" of CO_2 for solar energy capturing.

chemical reaction-based strategy which is considered energy consuming and costly process, and the only economical incentive is by claiming CO_2 credits to be generated under the Kyoto Protocol [Boom, 2001]. Lastly, the biological carbon mitigation by utilizing microalgae could be further made economical and environmentally sustainable, by combining it with other processes such as wastewater treatment. Combining this carbon bio-mitigation process with wastewater treatment will result in significant advantages: (1) microalgae have been shown to be effective in removing nitrogen and phosphorus removal, as well as in metal ion depletion, and combination of microalgae with wastewater treatment will significantly enhance the environmental benefit of this strategy, and (2) it will lead to savings in the consumption of nutrients for microalgae growing process and (3) it will definitely resulted in savings of the precious freshwater resources.

2.3 Growth method

In general, the production cost of microalgae biomass (and thus microalgae growing) is higher than the cost of growing crops. Photosynthetic growth need light, carbon dioxide, water and inorganic salts and the ideal growth temperature is between 20 to 30 °C.

For large scale production of microalgae biomass, it generally uses continuous culture during daylight. In this particular method, the amount of microalgae "soup" being withdrawn is the same quantity as the fresh culture being fed. In most cases, the feeding step is stopped during the night, but the mixing of "soup" must continue to prevent settling of the biomass. In general, around 25% of the biomass produced during the daylight may be lost during the night because of respiration. The loss amount mainly depends on the light level under which the microalgae were grown, the temperature during the day and the temperature at night. Nowadays, there are generally two methods of large-scale microalgae production; raceway ponds and tubular photobioreactors. The Blue Lagoon CO_2 bio-mitigation project is currently utilizing the tubular photobioreactors method with artificial illumination to replace the sunlight.

2.3.1 Raceway ponds

The first microalga' growing medium is raceway pond, which is made of a closed loop recirculation channel of stream. Mixing and circulation are done by a paddle wheel. In many cases, baffles are placed in the flow channel to guide around the bends. The channels are built in concrete or compacted earth and may be lined with plastic. During the day, the microalgae culture is fed continuously in front of the paddlewheel where the flow is initiated. Microalgae "soup" is usually being harvested behind the paddle wheel, on completion of the circulation loop. To prevent any sedimentation, the paddlewheel should be running all the time.

In this raceways method, cooling system is done only by evaporation. As the temperature fluctuates within a diurnal cycle and seasonally, evaporation water loss can be significant. Because of this occurrence, raceways use carbon dioxide much less efficiently than photobioreactors. It is not unusual that unwanted microalgae and other microorganisms contaminate the culture and thus lower the productivity.

Raceways pond is commonly identified as less expensive than the photobioreactors, because they cost less to build and operate. The low cost operation and investment is however at least partly compensated with low biomass productivity.

2.3.2 Photobioreactors

The second medium for microalgae is photobioreactors which allow essentially culture of single species microalgae for prolonged durations. This method has been successfully used for producing large quantities of microalgae biomass [Ugwu et. al., 2008].

A tubular photobioreactors usually consist of an array of straight transparent tubes that are usually made of plastic or glass. This tubular array is designed to capture the sunlight or any artificial sunlight. The diameter of the tubes is limited to a certain size because light will not penetrate too deep in the case of dense algae culture, which is necessary to ensure a high level of biomass productivity. Microalgae culture is circulated from a reservoir tank to the solar collector and back to the reservoir tank, continuously.

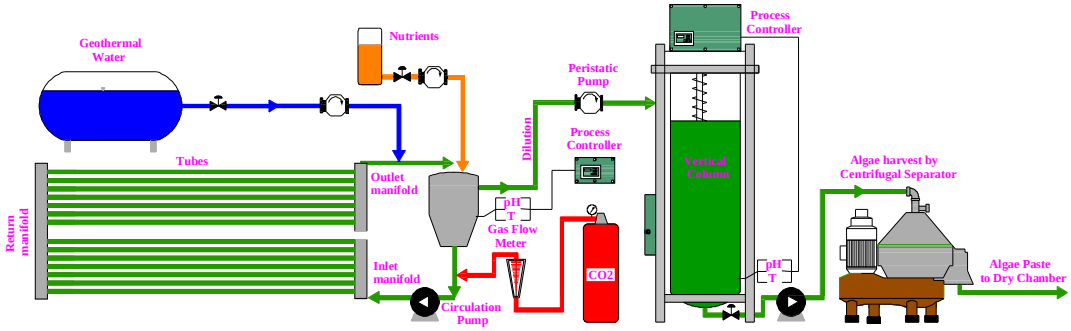


Figure 18. Set of equipments for microalgae project by using photobioreactors.

The photobioreactors unit is mainly consisting of: Plexiglas tubes, vertical manifolds, reservoir tank, pump and control panel unit (Figure 18.) The rate of circulation of the microalgae culture can be adjusted by selecting a different speed of the centrifugal pump. The carbon dioxide feed point should be located in the pipe section between the pump and the reservoir tank to ensure high mixing ratio of microalgae culture with the carbon dioxide. The flow nature of microalgae suspension should be turbulent to prevent the formation of biofilm on the walls of the tubes which can later slow down the photosynthesis process.

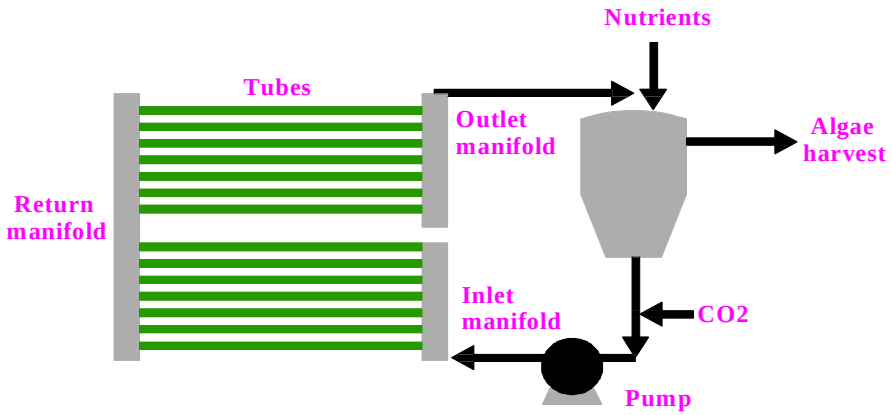


Figure 19. Reservoir tank of the photobioreactors.

The reservoir tank is considered as the most essential part of the photobioreactors system. Inside, there are several parts such as: temperature probe and pH probe to monitor the variables, and also a solenoid heater/radiator to maintain the temperature of the microalgae solution to a desired temperature by flowing either cold or hot water (Figure 19.)

Based on available literature, the maximum rate of oxygen generation in typical photobioreactors is approximately 10 gr-O_2.m^3.min^{-1}[Benemann, 1997]. If the level of oxygen is higher than the level of air saturation, most of the time it will restrain the photosynthesis process. To avoid this event, the returned microalgae solution is sprayed from the top of the reservoir tank where it falls down by a distance of about one fourth of the tank's height.

The Blue Lagoon CO_2 bio-mitigation project is currently utilizing the photobioreactors method (Figure 20) with artificial illumination to replace the sunlight. The horizontal tubes are stacked vertically to allow maximum exposure to the installed lights and also to optimize the space usage.

Figure 20. Photobioreactors used at Blue Lagoon R&D lab.

2.4 Experimental

Microalgae nutrition and its harvesting systems

Essentially, growth medium must provide sufficient nutrients for the growth of microalgae. Carbon, nitrogen, phosphorus, and sulfur are simply the most important elements constituting algae cells. Other essential elements include iron, magnesium, trace elements, and in some cases, silicon [Rebolloso-Fuentes et. al., 2001]. It is important to develop balanced media for optimal microalgae cultivation and CO_2 fixation [Mandalam and Palsson, 1998]. At the Blue Lagoon photobioreactors system, the concentration of nitrogen, phosphorus and sulfur is approximately 0.15% vol. of total microalgae solution.
Harvesting the microalgae is considered to be an expensive and problematic part of industrial production of microalgae biomass due to the low cell density achievable with microalgae cultures, which is typically in the range of 0.3–0.5 g dry cell weight per liter and with exceptional cases reaching 5 g dry cell weight per liter. There is no single harvest method that is suited to every case, and therefore, selecting the right technologies and optimizing the harvesting process are important [Molina et. al., 2003].

In the case of Blue Lagoon, the harvesting method is to utilize a centrifugal separation and later use the centrifugal sedimentation, which can yield in dry mass content of 2.5% wt. and 22% wt., respectively

Growth parameters

Depending on its species, microalgae growth rate may be not the same at different conditions. Some of the most important growing parameters are temperature, salinity, pH-value and level of illumination, have significant impact on growth productivity of algae. In many cases, series of experiments were carried out to examine the optimum condition for microalgae growth.

2.4.1 Parameters optimization

During the process of carbon dioxide bio-mitigation by utilizing microalgae, the Blue Lagoon R&D lab has also performed several scenarios of varying the parameters of microalgae growth, such as, pH level, growing temperature, salinity level and photoactive radiation levels. The experiment was carried out in small scale photobioreactor with a total volume of 10 liters. The reactor consists of four 2.5 liters glass bottles placed in water bath as seen in Figure 21 below.

Optimization of pH

The pH level depends on the amount carbon dioxide dissolved in the medium. Dissolution of carbon dioxide in water can be written as shown in Equation 3 below:

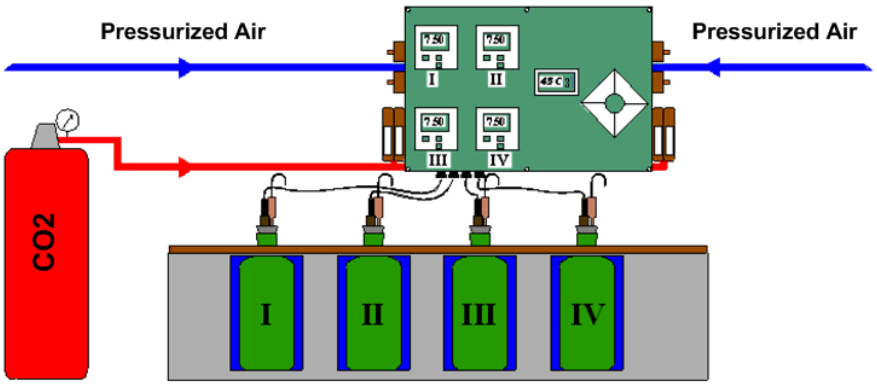


Figure 21. Diagram of small scale reactor with total volume of 10 liters.

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-} \quad \text{(Eq. 3)}$$

Equation 3. Carbon dioxide dissolved in water

The experiment to assess the pH variation was carried out at temperature of 43ºC, salinity 2% vol. and average photo-active radiation level of 180 µE/m^{2}sek. The results are presented in graph (Figure 22).

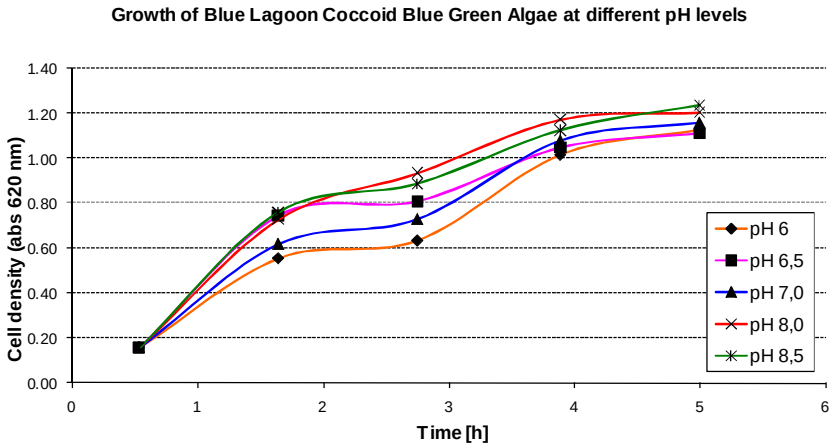


Figure 22. Growth characteristics dependent on pH level.

From the graph, it can be concluded that the pH level about 7.5 gave the highest growth efficiency for the Blue Lagoon blue-green microalgae.

Optimization of temperature

As stated in many technical publications, the optimum temperature for microalgae growth is highly dependent on the species being used. For most microalgae species it is recommended that the media temperature be kept near 28ºC. In the case of Blue Lagoon blue-green microalgae, which belong to the group of geothermal algae, the range of temperature occurs at approximately 40ºC. As natural respiration of the algae is not able to keep the temperature near this level and additional heat source is used. In small scale reactor a heating element is used to increase the medium tempera-

ture, in combination with temperature regulator. The experiment was carried out at pH level of 7.5 with salinity about 2% vol. and photoactive radiation level of 140 μE/m^{2}sek.

Based on the experiments (Figure 23), it can be concluded that the temperature which provides the optimum growth rate is 45ºC, which is a relatively high temperature as compared to other common algae species.

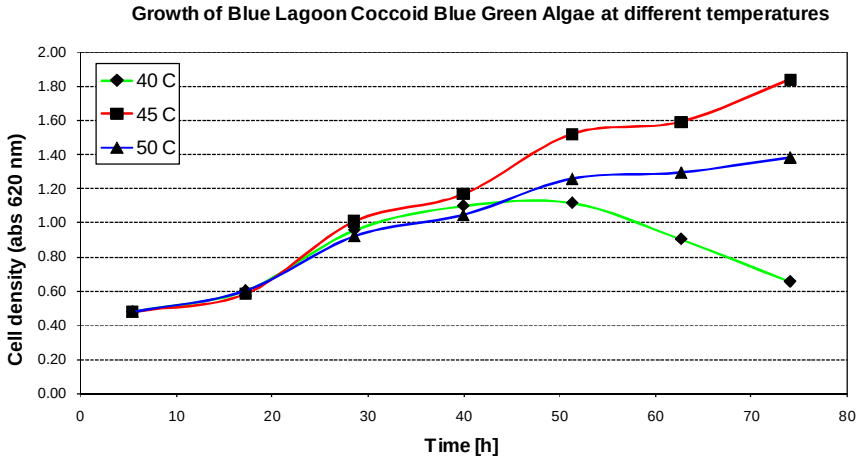


Figure 23. Growth characteristics dependent on temperature.

Optimization of salinity

In general, the marine microalgae (i.e. Blue Lagoon blue-green algae) will need seawater supplemented with commercial nitrate and phosphate fertilizers and few other micronutrients to grow. In many cases, fresh and brackish water from lakes, rivers and aquifers can also be used as a growth media. Thus, in general, the growth media is inexpensive. For blue green algae the natural environment is geothermal water with content 70% vol. of seawater and 30% vol. of freshwater. It could be expected that the optimal salinity of media for that kind of algae should be close to the level of seawater. The experiment for salinity effect was carried out at temperature 45ºC, pH = 7.5 and irradiation level of 140 μE/m^{2}sek.

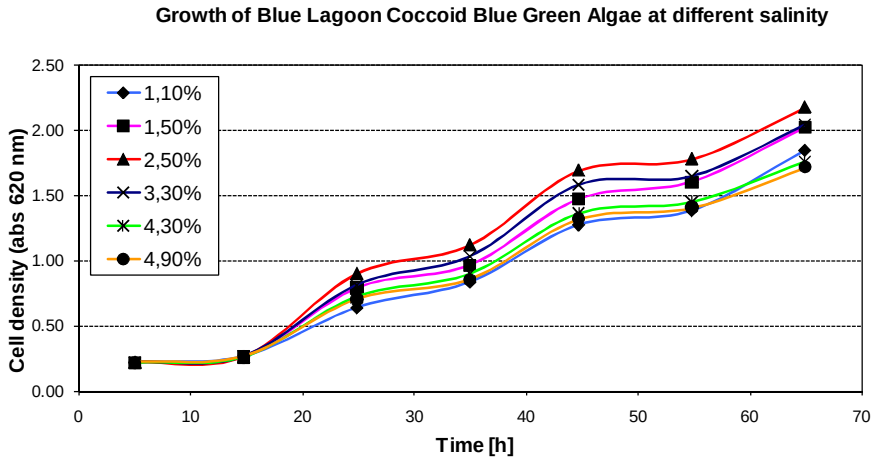


Figure 24. Growth characteristics dependent on salinity level.

The result in Figure 24 show that the highest growth rate for the blue green algae occurred at 2.5% vol. of salt content, which is comparable to the salinity of seawater. The conductivity of 2.5% vol. salt solution is about 50 mS/cm.

Optimization of illumination

Microalgae are considered as sunlight-driven cell factories that by photosynthesis reaction convert carbon dioxide to potential biomass. For that reason the light plays principle role during algae cultivation process and is considered as the most important parameter.

The optimization was carried out by changing the wattage of the light bulbs and varying the distance of the light bulbs to the photobioreactors which resulted in variance of illumination levels to the microalgae culture. Based on previous experiments the optimization of irradiation level was carried out at temperature 45ºC, pH = 7.5 and salinity level 2.5% vol.

The experiments revealed that irradiation level of 500 µE/m^{2}sek gives the optimal level of microalgae in respect to growth rate (Figure 25.) However, based on private conversation with Blue Lagoon senior scientist, Sigurbjörn Einarsson, when the costs of electricity is considered during the optimization the optimal irradiation level is estimated at level 200 µE/m^{2}sek.

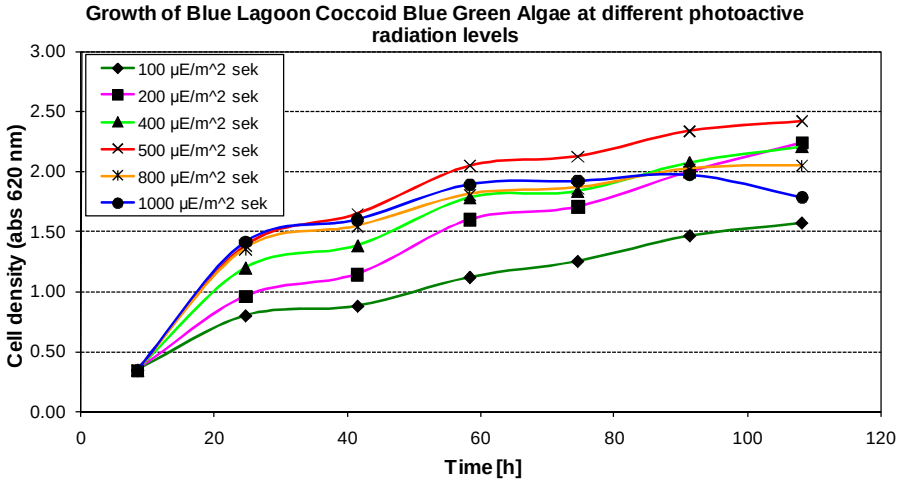


Figure 25. Growth characteristics dependent on irradiance level.

The required irradiation level is provided by placing two 400 W halogen lamps adjacent to the reactors. The heat needed to maintain the desired temperature was provided by heating element which placed in the water bath which then heats up the reactors by convection.

In summary, it can be concluded that the microalgae species that is being cultivated (Cyanobacteria/ blue-green algae) has reached the optimum growth with the following conditions:

- Salinity concentration of 2.5 % vol.
- Temperature of 45 deg-C
- Photoactive radiation of 500 µE/m^{2}sek (200 µE/m^{2}sek when electrical cost is taken into account)

pH of 7.5

2.4.2 Utilization of geothermal power plant's CO_2

The Blue Lagoon Company in partnership with HS-Orka power plant of Svartsengi has initiated a project to show the feasibility of utilizing the flue gas (non condensable gasses) from the geothermal power to feed the microalgae which so far has been cultivated by using commercial carbon dioxide gas. The commercial added value for HS-orka would be the carbon credits achieved by reducing the carbon emission, and for the Blue Lagoon Company this partnership could lower the carbon emission related to the production of Blue Lagoon products, not to mention the cost reduction of purchasing the commercial pure carbon dioxide.

The effect of supplying geothermal carbon dioxide was investigated on two types of algae: Blue Lagoon's blue green algae and diatoms algae. The first task was to collect the CO_2 into a gas container tank after initial preparation process. The flue gas, which is released as non-condensable gas from the condenser, contains approximately 2% vol of H_2S.

The gas collection set up is relatively simple as shown in Figure 26 below. A metal pipe is connected to power plant exhaust gas line. The pipe is then passing through a condenser in a helical form, which is simply a bucket of cold water. The condensed gasses/steam is then separated in a separator tank, before it is connected to a compressor and a gas container. The H_2S content and other toxic gasses are monitored during these steps.

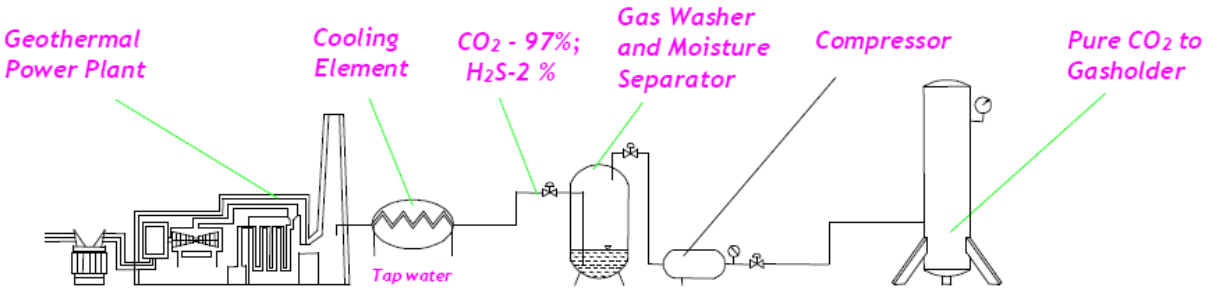


Figure 26. General setup for CO_2 collection process.

After the gas collection stage, the volume of the condensed water from crude gas was measured. In order to fill up the 200 liters gas tank to 8 bars, about 1.5 liters of water was collected. The collected water was analyzed and its measured pH was 4.5. This value, however, does not give direct information about H_2S content, because the amount of dissolved carbon dioxide was unknown and CO_2 also decreases the pH level. Two chemical sampling analyses (with Drager-Tubes®) were also carried out. First result showed that the water contained about 6 ppm of hydrogen sulfide, but second probe at the same conditions gave 2 ppm level. The level of H_2S content has dramatically dropped from 2% to 2 ppm which is suspected of some chemical reactions of the sulfuric gas to the inner material of the gas tank. However, the specific analysis of this occurrence will not be discussed further in this report.

The experiment was started by preparing the initial suspension for microalgae growing media. The small reactors of 10 liters in volume were filled with water and added with several chemicals as listed in Table 1 below.

Chemical species	*Amount, g/l*
NaCL	*27.98*
$MgCl_2 \cdot 6H_2O$	*5.20*
$MgSO_4 \cdot 7H_2O$	*7.12*
$CaCl_2 \cdot 2H_2O$	*1.54*
KCl	*0.78*
$NaHCO_3$	*0.20*
$NaSiO_3 \cdot 9H_2O$	*0.30*
HEPES	*1.19*

Table 1. Chemical species of artificial sea water [McLachlan, 1964].

Prior to use, the reactor bottles were sterilized by keeping the bottles at 120 ºC for 25 minutes. The setup, as shown in Figure 27 and 28 below, is mainly consist of four 10 liters-reactors, in which each of them has its own pH probe and feeder for air mixture with CO_2.

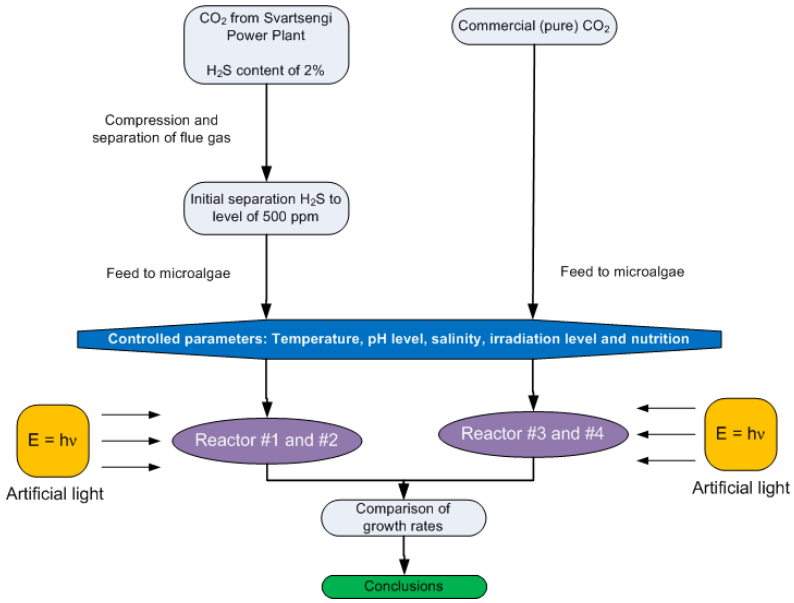


Figure 27. Schematic flow chart of geothermal biomitigation experiment by utilizing blue green microalgae.

Figure 28. Laboratory scale bioreactor used in the experiment.

Two types of gases were used for the algae growth: a gas from the geothermal power plant and a pure gas (commercial) as a reference. The algae were cultivated in laboratory-scale reactor (Figure 29) with total volume of 10 liters. The cell density was monitored two times per day in three ways: a) spectrophotometer, b) dry weight and c) turbidity.

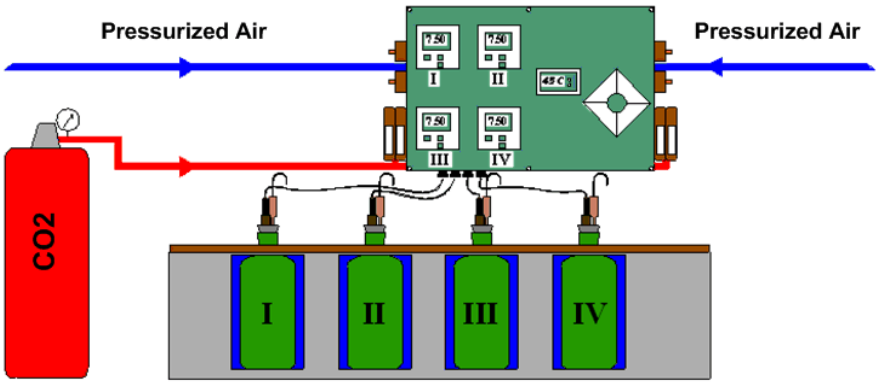


Figure 29. *Diagram of small scale reactor with total volume 10 liters.*

Spectrophotometer

First measurement was carried out in spectrophotometer, where the absorbance with light of wavelength 620 nm was used. This method is based on light absorption law (Beer–Lambert law), where some components absorb only selected wavelength of light spectra wave and the absorbance value of pick gives an equivalent of cell density. Absorbance transmissivity is considered following Figure 30 and Equation 4

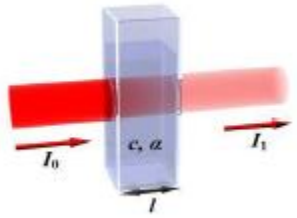


Figure 30. Diagram of Beer–Lambert absorption of a beam of light as it travels through a cuvette of width ℓ (Crouch).

The transmission (or transmissivity) is expressed in terms of an absorbance which for liquids is defined as

$$A = -\log_{10} \frac{I}{I_o} \qquad \text{(Eq. 4)}$$

where I_0 and I are the intensity (or power) of the incident light and the transmitted light, respectively.

Dry weight

A volume of 20 ml of suspension was filtered through pre-combusted (550ºC, 2h) and pre-weighted glass fiber filter. Later the filter with biomass was dried at 105ºC and reweighted. As shown in Figure 31, absorbance transmissivity and dry weight has relatively linear correlation which indicated the increase of dry weight as its absorption transmissivity is increasing.

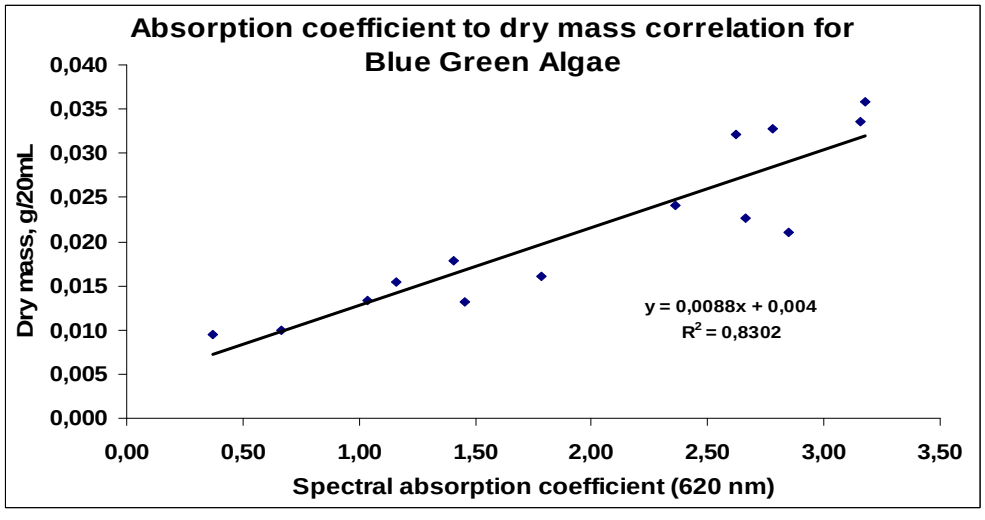
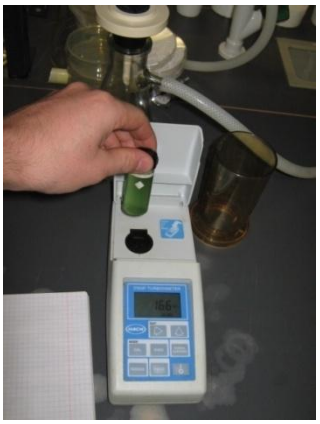


Figure 31. Correlation between absorbance transmissivity and dry weight of biomass.

Turbidity

The cell density was measured in portable turbidimeter shown in Figure 32 below. This method operates on the nephelometric principle of turbidity measurement [Collado-Fernández, 2000].



a)

b)

Figure 32.a) Turbidimeter during the experiment. b) Two solutions with different density which resulted in different turbidity readings.

Results shown in Figures 31 and 33 below strongly imply that all the three methods (spectrophotometer, dry weight and turbidity) are valid for monitoring the growth rate of microalgae.

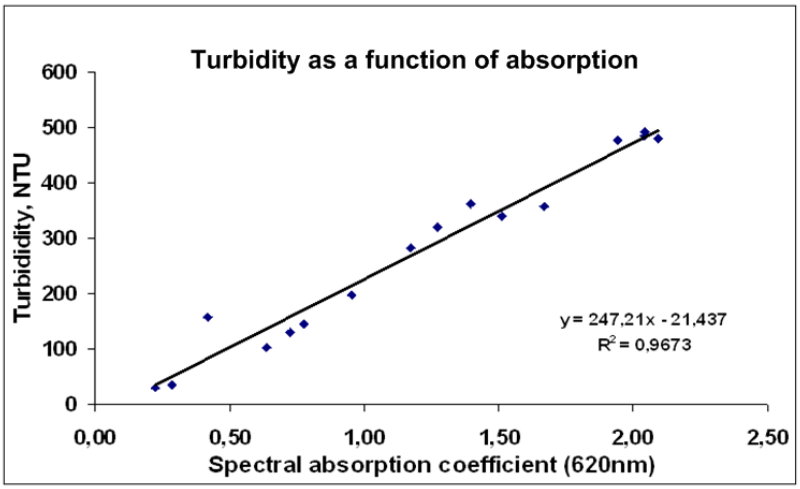


Figure 33. Absorbance and turbidity correlation for blue green algae.

2.5 Summary of Part II

The growth of blue-green and diatom microalgae were monitored for several days and samples were taken every day to measure the cell density in terms of its absorbance level.
The results as presented on Figure 34 and 35 below imply that feeding the microalgae with carbon dioxide from geo-thermal power plant does not lower its growth efficiency, as compared with pure (commercial) carbon dioxide gas. For the blue-green algae the average difference was only 2.41%, which is insignificant. For diatom algae the influence of geothermal gas is more visible, where the average different was 7.48%.

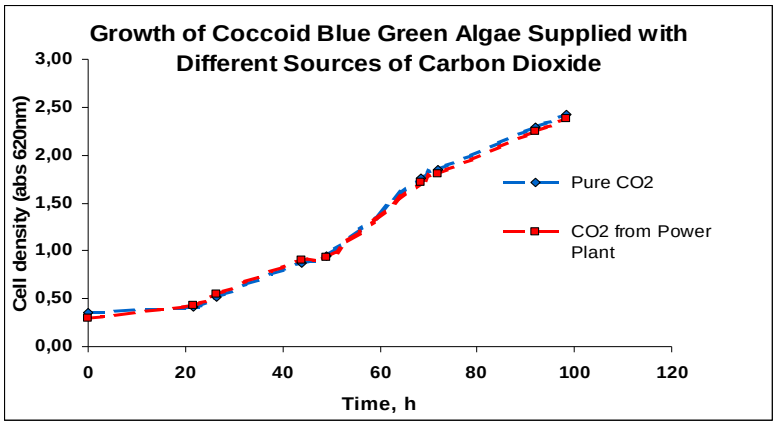


Figure 34. Growth comparison curve for Blue Green Algae.

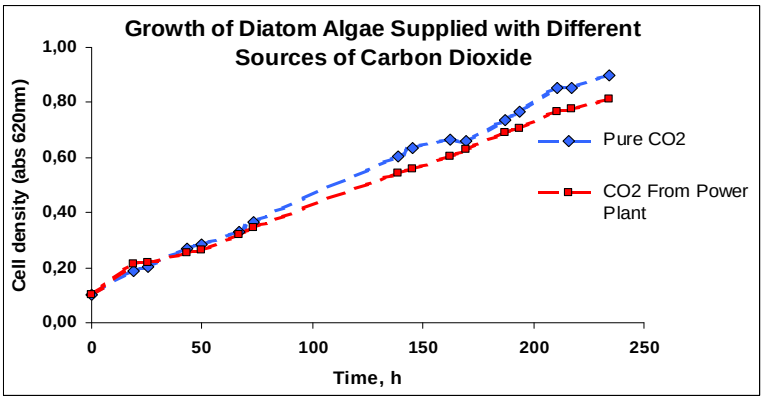


Figure 35. Growth comparison curve for Diatom Algae.

CO_2 fixation rate

From an economical point of view, it is crucial to identify the efficiency of microalgae CO_2 feeding process.

Microalga	CO_2%	T °C	P g l^{-1} per day	P_{CO2} g l^{-1} per day	Reference	Note
Chlorococcum littorale	40	30	N/A	1.0	Iwasaki et al. 1998; Murakami and Ikenouchi 1997	
Chlorella kessleri	18	30	0.087	0.163[a]	de Morais and Costa 2007b	
Chlorella sp. UK001	15	35	N/A	>1	Murakami and Ikenouchi 1997	
Chlorella vulgaris	15		N/A	0.624	Yun et al. 1997	Artificial wastewater
Chlorella vulgaris	air	25	0.040	0.075[a]	Scragg et al. 2002	Watanabe's medium
Chlorella vulgaris	air	25	0.024	0.045[a]	Scragg et al. 2002	Low-N medium
Chlorella sp.	40	42	N/A	1.0	Sakai et al. 1995	
Dunaliella	3	27	0.17	0.313[a]	Kishimoto et al. 1994	High salinity, ß-carotene
Haematococcus pluvialis	16–34	20	0.076	0.143	Huntley and Redalje 2007	Commercial scale, outdoor
Scenedesmus obliquus	Air	-	0.009	0.016	Gomez-Villa et al. 2005	Wastewater, outdoor, winter
Scenedesmus obliquus	Air	-	0.016	0.031	Gomez-Villa et al. 2005	Wastewater, outdoor, summer
Botryococcus braunii	–	25–30	1.1	>1.0	Murakami and Ikenouchi 1997	Accumulating hydrocarbon
Scenedesmus obliquus	18	30	0.14	0.26	de Morais and Costa 2007a	
[b]*Spirulina* sp.	12	30	0.22	0.413[a]	de Morais and Costa 2007a	

En-dash not specified or not controlled

[a] Calculated from the biomass productivity according to equation, CO_2 fixation rate (P_{co2})=1.88×biomass productivity (P), which is derived from the typical molecular formula of microalgal biomass, $CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$ (Chisti 2007).

[b] All species except *Spirulina* sp., which is a prokaryotic cyanobacteria (*Cyanophyceae*) species, are eukaryotic green algae (*Chlorophyta*) species (Bold and Wynne 1985).

Table 2. Some microalgae strains studied for CO_2 bio-mitigation [Wang et.al., 2008].

Carbon dioxide fixed through photosynthesis is converted to different organic cell components including carbohy-

drates, lipids, proteins, and nucleic acids [Spolaore et. al., 2006]. Although the cell carbon content varies with microalgae strains, media, and cultivation conditions, it changes in a relatively small range, and the law of material conservation allows us to calculate CO_2 fixation rate from biomass productivity at given cell carbon content. In Table 2, such calculations were conducted using a reported biomass molecular formula, $CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$[Chisty, 2007], when direct data on CO_2 fixation rate was not available, based on the assumption that CO_2 fixed in the form of extracellular products was negligible. The detailed calculation is presented as follow:

Biomass molecular formula:

$CO_{0,48}H_{1,83}N_{0,11}P_{0,01}$

$M_{\text{biomass}} = 12 + 0.48 \times 16 + 1.83 \times 1 + 0.11 \times 14 + 0.01 \times 15 = 23.2$ gr/mol

$$4CO_2 + \text{nutrients} + H_2O + h\nu\ (\text{light}) \rightarrow 4CO_{0.48}H_{1.83}N_{0.11}P_{0.01} + 3\frac{1}{2}O_2$$

$$M_{CO2} = 44\ gr/mol, \quad M_{biomass} = 23.2\ gr/mol, \quad F_{CO2} \Rightarrow \frac{44}{23.2} = 1.896 \qquad \text{(Eq. 5)}$$

The CO_2 fixation rate is given as $F_{CO2} = 1.89 \times$ biomass productivity
The biomass productivity is simply a portion of microalgae continuous production process as shown in Figure 36 below. Some of microalgae's growth ingredients such as nutrients, carbon dioxide and salt are supplied continuously into the process to keep the balance production while keeping the microalgae to its optimum conditions.

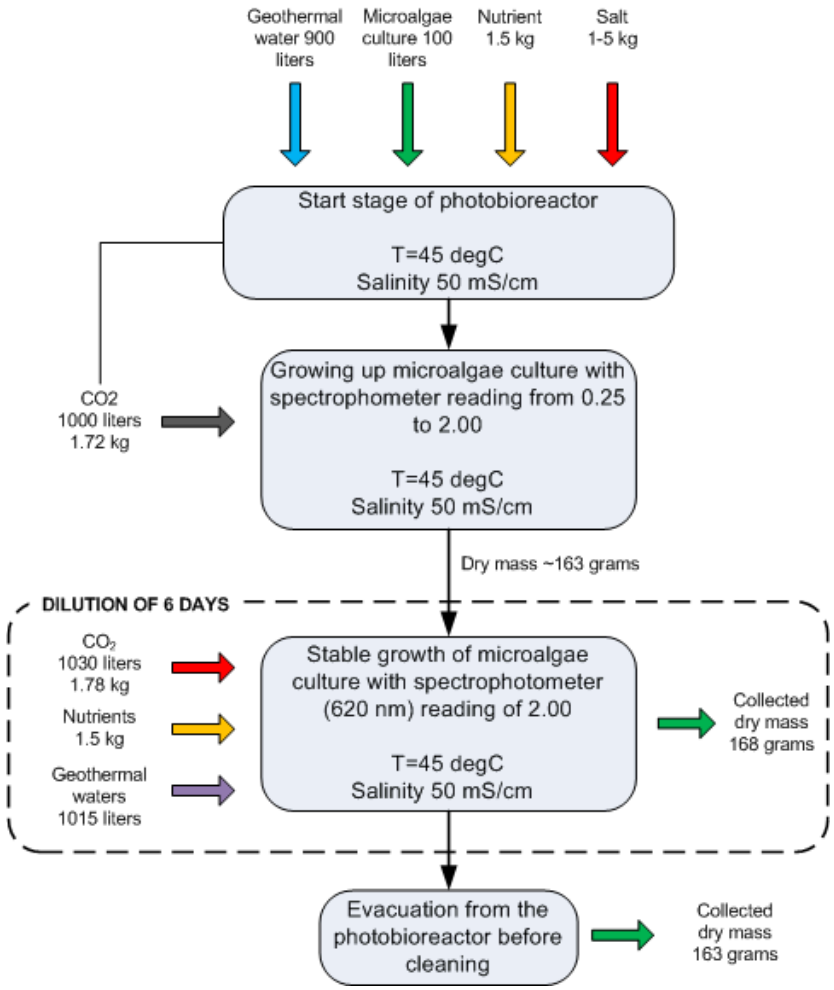


Figure 36. Microalgae continuous production process.

Referring to the biomass molecular formula, carbon dioxide flow rate measurement and continuous microalgae biomass dry weight monitoring, the approximate carbon dioxide fixation rate for Blue Lagoon blue-green microalgae is approximately **18%** vol. (only 18% vol. of the supplied CO_2 is absorbed by microalgae, while the remaining CO_2 gas

is has escaped from the system) as shown in Table 3 below.

Time hr	Time day	Dry biomass g	Dry biomass g (total)	CO2 Ltrs	CO2 g (total)	CO2 consumption ratio	Efficiency %	Cell density abs (620)
0	0,00			1000	1,72			2,259
19	0,79			1158	2,00			2,273
26	1,06			1220	2,10			2,269
44	1,83			1312	2,26			2,253
51	2,13			1312	2,26			2,226
67	2,79	72	235	1312	2,26	10	19	2,079
139	5,79			1867	3,22			1,045
142	5,92			1926	3,32			
150	6,23	94	329	2030	3,50	11	18	

Table 3. The calculated CO_2 fixation rate for blue-green microalgae.

RESULTS AND CONCLUSION

Part II of the report analyzes the feasibility of CO_2 fixation by using fast-growing blue green microalgae species which proved to provide a very promising alternative for mitigation of CO_2, the most prominent greenhouse gas. The primary merit of this strategy lays in the fact that, via the cultivation of microalgae, CO_2 mitigation and valuable biomass production could be combined in an economically feasible and environmentally sustainable manner. The feasibility of this strategy was also enhanced by fixing CO_2 from industrial exhaust gases such as geothermal power plant's flue gases.

The results from the experiments imply that feeding the microalgae with carbon dioxide from geothermal power plant does not lower its growth efficiency, as compared with pure (commercial) carbon dioxide gas. The average difference from the two sources of CO_2 gasses for the blue-green and diatom algae was only 2.41% and 7.48%, respectively.

The experiments also show that the approximate carbon dioxide fixation rate for Blue Lagoon blue-green microalgae is approximately **18% vol.** (only 18% vol. of the supplied CO_2 is absorbed by microalgae, while the remaining CO_2 gas is has escaped from the system).

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