Biodiesel production from microalgal culture

Master Thesis

Diplomarbeit zur Erlangung des akademischen Grades einer Diplom-Ingenieurin der Studienrichtung Biotechnologie an der Technischen Universität Graz

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Kurzfassung

Mikroalgen besitzen großes Potential für die Produktion von Biotreibstoffen. Die Algenbiomasse bindet zudem CO_2 und trägt zur Reduktion von Treibhausgasemissionen bei. In der vorliegenden Arbeit wurden die Auswirkungen unterschiedlicher CO_2 Konzentrationen und pH Werte auf das Wachstum und die biochemische Komposition der Mikroalge *Stichococcus bacillaris* 158/11 untersucht.

Zwei unterschiedliche Photobioreaktorkonzepte kamen hierbei zum Einsatz. Vertikale Blasensäulen wurden verwendet, um die Auswirkungen unterschiedlicher CO_2 Konzentrationen zu untersuchen: ~ 0.04 % (Luft), 5 %, 10 % und 15 % Volumenprozent CO_2. Geneigte Vierkant-Blasensäulen wurden eingesetzt, um die Auswirkungen von drei unterschiedlichen pH Werten zu untersuchen: pH 3, pH 7 und pH 9. Die geneigten Vierkant-Blasensäulen wurden mit 95 % Luft und 5 % CO_2 begast. *Stichococcus bacillaris* 158/11 erwies sich als tolerant gegenüber allen CO_2 Konzentrationen und pH Werten und konnte unter allen Prozessbedingungen wachsen.

Die höchste Fettsäureproduktivität zeigten Kulturen, die in den geneigten Vierkant-Blasensäulen bei pH 7 und mit 5 % zusätzlicher CO_2 Begasung kultiviert wurden (79.8 mg/L/d) und Kulturen, die in den vertikalen Blasensäulen bei 10 % zusätzlichem CO_2 kultiviert wurden (47.0 mg/L/d). Kulturen, die zusätzlich mit CO_2 (in allen Konzentrationen) begast wurden gingen schneller in exponentielles Wachstum über. Sie erreichten eine hohe Biomassekonzentration (~2.6 g/L), im Vergleich zu den mit Luft begasten Kulturen. Zusätzliches CO_2 hat außerdem Auswirkungen auf den ChlA Gehalt der Zellen (2.50 mg/g in den mit Luft begasten Kulturen, 0.30 mg/g in den mit zusätzlichem CO_2 begasten Kulturen).

Diese Auswirkungen könnten auf eine adaptive Reorganisation des Photosyntheseapparats bei CO_2 Stress hindeuten. Die Tests zeigten, dass eine erhöhte CO_2 Konzentration die Ausbeute an fettsäurereicher Algenbiomasse verbessert. Kulturen, die in Medium mit pH 9.0 kultiviert wurden hatten die niedrigste Fettsäureproduktivität (28.7 mg/L/d), außerdem reduzierte sich die Wachstumsrate bei pH 9 und pH 3 mit der Zeit.

Es scheint, als hätten auch die unterschiedlichen Reaktorkonzepte einen Einfluss auf das Wachstumsverhalten der Zellen. Speziell das Konzept des geneigten Vierkant-Blasensäulenreaktors zeigte vielversprechende Resultate.

Abstract

Biofuel production by microalgae has been considered a promising process both to produce liquid fuels and to capture and storage CO_2. The microalgal biomass fixes a large amount of CO_2 and strongly contributes to the reduction of greenhouse gas emissions. The effects of CO_2 concentrations and pH values on the growth and biochemical composition of the microalgae *Stichococcus bacillaris* 158/11 were investigated.

Two photobioreactor configurations were set-up and operated. A) Vertical bubble column photobioreactors were used to test effects of CO_2 concentration in the gas phase on process performances (~ 0.04 % (air), 5 %, 10 %, and 15 % volumetric percentage of CO_2 were investigated). B) Inclined square bubble column photobioractors, sparged with 5 % additional CO_2, were adopted to test three different pH values (3, 7, and 9). *Stichococcus bacillaris* 158/11 was able to grow in all investigated conditions.

The investigated strain resulted to be CO_2 and pH tolerant. The highest lipid productivity was at pH 7 and at both 5 % CO_2 (79.8 mg/L/d), and 10 % CO_2 (47.0 mg/L/d). The exponential phase of all cultures sparged with CO_2-added air (all concentrations) was faster than that sparged with fresh air. Moreover, these cultures achieved a high biomass concentration (~2.6 g/L) faster than the air sparged cultures. The additional CO_2 affected both the cell growth and the ChlA content. In particular, the ChlA content decreased with CO_2 concentration (2.50 mg/g in the air sparged cultures vs. 0.30 mg/g in the CO_2-added air).

The changes in both the amount of lipids and the content of ChlA indicate an adaptive reorganization of the photosynthetic apparatus at CO_2 stress. The tests showed that high CO_2 concentration improves the yield of algal biomass enriched in lipids. A significant decrease in the contents of lipids was measured during tests carried out at pH 9 (28.7 mg/L/d), regardless the microalgae tolerance to a broad pH spectrum. The growth rate of cultures at pH 3 and at pH 9 reduced with the cultivation time.

The reactor configuration seemed to affect the growth of microalgal cells. The performances assessed for cultures carried out in the inclined bubble column resulted very promising.

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1. Introduction

Continued use of petroleum derived fuels is nowadays widely recognized as unsustainable. Supplies are going to be depleted and furthermore there is a contribution of these fuels to the accumulation of CO_2 in the environment (Chisti, 2007). An intensive search for alternative energy sources is now under way, driven by different concerns including volatile oil prices, increased global demand, dependency on imports from politically unstable regions, and recognition of the harmful effects on the environment. The world's fossil reserves are limited and furthermore they are unevenly distributed in the world. The development of alternative energy sources is one of the most important priorities nowadays.

McKendry (2001) and Anderson and Fergusson (2006) showed that one of the most promising renewable energy sources is biomass. The use of biomass as energy source is housed in several areas including the transport sector (as a biofuel), the electricity generation and heat production. Two main goals define the transport and mobility field: one aim is the substitution of fossil fuels; another aim is the reduction of CO_2 emissions. According to Schenk et al. (2008) the climate change has been mainly attributed to the raising of CO_2 level in the atmosphere. The critic threshold of 450 ppm CO_2 has been overcome 10 years before the predicted instant. The reduction of the emissions is an appropriate strategy to face the problem.

Beside the substitution of fossil fuels, biomass is able to decrease the atmospheric CO_2 level by capturing. However, several studies (Blanco-Canqui and Lal, 2007; Field et al., 2007; Searchinger et al., 2008; Naik et al., 2010) show that the use of various plants for biofuel production has economic and environmental drawbacks. In contrast, second generation biofuel systems (such as microalgal biofuel systems) have the potential to overcome many of these drawbacks (Schenk et al., 2008). CO_2 addition even strongly stimulate the growth rate of many microalgae strains (Sergeenko et al., 2000; Chiu et al., 2009; Jiang et al., 2011; Kliphuis et al., 2011; Tang et al., 2011). Therefore the biofixation of CO_2 has been frequently proposed as a method to reduce the greenhouse effect utilizing a sustainable way. Following factors contribute to an increasing interest for microalgae cultivations (Schenk et al., 2008):

- the biomass growth is driven by photosynthesis;

- the lipid-enriched microalgae may be a potential source of biofuels;
- microalgal lipid content and composition are highly sensitive to both growth conditions and types of stress changes.

Microalgae couple CO_2 sequestration with bio-oil production. Moreover, they require less soil and water than crop cultures. The production of biodiesel using microalgae does not compromise production of food, fodder and other products derived from crops (Schenk et al., 2008). Considering different oil sources, Table 1 describes the oil yield, the land area needed, and the percentage of already existing cropping area in the US.

Table 1: Comparison of some sources of biodiesel

Crop	Oil yield (L/ha)	Land area needed (M ha)[a]	Percent of existing US cropping area[a]
Corn	172	1.540	846
Soybean	446	594	326
Canola	1.190	223	122
Coconut	2.689	99	54
Microalgae[b]	136.900	2	1.1
Microalgae[c]	58.700	4.5	2.5

[a] For meeting 50 % of all transport fuel needs of the United States.

[b] 70 % oil (by wt) in biomass.

[c] 30 % oil (by wt) in biomass.

Source: Chisti, 2007

Microalgae are unicellular organisms. They can have variable types of cell organisation: unicellular, colonial and filamentous (Tomaselli, 2004). There are two different groups with extraordinary potential for a variety of applications: heterotrophic microalgae and autotrophic microalgae. Spolaore et al. (2006) describes different application areas of microalgae. They are cultivated in large scale for human and animal nutrition, cosmetics, and they are a source of highly valuable molecules, such as polyunsaturated fatty acids and pigments.

Photosynthetic organisms, including plants, algae, and some photosynthetic bacteria, efficiently utilize energy from sun to convert water and CO_2 from air into biomass

(Campbell and Reece, 2006). The cellular structure of photosynthetic algae consists of (Masojídek et al., 2004):

- a cell wall which gives mechanical strength;
- the plasmatic membrane;
- a cytosolic compartment, which contains the cell organelles such as plastids, chloroplasts, the vacuole, and lipid globules;
- the nucleus.

The photosynthetic apparatus (inside the chloroplasts) is localized in the thylakoid membrane. The pigments present in the thylakoid membranes are responsible for the absorption of sunlight necessary for the photosynthetic process. Such pigments are chlorophyll (green), carotenoids (yellow-orange), xanthophylls (yellow), and phycobilins (red). The oxygenic photosynthetic process uses light energy to convert carbon dioxide and water into carbohydrates and oxygen (Masojídek et al., 2004).

$$6\ CO_2 + 6\ H_2O + \text{energy (sunlight)} \rightarrow C_6H_{12}O_6 + 6\ O_2$$

Photosynthesis is a two-step process: the light reactions and the Calvin cycle. The light reactions take place only in the presence of pigments and are characterized by the photosystems II and I, and the enzyme ATP synthase. Pigments absorb light energy (photons) and convert it into chemical energy in form of ATP and NADPH. Furthermore, O_2 is produced by photolysis and released into the atmosphere. The Calvin cycle uses the energy produced during light reactions (ATP and NADPH) to convert CO_2 captured from the atmosphere into glucose (Masojídek et al., 2004). Figure 1 illustrates the main steps of photosynthesis.

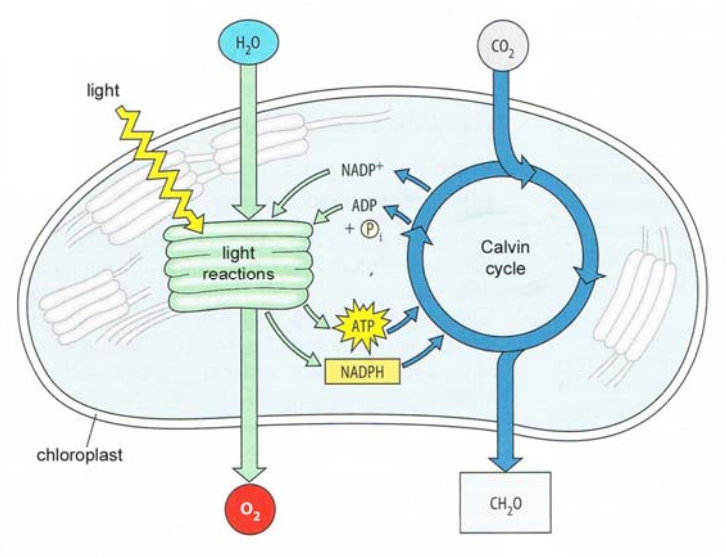


Figure 1: Interaction of the light reactions and Calvin cycle during photosynthesis.
Adapted from Campbell and Reece, 2006

Microalgal biomass growth is driven by photosynthesis. Therefore, an additional feed of organic compounds is not necessary. Microalgae have only minimal requirements to the medium (Grobnelaar, 2004). In addition, many microalgae have high lipid contents as energy storage. Microalgae, like higher plants, produce storage lipids in form of triacyglycerols (TAGs). TAGs could be used to produce fatty acid methyl esters (FAMEs) which can be used as a substitute for fossil-derived diesel fuel (Chisti, 2007; Posten and Schaub, 2009; Francisco et al., 2010). This fuel, known as biodiesel, can be synthesized from TAGs through a simple transesterification reaction in the presence of methanol in acidic or basic conditions (Rashid and Anwar, 2008a, 2008b). Chisti (2007) has reported that biodiesel production rate from microalgae cultures may be 1- up to 3-fold larger than that from oil crops. Microalgae grow fast and many are exceedingly rich in oil (50–80 $\%_W$). Compared with crop plants which are usually harvested once or twice a year, microalgae have a shorter harvesting cycle. Moreover, the microalgal biomass fixes a large amount of carbon dioxide (1.83 kg of CO_2 per kg of dry microalgae) and strongly contributes to the reduction of greenhouse gas emissions.

The growth rate and maximum biomass yield of microalgal strains are influenced by culture parameters (light, temperature, and pH) and nutritional status (CO_2, nitrogen, and phosphate concentrations). In addition to carbon provided in form of carbonates and carbon dioxide, nitrogen is one of the most important elements of an algal culture medium (Urabe and Sterner, 1996; Chiu et al., 2009; Figueroa et al., 2009). The nitrogen content in

biomass ranges from 1 % to more than 10 % of the total dry weight (Grobnelaar, 2004). The nitrogen is provided in concentrations below those required by the cell only for specific cases. Numerous studies (Khozin-Goldberg et al., 2002; Zhekisheva et al., 2002; Li et al., 2008; Solovchenko et al., 2008) show that nitrogen deficiency furthers lipogenesis and lipid accumulation. In such a condition the triacylglycerols are predominant compared to other lipids.

Nutrition access and light supply are important factors considering the growth of microalgae, and strongly depend on the applied cultivation method. Flickinger and Drew (1999) classified two methods: open and closed systems. According to Chisti (2007) only two methods of large-scale production of microalgae are practicable, raceway ponds (open system) and tubular photobioreactors (closed system).

Several types of open culture systems have been proposed. Open culture systems are the simplest method of algal cultivation. They offer advantages in construction cost and ease of operation. Open culture systems require a large surface area and a shallow depth to optimize light utilization. A mixing system distributes the cells and prevents their sedimentation. Contamination by different algal species and other organisms is a grave problem in open culture systems as well as the CO_2 transfer into the tank. Bubbling CO_2 gas in a trap placed under a paddle wheel optimizes the CO_2 transfer (Flickinger and Drew, 1999). Figure 2 shows raceway ponds agitated with paddle wheels.

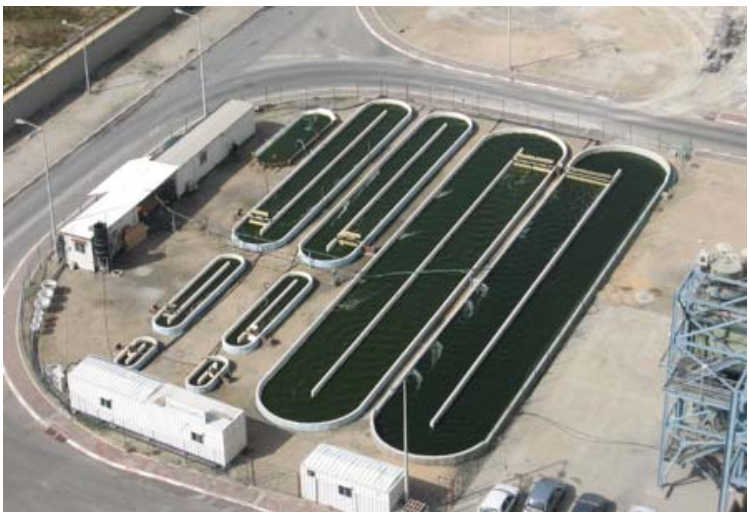


Figure 2: Open pond system in California

(http://algaeforbiofuels.com/algae-cultivation-systems-open-ponds-vs-pbr/ (03.11.2011))

Closed systems have been expected to overcome open culture system disadvantages. Photobioreactors are closed systems for the cultivation of photosynthetic organisms. In particular they allow to measure and to control operating conditions. Their advantages are:

- monoalgal cultures due to protection from contamination;
- avoidance of water loss and subsequent increase of salinity in the culture medium;
- higher productivity with increased cell densities, reducing harvesting costs;
- tuning culture conditions.

Several criteria need to be considered to maintain an efficient and reliable large-scale culture system: efficient light supply, effectual mixing (turbulence), low-shear environment, temperature control, and efficient gas transfer. The yield of algal biomass strongly depends on the light supply for each cell. The surface-to-volume ratio is an important factor for an efficient light utilization (Flickinger and Drew, 1999). Closed photobioreactors have been extensively studied for the reduction of greenhouse gases during the nineties in Japan (Maeda et al., 1995; Usui and Ikenouchi, 1997) and recently also in the United States (Bayless et al., 2001; Olaizola, 2003).

Figure 3 illustrates a typical photobioreactor system characterized by parallel horizontal tubes. Usually photobioreactors are tubular and consist of a set of transparent tubes ("array"). The tubes generally have a diameter less than 0.1 meter to allow a sufficient capture of light and ensure photosynthesis. Accumulation of biomass in the tubes is prevented by high turbulence. Either a mechanical pump (Figure 3), or an airlift pump is adopted (Chisti, 2007). Molina et al. (2000, 2001) successfully provided the turbulence by airlift.

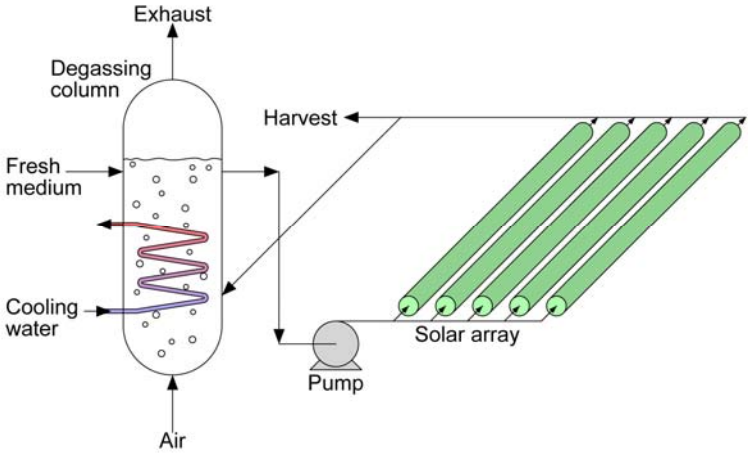


Figure 3: A tubular photobioreactor with parallel tubes.

Adapted from Chisti, 2007

A major problem in closed systems is photo-oxidative cell damage caused by accumulation of dissolved oxygen produced by photosynthesis during the light period. In open systems, evolved oxygen escapes to the atmosphere. Degasser systems are sometimes required in closed systems (Molina et al. 2001). Another relevant parameter in photobioreactor design is the increase of pH during photosynthesis due to CO_2 consumption. CO_2 injection prevents CO_2 limitation and pH increase (Camacho et al., 1999; Molina et al., 1999). Photobioreactors require a temperature control system. Water sprayed on the tubes cools the system through evaporation. This is a satisfactory strategy to maintain optimal temperatures (Tredici, 1999).

Different microalgae belonging to Cyanophyta, Dinophyta and Chlorophyta have been used for mass culture in closed systems and open ponds. Chlorophyta showed high CO_2 fixation rates. Members of this taxon could be efficiently employed for CO_2 capture and sequestration (Chisti, 2007). A recent study (Olivieri et al., 2011) evaluated the best performances in terms of growth rate, resistance to stress, and lipid production of different species and strains of the green alga *Stichococcus*. Strains belonging to the species *Stichococcus bacillaris* were grown under batch, fed-batch and semi-continuous conditions in a lab scale bubble column photobioreactor.

Stichococcus is a genus of green algae (Chlorophyta) characterized by a simple morphology, ranging from single unmotile cells to uniseriate, unbranched filaments (Pollio et al., 1997). The species belonging to this genus are widely distributed over different continents, at latitudes extending from polar and subpolar regions to the Equatorial belt (Broady, 1989). Some species such as *Stichococcus bacillaris* are tolerant to large variations of temperature, salinity, and pH, and are characterized by short life cycles (Pollio et al., 1997). Olivieri et al. (2011) could show promising results utilizing the strain *Stichococcus bacillaris* 158/11 of the ACUF collection (Algal Collection at University ”Federico II”, Naples). A biomass production of about 60 mg/L/d and a lipid productivity of 14 mg/L/d were obtained under semi-continuous conditions. Due to its lipid content (about 33 % of dry weight) and fatty acid distribution *Stichococcus bacillaris* 158/11 – along with other species of this genus – is a potential candidate for commercial-scale cultivations devoted to biofuel production.

Aim of the thesis

Several protocols for the cultivation of unicellular algae can be adopted depending on the cultivation strategy (batch, fed-batch, semi-continuous), the photobioreactor configuration (open systems, closed systems), and the chemical and physical parameters (such as light intensity, temperature, pH and composition of culture medium). The object is to establish cultivation conditions to obtain maximum yield with the best cost / benefit ratio.

Several studies (Sergeenko et al., 2000; Chiu et al., 2009; Jiang et al., 2011; Kliphuis et al., 2011; Olivieri et al., 2011; Tang et al., 2011) showed the prosperous production of lipids through microalgal strains. The successful cultivation and high lipid content of *Stichococcus bacillaris* 158/11 (Olivieri et al., 2011) was encouraging to pursue further studies.

The aim of the thesis is to optimize systems and technologies for the biodiesel production through *Stichococcus bacillaris* 158/11 (Naegeli genus). The work focused on identifying the optimal conditions in terms of cultivation medium and photobioreactor design for biomass growth and maximized lipid content. Tests operated with CO_2-enriched-air and with buffered medium were performed to optimize the O_2/CO_2 ratio in the gas phase and the pH level in the culture medium.

The steps of the project were:

1. Set-up of photobioreactors on laboratory scale for batch, fed-batch and semi-continuous operation;
2. Tests of algal growth at different conditions of CO_2 concentration in the gas phase and pH;
3. Lipid extraction and biodiesel characterization.

2. Materials and Methods

2.1 Organism and Medium

Stichococcus bacillaris 158/11 was from the ACUF collection of the Department of Biological Science at the University of Studies of Napoli "Federico II" (http://www.biologiavegetale.unina.it/acuf.html).

This alga has only minimal requirements to the medium for the growth. There is the need of inorganic ions and a minimal quantity of organic compounds, such as vitamins. Carbon, nitrogen and phosphor are the most important nutrients for the autotrophic algal growth (Grobnelaar, 2004). Bold Basal medium (Nichols, 1973) is an artificial freshwater medium, which is practical for growing green algae. Bold Basal Medium (BBM) supplemented with $NaNO_3$ (40 mg/L) as nitrogen source was adopted. Lacking an organic carbon source, BBM hardly allows bacterial growth.

After preparing the medium (Table 3) it was autoclaved for 20 minutes. The final pH should be 6.8. In one experiment the pH was fixed at values 3 and 9. So it was necessary to modify the Bold Basal medium changing the buffer system (Table 2). NaOH and NH_4Cl were added to obtain a pH value of 9. Glycylglycine and HCl were added to obtain a pH value of 3.

Table 2: Applied buffer systems to obtain the requested pH

pH in the final medium	component	Add quantity below per liter of medium	Molar concentration in final medium
6.8	K_2HPO_4	0.075 g	4.31×10^{-4} M
	KH_2PO_4	0.175 g	1.29×10^{-3} M
3	HCl (30 %)	1 mL	10^{-2} M
	Glycylglycine	1.320 g	10^{-2} M
9	NaOH	0.400 g	10^{-2} M
	NH_4Cl	0.535 g	10^{-2} M

Table 3: Composition of the Bold Basal Medium

Component	1 Liter stock solution	Add quantity below per Liter of medium	Molar concentration in final medium
Major stock solutions:			
$NaNO_3$	25.00 g/L	10 mL	2.94×10^{-3} M
$CaCl_2 \cdot 2\ H_2O$	2.50 g/L	10 mL	1.70×10^{-4} M
$MgSO_4 \cdot 7\ H_2O$	7.50 g/L	10 mL	3.04×10^{-4} M
K_2HPO_4	7.50 g/L	10 mL	4.31×10^{-4} M
KH_2PO_4	17.50 g/L	10 mL	1.29×10^{-3} M
NaCl	2.50 g/L	10 mL	4.28×10^{-4} M
EDTA anhydrous	5.00 g/L	10 mL	1.71×10^{-4} M
KOH	3.10 g/L	10 mL	5.52×10^{-4} M
H_3BO_3	11.42 g/L	1 mL	1.85×10^{-4} M
Acidified Iron stock solution:		1 mL	
$FeSO_4 \cdot 7\ H_2O$	4.98 g/L		1.79×10^{-5} M
H_2SO_4	1.00 mL		
Trace metal stock solution:		1 mL	
$ZnSO_4 \cdot 7\ H_2O$	8.82 g/L		3.07×10^{-5} M
$MnCl_2 \cdot 4\ H_2O$	1.44 g/L		7.30×10^{-6} M
MoO_3	0.71 g/L		4.93×10^{-6} M
$CuSO_4 \cdot 5\ H_2O$	1.57 g/L		6.29×10^{-6} M
$Co(NO_3)_2 \cdot 6\ H_2O$	0.49 g/L		1.68×10^{-6} M

2.2 Reactor Setup

Simple bubble column photobioreactors were adopted. Two different shapes were investigated: classic vertical bubble column and inclined square bubble column (Figures 4 and 5). The photobioreactors were located at the Department of Biological Science of the University Federico II (Orto Botanico, Naples).

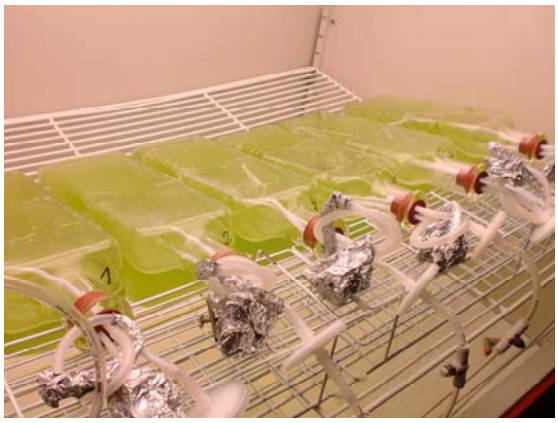


Figure 4: Inclined square bubble column photobioreactors

Figure 5: Vertical bubble column photobioreactors

2.2.1 Vertical bubble column photobioreactor

The 1 L bench scale photobioreactor had a cylindrical shape. It was made of glass (0.04 m ID, 0.80 m high) and closed at the top by a removable silicon cap. Twelve cylindrical photobioreactors were housed inside a climate chamber (M2M engineering, type: Solar neon) with a temperature set at 23±1 °C. Ten lamps (Philips Master, TL-D 90 de luxe, 36 W/940) continuously illuminated the front side of the photobioreactor. The climate chamber was equipped with a reflector to illuminate the back side of the reactor too. A schematic overview of the reactor shape and a description of the operating mode are shown in Figure 6.

Air was sparged at the bottom of the photobioreactor by means of a porous ceramic diffuser to provide the aeration and mixing of the bubble column. Different volumetric flow rates of CO_2 were fed from a tank to the photobioreactors to investigate the effect of different CO_2 levels in the gas phase. A flow meter (asa, Sesto S. Giovanni, model: E5-2600/H) was used to set and control the gas flow rate. A T-shaped valve connected and mixed the CO_2 and the air before entering the photobioreactor.

Three tubes were fixed in the silicon cap on the top of the reactor. Two of them were destined for the gas flow inlet and for the gas flow outlet. The third line was provided for the sampling operation and was closed by a clamp. The gas inlet tube was connected to a porous ceramic diffuser located at the bottom of the photobioreactor. A hydrophobic filter (0.2 µm) sterilized the gas flow inlet. Aluminium foil at the end of the off-gas tube protected the culture from contamination. The off-gas tube was knotted to keep the condensed water inside the reactors.

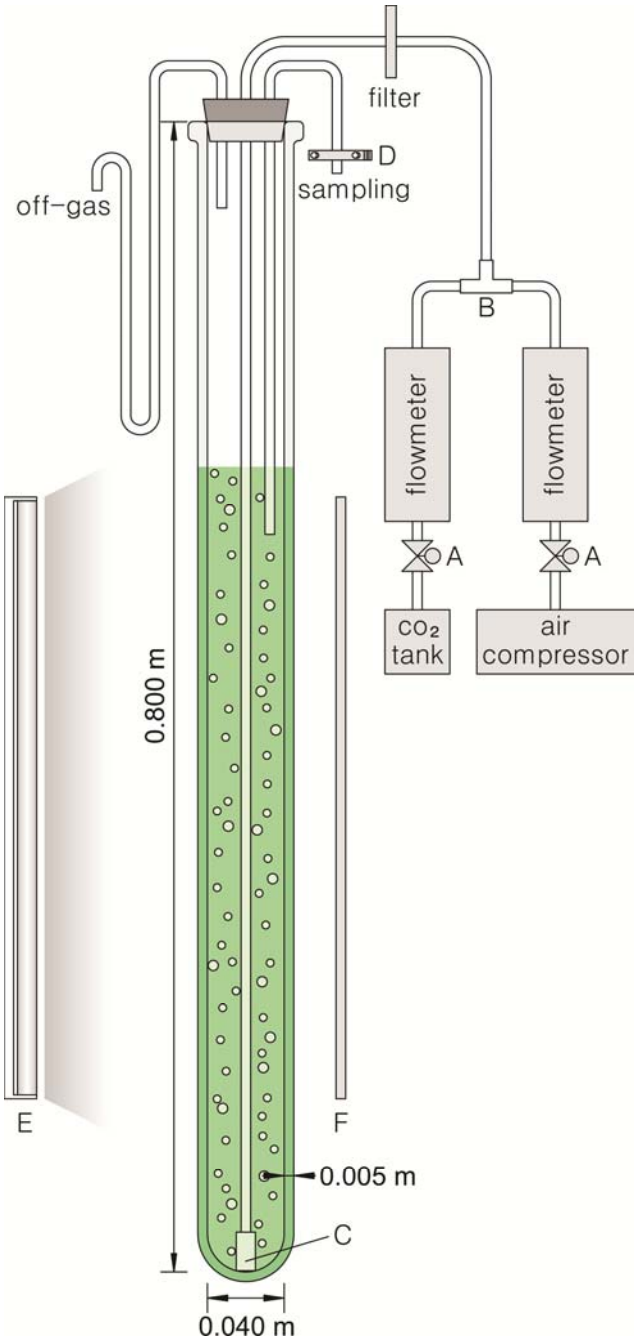


Figure 6: Schematic overview (not on scale) of the vertical bubble column photobioreactor.

A, valve, B, T-shaped valve, C, porous ceramic diffuser, D, sterile clamp, E, light source, F, reflector

The ratio and amount of CO_2 and air are regulated by flow meters and combined by a T-shaped valve. The mixture enters the photobioreactor by passing a sterile filter. It leaves the tube at the bottom through a porous ceramic diffuser. The generated bubbles lead to a sufficient aeration and mixing of the liquid volume. The excess gas leaves the photobioreactor through the off-gas tube.

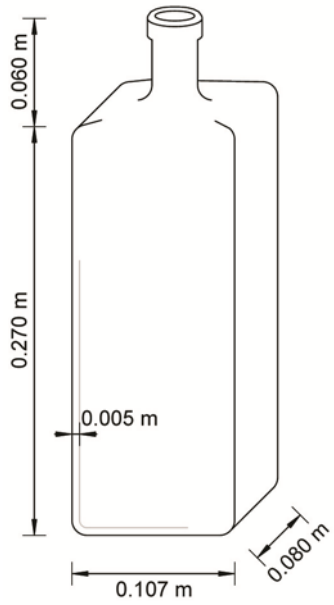
2.2.2 Inclined square bubble column photobioreactor

The 2 L bench scale inclined photobioreactor had a square shape. It was made of glass with a working volume of 1.7 L. The dimensions are shown in Figure 7 A. A removable silicon cap closed the reactor at the top. The six photobioreactors were housed in an inclined position inside a climate chamber (Heraeus Vötsch GmbH, type: HPS 500) with a temperature set at 23±1 °C. Lamps (M2M engineering) fixed on the upper part of the climate chamber continuously illuminated the upper side of the reactor from above. Air was sparged at the bottom of the photobioreactor by means of a plastic tube with many holes in a distance about 1 cm. Figure 7 illustrates a schematic overview of the reactor shape and of the operating mode.

Three tubes were fixed in the silicon cap on the top of the reactor, like it was done also in the vertical bubble column photobioreactor. Two of the tubes were destined for the gas flow inlet and for the gas flow outlet. The third line was provided for the sampling operation and was closed by a clamp.

One T-shaped valve mixed the inducted air of a compressor and the CO_2 from a tank to obtain the same concentration of the mixture in all photobioreactors. Another valve was used to adjust the volumetric flow rate for every reactor. A flow meter (asa, Sesto S. Giovanni, model: E5-2600/H) was used to measure the in-gas flow rate. A hydrophobic filter (0.2 µm) sterilized the inlet-gas. The off-gas line was connected to a gas fermenter analyser (Solaris biotechnology) to determine the concentration of CO_2 and air outgoing of the reactor. A cotton plug inside the gas-off tube absorbed the humidity and protected the gas fermenter analyser from condensing water running inside it. The cotton plug was replaced weekly.

A)



B)

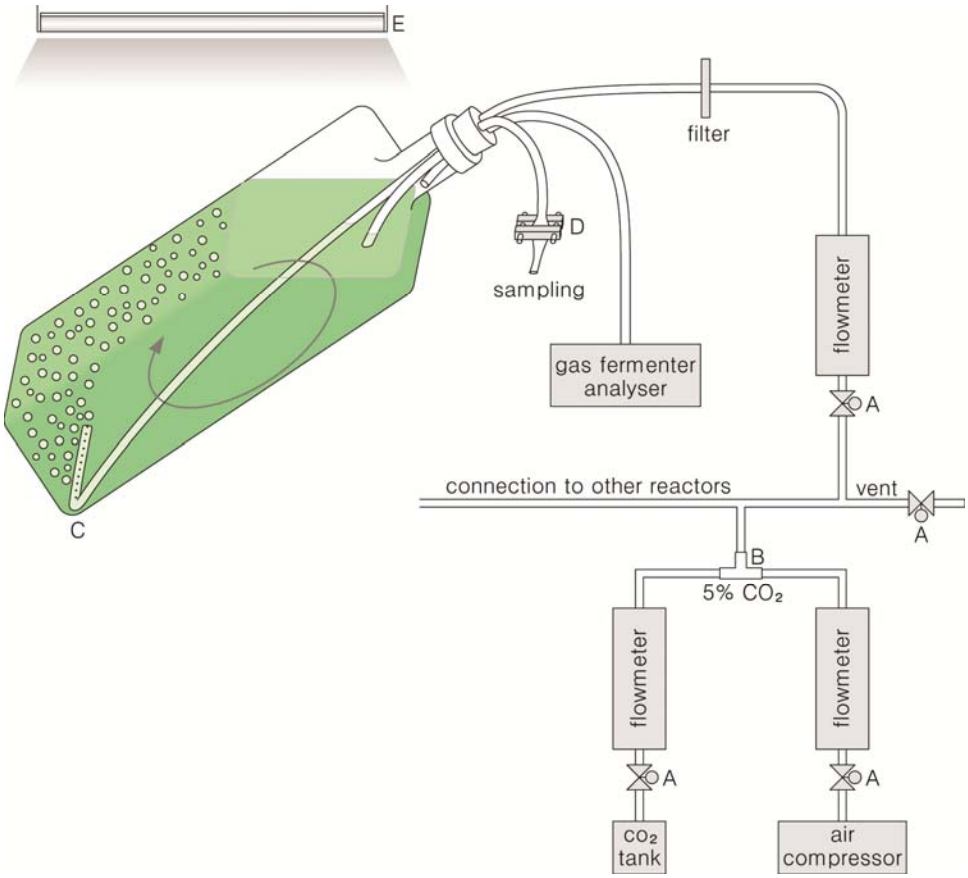


Figure 7: Sketch of the inclined square bubble column photobioreactor (not on scale). A) Dimensions, B) Current configuration. The arrow denotes the direction of mixing. A, valve, B, T-shaped valve, C, sparger, D, sterile clamp, E, light source.

2.3 Diagnostics

Some of the instruments for the analysis were located at the Department of Biological Science (Orto Botanico, Naples), some were located at the Department of Chemical Engineering in Naples. Some diagnostics (TOC, TN, IC, pH, protein assay) were performed using the cell-less supernatant. The samples were centrifuged for 30 minutes and 5000 rpm at 4 °C. Then, the supernatant was filtered with 0.2 µm filters to remove any solids. The pH was measured with a pH-meter (Crison, Basic 20) after filtering. All other diagnostics were made using the untreated sample.

The determination of the algal cell status and the control of bacterial and fungal contamination levels in the cultures were performed using a microscope with a magnification factor of 40 x and 100 x (Leitz Wetzler, 567146, Germany), respectively. A Nikon Eclipse 800 fluorescence microscope was applied to visualize the chlorophyll content in the algal cells.

2.3.1 Biomass determination

The concentration of algal biomass was estimated by measuring the optical density at 600 nm with a spectrophotometer (Specord 50 – Analytic Jena). The dry weight of the inoculum was used to establish the basis for converting the optical density in g/L of algal biomass. Therefore a Whatman filter was dried at 60 °C overnight and then weighed. A part of the inoculum was filtered using the dried Whatman filter. The wet biomass retained. The filter with the biomass on it dried overnight at 60 °C and was then weighed out. The microalgal dry weight (g/L) can be calculated from the difference in weight between the dry filter with and without biomass.

2.3.3 ChlA content

A fluorometer (AquaFluor^{TM}, Handheld Fluorometer/Turbidimeter, Turner Designs) was used to measure the content of in vivo ChlA in the untreated samples. Therefore the excitation light of the fluorometer passes through the medium and causes the ChlA inside the cells to fluoresce. This signal allows estimating the concentration of ChlA in the sample.

2.3.4 Photosynthesis and respiration rate

Photosynthesis is an indication of the health of algal cells and can be measured by means of an Oxygraph. The Oxygraph consists of two connected sections: the upper section of the Oxygraph which contains a transparent, thermostated sample chamber, and the lower electrode part. A thin teflon membrane separates the sample from the electrode compartment. A magnetic stirrer mixes the sample continuously. An adjustable stopper prevents room air dissolving during the experiments. A small polarising voltage is applied between the anode and the cathode to start the measurement. Then the oxygen diffuses through the teflon membrane and is converted into an electrical signal which can be detected. The oxygen control unit is connected to a PC, so it is possible to observe the oxygen gradient during the oxygen uptake (respiration) and the oxygen production (photosynthesis).

The light intensity during the analysis was set at 300 $\mu E/m^2/s$. That is the same light intensity like in the climate chamber where the reactors were housed. Three cycles of alternate light (about three minutes) and dark phases (about six minutes) for every sample were performed to have an average of every state. In the dark cycle the cells were not able to operate photosynthesis, but respiration. The Oxygraph of "Hansatech" continuously monitored the oxygen concentration in the chamber. If the photosynthesis was active (light phase), a positive slope (R_{Light}) in the oxygen vs. time plot could be measured. If only the respiration was active (dark phase) a negative slope (R_{Dark}) in the oxygen vs. time plot could be measured.

After the three cycles the average value in the light stage and in the dark stage was calculated. The real photosynthetic rate (R_{Ph}) and respiration rate (R_{Resp}) were calculated as following:

$$R_{Ph} = R_{Light} + R_{Dark}$$

$$R_{Resp} = R_{Dark}$$

2.3.5 Total organic carbon (TOC), total nitrogen (TN), inorganic carbon (IC)

The analysis of TOC, TN and IC was made with a "TOC-V CSH total organic carbon analyzer" of SHIMADZU (Model: TNM-1). Total organic carbon (TOC) is the amount of carbon bound in an organic compound. Inorganic carbon (IC) represents the content of

dissolved carbon dioxide and carbonic acid salts (IC = CO_2 + HCO_3^- + CO_3^{2-}). TN stands for total nitrogen (both organic and inorganic nitrogen).

2.3.6 Protein concentration

The Bradford protein assay was adopted to estimate the concentration of proteins in the medium. The colorimetric protein assay is based on a shift of absorbance under acidic conditions. While the dye Coomassie Brilliant Blue G-250 binds to cationic and non-polar side chains in the protein, its natural red form which has an absorbance maximum at 465 nm is converted into its bluer form. Hydrophobic and anionic interactions stabilize the dye. The sample can be measured by a photometer at an absorbance maximum at 595 nm wavelengths.

The micro assay was performed (1–10 mg/L). The standard, Bovine Serum Albumin (1 mg/mL BSA in 0.15 M NaCl, 0.05 % NaN_3, Sigma Aldrich) was prepared in the following concentrations: 1 mg/L, 5 mg/L and 10 mg/L. Bradford reagent (0.75 mL) was placed in a cuvette and 0.75 mL sample was added. After ten minutes incubation time the absorbance at 595 nm wavelengths was measured with a spectrophotometer (Varian Cary 50 Scan).

2.3.8 Fatty acid analysis

The lipids identification requires several steps:

- Biomass harvesting
- Biomass freeze-drying
- Lipid extraction and weight
- Lipid transesterification
- Methyl esters analysis

The samples were centrifuged (Eppendorf, Centrifuge 5804 R) for 20 minutes and 5000 rpm at 5 °C to separate the algal biomass from the medium. The obtained biomass pellet was collected and stored at -20 °C until the lyophilisation operation started.

Freeze-drying (lyophilisation) is usually used to conserve cells or other structures without the loss of biological activity and without destroying their structure. First the sample is

frozen. Then the surrounding pressure is reduced, so that the frozen water in the sample skips the liquid phase and goes directly from the solid phase into the gas phase. In this way sublimation takes place. The frozen biomass pellet was freeze-dried at a temperature around -50 °C and a pressure around 13.2 Pa. The process required about 12 hours. A freeze-dryer "Labconco Freezone® 1 L" with a vacuum pump of "Welch DirecTorr 8920" was applied. The freeze-dried pellet was ground to a fine powder which was used for further steps.

A modified method of De Swaaf et al. (2003) was performed to extract the lipids from the algal cells. A Soxhlet extractor is an apparatus typically used for the extraction of a compound which has a limited solubility in a solvent, in contrast to the unwanted material which is insoluble in that solvent. Figure 8 shows a schematic representation of a Soxhlet apparatus.

About 400 mg of freeze-dried and pounded algal biomass are placed inside a cellulose extraction thimble (25 x 80 mm, ID x length), which is loaded into the main chamber of the Soxhlet extractor. The Soxhlet extractor is then placed onto a weighed flask which contains the extraction solvents in a ratio about 2:1 (90 mL chloroform and 40 mL methanol). The flask with the solvent inside is heated. The solvent vapour travels up a distillation arm. It floods into the chamber housing the thimble with the biomass inside. A condenser on the top of the Soxhlet ensures that the solvent vapour cools, and drops down into the thimble containing chamber. The chamber with the cellulose thimble slowly fills with the condensed and warm solvent. During this step some of the desired algal compound which is placed inside the thimble dissolves in the warm solvent. A siphon side arm automatically empties the chamber when it is full and the solvent runs back down to the flask where it is heated again. Then the procedure starts again. With every cycle performed the desired compound gets more concentrated in the distillation flask. The non-soluble portion of the extracted biomass remains in the cellulose thimble and is then discarded. The Soxhlet extraction took about 7–8 hours. After extraction the solvent containing the lipids is ready for the evaporation procedure.

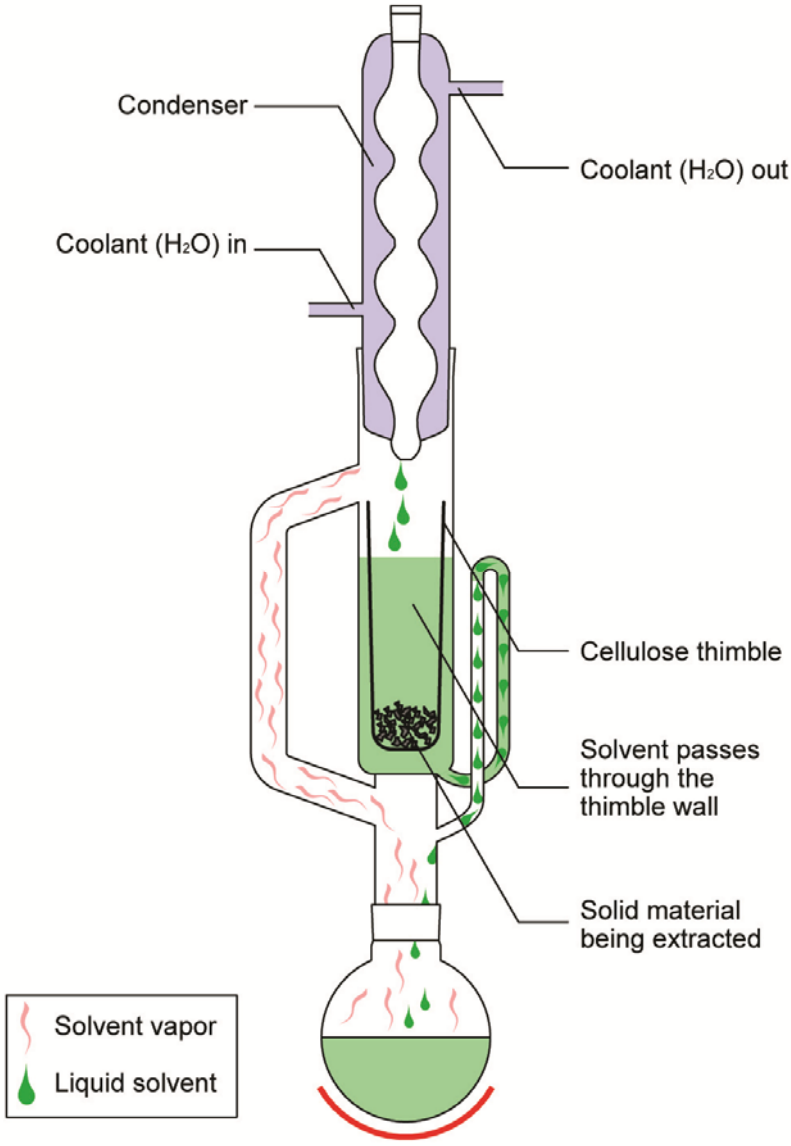


Figure 8: Schematic representation of a Soxhlet extractor.

(http://www.cremonatools.com/cremona_images/SOXLET%20scheme.jpg (22.01.2012))

After the Soxhlet operation it was necessary to remove the solvents. That was done by evaporation. The flask with solvents and extracted lipids was placed in a water bath at 60 °C. The solvents were removed by a vacuum pump, while the lipids remained in the flask. After a few hours, when the solvents were evaporated completely, the flask with adhering lipids was weighed. The full amount of extracted lipids is calculated by the difference between the weight of the empty flask before the extraction and the weight of the flask after evaporation with lipids inside.

The extracted lipids were converted to methyl esters by lipids transesterification (Figure 9). Transesterification is the process of exchanging an organic group of an ester with an organic group of an alcohol. Methanol (converted to sodium methoxide) is commonly used to produce the desired methyl esters. During the lipid transesterification process the base oil is converted to methyl esters. A by-product of the transesterification process is the production of glycerol (Rashid and Anwar, 2008a, 2008b).

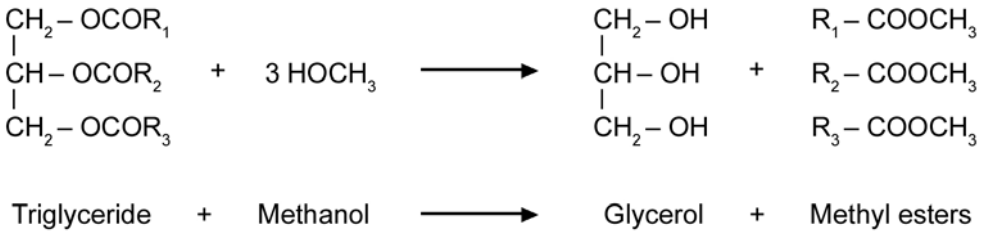


Figure 9: Transesterification mechanism.

A modified procedure described by Rashid and Anwar (2008) was applied for transesterification. 1.5 % w/w of sodium hydroxide dissolved in 12 mL of methanol was filled into the lipids containing flask. When the lipids went in solution with methanol, the flask was put in a water bath at 65 °C. After three minutes incubation the sample was stored at 4 °C and was ready for the analysis by HPLC.

High-performance liquid chromatography (HPLC) is an analytical method to separate, identify and quantify a mixture of non-volatile compounds, and was done according to Olivieri et al. (2011). The applied reversed HPLC (hp Hewlett Packard, Series 1100) utilizes different types of stationary phases, a pump which moves the mobile phases (H_2O and Acetonitrile) and the analyte through the column (Synergi 4u MAX-RP 80A 250*4.6mm 4 micron), and a detector (UV/Vis spectroscopic) at the end of the column. The sample was introduced in small volumes (20 µL) into the stream of the mobile phase. The velocity of the analyte passing the column depends on specific chemical or physical interactions with the stationary phase within the column. The time at which a specific sample elutes is called retention time. The gradient for reversed phase chromatography started at 10 % water for 25 minutes and proceeded to 1 % water in 95 minutes, and started

at 90 % acetonitrile for 25 minutes and proceeded to 99 % acetonitrile in 95 minutes. The peaks were analyzed by an UV detector at 210 nm.

The calibration was done using the following standards: stereate, decanoate, laurate, linolenate, linoleate, oleate, myristate, elaidate, palmitoleate, and palmitate dissolved in methanol (Table 4).

Table 4: Lipids used for the calibration

Standard	Chemical composition	Molecular mass (g/mol)	Conc. (mM)	Retention time (min)	Peaks area	Absorption coefficient (ε)
Decanoate	10:0	186.3	4.67	7.32	700.3	152.5
Laurate	12:0	214.4	4.06	10.94	358.0	86.4
Linolenate	18:3	292.5	1.48	14.07	14395.2	9680.6
Myristate	14:0	242.4	1.80	17.18	161.8	89.9
Palmitoleate	16:1	268.4	1.63	18.27	968.9	595.9
Linoleate	18:2	294.5	1.48	19.88	12753.0	8634.4
Palmitate	16:0	270.5	1.61	26.62	162.9	100.9
Oleate	18:1	296.5	1.47	27.44	825.6	561.6
Elaidate	18:1	296.5	1.47	28.02	1163.8	790.6
Stereate	18:0	298.5	16.75	32.38	1029.7	61.8

2.4 Operating conditions and procedure

2.4.1 “From stab to photobioreactor”

A high cell density in the preculture is necessary for the inoculation of the photobioreactors. The preculture was cultivated in a 250 mL Erlenmeyer flask. The inoculated flask was continuously illuminated and cultivated at 23±1 °C. After 4–5 days the flask containing an increased cell density was divided into two flasks and filled up again to the initial volume with Bold Basal medium. After another 4–5 days the 2 flasks were divided into further 4 flasks. This process was repeated up to a volume and high cell density efficiency for the inoculation of the reactors. The inoculum was ready for inoculation after 3–4 weeks. The risk of contamination after 3–4 weeks of cultivation was very high, so it was necessary to check the culture purity by microscopy. The flasks strongly contaminated and with not viable algal cells were separated out. The Erlenmeyer flasks containing the precultures were combined and mixed very well to have the same starting conditions in all the photobioreactors. The inoculation volume was 1/10 of the liquid working volume.

2.4.2 Operating methods

Three different steps characterized the culture strategy in the photobioreactors. The run started with the batch mode, and then switched into the fed-batch mode. The last step was the semi-continuous mode. The sampling operation took place two or three times a week. The following measurements were done periodically: biomass concentration by spectrophotometry, pH, contamination level by microscopy, ChlA content, photosynthesis rate and respiration rate, TOC, TN, and IC. The fatty acid analysis was done with the collected biomass of the semi-continuous culture. The protein assay was done once during the semi-continuous cultivation.

Batch culture: The batch reactor is a closed reactor system which is limited in the volume. All the necessary components for the fermentation are filled inside the reactor before the operation starts. It is not possible to remove material during the fermentation process or to fill in components; there is no input and output of materials. During the batch operation a consumption of substrates and a production of biomass and products take place. During the process there is a concentration change in terms of nutrients, cells and products over time. After degradation of the resources by the cells they start to die. The tests in batch mode

lasted about two weeks. The switch into the fed-batch mode was done when the nutrients were almost degraded.

Fed-batch culture: The system of the fed-batch reactor is similar to the batch-reactor. Like in the batch-operation there is no output of materials. In contrast to the batch operation the fed-batch reactor can be filled up continuously over time to its maximal liquid level after starting. The aim is to keep a constant substrate concentration within the reactor. The substrate inflow should compensate exactly the substrate uptake. The culture growth rate is prevented from substrate inhibition due to the constant low concentration of substrate.

In our experiments the addition of concentrated medium did not influence the volume of broth since the added liquid volume balanced the periodic culture sampling and the evaporation of water. When the nitrogen level achieved a critical value (< 15 mg/L) new medium was added to feed the cells well and to avoid nitrogen starvation. A fixed volume (1/10 of the total liquid amount inside the reactors) of 10x concentrated Bold Basal medium was added to the culture. In this way a high cell density was obtained. Tests lasted about two to four weeks.

Semi-continuous culture: During the semi-continuous mode a definite part of the broth is replaced periodically with fresh medium all at once. It is done by opening the reactor, throwing away a defined volume of liquid, and then refilling the reactor with fresh medium to the initial volume. There are two possibilities to operate in the semi-continuous mode:

- fixed period, where the replacing operation takes place e.g. once a week
- not time dependent, where the replacing operation takes place when a defined biomass concentration is achieved

During the semi-continuous mode the average of the cell concentration should be constant. Figure 10 shows an optimal growth curve of a semi-continuous reactor.

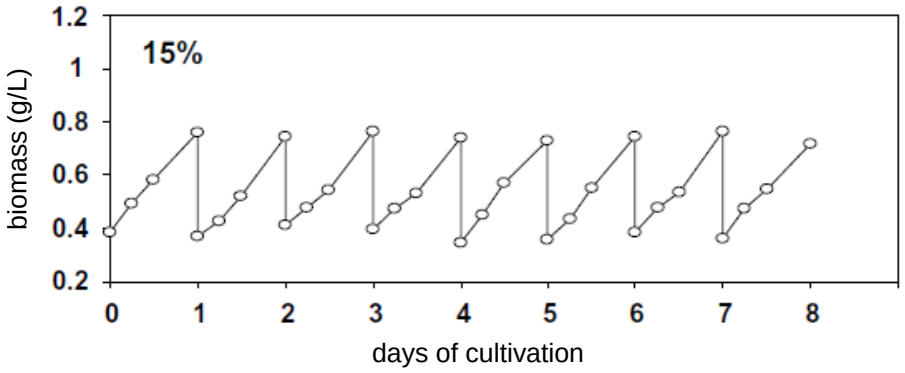


Figure 10: Growth profile of microalgae in the semi-continuous mode, aerated with 15 % CO_2.

Source: Chiu et al., 2009

Tests in the semi-continuous mode simulate closely a continuous culture. The main difference between the continuous reactor and the semi-continuous reactor is that in the continuous system the fresh medium is added constantly to the culture. A reason to choose the semi-continuous system could be the case of product inhibition. With the replacement of a partial liquid part in the reactor the product is prevented from being always at the highest possible concentration. On the contrary if there is the case of substrate inhibition a continuous reactor system should be preferred where the substrate concentration is kept on its minimum. A semi-continuous system was chosen also because of a low dilution rate. In relation to fast growing bacterial cells which have a very short doubling time algal cell growth is much slower and often almost unpredictable. A very low dilution rate was chosen to protect cells from being washed out. The average replacement of the medium in the experiments amounted 1.25 mL/h, which is less for a common pump.

Previously studies (Olivieri et al., 2011) show a maximum fatty acid production at a dilution rate of 0.05 d^{-1}, that means a broth replacement of 35 % weekly (fixed period). The average dilution rate (D) was assessed as the ratio between the weekly replaced suspension volume and the photobioreactor working volume, in our case 0.05 d^{-1} and 0.06 d^{-1}, respectively. The tests lasted about four weeks.

During the growth of algal cells the total organic carbon in the medium increases because of the secretion of molecules from algal cells (Olivieri et al., 2011). This organic carbon offers a nutrient source to bacterial cells or to other heterotrophic microorganisms. The risk of contamination increases in elder medium. Due to the partial exchange of medium with fresh medium in the semi-continuous mode the concentration of organic carbon was

diluted and in addition there was a supply of nutrients for the algal cells again. As a contamination in the reactors is likely it is important to provide optimum conditions for algal cell growth.

2.4.3 Test at different CO_2 levels

Tests were carried out in 1 L bench scale vertical bubble column photobioreactors with a working volume of 0.6 L. The experiment was started with 12 photobioreactors. The working temperature in the climate chamber was set at 23 ± 1 °C. The light irradiance (I_L) at the surface of the reactors was set continuously at 300 μE/m^2/s. Every reactor was filled up with 540 mL BBM and inoculated with 60 mL preculture. The aeration and mixing were carried out with air at a flow rate of 20 L/h. After a few days the aeration was reduced to 10 L/h due to the increased foam generation at higher biomass concentrations. So, the volumetric flow rate of the gas mixture was fixed at 10 L/h. CO_2 was started to be sparged one week after inoculation. The CO_2 was mixed with air to obtain different CO_2 concentrations. Three reactors were sparged with 5 % additional CO_2, other three reactors with 10 % additional CO_2, another three reactors with 15 % additional CO_2 and two reactors were sparged with air only.

2.4.4 Test at different pH values

Tests were carried out in 2 L bench scale horizontal bubble column photobioreactors made of glass with a working volume of 1.7 L. The experiments were started with 6 photobioreactors. The working temperature in the climate chamber was set at 23 ± 1 °C. The light irradiance (I_L) at the surface of the reactors was set continuously at 300 μE/m^2/s like in the other tests with different CO_2 concentrations. Every reactor was filled up with 1.53 L BBM and inoculated with 170 mL preculture to achieve a working volume of 1.7 L. The aeration and mixing were carried out with air at a gas flow rate in a range between 50 and 100 L/h. Additional carbon dioxide was started to be sparged on day 9. A carbon dioxide concentration of 5 % was chosen according to the previous experiment with different CO_2 levels. The pH in four reactors was adapted with modified medium (Table 2). Reactors 1 and 2 were cultivated at pH 6.8 (buffered from BBM). Reactors 3 and 4 were tested at a pH about 9 (NaOH + NH_4Cl buffer) and reactors 5 and 6 were cultivated at a pH about 3 (HCl + Glycylglycine buffer).

3. Results and discussion

3.1 Batch, Fed-Batch and Semi-continuous experiments

Tests were carried out in the first phase under batch conditions. They lasted about one or two weeks. In this phase the culture could adapt on the present cultivation conditions. The concentration of total organic carbon at the beginning of the experiments was low because the adopted medium contained almost no organic compounds, except of EDTA (0.17 mM) and low amounts of vitamins necessary for autotrophic growth. In this phase the risk of contamination was low. The concentration of total nitrogen decreased due to the algal uptake. A nutrient depletion was checked by periodical measurements of the medium composition. Due to the nutrients limitation in the batch operation it was not possible to achieve a high cell density, required for the experiments.

The shift to the fed-batch phase was done when the nitrogen concentration in the medium achieved a value below 15 mg/L. An addition of 10x concentrated medium took place, so that nutrient starvation was avoided and cell growth could continue. The liquid working volume never crossed the maximal capacity (0.6 L and 1.7 L in the vertical bubble column photobioreactors and in the inclined square bubble column photobioreactors, respectively): the medium addition was balanced by the water withdraw due to the samples and to the water stripping by gas bubbles. A disadvantage of the fed-batch operation was the increasing level of total organic carbon. Olivieri et al. (2011) assumes that this occurs due to the secretion of organic molecules from the algal cells during growth. In the fed-batch mode the cell density became very high and the cell death should be taken into account. Organic compounds were also released in the medium as a consequence of cell lysis. For this reason the risk of contamination increased.

In the semi-continuous phase the culture was periodically substituted with fresh medium and the TOC and, consequently, the contamination risk decreased. The semi-continuous operation involved a cycle with the algal growth followed by the sharp dilution. The semi-continuous phase was adopted to obtain a steady-state in all parameters monitored in the culture. This was necessary to determine accurately the differences in biomass composition under various conditions (influence of CO_2 concentration and pH). The collection of biomass for the lipid analysis was therefore made only when the steady-state was achieved.

A change in the environment can affect the content of lipids inside the cells as well as the composition of lipids, which is a quality characteristic of biodiesel.

Knothe (2005) showed the influence of fatty acid structures on biodiesel properties considering the cetane number, the heat of combustion, the cold flow properties, the oxidative stability, the viscosity, and the lubricity. As well Ramos et al. (2009) analyzed the influence of fatty acid composition on biodiesel properties regarding the International and European Standards ISO 5509 and EN 14103. Allen et al. (1999) developed a method for the prediction of biodiesel parameters by knowing the composition of the raw material.

3.2 Effect of CO_2

These tests were aimed to assess the effect of different CO_2 concentrations in the air fed to the culture on the photobioreactor performance.

Figures 11 and 12 report data regarding two cultures carried out adopting a strategy of progressive change of operating conditions. The culture in Figure 11 was sparged with air only. In contrast the culture shown in Figure 12 was sparged with 10 % additional CO_2. Both cultures were grown in vertical bubble column photobioreactors. The reactors ran two weeks in the batch mode. When the level of nutrients in the medium was low, the fed-batch cultivation started. The black dotted lines in Figures 11 and 12 mark the addition of concentrated medium. The biomass concentration increased from 0.02 g/L up to 0.50 g/L in Figure 11, and from 0.02 g/L up to 0.26 g/L in Figure 12 during the batch phase and achieved a steady-state value during the fed-batch phase.

On day 42 the system was switched into the semi-continuous cultivation by a weekly replacement of 35 % of the suspension, so that the dilution rate under semi-continuous conditions was 0.05 d^{-1}. The black dashed lines in the figures indicate the replacing operation. After achieving a steady state, the reactors were cultivated in the semi-continuous mode for about 6 weeks. The broth replaced with fresh medium was collected for the lipid analysis. At the end of the experiment the photobioreactors were cultivated under nitrogen starvation for one week. The beginning of the nitrogen starvation condition is indicated by the black continuous line. After 12 weeks of cultivation (from Batch to Semi-continuous mode) the reactors were stopped and the residual biomass was collected for the lipid analysis. Table 5 reports data of semi-continuous tests with different CO_2 concentrations.

The following effects were noticed by considering all cultures in the experiment.

3.2.1 Effects on the culture quality

Stichococcus bacillaris 158/11 was able to grow under all the investigated CO_2 concentrations. Differently from the culture grown with additional CO_2, the cultures aerated with air never achieved a steady-state in the biomass concentration, as indicated also in Figures 11 and 12. The biomass concentration achieved the highest value (5.40 g/L)

in a photobioreactor with 5 % additional CO_2. The specific biomass productivity ($D \cdot X$) was about 130 mg/L/d in all investigated conditions.

The value of the ChlA content was about 2 mg/g at the beginning of the experiments. Then the ChlA content almost continuously decreased in the cultures sparged with additional CO_2. These reactors showed always a lower ChlA content (about 0.3 mg/g) compared to the reactors sparged only with air (about 2.6 mg/g).

Huertas et al. (2000), Sergeenko et al. (2000) and Ge et al. (2011) report about a diminution in chlorophyll pigments in several algal strains cultivated under higher CO_2 concentrations. The chlorophyll content in the cells cultivated only with air sparging increased during the fed-batch cultivation from about 2.0 mg/g up to 6.5 mg/g. But the content of ChlA started to decrease rapidly when the biomass arrived to a concentration about 1.5 g/L. Messner and Ben-Shaul (1972) found that the ChlA content in young algae is relatively higher than in older cells. Vörös and Padisák (1991) showed a correlation of increasing cell concentration and decreasing ChlA content in phytoplankton. The studies of Li (2008) show a diminution in the chlorophyll content after the depletion of the external nitrogen pool. They hypothesize that algal cells are able to continue their growth after the exhaustion of external nitrogen sources by the consumption of the intracellular nitrogen pool such as chlorophyll molecules.

The OUR was at about the same value (about 14 $mg_{O2}/g_X/h$) in all reactors. However, the air sparged reactors showed the highest specific photosynthetic rate in steady state (35.9 $mg_{O2}/g_X/h$).

3.2.2 Effects on medium

The concentration of TOC increased constantly during the batch and the fed-batch phase. After the change to the semi-continuous phase the TOC concentration remained about constant.

The concentration of IC increased during the batch and the fed-batch phase and remained about constant in the semi-continuous phase. The IC obtained the highest value in the reactors with 15 % additional CO_2 and the lowest value in the reactors with only air sparging. The TN concentration approached a steady-state under semi-continuous conditions.

The average pH in the cultures with CO_2 addition settled down at about 7. The reactors with only air sparging had a higher pH of about 8. The lower value of pH in the reactors with CO_2 addition could be based on the CO_2 which dissolved in the medium and thereby generated carbonates (CO_3^{--}), bicarbonates (HCO_3^-) and H^+. This chemical reaction could have been also the reason for the high amount of inorganic carbon in the reactors with 5 %, 10 %, and 15 % CO_2 sparging (>120 mg/L), compared to the IC value (67.6 mg/L) of the air sparged reactors.

Table 5: Results obtained at different CO_2 concentrations in steady-state

	Air	5 % CO_2	10 % CO_2	15 % CO_2
Biomass (g/L)	No steady state	2.48	2.62	2.78
Biomass productivity (mg/L/d)	--	124	131	139
pH	8.2	7.1	7.0	7.0
TOC (mg/L)	188	192	182	229
TN (mg/L)	19.1	13.9	14.8	14.6
IC (mg/L)	67.6	128	133	146
ChlA (mg/g)	2.56	0.30	0.32	0.31
OUR ($mg_{O2}/g_X/h$)	13.7	15.0	16.9	13.6
Φ ($mg_{O2}/g_X/h$)	35.9	19.4	25.4	22.4

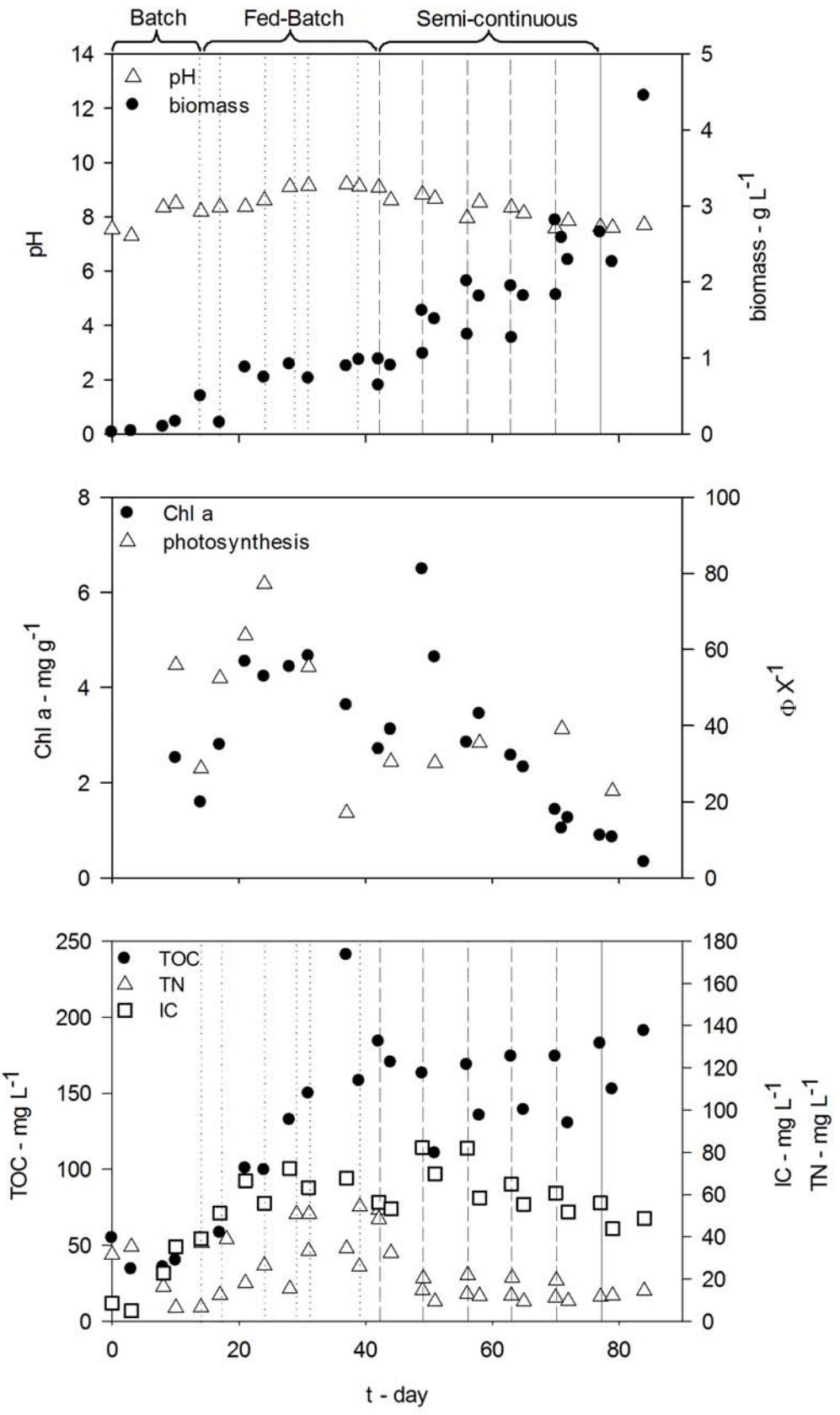


Figure 11: Characteristics of a *Stichococcus bacillaris* 158/11 culture running through batch, fed-batch, and semi-continuous conditions, sparged with air.

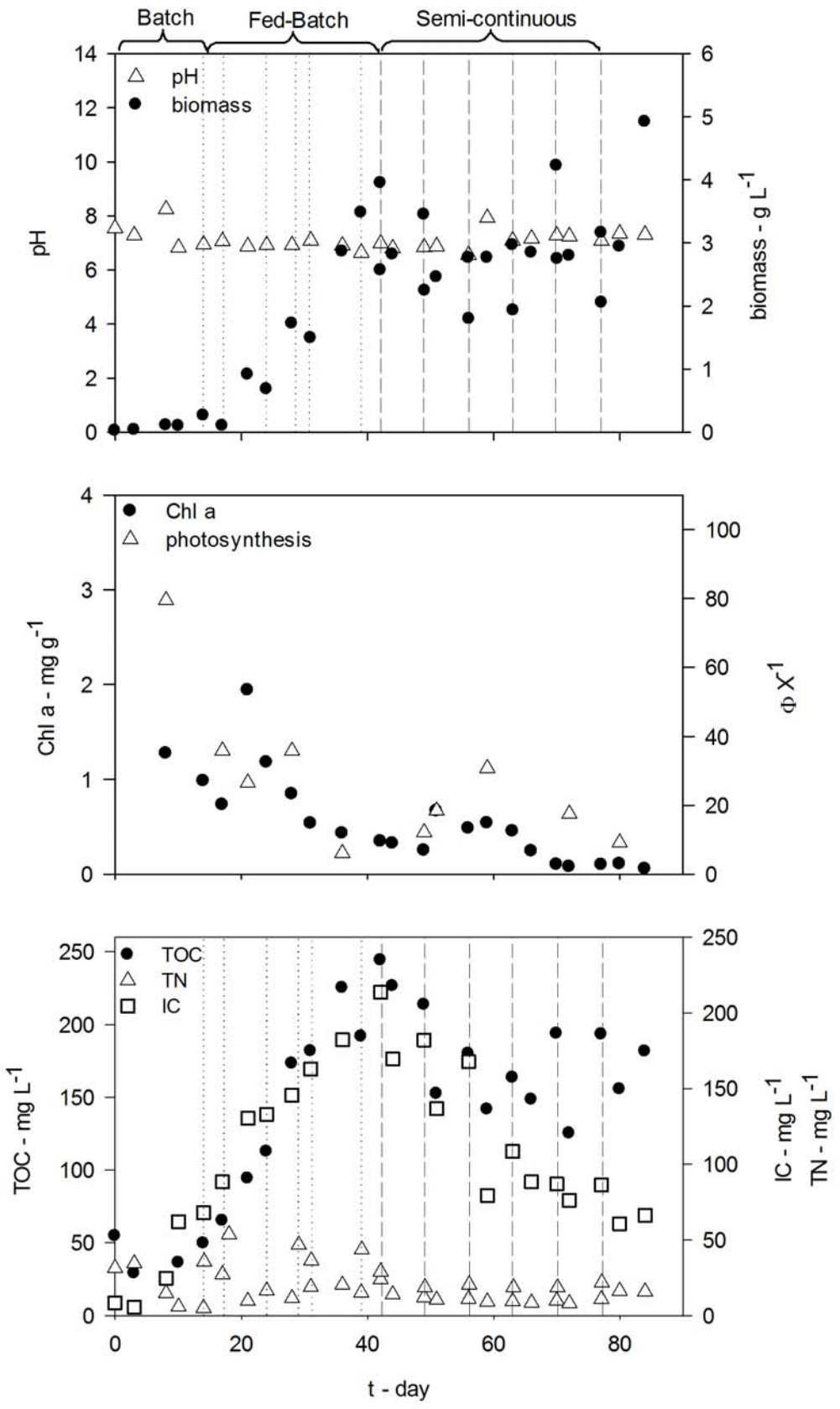


Figure 12: Characteristics of a *Stichococcus bacillaris* 158/11 culture running through batch, fed-batch, and semi-continuous conditions, sparged with 10 % additional CO_2.

3.2.3 Effects on lipid content and composition

The total lipid fraction and the specific lipid productivity achieved their maximum at 10 % additional CO_2 sparging with values of 35.9 %, and 47.0 mg/L/d, respectively. The values at 5 % additional CO_2 and 10 % additional CO_2 were similar. Definitely lower values were obtained with air sparging and with 15 % additional CO_2 sparging. Table 6 regards data obtained under different cultivating conditions. Different CO_2 conditions influenced the total amount of lipid fraction and also the amount of esterified lipids.

The total esterified lipids are the percentage of total lipids within algal cells which were esterified successfully by transesterification. They are defined as the amount of lipids which can be used as biodiesel. The highest amount of total esterified lipids was achieved in cultures grown at 10 % CO_2 and 15 % CO_2. More lipids (about 33 %) were esterified in the air run compared to the culture grown at 5 % additional CO_2 (about 24 %).

Different CO_2 conditions could have influenced also the composition of esterified lipids. Table 6 indicates the differences of lipid compositions under nitrogen sufficient conditions and nitrogen starvation conditions.

The amount of Linolenate was the highest (with about 4.5 %) in the cultures grown at 10 % CO_2 and 15 % CO_2. Linoleate occurred in higher CO_2 concentrations with about 4 %. Palmitate never appeared at these tests. Oleate was the dominating fatty acid in all cultures with a maximum at 10 % CO_2 sparging under nitrogen starvation (33 %). The culture grown at 5 % additional CO_2 showed the lowest Oleate content (about 18 %). Elaidate occurred only in the 15 % CO_2 sparged culture under nitrogen sufficient conditions.

Table 6: Lipid content and lipid composition of *Stichococcus bacillaris* 158/11 cells cultivated under different CO_2 conditions.

	Air		5 % CO_2		10 % CO_2		15 % CO_2	
	N +	N -	N +	N -	N +	N -	N +	N -
Total lipid %	28.8	28.2	34.3	27.4	35.9	25.4	26.9	26.7
Total lipid productivity (mg/L/d)	--	--	42.5	--	47.0	--	37.4	--
Linolenate*	3.1	3.0	2.8	3.0	4.1	4.4	5.6	4.7
Linoleate*	3.2	4.0	2.7	3.4	3.5	4.8	4.1	4.4
Palmitate*	--	--	--	--	--	--	--	--
Oleate*	26.5	26.8	18.0	18.2	22.0	33.0	32.6	24.2
Elaidate*	--	--	--	--	--	--	3.0	--
Total esterified lipids, %	32.8	33.8	23.5	24.5	29.5	42.2	44.6	33.2

*mass fraction with respect to the total amount of esterified lipids; %

N +: Nitrogen sufficient conditions

N -: Nitrogen starvation

The lipid analysis was operated in collaboration with Immacolata Gargano.

3.3 Effect of pH

Tests were aimed at assessing the effect of different pH values in the medium to the algal culture. The adopted gas mixture consisted of air with 5 % additional CO_2. The experiment based on the results of the previously performed tests with different CO_2 conditions. Inclined bubble column photobioreactors were adopted.

Figure 13 reports data regarding one of the cultures carried out adopting a progressive change of operating conditions. The culture was for 10 days under batch mode. When the level of nutrients in the medium was low, the fed-batch cultivation started and the pH changed. The grey dotted line in Figure 13 marks the addition of concentrated medium. The biomass concentration increased from 0.04 g/L up to 1.37 g/L during the batch phase and achieved its maximum (6.65 g/L) during the fed-batch phase. On day 16 the system was switched into the semi-continuous cultivation by a replacement of 35 % of the suspension, so that the average dilution rate under semi-continuous conditions was 0.06 d^{-1}. The black dashed lines in the figure indicate the replacing operation. The reactor was cultivated in the semi-continuous mode for about 4 weeks. The broth replaced with fresh medium was collected for the lipid analysis. At the end of the experiment the photobioreactor was cultivated in nitrogen starvation for four days. The beginning of the nitrogen starvation condition is indicated by the black continuous line. After 7 weeks of cultivation (running through all operating conditions) the reactor was stopped and the residual biomass was collected for the lipid analysis.

Table 7 reports data of semi-continuous tests with different pH values in the culture medium. The following effects could be noticed by considering all cultures in the experiment.

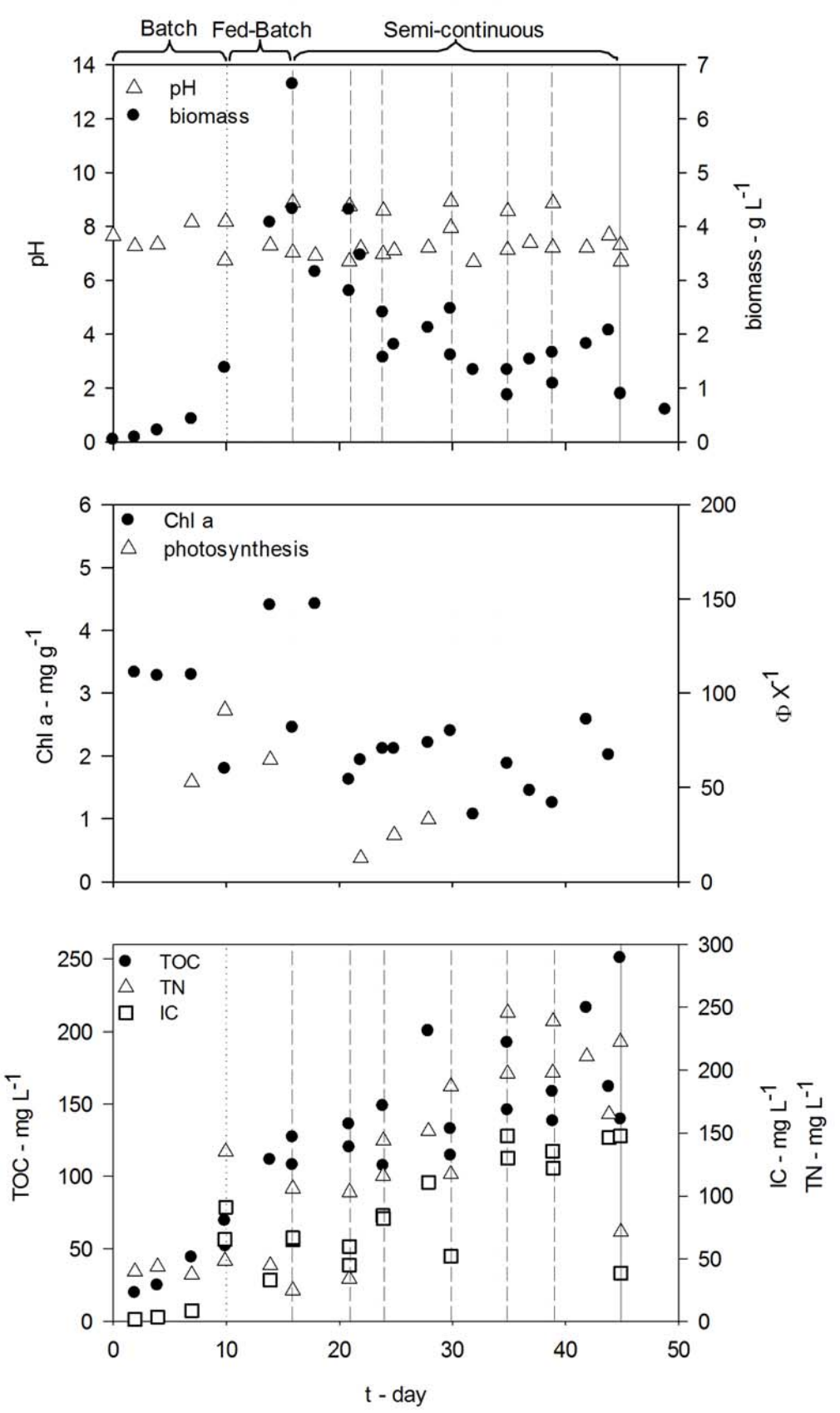


Figure 13: Characteristics of a *Stichococcus bacillaris* 158/11 culture running through batch, fed-batch, and semi-continuous conditions, grown at a pH about 9.

3.3.1 Effects on culture quality

Stichococcus bacillaris 158/11 was able to grow in all the investigated pH conditions. The cultures grown at pH 3 and at pH 9 never achieved a steady-state in the biomass concentration (Figure 13 illustrates the biomass trend at pH 9). The biomass concentration decreased continuously after changing to the semi-continuous mode. In contrast the cultures grown at pH 7 achieved the steady-state after an initial decrease of biomass from about 6 g/L down to about 4 g/L. The highest biomass concentration was achieved in all reactors on day 16 with about 7 g/L. The specific biomass productivity in the cultures grown at pH 3 and in the cultures grown at pH 9 was about 220 mg/L/d. The cultures grown at pH 7 achieved a biomass productivity about 260 mg/L/d.

Perhaps different pH conditions influenced the growth rate and also the appearance of the algal cells. Figures 14, 15 and 16 illustrate the appearance of *Stichococcus bacillaris* 158/11 cells grown under the adjusted pH conditions. The cells in the medium at pH 7 (Figure 14) seemed to be without any abnormality. A high cell density was visible with a lot of moving cells. They were uniform, small and short. Some cells agglomerated. The cells cultivated in the medium at pH 9 (Figure 15) were bigger than the cells at pH 7. They had various lengths and the filament forming cells predominated clearly. Filaments up to 18 cells in row were detected. Some cells were moving. Figure 16 shows *Stichococcus bacillaris* 158/11 cells grown in medium at pH about 3. The agglomerated cells were definitely not uniform. Different shapes were visible. They seemed to be distorted and curved, in some cases very plump. Cells were not moving.

Fungal contaminations were a problem in the cultures grown at pH 9 and at pH 3. Long branched hyphae were visible under the microscope. A weekly exploration by microscopy was an essential operation to control the level of contamination.

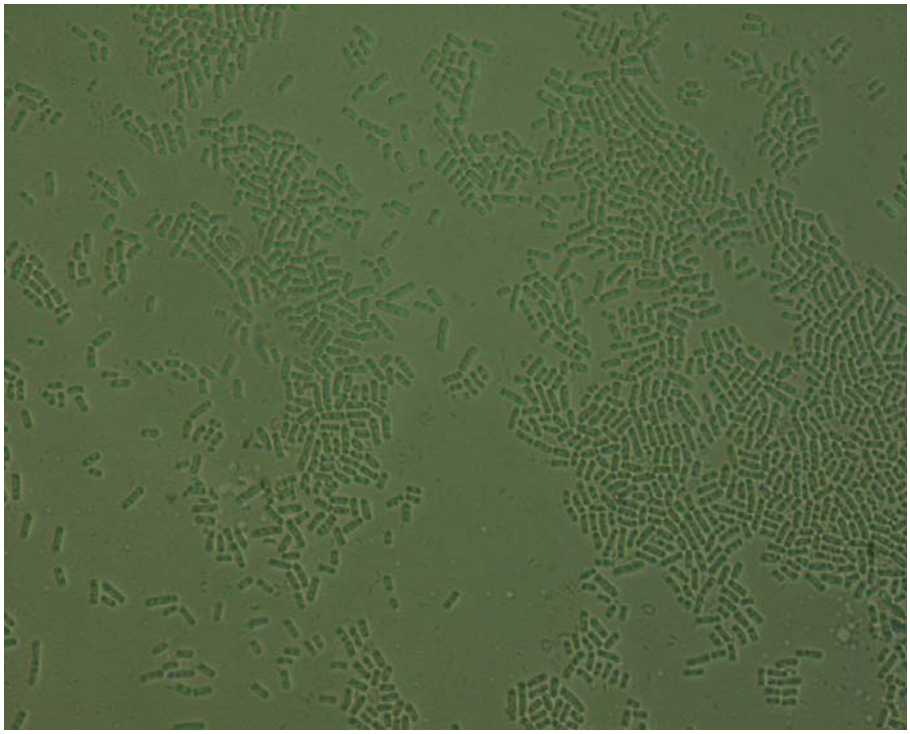


Figure 14: *Stichococcus bacillaris* 158/11 cells grown in BBM at pH 7

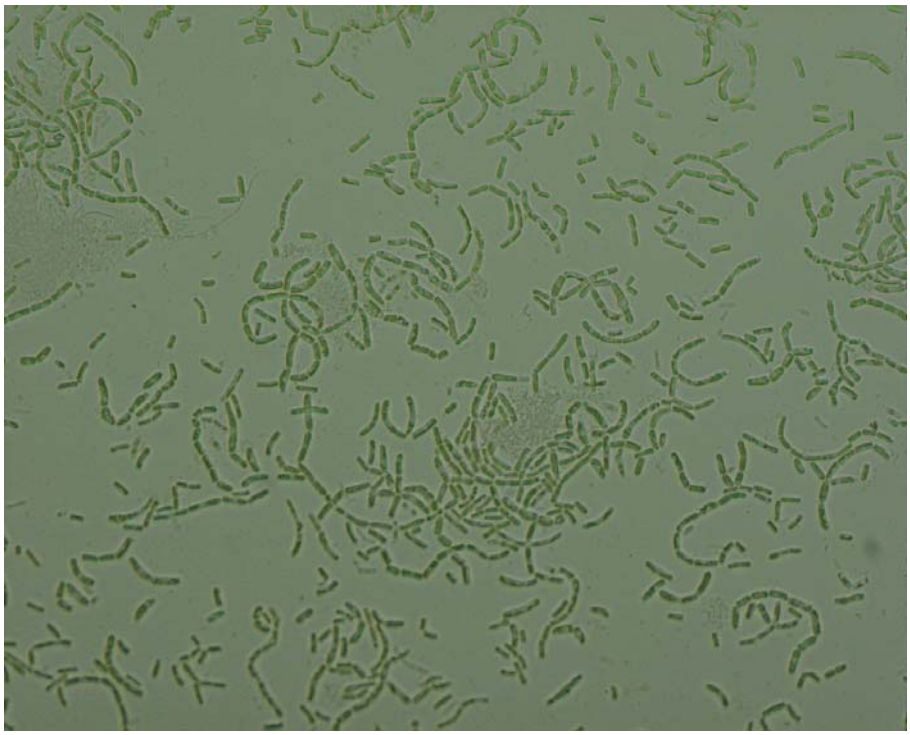


Figure 15: *Stichococcus bacillaris* 158/11 cells grown in modified BBM at pH 9

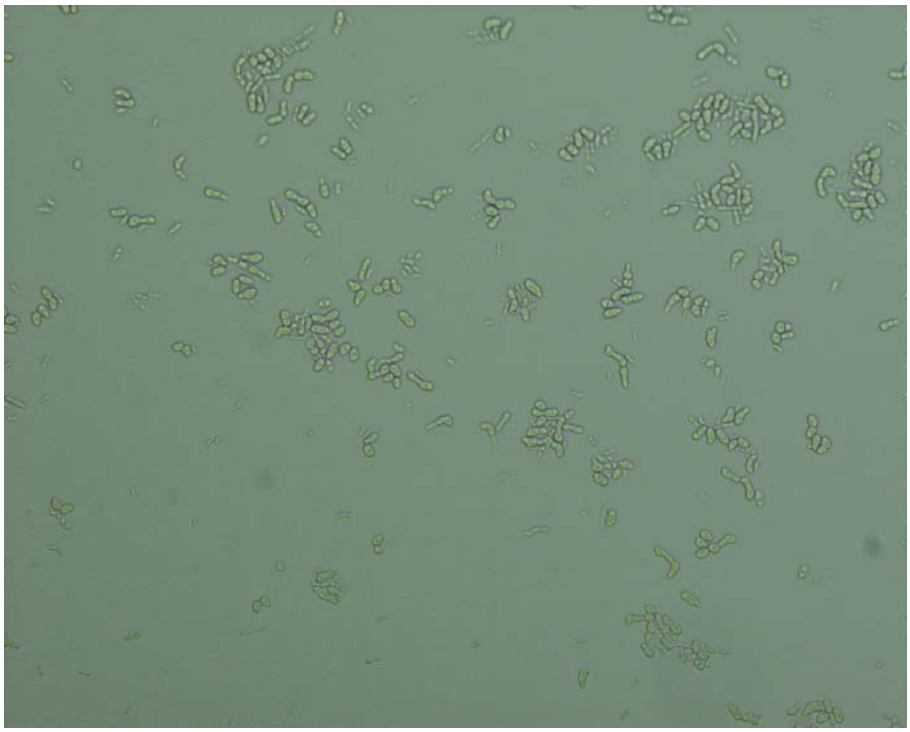


Figure 16: *Stichococcus bacillaris* 158/11 cells grown in modified BBM at pH 3

At the beginning of the experiments the ChlA content was at a value about 3.0 mg/g. It started to decrease rapidly at the same time when the biomass arrived to a concentration of about 1.5 g/L. The ChlA content decreased about continuously in the cultures grown at pH 7 and at pH 3 until it settled down at about 0.7 mg/g during the semi-continuous mode. In contrast the ChlA content in the cultures grown at pH 9 ranged between 1 mg/g and 3 mg/g during semi-continuous conditions.

The OUR was at about the same value (about 10 $mg_{O2}/g_X/h$) in all cultures. The cultures at pH 3 obtained the highest specific photosynthetic rate in steady state (23.2 $mg_{O2}/g_X/h$).

3.3.2 Effects on medium

The TOC concentration increased constantly during the batch and the fed-batch phase. It remained about constant in pH 7 and in pH 9 after the change to the semi-continuous phase with about 100 mg/L and 200 mg/L, respectively. The TOC concentration ranged between 100 mg/L and 1000 mg/L in the medium with pH 3.

The IC concentration in pH 7 increased during the batch and the fed-batch phase and remained about constant in the semi-continuous phase with a value of 64.4 mg/L. The concentration of IC at pH 9 increased during the batch and the fed-batch mode and ranged between 50 mg/L and 150 mg/L in the semi-continuous mode according to the fluctuating pH (shown in Figure 13). The medium at pH 3 showed a very low IC concentration of about 6 mg/L. So, the IC obtained its highest value in the reactors at pH 9, and its lowest value in the reactors at pH 3.

The TN concentration approached a steady-state under semi-continuous conditions at pH 7. In contrast it increased continuously in the semi-continuous mode at pH 9 and at pH 3 up to a value about 450 mg/L and 1000 mg/L, respectively. Consider that the modified buffer systems account for the high TN values.

The inorganic NH_4Cl buffer was added to keep the pH of the medium at a level of 9. The amount of total organic carbon in the medium was not influenced by adding this buffer. But the level of total nitrogen increased dramatically with respect to the ammonium in the buffer. The ammonium was a liked nitrogen source for *Stichococcus bacillaris* 158/11 cells. So, it was difficult to keep up the pH of about 9 due to the nitrogen uptake of the algal cells. The culture raised the ChlA production after addition of modified buffer. According to White and Payne (1977) an addition of nutrients stimulates the production of Chlorophyll.

The organic glycylglycine buffer was added to keep the pH of the medium at a value of 3. The amounts of TOC and TN in the medium increased by using this buffer. The glycylglycine seemed not to be a popular nitrogen source for *Stichococcus bacillaris* 158/11 cells as the TN remained in a high concentration during cell cultivation. The glycylglycine probably would be a carbon source for contamination but the low pH hardly allows bacterial growth.

The culture at pH 7 was grown in normal BBM without any modifications. Due to the additional CO_2 of 5 % the pH settled down at about 7.

Table 7 reports data of selected cultures obtained at different pH values under steady-state conditions.

Table 7: Results from different pH values in steady-state

	pH 6.8	pH 3	pH 9
Biomass (g/L)	4.29	3.89	3.59
Biomass productivity (mg/L/d)	257	233	215
pH	7.0	2.9	7.5
TOC (mg/L)	114	650	191
TN (mg/L)	26.6	311	139
IC (mg/L)	64.4	5.69	104
ChlA (mg/g)	0.50	0.63	1.88
OUR ($mg_{O2}/g_X/h$)	10.5	9.81	9.98
Φ ($mg_{O2}/g_X/h$)	16.6	23.2	19.9

3.3.2 Effects on lipid content and composition

The total lipid fraction and the specific lipid productivity achieved their maximum at pH 7 with 39.5 %, and 79.8 mg/L/d, respectively. Fewer lipids (about 14 %) were obtained in cultures grown at a pH about 9. Table 8 regards data obtained under different pH conditions. Perhaps different pH conditions influenced the total amount of lipid fraction as well as the amount of esterified lipids.

The total esterified lipids are the percentage of total lipids within algal cells which were esterified successfully. The highest amount of total esterified lipids was achieved in cultures grown at pH 7 (39.8 %). The culture grown at pH 3 showed a very low value (14.4 %) of esterified lipids. The amounts of esterified lipids in the culture grown at pH 9 were higher (27.0 %) than in pH 3, but you have to consider the low value of total lipids in cultures grown at pH 9.

Different pH conditions could have influenced also the composition of esterified lipids. Table 8 indicates the differences of lipid compositions under nitrogen sufficient conditions and nitrogen starvation conditions. The amount of Linolenate was the highest (about 5 %) in the cultures grown at pH 7 and pH 9. It was very low (about 1.5 %) at pH 3. Linoleate dominated at pH 7 with about 4 %. Palmitate appeared only at pH 7 under nitrogen

sufficient conditions. Under nitrogen starvation condition it was not detectable. Oleate was the dominating fatty acid in all cultures with a maximum (about 23 %) at pH 7. The culture grown at pH 3 showed the lowest Oleate content. Elaidate occurred in the cultures grown at pH 7 and at pH 9, not in cultures grown at pH 3.

The tests showed that *Stichococcus bacillaris* 158/11 cells are able to live in a low pH environment (about pH 3). It seems that the effect of low pH on total lipid content is acceptable. The cultures obtained a lipid content of almost 30 %. But consider that the total amount of esterified lipids was very low compared to the value achieved at pH 7. Furthermore, the biomass productivity was influenced by the low pH.

Table 8: Lipid content and lipid composition of *Stichococcus bacillaris* 158/11 cells cultivated under different pH conditions.

	pH 7		pH 3		pH 9	
	N +	N -	N +	N -	N +	N -
Total lipid %	31.2	39.5	28.5	24.3	14.1	--
Total lipid productivity (mg/L/d)	79.8	--	58.4	--	28.7	--
Linolenate*	5.6	5.0	1.6	1.3	5.4	--
Linoleate*	4.4	4.3	1.7	0.9	1.1	--
Palmitate*	5.6	--	--	--	--	--
Oleate*	23.2	22.4	11.1	3.9	18.8	--
Elaidate*	3.8	5.3	--	--	1.7	--
Total esterified lipids, %	39.8	36.9	14.4	6.1	27.0	--

*mass fraction with respect to the total amount of esterified lipids; %

N +: Nitrogen sufficient conditions

N -: Nitrogen starvation

The lipid analysis was operated in collaboration with Immacolata Gargano.

3.4 Effect of reactor shape

The results and observations of the performed tests were used to compare algal growth and also biomass composition in different reactor shapes under same cultivating conditions. Vertical bubble column photobioreactors and inclined bubble column photobioreactors were operated with 5 % CO_2 added to air and pH 7. The reactors followed a stepwise operation method (batch, fed-batch, and semi-continuous mode).

Figure 17 reports data comparing two cultures carried out adopting a progressive change of operating conditions. Figure 17A indicates a culture grown in vertical bubble column photobioreactors for a cultivation time about 12 weeks. The reactor ran two weeks in the batch mode. The first black dotted line in figure 17A marks the change to the fed-batch cultivation and the start of addition of concentrated medium. The biomass concentration increased from 0.02 g/L up to 0.12 g/L during the batch phase and achieved a steady-state value during the fed-batch phase. On day 42 the system was switched into the semi-continuous cultivation by a weekly replacement of 35 % of the suspension, so that the dilution rate under semi-continuous conditions was 0.05 d^{-1}. The black dashed lines in the figure indicate the replacing operation. After achieving a steady state, the reactor was cultivated in semi-continuous mode for about 6 weeks.

The graph in figure 17B refers to a microalgae growth in inclined square bubble column photobioreactors for about 6 weeks of cultivation time. The culture growth was one week under batch mode. The biomass concentration increased from 0.04 g/L up to 1.65 g/L during the batch phase and achieved a steady-state value during the fed-batch phase. On day 16 the system was switched to the semi-continuous cultivation by replacement of 35 % of the suspension with fresh medium each 5.8 days (average value). The dilution rate under semi-continuous conditions resulted 0.06 d^{-1}. After achieving a steady state, the reactor was cultivated under semi-continuous mode for about 3 weeks. The broth replaced with fresh medium was collected for the lipid analysis.

The beginning of the nitrogen starvation condition is indicated by the black continuous line. The residual biomass was collected for the lipid analysis after stopping the reactors.

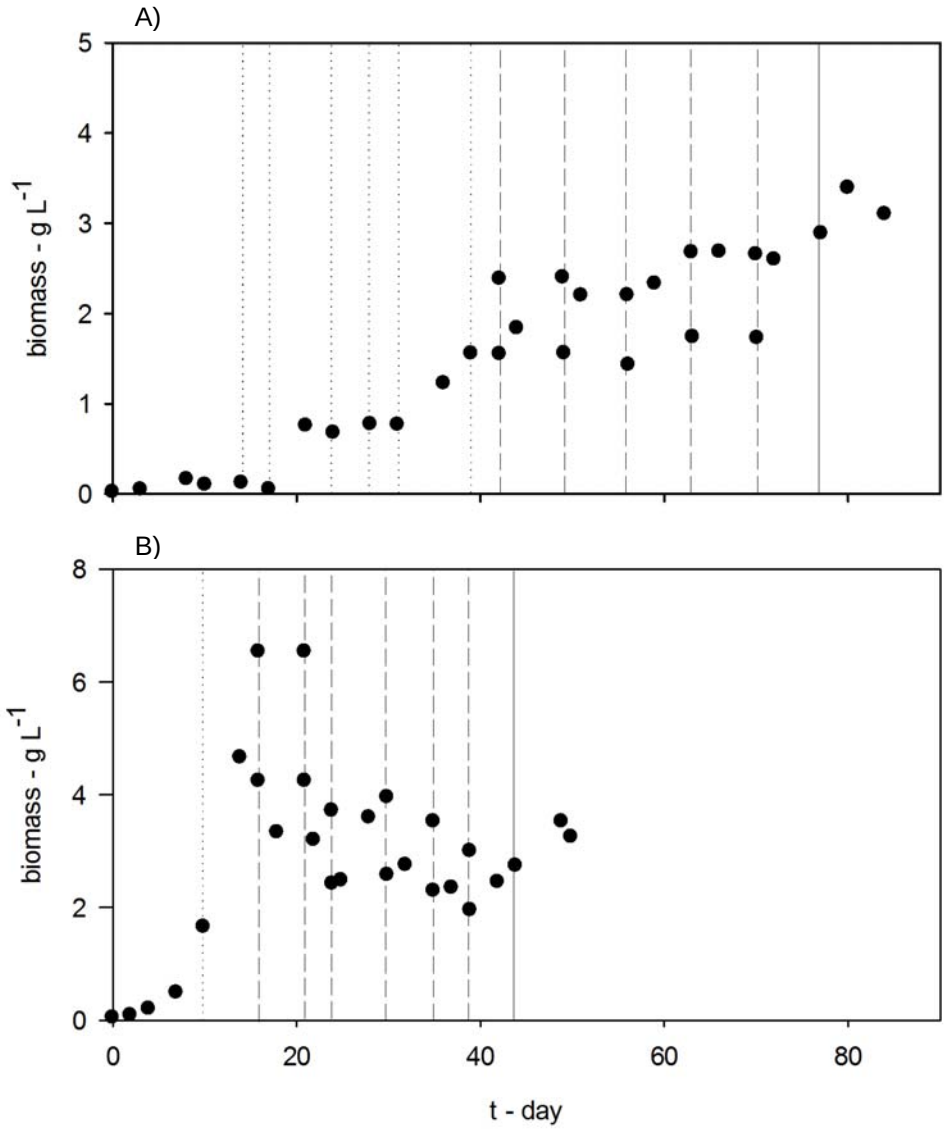


Figure 17: Biomass characteristics of *Stichococcus bacillaris* 158/11 cultures running through batch, fed-batch, and semi-continuous conditions, grown at pH 7, and 5 % additional CO_2 sparging. A) Culture grown in vertical bubble column photobioreactors; B) Culture grown in inclined square bubble column photobioreactors.

The culture grown in the inclined square bubble column photobioreactor achieved the highest biomass concentration with a value about 6.5 g/L at the beginning of the semi-coninuous mode on day 16. In contrast the culture in the vertical bubble column photobioreactor obtained the highest biomass concentration (about 3.4 mg/L) at the end of the run. Table 9 reports data concerning biomass and cell composition at semi-continuous tests in both reactor shapes.

Table 9: Results obtained in different reactor shapes in steady-state (5 % CO_2, pH 7)

	Vertical bubble column photobioreactors	Inclined square bubble column photobioreactors
Biomass (g/L)	2.48	4.29
Biomass productivity (mg/L/d)	124	257
Total lipid %	34.3 (N +)	39.5 (N -)
Total lipid productivity (mg/L/d)	42.5	79.8

N +: Nitrogen sufficient conditions

N -: Nitrogen starvation

Reactor shape may have an influence on the growth rate and the composition of algal cells. The effects of shear stress and light limitation due to the reactor design could be possible causes.

Microalgal cultures obtained their maximal biomass concentration at a defined value although enough nutrients were accessible in the medium. The biomass productivity could be an indicator for a light limitation due to the fact that it was approximately the same value in all vertical bubble column photobioreactors (Table 5). For example, at an optical density of 4 only 0.018 % of incident light passes through the first centimetre of algal culture. A much higher optical density than 4 was achieved during the tests. So, almost all the light was absorbed by the cells at the surface area without entering the centre of the reactors. A possibility to offer light also to inner layers would be an increase of light intensity. Optimizing the reactor shape would be another method.

The biomass productivity at pH 7 and with 5 % CO_2 was higher in the inclined square bubble column photobioreactors than in the vertical bubble column photobioreactors (Table 9). Perhaps the differential reactor shape and a better mixing mode were reasons for a higher biomass concentration (Figure 18). *Stichococcus bacillaris* 158/11 cells passed the light irradiation about every 2–3 seconds in the inclined square bubble column photobioreactors. The algal cells, which were grown in the vertical bubble column photobioreactors, probably spent more time (minutes) in the dark layer in the centre of the photobioreactors, where no light enters.

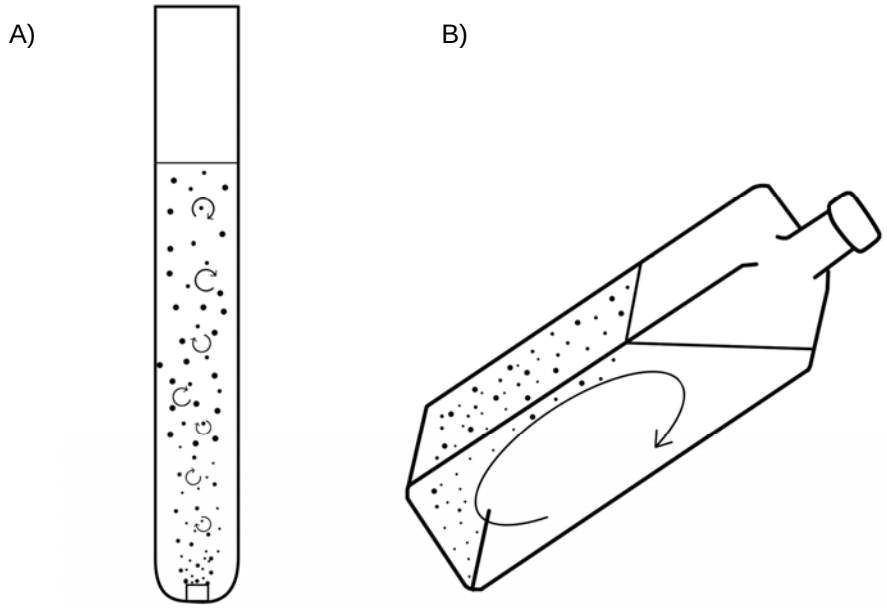


Figure 18: Schematic overview of the different reactor shapes and their bubbling mechanisms. The vertical bubble column photobioreactor (A) produces much turbulence, also in the middle of the reactor where the light is completely missing. An overall mixing takes place but needs more time compared to the inclined square bubble column photobioreactors (B) which generate a gentle mixing.

Shear stress due to the bubbling could be another influence for the algal growth rate. Figure 18 illustrates the mixing mode. The inclined square bubble column photobioreactors provided a carefully mixing without a lot of turbulences. The biomass constantly circled in one direction. Bubble size and strength of mixing seemed to be almost insignificant due to the soft stirring. In contrast the vertical bubble column photobioreactors generated much turbulence with different sized bubbles. The airstream went through the reactor distributing bubbles all over the liquid volume. Strong turbulences occurred everywhere inside the broth.

According to Barbosa et al. (2003) bubble rising and bubble bursting are not responsible for algal cell death, but the bubble formation at the sparger and the arising bubble size.

4. Conclusions

Stichococcus bacillaris 158/11 has shown to be a promising strain for the production of biodiesel. Tests showed the investigated strain to be CO_2 and pH tolerant over a broad range. Moreover carbon dioxide enriched air (all investigated concentrations) even stimulated algal growth and led to high fatty acid content. pH 3 and pH 9 negatively influenced the algal growth and the fatty acid content. However, low pH environment over short-time can be used as on-line sterilization strategy as described in Olivieri et al. (2011).

The best results were obtained performing semi-continuous conditions carried out at pH 7 and sparged with CO_2-enriched air (5 % CO_2). The biomass and lipid productivity exceeds results reported in literature. Rodolfi et al. (2008) compared several algal strains by analyzing the biomass productivity, lipid content as well as the lipid productivity. The author demonstrated *Nannochloropsis* with a high lipid productivity of 61 mg/L/d while our investigated strain *Stichococcus bacillaris* 158/11 achieved a productivity of 79.8 mg/L/d. The lipid productivity in our study was also higher than in the previous study of Olivieri et al. (2011), where they investigated the same strain. They achieved a lipid productivity of 14 mg/L/d. It should be noted, that a higher productivity was obtained in the inclined bubble column photobioreactors compared to the vertical bubble column photobioreactors.

Our results suggest that *Stichococcus bacillaris* 158/11 is a potential source of biodiesel. Improvements considering the lipid productivity could be achieved using increased cultivation volume and more efficient mixing. The high yield of biomass and lipids obtained in the experiments encourages one to pursue further studies. A new transesterification method is currently developed by our research group. The new method is supposed to reduce cost-determining factors by avoiding the use of expensive solvents, and/or recovery of solvents for further transesterification operations.

Another aim refers to scale-up the process to open raceway ponds with paddle wheels. Future studies could include the improvement of photosynthetic activity by genetic engineering of the photosynthetic complex to raise the light intensity threshold at which the photosynthetic machinery is light saturated.

5. Registers

5.1 Register of Tables

5.2 Register of Figures

5.3 Notation

ChlA	Chlorophyll A content, mg/g
°C	degree centigrade
D	dilution rate, d^{-1}
d	day
et al.	et alteri
FAME	fatty acid methyl ester
Φ	photosynthetic activity, $mg_{O2}/g_X\ h^{-1}$
ha	hectare
h	hour
IC	inorganic carbon, mg/L
k	kilo
L	liter
µ	micro
min	minute
m	milli
M	molar
OUR	specific respiration rate, $mg_{O2}/g_X/h$
Pa	Pascal
pH	negative decimal logarithm of the hydrogen ion activity in a solution
rpm	rounds per minute
s	second
TAG	triacylglycerol
TN	total nitrogen, mg/L
TOC	total organic carbon, mg/L
X	cell concentration, g/L

6. References

Allen C. A. W., Watts K. C., Ackman R. G. and Pegg M. J.: Predicting the viscosity of biodiesel fuels from their fatty acid ester composition. Fuel 78:1319-1326, 1999.

Anderson G. Q. A. and Fergusson M. J.: Energy from biomass in the UK: sources, processes and biodiversity implications. Ibis 148:180–183, 2006.

Barbosa M. J., Hadiyanto and Wijffels R. H.: Overcoming shear stress of microalgae cultures in sparged photobioreactors. Biotechnology and Bioengineering 85:78-85, 2004.

Bayless D. J., Kremer G. G., Prudich M. E., Stuart B. J., Vis-Chiasson M. L., Cooksey K. and Muhs J.: Enhanced practical photosynthetic CO_2 mitigation. Proceedings of the first national conference on carbon sequestration, 5A4, 1-14, 2001.

Benemann J. R., Weissmann J. C., Koopmann B. L. and Oswald W. J.: Energy production by microbial photosynthesis. Nature 268 (5615):19-23, 1977.

Blanco-Canqui H. and Lal R.: Soil and crop response to harvesting corn residues for biofuel production. Geoderma 141:355–362, 2007.

Broady P. A: Survey of algae and other terrestrial biota at Edward VII Peninsula, Marie Byrd Land. Antarctic Science 1 (3):215–224, 1989.

Camacho Rubio F., Acién Fernández F. G., Sánchez Pérez J. A., García Camacho F. and Molina Grima E.: Prediction of dissolved oxygen and carbon dioxide concentration profiles in tubular photobioreactors for microalgal culture, Biotechnology and Bioengineering, 62:71–86, 1999.

Campbell N. A. and Reece J. B., Biologie, 2006, Pearson Studium, Munich, Germany, ISBN 3-8273-7180-5

Chisti Y.: Biodiesel from microalgae. Biotechnology Advances 25:294–306, 2007.

Chiu S. Y., Kao C.Y., Tsai M.T., Ong S.C., Chen C.H. and Lin C.S.: Lipid accumulation and CO_2 utilization of *Nannochloropsis oculata* in response to CO_2 aeration. Bioresource Technology 100: 833–838, 2009.

De Swaaf M. E., Sijtsma L. and Pronk J. T.: High-cell-density fed-batch cultivation of the docosahexaenoic acid producting marine alga *Crypthecodinium cohnii*. Biotechnology and Bioengineering 81 (6):666-672, 2003.

Field C. B., Campbell J. E. and Lobell D. B.: Biomass energy: the scale of the potential resource. Trends in Ecology and Evolution 23 (2):65-72, 2007.

Figueroa F. L., Israel A., Neori A., Martínez B., Malta EJ., Ang P., Inken S., Marquardt R. and Korbee N.: Effects of nutrient supply on photosynthesis and pigmentation in *Ulva lactuca* (Chlorophyta): responses to short-term stress. Aquatic Biology 7:173-183, 2009.

Flickinger M. C. and Drew S. W., Algal culture, in Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis, and Bioseparation, Volumes 1-5, 1999, Wiley Biotechnology Encyclopedias, New York, USA, ISBN 0-471-13822-3

Francisco E. C., Neves D. B., Jacob-Lopes E. and Franco T. T.: Microalgae as feedstock for biodiesel production: Carbon dioxide sequestration, lipid production and biofuel quality. Journal of Chemical Technology & Biotechnology 85:395–403, 2010.

Ge Y., Liu J. and Tian G.: Growth characteristics of *Botryococcus braunii* 765 under high CO_2 concentration in photobioreactor. Bioresource Technology 102:130-134, 2011.

Grobnelaar J. U., Algal Nutrition – Mineral Nutrition, in Handbook of Microalgal Culture, 2004, Ed. A. Richmond, Blackwell Publishing, Iowa, USA, ISBN 0–632–05953–2

Huertas E., Montero O. and Lubia'n L. M.: Effects of dissolved inorganic carbon availability on growth, nutrient uptake and chlorophyll fluorescence of two species of marine microalgae. Aquacultural Engineering 22:181-197, 2000.

Jiang L., Luo S., Fan X., Yang Z. and Guo R.: Biomass and lipid production of marine microalgae using municipal wastewater and high concentration of CO_2. Applied Energy 88 (10):3336-3341, 2011.

Khozin-Goldberg I., Bigogn C., Shrestha P. and Cohen Z.: Nitrogen starvation induces the accumulation of arachidonic acid in the freshwater green alga *Parietochloris incisa* (Trebuxiophyceae). Journal of Phycology 38:991–994, 2002.

Kliphuis A. M. J., Martens D. E., Janssen M. and Wijffels R. H.: Effect of O_2:CO_2 Ratio on the Primary Metabolism of *Chlamydomonas reinhardtii*. Biotechnology and Bioengineering 108:2390-2402, 2011.

Knothe G.: Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. Fuel Processing Technology 86:1059-1070, 2005.

Li Y., Horsman M., Wang B., Wu N. and Lan C. Q.: Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochloris oleoabundans*. Applied Microbiology and Biotechnology 81:629-636, 2008.

Maeda K., Owada M., Kimura N., Omata K. and Karube I.: CO_2 fixation from the flue gas on coal-fired thermal power plant by microalgae. Energy Conversion and Management 36 (6-9):717-720, 1995.

Masojídek J., Koblízek M. and Torzillo G., Photosynthesis in microalgae, in Handbook of Microalgal Culture, 2004, Ed. A. Richmond, Blackwell Publishing, Iowa, USA, ISBN 0–632–05953–2

McKendry P.: Energy production from biomass (part 1): overview of biomass. Bioresource Technology 83:37–46, 2002

Molina E., Fernández J., Acién F. G. and Chisti Y.: Tubular photobioreactor design for algal cultures, Journal of Biotechnology 92:113–131, 2001.

Molina Grima E., Acién Fernández F. G., García Camacho F., Camacho Rubio F. and Chisti Y.: Scale-up of tubular photobioreactors. Journal of Applied Phycology 12:355–368, 2000.

Molina Grima E., Acién Fernández F. G., García Camacho F. and Chisti Y.: Photobioreactors: light regime, mass transfer, and scale-up. Journal of Biotechnology 70:231–247, 1999.

Naik S. N., Goud V. V., Rout P. K. and Dalai A. K.: Production of first and second generation biofuels: A comprehensive review. Renewable and Sustainable Energy Reviews 14:578–597, 2010.

Nichols H. W., Growth media – freshwater, in Handbook of Phycological Methods, 1973, Ed. Stein J. R. Cambridge University Press, USA, ISBN 0 521 20049 0

Olaizola M.: Microalgal removal of CO_2 from flue gases: Changes in medium pH and flue gas composition do not appear to affect the photochemical yield of microalgal cultures. Biotechnology and Bioprocess Engineering 8:360-367, 2003.

Olivieri G., Marzocchella A., Andreozzi R., Pinto G. and Pollio A.: Biodiesel production from *Stichococcus* strains at laboratory scale. Journal of Chemical Technology & Biotechnology 86:776–783, 2011.

Pollio A., Aliotta G., Pinto G., Paternò M. and Bevilacqua A.: Ecophysiological characters and biochemical composition of *Stichococcus bacillaris* Naegeli strains from low pH environments. Algological Studies 84:129-143, 1997.

Posten C. and Schaub G.: Review: Microalgae and terrestrial biomass as source for fuels – A process view. Journal of Biotechnology 142:64–69, 2009.

Ramos M. J., Fernández C. M., Casas A., Rodríguez L. and Pérez A.: Influence of fatty acid composition of raw materials on biodiesel properties. Bioresource Technology 100:261-268, 2009.

Rashid U. and Anwar F.: Production of biodiesel through base-catalyzed transesterification of Safflower oil using an optimized protocol. Energy & Fuels 22:1306-1312, 2008a.

Rashid U. and Anwar F.: Production of biodiesel through optimized alkaline-catalyzed transesterification of rapeseed oil. Fuel 87:265–273, 2008b.

Rebolloso Fuentes M. M., García Sánchez J. L., Fernández Sevilla J. M., Acién Fernández F. G., Sánchez Pérez J. A. and Molina Grima E.: Outdoor continuous culture of *Porphyridium cruentum* in a tubular photobioreactor: quantitative analysis of the daily cyclic variation of culture parameters. Journal of Biotechnology 70:271-288, 1999.

Rodolfi L., Zittelli G. C., Bassi N., Padovani G., Biondi N., Bonini G. and Tredici R.: Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnology and Bioengineering 102 (1):100-112, 2009.

Schenk P. M., Thomas-Hall S. R., Stephens E., Marx U. C., Mussgnug J. H., Posten C., Kruse O. and Hankamer B.: Second generation biofuels. High-efficiency microalgae for biodiesel production. Bioenergy Research 1 (1):20-43, 2008.

Searchinger T., Heimlich R., Houghton R. A., Dong F., Elobeid A., Fabiosa J., Tokgoz S., Hayes D. and Yu TH.: Use of U.S. croplands for biofuels increases greenhouse gases through emissions from land-use change. Science 319:1238-1240, 2008

Sergeenko T. V., Muradyan E. A., Pronina N. A., Klyachko-Gurvich G. L., Mishina I. M. and Tsoglin L. N.: The effect of extremely high CO_2 concentration on the growth and biochemical composition of microalgae. Russian Journal of Plant Physiology 47:722-729, 2000.

Solovchenko A. E., Khozin-Goldberg I., Didi-Cohen S., Cohen Z. and Merzlyak M. N.: Effects of light intensity and nitrogen starvation on growth, total fatty acids and arachidonic acid in the green microalga *Parietochloris incise*. Journal of Applied Phycology, 20:245–251, 2008.

Spolaore P., Joannis-Cassan C., Duran E. and Isambert A.: Review: Commercial Applications of Microalgae. Journal of Bioscience and Bioengineering 101 (2):87–96, 2006.

Tang D., Han W., Li P., Miao X. and Zhong J.: CO_2 biofixation and fatty acid composition of *Scenedesmus obliquus* and *Chlorella pyrenoidosa* in response to different CO_2 levels. Bioresource Technology 102:3071–3076, 2011.

Tomaselli L., The microalgal cell, in Handbook of Microalgal Culture, 2004, Ed. A. Richmond, Blackwell Publishing, Iowa, USA, ISBN 0–632–05953–2

Tredici M. R., Bioreactors, photo, in Encyclopedia of Bioprocess technology: Fermentation, Biocatalysis, and Bioseparation, Volumes 1-5, 1999, Wiley Biotechnology Encyclopedias, New Yeark, USA, ISBN 0-471-13822-3

Urabe J. and Sterner R. W.: Regulation of herbivore growth by the balance of light and nutrients. Proceedings of the National Academy of Sciences 93:8465-8469, 1996.

Usui N. and Ikenouchi M.: The biological CO_2 fixation and utilization project by RITE(1) – Highly-effective photobioreactor system. Energy Conversion and Management 38:487-492, 1997.

Vörös L. and Padisàk J.: Phytoplankton biomass and chlorophyll-a in some shallow lakes in central Europe. Hydrobiologia 215:111-119, 1991.

White E. and Payne G. W.: Chlorophyll production, in response to nutrient additions, by the algae in Lake Taupo water. New Zealand Journal of marine & freshwater research 11:501-507, 1977.

Zhekisheva M., Boussiba S., Khozin-Goldberg I., Zarka A. and Cohen Z.: Accumulation of the oleic acid in *Haematococcus pluvialis* (Chlorophyceae) under nitrogen starvation or high light is correlated with that of astaxanthin esters. Journal of Phycology 38:325-331, 2002.