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Growth physiology and photosynthetic performance of green microalgae mass culture grown in a thin-layer cascade  

Master’s Thesis  

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## Contents

1 Introduction 6

2 Motivation for microalgal mass production 7
   2.1 Technologies for microalgal mass production .......................... 7
   2.1.1 Raceway ponds and cascade systems ................................. 9
   2.1.2 Photobioreactors .................................................... 11
   2.2 Mixing and light/dark cycles in photobioreactors ....................... 13
   2.3 Supply of carbon dioxide .............................................. 14
   2.4 Photosynthetic efficiency in mass cultures ............................ 14

3 In vivo chlorophyll fluorescence 16
   3.1 Saturation pulse method of quenching analysis with the PAM fluorometer 17
   3.1.1 Photochemical processes ........................................... 19
   3.1.2 Nonphotochemical processes ....................................... 22
   3.1.3 Relative quenching coefficients ................................... 23

4 Material and methods 24
   4.1 Cultivation unit ....................................................... 24
   4.2 Organism and culture conditions ...................................... 25
   4.2.1 Supply of carbon dioxide ......................................... 26
   4.3 Sampling and measurements .......................................... 27
   4.3.1 Modulated fluorescence measurements .............................. 27
   4.4 Calculation of growth parameters ................................... 28
   4.5 Experiment for dark storage ......................................... 29

5 Results 30
   5.1 Biomass growth and yield ............................................ 30
   5.2 Culture pH ............................................................. 30
   5.3 Photochemical activity ............................................... 30
   5.4 Photosynthetic efficiency .......................................... 32
   5.5 Dark storage ......................................................... 33

6 Discussion 35
   6.1 Biomass yield ......................................................... 35
   6.2 Fluorescence parameters and changes in culture density ............ 36
      6.2.1 \( F_v/F_m \) for nutrient-replete culture ....................... 37
   6.3 Culture pH and CO\(_2\) availability ................................. 37
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.4 Dark storage</td>
<td>38</td>
</tr>
<tr>
<td>7 Conclusion</td>
<td>39</td>
</tr>
<tr>
<td>8 Rohevetika kasvufüsioloogia ja fotosünteesivõime</td>
<td>40</td>
</tr>
<tr>
<td>masskultiveerimisel &quot;õhukese kihi&quot; kaskaadil</td>
<td></td>
</tr>
<tr>
<td>9 Acknowledgements</td>
<td>41</td>
</tr>
<tr>
<td>References</td>
<td>42</td>
</tr>
</tbody>
</table>
Abbreviations:

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL</td>
<td>Actinic light</td>
</tr>
<tr>
<td>Chl</td>
<td>Chlorophyll a</td>
</tr>
<tr>
<td>DW</td>
<td>Dry weight</td>
</tr>
<tr>
<td>ML</td>
<td>Measuring light</td>
</tr>
<tr>
<td>PAM</td>
<td>Pulse-Amplitude-Modulation</td>
</tr>
<tr>
<td>PAR</td>
<td>Photosynthetically active radiation</td>
</tr>
<tr>
<td>PE</td>
<td>Photosynthetic efficiency</td>
</tr>
<tr>
<td>PS I</td>
<td>Photosystem I</td>
</tr>
<tr>
<td>PS II</td>
<td>Photosystem II</td>
</tr>
<tr>
<td>QA</td>
<td>Primary quinone-type electron acceptor of PS II</td>
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<tr>
<td>RC</td>
<td>Reaction center</td>
</tr>
<tr>
<td>SP</td>
<td>Saturation pulse</td>
</tr>
<tr>
<td>WW</td>
<td>Wet weight</td>
</tr>
</tbody>
</table>
1 Introduction

Cultivation of microalgae using man-made technology is not new. The concept of using microalgae as a source of fuel was proposed in the early 1950s when researchers visualized a process in which waste water could be used as a medium and source of nutrients for algae production. Since, devising and developing technical apparatus, cultivation procedures and choosing algal strains has been a major challenge for industrial microalgal culturing. The major goal is to achieve higher yields so that the energy output of algae cultivation exceeds the energy inputs.

I aim to correlate the culture’s productivity to photochemical activities of the freshwater microalga *Scenedesmus obliquus* (*Chlorophyceae*) grown in a thin-layer cascade in order to study the growth physiology of a mass culture. Green algae are preferred over other groups of algae as they are tolerant for a wide range of environmental conditions, they permit the use of non-arable land and non-potable water, have a fast growth rate, resistant cell wall and bear some turbulence. In fact, a large part of algal biotechnology is focused particularly on green algae. This study focuses on mass cultivation of microalgae for the production of biodiesel. *Scenedesmus obliquus* qualifies well as it presents an adequate biochemical composition, namely in terms of lipids and polyunsaturated fatty acids (Gouveia & Oliveira, 2009).

This experimental study aims to (i) evaluate approximate biomass yields (g dry weight m\(^{-2}\) d\(^{-1}\)) of *S. obliquus* mass culture grown in a prototype of a thin-layer open-culture system, (ii) investigate the significance of biomass loss during dark storage when instead of photosynthesis (and cell growth) dark respiration occurs and (iii) examine if and how changes in light availability (changes in the culture density) may influence chlorophyll \(\alpha\) fluorescence parameters obtained from the saturation pulse method of quenching analysis. This thesis was previousead by my bachelor thesis (Ojamäe, 2008), which gave an overview of the daily yields of three species of green microalgae grown in the batch cultures at different light intensities. Due to the high culture layer thickness (3.5 cm) it was difficult to evaluate the light effect on biomass yields as in thick cultures the light can penetrate approximately 1 cm deep. The middle layer of algae was in darkness and this could have reduced daily yields. In the present study the culture layer thickness has been brought to 1 cm and therefore equal diffusion of light to the cells is achieved better.

The future perspective is to develop technologies for commercial microalgal mass production in Estonia. Relatively suitable and unique conditions are in Northeastern Estonia near Narva power plants. In this region cheap non-arable land, concentrated source of
carbon dioxide (flue gases from power plants) and availability of cooling and warming water. Cool water is available from the river of Narva and warm cooling water from the power plant.

2 Motivation for microalgal mass production

Microalgae are a source for a vast array of valuable compounds: proteins, carotenoids, fatty acids, polysaccharides, lipids and microelements. Thus, algae have a large biotechnological potential for producing valuable substances for biotechnological processes — biofuels, pharmacy industries, cosmetics, food and feed. Current commercial microalgae production is focused on a few high-value products used mainly for human nutritional supplements. Most algal extracts produced for pharmacy and health food industry are supplied as tablets, capsules or health drinks (Pulz & Gross, 2004). Microalgae are also used as live feeds in aquaculture, and in waste-water treatment systems.

Our greatest challenge is to find means to reduce the extraction and combustion of fossil fuels. The ∼10 terrawatts year\(^{-1}\) (1 TW = 10\(^{12}\) W) of fossil fuels we use today is divided approximately 43% from petroleum, 40% from coal, and 17% from natural gas (Rittmann, 2008). From algae biomass three main options for fuel production exists: (i) production of methane gas, (ii) production of ethanol and (iii) production of biodiesel (Sheehan et al., 1998). Usually biodiesel is produced form oleaginous crops, such as rapeseed, soybean, sunflower and palm. However, the use of microalgae can be a suitable alternative feedstock for next generation biofuels because certain species contain high amounts of oil, which could be extracted, processed and refined into transportation fuels, using currently available technology. In addition, the concept of coupling a coal-fired power plant with algae cultivation provides an elegant approach to recycle CO\(_2\) from coal combustion into a useable liquid fuel.

For most of these applications, the market is still developing and the biotechnological use of microalgae will extend into new areas. Considering the enormous biodiversity of microalgae and recent developments in genetic engineering, this group of organisms represents one of the most promising sources for new products and applications.

2.1 Technologies for microalgal mass production

An efficient mechanism for algal mass production can not be properly designed without adequate knowledge of the physiology in mass culture of the organisms to be cultivated.
Primarily, microalgal mass production technologies can be divided into two large groups: open-culture systems and closed culture systems. Open-culture systems are almost always located outdoors and rely on natural light for illumination. Closed systems (in this context – photobioreactors) may be located indoors or outdoors, but outdoor location is more preferable because it can make use of sunlight. The main design criteria for culture systems include: light availability in the culture, orientation and inclination, surface-to-volume ratio and gas exchange.

The primary objective for photobioreactors is to expose all cells equally to intermittent illumination. This means to have cells move in and out of the illuminated volume (zone). Ideally, this approach would allow to use light most efficiently — cells in the illuminated part of the photobioreactor should be optimally exposed to light for duration required for the light reaction (ns to µs), then be replaced by cells from the dark zone. Upon being moved into the dark zone, the cells should optimally be returned into the illuminated zone when the dark reaction is complete (1 – 15 ms) and the photosynthetic units being, once again, receptive to photons. However, in practice, it is impossible to move every cell mechanically over a distance of the order of a few nm, within a few ns to a few µs. Cells in mass cultures are always exposed to wasteful dark periods. This subject is primarily targeted when designing a photobioreactor (also see Chap. 2.2) because improving the light regime will result in higher biomass yields (Richmond, 2004).

Photobioreactors may be oriented and inclined at various angels to the sun and thus offer the possibility to vary irradiance at the reactor surface. Generally, sun-oriented systems (south-facing and tilted so as to intercept maximum solar radiation) achieve higher cell concentrations. At low latitudes the influence of the angle of inclination with reactors placed in an east-west direction can be negligible (Lee & Low, 1991). On the other hand, it has been shown that the tilt angle exerts a significant effect on yields at higher latitudes (Tredici & Chini Zittelli, 1997).

The ratio between the illuminated surface area of a culture system and its volume (s/v) determines the amount of light that enters the system per unit volume. Generally, the higher the s/v, the higher the cell concentration and the higher the yields to harvest. High cell concentrations reduce the cost of harvesting. For this, culture systems with high s/v are generally preferred.

Carbon nutrition and removal of photosynthetically accumulated oxygen are also major factors that need to be taken into account when designing a system. In mass cultures cells easily suffer in carbon dioxide starvation as CO\textsubscript{2} diffuses from air to water slower than it can be assimilated by microalgae. Therefore, to receive higher yields, CO\textsubscript{2} is sup-
Figure 1: Open-culture systems for cultivation of microalgae. *Left panel:* a raceway pond with a depth of 20 – 30 cm. Culture flows along the surface of the earth and the flow is provided by the paddlewheels. *Right panel:* schematic diagram of a cascade where a thin layer (*ca.* 1 cm) of suspension flows along declined surface. The experimental unit consists of two declined cultivation lanes made of glass plates supported by a scaffolding.

Plyied from other sources, e.g. from power plants flue gases, which contain concentrated amount of carbon dioxide. In contrast, dissolved oxygen concentrations equivalent to 4–5 times saturation with respect to air are toxic to many phototrophs. The maintenance of $O_2$ levels below the toxic concentration requires frequent degassing (culture mixing) and thus high flow rates.

The size of culture systems is measured in terms of surface area (as opposed to volume), since surface area is critical to capturing sunlight. Their productivity is measured in terms of biomass produced per day per unit of available surface area. For mass cultivation autotrophic, heterotrophic and mixotrophic microalgae production is feasible (*Brennan & Owende, 2010*). In this thesis mechanisms used for photoautotrophic algae production are considered only.

### 2.1.1 Raceway ponds and cascade systems

The most simple example of a raceway pond consists of a shallow ditch dug into the ground and covered with plastic sheets draped up the sloping earth embankments. Water, algae and nutrients circulate around the racetrack and paddlewheels provide the flow (Fig. 1, left panel). This construction is relatively inexpensive and therefore is favoured in commercial plants. Large raceway ponds can not be operated at a water level much lower than 15 cm, otherwise a severe reduction of flow and turbulence would occur. Usually they are not deeper than 20 – 30 cm because of the need to keep the algae exposed to sunlight and
the limited depth to which sunlight can penetrate the pond water. Generally, in raceway ponds, cell concentrations of 0.1 – 0.5 g dw L\(^{-1}\) are achieved (Masojidek et al., 2011) and long-term yields in large commercial raceways rarely exceeds 12 – 13 g m\(^{-2}\) d\(^{-1}\) (Tredici, 2004).

The cost of microalgae biomass production in commercial raceway ponds ranges from $8 to $15 per kg of dry weight (van Beilen, 2010). This calculation goes back in ten years (Lee, 2001) and numbers are not corrected for inflation but is still two orders of magnitude more expensive than energy crop biomass from conventional agriculture or forestry (van Beilen, 2010). Over 90% of the world’s commercial microalgae production uses raceway type ponds. One of the first large commercial microalgae production plants using raceway ponds is located in California, USA, with 30 production ponds, each somewhat over 6000 m\(^{2}\), with a total pond area of about 20 ha (Earthrise Farms, www.earthrise.com). The plant grows *Arthrospira* (Cyanophyceae) for human consumption, with production estimated at over 500 t year\(^{-1}\). Production is mainly limited by the cold, nighttime temperatures that reduce the growing season for this warm-loving species to between 7 – 8 months a year (Spolaore et al., 2006).

In cascade systems, turbulence is created by gravity, the culture suspension flowing from the top to the bottom of sloping surfaces (Fig. 1, right panel). This type of systems allow adoption of very thin culture layers (less than 1 cm), facilitating higher cell concentrations and a higher surface-to-volume ratio (\(~ 100 \text{ m}^{-1}\)) compared to raceway ponds (<10 m\(^{-1}\)) (Richmond, 2004). Similarly to raceway ponds, these systems are limited by several problems, among which sedimentation of the cells at points of lower turbulence, strong evaporative losses, high rates of CO\(_2\) desorption, and considerable requirement of energy for continuously pumping the culture to the head of the declined surface. In a 224 m\(^{2}\) cascade of this type (thickness of algal culture layer 6 mm and s/v = 102 m\(^{-1}\)) operated in Trebon, Czech Republik, obtained yields of 23.5 and 11.1 dw m\(^{-2}\) d\(^{-1}\) with *Chlorella* (Chlorophyceae) in July and September, respectively. In a 2600 m\(^{2}\) plant installed in Roupite (Bulgaria), yields of 18 and 25 g dw m\(^{-2}\) d\(^{-1}\) were attained with *Arthrospira* and *Scenedesmus*, respectively (Fournadzhieva & Pillarsky, 1993).

Most microalgae cannot be maintained long enough in outdoor open systems because of the risk of contamination by fungy, bacteria and protozoa, and competition by other microalgae that tend to dominate regardless of the original species used as inoculum (Richmond, 1999). Excessive evaporative losses, particularly in hot dry climates, and lack of temperature control are other major drawbacks of open systems. However, raceway ponds are used by 98% of commercial microalgae production systems (Benemann, 2008).
2.1.2 Photobioreactors

Photobioreactors can be defined as culture systems for phototrophs in which a great proportion of the light (> 90%) does not impinge directly on the culture surface, but has to pass through the transparent reactor’s wall to reach the cultivated cells. Consequently, photobioreactors do not allow, or strongly limit, direct exchange of gases and contaminants (microorganisms, dust, etc.) between the culture and the atmosphere. On the basis of design, the main categories of reactors are: (i) flat or tubular; (ii) horizontal, inclined, vertical or spiral; and (iii) manifold or serpentine.

Tubular serpentine photobioreactors are systems in which several straight transparent tubes are connected in series by U-bends to form a flat loop that can be arranged either vertically or horizontally (Fig. 2, left panel). Gas exchange and nutrient addition normally take place in a separate vessel and culture is circulated by means of airlift. For temperature control water spraying can be used or a horizontal system can by immersed in a shallow water pond. Productivities from 20 to 25 g dw m\(^{-2}\) d\(^{-1}\) have been obtained with red alga *Porphyridium cruentum* (Tredici, 2004).

In manifold photobioreactors, a series of parallel tubes are connected at the ends by two manifolds, one for distribution and the other for collection of the culture suspension (Fig. 2, right panel). Length of the tubes is limited to about 40 m due to reduced mass transfer. In comparison to serpentine reactors in which the culture inverts the direction of motion at each turn, manifold reactors allow a significant saving of energy. Volumetric yields of up to 1.3 g dw L\(^{-1}\) d\(^{-1}\) and areal yields of more than 28 g dw m\(^{-2}\) d\(^{-1}\) have been obtained with *A. platensis* (Tredici & Zittelli, 1998). In some cases flexible tubes are spiral
or wound around a cylindrical support — these are called helical photobioreactors.

Flat plate photobioreactors are preferred due to their large illumination surface area. They are usually tilted at the proper angle to maximize solar radiation capture and in some cases they are vertical to the ground (Fig. 3). There are two kind of flat plate photobioreactors that have received much attention — alveolar panels and glass plates. Alveolar panels are constructed from transparent PVC or polycarbonate sheets that are internally partitioned to form narrow channels called alveoli. The panels are usually 3 – 4 cm thick and can be placed horizontally or vertically side by side ca. 20 cm apart to form a compact structure. Glass plates are glass chambers that can be even narrower and are similarly positioned as alveolar panels. This design may seem weak, at first sight, since in this configuration the panels shade each other. However, the point of these vertical "curtains" separated by a space of air is to bring diffused light from above to the bottom parts of the sheets. As discussed before, one of the objectives is to diffuse light equally to the cells and this is could be accomplished by the packed arrangement. Air bubble mixing is adopted by means of two perforated tubes running horizontally along the entire length of the reactor. Daily yields of 1.3 g dw L$^{-1}$ and 28 g dw m$^{-2}$ have been reported with *Chlorella* (Pulz & Scheibenbogen, 1998).

A relatively cheap and disposable vertical photobioreactors are bag or sleeve reactors. These are usually 2 m in height and 30 – 50 cm in diameter, transparent polyethylene bags, sealed at the bottom and hung on an iron structure. Similarly to the open ponds, the major drawback for sleeve reactors is a relatively large dark zone but they have been used to grow *Porphyridium* (Rhodophyceae) and *Dunaliella* (Chlorophyceae) and have obtained a significantly higher yields compared to open ponds (Cohen & Arad, 1989).

Figure 3: Flat plate photobioreactors. *Left panel*: glass plates packed into a compact unit. *Right panel*: alveolar panels tilted at a slight angle to maximize sunlight capture.
Photobioreactors are more expensive to build and operate than open-culture systems. The production cost of algal biomass is $30 – $70 kg$^{-1}$ (Molina Grima et al., 2003, Olaizola, 2003), which is at least an order of magnitude higher than fruit and vegetables produced in greenhouses (van Beilen, 2010). Thus, photobioreactors will not have a significant impact in the near future on any product that can be attained in large outdoor raceway technologies. However, photobioreactors and open ponds should not be viewed as competing technologies. The real competing technology is genetic engineering. Bringing into obligate photoautotrophs the capacity to use organic molecules as a source of energy and carbon is no more a remote target.

2.2 Mixing and light/dark cycles in photobioreactors

Mixing is necessary to prevent cells from settling, to avoid thermal stratification, to distribute nutrients, to improve gas exchange between the culture medium and the air and most importantly — to ensure that cells experience alternating periods of light and darkness of adequate length. The latter is most complicate to resolve and needs to be discussed in detail. The sunlight falling on the surface of the photobioreactor is absorbed by the algal culture inside, and depending on the biomass concentration, a part of the interior of the bioreactor can be considered a dark zone. If the culture is intensively mixed or aerated, cells travel through the dark and the illuminated zones, i.e. algae will experience a flashing light effect, first demonstrated by Kok (1974).

In a reactor with culture layer thickness of 1 cm, a typical length of the photic zone is 1 mm. On average, a cell would have to move 9 – 10 mm in about 10 ms (dark reactions take about 1 – 15 ms). This corresponds to a lateral velocity of about 90 – 100 cm s$^{-1}$. At best, the velocities in presently used photobioreactors are about 1/3 of that required. This is not enough to significantly reduce wasteful residence time in the dark zone if culture layer thickness is not reduced. On the other hand, too high velocities are harmful to many species, therefore, mixing rate cannot be increased without limit. Thus, reduction of the layer thickness represents the only practical mode by which to make the travel time through the dark zone as close as possible to the time needed for dark reactions.

Millisecond flashes of high intensity light, followed by an approximately five- to ten-fold longer dark period could enhance photosynthetic efficiency (PE). This is thought to result from the fast reduction of the electron acceptors associated to photosystem II (PS

$^{1}$Photic zone — the depth of the water that is exposed to sufficient illumination for photosynthesis to occur.
II) followed by their oxidation in the dark period (Matthijs et al., 1996). This will result in a maximum ‘photon use efficiency’ of PS II during light flashes. However, achieving the flashing light effect in algal cultures requires high mixing power inputs, which is not practical, in particular, for low-cost fuel production processes.

2.3 Supply of carbon dioxide

Carbon constitutes nearly 50% by weight of microalgal dry biomass (Carvalho et al., 2006), therefore, this element is a major nutrient for cell growth. High productivities in mass cultures often lead to carbon dioxide starvation in culture suspension. Fluxes of carbon transfer from open air to the culture are small, even in the presence of extended interface areas or enhanced mixing. Consequently, in common production units, CO$_2$ is fed into the algae growth medium from external sources such as power plants flue gases (Brown, 1996, Doucha et al., 2005), to force light to become the sole growth limiting factor. CO$_2$ capture from flue gas emissions from power plants that burn fossil fuels is adaptable for both raceway pond and photobioreactor systems.

Microalgae also take up inorganic carbon in the forms of HCO$_3^-$ and CO$_3^{2-}$. Detailed studies on the influence of the carbon source upon microalga productivity (Goldman et al., 1981) have indicated that, although HCO$_3^-$ is easily absorbed by cells, it is a poor source of carbon when compared with CO$_2$. Free CO$_2$ enters the cell with ease, whereas HCO$_3^-$ and CO$_3^{2-}$ must be actively transported. Transport of HCO$_3^-$ or CO$_3^{2-}$ and subsequent fixation as free CO$_2$ by ribulose bi-phosphate carboxylase-oxygenase also release 1 or 2 mol OH$^-$ ions, respectively. These ions must be actively transported out of the cell to maintain a neutral cytoplasmic pH. As a result, utilization of CO$_2$ rather than HCO$_3^-$ or CO$_3^{2-}$ is more efficient and would produce higher yields because it is not dependent on expenditure of ATP for active transport (Schiefer & Caldwell, 1982).

2.4 Photosynthetic efficiency in mass cultures

A prerequisite for the development of efficient photobioreactors is the efficient use of light. Photosynthetic efficiency is equal to the ratio between the caloric value of biomass produced and the light energy absorbed. It is usually expressed as percentage but can also be expressed as the biomass yield on light energy in grams of dry weight or protein produced per amount of light energy absorbed (Janssen, 2002). Only photosynthetically
active radiation (PAR\textsuperscript{2}) of wavelengths between 400 and 700 nm, representing 42.3% of the total energy from the light spectrum is captured. The captured energy is used in the Calvin cycle to produce carbohydrates by utilising CO\textsubscript{2} and H\textsubscript{2}O molecules in the process summarised by the reaction equation:

\[ 6\text{CO}_2 + 12\text{H}_2\text{O} + \text{photons} \rightarrow C_6\text{H}_{12}\text{O}_6 + 6\text{O}_2 + 6\text{H}_2 \]

A minimum of 8 light photons is required to generate one molecule of base carbohydrate (CH\textsubscript{2}O), one O\textsubscript{2} molecule and one H\textsubscript{2} (Bolton & Hall, 1991). The average energy content of PAR photons is roughly 218 kJ per mol and the total potential light energy captured by photosynthesis is 1744 kJ per mol of CH\textsubscript{2}O (Brennan & Owende, 2010). Given that the energy contained in one mole of CH\textsubscript{2}O is roughly 467 kJ (one-sixth of the energy content of glucose), the theoretical maximum efficiency of solar-to-chemical energy conversion is approximately 27%. However, since only the PAR (42.3%) can be utilised the maximum PE is estimated at 11.3%.

This theoretical ”upper limit” of PE, calculated by Bolton & Hall (1991) for a green-type plant in bright sunlight, does not account for factors that could reduce it. Theoretical maximum of PE assumes that (i) all available light is absorbed by photosynthetic apparatus without any losses, (ii) photosynthetic units work with 100% efficiency, i.e. every photon absorbed is used in photosynthesis and is not lost by fluorescence or heat dissipation, (iii) no energy is spent on cell maintenance. In reality, most plant’s PE falls well below this theoretical limit, with global averages estimated typically between 1 and 2%.

The objective of photobioreactor scaleups is to develop processes with efficiencies close to the theoretical maximum. In principle this could be done by reducing the amount of light — at low light intensities microalgae convert light with higher efficiencies. An alternative for reducing the light intensity is to increase the biomass concentration. The available light per algal unit is reduced and this results in higher efficiencies if the microalgae culture in the photobioreactor is well mixed (Wijffels, 2008). Studies by Doucha & Livansky (2006, 2009) and Hase et al. (2000) on Chlorella sp. recorded PAR-based PE values of 7%, 6% and 6.5%, respectively. Synechococcus sp. was found to have a PE of between 2% and 4% (Xia & Gao, 2003).

\textsuperscript{2}Photosynthetically active radiation — proportion of the spectrum that, on absorption by a photosynthetic organism, can promote photochemical reactions.
Figure 4: A simple model of the possible fate of light energy absorbed by photosystem II (PS II). Light energy absorbed by chlorophyll $a$ (Chl) molecules associated with PS II can be used to drive photochemistry in which an electron is transferred from the reaction center, P680, to the primary quinone acceptor of PS II, $Q_A$. Alternatively, absorbed light energy can be lost from PS II as Chl fluorescence or heat. The processes of photochemistry, Chl fluorescence, and heat loss occur in competition, such that any increase in the efficiency of one will result in a decrease on the yield of the other two. Modified from Baker (2008).

3 In vivo chlorophyll fluorescence

In vivo chlorophyll a (Chl) fluorescence was introduced in the 1960s as a means of estimating phytoplankton chlorophyll without extracting pigments (Lorenzen, 1966). It gained rapid acceptance and it is now routinely measured at sea using various commercial benchtop and in situ fluorometers. In the 1990s, Chl fluorescence measurements were employed to examine the photosynthetic performance of microalgal mass cultures (Vonshak et al., 1994). In particular, questions were studied associated with the relationship of fluorescence-based measures of PSII photobiochemical activity as a means to estimate primary productivity (Baker 2008, Genty et al. 1989).

Over time Chl fluorescence measurements have become one of the most common and useful monitoring techniques in microalgal photosynthesis research. Its sensitivity and non-intrusiveness makes it a convenient tool to investigate the utilization and dissipation of absorbed light energy in photosynthesis. However, it is much easier to accumulate fluorescence data than to interpret it in an unequivocal way.

Chl fluorescence reflects the performance of photochemical processes in photosystem
II (PS II); the contribution of photosystem I (PS I) emission to the total signal at ambient temperature is rather small and for practical purposes is often neglected. However, in cyanobacteria, the fluorescence of numerous PS I complexes and phycobilisomes contributes significantly to the total signal, which affects the correct determination of certain parameters, e.g. maximum photochemical yield (Masojidek et al., 2004).

Light energy absorbed by Chl molecules associated with PS II leads to the formation of singlet-excited Chl which returns to the ground state via three different pathways: photochemical reactions (P); radiationless energy dissipation into heat (D); and emission of fluorescence (F) (Fig. 4). At constant light intensity, the rate of these three pathways are complementary, i.e., fluorescence intensity is lowered by increased photochemical activity as well as by increased heat dissipation. At constant light intensity, the sum of all three pathways is one:

$$P + F + D = 1$$

Hence, by measuring the yield of Chl fluorescence, information about changes in the efficiency of photochemistry and heat dissipation can be gained (Maxwell & Johnson, 2000).

### 3.1 Saturation pulse method of quenching analysis with the PAM fluorometer

Pulse-Amplitude-Modulated (PAM) measuring principle is based on selective amplification of a fluorescence signal which is measured with the help of intense, but very short (μs-range) pulses of measuring light (ML\(^3\)) which can be applied repetitively at different frequencies. The relative quantum yield of Chl fluorescence is measured by applying low red measuring light with constant pulse amplitude. Each excitation pulse hitting a sample probes the probability of absorbed light energy being re-emitted in the form of fluorescence (Fig. 5). The key observation is that the redox state of the RCs in most photosystems (with the exception of PS I) controls the fluorescence yield. In particular, the way the fluorescence changes with time during continuous illumination, i.e. the fluorescence induction, carries information about the antenna organization. Also, the fluorescence yield at a given moment is related to the fraction of "closed" reaction centers. RCs are referred to as being "closed" if a primary quinone-type electron acceptor of PS II (Q\(_A\)) is reduced and unable to accept further electrons.

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3 Measuring light — short far-red pulses of light that does not induce photochemical reactions. Intensity of 1 μmol quanta m\(^{-2}\) s\(^{-1}\) was used in this thesis.
In photosynthesis research the decrease of the Chl fluorescence yield from its maximum is termed quenching. Thus, maximal fluorescence yield can be observed when various potentially quenching pathways are minimized. These can be separated into photochemical and nonphotochemical part. Photochemical quenching is defined as the decrease of fluorescence due to the increased photochemical reactions, i.e. a charge separation at the RCs of PS II (Schreiber et al., 1986). In photochemical charge separation energy is consumed that would otherwise be lost as fluorescence and/or heat. Nonphotochemical quenching is more generally defined as the decrease of fluorescence due to dissipating excess excitation energy as heat through molecular vibrations (see Chap. 3.1.2).

Under normal physiological conditions, the photosynthetic apparatus of most plants reaches a relatively stable state after dark-adaption, which is characterized by a fully oxidized state of QA (PS II RCs are ”open”), and absence of a transthylakoid proton gradient. Hence, in the dark-adapted state photochemical quenching is maximal and nonphotochemical quenching minimal. The fluorescence yield in this state is low, designated as \( F_0 \).

When PS II is exposed to a strong pulse of saturating light\(^4\) (SP), the RCs undergo charge separation and the electron is moved to QA. When QA is reduced, the photochemistry is transiently blocked. Since the yield of photochemistry becomes zero (\( P = 0 \)), the fluorescence yield and heat dissipation rise (proportionally to the level of the PS II closure) to the maximal value (\( F_m, D_m \)). Then the complementary relationship is simplified to: \( F_m + D_m = 1 \). \( F_m \) is fluorescence emitted from dark-adapted samples when probed with a weak pulse of ML before and after a strong saturating pulse has been given.

When the photosynthetic apparatus is exposed to actinic light\(^5\) (AL), QA can be fully reduced by a SP, such that photochemical quenching is completely suppressed. During the SP, a maximal fluorescence yield, \( F'_m \) is reached, which generally is lower than \( F_m \). \( F_m \) is also reached by applying SP but in a dark adapted state and being exposed to ML before the saturating flash. \( F'_m \) is lower than \( F_m \) due to nonphotochemical quenching which indicates an increased heat dissipation of excitation during continuous illumination. A prime notation (‘) used after a fluorescence parameter indicates that the sample is exposed to light that will drive photosynthesis, i.e., actinic light. The steady-state value of fluorescence during illumination is termed \( F'_t \), usually measured immediately prior to the saturating flash. After the flash, removal of AL and reapplying ML allows measure-

\(^4\)Saturating light — light sufficiently intense to maximally reduce the QA pool in the sample. Intensity of 2600 µmol quanta m\(^{-2}\)s\(^{-1}\) was used in this thesis.

\(^5\)Actinic light — light that is absorbed by the photosynthetic apparatus and will drive electron transport. Intensity of 190 µmol quanta m\(^{-2}\)s\(^{-1}\) was used in this thesis.
Figure 5: Fluorescence quenching analysis by the saturation pulse method. Typical fluorescence levels and definition of basic fluorescence parameters. $F_0$, fluorescence yield of dark-adapted sample; $F_m$, maximal fluorescence yield of dark-adapted sample, reached during a saturation pulse (SP); $F_v$, increase of fluorescence yield during SP (variable fluorescence); $F'_0$, steady-state fluorescence yield observed during illumination; $F'_m$, maximal fluorescence yield of light adapted sample, reached during a SP; $F'_0$, minimal fluorescence yield observed shortly after darkening of illuminated sample and reoxidation of the PS II acceptor side; ML, measuring light; AL, acitinic light; SP, saturation pulse. Modified from Schreiber (2004).

3.1.1 Photochemical processes

By definition, when all RCs are open the probability of excitation escape is low and therefore, fluorescence is minimal, i.e. the $F_0$ level. When the RCs become closed, absorbed excitation energy cannot be directed to photochemistry (i.e. no charge transfer occurs). Under such conditions, fluorescence rises to the maximal value, $F_m$. The maximum quantum yield of PS II photochemistry (described by the fluorescence parameter $F_v/F_m$) is related to the change in fluorescence by (Genty et al., 1989):
Thus, the ratio of the maximum change in variable fluorescence ($F_v$) to the maximum fluorescence yield ($F_m$) in the absence of any background light is a measure of the maximum quantum yield (also named as maximum quantum efficiency) of PS II photochemistry. This efficiency should not be confused with the quantum yield of photosynthesis\(^6\), rather it reflects the probability of PS II reaction centers to use the available excitation energy for photochemistry (reduction of $Q_A$). In healthy microalgal cultures, $F_v/F_m$ ranges from 0.6 for cyanobacteria, to 0.8 for green algae, and varies during the diurnal cycle, depending on the irradiance regime and treatment which determines the physiological status. It has been shown that in dense microalgal mass cultures a mid-day depression in $F_v/F_m$ occurs, because cells are shade-adapted and grow at sub-saturating irradiance and can be stressed at mid-day high irradiance (Masojidek et al., 2011). This is such a widespread phenomenon that $F_v/F_m$ measurements provide a simple and rapid way of monitoring stress. The reasons for stress-induced decreases in $F_v/F_m$ are often complex. Stressing photosynthetic tissues in the light can result in increase of dissipating excitation energy as heat rather than as photochemistry, and causes a decrease in $F_m$. Such quenching may not recover during a short period of dark adaption, or even overnight, and results in decreases in $F_v/F_m$ (Baker, 2008). $F_v/F_m$ can also be significantly lower if the cells are nutrient starved (Kolber et al., 1988, Lippemeier et al., 2001, 2003, Young & Beardall, 2003) or treated with copper for toxic effect assessment (Juneau et al., 2002).

During illumination (AL is on), the PS II quantum yield is lowered by closure of RCs (decrease in photochemical quenching) and by simultaneous heat dissipation (increasing nonphotochemical quenching). The resulting effective quantum yield of PS II corresponds to the fluorescence parameter $\Delta F/F'_m$ (Genty et al., 1989) which is calculated as:

$$\frac{\Delta F}{F'_m} = \frac{F'_{m} - F'_{t}}{F'_m}$$

$\Delta F/F'_m$ is the proportion of absorbed energy being used in photochemistry, i.e. it measures the operating efficiency of PS II, once steady-state electron transport has been achieved at any given light intensity. Closed RCs reduce PS II operating efficiency. Under laboratory conditions, $\Delta F/F'_m$ might correlate with the efficiency of carbon fixation or oxygen production.
Table 1: Chlorophyll (Chl) fluorescence parameters determined from the Chl fluorescence induction kinetics measured with the PAM fluorometer.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$F_0$</td>
<td>ground Chl fluorescence yield induced by ML in the dark-adapted state</td>
</tr>
<tr>
<td></td>
<td>(plastoquinone pool is fully oxidized and doesn’t accept any electrons)</td>
</tr>
<tr>
<td>$F_m$</td>
<td>maximum Chl fluorescence (induced by a saturating pulse, SP) in the dark</td>
</tr>
<tr>
<td></td>
<td>adapted state (PS II reaction centers oxidized, plastoquinone pool fully</td>
</tr>
<tr>
<td></td>
<td>reduced)</td>
</tr>
<tr>
<td>$F_v$</td>
<td>maximum efficiency of PS II reaction centers in dark adapted cells (variable</td>
</tr>
<tr>
<td></td>
<td>fluorescence, $F_m - F_0$)</td>
</tr>
<tr>
<td>$F'_m$</td>
<td>maximum Chl fluorescence in the light-adapted state (induced by an SP flash)</td>
</tr>
<tr>
<td>$F'_t$</td>
<td>steady-state Chl fluorescence yield induced by non-saturating irradiation (AL)</td>
</tr>
<tr>
<td>$F'_0$</td>
<td>ground fluorescence (minimum yield) after AL is turned off (usually measured</td>
</tr>
<tr>
<td></td>
<td>after a far-red pulse when PS II electron acceptors become maximally oxidized)</td>
</tr>
<tr>
<td>$F'_v$</td>
<td>efficiency of PS II reaction centers in light adapted cells (variable</td>
</tr>
<tr>
<td></td>
<td>fluorescence, $F'_m - F'_0$)</td>
</tr>
</tbody>
</table>

Another widely used fluorescence parameter, measuring photochemistry, is photochemical quenching, $q_P$. This parameter estimates the proportion of RCs that are open (Genty et al., 1989). This is calculated as:

$$q_P = \frac{F'_m - F'_t}{F'_m - F'_0}$$

$q_P$ is related to the probability by which excitons in the PS II antenna system will initiate a photochemical reaction. When all RCs are closed, $q_P = 0$, and all absorbed energy will be dissipated as heat or fluorescence. When all RCs are open (theoretical possibility), $q_P = 1$, and all absorbed energy goes to photochemistry, while heat dissipation and fluorescence are zero. The actual $q_P$ is determined by competing nonphotochemical processes, i.e. heat dissipation.

Whilst $\Delta F/F'_m$ gives us an estimation of the efficiency, $q_P$ and $F_v/F_m$ tell us if the efficiency has been altered. Closure of RCs as a result of a high intensity light will alter the value of $q_P$ (decreases) and therefore declines the efficiency of absorbed energy conversion in PS II (decrease in $\Delta F/F'_m$). Changes in the efficiency of nonphotochemical quenching will alter $F_v/F_m$, i.e. if heat dissipation of excessive light becomes less efficient, $F_v/F_m$ (as a proxy of the cell’s overall health) could decrease.
3.1.2 Nonphotochemical processes

Efficient photosynthesis by plants in their natural environment requires optimal utilization of absorbed photons when sunlight is limiting. On the other hand, plants must protect their photosynthetic apparatus against potentially damaging effects of excessive light. In full sunlight, absorption of photons may be several times higher than needed to drive photosynthesis at maximum capacity and therefore it is important to prevent the inactivated reaction centre becoming photodamaged. Nonphotochemical quenching is a process of getting rid of excess light energy that has already been absorbed. This excess light energy is dissipated as heat energy and it occurs in almost all photosynthetic eucaryotes. Heat dissipation helps to regulate and protect photosynthesis in environments in which light energy absorption exceeds the capacity for light utilization (Müller et al., 2001). Nonphotochemical quenching is given by the equation:

\[
q_N = 1 - \frac{F'_m - F'_0}{F_m - F_0}
\]

The difference between \(F'_m\) and \(F'_0\) is denoted as \(F'_v\).

Theoretically, three major components of \(q_N\) can be distinguished (Krause & Jahns, 2004):

1. Energy-dependent (or \(\Delta pH\)-dependent) quenching, \(q_E\), occurs when a high trans-thylakoid proton gradient (acidification of the thylakoid lumen) is built up in high light. When the lumen pH drops below \(ca.\ 6\), a xantophyll pigment, violaxanthin that is associated with the PS II antenna, is converted to zeaxanthin and a protein associated with the PS II, PsbS, becomes protonated (so-called xantophyll cycle). The zeaxanthin is an efficient quencher of excitation energy in the PS II antenna and the rate of heat loss from PS II increases, which increases \(q_E\). When light intensity decreases, deprotonation of PsbS occurs and zeaxanthin epoxidase converts zeaxanthin back to violaxanthin, which decreases \(q_E\). Under limiting light conditions, \(q_E\) is not induced.

2. Photoinhibitory quenching, \(q_I\), is related to photoinhibition of photosynthesis. It becomes prominent at light levels well in excess of that required to saturate photosynthesis and is able to compensate \(q_E\) when it becomes impaired. However, the interpretation of \(q_I\) is problematical since there is evidence for a relatively long-lived type of \(q_E\).
3. State-transition quenching, qT, is important only at low light levels. It is a regulatory process that balances the distribution of excitation energy between the two photosystems and is regulated by phosphorylation and following detachment of peripheral light-harvesting complexes of PS II.

In the literature, the term nonphotochemical quenching (‘NPQ’) is often used synonymously with qE, disregarding the complex nature of nonphotochemical quenching in vivo. Hence, it is usually assumed that the decrease of fluorescence due to nonphotochemical quenching is caused by increased loss of the energy taken up by absorption of photons via thermal dissipation.

3.1.3 Relative quenching coefficients

One would assume that if the fluorescence yield is 0, then the sum of the two quenching coefficients would lead to 1, i.e., that photochemical quenching and nonphotochemical quenching would stand for the full quenching. This is not the case for the most frequently used quenching coefficients qP and qN. Especially under stress conditions (e.g. high temperature or low irradiance), these parameters are not complementary to each other, i.e. the increase of qN does not always result to the decrease of qP. Therefore, the sum of these coefficients may lead to values greater than 1. The reason for this is partly because they refer to the different reference signal (different physiological states) — qP relates to the difference between $\text{F}'_\text{m}$ and $\text{F}'_\text{t}$ ($\text{F}'_\text{r}$) whereas qN relates to the difference between $\text{F}_\text{m}$ and $\text{F}_0$ ($\text{F}_\text{r}$) (Buschmann 1995, 1999) and partly because their derivations are based on different models of PS II connectivity (Rohácek 2002, Kramer et al., 2004). As a solution to that, Buschmann (1995) proposed two new relative quenching coefficients, called relative photochemical and nonphotochemical coefficient, $qP_{rel}$ and $qN_{rel}$ which represent better the balance between the energy dissipation pathways. Both, $qP_{rel}$ and $qN_{rel}$, are normalized to the total fluorescence quenched on going from the dark-adapted to the light-adapted state, i.e., having the same denominator, $\text{F}_\text{m} - \text{F}'_0$:

$$qP_{rel} = \frac{\text{F}'_\text{m} - \text{F}'_\text{t}}{\text{F}_\text{m} - \text{F}'_0}$$

$$qN_{rel} = \frac{\text{F}_\text{m} - \text{F}'_\text{m}}{\text{F}_\text{m} - \text{F}'_0}$$

Relative quenching coefficients can be used for the distinction of quenching mecha-
nisms and to follow the shifts from the de-excitation of energy from the photochemical to the nonphotochemical route. They can be summed up and compared on a common scale ranging between zero and one.

Juneau et al. (2005) proposed a new parameter, the realative unquenched fluorescence, $UQF_{rel}$, which takes into account the fraction of non-quenched fluorescence yield, $F'_{t}$. $UQF_{rel}$ is related to closed PS II RCs not participating in electron transport under continuous irradiation.

$$UQF_{rel} = \frac{F'_{t} - F'_{0}}{F_{m} - F'_{0}}$$

Since this parameter is normalized to $F_{m} - F'_{0}$ it is complementary to the other relative quenching components, $qP_{rel}$ and $qN_{rel}$, under continuous irradiation:

$$qP_{rel} + qN_{rel} + UQF_{rel} = 1$$

The concomitant use of the relative parameters with the usual fluorescence parameters should permit a better evaluation of the effect of any environmental factor on the primary photosynthesis and energy dissipation processes in plants when PAM fluorometry is used as a diagnostic tool to evaluate the physiological state of the plant.

4 Material and methods

4.1 Cultivation unit

The cultivation unit I used was a prototype of a flat plate open-culture system (Fig. 6A) which was conceptually designed to make efficient use of available light. It was made of plexiglass and consisted of two lanes (upper and lower) which were inclined in an opposite direction. The microalgal suspension circulated in a thin layer (10 mm) from the upper to the lower lane, and was collected into the retention tube where it was pumped back to the upper lane. The area exposed to light was estimated to be about 0.08 m$^2$ (two 10 cm $\times$ 40 cm lanes). The pump installed to the prototype was a 4W aquarium pump. The main parameter that affected the design of the prototype was a high surface-to-volume ratio which is crucial for achieving a high biomass density of microalgae.

Originally the cultivation unit design differed from what it had become for the final experiments (Fig. 6B). The large retention tank was exchanged for a tube, that collects
the culture from the lane and channels it directly to the pump. This allowed to decrease
the dark volume as cells accumulated to the corners and typically were exposed to com-
plete darkness. In addition, the aquarium pump was not suspended into the algal culture
anymore and therefore needless heating of the culture was decreased.

4.2 Organism and culture conditions

The microalgae strain used, *Scenedesmus obliquus*, was obtained from the SAG Culture
Collection maintained by the University of Goettingen (Germany). The medium com-
position was designed to reflect the mean content of basic chemical elements in the algal
biomass (*Doucha & Livansky, 2006*). Amounts of added nutrients per 1 kg (DW) of pro-
duced biomass were: NaNO$_3$ = 515.7 g; NaH$_2$PO$_4$·H$_2$O = 40.1 g; MgSO$_4$·7H$_2$O = 29 g;
FeCl$_3$·6H$_2$O = 4.8 g; H$_3$BO$_3$ = 137 mg; CuSO$_4$·5H$_2$O = 158 mg; CoCl$_2$·6H$_2$O = 84 mg;
MnCl$_2$·4H$_2$O = 537 mg; Na$_2$MoO$_4$·2H$_2$O = 25 mg; ZnSO$_4$·7H$_2$O = 440 mg; Na$_3$VO$_4$ = 2.7
mg. River water was used for preparation of the medium. Nutrient mixture was added in
quantities to keep the algae nutrient saturated throughout the experiment. It was assumed
that other nutrients were consumed in proportion to phosphate (P) and thus P concentration
(easy to determine) was used as a proxy to the overall nutrient availability.

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7http://www.uni-goettingen.de/de/45175.html
Algae were grown at room temperature with an irradiance of 200 µmol m$^{-2}$s$^{-1}$ above the culture and at 16-h-light/8-h-dark cycle. The experiments were based on the chemostat principle — while the culture circulated on the prototype, dispensing pumps simultaneously supplied fresh growth medium and removed the homogeneously mixed culture continuously. The approach is based on the observations that culture growth ceases due to the depletion of the growth limiting resource or accumulation of a growth-inhibiting product. To sustain cell growth, the growth-limiting resource needs to be replenished and the growth inhibitory product needs to be removed or diluted by adding fresh culture medium. The medium feed rate was 300 – 700 ml d$^{-1}$ which depended on water loss due to evaporation (195 – 460 ml d$^{-1}$ depending on room temperature) and the rate of harvesting which was 105 – 240 ml d$^{-1}$. The culture volume was kept constant (ca. 700 ml) and the s/v was 114 m$^{-1}$. Duration of the experiment was 22 days.

The biomass of *Scenedesmus* was harvested continuously and the process was fully automated. The pumps for dispensing the growth medium and harvesting the culture were identical Ismatec IPC-N (Switzerland) peristaltic tubing pumps that allowed to regulate the flow rate and dispensing volume with high precision.

### 4.2.1 Supply of carbon dioxide

To improve CO$_2$ availability to the cells, I added carbon dioxide to culture medium in two different experiment phases — when stable biomass concentration was achieved on days 10 to 14 and during P limitation on days 20 to 22 (Fig. 7). I used a 4 L medium bottle (filled with medium up to 2/3) and pressurised it with pure CO$_2$ to one extra bar. CO$_2$ originates from SodaStream licensed carbonator that are filled with beverage-grade CO$_2$ in compliance with standards for food and beverage safety. A stronger pressure that would have increased gas dissolution was not technically possible to apply. During solvation the bottle was left in the fridge and was re-pressurised when gas had dissolved. When pressure was endured, this indicated CO$_2$ saturated medium. The carbonated medium was dosed under 1 bar pressure to the algal culture by a dispensing pump. To keep the pressure while dosaging, a valve was connected to the tube. It was a pipe fitting, closed from one end and with a small opening on the side which was tightly covered by the tube as the valve was pushed deeply into the tube. The CO$_2$ enriched medium entered the valve from the front end, exited through the side opening and was pressed to move further through the space between the valve and the tube. This method allowed to supply the culture with carbonated medium under pressure which was necessary to ensure instant gas dissolution.
in the algal culture and to prevent losses to the atmosphere.

4.3 Sampling and measurements

I monitored the culture’s behaviour by cell count (cells ml\(^{-1}\)), biomass (g L\(^{-1}\)), pH and fluorescence parameters obtained from the saturation pulse method of quenching analysis. Cell densities were measured with an electronic particle counter (Casy TT, Schärfe System, Germany) which determines cell concentration and volume in the medium. Before taking measurements, samples were diluted with isotonic particle free solution. The measuring principle of the particle counter is based on measuring the electrical resistance caused by each cell when they pass a measuring pore. During the measurement process, a pulsed low voltage field with 1MHz is applied to the measuring pore via two platinum electrodes. The electrolyte-filled measuring pore represents a defined electrical resistance. The cells displace a quantity of electrolyte corresponding to their volume during their passage through the measuring pore. Intact cells are considered as isolators and an increased level of resistance is achieved over the measuring pore. The amplitude of the signal is scanned and the volume of the cells is calculated. Dead cells, whose membrane no longer acts as an electrical barrier, are recorded by the size of their cell nucleus. A range of the particle size distribution of 4 – 15 µm was used (i.e. all cells were counted and measured which diameter remained in that range). Each day three replicate samples were taken to account for the sampling error. Cell volume was converted to biomass (assuming the density of a cell is equal to 1). Biomass concentration and was measured as wet weight but in this thesis it is presented as dry weight to have a better comparison with the results from the literature. The conversion factor of wet weight to dry weight of 0.2 was used. The culture’s pH measurements (taken with Mettler Toledo pH Meter) started on the 2nd day of CO\(_2\) addition (11th experiment day) and lasted until the final experiment day. Measurements were automatically recorded with one minute interval.

4.3.1 Modulated fluorescence measurements

Fluorescence analysis was undertaken using the Walz PHYTO-PAM Phytoplankton Analyzer pulse amplitude modulated fluorescence system (Heinz Walz GmbH, Effeltrich, Germany) with Phyto-Win software version 1.45. During measurements ML and AL were 1 and 190 µmol quanta m\(^{-2}\)s\(^{-1}\), respectively, and saturating pulse (duration 200 ms) was 2600 µmol quanta m\(^{-2}\)s\(^{-1}\). The AL intensity was similar to the one used for the growth of algal cells in order to avoid photoinhibitory effects and to provide optimal conditions for
photosynthetic activity. The length of AL before measuring steady-state fluorescence was 25 s. It was fixed by visual inspection during pilot experiments and was considered to be enough to allow the fluorescence response to stabilize. The determination of $F_0$, $F_m$, $F'_m$, $F'_r$, and $F'_0$ allowed to calculate Chl fluorescence parameters $F_v/F_m$, $\Delta F/F'_m$, $qP_{rel}$, $qN_{rel}$ and $UQF_{rel}$. During measurements the samples were stirred in a 4 ml quartz kyvette with a magnetic stirrer and one measurement per sample was taken on each experiment day. Fluorescence parameters were calculated for every measurement and thereafter the mean values were calculated.

4.4 Calculation of growth parameters

Culture growth dynamics is shown by biomass yield and net biomass increase. Biomass yield per square meter was calculated as follows:

$$Y = \frac{F \times X}{0.08}$$

where $Y$ = biomass yield (g dw m$^{-2}$ d$^{-1}$), $F$ = culture outflow rate (L d$^{-1}$), $X$ = biomass concentration (g dw L$^{-1}$), 0.08 is the area of the cultivation unit (m$^2$).

In my experiment, the culture was harvested continuously. However, if culture removal was not installed, a linear increase in the cell density would follow (Ojamäe, 2008). The amount of algae removed per day was approximately equal to the amount of biomass gained during the same period by photosynthesis. So, due to harvesting, the biomass density is not allowed to increase continuously. Cell density on the experimental unit decreases if the culture is harvested and increases if not harvested. Biomass increase (or decrease) in the whole system (on the experimental unit and in the harvest, both summed up) between two measuring days is denoted as biomass net increase. This is calculated as follows:

$$\frac{dx}{dt} = (\mu + \frac{F}{V}) \times X$$

where $dx$ = increase in biomass concentration (g L$^{-1}$), $dt$ = time interval (d), $\mu$ = specific growth rate (d$^{-1}$), $F$ = culture outflow rate (L d$^{-1}$), $V$ = culture volume (L), $X$ = biomass concentration (g dw L$^{-1}$). $F/V$ is termed as the dilution rate, i.e. the proportion of the culture volume that was removed and exchanged by fresh medium during one experiment day. The specific growth rate for batch cultures can be calculated as follows:

$$\mu = \frac{ln(N_t/N_0)}{\Delta t}$$
where $\mu =$ specific growth rate ($d^{-1}$), $N_0 =$ cell concentration at time 0 (initial), $N_t =$ cell concentration at time $t$, $\Delta t =$ time interval (d). For chemostat cultures this way calculated $\mu$ is underestimated because $N_t$ is smaller due to culture removal. The growth rate for steady-state (cell density is constant) chemostat is determined by the dilution rate $F/V$. When not in steady-state (cell density varies) the growth rate can be calculated as $\mu + F/V$ and this is used in calculation for biomass net increase. The biomass net increase has a great importance if we consider that continuous biomass harvesting has a risk of culture depletion if for some reason the cells become in a physiologically bad shape and harvesting exceeds cell growth for prolonged periods. This parameter shows changes in biomass growth in the whole system.

4.5 Experiment for dark storage

In the absence of light energy, cells consume biomass (intracellularly stored carbohydrates) by respiration. This can significantly increase the costs for algae production if cell biomass is considerably lowered during night. Circulation and aeration of the large algal culture volume at night demands a high energy consumption for the operation of the pump. On the other hand, non-aeration could lead to settling and lowering the overall health of the cells. In algae mass production cells must be maintained in absence of light with as small losses as possible. Different solutions for maintenance can be tested — aeration (mixing), non-aeration, lowering the temperature etc. The goal is to find means for algae maintenance during night which is the least energy consuming and has small losses. In this experiment I expect biomass decrease after dark storage and larger biomass decrease to occur in aerated conditions as oxygen is needed for production of ATP.

The experiment was carried out with a healthy ($F_v/F_m = 0.6$) culture of microalga *S. obliquus* to measure biomass loss over a dark period. Two treatments were applied — culture aeration and non-aeration, in order to avoid oxygen depletion or incur anoxia, respectively. Two 160 ml culture flasks were filled up to the neck and incubated in complete darkness for 40 hours at 20 °C. For determination of biomass dry weight before and after treatments, 5 ml of culture was filtrated on previously dried (2 h at 65 °C) and weighed Whatman GF/C 25 mm filters in three replicates and were dried at 65 °C over night.
5 Results

5.1 Biomass growth and yield

The time course of algal biomass concentration, yield and biomass net increase during the 22 experiment days are shown in Fig. 7. On the first five days the biomass increase of the alga was almost linear. The rate of harvesting (i.e. the dilution rate) was increased gradually (up to 0.35) to keep the amount of harvested algae per day smaller compared to the new biomass gained by photosynthesis. This allowed an increase in biomass concentration both on the experimental unit and in the harvest. At 0.35 harvesting rate the culture’s density varied within a range of 0.2 – 0.6 g dw L\(^{-1}\). Biomass yields obtained values from 1 to 2 g dw m\(^2\) d\(^{-1}\) and followed the changes in biomass density.

A linear relationship was found between cell count and biomass density (Fig. 8). Both parameters describe the population density, therefore, data considering population size is presented only as biomass. Due to variations in cell volume within constant cell count, the R\(^2\) of the relationship is 0.97 and not exactly 1.

5.2 Culture pH

During the 22 experiment days culture’s pH varied within a range of 8.2 – 10.5. The diel pH amplitude was between 8.7 and 10.5 when non-carbonated medium was used. CO\(_2\) enriched medium significantly lowered culture’s pH maximal values and diel amplitudes, which remained between 8.2 – 9.2. During daytime pH levels rised and started to fall during night. CO\(_2\) addition to the culture media did not affect statistically significantly culture’s biomass density or yield (t-test, df = 5, p = 0.46) but had a clear effect on pH (Fig. 9).

5.3 Photochemical activity

The maximum quantum yield of PS II (F\(_v\)/F\(_m\)) showed values between 0.71 and 0.78 (Fig. 10), which indicates a healthy culture of *Scenedesmus*. On the other hand, low \(\Delta F/F'\_m\) on the first few days coincided with a phase of relatively dilute culture (biomass concentration 30 – 90 mg L\(^{-1}\)). Dynamics of nonphotochemical quenching, qN\(_{rel}\), in the culture was inversely related to the change of \(\Delta F/F'\_m\) and qP\(_{rel}\). A significant decrease in qN\(_{rel}\) and a complementary increase in qP\(_{rel}\) was found (Fig. 11) as the culture density increased. Overall, the relative quenching parameters, qN\(_{rel}\) and qP\(_{rel}\), were sensitive to
Figure 7: Time courses of biomass yield (upper panel), biomass net increase (lower panel) and biomass density of *Scenedesmus obliquus* in a continuous flow culture. Error bars show standard error of the mean. Biomass yield (upper panel) is the daily amount of harvested algae. Biomass net increase (lower panel) shows the biomass increase or decrease in the whole system (on the experimental unit + the harvest) between two days. The amount of harvest was increased gradually: 0, 15, 25 and 35% of total culture volume. Two treatments were applied — CO₂ enriched medium and phosphate (P) limitation.
Figure 8: Linear relationship between cell count and biomass density. The relationship can be expressed with a regression equation: cell count = $7.960 \times 10^5 \times$ biomass.

biomass density variations (which determines the average light availability to cells). Regression equations $q_N_{rel} = 0.4 - 0.08 \times$ biomass $(R^2 = 0.73, \text{df} = 20, p < 0.001)$ and $q_P_{rel} = 0.4 + 0.06 \times$ biomass $(R^2 = 0.53, \text{df} = 20, p < 0.001)$ were both highly significant. $UQF_{rel}$ was not sensitive to biomass variations $(R^2 = 0.09, \text{df} = 20, p = 0.1)$. If medium carbonization is added to the models as a covariate then neither $q_N_{rel}$ or $q_P_{rel}$ showed any significant response to increased inorganic carbon availability ($p = 0.45$ and $p = 0.64$, respectively).

5.4 Photosynthetic efficiency

The average energy content of 1 mole glucose ($C_6H_{12}O_6$) is about 2800 kJ (Brennan & Owende, 2010). The mass of $C$ in 1 mole of glucose is $6 \times 12$ (C atomic weight) = 72 g. The energetic value of 1 g organic $C$ is $2800/72 = 40$ kJ. About 50% of algal dry biomass is organic carbon and assuming that all the energy content is within the organic carbon, then the energetic value of 1 g algal dw is 20 kJ. The maximum yield achieved on the experimental unit was 2 g dw m$^{-2}$ d$^{-1}$, which makes 40 kJ m$^{-2}$ d$^{-1}$. The intensity of PAR radiation used in the experiment was 200 µmol m$^{-2}$ s$^{-1}$ that equals to 17.28 mol m$^{-2}$ d$^{-1}$. Assuming that that the energy content of 1 mole of PAR photons is 218 kJ, then the amount of energy falling on the experimental unit was $218 \times 17.28 = 3767$ kJ m$^{-2}$ d$^{-1}$. So, the photosynthetic efficiency of *Scenedesmus obliquus* was about 1.1%.
Figure 9: Culture pH during experiment days 11 to 22. Tick mark on the x-axis marks the midnight, i.e. the beginning of a new day which lasted to the next tick mark. Adding CO$_2$ affected pH significantly. Fluctuations in pH are regular with respect to the light cycle. When lights switch on, algae start CO$_2$ uptake and pH rises. As soon as the lights switch off pH decreases due to the gas exchange between the algae and culture media as CO$_2$ starts to dissolve. Without extra CO$_2$ the amplitude was 8.7 – 10.5, adding CO$_2$ decreased pH amplitude to 8.2 – 9.2.

5.5 Dark storage

The mean biomass (dw) before dark storage was 1560 (±52) mg and after 1547 (±31) mg and 1553 (±46) mg in aerated and non-aerated cultures, respectively. The non-aerated culture turned anoxic after a few hours of incubation. Decrease of biomass within the 5 ml algal suspension during the 48 h storage at 20°C was 13 (±81) mg and 6.7 (±90) mg in aerated and anoxic conditions, respectively. The biomass decreased less than 1% (0.9% and 0.4% in aerated and anoxic treatment, respectively). Statistically the biomass decrease was insignificant (t-test, df = 2, p = 0.80). Although the mean biomass loss in aerated conditions was higher than in anoxic conditions, this difference was not significant (t-test, df = 2, p = 0.84).

In this experiment I used three replicate samples for every group. Given the variability in the data, three replicates are enough to reveal a significant difference (at p = 0.05 level) between means of 132 mg with a probability of 0.8 (post-hoc power analysis, allowing a Type II error $\beta = 0.2$). Thus, with three replicates I should have been able to confidently
Figure 10: Time courses of maximum quantum yield of PS II, $F_{v}/F_{m}$ and effective quantum yield of PS II, $\Delta F/F_{m}^{'}$. Two treatments were applied — usage of medium which was enriched with extra CO$_2$ and phosphate (P) limitation. Error bars show standard error of the mean.

Figure 11: Relative photochemical quenching, $q_{P_{rel}}$, relative nonphotochemical quenching, $q_{N_{rel}}$ and relative unquenched fluorescence level, $UQF_{rel}$. Error bars show standard error of the biomass mean.
demonstrate up to 8% biomass loss. To show a statistically significant difference as small as the actual difference (13 mg), it would have taken 165 replicate samples, which is clearly more than feasible and practical.

6 Discussion

6.1 Biomass yield

The mass culture of microalgae in various production systems is primarily concerned with maximising daily yield of microalgal biomass in the condition of light limitation. In dense cultures light penetration is impeded by mutual shading and light absorption (Rabe & Benoit, 1962). Over 22 days of cultivation the values for biomass yield were between 1 and 2 g dw m\(^{-2}\) d\(^{-1}\) which is low compared to cutting edge technologies in the world. Masojidek et al. (2011) reached with *Chlorella* culture grown in a thin-layer cascade the optimal cell density of 6.5–13.5 g dw L\(^{-1}\) and at this concentration the yield reached as much as 55 g dw m\(^{-2}\) d\(^{-1}\). *Chlorella* culture was grown outdoors and exposed to available sun light, for this reason such high yields are possible. Indoors with low light conditions yields remain significantly lower.

I was able to continuously harvest 1/3rd of the culture on this type of cultivation unit without causing cell washout. This means that the net growth of the cells had to exceed 0.35 d\(^{-1}\) to compensate for the harvesting. Typically in steady-state chemostat cultures, the growth rate is constant and equals the dilution rate. The biomass concentration, which is determined by the limiting resource (nutrients or light) should also be constant. However, on such a small experiment device that I used, it was quite difficult to maintain a completely stable water level and prevent cells from settling. Lower water level and intermittent settling cause fluctuating cell concentrations on the experimental unit.

In continuous mass cultures the yield of cell mass per unit reactor area (or volume) is a function of the specific growth rate and cell concentration. The culture density at which the cell mass reaches its highest biomass yield for specific culture conditions is the optimal cell density (or biomass concentration). Very dilute or in contrast, very dense cultures, both yield below the possible maximum yield. This is because the growth rate is highest when sufficient cell mass is available to exert some mutual shading, protecting from photoinhibition that would take place if cell concentration is low and light irradiance is high. On the other hand, the growth rate in cultures of extremely high cell densities would be much reduced, due to a shortage of light available to the individual cells. Theoretically,
the optimal cell density when growth rate is the highest, falls somewhere between these extremes (Richmond, 2004). In my previous study (Ojamäe, 2008) I have shown that the maximum daily biomass yield can be achieved over a relatively wide range of biomass values (0.2 – 0.6 g dw L\(^{-1}\)). Similar results were shown by Sandnes et al. (2005) in the experiments with laboratory cultures with different artificial light intensity treatments were the maximum daily biomass yield was produced over a wide biomass density range (0.2 – 0.8 g dw L\(^{-1}\)). This simplifies the optimization of growth conditions in mass cultures.

6.2 Fluorescence parameters and changes in culture density

One of the primary aims of Chl fluorescence measurements was to find suitable monitoring parameters calculated from fluorescence quenching analysis to follow the status of the photosynthetic apparatus and its correlation to biomass productivity. Steady \(F_v/F_m\) values seen in Figure 10, show that cells were not physiologically stressed over the cultivation period. In outdoor mass cultures, \(F_v/F_m\) frequently exhibits a diurnal depression that is roughly symmetric with the irradiance intensity, showing a decrease at mid-day (Masojidek et al., 2011). The reason for this decline is explained in that even under intensive mixing the first layers of cells absorb light in excess to what they can use in photosynthetic processes, resulting in a need for dissipation of energy through nonphotochemical quenching. In my experiment the light radiation above the culture suspension layer was constantly 200 µmol m\(^{-2}\)s\(^{-1}\) which is ca. 5 – 10 times lower compared to mid-day outdoor irradiation. Such stable and moderate light conditions did not give any grounds for measuring the diel course of the \(F_v/F_m\).

Photosynthesis was limited on the first two days of the experiment due to optically very thin (low biomass) culture, which resulted exposing the cells to over-saturating light intensity. Firstly, this is shown by low \(\Delta F/F_m\) values that were only 0.15 and 0.2 on the first two days which is 60% less than the average on the rest of the experiment days. Secondly, low \(q_{P_{rel}}\) values on the first days of the experiment indicated to a closed state of large proportion of PS II RCs caused by excess energy absorption by PS II antennae system and therefore, the probability of excitation energy to initiate photochemical reactions was small. In this kind of excess light condition the nonphotochemical reactions were successfully facilitated which is proven by higher \(q_{N_{rel}}\) values on the first days of the experiment (0.73 and 0.65, Fig. 11). Stable values for \(F_v/F_m\) during high \(q_{N_{rel}}\) indicate good health of the cells during two days of extensive heat dissipation of excitation energy. Algal culture density increased to a ”safe concentration” level in two days which
was a short time period not to cause stress to the cells in the condition of extensive heat dissipation. At safe concentration level the cell density is such that mutual shading may greatly modify light availability for individual cells, resulting in cells receiving light intermittently and thus allowing \( q_{N\text{rel}} \) and \( q_{P\text{rel}} \) to stabilize for the rest 20 experiment days. At low biomass density the moderate light conditions (e.g. 200 \( \mu \text{mol m}^{-2}\text{s}^{-1} \)) had a clear effect on the fluorescence quenching parameters. As the biomass density increased, light started to limit the cell growth and thereafter, quenching parameters become insensitive to small biomass changes as the average available light per cells was low.

### 6.2.1 \( F_{v}/F_{m} \) for nutrient-replete culture

In my experiment the attempted phosphate limitation did not have any effect on the maximum quantum yield of PS II (\( F_{v}/F_{m} \)). Although phosphate was fully taken up from the medium, there is a possibility that actual nutrient limitation was never reached. Algae internal nutrient pools might have not been depleted yet by the end of the experiment. Another way to explain the lack of response of \( F_{v}/F_{m} \) to nutrient limitation is that \( F_{v}/F_{m} \) \textit{per se} is not a sensitive indicator of nutrient stress in mass cultures, which is also shown by Parkhill \textit{et al.} (2001) and Kruskopf & Flynn (2006). If the growth conditions in nutrient-replete culture are otherwise balanced (e.g. suitable irradiance, temperature and sufficient gas exchange are provided) then \( F_{v}/F_{m} \) can be shortly masked by those favourable conditions and values remain high and constant (Parkhill \textit{et al.}, 2001). However, contrasting results, where \( F_{v}/F_{m} \) has been accepted to be influenced by nutrient deficiency on batch and chemostat cultures have also been reported (Kolber \textit{et al.} 1988, Lippemeier \textit{et al.} 2001, 2003, Young & Beardall 2003). A slight decrease in biomass density on the last few experiment days can be an indicator of nutrient stress but in case of mass cultures a noticeable increase in cell settling and accumulation occurs which in this case was not visually observed.

### 6.3 Culture pH and CO\(_2\) availability

When lights switch on, algae start taking up CO\(_2\) which causes pH rise in the medium. After the lights switch off pH starts to decrease due to the dissolution of atmospheric CO\(_2\) into the undersaturated culture medium. A significant and fast decrease in pH occurred when carbonated medium was added (Fig. 9). In aqueous environments, inorganic carbon may exist in several alternative chemical forms, CO\(_2\), H\(_2\)CO\(_3\), HCO\(_3^-\) and CO\(_3^{2-}\) (Goldman \textit{et al.}, 1981), usually in an equilibrium as follows:

37
\[ CO_2(aq) + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-} \]

which are interconvertible via reactions controlled by temperature and pH. At pH 8.2 – 10.5, the inorganic carbon pool was primarily in the form of HCO_3^-. A decrease in pH amplitudes on experiment days of 12 – 14 and 20 – 22 resulted in increased level of CO_2 in the algal culture but pH did not decline to the level which could provide algae with needful amount of carbon dioxide. Therefore, significant biomass increase in the periods of carbonated medium addition was not possible to ascertain. Nevertheless, free CO_2 is not the only form of inorganic carbon that can be taken up by microalgal cells. There is good evidence that Scenedesmus obliquus takes up and accumulates inorganic carbon in a form of bicarbonate (Thielmann et al., 1990). For this reason, these cells can photosynthesize even when the pH is greater than 10 and HCO_3^- and CO_3^{2-} are the major inorganic carbon species in the medium. As Rubisco uses CO_2 and not HCO_3^-, the HCO_3^- must be converted to CO_2 for fixation. This is done near the location of Rubisco (Moroney & Somanchi, 1999). Free CO_2 enters the cell with ease by diffusion and unlike bicarbonate, it is not dependent on expenditure of ATP. So, utilization of CO_2 rather than HCO_3^- is more efficient and would theoretically produce higher yields (Schiefer & Caldwell, 1982). Quantum yields of PS II (F_v/F_m, \Delta F/F_m) and relative fluorescence quenching parameters (qP_{rel}, qN_{rel}) remained stable in response to extra carbon availability (Fig. 11).

### 6.4 Dark storage

In theory, the biomass loss could be attributed to respiration of the cells, both, algal and accompanying bacteria. To amplify the biomass loss signal, the incubations lasted for 40 h. This is 5 times longer than any practical need in mass production, where dark storage more than 8 h is not required. Thus, the actual biomass loss due to dark period respiration during one diel production cycle would be 20% of the biomass loss I achieved, if constant respiration loss in time is assumed. Moreover, respiration is temperature dependent (Ogbonna & Tanaka, 1996) and if the temperature of the culture suspension can be lowered during the dark period, respiration losses could be lowered even more. Therefore, with S. obliquus, dark storage does not pose a significant loss to the mass cultivation technology.
## 7 Conclusion

This study concerns cultivation of green microalgae *Scenedesmus obliquus* in an experimental unit designed to make efficient use of available light and achieve a high biomass yield of microalgae. The culture removal (harvesting) from the experimental unit was continuous. At the maximal harvesting rate of 0.35 d$^{-1}$ the biomass concentration varied within a range of 0.2 – 0.6 g dw L$^{-1}$ and biomass yields obtained values from 1 – 2 g dw m$^{-2}$ d$^{-1}$. Due to moderate light conditions (e.g. 200 µmol m$^{-2}$s$^{-1}$), yields much higher than that were difficult to reach with this experimental unit. In outdoor conditions where mid-day light irradiation is *ca.* 5 – 10 times higher, yields upto 55 g dw m$^{-2}$ d$^{-1}$ could be reached in a similar large-scale thin-layer open-culture system (Masojidek *et al.*, 2011). High pH of the culture indicated that cells suffered in CO$_2$ starvation and therefore, extra CO$_2$ was added to the growth medium. Carbonization affected the pH of the culture but did not have a significant effect on biomass density. This is because pH did not decline to the level which could provide algae with needful amount of carbon dioxide and *S. obliquus* was still forced to fulfill its inorganic carbon requirements by taking up HCO$_3^-$.

To observe biomass storage during dark period, the *S. obliquus* culture was incubated in darkness for 40 hours at 20 °C. During dark storage two treatments were applied — culture aeration and non-aeration (which became anoxic after a few hours of incubation). The biomass decreased less than 1% (0.9% and 0.4% in aerated and anoxic treatment, respectively) and was statistically insignificant. To show a statistically significant difference as small as the actual difference, it would have taken 165 replicate samples, which is clearly more than feasible and practical.

Variations in biomass concentration (changes in light availability per cell) significantly affected Chl fluorescence nonphotochemical quenching ($q_{N_{\text{rel}}}$) and photochemical quenching ($q_{P_{\text{rel}}}$). If there was less available light per cell due to culture growth, $q_{N_{\text{rel}}}$ started to decrease and was inversely related to $q_{P_{\text{rel}}}$ and to effective quantum yield of PS II ($\Delta F/F'_{\text{m}}$). The maximum quantum yield of PS II ($F_v/F_m$), showed steady values which indicates a physiologically healthy culture. The quenching parameters can be used to follow the status of the photosynthetic apparatus in low biomass density conditions. For example, light irradiation of 200 µmol m$^{-2}$s$^{-1}$ in optically thin (low biomass) culture was enough to expose cells to over-saturating light intensity (shown by low $\Delta F/F'_{\text{m}}$ and increased heat dissipation, $q_{N_{\text{rel}}}$). Further biomass density increase incurs light limitation and quenching parameters become insensitive to small biomass changes.
8 Rohevetika kasuvüsioloogia ja fotosünteesivõime masskultiveerimisel ”õhukese kihi” kaskaadil


Selgitamaks, kui suured võivad olla biomassikadu öössiti, korraldati katse kus Scenedesmus obliquus jäeti 40 tunniks pimedasse 20 °C juurde. Ühes pudelis kultuuri aereeriti, teises mitte (väärtused anoksiliseks paar tundi pärast pime-inkubatsiooni). Biomassikadu kum-maski puidel võrdelduna inkubatsioonise ajaga oli alla 1% (vastavalt 0.9% ja 0.4% aereeritud ja mitteaereeritud kultuuris) ja see oli statistiliselt mitteoluline erinevus. Näitamaks nii väikest biomassikadu statistiliselt olulisena, olnuks vaja biomassi mõõta 165 korda, mis on aga teostamiseks ebapraaktiline.

Muutused biomassitiheduses (valguse kättesaadavuse hulgas raku kohta) mõjutab statistiliselt oluliselt klorofüll a fluoresentsi parameetreid — fotosünteetilist (qP\(_{rel}\)) ja mittefotosünteetilist kustutust (qN\(_{rel}\)). Kultuuri tihenedes oli valgust raku kohta vähem ja see viis qN\(_{rel}\) languseni. Biomassi suurenedes tõusis qP\(_{rel}\) ja veel üks fluoresentsi parameeter — fotosüsteem II efektiivne kvantsaagis (\(\Delta F/F'_m\)). Fotosüsteem II maksimaalse kvantsaagise (\(F_v/F'_m\)) väärtused olid läbi kultuuritablesitus. Mis viitab füsioloogiliselt hea ter-mise juures olevale kultuurile. Klorofüll a fluoresentsi parameetreid saab kasutada fotosünteesiiparaadi tööefektiivsuse hindamiseks madala biomassitihedusega kultuuri puhul. Seda illustreeris suurenened sockuse eraldumine (qN\(_{rel}\)) ja langenud fotosüsteem II efekti-
ivne kvantsaagis ($\Delta F/F_m$) kui kultuur oli eksponeeritud 200 $\mu$mol m$^{-2}$s$^{-1}$, mis oli madala biomassitiheduse juures rakkudele üleküllastav valgusintensiivsus. Edasine biomassitõus viis rakud valguslimitatsioonini ja fluorestsentsi parameetrid ei olnud enam tundlikud väiksemate biomassitiheduse muutuste suhtes.

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References


