# Outdoor cultivation of *Chlorella vulgaris* - Assessment of yield influencing parameters and application as biogas substrate

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# List of Abbreviations

| Abbreviation | Full name |
|---|---|
| $A$ | Autotrophic growth |
| ALA | $\alpha$-Linolenic acid |
| ANOVA | Analysis of variance |
| ATP | Adenosine triphosphate |
| $cs$ | Closed system |
| Cel | Cellulase |
| Cel/Pec | Cellulase/Pectinase |
| CHP | Combined heat and power |
| DHA | Docosahexanoic acid |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DTT | Dithiothreitol |
| DWD | Deutscher Wetterdienst |
| EDTA | Ethylenediaminetetraacetic acid |
| F | Ratio of mean squares (ANOVA) |
| FAME | Fatty acid methyl ester |
| FPA | Flat-Plate Airlift |
| GC | Gas chromatography |
| GF | Glass fiber |
| GHG | Greenhouse gas |
| GMP | Good manufacturing practice |
| $H$ | Heterotrophic growth |
| $M$ | Mixotrophic growth |

| Abbreviation | Full name |
|---|---|
| Max | Maximum |
| Min | Minimum |
| N-Prot | Nitrogen-Protein |
| $os$ | Open system |
| $\omega$ | Omega |
| p | Probability |
| PBR | Photobioreactor |
| PCA | Principal component analysis |
| PC1 | Principal component 1 |
| PC2 | Principal component 2 |
| PC1ro | Principal component 1 after applying a varimax rotation |
| PC2ro | Principal component 2 after applying a varimax rotation |
| Pec | Pectinase |
| PUFA | Polyunsaturated fatty acid |
| r | Correlation coefficient |
| RNA | Ribonucleic acid |
| SAG | Culture Collection of Algae at Göttingen University |
| Std | Standard deviation |
| STP | Standard temperature and pressure |
| TAGs | Triacylglycerols |
| Tris | Tris(hydroxymethyl)aminomethane |
| TUHH | Hamburg University of Technology |
| VDI | Verein Deutscher Ingenieure |
| WTW | Wissenschaftlich-Technische Werkstätten GmbH |

| Parameter | Full name |
|---|---|
| $\alpha$ | alpha (Parameter in equation 3.2) |
| $B$ | Biomass concentration |
| $CellC_{Alg}$ | Cell counts (microalgae) |
| $CellC_{Bac}$ | Cell counts (bacteria) |
| $Cl$ | Cloudiness |
| $CO_{2,in}$ | Carbon dioxide content in the flue gas stream (inlet) |
| $CO_{2,out}$ | Carbon dioxide content in the flue gas stream (outlet) |
| $COD$ | Chemical oxygen demand |
| $Degree_{CO_2}$ | Degree of decarbonisation |
| $DIN$ | Dissolved inorganic nitrogen |
| $DIP$ | Dissolved inorganic phosphorus |

| **Parameter** | **Full name** |
|---|---|
| $DLI_{ver}$ | Vertically measured daily light intensity |
| $DOC$ | Dissolved organic carbon |
| $FA$ | Fatty acid |
| $F_{CO_2}$ | Carbon dioxide biofixation rate |
| $Frac_{Bac}$ | Total bacterial fraction |
| $LA$ | Light availability |
| $l_{Bac}$ | Length of a bacterial cell |
| $LI_{ver}$ | Vertically measured light intensity |
| $MaxL$ | Vertically measured maximum light intensity |
| $MaxT$ | Maximum temperature |
| $M_C$ | Molar mass of carbon |
| $M_{CO_2}$ | Molar mass of carbon dioxide |
| $M_N$ | Molar mass of nitrogen |
| $M_{NO_3}$ | Molar mass of nitrate |
| $M_P$ | Molar mass of phosphorus |
| $M_{PO_4}$ | Molar mass of phosphate |
| $MeanT$ | Mean temperature |
| $MinT$ | Minimum temperature |
| $OD$ | Optical density |
| $OD_{750}$ | Optical density at a wavelength of 750 nm |
| $OPD$ | Optimal population density |
| $OPP$ | Optimal population productivity |
| $OC$ | Organic carbon |
| $OLA$ | Optimal light availability |
| $ON$ | Organic nitrogen |
| $P$ | Productivity |
| $POC$ | Particulate organic carbon |
| $PON$ | Particulate organic nitrogen |
| $POP$ | Particulate organic phosphorus |
| $r_{Alg}$ | Radius of a microalgal cell |
| $r_{Bac}$ | Radius of a bacterial cell |
| $sCOD$ | Soluble chemical oxygen demand |
| $SD$ | Sun duration |
| $Surf_{Reactor}$ | Reactor surface area |
| $T$ | Temperature |
| $TOC$ | Total organic carbon |
| $TS$ | Total solids |

| Parameter | Full name |
|---|---|
| $TSV_{Alg}$ | Total specific biovolume (microalgae) |
| $TSV_{Bac}$ | Total specific biovolume (bacteria) |
| $TVar$ | Temperature variance |
| $V_{Alg}$ | Biovolume of a microalgal cell |
| $V_{Bac}$ | Biovolume of a bacterial cell |
| $V_{Reactor}$ | Reactor volume |
| $VS$ | Volatile solids |

| Units | Full name |
|---|---|
| d | Day |
| °C | Degree Celsius |
| g | Gram |
| h | Hour |
| Hz | Hertz |
| kg | Kilogram |
| KJ | Kilojoule |
| $km^2$ | Square kilometre |
| l | Length |
| L | Litre |
| m | Metre |
| M | Mol |
| $m^2$ | Square metre |
| $m^3$ | Cubic metre |
| µE | Microeinstein |
| µg | Microgram |
| µL | Microlitre |
| µm | Micrometre |
| $µm^3$ | Cubic micrometre |
| µmol | Micromole |
| mg | Milligram |
| min | Minute |
| mL | Millilitre |
| mm | Millimeter |
| mM | Millimolar |
| MW | Megawatt |
| nm | Nanometre |

| **Units** | **Full name** |
|---|---|
| % | Per cent |
| ppm | Parts per million |
| rpm | Rounds per minute |
| t | Time |
| x g | Times gravity |
| Vol.-% | Volume per cent |
| vvm | Volume per volume per minute |

# Chapter 1

# Introduction

The world energy consumption keeps rising since the industrial revolution and is expected to increase further by 53 % between 2008 and 2035 (EIA, 2011). A large part of the increase will be attributed to an increasing population and a fast economic growth of countries like China or India. Although renewable energy is the fastest growing energy source worldwide, it is predicted that fossil fuels will still cover 80 % of the overall energy demand in 2035 (EIA, 2011). However, fossil fuel reserves are limited and the production of oil and gas is becoming increasingly demanding and, thus, expensive (Murray and King, 2012; ter Veld, 2012). Securing energy supply will therefore become a major challenge in the future. Closely linked to the depleting resources of fossil fuels is the rising carbon dioxide concentration in the atmosphere, which contributes to anthropogenic global warming (IPCC, 2007; Murray and King, 2012).

Currently, many of the renewable energy sources under development focus on the electricity market (e.g. solar, wind). Nevertheless, there is also a high demand for biofuels (Schenk *et al.*, 2008). Therefore, $CO_2$-neutral, environmentally friendly, and sustainable alternatives (e.g. bioethanol, biodiesel, biogas, biohydrogen) have been developed and are still under development from a variety of plant feedstocks in recent years. In this context, especially the energetic use of biomass from land plants has been discussed controversially because of its potential competition with food production (Gomez *et al.*, 2008; Fargione *et al.*, 2008; Eisentraut, 2010). Thus, the search for alternatives gains more and more importance.

Microalgae show a number of potential advantages compared to higher plants: they (1) can produce cellulose, starch, and oils in large amounts in the absence of lignin, (2) have higher biomass yields per hectare, (3) can utilise salt, brackish, fresh, and waste water sources, (4) can be cultivated in non-arable areas, or in the water without food or feed competition (Chisti, 2008b; Schenk *et al.*, 2008; Rittmann, 2008; Stephens *et al.*, 2010; Wijffels *et al.*, 2010). So far, however, high microalgae production costs constrain the application of large-scale biomass cultivations to a small-scale high-value sector that produces mainly food supplements or ingredients for the cosmetic industry.

In recent years, research activities are ongoing in integrated algae-based biorefineries in order to benefit from synergistic effects (Mussgnug *et al.*, 2010). By producing multiple products (e.g. a low-volume of high-value and a high-volume of low-value products) the profitability can be enhanced, while the production costs can be reduced (Zinoviev *et al.*, 2007). Figure 1.1 shows a simplified and generalised biorefinery concept for the cultivation of microalgae and a simultaneous production of high-value products and energy from microalgae biomass. According to this figure, light, nutrients, and water are added to enable microalgae growth. Subsequently, the biomass is harvested and further processed into algae products. Residual biomass is converted into energy and arising flue gas $CO_2$ is recycled in a carbon capture and conversion step using microalgae as recipients.

However, the realisation of such a concept on an industrial scale is, at the current state of the scientific and technical knowledge, neither economically nor energetically viable (Johnson and Wen, 2010; Morweiser *et al.*, 2010; Wijffels *et al.*, 2010). As a consequence, the economic feasibility of microalgae cultivation and microalgae-based biorefinery concepts have to be substantially improved in the years to come. To achieve these goals, scientists are currently maximising microalgae productivity (Barbosa *et al.*, 2003; Tzovenis *et al.*, 2003; Doucha and Lívanský, 2012), minimising biomass production costs (Doucha *et al.*, 2005; Douskova *et al.*, 2009), and optimising energetic applications (Sialve *et al.*, 2009; Mussgnug *et al.*, 2010; Razon and Tan, 2011; Ehimen *et al.*, 2011).

## 1.1 Background

In this Chapter, a short overview is given about the backgrounds of the most important topics addressed in this investigation. The Chapter is subdivided into the Sections 'Optimisation of microalgae cultivation and productivity' and 'Biogas production'.

### Optimisation of microalgae cultivation and productivity

Microalgal growth is influenced by a multitude of biotic and abiotic factors. Thus, improving the productivity of an entire microalgae population requires an in-depth knowledge about individual and combined effects of key parameters on the dynamics of culture growth. A sound understanding of the role of light, temperature, and nutrients is therefore crucial to optimise the conditions of cultivation. Furthermore, two macro-nutrients carbon and nitrogen constitute about 60 % of the microalgae biomass. Alternative sources other than atmospheric $CO_2$ and industrial fertilizers have been considered to meet the required carbon and nitrogen demand and to achieve an optimal productivity. More importantly, however, alternative sources from waste bear the poten-

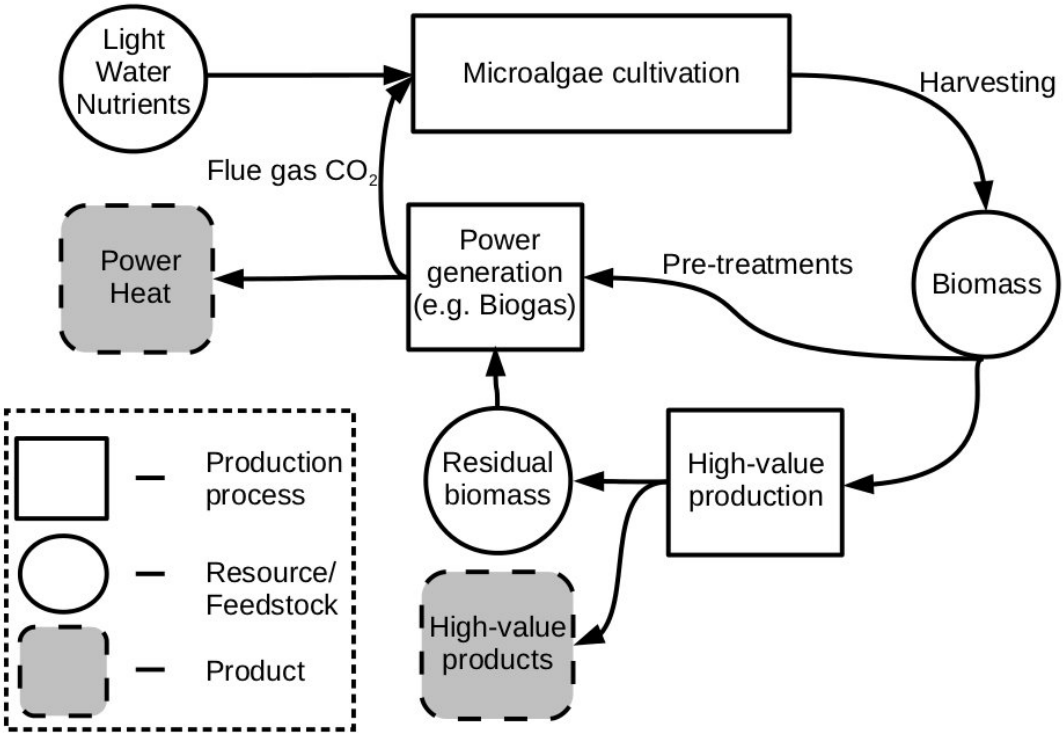


**Figure 1.1:** Simplified concept for the production of microalgae biomass and its use as feedstock for high-value products and energy

tial of a substantial reduction in biomass production costs. In the following, important aspects of microalgae cultivation are briefly introduced.

**Ambient light and temperature** Light and temperature are key for the productivity in photoautotrophic cultures (Goldman, 1979; Carvalho and Malcata, 2003). While light provides the energy source for photoautotrophic growth, temperature controls the rates of chemical reactions in the microalgal cell (Sandnes *et al.*, 2005). Ambient temperature and light are, however, highly variable on all temporal scales from seconds to years posing a major challenge for a stable and continuous outdoor cultivation of microalgae. Economic feasibility studies usually ignore such inherent stochasticity of outdoor conditions and base their conclusions on laboratory data instead. Consequently, their results tend to be too positive and not representative for real-world operations, as results gained from laboratory experiments are typically determined under constant and often optimal conditions (Sorokin and Krauss, 1958; Kessler, 1985; Renaud *et al.*, 1991; Carvalho and Malcata, 2003; Sandnes *et al.*, 2005; Trabelsi *et al.*, 2009; Carvalho *et al.*, 2009; Seyfabadi *et al.*, 2011; Li *et al.*, 2011; Khoeyi *et al.*, 2012).

**Nutrients** Maintaining algae in a replete state with respect to their main nutrients nitrogen and phosphorus is key for an undisturbed, reliable, and successful cultivation process. The complete conversion from nutrients into biomass is economically relevant, because both, excessive as well as insufficient nutrient supply, impairs the efficiency of cultivation. Continuous monitoring and adjusting is therefore indispensable. Nutrient sinks other than microalgae growth, e.g. consumption by other organisms (Joint *et al.*, 2002), may lower the conversion efficiency and directly increase the production costs.

**Dissolved organic carbon and bacteria** Algae typically exude a fraction of photosynthetically fixed organic matter into the surrounding water (Puddu *et al.*, 2003). Such a loss of organic matter in form of dissolved organic carbon ($DOC$) is, however, often ignored in microalgae systems (Hulatt and Thomas, 2010). Most of the current harvesting techniques only extract particulate matter (Molina Grima *et al.*, 2003) and dismiss dissolved organic matter. All organic carbon that is not harvested from the system is, however, a loss and decreases the conversion rate of solar energy to technically usable biomass. Besides, exudates of microalgae, especially $DOC$, provide an excellent substrate for bacterial communities (Obernosterer and Herndl, 1995; Lonborg and Sondergaard, 2009). Although the presence of bacteria in microalgae systems has been widely acknowledged (Croft *et al.*, 2005; Otsuka *et al.*, 2008; Lakaniemi *et al.*, 2012), little is known about their contribution to the total biomass and about their ecological role in microalgae production systems. In particular, bacteria may consume a substantial fraction of the available nutrients, and by this, impair the functioning of the microalgae culture as a production system.

**Alternative carbon and nitrogen source** Recently, a variety of artificial $CO_2$ sources has been tested as substrate for the build-up of algal biomass (Chiu *et al.*, 2008; Hsueh *et al.*, 2009; Borkenstein *et al.*, 2011) mainly for two reasons: (1) the potential reduction of biomass production costs, as the high costs for nutrients comprise a major barrier for the cultivation on an industrial scale (Sydney *et al.*, 2010) and (2) the generation of energy with a low fossil carbon footprint by recycling $CO_2$ from flue gas. Flue gas is an exhaust gas generated during combustion processes with a composition depending on the combusted fuel as well as the legal emission standards. While the main constituent is $N_2$, it also contains $CO_2$, $NO_x$, and $SO_x$. In particular, the application of flue gas as a carbon source has been a focus in recent research (Negoro *et al.*, 1991, 1992, 1993; Straka *et al.*, 2002; Doucha *et al.*, 2005; Douskova *et al.*, 2009). Occasionally occurring decreases in productivities were ascribed to the $SO_x$ fraction of the gas (Negoro *et al.*, 1991; Kumoro and Susanto, 2013). Likewise, the presence of $NO_x$ is often assumed toxic and inhibitory (Lee *et al.*, 2002), although Nagase *et al.* (1997) found that $NO_x$ was removed in a microalgae culture suggesting a $NO_x$ uptake by the algae. While considerable research efforts have been devoted to disentangle the effect of environmental factors on the productivity of mi-

croalgae culture systems in recent years, many questions remain unanswered and findings are often inconclusive.

### Biogas production

The interest for microalgae as a substrate for anaerobic fermentation, stems from their potential to exceed the productivity of terrestrial crop plants by 5 to 30 times (Sheehan *et al.*, 1998) and from their lack of lignin and high lipid and protein contents (Schenk *et al.*, 2008). Anaerobic fermentation, which produces biogas, is therefore a promising energy gaining process in microalgae biorefineries (Sialve *et al.*, 2009). Although in the past the production of biogas from microalgae has received only little attention in research (Golueke *et al.*, 1957; Samson and LeDuy, 1982; Bird *et al.*, 1990; Yen and Brune, 2007), recent theoretical calculations (Sialve *et al.*, 2009) and experimental studies (Mussgnug *et al.*, 2010; Ras *et al.*, 2011) eventually reveal the great potential of this process route. The experimental studies also unveil, however, that the conversion of microalgae into biogas involves species-specific approaches and is not free of complications. Mussgnug *et al.* (2010) indicate that even strain specific factors, in particular the composition of the cell wall, strongly influence the suitability for biogas production.

## 1.2 Objectives

The overall aim of this thesis is (1) to understand the complex biological processes in high-density microalgae cultures that affect productivity and biomass composition and (2) to evaluate and optimise the anaerobic digestion from microalgae biomass. To achieve these aims, microalgae cultivation experiments were conducted in an outdoor pilot plant in Northern Germany. The pilot plant was set-up as a technology platform for technical and biological experiments with the microalgae *Chlorella vulgaris*. The microalgae were cultivated in Flat-Panel Airlift (FPA) photobioreactors using either pure $CO_2$ or flue gas from a combined heat and power (CHP) plant. The harvested biomass then served as the substrate for anaerobic digestion experiments on a laboratory scale.

With reference to the aforementioned research areas in Chapter 1.1 ('Background') and the above mentioned overall aims, the following subordinate objectives were derived:

- understanding of the combined effect of light and temperature on productivity and biomass composition in outdoor microalgae cultures,
- assessment of nitrate and phosphate uptake, and of the fate of carbon in microalgae systems,

- evaluation of flue gas as a carbon and nitrogen source for the cultivation of microalgae,
- assessment of microalgae biomass as a feedstock for anaerobic digestion.

## 1.3 General approach

After the definition of the overall objectives in Chapter 1.2 ('Objectives'), the following Chapter summarises how to achieve these objectives.

Following Chapter 1 ('Introduction'), which provides a general overview of the potential of microalgae application on an industrial scale, Chapter 2 ('Fundamentals') addresses the biology of the cultivated microalgae *Chlorella vulgaris* (Figure 1.2). Particularly, growth requirements and microalgae products are reviewed and the various cultivation systems, including the process technology and operation modes are summarised.

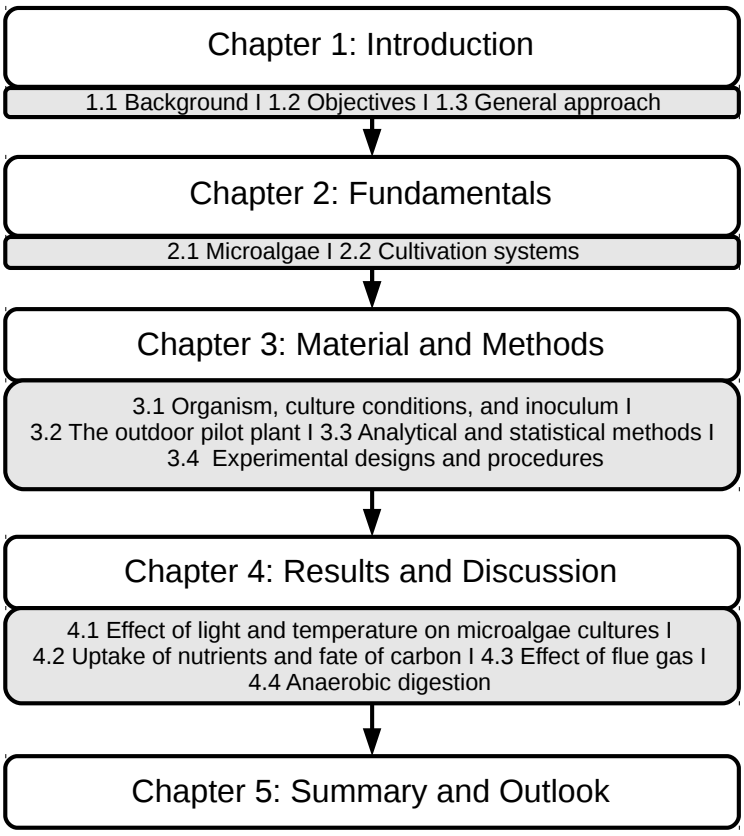


**Figure 1.2:** General approach

In Chapter 3 ('Material and Methods'), culture conditions and the microalgae pilot plant are described in detail, with special emphasis on the Flat-Panel Airlift (FPA) photobioreactor, the design and operation of the pilot plant, and potential sources of error. The 'Analytical and statistical methods' Section includes the definitions and explanations of the applied analytical and statistical methods. The analytical methods are further divided into biotic and abiotic parameters. Finally, the experimental designs and procedures of the four conducted experiments are outlined.

In Chapter 4 ('Results and Discussion'), the outcomes of the experiments are presented and different aspects of the cultivation of microalgae in outdoor FPA photobioreactors are thoroughly discussed (Experiments 1 to 3). The effect of light, temperature, and flue gas on microalgae growth as well as the nutrient uptake, and the fate of carbon in microalgae cultivation systems are addressed here. The results are discussed with respect to the current scientific understanding in this field and strategies for the optimisation of microalgae cultivation are indicated. Furthermore, optimisation strategies to increase the biogas potential from microalgae biomass are presented (Experiment 4). Different methods for the optimisation of the biogas potential, including thermal and enzymatic pre-treatments of the biomass and the application of residual biomass with a previous extraction of soluble proteins are compared. Results of this study are then compared with other studies on anaerobic digestion of microalgae biomass.

Chapter 5 ('Summary and Outlook') summarises the major findings of this thesis and gives suggestions for further research.

# Chapter 2

# Fundamentals

## 2.1 Microalgae

Microalgae are prokaryotic or eukaryotic photosynthetic microorganisms that form the base of the food chain in aquatic environments by building up organic biomass from inorganic $CO_2$. Microalgae, also called phytoplankton, can exist unicellular, in chains, or in groups and account for about 40 % to global photosynthesis (Andersen, 1992). While there are more than 50,000 microalgae species (Richmond, 2004), only very few of them are exploited biotechnologically so far. The green alga *Chlorella* and the cyanobacteria *Spirulina* clearly dominate the microalgal market, but *Dunaliella*, *Haematococcus*, and *Aphanizomenon* are also common species in commercial aquaculture (Spolaore *et al.*, 2006). From the estimated global production of 10,000 t dry biomass $a^{-1}$ half of the production is realised in China (Benemann, 2008).

### 2.1.1 *Chlorella vulgaris*

*Chlorella vulgaris* is a single-cell green algae and belongs to the phylum Chlorophyta. It reaches a size of 5 to 10 µm in diameter and has an almost spherical shape (Scragg *et al.*, 2003). Each individual cell is surrounded by a cell wall and contains one nucleus, one chloroplast, several mitochondria, vacuoles, and starch grains (Figure 2.1).

The species reproduces via autospores, which is a common mechanism of reproduction in unicellular microalgae (Yamamoto *et al.*, 2005). During the process two to eight daughter cells are formed within the mother cell (Figure 2.2) and after the disruption of the mother's cell wall, the daughter cells are released into the surrounding.

*Chlorella vulgaris* is a robust and fast growing algae that tolerates a wide range of environmental conditions (Liang *et al.*, 2009). It thrives in freshwater as well as under brackish water conditions, survives temperatures up to 38°C (Kessler, 1972), and may grow phototrophic, heterotrophic, or mixotrophic. It is this versatility and robustness that made *Chlorella vulgaris* one of the

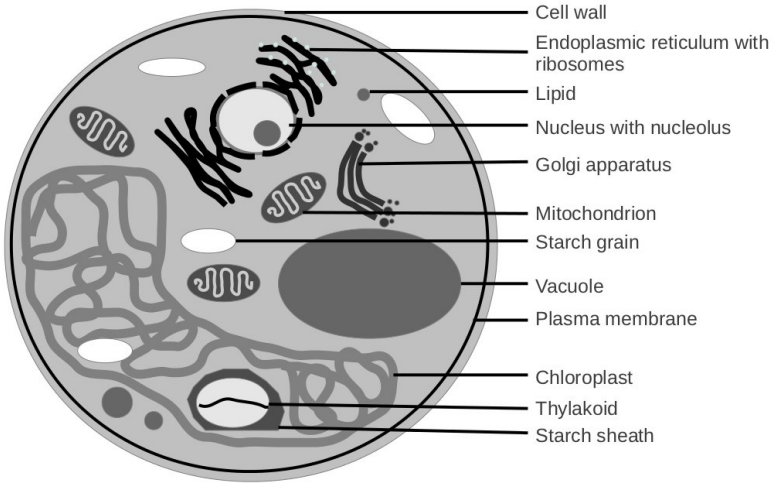


**Figure 2.1:** Schematic structure of a *Chlorella* cell modified from Vogel (2011)

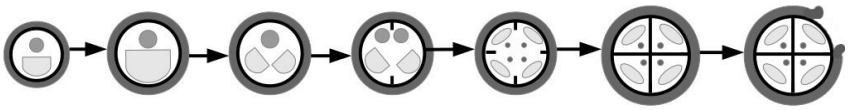

**Figure 2.2:** Timing and location of daughter cell wall synthesis in *Chlorella vulgaris* modified from Yamamoto *et al.* (2005)

most cultivated microalgae for commercial applications worldwide (Pulz and Gross, 2004; Liang *et al.*, 2009).

**Elemental composition** More than half of a *Chlorella* cell's dry weight typically consists of carbon followed by oxygen, whose fraction varies considerably between 11.6 and 28.5 % (Table 2.1). In comparison, nitrogen (6.2 to 7.7 %) and phosphorus (1.0 to 2.0 %) represent only small fractions. However, the uptake of nitrogen and phosphorus is critical for the economic cultivation of *Chlorella*, because in most culture systems these two elements are provided as nitrate and phosphate in mineral fertilizers, which present a major share of the total production costs. Furthermore, *Chlorella* is rich in potassium, magnesium, and iron (Table 2.1).

**Molecular composition** On a molecular level, microalgae mainly comprise of proteins, carbohydrates, and lipids:

- Proteins dominate the composition of macromolecules when microalgae grow under nutrient replete conditions, followed by carbohydrates and lipids. The average protein content

**Table 2.1:** Elemental composition of *Chlorella vulgaris* (by dry weight); values obtained from Oh-Hama and Miyachi (1988)

| Element | % Range |
|---|---|
| Carbon | 51.4 - 72.6 |
| Oxygen | 11.6 - 28.5 |
| Hydrogen | 7.0 - 10.0 |
| Nitrogen | 6.2 - 7.7 |
| Phosphorus | 1.0 - 2.0 |
| Potassium | 0.85 - 1.62 |
| Magnesium | 0.36 - 0.80 |
| Sulfur | 0.28 - 0.39 |
| Iron | 0.04 - 0.55 |
| Calcium | 0.005 - 0.08 |
| Zinc | 0.0006 - 0.005 |
| Copper | 0.001 - 0.004 |
| Manganese | 0.002 - 0.01 |

of *Chlorella* varies between 51 and 58 % (Becker, 2004). A comparison between standard human food items and selected microalgae species reveals why microalgae are generally considered a valuable protein source (Table 2.2). The protein content of the selected algae species exceeds the one found in conventional food sources by far.

- Carbohydrates are present in the form of starch, glucose, or sugar (Spolaore *et al.*, 2006). Their contribution to the cell composition varies between 12 and 17 %, whereas the lipid content ranges between 14 and 22 % (Becker, 2004).

- Microalgae also contain considerable amounts of saturated or unsaturated fatty acids. The latter are highly sought after products as dietary supplements or as additives in feed production. Especially $\omega$-3 (e.g. $\alpha$-linolenic acid (ALA - C18:3), eicosapentaenoic acid (EPA - C20:5), and docosahexaenoic acid (DHA - C22:6)) and $\omega$-6 families are economically relevant. Griffiths *et al.* (2011) analysed fatty acid profiles of eleven microalgae species grown under nutrient replete conditions and reported that the main fatty acids of *Chlorella vulgaris* are C16:0, C18:1, C18:2, and C18:3 (Table 2.3). *Chlorella vulgaris* contains high amounts of ALA; other important $\omega$-3 fatty acids (e.g. EPA and DHA) have not been observed (Griffiths *et al.*, 2011).

Finally, microalgae also comprise nearly all essential vitamins, more specifically A, $B_1$, $B_2$, $B_6$, $B_{12}$, C, E, nicotinate, biotin, folic acid, and pantothenic acid (Becker, 2004).

**Table 2.2:** Biochemical composition of different human food items and selected microalgae species modified from Becker (2004) and Spolaore *et al.* (2006); the values presented in this table are estimates, since the proportion of cell constituents depends on environmental parameters

| Commodity | Protein [% of biomass] | Carbohydrate [% of biomass] | Lipid [% of biomass] |
|---|---|---|---|
| Meat | 43 | 1 | 34 |
| Milk | 26 | 38 | 28 |
| Rice | 8 | 77 | 2 |
| *Chlorella vulgaris* | 51 - 58 | 12 - 17 | 14 - 22 |
| *Scenedesmus obliquus* | 50 - 56 | 10 - 17 | 12 - 14 |
| *Spirulina maxima* | 60 - 71 | 13 - 16 | 6 - 7 |

**Table 2.3:** Fatty acid profile of *Chlorella vulgaris* under nutrient replete conditions after Griffiths *et al.* (2011); the fatty acid content is given as a percentage of the total fatty acid content; fatty acids with a content < 2 % are grouped under 'Other'; blanks indicate levels below the detection limit

| Fatty acid | C14:0 | C14:1 | C15:0 | C16:0 | C16:1 | C16:2 | C16:3 | C18:0 |
|---|---|---|---|---|---|---|---|---|
| % | 0.5 | 0.5 | 0.6 | 23.1 | 0.2 | 7.4 | 5.8 | 5.2 |
| Fatty acid | C18:1 | C18:2 | C18:3 | C18:4 | C20:1 | C20:3 | C20:4 | Other |
| % | 16.1 | 20.9 | 18.0 | | | | | 1.7 |

Operating conditions affect the physiology of microalgae and potentially change their macromolecular composition. Nutrient starvation, in particular nitrogen limitation, can lead to an increase in lipid content (Richardson *et al.*, 1969; Olguín *et al.*, 2001; Wang *et al.*, 2008; Rodolfi *et al.*, 2009; Mata *et al.*, 2010), with lipid contents reaching values as high as 58 % of dry biomass (Mata *et al.*, 2010). Calorific values of *Chlorella vulgaris* cultured under nitrogen deplete conditions are substantially lower (18 KJ $g^{-1}$) than calorific values from cells grown under nitrogen replete conditions (23 KJ $g^{-1}$) (Illman *et al.*, 2000). Increased light intensities foster the formation of particular polyunsaturated C16 and C18 fatty acids in *Chlorella vulgaris* (Nichols, 1965; Pohl and Zurheide, 1979). However, the current understanding of the effect of light on the fatty acid composition of microalgae is contradictory. Seyfabadi *et al.* (2011), for instance, found that total saturated fatty acids increased, while monounsaturated and polyunsaturated fatty acids decreased with increasing irradiance. Moreover, temperature may also influence the relative share of the different macromolecules in the cell. Converti *et al.* (2009) for example demonstrated that the increase of temperature reduces the lipid content in *Chlorella vulgaris*. A change of

macromolecular composition is often the result of environmental stress and therefore usually accompanied by lower productivities (González-Fernández *et al.*, 2012).

**Cell wall structure** Generally, microalgae cell walls can be compared to plant cell walls with respect to their composition (Wang and Evangelou, 1995), both consisting mainly of polysaccharides and proteins. The average thickness of the cell wall is between 17 and 20 nm (Yamamoto *et al.*, 2005) and approximately 25 to 30 % of the cell wall is composed of cellulose, 15 to 25 % of hemicellulose, 35 % of pectin, and 5 to 10 % of glycoprotein. *Chlorella* and *Scenedesmus* are among the microalgae species with the most recalcitrant cell wall structure (González-Fernández *et al.*, 2012). The resistance of microalgae cell walls is often attributed to chemically very stable and, thus, hardly decomposable polymers, such as algaenan. The cell wall of *Chlorella vulgaris* presents an effective and robust barrier to the surrounding environment. Cell wall resistance hinders the accessibility of organic matter in the technical processing of algae biomass and may critically affect the efficiency of energetic processes like biomethane production (Mussgnug *et al.*, 2010).

### 2.1.2 Growth characteristics and requirements

In a homogeneous batch culture and, thus, in a resource limited environment, microalgae growth ideally undergoes the following, clearly distinguishable phases: (1) adaptation (or lag phase), (2) exponential growth (or log phase), (3) decreasing log growth (or linear growth), (4) stationary phase, and (5) decline (or death) phase (Becker, 2004; Mata *et al.*, 2010) (Figure 2.3).

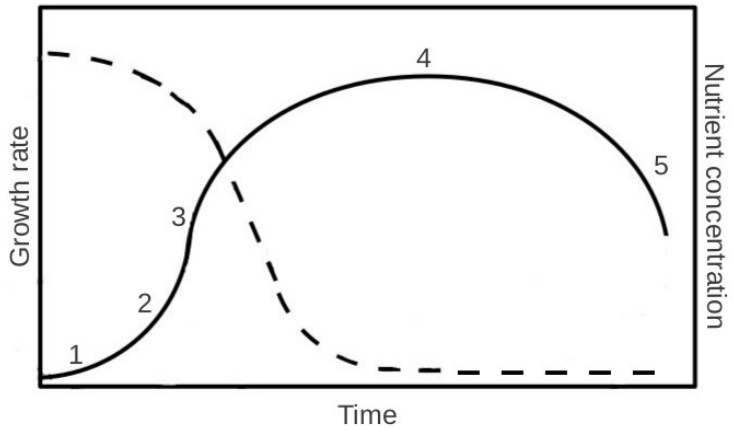


**Figure 2.3:** Growth phases (1 - 5) (solid line) and nutrient concentration (dashed line) of an algae batch culture modified from Mata *et al.* (2010); the dashed curve indicates the depletion of the nutrient concentration during the course of the experiment

After inoculation, microalgae cells have to adapt to the new environment. During this lag phase, the microalgae culture grows slowly or not at all, as the cells allocate most resources to the physiological adaptation induced by the new environment (Becker, 2004). The lag phase is followed by a phase of rapid, exponential (logarithmic) growth, because typically the initially small population is not resource limited. However, as resources become scarce (e.g. light, nutrients) the exponential growth is slowed down and the biomass of the culture increases only linearly. This is the reason for the name 'linear growth phase'. During the subsequent stationary phase, nutrients are almost depleted, nutrient supply per cell is low, and the birth rate equals the mortality rate. In the death phase, the mortality rate of the population exceeds its birth rate, because the latter is severely limited by the depleted nutrients. Hence, the biomass concentration decreases in the absence of new nutrient supply.

Note, that stationary growth can be the consequence of a multitude of factors. Unfavourable environmental conditions such as light or nutrient limitation, disadvantageous temperature conditions, or the accumulation of inhibitory substances, which can be produced and released into the surroundings by the algae themselves (Becker, 2004), may all substantially impair growth rates.

Microalgae growth can be distinguished by energy source into phototrophic, heterotrophic, and mixotrophic growth.

- Phototrophic microalgae use the energy from light and convert carbon dioxide into organic material, a process called photosynthesis. A major advantage of cultivating algae under conditions that allow for phototrophic growth are lower costs. Sunlight as the primary energy source and carbon dioxide from air or flue gas are free of charge or at least cheap to obtain. Therefore, open pond and most photobioreactor systems are usually operated under phototrophic cultivation conditions (Mata *et al.*, 2010).

- Heterotrophic microalgae use organic material, such as sugars and organic acids, as carbon source and typically maintain higher biomass and lipid productivities. Naturally, problems associated to light limitation are avoided (Liang *et al.*, 2009; Huang *et al.*, 2010; Chen *et al.*, 2011), but the organic material required for feedstock present a significant cost factor for the heterotrophic cultivation of microalgae. Therefore, the search for low cost feedstocks for heterotrophic growth has attracted considerable interest recently. One example for a low-cost organic carbon source is glycerol, which is a by-product in the biodiesel production (Liang *et al.*, 2009).

- Mixotrophic microalgae combine both processes for growth in one organism and can hence use both, energy from light and organic compounds, for their growth.

Plants need different macro- and micronutrients for growth. Macronutrients (i.e. carbon, nitrogen, phosphorus) comprise more than 60 % of a *Chlorella vulgaris* cell's biomass and have to be supplied in sufficient quantities to allow for the build-up of biomass.

Microalgae can fix carbon from the atmosphere, from artificial sources (such as flue gas or biogas), and from soluble carbonates ($NaHCO_3$ and $Na_2CO_3$) (Wang *et al.*, 2008). Nitrogen is most commonly fed as ammonium, nitrate, or urea (Lourenço *et al.*, 1998), with the first being the most readily assimilated source. The third macronutrient, phosphorus, is also critical for microalgae growth as it is contained in several essential molecules including DNA, RNA, ATP, and cell membrane materials (Wang *et al.*, 2008). Moreover, proteins are synthesised by phosphorus-rich ribosomes (Agren, 2004). It is preferentially taken up as $H_2PO_4^-$ and $HPO_4^{2-}$ (Gauthier and Turpin, 1997; Martínez *et al.*, 1999).

In contrast, micronutrients (including potassium, magnesium, iron, sulfur, zinc, copper, and other trace elements) are required in much smaller amounts, but may nevertheless limit growth if missing. The poster child example for the substantial effect a limitation by a trace-element may have is iron-limitation of phytoplankton communities in the so called high nutrient low chlorophyll regions of the global ocean (Boyd *et al.*, 2000). In these vast offshore regions terrigenous iron input is very low and artificial fertilization with iron hence provokes an outburst of phytoplankton growth that ceases once the excess iron is fully consumed.

*Chlorella vulgaris* tolerates temperatures up to 28°C (Kessler, 1985). Experiments are usually conducted at 25°C. Some *Chlorella* species are even able to grow at higher temperatures. *Chlorella sorokiniana* has an upper temperature limit of 38 to 42°C. The optimal pH for the growth of *Chlorella vulgaris* is between 7.0 and 7.5 (Nakamura and Imamura, 1985; Rachlin and Grosso, 1991; Wang *et al.*, 2010). Maintaining optimal temperature and pH conditions is essential for achieving high yields and therefore a primary operational goal in microalgae production (Grobbelaar, 2009).

### 2.1.3 Products

Microalgae products can be mainly divided into non-fuel products and biofuels as outlined in Figure 2.4.

**Non-fuel products** Besides the main components proteins, lipids, and carbohydrates, *Chlorella* comprises a wide range of biotechnologically interesting compounds. Examples for such compounds are carotenoids, vitamins, chlorophyll, or $\beta$-1,3-glucan (Spolaore *et al.*, 2006; Seyfabadi *et al.*, 2011). These compounds are mostly used for animal feed or human food, in pharmaceuticals, and cosmetics.

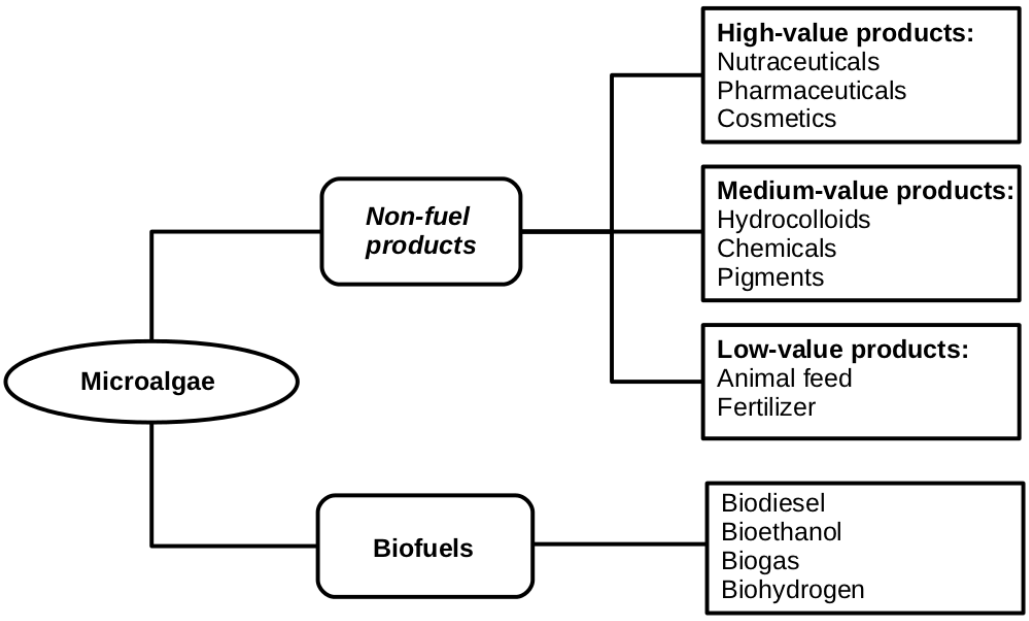


**Figure 2.4:** Classification of microalgae products

For human nutrition, *Chlorella* is nowadays marketed as pills, capsules, and liquids or added to pastas, sausages, snack foods, candy bars, and beverages (Liang *et al.*, 2004; Spolaore *et al.*, 2006). *Chlorella* has one of the highest chlorophyll contents found in nature (Burlew, 1953), which is used as an additive (e.g. natural colouring) for foods (Humphrey, 2004). It is also pharmaceutically important, as it is used in ointment, treatments for liver recovery, and ulcer treatment. Moreover, it repairs cells and increases haemoglobin in blood (Puotinen, 1997). The cultivation of *Chlorella vulgaris* plays an increasing role for aquaculture, because it represents an alternative to fish as the primary protein source for feed (Richmond, 2004). In particular, *Chlorella* can be used as livestock feed for zooplankton (e.g. rotifers and daphnia), fish, and mollusc larvae, as a 'greening effect' to improve pigmentation and growth conditions of oysters (Gastineau *et al.*, 2012), and as green-water technique to stabilize and improve the quality of the culture medium (Rodolfi *et al.*, 2003; Chuntapa *et al.*, 2003). About 30 % of the global algal production is sold to the feed sector (Becker, 2004).

**Biofuels** *Chlorella vulgaris* is a suitable feedstock for the production of different fuels including biogas, biodiesel, bioethanol, and biohydrogen.

Biodiesel is a commonly used biofuel, because a high share of vehicles are diesel-based and can therefore also utilise biodiesel (Chisti, 2008a). Microalgae with a high oil content and specifically with a high content of triacylglycerols (TAGs), which consist of three chains of fatty

acid attached to a glycerol backbone (Sheehan *et al.*, 1998), are suitable for the production of this biogenic fuel. The TAGs can be extracted and react, in a process called transesterification, with methanol to form fatty acid methyl ester (FAME), which is commonly known as biodiesel (Figure 2.5).

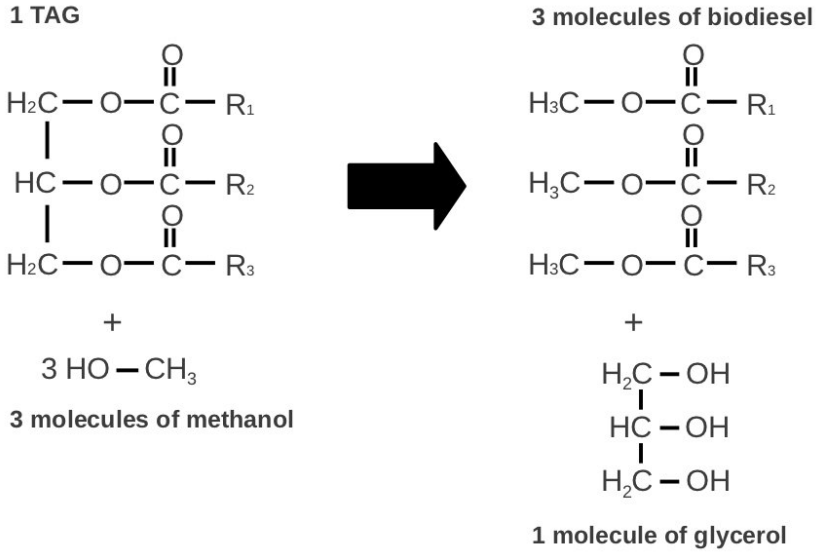


**Figure 2.5:** Biodiesel production: transesterification of triacylglycerols (TAGs)

Bioethanol is also well established as a fuel (Gray *et al.*, 2006) and is mainly derived from sugarcane or by the hydrolysis of starch (Gomez *et al.*, 2008). Thus, microalgae species that contain large quantities of starch as reserve polymers or of cellulose in the cell wall are of primary interest for ethanol production. Currently, there are two different ways of obtaining bioethanol from microalgae. The first method involves harvesting and decay of microalgae biomass and a subsequent addition of yeast, which ferments the microalgae biomass. The second method is a direct method using a specific bioreactor that collects the ethanol condensate from enhanced cyanobacteria (Algenol, 2013).

Algal biohydrogen production is attractive, because it uses sunlight to convert water to hydrogen and oxygen. It is therefore, at least theoretically, an emission-free process that can be coupled to atmospheric $CO_2$ sequestration (Schenk *et al.*, 2008). During photosynthesis, solar energy is used to extract protons and electrons from water via the water-splitting reaction. In green algae and under certain light and anaerobic conditions, these protons and electrons are recombined by a chloroplast hydrogenase to form molecular hydrogen (Zhang *et al.*, 2002). The hydrogen is then released by the cell as a gas. Currently, research on biohydrogen production is mainly focused on the green algae *Chlamydomonas reinhardtii*. However, the hydrogen production from microalgae biomass is still in its infancy.

The most simplistic approach to produce energy from algae is the anaerobic fermentation of microalgae biomass, because the process is not very sensitive to the biomass constitution (Sheehan *et al.*, 1998). Anaerobic fermentation breaks down most forms of organic carbon into methane. It comprises different stages and involves different microorganisms to convert the organic material into biogas, digestate, and new bacterial cells (Gerardi, 2003). The conversion is commonly known as a four-stage process with the stages hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Figure 2.6). The methane yield of microalgae depends on the cell composition, with the digestion of lipids resulting in the highest yields, followed by proteins and carbohydrates, and the digestibility of the microalgal cell wall. High lipid and protein contents and the absence of lignin make microalgae a highly suitable feedstock for the anaerobic digestion process (Schenk *et al.*, 2008).

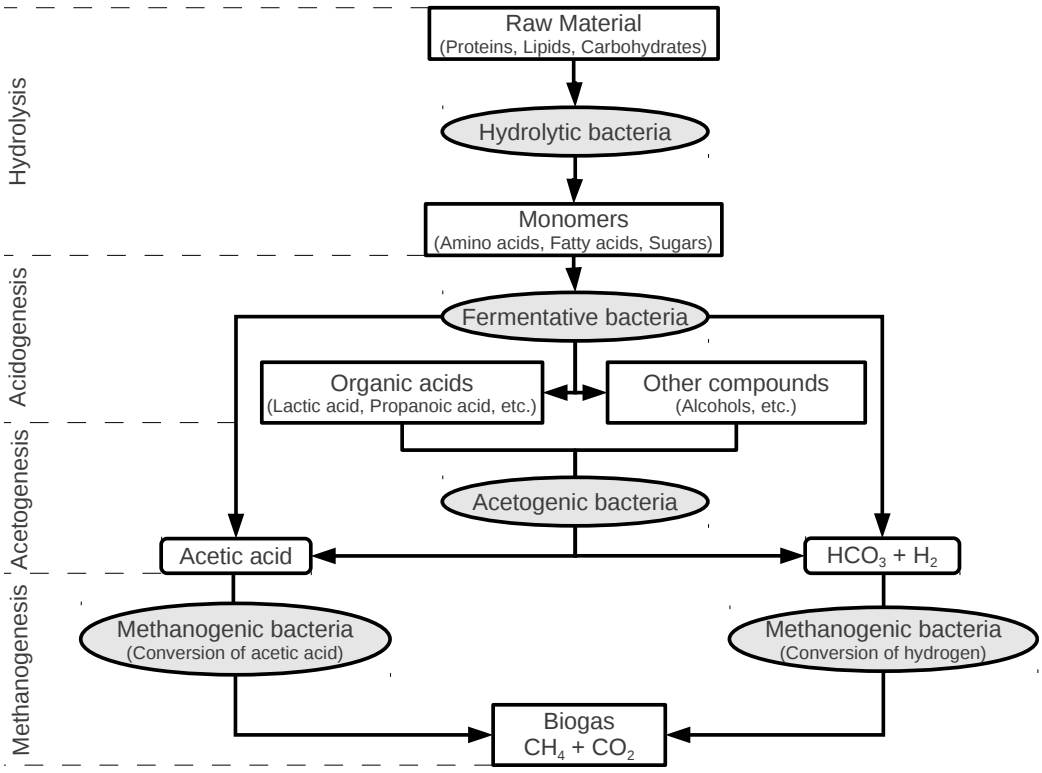


**Figure 2.6:** Anaerobic digestion of organic material modified from Scholwin *et al.* (2009); the four stages of the anaerobic fermentation process (hydrolysis, acidogenesis, acetogenesis, and methanogenesis) are highlighted in grey

## 2.2 Cultivation systems

Microalgae cultivation systems can be divided in open and closed systems. Applied phycologists define an open system as a cultivation system in which the culture is in direct contact with the atmosphere. Accordingly, in a closed system the culture is not directly in contact with the atmosphere and light does not impinge directly on the culture's surface (Tredici, 2004).

### 2.2.1 Process technology

**Open systems** Open systems require an ambient temperature continuously above the freezing point and optimally > 15°C. The pond depth is a compromise between an adequate light availability for algae cells (the shallower the better) and enough depth for mixing and avoidance of too high ionic fluctuation due to evaporation (Borowitzka, 1999). Most ponds are between 20 and 30 cm deep. Frequently, growth is light limited and typical biomass concentrations range between 0.1 and 0.5 g $L^{-1}$. Figure 2.7 shows a schematic diagram of an open pond. Algae, water, and nutrients are stirred by a paddle wheel and circulate around a raceway. $CO_2$ is constantly supplied to enhance the availability of carbon for the algae in the ponds.

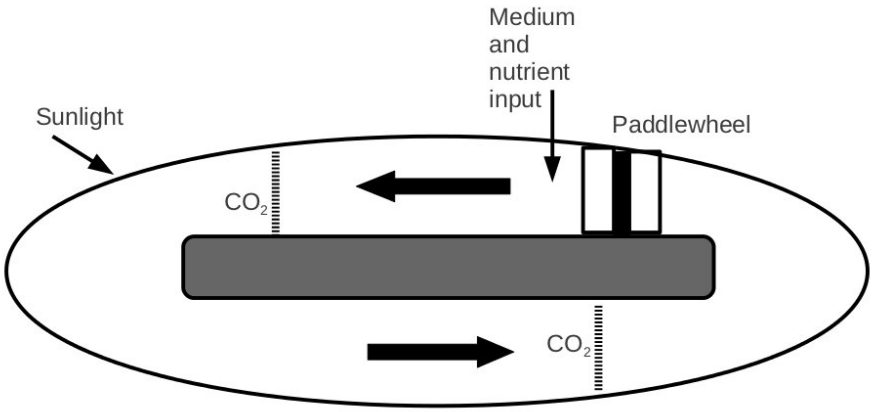


**Figure 2.7:** Schematic diagram of an open pond modified from Sheehan *et al.* (1998); bold arrows indicate the flow direction

The design of open ponds is simple. It can be distinguished between lakes, open ponds, closed open ponds, which are covered to reduce heat loss in cooler climates, raceway ponds, shallow big ponds, tanks, and circular ponds (Borowitzka, 1999; Mata *et al.*, 2010; Singh and Gu, 2010). Though there are many different pond systems, only three major designs have been operated in large scale: (1) circular ponds, (2) raceway ponds, and (3) inclined systems (Richmond, 2004).

Only very few microalgae species can be grown successfully in open ponds (e.g. *Spirulina*, *Dunaliella*). These species can cope with extreme growth conditions, for example a high pH, or have high growth rates and are therefore less affected by contamination (Pulz and Scheibenbogen, 1998). Despite perpetual contaminations by other microalgae, fungi, bacteria, or protozoa and the associated adverse effects on the efficiency of cultivation, the majority of the global annual microalgae production originates from open pond systems.

**Closed systems** Photobioreactors (PBRs) are fully closed systems, made of transparent material and specifically designed to meet the cultivation requirements of a specific algae. A PBR provides a controlled environment, especially with regard to temperature, culture density, pH levels, aeration rate, mixing regime, and the supplies of carbon dioxide, water, and light. The main issue of the PBR development is the surface-to-volume ratio. Increasing the surface-to-volume ratio leads to an improved light penetration, which in turn improves the photosynthetic efficiency (Carvalho *et al.*, 2006).

Following Olaizola (2003), the necessary characteristics of a PBR from a commercial point of view are:

- a high area productivity (g $m^{-2}$ $d^{-1}$),
- a high volumetric productivity (g $L^{-1}$ $d^{-1}$),
- a large volume (L $PBR^{-1}$),
- easily controllable culture parameters,
- a high reliability.

The most common closed reactor types are flat plate and tubular reactors (Carvalho *et al.*, 2006). These reactor types, which are described in detail in the following two Sections, achieve high surface-to-volume ratios.

***Flat plate reactor*** The flat plate reactor combines a high surface-to-volume ratio, which enables the efficient use of sunlight, with a simple setup (Morweiser *et al.*, 2010). The algae suspension is placed within narrow panels, which can be aligned horizontal, vertical, or at another angle (Figure 2.8). Main advantages of this design comprise high biomass concentrations, productivities, and the uniform distribution of light (Carvalho *et al.*, 2006). Water and nutrients are constantly pumped into the photobioreactor from a separate medium vessel and air and $CO_2$ are supplied via an aeration tube.

***Tubular reactor*** Tubular reactors consist of vertically, horizontally, or helically arranged transparent tubes, often installed as parallel loops (Morweiser *et al.*, 2010). Figure 2.9 shows a

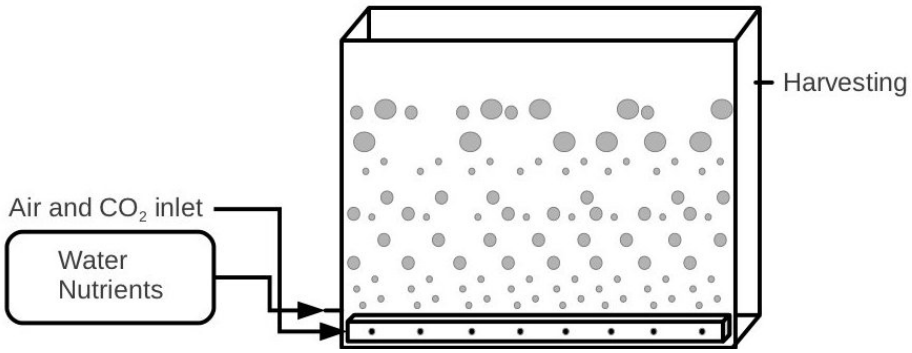


**Figure 2.8:** Schematic diagram of a flat plate photobioreactor

schematic diagram of a tubular PBR. The tubes are usually made of glass or plastic and generally less than 10 cm in diameter (Chisti, 2008a). They completely isolate the culture from the environment and thus from potential contaminants. Distribution of carbon dioxide and mixing is ensured by pumping the medium in a longitudinal laminar flow. High biomass concentrations of up to 6 g $L^{-1}$ can be achieved (Morweiser *et al.*, 2010). However, the scale-up of tubular photobioreactors entails a number of potential problems, e.g. high dissolved oxygen levels, suboptimum $CO_2$ supplies, and increase of pH levels, which all arise because of the disabled gas exchange with the environment over long time periods in systems with large tube lengths. Thus, the maximum length of a tube system is limited by the water chemistry of the cultivation medium.

Water, algae, and nutrients are constantly pumped through the tubular system, the nutrient tank, and back into the tubular system. Fresh nutrients are fed into the nutrient tank while the corresponding quantity of algae is harvested from the stream. The nutrient tank is aerated with air and $CO_2$, while $O_2$ is removed from the system.

**Open versus closed systems** The differences between open and closed systems lead to specific and significant advantages and disadvantages of both classes of systems, as summarised in Table 2.4.

Closed systems can reach higher yields per hectare, but cause higher set-up and operational costs compared to open systems. Culture conditions and growth parameters can be better controlled in closed systems, evaporation is reduced, and contamination is minimised. One of the main issues of flat plate reactors is the aeration, which is expensive, but essential to keep the suspension homogeneous, avoid microalgae settling, and provide algae with carbon dioxide. It is still unclear and, thus, calls for further research, whether higher productivities achieved in photobioreactors can compensate for the higher capital and operational costs (Mata *et al.*, 2010). Nevertheless,

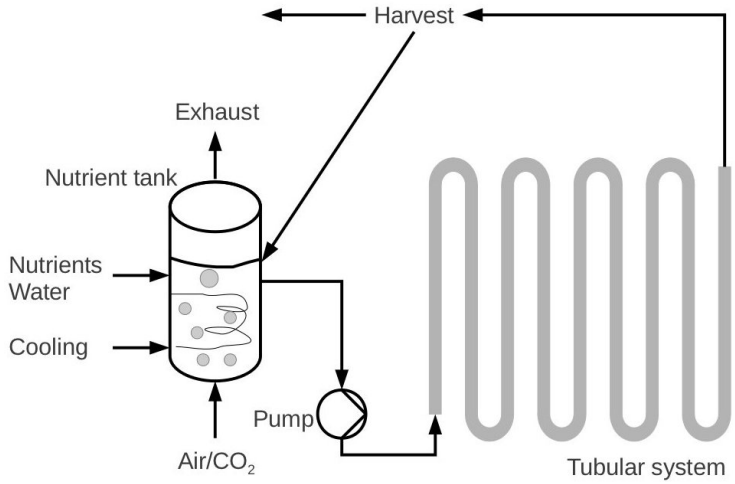


**Figure 2.9:** Schematic diagram of a tubular photobioreactor modified from Chisti (2008a)

**Table 2.4:** Main features of open and closed algal cultivation systems modified from Pulz (2001) and Grobbelaar (2009)

| Parameter | Open system | Closed system |
|---|---|---|
| Biomass concentration | Low, ~ 0.1 to 0.2 g $L^{-1}$ | High, ~ 2.0 to 8.0 g $L^{-1}$ |
| Productivity | Low | High |
| Contamination risk | Extremely high | Low |
| Capital and operating costs | Low | High |
| Evaporative losses | High | Low |
| Space required | High | Low |
| $CO_2$ losses | High | Low |
| Cultivatable species diversity | Cultivation restricted to a few species | Nearly all microalgae species cultivatable |
| Weather dependence | High | Low |
| Maintenance | Easy | Difficult |
| Overheating problems | Low | High |

there is a trend to use closed systems for the production of high-value products for application in pharmacy or cosmetics, because for these items a reliable and reproducible production and GMP (good manufacturing practice) conditions are critical.

Until now, however, most of the industrially produced microalgae biomass was and still is cultivated in open ponds, because of the simple set-up and operation of open ponds. Moreover, open pond systems may produce large quantities of microalgae, but for this they also occupy large

land areas (Mata *et al.*, 2010). Only specific microalgae can be grown in open ponds, whereas closed systems are advantageous for the cultivation of a variety of strains including sensitive algae. The cultivation of microalgae in open pond systems tends towards the production of biomass for commodities.

### 2.2.2 Operation modes

Open and closed microalgae cultivation systems can be operated continuously, semi-continuously, or in a batch operation mode.

- A continuous operation mode is characterised by a permanent flow through the culture, constantly supplying the algae with nutrients and harvesting the biomass. If the dilution rate is smaller than the maximum growth rate of the cultivated organisms, a steady state between production and losses of the population can be achieved. At steady state, the population density and the growth rate are determined by the substrate concentration in the medium and by the supply rate of new medium, respectively (Sciandra *et al.*, 2003). Before the steady state is reached the population undergoes a transient state (i.e. either increasing or decreasing). As a consequence, continuous PBRs have several advantages over batch systems: (1) growth rates can be maintained indefinitely and biomass concentrations can be controlled by varying the dilution rate, (2) continuously operated PBRs provide a better control than batch PBRs, (3) results are more reliable and reproducible, and (4) offer better possibilities for systematic investigations and improvements (Mata *et al.*, 2010).

- In semi-continuously operated PBRs, the culture medium is replaced with fresh medium at certain points in time, e.g. when a desired biomass or nutrient concentration is reached or before the culture reaches the stationary phase.

- In contrast, batch cultures are operated without addition of fresh medium for longer time periods than semi-continuously operated systems. Compared to continuous culture systems, semi-continuous and batch systems are technically simpler, but entail the disadvantage of ever-changing growth conditions (Jannasch, 1974).

# Chapter 3

# Material and Methods

In this Chapter, the growth conditions, experimental set-up, and the analytical procedures of this study are described in detail. More specifically, these comprise the growth conditions of the cultivated microalgae *Chlorella vulgaris*, the design of the outdoor pilot plant including the Flat-Panel Airlift (FPA) photobioreactor (PBR), the operation of the pilot plant, and potential sources of error. Moreover, the applied analytical and statistical methods are defined. Finally, the experimental designs and procedures for three microalgae cultivation and one anaerobic digestion experiment is presented.

## 3.1 Organism, culture conditions, and inoculum

*Chlorella vulgaris* (strain SAG 211-12 from the Culture Collection of Algae at Göttingen University) was cultivated in 180 L outdoor FPA photobioreactors. The algae were grown in an inorganic medium on the basis of a distilled water/seawater mixture (DS medium) modified from Pohl *et al.* (1987). Medium concentrations were 2.0 g $L^{-1}$ $KNO_3$, 3.5 g $L^{-1}$ aquarium synthetic sea salt (Instant Ocean), 20 mL $L^{-1}$ trace element solution A (DS-A solution), 0.15 g $L^{-1}$ $KH_2PO_4$, 20 mL $L^{-1}$ trace element solution B (DS-B solution), 1.2 g $L^{-1}$ $MgSO_4$, 0.56 g $L^{-1}$ $CaCl_2$, and 5 mg $L^{-1}$ Fe-Citrate. The DS-A solution contains 400 µg $L^{-1}$ $MnCl_2$, 100 µg $L^{-1}$ $ZnSO_4$, 100 µg $L^{-1}$ $CoSO_4$, 100 µg $L^{-1}$ $Na_2MoO_4$, and 10 µg $L^{-1}$ $CuSO_4$. The DS-B solution contains 0.4 g $L^{-1}$ $FeCl_3$, 0.55 g $L^{-1}$ $Na_2EDTA$, and 2.5 g $L^{-1}$ $K_2HPO_4$.

The inoculum for the outdoor cultivation was produced indoors in 6 L FPA photobioreactors followed by an upscaling step in 30 L FPA photobioreactors. Nutrient concentrations for the inoculum were identical to the ones for the outdoor pilot plant except for $MgSO_4$ and $CaCl_2$, which were not added to the inoculum. The pH was controlled and held close to 7.2 by constantly adding air and carbon dioxide at 24°C. Cultures were illuminated from one side only 24 h $d^{-1}$ with photosynthetically active radiation (PAR) between 50 and 400 µmol $m^{-2}$ $s^{-1}$ with halogen-metal vapour lamps (Betebe GmbH).

## 3.2 The outdoor pilot plant

The design and the functioning of the FPA photobioreactors are crucial for the pilot plant and therefore described in detail here. Furthermore, the outdoor pilot plant and its operation are explained in the following, as well as potential sources of error.

### 3.2.1 The 180 L Flat-Panel Airlift photobioreactor

The FPA photobioreactor is a vertical plate reactor consisting of two half-shells [Patent family EP 1 169428 B1 and EP 1 326959 B1]. It is 2,630 mm high and 1,900 mm wide. It has a layer thickness of 50 mm, and a surface area of about 4.5 $m^2$. Gas sparging provides for an upward movement of the fluid in parallel chambers, called risers (Figure 3.1 a)). The risers consist of single compartments that are interconnected by horizontally situated baffles (Figure 3.1 b)). These baffles are either connected to the front or the back of the reactor wall so that the aeration generates a circulation of the fluid within each compartment. The fluid, thus, moves swiftly between the light and the dark zone of a compartment exposing algae cells to a periodic light-dark cycle. By this means, the irradiance reaching the surface of the reactor is homogeneously distributed among algae cells. The downward circulation occurs in three downcomer zones. Because of the movement in the risers, downcomer zones, and compartments, an intense horizontal and vertical mixing can be achieved in the reactor.

The FPA reactor fulfills the needs for a commercial outdoor microalgae production by simultaneously allowing for high biomass concentrations and productivities. A key to accomplish these two seemingly conflicting aims is the flashing light effect, which refers to the frequent light-dark cycling of algae cells achieved by the small-scale flow pattern in the reactor. This effect enhances the conversion of light energy to biomass and hence increases the systems' productivity (Kok, 1956; Terry, 1986; Janssen *et al.*, 2000).

Microalgae cells repeatedly cycle between the lit exterior, with a high illumination intensity, and the dimly lit interior of a photobioreactor (Degen *et al.*, 2001) (Figure 3.2). The interior consists of a layer with optimal light intensity for microalgae growth, followed by a layer where light may be the limiting factor. Degen *et al.* (2001) reported that randomly mixing does not enhance the productivity as much as dark-light cycling with a frequency of 1 Hz. For the flashing light effect, which is applied in several photobioreactor designs (Laws *et al.*, 1983; Hu *et al.*, 1996), to work properly, the flow pattern and the geometry of the PBR have to be optimally matched.

The homogeneous distribution of microalgae within the FPA photobioreactor is a precondition for all further investigations with this reactor type. To prove the homogeneity within the reactor, the optical density ($OD_{750}$) of a microalgae suspension was measured at six locations evenly

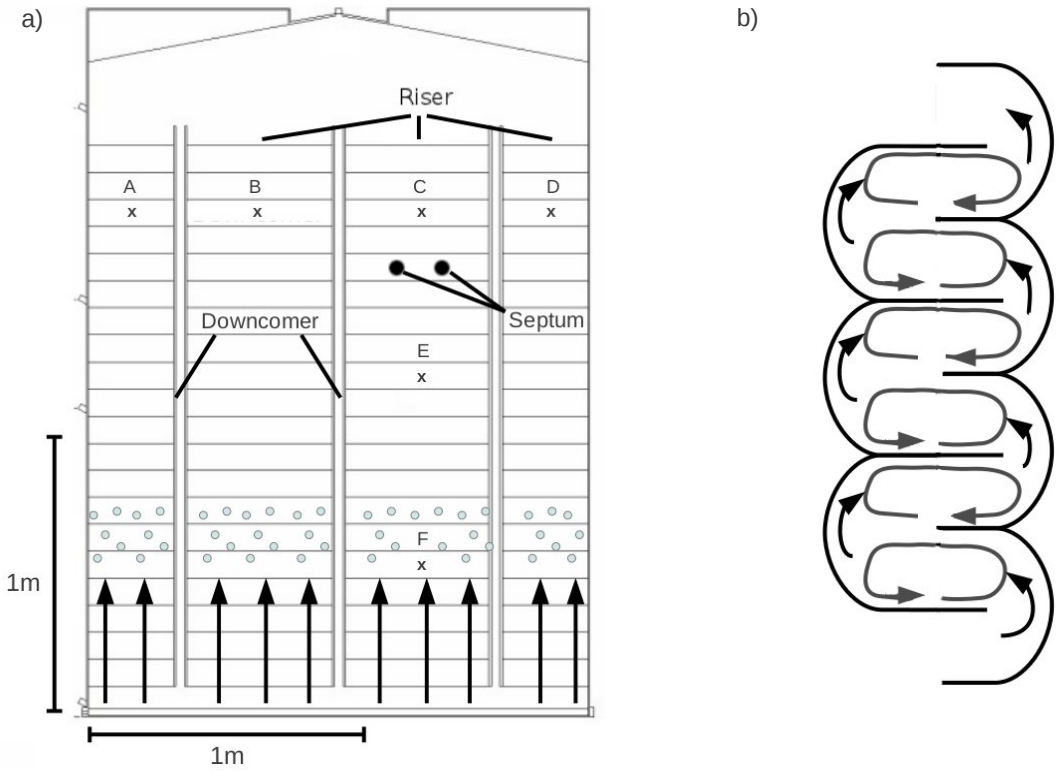


**Figure 3.1:** Diagram of the photobioreactor modified from Subitec GmbH (2012): a) 180 L FPA photobioreactor with dimensions; downcomer, riser, and septum for measurements and samplings are indicated; black arrows denote the gas sparging and the upward movement of the fluid; measurement locations (A - F) to test the homogeneity within the reactor are also included (Table 3.1); b) reactor profile with interconnected half shells and horizontally located baffles; arrows indicate the fluid flow in the riser and in each compartment

distributed over the reactor. Four measurement points were located in the upper part of the reactor (A, B, C, D), one in the middle part (E), and one in the lower part (F) (Figure 3.1 a)). To increase the robustness of the test, measurements were repeated under three different conditions at three different days.

The suspension of microalgae is regarded homogeneous here, if the standard deviation of the measurements of all six locations is 5 % smaller than the mean. According to this criterion, microalgae are distributed homogeneously within the photobioreactor, as the highest deviation from the mean is only 3.8 % (Table 3.1).

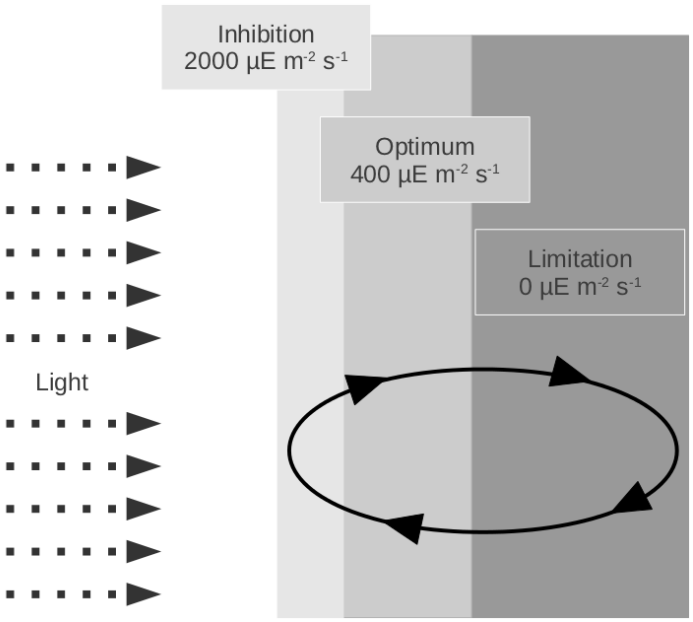


**Figure 3.2:** Profile of a FPA reactor, indicating the different zones of light distribution modified from Subitec GmbH (2012)

### 3.2.2 Design of the pilot plant

The microalgae pilot plant with a total volume of about 1.36 $m^3$ was located in Hamburg, Northern Germany (53°28'56"N, 10°7'44"E). Eight 180 L FPA photobioreactors (PBRs) were aligned in two parallel lines in East-West direction containing four PBRs in each line (Figure 3.3 a)). The front line (M1) was facing south and the back line (M2) was parallel to the first line in a distance of 2.5 m to M1 (Figure 3.3 b)). Both lines were operated individually.

The medium was prepared in tanks and the algae and medium flowed into the harvesting tanks via a sloping tube after opening a valve. In Figure 3.3 a), the locations of the vertical light sensor and the septum (used for pH and temperature measurements as well as samplings) are indicated.

### 3.2.3 Operation of the pilot plant

Before experiments started, the photobioreactors and the tube system were treated with hydrogen peroxide (3 %) overnight and washed three times with 0.2 µm filtered water (0.20 µm Polypropylen-Faltelement, Wolftechnik). After inoculation of the photobioreactors, it was aimed at keeping nutrient concentrations replete and the pH at approximately 7.2 by continuous addition of air and carbon dioxide or of flue gas. The reactors were filled with approximately 170 L medium.

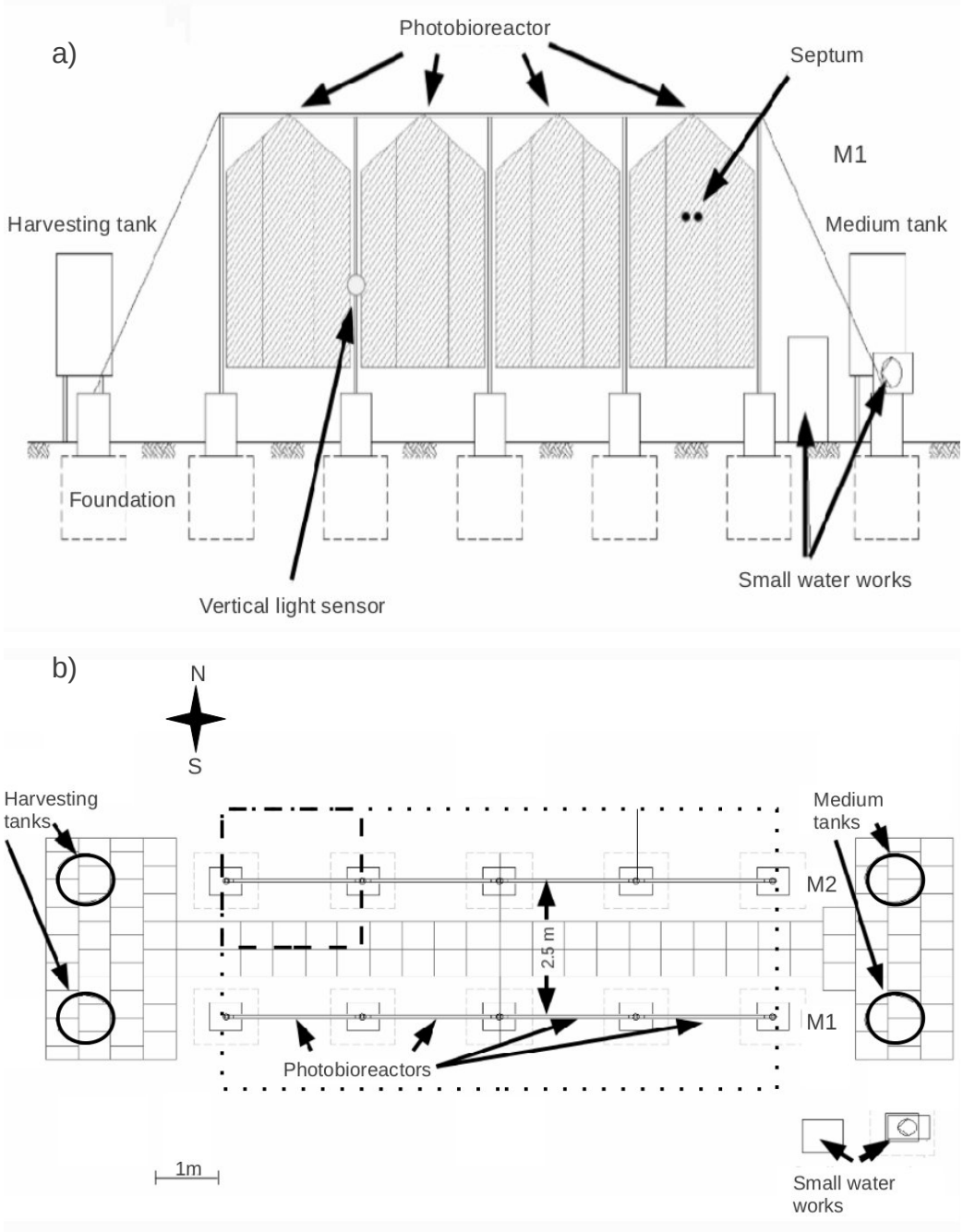


**Figure 3.3:** Schematic diagrams of the pilot plant: a) microalgae pilot plant with four aligned photobioreactors in line M1; b) top view of the microalgae pilot plant with lines M1 and M2 and the harvesting and medium tanks; quadratic structures on the ground indicate paving tiles; the pilot plant occupied a surface area of 75.32 $m^2$, of which 40.32 $m^2$ are reactor area (dotted area) and 35.00 $m^2$ are adjacent area; the minimum area to install a single reactor (without adjacent area) is 5.04 $m^2$ (dashed area)

**Table 3.1:** Optical density ($OD_{750}$) of microalgae suspension measured at six locations (A-F) and at three different time points (28-September 2009, 03-October 2009, 13-October 2009)

| Location | 28-September 2009 | 03-October 2009 | 13-October 2009 |
|---|---|---|---|
| | 5.60 | 5.88 | 3.00 |
| A | 5.60 | 5.92 | 3.20 |
| | 5.40 | 5.78 | 3.20 |
| | 5.40 | 5.76 | 3.00 |
| B | 5.60 | 5.80 | 3.20 |
| | 5.40 | 5.76 | 3.20 |
| | 5.60 | 5.74 | 3.00 |
| C | 5.40 | 5.84 | 3.00 |
| | 5.40 | 5.78 | 3.00 |
| | 5.40 | 5.74 | 3.00 |
| D | 5.60 | 5.82 | 3.20 |
| | 5.40 | 5.84 | 3.00 |
| | 5.60 | 6.00 | 3.20 |
| E | 5.60 | 6.02 | 3.20 |
| | 5.60 | 5.94 | 3.20 |
| | 5.60 | 5.78 | 3.20 |
| F | 5.80 | 5.84 | 3.20 |
| | 5.80 | 5.84 | 3.40 |
| Mean (A-F) | 5.54 | 5.84 | 3.13 |
| Standard deviation (A-F) | 0.13 | 0.09 | 0.12 |
| Deviation from the mean | 2.35 % | 1.54 % | 3.83 % |

Kessler (1985) found maximum growth temperatures for *Chlorella vulgaris* between 28 and 30°C. Therefore, automatic discontinuous irrigation of photobioreactors was activated at 27°C to prevent overheating. Irrigation stopped when temperature dropped to 26°C.

A semi-continuous harvesting regime was adopted in which roughly one third of the reactor volume was harvested and replaced by fresh medium approximately twice a week. Before harvesting, nitrate and phosphate concentrations were analysed to determine the optimal properties of the fresh medium to maintain nutrient concentrations replete. Gas exchange and mixing of the culture were achieved by continuously injecting a gas mixture into the reactor via a perforated membrane, which was integrated in the bottom of each photobioreactor. The injected gas, a mixture of compressed air and pure carbon dioxide or flue gas, was filtered through a 0.2 µm filter

(Wolftechnik). The aeration rate was maintained between 0.18 and 0.20 volume per volume per minute (vvm).

Sampling took place in the reactors of lines M1 and M2, which were equipped with a septum and a pH and temperature sensor (Figure 3.3 a)). Daily morning analyses included biomass concentration, optical density, inorganic nitrate and phosphate concentration, and microscopic examinations. Furthermore, bacterial and microalgal cell count samples were fixed and frozen. Organic carbon and nitrogen content samples were stored for later analysis. On harvesting days, the culture was sampled twice (before and after harvesting). Dissolved organic carbon and fatty acid samples were taken during harvesting events from the mixture of harvested medium of all reactors within a line.

### 3.2.4 Sources of error

In the following, potential sources of error that may occur during the cultivation process or during the sampling are listed.

Interrupted $CO_2$ or air supply, albeit rare, adversely affected the stability of the carbonate system and resulted in deviations from the target pH value of 7.2. This might happen because of malfunctioning devices, empty gas containers, or in case of maintenance measures that require an interruption of the gas supply. To minimize the impact of such events, only data that were not at all or only negligibly affected by interruptions in gas supply are presented an assessed. Nevertheless, the actual implications of such effects cannot be exactly determined. The same applies to failures of the discontinuous irrigation system and subsequent incidences of overheating.

The injected gas was constantly filtered through a 0.2 µm filter to remove suspended particles. Particularly when supplied with flue gas, the filter need to be changed in recurring intervals to prevent clogging and inadequately low aeration rates. While minor fluctuations in the aeration rates cannot be completely eliminated during cultivation, their effects on the growth conditions of the algae are assumed to be marginal.

Moreover, nutrient availability is controlled by regular measurements of nitrate and phosphate only. If required, the complete medium is replaced upon the results of these measurements. It is, thus, assumed that all other nutrients were available in sufficient quantities, because typically the macronutrients nitrate and phosphate are most critical for microalgae growth.

Regular sampling took place in a time range of approximately three hours between 9 am and 12 pm. Thus, calculated daily productivities may contain a certain error, because of the variations in sampling times. This error shrinks consequently for the mean productivity, i.e. the productivity calculated between two harvesting events.

Furthermore, samplings took place in the first reactor of each line, which is also equipped with a pH and a temperature sensor. Nevertheless, the results are regarded representative for the other reactors within a line. Light intensity is only measured regularly at one location in the middle of each line (Figure 3.3 a)). In a previous test, the differences between irradiances distributed over all reactors of line M1 were below 5 % and can thus be neglected. The differences between reactors of line M2 were slightly higher, which can be explained by the shadow cast of line M1, causing an inhomogeneous distribution of irradiance at times in line M2. Finally, the differences in irradiance between both line M1 and M2 are quantified in Chapter 4.3 using irradiances measured at the location in the middle of each line. To increase the reliability of these data, future investigations need to implement a higher resolution measuring the irradiance in each line and on each side of the photobioreactor.

## 3.3 Analytical and statistical methods

To characterise the growth and composition of microalgae, as well as the environmental parameters that led to the observed biotic dynamics, different analytical methods were applied. This Chapter gives an overview and definitions of the biotic and abiotic parameters and describes the applied statistical methods.

### 3.3.1 Biotic parameters

The biotic parameters considered here are listed alphabetically in Table 3.2 and explained below. The analytical methods to quantify these parameters are applied according to standardised, certified, and accredited laboratory guidelines. Following these guidelines, a maximum fault tolerance of 10 % can be maintained and generally a fault tolerance of 5 % can be assumed. It is discussed explicitly, when the error deviates from the above-mentioned range.

**Biomass concentration** Culture samples, which had a volume between 1 and 15 mL, were filtered through pre-weighed glass-fibre filters (Whatman GF/C; 25 mm) to determine the biomass concentration ($B$) [g dry weight $L^{-1}$] in the following referred to as [g $L^{-1}$]. Filters were washed twice with distilled water and dried overnight at 100°C until their weight remained constant and cooled down in a desiccator for a minimum of 15 min. The filter weight was determined on a precision balance (Mettler Toledo, EL 104).

**Carbon dioxide biofixation rate** The carbon dioxide biofixation rate ($F_{CO_2}$) [g $CO_2$ $L^{-1}$ $d^{-1}$] is calculated after De Morais and Costa (2007) using equation (3.1).

**Table 3.2:** Summary of biotic parameters that describe the microalgae cultivation and anaerobic digestion process; the column 'mc/ad' is short for 'microalgae cultivation/anaerobic digestion' and indicates the process in which the parameter is applied; indented parameters are explained in the previous parameter

| Biotic parameter | Symbol | Unit | mc/ad |
|---|---|---|---|
| Biomass concentration | $B$ | [g $L^{-1}$] | mc |
| Carbon dioxide biofixation rate | $F_{CO_2}$ | [g $CO_2$ $L^{-1}$ $d^{-1}$] | mc |
| Chemical oxygen demand and soluble chemical oxygen demand | $COD$, $sCOD$ | [g $L^{-1}$] | ad |
| Crude protein content | | [% of biomass] | mc |
| Dependency of the productivity on the light availability | | | mc |
| Dissolved and total organic carbon | $DOC, TOC$ | [g C $L^{-1}$] | mc/ad |
| Fatty acid content and composition | $FA$ content and composition | [% of biomass] | mc |
| Microalgal and bacterial cell counts | $CellC_{Alg}$, $CellC_{Bac}$ | [cells $mL^{-1}$] | mc |
| Microscopic examination | | | mc |
| Optical density | $OD_{750}$ | [] | mc |
| Optimal population productivity and density | $OPP, OPD$ | [g $L^{-1}$ $d^{-1}$], [g $L^{-1}$] | mc |
| Particulate organic carbon, nitrogen, and phosphorus | $POC, PON, POP$ | [g $L^{-1}$] or [mM] | mc |
| Organic carbon, nitrogen, and phosphorus | $OC, ON, OP$ | [% of biomass] | mc |
| Productivity (per volume, per area, per reactor surface area) | $P$ | [g $L^{-1}$ $d^{-1}$], [g $m^{-2}$ $d^{-1}$], [g $m^{-2}$ $d^{-1}$] | mc |
| Total bacterial fraction | $Frac_{Bac}$ | [%] | mc |
| Total specific biovolume of bacteria | $TSV_{Bac}$ | [µ$m^3$ $mL^{-1}$] | mc |
| Average biovolume of bacterial cells | $V_{Bac}$ | [µ$m^3$] | mc |
| Total specific biovolume of *Chlorella* | $TSV_{Alg}$ | [µ$m^3$ $mL^{-1}$] | mc |
| Average biovolume of a *Chlorella vulgaris* cell | $V_{Alg}$ | [µ$m^3$] | mc |
| Total solids and volatile solids | $TS, VS$ | [%], [% of $TS$] | ad |

$$F_{CO_2} = \frac{OC}{100} P \frac{M_{CO_2}}{M_C} \tag{3.1}$$

Here, $OC$, $P$, $M_{CO_2}$, and $M_C$ denote the carbon content of microalgal cells [% of biomass], the microalgal productivity [g $L^{-1}$ $d^{-1}$], the molar mass of carbon dioxide [g $mol^{-1}$], and the molar mass of carbon [g $mol^{-1}$], respectively.

**Chemical oxygen demand and soluble chemical oxygen demand** Samples for the soluble chemical oxygen demand ($sCOD$) [g $L^{-1}$] were centrifuged (12 min, 14,000 $min^{-1}$; Sigma 3K18) and the supernatant was filtered through 0.45 µm syringe filters (Whatman SPARTAN 30/0.45 RC). 10 mL of the filtered sample was acidified with 10 µL phosphoric acid (80 %, 1:1000) and stored at 4°C until further analysis.

Microalgae samples for the chemical oxygen demand ($COD$) [g $L^{-1}$] were acidified with phosphoric acid (80 %, 1:1000) without prior filtration and stored at 4°C until further analysis.

$COD$ and $sCOD$ samples were diluted 1:10 or 1:20 and then analysed in a photometer (Lange) with the test kits LCK014 and LCK114 (Lange), respectively.

**Crude protein content** Organic nitrogen ($ON$) contents were directly converted to crude protein [% of biomass] by applying the nitrogen-to-protein conversion factor (N-Prot factor) 4.78 (Lourenço *et al.*, 2004). This factor is derived from investigations of ten microalgae species including one *Chlorella* species.

**Dependency of the productivity on the light availability** The dependency of the productivity ($P$) on the light availability ($LA$) was approximated by Steele's function (Steele, J. H., 1962) using a nonlinear regression. This algorithm determines the optimal values for $OLA$ and $OPP$ in equation (3.2) that minimise the deviation between datapoints and model fit.

$$P(LA) \; = \; OPP \, \frac{LA}{OLA} \, e^{1 - \frac{LA}{OLA}} - \alpha \tag{3.2}$$

$OPP$ and $OLA$ denote the optimal population productivity and the optimal light availability, respectively. Note that the parameter $\alpha$ (0.15) was introduced here to allow for negative (net) productivities that occur when respiration or other losses exceed gross productivity.

**Dissolved and total organic carbon** Microalgae samples for the analysis of dissolved organic carbon ($DOC$) [g C $L^{-1}$] were centrifuged at 14,000 rpm for 12 min with a tabletop centrifuge (3K18, Sigma). The supernatant was then filtered through 0.45 µm syringe filters (Minisart (CA),

Sartorius). Samples (10 mL) were acidified with phosphoric acid (80 %, 1:1,000) and stored at 4°C until further analysis.

Microalgae samples for the analysis of total organic carbon ($TOC$) [g C $L^{-1}$] were acidified with phosphoric acid (80 %, 1:1,000) without prior filtration. Samples were stored at 4°C until further analysis.

$DOC$ and $TOC$ were analysed according to the thermocatalytic combustion principle (multi N/C 2000, Analytik Jena AG). Prior to analysis, samples were diluted 1:5. The sum of particulate and dissolved organic carbon measurements is the total organic carbon ($POC$ + $DOC$ = $TOC$). The $DOC$ content is also expressed as [% of $TOC$].

During microalgae cultivation experiments, only the $POC$ and $DOC$ contents were measured and, by definition, the sum of the two yields the $TOC$ content. Here, Whatman GF/C filters, which retain particles down to a minimum size of 1.2 µm, were used for $POC$ analysis.

The standard procedure is, however, to use GF/F filters that retain particles down to 0.7 µm. As $DOC$ is defined to be smaller than 0.45 µm, the calculation of $TOC$ does not take a range between 0.45 and 1.2 µm into consideration (instead of a standard range from 0.45 to 0.7 µm). This size range is neglected in the calculation of $TOC$ and, as a consequence, more particles remain uncounted than with the standard filter sizes. This leads to an underestimation of $TOC$ and also to a slight overestimation of $DOC$ when expressed as [% of $TOC$].

**Fatty acid content and composition** For fatty acid ($FA$) determination, algae cells were harvested by centrifugation (5,000 rpm, 12 min; Rotofix 32a, Hettich), washed twice with distilled water, and frozen at -28°C until further analysis.

Aliquots were lyophilised and the freeze-dried biomass was analysed by gas/liquid chromatography with prior transesterification (Lepage and Roy, 1984). The methyl esters were then injected in a gas chromatograph (GC, HP 5890 Series II Plus) provided with a column (Supelco SBP-PUFA 30 m · 0.32 mm), and a detector (HP Mass Selective 5972 Series). Temperature was maintained at 70°C for 3.5 min, then increased by a rate of 10°C $min^{-1}$ until it reached 160°C and subsequently increased further by a rate of 2°C $min^{-1}$ to 215°C. The maximum temperature was maintained for 13 min. Fatty acids were identified by similarities with retention times of known standards (C4:0, C6:0, C8:0, C10:0, C11:0, C12:0, C14:0, C14:1, C15:1, C16:0, C16:1(n-7), C17:1, C18:0, C18:1(n-9), C18:2(n-6), C18:3(n-6), C18:3(n-3), C20:0, C20:1(n-9), C20:2(n-6), C20:3(n-3), C20:3(n-6), C20:4(n-6), C20:5(n-3), C21:0, C22:0, C22:1(n-9), C22:2(n-6), C22:6(n-3), C23:0, C24:0, C24:1(n-9)). The $FA$ content is expressed as [% of biomass]. For the purposes of this investigation, the fatty acid content was taken as being equivalent to the lipid content.

**Microalgal and bacterial cell counts** Besides determining the biomass concentration, another way of quantifying a species is to count its cell numbers. Samples used to determine microalgal and bacterial cell numbers ($CellC_{Alg}$ and $CellC_{Bac}$) [cells $mL^{-1}$] were fixed with a preservative (1:30, Dekafald, Jan Dekker GmbH) and frozen at -28°C until measurement.

Frozen samples were thawed in a cold water bath, vortexed afterwards for 20 s and diluted (approx. 1:500) with distilled water until cell counts $s^{-1}$ were less than 1,000. Beads (Fluoresbrite Cat 18660, Polychromatic red 1.0 micron, Microspheres) were added as a standard (1:51.1). TruCount beads (Becton Dickinson) were used for calibration and absolute volume calculation. SybrGreenI and DMSO were mixed (1:25) and added to the sample (1:50). SybrGreenI (Lonza) was used as a nucleic acid stain. Bacterial and microalgal cell numbers were determined by flow cytometry (FACSCalibur, Becton Dickinson).

**Microscopic examination** The daily microalgae samples were examined for contamination and aggregation of microalgae cells under the light microscope. Alterations in the culture were noted and related to growth and biochemical parameter.

**Optical density** The optical density of a sample ($OD_{750}$) was determined spectrophotometrically (Spectroflex, WTW) at a wavelength of 750 nm against distilled water as blank. Samples with $OD_{750} > 0.4$ were diluted with distilled water until values fell within a range of 0.1 and 0.4. Mean values were calculated from triplicates.

**Optimal population productivity and density** The optimal population productivity ($OPP$) [g $L^{-1}$ $d^{-1}$] is defined as the maximum of the population productivity according to equation (3.2).

The optimal population density ($OPD$) [g $L^{-1}$] is defined as the cell density at which the culture reaches $OPP$. For a given ambient light intensity, the optimal light availability ($OLA$), which indicates the available light per unit of biomass and per unit of time and, hence, the $OPP$, can be achieved by manipulating the $OPD$. The $OPD$ can be calculated according to equation (3.3).

$$OPD = \frac{\frac{DLI_{ver}\, Surf_{Reactor}}{V_{Reactor}}}{OLA} \tag{3.3}$$

$DLI_{ver}$ is the vertically measured daily light intensity [µmol $m^{-2}$ $d^{-1}$], $V_{Reactor}$ is the reactor volume [L], $Surf_{Reactor}$ the reactor surface area [$m^2$], and $OLA$ is the optimal light availability.

**Particulate organic carbon, nitrogen, and phosphorus** Organic carbon ($OC$) and organic nitrogen ($ON$) contents [% of biomass] were analysed in duplicate from a microalgae suspen-

sion filtered onto pre-combusted glass fibre filters (GF/C, Whatman) with a Carlo Erba 1500 CNS analyser (Erba Science, Milan, Italy).

Organic phosphorus ($OP$) [% of biomass] was determined indirectly by assuming a ratio between organic nitrogen to phosphorus (N:P) of 16:1 (Redfield, 1958). The Redfield ratio represents the typical molar composition of phytoplankton (Hecky *et al.*, 1993).

Particulate organic nitrogen ($PON$), particulate organic carbon ($POC$), and particulate organic phosphorus ($POP$) contents [g $L^{-1}$] were determined by calculating the corresponding percentage of the $ON$, $OC$, and $OP$ with respect to the microalgae biomass concentration. Using the molar mass of nitrogen ($M_N$), carbon ($M_C$), and phosphorus ($M_P$), concentrations of $PON$, $POC$, and $POP$ were expressed as [mM].

To assess the nitrate and phosphate uptake, $PON$ and $POP$ production rates [mM $d^{-1}$] were integrated over time and denoted cumulated $PON$ and $POP$ production [mM], respectively.

**Productivity** The productivity ($P$) [g $L^{-1}$ $d^{-1}$] indicates the rate of biomass ($B$) change over time and is calculated according to equation (3.4).

$$P = \frac{B_{t1} - B_{t0}}{t1 - t0} \tag{3.4}$$

Here, $t0$ and $t1$ denote the chronologically ordered times of the daily biomass measurements. Thus, $P$ represents a net productivity between two time points (i.e. the minimum gross productivity in the respective period), because potential losses due to grazing, sinking, or respiration are unknown and hence not accounted for here.

The productivity can be also related to the land surface area that a single reactor occupies or the surface area of a reactor and expressed in units of [g $m^{-2}$ $d^{-1}$]. Here, the set-up of the pilot plant was provided. The entire pilot plant occupied a land surface area of 75.32 $m^2$, split into 40.32 $m^2$ reactor area and 35.00 $m^2$ adjacent area (Figure 3.3 b)). The distance between parallel reactor lines (2.5 m) has not been optimised with respect to potential shadowing on the reactor surface. The productivities in terms of the required land surface were obtained by using an area of 5.04 $m^2$ for a 180 L FPA photobioreactor ignoring additionally required adjacent area for auxiliary installations such as medium and harvesting tanks.

The productivities related to the reactor surface were determined by assuming a reactor surface area of 4.5 $m^2$.

**Total bacterial fraction** The total bacterial fraction ($Frac_{Bac}$) [%] of the total specific biomass volume is calculated using equation (3.5).

$$Frac_{Bac} = \frac{TSV_{Bac}}{(TSV_{Bac} + TSV_{Alg})/100} \tag{3.5}$$

$TSV_{Bac}$ and $TSV_{Alg}$ denote the total specific biovolume of bacteria [µm$^3$ mL$^{-1}$] and *Chlorella* cells [µm$^3$ mL$^{-1}$], respectively.

**Total specific biovolume of bacteria** The total specific biovolume of bacteria is calculated to estimate the often neglected bacterial fraction in microalgae cultures. The most prominent bacteria in *Chlorella* cultures belong to the class Alphaproteobacteria (Lakaniemi *et al.*, 2012), which are about the same size as average bacterial cell (Straza, 2010). Therefore, a rod-shaped bacterial cell with a length ($l$) of 1.2 µm and a diameter of 0.7 µm was assumed here to calculate the biovolume of bacterial cells in a *Chlorella vulgaris* culture. The biovolume was calculated after Hulatt and Thomas (2010) using equation (3.6).

$$V_{Bac} = 2\,(2/3\pi r_{Bac}^3) + \pi r_{Bac}^2 l_{Bac} \tag{3.6}$$

$V_{Bac}$ denotes the average biovolume of bacterial cells [µm$^3$], $r_{Bac}$ is the radius [µm], and $l_{Bac}$ is the length [µm] of the rod-shaped cell. The average volume ($V_{Bac}$) [µm$^3$] multiplied by the bacterial cell concentration ($CellC_{Bac}$) [cells mL$^{-1}$] is then the total specific biovolume ($TSV_{Bac}$) [µm$^3$ mL$^{-1}$] of bacteria (Gasol *et al.*, 1995). The $TSV_{Bac}$ was calculated using equation (3.7).

$$TSV_{Bac} = V_{Bac}\, CellC_{Bac} \tag{3.7}$$

**Total specific biovolume of *Chlorella*** The average biovolume of a *Chlorella vulgaris* cell ($V_{Alg}$) [µm$^3$] was calculated using equation (3.8).

$$V_{Alg} = 4/3\pi r_{Alg}^3 \tag{3.8}$$

$r_{Alg}$ indicates the radius [µm] of a spherical cell. A *Chlorella* cell is 5 to 10 µm in diameter (Scragg *et al.*, 2003) and in this study a spherical cell with an average diameter of 7.5 µm was assumed. The biovolume ($V_{Alg}$) [µm$^3$] multiplied by the microalgal cell concentration ($CellC_{Alg}$) [cells mL$^{-1}$] is the total specific biovolume ($TSV_{Alg}$) [µm$^3$ mL$^{-1}$]. The $TSV_{Alg}$ was calculated using equation (3.9).

$$TSV_{Alg} = V_{Alg}\, CellC_{Alg} \tag{3.9}$$

**Total solids and volatile solids** Standard procedures DIN EN 12880 and DIN EN 12879 were applied to respectively determine the amount of total solids ($TS$) [%] and volatile solids ($VS$) [% of $TS$] of inocula for biogas experiments.

Different from the DIN protocols, however, $TS$ in microalgal suspensions was measured following the above-described procedures for the biomass concentration ($B$). $VS$ was determined after combusting the pre-weighed glass fiber filters at 500°C for a minimum of 5 h.

### 3.3.2 Abiotic parameters

The abiotic parameters considered here are listed alphabetically in Table 3.3 and explained below. Again, the analytical methods are applied according to standardised, certified, and accredited laboratory guidelines. Following these guidelines, a maximum fault tolerance of 10 % can be maintained and generally a fault tolerance of 5 % can be assumed. It is discussed explicitly, when the error deviates from the above-mentioned range.

**Biogas yield** The suitability of *Chlorella vulgaris* as a substrate for biogas production was tested in anaerobic fermentation batch tests. Anaerobic digestion experiments were conducted under mesophilic conditions according to guideline Verein Deutscher Ingenieure (2006). The test system works according to volumetric principles as described in Heerenklage *et al.* (2000).

After cultivating in the pilot plant, the microalgae were harvested by centrifugation (10 min at 6,000 x g; Heraeus, Varifuge F) and stored at -28°C until analysis. Prior to anaerobic digestion experiments, the biomass was thawed at 4°C and diluted with distilled water to a biomass concentration of about 3 %. Microalgae suspension was then transferred into flasks and stored in the refrigerator until further usage.

The inoculum was obtained from digested sludge of the municipal wastewater treatment plant Köhlbrandhöft in Hamburg, Germany. Total solids ($TS$) in the inoculum were approximately 3 to 3.8 % and the volatile solids ($VS$) amounted to approximately 61 % of $TS$. Prior to use, the inoculum was stored at mesophilic conditions for one week.

Fermenters without the addition of substrate were used as blanks. Microcrystalline cellulose was used as reference substrate (3.2 mg cellulose was added $mL^{-1}$ inoculum). Blank and reference experiments were conducted to determine the net biogas production and to ensure the biological activity of the inoculum, respectively. For each sample, triplicate batch tests were conducted.

Generally, results from the biogas experiments presented here were compiled from a series of experiments that were conducted at different times. As a consequence, differences in the materials used during the experiments cannot be ruled out. For instance, the properties of the digested

**Table 3.3:** Summary of abiotic parameters that describe the microalgae cultivation and anaerobic digestion process; the column 'mc/ad' is short for 'microalgae cultivation/anaerobic digestion' and indicates the process in which the parameter is applied; indented parameters are explained in the previous parameter

| Abiotic parameter | Symbol | Unit | mc/ad |
|---|---|---|---|
| Biogas yield | | [mL $g^{-1}VS$] | ad |
| Methane yield | | [mL $CH_4$ $g^{-1}VS$] | ad |
| Carbon dioxide and methane concentration | $CO_2$, $CH_4$ concentration | [Vol.-%] | mc/ad |
| Degree of decarbonisation | $Degree_{CO_2}$ | [%] | mc |
| Dissolved inorganic nitrogen and phosphorus | $DIN$, $DIP$ | [mg $L^{-1}$] or [mM] | mc |
| Inorganic nitrate and phosphate concentration | $NO_3$, $PO_4$ concentration | [mg $L^{-1}$] | mc |
| Light availability | $LA$ | [µmol $g^{-1}$ $d^{-1}$] | mc |
| Optimal light availability | $OLA$ | [µmol $g^{-1}$ $d^{-1}$] | mc |
| Medium temperature and pH | $T$ | [°C] | mc |
| Mean, maximum, and minimum medium temperatures | $MeanT$, $MaxT$, $MinT$ | [°C] | mc |
| Temperature variance | $TVar$ | [$°C^2$] | mc |
| Nitrate and phosphate requirements | | [mg $NO_3$/$PO_4$ $g^{-1}$ biomass] | mc |
| Nitric oxide and carbon monoxide concentration | $NO_x$, CO concentration | [ppm] | mc |
| Sunshine duration and cloudiness | $SD$, $Cl$ | [h], [relative cloud cover] | mc |
| Theoretical methane yield | | [L $CH_4$ $g^{-1}VS$] | ad |
| Vertically measured light intensity and daily light intensities | $LI_{ver}$, $DLI_{ver}$ | [µmol $m^{-2}$ $s^{-1}$], [µmol $m^{-2}$ $d^{-1}$] | mc |

sewage sludge, which was received weekly from a municipal wastewater treatment plant, may vary over time. To proof the reproducibility of the experiments, the results of the cellulosic test runs were compared and showed no significant difference between samples (ANOVA; F = 0.37, p = 0.92). Experiments are hence comparable. Cellulose samples performed well and were almost fully degraded.

The pH (WTW pH 91) prior and after anaerobic digestion experiments, $TS$ and $VS$ contents from the substrate and inoculum, and the biogas composition (analysed every three to five days) were determined.

The biogas and methane yields are expressed in units of [mL $g^{-1}VS$] and [mL $CH_4$ $g^{-1}VS$], respectively, and analysed under standard temperature (273.15 K) and pressure (1013 hPa) conditions (STP) generated from one gram of volatile solids in anaerobic fermenters. The specific maximum methane yield was derived from the maximum biogas yield and the averaged methane concentration.

**Carbon dioxide and methane concentration** Carbon dioxide ($CO_2$) and methane ($CH_4$) concentrations were measured during microalgae and biogas experiments. In microalgae experiments, inlet and outlet gas mixtures of four interconnected photobioreactors were sampled by using gas sampling tubes and applying a flow period of 5 to 10 minutes. In anaerobic digestion experiments, gas mixtures were sampled from flask headspaces via a syringe.

The gas composition ($CH_4$ [Vol.-%], $N_2$ [Vol.-%], $O_2$ [Vol.-%], $CO_2$ [Vol.-%]) was analysed within 24 hours using a gas chromatograph (GC; 5890 series II, HP) with helium as carrier gas. The GC was calibrated with a gas mixture of 5 Vol.-% $H_2$, 34.9 Vol.-% $CO_2$, 10.0 Vol.-% $N_2$, and 50.1 Vol.-% $CH_4$.

**Degree of decarbonisation** The degree of decarbonisation ($Degree_{CO_2}$) [%] was calculated after Doucha *et al.* (2005) using equation (3.10).

$$Degree_{CO_2} = 100(1 - \frac{CO_{2,out}}{CO_{2,in}}) \tag{3.10}$$

Here, $CO_{2,in}$ and $CO_{2,out}$ are the $CO_2$ concentrations in the flue gas before entering and after passing through the four interconnected photobioreactors.

**Dissolved inorganic nitrogen and phosphorus** Inorganic nitrate ($NO_3$) and inorganic phosphate ($PO_4$) concentrations [mg $L^{-1}$] were analysed daily, as well as prior to and after harvesting by test kits (N2/25, P7/25; WTW) using a spectrophotometer (Spectroflex, WTW). Prior to analysis, samples were diluted with distilled water (1:20).

The $NO_3$ and $PO_4$ concentrations, respectively, comprise the dissolved inorganic nitrogen ($DIN$) and phosphorus ($DIP$) [mg $L^{-1}$] pools. Using the molar mass of $NO_3$ ($M_{NO_3}$) and $PO_4$ ($M_{PO_4}$), concentrations of $DIN$ and $DIP$ can also be expressed as [mM].

To assess the nitrate and phosphate uptake, $DIN$ and $DIP$ consumption rates [mM $d^{-1}$] were integrated over time and termed cumulated $DIN$ and $DIP$ consumption [mM], respectively.

**Light availability** The light availability ($LA$) [µmol $g^{-1}$ $d^{-1}$] depends on the vertically measured daily light intensity ($DLI_{ver}$) [µmol $m^{-2}$ $d^{-1}$] and the biomass concentration ($B$) [g $L^{-1}$]. It is given by equation (3.11).

$$LA = \frac{DLI_{ver}}{(\frac{BV_{Reactor}}{Surf_{Reactor}})} \tag{3.11}$$

$V_{Reactor}$ is the reactor volume [L] and $Surf_{Reactor}$ is the reactor surface area [$m^2$].

The optimal light availability ($OLA$) can be derived from equation (3.2). It is the x-value corresponding to the maximum y-value, which is the optimal population productivity ($OPP$).

**Medium temperature and pH** Medium temperature [°C] and pH were measured by one sensor (InPro3253, Mettler Toledo) that was positioned in the upper region of the first reactor of line M1 and M2 (Figure 3.3 a)). Temperature and pH were monitored every minute. Values for the pH and daily mean, maximum, and minimum medium temperatures ($MeanT$, $MaxT$, $MinT$), as well as the temperature variance ($TVar$), were derived from these data.

**Nitrate and phosphate requirements** Species-specific nitrate [mg $NO_3$ $g^{-1}$ biomass] and phosphate requirements [mg $PO_4$ $g^{-1}$ biomass] were determined assuming that the assimilation of 1 mM particulate organic nitrogen ($PON$) or particulate organic phosphorus ($POP$) requires 1 mM of dissolved inorganic nitrogen ($DIN$) or dissolved inorganic phosphorus ($DIP$), respectively. Additionally, it is assumed that the algae culture was aerated with a pure $CO_2$/air mixture. The required particulate organic nitrogen and phosphorus contents were derived from experiments conducted in spring 2011.

**Nitric oxide and carbon monoxide concentration** Nitric oxide ($NO_x$) [ppm] and carbon monoxide (CO) [ppm] concentrations were measured directly in the inlet and outlet gas mixtures of the four interconnected photobioreactors using a flue gas analysis device (ecom-EN2, rbr).

**Sunshine duration and cloudiness** The German Weather Service (Deutscher Wetterdienst, DWD) provided concurrent measurements of sunshine duration [h] and cloudiness [relative cloud cover] from the long-term weather station at Hamburg Fuhlsbüttel (DWD, 2012). The data are representative for the pilot plant site.

**Theoretical methane yield** The theoretical methane yield of organic matter [L $CH_4$ $g^{-1}$ volatile solids ($VS$)] can be calculated according to its composition. Several authors including Symons and Buswell (1933), Boyle (1976), Keymer and Schilcher (1999), and Weissbach (2008) have developed different approaches to calculate a theoretical yield. Here, it is calculated according to Sialve *et al.* (2009) who used an adapted formula developed by Symons and Buswell (1933), which is given by equation (3.12).

$$C_aH_bO_cN_d + \frac{4a - b - 2c + 3d}{4}H_2O \rightarrow \frac{4a + b - 2c - 3d}{8}CH_4 + \frac{4a - b + 2c + 3d}{8}CO_2 + dNH_3 \tag{3.12}$$

C, H, O, and N are the chemical elements carbon, hydrogen, oxygen, and nitrogen, respectively, and $a$, $b$, $c$, and $d$ indicate the quantity of the elements. Cell maintenance and anabolism are not taken into account here. The organic matter is stoichiometrically converted into $CH_4$, $CO_2$, and $NH_3$.

The average compositions of lipids, carbohydrates, and proteins are extracted from Angelidaki and Sanders (2004) and Becker (2007) (Table 3.4). After applying equation (3.12), the derived methane values are multiplied by the normal molar volume of methane and result in the specific methane yields per gram volatile solids (Table 3.4).

**Table 3.4:** Average compositions and specific methane yields of lipids, carbohydrates, and proteins (Angelidaki and Sanders (2004) and Becker (2007))

| Substrate | Composition | L $CH_4$ $g^{-1}$ $VS$ |
|---|---|---|
| Lipids | $C_{57}H_{104}O_6$ | 1.014 |
| Carbohydrates | $(C_6H_{10}O_5)n$ | 0.415 |
| Proteins | $C_6H_{13.1}O_1N_{0.6}$ | 0.851 |

Assuming a protein, lipid, and carbohydrate content of 51 to 58 %, 14 to 22 %, and 12 to 17 %, respectively (Becker, 2004), *Chlorella vulgaris* achieves a theoretical methane yield of 0.63 to 0.79 L $CH_4$ $g^{-1}$ $VS$.

**Vertically measured light intensity and daily light intensity** The light intensity ($LI_{ver}$) [µmol $m^{-2}$ $s^{-1}$] incident on the reactor surface was measured every minute by a light sensor (190 Quantum Sensor, LI-COR), vertically attached to the surface of a reactor. Each line (M1 and M2) was equipped with such a sensor in the lower third of one of the middle reactors (Figure 3.3 a)).

The measured light intensity was then summed up and multiplied by 60 to estimate the daily light intensity ($DLI_{ver}$) [µmol $m^{-2}$ $d^{-1}$].

The difference between $DLI_{ver}$ of line M1 and M2 was measured by subtracting the daily light intensity incident on line M2 from line M1.

### 3.3.3 Statistical methods

Statistical analyses were conducted using the software package Matlab (R2011b, MathWorks). If possible, data from microalgae cultivation and anaerobic digestion experiments were obtained from multiple measurements to improve robustness. Values determined from more than one measurement are then given as the mean ± standard deviation.

**Analysis of variance** The analysis of variance (ANOVA) is a statistical technique, which compares the differences between group means. It is one aim of ANOVA to test the null hypothesis that population group or treatment means are equal (Quinn and Keough, 2002). In this investigation, treatment effects between e.g. flue gas and pure $CO_2$ aerated microalgae cultures or between anaerobic digestion experiments were evaluated by applying ANOVA. A significance level of $p < 0.05$ was chosen.

**Linear correlation** The Pearson correlation coefficient (r) measures the strength of linear relationships. Note that r can be positive or negative with +1 and -1 indicating a perfect linear correlation and zero indicating no correlation.

**Principal component analysis** A principal component analysis (PCA) is a multivariate statistical tool that maps multidimensional data onto a set of linearly independent variables, the principal components (PCs). PCs are hence orthogonal to each other and sorted in descending order of their explanatory power, i.e. the first PC explains the largest part of the variance of the data. Therefore, a PCA can also be used to reduce the dimensionality of the data while minimizing the loss of information by considering only the first PCs of a data set (Quinn and Keough, 2002). A PCA can reveal patterns in the data that remain undetected when analysing

each variable separately. It is therefore a tool to extract relevant information from complex data sets. Standard PCA, however, only accounts for linear relationships in the data set.

Here, the PCA was applied to light variables (e.g. maximum light intensity, daily light intensity, light availability, sun duration, cloudiness) and temperature variables (mean, maximum, minimum temperature, and temperature variance), as well as to the biomass concentration and productivity. All data used were generated during exponential and early stationary growth phases and calculated as mean values between two harvesting events. Table 3.5 shows the cultivation periods considered for PCA.

**Table 3.5:** Cultivation periods considered for principal component analysis (PCA)

| Line | Spring period | Autumn period |
|---|---|---|
| M1 | 04-May - 08-June 2010 | 11-September - 20-September 2010 |
| M2 | 04-May - 08-June 2010 | 10-September - 20-October 2010 |
| M1 | 05-May - 07-June 2011 | 30-September - 02-November 2011 |
| M2 | 05-May - 07-June 2011 | 06-October - 25-October 2011 |

Data from cultures aerated with flue gas or pure $CO_2$ were treated equally. A varimax rotation of the eigenvectors (i.e. an orthogonal rotation of the factor axes) was applied to get a better structure for the components, because several variables in the first and second components (PC1 and PC2) revealed only moderate correlations (0.3 to 0.4).

Furthermore, eigenvectors were used to calculate a z-score of each component and for each object, which is the normalisation that makes variables comparable by dividing each element by the variable standard deviation (Abdi *et al.*, 2013). The objects were then positioned on a scatterplot.

## 3.4 Experimental designs and procedures

Here, the designs and procedures of four different experiments are presented. While three of them are concerned with the cultivation of microalgae, the fourth reports the application of microalgae biomass as biogas substrate.

### 3.4.1 Experiment 1: Effect of light and temperature on productivity and composition

To unravel the influence of temperature and light on the outdoor biomass productivity and composition, growth experiments were conducted in line M1 during spring 2011 (cf. Chapter 4.1.1). The aim was to rigorously characterise the cultivation system in terms of light, temperature, biomass concentration, productivity, carbon and nitrogen contents, crude protein contents, and fatty acid composition and contents. All relevant parameters of this experiment are shown in Figure 3.4.

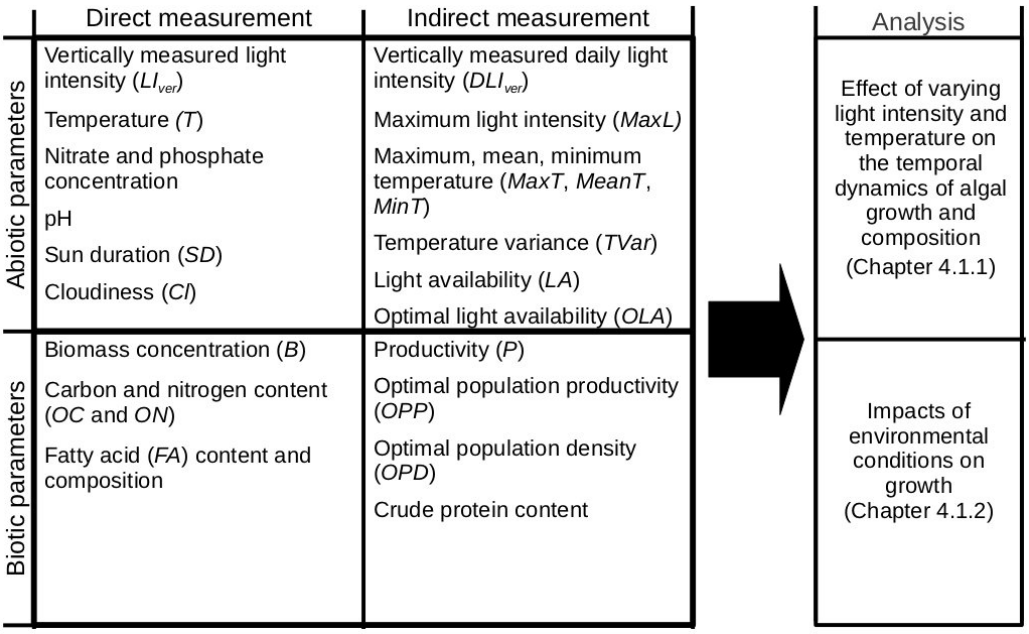


**Figure 3.4:** Biotic and abiotic parameters of Experiment 1, separated into direct and indirect measurements

A principal component analysis was applied to a large data set including data from cultivation periods in spring and autumn of the years 2010 and 2011 to identify the climatic parameters driving the measured productivity of the system (cf. Chapter 4.1.2). Furthermore, a non-linear relationship between light availability and productivity was investigated and the data set was approximated by Steele's equation (Steele, J. H., 1962). By this means, the optimal population productivity ($OPP$) and density ($OPD$) of the system could be derived. With these two parameters, the productivity in outdoor microalgae systems can be optimised by adapting the harvesting regime.

### 3.4.2 Experiment 2: Nitrate and phosphate uptake and the fate of carbon

First, the assimilation efficiencies for the macronutrients nitrogen and phosphorus were tested by comparing the cumulated particulate organic nitrogen ($PON$) and cumulated particulate organic phosphorus ($POP$) productions with the cumulated dissolved inorganic nitrogen ($DIN$) and cumulated dissolved inorganic phosphorus ($DIP$) consumptions, respectively (cf. Chapter 4.2.1). For this, two different cultivation periods (from May to June in 2010 and in 2011) in reactor lines M1 and M2 were analysed to evaluate the uptake and assimilation of nitrate and phosphate. The microalgae cultures were aerated with a $CO_2$/air mixture, except for line M2 in spring 2011, which was aerated with a flue gas/air mixture. This experimental set-up thus allowed for the determination of the system's nutrient conversion efficiency and of the utilisation of flue gas as a potential nitrogen source. Moreover, species-specific nitrate and phosphate requirements for *Chlorella vulgaris* were established.

The loss of dissolved organic carbon ($DOC$) was determined in line M1 during spring 2011 and related to microalgal and bacterial cell counts (cf. Chapter 4.2.2). Additionally, the total bacterial fraction was estimated during this cultivation period. To study the effect of $DOC$ on bacterial and microalgal growth in outdoor cultures and the relationship between microalgae and bacteria, glucose was added to the microalgae suspension of one photobioreactor. Glucose served as $DOC$ substitute, as it is the most simple $DOC$ form that can be exuded by microalgae. To ensure an adequate homogenisation within the photobioreactor, $DOC$ and bacterial and microalgal cell counts were measured 3 h after the glucose has been added. The measurements were repeated daily and compared to a control over a period of 13 days. All relevant parameters of these experiments are shown in Figure 3.5.

### 3.4.3 Experiment 3: Flue gas as a carbon and nitrogen source

Microalgae cultures were aerated with a flue gas/air mixture to investigate the effect of flue gas on microalgae growth and biochemical composition. Flue gas was supplied by a combined heat and power (CHP) plant (ECPower) that was driven by natural gas and located in the proximity of the microalgae pilot plant. The flue gas was cooled and dewatered via a dehumidification device (M-M-T Supergas) before it has been fed to the microalgae culture. The flue gas composition was then analysed directly after leaving the power plant using a flue gas analysis device (ecom-EN2, rbr).

Due to the specific lay-out of the pilot plant with the two parallel lines M1 (facing the sun) and M2 (situated in the shadow of line M1), differences in the solar radiation incident on the reactor walls of both lines occurred. Therefore, before assigning differences in the results of the two lines to the flue gas treatment, the comparability between line M1 and M2 was tested. Thus, two

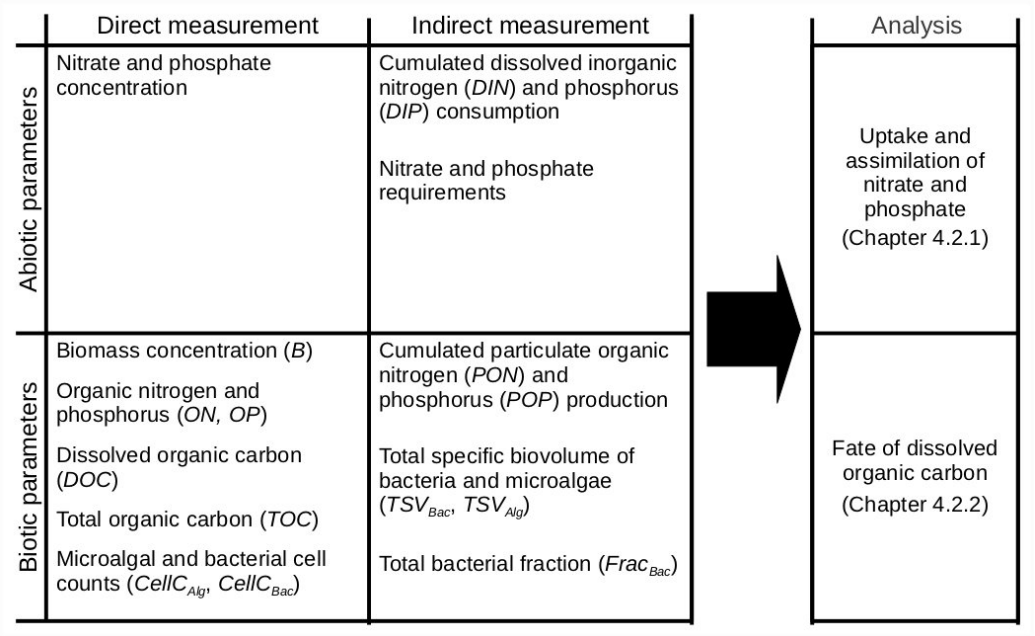


**Figure 3.5:** Biotic and abiotic parameters of Experiment 2, separated into direct and indirect measurements

sets of experiments were conducted: (1) proving the comparability of the system (cf. Chapter 4.3.1) and (2) analysing the effect of flue gas on algal growth and on the biochemical biomass composition (cf. Chapter 4.3.2).

To assess the comparability of the cultivation system, *Chlorella vulgaris* was grown in line M1 and M2 under identical pH and nutrient conditions and under ambient light and temperature conditions in May and June 2010. Both lines were aerated with an air/pure $CO_2$ mixture. Growth parameters (biomass concentration, productivity) and biochemical parameters (organic carbon and nitrogen content, crude protein content, and fatty acid content and composition) were determined. A summary of the applied conditions is presented in Table 3.6.

To investigate the long-term effect of flue gas on the growth and biochemical parameters of *Chlorella*, experiments with an air/flue gas mixture were conducted during May and June 2011. While weather conditions (cloudiness and temperature) differed between the years 2010 and 2011, the choice of a similar time period in May and June for the two experiments ensured similar astronomical forcing, i.e. similar day lengths and inclination of the incident irradiation. Nutrient concentrations and pH were kept similar in both lines and treated as in the control experiment of the preceding year. In contrast to the control experiment in 2010, however, line M1 was aerated with an air/pure $CO_2$ mixture and line M2 was aerated with an air/flue gas mixture. A summary of the experiment's conditions is presented in Table 3.6.

**Table 3.6:** Experimental set-up of experiments with pure $CO_2$ and flue gas (cf. Chapters 4.3.1 and 4.3.2)

| Variables | May/June 2010 | | May/June 2011 | |
|---|---|---|---|---|
| | Line M1 | Line M2 | Line M1 | Line M2 |
| Light | ambient | | ambient | |
| Temperature | ambient | | ambient | |
| Aeration mixture | air/pure $CO_2$ | air/pure $CO_2$ | air/pure $CO_2$ | air/flue gas |
| pH | constant | | constant | |
| Nutrients | identical addition | | | |

Furthermore, the removal of nitric oxide and carbon monoxide was quantified by comparing the measured outlet gas concentrations to the inlet gas concentrations, which were determined by the flue gas analysis device (cf. Chapter 4.3.3). The carbon dioxide removal was determined by comparison of inlet and outlet gas measurements and expressed as degree of decarbonisation and by calculating the carbon dioxide biofixation rate. All relevant parameters of these experiments are shown in Figure 3.6.

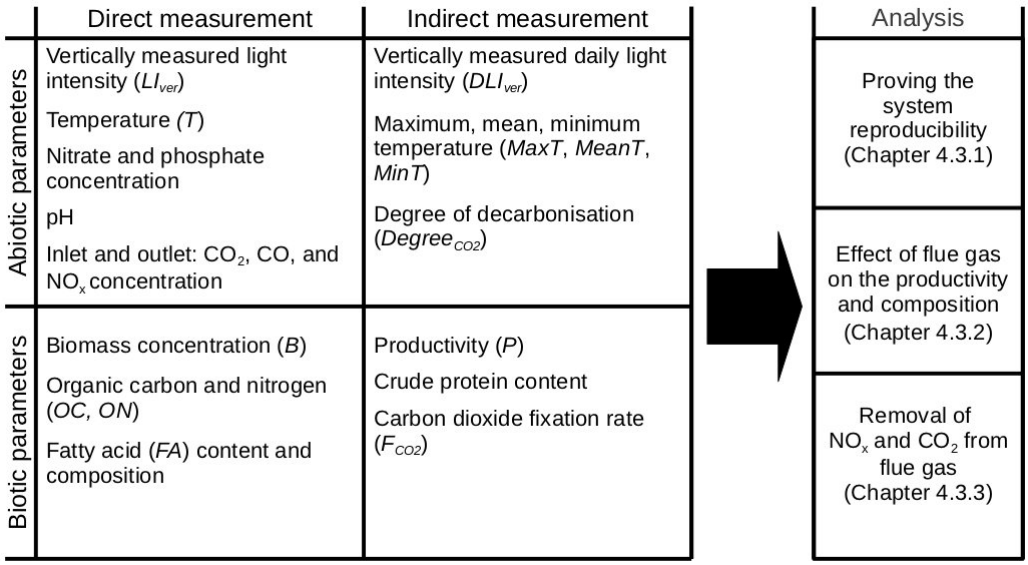


**Figure 3.6:** Biotic and abiotic parameters of Experiment 3, separated into direct and indirect measurements

### 3.4.4 Experiment 4: Microalgae as a feedstock for anaerobic digestion

Thermal and enzymatic biomass pre-treatments were tested with regard to the biogas yield from microalgae. Moreover, after extraction of soluble proteins, the suitability of residual biomass as feedstock for anaerobic digestion was evaluated. The experimental design is presented in Figure 3.7. An overview of the experiment's parameters is shown in Figure 3.8.

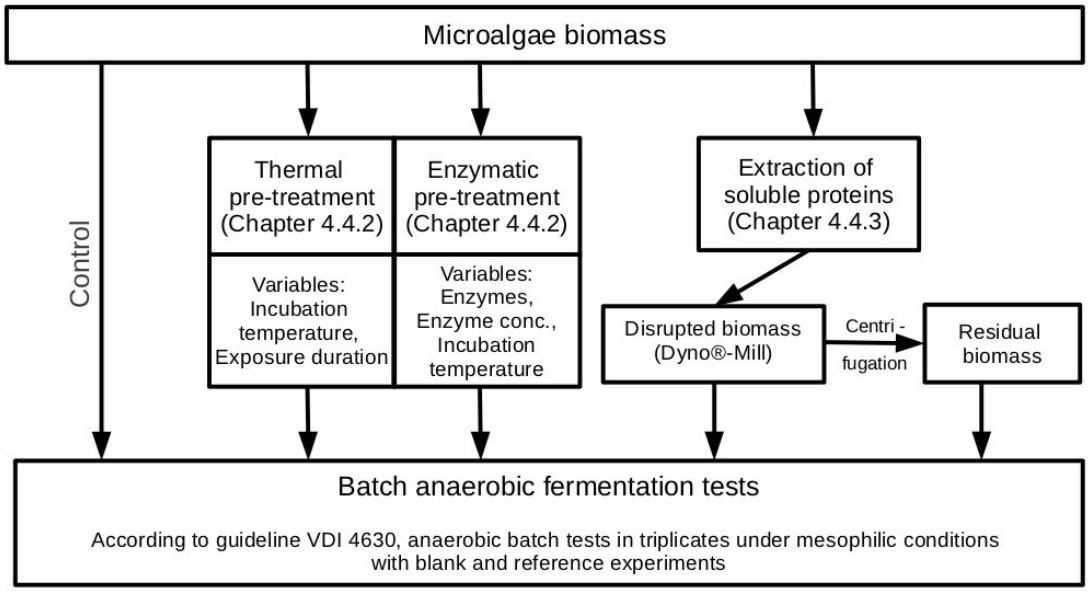


**Figure 3.7:** Experimental design of Experiment 4

**Thermal pre-treatment** Microalgae suspensions were thermally treated to assess the effect of temperature on the disintegration of *Chlorella vulgaris* cell walls prior to anaerobic digestion experiments (cf. Chapter 4.4.2). Therefore, microalgae suspension was filled into flasks, incubated at different temperatures (35, 55, 65, and 85°C), and continuously stirred. Additionally, different exposure durations (1, 6, 24 h) were applied. The incubation temperatures were checked regularly. Finally, batch anaerobic digestion experiments were performed to determine the biogas potential of the pre-treated substrate. The incubation conditions of the thermal experiments are shown in Table 3.7.

**Enzymatic pre-treatment** The effect of enzymes on the disintegration of *Chlorella vulgaris* cell walls depends on the enzyme type and the enzyme concentration as well as on the incubation parameters pH and temperature. Zapf (2009) tested the enzymes 'Onozuka R-10' (cellulase), 'Macerozyme R-10' (pectinase), and a mixture of both enzymes and successfully increased the

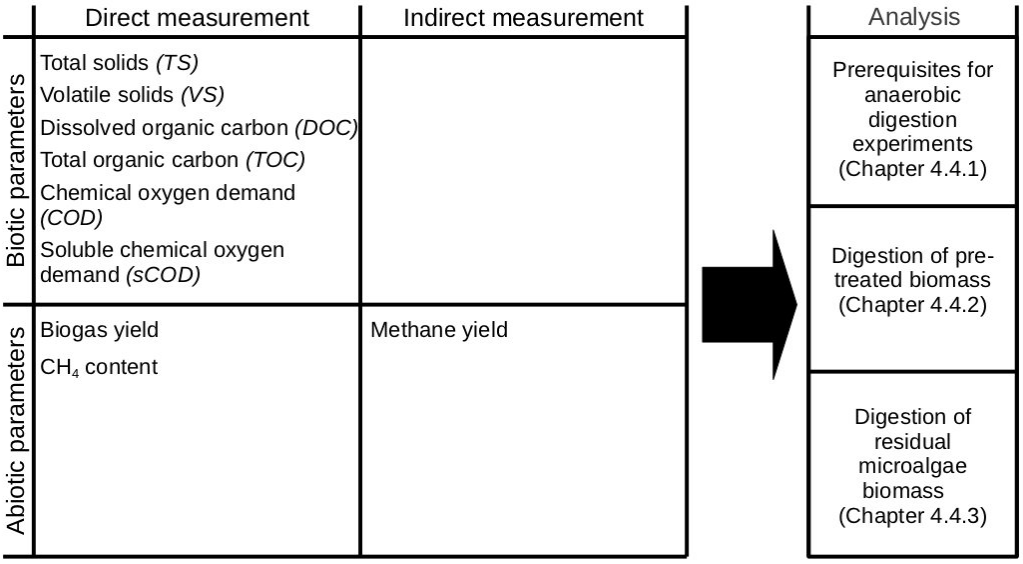


**Figure 3.8:** Biotic and abiotic parameters of Experiment 4, separated into direct and indirect measurements

**Table 3.7:** Incubation conditions for the determination of the optimal pre-treatment temperature and duration

| | Temperature [°C] | Exposure duration [h] |
|---|---|---|
| Microalgae suspension | 35 | 1, 6, 24 |
| | 55 | 1, 6, 24 |
| | 65 | 1, 6, 24 |
| | 85 | 1, 6, 24 |

biogas potential of *Chlorella*. Moreover, a pH of 5.0 showed the best results. To further improve the performance of pre-treatments with these enzymes, the enzyme concentration and incubation temperature were optimised here (cf. Chapter 4.4.2).

In order to achieve the optimal incubation temperature, a test series with different incubation temperatures and an addition of either 100 mg 'Onozuka R-10' or 100 mg 'Macerozyme R-10' to the sterile flasks was conducted.

Microalgae suspension was adjusted to a pH of 5.0 and homogenised. Then, 40 mL of the suspension was added to the enzymes. The suspension was incubated at 35, 40, 45, and 50°C (New Brunswick innova 44 and Heraeus Thermo Scientific) and shaken at 130 rpm for 24 h. A control sample without enzymes was set-up for each temperature. Enzyme concentrations are

expressed as [mg enzyme g$^{-1}$ volatile solids ($VS$)]. The incubation conditions of the first test series are shown in Table 3.8.

**Table 3.8:** Incubation conditions for the determination of the optimal incubation temperature for enzymatic substrate pre-treatment experiments; the pH of the microalgae suspension was set to 5.0 prior to experiments; samples were incubated for 24 h; enzymes were 'Onozuka R-10' (cellulase) and 'Macerozyme R-10' (pectinase)

| Enzyme | Temperature [°C] | Cellulase [mg enzyme g$^{-1}VS$] | Pectinase [mg enzyme g$^{-1}VS$] |
|---|---|---|---|
| Control | 35 | | |
| Pectinase | 35 | | 82.5 |
| Cellulase | 35 | 82.5 | |
| Control | 40 | | |
| Pectinase | 40 | | 82.5 |
| Cellulase | 40 | 82.5 | |
| Control | 45 | | |
| Pectinase | 45 | | 82.5 |
| Cellulase | 45 | 82.5 | |
| Control | 50 | | |
| Pectinase | 50 | | 82.5 |
| Cellulase | 50 | 82.5 | |

The set-ups with the highest increase in dissolved organic carbon ($DOC$) were chosen to conduct a second test series with different enzyme concentrations and an additional enzyme mixture, which consisted of equal amounts of cellulase and pectinase. The incubation conditions of the second test series are shown in Table 3.9. The total enzyme concentrations of the cellulase and the enzyme mixture ranged between 41.4 and 165.0 mg enzyme g$^{-1}VS$. To find the highest enzyme concentration still yielding a $DOC$ increase, the concentration of the enzyme mixture was increased up to 330.0 mg enzyme g$^{-1}VS$.

After the second test series, the six enzyme set-ups with the highest $DOC$ increase (bold highlighted in Table 3.9) were chosen as microalgae biomass pre-treatments for anaerobic digestion experiments. Prior to anaerobic digestion experiments the pH of the pre-treated microalgae suspension was raised from 5.0 to 7.0.

**Residual biomass for anaerobic digestion** Residual biomass from a protein extraction process was tested for its suitability as substrate for anaerobic digestion (cf. Chapter 4.4.3). Soluble proteins of *Chlorella vulgaris* were extracted according to Schwenzfeier *et al.* (2011).

**Table 3.9:** Incubation conditions for the determination of the optimal enzyme concentration in enzymatic pre-treatment experiments; the pH of the microalgae suspension was set to 5.0 prior to experiments; samples were incubated for 24 h; the applied enzymes were 'Onozuka R-10' (Cel - cellulase) and a mixture of 'Onozuka R-10' and 'Macerozyme R-10' (Pec - pectinase) at 35 and 45°C; Cel1 to Cel3 and Cel1/Pec1 to Cel4/Pec4 denote different enzyme concentrations; pre-treatments used in anaerobic digestion experiments are indicated in bold type

| Enzyme | Temperature [°C] | Cellulase [mg enzyme $g^{-1}VS$] | Pectinase [mg enzyme $g^{-1}VS$] | Total enzyme concentration [mg enzyme $g^{-1}VS$] |
|---|---|---|---|---|
| Cel1 | 35 | 41.4 | | 41.4 |
| **Cel2** | **35** | **82.5** | | **82.5** |
| **Cel3** | **35** | **165.0** | | **165.0** |
| Cel1 | 45 | 41.4 | | 41.4 |
| **Cel2** | **45** | **82.5** | | **82.5** |
| **Cel3** | **45** | **165.0** | | **165.0** |
| Cel1/Pec1 | 45 | 20.6 | 20.6 | 41.2 |
| Cel2/Pec2 | 45 | 41.4 | 41.4 | 82.8 |
| **Cel3/Pec3** | **45** | **82.5** | **82.5** | **165.0** |
| **Cel4/Pec4** | **45** | **165.0** | **165.0** | **330.0** |

The microalgae biomass, which had a dry weight of 32.4 ± 1.1 g $L^{-1}$, was kept frozen prior to use. After thawing, it was diluted 1:1 with 100 mM Tris/HCl buffer (pH 8.0), 1 mM EDTA, 50 mM DTT, and 5 mM $MgCl_2$. For cell disintegration the diluted algae paste was milled using a bead mill (Dyno®-Mill). The suspension was then centrifuged (4°C, 30 min, 40,000 x g). The pellet was washed with 200 mL water, and centrifuged again under the same conditions. The supernatant comprises the soluble protein fraction, while the residual biomass can be further processed (e.g. as biogas feedstock). The samples were frozen again at -28°C until the determination of the biogas potential.

To evaluate the viability of the process route, provided 1) untreated algae paste, 2) pre-treated algae paste (after milling), and 3) residual biomass (after removing the supernatant with the soluble protein fraction) were processed in an anaerobic digester and analysed qualitatively and quantitatively.

# Chapter 4

# Results and Discussion

This Chapter contains the results and discussions of the four experiments defined in Chapter 3.4. While three of them are concerned with the cultivation of microalgae, the last experiment considers the biogas production from microalgae biomass.

## 4.1 Experiment 1: Effect of light and temperature on productivity and composition

In this experiment, growth conditions and temporal dynamics of biomass and biochemical parameters of *Chlorella* cultivated in outdoor Flat-Panel Airlift (FPA) photobioreactors were monitored (Chapter 4.1.1). Furthermore, the effects of environmental parameters on the productivity were analysed statistically (Chapter 4.1.2). Eventually, an optimisation strategy to improve the productivity of microalgae in outdoor cultivation systems is established.

### 4.1.1 Effect of varying light intensity and temperature on the temporal dynamics of algal growth and composition

To assess the effect of light and temperature on the growth and biochemical composition of *Chlorella vulgaris*, growth experiments were conducted in line M1 during spring 2011. The aim was to rigorously characterise the cultivation system in terms of environmental, growth, and biochemical parameter.

#### 4.1.1.1 Results

**Light conditions** During the period of the experiment in May and June 2011, the light intensity ($LI_{ver}$) incident on the reactor surface was highly variable (Figure 4.1 a)). The substantial

differences in the maximum light intensity between 300 and 1,500 µmol $m^{-2}$ $s^{-1}$ were caused by the variability in the cloudiness, which is typical for Northern Germany in spring.

The daily light intensity ($DLI_{ver}$) ranged between 0.54 and 3.2·$10^7$ µmol $m^{-2}$ $d^{-1}$ (Figure 4.1 b)) with the highest values at the beginning of May and June. The lowest $DLI_{ver}$ was measured on 16-May and 29-May 2011.

**Temperature conditions** Likewise, the medium temperature in the photobioreactors exhibited a high variability that was caused by the irradiance pattern and the variable ambient air temperature. While mean values varied between 12 and 25°C (Figure 4.1 c)), the maximum differences within a day have been even larger. The differences between maximum day and minimum night temperatures in the reactor ranged between 3 and 20°C. The temperature control generally set in at a medium temperature of 27°C except for one incident on 03-June 2011 when the technical control failed. At that day, the temperature of the medium rose to 30°C and remained there for several hours.

**Other growth conditions** The pH of the medium was maintained at 7.11 ± 0.12 by adjusting the mixture of air and pure $CO_2$ throughout the cultivation period. On 11-May 2011, air supply was interrupted for approximately 1 h causing a decline in pH to values between 4 to 5. On 30-May 2011, an interrupted $CO_2$ supply resulted in a pH exceeding 8 for approximately 1 h.

Nutrients were added semi-continuously after each harvesting event. Mean nitrate concentration was 942 ± 344 mg $L^{-1}$ (range: 294 - 1,674 mg $L^{-1}$) and mean phosphate concentration was 109 ± 24 mg $L^{-1}$ (range: 63 - 156 mg $L^{-1}$).

**Biomass concentration and productivity** The biomass concentration ($B$) of the outdoor culture was maintained between 2.0 and 5.0 g $L^{-1}$ (mean biomass concentration over the entire growth period was 3.3 g $L^{-1}$) (Figure 4.2 a)). After harvesting, the removed algal suspension was replaced by new medium. During the cultivation period, the microalgae suspension showed neither cell aggregation nor contamination with protozoa or fungi. This was checked via regular and thorough microscopic examinations.

Mean and maximum productivities of 0.41 ± 0.28 and 1.01 g $L^{-1}$ $d^{-1}$, respectively, were achieved during the cultivation period (Figure 4.2 b)). On the basis of the volumetric productivity, the mean areal productivity was 13.83 ± 9.44 g $m^{-2}$ $d^{-1}$ and the maximum areal productivity was 34.07 g $m^{-2}$ $d^{-1}$. The mean and maximum volumetric productivities expressed per reactor surface area were then 15.49 ± 10.58 g $m^{-2}$ $d^{-1}$ and 38.16 g $m^{-2}$ $d^{-1}$, respectively.

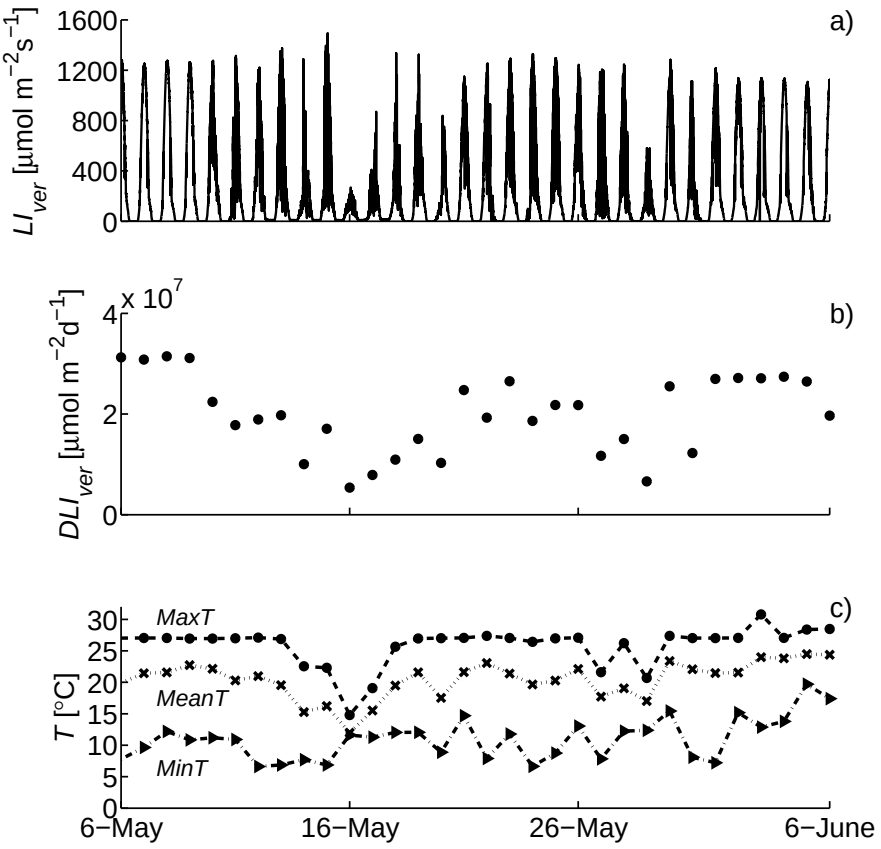


**Figure 4.1:** Ambient light and temperature conditions during May and June 2011: a) light intensity ($LI_{ver}$) measured vertically at the lower third of a reactor in line M1; b) daily light intensity ($DLI_{ver}$); c) maximum (Max), minimum (Min), and mean medium temperature ($T$)

**Organic carbon and nitrogen content** The organic carbon ($OC$) content of microalgae cells varied between 36.2 and 61.7 % of biomass (Figure 4.2 c)). Mean carbon content was 53.8 ± 4.8 % of biomass. The organic nitrogen ($ON$) content ranged from 6.2 to 11.1 % of biomass (Figure 4.2 c)) and the mean nitrogen content of all measurements was 9.1 ± 1.0 % of biomass. Nitrogen is one of the most essential nutrients and the nitrogen content is used to determine the main nitrogen demand in Chapter 4.2.

**Crude protein content** Results obtained during nutrient replete and ambient light and temperature conditions revealed a mean protein content of 43.6 ± 4.7 % of biomass (Figure 4.2 d)). The lowest protein content occurred on 17-May (30.0 ± 0.5 % of biomass). The highest protein content has been observed at the beginning of the cultivation period (52.4 ± 0.8 % of biomass).

**Fatty acid composition and content** The fatty acid ($FA$) content ranged between 7.5 and 8.8 % of biomass (Figure 4.2 e)). A temporal trend could not be detected over the course of the experiment.

The most abundant fatty acids were palmitic acid (C16:0) and $\alpha$-linolenic acid (C18:3(n-3)) followed by linoleic acid (C18:2(n-6)), oleic and elaidic acid C18:1(n-9) (Table 4.1). Linoleic acid is the only fatty acid with an increasing trend from about 0.7 to 2 % of biomass during the cultivation period. However, this increase is not apparent in the total amount of fatty acids.

**Table 4.1:** Fatty acid content and composition of *Chlorella vulgaris* grown in 180 L FPA photobioreactors outdoors in May and June 2011; only major fatty acids (> 0.2 % of biomass) are shown

| Fatty acid | 05-May | 10-May | 13-May | 17-May | 20-May | 24-May | 27-May | 31-May | 03-June |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | [% of biomass] | | | | |
| C16:0 | 1.61 | 1.89 | 2.06 | 1.84 | 1.93 | 2.03 | 1.94 | 1.99 | 1.91 |
| C18:1(n-9) | 0.17 | 0.18 | 0.26 | 0.16 | 0.23 | 0.16 | 0.16 | 0.18 | 0.17 |
| C18:2(n-6) | 0.73 | 1.43 | 1.56 | 1.55 | 1.98 | 2.15 | 2.00 | 2.04 | 1.80 |
| C18:3(n-3) | 4.77 | 4.11 | 4.57 | 4.01 | 4.15 | 4.32 | 4.15 | 4.17 | 4.13 |
| Other | 0.25 | 0.13 | 0.35 | 0.17 | 0.38 | 0.18 | 0.37 | 0.15 | 0.34 |
| Total | 7.53 | 7.74 | 8.80 | 7.73 | 8.67 | 8.84 | 8.62 | 8.53 | 8.35 |

#### 4.1.1.2 Discussion

**Cultivation mode and growth conditions** The results shown in Figure 4.2 a) are a typical example for the biomass dynamics of microalgae in a semi-continuous cultivation mode. Following Harrison *et al.* (1990), the stationary phase (i.e. the phase in which growth ceases) is typically induced through either light or nutrient limitation in a semi-continuous cultivation process. To prevent such limitations, the cultures are ideally supplied with additional nutrients or more light (via dilution) before a considerable slow-down of the growth rate. Here, nitrogen and phosphorus as the main nutrient constituents were measured almost every day and medium was exchanged regularly and replaced by new medium to ensure nutrient replete conditions. Moreover, the supply of carbon was assured by aerating the culture with an air/pure $CO_2$ mixture and by maintaining a constant pH. Microalgae cultivation systems require regular intervention by the operator to sustain microalgae growth with maximum productivity. The results shown here demonstrate that the long term cultivation of such a system is possible if the operational strategy accounts for technical and environmental conditions.

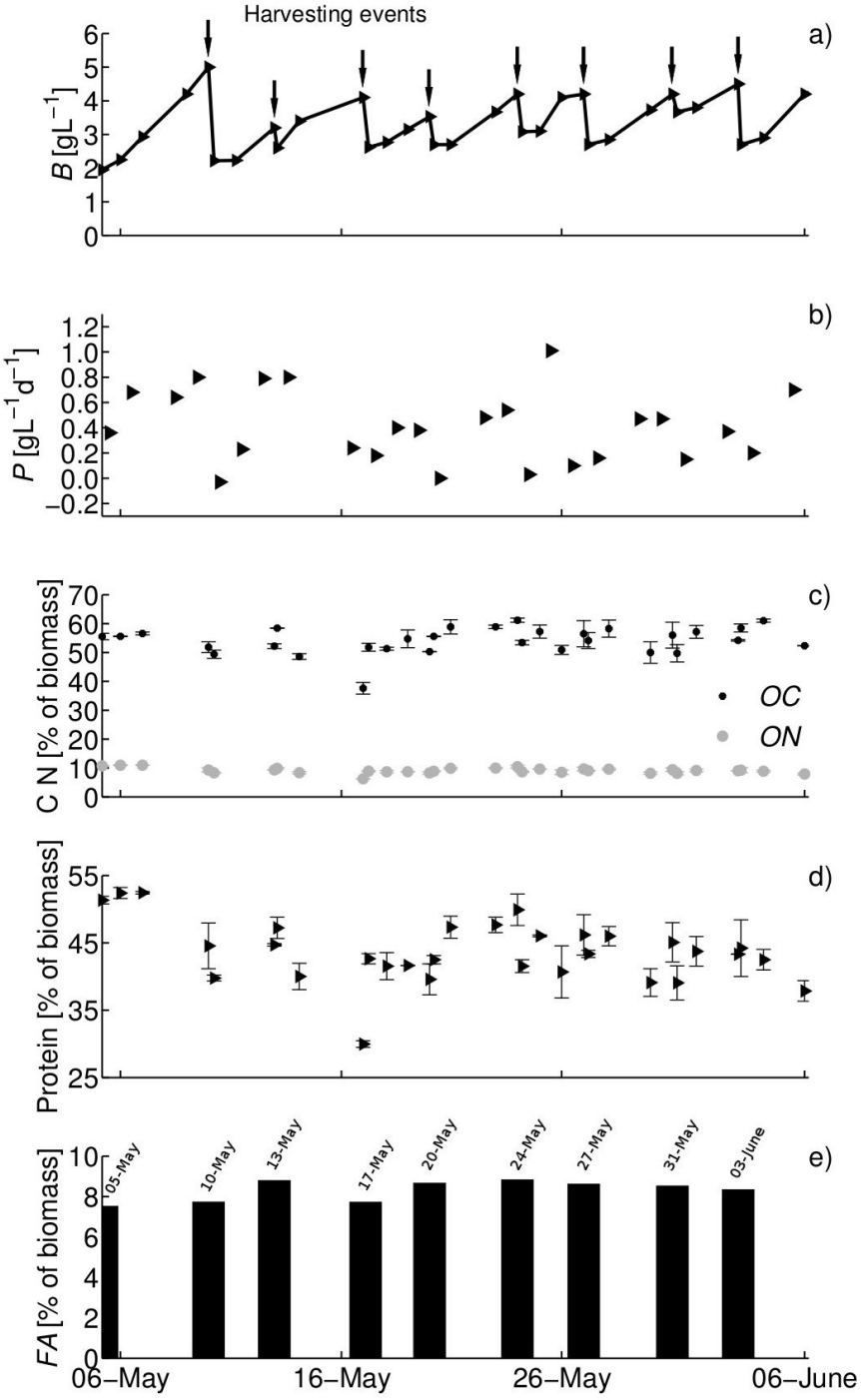


**Figure 4.2:** Growth and biochemical parameters during May and June 2011: a) biomass concentration ($B$) of *Chlorella vulgaris* grown outdoors in 180 L FPA reactors; black arrows indicate harvesting events; b) productivity ($P$); c) organic carbon ($OC$) and nitrogen ($ON$) content ± standard deviation; d) crude protein content (Protein) ± standard deviation; e) total fatty acid ($FA$) content; fatty acid samples were taken during harvesting events as a single sample from a mixture of medium from all reactors of line M1 (cf. Table 4.1 for the fatty acid composition)

**Productivity in outdoor FPA reactors** Biomass productivities of microalgae cultivated in outdoor FPA photobioreactors were monitored during May and June 2011. Despite the often suboptimal climatic conditions of Northern Germany, outdoor productivities measured in this experiment are comparable to data from several other studies conducted under laboratory and outdoor conditions (Degen *et al.*, 2001; De Morais and Costa, 2007; Ecke, 2008; Ripplinger, 2008; Liang *et al.*, 2009; Rodolfi *et al.*, 2009; Yoo *et al.*, 2010; Doucha and Lívanský, 2012; Petrick *et al.*, 2013) (Table 4.2). Generally, autotrophic microalgae productivities greatly vary ranging from 0.01 to 0.5 g $L^{-1}$ $d^{-1}$ and Degen *et al.* (2001) even reported a maximum productivity of 2.64 g $L^{-1}$ $d^{-1}$ during laboratory experiments with *Chlorella vulgaris*.

**Table 4.2:** Volumetric biomass production of *Chlorella vulgaris* under different cultivation conditions in the laboratory and outdoors; mean (Mean) and maximum (Max) values are marked if known; $^{A}$ denotes autotrophic growth, $^{H}$ heterotrophic growth, $^{M}$ mixotrophic growth, $^{cs}$ closed, and $^{os}$ open systems

| Laboratory Productivity [g $L^{-1}$ $d^{-1}$] | Reference | Outdoors Productivity [g $L^{-1}$ $d^{-1}$] | Reference |
|---|---|---|---|
| $0.1^{A/cs}$ | Yoo *et al.* (2010) | 1.5 - $4^{A/cs}$ | Doucha and Lívanský (2012) |
| $0.01^{A/cs}$ | Liang *et al.* (2009) | 12.5 - $36.3^{H/cs}$ | Doucha and Lívanský (2012) |
| $0.14^{A/cs}$ | De Morais and Costa (2007) | 0.145 - $0.148^{A/cs}$ | Petrick *et al.* (2013) |
| $0.2^{A/cs}$ | Rodolfi *et al.* (2009) | | |
| 0.08 - $0.15^{H/cs}$ | Liang *et al.* (2009) | | |
| $2.64^{A/cs}$ (Max) | Degen *et al.* (2001) | | |
| 0.2 - $0.3^{A/cs}$ | Ecke (2008) | | |
| 0.4 - $1.0^{M/cs}$ | Ecke (2008) | | |
| $0.5^{A/cs}$ (Mean) | Ripplinger (2008) | | |

In this experiment, mean and maximum productivities of the outdoor culture were 0.4 g $L^{-1}$ $d^{-1}$ and 1.0 g $L^{-1}$ $d^{-1}$, respectively. Both values compare well with the above mentioned autotrophic values proving the general suitability of Northern Germany as a location for the successful operation of intensive, outdoor microalgae cultivation.

Lowest productivities regularly occurred the day after a harvesting event. Most likely, the necessary physiological adaptation of the microalgae to the abrupt change of medium composition induced a short lag phase, in which growth was suspended.

Grown heterotrophically in fermenters, cultures of *Chlorella vulgaris* can reach mean productivities that are an order of magnitude higher than autotrophic productivities (12.5 to 36.3 g $L^{-1}$ $d^{-1}$) (Doucha and Lívanský, 2012). Heterotrophic cultures have the advantage of increased cell den-

sities and productivities because the microalgae can use organic carbon as their sole carbon and energy source and the culture is no longer in risk of light limitation (Chen, 1996). However, the cost of the organic carbon source (e.g. glucose) is high when compared to other nutrients (Liang *et al.*, 2009).

**Organic carbon and nitrogen content and $CO_2$ biofixation rate** The organic carbon ($OC$) content is required to calculate the $CO_2$ biofixation rate. Commonly, a carbon content of 48 or 50 % for microalgae species (Yoshihara *et al.*, 1996; Douskova *et al.*, 2009; Wang *et al.*, 2008) is used, resulting in a carbon dioxide fixation of 1.8 kg $CO_2$ $kg^{-1}$ microalgae biomass. Here, a mean carbon content of 54 % was measured for *Chlorella vulgaris* corresponding to a fixation of 2.0 kg $CO_2$ $kg^{-1}$ biomass. Thus, the $CO_2$ biofixation rates of *Chlorella* in this experiment slightly exceeded the typical range reported in the literature.

Wang *et al.* (2008) reported that while the carbon content varies with microalgal strains, media, and cultivation conditions, it is a rather stable, species-specific trait. Here, however, the carbon content ranged mainly between 50 and 60 % of biomass, but oscillated within this range in a relatively short time. This suggests that the highly variable conditions of an outdoor cultivation lead to high frequency fluctuations with a higher amplitude in the carbon content than the stable culture conditions in the laboratory under which most literature values are derived.

The lowest carbon and nitrogen content were measured on 17-May 2011. The minimum coincided with a pronounced drop in light intensity and temperature on the preceding day. This demonstrates the sensitivity of elemental composition on changes in light and temperature conditions. This finding corroborates Lourenço *et al.* (2004) who reported a strong effect of growth conditions and physiological state on nitrogen contents of microalgae biomass. Here, a mean nitrogen content of 9.1 ± 1.0 % was realised in the *Chlorella* culture, which is slightly higher than the 7.7 % observed by Tokuşoglu and Ünal (2003).

**Fatty acid composition and content** The fatty acid ($FA$) content in *Chlorella* samples varied between 7.5 and 8.8 % over the cultivation period of 32 days in spring 2011. Thus, despite the strong variations in ambient temperature and light, changes in the $FA$ content were small and correlations between the $FA$ content and environmental variables were weak. Other studies, however, established an effect of temperature and light on the lipid content (Guschina and Harwood, 2006; Hu *et al.*, 2008; Converti *et al.*, 2009). While a temperature increase from 25 to 30°C evoked a lipid content decrease from 14.7 to 5.9 % in *Chlorella vulgaris* cells (Converti *et al.*, 2009), lipid content in cells is known to increase with light intensity (Nichols, 1965; Pohl and Zurheide, 1979). These opposing effects probably cancelled each other out, which could explain the weak correlations between the $FA$ content and environmental variables.

A lipid content of 18.4 % was found by Rodolfi *et al.* (2009), who cultivated *Chlorella vulgaris* in 250 mL flasks in the laboratory at 25°C and constant illumination. Tokuşoglu and Ünal (2003) found a total lipid content of 13.3 % and Griffiths *et al.* (2011) measured a fatty acid content of 14 % in a nutrient replete *Chlorella vulgaris* culture. In this investigation, the observed $FA$ content in *Chlorella* cells grown outdoors is rather low compared to other studies. The cultures were grown under nitrogen replete conditions and harvested before stationary phase. Under these conditions high fatty acid contents are not formed (Dunstan *et al.*, 1992). This might explain the described results.

The $FA$ composition of *Chlorella vulgaris* cells is mainly comprised by C16:0, C18:1, C18:2, and C18:3 (Harris *et al.*, 1965; Hu *et al.*, 2008; Griffiths *et al.*, 2011; Khoeyi *et al.*, 2012), which is corroborated by the results of this study. While not observed here, fatty acids with a chain length greater than C18:3 were detected by Tokuşoglu and Ünal (2003). The variation in $FA$ compositions and contents between the studies may stem from different analytical methods or different environmental growth conditions. Especially changes in temperature, nutrient levels, light regime, or salinity affect the $FA$ content and composition (Ackman *et al.*, 1968; Seto *et al.*, 1984; James *et al.*, 1989; Lombardi and Wangersky, 1991). Another reason for considerable discrepancies between $FA$ contents and compositions of one and the same species might be the incorrect identification of such species. Biochemically different algae species sometimes have a superficial resemblance to each other (Dunstan *et al.*, 1992). For this reason, some *Chlorella* species were already mistakenly confused with *Nannochloropsis oculata*. Furthermore, the $FA$ composition of *Chlorella multimissima* and *Chlorella saccharophila* strongly resemble the composition of *Nannochloropsis oculata* but are very dissimilar to other *Chlorella* species (Dunstan *et al.*, 1992).

**Crude protein content** The high protein content of many microalgae species was one reason for considering microalgae as an alternative protein source. Becker (1994) compared different gross chemical compositions of human food sources and algae and found that *Chlorella vulgaris* has one of the highest crude protein contents (51 to 58 %) compared to other microalgae and food sources (cf. Chapter 2.1.1). Piorreck *et al.* (1984) and Tokuşoglu and Ünal (2003) reported a crude protein content of 54 and 48 %, respectively. In this investigation, a similar protein content was observed, which exceeds the protein content of the two aforementioned authors by 2.5 and 7.3 %, respectively.

The crude protein content is an estimation based on the analysis of the total nitrogen content and a subsequent multiplication of the value 6.25 (N-Prot factor). This factor is based on the assumption that biomass contains protein with a nitrogen content of 16 % and a low concentration of non-proteinaceous nitrogen is commonly used for many materials, even for microalgae samples. However, microalgae have a larger content of non-proteinaceous nitrogen and deviate from a

nitrogen content of 16 % in protein. Therefore, Lourenço *et al.* (2004) proposed a new and more adequate N-Prot factor (4.78) derived from 12 marine microalgae including a *Chlorella* species. In this investigation, the new factor was used to determine the crude protein content. The use of different N-Prot factors complicates the comparison of literature protein data. The comparison above already considers this circumstance.

Although it is known, that temperature and light have an effect on the protein content of microalgae, results are still contradictory (Renaud *et al.*, 1991; Carvalho *et al.*, 2009; Seyfabadi *et al.*, 2011). Seyfabadi *et al.* (2011) observed that the protein production increased with increase in light. Carvalho *et al.* (2009) studied the simultaneous effect of temperature and light and found that the protein content decreased with increasing temperature, increased with irradiance for a constant temperature of 18°C, but decreased with irradiance for a constant temperature of 22°C. Renaud *et al.* (1991) cultivated two microalgae species under four light regimes and the protein content showed little to no differences. Furthermore, not only light and temperature but also the nitrogen concentration in the medium affect the protein content. Low levels of nitrogen can cause a large decrease in microalgae protein (Dortch, 1982; Piorreck *et al.*, 1984). In this investigation, the crude protein content showed a high variability during the course of the experiment ranging between 30 and 52 % of biomass. The lowest protein content was measured on 17-May 2011. The minimum coincided with a pronounced drop in light intensity and temperature on the preceding day. This demonstrates the sensitivity of protein on changes in light and temperature conditions. Moreover, ever-changing nitrogen concentrations in the medium may also have an impact on the protein content of the microalgae.

### 4.1.2 Impacts of environmental conditions on growth

The effects of environmental parameters, such as light and temperature, on the productivity were analysed statistically using a principal component analysis. Eventually, an optimisation strategy to improve the productivity of microalgae in outdoor cultivation systems is established by means of key growth parameters.

#### 4.1.2.1 Results

A principal component analysis (PCA) was applied to identify linear relationships between microalgae productivity and environmental parameters, which characterise the temperature and light regime. These are cloudiness, daily light intensity, maximum light intensity, sun duration, light availability, temperature variance, minimum, maximum, and mean temperature. The first two principal components (PCs) combined already explain 72.8 %, i.e. the great majority of the variability inherent to the data set (Table 4.3). In other words, most of the variation of the

N-dimensional data set can be adequately explained by a projection onto the first two PCs.

**Table 4.3:** A PCA was performed on z-score standardised mean data from spring and autumn 2010 and 2011; 52 cases were included in the analysis; only the loading factors for the two first principal components (PC1/PC2) are shown; PC1ro and PC2ro denote the results after applying a varimax (orthogonal) rotation to the eigenvectors

| Variables | Symbol | PC1 | PC2 | PC1ro | PC2ro |
|---|---|---|---|---|---|
| Productivity [g $L^{-1}$ $d^{-1}$] | $P$ | 0.28 | -0.03 | 0.26 | 0.12 |
| Biomass concentration [g $L^{-1}$] | $B$ | 0.20 | 0.24 | 0.04 | 0.31 |
| Light availability [µmol $g^{-1}$ $d^{-1}$] | $LA$ | 0.27 | -0.33 | 0.40 | -0.13 |
| Sun duration [h] | $SD$ | 0.36 | -0.05 | 0.33 | 0.15 |
| Cloudiness [relative cloud cover] | $Cl$ | -0.31 | 0.24 | -0.40 | 0.04 |
| Daily light intensity [µmol $m^{-2}$ $d^{-1}$] | $DLI_{ver}$ | 0.38 | -0.13 | 0.39 | 0.10 |
| Max light intensity [µmol $m^{-2}$ $s^{-1}$] | $MaxL$ | 0.28 | -0.14 | 0.32 | 0.03 |
| Mean temperature [°C] | $MeanT$ | 0.30 | 0.46 | 0.00 | 0.55 |
| Max temperature [°C] | $MaxT$ | 0.36 | 0.26 | 0.16 | 0.41 |
| Min temperature [°C] | $MinT$ | 0.17 | 0.61 | -0.18 | 0.61 |
| Temperature variance [°$C^2$] | $TVar$ | 0.33 | -0.30 | 0.44 | -0.07 |
| Explained variation | | 55.1 % | 17.7 % | 55.1 % | 17.7 % |

By far most of the variation (55.1 %) occurs along PC1, which is closely associated to the light parameters cloudiness, sun duration, daily light intensity, and light availability as well as to the temperature variance. Thus, samples taken during days with little cloudiness and long sunshine duration, high daily light intensity and availability, as well as high temperature variance are located in the positive part of the first axis. In contrast, PC2 is related to the remaining temperature parameters (minimum temperature, mean temperature, and maximum temperature) and has significantly less explanatory power (17.7 %) than PC1. Accordingly, samples with low nighttime temperatures and high mean and maximum temperatures are located in the positive part of the second axis.

The graphical representation of the result in the PCA scaling plot, in which each variable is indicated by a vector, underlines the clear separation of temperature and light parameters and the association of productivity to the light parameters (Figure 4.3). From all light parameters, $DLI_{ver}$ has the highest correlation to productivity (Figure 4.4), but the correlation coefficient ($r = 0.68$, $p < 0.05$) indicates that either their relation is not perfectly linear or that other parameters also affect productivity. Relating productivity solely to light intensity neglects, besides other factors, the crucial role of biomass for the system. First, the available light per algal cell is

decreasing with increasing biomass concentration because of self-shading. Second, the productivity per volume is affected by the biomass concentration as *Chlorella vulgaris* grows by cell division.

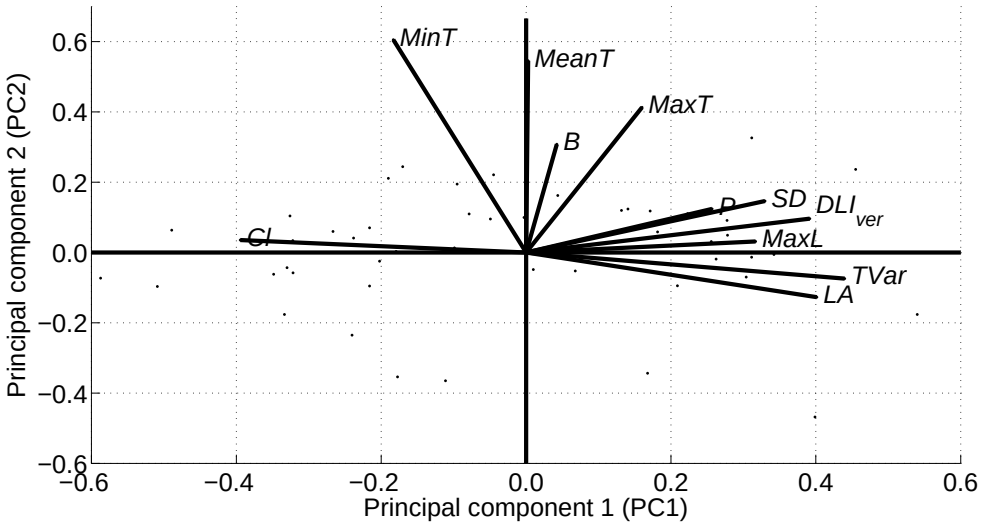


**Figure 4.3:** PCA scaling plot after applying a varimax rotation to the eigenvectors based on a correlation matrix of productivity ($P$), biomass concentration ($B$), light availability ($LA$), cloudiness ($Cl$), daily light intensity ($DLI_{ver}$), maximum light intensity ($MaxL$), sun duration ($SD$), mean temperature ($MeanT$), maximum temperature ($MaxT$), minimum temperature ($MinT$), and temperature variance ($TVar$)

To account for these two aspects, the available light per algal cell is determined and related to productivity. Since the measurement of cell numbers requires more effort than the routinely measured biomass concentration, daily light intensity per biomass concentration, termed light availability ($LA$) in the following, is used here instead.

Measured productivities peak above 1.0 g $L^{-1}$ $d^{-1}$ at intermediate light availabilities between 1 and $2{\cdot}10^5$ µmol $g^{-1}$ $d^{-1}$ (Figure 4.5). Lower as well as higher light availabilities lead to decreasing productivities, albeit for different reasons. While at the lower end of light availabilities the system is clearly light limited, this cannot explain below optimum productivities at very high light availabilities. Here, either very high light availabilities cause photoinhibition and thus effectively decrease the efficiency of photosynthesis or the biomass concentrations, indicated by different shades of grey in Figure 4.5, are too low to achieve high productivities on a per volume basis. The effect of biomass concentration is also apparent in the data-rich range between 0 and $2 \cdot 10^5$ µmol $g^{-1}$ $d^{-1}$, where highest productivities for a given light availability are only reached at biomass concentrations exceeding 3 g $L^{-1}$. The solid line in Figure 4.5 represents a least squares fit of Steele's function (modified from Steele, J. H. (1962)) to the observed data determining

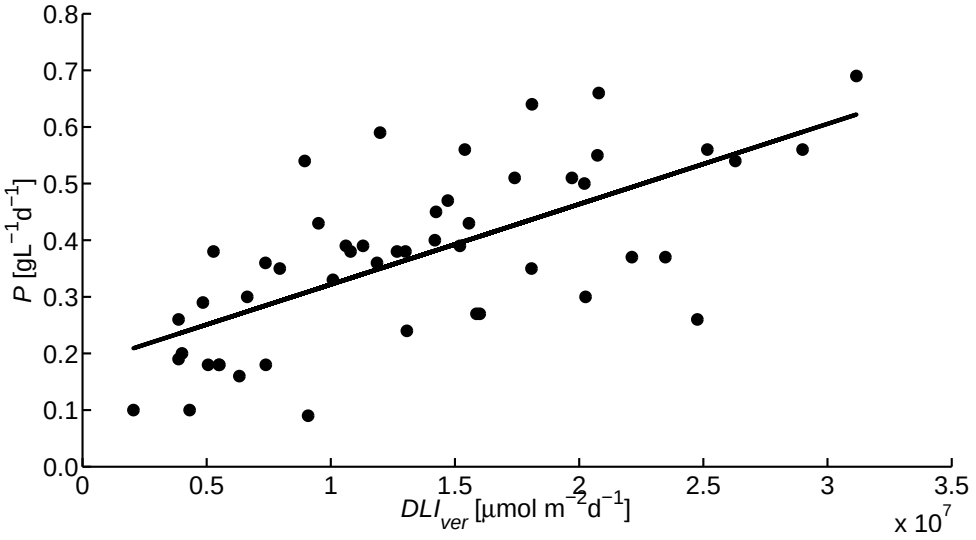


**Figure 4.4:** Relationship between productivity ($P$) and daily light intensity ($DLI_{ver}$)

the highest productivity of 0.54 g $L^{-1}$ $d^{-1}$ ($OPP$) at a light availability of $2.04 \cdot 10^5$ µmol $g^{-1}$ $d^{-1}$ ($OLA$). While this function represents a conservative estimate of the productivity, the upper envelope of the data can serve as an indicator for the maximum achievable productivity given a light availability.

The parameter $\alpha$ is introduced in equation (3.2) to allow for negative (net) productivities that occur when respiration or other losses exceed gross productivity. Here, $\alpha$ is conservatively chosen to be 0.15, although even lower productivities (up to -0.3) were observed. The $OPP$ does not react sensitive to changes in the y-intercept, which is shown by the following values of $\alpha$ and the corresponding $OPP$ ($\alpha$ = 0: $OPP$ = 0.52; $\alpha$ = 0.15: $OPP$ = 0.54; $\alpha$ = 0.3: $OPP$ = 0.56).

#### 4.1.2.2 Discussion

**Environmental parameters and productivity** The optimisation of biomass productivity and yield are major concerns in large outdoor microalgae cultures. The biomass productivity is, given unchanged fluid dynamics, constant nutrient concentration and a constant pH, mainly influenced by two factors: the amount of light available per cell and the temperature regime (Molina Grima *et al.*, 1999). Many studies focussed either on the effect of temperature or light, but the combined effect of light and temperature was often neglected (Sandnes *et al.*, 2005; Seyfabadi *et al.*, 2011; Khoeyi *et al.*, 2012; Trabelsi *et al.*, 2009; Renaud *et al.*, 1991; Sorokin and Krauss, 1958; Carvalho and Malcata, 2003; Li *et al.*, 2011; Kessler, 1985; Carvalho *et al.*,

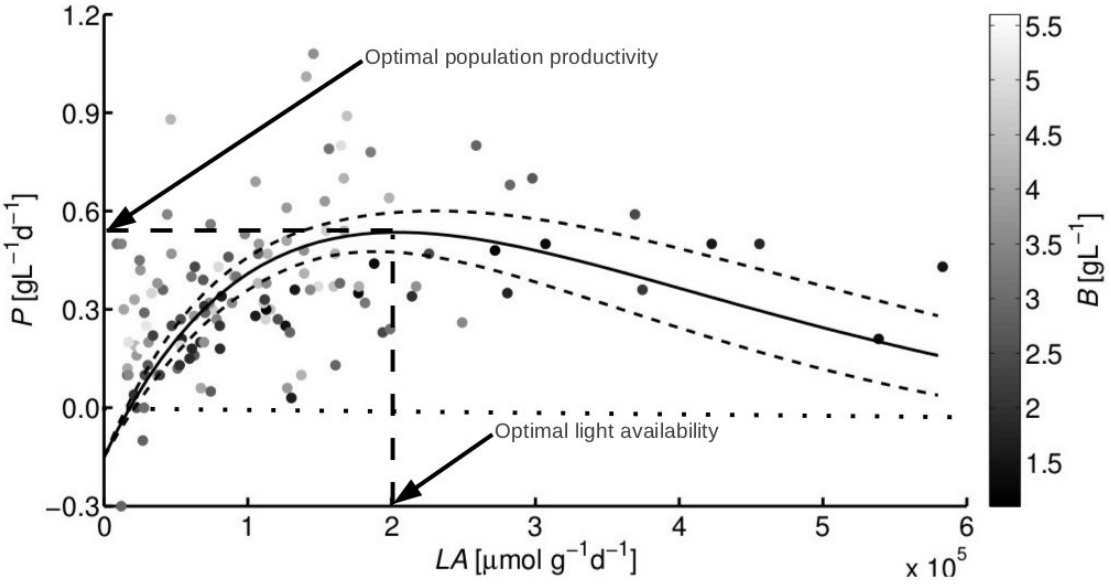


**Figure 4.5:** Relationship between productivity ($P$) and light availability ($LA$); the legend refers to the biomass concentration ($B$); the data were fitted by a model modified from Steele, J. H. (1962); dashed lines indicate 95 % confidence intervals

2009). Under non-laboratory conditions with highly variable environmental forcing, it is even more complicated to identify the driving growth parameters.

Outdoor cultivation is exposed to changes in the light and temperature regime on many different temporal scales, from minutes to seasons. In particular, fast changes between low and high light conditions generated by the succession of sunny and cloudy periods do not occur in the laboratory. Furthermore, the medium temperature is continuously changing and the day to night difference may exceed 25°C.

In this study, it was one aim to unravel the driving growth parameters in outdoor cultures. By means of a PCA, a clear separation of temperature and light parameters and the association of productivity to the light parameters was observed. According to the PCA, temperature has a minor effect on productivity. The productivity was best correlated with the daily light intensity, but nevertheless the correlation between the two variables is weak. Therefore, a parameter with more explanatory power, the light availability, which links the light intensity and the biomass concentration, was introduced (Figure 4.5).

A simple but powerful 'productivity/light availability' model is presented in Figure 4.6. The model shows, that the productivity increases with light availability up to an optimal point, the 'optimal population productivity' ($OPP$). At low light intensities (or very high biomass concentrations), the lack of light limits the productivity. Compared to other species, e.g. to *Scenedesmus*

*protuberans*, however, *Chlorella vulgaris* has a lower critical light intensity (Huisman *et al.*, 2002). In other words, growth can be maintained even at relatively low light intensities, a characteristic that makes *Chlorella* a good competitor and an ideal species for cultivation in lower light locations. At high light intensities (or very low biomass concentrations), photoinhibition or simply too low biomass for growth control the productivity on a per volume basis in microalgae systems.

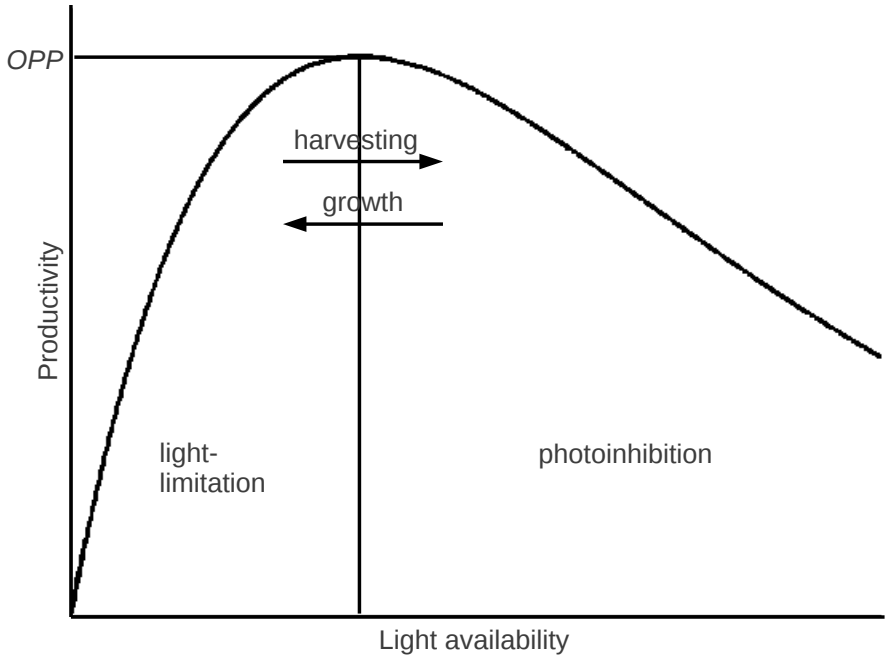


**Figure 4.6:** Simplified model of the dependency of productivity from available light; the $OPP$ denotes the optimal population productivity

The distribution of the biomass concentration in Figure 4.5 reveals that highest densities are mostly found at below optimum light availabilities (> 3 g $L^{-1}$ between 0 and $2{\cdot}10^5$ µmol $g^{-1}$ $d^{-1}$). The even distribution of low biomass concentrations over the whole range of light availabilities is more difficult to explain. It is assumed, that harvesting led to low biomass concentrations. These biomass concentrations may have been too low to achieve high productivities. At high light intensities, photoinhibition may then cause further damage to the culture. Though light, temperature, and biomass concentration are the major factors influencing growth in microalgae cultures, other factors (e.g. nutrient concentration, pH) that have not received special consideration here, because they have been maintained within non-limiting ranges, may have adverse effects on the growth of microalgae when occurring far from their optimum values.

**Increasing productivity using growth key parameters** This is one of the first investigation using outdoor cultivation data to obtain key growth parameters from a 'productivity/light availability' system with the objective of improving the harvest management and thus the productivity. The optimal population productivity of the system was determined to be 0.54 g $L^{-1}$ $d^{-1}$ and was achieved with an optimal light availability of 0.204 mol $g^{-1}$ $d^{-1}$.

Schmid-Staiger *et al.* (2009) observed a similar $OLA$ (0.1 to 0.2 mol $g^{-1}$ $d^{-1}$) in laboratory cultures of *Phaeodactylum tricornutum*. However, a generalisation of these observations and an application to other microalgae systems requires the investigation of a larger number of culture systems.

The $OPP$ and the $OLA$ can also be used to determine the optimal population density ($OPD$) for a given daily light intensity. Richmond (2004) found that at the $OPD$, the culture is most stable. One of the central aims of the harvesting management is therefore to maintain the $OPD$ in order to increase the biomass yield and improve the stability of the culture. The use of the 'productivity/light availability' system in combination with weather forecasts is a promising topic that deserves further investigation for its potential to optimise the productivity of a microalgae cultivation under real environmental conditions.

Table 4.4 shows mean daily light intensities of several months in 2011 and the corresponding $OPDs$, assuming an $OPP$ of 0.54 g $L^{-1}$ $d^{-1}$. For instance, in May 2011, the $OPP$ was achieved with an $OPD$ of 2.60 g $L^{-1}$. Thus, a productivity > 0.4 g $L^{-1}$ $d^{-1}$ could be maintained with a culture density ranging approximately between 1.4 and 5.3 g $L^{-1}$. Considering a harvesting volume of 60 L per event in 180 L FPA reactors, harvesting at a culture density between 2.2 and 5.3 g $L^{-1}$ was recommended during May 2011 in order to maintain a productivity > 0.4 g $L^{-1}$ $d^{-1}$.

**Table 4.4:** Optimal population densities ($OPDs$) calculated for several month of the year 2011 based on the mean daily light intensities ($DLI_{ver}$) and assuming an optimal population productivity of 0.54 g $L^{-1}$ $d^{-1}$

| Month 2011 | Mean $DLI_{ver}$ [$10^7$ µmol $m^{-2}$ $d^{-1}$] | $OPD$ [g $L^{-1}$] |
|---|---|---|
| May | 2.00 | 2.60 |
| June | 1.70 | 2.21 |
| July | 1.49 | 1.93 |
| August | 1.61 | 2.08 |
| September | 1.77 | 2.30 |
| October | 1.27 | 1.65 |

## 4.2 Experiment 2: Nitrate and phosphate uptake and the fate of carbon

The system's nutrient conversion efficiency was tested by comparing the consumption of dissolved inorganic nutrients and the production of the respective particulate organic matter. Moreover, species-specific nitrate and phosphate requirements for *Chlorella vulgaris* were established (Chapter 4.2.1).

Growing algae exude a fraction of photosynthetically fixed organic carbon into the medium, which is also an excellent substrate for bacteria. This potential loss of organic carbon is quantified in the following Chapter. Moreover, the amount of bacterial biomass is estimated, and an effort was made to unravel the relationship between microalgae and bacteria in microalgae systems (Chapter 4.2.2).

### 4.2.1 Uptake and assimilation of nitrate and phosphate

Nitrate and phosphate are, besides carbon compounds, the main nutrients required for algae growth and are here solely defined to comprise the dissolved inorganic nitrogen ($DIN$) and dissolved inorganic phosphorus ($DIP$) pools in the medium, respectively. To test this assumption and to assess the respective nutrient conversion efficiency, one can compare the consumption of dissolved inorganic nutrients and the production of the respective particulate organic matter.

#### 4.2.1.1 Results

**Nitrate** The consumption of dissolved inorganic nitrogen ($DIN$) and the production of particulate organic nitrogen ($PON$) are closely related in cultures aerated with a $CO_2$/air mixture (Figure 4.7 a)). In other words, nitrate from the medium was completely taken up and assimilated by algae cells. There is also a good relationship between the consumption of $DIN$ and the production of $PON$ in the flue gas culture, however, generally more $PON$ was produced than expected from the consumption of $DIN$. This indicates, that there must be an additional source of nitrogen in the cultivation system. A nitrate requirement of about 403.1 ± 44.3 mg $NO_3$ $g^{-1}$ biomass was determined.

**Phosphate** Since particulate organic phosphorus ($POP$) could not be measured directly, it was estimated by assuming a relationship of 16:1 for nitrogen:phosphorus (N:P) (Redfield, 1958). Figure 4.7 b) shows the relationship of the production of $POP$ and the consumption of dissolved inorganic phosphorus ($DIP$). Most of the data points lay above the line where $POP$

production resembles $DIP$ consumption. In contrast to nitrogen, there is no apparent difference between $CO_2$ and flue gas cultures for phosphorus. The phosphate requirement was 17.5 ± 0.2 mg $PO_4$ $g^{-1}$ biomass in this experiment.

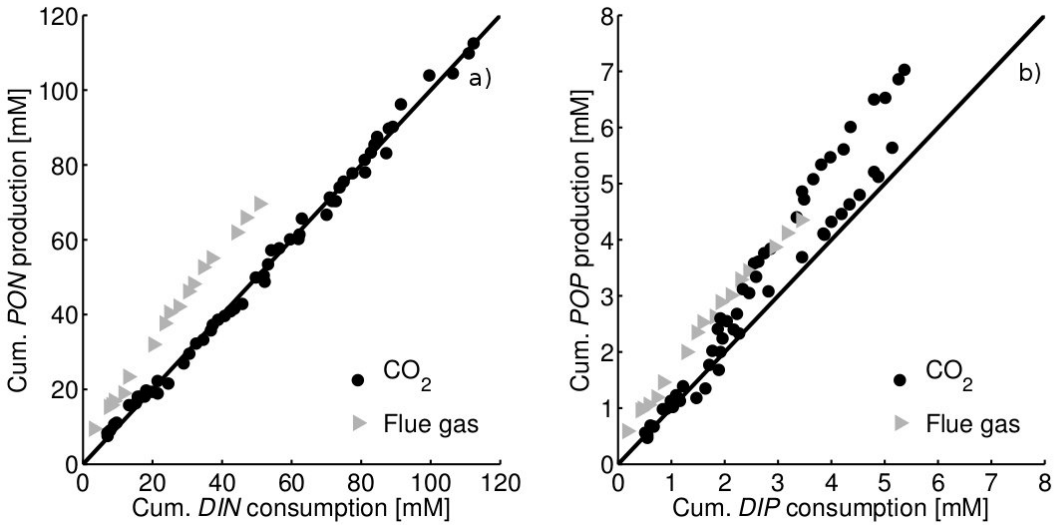


**Figure 4.7:** Relationship between consumption of nutrients and production of organic matter: a) cumulated dissolved inorganic nitrogen (Cum. $DIN$) consumption versus cumulated particulate organic nitrogen (Cum. $PON$) production; b) cumulated dissolved inorganic phosphorus (Cum. $DIP$) consumption versus cumulated particulate organic phosphorus (Cum. $POP$) production; cultures were aerated with a $CO_2$/air ($CO_2$) or a flue gas/air (Flue gas) mixture; the black line denotes the line where production resembles consumption

#### 4.2.1.2 Discussion

**Nitrate** In this investigation, the added nitrate is the most abundant nitrogen source. Therefore, it was assumed that $DIN$ comprises only nitrate neglecting other nitrogen ions such as nitrite and ammonium. The results demonstrate that this assumption is adequate for an air/pure $CO_2$ aerated culture, as the production of $PON$ closely resembles the consumption of $DIN$. Thus, an on-line measurement of $DIN$ is not only a good approximation of the nitrate concentration, but may also be used to estimate biomass production and concentration given a nitrogen cell content by simple book-keeping of $DIN$ fluxes. Similar to that, Münkel *et al.* (2012) reported that they could draw conclusions from the ammonium consumption (in their experiment ammonium was the major nitrogen source) on the biomass gain, which is part of an automatisation concept for the industrial outdoor production of microalgae biomass.

This approach is only viable, however, when the main nitrogen source is known. Naturally, multiple nitrogen sources may severely obscure the relationship between $PON$ production and $DIN$ consumption. The results of the flue gas treatment suggest that $NO_x$ from flue gas contributes substantially to the nitrogen nutrient pool and is assimilated by microalgae during their growth. As apparent from Figure 4.7 a), approximately one third of the cumulated $PON$ production cannot be explained by the consumption of $DIN$ (nitrate from the medium) and is therefore attributed to the useage of $NO_x$.

**Phosphate** The cumulated $POP$ production generally exceeded the $DIP$ consumption in this experiment. The determination of $POP$ was, however, afflicted with uncertainties. This variable was not analytically measured, but estimated from the nitrogen content of the algae by a fixed ratio. Since neither the flue gas nor the medium contained considerable sources of phosphorus (besides phosphate), the assumed and often used N:P ratio of 16:1 was most likely erroneous, i.e. too low and thus overestimating the organic phosphorus content of the algae.

### 4.2.2 Fate of dissolved organic carbon

The loss of dissolved organic carbon ($DOC$) was determined in line M1 during spring 2011. Additionally, the total bacterial fraction was estimated during this cultivation period. To study the effect of $DOC$ on bacterial and microalgal growth in outdoor cultures and the relationship between microalgae and bacteria, glucose was added to the microalgae suspension of one photobioreactor.

#### 4.2.2.1 Results

$DOC$ was measured in reactor line M1 during May and June 2011 to investigate the potential loss of carbon and its fate in microalgal systems (Figure 4.8 a)). Until day 50 of the cultivation period, $DOC$ contents in the *Chlorella* culture were relatively low ranging from 0.6 to 4.0 % of total organic carbon ($TOC$). Most values have been between 1 and 2.5 % of $TOC$ with a mean value of 1.8 ± 0.9 %. Then, however, $DOC$ increased rapidly to 13.8 % of $TOC$ during the last 10 cultivation days.

Algal cell counts were relatively constant until day 44 ($24.9 \pm 5.5 \cdot 10^7$ cells $mL^{-1}$) (Figure 4.8 b)). Thereafter, cell counts decreased rapidly to $3.6 \cdot 10^6$ cells $mL^{-1}$. In contrast, bacterial cell counts were constant until day 30 ($5.5 \pm 4.0 \cdot 10^7$ cells $mL^{-1}$), but increased steadily to $41.6 \cdot 10^7$ cells $mL^{-1}$ afterwards (Figure 4.8 b)). During phases of healthy algal growth, algal cell counts outnumbered bacterial cell counts by a factor of two to eight. At the end of the cultivation phase and during sub-

optimal growth conditions for microalgae, the algae culture collapsed and bacterial cell counts were up to 110-fold higher than algal cell counts. The increase of $DOC$ was hence accompanied by a rapid increase in bacterial cell numbers and a decrease in algal cell numbers at the end of the cultivation period.

In contrast to cell numbers, bacteria only accounted for a small part of the total biomass generated in the photobioreactors. In phases of healthy microalgae growth, the total bacterial biomass fraction comprises less than 1 % of the total specific biomass volume, but this fraction increased up to more than 20 % under suboptimal growth conditions for microalgae.

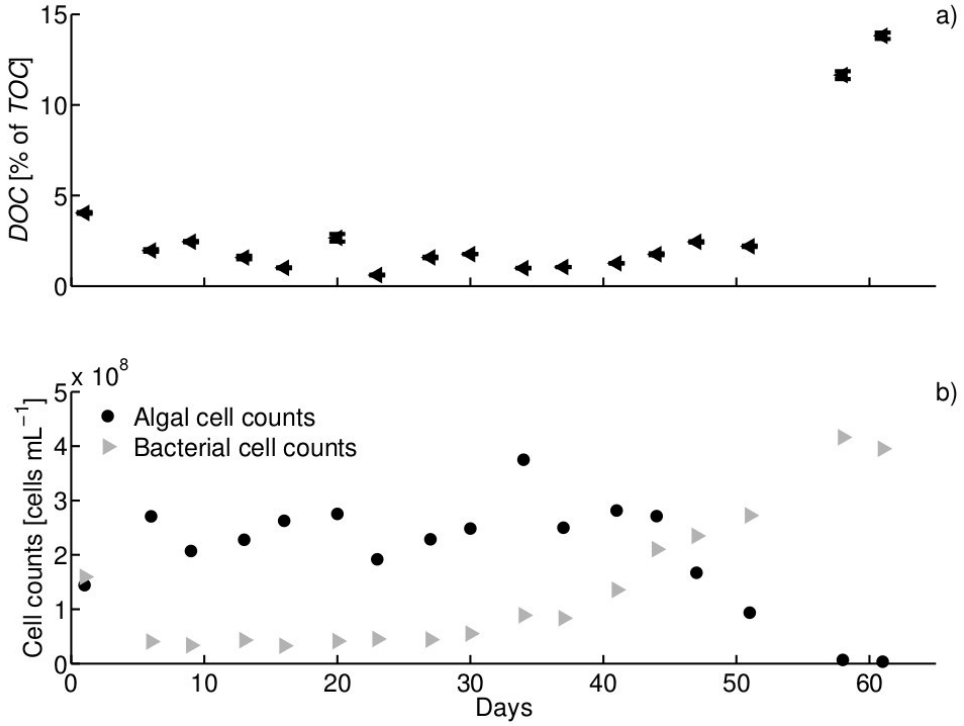


**Figure 4.8:** Dissolved organic carbon ($DOC$) content, as well as algal and bacterial cell counts; all samples were taken during harvesting events from pooled medium of all reactors from line M1 during May and June 2011; a) $DOC$ as a proportion of total organic carbon ($TOC$); error bars represent standard deviations; b) algal and bacterial cell counts

#### 4.2.2.2 Discussion

**Fate of carbon in microalgal culture systems** Hulatt and Thomas (2010) cultivated *Chlorella vulgaris* in 20 L photobioreactors under controlled conditions in the laboratory and measured an increase in the percentage of $DOC$ during algae growth. In the beginning, the mean $DOC$ content accounted for 2.0 ± 0.3 % of $TOC$ and at the end of the cultivation period, the $DOC$ fraction

rose to 6.4 ± 0.7 %. They suggested that the observed depletion of $DIN$ may have driven a part of the $DOC$ production during the late stages in their cultivation experiment. Moreover, mainly $HCO_3^-$ comprised the dissolved inorganic carbon pool due to an increase of pH throughout their experiment. Since the type and concentration of inorganic carbon influences the metabolism of microalgae (Falkowski and Raven, 2007), it can thus also affect the release of dissolved organic matter. Furthermore, they propose that exudation of dissolved organic matter under unstable outdoor conditions (i.e. with variable stress factors such as sunlight and temperature) may be higher than under stable indoor conditions (Hulatt and Thomas, 2010). In this investigation, very similar $DOC$ contents (mean value: 1.8 ± 0.9 % of $TOC$) were monitored during optimal microalgae growth, indicating that the exudation of carbon is similar under laboratory and optimal outdoor growth conditions. While the $DOC$ fraction in the former mentioned study only rose to 6.4 % at the end of the experiment, a rapid increase up to 13.8 % of $TOC$ was observed here.

The measured $DOC$ release falls within the ranges reported in other studies (Zlotnik and Dubinsky, 1989; Malinsky-Rushansky and Legrand, 1996). For instance, in *Chlorella vulgaris* cultures grown under different light conditions at 22°C in the laboratory, the excretion of $DOC$ was approximately 4 % of $TOC$ (Zlotnik and Dubinsky, 1989). Hellebust (1974) estimated that the fraction of the net photosynthesis released as $DOC$ ranges between 5 and 50 % in natural systems. Thus, extracellular release of $DOC$ is a normal process not only in natural aquatic systems (Kirchman *et al.*, 1991), but also in microalgae mass cultures.

In freshwater or marine systems, bacteria counts typically remain below $10^6$ counts $mL^{-1}$, while microalgae abundance varies between $10^2$ and $10^5$ counts $mL^{-1}$ (Cole, 1982). Naturally, microalgae are by far more abundant in photobioreactors than in natural waters (by three to six orders of magnitude), cell counts of bacteria in photobioreactors exceed the values found in natural systems by approximately one order of magnitude. Algal $DOC$ in microalgae cultivation systems comes from different sources, such as excretion of $DOC$ during photosynthesis or cell lysis. This carbon fraction can be rapidly metabolized by bacteria, which is why the gross amount of $DOC$ is larger than the measured amount (Cole, 1982; Malinsky-Rushansky and Legrand, 1996). In the experiment presented here, the amount of $DOC$ increased considerably after algal cell counts decreased. Different from Hulatt and Thomas (2010), it is assumed here, that increasing grazing pressure by protozoa (as suggested by the increase in protozoa abundance observed via microscopy after day 44) may have caused algae cells to enhance their exudation of $DOC$. Moreover, the lysis of algae cells may have also contributed to the $DOC$ pool.

**Relationship between bacteria and microalgae in microalgae cultivation systems** There is no simple explanation describing the relationship between microalgae and bacteria, but rather a multitude of inhibitory and stimulatory processes that may affect the thriving of microalgae and bacteria (Cole, 1982). On the one hand, bacteria can inhibit microalgae growth by adversely

altering the environment (consumption of oxygen at night in eutrophic ponds), via the lysis of algal cells, via competition for limiting nutrients (in particular for nitrogen and phosphorus), and via the excretion of inhibitory substances. On the other hand, bacteria can stimulate microalgal growth by means of nutrient regeneration (heterotrophic remineralisation) or by the production of vitamins (bacteria are the major source for vitamin $B_{12}$) and other stimulatory products. Bratbak and Thingstad (1985) reported that commensalism (relationship between two organisms where one organism benefits without affecting the other) and competition (a contest between organisms for resources) are likely the most suitable modes describing the cohabitation of microalgae and bacteria.

In a healthy culture and during photosynthesis, algae exudate organic matter (especially dissolved organic carbon), which facilitates the growth of bacteria (Grover, 2000). In most natural environments, bacteria depend on microalgae exudating carbon-rich substrate and are thus substrate limited, i.e. carbon limited (Rhee, 1972). Here, it is hypothesised that bacteria growing in outdoor photobioreactors were carbon limited under optimal growth conditions for microalgae. In the following, this hypothesis is tested.

To prove the carbon limitation of bacteria in a microalgae system, a photobioreactor with a *Chlorella* culture was inoculated once with glucose. Glucose served as a substitute for $DOC$, as glucose is the simplest $DOC$ molecule exuded by microalgae. The results of this experiment were compared to a control culture without glucose addition. Note that *Chlorella vulgaris* is mixotroph, i.e. the algae can also use glucose as a carbon source besides other inorganic substrates (Liang *et al.*, 2009).

Figure 4.9 a) shows that the $DOC$ concentration of the glucose culture and the control culture remained within a range between 17 and 67 mg C $L^{-1}$ throughout the cultivation period except for a short period after the addition of glucose at 12 am on day 6. The $DOC$ measurement was conducted 3 h after the addition to ensure an adequate homogenisation of the glucose in the photobioreactor. It is likely that glucose was already assimilated in the period between its addition and the first measurement. The measured concentration of 420 mg C $L^{-1}$ may therefore underestimate the peak value. The second $DOC$ measurement was scheduled 18 h after the first measurement. At this time, the glucose culture had already returned to normal $DOC$ concentrations, i.e. to concentrations comparable to the control experiment.

Figure 4.9 b) reveals that the cell counts of both, algal and bacterial communities, were similar at the beginning of the cultivation period and only evolved differently after the addition of glucose. Algal cell counts of the control and the glucose culture slightly increased until day 9. Afterwards cell counts in the control culture decreased again, while cell counts in the glucose culture kept growing. Apparently, the microalgae in the glucose culture also assimilated some of the readily available organic carbon.

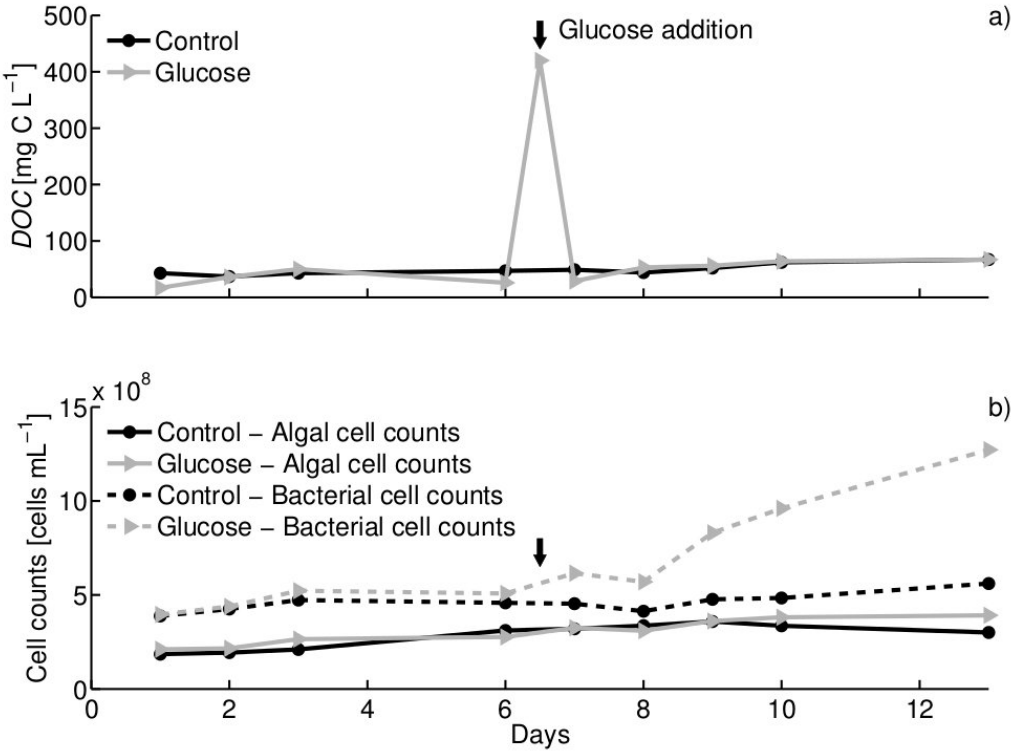


**Figure 4.9:** Dissolved organic carbon ($DOC$) concentration as well as algal and bacterial cell counts: a) $DOC$ concentration; b) algal and bacterial cell counts of a culture with a singular addition of glucose (grey) and of a control culture (black); cultivation took place in outdoor 180 L photobioreactors from 27-October to 08-November 2010; the black arrows indicate the singular addition of glucose on day 6

Bacterial cell counts of both cultures were similar until day 6. Afterwards, however, bacterial counts of the control culture only increased slightly until day 13, whereas the bacteria in the glucose culture thrived after the glucose addition. At the end of the cultivation phase bacterial cell counts exceeded algal cell counts by about 130 % in the glucose culture. Moreover, bacteria numbers were roughly 100 % higher in the glucose culture than in the control culture. The added glucose had an immediate effect on the bacterial community and caused a rapid reproduction. In conclusion, bacteria were, in fact, carbon limited under standard cultivation conditions, corroborating the commensal relationship between algae and bacteria.

Mayo and Noike (1994) studied the effect of glucose concentration, fed on a daily basis, on the growth of *Chlorella vulgaris* and heterotrophic bacteria under laboratory conditions. Specific growth rates for bacteria were much higher than specific growth rates for *Chlorella*. Heterotrophic bacteria are generally considered to be more efficient users of $DOC$ than microalgae (Kamjunke *et al.*, 2008). Many studies observed an enhancement of *Chlorella* growth when fed with glucose (Liang *et al.*, 2009; Doucha and Lívanský, 2012), albeit most of the studies were

conducted under axenic conditions (i.e. in the absence of bacteria). Whether there was also an effect of the added glucose on algal growth in this study could not be assessed in the presence of bacteria. Though algal cell counts slightly increased, this effect occurred not until four days after the glucose addition. However, it is possible that the suboptimal growth conditions (light and temperature regime in October and November) took a toll on the physiological state of the microalgae obscuring the potentially positive effect of glucose addition. Night temperatures already approached 0°C and 08-November 2010 was the last cultivation day before the operation was discontinued due to too low minimum temperatures. Unfortunately, time constraints precluded the verification of the glucose effect on microalgae growth under more favourable growth conditions. Moreover, the once added amount of glucose was rather small and it is clear that bacteria, due to their much higher specific growth rate, could assimilate the glucose faster than *Chlorella*.

**Axenic versus non-axenic microalgae cultures** Bacteria are often considered as contaminants in microalgae cultivation (Belay, 1997; Huntley and Redalje, 2007). Therefore, many experimental studies in photobioreactors use axenic microalgae cultures. However, the effort for sterilising the culture media and photobioreactors is high. The results presented here suggest that the relationship between bacteria and microalgae is one of commensalism, which means that bacteria benefit from microalgae exudates, but microalgae are not adversely affected by bacteria. In other words, microalgae manage to outcompete bacteria under regular culture conditions. Once these conditions become unfavourable for algae, however, bacteria fill the arising niche and rapidly multiply in number by using excess $DOC$ in the medium.

## 4.3 Experiment 3: Flue gas as a carbon and nitrogen source

In this Chapter, the effect of flue gas on the growth and biochemical composition of *Chlorella vulgaris* is investigated in more detail (Chapter 4.3.2). For this purpose, the comparability of the two parallel lines M1 and M2 is tested (Chapter 4.3.1). Moreover, the removal of $CO_2$ and $NO_x$ by microalgae is quantified (Chapter 4.3.3).

### 4.3.1 Proving the system comparability

To assess the comparability of line M1 and M2, both lines were aerated with a mixture of pure $CO_2$ and air, i.e. treated identically, from 05-May to 06-June 2010. Growth and biochemical parameters were monitored for a thorough analysis.

#### 4.3.1.1 Results

**Light conditions** The daily light intensity ($DLI_{ver}$) increased with time and ranged between 0.34 and 3.13·$10^7$ µmol $m^{-2}$ $d^{-1}$ in line M1 and between 0.19 and 2.49·$10^7$ µmol $m^{-2}$ $d^{-1}$ in line M2 (Figure 4.10 a)). Daily light intensities, measured on the surface of the unshadowed front line (M1), were significantly higher than the ones detected at the back line (M2) (ANOVA; F = 6.2, p = 0.016), which regularly experienced shadowing in the mornings and the evenings. Relative differences between both lines ranged between 20.1 and 45.6 % with the highest discrepancies generally occurring at low light intensities and vice versa.

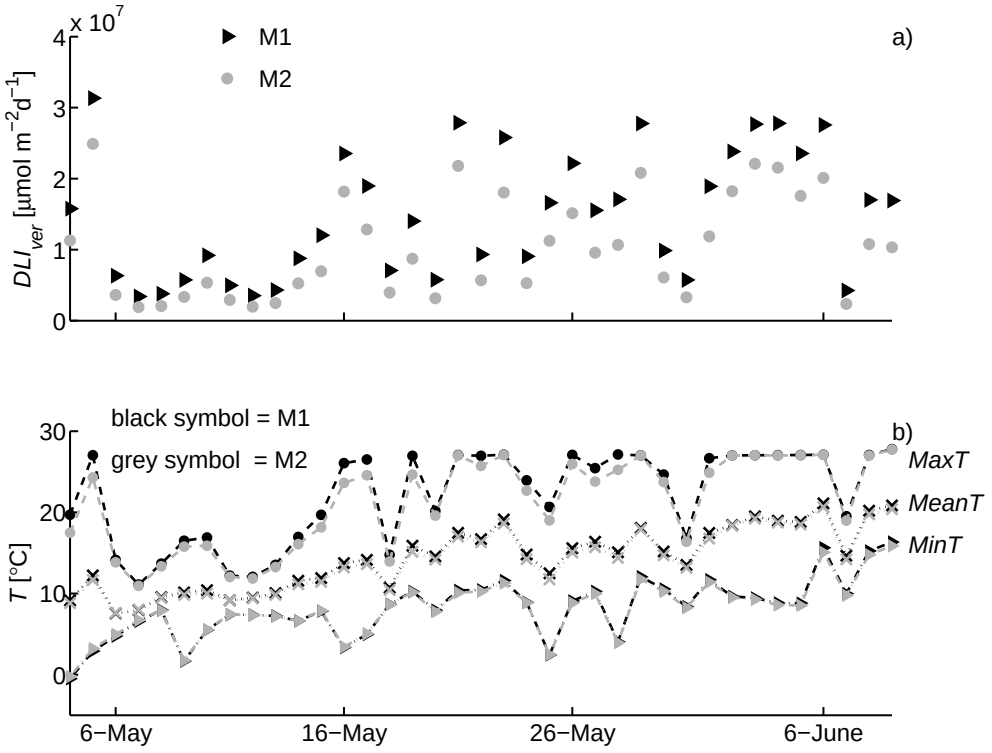


**Figure 4.10:** Ambient light and medium temperature of line M1 and M2 during May and June 2010: a) daily light intensity ($DLI_{ver}$) measured vertically at the lower third of two reactors, one in line M1 (black triangles) and one in line M2 (grey circles); b) maximum (Max), minimum (Min), and mean medium temperatures ($T$) of line M1 (black) and M2 (grey)

**Temperature conditions** Temperature variability in the medium of line M1 and M2 was high, fluctuating between 0 and 28°C (Figure 4.10 b)). Intraday, minimum, and maximum temperatures differed between 4.5 and 24.1°C and between 4.2 and 21.3°C in line M1 and M2, re-

spectively. Lower irradiance at the beginning of May resulted in lower maximum temperatures (below 15°C). If necessary, the temperature control set in at a medium temperature of 27°C, effectively maintaining the maximum temperatures between 27 and 28°C in both lines. Though line M2 received less irradiance than line M1, the temperatures of both reactor lines were not significantly different (ANOVA; F = 0.18, p = 0.67).

**Other growth conditions** The pH was maintained at 7.21 ± 0.19 and 7.27 ± 0.22 in line M1 and M2, respectively, by modifying the mixture of air and pure $CO_2$. At two incidents, both at night, $CO_2$ supply was interrupted for more than 30 min and, consequently, pH rose to a value above 8 (12-May 2010: pH increased up to 8.4 in M1 and M2 for approximately 4 h; 28-May 2010: pH increased up to 9.1 in M1 and M2 for approximately 6.5 h).

Nutrients were added semi-continuously after harvesting events. Nitrate and phosphate levels were never depleted during the cultivation period (Table 4.5). Thus, it was assumed that there was no significant nitrate or phosphate limitation.

**Table 4.5:** Nitrate and phosphate concentration ± standard deviation in the microalgae culture of line M1 and M2 during May and June 2010; minimum (Min), mean, and maximum (Max) values are denoted

| | Nitrate concentration | | Phosphate concentration | |
|---|---|---|---|---|
| | [mg $L^{-1}$] | | | |
| | M1 | M2 | M1 | M2 |
| Min | 56 | 56 | 30 | 34 |
| Mean | 686 ± 379 | 696 ± 407 | 94 ± 32 | 88 ± 35 |
| Max | 1,434 | 1,434 | 150 | 146 |

**Biomass concentration and productivity** The biomass concentration ($B$) in the outdoor photobioreactors of line M1 and M2 varied between 0.9 and 6.1 g $L^{-1}$ and 0.7 and 5.8 g $L^{-1}$, respectively, revealing a steady increase with time, only interrupted by harvesting events (Figure 4.11 a)). The different light regimes had no significant effect on the biomass concentration in line M1 and M2 (ANOVA; F = 0.27; p = 0.61). The mean productivities ($P$) calculated over a period of 36 days were identical in both lines and yielded 0.39 ± 0.23 g $L^{-1}$ $d^{-1}$.

**Organic carbon and nitrogen content** The organic carbon ($OC$) content of line M1 and M2 ranged between 47.0 and 63.5 % of biomass and 45.2 and 62.9 % of biomass, respectively

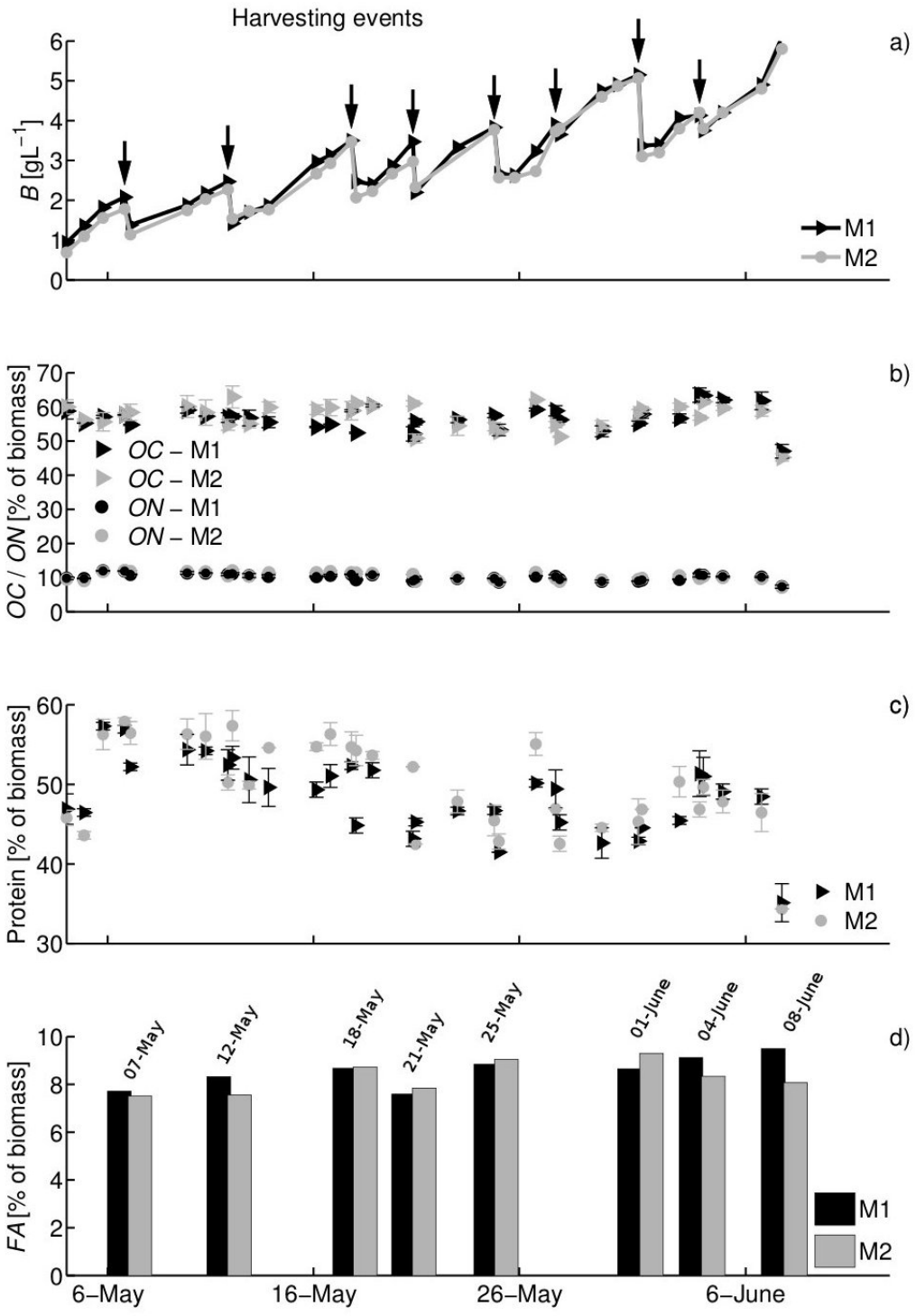


**Figure 4.11:** Growth and biochemical composition of *Chlorella vulgaris* grown in outdoor 180 L FPA photobioreactors with an air/pure $CO_2$ mixture (line M1 = black; line M2 = grey) during May and June 2010: a) biomass concentration ($B$); black arrows indicate harvesting events; b) mean organic carbon ($OC$) and nitrogen ($ON$) content ± standard deviation; c) mean crude protein (Protein) content ± standard deviation; d) fatty acid ($FA$) content

(Figure 4.11 b)). Mean organic carbon content has been 56.8 ± 3.5 % of biomass in line M1 and 57.7 ± 3.8 % of biomass in line M2.

The organic nitrogen ($ON$) content varied between 7.2 and 12.1 % and between 7.4 and 12.0 % of biomass in line M1 and M2, respectively (Figure 4.11 b)). Mean nitrogen content was 10.2 ± 1.0 % of biomass in line M1 and 10.6 ± 1.1 % of biomass in line M2.

No significant effects of the different light regimes were determined on the carbon and nitrogen contents in line M1 and M2 (ANOVA; $OC$: F = 0.32, p = 0.57; $ON$: F = 1.04, p = 0.31).

**Crude protein content** The protein content varied between 35.1 and 57.3 % of biomass in line M1 and between 34.4 and 57.9 % of biomass in line M2 (Figure 4.11 c)). No significant differences were detected between the two (ANOVA; F = 1.04, p = 0.31).

**Fatty acid composition and content** Also the fatty acid ($FA$) content revealed no significant differences between the two lines with mean values of 8.63 ± 0.67 % and 8.42 ± 0.73 % in line M1 and M2, respectively (Figure 4.11 d)). While variable, the time-series did not reveal a clear trend.

The most abundant fatty acids were C18:3(n-3) followed by C16:0 and C18:2(n-6) (Table 4.6). An increasing trend in fatty acid content was only observed for C18:2(n-6) in both lines, increasing from about 0.5 to 2.0 % of biomass. No significant differences between the single fatty acids of line M1 and M2 could be observed.

#### 4.3.1.2 Discussion

Results from the cultivation experiment conducted in May and June 2010 revealed that growth conditions were similar for both lines, except for the light regime. More specifically, line M1 received significantly more light than line M2. Despite these discrepancies, however, no significant differences in growth and biochemical parameters were observed. In other words, the comparability between both lines is given.

### 4.3.2 Effect of flue gas on the productivity and composition

In the next step, the effect of flue gas on the algae culture was assessed by aerating line M1 with a $CO_2$/air mixture and line M2 with a flue gas/air mixture in an experiment lasting from 05-May till 06-June 2011. Differences in the outcomes of these treatments were then attributed to the differing gases used for aeration.

**Table 4.6:** Fatty acid content and composition of *Chlorella vulgaris* cultivated in line M1 and M2 during May and June 2010; both lines were aerated with a $CO_2$/air mixture; only major fatty acids (> 0.2 % of biomass) are shown

| | Fatty acid | 07-May | 12-May | 18-May | 21-May | 25-May | 01-June | 04-June | 08-June |
|---|---|---|---|---|---|---|---|---|---|
| | | [% of biomass] | | | | | | | |
| M1 | C14:1 | 0.21 | 0.26 | 0.30 | 0.28 | 0.29 | 0.29 | 0.33 | 0.29 |
| | C16:0 | 1.53 | 1.66 | 1.88 | 1.87 | 1.98 | 1.90 | 2.14 | 2.19 |
| | C18:1(n-9c) | 0.10 | 0.11 | 0.23 | 0.19 | 0.19 | 0.20 | 0.20 | 0.2 |
| | C18:2(n-6) | 0.51 | 0.66 | 1.10 | 1.30 | 1.82 | 2.03 | 2.01 | 2.13 |
| | C18:3(n-3) | 5.26 | 5.36 | 5.04 | 3.56 | 4.33 | 4.00 | 4.19 | 4.46 |
| | Other | 0.11 | 0.27 | 0.24 | 0.35 | 0.23 | 0.24 | 0.25 | 0.25 |
| | Total | 7.71 | 8.32 | 8.67 | 7.59 | 8.84 | 8.65 | 9.12 | 9.50 |
| M2 | C14:1 | 0.19 | 0.23 | 0.28 | 0.27 | 0.28 | 0.29 | 0.29 | 0.28 |
| | C16:0 | 1.54 | 1.55 | 1.81 | 1.82 | 1.98 | 2.01 | 2.04 | 2.04 |
| | C18:1(n-9c) | 0.13 | 0.09 | 0.12 | 0.21 | 0.18 | 0.21 | 0.21 | 0.18 |
| | C18:2(n-6) | 0.52 | 0.56 | 1.02 | 1.34 | 1.98 | 2.32 | 2.01 | 1.95 |
| | C18:3(n-3) | 4.86 | 4.83 | 5.13 | 3.83 | 4.38 | 4.19 | 3.52 | 3.38 |
| | Other | 0.28 | 0.31 | 0.37 | 0.36 | 0.26 | 0.29 | 0.27 | 0.24 |
| | Total | 7.51 | 7.56 | 8.73 | 7.84 | 9.05 | 9.30 | 8.34 | 8.07 |

#### 4.3.2.1 Results

**Light conditions** Daily light intensity ($DLI_{ver}$) incident on the reactor surface was highly variable in May and June 2011. $DLI_{ver}$ ranged from 0.54 to 3.20·$10^7$ µmol $m^{-2}$ $d^{-1}$ in line M1 and from 0.33 to 2.99·$10^7$ µmol $m^{-2}$ $d^{-1}$ in line M2 (Figure 4.12 a)). Values measured for line M1 were again consistently higher (4.9 to 46.1 %) than corresponding ones for line M2 (ANOVA; F = 7.20, p = 0.009). Like in the experiment of the preceding year, the deviations between line M1 and M2 were high at low light intensities and low at high light intensities.

**Temperature conditions** The medium temperature of line M1 and M2 varied between 4.5 and 30°C (Figure 4.12 b)). Differences between minimum night and maximum day temperatures ranged from 4.5 to 24.1°C and from 4.2 to 21.3°C in M1 and M2, respectively. The cooling system prevented maximum temperatures to exceed 28°C. The temperature control failed once resulting in a rise of the medium temperature to 30°C for several hours on 03-June 2011. Though line M2 received significantly less light than line M1, the mean medium temperatures of both reactor lines were not significantly different (ANOVA; F = 0.02, p = 0.90).

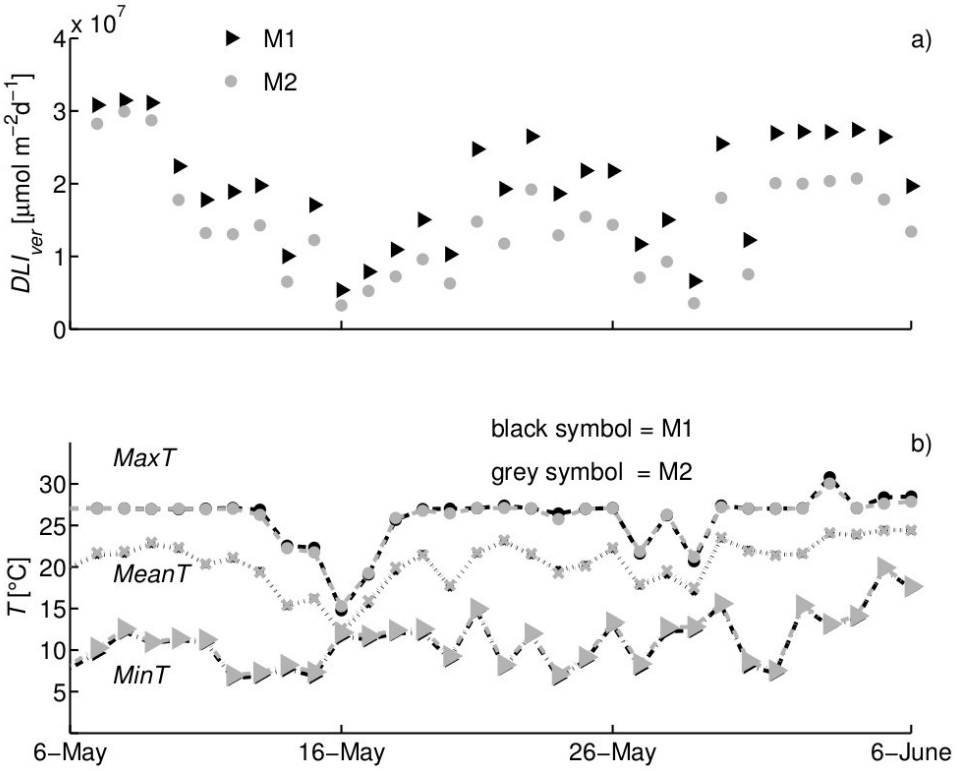


**Figure 4.12:** Ambient light and medium temperature of line M1 and M2 in May and June 2011: a) daily light intensity ($DLI_{ver}$) measured vertically at the lower third of two reactors, one in line M1 (black triangles) and one in line M2 (grey circles); b) maximum (Max), minimum (Min), and mean medium temperatures ($T$) of line M1 (black) and M2 (grey)

**Other growth conditions** Except for the two incidents described below, the pH values were maintained at 7.11 ± 0.12 and 7.18 ± 0.14 in line M1 and M2, respectively. On 11-May 2011, the air supply was interrupted and the pH declined to 4 in line M1 and M2 for approximately 1 h. Similarly, an interrupted $CO_2$ supply led to a pH increase to 8.7 in line M1 for approximately 1 h on 30-May 2011.

Nutrients were added semi-continuously after each harvesting event. Nitrate and phosphate remained well above 0 mg $L^{-1}$ during the experiment (Table 4.7). It is therefore assumed that there were no nitrate or phosphate limitations.

**Flue gas composition and concentration** Besides $N_2$, the main components of the flue gas were $O_2$, $CO_2$, $NO_x$, and CO. Table 4.8 shows mean concentrations of the main components, analysed on two days in 2011. The $CO_2$ concentration in the flue gas was 7.6 Vol.-% and $NO_x$ concentration was 600 ppm.

**Table 4.7:** Nitrate and phosphate concentration ± standard deviation in the medium of line M1 and M2 in May and June 2011; minimum (Min), mean, and maximum (Max) values are denoted

| | Nitrate concentration | | Phosphate concentration | |
|---|---|---|---|---|
| | [mg $L^{-1}$] | | | |
| | M1 | M2 | M1 | M2 |
| Min | 294 | 408 | 63 | 66 |
| Mean | 942 ± 344 | 1,035 ± 355 | 109 ± 24 | 108 ± 24 |
| Max | 1,674 | 1,856 | 156 | 158 |

**Table 4.8:** Gas composition ± standard deviation of flue gas from a combined heat and power (CHP) plant driven by natural gas; the flue gas was analysed directly after leaving the device on two days (12-May and 16-August 2011; n=8)

| $O_2$ [Vol.-%] | CO [ppm] | $NO_x$ [ppm] | $CO_2$ [Vol.-%] |
|---|---|---|---|
| 7.4 ± 0.3 | 229.1 ± 15.6 | 597.1 ± 92.1 | 7.6 ± 0.2 |

**Biomass concentration and productivity** The time series of the biomass concentration ($B$) of line M1 and M2 show the dynamics typical of a microalgae cultivation system that is operated semi-continuously (Figure 4.13 a)). The continuous increase of biomass concentration is interrupted by regular harvesting events. In this experiment, the biomass concentration of line M1 and M2 was maintained between 2 and 5 g $L^{-1}$. There was no significant effect of the flue gas treatment on the biomass concentration in line M2 (ANOVA; F = 0.06; p = 0.80). In both lines, very similar biomass concentrations were achieved. Mean productivities ($P$) yielded 0.41 ± 0.28 g $L^{-1}$ $d^{-1}$ and 0.42 ± 0.24 g $L^{-1}$ $d^{-1}$ in line M1 and M2, respectively.

**Organic carbon and nitrogen content** The organic carbon ($OC$) content of line M1 and M2 ranged from 37.6 to 61.2 % and from 45.8 to 64.3 % of biomass, respectively (Figure 4.13 b)). Mean values for the carbon content were 53.8 ± 4.8 % and 55.3 ± 4.6 % in line M1 and M2, respectively.

The organic nitrogen ($ON$) content ranged from 6.3 to 11.0 % and from 7.5 to 11.6 % of biomass in line M1 and M2, respectively, with mean values of 9.1 ± 0.9 % and 9.5 ± 0.9 % of biomass.

There was no significant effect of flue gas on the carbon and nitrogen content in line M2 (ANOVA; $OC$: F = 1.10, p = 0.30; $ON$: F = 3.10, p = 0.08).

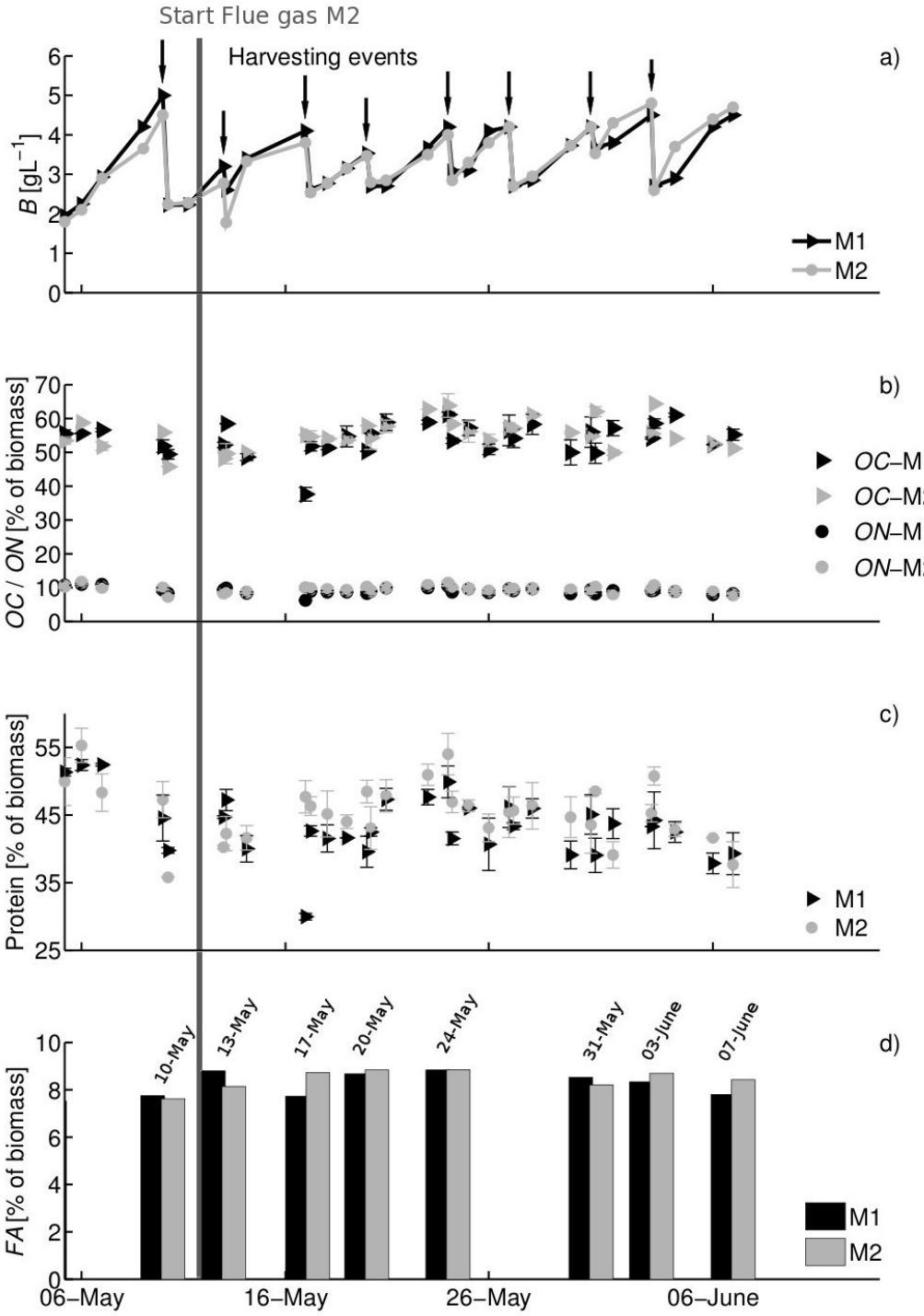


**Figure 4.13:** Growth and biochemical composition of *Chlorella vulgaris* grown outdoors in 180 L FPA photobioreactors with an air/pure $CO_2$ mixture (line M1 = black) and an air/flue gas mixture (line M2 = grey) in May and June 2011; the vertical grey line marks the beginning of flue gas aeration in M2: a) biomass concentration ($B$); black arrows indicate harvesting events; b) mean organic carbon ($OC$) and nitrogen ($ON$) content ± standard deviation; c) mean crude protein content ± standard deviation; d) fatty acid ($FA$) content

**Crude protein content** The protein content varied between 30.0 and 52.4 % of biomass in line M1 and between 35.8 and 55.3 % of biomass in line M2 (Figure 4.13 c)) with the respective mean values of 43.5 ± 4.6 % and 45.5 ± 4.3 %. The protein content of the control and the flue gas culture did not differ significantly (ANOVA; F = 3.10, p = 0.08).

**Fatty acid composition and content** The fatty acid ($FA$) content ranged from 7.7 to 8.8 % of biomass and from 7.6 to 8.9 % of biomass in line M1 and M2, respectively (Figure 4.13 d)). Mean $FA$ content was 8.31 ± 0.51 % of biomass in line M1 and 8.54 ± 0.50 % of biomass in line M2.

The most abundant fatty acid was C18:3(n-3) followed by C16:0 and C18:2(n-6) (Table 4.9). The time series of the individual fatty acid concentrations revealed no temporal trend in either of the lines. The concentration of the main fatty acids in the control and the flue gas culture was not considerably different.

**Table 4.9:** Fatty acid content and composition of *Chlorella vulgaris* cultivated in line M1 and M2 during May and June 2011; line M1 was aerated with a $CO_2$/air mixture, line M2 was aerated with a flue gas/air mixture; only the major fatty acids (> 0.2 % of biomass) are shown

| | Fatty acid | 10-May | 13-May | 17-May | 20-May | 24-May | 31-May | 03-June | 07-June |
|---|---|---|---|---|---|---|---|---|---|
| | | [% of biomass] | | | | | | | |
| M1 | C16:0 | 1.89 | 2.06 | 1.84 | 1.93 | 2.03 | 1.99 | 1.91 | 1.95 |
| | C18:1(n-9c) | 0.18 | 0.26 | 0.16 | 0.23 | 0.16 | 0.18 | 0.17 | 0.20 |
| | C18:2(n-6) | 1.43 | 1.56 | 1.55 | 1.98 | 2.15 | 2.04 | 1.80 | 2.02 |
| | C18:3(n-3) | 4.11 | 4.57 | 4.01 | 4.15 | 4.32 | 4.17 | 4.13 | 3.48 |
| | Other | 0.14 | 0.35 | 0.17 | 0.38 | 0.18 | 0.15 | 0.33 | 0.15 |
| | Total | 7.75 | 8.80 | 7.73 | 8.67 | 8.84 | 8.53 | 8.34 | 7.80 |
| M2 | C16:0 | 1.91 | 1.90 | 1.88 | 2.00 | 2.05 | 1.99 | 2.05 | 2.14 |
| | C18:1(n-9c) | 0.19 | 0.22 | 0.20 | 0.26 | 0.14 | 0.20 | 0.14 | 0.20 |
| | C18:2(n-6) | 1.30 | 1.28 | 1.42 | 1.76 | 1.85 | 1.70 | 1.66 | 1.88 |
| | C18:3(n-3) | 4.09 | 4.30 | 4.86 | 4.42 | 4.52 | 4.15 | 4.49 | 4.05 |
| | Other | 0.13 | 0.44 | 0.36 | 0.40 | 0.29 | 0.17 | 0.36 | 0.16 |
| | Total | 7.62 | 8.14 | 8.72 | 8.84 | 8.85 | 8.21 | 8.70 | 8.43 |

#### 4.3.2.2 Discussion

**Effect of flue gas on microalgae growth** A variety of artificial $CO_2$ sources for microalgae cultivation have been tested recently mainly by varying $CO_2$ concentrations, gas compositions,

or flow rates (Borkenstein *et al.*, 2011; Chiu *et al.*, 2008; Hsueh *et al.*, 2009). Although generally successful, the usage of flue gas as a $CO_2$ source also containing nitrogen oxides ($NO_x$) and sulfur oxides ($SO_x$) yielded inconclusive results so far (Negoro *et al.*, 1991, 1992, 1993; Doucha *et al.*, 2005; Douskova *et al.*, 2009; Straka *et al.*, 2002).

More specifically, Negoro *et al.* (1991) introduced flue gas in a pond system in which *Nannochloropsis* sp. and *Phaeodactylum tricornutum* were cultivated. In this experiment, algae growth was inhibited by $SO_x$ and $NO_x$ in the flue gas. In contrast to their previous study, however, Negoro *et al.* (1993) later claimed that productivity of flue gas cultures were similar to pure $CO_2$ cultures and barely influenced by the $SO_x$ and $NO_x$ components of the flue gas. Doucha *et al.* (2005) reported similar daily net productivities for algae cultivated with flue gas and with pure $CO_2$. Straka *et al.* (2002) demonstrated that flue gas from biogas, which was combusted in a boiler, can be used as carbon source without adverse effects on the production of *Chlorella* sp. in photobioreactors. Jander (2001) cultivated *Chlorella vulgaris* on a laboratory scale (8 L batch cultures) using flue gas from a natural gas driven CHP plant. Their results revealed that the flue gas culture and the control culture grew equally well. Likewise, Maeda *et al.* (1995) used flue gas from a thermal power plant and found no negative effects on algal growth. Doucha *et al.* (2005) proved that flue gas from the combustion of natural gas can be used to cultivate *Chlorella* in 400 L photobioreactors and Borkenstein *et al.* (2011) applied a complex flue gas from a cement plant to a *Chlorella emersonii* culture in a 5.5 L airlift photobioreactor and reported no adverse effect of the flue gas. Douskova *et al.* (2009) even reported that flue gas originating from a municipal waste incinerator increased the growth rate and the $CO_2$ fixation rate by 30 % compared to the control culture.

In this study and after ensuring the comparability between line M1 and M2, line M2 was aerated with flue gas from a natural gas driven CHP plant and compared to line M1, which was aerated with pure $CO_2$. In general, flue gas had no negative effect on microalgae growth when compared to a culture supplied with pure $CO_2$ over a cultivation period of about 25 days. No significant differences between the biomass concentrations of line M1 and M2 were observed and the mean productivities were very similar.

**Effect of flue gas on microalgae composition** While studies considering the effect of flue gas on the growth of different microalgae species are numerous (see above for references), only a few of them also investigated a potential effect on the composition of microalgae. This is particularly relevant, because flue gas from a natural gas driven CHP plant contains a variety of components, mainly nitrogen, water vapor, oxygen, but also other gases like carbon monoxide and nitrogen oxides that may have potential impacts on the cellular composition of microalgae.

In this experiment, the crude protein content of the flue gas culture of line M2 did not differ

significantly from to the one of the control culture. This result corroborates the finding of Jander (2001) who also compared the protein contents of *Chlorella vulgaris* in a control and a flue gas culture. They also observed that the application of flue gas from a natural gas driven CHP plant had no effect on the protein content.

Considering fatty acids, Jander (2001) observed a higher fatty acid content in a flue gas culture (7.8 % of biomass) than in a control culture (3.3 % of biomass). Despite the differences in absolute fatty acid content among the two treatments, their relative composition was remarkably similar. More specifically, Jander (2001) reported that there was no change in the percentage composition of the single fatty acids between treatments. These findings can be confirmed by the results of this experiment. The relative composition of the main fatty acids (C16:0, C18:1(n-9c), C18:2(n-6), and C18:3(n-3)) of the flue gas culture and the control culture did not differ significantly from each other. Here, however, also the fatty acid contents were similar in the control and flue gas culture throughout the experiment. In other words, there was no evidence that flue gas considerably affected the fatty acid content, as the variations of the fatty acid contents over time within one treatment were similar to the variation between treatments.

In summary, the results of this experiment are robust and consistent revealing no effect (i.e. neither positive nor negative) of the use of flue gas on *Chlorella vulgaris*' biochemical composition, which was characterised here by crude protein content, fatty acid content and composition, as well as organic carbon and nitrogen content.

### 4.3.3 Removal of $NO_x$ and $CO_2$ from flue gas

The removal of nitric oxide and carbon monoxide was quantified by comparing the inlet and outlet gas concentrations. The carbon dioxide removal was determined by comparing the inlet and outlet gas measurements expressed as degree of decarbonisation and by calculating the carbon dioxide biofixation rate.

#### 4.3.3.1 Results

Nitric oxide ($NO_x$) and carbon monoxide (CO) concentrations were measured in the flue gas inlet and outlet on three days (Table 4.10). $NO_x$ diminished substantially between inlet to outlet measurements. Between 28.6 and 48.3 % of the $NO_x$ was removed while passing through the microalgae suspension. In contrast, the CO content barely decreased between inlet and outlet measurements.

The carbon dioxide concentration in the gas inlet and outlet was measured at different times of the year and the degree of decarbonisation was determined. Figure 4.14 shows that the degree

**Table 4.10:** Carbon monoxide (CO) and nitric oxide ($NO_x$) content ± standard deviation measured in the flue gas inlet and outlet of the microalgae cultivation system on three different days (28-July 2011, 16-August 2011, 27-October 2011)

| Time | $NO_x$ | | | CO | | |
|---|---|---|---|---|---|---|
| | [ppm] | | [%] | [ppm] | | [%] |
| | Inlet | Outlet | △ | Inlet | Outlet | △ |
| 28-July 2011 (4pm) | 398.7 ± 17.4 | 206.0 ± 8.5 | -48.3 | 89.0 ± 2.6 | 85.0 ± 2.6 | -4.5 |
| 16-August 2011 (2pm) | 354.7 ± 19.7 | 207.0 ± 5.6 | -41.6 | 140.0 ± 3.5 | 130.7 ± 0.6 | -6.6 |
| 27-October 2011 (12am) | 562.0 ± 33.3 | 401.3 ± 26.0 | -28.6 | 207.0 ± 1.0 | 208.7 ± 2.1 | 0.8 |

varies between 3.5 and 36.3 % and is influenced by the productivity and the time of the day. In general, it increased with increasing productivity and was highest at noon. Note, that $CO_2$ measurements were only made at relatively small productivities (up to 0.3 g $L^{-1}$ $d^{-1}$). The highest degree of decarbonisation of 36.3 % was observed at a productivity of 0.3 g $L^{-1}$ $d^{-1}$ at noon.

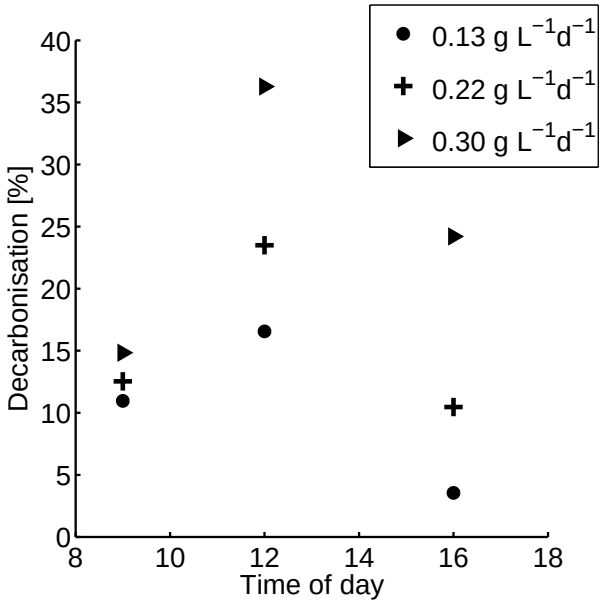


**Figure 4.14:** Degree of decarbonisation as a function of productivity and time of the day measured on three random days (16-August 2011: 0.13 g $L^{-1}$ $d^{-1}$; 15-June 2011: 0.22 g $L^{-1}$ $d^{-1}$; 06-June 2011: 0.30 g $L^{-1}$ $d^{-1}$) under optimal growth conditions

An average $CO_2$ biofixation rate of 0.85 ± 0.48 g $CO_2$ $L^{-1}$ $d^{-1}$ was calculated using the average productivity (0.42 ± 0.24 g $L^{-1}$ $d^{-1}$) and the average carbon content of 55 % measured in the flue gas culture.

#### 4.3.3.2 Discussion

**Removal of $NO_x$ and the effect on microalgae growth** There is broad consensus in the literature that $NO_x$, which mainly comprises nitric oxide (NO) (Nagase *et al.*, 1997), does not inhibit the growth of microalgae (Hauck *et al.*, 1996; Matsumoto *et al.*, 1997; Doucha *et al.*, 2005). More specifically, Yanagi *et al.* (1995) showed that *Chlorella* sp. is tolerant to up to 120 ppm $NO_x$, while Maeda *et al.* (1995) observed a tolerance of *Chlorella* sp. up to 60 ppm $NO_x$ in synthetic flue gas and up to 150 ppm $NO_x$ in coal-fired flue gas. Here, *Chlorella vulgaris* was tolerant to much higher concentrations than previously reported with peak values exceeding 500 ppm $NO_x$. Up to 48 % of $NO_x$ was transferred into the suspension. In contrast, Doucha *et al.* (2005) found that only up to 14 % of $NO_x$ was absorbed in the microalgae suspension. They used flue gas generated by combustion of natural gas in a boiler for the aeration of an outdoor culture of *Chlorella* sp.. Note that it does not necessarily mean that all of the $NO_x$ was taken up by algae.

Brown (1996) observed that the NO concentration in synthetic flue gas cultures was much higher than in cultures aerated with $CO_2$, $O_2$, and $N_2$. His finding suggests that NO dissolves in water and may be available as N-source for the microalgae. Following this reasoning, Nagase *et al.* (2001) investigated the uptake pathways of NO and demonstrated that NO in the gaseous phase dissolves in the medium and mainly permeates directly into the cells by diffusion. Furthermore, NO rather than nitrate was the N-source used preferentially by the microalgae. Nevertheless, the exact mechanisms that enable microalgae to use $NO_x$ are still to be proven (Van Den Hende *et al.*, 2012). But in general, the results of this study corroborate the findings of the above mentioned authors, since a substantial fraction of the $NO_x$ in the flue gas was transferred into suspension and may have been utilised as N-source by *Chlorella vulgaris*.

Taking also into account the results from Chapter 4.2.1, it can be concluded that flue gas can serve as a carbon as well as a nitrogen source for microalgae growth.

**Degree of decarbonisation and $CO_2$ biofixation rate** Here, the effect of productivity and the time of the day on the degree of decarbonisation was tested at a constant flow rate. The results led to the conclusion, that the removed inorganic carbon is assimilated by microalgae, as the degree of decarbonisation increased with productivity. It is, however, not possible to exactly quantify the amount of carbon converted into organic biomass, because of the dynamic equilibrium between $CO_2$, carbonic acid ($H_2CO_3$), bicarbonate ($HCO_3^-$), and carbonate ($CO_3^{2-}$) in water. Moreover, this equilibrium also depends on the pH. Therefore, the degree of decarbonisation is only an estimate for the amount of inorganic carbon converted into organic carbon.

The highest degree of decarbonisation of 36.3 % occurred at noon coinciding with the highest

observed productivity. Another relationship, in this case between the degree of decarbonisation and the flow rate, has been reported by Doucha *et al.* (2005). They showed that the degree of decarbonisation decreases with increasing flow rate and observed a maximum decarbonisation of 60 %. However, the degree depends on several factors including the state of algal physiology, the productivity (which itself mainly depends on ambient temperature, light regime, and biomass concentration), the aeration rate, and the cultivation system. Given the various parameters that can affect the degree of decarbonisation, comparability between studies is severely hindered.

The $CO_2$ biofixation rate quantifies the incorporation of inorganic carbon into microalgae biomass for a given time period by multiplying the productivity with the organic carbon content of the cell. Douskova *et al.* (2009) calculated a $CO_2$ biofixation rate of 3.0 g $CO_2$ $L^{-1}$ $d^{-1}$ in a control culture and 4.4 g $CO_2$ $L^{-1}$ $d^{-1}$ in a flue gas culture, indicating a potential increase of the $CO_2$ biofixation rate by aeration with flue gas. Other authors published biofixation rates ranging from 0.65 to 4.0 g $CO_2$ $L^{-1}$ $d^{-1}$ (Kurano *et al.*, 1995; Yoshihara *et al.*, 1996; Murakami and Ikenouchi, 1997). Kumoro and Susanto (2013) observed a maximum carbon biofixation rate of 1.84 g $CO_2$ $L^{-1}$ $d^{-1}$ in a *Chlorella sp.* culture aerated with synthetic flue gas. An exceptionally high carbon dioxide fixation rate was reported by Hu *et al.* (1998) measuring 16.7 g $CO_2$ $L^{-1}$ $d^{-1}$ in a high cell density reactor, sustained at a light intensity of 2,000 µmol $m^{-2}$ $s^{-1}$ at 25°C. This carbon dioxide fixation rate is not unusual considering that a cell density of over 80 g $L^{-1}$ was maintained. In this study, an average $CO_2$ biofixation rate of 0.85 ± 0.48 g $CO_2$ $L^{-1}$ $d^{-1}$ was observed in the flue gas culture. While this rate is within the range published by several authors, it is lower than the fixation rate observed by Hu *et al.* (1998). Note, that some studies did not account for nightly respiration losses of biomass or used cell carbon contents differing from 55 %, which was measured and used in this study.

## 4.4 Experiment 4: Microalgae as a feedstock for anaerobic digestion

The focus of this Chapter lies on the evaluation of *Chlorella* biomass as a substrate for the generation of biogas and its optimisation for the anaerobic digestion process. Therefore, microalgae biomass was exposed to different thermal or enzymatic pre-treatments (Chapters 4.4.1 and 4.4.2). Moreover, residual biomass, after extraction of soluble proteins, was tested as potential substrate for anaerobic digestion (Chapter 4.4.3).

### 4.4.1 Prerequisites for anaerobic digestion experiments

Before anaerobic digestion experiments started, the biogas potential of fresh and frozen microalgae biomass was compared. Furthermore, a new proxy for the degree of cell disintegration was evaluated.

#### 4.4.1.1 Results

**Fresh and frozen microalgae biomass** After harvesting the algae suspension, the microalgae biomass was centrifuged and stored at -28°C until further analysis. As freezing might also affect biomass characteristics, it was considered a pre-treatment. Fresh and previously frozen biomass (48 h) was compared in anaerobic fermentation batch set-ups according to guideline Verein Deutscher Ingenieure (2006). The results did, however, not reveal significant differences between the biogas potentials of fresh and frozen substrates (Figure 4.15). For the sake of simplicity, frozen biomass was used in all following experiments.

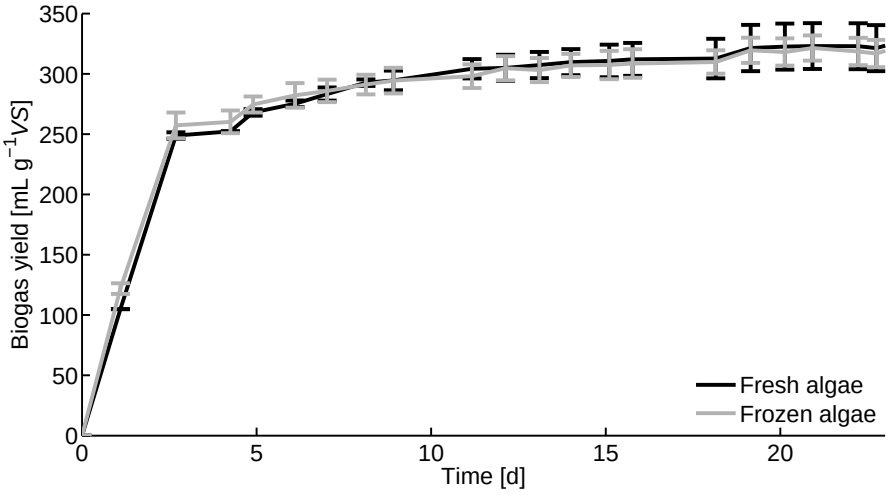


**Figure 4.15:** Specific biogas yield ± standard deviation from anaerobic digestion of fresh and frozen *Chlorella vulgaris* biomass; anaerobic batch tests were conducted in triplicates

**Microalgae cell disintegration** The percentage of soluble chemical oxygen demand ($sCOD$) to chemical oxygen demand ($COD$) has been used to evaluate substrate pre-treatments by indirectly measuring changes of dissolved organic compounds in water. This parameter is an indication for the degree of algae cell disintegration (Samson and Leduy, 1983; Bonmati *et al.*, 2001). In this study, however, the validity of the percentage of dissolved organic carbon ($DOC$)

to total organic carbon ($TOC$) as an easy to determine proxy for the degree of cell disintegration was evaluated. To establish this new proxy, the relationship between $DOC$ and $sCOD$ was studied. The results reveal a very good relationship (r = 0.97, p < 0.05) between $DOC$ and $sCOD$ (Figure 4.16) and indicate the suitability of $DOC$ for assessing algae cell disintegration.

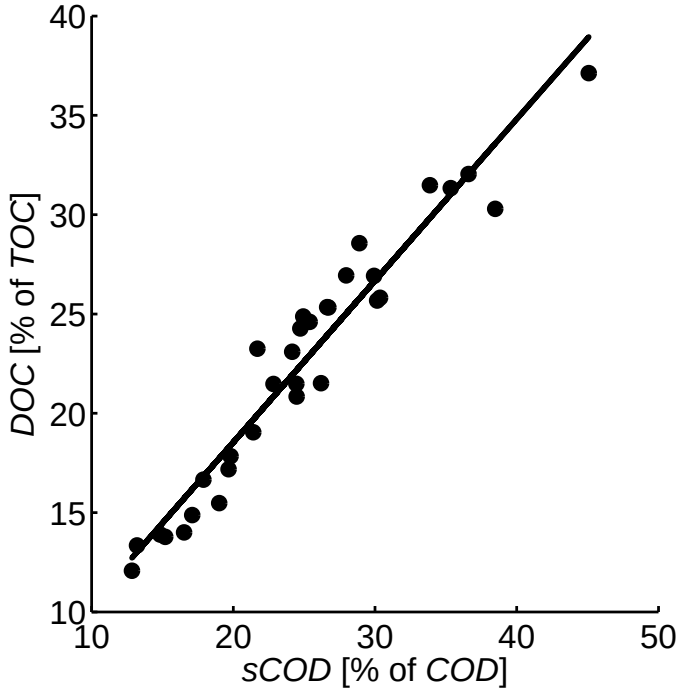


**Figure 4.16:** Relationship between soluble chemical oxygen demand ($sCOD$) and dissolved organic carbon ($DOC$); data were derived from enzyme pre-treatment experiments with microalgae biomass (n = 32)

#### 4.4.1.2 Discussion

**Freezing as pre-treatment** Babarro *et al.* (2001) showed that freezing and storage times influence the biochemical composition of microalgae biomass. While the lipid content remained constant, protein and carbohydrate contents decreased. Moreover, Babarro *et al.* (2001) stated that freezing affects the cell wall structure and attributed the loss of proteins and carbohydrates to the postulated alterations of the cell wall. In this investigation, a difference in the biogas yield of frozen and fresh biomass material could not be observed. In the following, experiments were generally conducted with frozen biomass. However, it cannot be ruled out that storing frozen material longer than two days could cause considerable biomass alterations and, hence, changes in the biogas yield.

**Disintegration of microalgae cells** Microbial cell walls release their intracellular organic compounds into the sludge water phase after their break-up (Neis *et al.*, 2000). Proxies can be used to quantify the release of intracellular organic compounds into the surroundings and, hence, the extent of cell disintegration. One method to determine the amount of cell disintegration measures the $COD$ increase in the sludge supernatant (Neis *et al.*, 2000). According to Bonmati *et al.* (2001) as well as Samson and Leduy (1983), an increase of soluble compounds after pre-treatments (e.g. $sCOD$) indicates the degradation of organic material. This study evaluated the increase of $DOC$ as a proxy for the cell disintegration. The very good relationship between $DOC$ and $sCOD$ (Figure 4.16) indicates the suitability of $DOC$ for this purpose. In comparison to $sCOD$, $DOC$ is an even a faster measurable parameter.

## 4.4.2 Digestion of pre-treated biomass

Microalgae were thermally and enzymatically pre-treated to assess the effect of temperature and enzymes on the disintegration of *Chlorella vulgaris* cells prior to anaerobic digestion experiments.

### 4.4.2.1 Results

**Thermal pre-treatment** Microalgae suspension was subjected to thermal pre-treatments at different temperatures (35, 55, 65, 85°C) and exposure times (1, 6, 24 h). Figure 4.17 shows that the highest $DOC$ content was achieved at the lowest temperature (35°C) after a 24 h exposure. Accordingly, the $DOC$ content decreased with increasing temperature except for the 6 h exposure.

The maximum biogas yields derived from pre-treated biomass decreased with increasing temperature, albeit only slightly (Figure 4.18). At 1 h exposure, the maximum biogas yield was 500.8 ± 11.1 mL $g^{-1}VS$ at 35°C. A temperature increase to 85°C resulted in a reduced yield of only 317.3 ± 6.7 mL $g^{-1}VS$, which is the minimum yield achieved at this exposure time. The highest maximum biogas yield of all pre-treatments, 623.8 ± 38.8 mL $g^{-1}VS$, was realised at 35°C and an exposure time of 24 h and exceeded the yield of the control culture (untreated microalgae biomass) by more than 50 % (cf. Table 4.11).

Mean $CH_4$ contents of the biogas ranged between 62.3 and 65.6 % (Table 4.11). Methane yields varied greatly between the treatments with an almost 100 % difference between the minimum of 203.1 ± 4.3 (1 h exposure at 85°C) and the maximum of 396.1 ± 24.6 mL $CH_4$ $g^{-1}VS$ (24 h exposure at 35°C).

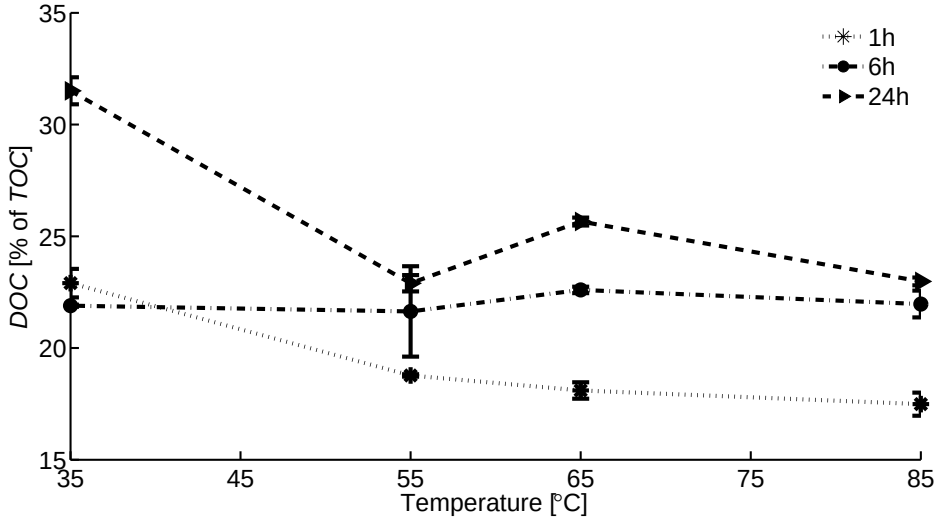


**Figure 4.17:** Dissolved organic carbon ($DOC$) ± standard deviation for different pre-treatment temperatures (35, 55, 65, 85°C) and exposure times (1, 6, 24 h)

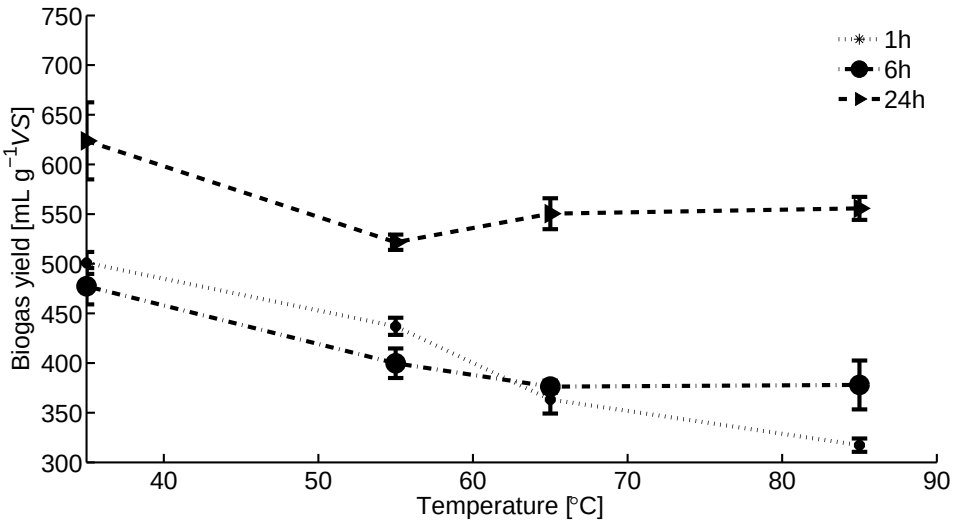


**Figure 4.18:** Biogas yield ± standard deviation from anaerobic digestion experiments with thermally pre-treated substrates; batch tests were conducted in triplicates; different exposure durations (1, 6, 24 h) and pre-treatment temperatures (35, 55, 65, 85°C) were applied

**Enzymatic pre-treatment** Finding optimal conditions for enzymes in pre-treatment experiments is crucial in order to increase the biogas yield of the substrate. For a systematic optimisation of these conditions screening experiments were conducted. Zapf (2009) reported, that the pectinase 'Macerozyme R-10' and the cellulase 'Onozuka R-10' are effective enzymes, which

**Table 4.11:** Specific maximum (Max) methane ($CH_4$) yields derived from the specific maximum (Max) biogas yields and the averaged $CH_4$ contents; biogas yields and $CH_4$ contents were derived from anaerobic digestion experiments with untreated biomass (Control) and pre-treated biomass; the anaerobic batch tests were conducted in triplicates; values denoted with a '*' are missing and replaced by the average $CH_4$ content of all experiments (63.5 %)

| Temperature | Exposure [h] | Max biogas yield [mL $g^{-1}VS$] | $CH_4$ content [%] | Max $CH_4$ yield [mL $CH_4$ $g^{-1}VS$] |
|---|---|---|---|---|
| Control | | 406 ± 28.3 | 62.8 | 255 ± 17.8 |
| 35°C | 1 | 500.8 ± 11.1 | 63.5* | 318.0 ± 7.1 |
| | 6 | 477.4 ± 18.3 | 63.7 | 304.1 ± 11.7 |
| | 24 | 623.8 ± 38.8 | 63.5* | 396.1 ± 24.6 |
| 55°C | 1 | 437.0 ± 8.6 | 62.3 | 272.3 ± 5.4 |
| | 6 | 399.8 ± 14.9 | 62.6 | 250.3 ± 9.3 |
| | 24 | 521.6 ± 7.7 | 65.6 | 342.2 ± 5.1 |
| 65°C | 1 | 363.3 ± 14.1 | 63.8 | 231.8 ± 9.0 |
| | 6 | 376.3 ± 6.0 | 62.7 | 235.9 ± 3.8 |
| | 24 | 550.4 ± 15.6 | 63.6 | 350.1 ± 9.9 |
| 85°C | 1 | 317.3 ± 6.7 | 64.0 | 203.1 ± 4.3 |
| | 6 | 377.9 ± 24.6 | 63.2 | 238.8 ± 15.6 |
| | 24 | 555.8 ± 11.6 | 63.7 | 354.1 ± 7.4 |

operated optimally at a pH of 5. To further improve the performance of pre-treatments with these enzymes, the enzyme concentration and incubation temperature were optimised here.

Figure 4.19 shows the $DOC$ contents of microalgae biomass pre-treated with two different enzymes at different incubation temperatures (35, 40, 45, 50°C) for 24 h. Generally, enzymatically pre-treated substrates had higher $DOC$ contents than the respective control cultures (only microalgae biomass without enzymes). More specifically, pectinase was less effective than cellulase in increasing $DOC$ contents. Incubation temperatures had only minor effects on the $DOC$ and therefore only two different temperatures (35 and 45°C) were chosen for further experiments.

Based on the results shown in Figure 4.19, three combinations were chosen for investigations with different enzyme concentrations: cellulase 'Onozuka R-10' at 35 and 45°C and an enzyme mixture of cellulase and pectinase at 45°C (Figure 4.20). Generally, $DOC$ increased with enzyme concentration and, in the cellulase treatments, also with incubation temperature. The highest cellulase concentration (Cel-3; 165 mg enzyme $g^{-1}VS$) improved $DOC$ contents by

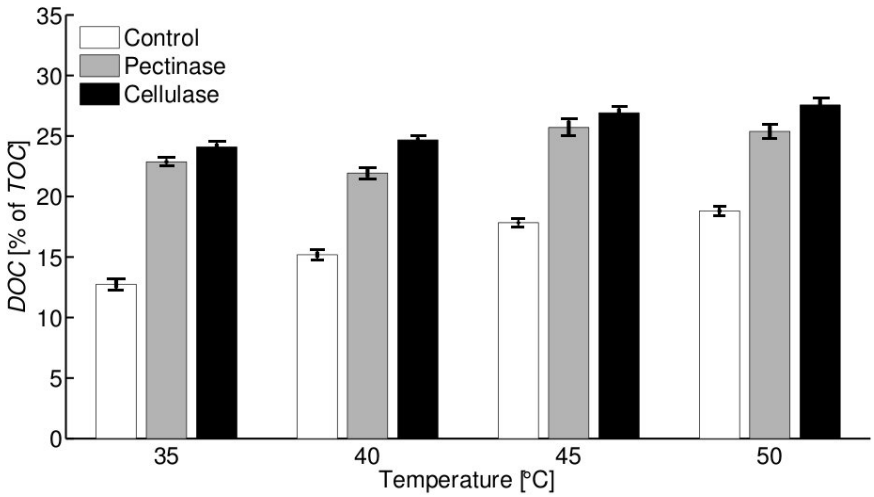


**Figure 4.19:** Dissolved organic carbon ($DOC$) content ± standard deviation of *Chlorella vulgaris* biomass pre-treated with different enzymes (cellulase 'Onozuka R-10' and pectinase 'Macerozyme R-10') at different temperatures (35, 40, 45, 50°C); pH was 5.0, incubation time was 24 h (cf. Table 3.8 for a detailed description)

75 % and 79 % at 35 and 45°C, respectively, compared to the control culture. The $DOC$ content achieved by the enzyme mixture Cel/Pec-3 (165 mg enzyme g$^{-1}VS$) also increased by 79 % compared to the control.

Enhancing the concentration of the enzyme mixture raised the $DOC$ release further. Thus, applying the highest Cel/Pec concentration (Cel/Pec-4; 330 mg enzyme g$^{-1}VS$) increased the $DOC$ release by 125 % compared to the control culture, containing also the highest total $DOC$ content of 45 % of $TOC$.

Subsequently, anaerobic digestion experiments with microalgae biomass pre-treated with cellulase (Cel-2/3) at 35 and 45°C and with an enzyme mixture (Cel/Pec-3/4) at 45°C were conducted. Figure 4.21 illustrates the cumulative specific biogas yields obtained under mesophilic conditions. Enzymatic pre-treatment generally enhanced the biogas production compared to a control with untreated microalgae biomass. The treatment with the lowest cellulase concentration resulted in a specific biogas yield of approximately 406 mL g$^{-1}VS$ at an incubation temperature of 35°C. Doubling the cellulase concentration, adding the same amount of pectinase, and increasing the pre-treatment temperature by 10°C resulted in a substantial increase in the biogas yield by approximately 110 %, corresponding to 860 mL g$^{-1}VS$.

Table 4.12 summarises the maximum biogas and methane yields of the enzyme experiments. The cumulated methane yield of the control experiment was 178.7 ± 10.3 mL $CH_4$ g$^{-1}VS$. The cellulase treatment (Cel-3) gained a higher yield than the cellulase/pectinase (Cel/Pec-3) mixture

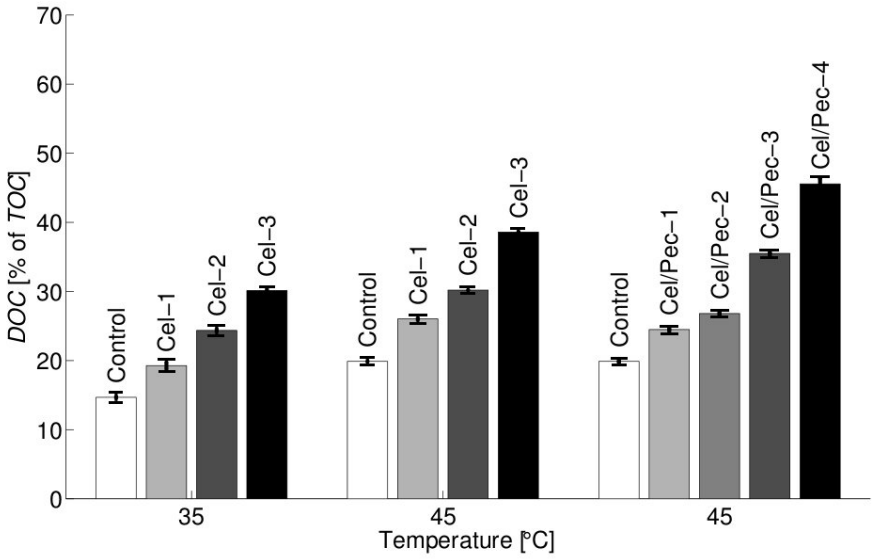


**Figure 4.20:** Dissolved organic carbon ($DOC$) ± standard deviation of *Chlorella vulgaris* biomass pretreated with different enzyme types, concentrations and temperatures (35 and 45°C); Cel-1..3 refer to different cellulase concentrations, Cel/Pec-1..4 refer to different concentrations of a cellulase/pectinase mixture; the incubation conditions were set to a pH of 5.0, incubation time was 24 h (cf. Table 3.9 for a detailed description)

at 45°C. The yield of the higher concentrated cellulase/pectinase mixture increased substantially by 65 % compared to the lower concentration, while the higher concentrated cellulase treatment at 45°C increased only marginal by 11 %. By far the highest methane yield was obtained by the highest concentration of the enzyme mixture.

#### 4.4.2.2 Discussion

**Theoretical and experimental methane yields** Many microalgae species do not fully unfold their biogas potential in experimental studies. A major reason for the deviation between experimental and theoretical methane yields is the resistance of algae cell walls, which is one of the limiting factors for cell digestibility (Angelidaki and Sanders, 2004; Sialve *et al.*, 2009; Schwede *et al.*, 2011). The rigid cell wall of most microalgae species generally contains approximately 25 to 30 % cellulose, 15 to 25 % hemicellulose, 35 % pectin, and 5 to 10 % glycoprotein (Wang and Evangelou, 1995). *Chlorella*'s polysaccharide-based cell wall contains glucosamine, mannose, and galactose and is therefore very recalcitrant to anaerobic digestion (Takeda, 1991).

Experimentally derived methane yields are generally highly variable. Angelidaki and Sanders (2004) reported conversion rates of up to 90 to 95 % of the theoretically achievable maximum

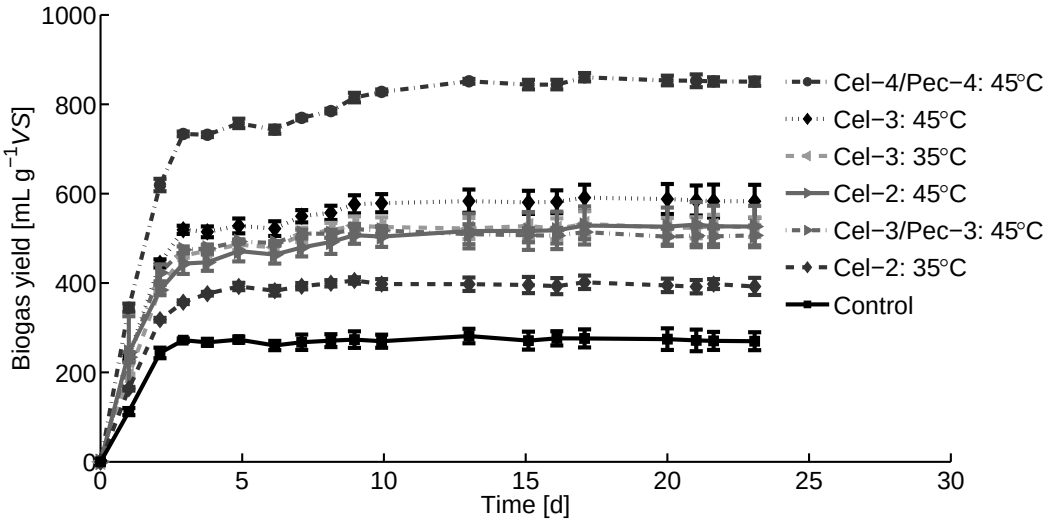


**Figure 4.21:** Biogas yield ± standard deviation from anaerobic digestion of *Chlorella vulgaris* biomass pre-treated with cellulase ('Cel', cellulase 'Onozuka R-10') and an enzyme mixture ('Cel/Pec', cellulase 'Onozuka R-10' and pectinase 'Macerozyme R-10'), at different concentrations and different incubation temperatures (35 and 45°C); anaerobic batch tests were conducted in triplicates; see Table 3.9 for a detailed description

**Table 4.12:** Specific maximum (Max) methane ($CH_4$) yield ± standard deviation derived from the specific maximum (Max) biogas yield ± standard deviation and the averaged $CH_4$ content (63.5 %, derived from Table 4.11); anaerobic batch tests were conducted in triplicates; prior to anaerobic digestion experiments, the biomass was pre-treated with cellulase ('Cel') or an enzyme mixture ('Cel/Pec', cellulase 'Onozuka R-10' and pectinase 'Macerozyme R-10') and different enzyme concentrations and incubation temperatures (35 and 45°C); batch experiments with untreated microalgae biomass served as control

| Temperature °C | Enzyme concentration [mg enzyme g$^{-1}VS$] | Symbol | Max biogas yield [mL g$^{-1}VS$] | Max $CH_4$ yield [mL $CH_4$ g$^{-1}VS$] |
|---|---|---|---|---|
| | Control | | 281.4 ± 16.2 | 178.7 ± 10.3 |
| 35 | 83.3 | Cel-2 | 405.7 ± 7.2 | 257.6 ± 4.6 |
| | 166.7 | Cel-3 | 532.3 ± 28.4 | 338.0 ± 18.0 |
| 45 | 83.3 | Cel-2 | 530.7 ± 50.5 | 337.0 ± 32.1 |
| | 166.7 | Cel-3 | 590.9 ± 29.2 | 375.2 ± 18.5 |
| 45 | 166.7 | Cel/Pec-3 | 520.6 ± 11.7 | 330.6 ± 7.4 |
| | 333.2 | Cel/Pec-4 | 860.5 ± 9.4 | 546.4 ± 6.0 |

under favorable conditions for mainly water-soluble matter. Much lower values between 30 to 60 % are typical degrees of conversion for structural organic matter such as manure (Angelidaki and Sanders, 2004). Likewise, Chen and Oswald (1998) showed that on average only 40 % of the energy in algal biomass was released by ordinary mesophilic methane fermentation. Hence, a biomass fraction of 60 % could not be converted into energy, as many of the cells and cell walls stayed intact during the digestion process.

Sialve *et al.* (2009) theoretically derived a maximum methane yield from microalgae biomass between 470 and 800 mL $CH_4$ $g^{-1}VS$ assuming a lipid content between 2 and 22 %. However, experimental data revealed a much lower realized yield between 170 and 450 mL $CH_4$ $g^{-1}VS$. The methane production of untreated microalgae biomass is hence comparable to experimental values reported from pig waste (190 mL $CH_4$ $g^{-1}VS$), wastewater sludge (230 mL $CH_4$ $g^{-1}VS$), and clover grass (340 mL $CH_4$ $g^{-1}VS$) (Hansen *et al.*, 1998; Sosnowski *et al.*, 2003; Amon *et al.*, 2007). Ehimen *et al.* (2013) found that only 39 to 60 % of the theoretical methane yield of the algae *Rhizoclonium* was achieved using different pre-treatments. The methane recovery without pre-treatments was even lower indicating the large methane potential of algae that typically remains unused. When comparing methane yields of various substrates, it is therefore helpful to determine experimental and theoretical values to unveil the hidden potential of the respective substrate. Table 4.13 shows a compilation of theoretically derived and experimentally realised data from various untreated feedstocks.

The large differences between experimental and theoretical methane yields for microalgae biomass illustrate the room for improvements, between 77 and 283 % according to the data shown in Table 4.13. Here, the mean biogas and methane yields of untreated *Chlorella vulgaris* were 343 ± 37 mL $g^{-1}VS$ and 218 ± 24 mL $CH_4$ $g^{-1}VS$, respectively, using the results of n = 10 experiments and an average $CH_4$ content of 63.5 %. The theoretical maximum of the methane yield is, thus, up to 260 % higher (630 to 790 mL $CH_4$ $g^{-1}VS$) than the experimental yield.

**Effect of thermal pre-treatments** Manipulating temperature is a simple and therefore often used pre-treatment that effectively alters biomass characteristics for the anaerobic digestion process. Several authors found that increasing temperature improves the bioavailability of sewage sludge, previous to its anaerobic digestion (Haug *et al.*, 1978; Pinnekamp, 1989). Heating also increased the methane production of mixed cattle, swine manure (Mladenovska *et al.*, 2006), and pig slurry (Bonmati *et al.*, 2001). In these experiments, treatment temperatures between 135 and 180°C maximised methane yields. At higher temperatures recalcitrant or toxic compounds that inhibit the anaerobic digestion process are formed leading to falling methane yields (Müller, 2000). Chen and Oswald (1998) explained decreasing methane yields at high temperatures with the excessive volatilisation of organic compounds and/or with increased denaturation of previously available substances. Schwede *et al.* (2011) reported that exposing *Nannochlorop-*

**Table 4.13:** Experimental and theoretical methane yields [mL $CH_4$ $g^{-1}VS$] of different untreated feedstocks

| Feedstock | Experimental yield [mL $CH_4$ $g^{-1}VS$] | Reference | Theoretical yield [mL $CH_4$ $g^{-1}VS$] | Reference |
|---|---|---|---|---|
| **Terrestrial feedstock** | | | | |
| Winter rye | 360 | Petersson *et al.* (2007) | 500 | Petersson *et al.* (2007) |
| Oilseed rape | 420 | Petersson *et al.* (2007) | 539 | Petersson *et al.* (2007) |
| Maize silage | 345 | Bauer *et al.* (2009) | 442 | Bauer *et al.* (2009) |
| Wheat straw | 276 | Bauer *et al.* (2009) | 436 | Bauer *et al.* (2009) |
| Dairy cattle manure | 148 | Moller *et al.* (2004) | 469 | Moller *et al.* (2004) |
| Pig manure | 200 - 250 | Hartmann *et al.* (2000) | 400 - 450 | Hartmann *et al.* (2000) |
| **Microalgae species** | | | | |
| *Dunaliella salina* | 320 | Mussgnug *et al.* (2010) | 680 | Sialve *et al.* (2009) |
| *Chlamydomonas reinhardtii* | 390 | Mussgnug *et al.* (2010) | 690 | Sialve *et al.* (2009) |
| *Chlorella kessleri* | 220 | Mussgnug *et al.* (2010) | 630 - 800 | Sialve *et al.* (2009) |
| *Euglena gracilis* | 320 | Mussgnug *et al.* (2010) | 500 - 800 | Sialve *et al.* (2009) |
| *Scenedesmus obliquus* | 180 | Mussgnug *et al.* (2010) | 590 - 690 | Sialve *et al.* (2009) |
| *Chlorella vulgaris* | 310 - 350 | Sanchez Hernandez and Cordoba (1993) | 630 - 790 | Sialve *et al.* (2009) |

*sis salina* biomass to 100°C for 8 h increased the cell disruption and the biogas production by 58 % to 549 mL $g^{-1}VS$.

In this study, highest biogas (624 mL $g^{-1}VS$) and methane yields (396 mL $CH_4$ $g^{-1}VS$) were derived at the lowest pre-treatment temperature (35°C) and an exposure time of 24 h. The biogas yield improved by 50 % with respect to the control, but only 50 to 63 % of the theoretical value were achieved and at higher temperatures the biogas potential even decreased. In conclusion, while adequate pre-treatments can substantially improve biodegradability and thus biogas yields, narrowing the large gap between observed and theoretically achievable yields remains the central and non-trivial challenge still requiring substantial research efforts in the future.

**Influence of enzymatic pre-treatments** Previous studies demonstrated that enzymatic pre-treatment of microalgae biomass improved its fermentability (Choi *et al.*, 2010; Fu *et al.*, 2010). In a similar study with several enzymes, Ehimen *et al.* (2013) reported that pre-treating the macroalgae *Rhizoclonium* with the enzyme cellulase was most effective in improving the anaerobic digestion process. However, only 39 to 60 % of the theoretical achievable methane yield was obtained using enzymatic and mechanical pre-treatments. In the present study, the digestibility of *Chlorella vulgaris* biomass was assessed systematically by varying the concentration of different enzymes as well as the incubation temperature. Corroborating previous works, the application of enzymatic pre-treatments generally increased methane yields by 40 to 200 % compared to the control culture. Highest methane yields observed here ranged between 70 to 86 % of the theoretical maximum.

The addition of enzymes increases the biomass in the anaerobic fermentation process, which is then digested as well. Hence, observed yields probably overestimate the sole activating effect of the added enzymes. The potential bias was not quantified here, but Suárez Quiñones *et al.* (2012) explicitly addressed this effect by adding inactivated enzymes to biomass samples and comparing these samples to controls without any enzyme additions. In their study, the addition of 40 mg enzyme $g^{-1}VS$ of inactivated enzymes (an enzyme mixture including cellulase and pectinase, among others) enhanced the methane yields of different substrates to a variable degree. While the increase was moderate for maize silage (methane production increased by 7 % compared to the control), the effect was considerable for feed residue and rye grain silage and increased up to 20 % (Suárez Quiñones *et al.*, 2012). However, adding inactivated enzymes to grass silage and solid cattle manure even slightly reduced the methane production compared to the control.

While the improvements in methane yields observed in this investigation are up to 200 % higher compared to the control and an effectiveness of enzymatic pre-treatments is likely, further investigations should precisely quantify this effect.

Previous studies suggest that most of the enzymatic hydrolysis occurs in less than 24 h (Yin *et al.*, 2010; Harun *et al.*, 2011). Thus, future studies should focus on improving the incubation duration. Therefore, refining, i.e. most likely shortening, incubation times appears promising. Suárez Quiñones *et al.* (2012) reported, that enzyme treatment of substrate reached maximum values after three hours.

### 4.4.3 Digestion of residual microalgae biomass

Residual microalgae biomass from a protein extraction process was tested for its suitability as substrate for anaerobic digestion. To evaluate the viability of the process route, original algae paste, pre-treated algae paste (after bead milling), and residues after pre-treatment and extraction of soluble proteins were processed in an anaerobic digester.

#### 4.4.3.1 Results

The cumulated biogas yields indicate that all substrates achieved high yields (Figure 4.22). The untreated algae paste yielded a biogas volume of 648.2 ± 3.7 mL $g^{-1}VS$ (Table 4.14). Bead milling improved the accessibility of organic matter and increased the biogas yield by 25 % (811.8 ± 33.6 mL $g^{-1}VS$). After the soluble proteins were removed, the yield increased by 8 % compared to the untreated algae paste.

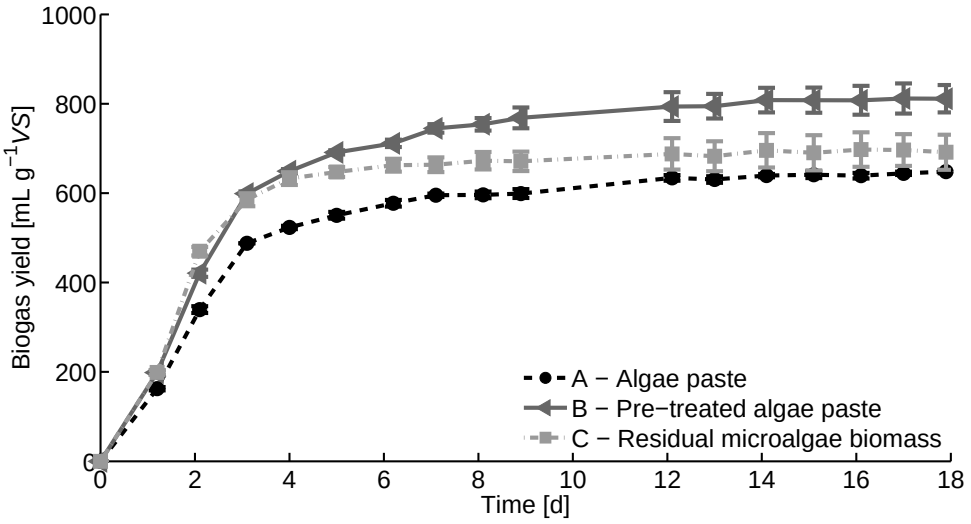


**Figure 4.22:** Cumulated biogas yields ± standard deviations from anaerobic digestion of *Chlorella vulgaris*; anaerobic batch tests were conducted in triplicates

The pre-treated algae paste (after milling) also produced the highest methane yield of all substrates (532.0 ± 22.0 mL $CH_4$ $g^{-1}VS$) (Table 4.14). Removing the supernatant including the soluble proteins slightly lowered the methane yield. The lowest methane yield was achieved by the untreated algae paste.

**Table 4.14:** Specific maximum (Max) methane ($CH_4$) yields ± standard deviations derived from the specific maximum (Max) biogas yields ± standard deviations and from averaged $CH_4$ contents; biogas yields and $CH_4$ contents originated from anaerobic digestion experiments with algae paste, pre-treated algae paste (after bead milling), and residual microalgae biomass (after milling and extraction of proteins); the anaerobic batch tests were conducted in triplicates

| Treatment | Max biogas yield [mL $g^{-1}VS$] | $CH_4$ content [%] | Max $CH_4$ yield [mL $CH_4$ $g^{-1}VS$] |
|---|---|---|---|
| A-Algae paste | 648.2 ± 3.7 | 67.0 | 434.0 ± 2.5 |
| B-Pre-treated algae paste | 811.8 ± 33.6 | 65.5 | 532.0 ± 22.0 |
| C-Residual microalgae biomass | 703.3 ± 32.5 | 67.5 | 474.4 ± 21.9 |

#### 4.4.3.2 Discussion

To recover proteins, lipids, or high-value products from microalgae biomass, various extraction processes, which typically induce physical cell disruption, have been proposed (Ehimen *et al.*, 2011; Schwenzfeier *et al.*, 2011). In most cases, the starting material for methane production is, thus, already pre-treated, rendering further pre-treatments unnecessary. A combination of the removal of high value products and the use of the residual biomass for biogas production benefits the renewable energy recovery and the recovery of nutrients. Moreover, several authors pointed out the necessity of a complete utilisation of microalgae biomass to achieve a sustainable operation (Sialve *et al.*, 2009; Mussgnug *et al.*, 2010; González-Fernández *et al.*, 2012; Acién Fernández *et al.*, 2012; Ehimen *et al.*, 2013). Although it appears to be too early to identify the most successful process route, the production of biogas seems to be the least complex one (González-Fernández *et al.*, 2012).

Supporting the results of this study, increased methane yields of residuals from an extraction step have been observed before (Mussgnug *et al.*, 2010; Ehimen *et al.*, 2009; Acién Fernández *et al.*, 2012). Here, a mildly isolated soluble protein was extracted (after Schwenzfeier *et al.* (2011)). Generally, proteins are often targeted in an extraction from microalgae biomass as they can be used as an alternative protein source or for applications in foods (Spolaore *et al.*, 2006; Schwenzfeier *et al.*, 2011). The recovery of methane from biomass residues following

soluble protein extraction is a new conversion route that has the potential to reduce costs and environmental impacts of the process.

The anaerobic digestion of untreated algae paste yielded 648 mL $g^{-1}VS$ and was much higher than in previous experiments with untreated algae (mean value: 343 mL $g^{-1}VS$; n = 10). One reason for this difference might be, that algae paste samples were frozen for several month (instead of some days) and thawed twice (instead of once). This probably led to an increased biodegradation of cell material and subsequently to a higher biogas yield during anaerobic digestion experiments.

After bead milling, 67 to 85 % of the theoretical maximum methane yield of *Chlorella* biomass was realised. This is comparable to the results of the best enzyme pre-treatment (cf. Table 4.12). After separating the supernatant, co-digestion of the residual biomass still yielded 474 mL $CH_4$ $g^{-1}VS$.

The results demonstrate the feasibility of an anaerobic digestion step with microalgae residuals after soluble protein extraction. Chemicals involved in this extraction process had no negative effect on the methane production.

# Chapter 5

# Summary and Outlook

Microalgae offer great potentials as feedstock for a variety of high-value products and biofuels owing to diverse and manipulable biochemical composition, high growth rates, and the ability to produce $CO_2$-neutral fuels on non-arable land. The production of microalgae biomass requires, however, high efforts, which have so far restricted commercial cultivations to a small high-value sector that produces mainly food supplements or ingredients for the cosmetic industry. Consequentially, commercial large-scale microalgae production facilities outdoors are still a rarity today and most scientific studies have been conducted with laboratory cultivation systems. The validity and generality of results obtained from such laboratory studies for large outdoor operating facilities are often uncertain or simply not given.

The aim of this investigation was therefore to set-up and operate an outdoor, high-density microalgae culture aerated with pure $CO_2$ as well as flue gas and to monitor, analyse, and interpret the complex biological processes that unfold in the system. In particular, the effects of a highly variable environmental forcing on productivity and stability of the cultivation system were of interest here as this variability is arguably a substantial difference between laboratory and outdoor operations. Moreover, the potential of biogas production from microalgae as a promising processing option was evaluated.

To achieve these aims, *Chlorella vulgaris*, a green microalgae, was cultivated outdoors during spring, summer, and autumn of the years 2010 and 2011 in Hamburg, Northern Germany. The pilot plant consisted of eight closed 180 L Flat-Panel-Airlift (FPA) photobioreactors (PBRs), which were aligned in two independent lines of four PBRs each allowing for parallel experiments. A semi-continuous cultivation mode (i.e. with discrete additions of nutrients and removals of algae biomass) was adopted.

This investigation advances the understanding of high-density cultivation of *Chlorella vulgaris* in closed photobioreactors under highly variable environmental conditions at a temperate location. More specifically, the research addressed important aspects and previously unresolved issues including the productivity and biochemical composition of microalgae cultivated outdoors, the effect of flue gas from a combustion process on the performance of cultivation, the nitrate

and phosphate uptake, the fate of dissolved inorganic carbon in microalgae systems, interactions between bacteria and microalgae in the non-sterile environment of a PBR, and the suitability of *Chlorella* biomass as a substrate for biogas production. The insights gained can also contribute to improve the realism and quality of environmental and economic assessments of large-scale systems.

The results of this thesis can be summarised in the following points:

1. To understand the effect of outdoor light and temperature variability, biomass productivities and biochemical parameters were monitored during May and June 2011 to provide an assessment of typical values under outdoor conditions. At biomass concentrations between 2.0 and 5.0 g $L^{-1}$, a mean productivity of 0.4 g $L^{-1}$ $d^{-1}$ was achieved, with 1.0 g $L^{-1}$ $d^{-1}$ as the maximum productivity. Mean carbon and nitrogen contents were 53.8 % and 9.1 % of biomass, respectively. A mean protein content of 43.6 % of biomass was obtained and the fatty acid content ranged between 7.5 and 8.8 % of biomass.

2. A principal component analysis of environmental parameters and productivity revealed a clear separation of temperature and light parameters and the association of productivity to the light parameters. Moreover, the relation between light availability and productivity is non-linear. It is shown that the productivity increases with light availability up to an optimum, the 'optimal population productivity'. At low light intensities (or very high biomass concentrations), the lack of light limits the productivity. At high light intensities (or very low biomass concentrations), photoinhibition or simply too low biomass for growth control the productivity. Important parameters, such as the optimal population productivity, the optimal population density, and the respective optimal light availability can be derived from this relation. These parameters are useful indicators to improve the harvest management and key for the systematic optimisation of productivities in the outdoor cultivation system. Accordingly, a productivity > 0.4 g $L^{-1}$ $d^{-1}$ can be maintained with a culture density ranging between 2.2 and 5.3 g $L^{-1}$ under the light conditions of May 2011.

3. Microalgae aerated with pure $CO_2$ and ambient air completely converted the inorganic nitrogen from the cultivation medium into biomass. Thus, an on-line measurement of dissolved inorganic nitrogen is not only a good approximation of the nutrient concentration, but may also help to estimate biomass concentration and production by simple book-keeping of dissolved inorganic nitrogen fluxes. In flue gas cultures, however, $NO_x$ existent within the flue gas is evidently assimilated by microalgae during their growth as approximately one third of the cumulated particulate organic nitrogen production cannot be explained by the consumption of inorganic nitrate originating from the medium.

4. The dissolved organic carbon released into the medium by microalgae, an often ignored carbon flux that can be metabolised by bacteria, ranged from 0.6 to 4.0 % of $TOC$ here. While bacteria are often considered as contaminants, results presented here suggest that the relationship between bacteria and microalgae is rather one of commensalism under optimal microalgae growth conditions. In other words, bacterial growth depends on organic carbon released by microalgae, which are not harmed from the relationship. In phases of healthy microalgae growth, the bacterial biomass fraction is low and comprises less than 1 % of the total specific biomass volume.

5. Flue gas from a natural gas driven combined heat and power plant had no negative effect on microalgae growth and composition when compared to a culture supplied with pure $CO_2$ over a cultivation period of about 25 days. The average $CO_2$ biofixation rate was 0.85 g $CO_2$ $L^{-1}$ $d^{-1}$. Furthermore, $NO_x$ from flue gas did not inhibit the growth of microalgae. Up to 600 ppm $NO_x$ were measured in the flue gas and tolerated by *Chlorella vulgaris*. Between 28.6 and 48.3 % of the flue gas $NO_x$ was transferred into suspension and potentially available as a nitrogen source for *Chlorella vulgaris*.

6. In the anaerobic fermentation experiments, the methane potential of untreated *Chlorella* yielded only approximately 28 to 34 % of the theoretical maximum yield, illustrating the hidden potential of this biogas substrate. Thermal and enzymatic pre-treatments increased this yield up to 50 to 63 % and 70 to 86 %, respectively, of the theoretical maximum. However, the effect of the additional enzyme biomass on the biogas yield is uncertain and requires further research. Moreover, microalgae residues from a soluble protein extraction process revealed 8 % higher biogas yields than the untreated algae biomass. Chemicals involved in this extraction process had no negative effect on the methane production. Thus, anaerobic fermentation is a suitable final step in a biorefinery primarily aiming at the extraction of soluble proteins.

In conclusion, this study demonstrated that a successful outdoor cultivation of *Chlorella vulgaris* in closed photobioreactors under the highly variable, and sometimes harsh, climatic conditions of Northern Germany from spring to autumn is possible. A year-round operation would clearly necessitate an additional heat source (e.g. waste heat). Encouraging results from the application of flue gas as a carbon and nitrogen source for microalgae growth indicate the potential for the integration of such devices into conventional power plants. Further research efforts are required to better constrain the balances of inorganic and organic carbon and nitrogen and the assimilation pathways, i.e. the formation of organic compounds from inorganic compounds present in the flue gas or other gas mixtures. Currently, the measuring techniques are inconsistent and thus hampering objective comparisons between studies.

Moreover, this study makes a valuable contribution to the often neglected topic of microalgae-bacteria interactions in cultivation systems by indicating commensalism under typical cultivation conditions. Nevertheless, more research is needed to better understand the conditions under which the relation between microalgae-bacteria breaks up, often to the disadvantage of the microalgae.

Future investigations should focus on further improving the stability and productivity of outdoor microalgae cultivation systems. Adopting and refining the 'productivity/light availability' system introduced in this study might be a good starting point. This system allows for the determination of key growth parameters at which the culture exhibits stable growth, such as optimal population productivity, optimal light availability, and optimal population density. Combining this method with high-precision weather forecasts to optimise the harvest management is a promising topic that deserves further investigation.

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