Effects of oxygen concentration on the growth of *Nannochloropsis* sp.

Sayam Raso

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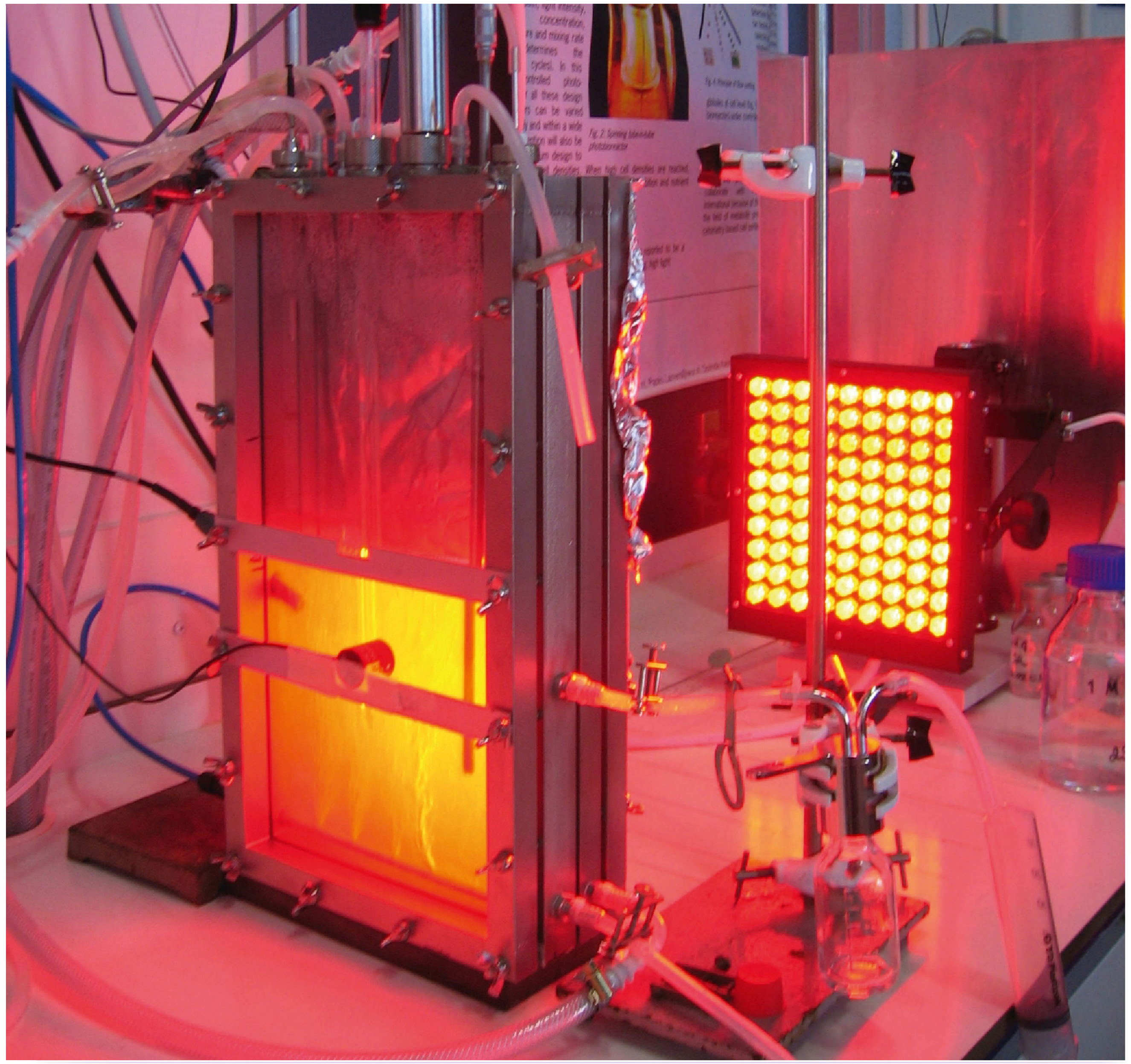
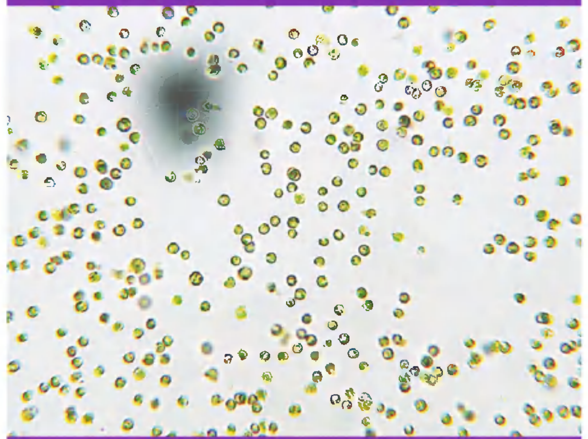


Sayam Raso

Invitation

You are cordially invited to attend the public defence of my PhD thesis entitled

Effects of oxygen concentration on the growth of *Nannochloropsis* sp.



The defence will take place on Wednesday, October the 9th 2013 at 11 a.m.,

Aula of Wageningen University,
Generaal Foulkesweg 1ª, Wageningen.

Directly afterwards, there will be a reception at the Aula.

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Effects of oxygen concentration on the growth of *Nannochloropsis* sp.

Sayam Raso

Thesis
submitted in fulfillment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
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Chapter 1

General Introduction and Thesis outline

INTRODUCTION

The decline in fish feed-stocks has resulted in an increasing interest in aquaculture which is subsequently associated with an increasing demand for aquaculture feed. Especially, microalgal feed sources rich in polyunsaturated fatty acids (PUFAs) are needed for the production of high quality feed (Patil et al. 2007). Due to the relatively high content of essential fatty acids (20:5n-3, EPA and 22:6n-3, DHA), many microalgae have already been widely exploited in aquaculture farming (Table 1).

The marine microalgae *Nannochloropsis* sp. is currently cultivated in aquaculture hatcheries to specifically provide live algal feed for juvenile shell fish and other marine animals (Volkman et al. 1993). This species is commercially produced for its relatively high eicosapentaenoic acid (EPA) content (Sukenik 1991; Sukenik 1997) and creates an attractive EPA source for human food supplements. It is also regarded as one of the promising alternative sources for biofuels (Rodolfi et al. 2008).

Cultivation of *Nannochloropsis* for fish feed

For years, *Nannochloropsis* sp. has been extensively commercially cultured in open raceway ponds and plastic bags (Chisti 2008; Zmora and Richmond 2004). Open culture systems are inexpensive, easy to operate, and require relatively low energy input. However, the quality and stability of the produced fish feed cannot be easily controlled, and there is an increased risk of bacterial contamination or sudden collapse of the culture due to predating protozoa (e.g. *Paraphysomona* sp.), being contaminated by bacteria (e.g. *Cytophaga* sp.), and competition of *cyanobacteria* and other microalgae (Zmora & Richmond 2004). The limitations of open systems can be eliminated by employing closed photobioreactors with controlled culture conditions, e.g., in closed tubular photobioreactors (Zittelli et al. 1999; Tredici et al. 2010). In closed systems, protection against contaminating microorganisms is easier, and much higher productivities can be achieved (Molina Grima et al. 2001; Wijffels et al. 2011). In addition, control of principal process parameters can be achieved to ensure an optimization of the cultivation process and to guarantee good quality. However, high investment and operation costs due to high energy input for mixing and degassing continue to limit commercial large-scale application of these closed systems for algal feed production.

Table 1 Types of microalgae used in aquaculture application.

Microalgae	Application	Remarks	References
Isochrysis aff. galbana	Aquaculture hatcheries	Hatcheries	Volkman et al. 1989; Brown 1991
Nannochloropsis sp.	Aquaculture hatcheries	Hatcheries	Richmond and Cheng-Wu 2001
Tetraselmis suicica	Aquaculture		Volkman et al. 1989; Brown 1991
Chaetoceros gracilis	Aquaculture		Volkman et al. 1989; Brown 1991
Dunaliella salina	β-carotene		Larmer et al. 2008
Chlorella sp.	Live feed enrichment	Aquaculture	Borowitzka 1999; Skaramuca 1998
Haematococcus sp.	Astaxanthin	Colour for Salmon	Beneman 1992;
Phaeodactylum tricornutum	Shellfish, live feed	Hatcheries	Patil et al. 2007; Brown 1991
Skeletonema costatum	Shellfish, live feed	Nurseries	Blnchemain and Grizeau 1999; Brown 1991
Pavlova lutheri	Shellfish, live feed	hatcheries	Patil et al. 2007; Volkman et al. 1989

While mixing in closed photobioreactors is mainly required to provide the microalgae with a sufficient amount of light, CO_2 and nutrients, the degassing is required in order to remove the oxygen produced during photosynthesis as high oxygen levels resulting from photorespiration and photoinhibition inhibit the growth of the microalgae (McMinn et al. 2005; Weissman and Goebel 1988).

Effects of oxygen on productivity

Algal biomass production in closed photobioreactors is accompanied by the accumulation of oxygen formed during photosynthesis (Figure 1). Oxygen concentrations above air saturation are known to inhibit the growth of microalgae. Previous studies have indicated that up to 400% air saturation causes a reduction in the growth rate of *Spirulina platensis* to 36% (Marquez et al. 1995). Molina Grima et al. (2001) reported that, in an outdoor tubular photobioreactor, accumulation of oxygen causes extremely lower productivity during hours of peak irradiance, however, photosynthetic activity of the cells recovered after a few hours, when the algae were exposed to lower light intensity. The synergetic effects of light and oxygen on algal growth have been extensively reported (Ugwu et al. 2007; Vonshak et al. 1996), however, far less literature is available on the effect of oxygen on algal growth as such. Very few studies have been published on the effect of oxygen at constant low light intensity, and these indicated that the algal growth rate significantly decreased due to photorespiration effects (Kliphuis et al. 2011; Sousa et al. 2012).

The term “photorespiration” is related to oxygen production that occurs during the dark reaction. The high oxygen concentration in the medium causes a high ratio in concentration of O_2 versus CO_2 within the chloroplast where the Rubisco enzyme is located. At these high

Textbox 1 Photosynthetic and Photorespiration Pathway

In the photosynthesis reaction (Figure 1, left hand side), water is oxidized and creates O_2. The energy of 8 photons is captured and exploited during the Calvin Cycle to fix one mole of CO_2 in the dark reaction with ribulose-biphosphate (RuBP) to form two molecules 1.3 PGA. This reaction of carbon dioxide with RuBP is termed carboxylation. During each cycle, 1 mol glyceraldehyde 3-phosphate (G3P) is formed at the expense of 3 mol of inorganic C in the form of CO_2 (as well as 9 mol ATP and 6 mol NADPH). G3P can be further converted into biomass.

If oxygen is present in sufficiently high concentrations, the photosynthesis switches to photorespiration (Figure 1, right hand side). Rubisco employs O_2 as a reactant with RuBP to produce 2-phosphoglycolate. The reaction of oxygen with RuBP at the Rubisco active site is termed oxygenation. Within each cycle, RuBP reacts with 1 mole of O_2 to form one molecule of 3PGA and one molecule of 2-phosphoglycolate. Phosphoglycolate is subsequently dephosphorylated to glycolate which cannot be further metabolized in the chloroplast. It is, therefore, excreted and enters the peroxisome. Glycolate in the peroxisome is first oxidized to glyoxylate with H_2O as a byproduct. Glyoxylate is transaminated to glycine. The glycine is transported to the mitochondrion where two molecules form one molecule of serine with CO_2 as a byproduct. The serine is transported back to the peroxisome where it is converted to OH-pyruvate which is subsequently converted to glycerate. At the expense of one ATP, this glycerate is converted into 3PGA. Overall, more ATP energy is expended during photorespitration in comparison with photosynthesis in order to form sugar.

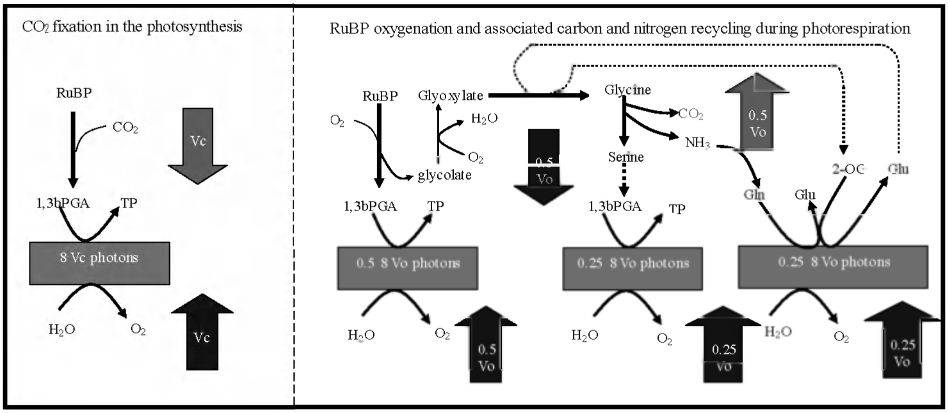

Figure 1 Photosynthesis and photorespiration pathways within photoautotrophic microalgae; left hand side: photosynthesis; right hand side: photorespiration (Raso et al. 2012).

oxygen conditions, Rubisco not only fixes carbon dioxide through photosynthesis but also catalyzes the reaction with oxygen in the photorespiration. As a result, less carbon dioxide is fixed and this ultimately results in loss of carbon from the organism (Figure 1). The relative rates of the carboxylase and oxygenase reactions depend on the concentrations of the two gaseous compounds at the Rubisco site and on the kinetic properties of the enzyme, which differs among microalgae (Bunt 1971; Pope 1975; Lívanský 1996).

With higher irradiance and high oxygen concentrations, the decrease in algal growth is not only caused by photorespiration but also by photoinhibition and photo-oxidative damage. Photoinhibition is associated with the formation of oxygen radicals and other reactive oxygen species (ROS) that damage the photosynthesis apparatus while photo-oxidative damage is associated with reactions of the highly reactive singlet oxygen[1].

In all light conditions, the light-harvesting complexes of Photosystem I (PSI) and Photosystem II (PSII) capture the light. At PSII, electrons are generated from water oxygenation while forming oxygen. The electrons are transported via the electron-transport chain to PSI where NADPH is formed. In this chain of reactions, light energy is transformed into chemical energy in the form of ATP.

With high light conditions, the photosynthetic apparatus cannot deal with the surplus of electrons generated at PSII, and the electrons react with oxygen to form superoxide radicals (O_2^-). This type of reactive oxygen species (ROS) can be addressed within the water-water cycle (Foyer et al. 2009, Figure 2). With the assistance of superoxide dismutase, the oxygen radicals disproportionate to H_2O_2 and O_2. The H_2O_2 is again a reactive oxygen species, but it is rapidly detoxified to water by the ascorbate peroxidase. With relatively low light conditions, the enzymes involved in the water-water cycle are able to cope with the surplus of

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[1] In literature, photo-inhibition as well as photo-oxidative damage are often employed to describe the combined effects of oxygen radicals and oxygen singlet molecules on algal growth. In this text, the term photo-inhibition is assigned to the damage by oxygen radical formation at PSII while photo-oxidative damage is associated with the damage by singlet oxygen formation.

electrons. With higher light conditions, however, the enzymes can no longer cope with the electron flow, and ROS begins to accumulate and damage cellular components such as protein D1 in the PSII center. This results in severe cell damage and causes growth inhibition.

Textbox 2 Water-water cycle

The water-water cycle is a defense mechanism that safeguards microalgae against reactive oxygen species formed at high light intensity. At photosystem II (PSII), water within the chloroplast is oxidized by light energy to form O_2 while electrons are released. In a series of subsequent enzyme reactions, oxygen absorbs the excessive electrons delivered to Photosystem I and forms superoxide radicals O_2^-. The reactive oxygen radicals are subsequently transformed into hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). The formed H_2O_2 is changed into water during a reaction in which the thylakoid-bound ascorbate peroxidase (tAPX) participates (Perelman et al. 2006; Ishikawa et al. 2009). This enzyme reduces Ascorbate (AsA) to monodehydroascorbate (MDA). The ferodoxin (Fd) finally catalyzes the regeneration of AsA.

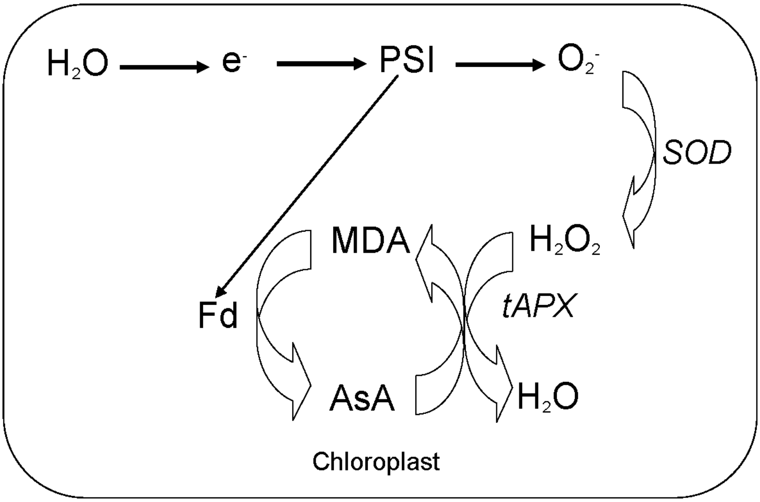

Figure 2 The water-water cycle occurs in the chloroplast of microalgae (adapted from Mittler 2002).

At high light intensity not only the formation of oxygen radicals, but also the formation of highly reactive singlet oxygen results in photo-oxidative damage (Figure 3). As the singlet oxygen rapidly transfers its energy to molecules within its vicinity, it particularly damages the venerable chlorophyll in the chloroplast (Asada 2006).

Textbox 3 Formation of singlet oxygen

During high light conditions, the visible light is absorbed by the photosynthetic reaction center in the chlorophyll (P680) which is in its ground state (S_o electronic state), and this results in excitation of P680 to its singlet form ($^1P680^*$) (S_1 electronic state). When the light conditions are too high, singlet-singlet energy transfer to neighboring chlorophyll is limited and triplet P680 ($^3P680^*$) is formed. The triplet excitation energy can be transferred to carotene (Car) or to molecular oxygen in its original ground state ($^3O_2$) subsequently forming highly reactive singlet oxygen ($^1O_2$). The singlet oxygen can be partly extinguished during the transfer to the triplet carotenoids ($^3Car^*$) but, in the event of a surplus of energy supply, the oxygen singlet will cause photo-oxidative damage, particularly to the photosynthetic active pigments within its vicinity.

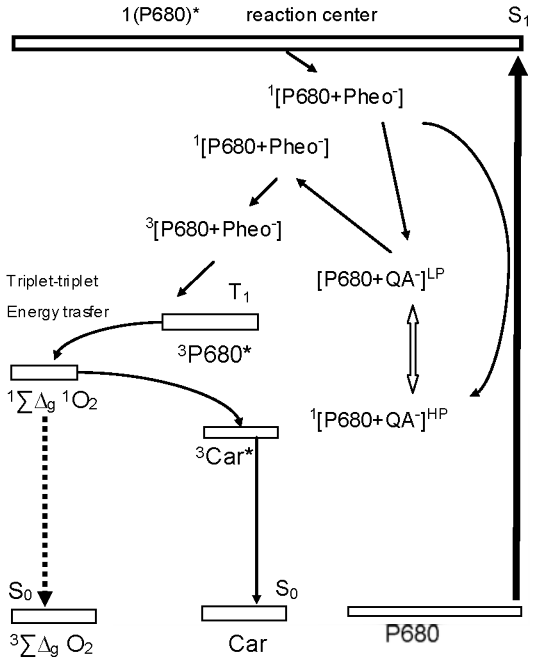

Figure 3 Formation of highly reactive singlet oxygen at high irradiance (adapted from Pospišil 2011).

Protection mechanisms against oxygen

Microalgae are able to contend with high oxygen concentrations at high light intensity to a certain extent. The first protective mechanism is the carbon concentrating mechanism (CCM) that is present in *Nannochloropsis* sp. (Sukenik et al. 1997; Tchernov et al. 1008). These microalgas have gradually developed an active carbon concentrating mechanism (CCM) in order to maintain a high CO_2 concentration at the Rubisco active site, and during evolution, also developed a form of Rubisco with low oxygenase affinity. They are able to accumulate CO2 around the Rubisco site whereby the increase in the local CO2 concentration suppresses photorespiration. This ensures that these algae do not suffer significant photorespiratory losses at moderate elevations in oxygen concentrations (up to two times air saturation).

As a second defense strategy at high irradiance, the microalgae can address the reactive oxygen species (ROS) in the water-water cycle (Mehler reaction). Enzymes in the water-water cycle, such as superoxide dismutase, hydrogen peroxidase, etc., catalyze a chain of reactions in which water reacts with oxygen radicals, thereby reducing protein damage due to ROS that is formed from the reactive radicals (see detail in chapter 3 and Textbox 2). Non-enzymatic mechanisms are also present to protect cells from damage by oxygen radicals as well as singlet oxygen. This protection involves carotenoid pigments for physical quenching, and they form the first line of protection at high irradiance. The carotenoids are able to absorb excess light energy, thereby protecting the chlorophyll.

In addition, other general protective mechanisms are reported such as an increase of cell size, cell density, and changes in biomass composition.The polyunsaturated fatty acids (PUFAs) are often specifically mentioned as beiing involved in the algal protection as they are able to scavenge the excessive electrons during high light conditions (Okuyama et al. 2007). It is uncertain, however, if all of these mechanisms are stimulated by light alone or if oxygen concentrations in the medium also stimulate these protective mechanisms.

Effects of oxygen on composition of *Nannochloropsis* sp.

Nannochloropsis sp. is a microalga that accumulates lipids with a relatively high content of 16:0, 16:1, and 20:5 fatty acids (Volkman et al. 1993); various light intensities are known to affect fatty acid compositions. At low light conditions, the lipid synthesis is mainly focused on membrane lipid synthesis, resulting in higher levels of elongated and desaturated fatty acids such as 20:5 (Sukenik et al. 1989). It was reported that, during low light conditions (35 μmol m^{-2} s^{-1}), *Nannochloropsis* sp. mainly synthesizes fatty acids primarily for esterification into glycerol-based membrane lipids, and these lipids constitute about 5-20% of dry weight and possess a relatively elevated content of PUFA (0.464 pg $cell^{-1}$; 3.71 mg l^{-1} day^{-1}) (Sukenik et al. 1989). At high light intensity and stress conditions for growth, many algae alter their lipid biosynthetic pathways toward the formation and accumulation of neutral lipids (20-50% of dry weight) mainly in the form of triacylglycerol (TAG) with a high content of saturated fatty acids. High oxygen levels have been suggested as such a stress factor and are expected to induce and alter the lipid content in microalgae and its fatty acid composition (Torzillo et al. 1998), thereby influencing the feed quality for shellfish or fish larvae.

It is clear that oxygen negatively effects the growth of *Nannochloropsis* sp, but it is not yet clear to what extent oxygen affects the growth and how it influences the biomass composition and EPA content. It is therefore crucial to investigate the effects of oxygen concentrations on both growth and biomass composition. As light is involved in all reactions of oxygen, the effect of oxygen at various light intensities should be investigated.

To be able to obtain high productivity in large scale closed photobioreactor, oxygen must be removed to limit growth inhibition. However, there has been no clear indication yet to what extent the accumulating oxygen is inhibiting the growth of *Nannochloropsis* sp. when cultured in closed photobioreactors and how this accumulating oxygen affects the productivity, and quality of the produced feed in terms of EPA content.

Objectives

The overall research goal of this thesis is to investigate the effect of oxygen on growth and fatty acid composition of *Nannochloropsis* sp. and ascertain how the quality of the microalgae in terms of EPA content is affected employing various light conditions.

Outline of thesis

In chapter 2, the effects of oxygen concentrations on the growth of *Nannochloropsis* sp. at low irradiance was studied in a flat panel photobioreactor operated in turbidostat mode. Based on theoretical quantum yield data on isolated Rubisco reported for *Nannochloropsis* sp., the effect of oxygen concentration on the rate of carboxylation (V_c) and the rate of oxygenation (V_o) could be predicted, and the growth rate based on quantum yield could be calculated for each applied oxygen concentration. The experimental results confirmed that, with the applied light conditions, the growth rate ceased at 300% air saturation.

Light is employed as an energy source and affects photosynthesis efficiency (PE) but also causes photo-oxidative damage. The synergistic effects of oxygen and light on the growth of *Nannochoropsis* sp. have been investigated and are described in Chapter 3. Also, in this chapter, the growth rate was determined at sub-saturating, saturating, and over-saturating light conditions. Chapter 4 addresses the defense mechanisms inherent in *Nannochloropsis* sp. These mechanisms are known to protect the algae against oxidative stress such as formation of reactive oxygen species (ROS) at a high irradiance level and high oxygen concentration. The effect of oxygen on the activity of superoxide dismutase, the formation of pigments and antioxidants such as fatty acid composition (EPA) inside the algal cells, was investigated. In Chapter 5, the effects of dynamic changes in oxygen concentrations that occur along the tube and between two compartments of a closed tubular photobioreactor system, the light harvesting unit, and the degasser tank have been studied, and the effect on the overall growth rate and quality of the algae was determined. In Chapter 6, the oxygen concentration is discussed as a key parameter in the design of large-scale microalgal production systems in order to control increased productivity of *Nannochloropsis* as well as to control its quality as fish feed.

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Chapter 2

Effects of oxygen concentration on the growth of *Nannochloropsis* sp. at low light intensity

The contents of this chapter have been published as: Raso S, Van Genugten B, Vermuë M, Wijffels RH. (2012) Effect of oxygen concentration on growth of *Nannochloropsis* sp. at low light intensity, J Appl Phycol, 24: 863-871.

ABSTRACT In large-scale micro-algal production in tubular photobioreactors the build-up of O_2 along the tubes is one of the major bottlenecks to obtain high productivities. Oxygen inhibits growth as it competes with carbon dioxide for the Rubisco enzyme involved in the CO_2 fixation to generate biomass. The effect of oxygen on the growth of *Nannochloropsis* sp. was experimentally determined in a fully controlled flat-panel photobioreactor operated in turbidostat mode employing an incident photon-flux density (PFD) of 100 μmol photons m^{-2} s^{-1} and with the oxygen concentration as the sole variable parameter. The dissolved oxygen concentration (DO) was varied from 20 to 250% air saturation. Results indicated that there was no clear effect of oxygen concentration on the specific growth rate (mean average of 0.48±0.40 day^{-1}) when increasing oxygen concentration from 20% to 75% air saturation. Upon further increasing the oxygen concentration, however, a linear decrease in specific growth rate was observed ranging from 0.48±0.04 day^{-1}at a DO concentration of 75% air saturation to 0.18±0.01 day^{-1} at 250% air saturation. *In-vitro* data on isolated Rubisco were employed to predict the quantum yield at various oxygen concentrations in the medium. The predicted decrease in quantum yield corresponds well with the observed decrease that was measured *in-vivo*. These results indicate that the effect of oxygen on the growth of *Nannochloropsis* sp. with low light intensity is only due to competitive inhibition of the Rubisco enzyme. At these sub-saturating light conditions, the presence of high concentrations of oxygen in the medium induced slightly higher carotenoid content, but the increased levels of this protective anti-oxidant did not diminish the growth-inhibiting effects of oxygen on the Rubisco.

KEYWORDS: Oxygen concentration, tubular photobioreactor, photorespiration, *Nannochloropsi*s sp.

INTRODUCTION

For large-scale cultivation of *Nannochloropsis* sp., tubular photobioreactor systems have been developed (Cheng-Wu et al. 2001; Richmond and Cheng-Wu 2001; Zittelli et al. 1999; Zittelli et al. 2003; Zou and Richmond 1999). In these photobioreactors, however, accumulation of oxygen is one of the major issues since it inhibits the growth of microalgae (Molina Grima et al. 2001; Livansky 1996; Ogawa et al. 1980; Pulz 2001; Vonshak et al. 1996; Weissman and Goebel 1988). There are two mechanisms largely responsible for the deleterious effects on the growth of microalgae: the competitive effect of O_2 on Rubisco (photorespiration) and photoinhibition which causes cell damage of photosystem II with the generation of reactive oxygen species (ROS) (Ohnishi et al. 2005). Photorespiration only occurs during the dark reaction of photosynthesis and is, therefore, independent of the exploited light conditions, while the ROS are formed if the algae are exposed to excessive amounts of light in the light reaction of photosynthesis. In this study, we will focus solely on the photorespiration reaction as we work with a low incident light irradiation of 100 μmol photons m^{-2} s^{-1} where photo-inhibition effects in *Nannochloropsis* sp. are negligible (Ogawa et al. 1980; Sukenik 1991, 1997).

The inhibiting effects of oxygen on the *in vivo* productivity are often referred to in literature (Kitaya et al. 2005; Livansky 1996; McMinn et al. 2005; Ogawa et al. 1980; Ugwu et al. 2007; Kliphuis et al. 2011). In almost none of the reported studies, however, was the effect of O_2 measured as an independent parameter in controlled conditions of light intensity, pH, temperature, and biomass concentration. The effects on growth, therefore, often reflect a combined effect of light and oxygen on photosynthesis. In addition, until recently, quantification of the direct effect of oxygen on *in-vivo* growth and productivity of

Nannochloropsis sp. has never been performed. In our study, the effects of oxygen levels on the growth of *Nannochloropsis* sp. have been determined in a flat-panel photobioreactor operating in turbidostat mode to ensure controlled conditions of sub-saturating light, pH, temperature, and mixing. These *in-vivo* growth rates of *Nannochloropsis* sp. at the various oxygen concentrations are compared with the growth rates and predicted employing the *in-vitro* kinetic parameters as determined by Tchernov et al. (2008) for isolated Rubisco from this species.

MATERIALS AND METHODS

Organisms and medium for pre-cultivation and growth

Nannochloropsis sp. (CCAP 211/78) was pre-cultured in an incubator (100 rpm), provided with a light-dark cycle of 18:6 h, a light intensity of 41 μmol photons m^{-2} s^{-1} at pH 7.8, and 2% of CO_2 enriched air for one week prior to the inoculation of the flat-panel photobioreactor. Modified Zou medium was utilized to pre-culture and cultivate *Nannochloropsis* sp.(Table 1). Stock solutions of the trace elements Fe-EDTA as well as the phosphate buffer solution were autoclaved separately and maintained at 4ºC. The medium was filter-sterilized using a 0.2 μm filter and, after adding the other stock solutions (phosphate, iron-EDTA), the pH was adjusted using NaOH or HCl.

Cultivation of the algae in the flat-panel photobioreactor

To measure the effect of oxygen with controlled light and culture conditions, a flat-panel photobioreactor with a culture volume of 1.5 L and a light path of 2.8 cm was employed. Continuous and homogeneous illumination at wavelength 637 nm was supplied by a Red-

Light Emitting Diode (PSI-Photon Systems Instruments, Kolackova 39, 621 00 Brno, Czech Republic). Following inoculation, the algae were allowed to grow batch-wise for nine days at controlled pH (pH 7.8) and temperature (25°C) and a total gas flow rate of 0.5 L L^{-1} min^{-1} using an average incident photon flux density (PFD) of 100 µmol photons m^{-2} s^{-1}. The light intensity at the back of the flat-panel photobioreactor was specified at 12 µmol photons m^{-2} s^{-1} using a light sensor (LICOR-190, Czech Republic company) that was connected to a control unit.

Table 1 Composition of the modified Zou medium exploited for the growth of *Nannochloropsis* sp.

Compounds	**Concentration (mM)**
NaCl	419.38
MgSO4	54.81
$MgCl_2.6H_2O$	27.55
$CaCl_2.2H_2O$	10.20
$CO(NH_2)$	19.98
$NaHCO_3$	0.476
EDTA-FE(III)-Na-salt	3.70
K_2HPO_4	57.41
KH_2PO_4	14.70
Trace elements	
Na_2EDTA	0.26
$CuSO_4.5H_20$	0.03
$ZnSO_4.7H_20$	0.04
$CoCl_2.2H_20$	0.03
$MnCl_2.4H_20$	0.46
$Na_2MoO_4.2H_20$	0.13

This unit controlled the medium in- and outflow pumps to set the turbidity. When the light intensity at the back achieved 12 µmol photons m^{-2} s^{-1}, the photobioreactor was switched into turbidostat mode and operated for 6 days with a DO concentration corresponding to 100% air saturation in order to allow the cells to adjust to turbidostat operation conditions and continuous illumination. A photosynthesis versus irradiance (PI) curve was measured for cells that had adapted to these light conditions and had achieved an oxygen concentration of 100% air saturation. Net photosynthesis rate was measured as oxygen evolution in a Liquid-Phase Oxygen Electrode Chamber (Hansatech, DW3) equipped with an oxygen control electrode unit (Hansatech, Oxy-lab) at 25°C. Light is supplied with a Red LED light source (Hansatech LH36/2R) with wave length of 637 nm. The PI-curve (Figure 1) confirmed that the algae indeed experience sub-saturating light conditions at photon flux densities < 200 μmol photons m^{-2} s^{-1}.

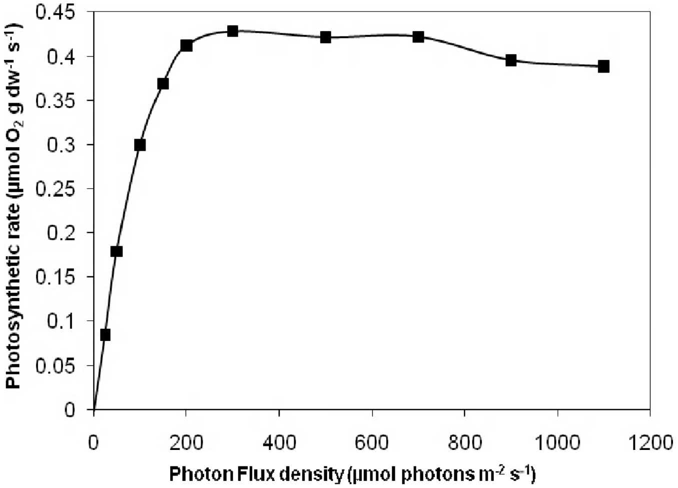

Figure 1 PI curve of *Nannochloropsis* sp. grown at 100% air saturation and adapted to an incident light intensity of 100 μmol photons m^{-2} s^{-1}.

During all runs, possible foam formation was controlled with an automatic foam control unit. Prior to entering the flat-panel photobioreactor, the incoming gas flow was humidified, and the outgoing gas flow was fed through a condenser to prevent loss of the medium by evaporation. The medium bottle was connected with a bag containing CO_2 to compensate for possible pH fluctuations during the turbidostat run. In the medium, 20 mM ureum was employed as a nitrogen source to ensure no pH fluctuations occurred due to utilization of the N-source.

The oxygen concentration during the turbidostat operation was monitored by a DO sensor (Applisens DO sensor 15 mm) and controlled by adjusting the concentration of oxygen in the incoming gas flow while the total gas flow (O_2 mixed with pure N_2 and pure CO_2) was maintained at a constant of 0.5 L L^{-1} min^{-1}. It was subsequently set for 4 days at 75%, 50%, 25% air saturation and with no additional O_2. A two-day time interval was specified to allow for adaptation of the algae to the new O_2 concentration before samples were taken. A second and third run were performed to test the effects of 125%, 150%, 175%, 200%, and 250% air saturation, respectively. Following the run at 250%, the oxygen concentration was increased to 300%. After one day at 300% air saturation, however, the cells became pale, indicating that they had lost chlorophyll, and a sudden increase in the OD_{680}/OD_{530} ratio indicated that contamination of the culture occurred with bacteria.

Off-line measurements

Biomass concentration and dry weight

Samples of approximately 100 mL were obtained aseptically each day during the subsequent turbidostat runs for off-line measurement of the biomass concentration and determination of the dry weight. The algal biomass concentration was monitored by the optical density at 530 nm (OD_{530}) and 680 nm (OD_{680}) utilizing a spectrophotometer (Spectronic ® Genesis^{Tm} spectrophotometer, UK). The ratio OD_{680}/OD_{530} was determined as an indicator for the amount of chlorophyll per amount of biomass and as an indicator for potential contamination. The biomass dry weight concentration was determined by filtering samples (in triplicate) of the algal culture using pre-weight glass microfiber filters (Whatman, Maidston, United Kingdom). The algae were washed three times with ammonium formate (0.5 M) to remove the precipitated salts and other non-organic substances and subsequently dried at 105ºC for 24 h, cooled overnight over silica gel in a desiccator, and weighed. In addition, the cell numbers and the distribution in the cell size was determined in a Coulter Counter (Beckman Multisizer 3^{Tm} Coulter Counter, USA) to reveal potential effects of oxygen on the cell size and cell density.

Elemental composition and pigment content

To assess possible effects of oxygen on the algal biomass composition, the elemental composition is determined for the algal cells growing at 100% and 300% air saturation. Liquid samples were centrifuged for 10 minutes at a 1750 rfc. The pellets were washed three times with de-mineralized water and re-suspended and centrifuged again. The remaining algae pellets were stored at -20°C. The frozen pellets were weighed, freeze dried and ground to a fine powder before further analyzing the biomass composition and moisture content. The C, H, and N contents of the freeze-dried samples of algae growing at 100% oxygen and 300% oxygen were measured at 925°C in the oven of an elemental analyzer (EA 1110, ThermoQuest CE Instruments). Ash content was determined by burning the freeze-dried algae samples in an oven at 550°C in order to oxidize all organic material, leaving the ash residue. From these determinations, the oxygen (O) content and the subsequent elemental composition of the algae could be calculated (C, H, O, N and ash) (Duboc et al. 1999).

Possible changes in pigment content due to increased oxygen concentrations were monitored by measuring the absorbance PAR spectrum (400-700 nm) in a spectrometer (Avants Fibre Optic Spectrometer, the Netherlands). Based on the spectra, the ratio of carotenoid and chlorophyll content in the samples were determined from the absorption at 490 nm and the absorption at 680 nm.

Specific growth rate and quantum yield

During the turbidostat operation, the specific growth rate of the algae (μ) was determined from the dilution rate whereby the medium output per liquid volume of reactor was weighed and recorded at one minute intervals.

The specific growth rate was used to calculate the biomass yield ($Y_{x,ph}$) on light energy which is defined as the amount of biomass (grams dry weight) produced per mole photon absorbed in the PAR range:

$$Y_{x,ph} = \mu * V * C_x / A * \Delta PFD \qquad (1)$$

where μ is the specific growth rate (day^{-1}), V is the culture volume of the photobioreactor (m^3), C_x is the biomass concentration (g dw m^{-3}), A is the illuminated area (m^2), and ΔPFD (moles photons m^{-2} day^{-1}) is the difference in incident photon flux density (PFD) and the PFD measured at the back of the flat panel.

The quantum yield (QY) (mol CO_2 fixed/mole photon absorbed) is defined as

$$QY = (Y_{x,ph} / M_{wx}) * Y_{CO2,x} \qquad (2)$$

where M_{wx} is the molar mass of the biomass (g dw/C-mole biomass) and $Y_{CO2,x}$ is the amount of carbon dioxide required to form one C-mole of biomass (mole CO_2/C-mole biomass).

RESULTS

Growth of *Nannochloropsis* sp. at controlled oxygen conditions

A typical growth curve of *Nannochloropsis* sp. during a run in the 1.5 L flat panel photobioreactor at different dissolved oxygen (DO) concentration is presented in Figure 2. It indicates that the algal cells were allowed to grow batch-wise until an optical density OD_{530} of 1.5 was achieved after which the turbidostat operation began, and the cells were allowed to adjust for 3 days to the set oxygen concentration of 100% air saturation, Off-line samples were then obtained during 3 subsequent days. This procedure was repeated for each tested oxygen concentration. The OD_{680}/OD_{530} ratio remained constant with the tested oxygen concentrations which indicates that the algal cells were healthy and no contamination by other micro-organisms had occurred.

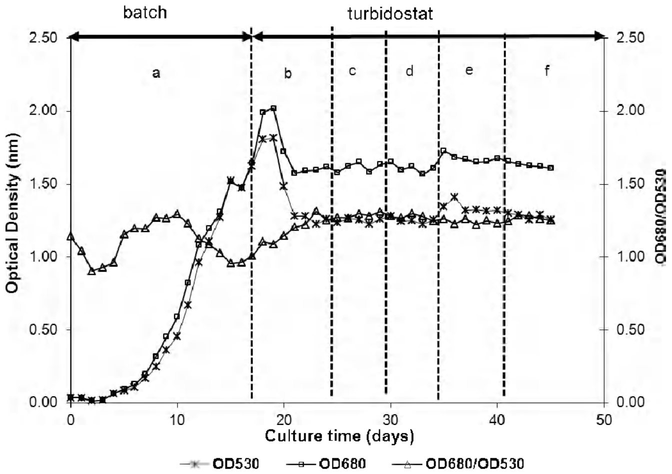

Figure 2 A typical growth curve of *Nannochloropsis* sp. during the batch run in 1.5 L flat panel photobioreactor followed by the turbidostat run at dissolved oxygen (DO) concentration ranging from 20-100 % air saturation; a= batch, b= 100% DO, c= 75% DO, d= 50% DO, e= 25% DO and f= 20% DO.

Figure 3 depicts the specific growth rate of the algae growing in sub-saturating light conditions with varying oxygen concentration from 20-250% air saturation. Upon increasing oxygen concentration from 20% up to 75% air saturation, no clear effect of oxygen on the specific growth rate was observed, and the growth rate demonstrated a mean average value of 0.48±0.04 day^{-1}. Upon further increase of the oxygen concentration, the specific growth rate of *Nannochloropsis* sp. linearly decreases ($R^2 = 0.95$) from 0.49±0.10 day^{-1} at DO of 75% air saturation to 0.18±0.01 day^{-1} at DO of 250% air saturation. This decrease in specific growth rate can be solely attributed to the effects of oxygen since all other conditions in the culture were kept constant.

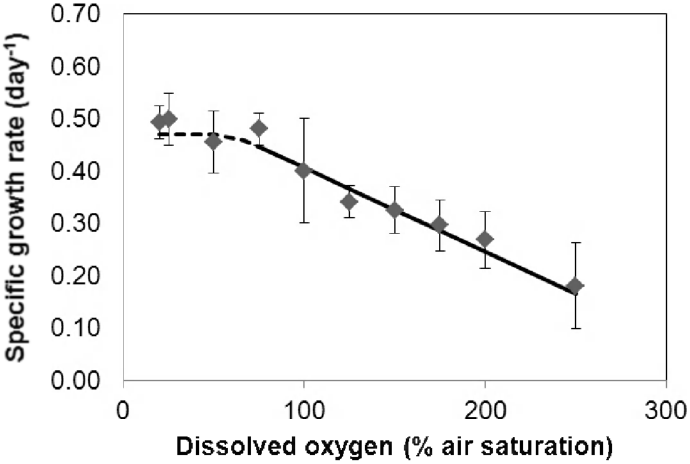

Figure 3 Effects of oxygen concentration on the specific growth rate (day^{-1}) of *Nannochloropsis* sp. at incident light intensity of 100 μmol photons m^{-2} s^{-1}.

Effects of oxygen concentration on cell concentration and cell composition

Off-line measurements of the cell concentration (cells mL^{-1}) and the cell dry weight (g L^{-1}) exhibited a minimal increase (Table 2) while total absorption area remained virtually constant at 0.10 m^2 L^{-1} in all experiments. This indicates that the biomass content per cell might have changed, but no change in light absorption characteristics occurred as an effect of modifying oxygen concentrations. The measurements of the ratio in the carotenoid and chlorophyll content of the algae with varied oxygen concentration again demonstrated little effect on increased oxygen levels (Figure 4).

Table 2 Off-line measurements of oxygen effects on the growth of *Nannochloropsis* sp. at various oxygen concentration.

Dissolved oxygen (% air saturation)	**Cell concentration (cells mL^{-1})** x10^7	**Cell dry weight (g L^{-1})**	**Total absorption area (m^2 L^{-1})**
20	7.78±0.20	0.46±0.01	
25	7.85±0.12	0.48±0.02	
50	7.40±0.22	0.45±0.00	
75	7.75±0.46	0.46±0.01	
100	7.64±0.36	0.44±0.01	0.08±0.01
125	7.82±0.15	0.54±0.12	0.11±0.03
150	7.57±0.72	0.66±0.13	0.11±0.04
175	9.08±0.29	0.57±0.05	0.10±0.01
200	8.05±0.44	0.60±0.02	0.09±0.01
250	9.48±0.45	0.65±0.05	

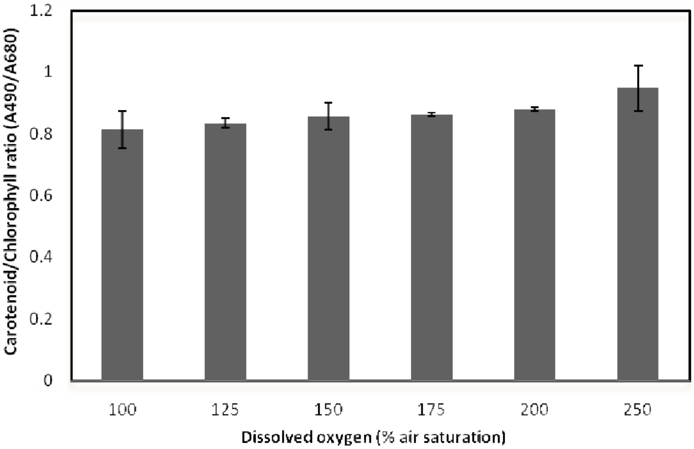

Figure 4 Effects of oxygen concentration on the ratio between carotenoids and chlorophyll of *Nannochloropsis* sp. cultured in incident light intensity of 100 μmol photons m^{-2} s^{-1}. The absorption at 490 nm (A490) is used as a measure for the carotenoid, and the absorption at 678 nm (A678) is employed as measure (n=6) for the chlorophyll.

The ratio remained constant up to an oxygen concentration of 200% air saturation, and only a slightly higher ratio was observed at 250% air saturation. The elemental composition of the algae also remained virtually the same (Table 3); at 100% O_2, the elemental composition was $CH_{1.77}\ O_{0.24}N_{0.15}$ and, at 300% O_2, it was $CH_{1.72}\ O_{0.24}N_{0.15}$.

Table 3 Measured elemental composition of *Nannochloropsis* sp. cultivated in incident light intensity of 100 μmol photons m^{-2} s^{-1} at DO concentration of 100% and 300% air saturation corrected for 3.63% ash and 3.38% moisture content.

Oxygen concentration (% air saturation)	Elemental composition (% w/w)			
	C	H	O	N
100	60.97	9.05	19.42	10.56
300	61.29	8.87	19.35	10.48

DISCUSSION

Quantification of the effects of oxygen concentration on specific growth rate

Upon increasing the oxygen concentration from 20 to 75% air saturation, the specific growth rate seems only minimally affected by the oxygen concentration (Figure 3). However, the specific growth rate decreases linearly with oxygen concentrations ranging from 75% to 250% air saturation. Similar effects of oxygen on the specific growth rate of microalgae were observed for *Phaeodactylum tricornutum* cultured in indoor conditions (Molina Grima et al. 2001), *Spirulina platensis* (Vonshak et al. 1996), *Chlorella sorokiniana* (Ugwu et al. 2007), *Euglena gracilis* (Kitaya et al. 2005) and for microalgae present in the sea ice community (McMinn et al. 2005). In most of these studies, the increase in oxygen concentration is claimed to be the major cause for inhibition of the growth of the microalgae, but the effects of oxygen where mainly measured while concurrently changing the light, temperature, and other culture conditions. Among the varying medium conditions, the pH is especially one of the parameters that directly affects the specific growth rate. In our experiment, the pH was controlled through the CO_2 supply, and this resulted in a constant concentration of CO_2 in the medium in all applied oxygen concentrations. This was also the case in the recent study of Kliphuis et al. (2011) who studied the effects of oxygen on the growth of *Chlamydomonas reinhardtii*. They measured the growth rate with two different oxygen concentrations with oxygen as the sole varying medium parameter and also reported that the growth rate decreased with increasing oxygen concentration (Kliphuis et al. 2011).

The specific growth rates of *Nannochloropsis* sp measured with oxygen concentrations below 100% exhibit a relatively high standard deviation, and it is not clear if the specific growth rate is indeed constant or decreasing with the oxygen concentration in these applied low O_2

concentrations. One should realize that the *in vivo* growth data always reflect the balance of the physical and many cellular processes in which oxygen participates. Especially with low oxygen concentrations in the medium, it is well feasible that the local concentration of oxygen at the Rubisco site is relatively high due to the photosynthesis activity of the cells. If the controlled oxygen concentration in the medium is lower than the oxygen concentration at the Rubisco site, the oxygen formed will diffuse from the cells into the medium which will result in a constant specific growth rate with the lower oxygen concentrations. With higher oxygen concentrations in the medium, the oxygen will diffuse from the high concentration through the cell membrane to the Rubisco site with the relative lower oxygen concentration. At the Rubisco site, the local CO_2 and O_2 concentrations will determine the balance between carboxylation (photosynthesis) and oxygenation (photorespiration).

Our results with oxygen concentrations above 75% air saturation prove that, indeed, an increase in oxygen concentration with constant CO_2 concentration in the medium leads to a linear decrease ($R^2 = 0.95$) of the specific growth rate of *Nannochloropsis* sp. with low light intensity. An increase in oxygen concentration from 75% to 250% air saturation results in a decrease of the specific growth rate of the algae from 0.48 ± 0.04 day^{-1} to 0.18 ± 0.01 day^{-1}.

Prediction of the ratio between carboxylation and oxygenation using *in-vitro* data

In the dark reaction of the photosynthesis, the enzyme Rubisco demonstrates carboxylase activity and is, therefore, responsible for fixation of CO_2. The carboxylase activity of Rubisco is in accordance with Michaelis-Menten kinetics, taking into account the competitive inhibition by oxygen (Tchernov et al. 2008).

$$v_c = \frac{v_{c\max} * C_{CO2}}{C_{CO2} + K_{mc}(1 + \frac{C_{O2}}{K_{mo}})} \quad (\mu\text{mol } CO_2 \text{ min}^{-1} \text{ mg}^{-1} \text{ protein})$$

where v_c and v_{cmax} represent the activity and maximum activity of the carboxylation reaction, respectively, while K_{mc} and K_{mo} represent the Michaelis-Menten constant of the Rubisco for CO_2 and O_2. In the presence of oxygen, the enzyme Rubisco exhibits oxygenase activity which suffers from competitive inhibition by CO_2. The oxygenase activity can be described with

$$v_o = \frac{v_{o\max} * C_{O2}}{C_{O2} + K_{mo}(1 + \frac{C_{CO2}}{K_{mc}})} \quad (\mu\text{mol } O_2 \text{ min}^{-1} \text{ mg}^{-1} \text{ protein})$$

where v_o and v_{omax} are the activity and maximum activity of the oxygenation reaction.

The rates of the carboxylation and oxygenation reactions can be combined to deduce that the ratio between both reactions linearly decreases with the ratio of CO_2 and O_2 concentration in the vicinity of the Rubisco enzyme:

$$\frac{v_c}{v_o} = \frac{v_{c\max} * K_{mo}}{v_{o\max} * K_{mc}} * \frac{C_{CO2}}{C_{O2}}$$

Tchernov et al. (2008) obtained K_{mc} values of 7-10 µM and a v_{cmax} between 0.85-1 µmol CO_2 min^{-1} mg^{-1} protein from *in-vitro* experiments on isolated Rubisco a K_{mo} value about 1 mM O_2 and a v_{omax} value of 3.9 µmol O_2 min^{-1} mg^{-1}. With these kinetic parameters determined for isolated *Nannochloropsis* sp. and the measured concentrations of dissolved oxygen and carbon dioxide in the medium, we were able to predict the ratio between carboxylation and oxygenation reaction.

The carbon dioxide concentration in the medium was calculated employing Henry's Law with the Henry coefficient of 161.3 MPa for pure CO_2 in water of 298K. The solubility of CO_2 in the aqueous modified Zou medium (Table 1) corresponds with 0.37% CO_2 in the gas phase that was used to maintain the pH in the medium at 7.8. The pKa value for CO_2 in water at 298K is adjusted according to Stumm and Morgan (1995) for the temperature and for the ions that were present in the modified Zou medium resulting in pKa = 6.16 which indicated that most of the CO_2 was present in the form of HCO_3^- . The resulting CO_2 concentration in the liquid was 97.7 µM. The oxygen concentration in the medium was also calculated using Henry's Law with the Henry coefficient for O_2 corrected for temperature and effects of solutes in the Zou medium (van 't Riet and Tramper 1991). The concentration of O_2 in the modified Zou medium at 100% air saturation was calculated to be 24.3 mM.

Prediction of the quantum yield from the ratio between carboxylation and oxygenation

The ratio between the carboxylation and oxygenation was used to predict the maximum possible quantum yield (QY) at the different oxygen concentrations employed in the experiments. Figure 5 provides an overview of the reactions involved in photorespiration. In this figure, the number of CO_2 molecules converted in the carboxylation reaction catalyzed by the Rubisco enzyme is depicted by v_c. Each mole of CO_2 used to convert the RuBP generates one mole of oxygen and, if v_c CO_2 molecules are fixed in this carboxylation reaction, $8*v_c$ photons/s are required. In the absence of oxygen (Figure 5, left-hand side), no photorespiration, theoretically, occurs and, at maximum, 8 photons are required to fix one mole of carbon dioxide while one mole of oxygen is released (Foyer et al. 2009; Tredici 2010; Wingler et al. 2009). The number of CO_2 molecules fixed (or oxygen evolved) per photon can be defined as the quantum yield. The theoretical maximum quantum yield is thus 0.125 mol CO_2 fixed (or oxygen evolved) mol^{-1} photons absorbed.

In the photorespiration pathway (Figure 5, right-hand side), the oxygenation reaction of oxygen competes with the carboxylation reaction of carbon dioxide with ribulose-biphosphate (RuBP).

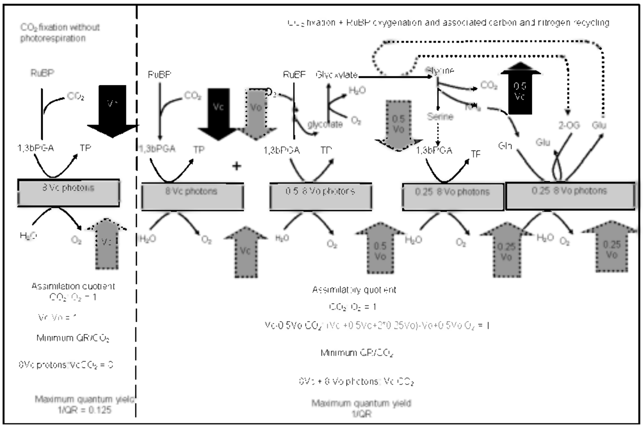

Figure 5 Photorespiration pathway of microalgae; left hand side: photosynthesis without photorespiration; right hand side: photorespiration (adapted from Foyer et al. 2009).

The number of oxygen molecules expended per second in the oxygenation reaction of the RuBP is depicted by v_o. Upon reacting with oxygen, one mole of RuBP will be converted into one mole of 1,3bPGA and one mole of glycolate. The 1,3bPGA re-enters the Calvin Cycle generating $0.5v_o$ moles of O_2 at the expense of $0.5v_o*8$ moles photons per second while the glycolate moves from the chloroplast into the peroxisome where it is converted into glyoxylate at the expense of $0.5v_o$ moles photons per second. The glyoxylate is converted into serine and, during this reaction, $0.5v_o$ molecules of CO_2 are generated and ammonium will form. In a series of reactions, 1,3bPGA is eventually produced which returns to the Calvin Cycle and results in formation of $0.25v_o$ of O_2. Another $0.25v_o$ mole/s is generated as a result of the formation of glutamate. Both reactions will expend $0.25v_o*8$ photons/s.

The oxygenation reaction of v_o moles of RuBP per second with v_o moles of O_2 overall yields 0.5 v_o moles of CO_2 and 0.5 v_o moles of O_2 per second and requires a total of 8 v_o moles photon s^{-1}.

The minimum quantum requirement (RQ) of the combined oxygenation and carboxylation reaction during photorespiration is $8\ v_c + 8\ v_o$ moles photon/s per v_c moles of CO_2 fixed s^{-1}. If the ratio between the carboxylation v_c and the oxygenation reaction v_o are known, the minimum quantum requirement can be predicted as well as the maximum quantum yield (1/RQ).

Comparison of in-vivo quantum yields with predicted yields using in-vitro data

In Figure 6, the quantum yield calculated from the *in-vivo* experiments is compared with the quantum yield that is predicted based on the *in-vitro* experimental data. These results demonstrate that, in both cases, the quantum yield decreases linearly with an increase in oxygen concentration in the medium. The calculated values for the *in-vivo* quantum yields are lower than the predicted values for isolated Rubisco, as expected. The model predicts the maximum possible photosynthetic yield and assumes that all absorbed photons are directed to the photosynthesis and photorespiration reactions. For *in-vivo*, a portion of the light is scattered by the cells and lost by diffusion. The resulting ATP and NADPH are not only exploited for growth but also for maintenance, therefore, much lower quantum yields are actually found. In addition, the model is based on data from *in-vitro* experiments on the Rubisco so it predicts the oxygenase and carboxylase activity of the Rubisco utilizing the dissolved O_2 and CO_2 concentration in the medium. The surplus of ions in the hypertonic cellular medium affects the solubility of CO_2 more than it affects the solubility of oxygen

(Stumm and Morgan 1995; Van 't Riet and Tramper 1991). The CO_2/O_2 ratio at the Rubisco in the living cells will thus be lower than in the medium, resulting in a lower ratio between the carboxylation/oxygenation and subsequent lower *in-vivo* quantum yields.

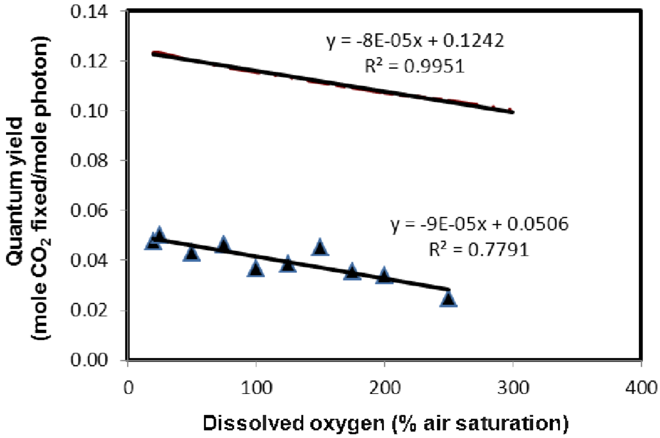

Figure 6 The effect of oxygen concentration on *in vivo* the quantum yield of *Nannochloropsis* sp. measured with incident light intensity of 100 μmol photons m^{-2} s^{-1} and compared with predicted quantum yields based on *in-vitro* data on the kinetics for carboxylation and oxygenation of Rubisco isolated from *Nannochloropsis* sp. (Tchernov et al. 2008).

To compare the observed quantum yields QY (mole of CO_2 fixed per mole of absorbed photons) with reported quantum yields of *Nannochloropsis* sp (Chini Zitelli et al. 1999), they have also been expressed in gram biomass obtained per mol photons in the PAR range in which the value of QY is multiplied by the average value of the measured molecular weight of the biomass ($CH_{1.75}O_{0.24}N_{0.15}$) of 19.7 g/C-mol (Table 2) and the theoretical yield of biomass on carbon dioxide. The elemental balance over the growth reaction of the algae indicates that a maximum of 1.08 mole of CO_2 can be fixed per C-mole biomass if ureum is exploited as the N-source for growth. The values range from 0.92 g/mol with an oxygen concentration of 20% air saturation to 0.45 g/mol at 250% air saturation. These values are comparable with the values obtained for *Nannochloropsis* sp. in outdoor tubular photobioreactors with oxygen concentrations above 100% air saturation, which range from 0.46–0.74 g/mol (Chini Zitelli et al. 1999).

The decrease in quantum yields with increasing oxygen concentration is similar to the decrease that is predicted based on the selectivity of the isolated Rubisco. The slope of both curves is virtually the same ($8*10^{-5}$ for the predicted values versus $9*10^{-5}$ for the measured values). The similarity in slope strongly indicates that oxygen mainly affects the carboxylase and oxygenase activity of Rubisco in these sub-saturating light conditions.

This linear decrease in quantum yield is also observed for *Phaeodactylum tricornutum* (Molina Grima 2001), for *Spirulina platensis* (Vonshak et al. 1996), *Chlorella sorokiniana* (Ugwu et al. 2007), and for *Euglena gracilis* (Kitaya et al. 2005) growing in controlled sub-saturating light conditions at oxygen concentrations beginning at 100% air saturation. The decrease in the quantum yield of *Spirulina platensis* (Vonshak et al. 1996) and *Chlorella sorokiniana* (Ugwu et al. 2007) proved to be accompanied by a gradual decrease in the ratio between variable and maximum fluorescence. This indicates that higher oxygen concentrations cause destruction of the chlorophyll via photo-oxidative inhibition. Similar effects are also observed in our experiments but only at an oxygen concentration of 250% air saturation. At this concentration, the carotene/chlorophyll ratio increases (Figure 4) indicating that a surplus of carotenoids is formed or that the chlorophyll is damaged at higher oxygen concentrations. The observed higher cell density and cell dry weight (Table 3) indicate extra formation of carotenoids to protect the cells against photo-oxidative damage of the chlorophyll

CONCLUSION

The effects of oxygen on the growth of *Nannochloropsis* sp in low irradiance have been determined in controlled culture and light conditions. An increase of the oxygen concentration from 20% to 75% air saturation exhibited no obvious effect of oxygen concentration on the specific growth rate. A linear decline in the specific growth rate and quantum yield was found when oxygen concentration increased from 75% to 250% air saturation. Our finding indicate that, with the employed sub-saturating light conditions, the oxygen as such mainly affects the carboxylation/oxygenation ratio of the Rubisco and only minimally influences the growth rate *c.q.* quantum yield through other photo-oxidative stress reactions.

To improve the productivity of *Nannochloropsis* sp. in large-scale, closed tubular photobioreactors, the oxygen level must be controlled. As light and O_2 simultaneously influence the photosynthesis, we need to further investigate synergetic effects of high irradiance and oxygen on growth for optimization of productivity in the closed photobioreactors.

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NOMENCLATURE

v_c	carboxylation rate
v_{cmax}	Maximum velocities of carboxylation
K_{mc}	Michaelis-Menten constant for carbon
v_o	oxygenation rate
v_{omax}	Maximum velocities of oxygenation
K_{mo}	Michaelis-Menten constant for oxygen
QR	Quantum requirement (moles photons/moles CO_2 fixed)
QY	Quantum yield (moles CO_2 fixed/moles photons absorbed)
μ	specific growth rate (day^{-1})
PFD	photon flux density (moles photons/m^2/day)
V	Culture volume (m^3)
$Y_{x,ph}$	photosynthetic yield (g dw/mole photons)
A	Areal illumination (m^2)
C_x	biomass concentration (g dw/m^3)

ABBREVIATIONS

DO	Dissolved oxygen (% air saturation)
ROS	Reactive oxygen species
LED	light emitting diode
OD	optical density

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Chapter 3

Synergistic effects of oxygen concentrations and light on the growth of *Nannochloropsis* sp.

The contents of this chapter have been submitted for publication as: Raso S, Vermuë M, Wijffels RH. Synergistic effects of oxygen concentrations and light on the growth of *Nannochloropsis* sp.

ABSTRACT The synergistic effects of oxygen and light on the growth of *Nannochloropsis* sp. were investigated in fully-controlled culture conditions in a flat-panel photobioreactor operated in turbidostat mode. Three different incident light intensities were employed: sub-saturating light of 100 µmol photons m^{-2} s^{-1}; near-saturating light of 400; and over-saturating light of 624 µmol photons m^{-2} s^{-1} with varying dissolved oxygen (DO) concentrations above air saturation. In all light intensities, a decrease in specific growth rate (day^{-1}) was observed with increasing DO concentrations. In sub-saturating light intensity, a linear decrease in the specific growth rate from 0.40±0.10 to 0.18±0.01 day^{-1} was found with DO concentrations ranging from 100-250% air saturation. A sharp decrease in the specific growth rate from 0.86±0.20 to 0.35±0.10 day^{-1} followed by a gradual decrease to 0.28±0.08 day^{-1} was detected in near-saturating light intensity with DO concentrations ranging from 100% to 400% air saturation. A similar pattern in the effect on oxygen concentration was observed in over-saturating light intensity; a sharp decrease followed by a gradual decrease in the specific growth rate from 0.37±0.06 to 0.17±0.02 day^{-1} with oxygen concentrations ranging from 100% to 175% air saturation. Our results indicate that an oxygen concentration above 100% air saturation inhibits growth of *Nannochloropsis* in all light intensities but that the effects of higher oxygen concentration are far less than the direct effect of modifying the light intensity. In low light intensity, the decrease in growth coinciding with increased oxygen concentration is a direct effect of oxygen on photorespiration. In higher light intensities, the combined effect of oxygen and light result in photo-inhibition, but an increase of the oxygen concentration only mildly affects the growth rate in those elevated light intensities. Our observations clearly demonstrate that optimal productivity of *Nannochloropsis sp.* in outdoor mass cultivation will be achieved if the oxygen concentration is maintained close to 100% air saturation but that it is more important to cultivate the algae in saturating light intensity to achieve high productivity.

KEYWORDS: O_2 accumulation, photobioreactor, *Nannochloropsis* sp., specific growth rate, photorespiration, photoinhibition

INTRODUCTION

Nannochloropsis sp. is an oleaginous microalga containing relatively large amounts of long-chain poly-unsaturated fatty acid, eicosahexaenoic acid (EPA) which makes this microalga an important source for EPA as a dietary supplement for human food (Bajpai and Bajpai 1993; Doughman et al. 2007). Additionally, due to its high lipid content, the microalgae is regarded a potential candidate for biofuel production (Rodolfi et al. 2008; Schenk et al. 2008; Pal et al. 2011). So far, *Nannochloropsis* has principally been cultured for fish larvae in aquaculture (Renaud et al. 1991), and a significant portion of the production is accomplished on a small scale in plastic bags or bubble columns (Raven and Sargeloos 1996). For large-scale cultivation of phototrophic microalgae, closed photobioreactors are often suggested (Janssen et al. 2003; Merchuk and Wu 2003; Molina Grima 2001; Pulz 2001; Zittelli et al. 1999), and the cultivation of *Nannochloropsis* sp in tubular photobioreactors, in particular, has been extensively studied (Cheng-Wu et al. 2001; Richmond and Cheng-Wu 2001; Zittelli et al. 2003; Zou and Richmond 1999). In these enclosed photobioreactors, it is easy to control culture conditions to maintain high productivity and to guarantee consistent quality of the product.

One of the major issues when employing closed photobioreactors, however, is the build-up of oxygen that is formed during the photosynthesis reactions. With high oxygen concentration, the Rubisco enzyme involved in photosynthesis not only binds carbon dioxide but also binds oxygen in the photo-respiration. With an increase in oxygen concentration, the rate of photo-respiration will increase and, as a result, the biomass productivity will decrease (Livansky 1996; Vonshak et al. 1996; Weissman and Goebel 1988; Yokota and Kitaoka 1987; Raso et al. 2011). Since the photo-respiration occurs in the dark reaction of the photosynthesis, it is expected to be the predominant mechanism involved in inhibition by oxygen in low irradiance (Raso et al. 2012).

In increasing light intensity, photo-inhibition will occur in addition to photorespiration. During photo-inhibition, the excess of oxygen generated at Photosystem II (PSII) in the light reaction of the photosynthesis results in formation of toxic reactive oxygen species such as oxygen radicals and H_2O_2 (Asada 2000; Asada 2006). In addition, photo-activation of triplet chlorophyll ($^3P_{680}^*$) occurs in these high irradiances which leads to a reaction from oxygen to form singlet oxygen, which is identified as one of the most harmful reactive oxygen species targeting the photosynthetic machinery (Triantaohylidès and Havaux 2009).

The algal cells can protect themselves against photo-inhibition as well as photo-activation effects by either channeling the excess electrons through the water-water cycle or with the reaction of the formed oxygen singlet with anti-oxidants such as carotenes, tocopherol, and unsaturated fatty acids which are present in the chloroplast (Triantaohylidès and Havaux 2009) and thus prevent the accumulation of ROS inside the algal cells. If the water-water cycle, however, is not able to handle the excess flow of electrons and the formed singlet oxygen cannot be addressed with sufficient speed, the ROS will accumulate and damage PSII, resulting in severe photo-inhibition effects on the growth of the algae (Asada 2000; Kitaya et al. 2005; Vonshak et al. 1996).

Although the synergistic inhibiting effects of oxygen and light on microalgal productivity are often referred to within literature (Marquez et al. 1995; Vonshak et al. 1996; Ogawa et al. 1980; Kromkamp et al. 2009; Molina Grima et al. 2001), the effect of oxygen has been only minimally investigated independently in different light intensities. This makes it impossible to predict to what extent build-up of oxygen concentration in the medium will affect the growth and productivity of the *Nannochloropsis* sp. in outdoor, closed photo-bioreactors where the solar irradiance varies throughout the day with low irradiance at dawn and dusk and often over-saturating light conditions around noon. In this paper, we describe the quantitative effects of oxygen on the growth of *Nannochloropsis* sp. in three different light intensities with fully controlled culture conditions in a flat-panel photobioreactor operated in turbidostat mode. The results will reveal the effect of oxygen build-up in closed photobioreactors and can be exploited to re-design these photobioreactor systems in order to optimize productivity of *Nannochloropsis* sp. for large-scale outdoor production.

MATERIALS AND METHODS

Organisms and medium

Nannochloropsis sp. (CCAP 211/78) was pre-cultured in an incubator (100 rpm), provided with a light-dark cycle of 18:6 h and a light intensity of 41 µmol photons m^{-2} s^{-1} in a modified Zou medium (Table 1) at pH 7.8 and with 2% of CO_2 enriched air. Stock solutions of the trace elements, Fe-EDTA, as well as the phosphate buffer solution were autoclaved separately and stored in the refrigerator at 4°C. The medium was filter-sterilized utilizing a 0.2 µm filter and, after adding the other stock solutions (phosphate, Fe-EDTA), the pH was adjusted to 7.8 using 0.5 M NaOH.

PI- curve to define light regime conditions

A PI-curve (photosynthetic activity versus irradiance curve) was measured to determine the irradiance levels at which *Nannochloropsis* sp. experience sub-saturating, near-saturating, and over-saturating light conditions. Cells were pre-cultured in the flat-panel photobioreactor at an average incident photon flux density (PFD) of 100 µmol photons m^{-2} s^{-1} and with a dissolved oxygen concentration of 100% air saturation. A sample of 3 mL at cell density of 0.17 g dw mL^{-1} was transferred from the photobioreactor into a Biological Oxygen Monitor (BOM), and the oxygen evolution rate (µmol g^{-1} s^{-1}) of the cells was monitored employing an oxygen sensor (Hansatech DW3) in light intensities ranging from 25 up to 1100 µmol photons m^{-2} s^{-1} using a red-LED light source (Hansatech LH36/2R).

Table 1 Composition of the modified Zou medium exploited for growth of *Nannochloropsis* sp. in different incident light intensities (100, 400 and 624 µmol photons m^{-2} s^{-1}).

Compounds	Concentration (mM)
NaCl	419.38
$MgSO_4$	54.81
$MgCl_2.6H_2O$	27.55
$CaCl_2.2H_2O$	10.20
$CO(NH_2)$Urea	19.98
$NaHCO_3$	0.48
Fe-EDTA	
EDTA-FE(III)-Na-salt	3.70
K_2HPO_4	57.41
KH_2PO_4	14.70
Trace element	
Na_2EDTA	0.26
$CuSO_4.5H_20$	0.03
$ZnSO_4.7H_20$	0.04
$CoCl_2.2H_20$	0.03
$MnCl_2.4H_20$	0.46
$Na_2MoO_4.2H_20$	0.13

Growth of *Nannochloropsis* sp. in the flat-panel photobioreactor

The synergistic effects of high oxygen concentrations and light on the growth of *Nannochloropsis* sp. were determined at controlled culture conditions using a flat-panel photobioreactor with a culture volume V_l of 1.5 L and a light path d of 2.8 cm. The medium bottle was connected with a bag containing CO_2 to compensate for possible pH fluctuations during the turbidostat operation. The 20 mM urea was used as a nitrogen source in the medium to prevent pH fluctuations due to utilization of the N-source.

Following inoculation, the algae were grown batch-wise for nine days in controlled culture conditions at pH 7.8 by CO_2 supply via the gas flow and a specified temperature of 25°C and a total gas flow rate of 0.5 $L L^{-1} min^{-1}$ using an average incident photon flux density (PFD) of 100, 400, and 624 μmol photons m^{-2} s^{-1} provided by continuous and homogeneous illumination with red-LED with a wave length of 637 nm (PSI-Photon Systems Instruments, Czech Republic) after which the flat-panel photobioreactor was switched to turbidostat mode.

According to Lambert-Beer, the light intensity at the back $I(z)$ is related to the incident light intensity I_{wall}, (μmol photons m^{-2} s^{-1}), the absorption coefficient α (m^2 kg^{-1}), the biomass concentration C_x (kg m^{-3}), and the light path z (m).

$$I(z) = I_{wall} \cdot e^{-\alpha C_x z} \qquad (\mu mol\ photons\ m^{-2}\ s^{-1}) \qquad (1)$$

The ratio between the incident light intensity and the light intensity at the back was kept constant for all experiments to ensure the same light gradients in each turbidostat run. In the event of an incident light intensity of 100, 400 and 624 μmol photons m^{-2} s^{-1}, this means that the light intensity at the back of the flat-panel photobioreactor was specified at 12, 48, and 75 μmol photons m^{-2} s^{-1} , respectively, resulting in a constant total absorption area $\alpha * C_x$ of 75.7 m^2 m^{-3} in all light intensities. The light at the back was monitored with a light sensor (LICOR-190, Czech Republic) connected to a control unit that controlled the medium in- and outflow pumps to set the turbidity of the culture. The average light intensity I_{ave} (μmol photons m^{-2} s^{-1}) experienced by the algal culture was calculated employing:

$$I_{ave} = \frac{\int_0^d I(z)dz}{d} \qquad (\mu mol\ photons\ m^{-2}\ s^{-1}) \qquad (2)$$

and was equal to 42, 168, and 262 μmol photons m^{-2} s^{-1}at sub-saturating, near-saturating, and over-saturating light intensity conditions, respectively.

Possible foam formation was controlled by an automatic foam control unit. Prior to entering the flat panel photobioreactor, the incoming gas flow was humidified and the outgoing gas flow was fed through a condenser to prevent losses of the medium. During the turbidostat operation, the on-line measured flow of biomass output was recorded by weighing the medium outflow (F_{out}), and the specific growth rate (μ) of the cells was determined from the dilution rate

($D = F_{out}/V_{1} = \mu$).

Off-line measurements

Biomass and growth monitoring

After the batch-wise growth and following each adjustment of the external oxygen concentration with an additional 50% air saturation, the culture was allowed to stabilize for a minimum of 2 days before the specific growth rate (day^{-1}) of the cells was determined and samples were withdrawn for off-line measurements.

To measure the biomass concentration, the number of cells, the cell dry weight, and the absorbance PAR spectrum, samples of approximately 100 mL were withdrawn from the photobioreactor each day during the subsequent turbidostat runs. The biomass concentration was monitored by the optical density at 530 nm (OD_{530}) and chlorophyll content at 680 nm (OD_{680}) in a spectrophotometer (Spectronic ® Genesis^{Tm} Spectrophotometer, UK). The ratio OD_{680}/OD_{530} was used as an indicator for the amount of evident chlorophyll per amount of biomass: a ratio above 1.0 indicates healthy and non-contaminated cells. Potential contamination by bacteria was assessed by plate count agar (PCA) with Zou medium and by observation employing a phase contrast of a microscope. The cell number (# mL^{-1}) and cell diameter were measured in a coulter counter (Beckman Multisizer 3^{Tm} Coulter Counter, USA). To determine the dry weight of the algae, samples (in triplicate) of the algal culture were filtered using pre-weighed glass microfiber filters (Whatman, Maidston, United Kingdom). The algae were washed three times with ammonium formate (0.5 M) to remove the precipitated salts and other non-organic substances and subsequently dried at 105°C 24 h, cooled over the silica gel in a desiccator, and weighed.

The absorbance PAR spectrum (400-700 nm) was determined in a spectrometer (Avants Fibre Optic Spectrometer, the Netherlands) to measure any changes in pigment content during each run and to verify if, indeed, the light gradient was constant for all runs.

Elemental composition

The elemental composition is determined for the algal cells growing at 100% and at 300% air saturation in sub-saturating and near-saturating light conditions. Liquid samples were centrifuged for 10 minutes at a 1750g. The pellets were washed three times with de-mineralized water and re-suspended and centrifuged again. The remaining algae pellets were stored at -20°C. The frozen pellets were weighed, freeze dried, and ground into a fine powder before further analysis of the biomass composition and moisture content occurred. The C, H, and N content of the freeze-dried samples of the algae growing at 100% and 300% oxygen was measured at 925°C in the oven of an elemental analyzer (EA 1110, ThermoQuest CE Instruments). Ash content was determined by burning the freeze-dried algae samples in an oven at 550°C to oxidize all organic material, leaving the ash residue. From these determinations, the oxygen (O) content and the subsequent elemental composition of the algae could be calculated (C, H, O, N, and ash) (Duboc et al. 1999).

RESULTS

PI-curve of low-light acclimated *Nannochloropsis* sp.

The PI-curve (Figure 1) measured cells of *Nannochloropsis* sp. that were acclimated to sub-saturating light conditions demonstrate that the photosynthesis activity increases almost linearly until 250 µmol photons m^{-2} s^{-1}, remains constant up to a light intensity of 600 µmol photons m^{-2} s^{-1} , and decreases in higher light intensities. This PI curve is similar to the PI-curve measured by Sukenik (1991) and confirms that the algae, indeed, grow in sub-saturating light conditions with an average light intensity of 42 µmol photons m^{-2} s^{-1}; they experience near-saturating light conditions at 168 µmol photons m^{-2} s^{-} ; and saturating light conditions at 262 µmol photons m^{-2} s^{-1} and corresponding with incident light intensity of 100, 400 and 624 µmol photons m^{-2} s^{-1}, respectively.

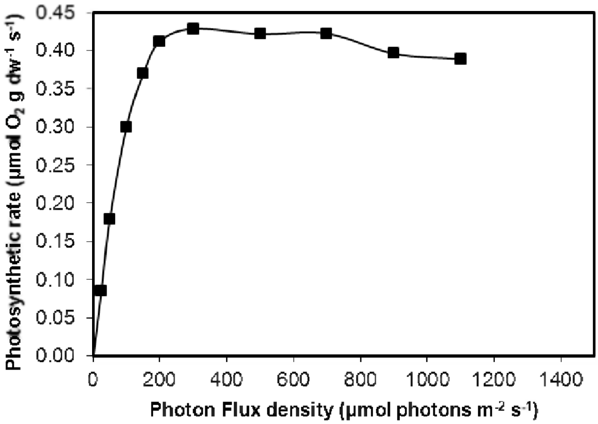

Figure 1 The photosynthetic-Irradiance (P-I) curve of *Nannochloropsis* sp using biological oxygen monitor (BOM) ranging from light intensity of 25-1100 µmol photons m^{-2} s^{-1} at different irradiances (100 and 400 and 624 µmol photons m^{-2} s^{-1}). The cells were acclimated to an incident light intensity of 100 µmol photons m^{-2} s^{-1}.

Typical growth curve of *Nannochloropsis* sp. in the flat-panel photobioreactor

Figure 2 depicts a typical growth curve of the algae in the flat-panel photobioreactor in near-saturating light condition. The algal cells were allowed to grow batch-wise until the light intensity at the back of the photobioreactor was 48 µmol photons m^{-2} s^{-1}. At that moment, the cell suspension achieved an optical density OD_{680} of 2.0, and the turbidostat operation was initiated. This typical growth curve exhibits that the OD_{680} remained constant with all tested oxygen concentrations and indicates that the turbidity of the cell suspension is only minimally influenced by the oxygen concentration in saturating light conditions. The OD_{680}/OD_{530} ratio remained constant which indicates that the algal cells were healthy and no contamination had occurred from other micro-organisms.

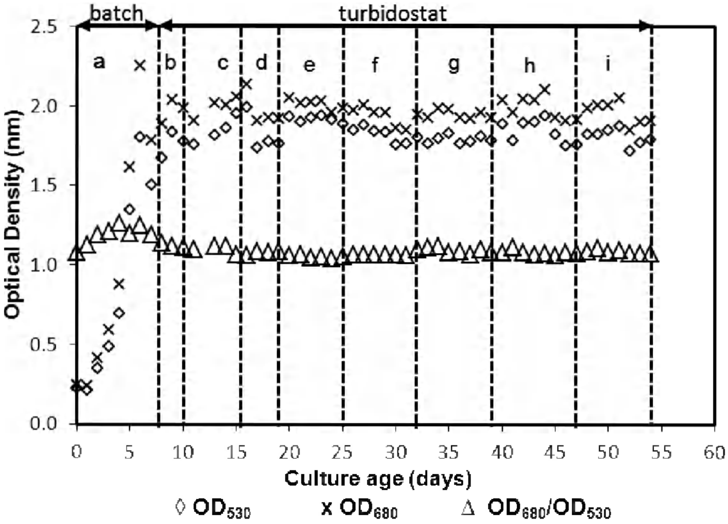

Figure 2 The typical growth curve of *Nannochloropsis* sp. in near-saturating light condition during the run of 1.5 L flat panel photobioreactor: a batch-wise growth at gradually increasing irradiances; b-h is turbidostat-operation at different oxygen concentrations ranging from 100-400% air saturation (b=100; c=125; d=150; e=175; f=200; g=250, h=300; i=400).

Effects of oxygen concentration on the growth in different light intensities

The specific growth rate μ was determined as a function of the oxygen concentration in the medium at sub-saturating, near-saturating, and over-saturating light intensity (Figure 3). In all light intensities, a decrease in μ of *Nannochloropsis* sp. with increasing oxygen concentration was observed. In sub-saturating light conditions, μ linearly decreased from 0.40±0.10 day^{-1} with an oxygen concentration of 100% up to 0.18±0.01 day^{-1} with 250% air saturation. A sharp decrease in μ from 0.86±0.20 to 0.5±0.10 day^{-1} was observed in near-saturating light conditions when the oxygen concentration was increased from 100% to 125% air saturation. When further increasing the oxygen concentration, the growth rate leveled off and remained constant at μ of 0.40±0.04 day^{-1} between 200% and 400% air saturation. In over-saturating light conditions, the growth rate was 0.37 day^{-1} with 100% air saturation, sharply decreased to 0.17± 0.01 day^{-1}, and remained constant with oxygen concentrations between 125% and 175% air saturation.

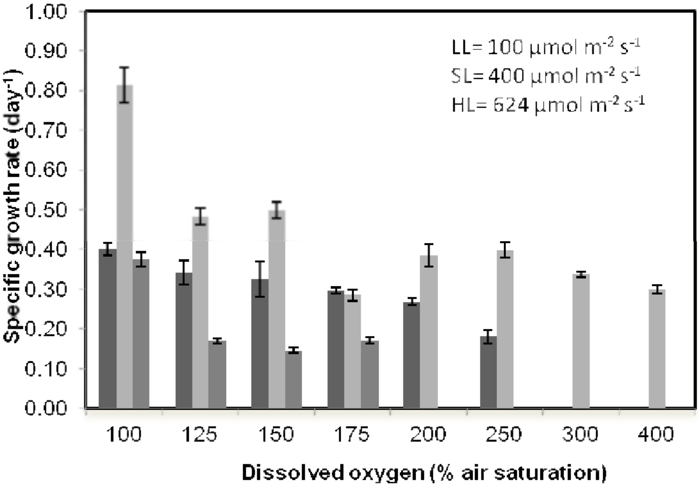

Figure 3 The effects of oxygen concentration on specific growth rate (day^{-1}) of *Nannochloropsis* sp. at different incident irradiances (100, 400 and 624 µmol photons m^{-2} s^{-1}).

Unfortunately, the effects of oxygen on the growth at concentrations above 200% air saturation could not be measured in over-saturating light conditions due to contamination of the culture with bacteria.

In near-saturating light conditions, the specific growth rate with 100% air saturation was approximately two times higher (0.86 ± 0.20 day^{-1}) than in sub-saturating light conditions (0.40 ± 0.10 day^{-1}). This increase in growth rate is similar to the increase in photosynthesis activity that is observed in the PI-curve (Figure 1) between 42 and at 168 µmol photons m^{-2} s^{-1}. In over-saturating light conditions when the algae are subjected to an average light intensity of 262 µmol photons m^{-2} s^{-1}, the growth rate again decreased (0.37 ± 0.06 day^{-1}). This significant decrease is not reflected in the PI curve. This difference in response to high light intensity is most probably caused by the differences in the level of adaptation of the cells to the new light conditions. The cells were pre-cultured in low light intensity and, during the PI-curve measurements, the cells were unable to adapt to the new high light intensity; they directly responded to higher irradiances with an increase of the rates of the reactions involved in photosynthesis. The cells in the photobioreactor, on the other hand, had ample time to adapt during the batch-wise cultivation prior to the turbidostat runs and to each new oxygen concentration. In these experiments, the growth rate represents a combination of the direct effects of light on the photosynthesis rate and adaptation of the cells to higher light irradiance. Phototrophic microalgae generally adapt to higher light intensities by decreasing the amount of chlorophyll and increasing their pigment content, e.g., carotenoids (Mimuro and Kotoh 1991). Higher oxygen concentrations in the medium may lead to formation of extra carotenoids since they can act as an anti-oxidant that quench singlet oxygen and react with oxygen radicals (Pospíšil 2011). These protective adaptation mechanisms to various oxygen and light conditions will affect the specific absorption area of the algae α (m^2 kg DM^{-1}) and evokes changes in the PAR absorption spectrum. In our experiments, a constant total absorption area $\alpha * C_x$ of 75.7 m^2 m^{-3} was applied during all turbidostat runs. A change in α due to different culture conditions of oxygen and light can thus be determined from the concentration of the cells C_x (kg DM m^{-3}). At sub-saturating light conditions, the biomass concentration remained constant at 0.58±0.06 g/L and did not exhibit a significant change when increasing the oxygen concentration ($P>0.05$) (Figure 4). In near-saturating light intensities, a higher biomass concentration is measured, but again, the change in biomass concentration is only minimally affected by the oxygen concentration. Apart from the slightly higher biomass concentration measured at an oxygen concentration of 125% air saturation, the biomass concentration remained constant at 0.81±0.08 g/L ($P>0.05$). Only in over-saturating light conditions was a significant ($P<0.05$) increase of the cell dry weight concentration observed with increasing oxygen concentration. A constant cell concentration, as observed in sub-saturating and near-saturating light conditions, should be accompanied by a constant specific absorption coefficient of the cells, whereas the increase in cell concentration with increasing oxygen concentration in over-saturating light conditions should be accompanied with a decrease in total absorption coefficient. This was confirmed in off-line measured absorbance spectra (data not shown).

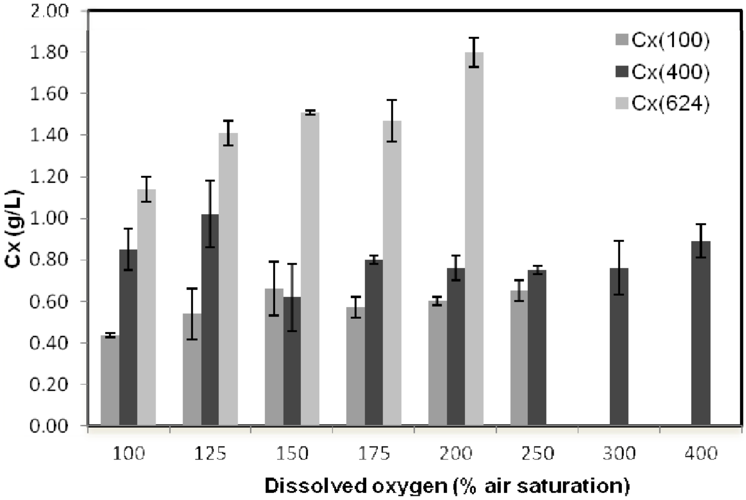

Figure 4 Effects of oxygen concentrations on *Nannochloropsis* sp. on cell concentration (g DW L^{-1}) in different incident irradiances (100, 400 and 624 μmol photons m^{-2} s^{-1}).

Figure 5 depicts the ratio between carotenoids and chlorophyll absorption with different oxygen and light conditions. As expected, an increase in light irradiance results in an increase in the ratio between carotenoids and chlorophyll peak. The graph exhibits that the ratio is not affected by the oxygen concentration in sub-saturating and near-saturating light conditions. Only in over-saturating light conditions was a significant increase of the amount of carotenoids evident with increasing oxygen concentration.

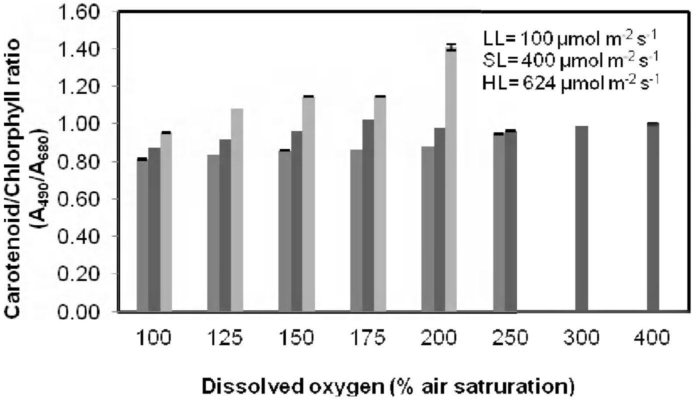

Figure 5 Effects of oxygen concentrations on the ratio between carotenoids and chlorophyll of *Nannochloropsis* sp. cultured at an incident irradiance of 100, 400, and at 624 µmol photons m^{-2} s^{-1}. The absorption at 490 nm (A490) is used as measure for the carotenoid, and the absorption at 678 nm (A678) is used as measure (n=6) for the chlorophyll.

DISCUSSION

Increases in dissolved oxygen (DO) concentration clearly cause a decrease in the specific growth rate (day^{-1}) of *Nannochloropsis* sp. in sub-saturating, near-saturating, as well as over-saturating light conditions. This effect was also observed for other microalgae such as *Phaeodactylum tricornutum* grown in indoor and outdoor conditions (Molina Grima et al. 2001); *Euglena gracilis* (Kitaya et al. 2005); *Chlorella sorokiniana* (Ugwu et al. 2007); *Chlorella pyrenoidosa* (Shelp and Canvin 1980); Cyanobacteria, *Spirulina plantensis*

(Vonshak et al. 1996); *Chlorella vulgaris* (Ogawa et al. 1980); and also in polar sea microalgae communities (McMinn et al. 2005). In most of these studies, however, the effects of oxygen interfere with light effects and, in most cases, also interfere with effects of changing culture conditions during batch cultivation. Only recently have studies been presented in which oxygen effects on growth in constant light and other culture conditions were performed on *Chlamydomonas reinhardtii* (Kliphuis et al. 2011), *Neochloris oleoabundans* (Sousa et al. 2012) and on *Nannochloropsis* sp. (Raso et al. 2012), however, in these studies, only the effects of oxygen in sub-saturating average light conditions were presented. In those light conditions, inhibition of growth resulting from oxygen can predominantly be ascribed to photorespiration which occurs when the ratio between the O_2 and CO_2 concentration inside the chloroplast of the algae cell changes, and the oxygenase activity of the Rubisco enzyme overtakes the carboxylase activity of the enzyme (Wingler et al. 2009; Kliphuis et al. 2011; Raso et al. 2012). Figure 3 certainly indicates that an increase in oxygen in the medium resulted in a linear decrease of the growth rate as predicted based on the inhibiting effect of oxygen through photorespiration.

In near-saturating and over-saturating light conditions, the increase in oxygen concentration from 100% to 125% air saturation evoked a sharp decrease in the growth rate followed by a more gradual decrease upon further increase of the oxygen concentration (Figure 3). At those high irradiance levels, photorespiration effects can still be expected, but photo-oxidative inhibitory effects caused by the combination of high irradiance and high oxygen concentrations will dominate (Asada 2006, Triantaphylides et al. 2008; Badger et al. 2000). At high irradiance, a surplus of electrons is generated at Photosystem II because the photon intensity is in excess of that which is required for the CO_2 assimilation. Through a sequence of reactions involved in the Mehler reaction (also called water-water cycle), the electrons arrive at the reducing side of Photosystem I where O_2 is generated by the oxidation of water (Falkowski and Raven 2007). In the Mehler reaction, ATP is generated at the expense of light energy but, ultimately, a portion of the absorbed light energy is dissipated as heat. The sequence of reactions in the reduction of O_2 to H_2O involves the formation of reactive intermediates such as superoxide and H_2O_2 catalyzed by the enzymes superoxide dismutase and peroxidases such as ascorbate peroxidase.

If one of the enzymes involved in the Mehler reaction cannot address the surplus of electrons generated at PSII quickly enough, accumulation of the reactive intermediates will occur which results in photo-oxidative damage (Asada 2006). Since the O_2 uptake due to the Mehler reaction of microalgae is much higher than the O_2 uptake by the oxygenase activity of Rubisco (Badger et al. 2000), minimal increases in oxygen concentration can result in more cell damage due to accumulation of these reactive intermediates.

In addition to the photo-oxidative damage caused by the accumulation of the ROS formed in the Mehler reaction, photo-oxidative damage by oxygen singlet molecules may occur at high irradiance levels. When exposed to excess light, absorption of the photons leads to generation of triplet excited P680 in the PSII reaction center with the formation of singlet oxygen as a consequence. This singlet oxygen is highly reactive and readily oxidizes pigments, lipids, and nucleic acids within its vicinity. In moderate light conditions, the carotenoids and tocopherols that are present in the chloroplasts of the algae can effectively suppress the singlet oxygen, however, when these antioxidant mechanisms are overwhelmed, the accumulation of intracellular ROS will stimulate cell damage and lead to cell death (Triantaphylides et al. 2008). Both photo-oxidation processes will occur in elevated light conditions, and the different ROS are produced simultaneously. The link between the accumulation of specific ROS in the cells and their effects on the decrease in the cells' growth rates is difficult to ascertain.

Further increase of the oxygen concentration in the medium to concentrations higher than 150% did not lead to a further decrease of the growth rate. This might be explained by considering the transfer of oxygen. With low oxygen concentration in the medium, the oxygen that is formed in the chloroplast during the photosynthesis reaction can diffuse from the chloroplast into the medium. As long as the transfer rate of the oxygen from the cells to the medium is higher than the rate of ROS formation, the damage from the reactants of the oxygen will be limited. With an increase of the oxygen concentration in the medium, the transfer rate of oxygen may be reversed. The higher oxygen concentration in the medium will evoke mass transfer from the medium to the active site of the photosynthesis and induce formation of the damaging ROS. Further increase of the oxygen concentration in the medium will increase the ROS formation until the ROS formation becomes rate limiting. At that point, a further increase in the oxygen concentration will no longer result in a further decrease of the growth rate (Ross et al. 2008; Badger et al. 2000).

The specific growth rate of *Nannochloropsis* sp. observed in our experiments is comparable with previous results obtained by Sukenik (1991) who observed the specific growth rate of 0.4 day^{-1} in sub-saturating light and specific growth rate of 0.8 day^{-1} in near-saturating light. At high light intensity, however, the specific growth rate by Sukenik (0.71 day^{-1}) is almost two fold higher than the growth rate of 0.40 day^{-1} that was found in this study. This is probably due to the difference in acclimation of the algae in the different light conditions employed in this study. While Sukenik worked with cells that were not acclimated to high light conditions, in this study, the cells were acclimated for at least 3 days to the higher light intensity. Figure 4 demonstrates that the cell concentration C_x significantly increased with higher irradiance while the total absorption area αC_x is constant for each experiment since the light gradient inside the photobioreactor is kept constant. The high-light acclimated cells thus have considerable lower absorption coefficient α. In a sub-saturating light condition, light is limited for growth which subsequently resulted in an increase in the number of photosynthetic units (PSU) while the PSU size remained constant (Fisher et al. 1996). There was also an increase in light-harvesting pigments such as chlorophylls, phycobinlin, fucoxanthin, and peridinin (Dubinsky and Stambler 2009). Obviously, the light-acclimated cells contain less chlorophyll content and will thus grow less quickly than non-acclimated cells.

From Figure 4, it can be deduced that the oxygen concentration at sub-saturating and near-saturating light intensity only minimally affects the absorption coefficient in the given light intensity. Only minimal increase in biomass concentration with oxygen concentration can be observed in over-saturating light conditions which is accompanied by minimal decrease of the absorption coefficient. The decrease of the absorption coefficient as an effect of increased oxygen levels is due to the extra formation of β-carotene at the expense of chlorophyll-a (Figure 5) that protect the algae cells from damage caused by the additional oxygen radicals formed by photo-activation of the oxygen that is present. Formation of additional carotenoids in the light-harvesting center quenches the formed oxygen singlets, thereby protecting the cells from further damage (Triantaphylides and Havaux 2009). In addition, other mechanisms to protect the algal cells from photo-activation effects at high irradiance induced by high oxygen concentration in the medium, may be active to quench possible photo-inhibition effects of high oxygen concentrations. Further research, however, is required to investigate if these mechanisms are indeed activated at elevated oxygen concentrations in the medium.

CONCLUSIONS

In our experiments, the synergistic effects of oxygen and light on growth of *Nannochloropsis* sp. in fully controlled culture conditions were revealed in sub-saturating, near-saturating as well as over-saturating light conditions. In all of the employed light conditions, a decrease in the specific growth rate was observed. In sub-saturating light conditions, the growth inhibition due to elevated oxygen concentrations was mainly caused by photorespiration. In near-saturating and over-saturating light conditions, the growth rate of *Nannochloropsis* sp. was mainly affected by photo-oxidation effects while photorespiration effects only played an insignificant role. In those conditions, a sharp decrease in the growth rate was observed when increasing the oxygen concentration form 100% to 125% air saturation. A further increase of the oxygen concentration did not result in a substantial further decrease in growth rate.

Our results indicate that high productivity of *Nannochloropsis sp*. in outdoor mass cultivation will only be possible if the oxygen concentration is maintained at around 100% air saturation and is cultivated in near-saturating light conditions. An increase in oxygen concentration leads to a decrease in growth rates due to effects of oxygen on the photorespiration and photo-oxidation. The effects of oxygen on growth, however, are outcompeted by the direct effects of limitation of light in sub-saturating light conditions and photo-inhibition effects in over-saturating light conditions.

ACKNOWLEDGEMENT This work was financially supported by The Royal Thai Government through the Office of Civil Service commission (OCSC), Thailand.

NOMENCLATURE

μ	specific growth rate (day^{-1})
α	absorption co-efficient ($m^2\ g^{-1}$)
C_x	Biomass concentration ($kg\ DM\ m^{-3}$)
z	position at light path (m)
d	total light path in the flat-panel photobioreactor (m)
$I(z)$	light intensity at the back of photobioreactor (μmol photons $m^{-2}\ s^{-1}$)
I_{wall}	light intensity at the front of photobioreactor (μmol photons $m^{-2}\ s^{-1}$)
F_{out}	flow of medium (m^3/day)
V_l	volume of liquid in the photobioreactor (m^3)
D	dilution rate (day^{-1})

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Chapter 4

Effects of oxygen on the molecular defense mechanisms during growth of *Nannochloropsis* sp. at different irradiances

The contents of this chapter have been submitted for publication as: Raso S, Vermuë M, Wijffels RH. Effects of oxygen on the molecular defense mechanisms during growth of *Nannochloropsis* sp. at different irradiances.

ABSTRACT *Nannochloropsis* sp. is an EPA rich microalga that is often cultured in closed photobioreactors where they are subjected to accumulating oxygen concentrations. High oxygen concentration causes photorespiration which inhibits growth. Oxygen is also involved in photo-inhibition caused by photo-oxidative stress, and several molecular mechanisms have evolved in the algae to protect them against this oxidative damage. In this study, the effect of the oxygen concentration on these molecular defense mechanisms against photo-oxidative stress is addressed, and the effect of oxygen on fatty acid content and composition are investigated in sub-saturating, saturating, and over-saturating light conditions.

Superoxide dismutase (SOD) is involved in protecting the cells against photo-oxidative stress caused by the oxygen radicals that are formed. The SOD activity was only minimally affected by oxygen, indicating that higher oxygen levels in the medium did not result in activation or induced production of this enzyme. Only in over-saturating light conditions was a slightly higher SOD activity observed with increased oxygen concentrations.

High oxygen concentrations coincide with an increase in the carotenoid content. This pigment is particularly involved in protection against highly reactive singlet oxygen that is formed at high irradiance. Our results indicate that this protection mechanism against photo-oxidative damage is provoked by oxygen that accumulates within the medium.

With an increase in light irradiance, the total fatty acid content significantly increased, and the percentage of unsaturated fatty acids and ratio of EPA content slightly decreased, which was expected. However, with these higher light intensities, high oxygen levels did not result in photooxidation of fatty acids which indicates that high oxygen concentrations inhibit the growth of *Nannochloropsis* sp. only through photorespiration and do not lead to additional oxidative damage by photo-inhibition. The only manner in which to prevent losses in productivity in closed photobioreactors is by minimizing photorespiration, i.e., by maintaining a sufficiently high CO_2/O_2 ratio in the medium.

KEYWORDS O_2, photo-oxidative damage, *Nannochloropsis* sp., molecular defense, SOD, carotenoids, fatty acid composition

INTRODUCTION

Nannochloropsis sp. is an alga that contains a relatively high lipid content rich in eicosapentaenoic acid (20:5n-3, EPA) (Mourente et al. 1990; Sukenik et al. 1993). This is why it is currently cultured worldwide as feed for fish larvae (Zitelli et al. 1999) and has also been recognized as a potential source for EPA production for human consumption (Rodolfi et al. 2008). For these high-value applications, production of *Nannochloropsis* in closed tubular photobioreactors is recommended to ensure high productivity with a continuous, high quality of the product (Zittelli et al. 1999). The production in tubular photobioreactors, however, is inhibited by O_2 that is produced by photosynthesis and accumulates during the algal cultivation (Molina et al. 2001; Kromkamp et al. 2009).

The inhibition of the growth rate due to oxygen was observed in various light conditions (Raso et al. 2012; McMinn et al. 2005; Sousa et al. 2012; Ugwu et al. 2007). In sub-saturating light intensity, Raso et al. (2012) reported that the specific growth rate of *Nannochloropsis* sp. decreased from 0.40±0.10 to 0.18±0.01 day^{-1} while increasing the dissolved oxygen (DO) concentration from 100% to 250% air saturation. A sharp decrease in the specific growth rate from 0.86±0.20 to 0.35±0.10 day^{-1} followed by a gradual decrease to 0.28±0.08 day^{-1} was ascertained in near-saturating light intensity when increasing the DO concentration from 100% to 400% air saturation. A similar pattern in the effect on oxygen concentration was observed in over-saturating light intensity; a sharp decrease followed by a gradual decrease in the specific growth rate from 0.37±0.06 to 0.17±0.02 day^{-1} with oxygen concentrations ranging from 100% to 175% air saturation (Raso et al. 2012). The accumulating oxygen was primarily affecting the growth through photorespiration, however, so far, it is unclear if increased oxygen concentrations also lead to additional photo-inhibition and photo-oxidative damage that is bound to happen in elevated light intensities.

During photosynthesis, O_2 plays a dual role. On the one hand side, O_2 inhibits growth through photorespiration and photo-oxidation, however, on the other hand, it is involved in protecting the photosynthetic apparatus by dissipating the excessive light energy through the water-water cycle (Figure 1).

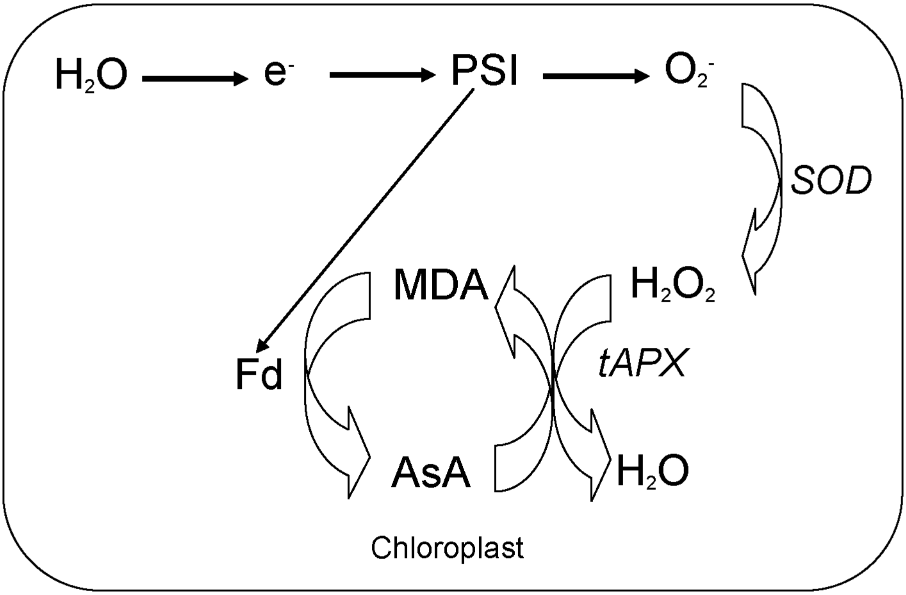

Figure 1 The water-water cycle occurs in the chloroplast of microalgae. At photosystem II (PSII), water within the chloroplast is oxidized by light energy to form O_2 while electrons are released. In a series of subsequent enzyme reactions, oxygen absorbs the excessive electrons delivered at Photosystem I and forms superoxide radicals O_2^-. The reactive oxygen radicals are transformed into hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). The formed H_2O_2 is transformed into water during a reaction in which the thylakoid-bound ascorbate peroxidase (tAPX) participates (Perelman et al. 2006; Ishikawa et al. 2009). This enzyme reduces Ascorbate (AsA) to monodehydroascorbate (MDA). The ferodoxin (Fd) finally catalysis the regeneration of AsA. (adapted from Mittler 2002).

In low light conditions, the algae are able to channel the excess electrons through the water-water cycle and actively remove the superoxide radicals and H_2O_2 that are formed (Asada 2006; Logan et al. 2006). In higher light intensities, however, the rate of the photo-reduction is faster than the rates of the subsequent reactions catalyzed by SOD and APX and the cells are no longer able to deal with the ROS that is formed, thereby experiencing photo-inhibition (Murata et al. 2007; Nishiyama et al. 2006). Superoxide radicals and H_2O_2 are not the only harmful ROS originating from oxygen. During high light conditions, photo-activation in PSII of the triplet chlorophyll $^3P_{680}{}^*$ occurs. Its energy is employed to generate the highly reactive singlet oxygen $^1O_2{}^{\cdot}$. The singlet oxygen can be partially quenched through transfer to the triplet carotenoids (^{3}Car*) (Figure 2), however, in the event of a surplus of energy supply, the oxygen singlet will cause photo-oxidative damage.

In addition to the protection resulting from the activity of enzymes during the water-water cycle, several other protective mechanisms to prevent cell damage by ROS to photosynthetic micro-organisms have evolved (Triantaphylides and Havaux 2009; Sabatini et al. 2009), and the carotenoid pigments are of particular importance in the scavenging of ROS. In *Nannochloropsis*, for example, the carotenoids violaxanthin and zeaxanthin are present in the thylakoid membranes (Pinto et al. 2000). Besides their well-established function as light-harvesting pigments and quenchers of singlet oxygen, they also protect the algae against peroxidative damage by regulating the thylakoid membrane fluidity (Triantaphylides and Havaux 2008). The latter is achieved by incorporating additional long-chain poly-unsaturated fatty acids (PUFA) such as EPA into the membrane phospholipids (Mallick and Mohn 2000). These molecules can serve as an anti-oxidant, and EPA has been especially mentioned as a stable anti-oxidant during oxidative stress caused by ROS in *Nannochloropsis* sp. (Okuyama et al. 2007; Triantaphylides and Havaux 2009). The photo-oxidation of the PUFA, however, may affect the PUFA content of the algae.

Although oxygen is clearly involved in photo-inhibition as well as in photo-oxidative damage, the effects of elevated oxygen levels on these processes in *Nannochloropsis* sp have not been studied independently from the employed light conditions (Sukenik 1989; Zittelli et al. 2000).

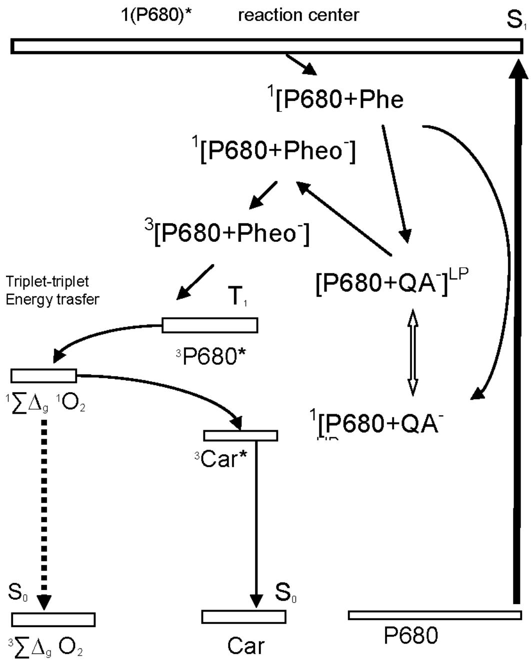

Figure 2 Under light stress, increasing amounts of singlet oxygen are generated in the attenna complex. The absorption of visible light by ground state chlorophyll (Chl) (S$_0$ electronic state) results in the formation of singlet chlorophyll (^{1}Chl*) (S$_1$ electronic state). When singlet-singlet energy transfer to neighboring chlorophyll is limited, triplet Chlorophyll (^{3}Chl*) is formed. The triplet excitation energy can be transferred to carotene or to molecular oxygen, forming highly reactive singlet oxygen ($^1O_2$). (adapted from Pospišil 2011).

This chapter focuses on the effects of oxygen on the molecular defense mechanisms against photo-inhibition and photo-oxidative stress in different light intensities to reveal if oxygen stimulates additional growth inhibition by ROS formation in elevated light irradiance. The effects on changes in activity of the enzyme superoxide dismutase (SOD) involved in the water-water cycle was investigated as well as the effect of oxygen on pigment content and on the role of PUFA as antioxidants in *Nannochloropsis* sp.

MATERIALS AND METHODS

Cultivation

Nannochloropsis sp. (CCAP 211/78) was pre-cultured in Erlenmeyer Flasks in an incubator (100 rpm) provided with a light-dark cycle of 18:6 h and a light intensity of 41 μmol photons m^{-2} s^{-1} in modified Zou medium (Table 1) at pH 7.8 and with 2% of CO_2 enriched air. Stock solutions of the trace elements, Fe-EDTA, as well as the phosphate buffer were prepared separately and added to the autoclaved medium. The pH of the medium was adjusted to 7.8 by 0.5 M NaOH. The medium was filter-sterilized through a 0.2 μm filter.

Experimental set-up

The effects of oxygen on the molecular defense mechanisms of *Nannochloropsis* sp. during growth in various light intensities were studied in a fully-controlled 1.5 L-flat panel photobioreactor with a light path of 2.8 cm operated in turbidostat mode as previously described by Raso et al. (2012). Three different light intensities were employed; 100, 400, and 624 μmol photons m^{-2} s^{-1}, which correspond to sub-saturating, saturating, and over-saturating light conditions according to the PI-curve of *Nannochloropsis* sp. (Raso et al. 2012).

During 3 days of turbidostat operation at specified oxygen concentration, the growth rate was monitored by measuring the outflow of the culture medium, and samples were withdrawn for off-line measurements of the biomass concentration, as well as the optical density at 530 nm (OD_{530}) and 680 nm (OD_{680}) (see Raso et al. 2012). In addition, the intracellular total SOD activity (U g cell dw^{-1}), the total absorption PAR spectrum, and the fatty acid content and composition (%w/w) as well as the elemental composition of the samples were determined.

Table 1 Composition of the modified Zou medium exploited for growth of *Nannochloropsis* sp. at different irradiances.

Compounds	**Concentration (mM)**
NaCl	419.38
$MgSO_4$	54.81
$MgCl_2.6H_2O$	27.55
$CaCl_2.2H_2O$	10.20
$CO(NH_2)$Urea	19.98
$NaHCO_3$	0.48
Fe-EDTA	
EDTA-FE(III)-Na-salt	3.70
K_2HPO_4	57.41
KH_2PO_4	14.70
Trace element	
Na_2EDTA	0.26
$CuSO_4.5H_20$	0.03
$ZnSO_4.7H_20$	0.04
$CoCl_2.2H_20$	0.03
$MnCl_2.4H_20$	0.46
$Na_2MoO_4.2H_20$	0.13

Superoxide dismutase (SOD) activity

Prior to the SOD determination, 10 mL of fresh algae samples were centrifuged at 3000 rpm for 15 minutes employing the Beckman Coulter (AllegraTm X-22R Centrifuge, Germany). Both supernatant and cell pellets were used to determine the SOD activity. The pellets were re-suspended in a phosphate buffer containing 137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, and a pH of 7.4; disrupted by bead milling for 30 minutes; and cooled on ice for 10 minutes between each subsequent bead-mill treatment. Samples were stored at -81°C prior to further analysis of the SOD activity.

For the determination of total SOD activity, the Cayman© Superoxide Dismutase Assay Kit was utilized (Ross et al. 2010). Results were expressed as the total SOD unit per g cell dry weight. One SOD unit was specified as the enzyme amount necessary to eliminate 50% of the superoxide radicals formed per gram of dry algae (U g cell dw^{-1}).

Absorption spectrum and changes in pigment composition

The absorption of the algae ABS$_\lambda$ was measured at intervals of 1 nm in the PAR range (400-700 nm) in an Avants Fibre Optic Spectrometer and corrected for scattering SCA at each wavelength. To determine the relative changes in pigment composition, the peak areas of the PAR spectrum were normalized by the chlorophyll-a peak (OD of 680 nm).

Fatty acid analysis

For analysis of the total fatty acid content (TFA) and fatty acid composition, 6 samples of 10 mL were centrifuged at 3000 rpm for 15 minutes, and the cell pellet was maintained at -81°C until the analysis. The lipid extraction and trans-esterification method described by Rodriguez-Ruiz et al. (1998) was used for fatty acid analysis. 3 mL of a 2:1 mixture of methanol (HPLC grade) and acetyl chloride (98%, Aldrich 29042-010) and 2 mL hexane with nonadecanoic acid methyl C19:0 as an internal standard were added to the frozen pellet of microalgae. The tubes were incubated at 122°C for 30 minutes and subsequently cooled to room temperature. An aliquot of the top phase was employed for GC analysis of the fatty acid composition. A gas-chromatography analysis was conducted with a Nukol™ column (30 m x 530 µm x 1.0 µm). The initial oven temperature was 90°C followed by a temperature program of 20°C min^{-1} to 200°C. The injector temperature was 250°C, flame ionization detector temperature was 270°C, and a split ratio of 0.1:1 was used. Fatty acid concentrations were calculated by comparing each peak area with that of the internal standard and corrected accordingly.

Elemental composition

The elemental composition is determined for the algal cells growing with 100% and 300% air saturation in sub-saturating and saturating light conditions. Liquid samples were centrifuged for 10 minutes at 1750g. The pellets were washed three times with de-mineralized water and re-suspended and centrifuged again. The remaining algae pellets were stored at -20°C. The frozen pellets were weighed, freeze dried, and ground to a fine powder prior to further analysis of the biomass composition and moisture content.

The C, H, and N content of the freeze-dried samples of algae growing with 100% and 300% oxygen was measured at 925°C in the oven of an elemental analyzer (EA 1110, ThermoQuest CE Instruments). Ash content was determined by burning the freeze-dried algae samples in an oven at 550°C to oxidize all organic material which resulted in ash residue. From these determinations, the oxygen (O) content and the subsequent elemental composition of the algae was calculated (C, H, O, N and ash) (Duboc et al. 1999).

Statistical analysis

The total superoxide dismutase (SOD) activity, total fatty acid (TFA), percentage of unsaturated fatty acid (%Unsat), and ratio of EPA content were statistically analyzed with ANOVA using a confidence interval of 95% with Tukey's b multiple comparisons. This statistical analysis was conducted to detect any significant changes in SOD, fatty acid compositions, and lipid content with the oxygen concentration and light intensity.

RESULTS AND DISCUSSION

Effect of elevated O_2 on SOD activity

To verify if the oxygen had an effect on the active enzyme in the water-water cycle, the total SOD activities were determined. Figure 3 depicts the total SOD activity as a function of the oxygen concentration in the medium in the various employed light intensities. As expected (Asada 2000; Asada 2006; Logan et al. 2006), the activity of the key enzyme SOD in the water-water cycle was lowest in low light intensity and only minimally increased with elevated oxygen concentration (Hirayama et al 1996). This indicates that superoxide radicals and H_2O_2 are only minimally formed in those light conditions, and elevated oxygen levels in the medium do not appear to stimulate the SOD activity. In low light conditions, the algae are capable of directly channeling the electrons captured at PSII through the photosynthesis pathway. This results in generation of NADPH and ATP that are required in the Calvin Cycle to fixate CO_2. It also generates sugars and ensures that oxygen concentrations in the medium do not interfere.

In saturating light irradiance, light is no longer limited, and an excess of electrons is generated at Photosystem II resulting in formation of oxygen radicals. To address the oxygen radicals, the enzymes in the water-water cycle are expected to become more active (Mallick and Mohn 2000; Ishikawa and Shigeoka 2008). Indeed, greater SOD activity is observed in these light conditions (Figure 3). This was also ascertained by Rossa et al. (2002) who studied the effects of light intensity on the SOD activity in the red alga *Gracilariopsis tenuifrons* in various PAR-light conditions. The SOD activity increased from 20-30 U mg^{-1} at 25 µmol m^{-2} s^{-1} to 160-200 U mg^{-1} at 250 µmol m^{-2} s^{-1} (Rossa et al. 2002).

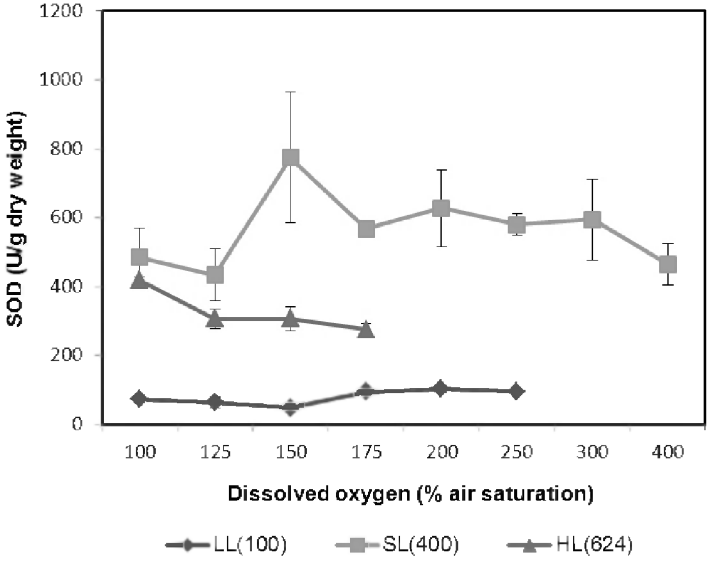

Figure 3 Effects of oxygen concentrations on total superoxide dismutase (SOD) activity (U g cell dw^{-1}) of *Nannochloropsis* sp. at various irradiances (LL = 100 µmol photons m^{-2} s^{-1} or sub-saturating, SL = 400 µmol photons m^{-2} s^{-1} or saturating and HL = 624 µmol photons m^{-2} s^{-1} or over-saturating light intensity).

Although a much higher SOD activity was ascertained, no significant change was observed with increasing oxygen levels in saturating light intensity which indicates that the light conditions, rather than the oxygen concentration, affect the activity of enzymes in the water-water cycle.

In over-saturating light conditions, the SOD activity at 100% DO level is similar to the SOD activity measured at this DO level in saturating light conditions, however, when increasing the oxygen concentration, the SOD activity slightly decreased. This indicates that high oxygen levels combined with an over-saturating light condition negatively affects the SOD activity. In those light conditions, the enzymes in the water-water cycle can no longer address the surplus of electrons generated at PSII. The oxygen radicals and H_2O_2 that are formed also accumulate and cause photo-inhibition (Murata et al. 2007; Nishiyama et al. 2006). Additionally, photo-activation will certainly occur, and the highly reactive oxygen singlet is formed (Triantaphylides and Havaux 2009). As the oxygen singlet has a relatively short life span of milliseconds, it is particularly harmful for biomolecules in the chloroplast at the PSII vicinity where it is formed (Triantaphylides and Havaux 2009). SOD appears to be one of the targets for the photo-oxidative damage caused by singlet oxygen; the enzyme activity decreases in over-saturating light conditions (Figure 3). The DO concentration in the medium, however, once again seems to only minimally contribute to the photo-inhibiting effect.

The induction of the SOD activity with light has been found for other microalgae as well (Janknegt et al. 2007; Perelman et al. 2006; Marshall et al. 2005). It is not clear, however, if the algae were subjected to saturating light conditions or over-saturated light conditions in all of these studies. Moreover, the effects of oxygen and light were not determined independently. Our study demonstrates that light influences the SOD activity but that the oxygen concentration in the medium only minimally attributes to the SOD activity.

Effects of O_2 on absorption and changes in pigment composition

Figure 4A-C illustrates the effects of oxygen concentrations on the normalized absorption spectra of *Nannochloropsis* sp. in different light conditions. In sub-saturating light (Figure 4A) and saturating light intensities (Figure 4B), a small modification in the normalized absorption spectra can be observed when increasing the oxygen concentration. While the chlorophyll peak at 680 nm remains constant, an increase in the peaks at 440 nm and at 490 nm can be observed when the oxygen concentration is increased from 100% to 300% air saturation. In over-saturating light intensity, the change in the absorption spectrum at 440 and 490 nm already begin to occur with oxygen concentrations of 175% air saturation (Figure 4C).

The shift in ratio of carotenoid and chlorophyll content is presented in the form of the ratio in absorbance measured at 490 nm and 680 nm (A_{490}/A_{680}) measured in different oxygen and light conditions (Figure 5). The graph illustrates that the ratio is not affected by the oxygen concentration in sub-saturating and near-saturating light conditions. It ranges from 0.81 up to 0.95 in sub-saturating light conditions and remained almost constant around 1 in near-saturating light conditions.

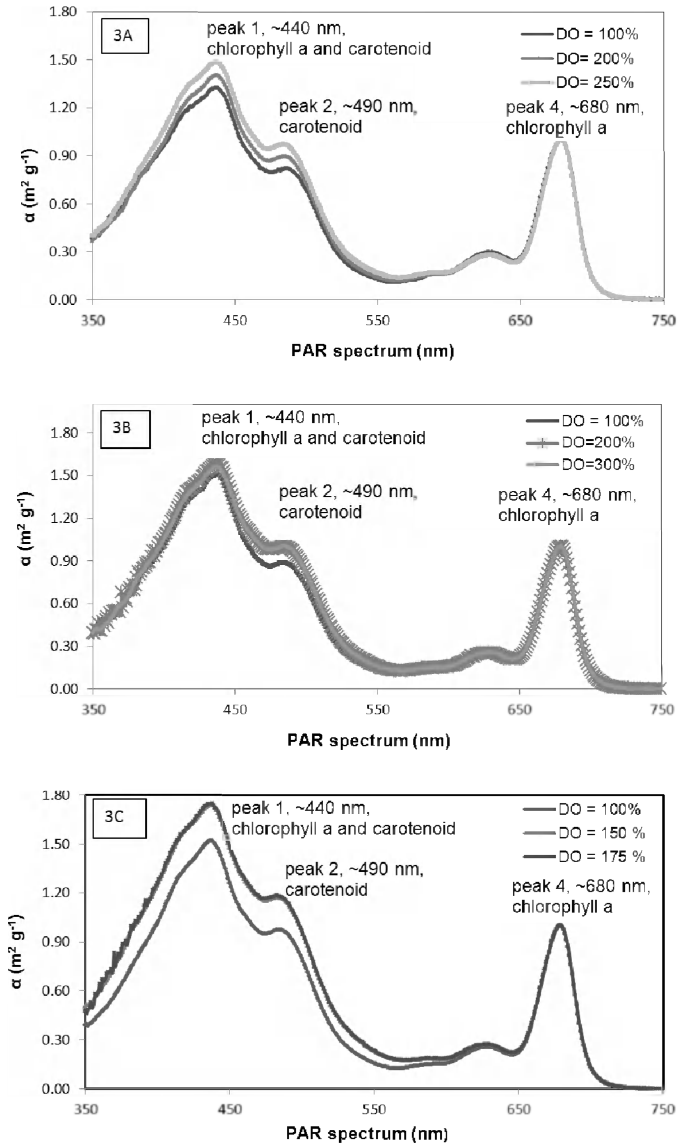

Figure 4 Effect of oxygen concentration on normalized absorption coefficient of *Nannochloropsis* sp. in different light intensities: (3A) sub-saturating light intensity; (3B) saturating light intensity; (3C) over-saturating light intensity.

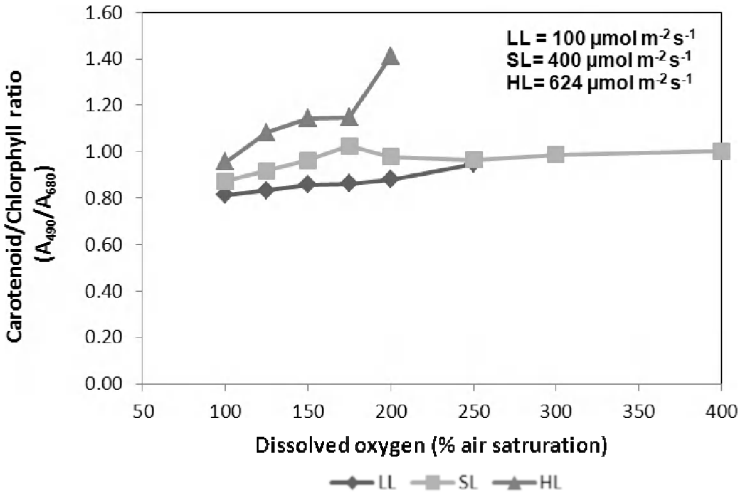

Figure 5 Effects of oxygen concentrations on the ratio between carotenoids and chlorophyll of *Nannochloropsis* sp. cultured at an incident irradiance of 100, 400 and at 624 µmol photons m^{-2} s^{-1}. The absorption at 490 nm (A490) is used as a measure for the carotenoid, and the absorption at 678 nm (A678) is used as a measure (n=6) for the chlorophyll.

In saturating conditions, the carotenoid/Chlorophyll ratio increases from 0.96 with 100% air saturation to 1.41 with 200% air saturation. Only in over-saturating light conditions does a significant increase in the amount of carotenoids occur with increasing oxygen concentration. In those high light conditions, greater total carotenoid content was also observed by Tada (1993) and Salguero et al (2003). In those light conditions, carotenoids are known to quench the electrons formed at PSII and prevent formation of harmful ROS such as oxygen radicals and, particularly, singlet oxygen (Asada 2006; Logan et al. 2006).

The carotenoids can serve as an anti-oxidant (Mallick and Mohn 2000; Pospíšil 2011), and the formation of extra carotenoids at elevated oxygen concentrations may protect the cells from oxidative damage. The oxygen itself combined with increased light conditions will induce extra formation of singlet oxygen. This will lead to more carotenoid formation to protect the photosystem in the cells from oxidative damage by the singlet oxygen that is formed.

The effect of oxygen on the pigment content is relatively small compared to the effects of elevated light conditions. In low light conditions, the algae exhibit typical photo-adaptation effects and form extra pigments in order to capture as much light as possible, and the carotenoid/chlorophyll ratio is smaller than in high light intensity (Tada 1993; Salguero et al. 2003). This is also reflected in the biomass concentrations measured when the cells were growing in turbidostat in the different light intensities. In low light conditions, the biomass concentration ranged from 0.53 to 0.66 kg m^{-3}, and no direct effects of oxygen on biomass concentration is observed (Table 2). In saturating and over-saturating light, higher biomass concentrations were expected as the cells exhibit photoadaptation and form less chlorophyll. The biomass concentration increased to values ranging from 0.62 to 1.02 kg m^{-3} in saturating light conditions and increased to values between 1.14 to 1.51 kg m^{-3}, but no relationship with the oxygen concentration could be ascertained.

Effects of O_2 on lipid content and fatty acid composition

Oxygen in combination with high light conditions may affect the fatty acid composition through the formation of reactive oxygen species. The long chain unsaturated fatty acids PUFA, such as EPA, are ascertained in the phospholipids fraction of the membrane of the algae. The PUFA content of the membrane lipids is expected to change in elevated light conditions. In high light intensity, the content of saturated fatty acids generally tends to increase as algae incorporate these in the form of triacylglycerides (TAG).

Table 2 Effects of oxygen concentration on the measured biomass concentration C_x (kg m^{-3}) of *Nannochloropsis* sp. during turbidostat operation in various light intensities. (LL = sub-saturating intensity, SL= saturating intensity and HL = over-saturating intensity).

DO (% air saturation)	C_x (LL) kg m^{-3}	C_x(SL) kg m^{-3}	C_x (HL) kg m^{-3}
	LL	SL	HL
100	0.55±0.04	0.77±0.23	1.14±0.06
125	0.53±0.02	1.02±0.16	1.41±0.06
150	0.66±0.13	0.62±0.16	1.51±0.01
175	0.57±0.05	0.80±0.02	1.47±0.10
200	0.60±0.02	0.66±0.06	
250	0.65±0.05	0.75±0.02	
300		0.76±0.12	
400		0.89±0.08	

In addition, carotenoids are formed to protect the algae against peroxidative damage by quenching the surplus of electrons that are generated at PSII and regulating the thylakoid membrane fluidity. This is achieved by incorporating more long-chain poly-unsaturated fatty acids (PUFA) such as EPA in the membrane phospholipids (Triantaphylides and Havaux 2008; Mallick and Mohn 2000). EPA has been specifically mentioned as a stable anti-oxidant during oxidative stress caused by ROS in *Nannochloropsis* sp. (Okuyama et al. 2008; Triantaphylides and Havaux 2009).

In this study, the effect of elevated oxygen concentrations on fatty acid composition and lipid content of *Nannochloropsis* sp. has been measured in sub-saturating, saturating, and over-saturating light intensity (Table 3A, B and C). The major fractions of long-chain fatty acids ascertained in *Nannochloropsis* sp. consist of C14:0, C16:0, C16:1 and the omega-3 poly-unsaturated fatty acid C20:5n-3 (EPA) while minor fractions of C18:0, C18:1, C18:2 and C20:3 are also evident.

In sub-saturating light conditions, the total fatty acid content remained constant at approximately 12% w/w. Only a minimal, but significant, increase in the total fatty acid content to 16.5% was ascertained with 250% air saturation. The increase in oxygen concentration only minimally affected the fatty acid composition, and the ratio between unsaturated and saturated fatty acids varied between 1 and 1.3. In near-saturating light conditions, the total fatty acid content was higher (22%), and the ratio of unsaturated and saturated fatty acids was lower and varied from 0.8 with 100% air saturation to 1.2 with 250% air saturation and then back to 0.9 with 400% air saturation. In saturating light conditions, the total fatty acid decreased to approximately 17% and, again, no effect from oxygen was detected. In these light conditions, the ratio range between unsaturated and saturated fatty acids remained constant between 1.0 and 1.2. These results indicate that the change in light conditions from sub-saturating to saturating light conditions have an effect on the total fatty acid content but minimal effect on the composition. They also demonstrate that oxygen concentration in the medium does not lead to changes in the fatty acid content and composition.

This is also reflected in Figure 6 where the EPA content (% of total fatty acid) is plotted for the different concentrations of dissolved oxygen. In low light intensity, a relatively high EPA content is ascertained, varying between 11% and 15% while, in near-saturating and saturating light conditions, the content varies between 7% and 10%; no effect of oxygen concentration on the EPA content could be identified.

The effects of light and oxygen concentrations on lipid content in *Nannochloropsis* sp. is reflected in the change of elemental composition (Table 3). The C/N ratio changed from 5.8 in low light intensity to 11.5 in saturating light conditions which coincides with the increase of total lipid content of the cells. No effect from oxygen on elemental composition could be detected. An increase in carotenoid/chlorophyll ratio was also observed in high light conditions (Figure 4). However, the difference in C/N ratios of chlorophyll and carotenoids is marginal, and any change in the ratio of pigments will only minimally contribute to differences in the C/N ratio of the algae.

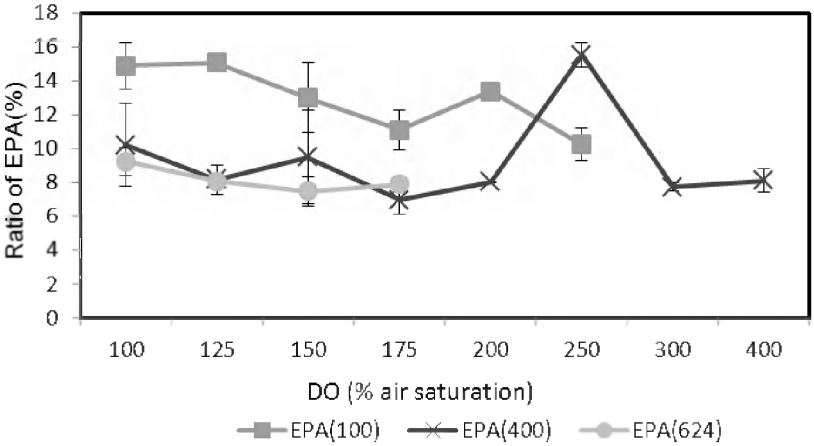

Figure 6 Effects of oxygen concentration on ratio of EPA content of *Nannochloropsis* sp. in different irradiances (100, 400 and 624 µmol m^{-2} s^{-1}).

Table 3 Measured elemental composition of *Nannochloropsis* sp. cultivated in incident light intensity of sub-saturating light (LL) 100 µmol photons m^{-2} s^{-1} and near-saturating light (SL) 400 µmol photons m^{-2} s^{-1} with DO concentration of 100% and 300% air saturation, corrected for 3.63% and 2.23% ash and 3.38% and 2.31% moisture content, respectively.

Oxygen concentration (% air saturation)	Light intensity	Elemental composition (% w/w)			
		C	H	O	N
100	LL	60.97	9.05	19.42	10.56
300	LL	61.29	8.87	19.35	10.48
100	SL	63.48	10.45	19.94	6.13
300	SL	64.31	10.58	20.13	4.98

In sub-saturating light conditions, the PUFA (including EPA) content is relatively high because relatively more thylakoid membrane with a relatively high content of polar lipids is generated (Thomson 1996; Wen and Chen 2003). The oxygen concentration in the medium does not influence the EPA content regardless of the employed light conditions. This indicates that EPA is not involved in the protection of the algae against oxidative stress caused by high oxygen levels in the medium as was suggested by Okuyama et al. (2008) and Triantaphylides and Havaux (2009).

Table 4A Effects of oxygen concentration on the fatty acid composition (% relative fatty acid) of *Nannochloropsis* sp. in sub-saturating light intensity.

Fatty acids	Dissolved oxygen (% air saturation) 100	125	150	175	200	250
Saturated						
14:0	5.90±0.20	8.30±0.10	5.72±0.01	4.89±0.14	4.27±0.03	3.67±0.16
16:0	35.41±0.68	30.33±0.13	26.96±0.01	39.48±0.89	37.05±0.39	43.56±0.02
18:0	0.94±0.11	11.71±0.22	11.21±0.14	1.27±0.14	1.53±0.15	1.26±0.02
%saturated	42.25±0.33	50.34±0.15	43.89±0.05	45.64±0.39	42.85±0.19	48.49±0.06
Unsaturated						
16:1n-7	30.85±0.22	24.11±0.03	19.51±0.09	31.52±0.25	31.90±0.06	29.06±0.30
18:1n-9	4.86±0.23	4.28±0.05	14.67±0.02	7.77±0.26	7.41±0.01	9.95±0.39
18:2n-6	3.94±0.35	5.23±0.03	6.78±0.02	2.38±0.20	2.53±0.05	1.88±0.08
20:3	2.51±0.14	1.64±0.00	1.55±0.01	2.51±0.09	1.93±0.04	1.21±0.09
20:5n-3	15.58±0.32	13.52±0.00	13.61±0.04	11.08±0.66	13.38±0.16	10.25±0.56
%unsaturated	57.74±0.25	48.78±0.02	56.12±0.03	55.26±0.29	56.34±0.06	52.35±0.28
Total	100	100	100	100	100	100
TFA (% w/w)	11.50±1.38	11.45±0.94	11.92±1.81	12.37±0.98	12.74±1.13	16.50±0.06

Table 4B Effects of oxygen concentration on the fatty acid compositions (% relative fatty acids) of *Nannochloropsis* sp. in saturating light intensity.

Fatty acids	Dissolved oxygen (% air saturation)							
	100	125	150	175	200	250	300	400
Saturated								
14:0	5.16±0.18	4.50±0.37	3.96±0.27	3.35±0.16	3.39±0.03	3.97±0.01	3.13±0.00	2.66±0.06
16:0	49.54±1.59	50.97±0.37	46.60±1.86	48.22±1.10	47.07±0.11	41.14±0.42	45.27±0.31	46.92±0.07
18:0	1.36±0.15	1.25±0.06	1.44±0.17	1.67±0.15	1.56±04	1.12±0.04	1.55±0.02	1.76±0.04
%saturated	56.06±0.64	56.72±0.26	52.00±077	53.24±0.66	52.02±	46.23±0.06	49.95±0.14	51.34±0.19
Unsaturated								
16:1n-7	26.77±0.69	27.62±0.33	29.55±0.27	29.99±1.28	30.72±0.10	28.12±0.10	28.78±0.15	28.71±0.64
18:1n-9	3.74±0.02	4.91±0.55	6.18±0.21	7.24±0.18	6.45±0.03	6.00±0.05	7.42±0.01	8.93±0.46
18:2n-6	1.37±0.13	1.23±0.02	1.68±0.16	1.56±0.03	1.48±0.00	2.19±0.05	1.72±0.01	1.63±0.08
20:3	1.85±0.09	1.37±0.15	1.11±0.05	1.02±0.01	1.33±0.13	2.25±0.03	1.59±0.01	1.24±0.01
20:5n-3	10.21±1.43	8.45±0.51	9.48±1.94	6.96±0.51	8.01±0.02	15.20±0.40	10.54±0.19	8.15±0.14
%unsaturated	43.94±0.36	43.58±0.26	48.00±0.50	46.77±0.17	47.99±0.04	53.76±0.11	50.05±0.04	48.66±0.14
Total	100	100	100	100	100	100	100	100
TFA (% w/w)	18.95±1.94	19.65±2.63	28.99±4.86	25.43±0.24	21.78±0.15	12.24±0.67	28.24±0.28	23.57±0.38

Table 4C Effects of oxygen concentration on the fatty acid composition (% relative fatty acids) of *Nannochloropsis* sp. in over-saturating light intensity.

Fatty acids	Dissolved oxygen (% air saturation)			
	100	125	150	175
Saturated				
14:0	4.13±0.05	3.52±0.06	3.50±0.12	3.09±0.06
16:0	43.41±0.05	41.48±0.39	41.70±0.94	41.09±0.02
18:0	1.37±0.02	1.26±0.01	1.28±0.02	1.35±0.00
%saturated	48.91±0.04	46.26±0.15	46.48±0.36	45.53±0.02
Unsaturated				
16:1n-7	31.53±0.37	34.31±0.31	34.65±0.26	34.51±0.05
18:1n-9	6.78±0.05	8.51±0.15	8.50±0.24	9.31±0.02
18:2n-6	2.00±0.08	2.06±0.05	2.10±0.06	2.08±0.04
20:3	1.15±0.01	0.80±0.01	0.80±0.04	0.87±0.03
20:5n-3	9.63±0.34	8.06±0.10	7.48±0.50	7.88±0.50
%unsaturated	51.09±0.17	53.74±0.12	53.53±0.22	54.65±0.12
Total	100	100	100	100
TFA (% w/w)	18.35±2.10	16.50±1.53	15.58±1.14	19.50±2.40

CONCLUDING REMARKS

Although accumulation of oxygen in closed photobioreactors causes severe inhibition of the productivity of *Nannochloropsis* sp., its effect on the fatty acid content as well as on the fatty acid composition and the EPA content is minimal. Elevated concentrations of dissolved oxygen in the medium also only minimally affected the SOD activity, and only when high light intensity combined with high oxygen concentrations was there relatively more carotenoid formation.

These results suggest that oxygen has minor effects on the product quality in terms of lipid content and fatty acid composition of *Nannochloropsis* sp. and only influences the productivity. Control of the oxygen concentration in the medium is required to merely overcome photorespiration effects. Control of light conditions in the photobioreactor below the photosaturation level is required to ensure production of high quality *Nannochloropsis* sp. with relatively high EPA content. Since the inhibition of the growth by oxygen appears to be principally caused by photorespiration effects, the only way to circumvent this growth inhibition is to prevent photorespiration by increasing the CO_2/O_2 ratio in the medium. This is currently achieved by degassing, but it can also be achieved by the addition of extra HCO_3^- (Sousa et al. 2012).

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NOMENCLATURE

I_{in}	Incoming light intensity (μmol photons m^{-2} s^{-1})
I_{out}	Outgoing light intensity (μmol photons m^{-2} s^{-1})
α_{λ}	absorption coefficient (m^2/kg)
C_x	dry weight concentration (kg m^{-3})
ABS_{λ}	measured absorbance of algae (nm)
SCA	Scattering absorbance of algae (nm)
α	absorption coefficient (m^2 g^{-1})
C_x	biomass concentration (g L^{-1})
z	light path (m)
$PFD_{ref}(\lambda)$	spectral photon flux density of the Red light LED source (nm)
$E_{n,PAR}(\lambda)$	the normalized emission (nm^{-1})
α_{PAR}	PAR absorption coefficient ($m^2 m^{-3}$)

ABBREVIATIONS

ROS	reactive oxygen species
SOD	superoxide dismutase
EPA	eicosahexanoic acid
PFD	photon flux density
LED	light emitting diode
PAR	Photosynthetic Active radiation
PUFA	polyunsaturated fatty acid
PSII	PhotoSystem II
NADPH	nicotinaminediphosphate hydrogenase
DO	dissolved oxygen
OD	optical density
GC	gas chromatography
TFA	total fatty acid
MGDG	monoglycerol diacylglyceride
TGA	Triacylglycerol
C14:0	Myristic acid
C16:0	Palmitic acid
C18:0	Stearic acid
C16:1n-6	Palmitoleic acid
C18:2n-6	Linoleic acid
C20:3	Homo-g-linolenic acid
C20:5n-3	Eicosapentaenoic acid (EPA)

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Chapter 5

Effects of dynamic changes in oxygen concentration on *Nannochloropsis* sp. cultured in different light conditions

The contents of this chapter have been submitted for publication as: Raso S, Abiusi F, Vermuë M, Wijffels RH. Effects of dynamic changes in oxygen concentration on *Nannochloropsis* sp. cultured in different light conditions.

ABSTRACT Phototrophic microalgae cultured in closed photo-bioreactors are exposed to accumulating oxygen concentrations which may inhibit the growth of the algae through photorespiration and photo-oxidative inhibition. The oxygen should, therefore, be removed by degassing. This means that the microalgae experience dynamically changing oxygen concentration when cultured in closed photobioreactors. This paper describes the effects of these changes on the growth rate and the fatty acid composition of *Nannochlropsis* sp. in two different light intensities.

The dynamic changes in oxygen concentration from 100% to 300% air saturation followed by subsequent degassing did not result in the expected decrease in the specific growth rate(day^{-1}). The specific growth rate (μ) in sub-saturating light intensity was 0.77±0.07 day^{-1} while μ was 0.76±0.01 day^{-1} with constant oxygen concentration at 100% air saturation. Similarly, in saturating light intensity, μ was 0.90±0.07 day^{-1} with dynamically changing oxygen concentration and 0.91±0.20 day^{-1} at a constant 100% air saturation. The absorption spectrum, the elemental composition, and total fatty acid content revealed that the composition of the algae was similar to the composition of algae cultured with a constant oxygen concentration of 100% air saturation.

These results indicated that oxygen accumulation in closed photobioreactor systems neither seemed to influence the growth nor the composition of the algae if the oxygen was removed by frequent degassing. Oxygen, as such, did not evoke additional photo-oxidative inhibition effects nor lead to extra stimulation of the molecular defense mechanisms; these are only stimulated by the employed light intensity. Obviously, the algae can withstand high oxygen concentrations when regular degassing occurs. As degassing expends energy, it is recommended to find out how much degassing is, in fact, required to suppress the inhibitory effects of oxygen.

KEYWORDS: Dynamic changes in oxygen concentration, specific growth rate, fatty acid composition, elemental composition and *Nannochoropsis* sp.

INTRODUCTION

Microalgae are considered to be one of the most promising renewable alternative feedstocks for replacing fossil fuels (Georgianna and Mayfield 2012, Wijffels et al. 2011). One of the candidates is the marine Eustigmatophyte, *Nannochloropsis* sp. which is currently mainly produced for its substantial nutritional quality of feed for fish and shellfish larvae (Benemann 1992; Duerr et al. 1998; Lubzens et al. 1997; Muller-Feuga 2000). The relatively high growth rate combined with high lipid content also make the algae a potential feedstock for biodiesel production and other bio-based commodities (Rodolfi et al. 2008; Schenk et al. 2008).

For large-scale outdoor production of *Nannochloropsis* sp., controlled, closed photobioreactors should be exploited to guarantee increased productivity in combination with consistent product quality (Zittelli et al. 1999; Cheng-Wu et al. 2001). In these closed systems, however, the microalgae are exposed to high levels of oxygen that is produced during photosynthesis. Continuous removal of oxygen by degassing is essential as excessive dissolved oxygen in the broth inhibits algae growth (Vonshak et al. 1996; Kliphuis et al. 2011; Sousa et al. 2012; Raso et al. 2012a, 2012b). For *Nannochloropsis* sp. cultivated in sub-saturating light conditions, it was ascertained that the specific growth rate decreases from 0.40±0.10 day^{-1} with a constant oxygen level of 100% air saturation to a growth rate of 0.18±0.08 with 250% air saturation and ceased at a growth rate of 0.02±0.01 day^{-1} with 300% air saturation (Raso et al. 2012a). These studies demonstrated that, in sub-saturating light conditions, oxygen inhibits the growth of *Nannochloropsis* sp. primarily during photorespiration. In higher light intensities, high oxygen levels induced additional photo-inhibition effects and stimulated the molecular defense mechanisms against photo-oxidative stress in the algae (Raso et al. 2012b).

The above mentioned studies on effects of oxygen on growth and molecular defense mechanisms of *Nannochloroposis* sp. were performed in constant controlled conditions of light and oxygen. In closed photobioreactors, however, microalgae do not experience constant oxygen levels but are subjected to dynamically changing oxygen concentrations. In tubular photobioreactors, for example, the algae experience an oxygen concentration of approximately 110% air saturation when exiting the degasser unit. Due to photosynthesis, the oxygen level in the tubes increases to levels of nearly 300% air saturation at the end of the tube and then the concentration sharply decreases to levels of 110% in the degasser unit.

Thus far, no information is known about the effects of these specific dynamic changes in oxygen concentrations on the growth and composition of *Nannochloropsis* sp. in various light intensities. To investigate these effects, algae were cultivated at constant controlled conditions of light, temperature, and pH employing a 1.5 L flat-panel photobioreactor in turbidostat mode while being exposed to a gradual increasing oxygen concentration from 100% to 300% air saturation followed by a quick decrease in oxygen concentration during degassing. The results of this study demonstrate the manner in which the algae respond to sudden alterations of oxygen in the medium and how the accumulation of oxygen in the medium will influence the overall productivity of *Nannochloropsis* sp. and the cell composition just as they would experience in large scale photobioreactors.

MATERIALS AND METHODS

Organism, culture conditions and medium preparation

Nannochloropsis sp. (CCAP 211/78) was pre-cultured in a shaking incubator (100 rpm) with a specified light dark cycle of 18:6 h, a light intensity of 41 μmol photons m^{-2} s^{-1} at pH 7.8, and with 2% of CO_2 enriched air for one week before inoculation of the flat panel photobioreactor. Modified Zou medium (Raso et al 2012a) was employed for pre-culturing and for growth of *Nannochloropsis* sp. in the photobioreactor. To prevent unwanted precipitation of nutrients, a stock solution of trace elements Fe-EDTA as well as the phosphate buffer solution was autoclaved separately prior to mixing with the other medium components and adjusting the pH to 7.8.

Photobioreactor set-up and light conditions

A flat-plate photobioreactor was constructed as illustrated in Figure 1 and filled with filter-sterilized modified Zou medium.

After inoculation, the algae were grown batch-wise for nine days in controlled culture conditions at pH 7.8, temperature (25ºC) and a total gas flow rate of 1.0 L L^{-1} min^{-1} with a dissolved oxygen (DO) concentration of 100% air saturation. A Red-Light Emitting Diode (Red-LED) (PSI-Photon Systems Instruments, Czech Republic) was utilized to provide continuous and homogeneous illumination with an average wave length of 637 nm. The light intensity at the back of the flat-panel photobioreactor was measured with a light sensor (LICOR-190, Czech Republic) which was connected to a control unit that controlled the medium in and outflow pumps to set the turbidity of the culture.

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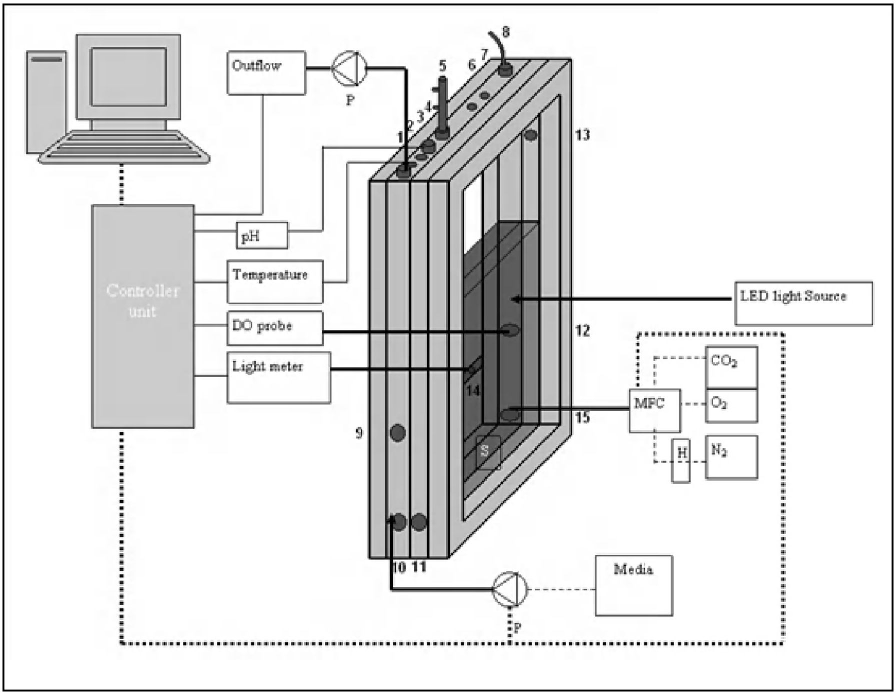

Figure 1 Schematic drawing of the experimental set-up of the flat-panel photobioreactor in turbidostat mode: (S = sparger, P = peristaltic pumps, H = gas humidifier, MFC = mass flow controllers for carbon dioxide (CO_2), nitrogen (N_2) and oxygen (O_2)
(1 = medium outflow, 2 = temperature probe, 3 = inoculum port, 4 = pH probe, 5 = condenser, 6,7 = spare ports, 8 = Antifoam unit, 9 = sampling port, 10 = medium inflow, 11 = water bath inlet, 12 = oxygen probe, 13 = outlet to water bath, 14 = light sensor, 15 = gas distributor).

Possible accumulation of foam was controlled by an automatic foam control unit. Before entering the flat panel photobioreactor, only nitrogen incoming gas flow was humidified, and the outgoing gas flow was fed through a condenser to prevent evaporation of the medium.

Following the batch-wise growth and adaptation phase at a DO of 100% air saturation, the flat-panel photobioreactor was switched to turbidostat mode. An average incident light irradiance of 100 µmol photons m^{-2} s^{-1} was employed to create sub-saturating light conditions, and 400 µmol photons m^{-2} s^{-1} was used for saturating light conditions (Raso et al. 2012b). Lambert Beers' Law was used to calculate the light gradient, and the light at the back was controlled at 12 µmol photons m^{-2} s^{-1} and 48 µmol photons m^{-2} s^{-1}, respectively, to ensure that the light gradient was the same in both light conditions. The photobioreactor was operated at turbidostat mode for 5 days with a constant O_2 concentration of 100% air saturation followed by 5 days with dynamically changing O_2 concentration before switching to another light intensity. The dissolved oxygen levels were monitored by a DO sensor (Applisens DO Sensor 15 mm). During all experiments, a constant total gas flow rate of 1.0 L L^{-1} min^{-1} was applied by a controlled supply of N_2, CO_2, and O_2.

To mimic the dynamic change in oxygen concentration that occurs in large scale tubular photobioreactor systems, the oxygen concentration was allowed to build up to 300% air saturation followed by degassing. Table 1 exhibits the time duration during oxygen build-up and degassing during the turbidostat operation.

Table 1 Times used for build-up oxygen from 100% to 300% air saturation (Ti) and time used for degassing from 300-100% air saturation (Td) in two different light intensities.

Light conditions	**Ti (s)**	**Td (s)**
Sub-saturating light	1240	140
Saturating light	365	140

Growth monitoring and off-line biomass analysis

During the turbidostat operation, the output flow of biomass was recorded and, after three days when the culture had achieved steady state conditions, the specific growth rate of the algae (μ) was determined from the dilution rate (D) for at least 3 days. Samples of approximately 100 mL were taken aseptically each day during the subsequent turbidostat runs to measure the optical density (OD_{530} for biomass and OD_{680} for chlorophyll), the cell concentration (g DW L^{-1}), the PAR spectrum, and the elemental and the fatty acid composition off line.

The ratio of OD_{680}/OD_{530} was employed as an indicator for the amount of chlorophyll per amount of biomass. A ratio above 1.0 indicates that the cells are healthy and that no contamination from bacteria or other non-algal micro-organisms occurred.

Determination of cell concentration and absorption coefficient

To measure the cell concentration (g DW L^{-1}), triplicate samples of the algal culture were filtered using pre-weighed glass microfiber filters (Whatman, United Kingdom). The algae were washed three times with 0.5M ammonium formate to remove precipitated salts and other non-organic substances, dried at 105ºC for 24h, cooled over silica gel in a desiccator, and weighed.

The effect of dynamically changing oxygen concentration on the specific absorption coefficient (α in m^2 g^{-1}) of *Nannochloropsis* sp. and the pigmentation of the cells was determined from the complete PAR spectrum (400-700 nm) according to Raso et al. (2012a).

Elemental composition

Algal samples were centrifuged for 10 minutes at 4500 rpm and washed three times with de-mineralized water by re-suspending and centrifuging. The algal pellets were stored at -20°C prior to freeze drying. The freeze dried algae were ground, and the algal powder was combusted at 925°C in the elemental analyzer (EA 1110, ThermoQuest CE Instruments) to measure C, H, and N content. A sample of the freeze dried algal powder was equilibrated at ambient condition for 6 hours, and the residual moisture was determined from the difference in the dry weight of the sample and the same sample dried for 24h in an oven at 105°C. Ash content was determined by burning the freeze-dried algae samples in an oven at 550°C. From these determinations, the O content and the subsequent dry biomass molar mass (g mol^{-1}) could be calculated (C, H, O, N, and ash) (Duboc et al. 1999).

Lipid content and fatty acid analysis

Prior to lipid content and fatty acid analysis, fresh algae samples were centrifuged at 3000 rpm for 15 minutes using a Beckman Coulter (AllegraTm X-22R centrifuge, Germany) and stored at -80ºC. For analysis of the lipid content and fatty acid composition, the lipid extraction and transesterification method described by Rodriguez-Ruiz et al. (1998) was employed. The frozen pellet of microalgae was re-suspended in 3 mL of a (2:1) mixture of methanol (HPLC grade), acetyl chloride (98%, Aldrich 29042-010), and 2 mL hexane including nonadecanoic acid methyl C19:0 as an internal standard. The tubes were incubated at 122°C for 30 minutes and subsequently cooled to room temperature. An aliquot of the top phase was used for gas chromatography (GC) analysis of the fatty acid composition. GC analysis was conducted with a Nukol^{TM} Column (30 m x 530 µm x 1.0 µm). The initial oven temperature was 90°C followed by a temperature program of 20°C min^{-1} to 200°C. The injector temperature was 250°C, flame ionization detector temperature 270°C, and a split ratio of 0.1:1 was used. Fatty acid concentrations were calculated by comparing each peak area with that of the internal standard and corrected accordingly.

RESULTS

Effect of dynamically changing oxygen concentration on the growth rate

The effect of dynamic changes in dissolved oxygen concentration on the specific growth rate of *Nannochloropsis* sp. was measured and compared with the growth rate measured with a constant controlled DO level of 100% air saturation, in both sub-saturating light conditions (LL) and saturating light conditions (SL). During the experiment with dynamically changing oxygen concentrations, the algae were subjected to a dissolved oxygen level which was gradually increased from 100% to 300% air saturation followed by a rapid decrease to 100% air saturation. The average oxygen concentration experienced by the algae would thus be approximately 200% air saturation. With this concentration, microalgae are expected to be inhibited by oxygen (Vonshak et al. 1996; Kliphuis et al. 2011; Sousa et al. 2012; Raso et al. 2012a, 2012b). Raso et al. (2012) demonstrated that the growth rate of *Nannochloropsis* sp.

linearly decreased with oxygen concentration in sub-saturating light conditions. They also investigated the effect of different constant oxygen concentrations on the growth rate of *Nannochloropsis* in saturating and over-saturating light conditions and discovered a sharp decrease in the growth rate when increasing the oxygen concentration from 100% to 200% air saturation (Raso et al. 2012b). This inhibiting effect of the oxygen concentration on the growth rate was not ascertained in these experiments.

The specific growth rate of *Nannochloropsis* sp. with the dynamically changing oxygen concentration was 0.77±0.07 day^{-1} (Figure 2), and it was not significantly different from the specific growth rate of 0.76±0.01 day^{-1} at a constant DO level of 100% air saturation. In saturating light intensity, the growth rate increased to 0.90±0.07 day^{-1}, however, again, there is no significant variance with the specific growth rate of 0.91±0.20 day^{-1} ascertained at a constant DO level of 100% air saturation.

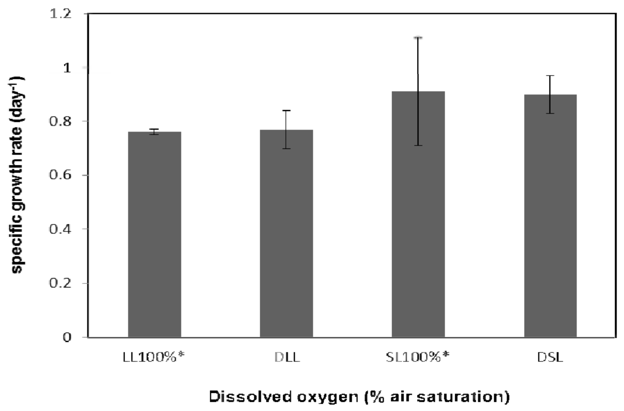

Figure 2 Effects of dynamic change in oxygen concentration on the specific growth rate (day^{-1}) of *Nannochloropsis* sp. in different light conditions in photobioreactor. Note: * data from Raso et al. 2012a.

Effect of dynamically changing oxygen concentration on light absorption and elemental composition of the algae

To ascertain if the dynamically changing oxygen concentration evoked additional changes in the light absorbance by the algae, the cell dry weight concentration and cell absorption coefficient were determined off line. Table 2 depicts the cell concentration (g DW/L) as well as the absorption coefficient α (m^2 g^{-1}) of the algae cultured with dynamically changing oxygen concentrations and of the algae cultured with a constant 100% air saturation. With a constant oxygen concentration of 100% air saturation and low light conditions (LL100%), the cell dry weight was 0.32±0.02 g L^{-1} while it increased to 0.65±0.09 g L^{-1} in saturating light conditions (SL100%). This increase in biomass concentration in higher light irradiance is the result of photoadaptation of the cells. In high light conditions, the cells form less pigments per cell, and this results in a lower absorption coefficient (Table 2).

Table 2 The effects of dynamic change in O_2 concentration on the cell concentration and absorption coefficient α of *Nannochloropsis* sp. in different irradiances. LL100%* and SL100%* represent data obtained with dissolved oxygen concentration of 100% air saturation in sub-saturating and saturating light intensity, respectively. DLL and DSL are data obtained with dynamically changing oxygen concentration in sub-saturating and saturating light intensity, respectively.

Dissolved oxygen conc. (% air saturation)	Cell concentration (g DW L^{-1})	Absorption coefficient α (m^2 g^{-1})
LL100%*	0.32±0.02	0.16±0.01
DLL	0.38±0.02	0.15±0.01
SL100%*	0.65±0.09	0.11±0.02
DSL	0.61±0.03	0.11±0.02

Note: * data from Raso et al. 2012a

As the photobioreactor was operated at turbidostat conditions, a decrease in absorption coefficient inevitably leads to a higher biomass concentration.

By dynamically changing oxygen concentration both in low light (DLL) and in high light conditions (DSL), the dry weight and the absorption coefficients were similar to those measured with a constant oxygen concentration of 100% air saturation (Table 2). This indicates that the absorption of light by the algae was only minimally affected by the changing oxygen concentrations in the medium.

To verify if the dynamic change in oxygen concentration affected the composition of the cells, the elemental composition of *Nannochloropsis* sp. was determined as well. Table 3 exhibits that the dynamically changing oxygen concentration induced no changes in elemental composition compared with the composition measured with a constant oxygen concentration of 100% air saturation as long as the same light intensity was applied. At saturating light conditions, however, the C/N ratio was approximately two times higher than in sub-saturating light conditions. The full PAR absorption spectra measured in both light conditions revealed.

Table 3 Effects of oxygen concentrations on elemental composition of *Nannochloropsis* sp. in different light intensities (LL = sub-saturating light condition of 100 μmol photons m^{-2} s^{-1} and SL = saturating light condition of 400 μmol photons m^{-2} s^{-1}).

Elements	DO (100% air saturation)		DO (300% air saturation)	
	LL	SL	LL	SL
C (mole)	1.77	1.95	1.74	1.95
N (mole)	0.15	0.08	0.15	0.07
O (mole)	0.24	0.24	0.24	0.23
CHNO (g mol^{-1})	19.70	18.92	19.60	18.67

that this change should be attributed to the lower chlorophyll and higher carotenoid content of the cells cultured in saturating light conditions (data not shown). As carotenoids do not contain nitrogen, as do the chlorophyll molecules, a higher C/N ratio in saturating light conditions is found.

Effect of dynamic oxygen concentration on fatty acid composition

The increase in the C/N ratio in high light conditions could also be attributed to an increase in lipid content and a possible change in fatty acid composition. In addition, oxygen may affect the composition of the algae. Especially in high light intensities, the unsaturated fatty acids can be involved in photo-oxidation (Okuyama et al. 2007 and, additionally, their formation may be induced as they can serve as molecular protection against photo-oxidation (Triantaphylides and Havaux 2009. Therefore, the lipid content and fatty acid composition of *Nannochloropsis* sp. was measured off-line.

Figure 3A demonstrates that the high light conditions indeed result in higher lipid content, but there is no significant difference in the lipid content of algae cultivated with constant low oxygen concentration (100%) and the lipid content of algae growing at dynamically changing oxygen concentration. The fatty acids composition also changed only with the light. The poly-unsaturated fatty acid (C18:2, C20:3 and C20:5) composition remained constant, and only an increase in C16:0 and C16:1 was observed when increasing the light intensity (Figure 3B). The fatty acid composition was not affected when changing from oxygen with a constant concentration of 100% air saturation to dynamically changing oxygen concentration.

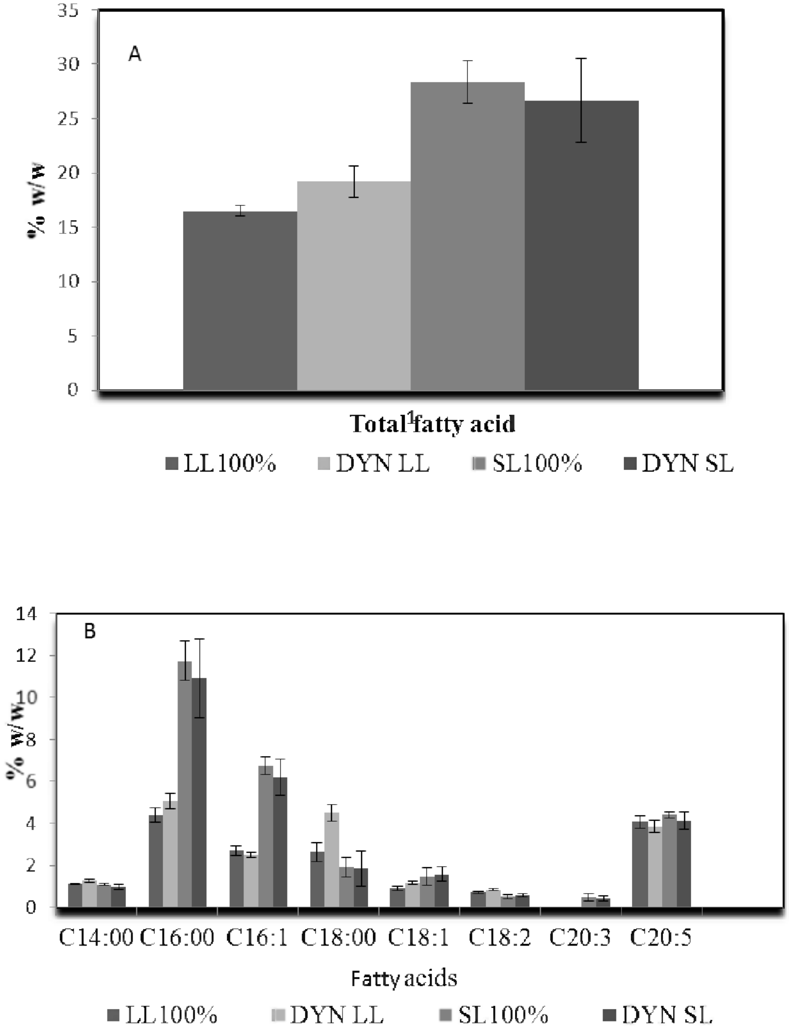

Figure 3 Effects of dynamic change in oxygen concentration on fatty acid composition of *Nannochloropsis* sp. cultured in different light intensities (A; total fatty acid content: B; fatty acid composition).

Only the C18:0 content increased in low light conditions and dynamically changing oxygen conditions, however, this is probably an artefact as it is not observed in elevated light conditions. In addition, an increase in C18:0 would be accompanied by a decrease in either the C16:0, the C18:1, or the C18:2 fraction as they are involved as precursor and successors, respectively, in the fatty acid synthesis pathway (Merchant et al. 2012).

DISCUSSION

Effects of dynamic oxygen concentration on growth of algae

Many studies indicate that oxygen affects the growth rate of micro algae through either photorespiration (Kliphuis et al. 2011; Raso et al. 2012a; Sousa et al. 2012) or a combined effect of photorespiration and photoinhibition (Raso et al, 2012b; Ugwu et al. 2007). Based on these studies, it has been suggested to maintain an oxygen level below 150-200% air saturation to ensure increased productivity (Molina Grima et al. 2001; Krompkam et al. 2009). All of the studies mentioned were performed with constant oxygen concentrations. In this study, however, it was ascertained that the expected decrease in the growth rate of *Nannochloropsis* sp. did not occur when applying oxygen concentration that gradually changed from 100% air saturation to 300% air saturation followed by subsequent degassing. In both sub-saturating and saturating light intensities, the specific growth rate of *Nannochloropsis* sp. was not significantly different from the growth rate measured with a constant low oxygen concentration of 100% air saturation.

Our findings indicate that the frequent oxygen removal by degassing is sufficient to prevent negative effects of the O_2 that accumulates inside closed photobioreactors in large-scale algal production systems. While the cells suffer from competitive inhibition of Rubisco with elevated oxygen concentrations (Raso et al. 2012a), they do apparently respond in a different manner to the average high oxygen concentration imposed by dynamically changing oxygen concentrations in the medium. It is possible that the carbon concentrating mechanism of *Nannochloroposis* sp. is responsible for this effect. The low ratio of CO_2/O_2 around the Rubisco stimulates the active absorption of inorganic carbon in the form of CO_2 or HCO_3^- from the medium. In *Nannochloropsis* sp., carbonic anhydrase activity converts much of the accumulated HCO_3^- to CO_2, concentrating this substrate around Rubisco, thereby optimizing photosynthetic efficiency (Raven 2003). During the 140 s of degassing, the oxygen diffuses from the cells into the medium while the carbon anhydrase activity remains high. During the degassing phase, the local CO_2/O_2 ratio will increase, resulting in a higher carboxylase activity of the Rubisco. During constant high oxygen concentrations, the carbon concentrating mechanism is active as well, however, without the stimulation of the carboxylase activity during the degassing phase, the observed growth rate of the cells is much lower (Sousa et al. 2012.

Effects of dynamic oxygen concentration on the algal composition

Neither the elemental composition of the algal cells (Table 3) nor the measured PAR spectrum was affected by the surplus of oxygen in the medium imposed by the dynamically changing oxygen concentrations. This indicates that no extra carotenoids were formed to protect the cells against any additional oxidative stress that were expected to occur with the applied dynamic oxygen concentrations (Ledford and Niyogi 2005; Zittelli et al. 1999). Only elevated light conditions evoked an increase in the carotenoid content of the cells which indicates that the cells only exhibited photoadaptation.

Furthermore, the fatty acid composition and lipid content remained unchanged with the dynamically changing oxygen concentrations (Figure 3A and 3B). This again demonstrates that oxygen, as such, did not influence the total lipid content or the fatty acid composition. Only in higher light conditions was an increase of the C16:0 and C16:1 content observed, indicating that light induced lipid synthesis in the form of neutral triglycerides (TAG). TAGs are generally formed by algae that experience light stress and consist of saturated and monoenoic C14 and C16 fatty acids (Kliphuis et al. 2012; Tonon et al. 2002; Sukenik et al. 1989). The content of polyunsaturated fatty acids such as EPA (C20:5) remained constant at around ~4% w/w (Zittelli et al. 1999). This indicates that the quality in terms of EPA content of the *Nannochloropsis* sp. is not affected by the oxygen accumulation that occurs in closed photobioreactors in large scale production systems.

CONCLUDING REMARKS

The dynamically changing oxygen concentrations that are imposed on *Nannochloropsis* sp. cultured in closed photobioreactors have no net effect on the growth rate and on the composition of the *Nannochloropsis* sp. in sub-saturating light intensity and saturating light intensity. As long as the oxygen is removed through regular degassing, the cells will be capable of coping with the accumulation of oxygen in the medium within closed systems. Degassing units are, therefore, required in large scale microalgae production systems to remove the accumulated oxygen. It is, however, still unclear how frequent the algal culture needs to be degassed. As the removal of oxygen in the degasser expends significant energy, it is recommended to investigate how the frequency of degassing can be limited while concurrently not compromising volumetric productivity.

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ABBREVIATIONS

PUFA	Polyunsaturated fatty acids
CCM	Carbon concentrating mechanism
DO	Dissolved oxygen
LED	Light emitting diode
D	Dilution rate
GC	Gas chromatography
TFA	total fatty acid
TAG	Triacylglycerol
C14:0	Myristic acid
C16:0	Palmitic acid
C16:1n-6	Palmitoleic acid
C18:0	Stearic acid
C18:1	Oleic acid
C18:2n-6	Linoleic acid
C20:3	Homo-g-linolenic acid
C20:5n-3	Eicosapentaenoic acid (EPA)

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Chapter 6

General Discussion and Future Prospective

INTRODUCTION

Microalgae are mainly commercially cultivated to be exploited as a supplement in food, cosmetics, and as feed in aquaculture, but they are gaining increasing attention as they are regarded as an attractive source of bio-based renewable feedstock for fuels, chemicals, materials, and commodities for food and feed (Wijffels et al. 2011; Wijffels and Barbosa 2010). The latter applications require large-scale production of microalgae and, in order to achieve this, cultivation in closed photobioreactors such as tubular and flat panel photobioreactors and raceway ponds is required (Zittelli et al. 1999; Tredici 2010; Molina Grima et al. 2001). In these systems, however, accumulation of oxygen almost certainly occurs, and this inhibits the growth of algae resulting from photorespiration (Kliphuis et al. 2011; Raso et al. 2012; Sousa et al. 2012), photoinhibition, and photo-oxidative damage (Mallick and Mohn 2000; Sousa et al. 2013a).

Photorespiration occurs in the dark reaction of the photosynthesis at a high O_2/CO_2 concentration ratio (Wingler et al. 2009) and is independent of the employed light intensity. At relatively high carbon dioxide levels, the Rubisco enzyme involved in the Calvin Cycle mainly catalyzes the fixation of CO_2 to ribulose-1,5-bisphosphate (RuBP) which results in production of 2 molecules 3-phosphoglycerate (1,3bPGA). When relatively high levels of oxygen are present, however, the Rubisco catalyzes the oxidation of RuBP and photorespiration subsequently occurs. This oxidation reaction yields one molecule of 1,3bPGA and one molecule of 2-phosphoglycolate (Chapter 2). With high oxygen concentrations, less carbon dioxide is bound in the Calvin Cycle of the photosynthesis, and more energy is expended to produce the same amount of building blocks for biomass formation.

Most microalgae have developed a tolerance to high O_2 levels by changing the O_2 affinity for the oxygenase activity of Rubisco or by developing some type of Carbon Concentrating Mechanism (CCM) to maintain a sufficiently high CO_2 concentration within the vicinity of the Rubisco enzyme (Tchernov et al. 2008).

When the light intensity is increased, not only photorespiration occurs but also photoinhibition and photo-oxidative damage takes place. In fact, light plays a dual role in algal cultivation. On the one hand, light is required for photosynthesis and, on the other hand, inhibits the growth of microalgae through photoinhibition and photo-oxidative damage (Ugwu et al. 2007). The same holds true for oxygen. On the one hand, oxygen is crucial in supplying energy and maintaining sufficient levels of 1,3bPGA for the Calvin Cycle in times of darkness, and, on the other hand, it inhibits cell growth.

Many studies have been conducted on the combined effects of oxygen and light on algal growth. The effect of elevated oxygen levels on the algal growth, however, has only been investigated in limited studies, and most of these were only performed in low light intensity (Vonshak et al. 1996; McMinn et al. 2005; Kliphuis et al. 2011; Raso et al. 2012; Sousa et al. 2012; Sousa et al. 2013a). In this thesis, the effect of oxygen on growth and cellular composition of *Nannochloropsis* sp. was investigated in different light conditions, and the concentration of oxygen at which it begins to inhibit the algal growth was quantified. Currently, the energy required for mixing and degassing is one of the main factors that makes outdoor large-scale applications of these closed photobioreactors for bulk production not economically feasible (Norsker et al. 2011). By quantifying the effect of elevated oxygen concentrations on algal growth, it is possible to ascertain at what point the algae actually require degassing. This knowledge can help to keep the overall productivity of the algae sufficiently high while still expending minimal energy on degassing and mixing to remove the oxygen.

In this discussion chapter, the effect of oxygen on the growth of *Nannochloropsis* sp. in various light conditions will be briefly reviewed and strategies toward further research to elucidate the effect of oxygen will be discussed. In addition, new strategies to change the design of photobioreactor systems to reduce the overall energy required for degassing will be examined.

Effect of oxygen on growth and quantum yield of *Nannochloropsis* at low light conditions

The impact of elevated oxygen levels on the growth of *Nannochloropsis* sp. was investigated in low light intensity at fully controlled cultured conditions in a flat panel photobioreactor operated as turbidostat. Upon increasing the dissolved oxygen (DO) concentration from 20% to 75% air saturation, no clear effect of oxygen on the specific growth rate of *Nannochloropsis* sp was observed. The growth rate showed a mean average value of 0.48±0.04 day^{-1}. When further increasing the oxygen concentration, the specific growth rate of *Nannochloropsis* sp. linearly decreased from 0.49±0.10 day^{-1} at DO with 75% air saturation to 0.18±0.01 day^{-1} at DO of 250% air saturation. These results were translated in terms of quantum yield, and the obtained values for the *in vivo* quantum yield were compared with predicted values for isolated Rubisco. As expected, the *in vivo* quantum yields were lower than the predicted values. The surplus of ions in hypertonic cellular medium affects the solubility of CO_2 more than it affects the solubility of oxygen. The CO_2/O_2 ratio at the Rubisco in the living cell will thus be lower than in the medium, causing a lower ratio between the carboxylation/oxygenation and thus a lower *in-vivo* quantum yield. The decrease in *in vivo* quantum yield measured with increasing oxygen concentrations, however, was similar to the decrease in the predicted quantum yield for the isolated Rubisco (chapter 2). This indicated that no additional damage from photoinhibition or photooxidative occurred in the employed low light intensity and that only photorespiration was responsible for the reduced productivity with high oxygen concentrations.

These photorespiration effects can be overcome by increasing the CO_2 concentration within the vicinity of the Rubisco. Recently, Sousa et al (2013a) indeed demonstrated that an increase in the CO_2/O_2 level in the medium restored the algal growth rate of *Neochloris oleoabundans*. Further research is required to test if this method can also be employed to overcome the photorespiration effects in *Nannochloropsis* sp..

Effect of oxygen on growth of *Nannochloropsis* at high light conditions

In photosynthesis, oxygen production is directly linked to the light irradiance. Higher light intensities increase photosynthesis, and this results in a higher oxygen production rate. The oxygen accumulation in higher light irradiance conditions not only causes additional photorespiration effects but will also lead to photoinhibition and photoxidative damage.

The synergetic effects of oxygen and light on the growth of *Nannochloropsis* sp have been investigated in a fully controlled flat panel photobioreactor operated at turbidostat mode, and the effects of elevated oxygen concentration were quantified in different light conditions (Chapter 3). This reactor set-up provided the possibility to study the effects of oxygen independently from the light in three different incident light intensities of 100, 400, and 624 μmol photons m^{-2} s^{-1}.

In all light intensities, a decrease in the specific growth rate (µ day^{-1}) was observed when increasing DO concentrations. In sub-saturating light conditions, a linear decrease in the growth rate from 0.40±0.10 to 0.18±0.01 day^{-1} was observed with increasing DO levels from 100% to 400% air saturation. In saturating light conditions, a sharp decrease in the growth rate from 0.86±0.20 to 0.35±0.10 day^{-1} followed by a gradual decrease to 0.28±0.08 day^{-1} was ascertained at DO levels ranging from 100% to 400% air saturation. A similar pattern was observed in over-saturating light conditions with a sharp decrease in the growth rate followed by a gradual decrease from 0.37±0.06 to 0.17±0.02 day^{-1} at DO concentrations ranging from 100% to 175% air saturation.

In high light intensity, a surplus of electrons is formed at PSII and transported to PSI where oxygen can accept the electrons to produce superoxide radicals. To address these harmful superoxide radicals, the enzyme SOD in the water-water cycle is activated (Textbox 1). Together with the other enzymes in the water-water cycle, SOD protects the cells by scavenging the superoxide radicals in a series of reactions to form water. Our experiments demonstrated that the SOD activity was indeed stimulated in the higher light conditions. Upon increasing the oxygen concentration in constant high light conditions, however, the SOD activity remained the same. Therefore, the oxygen concentration, as such, did not influence the SOD activity; only the high light conditions stimulated the enzymes in the water-water cycle.

In high light conditions, oxygen is not only involved in the formation of oxygen radicals and other reactive oxygen species. H_2O_2 formed in the water-water cycle, but oxygen is also transformed into highly reactive oxygen singlet (${}^1O_2$).

Textbox 1 Water-water cycle

The water-water cycle is a defense mechanism evident in microalgae against the reactive oxygen species formed in high light intensity. At photosystem II (PSII), water within the chloroplast is oxidized by light energy to form O_2 while electrons are released. In a series of subsequent enzyme reactions, oxygen absorbs the excessive electrons delivered at Photosystem I and forms superoxide radicals O_2^-. The reactive oxygen radicals are then transformed into hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). The formed H_2O_2 is turned into water during a reaction in which the thylakoid-bound ascorbate peroxidase (tAPX) participates (Perelman et al. 2006; Ishikawa et al. 2009). This enzyme reduces Ascorbate (AsA) to monodehydro-ascorbate (MDA). The MDA radicals produced by APX are converted to ascorbate (AsA) through the reaction with ferredoxin (Fd) (thylakoid). The ferodoxin (Fd) finally catalyzes the regeneration of AsA.

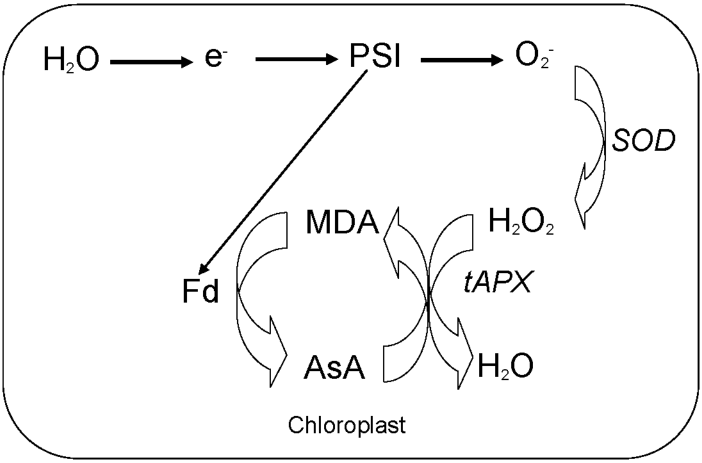

Figure 1 The water-water cycle occurs in the chloroplast of microalgae (adapted from Mittler 2002).

In high light, the photosynthetic reaction center in the chlorophyll (P680) absorbs the light energy and causes photo-activation in PSII, eventually resulting in formation of triplet chlorophyll $^3P_{680}^*$(Textbox 2). Its energy is used to generate the highly reactive singlet oxygen $^1O_2^·$. The cells are protected against singlet oxygen by the carotenoids (^{3}Car*) which can quench the singlet oxygen (Textbox 2) (Pinto et al. 2000), but in the event that a surplus of light energy is supplied, the oxygen singlet will cause photo-oxidative damage. The carotenoid/chlorophyll ratio in the *Nannochloropsis* indeed increased when increasing the light intensity (Chapter 4), indicating that more carotenoids were formed to protect the algal cells against the photo-oxidative damage by singlet oxygen. However, the oxygen concentration, as such, did not seem to affect the carotenoid content of the algal cell. Only in high light combined with high oxygen was minimal increase in carotenoid content observed. These results indicate that high light indeed stimulates formation of singlet oxygen but that higher oxygen concentration in the medium did not result in a significant increase in formation of singlet oxygen and does not result in additional photo-oxidative damage.

Textbox 2 Formation of singlet oxygen

During daylight, visible light is absorbed by the photosynthetic reaction center in the chlorophyll (P680) in its ground state (S_o electronic state), and this results in excitation of P680 to its singlet form ($^1P680^*$) (S_1 electronic state). When high light conditions are applied, singlet-singlet energy transfer to neighboring chlorophyll is limited and triplet P680 ($^3P680^*$) is formed. The triplet excitation energy is transferred to molecular oxygen in its original triple ground state ($^3O_2$) via the formation of highly reactive singlet oxygen ($^1O_2$). The singlet oxygen can also be quenched via transfer to the triplet carotenoids ($^3Car^*$). In the event of a surplus of energy supply, the quenching is not efficient enough, and the oxygen singlet will cause photo-oxidative damage, particularly to the photosynthetic active pigments within its vicinity.

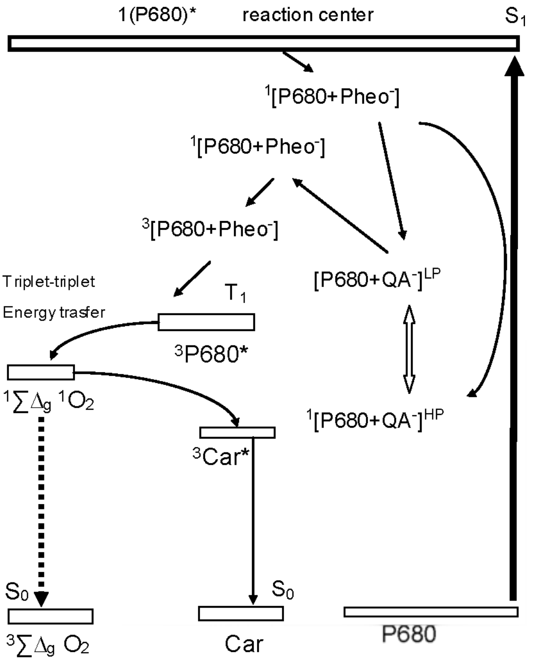

Figure 2 Formation of highly reactive singlet oxygen at high irradiance (adapted from Pospišil 2011).

Effects of oxygen on the composition of *Nannochloropsis* at high light conditions

The PUFAs have been mentioned in literature as an antioxidant to protect the photosynthetic pigments in cells against oxygen radicals and singlet oxygen (Okuyama et al. 2007, Triantaphylides and Havaux 2009). Therefore, the effect of oxygen on the FA composition was monitored in all applied circumstances. The results clearly indicated that the fatty acid composition was not influenced by elevated oxygen levels. Light played a far more important role regarding the fatty acids. This indicates that EPA is not involved in the protection of *Nannochloropsis* sp. against oxidative stress caused by high oxygen levels in the medium. In high light intensity, greater amounts of saturating FA where found, while the PUFA content remained the same. The quality of the algae in terms of the PUFA content did not vary when the oxygen concentrations increased but was only influenced by the light intensity (Chapter 3).

To achieve high productivity, light must be optimized to cultivate algae in sub-saturating light conditions inside the photobioreactors. These optimal light conditions are influenced by the biomass concentration in the tubes as these determine the light gradient in the tube. This implies that the biomass concentration is a parameter that should be controlled in large-scale production systems. Future research should be conducted to determine the biomass concentration that combines high growth rate with optimal composition of the algae in various incident light conditions.

Effects of dynamically changing oxygen on the growth of *Nannochloropsis*

In practice, algae cultivated in tubular photobioreactors do not experience constant oxygen concentrations. They experience dynamically changing oxygen concentrations with oxygen levels that gradually increase from 100% to about 300% air saturation. When algae reach the end of the tubes, they enter the degasser unit where quick removal of the oxygen occurs, and the oxygen level falls back to normal levels between 100% and 120 % air saturation (Molina Grima et al. 2001). The average oxygen concentration experienced by the algae in a tubular photobioreactor is thus expected to be approximately 250% air saturation. With a constant oxygen concentration of 250% air saturation and sub-saturating light conditions, we discovered that the growth of *Nannochloropsis* sp. was severely inhibited (Chapter 3). This inhibiting effect of the oxygen concentration on the growth rate, however, was not found when dynamically changing oxygen concentrations were applied (Chapter 5). The specific growth rate of *Nannochloropsis* sp. was 0.77±0.07 day^{-1} in sub-saturating light condition when dynamically changing oxygen concentrations were applied. The oxygen concentration gradually increased from 100% to 300% in 1240 seconds and subsequently decreased to 100% air saturation in 140 seconds. The measured growth rate was not significantly different from the specific growth rate of 0.76±0.01 day^{-1} measured at constant DO level of 100% air saturation. In saturating light intensity, the growth rate increased to 0.90±0.07 day^{-1}. If the oxygen concentration gradually changed from 100% to 300% in 365 seconds and subsequently decreased to 100% air saturation in 140 seconds, there was no significant difference with the specific growth rate of 0.91±0.20 day^{-1} measured at a constant DO level of 100% air saturation (Chapter 5).

It really surprised us to discover that dynamically changing oxygen concentrations in the medium did not affect the growth of *Nannochloropsis* sp.. After all, the growth of the algae decreased linearly with the oxygen concentration when constant elevated oxygen concentrations were applied (Chapter 2). It appears as though some type of protection mechanism has evolved in *Nannochloropsis* sp. against gradually increasing oxygen concentrations in the medium whereby the algae are able to withstand a brief exposure to high oxygen concentrations as long as the medium is regularly degassed.

This phenomenon was also observed for green microalgae *Neochloris oleoabundans* (Sousa et al. 2013b) which also exhibited severe inhibition of the growth rate with constant elevated oxygen concentrations (Sousa et al. 2012; Sousa et al. 2013a). However, when dynamically changing oxygen concentrations were applied, the growth rate remained unaffected. Even when the amount of exposure time to the elevated oxygen concentrations before degassing was increased 10 times, the growth rate of the algae remained constant. Moreover, the changing light conditions to which the algae were exposed when they were transferred from the light harvesting tubes into the dark degasser had far more impact on the productivity of the algal cells.

The fact that both *Nannochloropsis* sp. and *Neochloris oleoabundans* demonstrated similar responses to dynamically changing oxygen concentration indicates that we are dealing with a more general phenomenon. If that is indeed the case, it has far reaching implications for the future design and use of tubular photobioreactor systems for large-scale micro-algal culturing.

Oxygen as important parameter in design of photobioreactors

Oxygen accumulation has often been mentioned as the main bottleneck in tubular systems. Removal of oxygen in the degasser requires extensive energy which results in a negative energy balance for tubular systems (Norsker et al. 2011). To avoid oxygen accumulation, flat panel photobioreactors were designed in which higher degassing rates were applied, however, this resulted in even greater energy use for degassing (Norsker et al. 2011).

Our experiments on the effect of dynamic oxygen demonstrated that oxygen accumulation does not affect the growth rate as long as regular degassing is applied and that the algae can withstand longer residence time in the tubes.

This unexpected outcome of the research presented in this thesis can have significant impact on the design and future use of tubular photobioreactors. Longer residence times in the tubes before the oxygen must be removed indicates that the tubes can be made longer and that the number of degasser units can be decreased. It is also possible to apply lower liquid velocity in the tubes. Both methods will result in a substantial reduction of energy costs for mixing and degassing.

When decreasing the number of degasser units, the algae will spend relatively less time in the darkness of the degasser where no photosynthesis occurs. This will result in an overall higher productivity. Moreover, the algae will endure less shear stress due to the fact that less degassing is required which may also attribute to higher productivities. As a result, large-scale cultivation of shear-sensitive algae like *Chaetoceros muelleri* (Michels et al. 2011) or other shear-sensitive diatoms that are often exploited as PUFA-rich feed for (shell)fish become feasible in well controlled tubular photobioreactor systems.

Additional research needs to be conducted to test if optimization of the design of tubular photobioreactors in terms of energy savings can indeed be realized when the residence time in the tubes is elongated and/or the number of degassers is reduced. The effect of elongated residence times with a high oxygen concentration on the microalgal growth should be determined, and the reduction in energy required for mixing and degassing when applying these longer residence times in the tube need to be evaluated. As mixing is not only needed to prevent inhibition by oxygen but also to provide the algae with sufficient light and carbon dioxide, the effects of a longer residence time on light supply and carbon dioxide should be investigated as these parameters are crucial for the growth and productivity as well as for the quality of the algae. Our results on the effects of dynamically changing oxygen concentrations on the growth of microalgae in closed photobioreactor systems demonstrate that oxygen is less inhibiting than expected based on growth data obtained with constant elevated oxygen levels. In the design of closed tubular systems, the accumulation of oxygen should no longer be considered as a leading design constraint. Although degassing is still required, optimization in terms of energy use should focus preliminary on optimization of the use of light.

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SUMMARY

Microalgae are promising organisms for cultivation of food, feed, and biobased commodities such as biofuel. One of the candidate microalgae is *Nannochloropsis* sp. which can grow relatively quickly and has a high lipid content with a high content of eicosapentaenoic acid. High productivity with consistent product quality can be achieved in fully controlled closed photobioreactors. O_2 accumulation in these systems, however, is regarded as a major bottleneck as high oxygen concentration inhibits growth and may affect the lipid composition of *Nannochloropsis* sp. during cultivation. To remove the excessive oxygen, mixing and degassing are required which require high energy expenditures. Knowledge of oxygen inhibition is a topic of ongoing research toward optimizing the design of these closed photobioreactor systems in order to achieve high productivity combined with a positive energy balance for the production of algae.

During photosynthesis, O_2 is produced along the tubes of tubular photobioreactor. The accumulated O_2 increases the ratio of O_2/CO_2 which causes photorespiration resulting in growth inhibition. In high light intensities, the accumulated oxygen causes additional photo-inhibition effects due to the formation of singlet oxygen and oxygen radicals which damage the algae. Most microalgae have developed several molecular defense mechanisms to protect themselves against photo-oxidative damage. PUFA are mentioned in literature as being involved and may be affected with high oxygen concentrations.

This thesis focuses on the effects of oxygen concentrations on the growth and protective mechanisms of *Nannochloropsis* sp.. The research was performed in a fully-controlled flat panel photobioreacotor operated as a turbidostat in which the concentration of oxygen could be specified independent from the employed light conditions. **In chapter 2**, the effects of oxygen concentration on the growth of the microalgae was determined in constant low light

incident conditions (100 μmol photons m^{-2} s^{-1}) whereby only photorespiration occurs, and no additional photoinhibition effects are negligible. When increasing the oxygen concentration from 20-100% air saturation, no inhibition by oxygen was observed on the growth rate. When the oxygen level was increased from 100% to 300% air saturation, the growth rate and the associated quantum yield linearly decreased with the oxygen concentration. The linear decrease in quantum yield with oxygen concentration was similar to the quantum yields that were calculated based on the oxygenase and carboxylase activity data from the literature of isolated Rubisco. This indicated that the O_2/CO_2 ratio in the medium should be controlled to ensure that the Rubisco is deployed for biomass production. **Chapter 3** addresses the synergetic effect of oxygen and light on the growth of *Nannochloropsis* sp. when employing three light intensities; low light at 100 μmol photons m^{-2} s^{-1}; near-saturating light at 400; and over-saturating light at 624 μmol photons m^{-2} s^{-1}. As expected, a decrease in the growth rate was observed with increasing oxygen concentration in all employed light intensities. In high light intensity, a more severe decrease in growth rate was observed due to additional photoinhibition and photo-oxidative damage in high light intensity. The additional inhibiting effect of oxygen on the growth rate, however, was marginal compared to the stimulating effects of light in those elevated light intensities. Our observations clearly demonstrated that optimal productivity of *Nannochloropsis sp.* in outdoor mass cultivation can be achieved if the oxygen concentration is maintained close to 100% air saturation but that it is more important to ensure that the algae grow in saturating light intensity to achieve high productivity.

To gain more knowledge on how microalgae cope with high oxygen concentration in different light conditions, the effects of oxygen on the molecular defense mechanisms during the growth of this species were studied. In **chapter 4**, it was demonstrated that superoxide dismutase (SOD), one of the key enzymes in the water-water cycle, was activated when the cells were exposed to higher light conditions. This enzyme protects the cells against photo-oxidative stress caused by the oxygen radicals that are formed. The SOD activity was only minimally affected by oxygen, indicating that higher oxygen levels in the medium did not result in activation or induced production of this enzyme. Only in over-saturating light conditions was a slightly higher SOD activity observed with increased oxygen concentrations. In closed photobioreactors, algae do not experience constant high oxygen concentration, but they are subject to gradually increasing oxygen concentration when residing in the tubes followed by a quick decrease of oxygen concentrations in the degasser. The objective of **chapter 5** was to investigate the effect of these dynamically changing oxygen concentrations on the growth and the molecular defence mechanism of the algae. The algae were exposed to a gradual increase of the oxygen concentration from 100% to 300% followed by a quick decrease in oxygen to 100% air saturation. Although the algae were exposed to an elevated oxygen concentration, on average, neither the algal growth rate or the molecular defense mechanism was affected when applying the dynamically changing oxygen concentration; these were only stimulated by the employed light intensity. Obviously, the algae can withstand high oxygen concentrations when regular degassing occurs. As degassing expends energy, it is recommended to find out how much degassing is, in fact, required in order to suppress the inhibitory effects of oxygen**. Chapter 6** is a general discussion regarding the main findings of this thesis. While oxygen was always considered to be one of the main

parameters for designing closed photobioreactor systems, this role is questionable. The results obtained from our experiments on the effects of dynamically changing oxygen concentrations on the growth of microalgae indicated that oxygen is less inhibiting than expected based on growth data obtained at constant elevated oxygen levels. Although degassing is still required in closed photobioreactors, optimization of the design of these systems in terms of energy use should focus preliminary on optimization of the use of light rather than on the removal of accumulating oxygen.

Summary in Thai

สาหร่ายขนาดเล็กเป็นสิ่งมีชีวิตที่นับว่ามีศักยภาพสมควรให้มีการพัฒนาเพาะเลี้ยงอย่างเป็นระบบ เพื่อใช้เป็นอาหารเสริมของมนุษย์ อาหารสัตว์ และผลิตสารชีวผลิตภัณฑ์ เช่น น้ำมันเชื้อเพลิงชีวภาพ สาหร่ายสีเขียวแกมเหลือง *Nannochloropsis* sp. เป็นหนึ่งในกลุ่มสาหร่ายขนาดเล็กที่ได้รับความนิยมในการเพาะเลี้ยง เนื่องจากความสามารถในเจริญเติบโตอย่างรวดเร็ว และสามารถสังเคราะห์ไขมันภายในเซลล์ได้ในปริมาณสูง โดยเฉพาะกรดไขมันในกลุ่มโอเมกา 3 ได้แก่ กรดไอโคซาเพนทาอีโนอิก หรือ EPA

การเพาะเลี้ยงสาหร่ายขนาดเล็กเพื่อให้ได้เซลล์ในปริมาณมากและมีคุณภาพคงที่สามารถทำได้โดยใช้เทคนิคการเพาะเลี้ยงในระบบถังปฏิกรณ์ชีวภาพแบบใช้แสง (Photobioreactor) ที่มีการควบคุมปัจจัยต่างๆให้เหมาะสมต่อการเจริญของสาหร่ายชนิดนี้ แต่การเพาะเลี้ยงด้วยระบบนี้จะทำให้เกิดการสะสมของก๊าซออกซิเจนในปริมาณสูง ซึ่งนับเป็นปัญหาสำคัญในระบบการเพาะเลี้ยงแบบปิด ปริมาณก๊าซออกซิเจนที่สะสมจนสูงเกินไปจะมีผลทำให้การเจริญเติบโตของสาหร่าย *Nannochloropsis* sp. ลดลง และมีผลกระทบต่อองค์ประกอบไขมันที่สะสมภายในเซลล์ การลดปริมาณก๊าซออกซิเจนที่สะสมออกจากระบบถังปฏิกรณ์ชีวภาพจึงจำเป็นต้องใช้ระบบการตีผสม (mixing) ควบคู่กับระบบกำจัดก๊าซออกซิเจน (Degasser) ซึ่งทำให้ต้องสิ้นเปลืองพลังงานจำนวนมากในการเพาะเลี้ยงด้วยระบบนี้ ดังนั้นความรู้เกี่ยวกับผลของออกซิเจนต่อการเจริญเติบโตของสาหร่ายขนาดเล็กจึงเป็นหัวข้อที่ควรศึกษาวิจัยในปัจจุบัน เพื่อนำไปใช้ออกแบบการเพาะเลี้ยงในระบบถังปฏิกรณ์ชีวภาพแบบปิดให้มีความเหมาะสม เพื่อพัฒนาการเพาะเลี้ยงสาหร่ายขนาดเล็กให้ได้ผลผลิตในปริมาณสูง ทั้งในรูปแบบของเซลล์และสารชีวผลิตภัณฑ์จากเซลล์ ภายใต้เงื่อนไขของการใช้พลังงานในปริมาณที่เหมาะสมต่อกระบวนการผลิต

ในระหว่างปฏิกิริยาการสังเคราะห์ด้วยแสงของสาหร่ายการสะสมของก๊าซออกซิเจนจะสามารถพบได้ในระบบท่อต่าง ๆ ของถังปฏิกรณ์ชีวภาพ ปริมาณออกซิเจนที่สะสมมากขึ้นจะทำให้สัดส่วนของก๊าซออกซิเจน (O_2) ต่อก๊าซคาร์บอนไดออกไซด์ (CO_2) เพิ่มขึ้นตามไปด้วย ปรากฏการเช่นนี้จะไปกระตุ้นให้เซลล์เกิดกระบวนการหายใจแบบใช้แสง (Photorespiration) ซึ่งจะมีผลทำให้สาหร่ายมีการเจริญเติบโตลดลง ยิ่งไปกว่านั้นถ้ามีการสะสมของออกซิเจนในปริมาณสูงควบคู่ไปกับการได้รับแสงในช่วงความเข้มสูงในระหว่างการเพาะเลี้ยง จะมีผล

ทำให้เกิดการยังยั้งปฏิกิริยาการสังเคราะห์ด้วยแสง (Photo-inhibition) ซึ่งเป็นผลมาจากการเพิ่มปริมาณออกซิเจนอะตอมเดี่ยว (Singlet oxygen) และอนุมูลอิสระของออกซิเจน (oxygen radical) โดยสารเหล่านี้นับว่าเป็นอันตรายต่อเซลล์ของสาหร่ายขนาดเล็ก แต่มีการรายงานว่าสาหร่ายขนาดเล็กเกือบทุกชนิดมีการสังเคราะห์สารอินทรีย์บางชนิดเพื่อนำมาใช้ปกป้องเซลล์จากการทำลายของปฏิกิริยาออกซิเดชันโดยแสง (Photooxidative damage) ในระดับโมเลกุล ในงานวิจัยจำนวนมากได้มีการรายงานถึง กรดไขมันไม่อิ่มตัว (มีตำแหน่งพันธะคู่ ตั้งแต่ 2 ตำแหน่งขึ้นไป) หรือ PUFA ว่ามีส่วนเกี่ยวข้องและอาจส่งผลช่วยในกระบวนการปกป้องเซลล์จากการทำลายของปฏิกิริยาออกซิเดชันโดยแสงได้

วิทยานิพนธ์ระดับดุษฎีบัณฑิต สาขา วิศวกรรมกระบวนการชีวภาพ ฉบับนี้มีวัตถุประสงค์เพื่อศึกษาผลของปริมาณออกซิเจนต่อการเจริญเติบโตและกระบวนการปกป้องเซลล์ในสาหร่ายขนาดเล็ก *Nanochloropsis* sp. โดยดำเนินการเพาะเลี้ยงสาหร่ายในถังปฏิกรณ์ชีวภาพแบบใช้แสง สร้างด้วยแผ่นกระจกเรียบ (flat panel photobioreactor) ทำงานโดยการควบคุมอัตโนมัติเต็มรูปแบบด้วยระบบคอมพิวเตอร์ โดยสามารถควบคุมการเจริญของสาหร่ายให้คงที่โดยใช้ photoelectric cell ติดกับตัวถังปฏิกรณ์ชีวภาพ เพื่อติดตามวัดค่าความขุ่นของอาหารที่ใช้เพาะเลี้ยง ซึ่งสัมพันธ์โดยตรงกับอัตราความหนาแน่นของเซลล์โดยการควบคุมให้อาหารไหลเข้าและออกจากระบบอย่างสม่ำเสมอ (turbidostat) นอกจากนี้ระบบยังสามารถควบคุมปริมาณการไหลเข้า-ออกของออกซิเจนและความเข้มของแสงในระหว่างการทดลองให้มีปริมาณคงที่ตามที่กำหนดได้ **<u>เนื้อหาในบทที่ 2</u>** แสดงผลของปริมาณออกซิเจนต่อการเจริญเติบโตของสาหร่ายขนาดเล็กที่ปริมาณความเข้มของแสงต่ำ (100 ไมโครโมลต่อตารางเมตรต่อวินาที) โดยที่ปริมาณความเข้มของแสงต่ำจะทำให้เกิดกระบวนการหายใจด้วยแสงแต่จะไม่ยับยั้งกระบวนการสังเคราะห์ด้วยแสง เนื่องจากแสงยังมีผลเพียงเล็กน้อยในสภาวะความเข้มต่ำในระดับนี้ ในขณะที่มีการเพิ่มปริมาณออกซิเจนจาก 20-100 เปอร์เซ็นต์ที่ละลายน้ำปริมาณออกซิเจนยังไม่มีผลต่อการเจริญเติบโตของสาหร่ายขนาดเล็ก แต่เมื่อปริมาณออกซิเจนที่ละลายน้ำเพิ่มขึ้นจาก 100-300 เปอร์เซ็นต์ จะมีผลทำให้การเจริญเติบโตจำเพาะและปริมาณผลิตผลควอนตัมลดลงเป็นสัดส่วนโดยตรงต่อปริมาณออกซิเจน ความสัมพันธ์ของผลิตผลควอนตัมกับปริมาณออกซิเจนที่เพิ่มขึ้น ให้ผล

คล้ายคลึงกับผลิตผลควอนตัมที่คำนวณโดยใช้ข้อมูลของปริมาณเอนไซม์ในการเกิดปฏิกิริยาของเอนไซม์ออกซิเจนเนส (oxygenase) และการเกิดปฏิกิริยาของเอนไซม์คาร์บอกซิลเลส (Carboxylase) ซึ่งพบได้ในเอนไซม์ Rubisco ที่สกัดมาจากสาหร่ายชนิดนี้ ร่วมกับการใช้ข้อมูลประกอบอื่น ๆ ที่ได้มาจากการตรวจเอกสารที่เกี่ยวข้อง ผลที่ได้แสดงให้เห็นว่าควรจะมีการควบคุมปริมาณสัดส่วนของก๊าซออกซิเจนต่อก๊าซคาร์บอนไดออกไซด์ที่ละลายอยู่ในอาหารเพาะเลี้ยงสาหร่ายเพื่อให้แน่ใจว่าเอนไซม์ Rubisco อยู่ในสภาวะที่เหมาะสมต่อการเกิดปฏิกิริยาเพื่อสร้างสารที่ต้องการได้ **เนื้อหาในบทที่ 3** เกี่ยวข้องกับผลของปฏิสัมพันธ์ระหว่างออกซิเจนกับความเข้มของแสงต่อการเจริญเติบโตของสาหร่าย *Nannochloropsis* sp. โดยให้ระดับความเข้มของแสง 3 ชนิด ดังนี้คือ ความเข้มของแสงในระดับต่ำที่ 100 ไมโครโมลต่อตารางเมตรต่อวินาที ความเข้มของแสง ณ จุดอิ่มแสงที่ 400 ไมโครโมลต่อตารางเมตรต่อวินาที และปริมาณความเข้มของแสงในระดับสูงที่ 624 ไมโครโมลต่อตารางเมตรต่อวินาที ตามลำดับ ผลการทดลองที่ได้เป็นไปตามที่คาดการณ์ไว้คือที่การเจริญเติบโตของสาหร่ายลดลงเมื่อมีการเพิ่มขึ้นของปริมาณออกซิเจนที่ระดับความเข้มของแสงทุกชนิด โดยเฉพาะที่ระดับความเข้มของแสงสูง พบว่ามีสาหร่ายมีการเจริญเติบโตลดลงอย่างชัดเจน การเจริญเติบโตของสาหร่ายที่ลดลงที่ระดับนี้เป็นผลมาจากกระบวนการยังยั้งปฏิกิริยาการสังเคราะห์ด้วยแสงและการเกิดปฏิกิริยาออกซิเดชันโดยแสง อย่างไรก็ตามการเพิ่มปริมาณออกซิเจนต่อการยับยั้งการเจริญเติบโตของสาหร่ายถือว่ามีผลเพียงเล็กน้อยเมื่อเปรียบเทียบกับการถูกกระตุ้นด้วยปริมาณความเข้มแสงที่เพิ่มขึ้น ผลการทดลองนี้แสดงให้เห็นถึงความเป็นไปได้ในการจัดการสภาวะแวดล้อมในการผลิตสาหร่ายขนาดเล็ก *Nannochloropsis* sp. ในระบบการเพาะเลี้ยงขนาดใหญ่กลางแจ้งให้มีความเหมาะสมและประสบความสำเร็จได้ถ้ามีการควบคุมปริมาณออกซิเจนให้อยู่ในระดับที่ใกล้เคียงกับปริมาณออกซิเจนที่ละลายน้ำคือ 100 เปอร์เซนต์ แต่ที่สำคัญที่สุดคือต้องมีการควบคุมปริมาณของแสงให้อยู่ในระดับความเข้มของแสงที่จุดอิ่มแสงเพื่อให้ได้ปริมาณเซลล์สาหร่ายที่ผลิตได้สูงขึ้น **เนื้อหาในบทที่ 4** มีวัตถุประสงค์เพื่อต้องการศึกษาการปรับตัวของสาหร่ายขนาดเล็กภายใต้สภาวะออกซิเจนความเข้มข้นสูงเมื่อได้รับปริมาณความเข้มแสงในระดับต่างๆ ที่มีผลต่อกระบวนการปกป้องเซลล์ในระดับโมเลกุลในระหว่างที่สาหร่ายมีการเจริญเติบโต โดยพบว่าปริมาณเอนไซม์ superoxide dismutase (SOD) ซึ่งเป็นเอนไซม์หลักในกระบวนการ water-water cycle จะถูกกระตุ้นให้มีปริมาณเพิ่มมากขึ้นเมื่อ

สาหร่ายอยู่ในสภาวะที่มีปริมาณความเข้มของแสงสูง เอนไซม์ชนิดนี้จะช่วยป้องกันเซลล์ไม่ให้ได้รับอันตรายจากปฏิกิริยาออกซิเดชันโดยแสง โดยปฏิกิริยานี้ก่อให้เกิดอนุมูลอิสระของออกซิเจน จากการศึกษาพบว่าปริมาณออกซิเจนไม่มีผลต่ออัตราการเกิดปฏิกิริยา SOD แสดงให้เห็นว่าปริมาณออกซิเจนที่เพิ่มขึ้นในอาหารเพาะเลี้ยงสาหร่ายไม่มีผลกระตุ้นหรือเหนี่ยวนำให้เกิดปฏิกิริยา SOD แต่ที่ปริมาณความเข้มของแสงสูงเกินกว่าจุดอิ่มแสงจะมีผลกระตุ้นให้เกิดปฏิกิริยา SOD เพิ่มขึ้นเล็กน้อยเมื่อร่วมกับการเพิ่มปริมาณออกซิเจนในระบบ

การเพาะเลี้ยงสาหร่ายในถังปฏิกรณ์ชีวภาพแบบใช้แสงระบบปิดเช่นนี้ สาหร่ายจะไม่เผชิญกับสภาวะที่มีปริมาณออกซิเจนคงที่ แต่จะต้องปรับตัวให้เข้ากับสภาวะที่มีการเพิ่มของออกซิเจนอย่างช้าๆ แล้วตามด้วยการลดลงอย่างรวดเร็วโดยระบบกำจัดก๊าซออกซิเจนที่ติดตั้งไว้ตามจุดต่างๆตลอดความยาวของท่อในระบบเพาะเลี้ยง **เนื้อหาในบทที่ 5** มีวัตถุประสงค์เพื่อที่จะศึกษาผลของการเปลี่ยนแปลงของออกซิเจนแบบพลวัตต่อการเจริญเติบโตและการปกป้องเซลล์ของสาหร่ายขนาดเล็ก ผลการศึกษาพบว่าการเพิ่มขึ้นของออกซิเจนที่ละลายน้ำแบบพลวัตจาก 100-300 เปอร์เซ็นต์ แล้วตามด้วยการลดลงอย่างรวดเร็วจนถึงระดับ100 เปอร์เซ็นต์ไม่มีผลต่อการเจริญเติบโตและการปกป้องเซลล์ของสาหร่าย โดยแม้ว่าจะมีการเพิ่มขึ้นโดยเฉลี่ยของออกซิเจนในการเปลี่ยนแปลงแบบพลวัต แต่มีเพียงปริมาณความเข้มข้นแสงเท่านั้นที่มีผลต่อการเจริญเติบโตและการปกป้องเซลล์ของสาหร่าย จากการศึกษาที่ผ่านมาพบว่าสาหร่ายขนาดเล็กสามารถจะทนต่อสภาวะที่มีออกซิเจนในปริมาณสูงโดยเฉพาะเมื่อมีระบบกำจัดออกซิเจนออกจากระบบอย่างต่อเนื่อง แต่เนื่องจากว่าเครื่องกำจัดก๊าซออกซิเจนต้องใช้พลังงานค่อนข้างสูงในการทำงาน ดังนั้นจึงควรมีการศึกษาจำนวนจุดที่ต้องการกำจัดก๊าซออกซิเจนออกจากระบบตลอดความยาวของท่อเพื่อไม่ให้เกิดการยับยั้งการเจริญเติบโตของสาหร่าย **เนื้อหาในบทที่ 6** เป็นการอภิปรายเกี่ยวกับผลการศึกษาโดยรวมของวิทยานิพนธ์ฉบับนี้ ถึงแม้ว่าออกซิเจนถือว่าเป็นปัจจัยหลักสำหรับการออกแบบระบบการเพาะเลี้ยงสาหร่ายแบบปิดในถังปฏิกรณ์ชีวภาพแบบใช้แสง แต่ก็ยังไม่มีข้อสรุปเกี่ยวกับผลของออกซิเจนอย่างชัดเจน ผลที่ได้รับจากการศึกษาในครั้งนี้แสดงให้เห็นว่าปริมาณออกซิเจนแบบพลวัตไม่มีผลต่อการเจริญเติบโตของสาหร่ายขนาดเล็ก โดยให้ผลยับยั้งการเจริญเติบโตของสาหร่ายน้อยกว่าที่คาดไว้เมื่อเปรียบเทียบกับการทดลองที่ได้รับออกซิเจนในปริมาณคงที่ การนำออกซิเจน

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Finally, I would like to thank my **family in Thailand,** especially **my parents, Mrs. Boonmee and Mr. Muan, and my beloved wife and children, Narisara, Natchanon and Pattarapol. My** stay in the Netherlands must have been difficult for them sometimes as well, but they always encouraged me to continue. I am really grateful that you gave me the opportunity to do so.

Best Regards,
Sayam Raso
9th October, 2013

Curriculum Vitae

Publications

Training Activities

Curriculum Vitae

Sayam Raso was born in the Uttaradit province (Thailand) on the 30th of March 1974. In 1981, he began primary school education in Banwansamo Village, a small village, at the Banwansamo Primary School where he received his diploma in 1986. In 1987, he continued his secondary school education at Bandarapittayakom School and obtained his diploma in 1990 after which he completed his high school education at Phichai School in 1990-1993. In 1994, he continued his bachelor degree at Chiangmai University. He received a scholarship from the Australian Government called AusAid, (currently known as AIDAB) to finalize his Bachelor degree in Applied Science (Aquaculture) in 1994, leaving Chiangmai University after one year. He received his Bachelor of Applied Science diploma on 14th March, 1998, from the University of Tasmania, Australia.

One year after his return to Thailand, he received a scholarship from the Royal Thai Government to continue to pursue his Master of Science (MSc) and to conduct research in the field of Aquaculture at James Cook University of North Queensland, Townsville, Australia. His Msc thesis focused on the effects of different lipid sources on growth and fatty acid composition in juvenile barramundi (*Lates calcarifer*). He obtained his MSc degree on the 7th November 2002 and returned to Thailand to work as a university government official lecturer at Surindra Rajabhat Institute, now known as Surindra Rajabhat University. During that time, he married Naritsara Raso, and they now have two children, namely, Natchanon and Pattarapol Raso. He spent his teaching career for 3 years at this University before he again received a scholarship from the Royal Thai Government to begin his PhD research project at Wageningen University, the Netherlands on the 17th of May, 2005. His PhD research on interaction of microalgal feed for aquaculture of juvenile mussels did not fit well with his background education. After two years, the topic was, therefore, adjusted, and the focus was rearranged from the culture of mussels to the cultivation of microalgae. After he finalized all of the experiments, he returned to Thailand on the 24th of December 2010, to finalize his PhD thesis "Effects of oxygen concentration on the growth of *Nannochloropsis* sp." He continued his job as university government official lecturer in the Biological program, Faculty of Science and Technology, Surindra Rajabhat University with emphasis on microalgae research and development. Apart from his academic career, he is also a vice-director of Academic Services and Information Technology in the charge position of the Educational Services looking after the Central Library, Language Center and Neighboring Country Center.

Publications

Raso S and Anderson AT (2003) Effects of dietary fish oil replacement on growth and carcass proximate composition of juvenile barramundi (*Lates calcarifer*), *Aquaculture Research,* 34: 813-819.

Raso S (2003) Effects of different dietary lipids at high lipid level (30 %) on growth, fatty acid composition in muscle and flesh quality in juvenile barramundi (*Lates calcarifer*), In: 29th *Congress on Science and Technology of Thailand; 20-22 October 2003*, Convention Hall, Khon Kean University, Thailand, 14 p.

Raso S (2003) Effects of dietary lipids at high levels on growth in juvenile barramundi, In: *Thai Journal of Agricultural Science*, Bangkok, Thailand.

Raso S, Van Genugten B, Vermue M, Wijffels RH (2012) Effect of oxygen concentration on growth of *Nannochloropsis* sp. at low light intensity, J Appl Phycol, 24: 863-871.

Overview of completed training activities

Discipline specific activities

Courses

In vivo NMR course, 2005
Bioreactor Design and Operation, 2006
Introduction to Bioinformatics, 2006
Cell culture and Bioreactors, 2011

Conferences

Mini Symposium NMR, 2005
Bionanotechnology symposium, 88th Dies Natalis of Wageningen University, 2006
2nd Symposium BSDL, 2006
World Congress of Malacology, 2007
11th International Conference on Applied Phycology, 2008
International Algae Congress, 2008
International conference on Science and Technology for sustainable Development, 2011
International Seminar on research for sustainable Development for quality of life, 2012

General courses

Scientific Publishing Workshop, 2005
Time planning and project Management, 2005
Techniques for writing and Presentation a scientific paper, 2006
Philosophy and Ethics of Food Science and Technology course, 2006
PhD-week (Introductory course), 2006
Teaching and Supervising Thesis students, 2007
Basic Statistics Course, 2008
Academic Writing at Higher education level for manuscript, 2012
Using Latex program for academic and dissertation, 2012

Optionals

Preparation of project proposal, 2005
Process Engineering PhD excursion to Denmark and Sweden, 2006
Brainstorm week Process Engineering, 2006
Process Engineering PhD excursion to Japan, 2008
Oral presentation of PhD project, 2008
Group meetings, 2005-2011

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