On-line estimation of $O_2$ production, $CO_2$ uptake, and growth kinetics of microalgal cultures in a gas-tight photobioreactor

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Abstract Growth of the green algae Chlamydomonas reinhardtii and Chlorella sp. in batch cultures was investigated in a novel gas-tight photobioreactor, in which $CO_2$, $H_2$, and $N_2$ were titrated into the gas phase to control medium pH, dissolved oxygen partial pressure, and headspace pressure, respectively. The exit gas from the reactor was circulated through a loop of tubing and re-introduced into the culture. $CO_2$ uptake was estimated from the addition of $CO_2$ as acidic titrant and $O_2$ evolution was estimated from titration by $H_2$, which was used to reduce $O_2$ over a Pd catalyst. The photosynthetic quotient, $PQ$, was estimated as the ratio between $O_2$ evolution and $CO_2$ up-take rates. $NH_4^+$, $NO_2^-$, or $NO_3^-$ was the final cell density limiting nutrient. Cultures of both algae were, in general, characterised by a nitrogen sufficient growth phase followed by a nitrogen depleted phase in which starch was the major product. The estimated $PQ$ values were dependent on the level of oxidation of the nitrogen source. The $PQ$ was 1 with $NH_4^+$ as the nitrogen source and 1.3 when $NO_3^-$ was the nitrogen source. In cultures grown on all nitrogen sources, the $PQ$ value approached 1 when the nitrogen source was depleted and starch synthesis became dominant, to further increase towards 1.3 over a period of 3–4 days. This latter increase in $PQ$, which was indicative of production of reduced compounds like lipids, correlated with a simultaneous increase in the degree of reduction of the biomass. When using the titrations of $CO_2$ and $H_2$ into the reactor headspace to estimate the up-take of $CO_2$, the production of $O_2$, and the $PQ$, the rate of biomass production could be followed, the stoichiometrical composition of the produced algal biomass could be estimated, and different growth phases could be identified.

Key words Chlamydomonas reinhardtii · Chlorella sp. · photosynthetic quotient · lumostat · nitrogen limitation
Introduction

When microorganisms are grown in batch cultures, growth progresses through several phases, conventionally described as a lag phase, an exponential growth phase, and a stationary phase. However, this model is often too simplistic to give a reasonable description of batch growth in cultures of phototrophic algae and cyanobacteria. Growth is seldom exponential since increased biomass concentrations result in self-shading and decreased specific growth rates (Eriksen et al. 1996), and increased competition for light will often cause phototrophic microorganisms to increase their pigment contents (Richardson et al. 1983) and thereby change their composition.

Nutrient depletion, which is not necessarily followed by an immediate entry into a stationary phase, may also result in changes in biomass composition. During nutrient depleted growth phases, starch and other carbon and energy storage compounds may accumulate and constitute a major part of the biomass (Stenholm et al. 1998). Depletion of nutrients, in particular the nitrogen source, also results in break-down of the photosynthetic apparatus (Coleman et al. 1988), including the photosynthetic pigments (Eriksen and Iversen 1995).

As the composition of the produced biomass changes, so will the specific rates of substrate uptake and product formation. In phototrophs, CO₂ and O₂ are quantitatively the most important substrate and product, respectively. The ratio between O₂ evolution rate and CO₂ uptake rate (the photosynthetic quotient, PQ) depends on the composition of the produced biomass and the substrates that are utilised. Especially oxidised nitrogen sources, which must be reduced before they are incorporated into the biomass, affect the PQ. When biomass composition equals the Redfield ratio, CH₂O(NH₃)₀.₁₅ (Redfield et al. 1963), and NO₃ is the nitrogen source a PQ of 1.3 will be expected. With the lesser reduced NO₂ as nitrogen source, the expected PQ is 1.2. Growth on NH₄⁺,

\[
\text{CO}_2 + \text{H}_2\text{O} + 0.15\text{NO}_3^- + 0.15\text{H}^+ \\
\rightarrow \text{CH}_2\text{O(NH}_3)_0.15 + 1.3\text{O}_2
\]

(1)

which is of similar degree of reduction as nitrogen in the biomass, should result in a PQ of 1. Changes in PQ are therefore expected if especially NO₃⁻ or NO₂⁻ are depleted and reduction of nitrogen no longer takes place.

If the composition of the produced biomass is altered, the PQ is likely to change since different molecules, which are incorporated into the biomass, are not equally reduced. The PQ can be predicted from the degree of reduction of the biomass, γₓ (see Roels 1980), which describes the number of electrons per carbon atom available for oxidation reactions. When no organic products in addition to biomass are produced, and NH₄⁺ is the nitrogen source (Eq. 2), the molar ratio between oxygen production and carbon fixation, \( y_{\text{O}_2/x} \) can be found from a degree of reduction balance

\[
0 = -y_{\text{CO}_2} - y_{\text{H}_2\text{O}} - y_{\text{NH}_4^+} \cdot y_{\text{NH}_3} \\
+ y_x + y_{\text{O}_2} \cdot y_{\text{O}_2/x}
\]

(3)

where \( y_{\text{NH}_4^+} \) is the molar ratio between ammonium uptake and carbon fixation. The degrees of reduction of CO₂, H₂O and NH₃, \( y_{\text{CO}_2}, y_{\text{H}_2\text{O}}, \) and \( y_{\text{NH}_3}, \) respectively, all equal zero, and the degree of reduction of O₂, \( y_{\text{O}_2} = -4 \) (Roels 1980). For the biomass, \( y_x = 4 \) if carbohydrates are the only compounds formed, while synthesis of proteins and lipids results in higher \( y_x \) values. When no organic products are secreted from the cells, all CO₂ taken up is incorporated into the biomass, and the PQ is equal to \( y_{\text{O}_2/x} \). Equation 3 can then be rewritten

\[
PQ = y_{\text{O}_2/x} = \frac{y_x}{-y_{\text{O}_2}} = \frac{y_x}{4}
\]

(4)

Equation 4 predicts that synthesis of carbohydrates results in a \( PQ = 1 \), while synthesis of more reduced
cell components results in higher PQ values. The PQ in cultures using NO₂ or NO₃ as nitrogen source can also be predicted by Eq. 4, although γ in cultures using these nitrogen sources will be assigned slightly higher values (see Roels 1980).

In this paper, we have investigated the stoichiometry of gas exchange and biomass production in batch cultures of the green algae Chlamydomonas reinhardtii and Chlorella sp. grown on reduced and oxidised nitrogen sources, NH₄⁺, NO₂⁻, and NO₃⁻, respectively. All growth experiments were carried out in a novel gas-tight photobioreactor, in which CO₂ uptake was estimated from the addition of CO₂ as acidic titrant to maintain constant pH, and O₂ evolution was estimated from titration with H₂, which was used to reduce O₂ over a Pd catalyst and maintain constant dissolved oxygen partial pressure.

### Materials and methods

#### Strain and growth medium

*Chlamydomonas reinhardtii* UTEX2337 was obtained from the Culture Collection of Algae at The University of Texas at Austin, USA. *Chlorella* sp. was a gift from Dr. Šetlíc, Department of Autotrophic Microorganisms, Tréboň, Czech Republic. Both strains were maintained by sequential transfer into photoautotrophic batch cultures grown at room temperature in the growth medium for *Chlamydomonas* described by Starr (1978). For growth experiments, NH₄⁺, NO₂⁻, or NO₃⁻ was included in the growth medium as the final cell density limiting substrate.

#### Bioreactor

Batch cultures were grown in a 3.0 l Applikon BTS05 bioreactor (Applikon, The Netherlands) containing 2.1 L of culture. The bioreactor consisted of a cylindrical glass jar with a diameter of 13 cm fitted with a top plate of stainless steel. The bioreactor was placed in a cabinet of mirrors equipped with 12 Osram 18W/20 cool white fluorescent tubes. The temperature was maintained at 25°C and the culture was stirred at 500 rpm by a Rushton turbine and aerated with 1.4 L min⁻¹ of an aeration gas which was circulated through a closed loop of gas tight polypropylene tubing (Figure 1). The exit gas from the reactor was passed through a condenser at 4°C, a 0.25 L foam trap, a 50-ml glass column (inner diameter = 2.1 cm) containing 25 g of a palladium catalyst (Pd coated porous allumina pellets of approximately 1 × 3 mm supplied by Haldor Topsoe, Denmark) with a void fraction of 0.8 L, and a membrane compressor with a gas tight viton membrane, before it was re-introduced into the culture through a sparger placed below the impeller. An open-ended tubing submerged in 15 cm of a saturated NaCl solution served as a safety device against build-up of excess pressure in the headspace.

#### Headspace pressure

The pressure in the gas circulation loop was measured by a differential pressure transducer (Honeywell 26PC) and maintained at 1.0–1.6 kPa above ambient pressure. Fixed amounts of N₂ (129 ± 5 μmol) could be added to increase the pressure in the gas loop from a pressurised N₂ supply by opening a solenoid valve (Sirai z0300A, Italy) for 0.1 s. The pressure of the N₂ supply was 5.4 kPa above ambient pressure. Pressure could be released from the gas loop by a second solenoid valve. When opened for 0.1 s, the headspace pressure decreased by 0.1 kPa, and the amount of gas in the gas loop was lowered by 62 ± 3 μmol.

#### Dissolved oxygen tension and O₂ production

Dissolved oxygen tension in the growth medium was measured by an autoclavable oxygen electrode (Applisens), and maintained at 80% air saturation by reduction of O₂ in the gas loop on a Pd surface (see e.g., Nowakowski et al. 2002). Fixed amounts of H₂ (223 ± 13 μmol) were delivered through a third solenoid valve (opened for 0.1 s) located at a position just before the gas entered the Pd catalyst column (Figure 1). At the surface of the Pd catalyst, O₂ was reduced to H₂O by the consumption of 2 moles of H₂ per mol O₂. The pressure of the H₂ supply was 3.9 kPa above ambient pressure, and the H₂ additions were regulated by a dose-pause controller. The minimal pause between two H₂ additions was 10 s to allow time for the oxygen depleted aeration gas to dissipate oxygen from the liquid phase before a new dose of H₂ was added.
The number of additions of H$_2$ to the gas circulation loop was recorded by a computer and the molar amount of photosynthetically produced O$_2$, $\Delta m_{O_2}$, in time interval $\Delta t$ was estimated as

$$\frac{\Delta m_{O_2}}{\Delta t} = 0.5 \frac{\Delta n_{H_2}}{\Delta t} \cdot v_{H_2} + \frac{\Delta n_\text{H$_2$}}{\Delta t} \cdot v_\text{H}_2 \cdot \frac{P_{O_2}}{p}$$

(5)

where $\Delta n_{H_2}$ and $\Delta n_\text{H$_2$}$ are the number of H$_2$ additions and pressure releases, respectively, in time interval $\Delta t$, $v_{H_2}$ and $v_\text{H}_2$ are moles of H$_2$ added or gas molecules released from the headspace per H$_2$ addition or pressure release, respectively, and $P_{O_2}$ and $p$ are oxygen partial pressure and total pressure in headspace, respectively. It was assumed that O$_2$ in the headspace and dissolved O$_2$ in the culture were at equilibrium, and that $P_{O_2}$ measured in the medium therefore also represented $P_{O_2}$ in the gas loop. The total amount of O$_2$ produced was found by integration of Eq. 5.

**pH control and CO$_2$ uptake**

Culture pH was measured by an autoclavable pH electrode (Mettler Toledo). When pH increased above pH 7.5, a pulse of 101 ± 3 μmol of CO$_2$ was added as acidic titrant to the gas loop by opening a fourth solenoid valve for 0.1 s. In the growth medium, CO$_2$ dissolved partly as carbonic acid, resulting in a decrease in pH. Because CO$_2$ adsorbs reversibly onto Pd surfaces (see, e.g., Liu et al. 2001), the CO$_2$ addition valve was placed after the Pd catalytic column (Figure 1). The minimal pause between successive CO$_2$ additions was 30 s.

Uptake of CO$_2$ was the major process in the culture that affected medium pH, and the CO$_2$ uptake was estimated from the CO$_2$ addition rate. However, uptake of the nitrogen source also affected medium pH (see Eqs. 1 and 2). With NO$_3$ or NO$_2$ as nitrogen sources, more CO$_2$ than that removed by the cells had to be added to maintain constant pH, since reduction of 1 mol of NO$_3$ or NO$_2$ consumes 1 mol of protons (Eq. 1). These protons had to be replaced by dissociation of carbonic acid. The concentration of HCO$_3$ in the growth medium therefore increased in proportion to the decrease in the concentration of the nitrogen source, and this increase also increased other pools of inorganic carbon in the photobioreactor.

With NH$_4$ as nitrogen source, 1 mol of protons was excreted into the growth medium for each mol of NH$_4$ taken up by the cells (Eq. 2), and less CO$_2$ than that removed by the cells were needed to maintain constant pH. The concentration of HCO$_3$ therefore decreased in proportion to the decrease in concentration of NH$_4$, and therefore other pools of inorganic carbon in the photobioreactor also decreased when NH$_4$ was the nitrogen source.
The molar uptake of CO$_2$, $\Delta m_{\text{CO}_2}$ per time was also corrected for losses of CO$_2$ via ventilation of excess pressure, and calculated as

$$\frac{\Delta m_{\text{CO}_2}}{\Delta t} = \frac{\Delta n_{\text{CO}_2}}{\Delta t} \cdot v_{\text{CO}_2} - \frac{\Delta n_i}{\Delta t} \cdot v_i \cdot \frac{p_{\text{CO}_2}}{p} - \frac{\Delta N \cdot V_L}{\Delta t} \cdot \frac{\Delta m_{\text{GL}}}{\Delta N \cdot V_L}$$

(6)

where $\Delta n_{\text{CO}_2}$ is the number of CO$_2$ additions, $v_{\text{CO}_2}$ is the moles of CO$_2$ added per addition, $p_{\text{CO}_2}$ is CO$_2$ partial pressure, $\Delta N$ is the change in concentration of the nitrogen source, $V_L$ is the volume of the growth medium, and $\frac{\Delta m_{\text{GL}}}{\Delta N \cdot V_L}$ is the relationship between the overall change in total inorganic carbon content and nitrogen content in the photobioreactor. Calculations of $p_{\text{CO}_2}$ and $\frac{\Delta m_{\text{GL}}}{\Delta N \cdot V_L}$ are described in Appendix, and $\Delta N$ was estimated from Eq. 13 (see below). The photosynthetic quotient, $PQ$ was estimated by dividing Eq. 5 with Eq. 6

$$PQ = \frac{\Delta m_{\text{GL}}/\Delta t}{\Delta m_{\text{CO}_2}/\Delta t}$$

(7)

Since additions of CO$_2$ and H$_2$ as well as ventilation of excess pressure was not continuous functions but discrete events, a relatively large $\Delta t = 4$ h was used for estimation of the $PQ$ values.

Regulation of light intensity

The light intensity on the reactor, measured as the average light intensity at 6 positions on the reactor wall and at the bottom by a Licor LI-250 Light Meter fitted with a quantum sensor, could be controlled between 0 and 220 $\mu$mol photons m$^{-2}$ s$^{-1}$ by a dimming transformer. The dimming transformer was regulated by a 0–10 V analog signal controlled by the computer. The light intensity was automatically changed during batch cultivations in order to maximise the CO$_2$ addition rate and allow the culture to grow exponentially using a modified version of the approach described by Eriksen et al. (1996). The time taken for 15 CO$_2$ pulses to be added to the gas loop after a change in light intensity was compared to the time taken for the preceding 15 CO$_2$ additions. If the last 15 pulses were added during a shorter time interval than the preceding 15 pulses, the light intensity was changed by 1–8.5 $\mu$mol photons m$^{-2}$ s$^{-1}$ in the same direction as the previous change (the largest changes occurred at the lowest light intensities because of non-linearity between dimming transformer output and light intensity). If the time taken for the 15 CO$_2$ pulses to be added had increased, the light intensity was changed by 1–8.5 $\mu$mol photons m$^{-2}$ s$^{-1}$ in the direction opposite to the previous change. By this approach, the light intensity was automatically maintained at the optimal intensity resulting in the fastest CO$_2$ consumption rate, and was increased along with the increase in cell density.

Measurements of biomass, starch, chlorophyll a, and biomass composition

The concentration of biomass was measured spectrophotometrically at 750 nm (OD$_{750}$) at which wavelength there was no absorbance from pigments. If necessary, the samples were diluted to OD$_{750}$ values below 0.3. Biomass dry weight (DW) of samples was measured after filtration onto pre-dried, pre-weighed 0.22-$\mu$m Millipore filters and dried at 85°C overnight. The relationship between OD$_{750}$ and biomass dry weight was rectilinear at all cell densities.

Starch was extracted from $C.\ reinhardtii$ in 0.05 M acetate buffer, pH 5, after disruption of the cells in a B.Braun Mikro-Dismembrator S rotating at 2,500 rpm for 5 min. The cell homogenate was diluted in the same buffer in order to obtain a concentration of glucose equivalents not higher than 320 $\mu$g L$^{-1}$. The starch was hydrolysed to glucose by mixing 500 $\mu$L of the cell homogenate with 60 $\mu$L of a mixture containing 3 amylolytic enzymes (8 KNU of $\alpha$-amylase, BAN240L, 5 AGU of glucoamylase, AMG 300L, and 9 PUN of pullulanase, Promozyme 400L) from Novozymes, Denmark (KNU, AGU, and PUN are enzyme activity units used by Novozymes). The mixture was incubated for 1 h at 65°C followed by 5 min of centrifugation at 8,050 g. The starch content was measured as glucose equivalents, after subtraction of the glucose content in cell extracts not treated with amylolytic enzymes.

Chlorophyll a was measured spectrophotometrically after 24 h of dark extraction in 80% ethanol, followed by 5 min of centrifugation at 8,050 g using an extinction coefficient of 83 L g$^{-1}$ cm$^{-1}$ (Arvola 1981).
The composition of the major elements, carbon, hydrogen, nitrogen, and oxygen in samples of *C. reinhardtii* harvested from the photobioreactor was analysed on a Carlo Erba elemental analyser.

**Measurements of NH₄⁺, NO₂⁻, and NO₃⁻**

Concentrations of NH₄⁺, NO₂⁻, and NO₃⁻ in supernatants of samples removed from the cultures were measured using a Technicon Traacs 800 autoanlyser. NH₄⁺ was reacted with basic hypochlorite and salicylate to form a green indophenol complex, which could be measured at 660 nm. NO₂⁻ was reacted with sulphanilamide and N-(1-naphtyl)ethylenediamine dihydrochloride and the product was recorded at 520 nm. NO₃⁻ was reduced to NO₂⁻ over a Cd column, and otherwise detected as NO₂⁻.

**Modelling growth in nitrogen limited batch culture**

In order to describe observed changes in biomass composition during batch cultivations and the consumption of the nitrogen sources, a kinetic model describing growth of *Chlamydomonas reinhardtii* and *Chlorella* sp. in nitrogen limited, light sufficient batch conditions was developed as follows.
cultures, was developed. The specific uptake rate of the nitrogen source, $q_N$ (N-mol C-mol$^{-1}$ h$^{-1}$) per unit of non-starch biomass was modelled using saturation kinetics

$$q_N = \frac{q_{N,max} \cdot N}{K_N + N}$$

(8)

where $q_{N,max}$ is the maximal specific uptake rate of the nitrogen source, $N$ is the concentration of the nitrogen source in the growth medium (M), and $K_N$ is the affinity constant for the nitrogen source (M). The specific rate of fixation of CO$_2$ into the biomass, $q_C$ (C-mol C-mol$^{-1}$ h$^{-1}$) per unit of non-starch biomass in light sufficient cells, was negatively affected by the starch content per unit of biomass, $S$ (C-mol C-mol$^{-1}$) and was modelled using a logistic relationship

$$q_C = \frac{q_{C,max} \cdot S_{max} - S}{S_{max}}$$

(9)

where $q_{C,max}$ is the maximal specific carbon uptake rate per unit of non-starch biomass and $S_{max}$ is the maximum specific starch concentration per unit of biomass, respectively. The specific growth rate of the non-starch biomass, $\mu$ (h$^{-1}$) is controlled by $q_N$

$$\mu = q_N \cdot \frac{Y_{x/N}}{C_1}$$

(10)

where $Y_{x/N}$ is the molar ratio between carbon and nitrogen in the starch-free biomass (C-mol N-mol$^{-1}$) and accumulation of non-starch biomass, $x$ (C-mol L$^{-1}$) was described by

$$\frac{dx}{dt} = \mu x$$

(11)

Accumulation of starch in the culture, $s$ (C-mol L$^{-1}$) is described as the difference between the total carbon uptake rate and the production of non-starch biomass

$$\frac{ds}{dt} = q_c \cdot x - \frac{dx}{dt}$$

(12)

and the decrease in concentration of the nitrogen source depends on the production of non-starch biomass

$$\frac{dN}{dt} = -Y_{x/N} \frac{dx}{dt}$$

(13)

In order to describe $x$, $s$, and $N$ during batch cultures, Eqs. 11–13 were solved numerically. From Eq. 13, the amount of consumed nitrogen, $\Delta N$ was estimated and used in the calculation of the CO$_2$ uptake by Eq. 6.

**Results**

*Chlamydomonas reinhardtii*, NH$_4^+$ as nitrogen source

Figure 2 shows a batch culture of *Chlamydomonas reinhardtii* grown on NH$_4^+$ as the nitrogen source. Growth proceeded exponentially for approximately 100 h until NH$_4^+$ was depleted (Figure 2a) and the culture became nitrogen limited. Nitrogen limitation was clearly indicated also by the decrease of total and specific chlorophyll $a$ concentration in the culture (Figure 2b). However, the concentration of biomass dry weight was still increasing for the following 100 h despite nitrogen limitation.

| $t$ (h) | $x$ (g L$^{-1}$) | Biomass composition | $Y_{x}$ | $N_x$ (mM) | $\Delta t$ (h) | Biomass production | $\gamma \Delta x$ | Calculated $PQ$ | Measured $PQ^a$ |
|--------|----------------|---------------------|--------|----------|----------------|-------------------|---------------|----------------|----------------|
| 45     | 0.14           | CH$_1$.$77$O$_{0.53}$N$_{0.13}$ | 4.26   | 0.7      |                |                   |                |                |                |
| 77     | 1.08           | CH$_1$.$79$O$_{0.55}$N$_{0.09}$ | 4.38   | 3.6      | 45–77          | CH$_1$.$77$O$_{0.55}$N$_{0.09}$ | 4.40          | 1.10           | 1.13           |
| 97     | 1.65           | CH$_1$.$82$O$_{0.64}$N$_{0.05}$ | 4.39   | 2.9      | 77–97          | CH$_1$.$93$O$_{0.82}$ | 4.30          | 1.07           | 1.01           |
| 144    | 1.86           | CH$_1$.$82$O$_{0.60}$N$_{0.03}$ | 4.54   | 2.2      | 97–144         | CH$_1$.$88$O$_{0.42}$ | 5.04          | 1.26           | 1.16           |
| 161    | 2.20           | CH$_1$.$82$O$_{0.59}$N$_{0.02}$ | 4.59   | 1.7      | 144–161        | CH$_1$.$96$O$_{0.57}$ | 4.82          | 1.21           | 1.36           |

*Biomass dry weight, $x$ and biomass composition were measured on samples withdrawn from the culture at time $t$, $Y_x$ is the degree of reduction of the biomass, and $N_x$ is the total concentration of nitrogen in the biomass. Biomass production is the calculated composition of biomass produced in a time interval $\Delta t$, and $\gamma \Delta x$ is the degree of reduction of the produced biomass. Calculated $PQ$ values were obtained from Eq. 4. Measured $PQ$ values were obtained using Eq. 7.*

*$^a$The measured $PQ$ is from the experiment shown in Figure 2 while other data are from a replicate batch culture.*
Accumulation of starch in the cells accounted for the entire increase in biomass concentration during the NH$_4^+$ depleted growth phase. Before NH$_4^+$ was depleted, the specific starch concentration in the cells was 0.1 g g$^{-1}$ but increased and reached 0.6 g g$^{-1}$ (Figure 2b). In the same period, the specific nitrogen concentration decreased, partly due to the dilution effect caused by accumulation of the starch, but nitrogen was also lost from the biomass, indicating that lysis of some of the cells occurred during the nitrogen limited phase (Table 1). Additionally, foam development on the surface of the culture indicated accumulation of macromolecular lysis products in the growth medium.

In order to maintain constant pH and dissolved oxygen partial pressure during progress of the culture, CO$_2$ and H$_2$ were added to the closed gas loop during the nitrogen sufficient as well as during the nitrogen limited phase (Figure 2c). Gas also had to be released from the gas loop at a regular rate to avoid build up of pressure due to evaporation of water vapour from the culture medium and the catalytic column, and therefore parts of the O$_2$ produced or CO$_2$ added were lost via this route. Figure 2c also shows how the light intensity was automatically increased during the cultivation.

Figure 2d shows the integrated amounts of CO$_2$ and O$_2$ consumed or produced by the cells, respec-

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**Figure 3** Batch culture of *Chlamydomonas reinhardtii* grown on NO$_2^-$ as nitrogen source. (a) Concentration of biomass dry weight (□), starch (○), NO$_2^-$ (▿), and chlorophyll a (⋄). Symbols are experimental values, broken lines are calculated from Eqs. 11–13. Parameters used in Eqs. 11–13, $q_{N,\text{max}} = 0.01$ mol C mol$^{-1}$ h$^{-1}$, $K_N = 2.2$ mM, $q_{C,\text{max}} = 0.08$ C mol C mol$^{-1}$ h$^{-1}$, $S_{\text{max}} = 0.6$ C mol C mol$^{-1}$, $Y_{x/N} = 7$ C mol mol$^{-1}$. (b) Specific concentrations of chlorophyll a (⋄) and starch (○). Symbols are experimental values, broken line is calculated from Eqs. 11 and 12. (c) On-line measurements of pH, dissolved oxygen tension, headspace pressure, and number of pressure releases and additions of CO$_2$, H$_2$, and N$_2$. (d) Estimated amounts of produced O$_2$ and assimilated CO$_2$ from Eqs. 5 and 6, respectively (broken lines), and $PQ$ values estimated from Eq. 7 (solid line).
tively calculated from Eqs. 5 and 6, and the PQ value calculated from Eq. 7 using $\Delta t = 4$ h. During the nitrogen sufficient phase, $PQ = 1.08 \pm 0.13$. The PQ was hardly affected by nitrogen depletion, but increased gradually during nitrogen limitation. Since the $C. reinhardtii$ biomass during the nitrogen sufficient phase was more reduced ($\gamma_x = 4.26$–4.38; Table 1) than a biomass of composition corresponding to the Redfield ratio ($\gamma_x = 4$), the PQ value was approximately 10% higher than predicted by Eq. 2.

$Chlamydomonas reinhardtii$, NO$_2^-$ as nitrogen source

NO$_3^-$ did not support growth of $C. reinhardtii$, but NO$_2^-$ was used as nitrogen source by $C. reinhardtii$ although at a slower rate compared to NH$_4^+$ (Figure 3a). In contrast to when grown on NH$_4^+$, starch accumulated during the nitrogen sufficient phase, and no major increase in the specific starch concentration was seen after NO$_2^-$ was depleted (Figure 3b). Although the specific chlorophyll $a$ concentration was also low during the NO$_2^-$ sufficient growth phase (Figure 3b), a decrease of the specific chlorophyll $a$ concentration was still observed as the concentration of NO$_2^-$ approached depletion (Figure 4a,b). The PQ value for growth on NO$_2^-$ (Figure 3d) was higher ($PQ = 1.15 \pm 0.10$) than for growth on NH$_4^+$ (Figure 2d), as would be expected for a more oxidised substrate. After NO$_2^-$ was depleted, growth as well as the addition rates of CO$_2$ and H$_2$ ceased.

$Chlorella$ sp., NO$_3^-$ as nitrogen source

Since $C. reinhardtii$ did not grow on NO$_3^-$, growth experiments on this nitrogen source were carried out using $Chlorella$ sp. (Figure 4). After a lag phase of approximately 30 h, $Chlorella$ started to consume NO$_3^-$ and the concentrations of biomass and chlorophyll $a$ increased (Figure 4a). Starch measurements in this alga were highly irreproducible because its small cell size and solid cell wall resulted in incomplete cell disintegration and starch extraction. However, it was still clear that, after NO$_3^-$ was depleted after 90 h, the biomass concentration continued to increase and more than 50% of the total biomass was produced during the nitrogen limited phase.

Estimation of PQ values from the titrations with CO$_2$ and H$_2$, showed that in the period when NO$_3^-$ assimilated, PQ was high as would be expected from Eq. 1, but as soon as NO$_3^-$ was depleted, and reducing power was no longer needed for reduction of nitrogen, the PQ value decreased.

When $Chlorella$ was grown on NH$_4^+$ no major change in PQ values was observed when NH$_4^+$ was depleted (Table 2). Changes in chlorophyll $a$ concentration were similar to the changes observed when NO$_3^-$ was the nitrogen source, and the biomass concentration increased also during the nitrogen

![Figure 4](image-url)
limited phase. Chlorella was also grown on NO$_2^-$, but in contrast to C. reinhardtii, the uptake rate of NO$_2^-$ was of similar magnitude to the uptake rates of NH$_4^+$ and NO$_3^-$.

Starch accumulated predominantly during the nitrogen limited phase, and a clear decrease in PQ as NO$_2^-$ became depleted was observed (Table 2).

### Discussion

The batch cultures of Chlamydomonas reinhardtii and Chlorella sp. clearly demonstrated that growth of these species cannot simply be described by a lag phase, a growth phase, and a stationary phase. Large changes of biomass composition and kinetics of growth take place in response to changes in the availability of different nitrogen sources. Because the cultures were grown using the lumostat principle, where the light intensity on the surface of the culture automatically is maintained at the optimal intensity, which results in the maximal CO$_2$ titration rate (Eriksen et al. 1996), growth was exponential throughout most of the nitrogen sufficient growth phase. Only at points close to the time the nitrogen source in the cultures was depleted did the light intensity reach its maximal value, after which the cultures might have begun to also experience light limitation.

Linear relationships were found between the amounts of CO$_2$ and H$_2$ added and the biomass concentration in the bioreactor. More H$_2$ compared to CO$_2$ and N$_2$ was added per pulse because of the relatively low viscosity of H$_2$ gas compared to other
gasses (see e.g., Geankopolis 1978). The actual biomass concentrations were lower than expected from the gas titrations, probably as a result of cell lysis in the cultures. However, the estimation of PQ values based on on-line titrations with CO$_2$ and H$_2$ gas corresponded well to what was expected (see Tables 1 and 2). In Chlorella sp., growth on NO$_3^-$ resulted in PQ values 1.3 times higher compared to biomass formation in the nitrogen depleted growth phase. In both species, growth on NH$_4^+$ resulted in PQ values almost identical to the PQ values during the nitrogen depleted growth phase. Growth on NO$_2^-$ resulted in PQ values in between those of NO$_3^-$ and NH$_4^+$ in Chlorella sp. (Table 2).

The PQ estimates depend on the distribution of oxygen and inorganic carbon in the culture and in the headspace. The major part of the total oxygen content in the system (approximately 97%) was present in the headspace due to the low solubility of oxygen in water. Therefore, an amount of oxygen corresponding to 2–4% of the oxygen produced by the algae was lost when excess pressure was released from the closed gas loop, and had to be accounted for in Eq. 5 in order to estimate the O$_2$ production rate by the algae.

The distribution of inorganic carbon was very different from the distribution of O$_2$ since the major part (81–86% of the total inorganic carbon) dissolved in the growth medium as HCO$_3^-$.

Only 5% of the inorganic carbon was present as CO$_2$ in the headspace gas, and less than 0.2% of the added CO$_2$ was lost from the system via ventilations. Ventilation of excess pressure therefore had no significant effect on the estimations of CO$_2$ uptake. In contrast, uptake of the

### Table 2

| Biomass                          | PQ$_{N$-sufficient}$/PQ$_{N$-depleted} | NH$_4^+$ | NO$_2^-$ | NO$_3^-$ |
|---------------------------------|--------------------------------------|---------|----------|----------|
| CH$_2$O(NH$_3$)$_{0.15}$        | 1.0                                  | 1.2     | 1.3      |
| CH$_{1.8}$O$_{0.5}$N$_{0.2}$    | 1.05                                 | 1.35    | 1.45     |
| Chlamydomonas reinhardtii       | 1.05 ± 0.04, n = 2                   | n.d.$^a$| n.d.$^d$ |
| Chlorella sp.                   | 0.95 ± 0.01, n = 2                   | 1.14, n = 1| 1.31 ± 0.11, n = 3 |

Experimental PQ values are means ± standard deviations of values estimated either during the last 24 h before or the first 24 h after the nitrogen source was depleted. Number of replicate batch cultures indicated by n

$^a$ Biomass composition corresponding to the Redfield ratio (Redfield et al. 1963)

$^b$ Biomass composition corresponding to the average of microorganisms (Roels 1980)

$^c$ Biomass was not produced after depletion of NO$_2^-$ in C. reinhardtii cultures

$^d$ C. reinhardtii did not grow on NO$_3^-$.

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Eriksen et al. 1996
Geankopolis 1978
Redfield et al. 1963
Roels 1980
nitrogen sources affected the CO₂ titrations to a much greater extent. When NO₂ or NO₃ was the nitrogen source, approximately 8% of the added CO₂ during the nitrogen sufficient growth phase was added to replenish the protons consumed during nitrogen reduction. When NH₄⁺ was the nitrogen source, approximately 8% less CO₂ than that removed by cells was added to maintain constant pH. Besides leading to underestimation of the CO₂ uptake rate, uptake of NH₄⁺ and the subsequent excretions of protons into the medium also had the consequence that inorganic carbon in the photobioreactor was depleted, unless the HCO₃⁻ concentration in the growth medium was higher than the NH₄⁺ concentration at the beginning of the experiment.

CO₂ also adsorbed reversibly onto the surface of the Pd catalyst, and during growth experiments, 4–8% of the total inorganic carbon in the systems was stored in the catalytic column. Low CO₂ partial pressure resulted in a relatively high proportion of adsorbed CO₂, since the column then was far from being saturated. The total amount of CO₂ adsorbed onto the column increased by 40–60 μmol during NO₃⁻ or NO₂⁻ sufficient growth phases or decreased by 40–60 μmol during NH₄⁺ sufficient growth phases. During the same periods, 25–35 mmol of organic carbon was produced, and the changes in the amount of adsorbed CO₂ amounted to only 0.1%–0.25% of the amount of CO₂ incorporated into biomass during nitrogen sufficient growth phases. The estimates of CO₂ uptake were therefore not significantly influenced by the changes in the amount of CO₂ adsorbed onto the catalytic column.

The strain of *C. reinhardtii* used in this study, which had been sub-cultured for several years in our laboratory, was deficient in its uptake of NO₃⁻ and NO₂⁻. The strain was unable to grow on NO₃⁻, despite the fact that four nitrate transport systems (some of which also transport NO₂⁻) have been described in other strains of *C. reinhardtii* (Galván et al. 1996; Rexach et al. 2002). *C. reinhardtii* also had a very low affinity for NO₂⁻. In order to obtain an accurate description of growth and NO₂⁻ uptake by Eqs. 8–13 (Figure 3a), a *Kₚ* value of 2–3 mM for NO₂⁻ was needed. This *Kₚ* value is much higher than reported *Kₚ* values of 1–30 μM for nitrite and nitrate transporters in *C. reinhardtii* (Galván et al. 1996; Rexach et al. 1999; Navarro et al. 2000). Because of the low NO₂⁻ affinity, NO₂⁻ was taken up at a relatively low rate (maximal *qNO₂⁻* = 0.15 mmol g DW⁻¹ h⁻¹) compared to the uptake rate of NH₄⁺ (maximal *qNH₄⁺* = 0.34 mmol g DW⁻¹ h⁻¹). However, the low uptake rate of NO₂⁻ gave the opportunity to compare growth of *C. reinhardtii* under nitrogen sufficiency on NH₄⁺ and nitrogen limitation on NO₂⁻. Because the CO₂ assimilation rates during the growth phase were almost similar for both nitrogen sources (*qCO₂* = 1.5 and 1.8 mmol g DW⁻¹ h⁻¹ for growth on NO₂⁻ and NH₄⁺, respectively), the low NO₂⁻ uptake rate lead to a situation where the cells responded as if they had been nitrogen limited although NO₂⁻ was still present in the growth medium. Carbon was incorporated into starch simultaneously with growth of the non-starch biomass, and the specific concentration of chlorophyll *a* remained very low (Figure 2) compared to cells grown on NH₄⁺.

The uptake of CO₂ in *C. reinhardtii* appeared to be regulated by the specific starch concentration in the cells. When the specific starch concentration reached approximately 0.6 g g⁻¹, the increase in biomass dry weight ceased. With NH₄⁺ as nitrogen source, the specific starch concentration was less than 0.1 g g⁻¹ during the nitrogen sufficient growth phase (Figure 2b), and the cells were therefore able to synthesise and incorporate large amounts of starch after NH₄⁺ was depleted. With NO₂⁻ as the nitrogen source, the specific starch concentration in the cells was already close to 0.6 g g⁻¹ when NO₂⁻ was depleted (Figure 3b), and the culture therefore entered a stationary phase with no further increase in biomass dry weight.

The small amounts of starch also present in exponentially growing *C. reinhardtii* cells showed that the CO₂ fixation capacity of the cells exceeded the need for reduced carbon for synthesis of non-starch biomass, and it is therefore reasonable to assume that the specific growth rate of light sufficient cultures of *C. reinhardtii* is restricted by the specific nitrogen uptake rate as described by Eq. 10.

*Chlorella* was able to utilise all three nitrogen sources tested, and all *Chlorella* cultures went through a nitrogen sufficient growth phase characterised by a high specific chlorophyll *a* concentration, and a nitrogen depleted growth phase characterised by an increasing content of what presumably is starch and a decreasing specific concentration of chlorophyll *a*. Pronounced decreases in PQ values were observed when NO₂⁻ and especially NO₃⁻ were depleted (Figure 4 and Table 2).
Segregation of batch growth into an exponential growth phase where active biomass is formed followed by a second growth phase where predominantly storage compounds are formed is a well-known phenomenon in algal cultures (Rigano et al. 2000; Ball 2002; Zhila et al. 2005). In this paper, we have presented a kinetic growth model describing the major effects of nitrogen limitation in C. reinhardtii and Chlorella and a methodology which enables these effects to be demonstrated on-line. The principle of simultaneous CO2 and H2 titrations to maintain constant pH and dissolved oxygen partial pressure, respectively, in gas-tight photobioreactors can be used for estimation of photosynthetic activity and PQ values, and to predict the composition of the produced biomass in cultures of algae. Titration of CO2 uptake and O2 production may also be developed into control and regulation tools for commercial algal cultures, e.g., to monitor the synthesis of lipids, poly-unsaturated fatty acids and other energy and carbon storage compounds, a class of microalgal products offering interesting biotechnological potentials (see e.g., Molina Grima et al. 1995; Zittelli et al. 1999; Kalacheva et al. 2002). Microalgal cultures have also been suggested for production of isotope labelled fine chemicals from 13CO2 or 14CO2 in closed photobioreactors (Delente et al. 1992; Behrens et al. 1994). In such systems, it would be possible to use estimates of PQ to control addition of the isotope labelled CO2 exclusively during the nitrogen limited growth phase, in order to cost-optimise the production of isotope labelled carbohydrates and lipids.

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Appendix

Distribution of CO2 in gas tight photobioreactor

To account for the effect of uptake of the nitrogen source on the total content of inorganic carbon in the photobioreactor, the distribution of inorganic carbon between the Pd catalyst, the gas phase, and the liquid medium was calculated. Since batch cultures were grown over relative long periods of time (200–300 h), the calculations were based on pseudo-steady-state conditions

\[
\text{CO}_2\text{,Pd} \rightleftharpoons \text{CO}_2\text{,gas} \rightleftharpoons \text{H}_2\text{CO}_3^* \rightleftharpoons \text{HCO}_3^- \rightleftharpoons \text{H}_2\text{O} + \text{CO}_2
\]

\[
+ \text{H}_2\text{O} \rightleftharpoons \text{CO}_3^- \rightleftharpoons 2\text{H}_2\text{O} + \text{CO}_2
\]

(A1)

The total amount of inorganic carbon in the photobioreactor, \( m_C \), was the sum of all pools of inorganic carbon present in the system as described in Eq. A1

\[
m_C = m_{\text{H}_2\text{CO}_3},_{\text{aq}} + m_{\text{HCO}_3},_{\text{aq}} + m_{\text{CO}_3^-},_{\text{aq}} + m_{\text{CO}_2,\text{gas}} + m_{\text{CO}_2,\text{Pd}} \]  

(A2)

where \( m_{\text{H}_2\text{CO}_3},_{\text{aq}} \), \( m_{\text{HCO}_3},_{\text{aq}} \), and \( m_{\text{CO}_3^-},_{\text{aq}} \) are the amounts of dissolved H2CO3 (including dissolved CO2), HCO3−, and CO3−, respectively, \( m_{\text{CO}_2,\text{gas}} \) is the total amount of CO2 in the gas phase, and \( m_{\text{CO}_2,\text{Pd}} \) is the total amount of CO2 adsorbed to the Pd catalyst.

At pH 7.5, CO3− constituted only in the order of 0.1% of the total dissolved inorganic carbon, and the amounts of inorganic carbon in the different pools described in Eqs. A1 and A2 were essentially controlled by the concentration of HCO3− in the growth medium. Since 1 mol of H+ was consumed for each mol of NO3 or NO2 taken up by the cells, these protons were regenerated by addition of CO2, which dissolved as HCO3− and resulted in an equimolar increase of [HCO3−]. For each mol of NH4+ taken up, 1 mol of H+ was produced, and with NH4+ as the nitrogen source, less CO2 than taken up photosynthetically were therefore added to the photobioreactor resulting in an equimolar decrease of [HCO3−]. The amount of dissolved HCO3− was therefore estimated by

\[
m_{\text{HCO}_3^-},_{\text{aq}} = \left( [\text{HCO}_3^-]_{\text{Lt}} \pm \Delta N \right) \cdot V_L \]  

(A3)

where \( \Delta N \) is the total decrease in concentration of the nitrogen source due to consumption by the algae, and \( V_L \) is the volume of the liquid medium.

The amount of dissolved H2CO3* in the growth medium was described by

\[
m_{\text{H}_2\text{CO}_3^*},_{\text{aq}} = \left( \frac{[\text{HCO}_3^-] \cdot [\text{H}^+]}{K_1} \right) \cdot V_L \]  

(A4)

 Springer
where $K_1$ is the equilibrium constant between $\text{H}_2\text{CO}_3$ and $\text{HCO}_3^- + \text{H}^+$ ($10^{-6.3}$ M, Stumm and Morgan 1995). The amount of dissolved $\text{CO}_3^{2-}$ is described by

$$m_{\text{CO}_3^{2-},aq} = \left(\frac{K_2 \cdot [\text{HCO}_3^-]}{[\text{H}^+]}\right) \cdot V_L \quad (A5)$$

where $K_2$ is the equilibrium constant between $\text{HCO}_3^-$ and $\text{CO}_3^{2-} + \text{H}^+$ ($10^{-10.3}$ M, Stumm and Morgan 1995).

The relationship between the partial pressure of $\text{CO}_2$ in the headspace, $p_{\text{CO}_2}$, and the concentration of $\text{H}_2\text{CO}_3$ in the medium was described by Henry’s law

$$p_{\text{CO}_2} = K_H \cdot [\text{H}_2\text{CO}_3] \quad (A6)$$

where $K_H$ is Henry’s constant ($3.0 \cdot 10^3$ kPa M$^{-1}$, Atkins 1980). The total amount of $\text{CO}_2$ in the headspace was calculated from $p_{\text{CO}_2}$ using the gas law

$$m_{\text{CO}_2,\text{gas}} = \frac{p_{\text{CO}_2} \cdot V_G}{R \cdot T} \quad (A7)$$

where $V_G$ is the volume of the gas in the headspace and the closed gas loop, $R$ is the gas constant, and $T$ is the absolute temperature.

The amount of $\text{CO}_2$ reversibly adsorbed onto the Pd catalyst was described by a Langmuir binding isotherm

$$m_{\text{CO}_2,\text{Pd}} = \frac{c_{\text{CO}_2,\text{Pd, max}} \cdot W_{\text{Pd}} \cdot p_{\text{CO}_2,\text{gas}}}{a + p_{\text{CO}_2,\text{gas}}} \quad (A8)$$

where $c_{\text{CO}_2,\text{Pd, max}}$ is the maximal surface-cover of $\text{CO}_2$ on the Pd catalyst (60 $\mu$mol g$^{-1}$), $W_{\text{Pd}}$ is the mass of Pd catalyst in the catalytic column (25 g), and $a$ is the half saturation constant (2.1 kPa). The parameters, $c_{\text{CO}_2,\text{Pd, max}}$ and $a$ were estimated by measuring the increase of partial pressure after adding known amounts of $\text{CO}_2$ to a closed chamber containing the Pd catalyst.

If the nitrogen uptake is measured or modelled, it is now possible to calculate the relationship between the overall change in total inorganic carbon content and nitrogen content in the photobioreactor:

$$\frac{\Delta m_{\text{C}_i}}{\Delta N \cdot V_L} = \frac{m_{\text{C}_i} - m_{\text{C}_i,t_0}}{(N - N_{\text{ini}}) \cdot V_L} \quad (A9)$$

With NO$_3^-$ and NO$_2^-$ as nitrogen sources, $\frac{\Delta m_{\text{C}_i}}{\Delta N \cdot V_L}$ is negative. With NH$_4^+$ as nitrogen source, $\frac{\Delta m_{\text{C}_i}}{\Delta N \cdot V_L}$ is positive. In the experiments described in this paper, $\Delta N$ was estimated from Eq. 13.

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