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.

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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 production in green algae (Rigano et al. 2000; Ball 2002; Zhila et al. 2005). In nitrogen depleted and carbon sufficient bacteria, accumulation of carbon and energy storage compounds may account for all the produced 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, CO_2 and O_2 are quantitatively the most important substrate and product, respectively. The ratio between O_2 evolution rate and CO_2 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, $CH_2O(NH_3)_{0.15}$ (Redfield et al. 1963), and NO_3^- is the nitrogen source

$$CO_2 + H_2O + 0.15NO_3^- + 0.15H^+$$
$$\rightarrow CH_2O(NH_3)_{0.15} + 1.3O_2 \qquad (1)$$
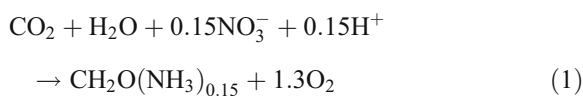
a PQ of 1.3 will be expected. With the lesser reduced NO_2^- as nitrogen source, the expected PQ is 1.2. Growth on NH_4^+,

$$CO_2 + H_2O + 0.15NH_4^+$$
$$\rightarrow CH_2O(NH_3)_{0.15} + 1.0O_2 + 0.15H^+ \qquad (2)$$
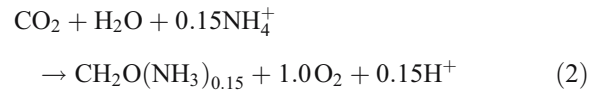
which is of similar degree of reduction as nitrogen in the biomass, should result in a PQ of 1. Changes in PQ are therefore expected if especially NO_3^- or NO_2^- are depleted and reduction of nitrogen no longer takes place.

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, γ_x (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 NH_4^+ is the nitrogen source (Eq. 2), the molar ratio between oxygen production and carbon fixation, $y_{O_2/x}$ can be found from a degree of reduction balance

$$0 = -\gamma_{CO_2} - \gamma_{H_2O} - y_{NH_3/x} \cdot \gamma_{NH_3}$$
$$+ \gamma_x + \gamma_{O_2} \cdot y_{O_2/x} \qquad (3)$$

where $y_{NH_3/x}$ is the molar ratio between ammonium uptake and carbon fixation. The degrees of reduction of CO_2, H_2O and NH_3, $\gamma_{CO_2}, \gamma_{H_2O}$, and γ_{NH_3}, respectively, all equal zero, and the degree of reduction of O_2, $\gamma_{O_2} = -4$ (Roels 1980). For the biomass, $\gamma_x = 4$ if carbohydrates are the only compounds formed, while synthesis of proteins and lipids results in higher γ_x values. When no organic products are secreted from the cells, all CO_2 taken up is incorporated into the biomass, and the PQ is equal to $y_{O_2/x}$. Equation 3 can then be rewritten

$$PQ = y_{O_2/x} = \frac{\gamma_x}{-\gamma_{O_2}} = \frac{\gamma_x}{4} \qquad (4)$$

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 NO_2^- or NO_3^- as nitrogen source can also be predicted by Eq. 4, although γ_x 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, NH_4^+, NO_2^-, and NO_3^-, respectively. All growth experiments were carried out in a novel gas-tight photobioreactor, in which CO_2 uptake was estimated from the addition of CO_2 as acidic titrant to maintain constant pH, and O_2 evolution was estimated from titration with H_2, which was used to reduce O_2 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, Třeboň, 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, NH_4^+, NO_2^-, or NO_3^- 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 N_2 (129 ± 5 μmol) could be added to increase the pressure in the gas loop from a pressurised N_2 supply by opening a solenoid valve (Sirai z0300A, Italy) for 0.1 s. The pressure of the N_2 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 O_2 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 O_2 in the gas loop on a Pd surface (see e.g., Nowakowski et al. 2002). Fixed amounts of H_2 (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, O_2 was reduced to H_2O by the consumption of 2 moles of H_2 per mol O_2. The pressure of the H_2 supply was 3.9 kPa above ambient pressure, and the H_2 additions were regulated by a dose-pause controller. The minimal pause between two H_2 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 H_2 was added.

Figure 1 Gas-tight photo-bioreactor, *r* reactor vessel, *s* sensors for pH and dissolved oxygen tension, *f* foam trap, *p* pressure gauge, *d* open ended tubing submerged in 15 cm of water, *c* catalytic column containing Pd catalyst, *a* air compressor, *v1–v4* solenoid valves controlling addition of N₂, release of excess pressure, addition of H₂, and addition of CO₂, respectively.
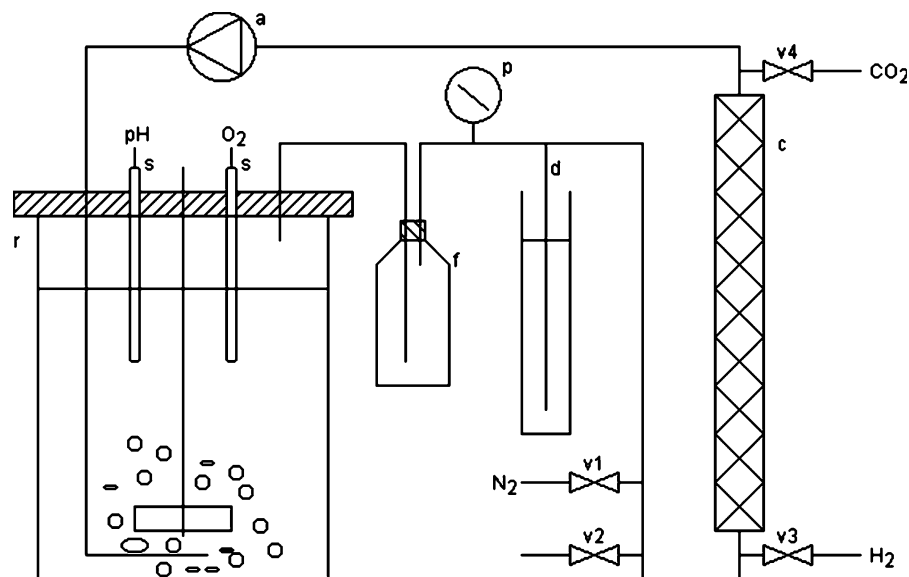
The number of additions of H_2 to the gas circulation loop was recorded by a computer and the molar amount of photosynthetically produced O_2, Δm_{O_2} in time interval Δt was estimated as

$$\frac{\Delta m_{O_2}}{\Delta t} = 0.5 \frac{\Delta n_{H_2}}{\Delta t} \cdot v_{H_2} + \frac{\Delta n_{\div}}{\Delta t} \cdot v_{\div} \cdot \frac{p_{O_2}}{p} \qquad (5)$$

where Δn_{H_2} and $\Delta n_{\div}$ are the number of H_2 additions and pressure releases, respectively, in time interval Δt, v_{H_2} and $v_{\div}$ 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 CO₂ 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, Δm_{CO_2} per time was also corrected for losses of CO_2 via ventilation of excess pressure, and calculated as

$$\frac{\Delta m_{CO_2}}{\Delta t} = \frac{\Delta n_{CO_2}}{\Delta t} \cdot v_{CO_2} - \frac{\Delta n_{\div}}{\Delta t} \cdot v_{\div} \cdot \frac{p_{CO_2}}{p} - \frac{\Delta N \cdot V_L}{\Delta t} \cdot \frac{\Delta m_{C_i}}{\Delta N \cdot V_L} \quad (6)$$

where Δn_{CO_2} is the number of CO_2 additions, v_{CO_2} is the moles of CO_2 added per addition, p_{CO_2} is CO_2 partial pressure, ΔN is the change in concentration of the nitrogen source, V_L is the volume of the growth medium, and $\frac{\Delta m_{C_i}}{\Delta N \cdot V_L}$ is the relationship between the overall change in total inorganic carbon content and nitrogen content in the photobioreactor. Calculations of p_{CO_2} and $\frac{\Delta m_{C_i}}{\Delta N \cdot V_L}$ are described in Appendix, and ΔN was estimated from Eq. 13 (see below). The photosynthetic quotient, PQ was estimated by dividing Eq. 5 with Eq. 6

$$PQ = \frac{\Delta m_{O_2}/\Delta t}{\Delta m_{CO_2}/\Delta t} \quad (7)$$

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 μmol 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 μmol 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 μmol 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-μm 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.Braunn 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 mg L^{-1}. The starch was hydrolysed to glucose by mixing 500 μL of the cell homogenate with 60 μL of a mixture containing 3 amylolytic enzymes (8 KNU of α-amylase, BAN240L, 5 AGU of glucoamylase, AMG 300L, and 9 PUN of pullunase, 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 NH_4^+, NO_2^-, and NO_3^-

Concentrations of NH_4^+, NO_2^-, and NO_3^- in supernatants of samples removed from the cultures were measured using a Technicon Traacs 800 auto-analyser. NH_4^+ was reacted with basic hypochlorite and salicylate to form a green indophenol complex, which could be measured at 660 nm. NO_2^- was

reacted with sulphanilamide and N-(1-naphtyl)ethylenediamine dihydrochloride and the product was recorded at 520 nm. NO_3^- was reduced to NO_2^- over a Cd column, and otherwise detected as NO_2^-.

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
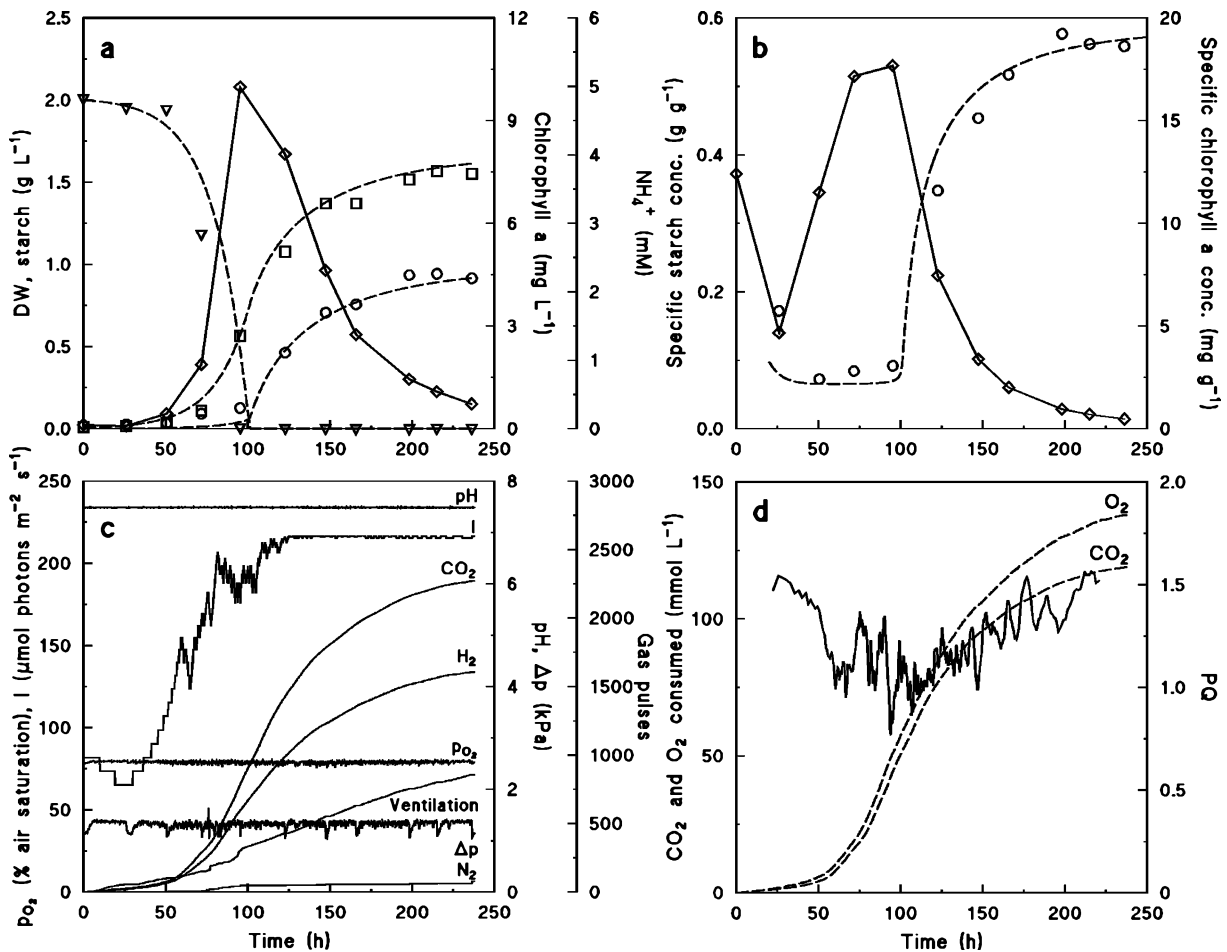
Figure 2 Batch culture of *Chlamydomonas reinhardtii* grown on NH_4^+ as nitrogen source. (a) Concentration of biomass dry weight (□), starch (○), NH_4^+ (▽), and chlorophyll *a* (◇). *Symbols* are experimental values, *broken lines* are calculated from Eqs. 11–13. Parameters used in Eqs. 11–13, $q_{N,max} = 0.01$ mol C-mol^{-1} h^{-1}, $K_N = 0.05$ mM, $q_{C,max} = 0.06$ C-mol C-mol^{-1} h^{-1}, $S_{max} = 0.6$ C-mol C-mol^{-1}, $Y_{x/N} = 5$ 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*).

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

$$q_N = \frac{q_{N,max} \cdot N}{K_N + N} \tag{8}$$

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

$$q_C = q_{C,max} \cdot \frac{S_{max} - S}{S_{max}} \tag{9}$$

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, μ (h^{-1}) is controlled by q_N

$$\mu = q_N \cdot Y_{x/N} \tag{10}$$

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

$$\frac{dx}{dt} = \mu x \tag{11}$$

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

$$\frac{ds}{dt} = q_c \cdot x - \frac{dx}{dt} \tag{12}$$

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

$$\frac{dN}{dt} = Y_{x/N}^{-1} \frac{dx}{dt} \tag{13}$$

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, ΔN 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.

Table 1 Biomass composition and *PQ* values during batch cultures of *Chlamydomonas reinhardtii* grown on NH_4^+ as nitrogen source

t (h)	x (g L^{-1})	Biomass composition	γ_x	N_x (mM)	Δt (h)	Biomass production	$\gamma\Delta x$	Calculated PQ	Measured PQ[a]
45	0.14	$CH_{1.71}O_{0.53}N_{0.13}$	4.26	0.7					
77	1.08	$CH_{1.76}O_{0.55}N_{0.09}$	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	$CH_{1.83}O_{0.60}N_{0.03}$	4.54	2.2	97–144	$CH_{1.88}O_{0.42}$	5.04	1.26	1.16
161	2.20	$CH_{1.85}O_{0.59}N_{0.02}$	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, γ_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 Δt, 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-
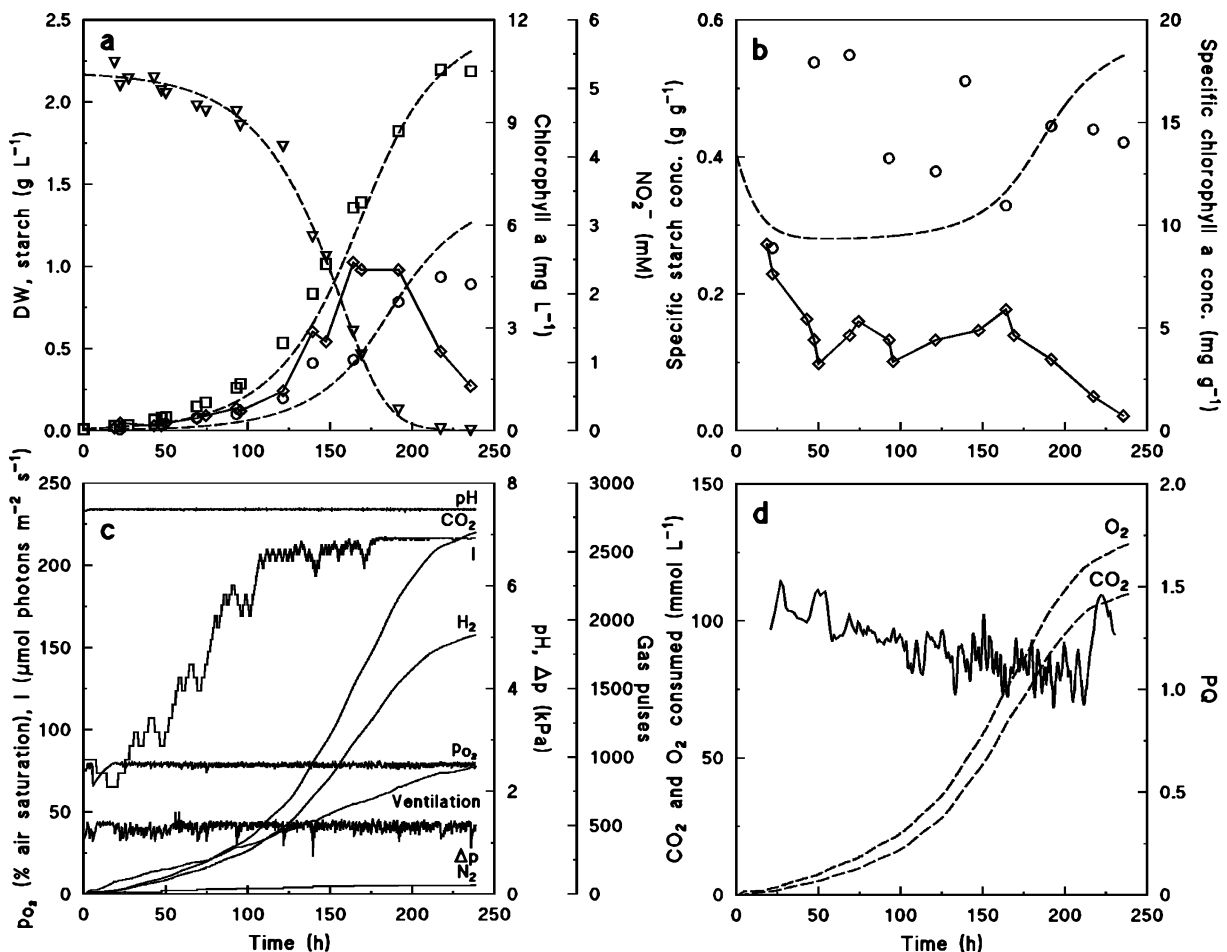
Figure 3 Batch culture of *Chlamydomonas reinhardtii* grown on NO_2^- as nitrogen source. (a) Concentration of biomass dry weight (□), starch (○), NO_2^- (▽), and chlorophyll *a* (◇). *Symbols* are experimental values, *broken lines* are calculated from Eqs. 11–13. Parameters used in Eqs. 11–13, $q_{N,max}$ = 0.01 mol C-mol^{-1} h^{-1}, K_N = 2.2 mM, $q_{C,max}$ = 0.08 C-mol C-mol^{-1} h^{-1}, S_{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 a $\Delta t = 4$ 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.
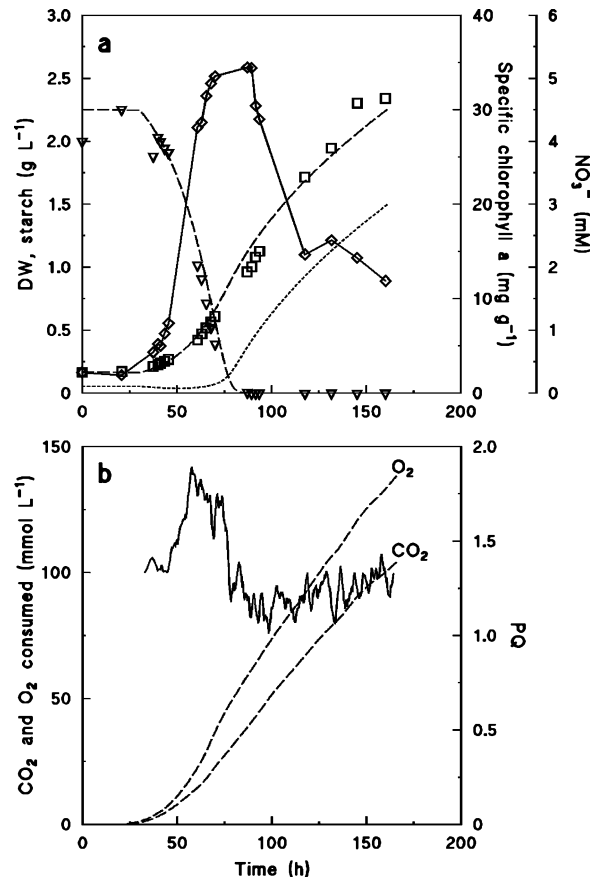
Figure 4 Batch culture of *Chlorella* sp. grown on NO_3^- as nitrogen source. (a) Concentration of biomass dry weight (□), NO_3^- (▽), and specific concentrations of chlorophyll *a* (◇). *Symbols* are experimental values, *broken lines* are calculated from Eqs. 11–13. Predicted concentration of starch (Eq. 12) indicated by *dotted line*. Parameters used in Eqs. 11–13, $q_{N,max} = 0.01$ mol C-mol^{-1} h^{-1}, $K_N = 0.5$ mM, $q_{C,max} = 0.05$ C-mol C-mol^{-1} h^{-1}, $S_{max} = 1$ C-mol C-mol^{-1}, $Y_{x/N} = 5$ C-mol mol^{-1}. (b) 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*).

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

Table 2 Ratio between PQ values during nitrogen sufficient and nitrogen depleted growth phases of different biomasses

Biomass	$PQ_{N\text{-sufficient}}/PQ_{N\text{-depleted}}$		
	NH_4^+	NO_2^-	NO_3^-
$CH_2O(NH_3)_{0.15}$[a]	1.0	1.2	1.3
$CH_{1.8}O_{0.5}N_{0.2}$[b]	1.05	1.35	1.45
Chlamydomonas reinhardtii	1.05 ± 0.04, $n = 2$	n.d.[c]	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^-

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

nitrogen sources affected the CO_2 titrations to a much greater extent. When NO_2^- or NO_3^- was the nitrogen source, approximately 8% of the added CO_2 during the nitrogen sufficient growth phase was added to replenish the protons consumed during nitrogen reduction. When NH_4^+ was the nitrogen source, approximately 8% less CO_2 than that removed by cells was added to maintain constant pH. Besides leading to underestimation of the CO_2 uptake rate, uptake of NH_4^+ and the subsequent excretions of protons into the medium also had the consequence that inorganic carbon in the photobioreactor was depleted, unless the HCO_3^- concentration in the growth medium was higher than the NH_4^+ concentration at the beginning of the experiment.

CO_2 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 CO_2 partial pressure resulted in a relatively high proportion of adsorbed CO_2, since the column then was far from being saturated. The total amount of CO_2 adsorbed onto the column increased by 40–60 μmol during NO_3^- or NO_2^- sufficient growth phases or decreased by 40–60 μmol during NH_4^+ sufficient growth phases. During the same periods, 25–35 mmol of organic carbon was produced, and the changes in the amount of adsorbed CO_2 amounted to only 0.1%–0.25% of the amount of CO_2 incorporated into biomass during nitrogen sufficient growth phases. The estimates of CO_2 uptake were therefore not significantly influenced by the changes in the amount of CO_2 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 NO_3^- and NO_2^-. The strain was unable to grow on NO_3^-, despite the fact that four nitrate transport systems (some of which also transport NO_2^-) 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 NO_2^-. In order to obtain an accurate description of growth and NO_2^- uptake by Eqs. 8–13 (Figure 3a), a K_s value of 2–3 mM for NO_2^- was needed. This K_s value is much higher than reported K_s 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 NO_2^- affinity, NO_2^- was taken up at a relatively low rate

(maximal $q_{NO_2^-} = 0.15$ mmol g $DW^{-1}h^{-1}$) compared to the uptake rate of NH_4^+ (maximal $q_{NH_4^+} = 0.34$ mmol g $DW^{-1}h^{-1}$). However, the low uptake rate of NO_2^- gave the opportunity to compare growth of *C. reinhardtii* under nitrogen sufficiency on NH_4^+ and nitrogen limitation on NO_2^-. Because the CO_2 assimilation rates during the growth phase were almost similar for both nitrogen sources ($q_{CO_2} = 1.5$ and 1.8 mmol g DW^{-1} h^{-1} for growth on NO_2^- and NH_4^+, respectively), the low NO_2^- uptake rate lead to a situation where the cells responded as if they had been nitrogen limited although NO_2^- 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 NH_4^+.

The uptake of CO_2 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 NH_4^+ 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 NH_4^+ was depleted. With NO_2^- as the nitrogen source, the specific starch concentration in the cells was already close to 0.6 g g^{-1} when NO_2^- 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 CO_2 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 NO_2^- and especially NO_3^- 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 CO_2 and H_2 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 CO_2 uptake and O_2 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 $^{13}CO_2$ or $^{14}CO_2$ 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 CO_2 exclusively during the nitrogen limited growth phase, in order to cost-optimise the production of isotope labelled carbohydrates and lipids.
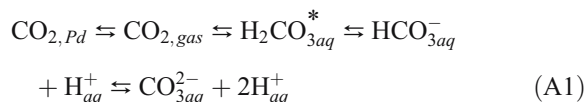
Appendix

Distribution of CO_2 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

$$CO_{2,Pd} \leftrightharpoons CO_{2,gas} \leftrightharpoons H_2CO_{3aq}^* \leftrightharpoons HCO_{3aq}^-$$
$$+ H_{aq}^+ \leftrightharpoons CO_{3aq}^{2-} + 2H_{aq}^+ \qquad (A1)$$

The total amount of inorganic carbon in the photobioreactor, m_{C_i} was the sum of all pools of inorganic carbon present in the system as described in Eq. A1

$$m_{C_i} = m_{H_2CO_3^*,aq} + m_{HCO_3^-,aq} + m_{CO_3^{2-},aq}$$
$$+ m_{CO_2,gas} + m_{CO_2,Pd} \qquad (A2)$$

where $m_{H_2CO_3^*,aq}$, $m_{HCO_3^-,aq}$, and $m_{CO_3^{2-},aq}$ are the amounts of dissolved H_2CO_3 (including dissolved CO_2), HCO_3^-, and CO_3^{2-}, respectively, $m_{CO_2,gas}$ is the total amount of CO_2 in the gas phase, and $m_{CO_2,Pd}$ is the total amount of CO_2 adsorbed to the Pd catalyst.

At pH 7.5, CO_3^{2-} 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 HCO_3^- in the growth medium. Since 1 mol of H^+ was consumed for each mol of NO_3^- or NO_2^- taken up by the cells, these protons were regenerated by addition of CO_2, which dissolved as HCO_3^- and resulted in an equimolar increase of $[HCO_3^-]$. For each mol of NH_4^+ taken up, 1 mol of H^+ was produced, and with NH_4^+ as the nitrogen source, less CO_2 than taken up photosynthetically were therefore added to the photobioreactor resulting in an equimolar decrease of $[HCO_3^-]$. The amount of dissolved HCO_3^- was therefore estimated by

$$m_{HCO_3^-,aq} = \left([HCO_3^-]_{t_0} \pm \Delta N \right) \cdot V_L \qquad (A3)$$

where ΔN 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 $H_2CO_3^*$ in the growth medium was described by

$$m_{H_2CO_3^*,aq} = \left(\frac{[HCO_3^-] \cdot [H^+]}{K_1} \right) \cdot V_L \qquad (A4)$$

where K_1 is the equilibrium constant between $H_2CO_3^*$ and $HCO_3^- + H^+$ ($10^{-6.3}$ M, Stumm and Morgan 1995). The amount of dissolved CO_3^{2-} is described by

$$m_{CO_3^{2-},aq} = \left(\frac{K_2 \cdot \left[HCO_3^- \right]}{\left[H^+ \right]_{t_0}} \right) \cdot V_L \qquad (A5)$$

where K_2 is the equilibrium constant between HCO_3^- and $CO_3^{2-} + H^+$ ($10^{-10.3}$ M, Stumm and Morgan 1995).

The relationship between the partial pressure of CO_2 in the headspace, p_{CO_2} and the concentration of $H_2CO_3^*$ in the medium was described by Henry's law

$$p_{CO_2} = K_H \cdot \left[H_2CO_3^* \right] \qquad (A6)$$

where K_H is Henry's constant ($3.0 \cdot 10^3$ kPa M^{-1}, Atkins 1980). The total amount of CO_2 in the headspace was calculated from p_{CO_2} using the gas law

$$m_{CO_2,gas} = \frac{p_{CO_2} \cdot V_G}{R \cdot T} \qquad (A7)$$

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 CO_2 reversibly adsorbed onto the Pd catalyst was described by a Langmuir binding isotherm

$$m_{CO_2,Pd} = \frac{c_{CO_2,Pd,max} \cdot W_{Pd} \cdot p_{CO_2,gas}}{a + p_{CO_2,gas}} \qquad (A8)$$

where $c_{CO_2,Pd,max}$ is the maximal surface-cover of CO_2 on the Pd catalyst (60 μmol g^{-1}), W_{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, $c_{CO_2,Pd,max}$ and a were estimated by measuring the increase of partial pressure after adding known amounts of 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, $\frac{\Delta m_{C_i}}{\Delta N \cdot V_L}$

$$\frac{\Delta m_{C_i}}{\Delta N \cdot V_L} = \frac{m_{C_i} - m_{C_i t_0}}{(N - N_{t_0}) \cdot V_L} \qquad (A9)$$

With NO_3^- and NO_2^- as nitrogen sources, $\frac{\Delta m_{C_i}}{\Delta N \cdot V_L}$ is negative. With NH_4^+ as nitrogen source, $\frac{\Delta m_{C_i}}{\Delta N \cdot V_L}$ is

positive. In the experiments described in this paper, ΔN was estimated from Eq. 13.

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