Assessment of Photosynthesis Regulation in Mixotrophically Cultured

Microalga *Chlorella sorokiniana*

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Abbreviations

Rubisco: ribulose-1, 5-bisphosphate carboxylase/oxygenase

PRK: phosphoribulokinase

PAM: pulse-amplitude modulation

rbcL: Rubisco large subunit

DCW: dry cell weight

RuBP: ribulose-1, 5-bisphosphate

I_k : the saturation light intensity

α -slope: the slope of the light-limited part of the $P-I$ curve

P_{max} : the light-saturated rate of photosynthesis

R_d : respiration rate

F_v/F_m : Potential maximum quantum efficiency

Φ_{PSII} : quantum yield of PSII photochemistry in the light

NPQ: non-photochemical quenching

qE: pH-dependent energy quenching

qL: the fraction of open PSII reaction centers

Q_A : quinone A

LHCSR3: light-harvesting complex stress-related protein 3

PQ pool: plastoquinone pool

1 **Abstract**

2 Mixotrophic growth of microalgae offers great potential as an efficient strategy for
3 biofuel production. In this study, photosynthetic regulation of mixotrophically cultured
4 *Chlorella sorokiniana* cells was systematically evaluated. Mixotrophic cells in the
5 exponential growth phase showed the highest photosynthetic activity, where maximum
6 photosynthetic O₂ evolution was approximately 3- and 4-fold higher than cells in the
7 same phase grown photoautotrophically in 1% CO₂ (in air) and air, respectively.
8 Additionally, characteristic chlorophyll fluorescence parameters demonstrated that no
9 limitation in electron transport downstream of PSII was detected in mixotrophic cells.
10 Up-regulation of photosynthetic activity was associated with high total ribulose-1, 5-
11 bisphosphate carboxylase/oxygenase (Rubisco) carboxylase activity and expression level
12 of phosphoribulokinase (PRK). After 3 days, photosynthetic O₂ evolution of mixotrophic
13 cells that went to the stationary phase, was strongly reduced, with reduced photochemical
14 efficiency and reorganization of the PSII complex. Simultaneously, enzymatic activity for
15 Rubisco carboxylase and mRNA levels of Rubisco and PRK diminished. Importantly,
16 there was almost no non-photochemical quenching for mixotrophic cells, whether grown
17 in log or stationary phase. A decline in the quantum efficiency of PSII and an oxidized
18 plastoquinone pool (PQ pool) was observed under N-depleted conditions during
19 mixotrophic growth. These results demonstrate that photosynthesis is regulated
20 differently in mixotrophically cultured *C. sorokiniana* cells than in cells grown under
21 photoautotrophic conditions, with a particularly strong impact by nitrogen levels in the
22 cells.

23 **Keywords:** microalgae; *Chlorella sorokiniana*; mixotrophic; photosynthesis.

1. Introduction

Mixotrophic growth of microalgae is gaining increasing interest, largely because it offers the promise of an efficient strategy for commercial production of lipids and other value-added products. The combination of organic carbon assimilation and simultaneous CO₂ fixation in the light leads to higher growth rates and biomass accumulation in algae cultured mixotrophically than the same strains cultivated photoautotrophically or heterotrophically [1, 2]. Mixotrophic growth also offers augmented lipid productivity [3], which is relevant for biofuel production from microalgae.

Our previous study demonstrated that the green alga *Chlorella sorokiniana* shows the best growth performance under mixotrophic conditions [1]. The mixotrophic growth of algal cells is based on CO₂ and organic carbon, thus both photosynthetic and organic substrate metabolic pathways have to operate concurrently. However, how this occurs, how it is regulated, and what the exact mechanism is that the mixotrophically grown cells use to accumulate biomass are largely unanswered questions. Of particular interest is determining how the photosynthetic and organic substrate metabolic pathways interact with each other. In photosynthetic pathway, light energy gathered as ATP and NADPH in light reactions is used to fuel cellular metabolism, in particular the reduction of CO₂ to three-carbon sugars. For the substrate metabolic pathway, glucose (as a model substrate) is metabolized to three carbon sugars following ATP-dependent entry into glycolysis. Thus, concurrent glucose metabolism may influence the utilization of carbon compounds arising from CO₂ fixation, and alter electron flux of photosynthesis by modulating the consumption rate of ATP and NADPH [4, 5].

46 It has been reported that acetate inhibits photosynthesis in *Chlamydomonas reinhardtii*,
47 and high acetate virtually abolishes photosynthetic carbon gain [6, 7].

48 Numerous groups have observed the influence of exogenous organic carbon on the
49 photosynthetic reaction centers of algal cells. Lewitus et al. (1991) showed that glycerol
50 supply in photoautotrophically grown *Pyrenomonas salina* led to a reduction of light-
51 harvesting related photosynthetic components in algal cells [8]. The addition of acetate to
52 *C. reinhardtii* grown photoautotrophically significantly reduced the fraction of carbon
53 biomass derived from photosynthesis, with dramatically declined net O₂ evolution rate
54 and CO₂ fixation rate [9]. Similarly, the mixotrophically cultured diatom, *Phaeodactylum*
55 *tricornutum*, supplemented with different kinds of organic substrate, showed a decline in
56 O₂ evolution accompanied by decreased PSII activity and reduced electron transport rate,
57 which demonstrated that organic carbon metabolism can have an adverse impact on
58 photosynthesis beyond the green algae [10]. However, glucose was shown to enhance the
59 net photosynthesis rate in the cyanobacterium *Synechococcus* sp., indicating that this is
60 not a universal situation [11].

61 Furthermore, exogenous organic substrate metabolism also affected the capacities of
62 photosynthetic enzymes.

63 Previously studies have reported that organic carbon represses the expression of some
64 proteins encoded in nucleus, such as chlorophyll a/b-binding proteins evolved in
65 photosystem II, and ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) in
66 Calvin cycle [12-14]. The addition of glucose lowered abundance of Rubisco and levels
67 of the PSII reaction center protein D1 in alga *Galdieria sulphuraria* [15]. Thus,

68 exogenous organic carbon exerts diverse influence on photosynthetic activity of algal
69 cells in mixotrophic growth.

70 In this study, we investigated the effect of glucose metabolism on photosynthesis and
71 related processes, and how microalgal cells respond under mixotrophic conditions
72 compared to photoautotrophic conditions. In particular, attention was paid to determine
73 how photosynthetic activity is regulated as the algae grow mixotrophically.

74

2. Materials and Methods

2.1 Organism and medium

The green microalga *Chlorella sorokiniana* (UTEX 1602) was purchased from the Culture Collection of Algae (UTEX). For algal seeds preparation, *C. sorokiniana* were cultured in Kuhl medium at 25 °C bubbled with 1% CO₂ in 250-ml Erlenmeyer flasks, and illuminated with fluorescent light to give constant light intensity of 100 μmol m⁻² s⁻¹. Except where indicated, the medium for photoautotrophic culture was Kuhl medium, agitated with 1% CO₂ in air (1% CO₂-conditioned) or supplied with only air (air-conditioned). For heterotrophic and mixotrophic culture, the Kuhl medium was supplemented with 6 g L⁻¹ glucose and the cells were grown with orbital shaking.

2.2 Growth analysis

Dry cell weight (DCW) was determined according to our previous methods [1]. Nitrate-nitrogen was determined with the H₂SO₄-salicylic acid method, as described by Cataldo et al. [16].

2.3 Photosynthetic Oxygen evolution

Measurement of photosynthetic O₂ evolution was conducted by a Clark-type oxygen electrode (Hansatech, UK), and 1.2 mL of cell suspension with the same chlorophyll concentration (20 μM) was loaded in the oxygen electrode chamber. Prior to the O₂ evolution measurements, 20 μL of a 0.5 M sodium bicarbonate solution (pH 7.4) was supplied to guarantee enough carbon source available to the cells. Chlorophyll extracted with 100% methanol from algal cells was quantified according to Porra et al. [17]. The parameters for the photosynthetic responses to irradiance curves were analyzed according to Henley [18]:

$$P = P_{max} \left(\alpha I / \sqrt{P_{max}^2 + (\alpha I)^2} \right) + R_d$$

$$I_k = P_{max} / \alpha$$

Where I ($\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) represents irradiance, P ($\text{mmol O}_2 \text{ mmol Chl}^{-1} \text{ h}^{-1}$) the photosynthetic rate at irradiance I , P_{max} ($\text{mmol O}_2 \text{ mmol Chl}^{-1} \text{ h}^{-1}$) the light-saturated rate of photosynthesis, I_k ($\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) the saturation light intensity, α ($(\text{mmol O}_2 \text{ (mmol Chl)}^{-1} \text{ h}^{-1}) (\mu\text{mol m}^{-2} \text{ s}^{-1})^{-1}$) the slope of the light-limited part of the P - I curve, and R_d the dark respiration rate ($\text{mmol O}_2 \text{ mmol Chl}^{-1} \text{ h}^{-1}$).

2.4 Photosynthetic Activity Measurements

Photosynthetic activities were measured by pulse-amplitude-modulation (PAM) fluorometry with a Hansatech system. Algal cell suspensions were loaded with chlorophyll concentration of 10 μM and kept in dark for 15 min before measurement. The minimal fluorescence yield (F_0) was measured by activating the modulated light which was too weak to close reaction center. A saturating light pulse (about 3000 $\mu\text{mol m}^{-2} \text{ s}^{-1}$) was then applied to determine the maximal fluorescence yield (F_m) of the dark-adapted samples. The actinic light from light-emitting diode was then turned on to provide continuous actinic light, and a steady fluorescence yield (F_s) could be obtained. Saturating pulses were applied to the sample at different intervals to obtain a stationary level of maximum fluorescence (F_m'). The fluorescence parameters mentioned in the following study were calculated according to Baker [19].

2.5 77K Fluorescence Spectroscopy

Fluorescence spectra at 77K were measured to analyze stoichiometry and composition of PSI and PSII. Low temperature fluorescence spectra were obtained as described by

Kirchhoff et al. [20] using a Horiba Jobin Yvon FluoroMax 4 spectrofluorimeter. Cells were adjusted to a low chlorophyll concentration of about 2 μ M to avoid fluorescence reabsorption, shock-frozen in liquid nitrogen, and excited at 435 nm (slit width 2 nm). Emission spectra were recorded in the spectral range of 650-800 nm.

2.6 Measurements of Rubisco activity

Rubisco is the initial enzyme of CO₂ fixation in the Calvin-Benson cycle, catalyzing the addition of CO₂ to ribulose-1, 5-bisphosphate (RuBP). Microalgae were grown photoautotrophically (1% CO₂, air), heterotrophically, and mixotrophically. After 1 and 3 days, the cells pellets after centrifugation were frozen immediately in liquid nitrogen, and subsequently ground on ice in 1 ml of extraction buffer with a mortar and pestle. The extraction buffer was modified based on Kodama et al. [21]. Briefly, it includes 50 mM Hepes, pH 7.8, 10 mM dithiothreitol (DTT), 1% polyvinylpolypyrrolidone (PVPP), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% Triton] with 10 μ L of protease inhibitor cocktail (Sigma, St. Louis, MO, USA). The supernatant of the extract was added with 15 mM MgCl₂ and 15 mM NaHCO₃ and incubated for 10 min at room temperature to activate enzymes. Total Rubisco activity was determined in activated supernatant in 1 mL of assay buffer (100 mM EPPS-NaOH pH 8.0, 20 mM MgCl₂, 1mM EDTA, 0.2 mM NADH, coupling enzymes, 20 mM NaHCO₃) as described previously [22]. The activity was determined by monitoring NADH consumption, following initiation with 0.5 mM ribulose bisphosphate (RuBP).

2.7 Quantitative RT-PCR conditions and analysis

The total RNA isolation and quantitative RT-PCR was performed as previously described [23]. Table 1 shows all primer sequences we have used in this work.

Table 1. Primers for quantitative real-time PCR detection of expression genes in *C. sorokiniana*

Gene	Primer sequence (5'→3')	Length (bp) of production
18S	F: ATCAACCTGACAAGGCAACC R: CCTGCGGCTTAATTTGACTC	150
rbcL	F: TGGTCACCACCTGATAAACG R: GCTCACTACTGCCGTGACAA	132
prk	F: TGAATGGCTGCTGCTATGAC R: GTTGAGGCATCTCCAATGCT	150

2.8 Statistical analysis

An analysis of presented data was statistically conducted using Student's *t* test, with a significance level of 0.05.

3. Results

3.1 Photoautotrophic, mixotrophic and heterotrophic cultivation of *C. sorokiniana*

Comparison of three different culture modes was carried out in 250-mL flasks. As a result, mixotrophy showed the most significant promotion in the cell growth (Figure 1A), with the specific growth rate and maximum biomass largely exceeding those of heterotrophy, 1% CO₂-conditioned photoautotrophy, and air-conditioned photoautotrophy (Table 2). After 1 day, algal cells grown under different conditions were at the exponential stage, with 90% nitrogen left in culture medium for photoautotrophic cells (1% CO₂-conditioned and air-conditioned) and 30% left for mixotrophic and heterotrophic cells; after 3 days, nitrogen was assimilated completely in the medium for both mixotrophic and heterotrophic cells, which went into a stationary phase; but nitrogen still remained in the medium of photoautotrophy, with 60% left in air-conditioned culture (Fig. 1B).

Table 2. Growth parameters of *C. sorokiniana* under different culture conditions

	Mixotrophy	Heterotrophy	Photoautotrophy (1% CO ₂)	Photoautotrophy (air)
Specific growth rate (d ⁻¹)	3.13 ± 0.22	1.56 ± 0.02	1.24 ± 0.04	0.60 ± 0.01
Max. biomass ^a (g L ⁻¹)	4.57 ± 0.12	2.78 ± 0.06	1.70 ± 0.22	0.36 ± 0.00

^a Cultivation for 4 days

Values shown are averages of triplicates ± standard deviation.

Figure 1. Cultivation of *C. sorokiniana* under different conditions. (A) Growth curves of *C. sorokiniana* under photoautotrophic (filled up-pointing triangle, in air; filled down-pointing triangle, in 1% CO₂), heterotrophic (open circle), and mixotrophic conditions (filled circle); (B) Time course of KNO₃ consumption under photoautotrophic, heterotrophic, and mixotrophic conditions. Symbols are the same as in Fig. 1A. All data are averages of triplicates $\pm$ standard deviation.

3.2 Comparison of photosynthesis and respiration for algal cells cultured mixotrophically and photoautotrophically

To determine the effect of glucose on photosynthesis, the net photosynthetic O₂ evolution rates as a function of the probing actinic light intensity (P - I curve) of mixotrophic and photoautotrophic cultures were compared. In the P - I curve, the O₂ evolution rates increased linearly with light intensity and then reached a plateau as the saturation light intensity (I_k) approached. This light-saturated O₂ evolution rates (P_{max}) measures the maximum capability of photosynthesis for the algal cells [24]. The slope of the initial linear increase (α -slope) reflects the photon use efficiency in photosynthesis.

When *C. sorokiniana* cells were cultured mixotrophically, there was a drastic change in the level of respiration and photosynthesis (Table 3) at different growth stages. Here mixotrophic cells cultured 1 day and 3 days represents the exponential phase and the stationary phase, respectively.

Mixotrophic cells in the exponential growth phase exhibited dramatic augmentation in respiration rate (R_d), which was 8- and 5-fold higher than 1% CO₂-conditioned cells and air-conditioned cells after growing for one day, respectively. At the same time, the photosynthetic O₂ evolution rate of mixotrophically grown cells was significantly higher than that of photoautotrophic cultures (Fig. 2). As shown in Table 3, mixotrophic cells reached the highest light-saturated rate of photosynthesis ($P_{max} \sim 300$ mmol O₂ (mmol Chl⁻¹) h⁻¹) that is approximately 3- and 4-fold higher than that of 1% CO₂-conditioned and air-conditioned cells, respectively. Moreover, the values of I_k and α -slope were also

much higher for mixotrophic cells, pointing to a high light requirement to obtain a high photosynthetic rate and more efficient light utilization, respectively.

However, mixotrophic cells at stationary growth phase (Fig. 1) exhibited considerably decreased photosynthetic capability, with the lowest P_{max} and I_k values (Fig. 2, Table 3). At the same time, the α -slope showed an obvious decrease, which suggests that not all chlorophyll molecules were photochemically competent in mixotrophic cells [25]. For photoautotrophic cells, 1% CO₂-conditioned cells had higher photosynthetic O₂ evolution rates than air-conditioned cells after growing either 1 day or 3 days, with higher values of P_{max} , I_k and α -slope.

Table 3. Characteristic P-I curve parameters (R_d , P_{max} , I_k and α -slope) of *C. sorokiniana* cultured under mixotrophic (M) and photoautotrophic (1% CO₂, air) conditions after 1 day and 3 days.

Time (d)	Mode	R_d	P_{max}	I_k	α -slope	R^2
1	M	62.00 ± 2.21	282.8 ± 8.85	310.8 ± 4.55	0.91 ± 0.03	0.99
	1% CO ₂	7.59 ± 0.39	99.02 ± 9.11	183.6 ± 19.65	0.54 ± 0.00	0.99
	Air	12.06 ± 0.45	68.49 ± 1.91	130.1 ± 7.30	0.53 ± 0.04	1
3	M	35.65 ± 0.64	27.93 ± 8.14	49.03 ± 7.78	0.56 ± 0.00	0.93
	1% CO ₂	13.71 ± 0.63	191.5 ± 8.49	250.1 ± 28.90	0.77 ± 0.10	0.99
	Air	12.47 ± 0.40	49.05 ± 3.51	149.2 ± 18.03	0.33 ± 0.05	0.99

Values are the means ± SE derived from the applied model.

Figure 2. Light-saturation curves of photosynthesis in mixotrophically (circle) and photoautotrophically (triangle up, air; triangle down, 1% CO₂) cultured *C. sorokiniana* after 1 day (filled) and 3 days (open), respectively. Rates of oxygen evolution were measured on a per chlorophyll basis, from single representative experiments.

3.3 Fluorescence characterization of mixotrophic and photoautotrophic cells

Light-dependent O₂ evolution is strictly dependent on PSII-associated water cleavage and concomitant linear photosynthetic electron flow. To characterize photosynthetic activity of mixotrophic and photoautotrophic cells, utilization of absorbed light energy was examined in more detail via measurement of chlorophyll fluorescence. Potential maximum quantum efficiency (F_v/F_m), quantum yield of PSII photochemistry in the light (Φ_{PSII}), non-photochemical quenching (NPQ), pH-dependent energy quenching (qE), and the fraction of open PSII reaction centers (qL) were determined (Fig. 3).

After 1 day of cultivation, F_v/F_m of mixotrophic cells was 0.81-0.86 (Fig. 3A), which indicated healthy cells and potentially efficient PSII primary activity (from water to quinone A (Q_A)). Moreover, Φ_{PSII} of mixotrophic cells tended to be slightly higher than photoautotrophic cells at all irradiance (Fig. 3B). No significant difference was observed between mixotrophic and 1% CO_2 -conditioned cells in qL (Fig. 3E), indicating no limitations in electron transport downstream of PSII in mixotrophic cells compared with photoautotrophic cells.

After 3 days, F_v/F_m of mixotrophic cells dramatically declined by nearly 60% compared with that after 1 day, but there was no difference for 1% CO_2 -conditioned cells (0.85-0.88) and air-conditioned cells (0.75-0.78) after cultivation for 1 day and 3 days (Fig. 3A, 3F). The Φ_{PSII} of mixotrophic cells also largely declined, and showed the lowest values (Fig. 3B, 3G). This could be caused by damage/degradation of PSII (F_v/F_m) and is in line with strong decline in oxygen evolution rate (Fig. 2).

In photoautotrophic conditions, 3 day cultured cells showed lower qL at low irradiances when compared with 1 day cultured cells, implying that the primary acceptor Q_A was more reduced after 3 day's growth. On the other hand, qL of mixotrophic cells was found to be less influenced after 3 days' culture, and tended to be higher than those of 3 day cultured photoautotrophic cells (Fig. 3E, 3J).

Figure 3. Photosynthetic characteristics of *C. sorokiniana* grown mixotrophically (filled circle) and photoautotrophically (filled up-pointing triangle, in air; filled down-pointing triangle, in 1% CO₂) after 1 day (A, B, C, D, E) and 3 days (F, G, H, I, J). The light dependency of each photosynthetic parameter is shown (A-J). (A, F) The potential maximum quantum efficiency (Fv/Fm); (B, G) the quantum yield of PSII (Φ_{PSII}); (C, H) the non-photochemical quenching (NPQ); (D, I) the energy-dependent quenching (qE); (E, J) the fraction of open PSII reaction centers (qL). Data of Fv/Fm, Φ_{PSII} , NPQ, qE and qL measured from single representative experiments.

NPQ, a measure of non-photochemical quenching [26], describes a collection of processes that are thought to protect the chloroplast from light damage. For mixotrophic growth, cells cultured for 1 day and 3 days displayed no NPQ at irradiance lower than 300 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (Fig. 3C, 3H); however, cells grown photoautotrophically in 1% CO₂ and air conditions had capacity for this process, yet this was markedly greater in air-conditioned cells than in 1% CO₂-conditioned cells. The air-conditioned cells cultured for 3 days exhibited increased levels of NPQ across the entire irradiance range. The pH-dependent quenching qE, part of NPQ resulting from an increase in the thermal dissipation capacity of the light-collecting apparatus, showed similar trends with NPQ in mixotrophic and photoautotrophic cells (Fig. 3D, 3I). Technically, qE is a widely adopted mechanism of coping with stressful light conditions in algae, through the qE effector light-harvesting complex stress-related protein 3 (LHCSR3) [27]. Overall data indicate that photoprotective qE is not upregulated under mixotrophic conditions.

3.4 Φ_{PSII} vs. qE and Φ_{PSII} vs. qL

To further characterize the influence factor of Φ_{PSII} , we analyzed the dependence of Φ_{PSII} on q_E and on the redox level of the PQ pool (q_L). For mixotrophic cells, there was at most a small amount of q_E in the range of Φ_{PSII} , and the two factors were not significantly correlated (Fig. 4A, 4B). On the other hand, photoautotrophic cells, in particular under air, generally showed a stronger correlation of Φ_{PSII} on q_E .

Figure 4. Φ_{PSII} vs. q_E (A, B) and Φ_{PSII} vs. q_L (C, D) of *C. sorokiniana* grown mixotrophically (filled circle) and photoautotrophically (filled up-pointing triangle, in air; filled down-pointing triangle, in 1% CO_2) after 1 day (A, C) and 3 days (B, D). Data from single representative experiments.

284

285 We also studied the dependency of Φ_{PSII} on the redox level of the PQ pool, which are
286 generally correlated directly. Under all cultivation conditions, Φ_{PSII} showed the expected
287 positive relationship with qL, which indicates PSII can convert light energy more
288 efficiently with a more oxidized PQ pool (higher Φ_{PSII} if qL is higher). After cultivation
289 for 1 day, mixotrophic and 1% CO₂-conditioned cells had similar trends of dependency of
290 Φ_{PSII} on qL (Fig. 4C). However, after cultivation for 3 days, the augmentation of Φ_{PSII} in
291 mixotrophic cells was less pronounced in response to qL. Thus, compared with
292 photoautotrophic cells, Φ_{PSII} in mixotrophic cells is less sensitive to qE and qL. This
293 could be caused by an increased number of damaged PSII centers in 3 day cultured cells.

294 **3.5 77K fluorescence spectra**

295 The above results of photosynthetic regulation prompted us to analyze PSI and PSII with
296 associated antenna in mixotrophic and photoautotrophic cells. 77K fluorescence spectra
297 has been widely used to indicate the abundance of photosystems with associated antenna
298 proteins. Our results show that the spectra of the mixotrophic and photoautotrophic cells
299 had major emission peaks around 688 nm (F688) and 722 nm (F722), and shoulder peaks
300 around 695 nm (F695) (Fig. 5). Among these, F688 and F695 are emitted from PSII,
301 F722 is from PSI [28]. The fluorescence ratio of F722 to F688 was used to examine
302 variations in the redistribution of excitation energy between PSI and PSII. This value was
303 0.97 for 1 day cultured mixotrophic cells, which was much less than that of 1% CO₂-
304 conditioned cells (1.63) and air-conditioned cells (1.32). The low F722 to F688 ratio for
305 mixotrophic cells indicates that the PSII antenna system is enlarged relative to the PSI
306 antenna. For 3 day cultured mixotrophic cells, the fluorescence peak at 688 nm shifted to

685 nm. The observed shift is a sign of increased free LHCII component, which has a characteristic emission around 680 nm [29], and indicates that more LHCII is not functionally coupled to PSII or PSI. This free LHCII observed for 3 day cultured mixotrophic cells could be released from damaged PSII (see above).

Figure 5. 77K fluorescence emission spectra of mixotrophic (solid line) and photoautotrophic (dotted line, 1% CO₂; dash line, air) cells after excitation of chlorophylls at 435 nm. Spectra are normalized to the main PSII emission peak. A, 1 day; B, 3 days.

3.6 Adjustment of Rubisco and PRK

For efficient utilization of chemical energy generated from light reactions, the activation degree of the Calvin-Benson cycle must be coordinated, which is the major sink for reductants and ATP. Activities and expression levels of enzymes involved in the Calvin-Benson cycle were compared in order to determine whether the regulation of photosynthesis was accompanied by major changes in the protein levels.

Total Rubisco carboxylase activity of 1 day cultured mixotrophic cells was the highest, yet it declined by 87% and became the lowest after 3 days' cultivation (Table 4). For photoautotrophic conditions, 1% CO₂-conditioned cells showed much higher Rubisco carboxylase activities than air-conditioned cells after cultivation for 1 day and 3 days. Instead of keeping similar Rubisco carboxylase activity in air-conditioned cells, activity in 1% CO₂-conditioned cells declined by 30% after 3 days.

Table 4. Effects of different growth conditions on total Rubisco carboxylase activity and chlorophyll to protein ratio.

Culture condition	Rubisco carboxylase activity ($\mu\text{mol CO}_2 \cdot [\text{mg chlorophyll}]^{-1} \cdot \text{s}^{-1}$)	
	1 d	3 d
Mixotrophy	48.02 ± 3.62	6.40 ± 0.24
Photoautotrophy (1% CO ₂)	41.12 ± 1.61	28.66 ± 0.19
Photoautotrophy (air)	15.76 ± 0.03	17.91 ± 0.03

Values shown are averages of triplicates $\pm$ standard deviation.

The expression levels of Rubisco and phosphoribulokinase (PRK) were examined by quantitative RT-PCR (Fig. 6). Rubisco expression levels in mixotrophic cells were similar to 1% CO₂-conditioned cells for 1 day and 3 days, respectively. On the other hand, PRK expression level in mixotrophic cells was the highest after 1 day. Impressively, air-conditioned cells exhibited dramatically high values of Rubisco expression level, indicating that the *rbcL* gene was obviously up-regulated under this condition.

Figure 6. RT-PCR analysis of Rubisco (A) and PRK (B) in mixotrophically (black bar) and photoautotrophically (light grey bar, 1% CO₂; dark grey bar, air) cultured cells. All data are averages of triplicates $\pm$ standard deviation.

4. Discussion

In this study, we comparatively analyzed the response of photosynthetic characteristics of mixotrophic algal cells at different growth stages. Compared with photoautotrophic cells, the mixotrophically grown *C. sorokiniana* cells exhibited an increase in respiratory metabolism, as indicated by the large respiratory O₂ consumption rates (Table 3). This result was in agreement with glucose-grown *C. vulgaris* UAM 101 cells [30], fructose-grown *Anabaena variabilis* cells [31], acetate-grown *Chlamydomonas reinhardtii* cells [32], and *Phaeodactylum tricornutum* cells supplied with several organic carbons [10].

Meanwhile, mixotrophic cells in the exponential phase showed the greatest photosynthetic activity, which was corroborated by the highest photosynthetic O₂ evolution rate. Additionally, the result of 77K fluorescence spectra was consistent with the results of photosynthetic O₂ evolution rate. It has been widely accepted that 77K fluorescence spectra can be used to measure the distribution of absorbed light energy between PSI and PSII [33, 34]. In 1 day cultured mixotrophic cells, the ratio of PSI fluorescence to PSII fluorescence was reduced. This is a first indication of increased linear electron transport, i.e., more excitation energy is directed to that photosystem than generates electrons used for NADP⁺ reduction. This is further supported by the high oxygen evolution rate and the increased Φ II values for 1 day cultured mixotrophic cells. These results proved that PSII activity increased in mixotrophic cells. The augmentation in photosynthetic efficiency of mixotrophic cells was associated with an increased

Rubisco carboxylase activity (Table 4). Combined with the highest expression level of PRK, 1 day cultured mixotrophic cells exhibited the highest activation degree of the Calvin-Benson cycle to support CO₂ assimilation.

Based on current knowledge about the process of photosynthesis adjustment in algal cells, photosynthetic activity may be down-regulated by exogenous carbon sources [6-8, 10, 35], although this condition generally increases specific growth rates and biomass concentration [36, 37]. Photoautotrophically grown algae often have decreased photosynthetic capacity and reduced Rubisco carboxylase activity after supplementation with glucose [10, 30]. Also, in these cases, genes related to photosynthesis were down-regulated, including those for chlorophyll-binding proteins and Rubisco.

In our study, enhanced photosynthetic capacity was observed in mixotrophic cells at the exponential growth stage. On the other hand, mixotrophic cells at the stationary growth stage showed largely decreased photosynthetic efficiency. Lowered Φ_{PSII} and F_v/F_m are indicators of a reduced photochemical efficiency by damage to PSII, which are in agreement with the results of dramatically decreased photosynthetic O₂ evolution rate. This conclusion was further supported by the 77K fluorescence spectra. The shift of F688 to F685 points to changes in the organization of the components of PSII complex. It strongly suggests that the light energy absorbed by LHCII is not properly transferred to the PSII reaction center. The major difference of the photosynthetic regulation strategy in mixotrophic cells from the exponential phase and the stationary phase is correlated with the different physiological state, especially from the aspect of nutrient availability, like nitrogen source, which was completely assimilated from the medium at the stationary stage (Fig. 1). It has been proven that in algae nitrogen assimilation is directly coupled to

photosynthetic electron flow and 20% of the photosynthetic electron flow would be in support of nitrogen assimilation for amino acids synthesis [38]. In *Chlamydomonas* at low light intensities, up to 40% of photogenerated reductant is allocated to nitrate reduction [39]. As growth becomes limited by nitrogen, there are large changes in cellular metabolic ATP and NADPH demands in algal cells. Thus, the down-regulation of photosynthesis in mixotrophic cells probably serves as a feedback control to guarantee growth and survival of algal cells.

In plant leaves after long-term growth at elevated CO₂, down-regulation of the photosynthetic machinery also occurs and is called “sugar signalling” [40]. Glucose concentrations always increase in plants grown with high CO₂, whereas the expression of genes involved in photosynthesis is reduced, yet no loss appeared if replete nitrogen is supplied [41-43]. It is proved that the photosynthetic capacity of plants can be stimulated or reduced by controlling replete or limited nitrogen supply under high CO₂ condition [44].

It seems that *C. sorokiniana* cells follow the same photosynthesis regulatory patterns. Here we further tested this regulation strategy through fluorescence characterization of algal cells after cultivation under nitrogen-depleted or nitrogen-replete conditions (Table 5). Compared with control values, Φ_{PSII} , F_v/F_m and qL in nitrogen-depleted conditions were clearly reduced by glucose supplementation. On the other hand, they were largely enhanced in nitrogen-replete conditions, even with glucose addition. Therefore, sugar-signalling appears to be operating under nitrogen-depleted conditions, whereas under nitrogen-replete conditions, no down-regulation of photosynthesis was observed.

411 Collectively, the carbon to nitrogen balance rather than purely high carbon levels appear
412 to be a central to component controlling photosynthesis.

413

414 **Table 5.** Chlorophyll fluorescence characteristics of algal cells.

Parameter	Control	+ N			– N		
		Glucose content (g L ⁻¹)			Glucose content (g L ⁻¹)		
		0	6	20	0	6	20
F _v /F _m	0.77 ± 0.01	0.81 ± 0.01	0.83 ± 0.02	0.84 ± 0.02	0.74 ± 0.01	0.73 ± 0.01	0.72 ± 0.02
Φ _{PSII}	0.57 ± 0.01	0.72 ± 0.02	0.74 ± 0.00	0.72 ± 0.01	0.62 ± 0.02	0.48 ± 0.01	0.45 ± 0.00
qL	0.49 ± 0.00	0.67 ± 0.02	0.66 ± 0.01	0.76 ± 0.01	0.52 ± 0.01	0.34 ± 0.00	0.34 ± 0.00

415

416 Values are the means ± standard deviation. Standard deviation was calculated based on three independent measurements from single
 417 representative experiment.

418 Algal cells were grown photoautotrophically in Kuhl medium under light intensity of 100 μmol m⁻² s⁻¹, agitated with 1% -CO₂. When
 419 grown to the early stationary phase, algal cells were utilized as control and inoculated into Kuhl medium with (+ N) or without (– N) 1
 420 g L⁻¹ KNO₃, supplemented with 0, 6, and 20 g L⁻¹ glucose, respectively. After inoculation, cells were grown for 9 hrs with orbital
 421 shaking, under light intensity of 100 μmol m⁻² s⁻¹. For fluorescence measurement, the actinic light intensity was 100 μmol m⁻² s⁻¹.

5. Conclusions

By systematically analyzing photosynthetic regulation in mixotrophic cells of *C. sorokiniana*, we found that photosynthesis is dynamically adjusted at different growth stages. Additionally, nitrogen limitation is correlated with down-regulation of photosynthesis in mixotrophic cells. Although elucidation of the precise mechanisms that give rise to feedback control of photosynthesis in algae still remains to be completed, our results provide clues about how to keep high photosynthesis efficiency for carbon acquisition and biomass accumulation, whether through nutrient supply schemes or through molecular engineering.

Authors' contributions

TL participated in the study design, data collection and analysis, and drafted the manuscript. HK assisted the results interpretation and the manuscript revision. MG, JF, ABC and DRG participated in RT-PCR and Rubisco carboxylase activity analysis, and helped to draft the manuscript. PTP and SC participated in the results discussion and critical review of the manuscript. All authors read and approved the final manuscript.

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