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Balancing photosynthesis, O₂ consumption, and H₂ recycling for sustained H2 photoproduction in pulse-illuminated algal cultures†

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Photosynthetic H₂ production in unicellular green alga Chlamydomonas reinhardtii is catalysed by O₂sensitive [Fe-Fe]-hydrogenase (H2ase) enzymes located in the chloroplast. The process is difficult to sustain due to (i) the inactivation of H₂ase enzymes by O₂ coevolved in photosynthesis and (ii) the competition of H₂ases with the Calvin-Benson-Bassham (CBB) cycle for photosynthetic reductants. Our previous studies revealed that H2 production in nutrient-replete algal cultures could be sustained by applying a train of strong but short (1-5 s) light pulses interrupted by longer (3-9 s) dark periods. This limits O2 accumulation produced by photosystem II, prevents activation of the CBB cycle and redirects photosynthetic electrons to H₂ase. In the present research, we demonstrate that the combination of strong light pulses with continuous low background illumination gives a significant gain in the net H₂ photoproduction yield by pulse-illuminated algae but only for the first 24 h. We bring evidence that the attenuation of H2 evolution is primarily caused by the accumulation of H2 in the headspace of vials rather than O2 inhibition of the H2ase, whereas an increase in the H2 partial pressure leads to activation of H₂ recycling and noticeable H₂ uptake, which is accelerated by O₂. We predicted that sustained H₂ production in pulse-illuminated algae, which are additionally exposed to continuous low background light, could be achieved by decreasing the H2 partial pressure in cultures and preventing excessive accumulation of O2. Indeed, the application of periodic refreshments of a headspace atmosphere with argon and the introduction of O₂ scavenger L-cysteine allowed the H₂ photoproduction activity in algal cultures to be sustained for more than 10 days both under photoheterotrophic and photoautotrophic conditions, and yielding at least 6-times more H2 per litre of the culture than the standard pulseillumination protocol.

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Introduction

Molecular hydrogen (H2), when produced from renewable sources, is regarded as the cleanest energy carrier for the future circular economy with significant demands from the global fuel market. In addition, it serves as a crucial feedstock for a variety of industrial activities, including the manufacturing of fertilisers and the refining of petroleum. The photobiological water splitting (water biophotolysis) process, which is inherent to many species of cyanobacteria and green algae, is the most promising and environmentally friendly way for the generation of H₂.²⁻⁴ In comparison with inorganic photocatalysts for water oxidation, CO2 reduction and H2 generation,5-7 the photobiological approach considers the natural and engineered photosynthetic organisms as whole-cell biocatalysts, which provide a fully renewable alternative to the traditional chemical

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synthesis and which are capable of self-repairing, operating in a wide range of the light spectrum, and utilizing cheap and abundant raw materials such as water, mineral nutrients, CO2 and some organic substrates.8-10

In green algae, H₂ photoproduction occurs in two steps with the involvement of the photosystem II (PSII) water oxidizing complex and the proton-reducing [Fe-Fe]-hydrogenase (H₂ase) enzyme associated with the photosynthetic electron-transport chain:

$$2H_2O \rightarrow 4H^+ + O_2 + 4e^-$$
; (Step 1, PSII-dependent reaction) (1)

$$4H^+ + 4e^- \rightleftharpoons 2H_2$$
; (Step 2, H_2 as e-dependent reaction) (2)

Green algae typically produce H2 on exposure to light after a period of dark anaerobic adaptation. The process is very efficient but short due to a fast accumulation of O2 co-evolved in step 1.11,12 Accumulated O2 leads to the inactivation of O2sensitive H2ase in cells.13,14 Another reason for the fast termination of H₂ evolution in algae is a competition of H₂ase with the Calvin-Benson-Bassham (CBB) cycle for photosynthetic reductants, which occurs just before the inactivation of H2ase by O2. 15,16 Activation of CO2 fixation coincides with a pronounced H2 uptake that also terminates with the accumulation of O2 in cells.17

Sustained H₂ photoproduction in green algae is typically achieved by nutrient deprivation.18 In this approach, algal cultures are transferred to the medium depleted of an essential nutrient such as sulfur, nitrogen, phosphorus, magnesium.19-22 Typically, under nutrient deprivation cells stop dividing, accumulate significant amounts of starch and partially degrade PSII reaction centres. The partial loss of wateroxidizing activity in algae results in the establishment of anaerobiosis in cultures due to efficient respiration and expression of H2ase enzymes in cells leading to sustained H2 photoproduction for a few days. 23,24 Despite substantial improvement in the H₂ production yield, the nutrient deprivation approach is not scalable for commercial purposes due to its low photosynthetic efficiency.²⁵ Therefore, the current research efforts are primarily concentrated on the improvement of H₂ photoproduction activity in nutrient-replete algae.26

Our previous studies revealed that H2 production in nutrient-replete Chlamydomonas reinhardtii algae could be sustained by a train of strong white-light pulses interrupted by longer dark phases. 17,27,28 In this protocol, the duration of each light pulse in the light/dark sequence is short enough to prevent activation of the CBB cycle and limit the accumulation of O2 in cells. The pulse-illumination protocol represents a way to redirect photosynthetic electron flow to the green algal H₂ase, thus resulting in increased H2 photoproduction yields. Under these conditions, algae produce H2 via the most efficient mechanism of direct water biophotolysis, which has a theoretical light energy to H₂ energy conversion efficiency (LHCE) of around 11-13%.29,30 Although in the best case up to 8% LHCE has been achieved during a short period in photoheterotrophic algal cultures³¹ and up to 4% under photoautotrophic conditions, ¹⁶ photosynthetic H2 production in the current state is not yet efficient enough for industrial applications. There are a number of physiological, biochemical and technological barriers that limit the H₂ production yield in algal cultures.³² Thus, further research efforts on improving the algal capacity to produce molecular hydrogen are indispensable.

In the present study, we identify the key factors affecting H₂ photoproduction in green alga C. reinhardtii exposed to pulseillumination under photoautotrophic and photoheterotrophic conditions and determine the major requirements for algae cultivation, which are important for supporting the long-term H₂ photoproduction activity in nutrient-replete algal cultures and leading to improved H₂ production yields.

Experimental

Algal growth conditions

The wild-type green alga Chlamydomonas reinhardtii strain CC-124 (mt-, nit-), which is a common model organism for studying photobiological H₂ production, was obtained from the Chlamydomonas Resource Center at the University of Minnesota, USA. The stock cultures were grown in 150 ml Erlenmeyer flasks containing 50 ml of either Tris-acetate-phosphate (TAP) medium (pH 7.0) for photoheterotrophic growth or Trisphosphate-HCl (TP) medium (pH 7.0) for photoautotrophic growth. The culture flasks were placed in a growth chamber at 25 °C on a rotary shaker (120 rpm) and cultivated under 70-75 μmol photons m⁻² s⁻¹ of photosynthetically active radiation (hereafter μ mol m⁻² s⁻¹) provided by cool-white, fluorescent lamps (Philips Master TL-D T8 15W/840) and a 14 h photoperiod. The stock cultures were maintained by weekly dilutions. The experimental cultures were grown under the same growth conditions in 500 ml conical flasks containing 200 ml of TAP medium. These cultures were continuously sparged with sterile filtered air (filter with a pore-size 0.2 µm, Acro 37 TF, Gelman Sciences, USA).

Hydrogen photoproduction experiments

All H₂ photoproduction experiments were performed with nonstressed nutrient-replete algae. No centrifugation was applied for cell harvesting. Simply, 10 ml cell suspensions were transferred from cultivation flasks into 73.5 ml gas-tight vials within 6 h from the start of the photoperiod on the 2nd, 3rd, and 4th day of growth depending on the required cell density. The vials were sealed with butyl rubber stoppers, flashed with argon (Ar) for 30 min and placed on a rotary shaker (120 rpm) in a growth chamber (AlgaeTron AG 130-ECO, PSI) at 25 °C. To initiate H₂ photoproduction in algal suspensions, a train of 1 s white light pulses (280 μmol m⁻² s⁻¹) was applied to the surface of the vials.27 The pulses were either interrupted by 9 s dark periods (the control sample) or superimposed at the same 9 s intervals on white background illumination of different intensities: 2, 4, 12, 20 and 30 μmol m⁻² s⁻¹. Pulses and background illumination were provided by the top LED panel of the growth chamber. For long-term H₂ production, the headspace of culture vials was replaced with pure Ar every 24 h by purging the gas through the suspensions for 30 min. For O2 scavenging, a fresh solution of 500 mM L-cysteine was prepared with deoxygenated water, filter sterilized and then introduced into the vials in the final concentration of 4 and 8 mM at the beginning of the experiment. The amounts of H₂ and O₂ in the headspace of vials were monitored once a day by injecting 150 µl gas samples into a gas chromatograph (Clarus 500, PerkinElmer, Inc.) equipped with a thermal conductivity detector and a molecular sieve 5A column (60/80 mesh). Ar was used as a carrier gas. The amounts of H2 and O2 dissolved in the liquid phase were calculated based on the partial pressure of the corresponding gas at the time of sampling and the solubility coefficients, which are 713 μmol H₂ $l^{-1} H_2 O (0.01744 \text{ ml ml}^{-1}) \text{ under 1 atm and 258 } \mu \text{mol } O_2 l^{-1} H_2 O$ $(0.00632 \text{ ml ml}^{-1})$ under 0.21 atm at 25 °C.^{33,34} These values as well as amounts of gases withdrawn for sampling were considered in the final production yields. The H₂ and O₂ yields in the cultures with daily renewals of the headspace atmosphere to Ar are shown as cumulative yields. The specific yields were calculated based on the initial total (a + b) chlorophyll (Chl) content in the samples. The changes in the Chl content throughout long-term (240 h) experiments, if any, have not been considered in these calculations. The Chl content in algal

suspensions was assayed spectrophotometrically in 95% ethanol extracts by the method of Spreitzer.³⁵

Hydrogenase activity assay

In vitro hydrogenase activity during H2 photoproduction was determined in photoheterotrophic and photoautotrophic algal samples. The assay was performed in 10 ml serum vials containing 900 µl of the reaction mixture consisting of 50 mM potassium-phosphate buffer (pH 6.9), 10 mM oxidized methyl viologen and 0.2% (w/v) Triton X-100. The vials were tightly sealed and flushed with Ar for 30 min. Then, 100 µl of anaerobic 100 mM Na-dithionite solution (prepared by adding anaerobic water to anaerobic vials with the Na-dithionite salt) was introduced into the vials to reduce the methyl viologen. The reaction was started by injecting 1 ml of the cell suspension into the reaction mixture and performed at 37 °C. The level of H2 in the headspace of the vials was measured by gas chromatography (as described above) for 40 min and the activity (µmol H₂ (mg Chl h)⁻¹) was calculated for the maximum H₂ production rate based on the total Chl content in the sample.

Membrane inlet mass spectrometry measurements

In vivo CO_2 (m/z=44) exchange in algal cultures was measured by membrane inlet mass spectrometry (MIMS) using a modified DW1 (Hansatech Instruments) electrode chamber connected to a Prima PRO mass spectrometer (Thermo ScientificTM) via a refrigerated cooling trap (-65 °C; EtOH; Julabo FT 902) as previously described. ^{17,36} Briefly, 2 ml algal samples were placed in an MIMS chamber, and a microoxic environment inside the chamber was achieved by purging Ar gas through the suspension for about 2–3 min in the dark. The white light-emitting diode light pulses ($\sim 1000~\mu \text{mol m}^{-2}~\text{s}^{-1}$) were applied using an STM32F103 micro-controller board. The final curves were obtained after correction for gas consumption by using the mass spectrometer during the dark periods in the beginning and at the end of each experiment. This correction also included the CO_2 release by the culture in the dark.

Results and discussion

Effect of low background illumination on H_2 photoproduction by algal cultures exposed to the pulse-illumination protocol

Recently, we demonstrated that the induction of sustained H_2 photoproduction in anaerobic *C. reinhardtii* algae under pulse-illumination occurs not only when the train of strong light pulses is superimposed on darkness (1 s light-on/9 s light-off regime, hereafter 1 s/9 s) but also when 1 s/9 s pulses are applied atop a very low (3 μ mol m⁻² s⁻¹) background illumination.²⁷ The appearance of H_2 in pulse-illuminated cultures exposed to 3 μ mol m⁻² s⁻¹ continuous background light was not surprising since even if this condition activates the CCB cycle, co-produced O_2 is efficiently removed by respiration. Hydrogen photoproduction in green algae depends on photosynthetic activity and increases with an increase in light intensity.³⁷ The increase in light intensity, though, enhances O_2 accumulation. Therefore, we proposed that an increase in the

intensity of the background illumination should also enhance H_2 photoproduction activity in algal cultures, until the point where respiration could not adequately cope with the accelerated evolution of O_2 leading to the inactivation of H_2 as enzymes in algae.

To verify this hypothesis, 1 s light pulses were superimposed with a 10 s frequency either on the dark background (the control cultures) or on the low background illumination (2–20 μ mol m⁻² s⁻¹). As shown in Fig. 1A, the control cultures produced around 140 μ mol H₂ (mg Chl)⁻¹ in 24 h. Increasing the background illumination from 2 to 12 μ mol m⁻² s⁻¹ resulted in a gradual increase in the maximum net H₂ photoproduction yield from ~200 to ~380 μ mol H₂ (mg Chl)⁻¹, while cultures exposed to 20 μ mol m⁻² s⁻¹ showed a slight decrease in the net H₂ production yield compared to cells illuminated by 12 μ mol m⁻² s⁻¹ (Fig. 1A). The inhibition of the H₂ production rate under 20 μ mol m⁻² s⁻¹ light was likely caused by the excess content of intracellular O₂ in algae, even though the net release of O₂ in the headspace of vials occurred only after 24 h (Fig. 1B).

In line with our previous experiments, 27,28 pulse-illuminated cultures produced the most H_2 during the first 24 h. The application of background illumination resulted in a noticeable H_2 uptake after 24 h, which became very pronounced at the light intensities above 4 µmol m^{-2} s⁻¹ (Fig. 1A). This experiment demonstrated that the application of low background light in the pulse-illumination protocol improves the yield of H_2 photoproduction, while still preventing O_2 accumulation. In fact, most of the cultures yielded around $10 \, \mu \text{mol } O_2 \, (\text{mg Chl})^{-1}$ by the end of the experiment, except algae exposed to $20 \, \mu \text{mol } m^{-2} \, \text{s}^{-1}$ light where they produced above 440 $\mu \text{mol } O_2 \, (\text{mg Chl})^{-1} \, (\text{Fig. 1B})$.

To check whether H₂ uptake in pulse-illuminated algae is linked to CO₂ photoreduction in the CBB cycle, we introduced 20 mM NaHCO3 into the cultures at the beginning of the experiment. As shown in ESI Fig. 1,† the supplementation of pulse-illuminated algae with 20 mM NaHCO3 did not affect net H₂ and O₂ production yields in the control cultures throughout the experiment, and during the first 24 h in cultures exposed to $20 \mu mol m^{-2} s^{-1}$ background light (ESI Fig. 1†). After 24 h, the algae exposed to 20 µmol m⁻² s⁻¹ background illumination accelerated O2 production in the presence of bicarbonate compared to the cultures without added bicarbonate (ESI Fig. 1B†). The appearance of O2 in both cultures exposed to 20 μmol m⁻² s⁻¹ light indicated acceleration of the CBB cycle at around 24 h. This resulted in the pronounced H2 uptake (ESI Fig. 1A†). Interestingly, the addition of bicarbonate reduced H₂ uptake under 20 μmol m⁻² s⁻¹ background illumination most probably due to the excessive production and accumulation of O₂, leading to inhibition of the H₂ase. Thus, the response of H₂ uptake to the acceleration of the CBB cycle is rather complex. The enhanced CO₂ fixation may indeed enhance H₂ uptake (depending on the H₂ partial pressure) but leads to excessive production of O_2 that inactivates the H_2 as enzyme.

Substantial H_2 production during the first 24 h also occurred in cultures exposed to continuous light (without pulses) under pre-established anaerobic conditions (Fig. 1C). Similar observations of prolonged H_2 production under low light intensities

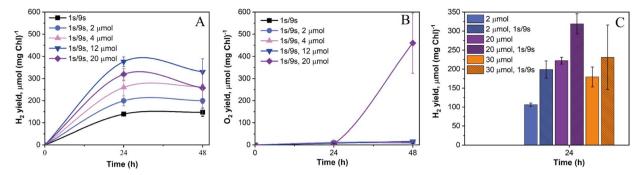


Fig. 1 Effect of low background illumination on net H₂ photoproduction and net O₂ evolution by wild-type C. reinhardtii cells exposed to the pulse-illumination protocol. (A) Net H_2 photoproduction and (B) net O_2 evolution yields under 1 s/9 s pulse illumination (280 μ mol m⁻² s⁻¹) applied atop darkness (the control sample) or continuous background illumination of 2 to 20 μ mol m⁻² s⁻¹. (C) Comparison of net H₂ photoproduction yields after 24 h illumination under continuous light and light pulses superimposed on continuous light. The initial experimental cultures contained \sim 10 (A and B) and \sim 8 (C) μ g Chl (a+b) ml⁻¹. The H₂ and O₂ yields were normalized to the initial total Chl content. The experiments were performed under photoheterotrophic conditions. Values are the mean of 6 to 15 independent replicates \pm SD

have been previously reported.^{38,39} However, it should be noted that the application of strong light pulses to cultures exposed to low continuous light always led to improved net H₂ production yields in the range of tested conditions (Fig. 1C). It is important to note here that 280 µmol m⁻² s⁻¹ light pulses, even if applied only for 1 s in the 10 s pulse-illumination sequence, bring a significant fraction of light energy to drive photosynthetic H₂ production, especially under the low background illumination. For example, under 2 μmol m⁻² s⁻¹ of continuous background light the applied 280 μ mol m⁻² s⁻¹ light pulses bring up to 93% of the total light energy, while under 30 μmol m⁻² s⁻¹ background light, this value is around 48%. As shown in Fig. 1C, the net H₂ photoproduction yield in algal cultures started decreasing after their exposure to 30 $\mu mol \; m^{-2} \; s^{-1}$ light, which is just below the compensation point (when the rate of photosynthesis becomes equal to the rate of respiration) in the wildtype culture grown under photoheterotrophic conditions. 40 Thus, the results revealed that efficient photosynthetic H₂ production occurs when cellular respiration overtakes or minimizes accumulation of photosynthetic O2 and creates a microoxic environment inside algal chloroplasts. The latter is important for preventing inactivation of the O2-sensitive H2ase enzymes.41 Overall, the highest net H2 production yield in pulseilluminated algae could be observed in cultures exposed additionally to around 10-12 µmol m⁻² s⁻¹ of background light (Fig. 1).

Thus, despite the limited accumulation of O₂ in most of the cultures, the application of low intensity background light in the pulse-illumination protocol did not allow us to sustain H₂ photoproduction for more than 24 h mainly due to activation of H₂ uptake after that point.

Impact of cultivation conditions on pulse-illuminated algae under low background illumination

Similar to photoheterotrophic cultures, algae pre-grown under strict photoautotrophic conditions showed the capacity to sustain H₂ photoproduction under pulse-illumination.^{27,28} Therefore, it was of interest to check whether pulse-illuminated

photoautotrophic algae could also produce H2 under low background illumination. For this purpose, low cell density C. reinhardtii cultures (\sim 5 µg total Chl ml $^{-1}$) were exposed to 1 s/ 9 s pulses superimposed on 12 μmol m⁻² s⁻¹ of continuous background light. The low cell density cultures were applied to minimize the self-shading effect, which extends the "dark zone" in algal suspensions, leading to the decreased photosynthetic O₂ production and the enhanced O₂ consumption by cells.^{39,42} As shown in Fig. 2A, photoautotrophic algal cells could produce H₂ under this condition, albeit giving much lower yields compared to photoheterotrophic cultures (0.46 vs. 2.9 mmol H₂ l^{-1} , respectively). Predictably, H_2 production in photoautotrophic algae proceeded with a noticeable release of O2 into the headspace of the vials (Fig. 2A), though the O_2 content in vials did not exceed 0.1% by the end of the experiment (as calculated from the original GC data). Slight accumulation of O2 in cultures resulted in very low activities of the H2ase enzyme in cells (Fig. 2B). Similarly, Lee and Greenbaum observed 50% inhibition of H₂ photoproduction in photoautotrophic algae after the introduction of 0.05% O2 in vials.43 However, the intracellular H₂ase could tolerate up to 0.5% O₂.43 Thus, the low level of the H2ase activity in photoautotrophic algae, which is observed in our case (Fig. 2B), could be caused not only by the inactivation of the H2ase enzyme itself but by its impaired biosynthesis. Indeed, Forestier et al. observed a much slower accumulation of H₂ase transcripts and induction of the H₂ photoproduction activity in photoautotrophically pre-grown algae compared to in photoheterotrophic cultures.44 In the presence of acetate in the medium (Fig. 2A), the concentration of O2 in vials was noticeably lower than that in photoautotrophic algae and is retained at a level sufficient for preventing H_2 as enzyme inactivation by intracellular respiration (Fig. 2B).

In our experimental setup, the highest level (476 µmol H₂ $(mg \, Chl \, h)^{-1})$ of the *in vitro* H_2 as activity in photoheterotrophic algae was detected 24 h after application of the pulseillumination protocol (Fig. 2B), and close to the point where cells began consuming H2 (Fig. 2A). The H2ase activity in photoheterotrophic algae was comparable to the one observed in S-

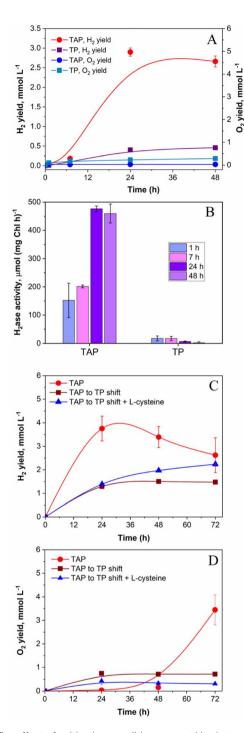


Fig. 2 The effect of cultivation conditions on net H_2 photoproduction, net O_2 evolution and the H_2 ase activity in pulse-illuminated algae exposed to $12~\mu mol~m^{-2}~s^{-1}$ of background illumination. (A) Net H_2 and O_2 yields in photoheterotrophic (TAP) and photoautotrophic (TP) cultures. (B) Changes in the *in vitro* H_2 ase activity in the same cultures throughout the experiment. A comparison of net H_2 photoproduction (C) and net O_2 evolution (D) yields in photoheterotrophic cultures (TAP) and photoheterotrophically pre-grown cultures after their shift at t=0 h to photoautotrophic conditions (TAP to TP shift) in the presence and absence of 4 mM L-cysteine. Experimental cultures contained around 5 (A, B) and 40 (C, D) μ g Chl $(a+b)~ml^{-1}$ at the beginning of the experiment. Values are the mean of 3 independent replicates \pm SD.

deprived cells during the period of efficient H_2 production.^{23,45} Even at 7 h, the H_2 ase activity was around 200 µmol H_2 (mg Chl h)⁻¹ (Fig. 2B; the 7 h TAP sample), which significantly exceeded the rate of photosynthetic H_2 production (42 µmol H_2 (mg Chl h)⁻¹) determined for the interval between 7 and 24 h (Fig. 2A; the TAP sample). Thus, the H_2 ase activity could not be the limiting factor for H_2 production in photoheterotrophic algae. It is important to note that the H_2 ase assay evaluates the maximum enzymatic capacity *in vitro*, while the situation *in vivo* might be more complex. On the other hand, photoautotrophic algae possessed a very low H_2 ase activity (maximum 18 µmol H_2 (mg Chl h)⁻¹ at 7 h) that led to the low net H_2 photoproduction yield in cultures (Fig. 2, panels A and B; TP samples).

The presence of acetate in the medium during algae growth results in the accumulation of intracellular storage reserves in the form of starch and proteins. 46 During H₂ production, these reserves can be further utilized either for supporting anaerobiosis in algal cultures as substrates for respiration or for providing reductants to H2ase via the indirect H2 production pathway. 47,48 Considering this information and attempting to improve the H₂ photoproduction yield under photoautotrophic conditions, we pre-grew algae in the presence of acetate and before the investigation shifted them into photoautotrophic conditions. To ensure high respiratory activity in cultures, experiments were performed at high cell density (\sim 40 µg Chl (a + b) ml⁻¹). This approach, though, did not enhance the net H₂ photoproduction yield in the absence of acetate. As shown in Fig. 2C, the shifted algae produced \sim 2.5 times lower H₂ than the non-shifted cultures (1.5 νs . 3.75 mmol l^{-1} , respectively). The shifted photoautotrophic algae, even at high cell density, still suffered from the excessive production of O2 (Fig. 2D). For reducing the O₂ content in the medium, we applied L-cysteine, which is an efficient O₂ scavenger. It shows reducing properties attributed to its thiol group, which in the presence of O2 reacts with the thiol group of another cysteine molecule to generate a disulphide bond and water. Furthermore, L-cysteine does not have inhibitory effects on algal growth and even slightly stimulates it.49 As shown in ESI Fig. 2,† the introduction of 4-8 mM L-cysteine into the cultures is sufficient for supporting H₂ photoproduction in pulse-illuminated photoheterotrophic cultures under 12 µmol m⁻² s⁻¹ of background illumination. Similarly, the addition of 4 mM L-cysteine in photoautotrophic cultures slightly improved the net H2 photoproduction yield (Fig. 2C). These results indicated the essential role of acetate in supporting anaerobiosis and sustaining H2 production in pulseilluminated algae under low background illumination. In the absence of acetate, the application of extra O2 scavengers is required for sustaining the H2 production activity in algal cultures.

Effect of H₂ partial pressure on the photosynthetic production of H₂ by pulse-illuminated algae

Vials with pulse-illuminated algae, which were additionally exposed to low background light, showed a pronounced H₂ uptake after 24 h of the experiment (Fig. 1A). The effect was more pronounced in cultures that produced considerable

amounts of H_2 during the first 24 h (Fig. 1A, 12 and 20 μ mol m⁻² s⁻¹ trends) just before H₂ started to decline. Since the experiments were performed in gas-tight vials, it is unlikely that the effect is caused by the inhibition of H2ase in the presence of coevolved O2 and the passive leak of H2 from the vials. We suggested the catalytic nature of H2 uptake in algae that occurs simultaneously with H2 release and becomes more pronounced under high H₂ partial pressure. Indeed, the reaction of H₂ production driven by algal H2ases is highly reversible. 50-52 Depending on the redox state of the cells and the partial pressure of H2 in the environment, algae could either produce or oxidize H2. Hydrogen assimilation could be either the result of the oxy-hydrogen reaction, CO₂ photoreduction in the CBB cycle or another unknown pathway.26,53,54

To confirm the catalytic nature of H2 uptake in pulseilluminated algae, we first checked the ability of algal cultures that were exposed to the standard 1 s/9 s pulse-illumination protocol (without background illumination) to photoproduce H₂ in the presence of 3% H₂ in the headspace of vials. As shown in Fig. 3, under these conditions a very minor H₂ production was observed. In the next step, we checked if periodic replacements of headspace gases with Ar without changing the medium could improve H2 photoproduction yield. This approach indeed extended the period of efficient H2 photoproduction by pulse-illuminated C. reinhardtii from 48 h (observed in the control) to above 120 h, yielding 440 µmol H₂ (mg Chl)⁻¹ at that point. It should be noted, however, that periodic replacements of headspace gases with Ar remove not only H₂ but O₂ as well. As we demonstrated above (ESI Fig. 1†), accumulation of O2 in vials may either accelerate H2 uptake by algae or inhibit the H₂ase activity in cells depending on the produced O2 level. The addition of an O2 scavenger (8 mM Lcysteine) to the cultures stopped H₂ uptake, though without any

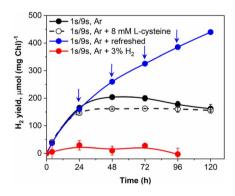


Fig. 3 Effect of H₂ partial pressure on net H₂ photoproduction under pulse-illumination. The following conditions were tested: the control sample (1 s/9 s, Ar); 8 mM L-cysteine was introduced into anaerobic vials at t = 0 h (1 s/9 s, Ar + 8 mM L-cysteine); headspace gases in the vials were replaced with Ar every 24 h (1 s/9 s, Ar + refreshed); 3% H₂ was introduced into the headspace of anaerobic vials at t = 0 h (1 s/9 s, $Ar + 3\% H_2$). Arrows indicate the points where the atmosphere of vials was replaced with Ar. The H₂ yields were normalized to the initial total Chl content, which was around 9 μ g ml⁻¹. The H₂ yield in the culture with daily renewals of headspace gases (1 s/9 s, Ar + refreshed) is represented as the cumulative yield. Values are the mean of 3 independent replicates \pm SD.

improvement in the H2 yield (Fig. 3; ESI Fig. 2†), while the addition of the O2 scavenger under low background illumination decreased the rate of H2 consumption by the cultures (ESI Fig. 2†). This indicates that the H₂ uptake component depends on the H2 partial pressure in vials and the level of intracellular O2. The dependence of H2 uptake on O2 has also been demonstrated by Milrad et al.54 who observed elevated consumption of H₂ in the absence of an O₂-scavenging system in contrast to the sample with added glucose, glucose oxidase and catalase. Though, it should be noted that the excessive production of O2 inhibits H2 uptake (ESI Fig. 1†).

Maximizing H₂ photoproduction yields in pulse-illuminated cultures under photoheterotrophic and photoautotrophic conditions

As discussed above, the application of the low-intensity background light to pulse-illuminated C. reinhardtii cultures dramatically improves the H2 photoproduction rate but the process could not be sustained for longer than 24 h (Fig. 1A) due to the activation of H₂ uptake under increasing H₂ partial pressure (Fig. 3). Meanwhile, simultaneous accumulation of O2 in vials due to water-oxidation in PSII may partly accelerate H2 assimilation by the cells (ESI Fig. 1 and 2†) and lead to inactivation of the H2ase enzyme if the O2 content reaches the critical level. Since daily re-flushing of cultures with Ar replaces both H₂ and O2 produced by algae, this approach should improve H2 photoproduction not only in cultures exposed to the standard 1 s/9 s pulse-illumination protocol (Fig. 3) but also in algae exposed to the same protocol under low background illumination. To check this, we applied 12 µmol m⁻² s⁻¹ background light. As expected, daily replacements of headspace gases to Ar indeed allowed the process to be sustained for longer than 200 h (Fig. 4A). Moreover, the pulse-illuminated algae exposed to 12 μ mol m⁻² s⁻¹ continuous light significantly outperformed (P < 0.001) cultures without background illumination; H₂ photoproduction yields at 216 h were 2.7 and 1.3 mmol H_2 (mg Chl)⁻¹, respectively, whereas the pulse-illuminated culture exposed to background light but without headspace refreshments could not sustain the process (Fig. 4A). As shown in the figure, the short (24 h) period of initial H₂ production was followed by a long period of H₂ uptake.

The net release of O2 in pulse-illuminated algal cultures, which were exposed to continuous 12 µmol m⁻² s⁻¹ background light, occurred only after 72 h of cultivation (Fig. 4B, except the sample with 4 mM L-cysteine). To check whether the effect is linked to activation of the CBB cycle in cells at that point, real-time CO₂ exchange measurements using MIMS were performed. As expected, the application of continuous light to pulse-illuminated algae at any stage of the long-term experiment led to activation of CO₂ fixation in the CBB cycle (Fig. 5B). In contrast, the control algae that were exposed only to pulse-illumination release CO2. The CO2 release under pulse-illumination has been already explained,17,55 and therefore, will not be discussed in this work. Since the pulseilluminated algae perform CO2 fixation under continuous low background light from the beginning of the experiment,

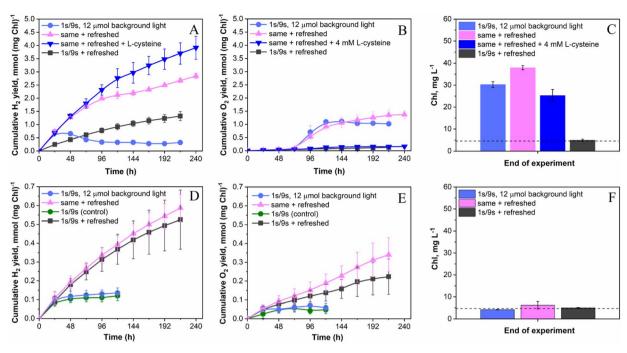


Fig. 4 Sustaining H₂ photoproduction in pulse-illuminated algae by the periodic replacement of headspace gases. The experiments were performed under photoheterotrophic (A-C) and photoautotrophic (D-F) conditions. The panels show: (A and D) the cumulative H₂ photoproduction yield, (B and E) the cumulative O_2 evolution yield, and (C and F) the total Chl (a + b) contents at the end of the experiment compared to the initial total Chl (a + b) contents (shown as dashed lines). For sustained H₂ photoproduction, the atmosphere of the culture vials was replaced with Ar every 24 h. For O2 scavenging, 4 mM L-cysteine was introduced directly into the medium at the beginning of the experiment. The H_2 and O_2 yields were normalized to the initial total Chl content, which was around 5 μ g ml⁻¹. The H_2 and O_2 yields of periodically refreshed cultures are represented as cumulative yields. Values are the mean of 3–9 independent replicates \pm SD.

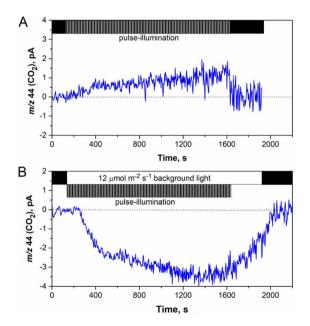


Fig. 5 The effect of background illumination on CO₂ exchange in pulse-illuminated algae. (A) The standard (1 s light/9 s dark) pulseillumination protocol was applied to cultures after 2 min of dark adaptation. (B) The same pulse-illumination was employed simultaneously with continuous 12 $\mu mol\ m^{-2}\ s^{-1}$ background light but background light was stopped 5 min before exposure of the sample to darkness. Both protocols were applied to microoxic algal samples, which were handled as described in the Experimental section.

the net release of O2 observed in cultures after 72 h (Fig. 4B) is likely caused by activation of aerobic metabolism in algal cells at that point leading to enhanced consumption of acetate and further acceleration of CO2 fixation. The latter resulted in a significant culture growth noticed from the increased Chl content (Fig. 4C). In contrast, algal cultures exposed to the standard pulse-illumination protocol did not show any noticeable growth (Fig. 4F). These data bring another piece of evidence on the absence of CO2 fixation in algal cultures just under the train of 1 s/9 s pulses, now demonstrated in the longterm (240 h) process.

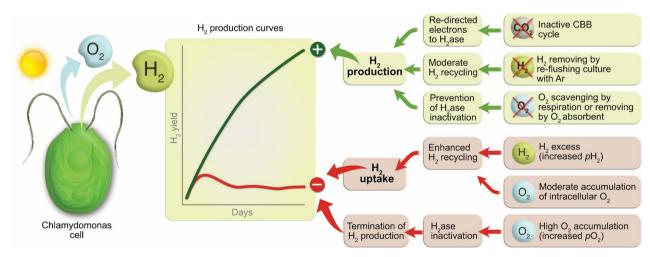
To determine the impact of O₂ on H₂ photoproduction by pulse-illuminated algae during periods between replacements of headspace gases with Ar, we introduced 4 mM L-cysteine into the cultures at the beginning of the experiment. This amount of L-cysteine was sufficient for retaining a microoxic environment in the cultures exposed to background light at least for 240 h (Fig. 4B). The final cumulative O_2 yield was \sim 8-times lower than that in the similar cultures without 1-cysteine (0.16 vs. 1.37 mmol O_2 (mg Chl)⁻¹, respectively). Daily levels of O_2 in Lcysteine-treated vials were stable and did not exceed 0.03% by the end of each cycle on an average basis. As a result, L-cysteinetreated algae further improved the H₂ photoproduction yield to above 3.9 mmol H_2 (mg Chl)⁻¹ (Fig. 4A) or \sim 20 mmol l⁻¹. This amount of H₂ was at least 6-times higher than that in the standard 1 s/9 s pulse-illumination protocol (1.5–3 mmol l^{-1}) as demonstrated previously27 and calculated from data shown in

Fig. 1A, 2A and 3. Due to excessive growth (Fig. 4C), the H₂ photoproduction activity in L-cysteine-treated cultures still suffered from competition with the CBB cycle. Therefore, we expect that the improved H2 production yield under this condition can be achieved by further limitation of photosynthetic electron flow to the CBB cycle, for example, in Rubiscodeficient C. reinhardtii mutants with impaired CO2 fixation capacity. Working with the Rubisco-deficient CC-2803 strain, Hemschemeier and co-authors48 demonstrated that this mutant can produce H2 without S-deprivation, but alga experiences enhanced photoinhibition under H2 production conditions. The latter might be resolved by the application of the pulseillumination approach.

Thus, the major trigger for the induction of efficient H₂ photoproduction in anaerobic algae seems to be the limitation of the CBB cycle. In the absence of CO₂ fixation, H₂ generation is one of the major electron sinks for supporting the PSII activity in an anaerobic environment.48 For example, in S-deprived algae, a major degradation of the Rubisco enzyme occurs before the onset of efficient H₂ production, ^{24,56} while restoration of the linear photosynthetic electron flow from PSII coincides with the appearance of H₂ in cultures.^{57,58} In nutrient-replete dark-adapted algae, the activation of CO2 fixation results in the loss of H2 production activity and initiates H2 uptake.15 If activation of the CBB cycle is prevented by substrate-limitation16 or pulse-illumination (as discussed in this paper), the cultures are capable of sustaining H2 photoproduction for a prolonged period. All these data indicate the strong competition between these two metabolic pathways. Nevertheless, our current experimental data suggest the existence of the steady-state condition in which H₂ photoproduction and CO₂ fixation operate simultaneously (Fig. 4, A-C panels). Here, algal growth is supported by the assimilation of acetate in the presence of photosynthetically produced O2. On the other hand, respiration of acetate retains an anaerobic environment in vials, and protects H2ase from O2 inactivation. 59-61

H₂ photoproduction, O₂ evolution and Chl content profiles noticeably changed when the same experiments were

performed in photoautotrophic algae (Fig. 4, D-F panels). First, all cultures produced significantly (P < 0.01 for the worst pair) less H₂ than that under photoheterotrophic conditions (Fig. 4, A and D panels). Second, in the absence of acetate, the pulse-illuminated algae released H₂ and O₂ in a stoichiometry close to that of direct water biophotolysis (2 mol H₂ to 1 mol O₂) in all daily refreshed cultures throughout the experiment (Fig. 4, compare D and E panels). Third, all cultures did not show any pronounced growth under any condition tested (Fig. 4F). As previously mentioned, the H₂ photoproduction activity in photoautotrophic algae is limited by the H2ase activity (Fig. 2B), which suffers from the excessive net O2 produced by algae. As a result, the application of background illumination did not show any significant effect on H2 photoproduction yield both in daily refreshed and non-refreshed algae (Fig. 4D), though daily replacement of headspace gases with Ar did improve H2 photoproduction, as expected. The final H₂ photoproduction yields in these cultures were around 0.5 and 0.6 mmol (mg Chl)⁻¹ or 2.5 and 2.9 mmol l⁻¹ under the standard pulse-illumination protocol and the same protocol with 12 μmol m⁻² s⁻¹ of background light, respectively. The corresponding non-refreshed cultures produced approximately the same amount of H2 of around 0.12 mmol H2 (mg Chl)⁻¹ or around 0.6 mmol H₂ l⁻¹. In contrast to photoheterotrophic algae, photoautotrophic cultures placed in sealed vials under an Ar atmosphere were limited not only by acetate but CO2 as well. As a result, they did not show any significant growth. The conditions were similar to ones applied in the substrate (CO₂) limitation protocol for initiation of sustained H₂ photoproduction in photoautotrophic cultures under continuous light.16 It is important to note though, that the original CO₂ limitation protocol is performed at high cell density (up to 150 μ g total Chl l⁻¹) and initiated by the exposure of algae to light after dark anaerobic adaptation for up to 4 h. 16,62 The latter creates conditions for full expression of H₂ase in algal cells, while the former prevents the inactivation of H₂ase by O₂ throughout H₂ production due to the high respiratory activity in the dense algal cultures.



Factors affecting H₂ photoproduction in pulse-illuminated algal cultures.

In the absence of CO₂ and acetate, anaerobic algae could not perform normal photosynthesis that links the water oxidation reaction to CO₂ fixation, and therefore algal cells predominantly derive O_2 via the water-splitting reaction (see eqn (1) and (2) in the Introduction section). Since respiration is also limited by the microoxic environment (photosynthetically produced O₂ is diluted in an Ar atmosphere), it is not surprising that under these conditions we observed the stoichiometry of 2 mol H₂ to 1 mol O₂ during almost the whole period of H₂ production. It is clear that H2 photoproduction in photoautotrophic cultures could only be sustained if O2 is efficiently removed from the cultures by periodic purging with Ar (Fig. 4D) and/or introduction of O2 scavengers and O2 absorbents. 62-64 Nevertheless, our data show that substantial amounts of H2 can be produced by photoautotrophic algae in the long-term process since the water-splitting reaction, driven by the photosynthetic apparatus in tight connection with the H₂ase enzyme, seems to provide cells with the energy for supporting their metabolic activity at a minimal level.

Conclusions

The factors affecting the long-term H_2 photoproduction activity in pulse-illuminated green algae are summarized in Fig. 6. Among positive factors leading to the increased H_2 photoproduction yield are inactivation of CO_2 fixation by algal cells, no (or moderate) H_2 recycling by the cells under high H_2 partial pressure, and an anaerobic or microoxic environment preventing inactivation of the H_2 ase in algal cells. Non-compliance with these factors causes the activation of H_2 uptake by algae and, in the worst case, the termination of H_2 photoproduction in algal cultures due to activation of CO_2 fixation and/or inactivation of the H_2 ase enzyme in cells.

Thus, for achieving efficient and sustained H_2 photoproduction in nutrient-replete pulse-illuminated algae, our research suggests the accomplishment of the following three requirements:

- (1) O_2 accumulation in cultures should be contained within the levels favourable to the induction and operation of the H_2 ase enzyme in algal cells. Under photoautotrophic conditions, this requirement could be satisfied by periodic purging of the cultures with Ar and/or by introducing O_2 scavengers and O_2 absorbents into the cultures. In photoheterotrophic algae, respiratory activity in cultures should be sufficient for removing photosynthetically produced O_2 . Thus, the limitation of respiratory activity by substrates (acetate in the case of C. reinhardtii) should be avoided.
- (2) The produced H_2 should be kept at sufficiently low levels for preventing H_2 recycling by algal cells. For example, the produced H_2 can be re-utilized by coupling H_2 -producing algal cultures to a H_2 -consuming fuel cell in a hybrid system to produce power.
- (3) H_2 photoproduction should not compete with CO_2 fixation. In the future, this requirement could be resolved by metabolic engineering of production strains, while in the current state, pulse-illumination, and substrate (CO_2) limitation

protocols could be sufficient for preventing competition with the CBB cycle.

Author contributions

S. K. and Y. A.: conceptualization, methodology, funding acquisition and project management; S. V. and S. K.: investigation and formal analysis; S. K. and Y. A.: supervision and data validation; S. V. and S. K.: writing – original draft; S. K. and Y. A.: writing – review and editing.

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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