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## Balancing photosynthesis, O<sub>2</sub> consumption, and H<sub>2</sub> recycling for sustained H<sub>2</sub> photoproduction in pulse-illuminated algal cultures†

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Photosynthetic H<sub>2</sub> production in unicellular green alga *Chlamydomonas reinhardtii* is catalysed by O<sub>2</sub>-sensitive [Fe–Fe]-hydrogenase (H<sub>2</sub>ase) enzymes located in the chloroplast. The process is difficult to sustain due to (i) the inactivation of H<sub>2</sub>ase enzymes by O<sub>2</sub> coevolved in photosynthesis and (ii) the competition of H<sub>2</sub>ases with the Calvin–Benson–Bassham (CBB) cycle for photosynthetic reductants. Our previous studies revealed that H<sub>2</sub> 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 O<sub>2</sub> accumulation produced by photosystem II, prevents activation of the CBB cycle and redirects photosynthetic electrons to H<sub>2</sub>ase. 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 H<sub>2</sub> photoproduction yield by pulse-illuminated algae but only for the first 24 h. We bring evidence that the attenuation of H<sub>2</sub> evolution is primarily caused by the accumulation of H<sub>2</sub> in the headspace of vials rather than O<sub>2</sub> inhibition of the H<sub>2</sub>ase, whereas an increase in the H<sub>2</sub> partial pressure leads to activation of H<sub>2</sub> recycling and noticeable H<sub>2</sub> uptake, which is accelerated by O<sub>2</sub>. We predicted that sustained H<sub>2</sub> production in pulse-illuminated algae, which are additionally exposed to continuous low background light, could be achieved by decreasing the H<sub>2</sub> partial pressure in cultures and preventing excessive accumulation of O<sub>2</sub>. Indeed, the application of periodic refreshments of a headspace atmosphere with argon and the introduction of O<sub>2</sub> scavenger L-cysteine allowed the H<sub>2</sub> 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 H<sub>2</sub> per litre of the culture than the standard pulse-illumination protocol.

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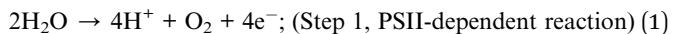
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## Introduction

Molecular hydrogen (H<sub>2</sub>), 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.<sup>1</sup> 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 H<sub>2</sub>.<sup>2–4</sup> In comparison with inorganic photocatalysts for water oxidation, CO<sub>2</sub> reduction and H<sub>2</sub> generation,<sup>5–7</sup> the photobiological approach considers the natural and engineered photosynthetic organisms as whole-cell biocatalysts, which provide a fully renewable alternative to the traditional chemical

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, CO<sub>2</sub> and some organic substrates.<sup>8–10</sup>

In green algae, H<sub>2</sub> photoproduction occurs in two steps with the involvement of the photosystem II (PSII) water oxidizing complex and the proton-reducing [Fe–Fe]-hydrogenase (H<sub>2</sub>ase) enzyme associated with the photosynthetic electron-transport chain:



Green algae typically produce H<sub>2</sub> on exposure to light after a period of dark anaerobic adaptation. The process is very efficient but short due to a fast accumulation of O<sub>2</sub> co-evolved in step 1.<sup>11,12</sup> Accumulated O<sub>2</sub> leads to the inactivation of O<sub>2</sub>-sensitive H<sub>2</sub>ase in cells.<sup>13,14</sup> Another reason for the fast termination of H<sub>2</sub> evolution in algae is a competition of H<sub>2</sub>ase with the Calvin–Benson–Bassham (CBB) cycle for photosynthetic

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reductants, which occurs just before the inactivation of H<sub>2</sub>ase by O<sub>2</sub>.<sup>15,16</sup> Activation of CO<sub>2</sub> fixation coincides with a pronounced H<sub>2</sub> uptake that also terminates with the accumulation of O<sub>2</sub> in cells.<sup>17</sup>

Sustained H<sub>2</sub> photoproduction in green algae is typically achieved by nutrient deprivation.<sup>18</sup> In this approach, algal cultures are transferred to the medium depleted of an essential nutrient such as sulfur, nitrogen, phosphorus, or magnesium.<sup>19–22</sup> Typically, under nutrient deprivation cells stop dividing, accumulate significant amounts of starch and partially degrade PSII reaction centres. The partial loss of water-oxidizing activity in algae results in the establishment of anaerobiosis in cultures due to efficient respiration and expression of H<sub>2</sub>ase enzymes in cells leading to sustained H<sub>2</sub> photoproduction for a few days.<sup>23,24</sup> Despite substantial improvement in the H<sub>2</sub> production yield, the nutrient deprivation approach is not scalable for commercial purposes due to its low photosynthetic efficiency.<sup>25</sup> Therefore, the current research efforts are primarily concentrated on the improvement of H<sub>2</sub> photoproduction activity in nutrient-replete algae.<sup>26</sup>

Our previous studies revealed that H<sub>2</sub> production in nutrient-replete *Chlamydomonas reinhardtii* algae could be sustained by a train of strong white-light pulses interrupted by longer dark phases.<sup>17,27,28</sup> 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 O<sub>2</sub> in cells. The pulse-illumination protocol represents a way to redirect photosynthetic electron flow to the green algal H<sub>2</sub>ase, thus resulting in increased H<sub>2</sub> photoproduction yields. Under these conditions, algae produce H<sub>2</sub> via the most efficient mechanism of direct water biophotolysis, which has a theoretical light energy to H<sub>2</sub> energy conversion efficiency (LHCE) of around 11–13%.<sup>29,30</sup> Although in the best case up to 8% LHCE has been achieved during a short period in photoheterotrophic algal cultures<sup>31</sup> and up to 4% under photoautotrophic conditions,<sup>16</sup> photosynthetic H<sub>2</sub> 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 H<sub>2</sub> production yield in algal cultures.<sup>32</sup> 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 H<sub>2</sub> photoproduction in green alga *C. reinhardtii* exposed to pulse-illumination under photoautotrophic and photoheterotrophic conditions and determine the major requirements for algal cultivation, which are important for supporting the long-term H<sub>2</sub> photoproduction activity in nutrient-replete algal cultures and leading to improved H<sub>2</sub> 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 H<sub>2</sub> 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 Tris-phosphate-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<sup>−2</sup> s<sup>−1</sup> of photosynthetically active radiation (hereafter μmol m<sup>−2</sup> s<sup>−1</sup>) 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 H<sub>2</sub> photoproduction experiments were performed with non-stressed 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, flushed 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 H<sub>2</sub> photoproduction in algal suspensions, a train of 1 s white light pulses (280 μmol m<sup>−2</sup> s<sup>−1</sup>) was applied to the surface of the vials.<sup>27</sup> 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<sup>−2</sup> s<sup>−1</sup>. Pulses and background illumination were provided by the top LED panel of the growth chamber. For long-term H<sub>2</sub> 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 O<sub>2</sub> 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 H<sub>2</sub> and O<sub>2</sub> 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 H<sub>2</sub> and O<sub>2</sub> 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 H<sub>2</sub> l<sup>−1</sup> H<sub>2</sub>O (0.01744 ml ml<sup>−1</sup>) under 1 atm and 258 μmol O<sub>2</sub> l<sup>−1</sup> H<sub>2</sub>O (0.00632 ml ml<sup>−1</sup>) under 0.21 atm at 25 °C.<sup>33,34</sup> These values as well as amounts of gases withdrawn for sampling were considered in the final production yields. The H<sub>2</sub> and O<sub>2</sub> 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.<sup>35</sup>

### Hydrogenase activity assay

*In vitro* hydrogenase activity during H<sub>2</sub> photoproduction was determined in photoheterotrophic and photoautotrophic algal samples. The assay was performed in 10 ml serum vials containing 900  $\mu$ l 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  $\mu$ l 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 H<sub>2</sub> in the headspace of the vials was measured by gas chromatography (as described above) for 40 min and the activity ( $\mu$ mol H<sub>2</sub> (mg Chl h)<sup>-1</sup>) was calculated for the maximum H<sub>2</sub> production rate based on the total Chl content in the sample.

### Membrane inlet mass spectrometry measurements

*In vivo* CO<sub>2</sub> ( $m/z$  = 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 Scientific™) via a refrigerated cooling trap (−65 °C; EtOH; Julabo FT 902) as previously described.<sup>17,36</sup> 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  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>) 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<sub>2</sub> release by the culture in the dark.

## Results and discussion

### Effect of low background illumination on H<sub>2</sub> photoproduction by algal cultures exposed to the pulse-illumination protocol

Recently, we demonstrated that the induction of sustained H<sub>2</sub> 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  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>) background illumination.<sup>27</sup> The appearance of H<sub>2</sub> in pulse-illuminated cultures exposed to 3  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> continuous background light was not surprising since even if this condition activates the CCB cycle, co-produced O<sub>2</sub> is efficiently removed by respiration. Hydrogen photoproduction in green algae depends on photosynthetic activity and increases with an increase in light intensity.<sup>37</sup> The increase in light intensity, though, enhances O<sub>2</sub> accumulation. Therefore, we proposed that an increase in the

intensity of the background illumination should also enhance H<sub>2</sub> photoproduction activity in algal cultures, until the point where respiration could not adequately cope with the accelerated evolution of O<sub>2</sub> leading to the inactivation of H<sub>2</sub>ase enzymes in algae.

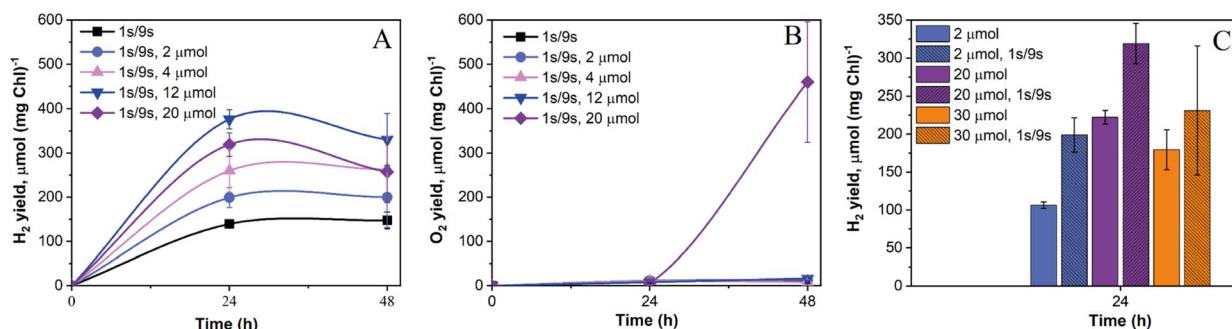
To verify this hypothesis, 1 s light pulses were superimposed with a 10 s frequency either on the dark background (the control cultures) or on the low background illumination (2–20  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>). As shown in Fig. 1A, the control cultures produced around 140  $\mu$ mol H<sub>2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

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

To check whether H<sub>2</sub> uptake in pulse-illuminated algae is linked to CO<sub>2</sub> photoreduction in the CBB cycle, we introduced 20 mM NaHCO<sub>3</sub> into the cultures at the beginning of the experiment. As shown in ESI Fig. 1,† the supplementation of pulse-illuminated algae with 20 mM NaHCO<sub>3</sub> did not affect net H<sub>2</sub> and O<sub>2</sub> production yields in the control cultures throughout the experiment, and during the first 24 h in cultures exposed to 20  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> background light (ESI Fig. 1†). After 24 h, the algae exposed to 20  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> background illumination accelerated O<sub>2</sub> production in the presence of bicarbonate compared to the cultures without added bicarbonate (ESI Fig. 1B†). The appearance of O<sub>2</sub> in both cultures exposed to 20  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> light indicated acceleration of the CBB cycle at around 24 h. This resulted in the pronounced H<sub>2</sub> uptake (ESI Fig. 1A†). Interestingly, the addition of bicarbonate reduced H<sub>2</sub> uptake under 20  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> background illumination most probably due to the excessive production and accumulation of O<sub>2</sub>, leading to inhibition of the H<sub>2</sub>ase. Thus, the response of H<sub>2</sub> uptake to the acceleration of the CBB cycle is rather complex. The enhanced CO<sub>2</sub> fixation may indeed enhance H<sub>2</sub> uptake (depending on the H<sub>2</sub> partial pressure) but leads to excessive production of O<sub>2</sub> that inactivates the H<sub>2</sub>ase enzyme.

Substantial H<sub>2</sub> 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<sub>2</sub> production under low light intensities





**Fig. 1** Effect of low background illumination on net  $\text{H}_2$  photoproduction and net  $\text{O}_2$  evolution yields by wild-type *C. reinhardtii* cells exposed to the pulse-illumination protocol. (A) Net  $\text{H}_2$  photoproduction and (B) net  $\text{O}_2$  evolution yields under 1 s/9 s pulse illumination ( $280 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) applied atop darkness (the control sample) or continuous background illumination of 2 to 20  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . (C) Comparison of net  $\text{H}_2$  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)  $\mu\text{g Chl (a + b) ml}^{-1}$ . The  $\text{H}_2$  and  $\text{O}_2$  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.<sup>38,39</sup> However, it should be noted that the application of strong light pulses to cultures exposed to low continuous light always led to improved net  $\text{H}_2$  production yields in the range of tested conditions (Fig. 1C). It is important to note here that 280  $\mu\text{mol m}^{-2} \text{s}^{-1}$  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  $\text{H}_2$  production, especially under the low background illumination. For example, under 2  $\mu\text{mol m}^{-2} \text{s}^{-1}$  of continuous background light the applied 280  $\mu\text{mol m}^{-2} \text{s}^{-1}$  light pulses bring up to 93% of the total light energy, while under 30  $\mu\text{mol m}^{-2} \text{s}^{-1}$  background light, this value is around 48%. As shown in Fig. 1C, the net  $\text{H}_2$  photoproduction yield in algal cultures started decreasing after their exposure to 30  $\mu\text{mol m}^{-2} \text{s}^{-1}$  light, which is just below the compensation point (when the rate of photosynthesis becomes equal to the rate of respiration) in the wild-type culture grown under photoheterotrophic conditions.<sup>40</sup> Thus, the results revealed that efficient photosynthetic  $\text{H}_2$  production occurs when cellular respiration overtakes or minimizes accumulation of photosynthetic  $\text{O}_2$  and creates a microoxic environment inside algal chloroplasts. The latter is important for preventing inactivation of the  $\text{O}_2$ -sensitive  $\text{H}_2$ ase enzymes.<sup>41</sup> Overall, the highest net  $\text{H}_2$  production yield in pulse-illuminated algae could be observed in cultures exposed additionally to around 10–12  $\mu\text{mol m}^{-2} \text{s}^{-1}$  of background light (Fig. 1).

Thus, despite the limited accumulation of  $\text{O}_2$  in most of the cultures, the application of low intensity background light in the pulse-illumination protocol did not allow us to sustain  $\text{H}_2$  photoproduction for more than 24 h mainly due to activation of  $\text{H}_2$  uptake after that point.

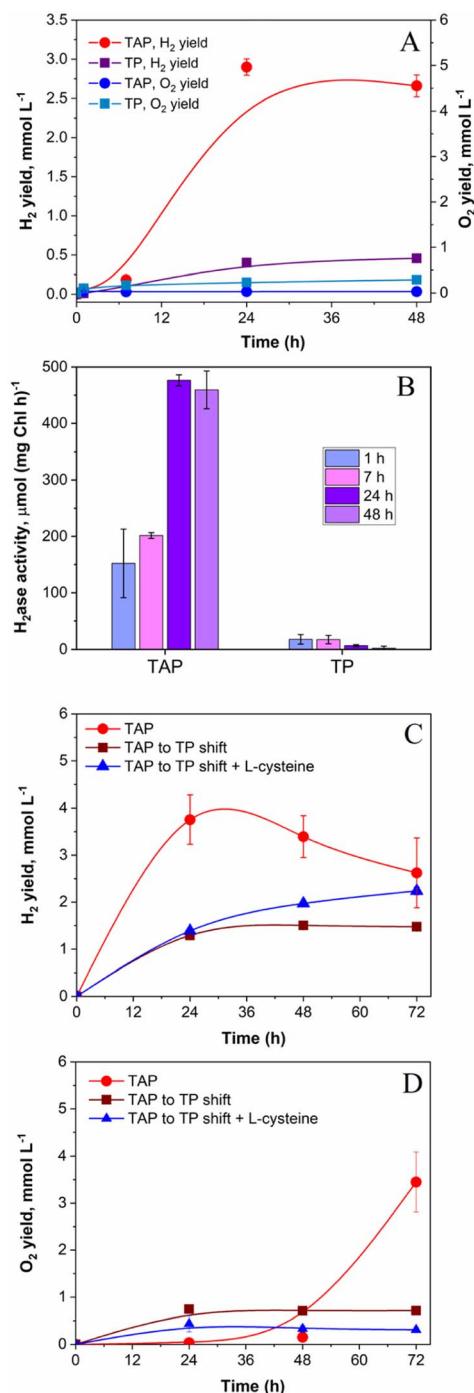
### Impact of cultivation conditions on pulse-illuminated algae under low background illumination

Similar to photoheterotrophic cultures, algae pre-grown under strict photoautotrophic conditions showed the capacity to sustain  $\text{H}_2$  photoproduction under pulse-illumination.<sup>27,28</sup> Therefore, it was of interest to check whether pulse-illuminated

photoautotrophic algae could also produce  $\text{H}_2$  under low background illumination. For this purpose, low cell density *C. reinhardtii* cultures ( $\sim 5 \mu\text{g total Chl ml}^{-1}$ ) were exposed to 1 s/9 s pulses superimposed on 12  $\mu\text{mol m}^{-2} \text{s}^{-1}$  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  $\text{O}_2$  production and the enhanced  $\text{O}_2$  consumption by cells.<sup>39,42</sup> As shown in Fig. 2A, photoautotrophic algal cells could produce  $\text{H}_2$  under this condition, albeit giving much lower yields compared to photoheterotrophic cultures (0.46 vs. 2.9  $\text{mmol H}_2 \text{l}^{-1}$ , respectively). Predictably,  $\text{H}_2$  production in photoautotrophic algae proceeded with a noticeable release of  $\text{O}_2$  into the headspace of the vials (Fig. 2A), though the  $\text{O}_2$  content in vials did not exceed 0.1% by the end of the experiment (as calculated from the original GC data). Slight accumulation of  $\text{O}_2$  in cultures resulted in very low activities of the  $\text{H}_2$ ase enzyme in cells (Fig. 2B). Similarly, Lee and Greenbaum observed 50% inhibition of  $\text{H}_2$  photoproduction in photoautotrophic algae after the introduction of 0.05%  $\text{O}_2$  in vials.<sup>43</sup> However, the intracellular  $\text{H}_2$ ase could tolerate up to 0.5%  $\text{O}_2$ .<sup>43</sup> Thus, the low level of the  $\text{H}_2$ ase activity in photoautotrophic algae, which is observed in our case (Fig. 2B), could be caused not only by the inactivation of the  $\text{H}_2$ ase enzyme itself but by its impaired biosynthesis. Indeed, Forestier *et al.* observed a much slower accumulation of  $\text{H}_2$ ase transcripts and induction of the  $\text{H}_2$  photoproduction activity in photoautotrophically pre-grown algae compared to in photoheterotrophic cultures.<sup>44</sup> In the presence of acetate in the medium (Fig. 2A), the concentration of  $\text{O}_2$  in vials was noticeably lower than that in photoautotrophic algae and is retained at a level sufficient for preventing  $\text{H}_2$ ase enzyme inactivation by intracellular respiration (Fig. 2B).

In our experimental setup, the highest level (476  $\mu\text{mol H}_2 (\text{mg Chl h})^{-1}$ ) of the *in vitro*  $\text{H}_2$ ase activity in photoheterotrophic algae was detected 24 h after application of the pulse-illumination protocol (Fig. 2B), and close to the point where cells began consuming  $\text{H}_2$  (Fig. 2A). The  $\text{H}_2$ ase activity in photoheterotrophic algae was comparable to the one observed in S-





**Fig. 2** The effect of cultivation conditions on net H<sub>2</sub> photoproduction, net O<sub>2</sub> evolution and the H<sub>2</sub>ase activity in pulse-illuminated algae exposed to 12  $\mu\text{mol m}^{-2} \text{s}^{-1}$  of background illumination. (A) Net H<sub>2</sub> and O<sub>2</sub> yields in photoheterotrophic (TAP) and photoautotrophic (TP) cultures. (B) Changes in the *in vitro* H<sub>2</sub>ase activity in the same cultures throughout the experiment. A comparison of net H<sub>2</sub> photoproduction (C) and net O<sub>2</sub> evolution (D) yields in photoheterotrophic cultures (TAP) and photoautotrophically 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)  $\mu\text{g Chl (a + b) ml}^{-1}$  at the beginning of the experiment. Values are the mean of 3 independent replicates  $\pm$  SD.

deprived cells during the period of efficient H<sub>2</sub> production.<sup>23,45</sup> Even at 7 h, the H<sub>2</sub>ase activity was around 200  $\mu\text{mol H}_2$  (mg Chl h)<sup>-1</sup> (Fig. 2B; the 7 h TAP sample), which significantly exceeded the rate of photosynthetic H<sub>2</sub> production (42  $\mu\text{mol H}_2$  (mg Chl h)<sup>-1</sup>) determined for the interval between 7 and 24 h (Fig. 2A; the TAP sample). Thus, the H<sub>2</sub>ase activity could not be the limiting factor for H<sub>2</sub> production in photoheterotrophic algae. It is important to note that the H<sub>2</sub>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<sub>2</sub>ase activity (maximum 18  $\mu\text{mol H}_2$  (mg Chl h)<sup>-1</sup> at 7 h) that led to the low net H<sub>2</sub> 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.<sup>46</sup> During H<sub>2</sub> production, these reserves can be further utilized either for supporting anaerobiosis in algal cultures as substrates for respiration or for providing reductants to H<sub>2</sub>ase *via* the indirect H<sub>2</sub> production pathway.<sup>47,48</sup> Considering this information and attempting to improve the H<sub>2</sub> 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  $\mu\text{g Chl (a + b) ml}^{-1}$ ). This approach, though, did not enhance the net H<sub>2</sub> photoproduction yield in the absence of acetate. As shown in Fig. 2C, the shifted algae produced  $\sim$ 2.5 times lower H<sub>2</sub> than the non-shifted cultures (1.5 vs. 3.75 mmol L<sup>-1</sup>, respectively). The shifted photoautotrophic algae, even at high cell density, still suffered from the excessive production of O<sub>2</sub> (Fig. 2D). For reducing the O<sub>2</sub> content in the medium, we applied L-cysteine, which is an efficient O<sub>2</sub> scavenger. It shows reducing properties attributed to its thiol group, which in the presence of O<sub>2</sub> 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.<sup>49</sup> As shown in ESI Fig. 2,† the introduction of 4–8 mM L-cysteine into the cultures is sufficient for supporting H<sub>2</sub> photoproduction in pulse-illuminated photoheterotrophic cultures under 12  $\mu\text{mol m}^{-2} \text{s}^{-1}$  of background illumination. Similarly, the addition of 4 mM L-cysteine in photoautotrophic cultures slightly improved the net H<sub>2</sub> photoproduction yield (Fig. 2C). These results indicated the essential role of acetate in supporting anaerobiosis and sustaining H<sub>2</sub> production in pulse-illuminated algae under low background illumination. In the absence of acetate, the application of extra O<sub>2</sub> scavengers is required for sustaining the H<sub>2</sub> production activity in algal cultures.

### Effect of H<sub>2</sub> partial pressure on the photosynthetic production of H<sub>2</sub> by pulse-illuminated algae

Vials with pulse-illuminated algae, which were additionally exposed to low background light, showed a pronounced H<sub>2</sub> uptake after 24 h of the experiment (Fig. 1A). The effect was more pronounced in cultures that produced considerable



amounts of  $\text{H}_2$  during the first 24 h (Fig. 1A, 12 and 20  $\mu\text{mol m}^{-2} \text{s}^{-1}$  trends) just before  $\text{H}_2$  started to decline. Since the experiments were performed in gas-tight vials, it is unlikely that the effect is caused by the inhibition of  $\text{H}_2\text{ase}$  in the presence of co-evolved  $\text{O}_2$  and the passive leak of  $\text{H}_2$  from the vials. We suggested the catalytic nature of  $\text{H}_2$  uptake in algae that occurs simultaneously with  $\text{H}_2$  release and becomes more pronounced under high  $\text{H}_2$  partial pressure. Indeed, the reaction of  $\text{H}_2$  production driven by algal  $\text{H}_2\text{ases}$  is highly reversible.<sup>50–52</sup> Depending on the redox state of the cells and the partial pressure of  $\text{H}_2$  in the environment, algae could either produce or oxidize  $\text{H}_2$ . Hydrogen assimilation could be either the result of the oxy-hydrogen reaction,  $\text{CO}_2$  photoreduction in the CBB cycle or another unknown pathway.<sup>26,53,54</sup>

To confirm the catalytic nature of  $\text{H}_2$  uptake in pulse-illuminated 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  $\text{H}_2$  in the presence of 3%  $\text{H}_2$  in the headspace of vials. As shown in Fig. 3, under these conditions a very minor  $\text{H}_2$  production was observed. In the next step, we checked if periodic replacements of headspace gases with Ar without changing the medium could improve  $\text{H}_2$  photoproduction yield. This approach indeed extended the period of efficient  $\text{H}_2$  photoproduction by pulse-illuminated *C. reinhardtii* from 48 h (observed in the control) to above 120 h, yielding 440  $\mu\text{mol H}_2 (\text{mg Chl})^{-1}$  at that point. It should be noted, however, that periodic replacements of headspace gases with Ar remove not only  $\text{H}_2$  but  $\text{O}_2$  as well. As we demonstrated above (ESI Fig. 1†), accumulation of  $\text{O}_2$  in vials may either accelerate  $\text{H}_2$  uptake by algae or inhibit the  $\text{H}_2\text{ase}$  activity in cells depending on the produced  $\text{O}_2$  level. The addition of an  $\text{O}_2$  scavenger (8 mM L-cysteine) to the cultures stopped  $\text{H}_2$  uptake, though without any

improvement in the  $\text{H}_2$  yield (Fig. 3; ESI Fig. 2†), while the addition of the  $\text{O}_2$  scavenger under low background illumination decreased the rate of  $\text{H}_2$  consumption by the cultures (ESI Fig. 2†). This indicates that the  $\text{H}_2$  uptake component depends on the  $\text{H}_2$  partial pressure in vials and the level of intracellular  $\text{O}_2$ . The dependence of  $\text{H}_2$  uptake on  $\text{O}_2$  has also been demonstrated by Milrad *et al.*<sup>54</sup> who observed elevated consumption of  $\text{H}_2$  in the absence of an  $\text{O}_2$ -scavenging system in contrast to the sample with added glucose, glucose oxidase and catalase. Though, it should be noted that the excessive production of  $\text{O}_2$  inhibits  $\text{H}_2$  uptake (ESI Fig. 1†).

### Maximizing $\text{H}_2$ 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  $\text{H}_2$  photoproduction rate but the process could not be sustained for longer than 24 h (Fig. 1A) due to the activation of  $\text{H}_2$  uptake under increasing  $\text{H}_2$  partial pressure (Fig. 3). Meanwhile, simultaneous accumulation of  $\text{O}_2$  in vials due to water-oxidation in PSII may partly accelerate  $\text{H}_2$  assimilation by the cells (ESI Fig. 1 and 2†) and lead to inactivation of the  $\text{H}_2\text{ase}$  enzyme if the  $\text{O}_2$  content reaches the critical level. Since daily re-flushing of cultures with Ar replaces both  $\text{H}_2$  and  $\text{O}_2$  produced by algae, this approach should improve  $\text{H}_2$  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  $\mu\text{mol m}^{-2} \text{s}^{-1}$  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  $\mu\text{mol m}^{-2} \text{s}^{-1}$  continuous light significantly outperformed ( $P < 0.001$ ) cultures without background illumination;  $\text{H}_2$  photoproduction yields at 216 h were 2.7 and 1.3  $\text{mmol H}_2 (\text{mg Chl})^{-1}$ , 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  $\text{H}_2$  production was followed by a long period of  $\text{H}_2$  uptake.

The net release of  $\text{O}_2$  in pulse-illuminated algal cultures, which were exposed to continuous 12  $\mu\text{mol m}^{-2} \text{s}^{-1}$  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  $\text{CO}_2$  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  $\text{CO}_2$  fixation in the CBB cycle (Fig. 5B). In contrast, the control algae that were exposed only to pulse-illumination release  $\text{CO}_2$ . The  $\text{CO}_2$  release under pulse-illumination has been already explained,<sup>17,55</sup> and therefore, will not be discussed in this work. Since the pulse-illuminated algae perform  $\text{CO}_2$  fixation under continuous low background light from the beginning of the experiment,

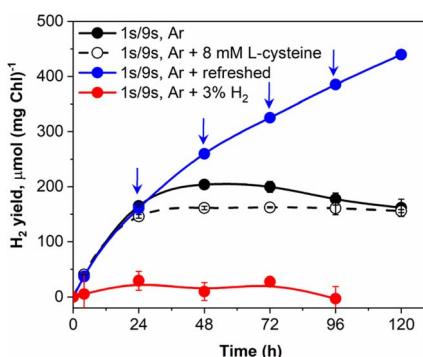
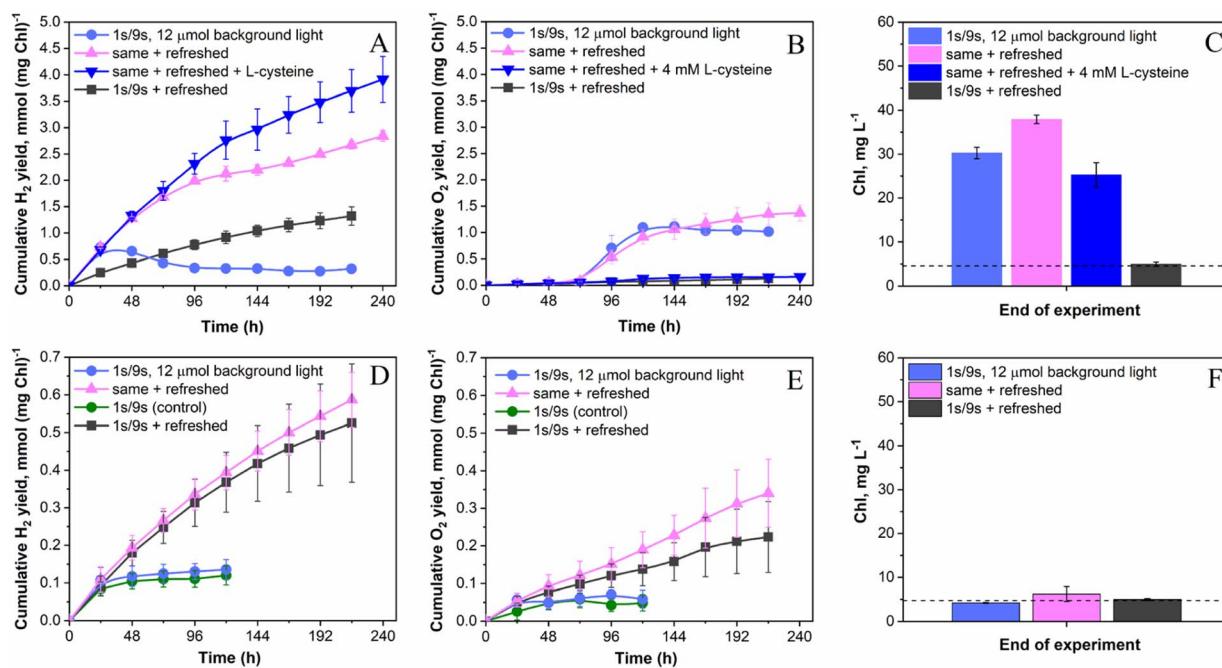
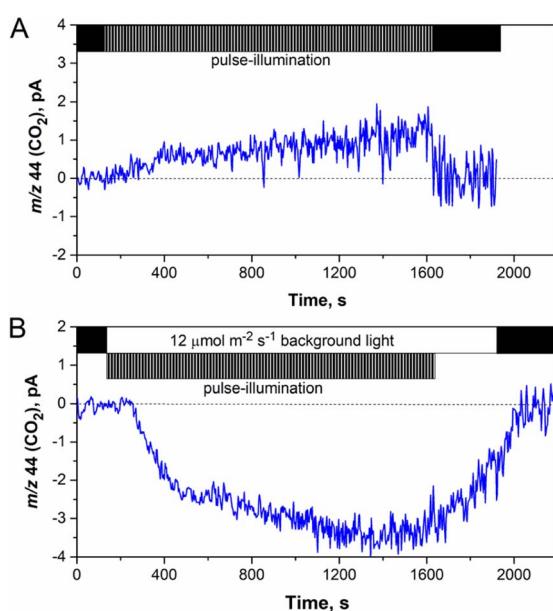


Fig. 3 Effect of  $\text{H}_2$  partial pressure on net  $\text{H}_2$  photoproduction under pulse-illumination. The following conditions were tested: the control sample (1 s/9 s, Ar); 8 mM L-cysteine was introduced into anaerobic vials at  $t = 0$  h (1 s/9 s, Ar + 8 mM L-cysteine); headspace gases in the vials were replaced with Ar every 24 h (1 s/9 s, Ar + refreshed); 3%  $\text{H}_2$  was introduced into the headspace of anaerobic vials at  $t = 0$  h (1 s/9 s, Ar + 3%  $\text{H}_2$ ). Arrows indicate the points where the atmosphere of vials was replaced with Ar. The  $\text{H}_2$  yields were normalized to the initial total Chl content, which was around 9  $\mu\text{g ml}^{-1}$ . The  $\text{H}_2$  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.





**Fig. 4** Sustaining  $\text{H}_2$  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  $\text{H}_2$  photoproduction yield, (B and E) the cumulative  $\text{O}_2$  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  $\text{H}_2$  photoproduction, the atmosphere of the culture vials was replaced with Ar every 24 h. For  $\text{O}_2$  scavenging, 4 mM L-cysteine was introduced directly into the medium at the beginning of the experiment. The  $\text{H}_2$  and  $\text{O}_2$  yields were normalized to the initial total Chl content, which was around  $5 \mu\text{g ml}^{-1}$ . The  $\text{H}_2$  and  $\text{O}_2$  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  $\text{CO}_2$  exchange in pulse-illuminated algae. (A) The standard (1 s light/9 s dark) pulse-illumination protocol was applied to cultures after 2 min of dark adaptation. (B) The same pulse-illumination was employed simultaneously with continuous  $12 \mu\text{mol m}^{-2} \text{s}^{-1}$  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  $\text{O}_2$  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  $\text{CO}_2$  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  $\text{CO}_2$  fixation in algal cultures just under the train of 1 s/9 s pulses, now demonstrated in the long-term (240 h) process.

To determine the impact of  $\text{O}_2$  on  $\text{H}_2$  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  $\text{O}_2$  yield was  $\sim$ 8-times lower than that in the similar cultures without L-cysteine ( $0.16$  vs.  $1.37 \text{ mmol O}_2 (\text{mg Chl})^{-1}$ , respectively). Daily levels of  $\text{O}_2$  in L-cysteine-treated vials were stable and did not exceed 0.03% by the end of each cycle on an average basis. As a result, L-cysteine-treated algae further improved the  $\text{H}_2$  photoproduction yield to above  $3.9 \text{ mmol H}_2 (\text{mg Chl})^{-1}$  (Fig. 4A) or  $\sim$ 20  $\text{mmol l}^{-1}$ . This amount of  $\text{H}_2$  was at least 6-times higher than that in the standard 1 s/9 s pulse-illumination protocol ( $1.5$ – $3 \text{ mmol l}^{-1}$ ) as demonstrated previously<sup>27</sup> and calculated from data shown in



Fig. 1A, 2A and 3. Due to excessive growth (Fig. 4C), the H<sub>2</sub> photoproduction activity in L-cysteine-treated cultures still suffered from competition with the CBB cycle. Therefore, we expect that the improved H<sub>2</sub> production yield under this condition can be achieved by further limitation of photosynthetic electron flow to the CBB cycle, for example, in Rubisco-deficient *C. reinhardtii* mutants with impaired CO<sub>2</sub> fixation capacity. Working with the Rubisco-deficient CC-2803 strain, Hemschemeier and co-authors<sup>48</sup> demonstrated that this mutant can produce H<sub>2</sub> without S-deprivation, but alga experiences enhanced photoinhibition under H<sub>2</sub> production conditions. The latter might be resolved by the application of the pulse-illumination approach.

Thus, the major trigger for the induction of efficient H<sub>2</sub> photoproduction in anaerobic algae seems to be the limitation of the CBB cycle. In the absence of CO<sub>2</sub> fixation, H<sub>2</sub> generation is one of the major electron sinks for supporting the PSII activity in an anaerobic environment.<sup>48</sup> For example, in S-deprived algae, a major degradation of the Rubisco enzyme occurs before the onset of efficient H<sub>2</sub> production,<sup>24,56</sup> while restoration of the linear photosynthetic electron flow from PSII coincides with the appearance of H<sub>2</sub> in cultures.<sup>57,58</sup> In nutrient-replete dark-adapted algae, the activation of CO<sub>2</sub> fixation results in the loss of H<sub>2</sub> production activity and initiates H<sub>2</sub> uptake.<sup>15</sup> If activation of the CBB cycle is prevented by substrate-limitation<sup>16</sup> or pulse-illumination (as discussed in this paper), the cultures are capable of sustaining H<sub>2</sub> 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 H<sub>2</sub> photoproduction and CO<sub>2</sub> fixation operate simultaneously (Fig. 4, A–C panels). Here, algal growth is supported by the assimilation of acetate in the presence of photosynthetically produced O<sub>2</sub>. On the other hand, respiration of acetate retains an anaerobic environment in vials, and protects H<sub>2</sub>ase from O<sub>2</sub> inactivation.<sup>59–61</sup>

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

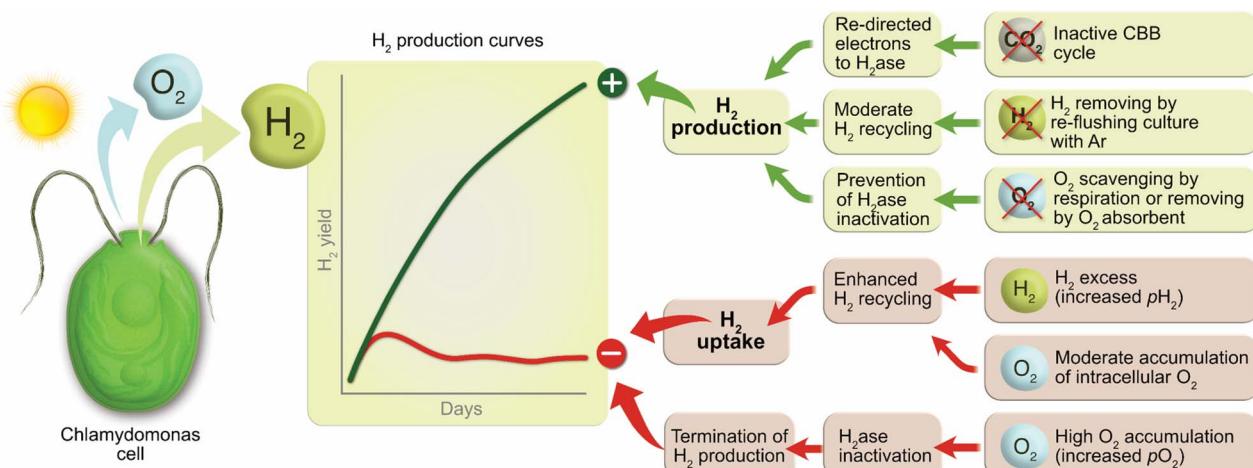


Fig. 6 Factors affecting H<sub>2</sub> photoproduction in pulse-illuminated algal cultures.



In the absence of CO<sub>2</sub> and acetate, anaerobic algae could not perform normal photosynthesis that links the water oxidation reaction to CO<sub>2</sub> fixation, and therefore algal cells predominantly derive O<sub>2</sub> *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 O<sub>2</sub> is diluted in an Ar atmosphere), it is not surprising that under these conditions we observed the stoichiometry of 2 mol H<sub>2</sub> to 1 mol O<sub>2</sub> during almost the whole period of H<sub>2</sub> production. It is clear that H<sub>2</sub> photoproduction in photoautotrophic cultures could only be sustained if O<sub>2</sub> is efficiently removed from the cultures by periodic purging with Ar (Fig. 4D) and/or introduction of O<sub>2</sub> scavengers and O<sub>2</sub> absorbents.<sup>62–64</sup> Nevertheless, our data show that substantial amounts of H<sub>2</sub> 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 H<sub>2</sub>ase 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<sub>2</sub> photoproduction activity in pulse-illuminated green algae are summarized in Fig. 6. Among positive factors leading to the increased H<sub>2</sub> photoproduction yield are inactivation of CO<sub>2</sub> fixation by algal cells, no (or moderate) H<sub>2</sub> recycling by the cells under high H<sub>2</sub> partial pressure, and an anaerobic or microoxic environment preventing inactivation of the H<sub>2</sub>ase in algal cells. Non-compliance with these factors causes the activation of H<sub>2</sub> uptake by algae and, in the worst case, the termination of H<sub>2</sub> photoproduction in algal cultures due to activation of CO<sub>2</sub> fixation and/or inactivation of the H<sub>2</sub>ase enzyme in cells.

Thus, for achieving efficient and sustained H<sub>2</sub> photoproduction in nutrient-replete pulse-illuminated algae, our research suggests the accomplishment of the following three requirements:

(1) O<sub>2</sub> accumulation in cultures should be contained within the levels favourable to the induction and operation of the H<sub>2</sub>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<sub>2</sub> scavengers and O<sub>2</sub> absorbents into the cultures. In photoheterotrophic algae, respiratory activity in cultures should be sufficient for removing photosynthetically produced O<sub>2</sub>. Thus, the limitation of respiratory activity by substrates (acetate in the case of *C. reinhardtii*) should be avoided.

(2) The produced H<sub>2</sub> should be kept at sufficiently low levels for preventing H<sub>2</sub> recycling by algal cells. For example, the produced H<sub>2</sub> can be re-utilized by coupling H<sub>2</sub>-producing algal cultures to a H<sub>2</sub>-consuming fuel cell in a hybrid system to produce power.

(3) H<sub>2</sub> photoproduction should not compete with CO<sub>2</sub> 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<sub>2</sub>) 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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