

Coupling microalgal bioremediation of recirculating aquaculture effluents with photobiological hydrogen production

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ABSTRACT

Recirculating aquaculture system (RAS) effluents contain substantial nutrient loads, posing a eutrophication risk to aquatic ecosystems. Microalgal bioremediation is a promising strategy, but further valorisation of this process is required. This study couples the cultivation of nitrate-utilizing *Chlamydomonas reinhardtii* strain CC-1690 in RAS effluent with photobiological hydrogen (H₂) production to achieve nutrient removal, bioenergy production, and high-quality biomass. Within 70 h, CC-1690 achieved near-complete depletion of nitrate and phosphate from the effluent. Subsequently, H₂ production was induced via carbon limitation and anoxia, yielding 49 μmol H₂ mg⁻¹ Chl over 72 h. Although signs of physiological stress were observed, key photosynthetic subunits (PsbA, PSBO, CP47, PetB and PsaA) remained largely stable. Total lipid content increased slightly, enriched in palmitic and α-linolenic fatty acids, while protein content was unaffected. These results demonstrate that integrating H₂ production with RAS effluent remediation offers a robust approach, valorising waste into bioenergy and high-quality biomass.

1. Introduction

Over the past decades, global aquaculture production has undergone rapid growth as a means of satisfying rising protein demand [1]. Recirculating aquaculture systems (RAS) have emerged as a sustainable approach to intensive aquaculture, in which closed-loop systems facilitate water use efficiency, improve biosecurity, and enable the optimization of production conditions [2]. Although water recirculation rates can reach 90–99% [3], wastewater generation is unavoidable, and the accumulated volume becomes substantial in medium-to large-scale operations. This effluent contains high levels of nitrate and phosphate, primarily derived from metabolic waste and the dissolved portion of uneaten feed [4]. Currently, nutrient-rich effluents from RAS are often released into the environment, presenting a significant eutrophication risk to aquatic ecosystems. While typically viewed as waste, these nutrients represent valuable resources. For instance, nitrogen fertilizers are energy-intensive to produce, contributing to greenhouse gas emissions, while phosphorus is sourced from finite phosphate rock reserves that are

being rapidly depleted [5]. Recovering these nutrients from RAS effluents, therefore, offers a dual benefit: mitigating environmental pollution and recycling critical nutrients for further utilization.

Among various wastewater treatment strategies, microalgal bioremediation is a promising, eco-friendly solution for nutrient recovery and valorisation [6–8]. Most microalgae are capable of assimilating nitrate and other inorganic nutrients present in RAS effluents into biomass through photosynthetic growth. Compared to established technologies for the treatment of RAS effluents, such as anaerobic digestion and pyrolysis, microalgal bioremediation does not require dewatering of the effluent [9], thereby reducing energy input and increasing overall process sustainability. Due to its high nutrient content and ratios compatible with microalgal requirements, as well as the absence of toxic chemicals, RAS effluent has been shown to outperform other waste streams, such as municipal or industrial wastewater, when used as a growth medium [10]. Microalgal production utilizing aquaculture effluents represents a multifunctional opportunity with diverse potential applications in the bioeconomy [8]. Untreated biomass can be used as a

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biofertilizer [11], while high-value pigments, proteins, fatty acids, polysaccharides, and bioactive metabolites can be extracted for use in a wide range of bio-based products, including plant biostimulants and aquafeed additives [12,13]. Biomass quality is therefore key to enabling the valorisation of the recovered nutrients from RAS effluents.

Photobiological hydrogen (H_2) production represents a promising strategy to expand the biotechnological application of photosynthetic microorganisms, particularly microalgae. H_2 is a versatile energy carrier with a wide variety of industrial applications. Certain green microalgae species, such as *Chlamydomonas reinhardtii*, are able to produce H_2 utilizing the photosynthetic electron transport chain. This reaction is mediated by [FeFe]-hydrogenase enzymes (HydA1 and HydA2), which are positioned on the acceptor side of photosystem I (PSI) [14–16]. These hydrogenases act as electron sinks, alleviating excess reductant accumulation and preventing over-reduction of the photosynthetic apparatus during dark-to-light transitions [17]. However, once the Calvin-Benson cycle is operative under light, electrons originating from water splitting in photosystem II (PSII) are primarily utilized for carbon fixation [17], while oxygen (O_2) buildup irreversibly inactivates the hydrogenases [18].

Strategies such as nutrient deprivation (typically sulphur) [19], balancing cellular respiration and O_2 production [20], and establishing algae-bacteria co-cultures [21] maintain low O_2 concentrations under photoheterotrophic conditions. Meanwhile, pulse illumination decreases the activity of the Calvin-Benson cycle under both photoautotrophic and photoheterotrophic conditions [22]. Direct removal of O_2 via scavengers coupled with substrate limitation of the Calvin-Benson cycle (acetate- and CO_2 -free conditions) is another strategy for sustained H_2 production under photoautotrophic conditions [23]. This approach minimizes both O_2 accumulation and the activity of the Calvin-Benson cycle, offers a high theoretical efficiency of converting light into chemical energy (11–13%), is applicable at sunlight intensities [24], and can avoid the degradation of photosynthetic complexes or cell death associated with nutrient-deprivation strategies.

In addition to the competition of the Calvin-Benson cycle with H_2 production, nitrate assimilation acts as a potent alternative electron sink, specifically during the reduction of nitrate and nitrite, thereby suppressing H_2 evolution [25,26]. Given that only a subset of *C. reinhardtii* strains are capable of assimilating nitrate [27] and that H_2 production studies are typically carried out in ammonium-containing media, the feasibility of coupling nitrate-containing media with H_2 production has remained largely unexplored.

In this study, we demonstrate that the nitrate-utilizing *C. reinhardtii* strain CC-1690 efficiently removes nitrate and phosphate present in RAS effluents, unlike standard H_2 -producing strains (CC-124, T222⁺, *pgr5*) which lack this capability. Following nutrient removal, H_2 production was induced directly in the effluent using the anaerobiosis-induced photoautotrophic protocol [23]. Although H_2 yields were slightly lower compared to those in standard algal media, substantial production was obtained. Furthermore, despite the observed stress responses, the biomass quality was preserved, supporting downstream valorisation. These findings demonstrate the feasibility of integrating microalgal bioremediation of RAS effluents with photobiological H_2 production using a nitrate-assimilating *C. reinhardtii* strain.

2. Materials and methods

2.1. Recirculating aquaculture system effluent

Recirculating aquaculture system (RAS) effluent (20 l) was obtained from the Laukaa fish farm in central Finland [28], which is part of the Natural Resources Institute Finland (LUKE) research infrastructure. Effluent samples were collected during May of 2025 from the water outlet after mechanical (drum filtration) and biological (fixed bed bioreactor) treatment. The fish farm obtains its water from an oligotrophic lake (lake Peurunka; 62.44886, 25.85201), and the sampled

effluent originates from a RAS system, which at the time of sampling, was used for low-density rearing of rainbow trout (*Oncorhynchus mykiss*). Effluent was filtered with a tangential-flow filtration system (0.22 μm Millipore Durapore cassette) and UV-sterilized for 45 min before being stored at 4 °C.

2.2. Algal culturing and H_2 production

C. reinhardtii wild-type strains CC-1690 and CC-124 were purchased from the Chlamydomonas Resource Center (University of Minnesota, USA) while T222⁺ and the *pgr5* mutant strain were provided by Prof. Dr. Michael Hippler (University of Münster, Germany). All strains were initially grown in Tris-acetate-phosphate (TAP) medium for 3 days at 23 °C under continuous shaking and constant illumination at an intensity of 80–100 μmol photons $m^{-2} s^{-1}$ (white fluorescent light). Cells were then centrifuged at 2900 rpm for 10 min at 20 °C and cell pellets were resuspended in their respective treatment media as described below.

To test the nitrate removal capacity of CC-1690, CC-124, T222⁺ and *pgr5*, cell pellets were resuspended in either Tris-phosphate (TP) medium or RAS effluent at an optical density (OD_{680}) of 0.05. Cells were then grown for 7 days in 600 mL flasks containing 400 mL of culture at 20 °C under continuous bubbling with room air and illuminated at 100–120 μmol photons $m^{-2} s^{-1}$. Growth curves in each medium, together with nitrate and nitrite removal from RAS effluent, were used to assess the potential of each strain for effluent bioremediation.

To evaluate the H_2 production capacity of CC-1690 and compare it with that of CC-124, cell pellets were resuspended in high-salt (HS) medium with a Chlorophyll (Chl) content adjusted to 20 μg Chl (a + b) mL^{-1} .

For integrating bioremediation of RAS effluent with H_2 production, CC-1690 cell pellets were resuspended in RAS effluent and Chl content adjusted to 1 μg Chl (a + b) mL^{-1} . Cells were then grown in a Multi-Cultivator MC 1000-OD bioreactor (Photon Systems Instruments, Czech Republic) for approx. 70 h at 23 °C under continuous bubbling with CO_2 -enriched air (0.5% v/v) and illuminated at 90 μmol photons $m^{-2} s^{-1}$. Growth rate was calculated from the change in OD_{680} during exponential growth using the following equation:

$$\text{Growth rate} = \frac{\ln(OD_2/OD_1)}{(t_2 - t_1)}$$

where OD_2 and OD_1 represent the OD_{680} at the end (t_2) and beginning (t_1) of the cultivation period, respectively [29].

To assess the H_2 production capacity of CC-1690 following cultivation in RAS effluent, cultures were centrifuged at 2900 rpm for 10 min at 20 °C. The supernatant from each culture was collected and retained separately. Cell pellets were then resuspended in an aliquot of their corresponding supernatant to achieve a final Chl concentration of 20 μg Chl (a + b) mL^{-1} . Thus, post-cultivation RAS effluent was partially retained and used as H_2 production medium.

A previously established protocol for inducing photoautotrophic, anaerobiosis-driven H_2 evolution was employed [23]. Briefly, once the Chl content was set to 20 μg Chl (a + b) mL^{-1} , 30 mL of culture were transferred to sterile glass serum bottles (120 mL, 52 mm $\times$ 95 mm) closed by a rubber septum. Approximately 2 g of the commercial iron-salt-based O_2 absorbent O2Zero-50 (Global Reach Ltd, London, UK) was added to the headspace of serum bottles to minimize the O_2 concentration (under 0.05%). This O_2 absorbent is composed of a mixture of ferrous iron powder (55%), silicon dioxide hydrate (25%), water (15%) and sodium chloride (5%), and does not produce volatile organic compounds upon interaction with O_2 . A dark anaerobic incubation was then carried out by purging the headspaces with N_2 gas for 15 min and leaving the cultures in darkness for 3 h with constant shaking (120 rpm). The headspaces were then purged again with N_2 gas for 15 min before starting H_2 production under constant light at either 100 or 350 μmol

photons $\text{m}^{-2} \text{s}^{-1}$. Sampling took place prior to applying the dark anaerobic incubation (control, aerobic samples), after the dark anaerobic incubation, and after 24, 48 and 72 h of H_2 production.

2.3. H_2 quantification

The H_2 concentration in the headspace of the serum bottles was determined by collecting a 200 μL aliquot with a gas-tight Hamilton microsyringe. Headspace samples were manually injected into a gas chromatograph (Hewlett Packard 5890) equipped with a HP-PLOT Molesieve column (30 m $\times$ 0.53 mm $\times$ 0.25 μm). The column oven was maintained at 40 $^\circ\text{C}$, and the thermal conductivity detector was operated at 160 $^\circ\text{C}$. Argon served as the carrier gas at a linear velocity of 115 cm s^{-1} . Following each 24 h gas sampling interval, the serum bottles were purged with N_2 gas to prevent H_2 accumulation from exceeding 5% [30].

2.4. *Chl a* fluorescence analysis

Before measurements, *C. reinhardtii* cultures were kept in darkness for 10 min. Subsequently, 60 μL of culture was placed onto a GF/B filter (Whatman glass microfibre) and *Chl a* fluorescence was measured with a HandyPEA (Hansatech Instruments Ltd, UK). The 24, 48, and 72 h H_2 production samples were placed under moderate light intensity and an ambient atmosphere for 2 h upon opening of the serum bottles to allow the cells to recover before measurement.

2.5. Immunoblot analysis

At each time point, 2 mL of culture was centrifuged (15,000 rcf, 3 min, 4 $^\circ\text{C}$), the supernatant was removed, and the obtained pellet was flash-frozen in liquid nitrogen and stored at -80°C . Immunoblotting was carried out as described in Vidal-Meireles et al. [31] with minor changes. Briefly, a cell extract corresponding to 0.5 million cells was combined with 6x Laemmli buffer (375 mM Tris/HCl [pH 6.8], 600 mM dithiothreitol, 12.6% [w/v] sodium dodecyl sulfate, 60% [v/v] glycerol and 0.09% [w/v] bromophenol blue) incubated at 75 $^\circ\text{C}$ (10 min), and subsequently loaded. Protein separation conditions and Western blot analysis were performed as previously described by Podmaniczki et al. [32]. Polyclonal antibodies against PsbA, PSBO, CP47, PetB, and PsbA (Agrisera AB, Sweden) were produced in rabbits. Densitometry analysis of the immunoblots was carried out using ImageJ (v1.54p).

2.6. Pigment analysis

Chl (a + b) content of cultures was determined as described by Porra et al. [33]. For pigment analysis, algal cells were centrifuged (15,000 rcf, 3 min, 4 $^\circ\text{C}$), the supernatant was removed, and the obtained pellet was flash-frozen in liquid nitrogen and stored at -80°C . HPLC analysis was carried out as previously described [34].

Briefly, frozen cells were resuspended in 1 mL of solvent mix (80% acetone, 20% methanol; v/v) and left in the darkness for 30 min at 25 $^\circ\text{C}$ under continuous shaking (1000 rpm) to facilitate pigment extraction. Samples were then centrifuged (11,500 g, 10 min, 4 $^\circ\text{C}$) and the supernatants were filtered through a 0.22 μm PTFE syringe filter and collected in vials. Carotenoids were separated with a Prominence HPLC system (Shimadzu, Kyoto, Japan) coupled with an Acclaim reverse-phase C30 column (3 $\times$ 150 $\times$ 4.6, Thermo Fisher Scientific). A linear gradient from 100% acetonitrile:water:trimethylamine (90:10:0.1) to 60% ethyl acetate was used. Retention time was used for pigment identification while peak area, at the wavelength of maximum absorbance, was used for quantification. The de-epoxidation index was calculated as the molar ratio of between de-epoxidized pigments and the sum of all xanthophyll cycle pigments using the following formula:

$$\text{De-epoxidation index} = \frac{(\text{Zeaxanthin} + 0.5 \times \text{Antheraxanthin})}{(\text{Zeaxanthin} + \text{Antheraxanthin} + \text{Violaxanthin})}$$

2.7. Total carbon, protein and lipid analysis

At each sampling point, cultures were centrifuged at 15,000 rpm at 4 $^\circ\text{C}$ for 3 min, the supernatant was discarded, and the samples were flash-frozen in liquid N_2 before being stored at -80°C . Samples were then freeze-dried overnight before analysis. For total carbon and nitrogen analysis, 1 mg of dry biomass was weighed in a smooth-wall tin cup (D4057 Elemental Microanalysis). Samples were left in a desiccator until analysis (no longer than 24 h). Carbon and nitrogen contents were quantified with a Thermo Finnigan DELTAplusAdvantage mass spectrometer (Thermo Electron) connected to a FlashEA 1112 Elemental Analyser. An internal standard (*Betula pendula*) was used during the run to correct for the drift in obtained values. Conversion of total nitrogen to protein content was carried out using a conversion factor of 4.08 [35]. For total lipid quantification, 2.2 mg of dry biomass was transferred to a 10 mL Kimax tube. Total lipids were then extracted with 3.75 mL chloroform/methanol/water (4:2:1) and sonication (10 min). After phase separation, the lipid-containing lower phase was collected in a clean Kimax tube and solvents were evaporated under a N_2 stream. Dried lipids were resuspended in 150 μL of chloroform and transferred to pre-weighed smooth-wall tin cup (D4057 Elemental Microanalysis). The remaining solvents were left to evaporate overnight at room temperature and total lipid content was calculated as the change in weight of the tin cups.

2.8. Fatty acid analysis

Approximately 1 mg of dry biomass was placed into a 10 mL Kimax tube and lipids were extracted as described for total lipid analysis. After phase separation and evaporation of solvents, in 1 mL of toluene was added, and fatty acids were transesterified overnight at 50 $^\circ\text{C}$ using 2 mL of methanolic H_2SO_4 (1%, v/v). Fatty acid methyl esters were separated by the addition of 2 mL of H_2O and 2 mL of hexane; the fatty-acid-containing upper phase was transferred to a clean Kimax tube where solvents were evaporated under a N_2 stream. Samples were resuspended in 300 μL of hexane and transferred to vials. Fatty acid analysis was carried out using a gas chromatograph coupled with a flame ionization detector (FID-2010 Plus, Shimadzu). A DB-FastFAME column (30 m $\times$ 0.25 mm $\times$ 0.25 μm ; Agilent) with a previously described temperature program [36] was used for fatty acid separation. Identification of fatty acids was done using retention times of an external fatty acid standard (GLC reference standard 556C, Nu-Chek Prep, Elysian) and quantification was based on peak integration using a three-point calibration curve using the same external standard. Fatty acid losses during the extraction process were accounted for by using the internal standard (free fatty acid C23:0; Larodan), which was added to all samples before lipid extraction.

2.9. Nutrient analysis

Water samples were filtered through nylon syringe filters (0.45 μm , Whatman), and stored at -20°C . Nutrient analysis of the samples (nitrate + nitrite, phosphate, and ammonium) was conducted by KVVY Tutkimus Oy (Finland). Briefly, nutrients were quantified spectrophotometrically following reference methods (SFS Finnish standards). The reference methods employed were the ammonium molybdate spectrophotometric method for phosphate determination (ISO 15923-1:2018; detection limit: 0.001 mg L^{-1}), the indophenol blue method for ammonium determination (ISO 15923-1:2013; detection limit: 0.01 mg L^{-1}), and the hydrazine reduction method for the determination of nitrate and nitrite (ISO 20236:2021; detection limit: 0.2 mg L^{-1}).

2.10. Statistical analysis

When applicable, the treatment effect was first tested using one-way ANOVA or the Kruskal-Wallis test depending on the Levene's test results. If the treatment effect was significant, Dunnett's or Dunn's post-hoc tests were used to compare the initial conditions with the rest of the treatments ($p < 0.05$). The presented data represent at least three independent experiments, with figure captions presenting the exact number of biological replicates. All statistical analyses were conducted using the R programming language (v4.5.1) within the RStudio environment (v2025.09.2).

3. Results and discussion

3.1. H_2 production and nutrient remediation by nitrate-assimilating *C. reinhardtii* CC-1690

For this study, we selected the *C. reinhardtii* CC-1690 strain, which possesses wild-type alleles at the NIT1 and NIT2 loci [27] and can therefore utilize nitrate for growth. To our knowledge, the H_2 production capacity of this strain has not been previously evaluated; hence we first characterized its photobiological H_2 evolution potential under conditions comparable to those previously described, namely using anaerobic induction, carbon limitation, and the inclusion of an O_2 scavenger in the headspace [23].

CC-1690 cultures were grown in TAP medium for three days and subsequently transferred to HS medium at a Chl (a+b) concentration of $20 \mu\text{g mL}^{-1}$. Hydrogenase expression was induced by a 3 h dark

anaerobic incubation as described previously [23], after which cultures were exposed to continuous illumination at $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. In agreement with previous results [23], the inclusion of an O_2 scavenger in the headspace approx. doubled the H_2 yield of CC-1690 (Fig. S1). Daily H_2 production ranged from 13 to $26 \mu\text{mol H}_2 \text{mg}^{-1}$ Chl, reaching a cumulative total of $61 \mu\text{mol H}_2 \text{mg}^{-1}$ Chl over 72 h (Fig. S1). Compared with the widely studied CC-124 strain, CC-1690 exhibited lower daily H_2 production, resulting in 38% less cumulative H_2 production after 72 h under anaerobiosis-induced, carbon-limited conditions (Fig. 1a and b).

The bioremediation performance of CC-1690 was then tested by cultivating the strain in RAS effluent. CC-1690 exhibited robust growth (Fig. S2), whereas strains previously reported to have high H_2 production capacity (CC-124, T222⁺, and pgr5; [24,37]), but lacking a functional nitrate-assimilation pathway [27], showed little or no growth. These strains were unable to effectively assimilate the nitrate and nitrite present in the RAS effluent, while the low concentrations of ammonium available were insufficient to support growth (Table 1; Fig. S2). Under optimized growth conditions, CC-1690 reached a growth rate of 0.85 day^{-1} and removed 99% of the available nitrate, nitrite, and phosphate from RAS effluent within approx. 70 h of cultivation (Fig. S2; Table 1; Fig. 1c and d). The observed growth rate was comparable to that of the fast-growing *Chlorella vulgaris* [38] and higher than other tested species grown in similar RAS effluent [6,39].

To avoid transferring cells into fresh HS medium, we tested the capacity of CC-1690 to produce H_2 directly in RAS effluent following cultivation and dark-anaerobic incubation. Daily H_2 production ranged from 13 to $18 \mu\text{mol H}_2 \text{mg}^{-1}$ Chl, reaching a total of approx. $49 \mu\text{mol H}_2$

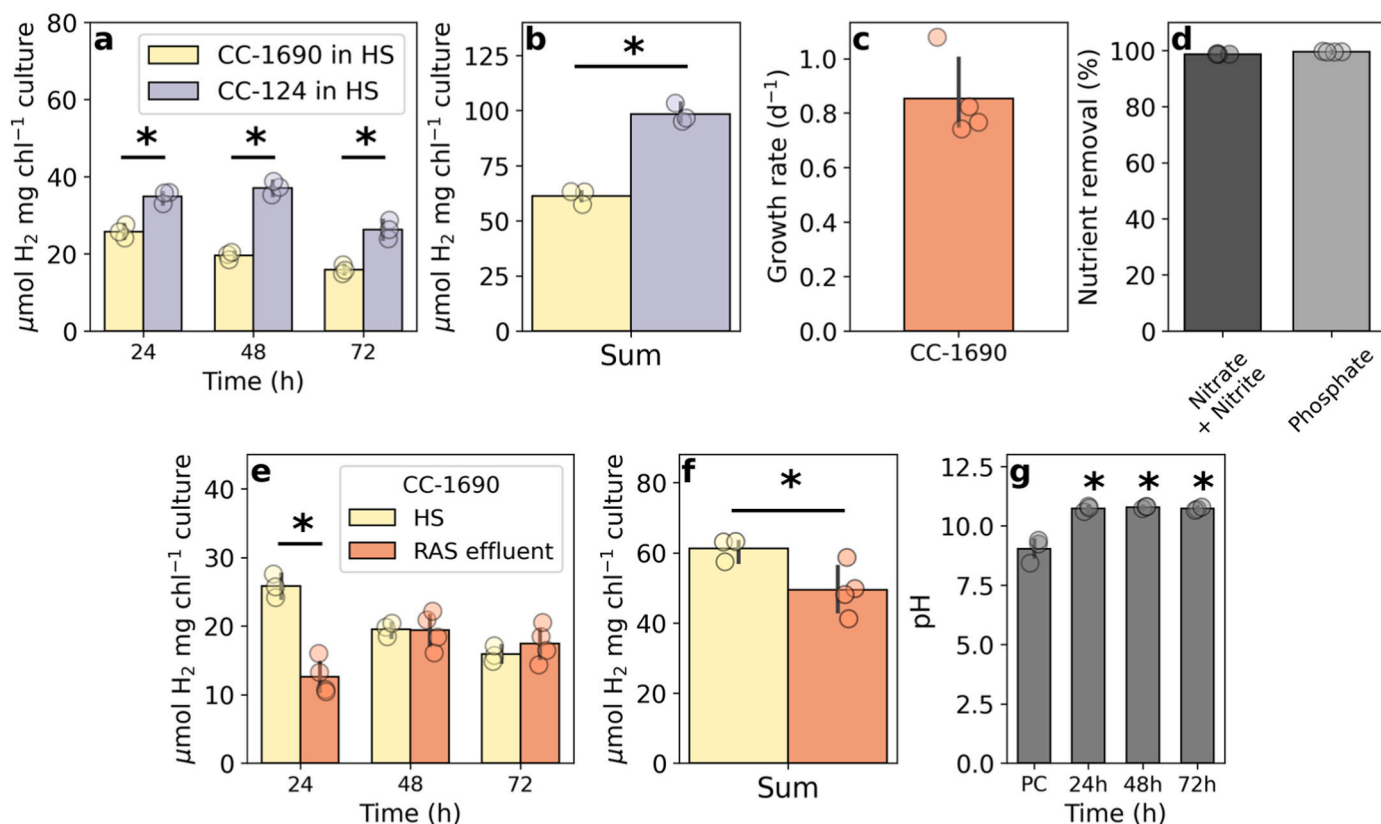


Fig. 1. Anaerobiosis-induced H_2 production by *C. reinhardtii* under continuous light at $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and a Chl (a + b) content of $20 \mu\text{g mL}^{-1}$. Daily (a) and cumulative 72 h (b) H_2 production in HS medium for CC-124 and CC-1690. Growth rate (d⁻¹) (c) and total nutrient (%) removal (d) of CC-1690 after approx. 70 h of cultivation in RAS effluent. Daily (e) and cumulative 72 h (f) H_2 production in RAS effluent and HS medium for CC-1690. RAS effluent pH (g) post-cultivation of CC-1690 (PC) and at 24, 48, and 72 h of H_2 production. The data represent means and standard deviation of at least three independent biological replicates and asterisks indicate statistically significant differences ($p < 0.05$). In H_2 production subfigures (a-b, e-f), asterisks indicate significant differences in daily or cumulative production based on one-way ANOVA. For pH comparison (g), asterisks indicate significant differences between the initial conditions (PC) and the subsequent treatment conditions, determined using Dunnett's test following a significant one-way ANOVA.

Table 1

Chemical characteristics of filtered (0.22 μm), UV-sterilized RAS effluent prior and after the cultivation of *C. reinhardtii* CC-1690 for approx. 70 h and after 72 h of H_2 production under continuous light (100 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$). Post-cultivation nutrient values represent the average of eight independent biological replicates, while values after 72 h of H_2 production represent the average of four independent biological replicates.

	RAS effluent	Post-cultivation	72 h H_2 production
Ammonium (mg L^{-1})	2.0	3.9	5.0
Phosphate (mg L^{-1})	1.1	0.005	0.018
Nitrate + nitrite (mg L^{-1})	25	0.3	<0.2 ^b
N:P ratio ^a	24.5	840	288.9
pH	7.31	9.02	10.72

^a Calculated as the ratio of nitrogen to phosphate containing compounds in mg L^{-1} .

^b Below the detection limit.

mg^{-1} Chl after 72 h (Fig. 1e and f). Compared with HS medium, H_2 production in RAS effluent was approx. 25% lower, primarily due to reduced production during the first 24 h of illumination (Fig. 1e and f). The pH of RAS effluent increased by 3.4 units after 24 h of H_2 production

relative to post-cultivation conditions (Fig. 1g). Notably, the use of higher light intensities (350 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) did not increase H_2 production, yielding 46 $\mu\text{mol H}_2 \text{ mg}^{-1}$ Chl after 72 h (Fig. S3).

The observed difference in H_2 production between RAS effluent and HS medium during the first 24 h is likely a consequence of differences in the chemical conditions of the two media, including pH, inorganic carbon availability, and residual nitrogen compounds. The increase in pH observed during the first 24 h of continuous illumination may be partially explained by inorganic carbon uptake and fixation. Inorganic carbon can promote Calvin–Benson cycle activity and potentially divert some photosynthetic electron flux toward carbon fixation rather than H_2 production [23,40]. More directly, the increase in pH itself may have negatively affected H_2 production, as alkaline conditions can reduce hydrogenase activity [41]. During the cultivation stage, most of the removed N and P are assimilated into algal biomass leaving only small amounts of these nutrients in the medium. Although nitrate and nitrite were present at low concentrations after cultivation (Table 1), H_2 evolution can occur only after their complete depletion [26]. Their continued reduction may therefore have also contributed to the lower H_2 production during the first 24 h. This is partially supported by the observed increase in ammonium concentration following the onset of H_2

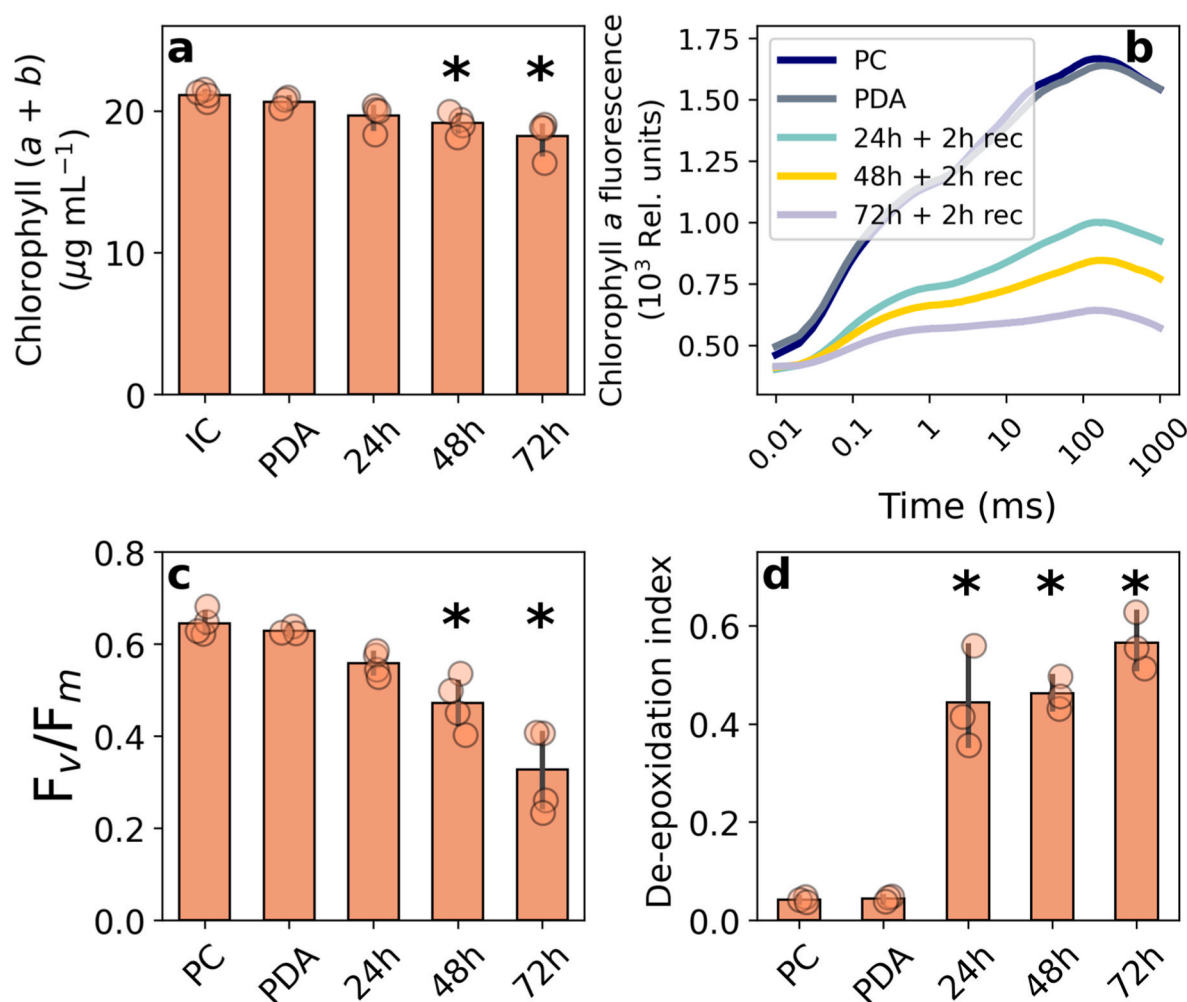


Fig. 2. Characterization of the photosynthetic apparatus and pigment composition of CC-1690 before and during anaerobiosis-induced H_2 production under continuous light at 100 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$. Chl (a + b) content (a), fast Chl a fluorescence transient (OJIP) (b), F_v/F_m values derived from the OJIP transient (c), and de-epoxidation index (d) were analysed for the starting conditions, after dark anaerobic incubation (PDA) and 24, 48 and 72 h of H_2 production. Given that the Chl (a + b) concentration was adjusted to 20 $\mu\text{g mL}^{-1}$ in serum bottles prior to H_2 production, such Chl content was considered the initial conditions (IC), while all other starting measurements were taken immediately post-cultivation (PC) in RAS effluent. The data represent means and standard deviation of at least three independent biological replicates. Asterisks indicate a significant difference between the treatment group and the starting conditions (PC or IC) by either Dunnett's or Dunn's post-hoc test following a significant one-way ANOVA or Kruskal–Wallis test ($p < 0.05$).

production (Table 1; [26]).

3.2. Changes in the photosynthetic apparatus and *HydA* during the H_2 production phase

Next, we examined the response of the photosynthetic apparatus to the H_2 production conditions in RAS effluent. Anaerobiosis-induced H_2 production led to a small but progressive daily decrease in the Chl content relative to the initial Chl concentration. After 48 h, the Chl content decreased by approx. 10%, with losses reaching 14% after 72 h compared to the initial levels (Fig. 2a). Fast Chl *a* fluorescence (OJIP), a diagnostic method for assessing the photosynthetic electron transport chain [42], revealed a progressive attenuation in intensity and substantial changes in its kinetics (Fig. 2b). This effect was observed even though the 24, 48 and 72 h samples underwent a 2 h incubation under standard growth conditions (moderate light intensity and ambient atmosphere) prior to measurement, a step designed to enable the recovery of the electron transport chain following reversible downregulation [43]. The F_V/F_M value, an indicator of PSII efficiency, declined from 0.65 post-cultivation to 0.47 at 48 h, and finally to 0.33 at 72 h (Fig. 2c). Although decreases in F_V/F_M have been observed with prolonged H_2 production [43], the rapid increase in pH during H_2 evolution (Fig. 1g) may also contribute to the observed decline [44], warranting further investigation into its potential effects on cell viability.

Given that light stress may lead to the conversion of violaxanthin to zeaxanthin in *C. reinhardtii* [45], we calculated the degree of violaxanthin cycle de-epoxidation during the H_2 production phase. The de-epoxidation index increased 10-fold after 24 h of light exposure and reached its maximum after 72 h, exhibiting an approx. 13-fold increase relative to post-cultivation and post-dark incubation levels (Fig. 2d).

Significant increases in the Chl *a/b* ratio and the lutein content (normalized to Chl *a*) after light exposure further suggest the occurrence of light stress during the H_2 production phase (Fig. S4). Additionally, the increase in lutein coincides with a decrease in luteoxanthin (Fig. S4), suggesting the activation of the luteoxanthin cycle [46]. Such changes in the composition of pigments are consistent with a reduction in light-harvesting antenna size and an increase in non-photochemical quenching [47].

To investigate whether key components of the photosynthetic electron transport chain were affected by the anaerobiosis-induced H_2 production, immunoblot analysis was carried out. The relative amounts of PSII subunits responded differently to the experimental conditions: PsbA levels were maintained, whereas the CP47 content increased by 40% and PSBO decreased by 60% after 72 h compared to the initial conditions (Fig. 3a–c). The cytochrome *b₆f* subunit PetB was maintained, while the PSI subunit PsaA exhibited some variation (Fig. 3e). Under our carbon- and oxygen-limited H_2 -production conditions, the restricted availability of alternative electron sinks may have altered the redox state and electron flux through the photosynthetic electron transport chain [23], thereby promoting a selective remodelling of the photosynthetic apparatus [48]. Furthermore, we suggest that the substantial alkaline shift observed in the medium during H_2 production may have directly contributed to this process. Elevated pH is known to destabilize the oxygen-evolving complex (OEC), leading to the inactivation of oxygen evolution and the subsequent release or degradation of extrinsic proteins such as PsbO [49]. On the other hand, the increase in CP47 may represent retention or compensatory accumulation of the PSII core antenna during these changes in the pH of the medium. The relatively stable PsbA content further supports the interpretation that these changes do not represent generalized degradation of PSII.

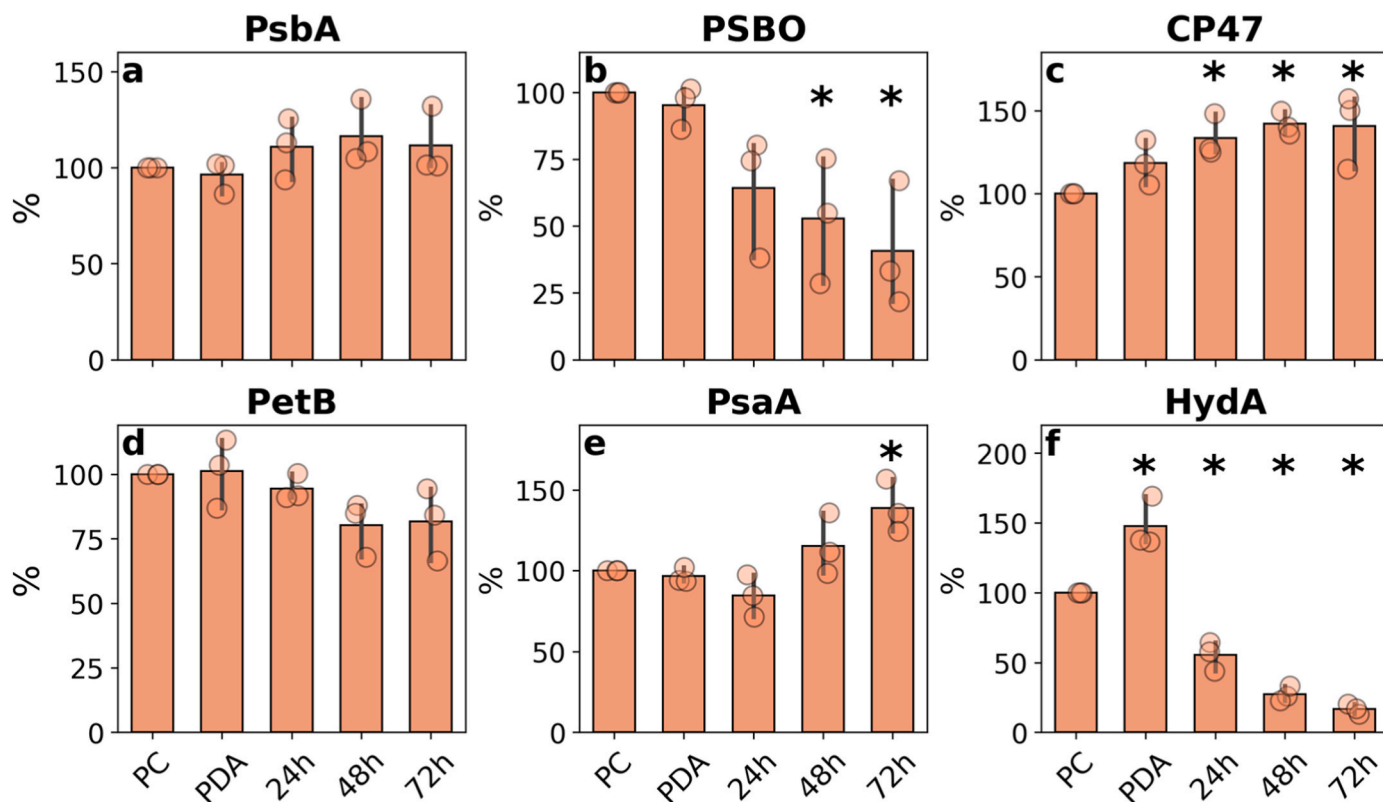


Fig. 3. Immunoblot analysis for the semi-quantitative determination of key components of the electron transport chain and *HydA* of CC-1690 before and during anaerobiosis-induced H_2 production under continuous light at $100 \mu\text{mol photons m}^{-2}\text{s}^{-1}$. The relative abundance of the PSII subunits PsbA (a), PSBO (b), CP47 (c); the cytochrome *b₆f* PetB (d); the PSI subunit PsaA (e); and the hydrogenase *HydA* (f) were analysed. Samples were taken post-cultivation (PC) in RAS effluent, post-dark anaerobic incubation (PDA) and after 24, 48 and 72 h of H_2 production. The data represent means and standard deviation of three independent biological replicates. Data are expressed as percentage (%) normalized to PC conditions. Asterisks indicate a significant difference between the treatment group and the PC conditions by either Dunnett's or Dunn's post-hoc test following a significant one-way ANOVA or Kruskal-Wallis test ($p < 0.05$).

The amount of hydrogenase HydA was maximal after the dark anaerobic incubation, but decreased sharply upon light exposure to levels lower than those observed post-cultivation, retaining only 17% of the initial level after 72 h (Fig. 3f). Such a reduction of HydA has been previously observed under similar conditions in the CC-124 strain [24, 43], and is most likely due to the inhibitory effect of the small amounts of O_2 inevitably generated in the chloroplasts by PSII activity. Although H_2 production remained relatively stable up to 72 h, the reduction in the amount of hydrogenase and the F_V/F_M values suggest that further improving the removal of O_2 from the cultures could contribute to sustaining electron transfer to the hydrogenase and, consequently, photosynthetic activity.

Our results indicate that CC-1690 experienced a degree of light stress under the applied conditions. This interpretation is further supported by the more pronounced reductions in Chl content and F_V/F_M , together with the higher de-epoxidation index and associated changes in pigment profiles and components of the photosynthetic electron transport observed at the higher light intensity ($350 \mu\text{mol photons m}^{-2} \text{s}^{-1}$), which did not result in an increase in H_2 yield (Figs. S3, S5, S6). These observations suggest that the enhanced light stress responses limited photosynthetic electron transport. Additionally, the continuous decrease in hydrogenase content during H_2 production likely acted as a limiting factor, preventing the higher light intensity from enhancing H_2 yield. Consequently, under these conditions, electron transport and/or hydrogenase activity contributed to the reduced H_2 yield, rather than the lack of excitation energy.

Altogether, our findings suggest that minimizing light stress and the associated photoprotective energy-dissipation responses, together with pH regulation and ensuring the complete depletion of inorganic carbon, nitrate, and nitrite prior to H_2 production may provide a means of increasing H_2 yield, either by prolonging the period of H_2 production or

by increasing the H_2 production rate. In addition, the use of photobioreactors with improved light-to-surface-area ratio [43] has been shown to substantially increase total H_2 yield by improving the removal of O_2 and H_2 from the culture. Alternatively, genetic engineering of the CC-1690 strain to suppress competing electron sinks downstream of PSI, including cyclic electron transport and flavodiiron proteins, is a potential avenue to enhance H_2 production [37,50].

3.3. Effects of H_2 production on CC-1690 biomass composition

Recovery of high-quality microalgal biomass following the H_2 production phase is critical for the integration of downstream applications. Traditional methods, such as the use of sulphur deficiency to promote H_2 evolution, lead to a stress-induced metabolic shift that decreases the quality of the resulting biomass by lowering protein content [51,52]. Hence, we investigated whether CC-1690 biomass is altered during and after the H_2 production phase. On a dry weight basis, the post-cultivation biomass was composed of approx. 50% carbon, 25% protein, and 21% lipids. (Fig. 4a–c). Total carbon and protein content were not significantly affected by the dark anaerobic incubation or by the H_2 production conditions.

Total lipid content increased slightly (3%) upon 48 and 72 h of illumination (Fig. 4c), driven by the accumulation of fatty acids (Table S1; Fig. 4d–f). Saturated (SFA) and polyunsaturated fatty acids (PUFA) increased by 49% and 41%, respectively, after 72 h of H_2 production compared to the post-cultivation conditions (Fig. 4c–f). Mono-unsaturated fatty acids (MUFA) remained largely unchanged during the H_2 production phase. The changes in SFA were predominantly a result of increases in palmitic acid (16:0), which accounted for 20% of all fatty acids, while the increase in PUFA was primarily driven by α -linolenic acid (18:3 ω -3), which accounted for 26% of all fatty acids (Fig. S7).

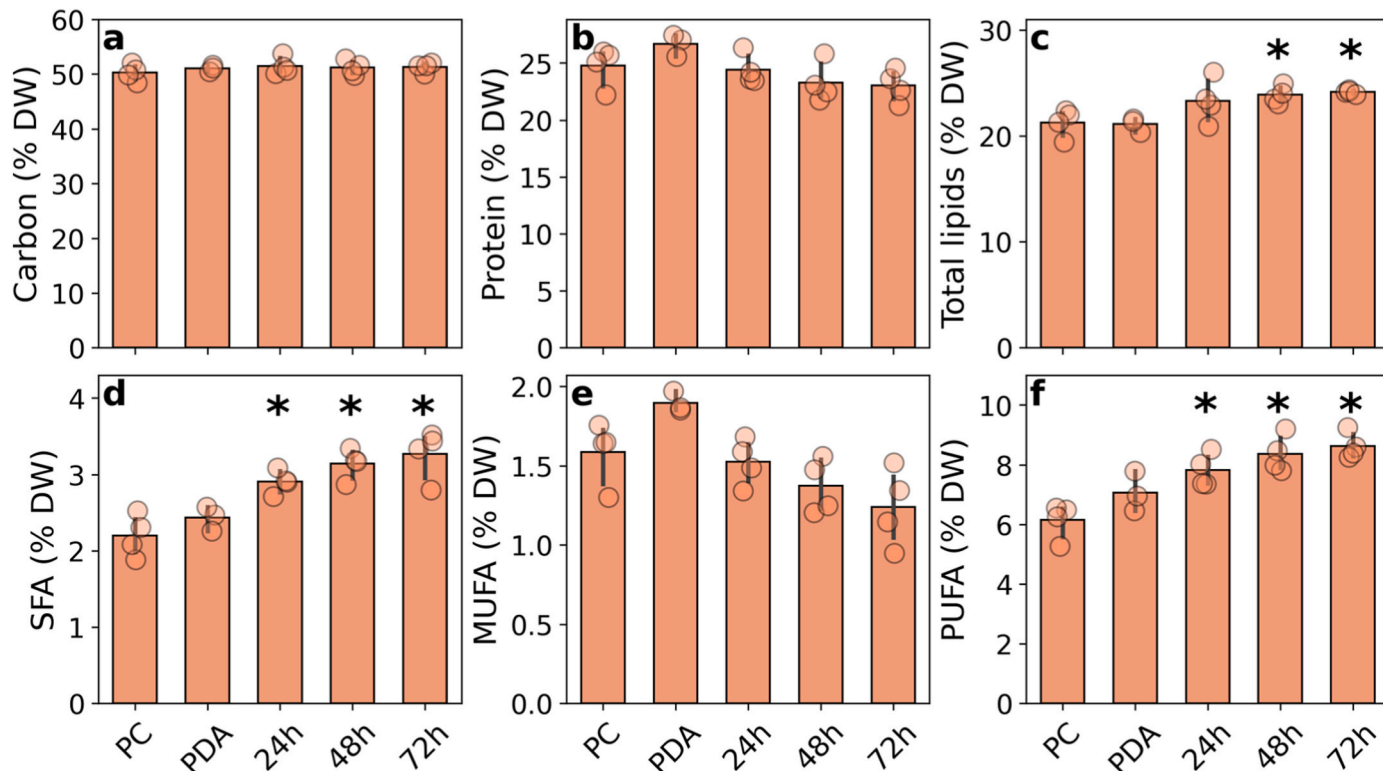


Fig. 4. Biomass composition of CC-1690 before and during anaerobiosis-induced H_2 production under continuous light at $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. Total carbon (a), protein (b), and lipid (c) contents, as well as saturated (SFA) (d), monounsaturated (MUFA) (e) and polyunsaturated (PUFA) (f) fatty acids, expressed as a percentage of dry weight (DW). Samples were taken post-cultivation (PC) in RAS effluent, post-dark anaerobic incubation (PDA), and after 24, 48 and 72 h of H_2 production. The data represent means and standard deviation of three independent biological replicates. Asterisks indicate a significant difference between the treatment group and the PC conditions by either Dunnett's or Dunn's post-hoc test following a significant one-way ANOVA or Kruskal-Wallis test ($p < 0.05$).

Due to the complexity of the conditions to which CC-1690 was exposed to during the H₂ production phase, it is difficult to link the observed changes in biomass to specific metabolic pathways. During the H₂ production phase, the nitrogen concentration in RAS effluent was negligible, suggesting a possible metabolic response to nitrogen deprivation by CC-1690. Such response usually leads to the degradation of Chl and protein and the initial channelling of CO₂ fixation towards lipid production [53,54]. However, due to the substrate limitation of the Calvin-Benson cycle and the expression of HydA, only the degradation of macromolecules could provide carbon for lipid synthesis under the tested conditions. Although we did not observe gross alterations in the protein content typical of nitrogen deprivation studies in *C. reinhardtii* [54,55], it is likely that the degradation of a small fraction of proteins and other macromolecules were responsible for fuelling the observed increase in lipid content.

Overall, the biomass composition indicates potential for its valorisation as a feed ingredient in aquaculture [56]. The observed protein content suggests that the biomass could contribute to the protein fraction of feeds, particularly for herbivorous and omnivorous fish, for which dietary protein requirements are generally in the range of 25–35% [57]. In addition, the relatively high proportion of α -linolenic acid may increase the value of the biomass as a source of essential fatty acids. α -linolenic acid is an established dietary essential fatty acid for several freshwater fish species, including Arctic charr and whitefish, for which dietary requirements of approximately 1–2% of dry diet have been reported [58].

4. Conclusion

Bioremediation of RAS effluents using the nitrate-assimilating *C. reinhardtii* strain CC-1690 can be effectively coupled with H₂ production for further valorisation of the process. Within approx. 70 h of cultivation, CC-1690 achieved near-complete removal of all nitrate and phosphate from the RAS effluent, while standard H₂-producing strains (CC-124, T222⁺, *pgr5*) were unable to remove nitrate. Post-cultivation induction of H₂ evolution directly in RAS effluent through anaerobiosis-induced photoautotrophic conditions sustained H₂ production for 72 h, yielding 49 $\mu\text{mol H}_2 \text{ mg}^{-1}$ Chl. Although indicators of light stress such as a reduction of F_v/F_m and an increase in the depoxidation index were observed, major photosynthetic subunits remained largely intact. Biomass composition presented only minor alterations during the H₂ production phase, mostly driven by increases in palmitic and α -linolenic fatty acids. The presented methodological approach serves as a proof of concept of RAS effluent bioremediation for the recovery of nitrogen and phosphorus to produce H₂ and valuable microalgal biomass. Given that the experiments were conducted at a small scale and a single nitrate-utilizing *Chlamydomonas* strain was tested, future studies should therefore focus on assessing the H₂ production capacity of other nitrate-utilizing strains and scaling-up production systems to further test the robustness and broader applicability of this approach. In particular, scale-up studies should evaluate photobioreactor configurations that provide efficient light distribution and mixing, such as flat-panel systems with short light paths, which have been shown to improve photobiological H₂ production in *C. reinhardtii*. In addition, strategies for continuous or periodic removal of H₂ from the reactor headspace should be investigated to prevent H₂ accumulation and maintain conditions favourable for sustained H₂ evolution. Optimizing gas exchange, light availability, mixing, and the maintenance of low-O₂ and carbon-limited conditions at larger cultivation volumes will therefore be important for determining whether the H₂ production observed here can be maintained during scale-up.

CRedit authorship contribution statement

Marco L. Calderini: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Conceptualization. **Valéria Nagy:**

Writing – review & editing, Methodology, Formal analysis. **Szilvia Z. Tóth:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition. **László Kovács:** Writing – review & editing, Methodology. **Soujanya Kuntam:** Writing – review & editing, Methodology. **Paulina Salmi:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Data statement

All data and data analysis files necessary to replicate the results presented in this study are available at <https://doi.org/10.17011/jyx/dataset/114110>. A comprehensive description of the content of each file can be found in the same DOI under the name “Metadata file.”

Declaration of competing 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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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijhydene.2026.157939>.

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