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ORIGINAL PAPERS

# Photosynthesis and biochemical characterization of the green alga *Chlamydopodium fusiforme* (Chlorophyta) grown in a thin-layer cascade

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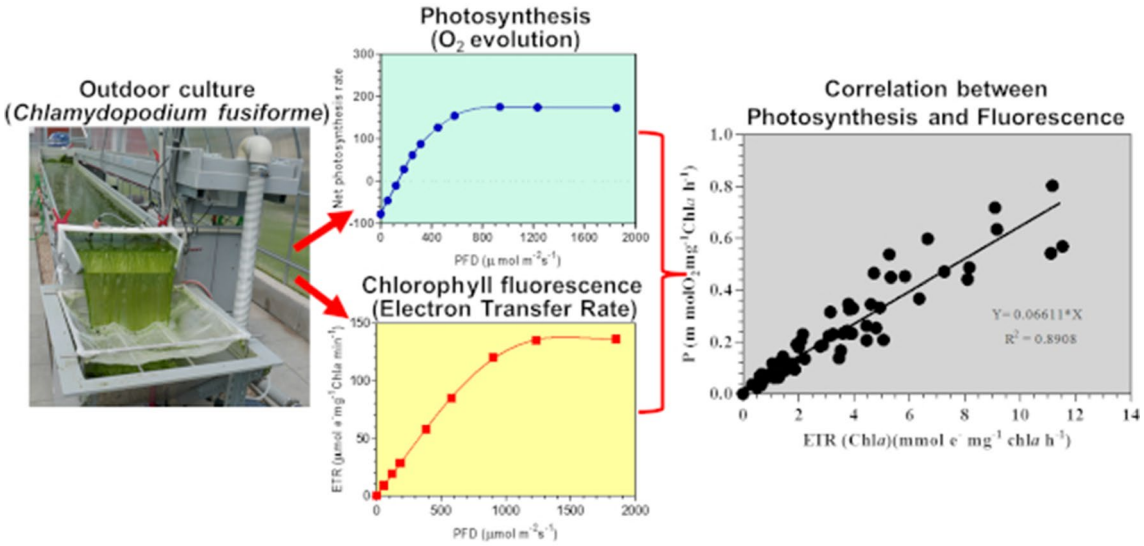
**Abstract**

Photosynthesis, growth and biochemical composition of the biomass of the freshwater microalga *Chlamydopodium fusiforme* cultures outdoors in a thin-layer cascade were investigated. Gross oxygen production measured *off-line* in samples taken from the outdoor cultures was correlated with the electron transport rate estimated from chlorophyll *a* fluorescence measurements. According to photosynthesis measurements, a mean of 38.9 ± 10.3 mol of photons were required to release one mole of $O_2$, which is 4.86 times higher than the theoretical value (8 photons per 1 $O_2$). In contrast, according to the fluorescence measurements, a mean of 11.7 ± 0.74 mol of photons were required to release 1 mol of $O_2$. These findings indicate that fluorescence-based photosynthesis rates may not be fully replace oxygen measurements to evaluate the performance of an outdoor culture. Daily gross biomass productivity was 0.3 g DW $L^{-1}$ $day^{-1}$ consistently for 4 days. Biomass productivity was strongly affected by the suboptimal concentration at which the culture was operated and by the respiration rate, as the substantial volume of culture was kept in the dark (about 45% of the total volume). As the cells were exposed to excessive light, the photosynthetic activity was mainly directed to the synthesis of carbohydrates in the biomass. In the morning, carbohydrate content decreased because of the dark respiration. Per contra, protein content in the biomass was lower at the end of the day and higher in the morning due to carbohydrate consumption by respiration. The data gathered in these trials are important for the future exploitation of *Chlamydopodium fusiforme* as a potential novel species in the field of microalgae for the production of bio-based compounds.

Extended author information available on the last page of the article

**Graphical abstract**




**Keywords** *Chlamydopodium fusiforme* · Photosynthesis · Chlorophyll fluorescence · Microalgae · Biomass composition

## 1 Introduction

Microalgae are photosynthetic microorganisms capable of acclimating to a variety of environmental conditions. They have been identified as efficient biological cell factories that produce large amounts of biochemical products such as carbohydrates, proteins and lipids as well as various high-value secondary metabolites in a controlled cultivation process [1]. For this reason, these microorganisms are increasingly employed as a source of dietary supplements and biomaterials [2–4]. Microalgal strains can be selected for the production of biostimulants such as auxins and cytokinins [5], biopesticides [6], and antioxidants [7, 8]. They may contain several classes of flavonoids, such as isoflavones, flavanones, flavanols and dihydrochalcones [9] indicating that microalgae, like terrestrial plants, can produce relatively complex polyphenols. Since most synthetic antioxidants are suspected of being carcinogenic, it is mandatory to look for natural sources of antioxidants [7]. In this regard, microalgae can play an important role in the production of these antioxidants in the future, as they grow faster compared to higher plants and do not compete with crops for arable land, as they can be grown on marginal areas not suitable for agriculture [10–12].

In order to expand microalgae industry, new species suitable for the production of renewable biological resources and high-value products, such as food, feed, bio-based products and bioenergy need to be tested to meet societal demands [13].

The freshwater microalga *Chlamydopodium fusiforme* is new to industrial utilization and could contribute to the production of bio-based products, such as proteins, lipids, and bioactive substances [5]. However, information on its physiology and outdoor growth is still limited [14]. Recently, researchers investigated the cultivation of *Chlamydopodium fusiforme* outdoors in a case study with the aim of scaling up the process to produce biomass for agricultural purposes [15]. In a recent article, we studied the productivity and nutrient removal of *Chlamydopodium fusiforme* grown in a synthetic medium and diluted pig wastewater in outdoor mini-ponds [16].

In recent years, chlorophyll fluorescence quenching has been increasingly used to assess the physiological status of microalgal cultures and many attempts have been made to correlate fluorescence yields to growth (and biomass productivity) [17, 18]. However, to prove the usefulness of chlorophyll fluorescence monitoring as an indicator of photosynthesis performance of microalgal cultures, the relationship between chlorophyll fluorescence and quantum yield of gas evolution or uptake ($O_2$, $CO_2$) needs to be demonstrated in the largest possible number of microalgae species [19–21]. Current mass cultures of microalgae generally operate at very low light conversion efficiencies, close to 1% on a solar basis. Online measurements of chlorophyll fluorescence have shown that the amount of absorbed light that can be dissipated by non-photochemical quenching (NPQ, heat) and fluorescence can be as high as 80% at noon time.

Monitoring photosynthesis and chlorophyll fluorescence behavior of mass cultures may be a way to better understand their limitations and thus bridge the gap between theoretical and actual light conversion efficiency.

In this work, rapid Light Response Curves (LRC) of electron transport rates (ETR) were correlated with steady-state LRC of photosynthetic oxygen evolution under the same light irradiances to determine whether fluorescence and oxygen measurements are interchangeable measurement techniques to evaluate the performance of an outdoor culture of microalgae. Both were measured in the laboratory on culture samples of *Chlamydopodium fusiforme* and during a 4-day experiment in an outdoor thin-layer cascade. Changes in gross and net productivity and biochemical composition of the biomass produced were also investigated. The development of a reliable and fast monitoring technique to evaluate the performance of an outdoor culture is one of the basic requirements for the optimization of growth and for the scaling up to an industrial level.

## 2 Materials and methods

### 2.1 Strain, laboratory culture preparation and outdoor trial

The freshwater microalga *Chlamydopodium fusiforme* MACC-430 (further abbreviated as *C. fusiforme*), Class, Chlorophyceae; Order, Chlamydomonadales; Family, Characiochloridaceae, Genus, *Chlamydopodium,* obtained from the Algae Culture Collection of the Szechényi István University in Mosonmagyaróvár, Hungary, was used in these trials (Fig. 1).

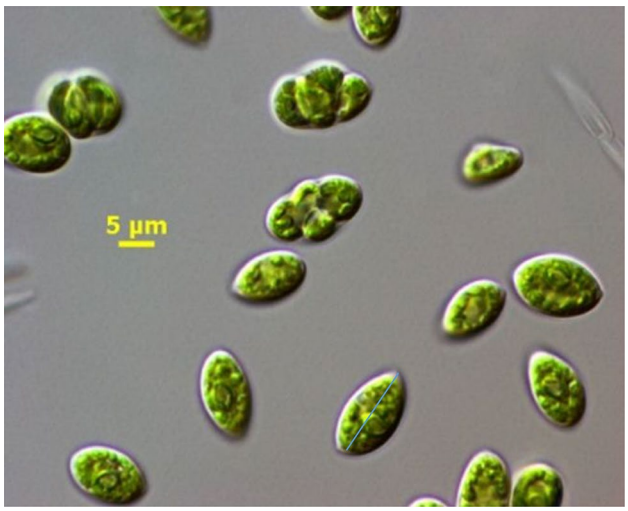



**Fig. 1** Light microscopy images of Chlamydopodium fusiforme (taken by dr. Claudio Sili, CNR)

The inoculum was grown in BG11 medium bubbled with a mixture of air and 2% $CO_2$ in a 100-L column photobioreactor with internal illumination by neutral-white and warm-white light emitting diodes (LEDs) [22], with irradiance increasing according to biomass density. When the culture reached a biomass density of about 2 g DW $L^{-1}$, it was transferred to an outdoor 5 $m^2$ thin-layer cascade (TLC) with 74 L of working volume which was placed in a polycarbonate greenhouse for better control of cultivation conditions (Centre Algatech, Třeboň, CZ) (Fig. 2). The system has been described in detail elsewhere [23]. The mean culture layer in the TLC unit was 8 mm, and the flow velocity was set at 0.5 m $s^{-1}$ using a centrifuge pump; the ratio of exposed surface to total volume was about 67 $m^{-1}$. The unit was equipped with automatic addition of $CO_2$ based on pH changes (set point = 7.5 ± 0.2).

### 2.2 Location and weather conditions

Experiments were performed in 2017 at the Algatech Centre in Třeboň, Czech Republic (GPS coordinates – 48°59′15" N; 14°46′40.630" E). Daytime in the figures corresponds to CEST (GMT + 1). The cascade was inoculated on August

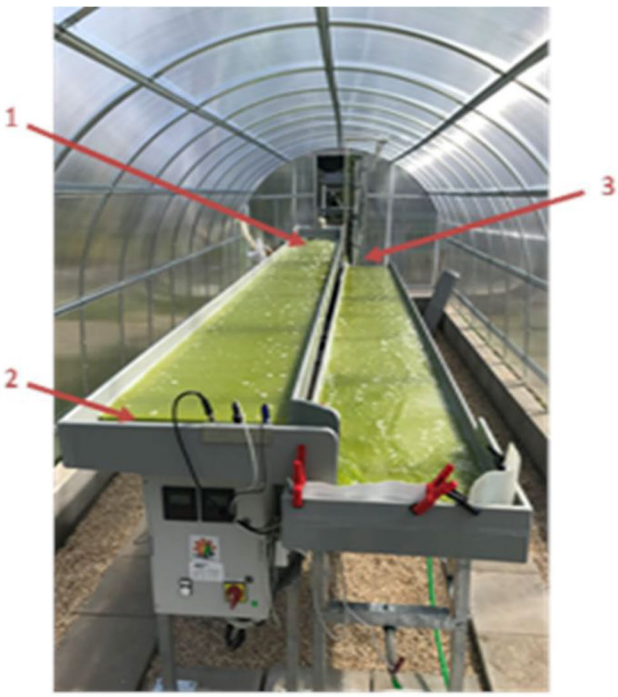



**Fig. 2** General view of the thin-layer cascade used for the experiments. Measurements of the main physico-chemical variables (dissolved oxygen concentration (DO), pH, and temperature) were performed in three points of the culture system, (1) 20 cm from the culture inlet, (2) in the channel connecting the two culture lanes, and at the end, i.e., 5 cm before the culture falls into the retention tank. Culture probes (pH, T, DO) were placed in the channel (3) connecting the two lanes

21, and after 2 days of acclimation of the culture to outdoor conditions, measurements were started (Day 1). Weather conditions (solar irradiance in PAR and ambient temperature) were monitored by a local meteorological station.

## 2.3 Growth and chlorophyll measurements

Determination of dry biomass weight (DW) was performed in triplicate. Culture samples were taken from the outdoor culture at 9.00 and 17.00 h, filtered using pre-weighted glass microfiber filters (Whatman GF/F, Maidstone, England), washed twice with deionized water, oven dried at 105 °C for 3 h and after equilibration to room temperature (10 min) in a desiccator, they were weighed [24]. Total chlorophyll content was determined spectrophotometrically according to the method described by Lichtenthaler [25].

## 2.4 Photosynthesis and fluorescence measurements

For both photosynthesis and chlorophyll measurements, culture samples were taken from the outdoor culture at three-time intervals (09.00, 14.00, and 17.00 h). In order to make comparable photosynthesis and fluorescence measurements, they were carried out using the same cuvette geometry (Hansatech DW2/2), same mixing rate and light source (white light LED 1/W), driven by Hansatech, Oxygen + control box. For $F_m$' measurements (maximal fluorescence yield in the light-adapted state), high-intensity light pulses were supplied by PAM-2100 white light source. To facilitate comparison between fluorescence and photosynthesis measurements, both ETR and photosynthesis oxygen evolution were expressed on a chlorophyll *a* basis.

### 2.4.1 Measurement of LRC

Rates of photosynthetic oxygen evolution were measured as a function of irradiance using a Clark-type oxygen electrode (mounted in the temperature-controlled and mixed by a magnetic stirrer DW2/2 chamber, Hansatech Instrument Ltd., Norfolk, UK) connected to an Oxylab + control box with the Oxytrace + software (Hansatech Instrument Ltd., Norfolk, UK). Respiration rate ($R_d$) was measured at the start of the measurements. Microalgal samples (2 mL) were inserted into the chamber with mixing and the oxygen evolution rate was recorded. Oxygen evolution rates (average of at least three repetitions) were calculated in µmoles of $O_2$ evolved per unit of time and chlorophyll. Steady-state LRCs were recorded using a stepwise increasing light intensity (0–1800 µmol $m^{-2}$ $s^{-1}$), each step lasting 2 min. Each P vs I curve was generated according to the equation $P = (I\text{-}I_c)/a(I\text{-}I_c)^2 + b(I\text{-}I_c) + C$ [26] according to which several photosynthetic variables such as the dark respiration ($R_d$), the compensation point ($E_c$), i.e., when the photosynthetic rate is just balanced by respiration, maximum rate of light-saturated photosynthesis ($P_{max}$), irradiance at the onset of light saturation ($E_k$), and the light utilization efficiency as determined by the initial slope (α) were determined. All symbols and abbreviations of the photosynthesis and fluorescence variables are shown in Table 1.

### 2.4.2 Measurement of specific chlorophyll optical absorption cross-section

The average specific chlorophyll optical absorption cross-section (*a**) of cells ($m^2$ $mg^{-1}$ Chl *a*) was determined from in vivo absorption spectra (in the range from 400 to 750 nm) according to [27], using a double-beam spectrophotometer

**Table 1** Symbols and abbreviations of the photosynthesis and fluorescence variables

| Symbols | Abbreviations (units) |
|---|---|
| PFD | Photon flux density (400–700 nm) (µmol photons $m^{-2}$ $s^{-1}$) |
| Chl a | Chlorophyll *a* (mg Chl *a* $L^{-1}$) |
| a* | In vivo optical absorption cross-section ($m^2$ $mg^{-1}$ Chl *a*) |
| α per Chl *a* | Light utilization efficiency per Chl *a* (µmol $O_2$ $mg^{-1}$ Chl *a* $\mu mol^{-1}$ photons $m^2$) |
| $\Phi_{max}$ | Maximum quantum yield (mol $O_2$ evolved per mol photons absorbed) |
| $\Phi_{max}^{-1}$ | Minimum quantum requirement (mol photons $mol^{-1}$ $O_2$ evolved) |
| $E_k$ | Irradiance at the onset of light saturation (µmol photons $m^{-2}$ $s^{-1}$) |
| $P_{max}$ per Chl *a* | Maximum photosynthesis rate per Chl *a* (µmol $O_2$ $mg^{-1}$Chl *a* $h^{-1}$) |
| $R_d$ per Chl *a* | Dark respiration rate per Chl *a* (µmol $O_2$ $mg^{-1}$chl*a* $h^{-1}$) |
| $E_c$ per Chl *a* | Compensation point. Light intensity at which photosynthesis rate is zero (i.e. Photosynthesis = Respiration) (µmol photons $m^{-2}$ $s^{-1}$) |
| $F_v/F_m$ | Maximum photochemical quantum yield of PSII |
| ETR | Electron transfer rate (µmol e- $mg^{-1}$ Chl *a* $s^{-1}$) |
| qN | Non-photochemical quenching |

(Shimadzu UV-3000). To minimize the impact of the light scattering effect from the cell surface, the sample cuvette was placed close to the detector window with standard white printer paper as a light diffuser placed in between.

The maximum quantum yield $\theta_{max}$, i.e., the ratio between the rate of oxygen evolution and light absorption under limiting light, was calculated according to the following equation [28]:$\theta_{max} = \alpha /$ a*α and a* were expressed per Chl *a*.

#### 2.4.3 Fluorescence measurements

Rapid LRCs of *C. fusiforme* cultures were measured using 2 mL samples (4 µg Chl *a* $mL^{-1}$) placed in a Liquid-Phase Oxygen Electrode Chamber (DW2/2, Hansatech) thermoregulated at 25 °C using a pulse-amplitude-modulation fluorimeter (PAM-2100, H. Walz, Germany). A series of stepwise increasing irradiance intensities (0–1850 µmol photons $m^{-2}$ $s^{-1}$) provided by a light source (LED 1/W white light, Hansatech, UK) were applied at 30 s intervals to obtain the light-adapted fluorescence level F' (steady-state fluorescence yield in light), and at the end of each step a saturation pulse (> 6,000 µmol photons $m^{-2}$ $s^{-1}$, 0.6 s duration, white light, supplied by PAM-2100) was triggered to reach the maximum fluorescence level $F_m$' (steady-state maximum fluorescence in the light). The actual photochemical quantum yield of PSII in light, $Y_{II}$ was determined as ($F_m$'- F')/$F_m$' in the light-adapted state at the respective irradiance level. The electron transport rate ETR = PFD × YII × 0.5 × a* (µmol $e^-$ $mg^{-1}$Chl *a* $s^{-1}$) was calculated according to [29]. Although the measurement of a* is based on the extinction coefficient of chlorophyll *a* and, therefore, cannot be considered specific to PSII, it provides a more accurate measurement of ETR in the microalgal suspension compared to that directly extracted from the PAM Software developed for fluorescence measurements on leaves.

Analysis of the LRCs was used to calculate changes in important parameters, such as the maximum electron transport rate through PSII, $ETR_{max}$, the initial slope of the ETR vs. PFD curve, α, the saturation irradiance, $E_{k,}$ given as the intercept between α, and $ETR_{max}$, the maximum quantum yield of photosynthesis $\Phi_{max}$. The curves were fitted to the non-linear least-squares regression model by Eilers and Peeters [26], using PamWin-3 software. Non-photochemical quenching ($qN = (F_{m\_}F_m')/(F_m\text{-}F_0)$) was automatically calculated by the PAM software and recorded with all fluorescence measurements performed at a given light irradiance. This coefficient ranges from 0 to 1.

#### 2.4.4 Light measurements

Photosynthetically active radiation (PAR) values were measured for both fluorescence and photosynthesis measurements using a LI-250A light meter (Li-Cor, USA) equipped with a spherical sensor model SQSA0191 (H. Walz, Germany), which was placed in the cuvette, or with a flat LI-190SA quantum sensor (cosine-corrected up to 80° angle of incidence) for the cultures grown outdoors.

### 2.5 Analytical measurements

#### 2.5.1 Total phenolic compound

Phenolic compounds (PC) were determined colorimetrically using Folin-Ciocalteu reagent. The calibration curve (0–300 µg $mL^{-1}$) was prepared using phloroglucinol (1.3.5-trihydroxybenzene, Sigma P-3502) [30, 31]. Then, 30–50 mg of lyophilized microalgal biomass were taken and homogenized with 4 mL of methanol (80%) using an ultra-turrax. They were then left to extract for 24 h in the dark at 4 ºC. The extract was then centrifuged at 10,000 g for 15 min at 4 ºC. 100 µL of the supernatant were taken and mixed with 6 mL of Milli-Q water and 0.5 mL of Folin-Ciocalteu reagent and shaken vigorously, and 1.5 mL of 20% $Na_2CO_3$ was quickly added. The test volume was completed with 1.9 mL of Milli-Q water to reach 10 mL. The solution for 2 h in the dark at 4 °C, and the absorbance was measured at 760 nm. The PC content was expressed in mg DW $g^{-1}$ using the calibration curve.

#### 2.5.2 Antioxidant capacity

Antioxidant activity was evaluated with the ABTS method using a spectrophotometer [32, 33]. The ABTS, 2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) reagent was prepared in sodium phosphate buffer (0.1 M, pH 6.5), ABTS (7 mM) and potassium persulfate ($K_2S_2O_8$, 2.45 mM). Then, the reagent was incubated in the dark at laboratory temperature for 12 h to allow the complete formation of the radicals. Thereafter, the reaction was performed by adding 940 µL of sodium phosphate buffer (0.1 M, pH 6.5), 10 µL of ABTS and 50 µL of microalgal extract. Samples were shaken and absorbance was recorded using a UV–VIS spectrophotometer (Shimadzu UV mini-1240) at 727 nm (abs727), at time 0 (immediately at the beginning of reaction) and after 8 min of incubation. The blank sample was phosphate buffer. The antioxidant activity (AA) of the free radicals was calculated according to the following formula:

$$AA\% = [(\text{abs727atinitialtime} - \text{abs727after8min})/\text{abs727atinitialtime}] \times 100$$

Quantification of antioxidant capacity was determined by constructing a standard curve using different concentrations of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). Values were normalized to volume and biomass. Results were expressed as mg DW $g^{-1}$ of TE (Trolox equivalents).

#### 2.5.3 Protein analysis

Soluble proteins were determined according to procedure previously described [34] from the extract prepared for the evaluation of antioxidant capacity using the ABTS method. For the quantification of the soluble protein content, the Bio-Rad® reagent was used. Thus, 50 µL of the extract was added to 750µL of the extraction buffer (Phosphate Buffer Solution; PBS). The reaction begins after completing 1 mL of the reaction with 200 µL of Bio-Rad® reagent. After shaking the samples, they were kept for 15 min at room temperature and then the absorbance at 595 nm was measured. Bovine albumin was used as a standard in a concentration range of 0 to 20 µg DW $mL^{-1}$ to generate the calibration curve.

#### 2.5.4 Carbohydrate analysis

Soluble carbohydrates were evaluated according to [35]. For this purpose, 5 mg of lyophilized algal biomass were dissolved in 6 mL of $H_2SO_4$ (1 M) in a water bath and kept at 100 °C for 1 h. After this step, the sample was centrifuged at 4,000 g for 15 min at laboratory temperature. The amount of 250 µL were then taken from the supernatant and mixed with 750 µL of $H_2SO_4$ (1 M) and 1 mL of phenol (5%), allowing the reaction to proceed for 40 min. Then, 5 mL of $H_2SO_4$ (96%) was slowly added. As the blank, 1 mL of $H_2SO_4$ (1 M) was used. The absorbance was measured at 485 nm. To obtain the standard curve, D-glucose was used as a standard at concentrations ranging from 0 to 100 mg DW $L^{-1}$.

#### 2.5.5 Lipid analysis

Total lipid content was quantified according to Sulfo-Phospho-Vanillin (SPV) photocolorimetric method [36]. The required phosphovanillin reagent was prepared by dissolving 0.6 g of vanillin in 100 mL of Milli-Q water (PV reagent) and adding phosphoric acid (85% $H_3PO_4$) to a total volume of 500 mL. Subsequently, 50 µL of sample were taken (100 mg of lyophilized microalgal biomass extracted in 1 mL of a 2:1 solution of chloroform and methanol), and 1 mL of $H_2SO_4$ (96%) was added. Then, the samples were incubated for 30 min at 100 °C in a thermostatic bath. When the samples cooled down, 2 mL of the PV reagent was added. Finally, the absorbance was measured at 625 nm. To obtain the calibration curve, a triglyceride derived from glycerol (triolein) at concentrations between 0.1 and 0.6 mg DW $L^{-1}$ was used.

### 2.6 Statistical analyses

Data are presented as mean ± standard deviation (SD). The effect of the treatments on the dependent variables was evaluated using the analysis of variance (ANOVA) [37]. Student–Newman–Keuls (SNK) post hoc tests were performed after significant ANOVA interactions and are shown in the figures and tables as lowercase letters in *C. fusiforme*. Homogeneity of variance was tested using Cochran tests and by visual inspection of the residuals. Correlations among data obtained were calculated using Pearson´s correlation coefficient (r) using an $N = 11$ [38]. The software SPSS for Windows (version 21, IBM) was used for the analyses.

## 3 Results

The culture was started with a biomass concentration of 0.3 g DW $L^{-1}$ and operated in batch regime for a period of 4 days. Changes in irradiance and ambient temperature were recorded at 10 min intervals, while the culture was sampled every 4 h (Fig. 3). The second and third day were characterized by variable irradiance conditions. Both minimum and maximum ambient temperatures occasionally increased; on day 3 and particularly on day 4, it surpassed the optimum (28 °C) for this species, reaching 36 °C (Fig. 3). Dissolved oxygen concentration increased during the cultivation period up to 135% of saturation (Fig. 4).

### 3.1 Photosynthesis changes

Photosynthetic variables estimated from LRC were measured as photosynthetic oxygen production/respiration in

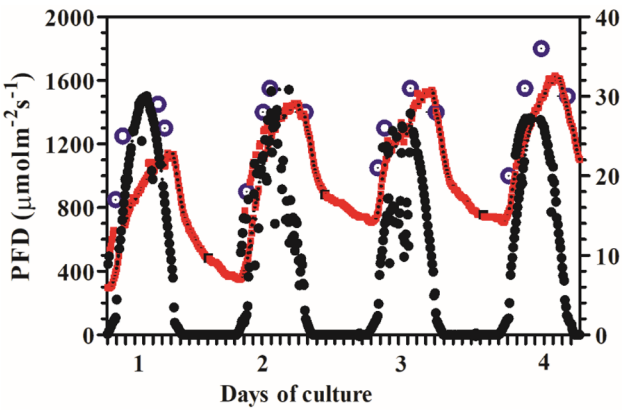



**Fig. 3** Time course of light irradiance (black symbols), ambient temperature (red symbols), and culture temperature (blue symbols)

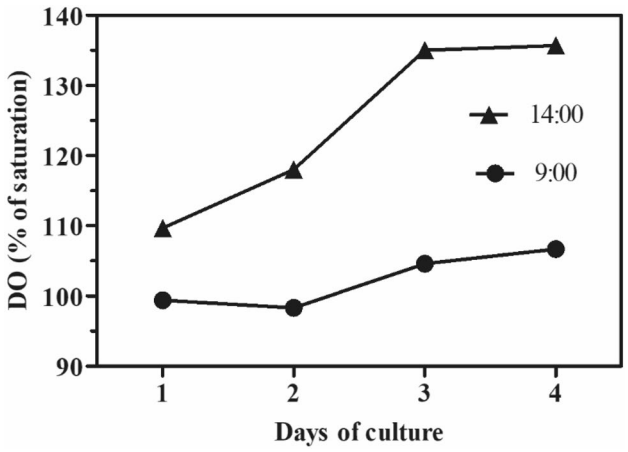



**Fig. 4** Dissolved oxygen concentration measured at the end of the cultivation lane just before the culture falls into the retention tank

samples taken from the outdoor culture during the daylight period (Table 2). Maximum photosynthesis rate ($P_{max}$) was higher during the first days of measurements, and occasionally decreased as the culture acclimated to the lower light conditions and due to the increased cell density. $P_{max}$ was lower in the morning as a result of the acclimation to low irradiance during the night time. On day 4, the lowest $P_{max}$ rates were recorded at times 9.00 and 14.00 h (244 and 238 µmol $O_2$ $mg^{-1}$ Chl *a* $h^{-1}$, respectively), most likely due to the high temperature and dense culture acclimation to the low irradiance during the experiment. Indeed, the temperature reached 36 °C at 14.00 h on day 4 (Fig. 3), which is supra-optimal for this species. The initial slope α (Table 2) showed the highest values on day 1 (mean $0.3226 \times 10^{-3}$) when the cells were acclimating to the high irradiance, while it stabilized around an average value of $0.203 \times 10^{-3}$ in the following days, with the minimum value observed on the last day ($0.163 \times 10^{-3}$) in coincidence of high culture temperature (Fig. 2). The saturation irradiance $E_k$ was generally higher in the middle of the day and decreased over the course of the experiment.

The compensation point ($E_c$), i.e. the value of irradiance at which oxygen evolution becomes equal to oxygen uptake, was higher on the first day (380 µmol photons $m^{-2}$ $s^{-1}$), in coincidence with the high respiration rate recorded at 9.00 h on day 1, and in the following days it stabilized within 90 and 150 µmol photons $m^{-2}$ $s^{-1}$. Respiration rates $R_d$ were rather high during the first day of the trial, peaking at 0.0972 µmol $O_2$ $mg^{-1}$ Chl *a* $s^{-1}$, after which became lower and ranged between 0.0175 and 0.0269 µmol $O_2$ $mg^{-1}$ Chl *a* $h^{-1}$. The contribution of respiration to the gross photosynthesis rate (photosynthesis plus respiration) reached 55% on the first day of the measurement (9.00 h). The maximum quantum yield of photosynthesis $\Phi_{max}$ ranged from a maximum of 0.0344 µmol $O_2$ µmol$^{-1}$ photons (9.00 h on day 1) to a minimum of 0.0164 (14.00 h on day 4) in coincidence with the attainment of the aforementioned excessive temperature in the culture. The corresponding quantum requirements $\varphi_{max}^{-1}$ ranged from a minimum of 29 to a maximum of 61 mol photons$^{-1}$ per 1 mol $O_2$ produced when the temperature exceeded the optimal value.

## 3.2 Fluorescence changes

In vivo fluorescence measurements were carried out on culture samples taken from the outdoor culture at the same time (9.00, 14.00, 17.00 h) as those used for photosynthesis measurements. Samples were exposed to the same light

**Table 2** Variables calculated from photosynthesis LRC measured in the *C. fusiforme* cultures grown outdoors in the thin-layer cascade. The units of the variables are reported in Table 1

| Day | Hour | $P_{max}$ µmol $O_2$ $mg^{-1}$ Chl *a* $s^{-1}$ ($10^{-3}$) | α (µmol $O_2$ $mg^{-1}$ Chl *a* µmol$^{-1}$ photons $m^2$) ($10^{-3}$) | $E_k$ µmol photons $m^{-2}$ $s^{-1}$ | a* $m^2$ $mg^{-1}$ Chl *a* | Rd µmol $O_2$ $mg^{-1}$ Chl *a* $s^{-1}$ ($10^{-3}$) | $E_c$ µmol photons $m^{-2}$ $s^{-1}$ | Rd/$P_{max}$ (%) | $\varphi_{max}$ mol $O_2$ mol$^{-1}$ photons | $\varphi_{max}^{-1}$ mol photons mol$^{-1}$ $O_2$ |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 09.00 | 176.9 | 0.378 | 467 | 0.011 | − 97.2 | 380 | 54.9 | 0.0344 | 29 |
| | 14.00 | 233.8 | 0.295 | 793 | 0.010 | − 58.8 | 220 | 28.5 | 0.0295 | 34 |
| | 17.00 | 276.6 | 0.295 | 936 | 0.010 | − 52.2 | 180 | 18.8 | 0.0295 | 34 |
| 2 | 09.00 | 140.0 | 0.195 | 718 | 0.009 | − 17.5 | 90 | 12.5 | 0.0216 | 46 |
| | 14.00 | 135.3 | 0.168 | 802 | 0.008 | − 18.0 | 110 | 13.3 | 0.0211 | 47 |
| | 17.00 | 151.6 | 0.224 | 675 | 0.009 | − 19.8 | 95 | 13.0 | 0.0249 | 40 |
| 3 | 09.00 | 72.7 | 0.197 | 368 | 0.007 | − 21.3 | 125 | 29.3 | 0.0282 | 35 |
| | 14.00 | 158.0 | 0.219 | 720 | 0.010 | − 26.1 | 120 | 16.5 | 0.0219 | 46 |
| | 17.00 | 129.1 | 0.195 | 661 | 0.005 | − 26.9 | 150 | 20.8 | 0.0391 | 26 |
| 4 | 09.00 | 67.7 | 0.268 | 252 | 0.008 | − 26.9 | 140 | 39.7 | 0.0336 | 30 |
| | 14.00 | 66.1 | 0.163 | 404 | 0.010 | − 24.2 | 150 | 36.5 | 0.0164 | 61 |

**Table 3** Variables estimated from LRC of Chl fluorescence measured in cultures of *C. fusiforme* grown outdoors in a thin-layer cascade

| Day | Hour | $F_v/F_m$ | $qN_{max}$ | $ETR_{max}$ µmol e- $mg^{-1}$ Chl *a* $s^{-1}$ | α mol e- $mg^{-1}$ chl a $mol^{-1}$ photons $m^2$ | $E_k$ µmol photons $m^{-2}$ $s^{-1}$ | a* $m^2$ $mg^{-1}$ Chl*a* | $\Phi_{max}$ mol *e*- $mol^{-1}$ photons (1) | $\Phi_{max}^{-1}$ mol photons $mol^{-1}$ *e*- (1) | $\Phi_{max}$ mol $O_2$ $mol^{-1}$ photons (2) | $\Phi_{max}^{-1}$ mol photons $mol^{-1}$ $O_2$ (2) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 09.00 | 0.702 | 0.083 | 3.716 | 0.00376 | 988 | 0.011 | 0.341 | 2.93 | 0.0850 | 11.7 |
| | 14.00 | 0.714 | 0.010 | 3.616 | 0.00349 | 1036 | 0.010 | 0.349 | 2.86 | 0.0872 | 11.4 |
| | 17.00 | 0.696 | 0.175 | 1.90 | 0.00307 | 618 | 0.010 | 0.307 | 3.25 | 0.0767 | 13.0 |
| 2 | 09.00 | 0.703 | 0.107 | 1.95 | 0.00281 | 692 | 0.009 | 0.313 | 3.20 | 0.0782 | 12.8 |
| | 14.00 | 0.717 | 0.062 | 2.3 | 0.00271 | 848 | 0.008 | 0.339 | 2.95 | 0.0847 | 11.8 |
| 3 | 09.00 | 0.724 | 0.212 | 1.15 | 0.00247 | 465 | 0.007 | 0.353 | 2.83 | 0.0883 | 11.3 |
| | 14.00 | 0.720 | 0.075 | 3.25 | 0.00344 | 945 | 0.010 | 0.344 | 2.91 | 0.0860 | 11.6 |
| | 17.00 | 0.733 | 0.147 | 1.333 | 0.00168 | 789 | 0.005 | 0.337 | 2.96 | 0.0840 | 11.9 |
| 4 | 09.00 | 0.705 | 0.375 | 1.075 | 0.00302 | 355 | 0.008 | 0.378 | 2.64 | 0.0945 | 10.6 |
| | 14.00 | 0.666 | 0.321 | 1.176 | 0.00363 | 324 | 0.010 | 0.363 | 2.75 | 0.0907 | 11.0 |

$\Phi_{max}$ (1), mol *e*- $mol^{-1}$ photons; $\Phi_{max}^{-1}$(1) = mol photons $mol^{-1}$ $e^-$, near 3 (2.928 ± 0.185) mol photons are required to move 1 mol *e*- across the photosynthetic chain). $\Phi_{max}$ (2), quantum yield (mol $O_2$ $mol^{-1}$ photons) and. $\Phi_{max}^{-1}$(2) quantum requirement (mol photons $mol^{-1}$ $O_2$). It is assumed that 4 $e^- = 1O_2$

source and protocol using a cuvette with the same geometry as used for the photosynthesis measurements. $ETR_{max}$ were generally higher in the middle of the day. The maximum value was recorded on the first day and occasionally varied throughout the experiment. The mean $F_v/F_m$ ratio was 0.705 ± 0.02 (Table 3). Similar to photosynthesis measurements, $E_k$ was higher in the middle of the day. Non-photochemical quenching (qN) was scarcely induced during the LRC measurements, and the highest value was found on the last day when the temperature exceeded the physiological value of 28 °C. The qN value was already high in the early morning hours confirming that the culture had already been exposed to temperature stress the previous day. On average, nearly 3 (2.99 ± 0.25) photons were required to move 1 $e^-$ across the photosynthetic chain. The quantum yield determined by fluorescence measurements, assuming a minimum requirement of 4 e- = 1 mol of $O_2$, was 0.08 ± 0.007 mol $O_2$ mol photons$^{-1}$, and the mean quantum requirement was 11.9 ± 1.04 mol photons $mol^{-1}$ $O_2$, which is much closer to the theoretical value than that calculated from the LRC (Table 3).

The relationship between the photosynthesis rates (P) and ETR recorded at the different irradiance intensities (0 to 1850 µmol photons $m^{-2}$ $s^{-1}$) was illustrated plotting all the measurements performed during a 4-day experiment using a linear regression curve ($r^2 = 0.8908$) (Fig. 5). Data scattering was observed mainly for measurements with higher light intensity. As can be deducted from Fig. 5, the relationship between ETR and P deviates strongly from the theoretical value of 4e- = 1 $O_2$. Indeed, about 15 e- are needed to produce 1 $O_2$ (i.e. 3.75 higher than the theoretical value).

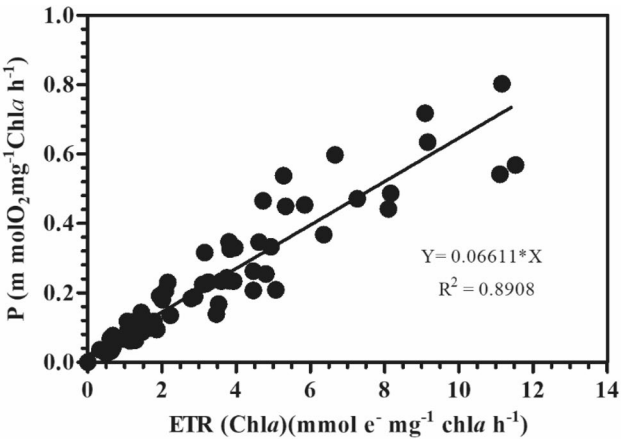



**Fig. 5** Gross oxygen evolution rates versus electron transfer rates gathered over ten measurements of P/I and ETR/I curves

Both quantum yields (moles of $O_2$ per mol of photons) calculated using oxygen evolution and fluorescence measurements were compared (Fig. 6). It is assumed that, theoretically, a minimum of 4 e- are transported through the photosynthetic chain for each molecule of $O_2$ evolved [39]. Fluorescence measurements gave a minimum quantum yield of 0.094 mol of $O_2$ per mole of photons, corresponding to a quantum requirement of about 10.6 photons per mole of oxygen released, which is not too far from the theoretical value (8 mol of photons per mole of $O_2$), with a maximum of 0.077 mol of $O_2$ per mole of photons, corresponding to nearly 13 photons required per mole of oxygen released. On average, the quantum yield values gathered via fluorescence measurements were about three times higher than those

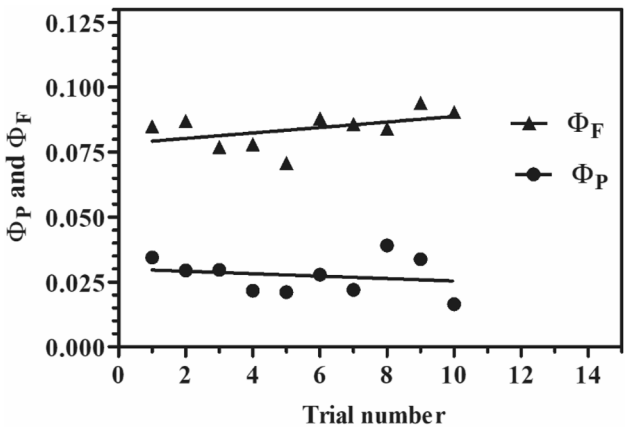



**Fig. 6** Comparison of quantum yields obtained through fluorescence and oxygen evolution measurements in cultures of C. fusiforme grown outdoors on a thin-layer cascade

obtained from the measure of oxygen evolution. However, the difference between quantum yield gathered through fluorescence and oxygen evolution measurements was lower in the beginning (2.7) and gradually increased over the course of the experiment, most likely due to the unstable irradiance that occurred particularly during the second and third day of the measurements (Fig. 6).

### 3.3 Growth

The changes in DW of the *C. fusiforme* culture grown in the thin-layer cascade are shown in Fig. 7. DW during the light period increased linearly by 0.3 g $L^{-1}$ $day^{-1}$ (black columns), however, due to overnight biomass loss, the mean net productivity increase was lowered to 0.2 g $L^{-1}$ $day^{-1}$ (white columns). Nightly biomass losses were relevant and accounted for 1/3 of the biomass produced during the day. Considering that the total volume of the culture was 74 L and the illuminated area of the cascade was 5 $m^2$, the average gross daily yield was 4.81 g DW $m^{-2}$ $day^{-1}$ with a net yield of about 3 g DW $m^{-2}$ $day^{-1}$.

### 3.4 Biomass composition

The protein content of the biomass harvested in the morning and at the end of the day is shown in Fig. 8. The average protein content was significantly higher in the biomass harvested in the morning (55.4% ± 8.14 of DW) than in that collected in the evening (43.21% ± 6.38). The largest differences were observed on the first day of measurements (65.03% morning vs 45.78% evening) and on day 4 (51.7% in the morning vs 38.11% in the evening). The protein content, particularly in the morning, steadily decreased throughout the experiment, going from 65% of DW at the start of the experiment to 45% at the end, as a result of a more balanced growth of the biochemical components and the increased carbohydrate content. In contrast to protein content, carbohydrates showed a light vs. dark trend with percentages on total DW ranging from a minimum value of 18.7% ± 1.40 in the morning, to 26.2% ± 1.36 in the afternoon. No significant differences were found between the different culture days in both the morning and afternoon (Fig. 9).

The lipid content measured in the morning and at the end of the day is shown in Fig. 10. No significant differences were found between the morning and end-of-day samples, but there was a tendency to decrease during the day.

Total phenolic content (PC) of the biomass harvested in the morning and in the evening was significantly ($p < 0.05$) higher on the third day, with maximal values around

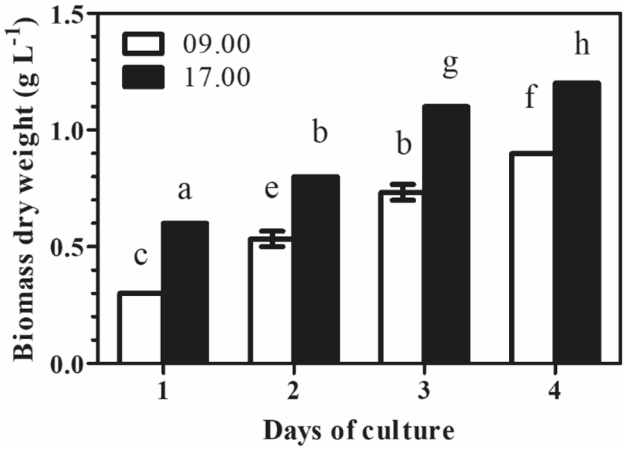



**Fig. 7** Dry weight (g L-1) increase recorded during the course of experiments with cultures of C. fusiforme grown in a thin-layer cascade

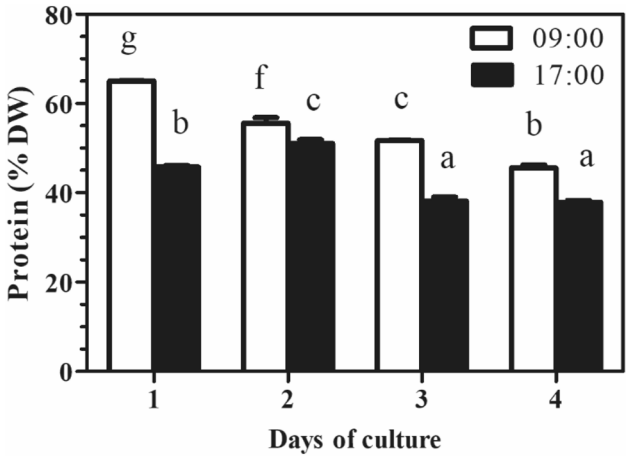



**Fig. 8** Protein content of C. fusiforme biomass grown in a thin-layer cascade during a 4-day experiment

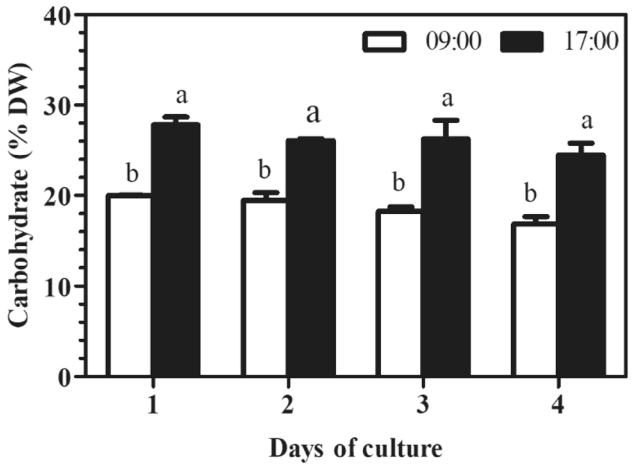



**Fig. 9** Carbohydrate content of C. fusiforme biomass grown in a thin-layer cascade during a 4-day experiment

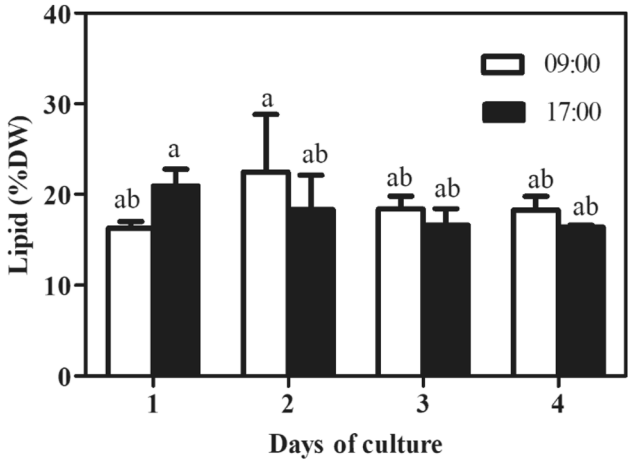



**Fig. 10** Lipid content of the C. fusiforme biomass grown in a thin-layer cascade during a 4-day experiment

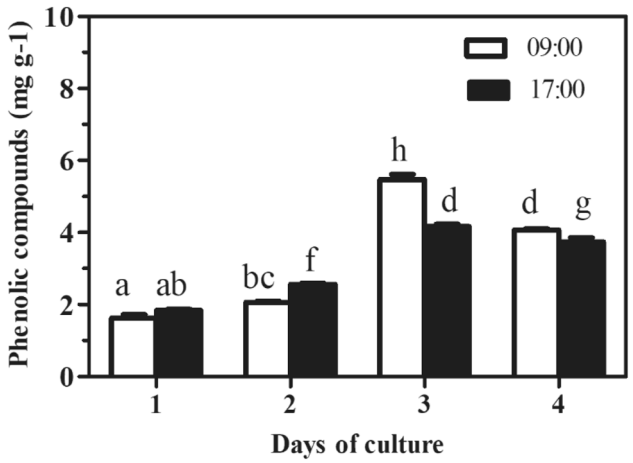



**Fig. 11** Total phenolic content of C. fusiforme cells grown outdoors in summer in a thin-layer cascade

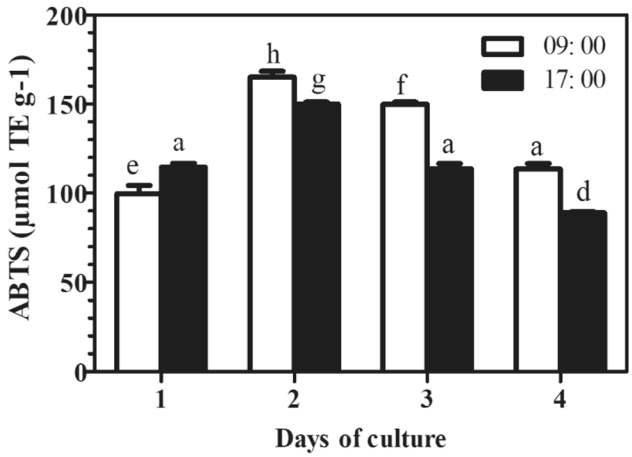



**Fig. 12** Antioxidant activity recorded in cell of C. fusiforme cells grown outdoors in summer in a thin-layer cascade

5–6 mg DW $g^{-1}$ in the morning. (Fig. 11). In general, PC increased during the experiment.

The antioxidant activity measured with the ABTS method changed significantly ($p < 0.05$) during the experiment. On day 2 in the morning, the antioxidant activity showed the maximal values reaching 175 µmol TE $g^{-1}$ DW (Fig. 12). Thus, the antioxidant capacity increased in the middle of the experiment (day 2, 3), and the same trend was observed in phenolic compound content.

Both compounds had a positive Pearson correlation value ($p < 0.02$), indicating that both variables increased together. In general, significantly higher antioxidant activity was observed in the morning samples.

## 4 Discussion

The microalga *C. fusiforme* was grown in a thin-layer system and studied in detail by evaluating its photosynthetic performance, growth, and biochemical composition. Thin-layer systems combine the positive aspects of closed systems (operation at high biomass densities which facilitates harvesting) with the advantages of open systems (evaporative self-cooling, and an easier oxygen stripping). A methodological comparison of photosynthetic oxygen evolution and estimated electron transport rates was carried out in order to explore the usefulness of chlorophyll fluorescence techniques as an indicator of photosynthetic performance (and productivity) of outdoor cultures of this microalga.

A linear relationship was found between two photosynthetic measurement techniques—oxygen production and fluorescence quenching analysis (ETR). However, when all the measurements were compared, a high degree of data scatter was observed above the limiting light conditions (Fig. 5). In other words, above the limiting light level, several largely

different values of photosynthetic rates corresponded to the given ETR. This was most likely due to the different light acclimation (history) of the cell samples, collected on different days and at different times of the day, which significantly affected the photosynthetic capacity of the cells. Indeed, photosynthesis (oxygen evolution) showed lower saturation intensities ($E_k$) than that of ETR.

The mean quantum yields measured by oxygen evolution techniques were about three times lower than those estimated by fluorescence measurements (Fig. 6). Several factors may be responsible for these differences. First, fluorescence measurements do not consider the respiration (oxygen consumption), and although the calculation of quantum yields for oxygen evolution was performed using gross photosynthesis rates, the respiration rate can vary during the measurement, ranging from limiting to saturating light conditions, and can increase with increasing irradiance [40]. Second, oxygen measurements require much more time compared to fluorescence measurements. Typically, rapid fluorescence measurements take about 20 s for each step, while photosynthesis measurements require at least 1 min per step to obtain a reliable measurement. This increases the time that cells spend under actinic light and causes an increase in dissolved oxygen in the chamber, leading to the development of non-photochemical quenching. Moreover, the high oxygen concentration can increase the contribution of photorespiration, and trigger the Mehler reaction, all processes that consume oxygen [41, 42]. Current understanding of light-dependent oxygen uptake rates is limited by the small number of species studied to date. Microalgae show a similar range of light-dependent $O_2$ uptake rates as C4 plants. As with C4 plants, $O_2$ uptake appears to be largely insensitive to $CO_2$ levels, even in species lacking $CO_2$ concentration mechanisms and under conditions that are clearly limiting with respect to inorganic carbon supply [41]. A partial explanation for this behavior may lie in the fact that Rubisco may have considerably different oxygenase kinetics and much lower oxygenase potential in air in many algal species. This could lead to a greater proportion of observed $O_2$ uptake attributable to the Mehler reaction instead than Rubisco compared to C3 plants. It is estimated that $O_2$ uptake accounts for 15–30% of the total oxygen formed at both high and low $CO_2$ concentrations. Therefore, it remains unlikely that photorespiration and the Mehler reaction are responsible for the strong difference between quantum yields from oxygen measurements and fluorescence that we found in our experiments. Microalgae, like C4 plants, usually have a well-functioning xanthophyll cycle. It is responsible for the development of the non-photochemical quenching mechanism and can efficiently dissipate excess of light [43–45]. In contrast to higher plants, where the amplitude of NPQ is proportional to the light intensity to which they are exposed, microalgae strongly upregulate their ability to switch on NPQ even under moderate light irradiance, resulting in a remarkable decrease in light conversion efficiency [46] and the ability to dissipate photons absorbed by light-harvesting complexes under excess light. These facts indicate that fluorescence-based photosynthesis rates may not be fully used as an alternative to oxygen measurements. Further trials in microalgal mass cultures may be useful to obtain a more accurate estimate of photosynthesis from fluorescence measurements.

However, several authors have proved a correlation between algal productivity and daily integrated electron transfer rate. In an outdoor culture of microalgae [17, 18], algal productivity depends primarily upon light energy conversion efficiency, that is the efficiency of an algal culture in capturing, absorbing and utilizing light to assimilate $CO_2$ into dry matter. This simple concept predicts a linear relationship between the absorption of light and the synthesis of algal biomass. Photosynthesis in an algal system should therefore be predominantly light-limited and rather than operating at light-saturated rates. Consequently, the photosynthetic performance of an algal culture depends on the initial slope of the P/I curve, that is, on the light-limited region, rather than the light-saturated part. Therefore, a deviation from the linearity of the ETR vs P curve out at high light irradiance as found in this study, should not affect the correlation of productivity and fluorescence (ETR).

The biomass productivity attained with *C. fusiforme* cultures, was relatively lower (0.2–0.4 g DW $L^{-1}$ $day^{-1}$, corresponding to 3–6 g $m^{-2}$ $d^{-1}$), compared to that achieved with the same strain grown in open mini-ponds (about 14 g $m^{-2}$ $s^{-1}$) [16]. This difference can be justified by the fact that in the second case the cultures were thermostated at the optimal temperature of 30 °C and exposed to a higher solar light energy, since the experiments were carried out in July in central Italy, when the mean light energy reaches 25 MJ $m^{-2}$ $d^{-1}$, while the experiments with *Chlamydopodium* on thin-layer cascade were performed in August in central Europe with an average solar light energy of 15 MJ $m^{-2}$ $d^{-1}$ [47]. Comparing the biomass yields of *C. fusiforme* with those of *Chlorella sorokiniana R*117 grown at the same location and in a similar culture system, the yield of *Chlorella* was 50% higher [23]. However, it must be pointed out that *Chlorella sorokiniana* has been cultivated in thin-layer cascades since 1963 [23], and thus its growth has been fully optimized. Moreover, it has a wide temperature range for growth (20–40 °C), which makes it an optimal strain for cultivation in the climatic conditions of Central Europe [48]. Finally, it should be noted that the experiments were not aimed at optimizing productivity, but at studying the correlation between fluorescence and photosynthesis measurements. For this purpose, the culture was started with a very low biomass concentration to better investigate the acclimation phenomenon in this species. Therefore, the cells were

exposed to high light irradiance which caused downregulation of the photosynthetic apparatus, as shown by the strong reduction in the quantum yield of photosynthesis gathered by photosynthesis measurements. Another very important factor that must have affected productivity was the high volume of the culture (about 45% of the total volume) that remained in the dark [49]. This percentage of the culture in the dark may have determined a high energy loss due to a continuous phenomenon of regulation/deregulation of the photosynthetic apparatus caused by continuous shift of cells from light to dark conditions.

Light stress also resulted in biomass production strongly imbalanced toward carbohydrate synthesis, particularly on the first day, which most likely acted as a sink of the photosynthate produced during excessive light excitation. The carbohydrate content was always higher at the end of the light period. Overnight, a large amount of carbohydrate was lost via dark respiration. A similar behavior was frequently reported in the literature for both microalgae [50, 51].

An opposite behavior was observed for the protein content of the biomass. The protein content was lower at the end of each day and always higher in the morning. Mass balance of these biochemical components, performed both in the evening and the next morning, showed that the higher amount in the morning was simply due to changes in the relative amount of major cellular components and not due to nocturnal protein synthesis [51].

A correlation was found between antioxidant activity and polyphenol content, as well as between antioxidant activity and the content of other substances such as chlorophyll *a* or other accessory pigments that can contribute to the protection the cells from radical oxygen substances (ROS).

The process of stress acclimation involves several photoprotective mechanisms, but the strategies of antioxidant capabilities of microalgae are not well understood [52]. The phenolic compounds increased during the experiment, with a positive relationship with biomass [31]. High photosynthetic activity is associated with high oxygen production, which can lead to oxidative stress and consequently to an increase in antioxidant activity measured by the ABTS method, as well as to a positive correlation with both biochemical parameters [53]. The increase in polyphenols, which coincided with the increase in dissolved oxygen concentration in the culture, may indicate that they play a role in protecting against increased formation of ROS.

## 5 Conclusions

The findings reported in this work can have an important impact on the optimization of outdoor *C. fusiforme* cultures, since this microalga is not yet well known in the panorama of microalgal mass cultivation. Due to its high protein content, this microalga is suitable for large-scale production, thus expanding the microalgae industry for bio-based products to meet the requirements of the bioeconomy. The cells of *Chlamydomodium fusiforme* are at least twice as long as those of many *Chlorella* species, and this is an important feature appreciated in mass cultures as it facilitates biomass harvesting. Outdoor biomass growth can be optimized by removing the physical causes that reduce light conversion efficiency, for example the dark zone of the reactor, and by using denser cultures than those used in this study, which was mainly aimed at understanding the acclimation process. The high quantum yield obtained through fluorescence measurements was comparable to that of other microalgal species, indicating that the productivity of this microalga can be strongly improved. The close relationship between chlorophyll fluorescence and oxygen evolution was confirmed, although the discrepancy between these two measurements is very large. However, the quantum yields obtained with fluorescence technique, which are about three times higher, indicates that it is necessary to use both techniques to have a more realistic picture of the photosynthetic performance of this species, particularly outdoors where temperature and light are subject to continuous changes. Fluorescence measurements must always be accompanied by dry weight measurements to obtain a more accurate estimate of biomass productivity.

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**Data availability** The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Declarations

**Conflict of interest** The authors declare that they have no conflict of interest.

**Informed consent, human/animal rights** No conflicts, informed consent, human/animal rights applicable.

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