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Differences in susceptibility to photoinhibition do not determine growth rate under moderate light in batch or turbidostat – a study with five green algae

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Abstract

To understand growth limitations of photosynthetic microorganisms, and to investigate whether batch growth or certain photosynthesis-related parameters predict a turbidostat (continuous growth at constant biomass concentration) growth rate, five green algal species were grown in a photobioreactor in batch and turbidostat conditions and their susceptibilities to photoinhibition of photosystem II as well as several photosynthetic parameters were measured. Growth rates during batch and turbidostat modes varied independently of each other; thus, a growth rate measured in a batch cannot be used to determine the continuous growth rate. Greatly different photoinhibition susceptibilities in tested algae suggest that different amounts of energy were invested in repair. However, photoinhibition tolerance did not necessarily lead to a fast growth rate at a moderate light intensity. Nevertheless, we report an inverse relationship between photoinhibition tolerance and minimum saturating irradiance, suggesting that fast electron transfer capacity of PSII comes with the price of reduced photoinhibition tolerance.

Keywords: *Chlorococcum novae-angliae*; *Desmodesmus quadricauda*; *Ettlia oleoabundans*; microalga; photodamage; photoinactivation; rapid light curve; *Scenedesmus ecornis*; *Scenedesmus obliquus*.

Introduction

The potential of microalgae or cyanobacteria to produce food, biomass, fuel, various high-value compounds, and electricity or to clean wastewater is intensively studied (for reviews, *see* Borowitzka and Vonshak 2017, Stensjö *et al*. 2018). To obtain a commercially viable yield of the desired product and to keep contamination in control, these

Highlights

- Growth rate of a dilute batch does not predict growth rate in a dense turbidostat
- Susceptibility to PSII photoinhibition does not necessarily limit growth
- Fast forward electron transfer of PSII may lead to fast photoinhibition

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Abbreviations: ETR – electron transfer rate; ETR(II) – electron transport rate calculated by taking into account the relative absorption cross-section of PSII; ETR_{MAX} – maximum electron transport rate; F_v/F_m – ratio of variable to maximum fluorescence; I_k – minimum saturating light intensity; OD – optical density; RLC – rapid light curve; α – light-use efficiency; $\sigma_{II}(\lambda)$ – wavelength-dependent functional cross-section of PSII.

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applications often require a continuously high growth rate. The simplest way to determine the growth rate is to use a batch culture, in which a dilute algal suspension is prepared in a suitable growth medium, usually in a cultivation flask. The culture is stirred, possibly in an atmosphere enriched with CO_2, and growth of the algae is followed for a few days until their growth slows down and finally ceases, while cell density becomes very high. If it is possible to discern a phase at which the growth in a very dilute culture is exponential, the maximum specific growth rate is often calculated. In batch growth, the cell density increases during the whole experiment, increasing attenuation of light within the culture. The light attenuation is assumed to be at a minimum during an apparently exponential growth phase, but there is no factual proof for this assumption. In industrial systems, on the other hand, microalgal cultures are often continuously maintained at a constant cell density by frequent additions (influent) of culture medium and simultaneous removal (effluent) of the same amount of culture (*see*, *e.g.*, Valev *et al*. 2020). Cell density is commonly followed by measuring the turbidity of the culture, and therefore this cultivation system is called a turbidostat. In a dense culture, light availability is a major factor limiting algal growth. Nevertheless, the turbidostat is usually kept at a relatively high cell density, as a compromise between growth rate and algal concentration, to maximize the productivity of the system (*see* García-Cubero *et al*. 2021). Due to the higher cell density, mutual shading is typically much stronger in continuous cultures than in a dilute batch. Also, when in excess, light may inhibit growth (*see*, *e.g.*, Hindersin *et al*. 2013). In contrast to a batch culture, in a turbidostat, mutual shading does not vary with time, and cells do not need to acclimate to changing light conditions over the growth period of the culture.

In addition to different environmental conditions, growth rates of photosynthetic organisms may vary because of intrinsic differences in their (photosynthetic) characteristics. For example, different organisms have very different capacities to utilize low or high light or to combat stress (Flynn 2009). Photosystem II (PSII) of algae and other photosynthetic organisms is continuously damaged in the light and the damage needs to be constantly repaired, to keep photosynthesis going (for reviews, *see* Nath *et al*. 2013, Tyystjärvi 2013). If the rate of the damage is faster than the rate of the repair, *e.g.*, under high light, nonfunctional PSII units accumulate, net photoinhibition occurs, and possibilities to fix carbon may be lost due to the decreased photosynthetic capacity (Raven 2011). In addition, even if an organism manages to compensate the damage by efficient repair, the repair reactions consume a lot of energy (Raven 2011, Miyata *et al*. 2012, Murphy *et al*. 2017, Murata and Nishiyama 2018). The vulnerability of PSII to light-induced damage and the repair capacity varies among different algal species (*see*, *e.g.*, Campbell and Serôdio 2020). Thus, differences in the rates of photoinhibition or in other photosynthetic properties in different algal species may explain, at least partly, their varying growth rates.

In the present study, we set out to investigate whether the maximum specific growth rate measured from a dilute batch culture, photoinhibition tolerance, and/or specific photosynthetic parameters of the algae could predict their growth rates in continuous cultivation. For this, five species of green algae, *Scenedesmus obliquus*, *Scenedesmus ecornis*, *Desmodesmus quadricauda*, *Chlorococcum novae-angliae*, and *Ettlia oleoabundans*, were used. These are unicellular freshwater species (except *C. novae-angliae*, which has been isolated from bog soil) although *S. obliquus*, *S. ecornis*, and *D. quadricauda* can also form aggregates, surrounded by a single cell wall (called coenobia), of four or eight cells. These five algal species were cultivated in a phototrophic liquid medium and tested for their growth rates, photoinhibition tolerance, and several other photosynthesis-related processes. To focus on the differences between the growth modes, both the batch and the continuous growth experiments were done as a part of the same experiment, in the same device. We show an inverse relationship between photoinhibition tolerance and the minimum saturating irradiance of PSII. However, based on our results, a continuous culture must be used to measure the growth rate for continuous production.

Materials and methods

Algal and cyanobacterial strains and culture conditions: *S. cf. obliquus* (strain #455 of the collection), *S. cf. ecornis* (444), and *D. quadricauda* (463) were obtained from CCALA (Culture Collection of Autotrophic organisms, Třeboň, Czech Republic), and *C. novae-angliae* (1778) and *E. oleoabundans* (1185; formerly called also *Neochloris oleoabundans*) from UTEX (Culture Collection of Algae at the University of Texas at Austin, USA). The algal cultures and the cyanobacterium *Synechocystis* sp. PCC 6803 were maintained on agar plates made in BG-11 growth medium (Rippka *et al*. 1979) with 20 mM Hepes–NaOH (pH 7.5). Prior to the experiments, if not mentioned otherwise, the algae were grown in 100-ml flasks in BG-11 at the volume of 30 ml, at 25–30°C with mild shaking in continuous light under the PPFD of ~50 μmol(photon) m^{-2} s^{-1}. Cyanobacteria were grown under similar conditions, but in 250-ml flasks at the volume of 50 ml, at 32°C.

Growth rate measurements: Inocula for the photobioreactor growth experiments were cultured as described above but at 20°C at the PPFD of 30 μmol(photon) m^{-2} s^{-1} on a shaker set at 100 rpm. A 400-ml flat panel photobioreactor (*FMT-150*, *PS Instruments*, Czech Republic) was used for growth rate measurements. During the experiments the algal cultures were continuously sparged with a mixture of air and 9,600 ppm CO_2 (1% CO_2) at 0.15 ml min^{-1} flow rate, obtained with a gas mixer unit (*GMS 150*, *PS Instruments*). Algae were grown under continuous illumination with white LEDs of the photobioreactor. PPFD was measured from inside of the water-filled bioreactor with a PPFD meter (*LI-250A*,

LI-COR, USA) equipped with a submersible sensor (*US-SQS/L*, *Heinz Walz*, Germany) and adjusted to the PPFD of 200 µmol(photon) m^{-2} s^{-1} (averaged over the panel surface) prior to each experiment. Temperature was maintained at 25°C. Each experiment consisted of an initial batch phase and a subsequent turbidostat phase. The batch phase was used to calibrate an OD (optical density) 735-nm sensor with measurements of dry algal biomass (*see* below; Fig. 1S, *supplement*). OD at 680 nm was also recorded. The maximum specific growth rate (based on the increase in OD at 735 nm) was calculated by finding the best fit (least squares method), during an apparent exponential phase (lasting 15–25 h) of the batch culture, to an exponential equation in *Microsoft Excel*. In addition, the linear growth rate (when OD at 735 nm increased linearly with time), observed at a later phase of each batch culture, was calculated at the time when the culture had reached the biomass concentration chosen for the turbidostat mode (~0.43 g L^{-1}) by fitting the growth curve (during 7 h) to a linear equation in *Microsoft Excel*.

Turbidostat operation was enabled with the OD 735-nm sensor and a peristaltic pump (*PP 500*, *PS Instruments*); fresh BG-11 growth media was pumped into the photobioreactor on demand, in order to dilute the culture and acquire a stable concentration of algal biomass. The dilution rate (which corresponds to the growth rate in the turbidostat) was then measured with a weight balance (*CBK 8H*, *Adam DU*, UK) from the harvest (effluent). At the initial part of the turbidostat phase, the OD value at 735 nm was adjusted, based on dry mass measurements (*see* below), and then kept stable so that all the cultures were grown at an almost equal biomass concentration of ~0.43 g L^{-1} (Table 1). During a turbidostat run, the growth rate was calculated every six hours, over a time period ≥ 3 × (the specific growth rate of the alga)$^{-1}$, and averaged. The photobioreactor was checked daily for biofouling, and when needed, the cultures were mixed mechanically. The biomass concentration was chosen from the 0.1 to 2 g L^{-1} harvest density range often used in biofuel research (for a review, *see* Su *et al.* 2017).

Light attenuation by cells inside the photobioreactor was estimated by measuring light intensity, with the *LI-COR* light meter using the submersible sensor, from separate cultures (pre-grown in flasks as described above) of *S. obliquus*, *C. novae-angliae*, and *E. oleoabundans*. The algal suspensions were placed in a 30-ml cylinder (diameter of 5 cm) and light was measured from 1.2 cm below the illuminated surface (the midway of the 2.4 cm deep culture in the cylinder, corresponding to the midway of the light path in the photobioreactor). After the first PPFD measurement, a part of the sample was collected for dry mass measurements (*see* below) to calculate the biomass concentration. After that the algal cultures were diluted with BG-11 to measure light availability inside the culture as a function of cell density. The cyanobacteria were grown only as batch cultures in flasks, as above described, and their growth rate was monitored by measuring OD at 730 nm with a spectrophotometer from aliquots of the culture. PPFD inside the flasks was measured with the *LI-COR* light meter using the submersible sensor. Transmission of light through an algal suspension (*S. obliquus*) was measured with an *STS-VIS* spectrometer (*Ocean Insight*).

Dry mass measurements: Samples for dry mass analysis were collected daily from the photobioreactor. Three 5-ml samples were diluted with 0.5 M ammonium formate to a final volume of 10 ml and then filtered through pre-weighed 1.6-µm retention glass fiber filters (*VWR*, USA). Samples were dried for 24 h at 96°C on the filters. After cooling for 1 h in a desiccator at room temperature, the filters were weighed with an analytical balance (*MC1 RC 210-P*, *Sartorius*, Germany). Biomass density was calculated as $(m_2 - m_1)$/sample volume, where m_1 is the mass of the filter and m_2 is the mass of filter with dried biomass.

Photoinhibition experiments: After 5−20 d of preliminary batch culture in flasks, 35 ml of algal suspension, diluted with fresh BG-11 growth medium to the chlorophyll concentration of ~8 µg ml^{-1}, was illuminated at 25°C with constant mixing in the PPFDs of 500 µmol(photon) m^{-2} s^{-1} or 1,500 µmol(photon) m^{-2} s^{-1} for 0−90 min in the absence or in the presence, as indicated, of 0.4 mg ml^{-1} lincomycin to block the repair of PSII. The cultures used were in the late exponential growth phase or linear growth phase. In some cases, as indicated, the algae were let to recover after the light treatment for two days at their growth conditions (in the light). Chlorophylls (Chl) *a* and *b* were extracted from separate cultures by at least overnight incubation in methanol at 4°C in the dark and quantified according to Inskeep and Bloom (1985); the extraction was considered complete if the pellet had no green tint after centrifugation. PSII activity was assayed by measuring the Chl *a* fluorescence parameter variable to maximum fluorescence $F_v/F_m = (F_m - F_0)/F_m$ with *Multi-Color-PAM* fluorometer (measuring beam: 625 nm; *Heinz Walz*, Effeltrich, Germany) after 10 min in darkness. Minimum fluorescence, F_0, was measured using only very low intensity measuring flashes, while F_m was induced with 800-ms saturating pulses (PPFD of 10,000 µmol m^{-2} s^{-1}).

Rapid light curves (RLCs) were measured from all species using the *Multi-Color PAM* fluorometer (*Heinz Walz*). All cultures used in RLC measurements were in the late exponential growth phase, and samples from batch cultures (in flasks) were normalized to OD at 680 nm = 0.2, to obtain similar, healthy algal samples, by diluting with fresh BG-11 growth medium. OD at 680 nm corresponds to the peak of Chl *a* absorption and gives a rough estimate of the Chl concentration. After dilution, the samples were acclimatized for 15 min in the growth conditions (in the light) prior to the measurements. The measuring protocol consisted of 13 steps of increasing intensities of white light, up to PPFD of 1,115 µmol m^{-2} s^{-1}, with each step lasting for 90 s. The intensity of the 440-nm measuring

light (modulation frequency 20,000 Hz) was adjusted for each species separately to achieve comparable fluorescence values. The temperature was maintained at 27°C using the temperature control unit (*US-T*) accessory of *Multi-Color PAM*. The samples were agitated with a magnetic stirrer during the measurements. The parameters α (light-use efficiency), ETR_{MAX} (maximum electron transport rate), and I_k (minimum saturating irradiance) were derived by analyzing the RLCs according to the model described by Eilers and Peeters (1988) using the in-built fitting protocol of the *PamWin-3* (v3.22) software of *Multi-Color PAM*.

Wavelength-dependent functional cross-sections of PSII [$\sigma_{II}(\lambda)$] were measured as described by Schreiber *et al*. (2012) from samples that had been dark-acclimated for 15 min but were otherwise identical to the ones used in the RLC measurements. This method allowed the estimation of photons absorbed by PSII *vs*. photons absorbed by all photosynthetic pigments [PPFD(II)] and, therefore, also the estimation of PSII-specific (absolute) electron transport rate [ETR(II)]. The $\sigma_{II}(\lambda)$ values obtained with a combination of white light and 440-nm measuring light were used to calculate PPFD(II) and ETR(II) during the RLC measurements in all algae strains using the *PamWin-3* software.

Q_A^- reoxidation: Kinetics of Q_A^- reoxidation were measured with an *FL 200* fluorometer with a *SuperHead* optical 525 unit (*PS Instruments*) after a strong single turnover flash (530 nm). Prior to the measurements, the algae were diluted to OD at 680 nm = 0.3 with fresh BG-11 growth medium and dark-acclimated for 5 min. The first measuring point was recorded 150 µs after the flash. The decay of Chl *a* fluorescence yield (f) was fitted to the equation $f(t) = a \times \exp(-b \times t) + c \times \exp(-d \times t) + g \times \exp(-h \times t)$, where t is time, in *SigmaPlot* (*Systat Software*, *Inc.*) to calculate the rate constants for electron transfer from Q_A to Q_B (b), an apparent first-order rate constant for PQ binding to an empty Q_B site and possible PQH_2 release (d) and recombination of $S_2Q_A^-$ to S_1Q_A (h), and the proportions of functional PSII units (a), PSII units with an empty Q_B site (c), and Q_B nonreducing PSII reaction centers (g).

Results

Fast batch growth rate did not always predict fast continuous growth

Growth rates of five green algal species at moderate light [200 µmol(photon) m^{-2} s^{-1}] were compared by cultivating them in a flat panel photobioreactor, first as a batch culture, and then in a continuous mode (turbidostat) where the biomass concentration was kept constant (at ~0.43 g L^{-1}) by frequent replacement of part of the culture with fresh growth medium. Growth was followed by recording optical density (OD) at 735 nm. Relatively little variation was observed in the growth rates of the different algal species during the turbidostat mode (Fig. 1, Table 1); *E. oleoabundans*, *C. novae-angliae*, and *S. ecornis* had almost the same duplication rates (0.68, 0.71, and 0.72 d^{-1}, respectively), whereas the growth rates of the other two species, *D. quadricauda* and *S. obliquus*, were slightly higher (0.85 and 0.94 d^{-1}, respectively). In the batch mode, on the other hand, the growth rates of the algae differed more from each other; the maximum specific growth rate, measured during an initial exponential growth phase, of *D. quadricauda* was approximately three times as high as that of *E. oleoabundans* (Fig. 1, Table 1).

It was not feasible to measure how light availability is changing inside the used flat panel photobioreactor during algal growth; therefore, light intensity inside an optically similar setting was measured from different dilutions of *E. oleoabundans*, *C. novae-angliae*, and *S. obliquus* (Fig. 2*A*). Unexpectedly, light that passed through an *S. obliquus* culture was enriched in green (~520–580 nm) and far-red wavelengths (Fig. 2*B*), as these wavelengths are not well absorbed by chlorophylls. We also measured light intensity inside 250-ml flasks during a 50-ml cyanobacterial batch culture. In both cases, light availability decreased drastically when cell density increased (Fig. 2). The effects of cell density on light availability slightly differed in the different organisms, probably due to different cell sizes, as the used flat panel bioreactor had a relatively short light path of 24 mm, which is rather close to what the 50-ml cyanobacterial cultures experienced in the 250-ml flasks. Based on the light availability measurements (Fig. 2*A*), it is clear that during the turbidostat culture the algae received less light than a dilute culture (at its exponential growth phase). Consequently, it is unsurprising that the specific growth rates measured during the exponential batch growth were higher than the growth rates during the turbidostat mode (Fig. 1*F*). However, these growth rate measurements also did not correlate very well (Fig. 1*F*; $r^2 = 0.608$) with each other, as different species grew fast during the batch and different ones during the continuous growth.

However, to achieve a fairer comparison for batch and turbidostat growth modes, growth rates in the batch culture were calculated (by linear fitting) also during the phase when the algae had achieved the biomass concentration selected for turbidostat mode (~0.43 g L^{-1}); at that point the increase in the algal concentration in the batch culture was rather linear. The change in the concentration of *S. obliquus* at that point, however, slightly resembled an exponential increase, which may partially explain its high turbidostat growth rate. However, even in this comparison the batch and turbidostat growth rates did not correlate (Fig. 1*F*, Table 1; $r^2 = 0.038$), not even for *S. obliquus*.

Susceptibility to photoinhibition varied among algal species but showed no general correlation with the growth rate

To see if susceptibility to photoinhibition of PSII could explain the differences in the growth rates, cells of the five green algal species were illuminated with high light [PPFD of 500 µmol(photon) m^{-2} s^{-1}] in the presence of lincomycin, an antibiotic that blocks translation in

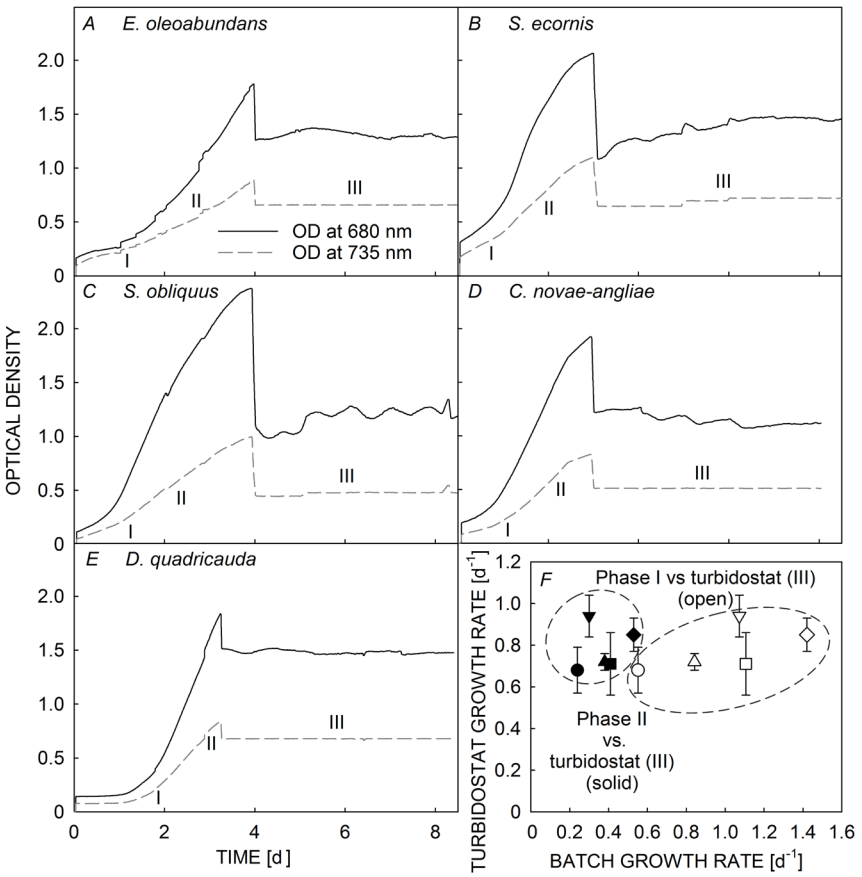

Fig. 1. Growth of five species of green algae in a flat panel photobioreactor. (*A–E*) The indicated algae were grown first in batch mode and then in turbidostat mode [the change between the growth modes is seen as a drop in both the optical density (OD) values at the 3rd or 4th growth day], where biomass concentration was kept constant by dilutions with fresh growth medium. Different growth phases are highlighted with Roman numerals; I: an initial exponential growth phase in batch mode, II: a linear growth phase in batch mode, III: turbidostat mode. Growth was measured by recording OD of the culture at 680 and 735 nm. (*F*) Comparison of growth rates during the batch and turbidostat modes. The batch growth rate was measured during an initial exponential growth phase (phase I, *open symbols*) and during a linear growth phase, *i.e.*, when the algae had reached the same biomass concentration as used in the turbidostat mode (phase II, *solid symbols*), from *Ettlia oleoabundans* (*circles*), *Scenedesmus ecornis* (*upward triangles*), *Scenedesmus obliquus* (*downward triangles*), *Chlorococcum novae-angliae* (*squares*), and *Desmodesmus quadricauda* (*diamonds*). Turbidostat growth rate measurements (*F*) are averaged over a single photobioreactor experiment (growth rate calculations were conducted every 6 h). Samples within the same encirclement can be compared with each other. Error bars show SD.

Table 1. Biomass concentrations and growth rates during continuous (turbidostat) growth, and batch growth, at the PPFD of 200 µmol m^{-2} s^{-1} at 25°C of five species of green algae. SD values are calculated from turbidostat growth rate measurements, conducted every 6 h over a single photobioreactor experiment. Growth rates during batch were measured during an initial exponential growth in a dilute culture (maximum specific growth rate) and during the time point the algae had reached the concentration used for the turbidostat mode (when the algal concentration increased linearly).

Species	Turbidostat		Batch	
	Biomass [g L^{-1}]	Growth rate [d^{-1}]	Growth rate, exponential phase [d^{-1}]	Growth rate, linear phase [d^{-1}]
Ettlia oleoabundans	0.434 ± 0.010	0.68 ± 0.11	0.55	0.24
Scenedesmus ecornis	0.426 ± 0.004	0.72 ± 0.04	0.84	0.38
Scenedesmus obliquus	0.434 ± 0.012	0.94 ± 0.10	1.07	0.30
Chlorococcum novae-angliae	0.420 ± 0.005	0.71 ± 0.15	1.11	0.41
Desmodesmus quadricauda	0.421 ± 0.007	0.85 ± 0.08	1.42	0.53

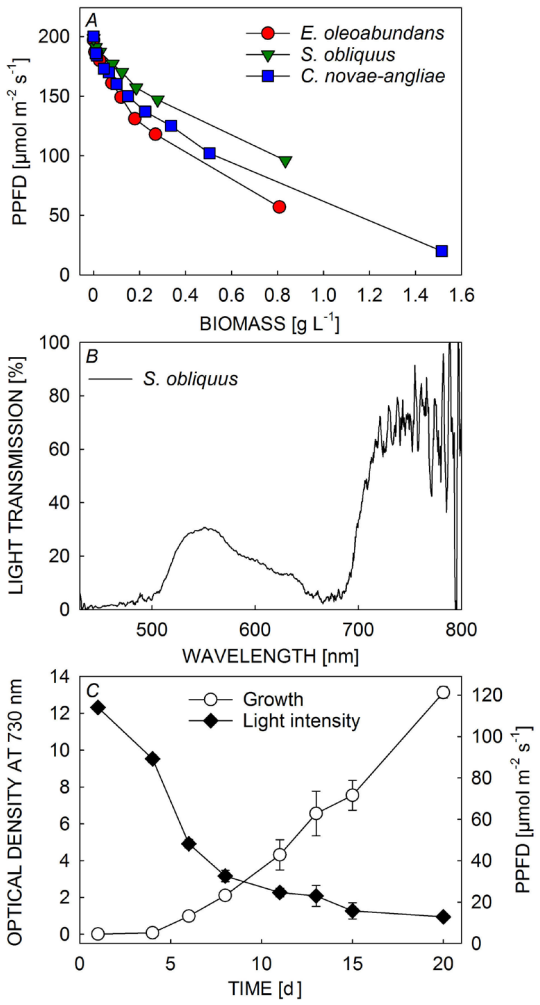

Fig. 2. Effects of cell density on light availability inside cultures of photosynthetic organisms. (*A*) PPFD values at 1.2 cm depth inside a cylinder filled with different dilutions of cultures of the indicated green algae. Biomass concentrations were calculated from dry mass measurements of the undiluted samples (three technical repetitions, error bars are smaller than the symbols). (*B*) A spectrum of light transmitted through an algal (*Scenedesmus obliquus*) culture (2.4-cm light path) with a biomass concentration of 0.834 ± 0.008 g L^{-1} [the highest cell density shown in (*A*)]. (*C*) Light intensity (*black symbols*) inside 50-ml cyanobacterial batch cultures after 1 to 20 d of growth (*open symbols*). Error bars show SD from three experimental repetitions.

chloroplasts and thereby inhibits the repair of damaged PSII units. A relatively high light intensity, instead of the growth light intensity, was used to observe clear photoinhibition. The direct proportionality between light intensity and the rate constant of photoinhibition (for a review, *see* Tyystjärvi 2013) guarantees that the results predict photoinhibition well at lower light intensities, too. Photoinhibition of PSII was monitored with the F_v/F_m parameter; a decrease in F_v/F_m is known to correlate well with the loss of oxygen evolution in photoinhibition experiments (for a review, *see* Tyystjärvi 2013). Clear differences in the rates of PSII photoinhibition were observed: after the 40-min illumination, 89, 80, 76, 54, and 39% of PSII remained active in *S. ecornis*, *D. quadricauda*, *S. obliquus*, *C. novae-angliae*, and *E. oleoabundans*, respectively (Fig. 3*A*). *E. oleoabundans*, the slowest grower in both the batch and turbidostat cultivation modes (Table 1), was indeed highly susceptible to photoinhibition of PSII, and *D. quadricauda*, the alga that was the most tolerant against the high-light-induced inactivation of PSII, exhibited high batch growth rates (but not the highest growth rate in the turbidostat mode; Table 1). However, only a weak correlation between photoinhibition and growth rates, in either batch or continuous culture, was observed (r^2 = 0.25–0.28; Fig 3*B*). The susceptibility of the algae to the damaging reaction of photoinhibition of PSII did not seem to be a decisive factor in determining the growth rate here.

If lincomycin did not completely inhibit the repair of damaged PSII units in the used algal species, the observed photoinhibition rates would not have been comparable. Therefore, we tested whether lincomycin blocked PSII repair in the used algal species (Fig. 3*C*). In all the cases, the F_v/F_m values increased when the algae were let to recover for two days at their growth light [PPFD of 40 µmol(photon) m^{-2} s^{-1}] after a strong high light illumination [PPFD of 1,500 µmol(photon) m^{-2} s^{-1} for 90 min] in the presence of lincomycin. However, the recovery was much faster and essentially complete in the absence of lincomycin (tested in all the algal species except *D. quadricauda*), indicating that the algae were not resistant to lincomycin (Fig. 3*D*). It has been shown that F_m is not reached simply by the reduction of the Q_A electron acceptor of PSII, but formation of a distinctive light-adapted closed state of PSII reaction center, formed *via* conformational changes in PSII, is required to obtain F_m (Sipka *et al.* 2021). The finding that the F_v/F_m values of PSII preparations isolated from healthy plant leaves or cyanobacteria are largely determined by a conformational change of the PSII reaction center may suggest that the lowering of F_v/F_m in photoinhibition is partly caused by a conformational change and partly by lowering of the photochemical efficiency of PSII. Therefore, an increase in F_v/F_m after photoinhibition in the presence of lincomycin (Fig. 3*C*) may not indicate an increase in PSII yield.

Light utilization capabilities and PSII electron transfer kinetics differed between the algae

To understand the limitations behind algal growth, we analyzed photosynthetic reactions of the algae by Chl *a* fluorescence measurements. The five green algae were illuminated with stepwise increasing intensities of white light, to record light-response curves (Fig. 4*A*). Based on the measurements, *E. oleoabundans*, *S. ecornis*, and *C. novae-angliae* were observed to have slightly higher light-use efficiencies (α; measured as the slope of the initial linear rise in the relative electron transfer rate in low light) than *D. quadricauda* or *S. obliquus*. *E. oleoabundans* had both the highest maximum (relative) electron transport rate (ETR_{MAX}) and minimum saturating irradiance (I_k) and *D. quadricauda* the lowest ones (Fig. 4*B*). In all the

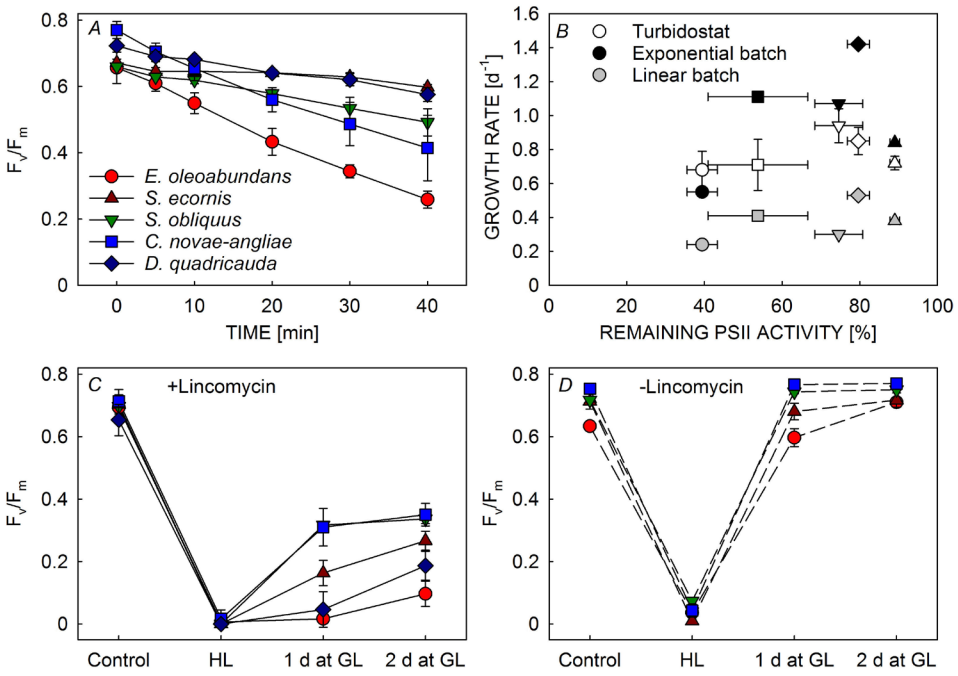

Fig. 3. Photoinhibition of PSII. (*A*) Changes in F_v/F_m, measured from five species of green algae during 0–40 min high light illumination [PPFD of 500 µmol(photon) m^{-2} s^{-1}] at 25°C in the presence of lincomycin. F_v/F_m values were measured after 10 min of darkness. Error bars, shown when larger than the symbol, show SD calculated from at least three experimental repetitions. (*B*) Growth rates of the algae measured during continuous culture (turbidostat; *open symbols*), and during batch culture, either during an initial exponential growth (*solid symbols*) or during a linear growth phase (when the algae had reached the same biomass concentration as used in the turbidostat mode; *grey symbols*), plotted against photoinhibition tolerance (remaining PSII activity after the 40-min illumination). The symbol shapes are the same as in (*A*). Growth data are from Fig. 1. (*C*) The algae were illuminated for 90 min, at the PPFD of 1,500 µmol(photon) m^{-2} s^{-1} at 25°C (HL, high light), in the presence of lincomycin. After the high light, the algae were let to recover for 2 d at their growth conditions [GL, growth light; PPFD 40 µmol(photon) m^{-2} s^{-1}]. (*D*) The algae (except *Desmodesmus quardicauda*) were illuminated as in (*C*) but in the absence of lincomycin. F_v/F_m values were measured after 20 min of darkness. The symbol shapes and colours are the same as in (*A*). Error bars, shown when larger than the symbol, show SD calculated from three experimental repetitions.

wavelengths measured, *E. oleoabundans*, *S. ecornis*, and *D. quadricauda* had bigger functional cross-sections of PSII [$\sigma_{II}(\lambda)$] than *S. obliquus* and *C. novae-angliae* (Fig. 4*C*). In line with the low $\sigma_{II}(\lambda)$, PSII specific electron transfer rates [taking into account the measured values for absorption cross-sections; ETR(II)] of *C. novae-angliae* were low (Fig. 4*D*) compared to the relative electron transport rates of the same species (Fig. 4*A*).

The measured light-use efficiencies (α parameter) did not, in general, correlate either with electron transfer rates, or with growth rates. Neither did the functional cross-sections of PSII correlate with the other measured photosynthetic parameters. Instead, minimum saturating irradiances (and to a lesser degree, ETR_{MAX}) correlated negatively with photoinhibition tolerance of the species ($r^2 = 0.86$; Fig. 4*E*). However, a low minimum saturating irradiance of an alga did not lead to a high continuous growth rate ($r^2 = 0.13$; Fig. 4*F*). Relatively low correlations were found also between minimum saturating irradiance and batch growth rate during the exponential ($r^2 = 0.40$) or the linear ($r^2 = 0.59$) growth phases.

To understand if the correlation of low minimum saturating irradiance for electron transfer rate with photoinhibition tolerance or growth rate could be explained by electron transfer kinetics of PSII, reoxidation of Q_A^- (an electron acceptor of PSII) was measured from the algae after a short dark-acclimation to oxidize Q_A (Fig. 5). The Q_A^- reoxidation kinetics in *S. obliquus* and *C. novae-angliae* were visually different from the kinetics in the other three algae (*D. quadricauda*, *E. oleoabundans*, and *S. ecornis*). The data were fitted to a model with three exponential components corresponding to $Q_A^-/Q_B \rightarrow Q_A/Q_B^-$ electron transfer (fast component), binding of a plastoquinone (PQ) molecule to an empty Q_B site, possibly including also the release of PQH_2 from the Q_B site (middle component), and charge recombination of $S_2Q_A^- \rightarrow S_1Q_A$, occurring in inactive PSII centers (slow component). The results show that the binding of PQ to PSII or the release of PQH_2 was slow in *S. obliquus* and *C. novae-angliae*, compared to the other algae, and the amount of inactive, Q_B nonreducing PSII centers was high in these two algae (Table 2).

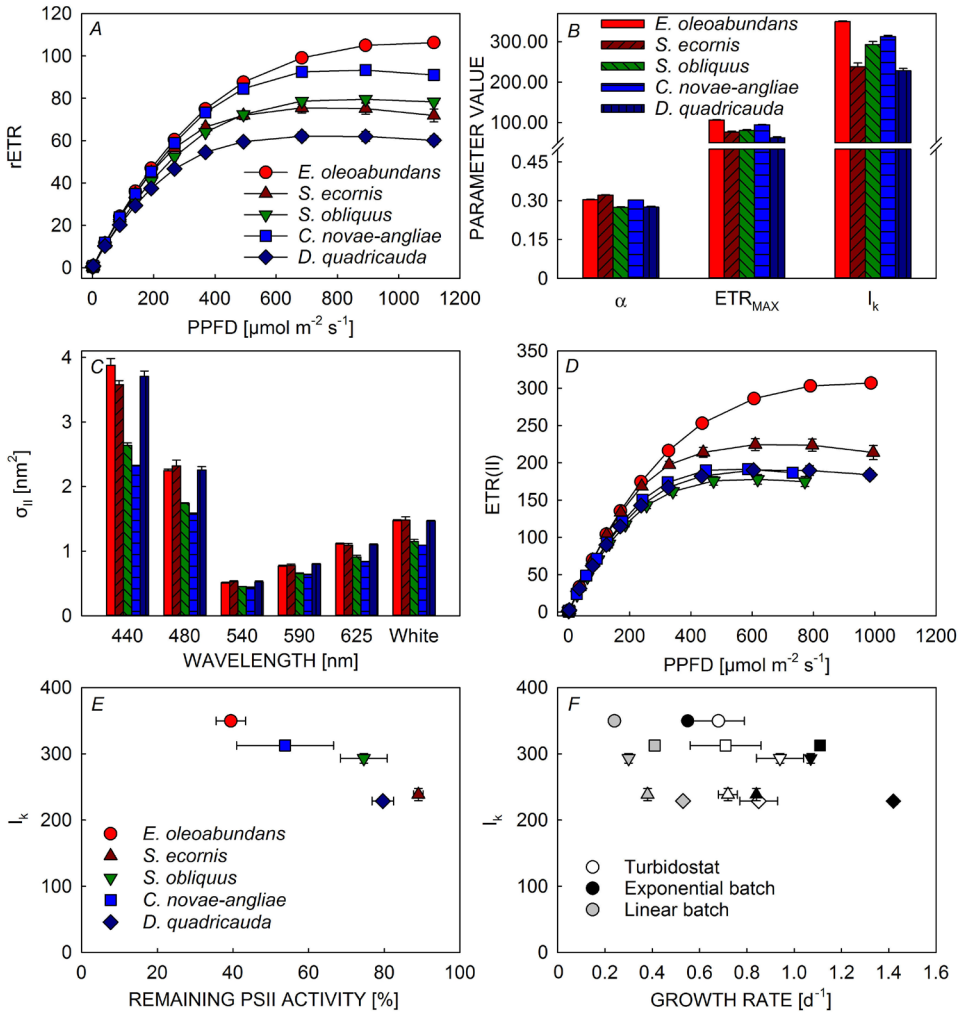

Fig. 4. Photosynthetic parameters in five species of green algae. (*A*) Relative electron transport rates (rETR) were measured by illuminating light-acclimated algal suspension for 90 s with the indicated intensity of light before firing a saturating pulse. (*B*) Parameters [α: light-use efficiency, ETR_{MAX}: maximum (relative) electron transport rate, I_k: minimum saturating irradiance] were calculated from the light curves. (*C*) Wavelength-dependent functional cross-sections of PSII [$\sigma_{II}(\lambda)$] were measured from dark-acclimated algae. (*D*) PSII specific electron transport rates [ETR(II)] were calculated by using rETR data from (*A*) and the white light specific σ_{II} values from (*C*). All data points are averages from three experimental repetitions and error bars, shown when larger than the symbol, indicate SD. (*E*,*F*) Comparison of I_k with photoinhibition tolerance (*E*) and growth rates (*F*) of the five algae. Growth rates of the algae were measured during continuous culture (turbidostat; *open symbols*), and during batch culture, either during initial exponential growth (*solid symbols*) or during linear growth (when the algae had reached the same biomass concentration as used in the turbidostat mode; *grey symbols*), the symbol shapes and colours (*E*) are the same as in (*A–E*). Growth data are from Fig. 1.

Discussion

Why do growth rates differ between batch and turbidostat modes?

The maximum specific growth rate, representing the highest cell division rate the organism is capable of, is usually (as well as here) calculated based on a dilute batch culture. However, to select the most suitable species for a biotechnological application that uses continuous culture, a test with batch culture may not reveal the fastest growing candidates; we observed poor correlations between growth rates in batch and turbidostat cultures in the tested five green algal species *S. obliquus*, *S. ecornis*, *D. quadricauda*, *C. novae-angliae*, and *E. oleoabundans* (Fig. 1). It has been proposed that even measurements of the maximum specific growth rate would be more reliable if done in a continuous culture (Barbera *et al*. 2019). However, the conditions selected for our turbidostat cultivation would not reveal the maximum rate of cell division.

The observation that one species excels (regarding its growth rate) in a batch culture and a different species excels in a continuous culture, may not be surprising. An obvious difference between the growth modes is that during the initial phase of batch culture, the algae experience (continuous) high irradiances when there is

little shading by other cells (*see* Fig. 2). *E. oleoabundans* grew slowly in the batch mode but showed an average growth rate in the turbidostat mode (Fig. 1). The high sensitivity of this species to photoinhibition of PSII (Fig. 3) may have been less important during continuous culture at a high cell density (and consequently lower light intensity) than at the beginning of the batch. Accordingly, algae cultured in an outdoor bioreactor were more prone to photoinhibition when their concentration was kept low (Hindersin *et al*. 2013). In general, attenuation of light by the antenna system of the organism itself or by a thick suspension of microalgae strongly protect against photoinhibition (Pätsikkä *et al*. 2002, Serôdio and Campbell 2021). Therefore, although we did not observe a general correlation between photoinhibition tolerance and growth rate (Fig. 3*B*), susceptibility to photoinhibition may be expected to limit growth under certain conditions, at least in certain species.

Small antenna size is a common acclimation response to high light (*e.g.*, Neale and Melis 1986, Treves *et al*. 2016). Therefore, the observed small PSII cross-sections of *S. obliquus* and *C. novae-angliae* (Fig. 4*C*) may have contributed to their relatively fast batch growth rates (Table 1). However, the slow forward electron transfer in these two algae (Fig. 5) may have affected the calculations of the cross-sections (which employs Q_A^- reoxidation parameters; Schreiber *et al*. 2012).

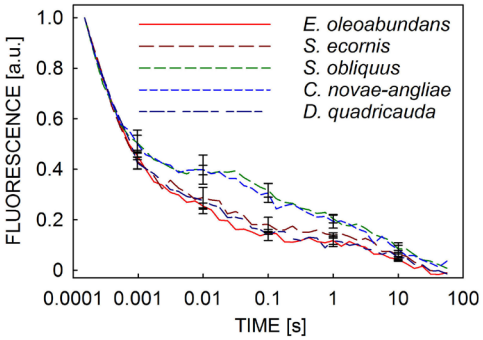

Fig. 5. Q_A^- reoxidation kinetics in five species of green algae. Dark-acclimated algae were given a single-turnover saturating flash, after which the decay in fluorescence yield was recorded (first data point 150 µs after the flash). To facilitate comparison, the lines are normalized to the first data point. The lines show averages from three experimental repetitions and error bars show SD.

In a dense continuous culture, the ability to utilize all light efficiently may be more important than during batch growth. However, as *D. quadricauda* and *S. obliquus*, the two fastest growers under the turbidostat mode (Table 1), had the lowest light-use efficiencies (Fig. 4*B*), it seems reasonable to assume that under our conditions, the biomass concentration during the turbidostat mode was low enough to prevent serious limitations in light availability. The fact that, in general, we only found very weak correlations between electron transfer parameters and growth rates (Fig. 4*F*) may not be surprising. Even though the fluorescence parameters usually correlate well with carbon fixation in different algae (Barranguet and Kromkamp 2000), the assimilated carbon may be used, instead of growth, to synthesize different (secondary) metabolites or storage compounds.

Dilute and dense cultures (whether batch or turbidostat) are also not directly comparable due to the fact that in a dilute culture, each algal cell continuously receives the same light intensity (at least when the light path is relatively short) while in a dense culture, mixing exposes the cells to rapidly changing light conditions, which has been shown to affect algal growth (Grobbelaar *et al*. 1996, Levasseur *et al*. 2018). In our case, however, growth rates even between dense batch and turbidostat cultures showed a weak correlation (Fig. 1*F*), although both culture types were mixed similarly.

Besides the difference in the attenuation of light, the growth medium develops differently during a batch than during a continuous growth experiment and many of these differences are likely to be strongly dependent on the experimental conditions and species studied. In the batch mode, main nutrients are usually consumed in a few days (*e.g.*, Valev *et al*. 2020), whereas in the turbidostat mode, the growth medium is continuously replenished. Depletion of nutrients may partially explain why the batch growth rates during the time the algae had reached the concentration that was later used for the turbidostat mode were slower than the growth rates during the turbidostat cultivation phase (Fig. 1*F*). The different nutrient contents in batch and turbidostat modes may also favor different algal species. However, Valev *et al*. (2020) observed that even though nutrient contents in the effluent were stable during a turbidostat run, ammonium contents were (constantly) low, compared to the influent, and no

Table 2. Kinetic parameters of Q_A^- reoxidation in five species of green algae, calculated by fitting the data shown in Fig. 5 to a model with three exponential components.

Species	$Q_A^- \rightarrow Q_B^-$ [µs]	PQ binding/PQH_2 release [ms]	$S_2Q_A^- \rightarrow S_1Q_A$ [s]	Functional PSII [%]	PSII with an empty Q_B site [%]	Q_B nonreducing [%]
Ettlia oleoabundans	380	15	7.4	71	18.5	10.5
Scenedesmus ecornis	352	24	9.6	72	15	13
Scenedesmus obliquus	312	138	12.4	67	15	17
Chlorococcum novae-angliae	330	84	9.8	67	15.5	17.5
Desmodesmus quadricauda	410	27	9.6	71	18	11

phosphate was detected in the effluent, indicating efficient nutrient uptake by algae cultured in a turbidostat.

What is the relationship between PSII photoinhibition and growth rate during continuous culture?

Some of the green algae tested here seemed very vulnerable to PSII damage while others were considerably more resistant (Fig. 3*A*). However, these differences did not in general lead to different growth rates in continuous culture (Fig. 3*B*). The highest light intensity at which a phototroph (an alga in this case) gains energy (*i.e.*, the highest intensity at which the costs of PSII repair do not exceed the energy that can be obtained by photosynthesis) depends on the species (Murphy *et al*. 2017). In addition, acclimation allows for example the green alga *Chlamydomonas reinhardtii* to grow at the extreme PPFD of 3,000 µmol(photon) m^{-2} s^{-1} (Virtanen *et al*. 2021). It is concluded that all the tested algal species were able to grow under the light intensity used in the present study [200 µmol(photon) m^{-2} s^{-1}]. A *Scenedesmus* species has previously been shown to possess an efficient repair cycle and to be able to maintain almost all PSII units functional even at 500 µmol(photon) m^{-2} s^{-1} (Komenda 1998), indicating that most probably the algae here did not experience net photoinhibition during the continuous culture. Also, here most of the algal species were able to repair their PSII units in less than 24 h, after a high light illumination that had led to a complete loss of PSII activity (Fig. 3*D*).

The efficient repair cycle indicates that at the PPFD of 500 µmol(photon) m^{-2} s^{-1} it is beneficial to invest the energy to keep PSII functional (rather than to wait for better times). Indeed, 500 µmol(photon) m^{-2} s^{-1} is oversaturating for all the studied algae (Fig. 4*B*); when (light) energy is not a limiting factor, it can be used to constantly repair PSII. The same situation may apply to the continuous culture here, where the used PPFD of 200 µmol(photon) m^{-2} s^{-1}, was close to saturating (Fig. 4*B*).

We observed that photoinhibition tolerance had a rather linear relationship with minimum saturating irradiance [or to a lesser degree with maximum (relative) electron transport rate]; the lower the saturating irradiance, the higher was the alga's tolerance to photoinhibition (Fig. 4). Does, therefore, the capacity for a high electron transfer rate under high light have something to do with the alga's susceptibility to PSII damage? Our result is in agreement with the suggestion that by increasing the rate of certain backward electron transfer reactions in PSII (on the expense of forward reactions), a desert alga *Chlorella ohadii* is able to acquire exceptional tolerance against high light, as these backward reactions may decrease singlet oxygen production by PSII (Treves *et al*. 2016). In addition, a high light-tolerant mutant of *C. reinhardtii* showed slower electron transfer from Q_A to Q_B and tolerance to PSII photoinhibition (Virtanen *et al*. 2019). What are the benefits of a slow forward electron transfer, one may wonder, as it could be assumed to lead to a slow growth rate? However, when light is not limiting, slow forward electron transfer capacity of PSII may not be critical; indeed, *C. ohadii* and the *C. reinhardtii* mutant grow and accumulate biomass efficiently under high light (Treves *et al*. 2016, Virtanen *et al*. 2019). Here, at 200 µmol(photon) m^{-2} s^{-1}, different electron transfer capacities of the algae (Fig. 4) did not translate to different turbidostat growth rates (Table 1). We also did not observe a correlation between Q_A^- reoxidation rates (Fig. 5) and photoinhibition tolerance of the algae (Fig. 3). Still, the hypothesis that gained photoinhibition tolerance comes with the price of a slower forward electron transfer is intriguing, as it could be yet another regulatory point that maintains the cost to benefit ratio of PSII repair and electron transfer to carbon fixation optimal. Indeed, Li and Campbell (2013) showed that while growth light is increased, which increased the growth rate, *Thalassiosira pseudonana* became more susceptible to photoinhibition.

In the present study, the photobioreactor experiments were conducted once with each algal species. Calculations of turbidostat growth rates and error bars associated with the calculated values are based on measurement of the accumulation of effluent every 6 h of growth and do not represent true biological repetitions. The turbidostat growth rates were 0.68–0.94 d^{-1}, and therefore the cell population in the reactor turned over in 1.1 to 1.5 d.

Conclusions: We have shown that batch growth is an inadequate method to predict the growth rate during a continuous culture, due to, *e.g.*, different light conditions. *E. oleoabundans* was very susceptible to photoinhibition of PSII and grew slowly under batch culture but otherwise tolerance to photoinhibition did not automatically increase the growth rate. We found a correlation between photoinhibition tolerance and fluorescence parameters indicating a slow forward electron transport of PSII. However, the observation requires further studies.

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