Elsevier Editorial System(tm) for Plant Physiology and Biochemistry

Manuscript Draft

Manuscript Number: PLAPHY-D-18-00528

Title: Enhanced photosynthetic linear electron flow in mixotrophic green microalga Ettlia oleoabundans UTEX 1185

Article Type: Research Paper

Keywords: Ettlia oleoabundans; Chlorophyll fluorescence; Photosystem II; Mixotrophy; Light energy use

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Università degli Studi di Ferrara | Dipartimento di Scienze della Vita e Biotecnologie

Ferrara, the 10th of April 2018

For the attention of the Editors
of *Plant Physiology and Biochemistry*

Dear Editors,

please find our manuscript entitled "*Enhanced photosynthetic linear electron flow in mixotrophic green microalga Ettlia oleoabundans UTEX 1185"*, which we are submitting for consideration to Plant Physiology and Biochemistry.

The manuscript deals with the photosynthetic physiology of a most studied green oleagineous microalga, as assessed in a modulated fluorescence study. The paper presents novel results revealing an improved light energy use in the alga grown in mixotrophic conditions in comparison with the autotrophic mode of cultivation.

The work described in this manuscript has not been published previously and is not under consideration for publication elsewhere. All authors have approved the submitted final version.

Hoping that the manuscript can meet the high quality requirements of your Journal, we convey you our best regards.

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Highlights

- Mixotrophic *E. oleoabundans* down-regulates dark chlororespiratory electron recycling.
- Under mixotrophy, linear electron flow from PSII to PSI is enhanced.
- A small, but significant, gain in PSII photochemical yield occurs in mixotrophic cells.
- Improved light energy use can depend on internal recycling of CO_2 generated by respiration of the taken up glucose.

Enhanced photosynthetic linear electron flow in mixotrophic green microalga *Ettlia oleoabundans* UTEX 1185

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Abstract

Basic understanding of the photosynthetic physiology of the oleaginous green microalga *Ettlia oleoabundans* is still very limited, including the modulation of the photosynthetic membrane upon metabolism conversion from autotrophy to mixotrophy. It was previously reported that, upon glucose supply in the culture medium, *E. oleoabundans* preserves photosystem II (PSII) from degradation by virtue of a higher packing of thylakoid complexes. In this work, it was investigated whether in the mixotrophic exponential growth phase the PSII activity is merely preserved or even enhanced. Modulated fluorescence parameters were then recorded under short-term treatments with increasing irradiance values of white light. It was found that the mixotrophic microalga down-regulated the chlororespiratory electron recycling from photosystem I (PSI), but enhanced the linear electron flow from PSII to PSI. Ability to keep PSII more open than in autotrophic growth conditions indicated that the respiration of the glucose taken up from the medium fed with CO_2 the carbon fixing reactions. The overall electron poise was indeed well regulated, with a lesser need for thermal dissipation of excess absorbed energy. It is proposed that the small, though significant, increase in PSII maximum quantum yield in mixotrophic cells just reflects an improved light energy use and an increased photochemical capacity as compared to the autotrophic cells.

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1. Introduction

Starting from a seminal paper by Tornabene et al. (1983), for 35 years the unicellular green microalga *Ettlia oleoabundans* (synonym of *Neochloris oleoabundans*) has become the subject of several studies aimed at understanding and improving the capacity of this organism to accumulate large amounts of intracellular neutral lipids (reviewed by Abu Hajar et al., 2017). In fact, research has primarily been focused on the alga as a potential source of biofuels, in particular biodiesel (among others, Hegel et al., 2017; Levine et al., 2011; Popovich et al., 2012; Pruvost et al., 2009; Santos et al., 2012; Yoon et al., 2015). Among the diverse modes of *E. oleoabundans* cultivation, mixotrophy has gained some special attention. Mixotrophy is defined as "the physiological feature of an organism whose cells use both photosynthesis and external organic matter as a source of carbon and/or non-carbon elements" (Selosse et al., 2017). In *E. oleoabundans* the mixotrophic mode of growth can lead to high yields of lipid-rich biomass (Baldisserotto et al., 2014; Giovanardi et al., 2013, 2014; Sabia et al., 2015; Silva et al., 2016).

A batch culture of *E. oleoabundans* supplied with glucose grows through two steps (Giovanardi et al., 2014a). The exponential growth phase is characterized by rapid cell divisions, with an increase in the maximum quantum yield (F_V/F_M) of photosystem II (PSII), the complex machinery initiating the electron transport chain in the photosynthetic membrane. During the subsequent stationary phase, cell divisions slow down until they stop, cell volume increases and neutral lipid droplets accumulate in the cytoplasm, while a progressive decline in F_V/F_M occurs (Baldisserotto et al., 2016; Giovanardi et al., 2014a). The increase in F_V/F_M of *E. oleoabundans* during the exponential phase is interesting, because in green algae mixotrophy often leads to an early down-regulation of photosynthesis, with a decrease in PSII photochemical yield (Giovanardi et al., 2016; Martinez and Orus, 1991; Valverde et al., 2005). A reduced activity of PSII caused by the mixotrophic growth can indeed be interpreted in terms of a feedback inhibition loop on the photosynthetic machinery by the supplied organic carbon (Burch et al., 2015; Demmig-Adams et al., 2014). Giovanardi et al. (2017) discovered that the preservation of PSII activity in mixotrophic *E. oleoabundans* was related to a modified supramolecular organization of the thylakoid complexes. In particular, PSII tended to organize with photosystem I (PSI) and the light harvesting complex II (LHCII) in large megacomplexes, which

were instead not resolved in the autotrophic cells. However, it was also found that a higher F_V/F_M ratio of mixotrophic cells actually resulted from a down-regulated chlororespiration (Giovanardi et al., 2017). This last process depends on alternative photosynthetic electron pathways, recycling electrons from stromal reducing equivalents to the plastoquinone pool in darkness (Bennoun, 1983; Feild et al., 1998). Operation of such electron recycling route is deemed to be important as a safety valve against an excess of electron pressure in the light-exposed samples (reviewed by Alric and Johnson, 2017). In fact, electrons recycled from NAD(P)H to the plastoquinone pool can then be passed to molecular oxygen in a reaction catalysed by a plastoquinol terminal oxidase, PTOX, while contributing to the generation of the trans-thylakoidal ΔpH (Alric and Johnson, 2017). Electron recycling routes can be quite active also in green algae (Peltier et al., 2010). In darkness, effects of an operative electron recycling are an increased basal fluorescence of PSII (F_0), because plastoquinone is not fully re-oxidized and a population of PSII remains closed, i.e., with reduced Q_A (Plyusnina et al., 2013). Moreover, another effect is a decreased maximum PSII fluorescence (F_M) in darkness, because protons are accumulated in the lumen, leading to activation of thermal de-excitation as non-photochemical quenching (*NPQ*; Cruz et al., 2011). Consequently, the F_V/F_M calculated in a dark-acclimated sample is underestimated. In *E. oleoabundas*, a correction of F_V/F_M taking into account the occurrence of chlororespiration led to comparable values between autotrophic and mixotrophic cells (Giovanardi et al., 2017). However, the impact of mixotrophy on the photosynthetic electron flow in *E. oleoabundans* remains elusive.

Using a formalism based on energy partitioning models (reviewed by Lazár, 2015), *Y(NO)* is a parameter related to the non-regulatory dissipation of absorbed energy. Importantly, *Y(NO)* gives information on the state of the electron flow between PSII and PSI (Grieco et al., 2012; Tikkanen et al., 2017). An efficient regulation of the electron flow depends on the capacity of PSI to receive the electrons that then feed the dark reactions of photosynthesis and, at the same time, on the capacity to alleviate the electron pressure on PSI by recirculating excess electrons from the stromal acceptors to the Cytochrome b_6f complex, which is interposed between PSII and PSI in the electron transport chain (Chaux et al., 2015). A less fluent electron flow towards PSI causes the accumulation of reduced plastoquinone at the acceptor side of PSII, with an increase in *Y(NO)*. With respect to *Y(NO)*, a comparison between autotrophic and

mixotrophic *E. oleoabundans* cells is presently not conclusive about electron transport efficiency. In fact, in previous works probing *E. oleoabundans* with high irradiance, it was found either a decrease, or no variation, or an increase in *Y(NO)* (Baldisserotto et al., 2014, 2016; Giovanardi et al., 2017, respectively).

A plant organism can preserve the photosystems against photodamage through concerted types of short-term photoregulation, i.e., the regulation of the electron transport to reduce the electron pressure on PSI (Chaux et al., 2015) and the regulation of light energy harvesting and funnelling to photosystems, including the de-excitation as thermal dissipation of excess absorbed energy, meant by *NPQ* (Demmig-Adams et al., 2014). *NPQ* is activated upon the creation of a trans-thylakoidal ΔpH during the electron flow in the membrane and basically depends on the balance between the proton pumping into the thylakoid lumen during the electron flow and the proton use for the ATP synthesis catalyzed by the chloroplastic ATP synthase. Ability to generate *NPQ* also depends on structural changes and specific modulators, including regulatory thylakoid proteins and carotenoids (reviewed by Goss and Lepetit, 2015; Ruban, 2016).

In spite of an ever increasing interest in *E. oleabundans* for its biotechnological potential as a source of lipids, the basic understanding of the microalgal photosynthetic physiology is still very limited. In particular, the modulation of the photosynthetic function upon metabolism conversion from autotrophy to mixotrophy is obscure in many instances. In particular, we consider of outmost importance to understand whether under mixotrophy the PSII activity is enhanced (Baldisserotto et al., 2016) or not (Giovanardi et al., 2017). To this aim, we recorded fluorescence parameters under short-term treatments with increasing irradiance values of white light, taking into due account the impact of chlororespiration as a non-negligible variable for a correct comparison of fluorescence parameters between autotrophic and mixotrophic cells.

2. Material and methods

Ettlia oleoabundans (syn. *Neochloris oleoabundans*) (S. Chantanachat & Bold) J. Komárek, strain UTEX 1185 (Trebouxiophyceae, Chlorellales - taxonomic position according to Garibay-Hernández et al., 2017) was maintained in static liquid culture in BM medium (Baldisserotto et al., 2012) in a growth chamber at 24 ± 1°C, 80 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR), 16:8 h light:darkness photoperiod. For experiments, inoculum of the microalga was done according to Giovanardi et al. (2017), so as to have an

initial cell density of 0.6 ± 0.1 ×10^6 cells mL^{-1} in fresh BM medium for autotrophic cultures, or in fresh BM medium supplied with 2.5 g L^{-1} glucose for mixotrophic cultures. Experimental cultures were set up in 500 mL-Erlenmeyer flasks with a culture volume of 300 mL and maintained under constant shacking at 80 rpm, the other culture conditions described above being the same. During the growth of cultures, cell density was checked by both cell density counts with a Thoma haemocytometer and optical density with a Pharmacia Ultrospec spectrophotometer (Baldisserotto et al., 2012). In order to have an experimental material fully comparable to previous reports, cultures were sampled in the late exponential phase of growth at the fifth or sixth day from the inoculum, i.e., when the F_V/F_M showed the maximum difference between autotrophic and mixotrophic cultures (Baldisserotto et al., 2016; Giovanardi et al., 2014, 2017).

For fluorescence analysis, cells were collected by centrifugation at 8000 *g* for 10 min. The resulting cell pellet was deposited as a single drop onto small strips of filter paper soaked with BM medium (Ferroni et al., 2011). The strips were kept in darkness for 10 min for dark acclimation of the cells. Subsequently, a pulse amplitude modulated fluorimeter (ADS-OS1-FL, ADC Bioscientific Ltd., Herts, UK) was used for fluorescence analysis. The fiberoptic was driven to the algal pellet spot for determination of the basal fluorescence F_0 and then a saturation pulse (0.6 s) was applied for determination of the maximum fluorescence F_M. The values were used to calculate the PSII fluorescence yield in the dark-acclimated state as $F_V/F_M = (F_M – F_0)/F_M$. For the induction curves, a halogen lamp was used as the source of white actinic light, which was driven to the sample through fiberoptics. The PAR irradiance reaching the sample was accurately set as uniform as possible in the measuring spot of the sample clip and its precise value was checked with a quanto-photoradiometer (Delta Ohm HD9021). Actinic light was driven to the sample at the irradiance of 12.5, 25, 50, 100, 200, 400 and 800 μmol m^{-2} s^{-1}. After turning on the actinic light, the sample was probed applying a saturation pulse every min for 10 min. The fluorescence value at each time *Ft* and the maximum value F_M' in the light-acclimated state were measured. Then, each sample was analysed for dark relaxation: actinic light was turned off and a saturation pulse was applied after 1, 2, 5, 10 and 20 min of darkness. The values obtained at the end of the induction phase (10^{th} min) were used as an approximation of steady state fluorescence values to build light-response curves.

The formalism of the energy partitioning proposed by Hendrickson et al. (2004) was used to

combine the fluorescence values into informative parameters. The photochemical yield of PSII was calculated as $Y(PSII) = (F_M' – Ft)/F_M'$ (Genty et al., 1989). $Y(NO) = Ft/F_M$ was used to evaluate the electron poise of the plastoquinone pool (Grieco et al., 2012). $Y(NPQ) = Ft/F_M' - Ft/F_M$ was used to evaluate the regulatory thermal dissipation. In the formalism of Hendrickson et al. (2004), the three parameters are interpreted as complementary quantum yield of competing processes, so that $Y(PSII) + Y(NO) + Y(NPQ) = 1$. The yields can be combined to obtain:

$$NPQ = Y(NPQ)/Y(NO) = (F_M - F_M')/F_M' \quad \text{(eq. 1)}$$

which corresponds to the common Stern-Volmer formulation of the non-photochemical quenching (Bilger and Björkmann, 1991; Ferroni et al., 2014; see Lazár, 2015 for equation derivation).

By analogy, a synthetic index of the photochemical capacity was calculated as (Ferroni et al., 2016; Lazár, 2015; Porcar-Castell et al., 2014):

$$PQ = Y(PSII)/Y(NO) = F_M/Ft – F_M/F_M' \quad \text{(eq. 2)}$$

which, in dark-acclimated samples, reduces to:

$$PQ_{darkness} = F_M/F_0 – F_M/F_M = (F_M \cdot F_M - F_0 \cdot F_M)/(F_0 \cdot F_M) = F_M \cdot (F_M - F_0)/(F_0 \cdot F_M) = F_V/F_0 \quad \text{(eq. 3)}$$

F_V/F_0 is a very sensitive parameter with respect to the maximum photochemical capacity of a plant sample (Lichtenthaler et al., 2005).

Samples induced with the lowest irradiance of 12.5 μmol m^{-2} s^{-1} were used to measure the impact of chlororespiration on fluorescence values, in particular the maximum F_M' was used as an approximation of the true F_M to correct all other F_M-dependent parameters (Cruz et al., 2011; Kalaji et al., 2014).

Relative linear electron transport rate was calculated as $rETR = Y(PSII) \cdot Irradiance \cdot \alpha_{PSII}$, where α_{PSII} is the fraction of energy absorbed by PSII. In most cases, it is commonly assumed the absorbed photons are equally distributed between PSII and PSI, i.e., α_{PSII} = PSII/(PSI + PSII) = 0.5 (Baker, 2008; Miyake et al., 2005; Schreiber, 2004;). We accepted this assumption for autotrophic cells. In mixotrophic cells, we previously reported a decrease in PSI amount by 40% and conversely an increase in PSII by 47% (evidence from relative quantification by immunoblot in gels loaded on the same chlorophyll basis; Giovanardi et al., 2017). Therefore, the α_{PSII} was corrected to 0.7. However, the absolute amount of photosystems, the actual energy distribution between PSI and PSII, and also the PAR absorption of cells remain unknown; therefore, the rETR

was only used in a comparative way between autotrophic and mixotrophic, without analysis of parameters that can be derived from rETR-light curves.

3. Results

3.1 Impact of electron recycling in darkness in autotrophic and mixotrophic cells

F_V/F_M of mixotrophic cells confirmed to be higher than in autotrophic cells by ca. 9%, in agreement with previous reports (Baldisserotto et al., 2016; Giovanardi et al., 2014) **(Fig. 1A).** Based on Giovanardi et al. (2017), this increase is the result of the operation of an electron recycling in darkness, which impacts on the determination of the fluorescence parameters. To evaluate the fraction of F_V/F_M hidden by dark chlororespiration, we exposed the cells to a low light intensity of 12.5 μmol m^{-2} s^{-1} (white light <16% of the growth PAR) for 10 min, in order to activate the electron transport up to final acceptors and, at the same time, allow the light-induced activation of the ATP synthase for the consumption of protons accumulated in the lumen. In autotrophic cells, the F_t value did not increase beyond F_0 during the first min of illumination **(Fig. 1B)**, indicating that in this time interval the electron flow allowed by PSI oxidized a partially dark-reduced plastoquinone. Subsequently, PSII progressively fed the pool. However, the effect of illumination was much more evident on F_M, which increased progressively up to a plateau level after the 7^{th} min of induction **(Fig. 1B)**. This attested the light-induced reversal of a non-photochemical quenching of fluorescence developed in darkness, which is a typical effect of the chlororespiratory accumulation of protons in the lumen (Cruz et al., 2011). As soon as light was turned off, protons again accumulated because of chlororespiration, causing a new decrease in F_M. In terms of NPQ, the result of this kinetics is shown in **Fig. 1C**, i.e., negative values of this parameter upon light induction.

In the mixotrophic cells, the same protocol did not show a major effect of a chlorespiratory electron recycling on F_0 and of proton accumulation on F_M. However, *NPQ* showed that the electron recycling was not completely absent, in fact an increasing trend was maintained during dark relaxation, though much slower than in autotrophic samples **(Fig. 1 B, C)**.

Based on the above evidence, for a correct comparison of the maximum photochemical activity of PSII in autotrophic and mixotrophic cells, for each sample the highest F_M' obtained during the low light

induction was used to recalculate the best approximation of the true F_V/F_M. In autotrophic cells, the difference between the corrected F_V/F_M and the dark F_V/F_M represents the yield of PSII photochemistry "lost" because of the ΔpH-activated thermal dissipation in darkness, which we call "missing quantum yield" *Y(miss*). Within a range of dark F_V/F_M of 0.680-0.740, this *Y(miss)* was independent of the dark F_V/F_M value itself, and its average value was 0.034 ± 0.003 (SE, *n=7*), corresponding to ca. 5% of the corrected F_V/F_M. The same calculations done for the mixotrophic cells confirmed an average impact on F_V/F_M of ca. 0.1%, i.e., completely negligible *Y(miss)*.

The average values of F_V/F_M obtained for mixotrophic samples and autotrophic samples, with and without the correction for the chlororespiration, were then compared. The determinations obtained from 21 independent mixotrophic and autotrophic cultures were used. Upon the correction of F_V/F_M for *Y(miss)*, in the autotrophic cells the F_V/F_M was still significantly lower than in the mixotrophic (*t*-test, $P<10^{-4}$), although in absolute terms the difference was very small (-2,6%; **Fig. 1A**).

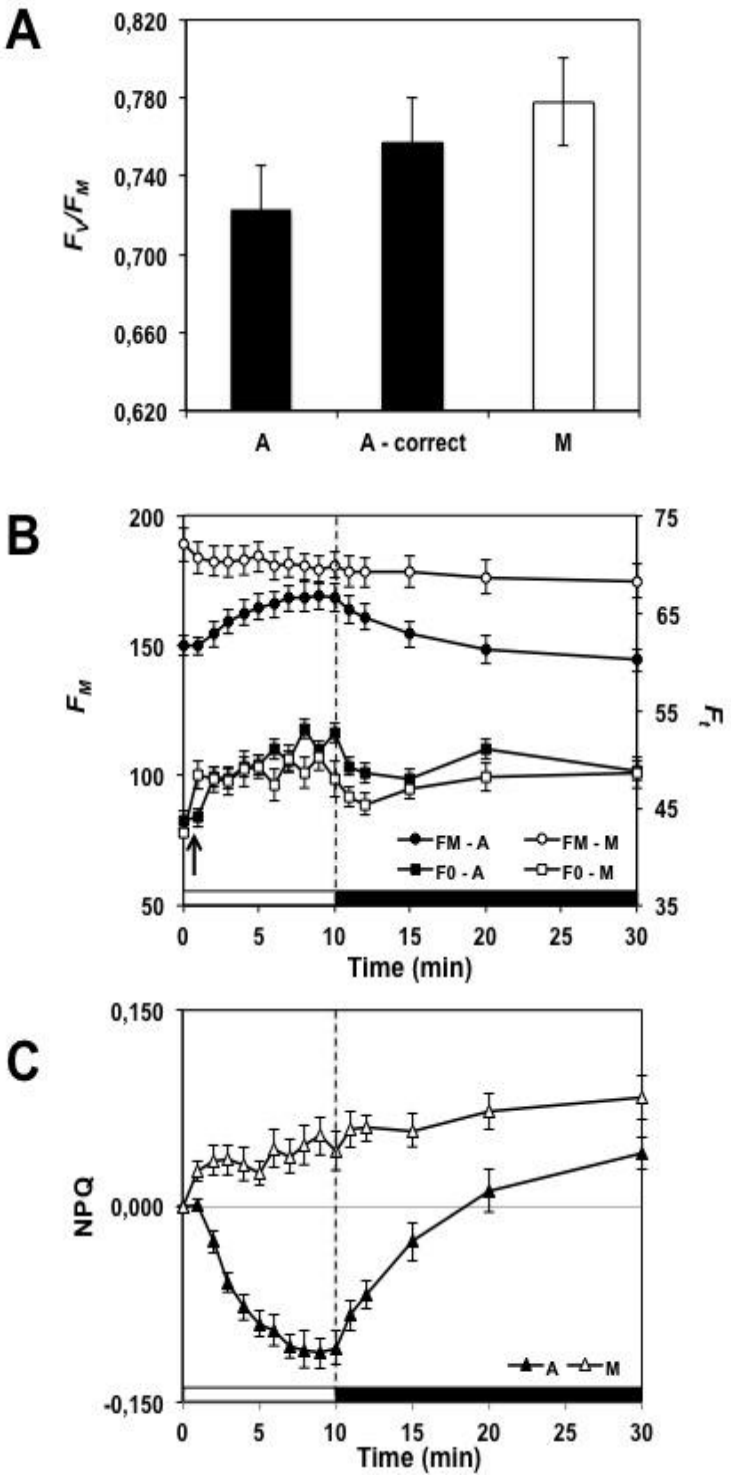

Figure 1. Effects of dark electron recycling in *Ettlia oleoabundans* grown autotrophically or mixotrofically.

A. Maximum PSII quantum yield F_V/F_M in autotrophic cells (A), in autotrophic cells after correction for dark chlororespiration (A – correct) and in mixotrophic cells (M). Mean values with SE of *N>40.*

B. Effect of induction with 12.5 μmol m^{-2} s^{-1} and subsequent relaxation in darkness on F_M and *Ft*. The arrow points to the different behavior in the rise from F_0 to *Ft* in autotrophic and mixotrophic cells. Mean values with SE of *N*=7-9.

C. *NPQ* induction and relaxation under the same conditions as in B.

3.2 *Induction and relaxation curves*

Upon exposure to light, a dark-acclimated sample initiates the electron transport chain, with a reduction of the plastoquinone pool that is related to the functioning of the entire photosynthetic machinery (Kalaji et al., 2014). The fluidity of the electron flow and the occurrence of thermal de-excitation processes decides about the fraction of PSII that can remain open under each irradiance. **Fig. 2A** reports the kinetics of changes in Y(PSII) upon 10 min induction with different irradiance levels and during the subsequent relaxation in darkness. In all samples and for each irradiance level, Y(PSII) promptly decreased to a quasi-steady state value, generally lower in autotrophic than in mixotrophic cells. After turning off the actinic light, Y(PSII) rapidly recovered to values similar to those of a dark-acclimated sample, except under the two higher irradiances, in which recovery was slower and incomplete.

The capacity to keep the electron transport chain under control was analysed using Y(NO), i.e., Ft/F_M. This parameter normalizes the *Ft* against the maximum F_M and is very sensitive to conditions in which the electron transport chain is affected by an imbalance that a plant organism is not able to adequately adjust (Grieco et al., 2012). To avoid distorted values of this parameter, it is necessary to know the true maximum value of F_M. However, we showed in 3.1 that, in the autotrophic cells, the dark chlororespiration led to an underestimation of F_M. Different from the samples irradiated with 12.5 μmol m^{-2} s^{-1}, the "best F_M" could not be approximated for each independent set of data in the induction experiments with higher irradiance, because the accumulation of protons determined by the photosynthetic electron flow prevailed over the reversal of the chlororespiratory proton gradient. In order to take into account the F_M underestimation due to ΔpH occurring in darkness, we used the formalism of the energy partitioning (Hendrickson et al., 2004). *Y(NO)* is represented as a quantum yield of dissipative processes without a regulatory role and, together with *Y(PSII)*, the complement to unity is obtained with the third quantum yield *Y(NPQ)*, representing the regulatory thermal dissipation (Hendrickson et al., 2004). Since *Y(miss)* also relates to a yield of regulatory non-photochemical quenching, it was incorporated into *Y(NPQ)* and, conversely, subtracted from *Y(NO)*. Consequently, the whole equivalence became the following:

$$Y(PSII) + [Y(NO) - Y(miss)] + [Y(NPQ) + Y(miss)] = 1 \quad \text{(eq. 4)}$$

Therefore, we used the best estimated value of *Y(miss)* = 0.034 calculated from the autotrophic samples induced with low light (section 3.1) to correct *Y(NO)* and *Y(NPQ)* in autotrophic samples under any irradiance.

In the autotrophic cells and for each irradiance, the Y(NO) value promptly increased soon after light was turned on, and was maintained almost constant during the entire 10 min induction. In the mixotrophic cells the response of *Y(NO)* was similar, but during induction with 400-800 μmol m^{-2} s^{-1} a decreasing trend after the peak occurred, showing the activation of a mechanism capable to relief the electron poise within some ten min (**Fig. 2B**).

The *Y(NPQ)* of autotrophic cells started from the value of *Y(miss)* in darkness, as a consequence of the correction. With the exception of the lowest irradiance (see 3.1), all irradiance levels attested the fast accumulation of protons within the first min of induction, with a peak in *Y(NPQ)*. Subsequently, for inductions at 200 μmol m^{-2} s^{-1}, *Y(NPQ)* underwent a decrease because of the light-induced activation of the ATP synthase and the Calvin-Benson-Bassham cycle (Tikhonov, 2015). Only at 800 μmol m^{-2} s^{-1} *Y(NPQ)* showed a slow increase after the peak. Extent of recovery in darkness was clearly related to the intensity of light used during the previous irradiation and its kinetics was not merely decreasing, but also affected by the operation of the electron recycling, which explains some slowly increasing trend in the dark (**Fig. 2C**). In mixotrophic cells, *Y(NPQ)* was visibly less induced than in the autotrophic counterpart. After the initial induction, no evident decrease in *Y(NPQ)* occurred, but instead a prominent further rise characterized the samples exposed to 400 and 800 μmol m^{-2} s^{-1}. Moreover, at the highest irradiance, *Y(NPQ)* relaxation in darkness appeared modified in its kinetics as compared to the autotrophic counterpart.

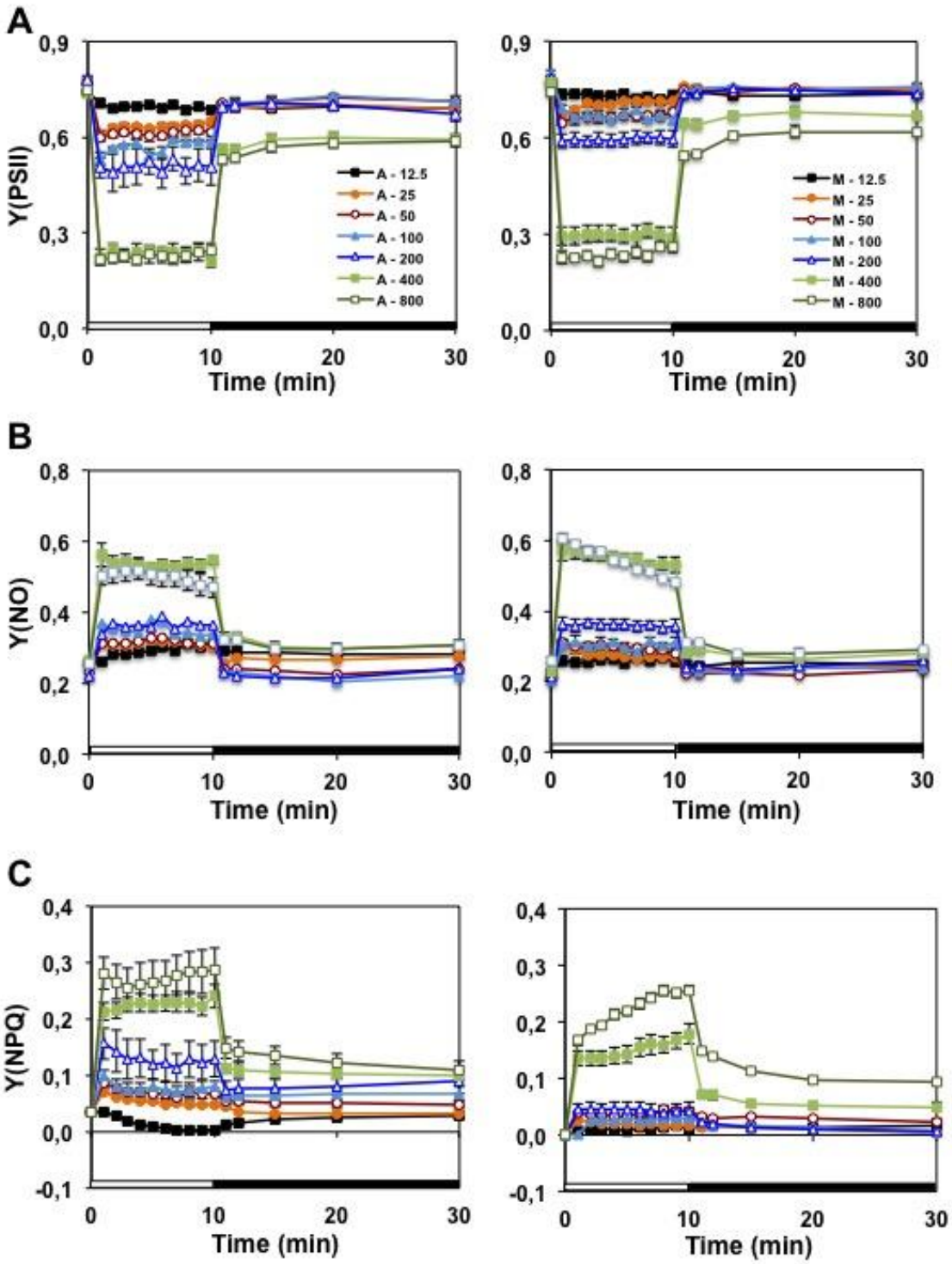

Figure 2. Light induction and dark relaxation of quantum yields in *Ettlia oleoabundans* grown autotrophically or mixotrofically. In each row, the left graph shows trends obtained from autotrophic cells, the right graph the mixotrophic counterpart. Kinetics were recorded upon short-term treatment with irradiance levels as reported in the legend. Mean values with SE of *N=3-9.*

A. Yield of PSII photochemistry, *Y(PSII)*.

B. Yield of non-regulatory energy dissipation, *Y(NO)*.

C. Yield of regulatory thermal dissipation, *Y(NPQ)*.

3.3 Light curves of fluorescence parameters

Light-response curves of fluorescence-derived parameters provide a straightforward tool to compare samples possibly differing in their use of light. Curves were drawn using the values recorded at the end of the light inductions shown in **Fig. 2**. Therefore a condition of steady state photosynthesis was approximated, in which the most important regulatory mechanisms had been activated during the 10 min-long exposure to white light.

In the *Y(PSII)*-light curve, the first data point corresponds to the F_V/F_M, in particular the value corrected for *Y(miss)* was used for the autotrophic cells (**Fig. 3A**). Values in the light-acclimated state do not need any correction, *Y(PSII)* being independent of F_M (Genty et al., 1989). Interestingly, *Y(PSII)* was consistently higher in mixotrophic than in autotrophic cells, except at the highest irradiance level of 800 μmol m^{-2} s^{-1}. In a range of 50-400 μmol m^{-2} s^{-1}, the difference in photochemical quantum yield between the two types of samples was nearly constant around 0.07.

The higher ability of mixotrophic cells to maintain PSII open could depend on a more fluid electron flow. The light dependency of *Y(NO)* illustrates that the plastoquinone pool in the photosynthetic membrane of the microalga underwent a steady rise upon increasing irradiance, up to reaching a plateau at 400-800 μmol m^{-2} s^{-1}. The divergence in *Y(NO)* between autotrophic and mixotrophic cells was specific to the range of the low light intensities, specifically, lower than the growth light irradiance. At higher irradiance, *Y(NO)* curves tended instead to overlap (**Fig. 3B**).

Analysis of *Y(NPQ)*-light curves showed the progressive activation of thermal dissipation mechanisms for increasing levels of light intensity in both types of samples (**Fig. 3C**). Thanks to the correction of *Y(NPQ)* for *Y(miss)* in autotrophic cells, it was clear that the extent of regulatory thermal dissipation was larger in the autotrophic cells as compared to the mixotrophic. The divergence between autotrophic and mixotrophic samples was wider at irradiance in the range of growth light and just above it (200 μmol m^{-2} s^{-1}), while for higher values they tended to converge to similar values. Therefore, *Y(NO)* and *Y(NPQ)* revealed their different relevance for the regulation of the electron flow in relation to the light intensity.

Stern-Volmer *NPQ* expression for the thermal dissipation was also calculated to allow an easier

comparison with literature data (e.g., Serôdio and Lavaud, 2011; **Fig. 3D**). Conclusions about trends of *NPQ* were essentially similar to those obtained for *Y(NPQ)*, being *NPQ = Y(NPQ)/Y(NO)*. However, it is interesting to observe that, with this representation, the total thermal dissipation capacity of *E. oleoabundans* was quite low even at the highest irradiance for both samples (*NPQ* < 0.8). This clearly depended on the progressively higher values reached by *Y(NO)* in this microalgal species upon increasing irradiance.

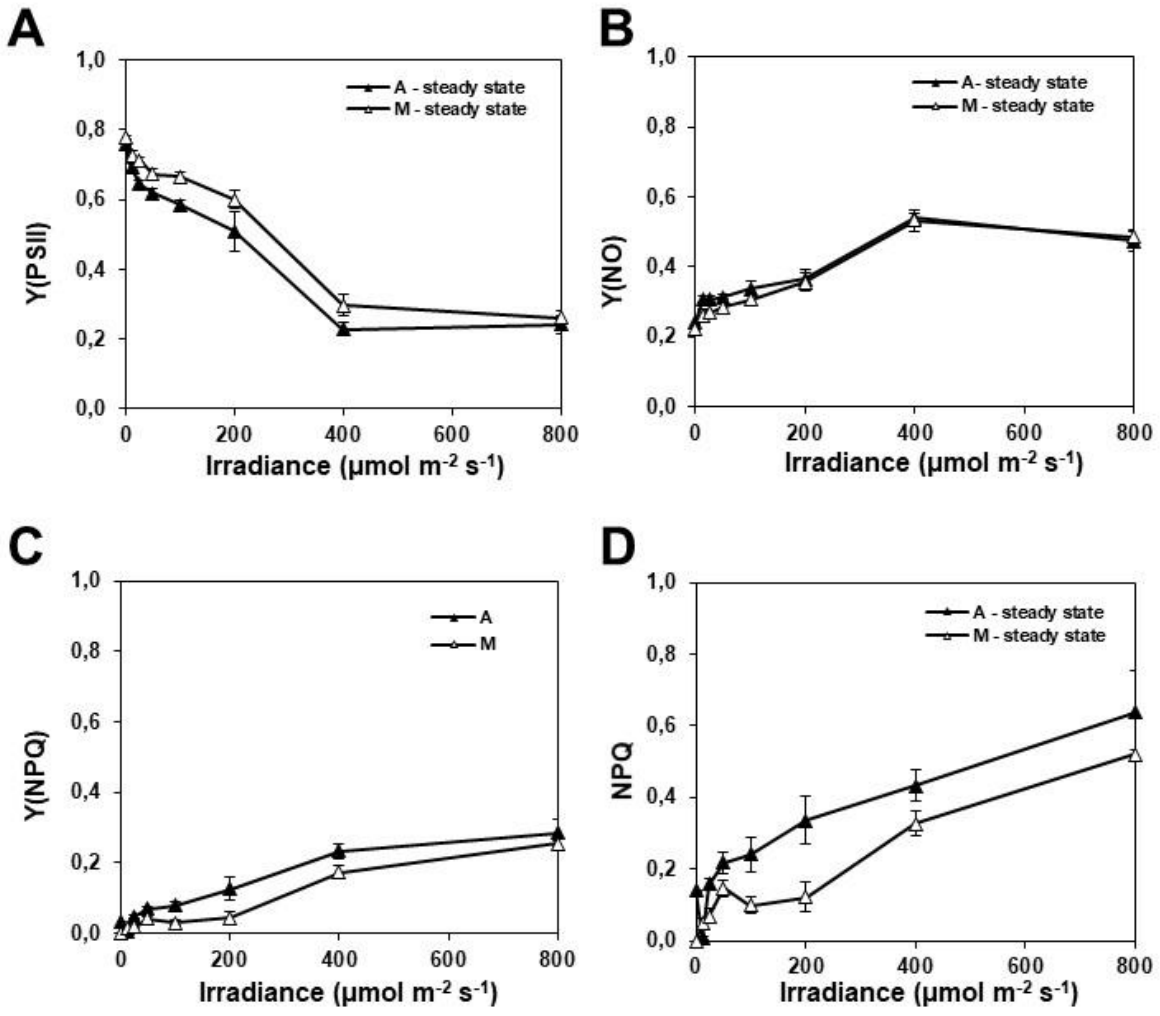

Figure 3. Light curves of fluorescence-derived parameters in *Ettlia oleoabundans* grown autotrophically or mixotrofically. Mean values with SE of *N=3-9.*

A. Yield of PSII photochemistry, *Y(PSII)*.

B. Yield of non-regulatory energy dissipation, *Y(NO)*.

C. Yield of regulatory thermal dissipation, *Y(NPQ)*.

D. Non-photochemical quenching, *NPQ*.

3.4 Photochemical capacity, electron transport, PSII photoinhibition

In this paragraph, we present some additional information base on the recorded fluorescence data. First of all, let us consider the result about F_V/F_M and *Y(PSII)* (**Fig. 1A** and **3A**), in particular the fact that a very small difference in F_V/F_M resulted in a quite important difference in Y(PSII) in the light-acclimated state. The photochemical capacity of PSII was proposed to be better represented by the F_V/F_0 ratio, which is not a yield, but is characterized by a higher dynamic range than F_V/F_M (Lichtenthaler et al., 2005). F_V/F_0 results from the *PQ* parameter calculated in the dark-acclimated state ($PQ_{darkness}$, see eq. 3) and, according to the formalism of energy partitioning (Lazár, 2015), is the yield ratio *Y(PSII)/Y(NO)* in the case of a dark-acclimated sample. In general, under any light condition *PQ* normalizes the *Y(PSII)* on the electron traffic at the level of the plastoquinone pool in terms of *Y(NO)*. The *PQ*-light curve of **Fig. 4A** shows that F_V/F_0 in mixotrophic cells was 12% higher than in autotrophic cells. In mixotrophic cells in the light-acclimated state, the steady state PQ was maintained higher than in autotrophic cells by a nearly invariable percentage of 20-25%, until the two types of samples decayed to the same low *PQ* value at 400-800 μmol m^{-2} s^{-1}. Starting from *Y(PSII)*-light curves (**Fig. 3A**), it is also possible and very common to make evaluations about the relative electron transport, rETR. Since rETR is merely a mathematical transformation of *Y(PSII)*, it is obvious that it reflects the difference between autotrophic and mixotrophic cells already shown for *Y(PSII)*. However, in a previous work, we thoroughly characterized the thylakoid membrane composition of *E. oleabundans* and found that the relative amounts of PSI and PSII neatly changed in favour of PSII in mixotrophic cells (Giovanardi et al., 2017). The data points shown in **Fig. 4B** represent the rETR under the assumption that the fraction of energy absorbed by PSII has changed from 50% in autotrophic cells to 70% in mixotrophic cells by virtue of a changed PSII/PSI stoichiometry. Results with *PQ* and rETR are strongly indicative that the linear electron flow was enhanced in the mixotrophic cells.

The third element we analysed was the degree of PSII photoinhibition experienced by *E. oleoabundans* cells. In this case, the values of PSII photochemistry measured in the dark-acclimated state were compared after the induction/relaxation protocol. The dark-acclimated F_V/F_M was not corrected for the autotrophic cells, because it was assumed that the chlororespiratory electron recycling had a

comparable impact on the values after the first dark-acclimation and, following the light induction, after the 20 min dark relaxation. The results in **Fig. 4C** show that the capacity of PSII photoprotection in mixotrophic and autotrophic cells was very similar.

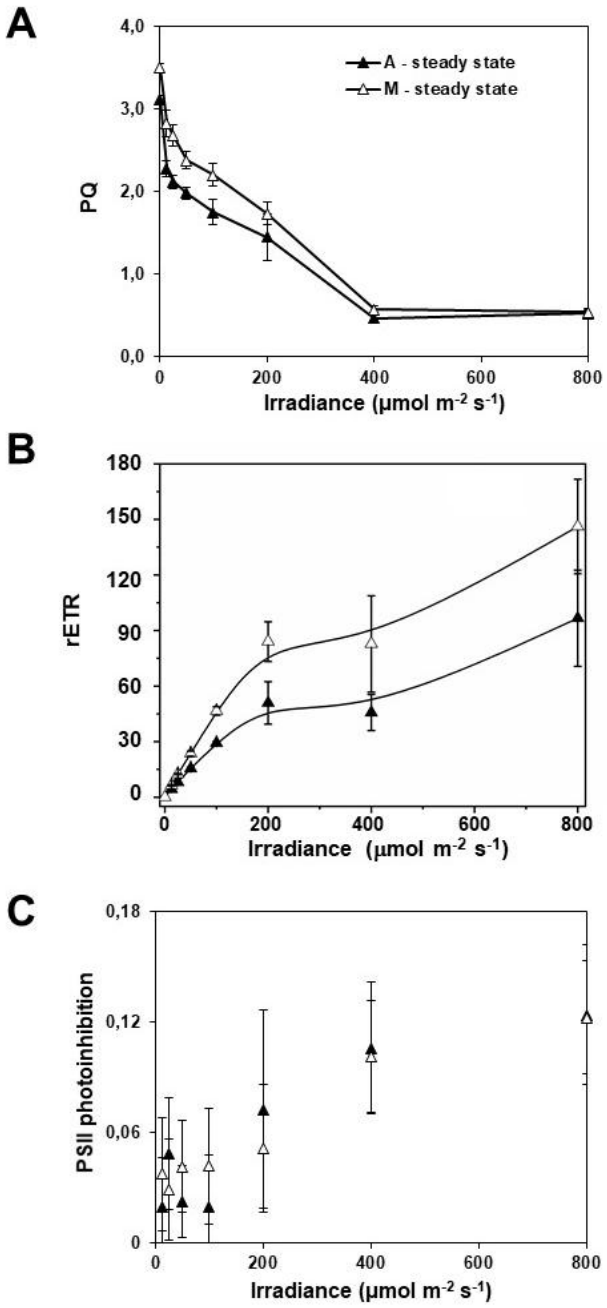

Figure 4. Light curves of fluorescence-derived parameters in *Ettlia oleoabundans* grown autotrophically or mixotrofically. Mean values with SE of *N=3-9.*

A. Photochemical capacity, *PQ*.

B. Relative electron transport rate, *rETR*, calculated assuming a change in PSI/PSII stoichiometry in mixotrophic cells as reported in Giovanardi et al. (2017).

C. PSII photoinhibition, as difference between dark-acclimated maximum PSII quantum yield before light induction and after 10 min light induction and 20 min dark relaxation.

4. Discussion

Ettlia oleoabundans was already reported to increase the dark-acclimated PSII photochemical yield in the early phase of mixotrophic growth (Giovanardi et al., 2014). In a first instance, we attributed the high F_V/F_M to an enhanced photosynthetic capacity, co-operating with the oxidative phosphorylation to burst the culture growth (Baldisserotto et al., 2014; Giovanardi et al., 2014). However, a comprehensive characterization of the thylakoid complexes suggested that high F_V/F_M should be interpreted as a consequence of down-regulated chlororespiration, rather than of enhanced PSII activity (Giovanardi et al., 2017). Therefore, we proposed that under mixotrophy PSII is simply preserved against degradation (Giovanardi et al., 2017). Nevertheless, taking into account that Y(PSII) under high light was slightly higher in mixotrophic cells (Baldisserotto et al., 2016) and that the re-oxidation kinetics of reduced primary electron acceptor Q_A was nearly unaffected (Giovanardi et al., 2017), the problem about PSII function and electron transport chain in mixotrophic *E. oleoabundans* was not yet completely resolved. In this report, we show that a higher F_V/F_M cannot be merely explained by a down-regulated electron recycling from PSI to plastoquinone, but instead it is at least partly due to a genuinely superior PSII activity allowed by a more fluent electron transport chain.

The operation of electron transport pathways alternative to the linear electron flow from PSII to PSI to the Calvin-Benson-Bassham cycle is considered of great relevance for plants to effectively respond to environmental changes (for review, Alric and Johnson, 2017). Autotrophic *E. oleoabundans* is not an exception in this respect. In a membrane proteomic study of nitrogen-starved *E. oleoabundans*, Garibay-Hernández et al. (2017) evidenced the expression of key components of the two main cyclic electron flows, in particular of PGR5-like protein 1 (PGRL1) and Type-II NAD(P)H dehydrogenase (NDH2). The former belongs to the PGR5-PGRL1 pathway, which recycles electrons from ferredoxin to plastoquinone (Munekage et al., 2002). Relevance of this pathway is greater under high light and especially under fluctuating light regimes, which need a very strict control of the membrane redox poise to protect PSI from an excess of electron pressure coming from PSII (Suorsa et al., 2012, 2016). Moreover, co-operating to the generation of trans-thylakoid ΔpH, this route sustains ATP synthesis and also lowers excitation energy pressure through *NPQ* (Munekage et al., 2002). The NDH2 route works in concert with PTOX and uses electrons of NAD(P)H to

reduce plastoquinone and then molecular oxygen to water, thus relieving the electron poise at the acceptor side of PSII, while also contributing to the generation of the ΔpH (reviewed by Peltier et al., 2016). Similar to the NDH1-like pathway present in land plants (Kou et al., 2015), the NDH2 route could be more relevant under low light. The NDH2/PTOX system is deemed responsible for dark chlororespiration in green algae, leading to the effects we have characterized in autotrophic *E. oleoabundans*, principally the dark induction of *NPQ* (Fig. 1C). In fact, based on the current knowledge, it is not difficult to accept that autotrophic *E. oleoabundans* can get some benefits from electron recycling around PSI. Conversely, the same benefits seem to be less important under mixotrophy (Giovanardi et al., 2017). A depressed activity of electron recycling is expected to result in a decreased population of PSII in the closed state, i.e., lower *Ft* and *Y(NO)*. In mixotrophic cells, such a decrease is specific to irradiances lower than growth light (Fig. 3B), suggesting that electron recycling is down-regulated with respect to the pathway(s) operating at low light, thus probably the NDH2 route. Interestingly, Huang et al. (2010, 2018) suggested that the physiological importance of electron recycling under low light is to secure an extra ATP supply so as to sustain PSII repair. Since mixotrophic *E. oleoabundans* can rely on the ATP derived from the respiration of the taken up glucose, a down-regulation of the chlororespiratory ATP production is in line with that hypothesis.

A comparative view of *Y(NO)* excludes any impairment in plastoquinone redox state regulation in mixotrophic *E. oleoabundans* (Fig. 3B). The slightly lower *Y(NO)* previously reported by Baldisserotto et al. (2016) is consistent with the still missing F_M correction for chlororespiration in darkness in autotrophic cells. Conversely, Giovanardi et al. (2017) took into account the correction, but found a higher *Y(NO)* (there indicated as Ft/F_{Mtrue}) in mixotrophic cells treated with high light. However, results are only seemingly contrasting and can be explained by the different actinic lights used in the experiments, either blue (Giovanardi et al., 2017) or white (this report) (for the impact of actinic light wavelength, see e.g. Tikkanen et al., 2017). Therefore, with a balanced excitation of both photosystems through actinic white light, autotrophic and mixotrophic cells reach the same goal of keeping the plastoquinone redox state under control. In mixotrophic cells, the light dependency of *Y(PSII)* and *PQ* clearly show that electrons move more easily towards their final acceptors than in autotrophic cells (Fig. 3A). In the green microalga *Chlorella vulgaris*, under mixotrophy the respiration of the taken up glucose results in the intracellular production of

CO_2, which can feed the Calvin-Benson-Bassham cycle (Villarejo et al., 1995). As a consequence, mixotrophic *C. vulgaris* strongly enhances the PSII activity in terms of O_2 evolution (Spijkerman et al., 2017). Accordingly, in mixotrophic *E. oleoabudans* the more fluid linear electron flow from PSII to PSI reflects a stronger downstream electron sink, i.e., a more active CO_2 fixation (Fig. 4B). Since the membrane is less prone to excess electrons in mixotrophic cells, some auxiliary electron routes working as safety valves can be down-regulated.

Another consequence of a more fluid linear electron transport to the Calvin-Benson-Bassham cycle is the lower thermal dissipation of excess absorbed energy in mixotrophic cells (Fig. 3C). Garibay-Hernández et al. (2017) showed that, for the *NPQ* induction, *E. oleoabundans* can rely on the Photosystem II Subunits S (PSBS) protein (Li et al. 2000), while it lacks the LHC-like Protein Stress Related (LHCSR) protein, on which *NPQ* typically depends in eukaryotic microalgae, possibly in cooperation with PSBS (Correa-Galvis et al., 2016; Peers et al., 2009). Moreover, the microalga also expresses other key enzymes for the modulation of light harvesting, especially enzymes involved in xanthophyll cycle and state transitions (Garibay-Hernández et al., 2017). Especially under high light, the different induction and relaxation kinetics of *Y(NPQ)* between autotrophic and mixotrophic cells indicate that the above mentioned mechanisms are strongly impacted by the mode of cultivation.

In mixotrophic *E. oleoabundans*, the enhanced PSII activity is even more interesting because, through protein immunodetection and low temperature fluorescence spectra, Giovanardi et al. (2017) showed a change in PSII/PSI ratio in favour of PSII. The rETR comparison in Fig. 4B emphasizes the gain in linear electron flow in mixotrophic cells just taking into account the change in photosystem stoichiometry. How this can occur in spite of a lower relative amount of PSI is certainly problematic. However, an explanation can be found in the supramolecular organization of thylakoid complexes, which was characterized previously (Giovanardi et al., 2017). In both *Chlorella vulgaris* (Orus and Martinez, 1991) and *E. oleoabundans* (Baldisserotto et al., 2016), the uptake of glucose results in a higher degree of thylakoid appression, which is the reflection of a higher packing of photosystems, possibly determined by changed membrane lipid pattern (Spijkerman et al., 2017) and low levels of LHCII phosphorylation (Giovanardi et al., 2017). In mixotrophic *E. oleoabundans* PSI and PSII, together with LHCII, tend indeed to organize into stable

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large megacomplexes (Giovanardi et al., 2017). In land plants, similar PSII-LHCII-PSI large associations are currently attributed important physiological roles for the regulation of energy distribution between PSI and PSII (Ferroni et al., 2016, 2018; Suorsa et al., 2015; Yokono et al., 2015). A most recent report by Rantala et al. (2017) suggests that in land plants they could be treated as a functional unit "photosystome", which would specifically allow a fluent and balanced linear electron flow from PSII to PSI. This idea perfectly fits our observations in mixotrophic *E. oleoabundans* and can solve a potential contradiction between high *Y(PSII)* (or rETR) and high PSII/PSI ratio.

In conclusion, during the exponential growth phase the photosynthetic membrane of mixotrophic *E. oleoabundans* is fully functional and well protected against photodamage. A more efficient electron sink sustains a fluent linear electron flow from PSII to PSI, which in turn results in the down-regulation of some auxiliary electron recycling routes and of thermal dissipation of excess energy. Effectiveness of such modulation is patent when comparing the level of photoinhibition between autotrophic and mixotrophic samples, which is nearly identical. Actually, it is very likely that PSII protection is even higher in mixotrophic than in autotrophic cells. In fact, in spite of the difference in *Y(NO)* being inconspicuous between autotrophic and mixotrophic cells under low light, during a daily light period even a small relief in the plastoquinone reduction state can have a measurable effect. We propose that the small, though significant, increase in PSII maximum quantum yield in mixotrophic cells may depend on such small difference in *Y(NO)* and, possibly, also on efficient PSII repair. These would be the ultimate beneficial effects of the internal CO_2 recycling and extra ATP supply allowed by the glucose uptake and respiration. This finding interprets our previous reports about PSII preservation against degradation (Giovanardi et al., 2017) contextualizing them in a framework of improved light energy use under mixotrophy.

Contributions

LF and MG designed the experiment and performed data analysis; MG, MP and CB performed experiments; SP supervised the research; LF wrote the manuscript and all authors read and approved the manuscript.

Acknowledgements

This work was supported by the University of Ferrara [Fondo di Ateneo per la Ricerca Scientifica FAR2017 granted to SP and LF].

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***Contribution**

Authors' contributions

LF and MG designed the experiment and performed data analysis; MG, MP and CB performed experiments;

SP supervised the research; LF wrote the manuscript and all authors read and approved the manuscript.