Photosynthesis regulation in response to fluctuating light in the secondary endosymbiont alga *Nannochloropsis gaditana*

Running head: Response to light fluctuations in eukaryotic alga

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Photosynthesis regulation in response to fluctuating light in the secondary endosymbiont alga *Nannochloropsis gaditana*

Running title: Response to light fluctuations in eukaryotic alga

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ABSTRACT

In nature, photosynthetic organisms are exposed to highly dynamic environmental conditions where the excitation energy and electron flow in the photosynthetic apparatus need to be continuously modulated. Fluctuations in incident light are particularly challenging since they drive oversaturation of photosynthesis, with consequent oxidative stress and photoinhibition. Plants and algae have evolved several mechanisms to modulate their photosynthetic machinery to cope with light dynamics, such as thermal dissipation of excited chlorophyll states (Non-Photochemical Quenching, NPQ) and regulation of electron transport.

The regulatory mechanisms involved in the response to light dynamics have adapted during evolution and exploring biodiversity is a valuable strategy for expanding our understanding of their biological roles. In this work, we investigated the response to fluctuating light in *Nannochloropsis gaditana*, a eukaryotic microalga of the phylum *Heterokonta* originating from a secondary endosymbiotic event. *N. gaditana* is negatively affected by light fluctuations, leading to large reductions in growth and photosynthetic electron transport. Exposure to light fluctuations specifically damages photosystem I, likely because of ineffective regulation of electron transport in this species. The role of Non-Photochemical Quenching, also assessed using a mutant strain specifically depleted of this response, was instead found to be minor, especially in responding to the fastest light fluctuations.

Keywords : Photosynthetic electron transport, photoprotection, photosynthesis, biodiversity, algae, Nannochloropsis

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INTRODUCTION

Oxygenic photosynthesis enables plants, algae and cyanobacteria to convert light into chemical energy due to the activity of two photosystems, PSII and I, with the generation of NADPH and ATP, which sustain cell metabolism. Sunlight provides energy supporting the life of photosynthetic organisms, but excess sunlight leads to the formation of reactive oxygen species (ROS) and photoinhibition (Eberhard et al., 2008; Li et al., 2009). In their natural environment, all photosynthetic organisms are exposed to continuous fluctuations in light intensity; sun and cloud positions, wind, waves and phytoplankton movement can all cause changes in incident light by orders of magnitude (Allahverdiyeva et al., 2015; Ganeteg et al., 2004), greatly affecting excitation energy and electron flow. Photosynthesis therefore requires continuous modulation to adjust light harvesting and electron transport efficiency to dynamic environmental conditions and metabolic demand (Eberhard et al., 2008; Peltier et al., 2010).

Fast changes in illumination are particularly stressful for photosynthetic organisms since they readily cause overreduction of electron transporters, which in turn drives the production of harmful reactive oxygen species. One mechanism for responding to light changes is Non Photochemical Quenching (NPQ), where excess chlorophyll excited states are dissipated as heat (Goss and Lepetit, 2015). NPQ is critical for plant fitness under natural variable conditions (Kulheim et al., 2002) and has a major impact on productivity under open-field cultivation (Kromdijk et al., 2016). Some eukaryotic algae such as the diatom *Phaeodactylum tricornutum* modulate NPQ intensity under fluctuating light (Lepetit et al., 2017) via transcriptional and posttranscriptional regulation of the specific light-harvesting complexes responsible for its activation (i.e., LHCSR/LHCXs antenna proteins) (Maruyama et al., 2014; Peers et al., 2009; Taddei et al., 2016).

Photosynthetic electron transport is also regulated in response to light dynamics, and genetic approaches have allowed the identification of multiple auxiliary electron transport pathways involved (Peltier et al., 2010; Shikanai, 2014). Proton gradient regulation 5 (PGR5) and PGR5-like 1 (PGRL1) are involved in cyclic electron transport around PSI, although the precise mechanism is still under debate (Hertle et al., 2013; Nawrocki et al., 2019). *Arabidopsis thaliana pgr5* mutant lines are particularly sensitive to light damage if exposed to light fluctuations (Suorsa et al., 2012; Tikkanen et al., 2012). Additionally, the pseudo-cyclic electron transport mediated by Flavodiiron (FLV) proteins, which catalyze the light-dependent reduction of O_2 to water, was found to be relevant upon exposure to light fluctuations in cyanobacteria, green algae and mosses, protecting PSI from overreduction and damage (Allahverdiyeva et al., 2013; Chaux et al., 2017; Gerotto et al., 2016).

Photochemical reactions are conserved in all organisms performing oxygenic photosynthesis, while regulatory mechanisms are diversified in various phylogenetic groups (Alboresi et al., 2019; Eberhard

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et al., 2008), likely as a result of adaptation to different environmental niches. The investigation of the biological role of the regulatory mechanisms of photosynthesis in diverse organisms can therefore complement the information obtained from the characterization of specific mutants. In this context, eukaryotic microalgae, which present wide biodiversity, can be highly informative. *Nannochloropsis gaditana* is a microalga of the class *Eustigmatophyceae* within *Heterokonta*, a group that also includes diatoms and brown algae (Cavalier-Smith, 2004; Riisberg et al., 2009). These species originated from a secondary endosymbiotic event in which a eukaryotic host cell engulfed a red alga with a very different evolutionary history from those of plants and green algae (Archibald and Keeling, 2002). *Nannochloropsis* species in fact exhibit a peculiar photosynthetic apparatus with a unique pigment composition, presenting only Chl *a* and lacking any other accessory Chl molecules (Basso et al., 2014; Sukenik et al., 1992). In addition to the interest in their evolutionary origin, species belonging to the *Nannochloropsis* genus have also gained increasing attention related to industrial applications in recent years, especially regarding their ability to accumulate lipids (Ajjawi et al., 2017; Li et al., 2014).

Here, we investigated the response of *Nannochloropsis gaditana* to light fluctuations to assess the role of NPQ and the regulation of electron transport in this species. *N. gaditana* was particularly susceptible to light changes because of ineffective protection of PSI from overreduction, whereas the impact of NPQ in these conditions was found to be minor.

RESULTS

Light fluctuations have a major impact on N. gaditana growth.

Nannochloropsis gaditana cultures were exposed to four different fluctuating light cycles (FL1-4, Figure S1) designed to alternate saturating and limiting illumination (1000 and 10 µmol of photons m^{-2} s^{-1}, respectively (Sforza et al., 2012)). The high light conditions were chosen to be saturating but not so strong as to impair growth (high light, HL, 1000 µmol of photons m^{-2} s^{-1}; Figure S2).

In all cultures exposed to fluctuations, the limiting light treatment always lasted 6 times longer than the saturating illumination, making the total amount of energy provided equivalent, corresponding to continuous illumination of 150 µmol of photons m^{-2} s^{-1} (CL), which is an optimal intensity for *N. gaditana* growth (Sforza et al., 2012). The various FL cultures, however, differed in the duration of the saturating light treatment received, which lasted for 600, 60, 10 and 1 seconds in FL1-4, respectively, as shown in Figure S1. Since the maximal light intensity and the total amount of energy provided were equivalent, the comparison of the FL1-4 cultures highlighted the impact of the frequency of light changes.

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The experimental data showed that fluctuating light had a major impact on *N. gaditana* cultures, resulting in strongly reduced growth compared to control conditions (Figure 1A). The largest growth reduction occurred under FL3, when 10 seconds of saturating light were alternated with 60 seconds of limiting illumination. It is worth highlighting that the cultures exposed to constant saturating illumination (high light, HL, 1000 µmol of photons m^{-2} s^{-1}) were able to sustain a good growth rate, demonstrating that the strong inhibition observed was not due to the light intensity *per se* but rather to a negative effect of the fluctuations (Figure S2).

Effect of fluctuating light on the N. gaditana photosynthetic apparatus

The cultures presented in Figure 1 were grown under full photoautotrophy in the presence of excess inorganic nutrients; thus, the differences in the growth rates are expected to be due to the photosynthetic efficiency and alterations in the photosynthetic apparatus.

The Chl contents per cell in FL1-4 were not altered with respect to that in CL, suggesting the absence of any significant regulation of pigment synthesis (Figure 2). This was surprising since modulation of pigment content is a very common response in photosynthetic organisms exposed to different light regimes (Falkowski and Owens, 1980), including *N. gaditana* (Meneghesso et al., 2016). Indeed, the same *N. gaditana* cells cultivated in either constant low light (LL, 10 µmol of photons m^{-2} s^{-1}) or HL conditions showed a large increase or decrease in Chl content (Figure 2), respectively.

The effects of light fluctuations on photosynthetic apparatus composition were evaluated in more detail spectroscopically. The PSI content per cell was quantified from the P_{700}^{+} absorption signal in cells exposed to saturating light in the presence of DCMU and DBMIB to inhibit rereduction and showed a significant reduction in FL2-4, with the largest effect observed in FL3 (Figure 3A). Indirect information on PSII content can be obtained from the determination of the PSI/PSII ratio measured from the electro-chromic shift (ECS) signal upon exposure to a single turnover flash in the presence/absence of PSII inhibitors (Bailleul et al., 2010a). PSI/PSII was reduced, especially in FL3, where it exhibited a 45% decrease compared to CL conditions (Figure 3B), showing that PSI was more sensitive than PSII to fluctuating light. The estimation of the PSII maximum quantum yield from Chl fluorescence (Fv/Fm) showed only minor photoinhibition, limited to FL3. This parameter did not correlate with the major impact on growth observed in all cultures exposed to light fluctuations and confirmed the limited impact of light fluctuations on PSII.

The presence of eventual alterations in the photosynthetic apparatus composition was also assessed by immunoblot analysis against several photosystem components (Figure 3D). Overall, the results showed conservation of the photosystem composition in all FL cultures, with PsaA, a PSI core complex subunit, showing only a slight decrease, which was much lower than the observed reduction

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in activity. These results suggest that PSI is inactivated but not broken down, consistent with earlier observations in plants that showed a lag between inactivation and degradation in the case of PSI damage (Erling Tjus et al., 1999). This result also explains why pigment content remained stable even in cells showing significant PSI inactivation.

Role of NPQ in response to fluctuating light

NPQ plays a major role in response to changes in light intensity in plants (Kromdijk et al., 2016; Kulheim et al., 2002) as well as in some eukaryotic microalgae (Lepetit et al., 2017). In *Nannochloropsis* cultures exposed to FL, instead, NPQ response showed no significant alteration with respect to the control condition (Figure S3). NPQ activation was monitored in *N. gaditana* cells upon exposure to light fluctuations using Chl fluorescence to assess its activation in the conditions tested here (Figure S1). NPQ was fully activated in samples exposed to 600 seconds of strong illumination (Figure 4A), and a significant response was also present when the saturating light exposure lasted 60 seconds (Figure 4B). In contrast, when the high light treatments were as short as in FL3 (i.e., 10 seconds), NPQ activation remained negligible after 45 minutes of light treatment (Figure 4C). This observation suggests that if the time of exposure to saturating light is short (i.e., 10 seconds or less), NPQ is not significantly activated and does likely not play a significant physiological role.

NPQ kinetics upon exposure to fluctuating light were also monitored in cells treated with DTT, an inhibitor of zeaxanthin biosynthesis. The results in all cases showed that NPQ was largely depleted in the presence of the inhibitor, indicating that its activation is dependent on the xanthophyll cycle in all tested conditions, as expected for this species (Cao et al., 2013; Chukhutsina et al., 2017; Laavi et al., 2013; Park et al., 2019).

A genetic approach for assessing the role of NPQ in the response of *N. gaditana* to light fluctuations was pursued by analyzing the mutant strain I48 (hereon '*npq-less*'), which exhibits severe NPQ reduction (Figure 5). This *npq-less* strain was isolated during the screening of a collection of random mutants (Perin et al., 2015). Here, genome resequencing enabled the identification of a single-base mutation in the gene encoding LHCX1 (Naga_100173g12, (Corteggiani Carpinelli et al., 2014)), which modifies the 5'-donor splicing site of the fourth intron (GT to GA) and causes its retention in the mature mRNA. This modification alters the amino acid sequence of the protein, also introducing a premature stop codon (Figure S4). LHCX1 is a member of the LHC multigenic family and is known to be required for NPQ activation in eukaryotic microalgae, including *Nannochloropsis* (Park et al., 2019; Taddei et al., 2016). Although this was not the only mutation identified in the genome, western blotting confirmed that the *npq-less* strain

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did not accumulate LHCX1 (Figure 4), strongly suggesting that the decreased activation of NPQ was indeed due to the lack of accumulation of this protein.

The availability of a strain depleted in NPQ allowed us to directly investigate the role of this mechanism in the response to fluctuating light. As shown in Figure 5, the *npq-less* strain was tested under FL2 and FL3, which were the conditions showing the largest growth effects on the WT strain. In CL conditions, the *npq-less* strain showed a growth rate in the exponential phase equal to that of WT, although it reached a higher cell density after 9 days of cultivation, consistent with a previous analysis showing an advantage of the mutation when cultures reach higher densities (Perin et al., 2017a). In fluctuating light conditions, there was no significant difference from WT in terms of growth (Figure 5C) or Fv/Fm (Figure S5). The inability to activate NPQ therefore does not have any exacerbating effect on the *Nannochloropsis* response to light fluctuations.

Effect of fluctuating light on PSI and photosynthetic electron transport

The impact of light fluctuations on photosynthetic activity was assessed by monitoring P_{700} rereduction kinetics after saturating light treatment that allowed the evaluation of electron transport rates at the level of PSI (Simionato et al., 2013). Rereduction kinetics were not affected in FL cultures, suggesting that residual active PSI was fully functional (Figure S6). However, because of the overall reduced content of active PSI, the total electron transport capacity per cell was decreased in FL cultures, in agreement with the observed growth reductions (Figure 6A).

The contribution of linear (LEF) and cyclic electron flow (CEF) pathways to the total electron transport was also quantified using specific inhibitors of PSII and cytochrome b_6f, respectively (Figure 6B). Under all conditions, CEF was barely detectable, and LEF was responsible for total electron transport activity, confirming earlier results in *Nannochloropsis* (Perin et al., 2017b; Simionato et al., 2013).

Conservation of mechanisms for the regulation of electron transport in N. gaditana

Mechanisms for the regulation of electron transport have been shown to be relevant for the protection of PSI from light fluctuations in plants, algae and cyanobacteria. Two molecular mechanisms in particular have been identified to play a significant role in light fluctuations: cyclic electron transport dependent on PGR5/PGRL1 and pseudocyclic electron transport dependent on FLV (Alboresi et al., 2019; Yamori and Shikanai, 2016). The *N. gaditana* genome was therefore analyzed to assess the presence of these proteins, comparing the results with sequences from other species.

A putative homologue of PGR5 was found in the *N. gaditana* genome, as in other secondary endosymbionts such as diatoms (Figure 7A). However, these sequences are clearly divergent from

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others found in plants and green algae, and the biological activity of the encoded proteins remains uncertain. PGRL1 has not been identified in *Nannochloropsis* genomes but is present in other secondary endosymbiotic algae (Figure 7B). This is particularly significant since PGRL1 has been suggested to be indispensable for PGR5 activity in CEF by driving its association with the thylakoid membranes (Jokel et al., 2018). The absence of PGRL1 therefore raises additional doubts about the functionality of endogenous PGR5 in *Nannochloropsis*.

Conserved FLV sequences are found in cyanobacteria, green algae, bryophytes and gymnosperms but not in any angiosperm. It is interesting that no FLV sequence has been identified in either *Nannochloropsis* or other secondary endosymbionts such as *Phaeodactylum tricornutum*, suggesting the possibility that a common ancestor of these secondary endosymbiotic algae lost this gene.

DISCUSSION

Light fluctuations have a major impact on Nannochloropsis gaditana growth.

Photosynthesis is continuously adjusted to environmental cues due to a complex regulatory response. This response involves multiple overlapping mechanisms (Alboresi et al., 2019; Eberhard et al., 2008; Shikanai and Yamamoto, 2017) that provide protection from potential excess light but also allow the maintenance of light use efficiency when illumination is limiting. Facing light fluctuations is particularly challenging because the balance between protection and efficiency must be continuously adjusted. The impact of such regulatory mechanisms in the outdoor environment was well demonstrated by a recent study showing that optimization of the NPQ response to light fluctuations yields a significant improvement in biomass yield in tobacco plants cultivated in the field (Kromdijk et al., 2016). The response to light dynamics is also a challenge for aquatic organisms that are exposed to fluctuations because of, for instance, vertical migrations in the water column. Waves can also have a lens effect and concentrate sunlight, resulting in peak values as high as 9000 μmol of photons m^{-2} s^{-1} (Schubert et al., 2001).

In the present work, we investigated the impact of light fluctuations in the heterokont *N. gaditana*, a secondary endosymbiont (Alboresi et al., 2017; Basso et al., 2014) that contributes to the understanding of how evolution has shaped the regulatory mechanisms of photosynthesis. *N. gaditana* cultures were exposed to fluctuating illumination conditions designed to highlight the effect of light kinetics. All cultures received an optimal average photon flux with an illumination intensity oscillating between saturating and limiting, thus challenging cells to protect themselves from excess illumination while also maintaining efficiency under dim illumination. The only variable between the different conditions was the frequency of illumination changes, since the saturating light treatments lasted for 1, 10, 60 or 600 seconds, while the limiting light was correspondingly six times longer.

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Light fluctuations showed a major impact, resulting in an over four-fold decrease in the growth rate and a reduction in the biomass produced of ≈ 85%. It is worth highlighting that the same cells maintain a good growth rate if exposed to constant high light, demonstrating that the observed inhibition was specifically due to dynamic fluctuations and that cells can deal much better with a strong saturating illumination if it is constant (Figure S2).

It is interesting to compare these results with earlier studies assessing the impact of light fluctuations with a higher frequency where the duration of saturating illumination was 0.01 – 0.1 seconds (Sforza et al., 2012). Merging of the data clearly demonstrated some common trends, showing that very short light flashes do not cause significant damage and that the photons provided can even be exploited with good efficiency. Similar results have been obtained for *Chlamydomonas* (Vejrazka et al., 2011), suggesting that this is not a species-specific characteristic. In contrast, when the fluctuations are slower and saturating light exposure lasts for 1-100 seconds, there is a major negative impact, leading to a 4-5-fold reduction in the growth rate. At the other extreme, when light fluctuations are much slower, the negative impact decreases, and the cultures show behavior closer to that under continuous mild illumination.

A mechanistic explanation can be found considering that intense light flashes can initiate photochemical reactions, with the electron flux becoming limited at the level of diffusion-dependent transporters such as plastoquinone (Kirchhoff, 2014; Srivastava et al., 1995). However, when light is quickly switched off, there is available time for the slower diffusion of electron transporters and their reoxidation. At the other extreme, when intense light treatments last for several minutes or hours, there is time for the activation of regulatory mechanisms and the modulation of the photosynthetic efficiency in relation to incident light, such as the dissipation of eventual excesses as heat.

The most challenging conditions are therefore those in which strong light exposure lasts long enough to cause damage but not long enough for the activation of protection mechanisms. This is well exemplified in the case of NPQ presented in Figure 5, where at least 60 seconds of intense light are needed to induce this mechanism for the regulation of photosynthesis, and if saturating light exposure occurs for a shorter time, there is no significant response. Fluctuating light is therefore particularly damaging if strong light lasts for 1-60 seconds when the electron transport chain is saturated but there is not enough time to activate a significant regulatory response.

Photosynthetic organisms also respond to environmental conditions by adjusting the composition of the photosynthetic apparatus through the regulation of gene expression and protein accumulation. The capacity to acclimate in the long term is conserved in all photosynthetic organisms and is readily demonstrated by changes in pigment content (Falkowski and Owens, 1980). It was surprising to observe here that cells maintained a stable pigment composition upon exposure to fluctuating light,

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even though *N. gaditana* clearly presents this capacity (Figure 2 (Meneghesso et al., 2016)). Even when exposed to light fluctuations, the cells seemed to respond to the average illumination in the regulation of pigment content. The data therefore suggest that fluctuating light treatments do not activate the specific signals modulating acclimation despite the stress perceived and the growth reduction experienced by the cells.

Regulation of electron transport and PSI photoprotection are fundamental to responding to light fluctuations.

Considering the major impact of light fluctuations on algal growth, it is interesting to compare the timescales with the kinetics of different regulatory mechanisms involved in the photosynthetic apparatus to obtain information on which mechanisms are most impactful. In cultures grown under full photoautotrophy, all reduced carbon present in the cells originates from carbon dioxide fixation in the Calvin-Benson cycle, which represents the major sink for energy and the reduction in power produced by photochemical reactions. The Calvin-Benson cycle is inactive in the dark and takes a few minutes to be activated when light is switched on (Zaffagnini et al., 2018). The regulation of carbon fixation depends on the chloroplast redox state, and it was recently shown that it also responds to light intensity fluctuations, again in a timeframe of minutes (Howard et al., 2008; Thormählen et al., 2017). Based on presently available knowledge, short light fluctuations at the 1-60 second scale are too fast to allow adjustment of the CO_2 fixation rate to the photon flux. This likely makes CO_2 fixation less efficient, with a negative impact on growth. In addition, if the major pathway consuming products of the light phase of photosynthesis is less active, the effects of potential future excess light are exacerbated.

To respond to excess light, photoprotection mechanisms are needed to dissipate energy and reduce the resulting stress. NPQ, by inducing the dissipation of Chl excited states as heat, is active in reducing the production of oxidative species, which protects PSII in particular. This activity was shown to be important in the response to light variations typical of a natural environment in plants (Kulheim et al., 2002) with a significant impact on biomass productivity in the field (Kromdijk et al., 2016). In the present work, however, the influence of NPQ appeared to be minor in *N. gaditana* under conditions in which light fluctuations were more damaging, such as saturating light lasting 1-60 seconds. Indeed, as shown in Figure 5, light fluctuations in this time range are unable to trigger NPQ activation. As shown by the effect of inhibition with DTT, the largest fraction of NPQ in *Nannochloropsis* depends on zeaxanthin synthesis, which takes a few minutes to achieve significant accumulation (Murchie and Niyogi, 2011; Nilkens et al., 2010); thus, fast light transitions do not allow enough time for zeaxanthin accumulation to trigger significant photoprotection. Consistent with

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this hypothesis, a strain that was unable to activate NPQ did not show any additional sensitivity with respect to WT in any of the FL conditions tested. In this context, it is interesting to emphasize that while in most eukaryotic algae, LHCX/LHCSR are key activators of NPQ (Bailleul et al., 2010b; Peers et al., 2009), vascular plants rely on PSBS for NPQ activation, and its activity is less dependent on zeaxanthin accumulation (Li et al., 2000). This mechanistic difference could make NPQ in plants faster and, thus, more effective in responding to light fluctuations. Comparison of *P. patens* mutants in which NPQ is dependent only on either PSBS or LHCSR indeed showed that activation is faster when dependent on the former (Gerotto et al., 2012)

The dissipation of excess excitation energy by NPQ is known to be particularly important for the protection of PSII (Peers et al., 2009; Tian et al., 2019). *N. gaditana* cells under fluctuating light showed extensive damage to PSI, with a reduction of active P700 per cell and a decrease in the PSI/ PSII ratio, as in other organisms exposed to light fluctuations (Alboresi et al., 2019; Shikanai and Yamamoto, 2017). The mechanistic explanation for PSI impairment under these conditions is that upon sudden increases in illumination, PSI becomes limited on the acceptor side, and excess electrons are accumulated at the level of iron-sulfur clusters, which are consequently damaged (Tiwari et al., 2016). A substantial difference between the two photosystems is that while even extensive damage to PSII can be overcome with limited impact due to effective repair mechanisms, if PSI is inactivated, recovery is slow, with a direct negative effect on electron transport capacity and growth (Larosa et al., 2018).

It is important to emphasize that the impact of fluctuating light on PSI stability in plants, algae and cyanobacteria has been similarly observed in several mutants in which cyclic and pseudocyclic electron transport are affected (Allahverdiyeva et al., 2013; Chaux et al., 2017; Gerotto et al., 2016), whereas PSI normally shows a remarkable stability in WT. The strong influence of FL on PSI and growth observed here in WT *N. gaditana* is, however, consistent with the absence in its genome of several of the proteins known to be involved in the mechanisms for the regulation of electron transport. Only a putative PGR5 protein was identified in the *Nannochloropsis* genome, while PGRL1 and FLV were absent. A similar situation is found in other secondary endosymbiotic organisms that lack FLV such as diatoms (Figure 7). In this context, it is interesting that FLV was shown to be active between 5 and 60 seconds after a light increase (Gerotto et al., 2016), which is precisely the timescale at which *N. gaditana* PSI has been found to be particularly sensitive.

If the hypothesis that the strong impact of light fluctuations observed here is correlated with the absence of some of the mechanisms regulating electron transport and protecting Photosystem I is correct, it is interesting to consider why several secondary endosymbionts are missing these genes. FLV proteins are widespread in cyanobacteria, where they play a relevant role in photoprotection

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(Allahverdiyeva et al., 2013). The most parsimonious hypothesis is therefore that FLV proteins were present in ancestors of *N. gaditana* and other eukaryotic algae but were lost in a later stage, which would suggest some selective advantage associated with the loss of FLV. This possibility is also more likely considering that FLV proteins were also lost by angiosperms.

One potential explanation is that FLV, by donating electrons back to oxygen, closing the water-water cycle, causes an energy loss that could be detrimental to efficiency, overshadowing the advantage in terms of photoprotection (Alboresi et al., 2019; Storti et al., 2019). In the case of algae, one additional factor could be correlated with their life in the sea, where photosynthetic organisms are often limited by iron availability (Boyd et al., 2007; Buesseler et al., 2008). FLV proteins bind iron, and to provide a significant contribution as electron acceptors, they should accumulate in significant amounts; thus, it is plausible that their activity requires a significant investment in a limiting nutrient that could become disadvantageous. PSI itself binds 12 iron atoms and represents a major sink for this nutrient in photosynthetic organisms, and its content is reduced in limiting conditions (Morrissey and Bowler, 2012). If PSI content is reduced under iron limitation, however, it should also decrease the probability that its electron transport activity is limited by the availability of electron acceptors (Lommer et al., 2012). It is therefore possible that in a natural environment limited by iron, PSI would be less prone to acceptor side limitation and the consequent damage, reducing the need for additional mechanisms for its regulation.

MATERIALS AND METHODS.

Microalgae growth. Nannochloropsis gaditana (strain 849/5) was obtained from the Culture Collection of Algae and Protozoa (CCAP). Cells were grown in sterile F/2 media with sea salts (32 g/l, Sigma Aldrich), 40 mM Tris–HCl (pH 8) and Guillard's (F/2) marine water enrichment solution (Sigma Aldrich). The F/2 medium was enriched with 10 times more $NaNO_3$ (0.75 g/L) and NaH_2PO_4 (0.05 g/L) and 2 times more $FeCl_3 \cdot 6H_2O$ (0.0063 g/L) to avoid nutrient limitation. Growth experiments were performed using a Multicultivator MC 1000-OD system (Photon Systems Instruments, Czech Republic) at 21°C, and the culture was mixed and aerated through air bubbling. Illumination was provided with a 16/8 light/dark photoperiod with an array of white LEDs. Control samples (CL) were exposed to continuous light at 150 µmol of photons m^{-2} s^{-1}. In the fluctuating light conditions (FL), the cells were exposed alternatively to 10 and 1000 µmol of photons m^{-2} s^{-1}. The low light exposure time was always 6 times longer than the strong light exposure time to achieve an average of 150 µmol of photons m^{-2} s^{-1} in all conditions. The different culture treatments differed only for the duration of the light treatments, specifically: FL1: 10/ 60 minutes at 1000/ 10 µmol of photons m^{-2} s^{-1}, respectively; FL2: 1/ 6 minutes at 1000/ 10 µmol of photons m^{-2} s^{-1}; FL3: 10/ 60

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seconds at 1000/ 10 µmol of photons m^{-2} s^{-1}; FL4: 1/ 6 seconds at 1000/ 10 µmol of photons m^{-2} s^{-1}. Growth was measured using a cell counter (Cellometer Auto X4, Nexcelom Bioscience). The growth rate was calculated from the slope of the logarithmic phase for the number of cells.

Pigment analysis. Chlorophyll a and total carotenoids were extracted using a 1:1 biomass to solvent ratio of 100% N, N dimethylformamide (Sigma Aldrich) at 4°C in the dark for at least 24 h. The concentration was spectrophotometrically determined using specific extinction coefficients (Wellburn, 1994).

Spectroscopic analysis. Photosynthesis monitoring was performed by measuring *in vivo* Chl fluorescence using a PAM 100 fluorimeter (Heinz-Walz, Effeltrich, Germany). PSII functionality was expressed as the PSII maximum quantum yield (Fv/Fm) according to a previous report (Maxwell and Johnson, 2000). The samples were exposed to either constant illumination for 8 minutes or to an increasing light intensity up to 2000 µmol of photons m^{-2} s^{-1}, and the light was then switched off to evaluate NPQ relaxation kinetics. NPQ values were calculated as previously described (Maxwell and Johnson, 2000).

Electrochromic shift (ECS) measurements were performed in intact cells using a final concentration of 200* 10^6 cells/ml. Data were collected as the difference between the signals at 520 and 498 nm (which represent the positive and negative peaks of the ECS signal in *Nannochloropsis*, respectively) to deconvolute this signal from other spectral changes unrelated to the building of the transmembrane potential (Simionato et al., 2013).

The spectroscopic quantification of P700 and the total electron flow (TEF) was performed by measuring P700 (the primary electron donor to PSI) absorption at 705 nm in intact cells. The analyses were conducted by exposing the samples (300 * 10^6 cells/ml final concentration) to saturating actinic light (2050 µmol of photons m^{-2} s^{-1}, at 630 nm) for 15 seconds to maximize P700 oxidation and reach a steady state. Then, the light was switched off to follow the P700 rereduction kinetics in the dark for 5 seconds. The total electron flow (TEF) was calculated from the monitoring of the P700 rereduction rates after illumination in untreated cells. The electron transport rate was calculated assuming single exponential decay of P700. This allowed us to calculate the rate constant of P700 reduction as $1/\tau$. By multiplying the rate constant by the fraction of oxidized P700 and considering this value as 1 in DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea)- and DBMIB (dibromothymoquinone)-treated cells, we could evaluate the number of electrons transferred per unit of time per PSI unit(Meneghesso et al., 2016). The PSI content was evaluated from the maximum change in the absorption of P700 in cells treated with DCMU (80 µM) and DBMIB (300 µM) under saturating actinic light (2050 µmol of photons m^{-2} s^{-1} at 630 nm). Under these conditions, the rereduction of P700 through photosynthetic

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electron flow is considerably slowed, allowing us to evaluate the full extent of photooxidizable P700 (Simionato et al., 2013).

Sequence analysis. Sequences were searched with BLASTp and tBLASTn in the UniProt and NCBI databases and then aligned using MUSCLE version v3.8.31 (Edgar, 2004) in automatic mode. The transit peptides were manually deleted from the alignments using Jalview version 2.8 (Clamp et al., 2004). Mr. Bayes was run to build a phylogenetic tree that had a standard deviation of split frequencies below 0.01 (Ronquist et al., 2012), which was visualized using FigTree.

SDS-PAGE electrophoresis and western blotting. Samples were collected from 9-day cultures in the exponential phase. Cells were disrupted with a Mini Bead Beater (Biospec Products) at 3500 RPM for 20 seconds in the presence of glass beads (150–212 μm diameter), B1 buffer (400 mM NaCl, 2 mM $MgCl_2$, and 20 mM Tricine–KOH, pH 7.8), 0.5% milk powder, 1 mM PMSF, 1 mM DNP-ε-amino-n-caproic acid and 1 mM benzamidine. The ruptured cells were then solubilized in 10% glycerol, 45 mM Tris (pH 6.8), 30 mM dithiothreitol, and 3% SDS at RT for 20 min. Western blot analysis was performed by transferring the proteins to nitrocellulose (Bio Trace, Pall Corporation) and detecting them with alkaline phosphatase-conjugated antibodies. The antibodies recognized the PSI subunit PsaA (Agrisera), Cytochrome b6f, D2, LHCf1 and LHCX1 proteins (antibodies produced by immunizing New Zealand rabbits with purified spinach protein (Cytochrome b6f and D2) or recombinant *N. gaditana* proteins (LHCf1 and LHCX1) obtained from cDNA overexpression in *E. coli*).

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FIGURE LEGENDS.

Figure 1. *Nannochloropsis* growth under different fluctuating light conditions. *N. gaditana* cells exposed to 150 μmol of photons m^{-2} s^{-1} (Control Light, CL, black squares) are compared with the FL1-4 treatments (red triangles, green circles, blue triangles and purple diamonds, respectively). Growth curves are shown in A, while B provides the calculated growth rates. In all cases, cells were grown with a 16/8 hour light/dark photoperiod. The average and standard deviation (SD) are reported ($n > 5$). A, b, c, and d indicate statistically significant differences from CL, FL1, FL2, and FL3, respectively (one-way ANOVA, p-value < 0.05).

Figure 2. Effect of light conditions on pigment content. The Chl content (pg Chl / cell) determined after 9 days of culture is shown for all growth conditions tested in this work (CL, FL1-4). LL and HL cultures, where cells were exposed to constant 10 and 1000 μmol of photons m^{-2} s^{-1} (figure S2), are also shown. Asterisks indicate statistically significant differences between LL, HL and CL conditions (one-way ANOVA, p-value < 0.05, n = 5).

Figure 3. Effect of fluctuating light on photosystems. A) PSI content per cell quantified from $P700^{+}$ maximum oxidation. Equal numbers of cells (300 10^6 / ml) were always used for the measurements (n=8). WT is shown in black, FL1-4 in red, green, blue, and magenta. B) PSI/PSII ratio obtained by ECS measurement (n=4). C) PSII efficiency in cultures evaluated from the fluorescence parameter Fv/Fm, measured after 9 days of growth (n=4). In all panels, samples that significantly differed from CL are indicated with a (one-way ANOVA, p-value < 0.05). D) Western blotting targeting different

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components of the photosynthetic apparatus from PSI (PsaA), PSII (D2), antenna complexes (LHCX1 and VCP) and Cytochrome f. Total cell extracts containing 0.5 µg of Chl were loaded for each sample (or 0.2 µg in the case of VCP). On the left, different dilutions of the control samples were loaded to check for signal linearity. MW indicates the lane in which the molecular weight standards were loaded.

Figure 4. NPQ activation in *N. gaditana* cells upon exposure to FL. NPQ kinetics upon exposure of cells grown in CL to the light conditions of FL 1-3, where illumination with saturating light lasted for 600, 60 or 10 seconds, respectively, for A, B and C. Light gray squares indicate the dark periods of the kinetics. Note that the y axis scale is different in different panels. The light fluctuations in FL4 were too fast to be simulated in this experimental setup. Saturating flashes were provided regularly for NPQ value determination (n=3). Black and red circles refer to samples that were not treated/treated, respectively, with DTT.

Figure 5. Effect of fluctuating light conditions on an *N. gaditana* strain depleted in NPQ. A) NPQ activation in WT (black) and an *npq-less* mutant (red) upon exposure to saturating light. Most of the decrease in NPQ is attributable to the fast qE component. The yellow/black box indicates when light was on/off. B) Immunoblotting against D2, LHCX1 and LHCF1 showing that the *npq-less* strain is specifically depleted in LHCX1. Growth curves (C) and kinetics (D) of WT (black) and the *npq-less* mutant (red) upon exposure to FL2 (circle) and FL3 (triangle) compared to the control conditions (CL, square), (n=3).

Figure 6. TEF and CEF measurements. A) Total (TEF) and B) cyclic (CEF) electron flow determination from the kinetics of $P700^+$ rereduction after light was switched off (n>4). Data are normalized to the cell concentration if normalized to the PSI TEF was indistinguishable for all cells.

Figure 7. Distribution of cyclic / pseudocyclic mechanisms in *Nannochloropsis*. A) PGRL1, B) PGR5 and C) FLV. Sequences were aligned using MUSCLE version v3.8.31 (Edgar, 2004) in automatic mode, and transit peptides were manually deleted from the alignments using Jalview version 2.8 (Clamp et al., 2004). Mr. Bayes was run to build a phylogenetic tree that had standard deviation of split frequencies below 0.01 (Ronquist et al., 2012), which was visualized using Figtree. Arrows indicate genes identified in *Nannochloropsis*, if present.

Figure 8. Impact of light fluctuations with different frequencies on *N. gaditana* growth. Data from the present work (black squares) are compared with other data from the same species (Sforza et al., 2012), shown in red circles. Growing rate data were all normalized to the maximal value. In(Sforza et al., 2012), the growth conditions were slightly different since the cultures were performed in a flat photobioreactor with excess CO_2 and continuous illumination, which are conditions that are expected to result in higher potential growth than in the culture conditions used

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here. In all cases, the light fluctuations were designed to expose cells to an optimal average photon flux corresponding to 150 μmol of photons m^{-2} s^{-1}. Cultures exposed to continuous illumination are represented as a 24-hour fluctuation treatment.

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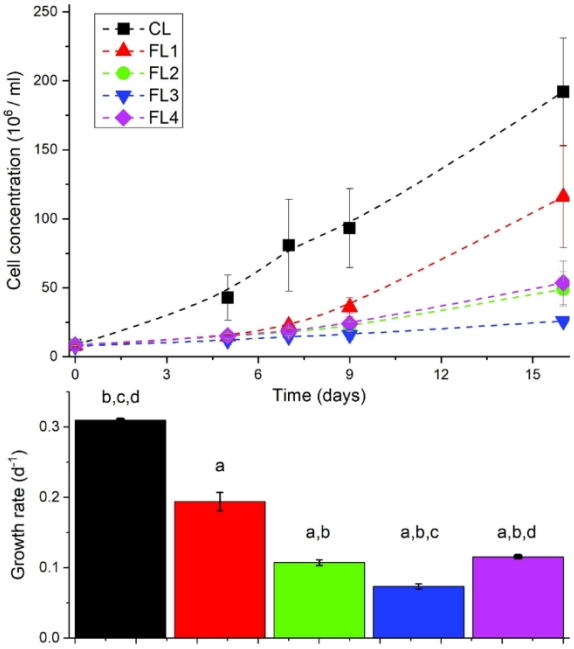

Figure 1. Nannochloropsis growth under different fluctuating light conditions. N. gaditana cells exposed to 150 µmol of photons m-2 s-1 (Control Light, CL, black squares) are compared with FL1-4 (red triangles, green circles, blue triangles and purple diamonds, respectively). Growth curves are shown in A while B reports the calculated growth rates. In all cases cells were grown with a 16/ 8 hours light / dark photoperiod. Average and standard deviation (SD) are reported (n > 5). a, b, c, d indicates statistically significant differences from CL, FL1, FL2, FL3 respectively (One-way ANOVA, p-value < 0.05).

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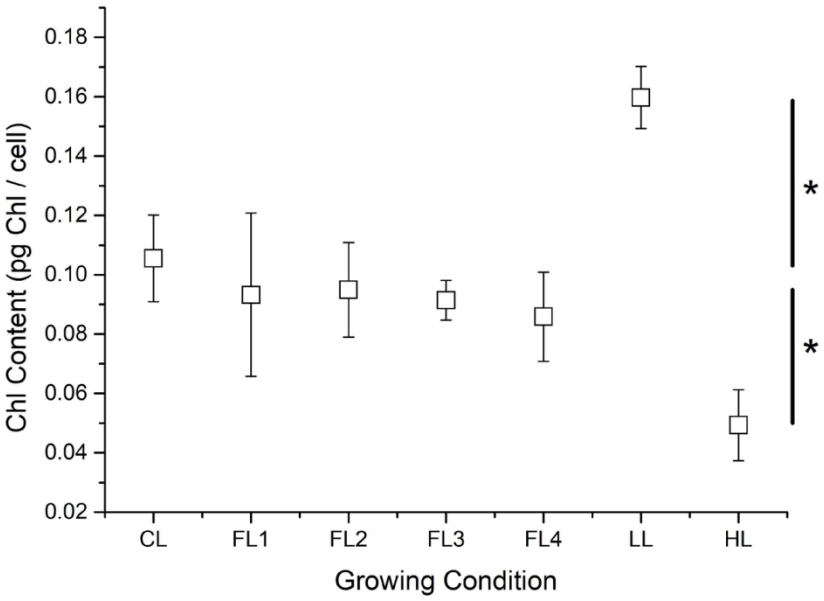

Figure 2. Effect of light conditions on pigment content. Chl content (pg Chl / cell) determined after 9 days of culture is shown for all growth conditions tested in this work (CL, FL1-4). LL and HL cultures, where cells were exposed to constant 10 and 1000 µmol of photons m-2 s-1 (figure S2), are also shown. Asterisks indicate statistically significant differences between LL, HL and CL conditions (One-way ANOVA, p-value < 0.05, n = 5).

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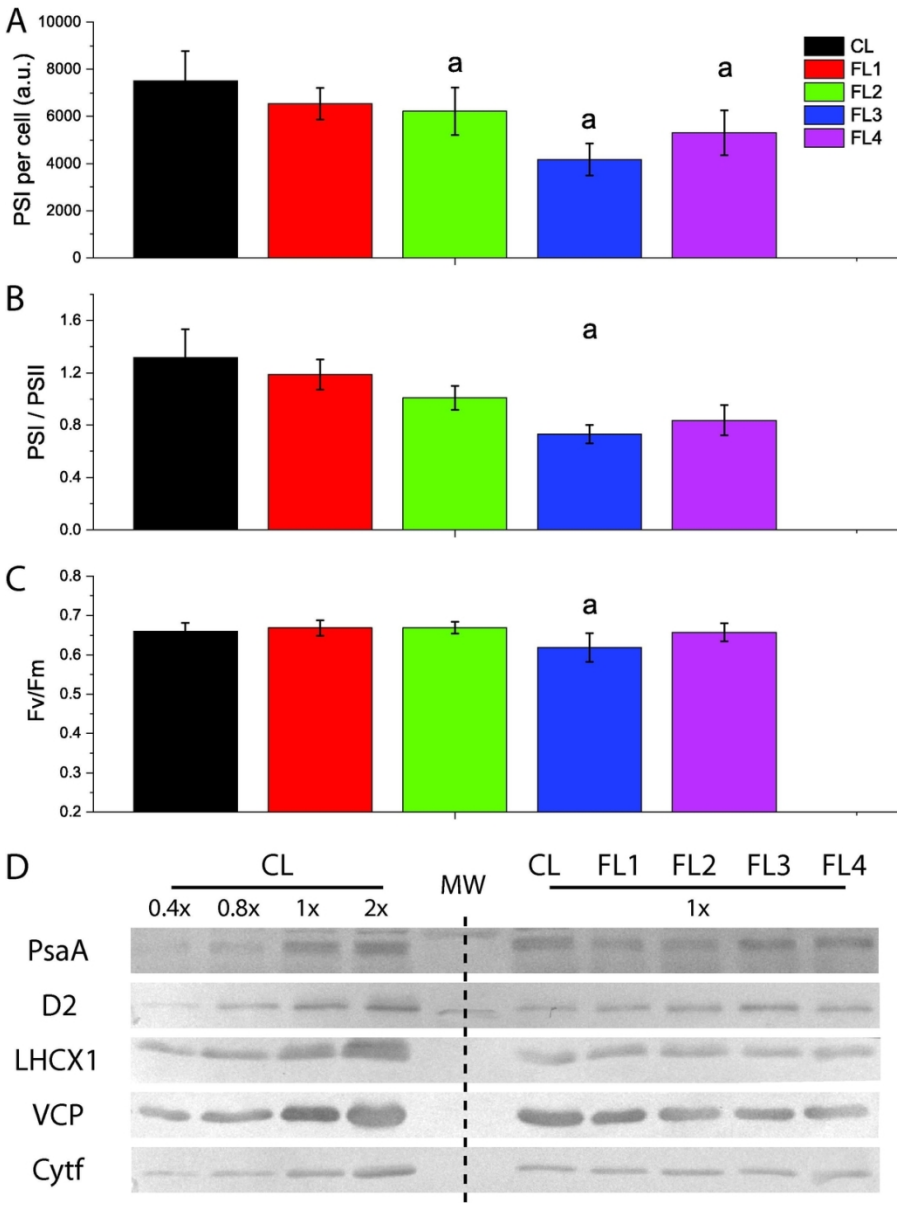

Figure 3. Effect of Fluctuating light on Photosystems. A) PSI content per cell quantified from P700+ maximum oxidation. Equal number of cells (300 106 / ml) was always used for the measurements (n=8). WT is shown in black, FL1-4 in red, green, blue, magenta respectively. B) PSI/PSII ratio obtained by ECS measurement (n=4). C) PSII efficiency in cultures evaluated from the fluorescence parameter Fv/Fm, measured after 9 days of growth (n=4). In all panels samples statistically different from CL are marked with a (one-way ANOVA, p-value < 0.05). D) western blotting against different components of photosynthetic apparatus from PSI (PsaA), PSII (D2), antenna complexes (LHCX1 and VCP) and Cytochrome f. Total cell extract containing 0.5 µg of Chl were loaded per each sample (whereas 0.2 µg in case of VCP). On the left different dilutions of the control samples were loaded to check for signal linearity. MW indicate lane where molecular weight standards were loaded.

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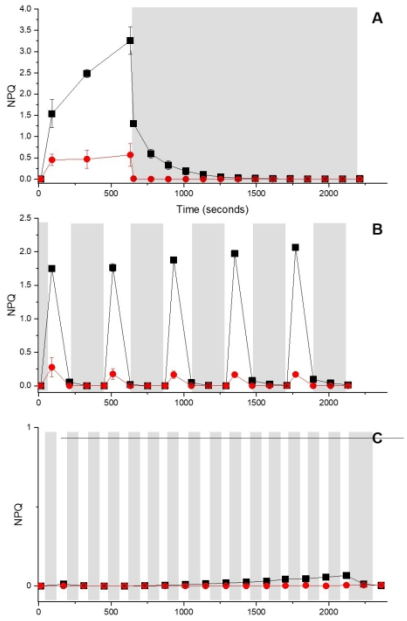

Figure 4. NPQ activation in N. gaditana cells upon exposure to FL. NPQ kinetics upon exposure of cells grown in CL to light of FL 1-3, where illumination with saturating light lasted for 600, 60 and 10 seconds, respectively for A, B and C. Light gray square indicate the dark periods of the kinetics. Note that y axis scale is different in various panels. Light fluctuations in FL4 were too fast to be simulated in this experimental setup. Saturating flashes were provided regularly for NPQ values determination (n=3). Black and red circles refer to samples not treated/treated, respectively, with DTT.

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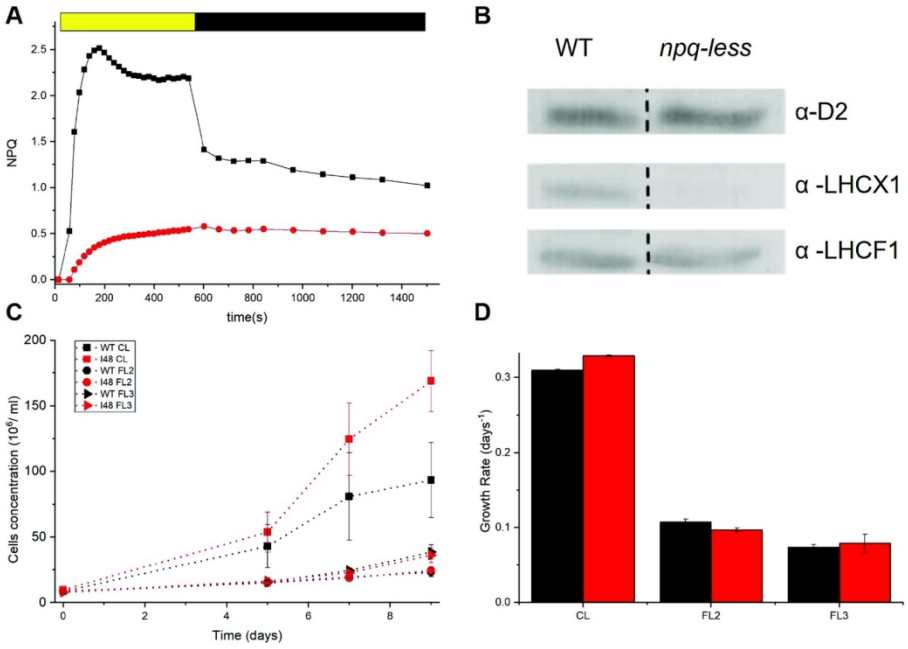

Figure 5. Effect of fluctuating light conditions on a N. gaditana strain depleted in NPQ. A) NPQ activation of WT (black) and npq-less mutant (red) upon exposure to saturating light. Most of the decrease in NPQ is attributable to the fast qE component. Yellow/ black box indicates when light was on/ off. B) Immunoblot against D2, LHCX1 and LHCF1 showing that npq-less strain is specifically depleted of LHCX1. Growth curves (C) and kinetics (D) of WT (black) and npq-less mutant (red) upon exposure to FL2 (circle) and FL3 (triangle), compared to the control conditions (CL, square), (n=3).

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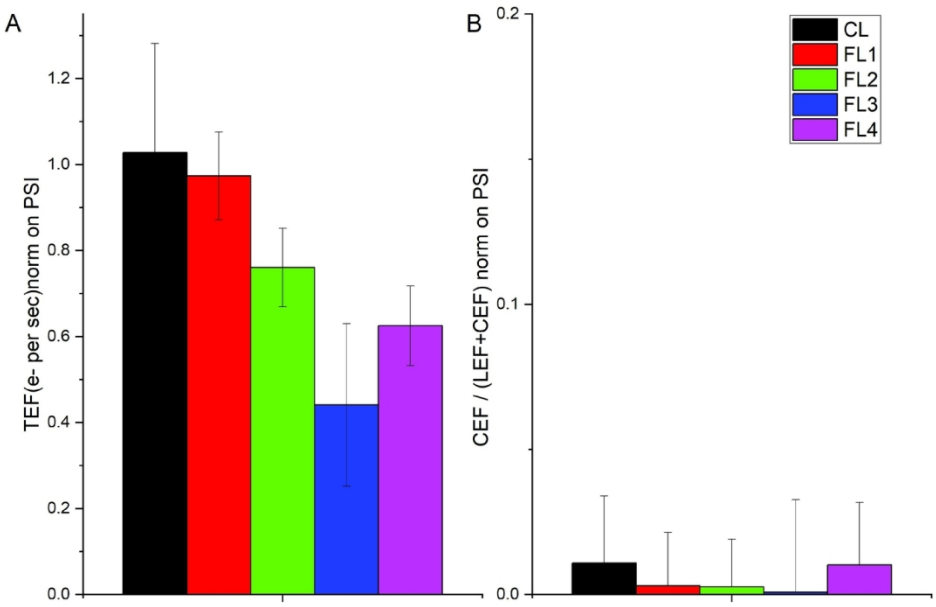

Figure 6. TEF and CEF measurements. A) Total (TEF) and B) cyclic (CEF) electron flow determination from the kinetics of P700+ re-reduction after light switched off (n>4). Data are normalized to the cell concentration, if normalized to PSI TEF is indistinguishable for all cells.

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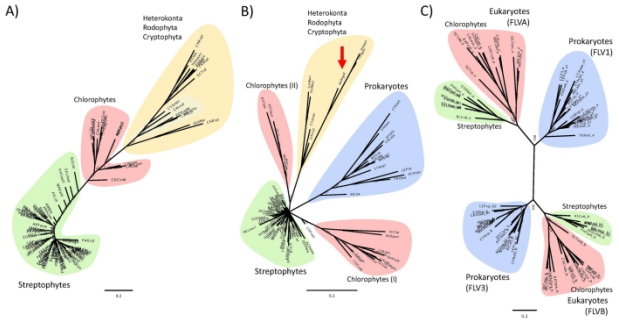

Figure 7. Distribution of mechanisms for cyclic / pseudo-cyclic in Nannochloropsis. A) PGRL1, B) PGR5 and C) FLV. Sequences were aligned using MUSCLE version v3.8.31 (Edgar, 2004) in automatic mode and transit peptides were manually deleted from the alignments using Jalview version 2.8 (Clamp et al., 2004). Mr. Bayes was run to build a phylogenetic tree that had standard deviation of split frequencies below 0.01 (Ronquist et al., 2012) and that was visualized using Figtree. Arrow indicates genes identified in Nannochloropsis, if present.

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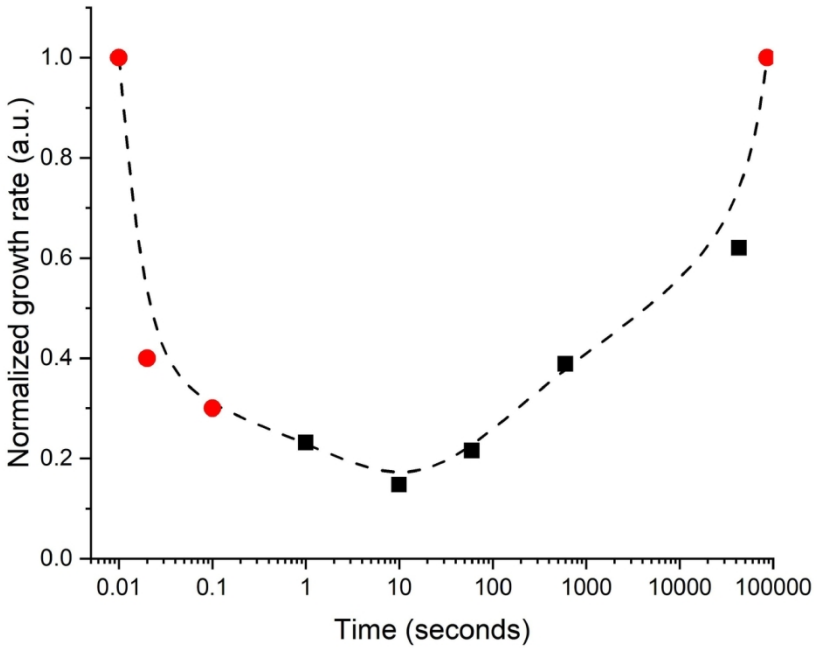

Figure 8. Impact of light fluctuations with different frequencies on N. gaditana growth. Data from present work (black squares) are compared with others from the same species (Sforza et al., 2012) shown in red circles. Growing rates data were all normalized to the maximal value. In (Sforza et al., 2012) growth conditions were slightly different since cultures were performed in flat photobioreactor with excess CO2 and continuous illumination, which are expected to result in a higher potential growth than in the culture conditions used here. In all cases light fluctuations were designed in order to expose cells to optimal average photon flux, corresponding to 150 µmol of photons m-2 s-1. Cultures exposed to continuous illumination are represented as a 24 hours fluctuation.

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