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The regulation of xanthophyll cycle activity and of non-photochemical fluorescence quenching by two alternative electron flows in the diatoms *Phaeodactylum tricornutum* and *Cyclotella meneghiniana*

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ABSTRACT

Intact cells of diatoms are characterized by a rapid diatoxanthin epoxidation during low light periods following high light illumination while epoxidation is severely restricted in phases of complete darkness. The present study shows that rapid diatoxanthin epoxidation is dependent on the availability of the cofactor of diatoxanthin epoxidase, NADPH, which cannot be generated in darkness due to the inactivity of PSI. In the diatom *Phaeodactylum tricornutum*, NADPH production during low light is dependent on PSII activity, and addition of DCMU consequently abolishes diatoxanthin epoxidation. In contrast to *P. tricornutum*, DCMU does not affect diatoxanthin epoxidation in *Cyclotella meneghiniana*, which shows the same rapid epoxidation in low light both in the absence or presence of DCMU. Measurements of the reduction state of the PQ pool and PSI activity indicate that, in the presence of DCMU, NADPH production in *C. meneghiniana* occurs via alternative electron transport, which includes electron donation from the chloroplast stroma to the PQ pool and, in a second step, from PQ to PSI. Similar electron flow to PQ is also observed during high light illumination of DCMU-treated *P. tricornutum* cells. In contrast to *C. meneghiniana*, the electrons are not directed to PSI, but most likely to a plastoquinone oxidase. This chlororespiratory electron transport leads to the establishment of an uncoupler-sensitive proton gradient in the presence of DCMU, which induces diadinoxanthin de-epoxidation and NPQ. In *C. meneghiniana*, electron flow to the plastoquinone oxidase is restricted, and consequently, diadinoxanthin de-epoxidation and NPQ is not observed after addition of DCMU.

1. Introduction

Diatoms represent the most important algal group in terms of primary production in the oceans [1]. They are unicellular photosynthetic organisms that possess fucoxanthin (Fx) as their main light-harvesting xanthophyll and diadinoxanthin (Ddx) and diatoxanthin (Dtx) as xanthophyll cycle pigments. In diatoms, the Ddx cycle is the most important short-term photo-protective mechanism. It consists of a forward reaction that converts the light harvesting pigment Ddx into the photoprotective pigment Dtx under conditions of high light illumination [2]. Ddx de-epoxidase, the enzyme catalyzing the forward reaction of the cycle, can be activated at almost neutral pH-values [3] and has a significantly lower apparent K_M-value for its cosubstrate ascorbate compared with the violaxanthin (Vx) de-epoxidase of vascular plants [4]. The reverse reaction of the diatom xanthophyll cycle, the epoxidation of Dtx to Ddx, is extremely rapid, in contrast to the slow zeaxanthin (Zx) epoxidation observed in vascular plants and green algae [5]. Rapid epoxidation of Dtx can take place, however, only under conditions of illumination with low light intensities, and is severely restricted in complete darkness. The absence of efficient epoxidation during dark periods has been attributed to a shortage in NADPH [5], which is an essential cofactor of Dtx epoxidase and the Zx epoxidase of vascular plants [6]. In addition to the NADPH requirement for Dtx epoxidation, there exists a direct control of the enzyme Dtx epoxidase by the proton gradient. In the presence of a high light-driven ΔpH Dtx epoxidase is almost completely inhibited and the rapid epoxidation rates observed at low light intensities are reduced by a factor of 10 to 20 [5,7].

Among the multitude of diatom species, *Phaeodactylum tricornutum* represents the most extensively studied. Its genome has recently been sequenced [8] and unique photo-protective features have been revealed in this diatom [5,9–12]. In addition, *P. tricornutum* displays chlororespiratory activity during periods of prolonged darkness [3,13], leading

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Abbreviations: Chl *a*, chlorophyll *a*; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DDE, diadinoxanthin de-epoxidase; Ddx, diadinoxanthin; DES, de-epoxidation state of the Ddx cycle pigment pool; Dtx, diatoxanthin; Fo, Fm, fluorescence intensity measured with all reaction centers open (Fo) or closed (Fm) by a saturation pulse; FNR, ferredoxin-NADP$^+$-oxidoreductase; HL, high light; LL, low light; NADH, nicotinamide adenine dinucleotide, reduced form; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; Ndh, NAD(P)H dehydrogenase; NPQ, non-photochemical chlorophyll fluorescence quenching; OJIP curve, fast rise in fluorescence defined by its transient states; P700, reaction centre pigments of PS I (primary electron donor of PS I); PAR, photosynthetically active radiation; PS, photosystem; PQ, plastoquinone; Vx, violaxanthin; Zx, zeaxanthin

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to the build-up of a proton gradient and Dtx accumulation without the participation of PSII electron transport.

Evidence for the existence of a respiratory electron transport chain inside the chloroplast was first presented for vascular plants [14], but was later also found in the green alga *Chlamydomonas reinhardtii* [15] and the diatom *P. tricornutum* [16]. In another diatom, *Thalassiosira weissflogii*, chlororespiratory reduction of the PQ pool was observed after a light-to-dark transition of the algal cultures [17]. It was suggested that an NAD(P)H-PQ oxidoreductase [18], alternatively termed plastidal NAD(P)H dehydrogenase (Ndh) complex, and a plastidal terminal oxidase (PTOX), responsible for the non-photochemical re-oxidation of PQ [19], are involved in the electron transport from reducing equivalents to oxygen via the PQ pool. This electron transport was termed chlororespiration (for a review see [20]). The significance of chlororespiration for photosynthesis and plant stress responses has recently been addressed [21]. Chlororespiration was also observed in illuminated cells of the prasinophycean alga *Mantoniella squamata* in the presence of DCMU. In *M. squamata* it was thought to be responsible for the establishment of a proton gradient without the contribution of PSII. This ΔpH induced non-photochemical quenching of chlorophyll fluorescence (NPQ) which persisted during dark periods following illumination of the cells [22]. Interestingly, an establishment of NPQ in the presence of DCMU was also found in intact cells of *P. tricornutum* [23].

It was our intention in the present study to understand, in greater detail, why Dtx epoxidation is strongly restricted during dark periods following high light illumination, and why low light conditions are needed for efficient Dtx epoxidation. For this purpose the status of the two regulatory elements of Dtx epoxidation, i.e. the availability of NADPH and the extent of the trans-membrane proton gradient, were investigated during periods of high light illumination followed by phases of low light or complete darkness. With the combination of fast Chl fluorescence induction and P700 absorbance change measurements, we obtained information about the reduction state of the PQ pool and the activity of the PSI donor and acceptor sides, allowing estimation of the electron pressure on the PQ pool and the capacity of PSI to reduce NADP$^+$, respectively. Inhibition of PSII electron transport by DCMU was used to gain information about the possible existence of alternative electron transport pathways, i.e. chlororespiration, under the different light treatments used in the present study. Finally, manipulations of the proton gradient by addition of DCMU and the uncoupler NH_4Cl were performed. The effects on Dtx epoxidation by either the presence or absence of the ΔpH were analyzed by determinations of the de-epoxidation state of the Ddx cycle pigment pool and by measurements of NPQ.

The present study includes a detailed comparison of *P. tricornutum* and *Cyclotella meneghiniana* because previous studies have noted that these diatom species differ with regard to the kinetics of the Ddx cycle and NPQ. These differences include the presence of a transient NPQ component in *C. meneghiniana* which induces a very rapid quenching of Chl fluorescence immediately upon illumination with high light intensities [12]. Furthermore, *C. meneghiniana* is characterized by significantly faster Dtx epoxidation compared with *P. tricornutum* [5]. Therefore, our second goal in the present study was to examine whether the differences in the Dtx epoxidation kinetics are based on a different regulation of the Ddx cycle with respect to its NADPH requirement and pH-dependence.

2. Materials and methods

2.1 Algal cultures, preparation of samples

Airlift cultures of *C. meneghiniana* (strain 1020_1a) and batch cultures of *P. tricornutum* (strain 1090_1a) obtained from the Culture Collection of Algae Göttingen (SAG), Germany, were grown at 20 °C with a light to dark cycle of 14:10 h at a light intensity of 50 µmol photons m^{-2} s^{-1} (PAR). *C. meneghiniana* was cultivated in silica-enriched f/2 medium according to [24] with a modification of the salt content to match that of brackish water (half the sea salt content of the original f/2 medium). *P. tricornutum* was grown in modified ASP-2 medium according to [25]. All measurements in the present study were performed with cells that were harvested 15 min after the onset of the daily light period and concentrated to a chlorophyll *a* content of 30 µg mL^{-1} by a 10 min centrifugation at 2500 *g* and 10 °C (Varifuge, Heraeus, Germany). The cells were then supplemented with 10 mM $KHCO_3$ in order to prevent carbon limitation during the actinic high light (HL) illumination used in the experiments and kept in actinic low light (LL, 25 µmol photons m^{-2} s^{-1}) before the start of the measurements. All experiments were performed at a constant temperature of 21 °C with algae harvested during the logarithmic growth phase.

2.2 Chlorophyll *a* fluorescence/830 nm absorption changes measurements

Measurements of slow changes of variable fluorescence, fast fluorescence induction kinetics (time scale of ms) and absorption changes at 830 nm were carried out simultaneously with a Dual-PAM-100 (Chlorophyll fluorescence and P700 photosynthesis analyzer equipped with a P700-dual-wavelength-emitter at 830 and 875 nm, Walz GmbH, Germany). Note that the absorption changes at 830 nm are displayed as the photocurrent measured with the Dual-PAM-100 in the respective figures. In the Dual-PAM-100 P700 oxidation/reduction is measured in transmission mode and is calculated as the signal at 875 nm minus the signal at 830 nm. The signal difference is proportional to the absorbance change at 830 nm. Thus oxidation of P700 becomes visible as an increase of P700$^+$ (relative), whereas re-reduction of P700 leads to a decrease of P700$^+$. The illumination protocol used consisted of 5 min dark-adaptation, a 10 min period of actinic HL illumination with a light intensity of 820 µmol m^{-2} s^{-1}, followed by another 10 min of darkness or LL illumination with a light intensity of 25 µmol m^{-2} s^{-1}. The slow changes of variable fluorescence were recorded using the saturating pulse method according to [26]. Saturating pulses with a duration of 900 ms and a light intensity of 5000 µmol m^{-2} s^{-1} were applied with a frequency of 1 pulse per min. The same saturating pulses also induced both the fluorescence transient at PSII and the photo-oxidation of PSI. The first saturating pulse was applied after 5 min dark-adaptation right before the onset of the actinic HL illumination. Additional pulses were applied every minute during the 10 min period of darkness or LL illumination following the HL illumination. The first pulse during the dark or LL phase was applied 2 s after the transition from HL to darkness or LL. Data for the fast fluorescence induction and the 830 nm absorbance changes were recorded during the 900 ms saturating pulse and for another 500 ms after the end of the saturating flash. Stirring of the samples was avoided during data recording in order to minimize disturbances of the P700 absorbance signal.

Fast fluorescence induction curves were additionally recorded with a Plant Efficiency Analyzer (PEA, Hansatech, Norfolk, England) as described in [27]. In these measurements the light intensity of the 1 s flash was set to 3500 µmol m^{-2} s^{-1}. The results from the PEA analyses were comparable to the results achieved with the Dual-PAM-100.

NPQ was calculated as $(Fm - Fm')/Fm'$ according to [28]. In the present measurements, Fm stands for the maximum fluorescence of dark-adapted intact cells before the onset of actinic HL illumination, whereas Fm′ denotes the maximum fluorescence of cells illuminated by actinic HL or exposed to periods of LL or darkness following HL illumination.

2.3 Pigment data

For the analysis of Ddx de-epoxidation pigment samples were collected before and after 10 min of the actinic HL period under the different experimental conditions used in the present study (for details see the Results section). Dtx epoxidation was studied during the dark or LL phase following the HL illumination and samples were typically collected after 10 min of the respective period. For pigment analysis the algal cells were rapidly transferred onto a glass fiber filter and frozen in liquid nitrogen. For the HPLC analysis the pigments were extracted with a medium consisting of 90% methanol/0.2 M ammonium acetate (90/10, v/v) and 10% ethyl acetate. The extracts were then centrifuged for 2 min at 13,000 g (Centrifuge 5417C, Eppendorf, Germany) and injected into the HPLC column. Pigment analysis was carried out on a Waters HPLC system (Waters, Millipore, Germany) equipped with a Nucleosil ET 250/8/4, 300-5, C18 column (Macherey and Nagel, Germany). Pigments were analyzed and quantified according to [25,29]. Ddx de-epoxidation and Dtx epoxidation are depicted as changes in the de-epoxidation state (DES) of the Ddx cycle pigment pool calculated as Dtx $(\mathrm{Ddx}+\mathrm{Dtx})^{-1}$.

2.4 Inhibition of linear electron transport and Ddx de-epoxidation, uncoupling of the ΔpH

40 μM of DCMU was used to inhibit PSII and linear electron transport. DCMU was either added to the algal samples before the 10 min period of actinic HL illumination or immediately before the 10 min LL period following HL illumination. NH_4Cl (20 mM) was used to uncouple the trans-membrane proton gradient as nigericin was unable to penetrate the intact cells of *P. tricornutum* (not shown). NH_4Cl was added either immediately before the start of actinic HL illumination or before the following LL period.

The concentrations of DCMU and NH_4Cl used in this study were approximately twice as high as those in other studies. This was done because of the high Chl *a* content of the cell suspensions that were needed for the measurement of fast Chl fluorescence and P700 absorbance changes. High inhibitor concentrations were also used to assure the complete inhibition of PSII electron transport by DCMU and a total uncoupling of the ΔpH by NH_4Cl.

3. Results

3.1. Non-photochemical fluorescence quenching and Ddx de-epoxidation

Fig. 1 compares the NPQ of the diatoms *P. tricornutum* and *C. meneghiniana* during a period of actinic HL illumination and a subsequent transition to either complete darkness or LL. Although both algae showed high NPQ values during the HL period, visible as reduction of the fluorescence signal, significant differences were observed. The extent of NPQ after 10 min of actinic illumination (820 μmol photons m^{-2} s^{-1}) was much higher in *C. meneghiniana* (Fig. 1B) than in *P. tricornutum* (Fig. 1A). For *C. meneghiniana*, NPQ values between 3 and 4 were typically observed, whereas *P. tricornutum* exhibited NPQ values between 1.2 and 1.3. After a transition of the intact *P. tricornutum* cells from HL to complete darkness (Fig. 1A), a very slow increase of Fm and decrease of Fo was observed, and the NPQ value remained at a high level during the 10 min period of darkness used in the present measurements. *C. meneghiniana* showed similar behavior (Fig. 1B), with the exception of the relaxation of a fast Ddx cycle-independent NPQ component directly after the transition from HL to darkness (see also [12]), which led to an increase in the fluorescence signal. The existence of high NPQ in periods of darkness after preceding HL illumination was in line with the data on the de-epoxidation state of the Ddx cycle pigment pool given as Dtx $(\mathrm{Ddx}+\mathrm{Dtx})^{-1}$ (Fig. 3). In *P. tricornutum* the de-epoxidation state reached a value of around 0.55 after 10 min of HL illumination and remained almost identical for an additional 10 min of darkness. *C. meneghiniana* was characterized by a slightly

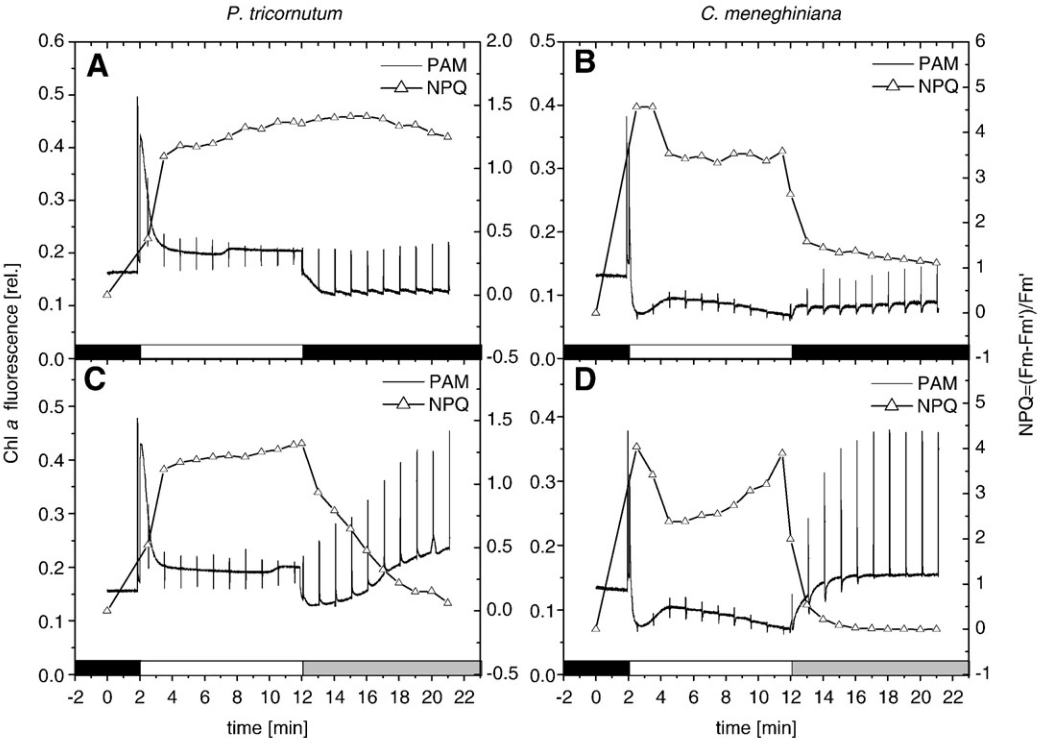

Fig. 1. Time-course of slow chlorophyll *a* fluorescence induction (PAM) and NPQ of untreated cells of *P. tricornutum* and *C. meneghiniana*. Samples were adjusted to 30 μg Chl *a* mL^{-1}. Panels A and B depicts a dark to HL (820 μmol photons m^{-2} s^{-1}) to dark transition as indicated at the bottom of the figures. Panels C and D shows a dark to HL to LL (25 μmol photons m^{-2} s^{-1}) transition. The Fv/Fm values of dark-adapted cells were 0.68 for *P. tricornutum* and 0.67 for *C. meneghiniana*. Representative measurements are shown; four measurements per condition were carried out in total to confirm the results. The first saturating pulse was applied after 5 min of dark adaptation just before the start of HL illumination and corresponds to the condition "dark" in Fig. 4A and B. Pulse 1 after the termination of the HL phase was applied after 2 s and corresponds to the condition "dark after HL" in Fig. 4E and F. Pulse 5 after switching off the HL illumination corresponds to the condition "4′ dark after HL" in Fig. 5A and B. Note that the fluorescence data and NPQ are scaled differently in order to demonstrate the effects of the different measurement conditions.

higher Ddx conversion, resulting in a DES of around 0.6 at the end of the HL period. Comparable to what was observed in *P. tricornutum*, the DES remained high during the dark period following actinic illumination, demonstrating that the fast relaxing component of NPQ was not accompanied by a concomitant decrease in the Dtx content. When both cultures were transferred from HL to LL illumination (25 μmol photons m^{-2} s^{-1}), a rapid increase of Fm was observed. Whereas for *P. tricornutum* 10–15 min in LL was needed until a steady-state was reached (Fig. 1C), in *C. meneghiniana*, the decrease of NPQ during the LL phase was faster and was completed after 5 min (Fig. 1D). In *P. tricornutum* we observed a short decrease in Fo upon the HL to LL transition followed by a pronounced increase, in *C. meneghiniana* the Fo increase was direct and rapid. The decrease of NPQ was correlated with a rapid decrease in the DES of the Ddx cycle pigment pool in both algae. After 10 min of the LL period, Dtx, which had been accumulated during the HL period, was epoxidized to Ddx, resulting in a DES value of around 0.1 for both algae (Fig. 3).

To investigate whether the strong difference in Dtx epoxidation and NPQ decrease during the dark and the LL phase was caused by a shortage in the NADPH supply during complete darkness, linear electron transport was inhibited by addition of DCMU during the LL period following actinic HL illumination. Surprisingly, *C. meneghiniana* and *P. tricornutum* showed opposite reactions to the addition of DCMU. In *P. tricornutum*, the addition of DCMU immediately before the transition from HL to LL completely blocked the increase of Fm and Fo (Fig. 2A). This inhibition was accompanied by an almost complete absence of Dtx epoxidation, as illustrated by a DES of around 0.5 at the end of the 10 min LL period (Fig. 3). However, in *C. meneghiniana*, epoxidation and NPQ decrease were completely unaffected by the addition of DCMU (Figs. 2B and 3).

Drastic differences between *P. tricornutum* and *C. meneghiniana* were also clear when DCMU was added to the intact cells before the start of the 10 min period of actinic HL illumination. In DCMU-treated cells of *P. tricornutum* NPQ was established with similar kinetics and to a similar extent as in control cells (Fig. 2C). This NPQ was accompanied by a corresponding de-epoxidation of Ddx to Dtx, leading to a high DES value of around 0.5 (Fig. 3). DCMU-treated cells of *C. meneghiniana*, however, did not show NPQ and Fm remained on a high level (Fig. 2D). In accordance with the absence of NPQ, DCMU inhibited the formation of Dtx, and the DES of around 0.15 after the HL period was comparable to dark-adapted control cells (Fig. 3).

In order to test whether a proton gradient was responsible for NPQ and Ddx de-epoxidation in DCMU-treated *P. tricornutum* cells, the uncoupler NH_4Cl was added before the start of actinic illumination. In the presence of both NH_4Cl and DCMU *P. tricornutum* did not exhibit NPQ (not shown) and the DES of the Ddx cycle pigment pool remained on a low level (less than 0.1) during HL illumination, comparable to that of dark-adapted control cells (Fig. 3). NH_4Cl in combination with DCMU was also added to *P. tricornutum* cells before the transition from HL to LL, resulting in a lower DES of the Ddx cycle pool (around 0.25) at the end of the LL phase compared with cells where only DCMU was used (DES value around 0.45, Fig. 3). This measurement indicates, that during the LL period in the presence of DCMU, a proton gradient persisted that kept the DDE in an active state, thereby preventing a decrease in the Dtx content.

To obtain further experimental evidence that, in the presence of DCMU, alternative electron transport pathways were responsible for the generation of the ΔpH in *P. tricornutum* and the reduction of $NADP^+$ in *C. meneghiniana*, measurements of fast fluorescence induction and P700 oxidation were performed.

3.2. Fast fluorescence induction curves and P700 absorption

Measurements and nomenclature of fast Chl fluorescence induction [30] provide information about the reduction state of Q_A, the

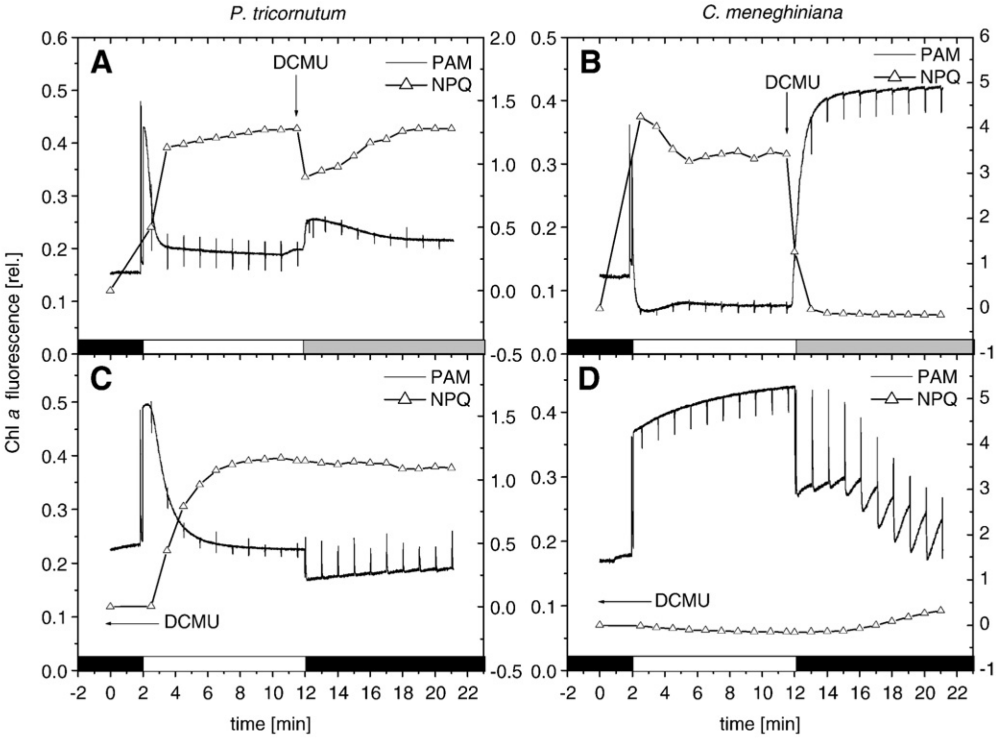

Fig. 2. Time-course of slow chlorophyll *a* fluorescence induction (PAM) and NPQ of intact cells of *P. tricornutum* and *C. meneghiniana*. Samples were adjusted to 30 μg Chl *a* mL^{-1}. Panels A and B shows a dark to HL (820 μmol photons m^{-2} s^{-1}) to LL (25 μmol photons m^{-2} s^{-1}) transition where 40 μM DCMU was added directly before the transition from HL to LL. Panels C and D depicts a dark to HL to dark transition where DCMU was added during the dark adaptation preceding the start of the HL illumination. Representative measurements are shown; four measurements per condition were carried out in total to confirm the results. The first saturating pulse was applied after 5 min of dark adaptation just before the HL illumination and corresponds to condition "dark DCMU" in Fig. 4C and D. Pulse 5 after switching off the HL illumination corresponds to the condition "DCMU 4′ dark after HL" in Fig. 5A and B. Note that the fluorescence data and NPQ are scaled differently in order to demonstrate the effects of the different measurement conditions.

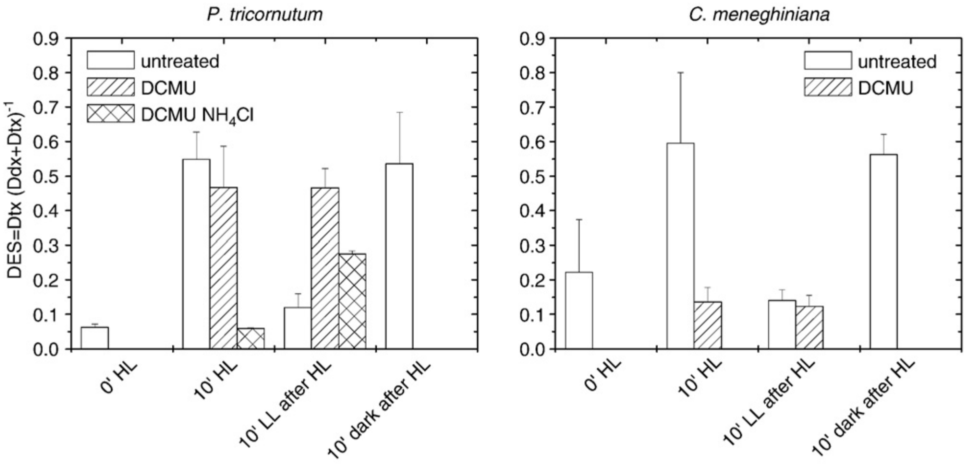

Fig. 3. De-epoxidation state (DES, given as Dtx $(Ddx+Dtx)^{-1}$) of control and DCMU treated cells of *P. tricornutum* and *C. meneghiniana* in the dark-adapted state (0′ HL), after 10 min of HL illumination with a light intensity of 820 μmol photons m^{-2} s^{-1} (10′ HL) and after 10 min of darkness (10′ dark after HL) or low light illumination (25 μmol photons m^{-2} s^{-1}) following a period of 10 min HL illumination (10′ LL after HL). For the condition "10′ LL after HL" cells were also treated with DCMU and a combination of DCMU and NH_4Cl. DCMU and DCMU/NH_4Cl were added at the end of the HL phase, after the accumulation of Dtx had taken place. Mean values of three to eight samples are depicted with the respective standard deviations.

primary electron acceptor of PSII. In principle, the reduction state of Q_A is indicated by the J-level of the fast fluorescence induction curve which is visible 2 to 3 ms after the onset of the saturating light pulse [31,32]. Since Q_A is in redox equilibrium with the PQ pool, this method also represents an estimate of changes in the reduction state of the PQ pool [33]. An increase in the J-level of the transient fluorescence is then assumed to be correlated with a more reduced Q_A/PQ pool due to electron inflow, either from PSII or from electron sources located in the chloroplast stroma [34,35]. To gain further insight into the electron flow from the PQ pool to PSI and the electron transfer steps after PSI, we performed measurements of P700 absorbance changes upon illumination by a saturating light pulse. The P700 absorbance changes provide information about the kinetics and extent of P700 oxidation and reflect both the efficiency of charge separation of P700 and the capacities of the donor and acceptor sides of PSI, respectively. Three different phases can be distinguished from the measurement of P700 absorbance changes: i) the oxidation of P700 during the first 20 to 30 ms of the saturating illumination, ii) a transient reduction of P700 between the time points of 30 and 150 ms and iii) the final oxidation of P700 after 300–400 ms. It must be emphasized that the transient reduction of P700 in general coincides with the I to P transition of the fast fluorescence induction curve and is due to an inactivation of the PSI acceptor side which inhibits the electron transfer to $NADP^+$ [35–37]. Thus, the transient reduction of P700 reflects electrons originating from PSII. Consequently, the extent of the transient reduction can be seen as an indicator of the electron pressure on PSI. Once the FNR is activated (after approximately 150 ms of saturating illumination) the acceptor side of PSI has sufficient capacity to completely oxidize P700.

Fig. 4A and B illustrates the fast fluorescence and P700 absorbance measurements of dark-adapted control cells of *P. tricornutum* and *C. meneghiniana*. In both diatoms the dark-adapted cells exhibited the typical OJIP-shape of fast transient fluorescence measurements. However, in the comparison of both diatoms, the reduction state of the PQ pool (indicated by the J-level) of *P. tricornutum* appeared to be higher than that of *C. meneghiniana*. Interestingly, this increased reduction state of the PQ pool in *P. tricornutum* was already induced after a short dark adaptation of 5 min. When DCMU was added (Fig. 4C and D), the fluorescence signal reached the highest level after only 2–3 ms in both algae. This time point coincided with the J-level and indicated the complete inhibition of electron transfer after Q_A. In the P700 absorbance measurements the transient reduction of P700, visible in the absence of DCMU (Fig. 4A and B), was completely abolished. Thus, dark adaptation under DCMU can be assumed to oxidize the electron transport system between Q_A and PSI, meaning that under these conditions no electrons can be donated to PSI to induce its transient reduction.

A different situation was observed in samples after 10 min of actinic illumination with 820 μmol photons m^{-2} s^{-1} (Fig. 4E and F). Immediately after a transition from HL to darkness a strong reduction in the PQ pool of *P. tricornutum* was detected (Fig. 4E). This was evident from the higher J-level of the fast fluorescence induction curve in comparison to the dark-adapted control sample (Fig. 4A). However, the transient reduction of P700 after 2 s of the dark period following HL illumination (Fig. 4E) was not as pronounced as in the control sample (compare to Fig. 4A). This was most likely due to a full activation of the PSI acceptor side after the HL period in contrast to the inactivated electron transfer steps after PSI during dark adaptation. The transient fluorescence induction curves and P700 absorbance measurements obtained for *C. meneghiniana* cells after 2 s of the dark period following HL illumination (Fig. 4F) showed comparable results to those recorded for *P. tricornutum* (Fig. 4E). The increase in the J-level of the fluorescence induction curve was, however, significantly less pronounced than in *P. tricornutum*. This indicates that HL illumination did not lead to a similarly high reduction state of the PQ-pool in *C. meneghiniana* as in *P. tricornutum*.

During later stages of the dark period, after 4 min of complete darkness, only a slight decrease of the reduction state of the PQ pool was observed in *P. tricornutum* (data not shown). This shows that, even 4 min after the transition from HL to darkness a high electron pressure on the PQ pool persisted. However, in the measurements of the P700 absorbance changes, a stronger transient reduction of P700 was observed after 4 min of the dark period (Fig. 5A) compared with the reduction visible after a dark adaptation of 2 s (Fig. 4E), implying increased inactivation of the PSI acceptor side during later stages of the dark phase following HL illumination.

As already observed for the fluorescence, NPQ and Ddx de-epoxidation measurements (shown in Figs. 2 and 3), the addition of DCMU to HL illuminated intact cells revealed striking differences between the two diatoms with respect to the P700 oxidation kinetics. In *P. tricornutum* a transient reduction of P700 was almost completely absent after 4 min of the dark period following HL illumination in the presence of DCMU (Fig. 5A). Thus, even in the presence of high electron pressure on the PQ pool (as deduced from the high J-level in the fast fluorescence induction measurements of control samples), there was only very weak electron flow from the PQ pool to PSI during

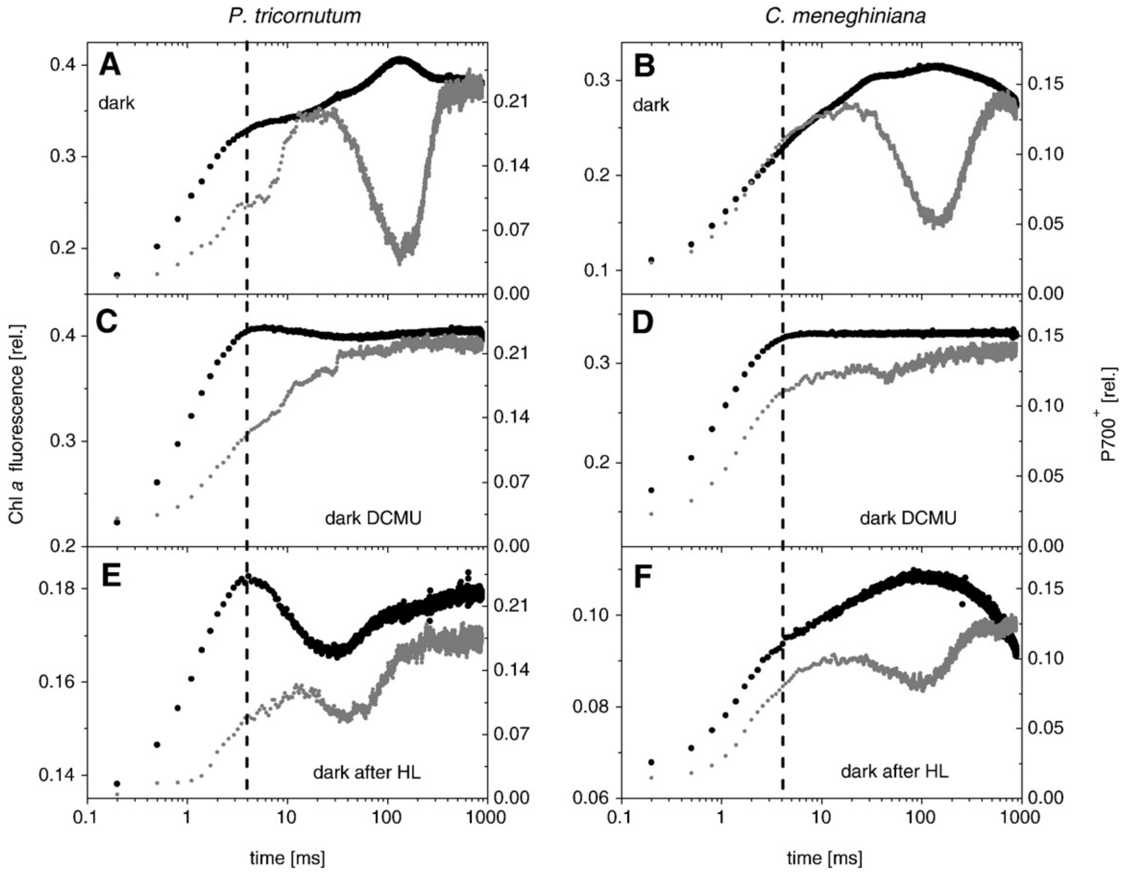

Fig. 4. Simultaneous measurements of fast Chl *a* fluorescence induction (black symbols) and absorption changes at 830 nm (photo-oxidation of P700, grey symbols) in *P. tricornutum* and *C. meneghiniana* induced by a saturating light pulse of 5000 µmol photons m^{-2} s^{-1}. Note that oxidation of P700 becomes visible as an increase in the signal of the photocurrent (depicted as $P700^{+}$) measured with the PAM-100 (for further explanations see the Materials and methods section). Time is plotted on a logarithmic scale. Measurements were performed at different time points of the illumination program (see Figs. 1 and 2 and the respective figure legends). Panels A and B (dark): the saturating light pulse was applied after a 5 min dark-adaptation (before a HL phase) to untreated samples. Panels C and D (dark DCMU): the saturating light pulse was applied after a 5 min dark-adaptation (before a HL phase) to samples incubated with 40 µM DCMU. Panels E and F (dark after HL): the saturating pulse was applied immediately (within 2 s) after a transfer of untreated samples from HL to darkness. Note that the absorption changes at 830 nm have been normalized to zero and the fast fluorescence signals are scaled differently. Data are the average of four measurements with a single culture; up to 15 measurements were carried out with different cultures on different days to confirm the results.

the saturating light flash applied for the P700 absorbance change measurement. In *C. meneghiniana*, on the other hand, the transient reduction of P700 measured after 4 min of the dark period was even stronger in the presence of DCMU compared to the control measurements where DCMU was omitted (Fig. 5B), suggesting a strong electron donation from PQ to PSI.

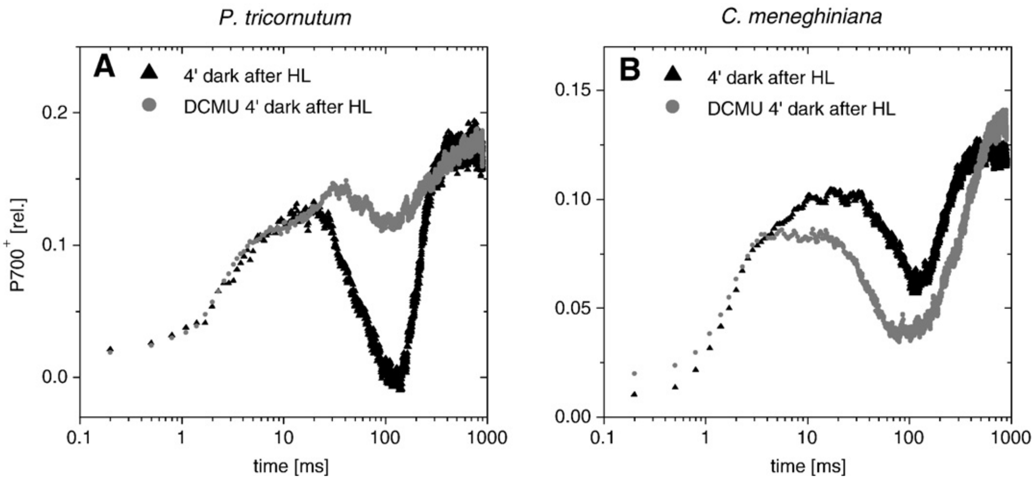

Fig. 5. P700 absorption changes at 830 nm in *P. tricornutum* and *C. meneghiniana* induced by a saturating light pulse of 5000 µmol photons m^{-2} s^{-1}. Note that oxidation of P700 becomes visible as an increase in the photocurrent (depicted as $P700^{+}$) measured with the PAM-100. Cells were measured in the absence or presence of 40 µM DCMU 4 min after a transfer from HL (10 min illumination with 820 µmol m^{-2} s^{-1}) to darkness. DCMU was added before the HL phase. Data are the average of four measurements with a single culture; up to 15 measurements were carried out with different cultures on different days to confirm the results.

4. Discussion

The objective of the present study was to investigate why Dtx epoxidation proceeds with high efficiency during low light periods following high light illumination. We also examined why Dtx epoxidation is, on the other hand, severely restricted during phases of complete darkness. To elucidate the factors controlling Dtx epoxidation, three different experimental conditions were established and investigated with respect to NPQ, the de-epoxidation state of the Ddx cycle, the reduction state of the PQ pool and the kinetics of P700 oxidation: i) intact cells were transferred from HL to LL and were compared with cells subjected to a transition from HL to darkness, ii) the transition from HL to LL was also carried out in the presence of DCMU (given at the end of the HL period) and iii) intact cells of *P. tricornutum* and *C. meneghiniana* were illuminated with HL intensities in the presence of DCMU.

4.1. Transition from HL to LL and from HL to darkness

After the transition from HL to LL, rapid decreases of NPQ as well as rapid Dtx epoxidation were observed in both diatoms (Fig. 1C and D). On the other hand, during periods of darkness following HL illumination, both the decrease of NPQ and Dtx epoxidation were strongly inhibited (Fig. 1A and B). These results confirm earlier studies [5] and lead to the conclusion that the cosubstrate of Dtx epoxidase, NADPH, was available in sufficient concentration during the LL period following HL illumination but was absent during the phase of complete darkness. Possible candidates for an increased oxidation of NADPH after the transition from HL to darkness are the electron consumption by ongoing CO_2-fixation in the Calvin-Cycle or a chlororespiratory electron flow.

In both diatoms, measurements of the fast fluorescence kinetics showed an increase of the reduction state of the PQ pool at the beginning of the dark period following HL illumination which persisted for at least 4 min in darkness (Figs. 4 and 5). This dark reduction of the PQ pool is generally believed to result from a chlororespiratory electron donation to the PQ pool by the oxidation of NADPH or NADH via an NAD(P)H dehydrogenase (Ndh) [13,14,16,17]. Further evidence for the reduction of PQ by stromal electron sources was derived from measurements of the P700 absorbance changes, where directly after the transition from HL to darkness a transient reduction of P700 was found (Fig. 4E and F). Such a transient reduction is normally detected only during a saturating pulse applied to dark-adapted cells (Fig. 4A and B), where it can be explained by the presence of an inactive PSI acceptor side, leading to an accumulation of electrons delivered from PSII [38,39]. Since the transient reduction of P700, observed in both *P. tricornutum* and *C. meneghiniana* after a period of HL illumination, took place in the presence of a still active PSI acceptor side, it is likely that a strong alternative electron flow from the chloroplast stroma to the PQ pool existed in both diatoms. The reduction state of the PQ pool after HL illumination was higher in *P. tricornutum* than in *C. meneghiniana* (Fig. 4E and F). Taking into account the comparable transient P700 reduction this implies a stronger electron pressure on the PQ pool in *P. tricornutum*, which could have been due to increased activity of the Ndh.

4.2. Transition from HL to LL in the presence of DCMU

Considering the assumption that the inhibition of Dtx epoxidation after the transition from HL to darkness is caused by a shortage of NADPH, inhibition of linear electron transport during illumination should block the reduction of $NADP^+$ to NADPH and finally result in a suppressed conversion of Dtx during the LL phase. To test this hypothesis, DCMU was added at the end of the HL period just before the transition to LL illumination. Consistent with the hypothesis, epoxidation of Dtx and decrease of NPQ in the LL phase were inhibited in DCMU-treated cells of *P. tricornutum* (Figs. 2A and 3). This leads to the conclusion that, immediately after the HL period, NADPH is not available in sufficient amounts to allow efficient Dtx epoxidation and LL illumination is required for the reduction of $NADP^+$ to NADPH. It could also be argued that the NADPH shortage after HL illumination is due to a small pool size of NADPH in the chloroplast and/or to a very active NADPH-consuming reaction, like the NAD(P)H dehydrogenase involved in the chlororespiratory electron flow or the ongoing CO_2-fixation by the Calvin-Cycle. NADPH consumption by the Calvin-Cycle is likely to proceed until Rubisco has been inactivated. Inactivation of Rubisco was shown to take place on a time scale of 12 to 18 min after the termination of a HL period in the diatoms *T. pseudonana* and *T. weissflogii* [40].

As chlororespiration is able to generate a trans-membrane proton gradient [13], a second factor was established during the LL period in the presence of DCMU that acted as a regulatory element for the Ddx cycle. In cells of *P. tricornutum* treated with a combination of DCMU and the uncoupler NH_4Cl a decrease of the DES was observed during the LL period after HL illumination, though not as pronounced as in untreated cells (Fig. 3). This confirms observations [16] that, in *P. tricornutum*, NPQ in the presence of DCMU can be partly abolished by addition of NH_4Cl. It also supports the assumption that in LL-illuminated cells, a ΔpH persisted even in the presence of DCMU, keeping the Ddx de-epoxidase in an activated state. It is important to note that the extent of the proton gradient did not need to be high since it is known from previous studies that the Ddx de-epoxidase in diatoms can be activated at almost neutral pH-values [3,4]. In addition, the possibility that the ΔpH at least partially inhibited the proton gradient sensitive Dtx epoxidase cannot be excluded [5,7]. Taken together it must be concluded, that an effective Dtx epoxidation in DCMU-treated cells of *P. tricornutum* after a HL to LL transition was inhibited by a combination of a decreased NADPH availability and an ongoing Ddx de-epoxidase reaction.

In *C. meneghiniana*, however, both Dtx epoxidation and NPQ decrease during the LL phase showed the same kinetics in the presence or absence of DCMU (Figs. 1D, 2B and 3). Based on the observation that Dtx epoxidation was inhibited during darkness after actinic HL illumination, decreased availability of NADPH after HL illumination must also be assumed for *C. meneghiniana*. However, to support the fast Dtx epoxidation observed in *C. meneghiniana* during the LL phase, the NADPH supply must have been sufficient, both in the presence or absence of DCMU. Inevitably, this leads to the conclusion that in *C. meneghiniana* $NADP^+$ was reduced by an alternative electron donor other than PSII and that these electrons were shuttled to the active PSI via the PQ pool. The production of NADPH by this alternative electron transport finally enabled Dtx epoxidation during LL illumination in DCMU-treated cells. To further investigate the reasons for the differences between *P. tricornutum* and *C. meneghiniana* with respect to the pathways of alternative electron transport, the Ddx cycle activity, NPQ, the reduction state of PQ and P700 absorbance changes were analyzed under conditions in which DCMU was added before the start of actinic HL illumination.

4.3. Illumination with HL in the presence of DCMU

As observed in a previous study [23], DCMU-treated cells of *P. tricornutum* showed a similar extent of NPQ and DES of the Ddx cycle pigment pool during HL illumination as control cells in the absence of DCMU (Figs. 1A, 2C and 3). This means that a Ddx de-epoxidation and NPQ driving proton gradient must have been established, although DCMU completely inhibited the PSII electron transport (as evidenced by the measurements of fast fluorescence induction depicted in Fig. 4C). The build-up of a ΔpH in the presence of DCMU was further corroborated by the addition of a combination of the uncoupler NH_4Cl and DCMU before the start of the HL illumination. *P. tricornutum* cells treated in this way were unable to show NPQ and the DES was comparable to dark-adapted control cells (Fig. 3) due to the absence of a ΔpH-dependent activation of Ddx de-epoxidation.

A proton gradient in DCMU-treated *P. tricornutum* cells could have been established either by a chlororespiratory electron transport or by a cyclic electron transfer around PSI, as originally suggested by [23]. Both processes would have been able to co-translocate protons across the thylakoid membrane. Chlororespiration seems more likely than a PSI electron cycle, since the addition of methylviologen (MV) was shown to be unable to affect NPQ or the DES of the Ddx cycle pool in DCMU-treated cells [23]. MV is expected to draw electrons from PSI and should inhibit an electron cycle around PSI and thus, prevent the acidification of the thylakoid lumen required for xanthophyll cycle activity.

In contrast to *P. tricornutum*, cells of *C. meneghiniana* did not show NPQ and did not activate the Ddx de-epoxidase in the presence of DCMU (Figs. 2D and 3), although stromal electron sources that should have contributed to an increased reduction state of the PQ pool existed. In *C. meneghiniana*, these alternative electrons were, however, donated to PSI without the establishment of a ΔpH. This conclusion is supported by the measurements of the P700 absorbance changes where a very strong transient reduction of P700 was observed 4 min after the transition from HL to darkness in the presence of DCMU (Fig. 5B).

In *P. tricornutum* on the other hand, the transient reduction of P700 after the transition from HL to darkness was strongly diminished in the presence of DCMU (Fig. 5A). This result is remarkable in the light of the strong reduction of the PQ pool, as indicated by the measurements of the fast fluorescence induction in the absence of DCMU and the inactivated PSI acceptor side. Thus, during the saturating light pulse used to determine the P700 absorbance changes, electrons delivered from stromal sources must have been directed preferentially to electron sinks other than PSI. It must be assumed that in *P. tricornutum* these alternative electrons were consumed by an oxygen-reducing PQ oxidase involved in chlororespiratory electron transport that is not present in *C. meneghiniana*. Evidence for the existence of a chlororespiratory proton gradient in *P. tricornutum* was then derived from the strong de-epoxidation of Ddx in DCMU-treated cells and its sensitivity to the addition of the uncoupler NH_4Cl (see above).

4.4. A model for the regulation of the diadinoxanthin cycle by alternative electron flow in P. tricornutum and C. meneghiniana

A working hypothesis representing the interaction of alternative electron pathways and xanthophyll cycle activity in *P. tricornutum* and

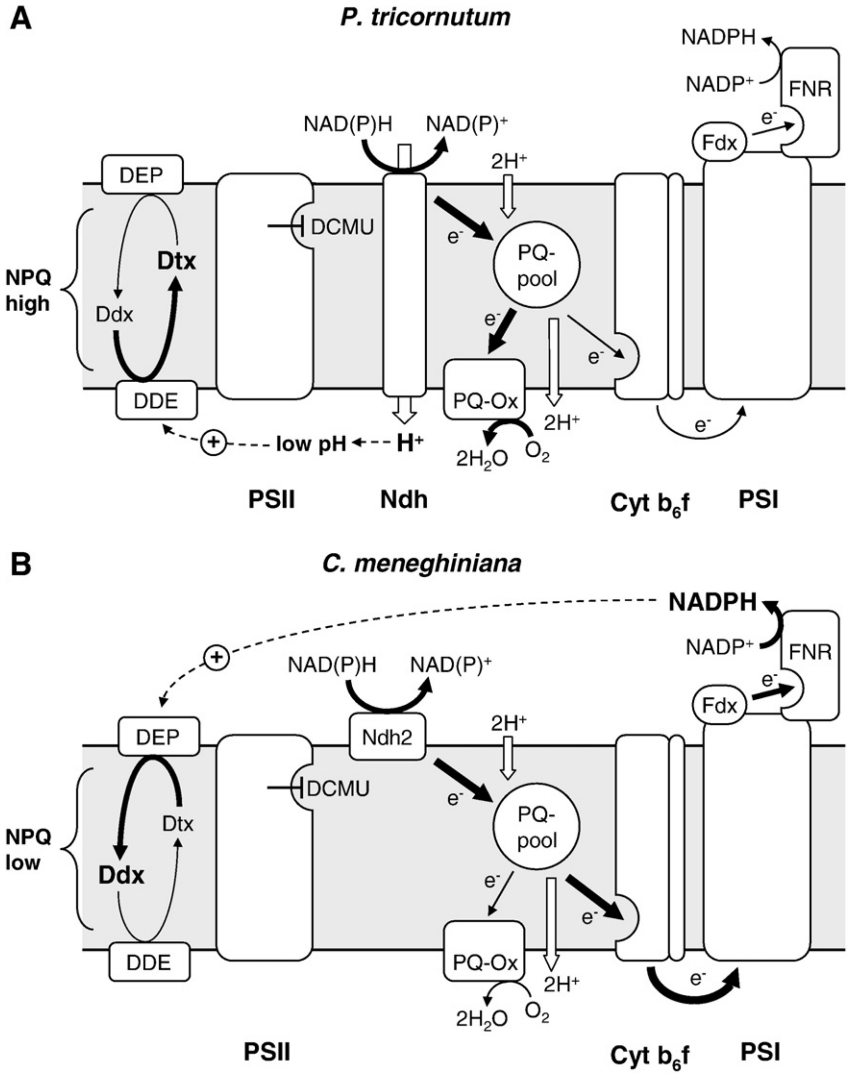

Fig. 6. A model depicting the alternative electron pathways in the diatoms *P. tricornutum* (A) and *C. meneghiniana* (B) during a period of LL illumination in the presence of DCMU after a preceding HL illumination in the absence of DCMU. The importance of the alternative electron transport for the generation of NADPH and the regulation of the Ddx cycle are illustrated (for details refer to the text). Abbreviations: Ndh: NADPH-dehydrogenase (type 1 and 2); PQ: plastoquinone; PQ-Ox: plastoquinone oxidase; Fdx: ferredoxin; FNR: ferredoxin-NADP$^+$ oxidoreductase; Ddx: diadinoxanthin; Dtx: diatoxanthin, DDE: diadinoxanthin de-epoxidase, DEP: diatoxanthin epoxidase.

C. meneghiniana is shown in Fig. 6. The model depicts the electron flow in algal cells during a low light period in the presence of DCMU following HL illumination. As DCMU is an inhibitor of PSII-electron transport at the Q_A site, all electrons leading to the observed changes in the reduction state of PQ and the oxidation kinetic of P700 must have been provided by stromal reductant sources. In *P. tricornutum* (Fig. 6A), these PQ-reducing alternative electrons are guided preferentially to a PQ-oxidase, although PSI is simultaneously excited by the LL illumination. In the alternative electron transport of *P. tricornutum*, the reduction of $NADP^+$ to NADPH is prevented to a large extent, as indicated by the inhibition of Dtx epoxidation during the low light phase in the presence of DCMU. The alternative electron transport via the PQ pool is coupled to a co-translocation of protons. However, it must be assumed that these protons are consumed by the PQ oxidase after the reduction of molecular oxygen. This means that a chlororespiratory proton gradient can only be established when a membrane-intrinsic, proton-pumping Ndh comparable to the mitochondrial complex I is involved [41,42]. Although the results of the present study do not provide direct evidence for the involvement of a thylakoid type I NAD(P)H dehydrogenase in the alternative electron transport of *P. tricornutum*, they clearly demonstrate that a chlororespiratory proton gradient is established. This ΔpH plays an important role in the regulation of the Ddx cycle through the activation of Ddx de-epoxidation and the simultaneous inhibition of Dtx epoxidation.

In contrast to our knowledge about the Ndh as the PQ-reducing enzyme complex, the nature and localization of the PQ oxidizing enzyme remains largely enigmatic. For vascular plants, the involvement of the PSII associated Cyt *b*-559 has been suggested in the PQ pool oxidizing reaction [43,44]. Cyt *b*-559 is also known as a basic component of the cyclic electron transport around PSII (for a review see [45]) which may play an important role in the alternative electron pathways of intact cells of *P. tricornutum* during illumination with high light intensities [46].

For *C. meneghiniana*, we propose a different pathway of alternative electron transport in which the electrons donated by stromal reductants are not consumed by a PQ oxidase, but are guided to PSI with high priority (Fig. 6B). These alternative electrons are then used for NADPH production, which enables the rapid Dtx epoxidation observed in DCMU-treated *C. meneghiniana* cells under LL illumination. Reduction of $NADP^+$ to NADPH is possible, however, only if additional alternative electrons are provided by, e.g., cytosolic reductants. In contrast to *P. tricornutum*, no indication of the establishment of a transmembrane proton gradient was found in intact cells of *C. meneghiniana* during LL illumination in the presence of DCMU. This may indicate the involvement of a type 2 NAD(P)H dehydrogenase (Ndh2) in the alternative electron transport of *C. meneghiniana*. This enzyme, which consists of a single subunit, is not coupled to a proton translocation across the thylakoid membrane [20,47].

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