The diatom *Phaeodactylum tricornutum* adjusts nonphotochemical fluorescence quenching capacity in response to dynamic light via fine-tuned Lhcx and xanthophyll cycle pigment synthesis

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**Summary**

- Diatoms contain a highly flexible capacity to dissipate excessively absorbed light by nonphotochemical fluorescence quenching (NPQ) based on the light-induced conversion of diadinoxanthin (Dd) into diatoxanthin (Dt) and the presence of Lhcx proteins. Their NPQ fine regulation on the molecular level upon a shift to dynamic light conditions is unknown.
- We investigated the regulation of Dd + Dt amount, Lhcx gene and protein synthesis and NPQ capacity in the diatom *Phaeodactylum tricornutum* after a change from continuous low light to 3 d of sine (SL) or fluctuating (FL) light conditions. Four *P. tricornutum* strains with different NPQ capacities due to different expression of Lhcx1 were included.
- All strains responded to dynamic light comparably, independently of initial NPQ capacity. During SL, NPQ capacity was strongly enhanced due to a gradual increase of Lhcx2 and Dd + Dt amount. During FL, cells enhanced their NPQ capacity on the first day due to increased Dd + Dt, Lhcx2 and Lhcx3; already by the second day light acclimation was accomplished. While quenching efficiency of Dt was strongly lowered during SL conditions, it remained high throughout the whole FL exposure.
- Our results highlight a more balanced and cost-effective photoacclimation strategy of *P. tricornutum* under FL than under SL conditions.

**Introduction**

Diatoms are unicellular microalgae constituting one of the most important phytoplankton groups in terms of biodiversity (Mann & Vanormelingen, 2013) and productivity (c. 45% of marine carbon fixation) (Geider et al., 2001). They participate strongly in the biological carbon pump and the functioning of contemporary aquatic ecosystems (Armburst, 2009). Due to their high productivity and high lipid content, diatoms could potentially replace American fossil oil consumption in the future (Levitan et al., 2006; Kroth et al., 2008; Lepetit et al., 2012; Bailleul et al., 2015) and effective photoprotection mechanisms that include: (1) a fast operating photosystem II (PSII) electron cycle (Lavaud et al., 2002c; Wagner et al., 2016), (2) a tuneable amount of membrane-dissolved xanthophylls diadinoxanthin (Dd) and diatoxanthin (Dt) acting as antioxidants (Lepetit et al., 2010), and (3) a high capacity for dissipation of excess excitation energy, illustrated by nonphotochemical fluorescence quenching (NPQ) (Lavaud & Goss, 2014). NPQ in plants and green algae is divided into three to four subtypes, which are not similarly well defined in diatoms (Lavaud & Goss, 2014; Goss & Lepetit, 2015). Here we will refer to NPQ as a photoprotective
mechanism whose induction depends upon three regulatory components: (1) the proton gradient generated between the thylakoid lumen and the chloroplast stroma during light exposure (ΔpH), (2) a fast operating xanthophyll cycle (XC) through enzymatic conversion of Dd to Dt in the presence of the ΔpH and the back conversion in its absence (i.e. typically in the dark), and (3) chloroplast located, but nuclear-encoded antenna proteins of the light-harvesting complex (LHC) superfamily. While Lhcf proteins build up the peripheral light harvesting antenna (called ‘FCP’, probably predominantly associated with PSII in vivo; Grouneva et al., 2011; Gundermann et al., 2013; Nagao et al., 2013; Schaller-Laudel et al., 2015), Lhc proteins form the PSI antenna (Veith et al., 2009; Lepetit et al., 2010; Grouneva et al., 2011; Ikedo et al., 2013; Bina et al., 2016). By contrast, Lhcx proteins are involved in NPQ in the pennate diatom Phaeodactylum tricornutum (Bailleul et al., 2010; Lepetit et al., 2013) and the centric Thalassiosira pseudonana (Zhu & Green, 2010; Wu et al., 2012; Dong et al., 2015). Involvement of Lhcx proteins in photoprotection in other diatoms is also very likely (Beer et al., 2006; Park et al., 2010; Laviale et al., 2015; Ghazaryan et al., 2016). It is assumed that Lhcx proteins bind Dd and Dt (Beer et al., 2006; Lepetit et al., 2013) and they apparently influence the supramolecular organisation of the antenna complexes (Ghazaryan et al., 2016). The location of Lhcx within thylakoids remains ambiguous as based on contrasting reports of both FCP (Beer et al., 2006; Lepetit et al., 2010; Grouneva et al., 2011; Nagao et al., 2013; Schaller-Laudel et al., 2015) and PSI protein (Grouneva et al., 2011). The current NPQ model proposes two major quenching sites in diatoms (Miloslavina et al., 2009; Chukhutsina et al., 2014; Lavaud & Goss, 2014; Derks et al., 2015; Goss & Lepetit, 2015): Quenching site 1 is mechanistically independent of Dt (Chukhutsina et al., 2014) and is formed rapidly mainly by detached, oligomeric antenna complexes due to build-up of the ΔpH, while quenching site 2 is located close to the PSII reaction centres and is directly dependent on Dt formation. Quenching site 1 also exists in P. tricornutum (Miloslavina et al., 2009), but in this species NPQ always relies on Dt (Lavaud et al., 2002a; Goss et al., 2006), except under special (i.e. artificial) conditions (Lavaud & Kroth, 2006; Eisenstadt et al., 2008; Lepetit et al., 2013). This apparent contradiction has not yet been fully resolved, but an indirect influence of Dt also on formation of quenching site 1 has been postulated (Lavaud & Goss, 2014; Goss & Lepetit, 2015).

In different diatoms, NPQ capacity, in relation to the light environment of the respective planktonic and benthic habitat, can be rather variable. Diatoms that cope with sudden light exposures, for example coastal planktonic and immotile estuarine sediment-inhabiting diatoms, show a higher NPQ capacity than diatoms living in more stable water bodies (e.g. semi-enclosed bays, open ocean) and the motile and photophobic sediment-inhabiting forms (Lavaud et al., 2007; Dimier et al., 2009; Barnett et al., 2015). The same holds true for diatom species adapted to the seasonally successive polar habitats (Petróu et al., 2011). P. tricornutum is cosmopolitan, but prefers habitats where light climate is unstable and reaches punctual but regular high intensities, such as coasts, estuaries or rocky pools (De Martino et al., 2007). Different P. tricornutum ecotypes have different and variable NPQ capacities (Lavaud & Lepetit, 2013) that depend largely on the amount of Lhcx1 (Bailleul et al., 2010). Additionally, there is growing evidence that two other light-regulated isoforms, Lhcx2 and Lhcx3, may also participate in NPQ under prolonged light stress (Lepetit et al., 2013; Taddei et al., 2016).

For several diatom species the influence of near-natural light conditions on photosynthetic performance and on growth has been thoroughly investigated (Kromkamp & Limbeck, 1993; Litchman, 2000; Fietz & Nicklisch, 2002; Wagner et al., 2006; Jakob et al., 2007; van de Poll et al., 2007; Kroopenske et al., 2009; Su et al., 2012; Jallet et al., 2016). As P. tricornutum is one of the best characterized diatoms on the molecular level, we investigated its photophysiology during acclimation from stable low light to dynamic and potentially stressful light conditions with respect to the interplay between Dd + Dt synthesis, Lhcx expression and NPQ capacity. Sine light (SL) conditions simulated the rise and decline of the sun during a cloudless day in a stable water body. Fluctuating light (FL) conditions superimposed the effect of vertical cell movement along the water column in an idealized manner with a mixing from and to the aphotic zone via the surface within periods of 30 min. We also investigated the influence of the initial photoprotection capacity on the adjustment of NPQ capacity to dynamic light. Therefore, we used two P. tricornutum ecotypes with different initial NPQ capacities (i.e. low and high natural NPQ phenotypes, see Bailleul et al., 2010), but also strains with silenced or overexpressed Lhcx1 protein. Our results contribute to a better understanding of the molecular fine-tuning of NPQ capacity during acclimation to dynamic light conditions in pennate diatoms.

Materials and Methods

Cell culturing and light treatments

Experiments were performed in four P. tricornutum strains with different NPQ capacities: (1) P. tricornutum strain 1 (Pt1, CCAP 1055/1); (2) Pt1sil, a Pt1 strain which contains an antisense construct against the Lhcx1 gene, leading to reduced Lhcx1 protein synthesis (Bailleul et al., 2010); (3) P. tricornutum strain 4 (Pt4, UTEX 646); and (4) Pt4ov, a Pt4 strain which overexpresses the Lhcx1 gene. The full length cDNA of the Lhcx1 gene (GI ID: 27278) was cloned downstream of the FcpA (Lhef1) promoter into the pPha-T1 transformation vector (Zaslavskaya et al., 2000). Pt4 cells were biolistically transformed with this construct according to Kroth (2007). Positive clones were selected on Zeocin™ (Thermo Fisher Scientific, Waltham, MA, USA) containing solid medium plates. Lhcx1 overexpressing clones were screened based on their NPQ capacity and amongst several clones showing increased NPQ capacity the one with the highest NPQ was selected for the present experiments (Pt4ov). This clone has an identical photosynthetic yield to the wild-type under low light conditions (data not shown), but shows strongly increased Lhcx1 gene expression (Supporting Information Fig. S1). All four strains were grown in airlift tubes (4 cm diameter) at 20°C in a
16 h : 8 h, day : night rhythm with a light intensity of 50 μmol photons m$^{-2}$s$^{-1}$ (onset at 08:00 h) defined as low light (LL). Light was provided by computer-controlled flora LED units (CLF Plant Climatics, Wertingen, Germany) with all LEDs (white, blue, red and far red) switched on. Cells were cultured in sterile Provasoli’s enriched F/2 seawater medium. Chlorophyll a (Chla) concentration was determined as described by Lepetit et al. (2013). Cells in logarithmic growth phase were adjusted to a Chl$\alpha$ concentration was determined daily at 18:00 h and cultures were diluted with fresh F/2 medium to a concentration of 1.4 μg Chla ml$^{-1}$ to prevent nutrient limitation and self-shading.

**Sampling**

Cells were harvested with a sterile syringe via a tube drawn in the airlift flask which was sealed except for sampling. For pigment analyses, 500 μl of cells was filtered each day at 11:00, 14:00 and 17:00 h on an Isopore Polycarbonate filter 1.2 μm (Millipore, Billerica, MA, USA) and immediately frozen in liquid nitrogen. Cells exposed to FL were additionally harvested during the light maxima directly before the three indicated time points. For gene expression and protein analyses, 15 and 23 ml of cell suspension, respectively, was harvested each day at 14:00 h and centrifuged for 4 min at 4°C and 4000 g. The precipitated cells were resolved in 1 ml ice-cooled phosphate-buffered saline and centrifuged at 14 000 g for 1 min. The pellet was frozen in liquid nitrogen and stored at −80°C until further analysis.

**Fluorescence analyses**

Cells harvested each day at 11:00, 14:00 and 17:00 h were acclimated to 30 μmol photons m$^{-2}$s$^{-1}$ for 30 min before measuring the maximum photosynthetic efficiency of PSII as ($F_{m'}/F_{m}$). $F_{m}$ = $F_{m}/F_{m'}$ with an Aqua Pen (Photon Systems Instruments, Brno, Czech Republic). To take into account slower relaxing NPQ processes and to assess the maximum NPQ capacity, rapid light curves, measured with a Water PAM and an Imaging PAM (Walz), were recorded after 45 min acclimation to 30 μmol photons m$^{-2}$s$^{-1}$, by applying 13 steps of increasing light intensity up to 1250 μmol m$^{-2}$s$^{-1}$ with a respective duration of 30 s at 455 nm. Before the onset of the actinic light and during each rapid light curve, an 800 ms pulse of 4000 μmol photons m$^{-2}$s$^{-1}$ was applied to determine the maximum fluorescence $F_{m}$ and $F_{m'}$, respectively. Maximum relative electron transport rates (rETR$_{max}$) and other photosynthetic and photoprotective parameters were obtained by fitting the obtained values according to Eilers & Lavaud (2011). A description of these parameters can be found in Table S1.

**Pigment, transcript and protein analyses**

Pigment extraction and high-performance liquid chromatography (HPLC) analysis were performed as described in Lepetit et al. (2013). The de-epoxidation state was calculated as DES = Dr/(Dd + Dr).

RNA extraction, cDNA synthesis, quantitative PCR (qPCR) and quantification followed the protocol in Lepetit et al. (2013), except that RPS (ribosomal protein S1, JGI ID: 44451) was used as the reference gene instead of RPS. The primer sequences for Lhcx1, Lhcx2 and Lhcx3 are listed in Lepetit et al. (2013). For RPS, we used 5′-AATTCTCTGAAGTCAACCA GG-3′ and 5′-GTGCAGAAGACCGACATAC-3′ as forward and reverse primer, respectively, and for Lhcx2 the forward and

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**Fig. 1** Dynamic light conditions used in the experiments. One sine light (SL) and two fluctuating light (FL) conditions with different intensities were applied during the daily phases of the light exposure and light intensity was measured every minute.
reverse primers were 5'-GCCGATATCCCCAATGGATTT-3' and 5'-CTTGTTGCAAGGACTCCCATC-3', respectively.

Protein extraction and Western blot analysis followed the protocol described in Coesel et al. (2009), but using a 14% lithium dodecylsulfate polyacrylamide gel electrophoresis for protein separation. Samples corresponding to an amount of 1 μg Chl a were loaded on the gel. Anti-FCP6 (Westermann & Rhiel, 2005) was kindly provided by Dr Erhard Rhiel (University of Oldenburg, Germany). This antibody detects all Lhcx isoforms in P. tricornutum (Laviale et al., 2015), but based on its sequence it has the highest affinity for Lhcx3 and the lowest affinity for Lhcx2. Anti-FCP6 was applied at a 1 : 5000 dilution overnight. Accurate loading and blotting was verified by correct transfer of pre-stained protein markers (Roti-Mark Bicolor, Roth, Germany) on the PVDF membrane (Amersham Hybond-P; GE Healthcare, Piscataway, NJ, USA), by staining gels with the Coomassie R-250 Pierce Imperial Protein stain (Thermo Fisher Scientific) and by incubating the blot membrane with anti-PsbB (CP47; Agrisera, Vännäs, Sweden). Anti-PsbB detection was only used as a proxy for correct loading and blotting, as the amount of CP47 has a high turnover under light stress conditions (Wu et al., 2011). Antibody signals were detected using the ECL Plus chemiluminescence system (GE Healthcare) followed by X-ray film exposure. To compare relative Lhcx protein expression within the time-course of the experiments, protein samples of each P. tricornutum strain of either the SL or the FL 1000 experiment were loaded on a single gel. Antibody signal intensities were quantified using IMAGEJ (http://imagej.nih.gov/ij/). For each blot several films with different exposure and development times were produced, to avoid saturation of the immunodetection signal for Lhcx1 (due to its high abundance) and Lhcx3 (due to its highest affinity to anti-FCP6), while also obtaining a signal for the weakly visible Lhcx2 protein (due to its lowest affinity to anti-FCP6). Relative quantification of Lhcx1 and Lhcx3 was based on the corresponding signal at unstressed conditions (day 0). As Lhcx2 could not be detected at day 0, relative quantification was performed based on comparison of Lhcx2 to the Lhcx3 value at day 0. For each experimental point (SL and FL 1000), the mean of all strains was calculated, except that Pt4ov was omitted from the analysis of Lhcx1 expression due to its artificial regulation by the overexpressing Lhcf1 promoter (see section ‘Cell culturing and light treatments’).

Statistics
Significance at a P value ≤ 0.05 was determined with Student’s t-test calculated with SIGMAPLOT (Systat Software Inc., Chicago, IL, USA), but for gene expression we used the Pairwise Fixed Reallocation Randomization Test performed by REST according to Pfaffl et al. (2002).

Results
NPQ capacity and photosynthetic parameters during SL and FL conditions
Before exposing cells to dynamic light conditions, we analysed their NPQ capacity under stable low light growth conditions. NPQ capacity was highest in the Lhcx1 overexpressing strain Pt4ov with values between 4 and 5 (Fig. 2, day 0). Pt1 showed an NPQ capacity of c. 2.5, Pt1sil of c. 2 and Pt4 of c. 1.8 (Fig. 2). These different NPQ capacities were mainly due to different expression of the Lhcx1 gene (Fig. S1), as already shown in Baileul et al. (2010). The very high NPQ capacity of the Pt4ov strain was achieved by driving Lhcx1 gene expression by the Lhcf1 promoter.
promoter, resulting in a >10-fold higher Lhcx1 transcript amount (Fig. S1).

The rationale of working with *P. tricornutum* strains showing naturally or genetically manipulated differential NPQ capacities was to investigate whether these differences would influence their acclimation to dynamic light conditions. There was a strong continuous rise of NPQ capacity during the SL treatment (except in Pt4ov, see later), while under FL conditions NPQ increased on day 1 similarly as under SL conditions, but then slowed down (Fig. 2). Importantly, the high NPQ strains (Pt1 and Pt4ov) still exhibited the highest NPQ at the end of both dynamic light treatments. Pt4 eventually reached a higher or similar NPQ as Pt1sil under SL and FL conditions, respectively. The Pt4ov strain behaved somewhat differently, as there was no increase of NPQ on day 1 under SL. This is probably due to the pronounced decrease of *Lhcx1* gene expression (Fig. S1), as its overexpressing Lhef1 promoter is repressed under light stress (Nymark et al., 2009). However, from day 2 onward Pt4ov also started to increase NPQ capacity. Interestingly, although under stable LL conditions (i.e. day 0) Pt4ov had already higher NPQ values than the other strains at the end of FL treatment, it further increased NPQ during FL exposure (Fig. 2). All these results failed to indicate a strong influence of initial NPQ capacity on the NPQ adjustment to dynamic light; instead, all strains responded in a similar manner by increasing their NPQ capacity. As the standard errors were rather high due to the dynamic nature of the experiment, in the following we combined results of the different strains to better reveal the specific response of *P. tricornutum* to the very distinct dynamic light conditions. In this way, it became directly apparent that the doubling of light intensity under FL 1000 conditions led to no further increase of NPQ compared to FL 500 conditions. In fact, with a final value of c. 3.2 the NPQ was almost identical at both FL conditions, while it was 5.8 under SL conditions (Fig. 3).

The maximum photosynthetic yield of PSII gradually decreased during SL conditions, especially during day 3, to 0.55 (20% decrease), illustrating the appearance of photoinhibition (Fig. 4a). By contrast, Fv/Fm remained high (c. 0.65) during FL conditions. Again, no differences between FL 500 and FL 1000 conditions were observed (data not shown, but can be seen from the minimal error bars in Fig. 4a). Because also no major differences between FL 500 and FL 1000 conditions could be observed during the follow-up experiments, data were combined (unless otherwise noted) to highlight significant differences compared to SL.

Under SL conditions, rETRmax (see Table S1 for definitions of the parameters) dropped during the first day and increased slightly over the next 2 d compared to LL (Fig. 4b). By contrast, rETRmax did not change during the first day of FL, but increased strongly during days 2 and 3. It decreased by roughly 20% even during the first day of SL and then stabilized (Fig. 4c). It did not change to any great degree under FL conditions. Interestingly, there was no major change in E50 between SL and FL conditions (Fig. 4d). It increased by c. 50% during day 2 and stabilized over day 3. The apparent low E50 values were probably due to the blue light of the Imaging-PAM excitation beam and are in line with previous E50 values obtained in the same way (Serôdio & Lavaud, 2011). Huge differences were observed in NPQ confess/NPQmax. It increased from c. 7% in LL to almost 20% under SL conditions, while under FL it even decreased (Fig. 4e). In line with this, E50NPQ also behaved differently: at first it decreased, but then increased under both conditions. However, while the final E50NPQ value under SL conditions was similar as under LL, it was >1.5-fold higher under FL conditions (Fig. 4f).

**Pigment stoichiometry during SL and FL**

The Chla increase per day per culture volume (μChla) differed strongly under both dynamic conditions. Under SL conditions, μChla decreased dramatically by 50% on day 1 (compared to LL) and by 80% over the rest of the experiment (Fig. 5). By contrast, under FL conditions a slight decrease of μChla occurred during the first day, while even from the second day onward the cultures produced as much Chla per day as during LL conditions. For both the Chlc to Chla and the Fx to Chla ratio, no remarkable differences between SL and FL conditions were observed (Fig. S2).

In general, changes of the NPQ capacity were well reflected by changes in the amount of XC pigments. Dd + Dt increased 3.4-fold during SL treatment, reaching its maximum on the last day (Fig. 6). Although most of the increase had occurred by day 1, the XC pool size increased steadily during days 2 and 3 with repeated drops at night. By contrast, under FL conditions the
increase of XC pigments was only 1.7-fold, and the largest part of XC pigments was also synthesized on day 1. The observed trend was identical in all strains under both light conditions, although Pt1 strains synthesized more Dd + Dt than Pt4 strains (Fig. S3).

DES (Dt/(Dd + Dt)) values reached c. 50% during the first day of SL at 14:00 h (6 h after light onset) and 17:00 h (9 h after light onset) (Fig. S4). During the following SL days and during all 3 d of FL conditions, DES was lowest in the morning (11:00 h, 3 h after light onset) and highest in the afternoon (17:00 h), in line with the respective light intensities. During the light intervals of FL conditions, DES reached similar values as under SL conditions, but always decreased below 10% during the subsequent low light/dark phases, indicating that Dt epoxidation took place rapidly during the decline of light intensity.

NPQ vs Dd + Dt relationship

The ratio of NPQ to Dd + Dt is a robust indicator of the efficiency of the XC pigments to confer NPQ (Lavaud & Lepetit, 2013; Lepetit et al., 2013). NPQ/(Dd + Dt) decreased strongly during the first day of SL treatment and recovered during the following days (Fig. 7). At the last time point (day 3, 17:00 h), it became statistically indistinguishable from day 0, indicating that
eventually Dt quenching efficiency reached those of LL-acclimated cells. By contrast, NPQ/(Dd + Dt) remained high under FL conditions, highlighting a high quenching efficiency of Dt. During the second and third days it was statistically significantly higher at some time points than under LL conditions.

Lhcx gene expression and protein synthesis

In agreement with previous analyses for low light to high light shifts (Nymark et al., 2009; Lepetit et al., 2013), cells of all Phaeodactylum tricornutum strains strongly increased the transcript amounts of Lhcx2 and Lhcx3 under both SL and FL conditions, while Lhcx1 was only slightly more transcribed (Fig. S1; note that Lhcx1 transcription in Pr4ov reacted differentially due to regulation of the Lhcx1 gene by the Lhcf1 promoter). This became even more obvious when comparing the mean values of all strains (Fig. 8a–c). Intriguingly, there was a strong difference in Lhcx2 and Lhcx3 transcript amounts depending on the light climate: under SL conditions, Lhcx2 transcription was much more pronounced than under FL. By contrast, Lhcx3 transcript amount was higher under FL conditions. Transcription of Lhcx2 and Lhcx3 increased throughout SL treatment, while under FL conditions the maximum transcript content was already reached on day 1 (but note the decrease of Lhcx3 transcription on days 2 and 3). Lhcf2 is one of the major classical light-harvesting antenna proteins of the FCP under low light conditions (Lepetit et al., 2010; Grouneva et al., 2011; Gundermann et al., 2013), and thus under stressful light conditions an expression pattern opposite to Lhcx genes was expected. Indeed, there was a strong Lhcf2 transcript reduction throughout the whole SL treatment (Fig. 8d). By contrast, under FL conditions Lhcf2 transcript dropped only during day 1, but reached almost initial values by day 2.

To investigate whether the differences in Lhcx transcription between SL and FL conditions were also reflected by the protein content, the Lhcx proteins were quantified. No significant changes could be observed for Lhcx1 compared to LL conditions either in SL or in FL (Fig. 9a). There was a gradual increase of Lhcx2 protein synthesis throughout the whole SL experiment (Figs 9b, S5 for an example of the Western blots obtained for Pr4), while under FL conditions it reached maximum values even on the first day and was much lower compared to SL conditions on days 2 and 3. By contrast, Lhcx3 content similarly increased during day 1 under SL and FL, and declined during the following days (Fig. 9c). Hence, only the Lhcx2 protein content correlated with the respective amounts of transcripts, and both paralleled the increase in NPQ capacity under SL exposure (see Fig. 3). This is better illustrated by plotting the mean relative amount of Lhcx2 vs the mean increase of NPQ capacity under SL conditions of all strains (except Pr4ov due to its unusual NPQ behaviour caused by the Lhcx1-overexpressing Lhcf1 promoter), yielding a linear correlation with an $R^2$ of 0.997 (Fig. 10).
Insights into the regulation of NPQ actors in diatoms have been gained recently, and clearly photoreceptor-mediated processes influence NPQ capacity (Schellenberger Costa et al., 2013; Brunet et al., 2014). In line with this, the Lhcx1 gene promoter has a binding motif for a blue light receptor, aureochrome 1a (Schellenberger Costa et al., 2013). The cryptochromes CPF (Coesel et al., 2009) and CryP (Juhas et al., 2014) regulate expression of Lhcx genes. Interestingly, in Chlamydomonas reinhardtii the cryptochrome aCRY, which is related to CryP, seems to react on light intensity rather than on light quality (Beel et al., 2012), and hence CryP could modulate NPQ capacity in diatoms in response to different light intensities.

Besides the influence of photoreceptors on NPQ adjustment, we previously demonstrated that Dd + Dt increase and Lhcx2 expression rates are controlled by changes in the redox state of the PQ-pool, while Lhcx3 expression may be regulated via ROS (Lepetit et al., 2013; Lepetit & Dietzel, 2015). In the present study, Lhcx2 expression and Dd + Dt content correlated well under both dynamic light conditions, indicating the presence of a common trigger. Clearly, the expression of Lhcx2 and Lhcx3 differed under SL and FL, suggesting that both light conditions elicit two different regulation pathways, probably due to the different characteristics of SL and FL in combination with their different total light doses. The PQ-pool redox state responsive Lhcx2 and Dd + Dt reacted rather gradually to long-lasting light stimuli under SL, which generated a higher cumulative photon amount per day. By contrast, Lhcx3 expression was regulated by short but intense light intervals, conditions which are expected to generate pronounced amounts of ROS. Because NPQ capacity has an influence on energy flow into the electron transport chain and thus on the redox state of the PQ-pool and on ROS generation (Triantaphylides et al., 2008; Kruk & Szymańska, 2012), the concentration of the reduced PQ-pool and ROS should be different in the four Phaeodactylum tricornutum strains under dynamic light conditions. Still, the four strains adjusted their NPQ capacity in a similar way under dynamic light conditions. The fact that NPQ capacity increases even in the highest NPQ strain (Pt4ov) illustrates that initial NPQ capacity was not sufficient to avoid partial over-reduction of the electron transport chain. Sensitive PQ-pool redox state and ROS responding promoter elements may already react to a partly reduced PQ-pool and small amounts of ROS, so that minor differences in these triggers would hardly differentially affect the expression of Lhcx genes and the synthesis of Dd + Dt pigments. Moreover, the redox state of the PQ-pool can be influenced by changes of the metabolome (Jung Andreas et al., 2014; Wilhelm et al., 2014; Levitan et al., 2015). Such influences would be fairly independent of the NPQ capacity and could also explain the similar NPQ response in the four Phaeodactylum tricornutum strains.

Lhcx2 in combination with the amount of XC pigments probably increases NPQ capacity under dynamic light conditions.

In diatoms, the increase of XC pigments does not necessarily lead to a higher NPQ (Schumann et al., 2007). Specific proteins must be synthesized to bind these pigments for an effective

Discussion

Up-regulation of NPQ capacity is independent of initial NPQ capacity during acclimation to dynamic light conditions.

We could not observe major differences in adjustment of NPQ capacity during dynamic light conditions in the low NPQ strains (Pt1sil and Pt4) compared to the high NPQ strains (Pt1 and Pt4ov). In line with this result, there was also no correlation between initial NPQ capacity and XC pigment synthesis, the latter being strain dependent: Pt1 and Pt1sil showed a stronger increase of Dd + Dt pool size than Pt4ov and Pt4 (Fig. S3). Furthermore, the Pt4ov strain, which under LL already possessed an NPQ capacity as high as Pt1sil at the end of the SL treatment (Fig. 2), similarly increased Lhcx2 and Lhcx3 gene expression as well as the Dd + Dt pool size (Figs S1, S3). The apparently small influence of the initial NPQ capacity on the subsequent NPQ adjustment during dynamic light conditions may be due to the fact that initial NPQ capacity under stable LL conditions is first determined by Lhcx1 and Dd + Dt amount (Bailleul et al., 2010). As reported here for dynamic light and before for prolonged high light conditions (Lepetit et al., 2013), a higher NPQ capacity is obtained by the combined increase of Dd + Dt pool size and primarily Lhcx2 expression.
involvement in NPQ (Lepetit et al., 2013). Lhcx1 does not increase significantly during dynamic light conditions (Fig. 9a), and hence confers only basal NPQ capacity. In Lhcx2 or Lhcx3 overexpression lines, both proteins provide additional NPQ capacity (Taddei et al., 2016). Lhcx2 content similarly increased on day 1 in SL and FL conditions, and remained stable during the following days in FL, while it increased in SL. These features were paralleled by the NPQ capacity. In fact, Lhcx2 amount was linearly correlated with NPQ capacity increase under SL conditions (Fig. 10). Although this correlation is based only on a few data points, it suggests Lhcx2 as a major actor in modelling NPQ capacity under dynamic light conditions, together with the size of the XC pigment pool and the degree of de-epoxidation.

The impact of enhanced Lhcx3 on NPQ capacity is difficult to deduce. Our data suggest that Lhcx3 was less responsible for NPQ increase during days 2 and 3 in SL and FL, as its protein content was rather decreasing (Fig. 9c). Instead, the prompt increase of Lhcx3 on day 1 under both dynamic conditions may provide a fast increase of photoprotection capacity, while Lhcx2 continues to add up during prolonged light stress conditions as under SL treatment. Interestingly, there was a significant difference in Lhcx3 transcript levels between FL and SL on day 1, which was not reflected by the protein level. This suggests that post-transcriptional control mechanisms that specifically respond to fast light intensity fluctuations partially prevent Lhcx3 protein synthesis. In line with this, it was recently shown in Arabidopsis thaliana that high light regulation of several high light responsive target proteins often occurs differentially on the transcriptional and translational level (Oelze et al., 2014). An additional control point at the Lhcx3 translational level might be a strategy to acclimate the NPQ system to an average light intensity delivered by light fluctuations, while keeping a high Lhcx3 transcript reservoir in case of prolonged light stress. This would avoid a too strong down-regulation of photochemistry during low light periods, while ensuring sufficient excess energy dissipation during high light regular peak exposures, a feature in line with the fast on/off switch of the NPQ system (Lavaud et al., 2007) and the fine regulation of Dd + Dt synthesis vs the velocity of light fluctuations (Giovagnetti et al., 2014) in diatoms.

Besides Lhcx2, and to some extent Lhcx3, other proteins could contribute to additional NPQ capacity under dynamic light, especially to the slight increase of NPQ during FL on days 2 and 3. The Lber gene family contains a phylogenetically separated clade (Nymark et al., 2013), which genes are transcriptionally up-regulated during high light stress (Nymark et al., 2009). The corresponding proteins may be at least partially involved in modulating NPQ capacity. Another possible candidate is Lhcf15, which is the only Lhcf gene that shows up-regulation during short-term light stress (Nymark et al., 2009), but which especially responds to red light (Schellenberger Costa et al., 2013; Valle et al., 2014; Herbstová et al., 2015). Lhcf15 can build up specific antennae complexes with a red-shifted fluorescence emission (Herbstová et al., 2015) that could be correlated to the NPQ capacity (Lavaud & Lepetit, 2013).
FL triggers a very effective photoprotective response

Under SL conditions, the cells first synthesized much more Dt than could be used for NPQ. Eventually, the NPQ/(Dd + Dt) ratio was better adjusted towards a higher quenching efficiency of Dt due to a slowdown of Dd + Dt synthesis and a concomitant catch-up of Lhcx2 synthesis. Despite a strongly increased NPQ capacity as well as a massive reduction of Chl a synthesis and Lhcf2 transcription, a decrease in photosynthetic efficiency could not be completely prevented. By contrast, cells under FL conditions kept the NPQ/(Dd + Dt) efficiency comparable to that of LL conditions, that is they synthesized only as many Dd + Dt as actually could be used to provide an optimal effective involvement of Dt in NPQ. Parallel to enhancing NPQ capacity, FL cells adjusted the reactivity of NPQ activation. The light intensity, for which 50% of the maximum NPQ capacity was reached, was shifted to much higher values (Fig. 4f). Simultaneously, despite increasing $E_k$, FL cells kept the level of NPQ activation at $E_k$ very low (i.e. a few per cent of NPQmax, Fig. 4e). This is noteworthy because although $E_k$ increased similarly, SL cells were unable to adjust the threshold for NPQ onset and activated a pronounced NPQ even at $E_k$. Ultimately, FL cells strongly increased rETRmax, but kept $\alpha$ high as in LL cells, in line with previous results in Skeletonema costatum (Kromkamp & Limbeek, 1993). All these changes enabled FL cells to use absorbed light efficiently until $E_k$ was reached and even beyond due to the moderate switch-on of NPQ. Hence, they exploited as much light as possible for photochemistry during the short light periods thanks to the adjustment of NPQ capacity and kinetics. Consequently, after 1 d of acclimation, $\mu_{Chl a}$ and Lhcf2 transcription reached similar values as under LL conditions. As FL cells developed a much higher NPQ capacity than LL cells, altogether FL acclimation does not correspond to either a low or high light type strategy, but shows peculiar characteristics. Such a particular fluctuating light acclimation strategy has also been observed in the diatom Stephanodiscus neoastraea (Fietz & Nicklisch, 2002).
We note that the fine acclimation to FL conditions with different intensity maximums may be limited. Although FL 1000 cells faced much higher light intensities, steeper light gradients and the double daily photon dose, NPQ capacity increased similarly as in FL 500 cells. On the one hand, this very similar NPQ pattern may be co-initiated by an internal trigger such as the circadian clock. The influence of the circadian clock on regulation of the XC pigments in *P. tricornutum* has already been demonstrated (Ragni & d’Alcalà, 2007). On the other hand, Giovangnetti *et al.* (2014) showed that the light acclimation response in *Pseudo-nitzschia multistriata* is triggered by light intensity, and also by the velocity of the light increase. Moreover, in *Skeletonema marinoi*, NPQ capacity is not directly correlated to the total daily photon dose (Orefice *et al.*, 2016). Our results suggest that, below a certain duration threshold of regular light periods, different high light intensities only trigger an efficient on/off reaction of NPQ capacity adjustment, but no fine-tuned response. This is possibly due to the lack of the gradual PQ-pool signal. Interestingly, when the light intervals become even shorter than the dark intervals in a fluctuating light regime, *P. tricornutum* is able to generate a very high NPQ (Lavaud *et al.*, 2002b; Ruban *et al.*, 2004), which may not be triggered by light, but may be elicited by darkness alone. As only transcription of the Lhcx4 isoform is stimulated in the dark (Lepeit *et al.*, 2013; Nymark *et al.*, 2013) and the respective protein can induce NPQ (Taddei *et al.*, 2016), Lhcx4, together with the increased Dd + Dt content (Lavaud *et al.*, 2002b), may be responsible for this particular increased NPQ capacity.

**Conclusion**

Our study highlights the importance of investigating the influence of dynamic light conditions on NPQ in diatoms. Recently, it was shown that *P. tricornutum* is coping well with fluctuating light conditions by possessing a low-cost PSII repair cycle compared to diatoms living in the open ocean in more stable light conditions (Lavaud *et al.*, 2016). A comprehensive study by Wagner *et al.* (2006) demonstrated that in *P. tricornutum* absorbed photons are converted to a much higher extent into biomass in FL than in SL conditions, which is due to a strongly decreased amount of alternative electrons and a lowered quantum requirement per molecule of oxygen evolved. The finely adjusted regulation of NPQ capacity by balanced Lhcx2/Lhcx3 and Dd + Dt synthesis under FL has three consequences which support the observations of Wagner *et al.* (2006): (1) investment costs for photoprotection mechanisms are lower than in SL; (2) photodamage is reduced, keeping costs for repair processes low; and (3) too much photoprotection, leading to a poor light energy to chemical energy conversion rate and hence to a high quantum requirement for carbon fixation, is prevented. This last feature has been recently demonstrated to be of global importance in the upper ocean (Lin *et al.*, 2016). The better balanced photoacclimation strategy under FL compared to SL conditions may be one reason why diatoms dominate in habitats where the light climate is regularly punctuated with high-intensity exposure periods, such as coastal waters and estuarine intertidal sediments (Strzepek & Harrison, 2004; Lavaud *et al.*, 2007; Dimier *et al.*, 2009; Petrou *et al.*, 2011; Barnett *et al.*, 2015).

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**Author contributions**

B.L., P.G.K., A.F. and J.L. designed research; B.L., G.G., M.L., S.S., S.V., A.R. and J.L. performed experiments; B.L. and J.L. interpreted results; B.L., A.R., P.G.K., A.F. and J.L. wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

**Fig. S1** Transcript levels of *Lhcx1*–*Lhcx3* and *LhcB2* genes under SL and FL conditions in all four *P. tricornutum* strains.

**Fig. S2** Fx/Chla and Chl/c/Chla content of all four *P. tricornutum* strains under SL and FL conditions.

**Fig. S3** Dd+Dt pool size in the four individual *P. tricornutum* strains during SL and FL conditions.

**Fig. S4** De-epoxidation state of all four *P. tricornutum* strains under SL and FL conditions.

**Fig. S5** Lhcx protein expression in Pr4 during SL and FL conditions.

**Table S1** Photophysiological parameters used in this study

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