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An explanation for the inter-species variability of the photoprotective non-photochemical chlorophyll fluorescence quenching in diatoms

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ABSTRACT

Diatoms are a major group of microalgae whose photosynthetic productivity supports a substantial part of the aquatic primary production. In their natural environment they have to cope with strong fluctuations of the light climate which can be harmful for photosynthesis. In order to prevent the damage of their photosynthetic machinery, diatoms use fast regulatory processes among which the non-photochemical quenching of chlorophyll *a* fluorescence (NPQ) is one of the most important. In a previous work, we highlighted differences in the kinetics and extent of NPQ between diatom species/strains originating from different aquatic habitats. We proposed that the NPQ differences observed between strains/species could potentially participate to their ecophysiological adaptation to the light environment of their respective natural habitat. In order to better understand the molecular bases of such differences, we compared the NPQ features of four strains/species of diatoms known for their NPQ discrepancy. We could identify new spectroscopic fingerprints concomitant to NPQ and the related xanthophyll cycle. These fingerprints helped us propose a molecular explanation for the NPQ differences observed between the diatom species/strains examined. The present work further strengthens the potential role of NPQ in the ecophysiology of diatoms.

1. Introduction

Diatoms are a major group of microalgae ubiquitous in all marine and freshwater ecosystems. They are among the most significant photosynthetic organisms contributing to about 40% of the aquatic primary production, especially in coastal ecosystems [1,2]. Their biological characteristics have largely shaped the structure of contemporary aquatic ecosystems [1,3]. As for most microalgae, the photosynthetic productivity of diatoms strongly depends on the aquatic light climate [4–6]. Planktonic as well as benthic diatoms tend to dominate ecosystems (coasts and estuaries) where they have to cope with a light climate with high-frequency irradiance fluctuations coupled with large amplitudes. Depending on the rate of water mixing, on the tidal cycle, on the daily/seasonal changes of solar irradiation, diatoms can be exposed to punctual or chronic excess light, possibly generating stressful conditions that impair their photosynthetic efficiency and their productivity (i.e. photoinhibition/photoinactivation) [7–10]. In order to prevent such situation, diatoms [11,12], and to a larger extent microalgae and other photosynthetic organisms [13–17], have evolved fast regulatory physiological processes which compose a 'photoprotective network'. They help to safely dissipate the excess of absorbed light energy as heat and/or to balance the excitation energy within the photosynthetic apparatus thus preventing/lowering the potential oxidative damages to the photosynthetic machinery. Additional mechanisms help repair the unavoidable photodamages [10,18]. In diatoms, two fast processes are believed to be essential for the photoprotection: the electron cycle of the photosystem II reaction center (PS II CET) and the non-photochemical quenching of chlorophyll *a* (Chl *a*) fluorescence (NPQ) [11,12,14,19]. NPQ is composed of three components, namely the high-energy state (qE), the state-transition (qT) and the 'photoinhibitory' (qI) quenchings [13,17]. In diatoms, qT does not exist and the origin of qI remains unclear [11,12]. Therefore, the diatom NPQ mainly relies on qE, a quenching mechanism which is controlled by i) the build-up of the transthylakoidal proton gradient (ΔpH), ii) the conversion of the xanthophylls diadinoxanthin (DD) into diatoxanthin (DT) called 'xanthophyll cycle' (XC), and iii) the presence of specific polypeptides of the light-harvesting complex (LHC) antenna named Lhcx [11,12,14,20–23]. Additionally, there exists a PS II reaction center quenching which might be related to the PS II CET [24,25]. These fast regulatory mechanisms proved to be important in field situation for maintaining the photosynthetic efficiency and productivity of diatoms [15,21]. NPQ was shown to significantly participate in the cellular response under fluctuating light conditions [26–28]. Recently, an

Abbreviations: Chl *a*, Chlorophyll *a*; Chl *a* FIKs, Chl *a* fluorescence induction kinetics; Chl a_{711}, Chl *a* fluorescence emission band at 711 nm; DD, diadinoxanthin; DES, de-epoxidation state; DT, diatoxanthin; FCP, fucoxanthin-chlorophyll protein; LHC, light-harvesting complex; NPQ, non-photochemical Chl *a* fluorescence quenching; PAM, Pulse-amplified modulation; PS II, Photosystem II; PS II CET, PS II cyclic electron cycle; ΔpH, transthylakoidal proton gradient; Δ522 nm, absorption change at 522 nm

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increasing number of reports suggested that the diatom/microalgae inter-species differences in NPQ are involved in the differential colonization of aquatic habitats depending on the rate of light fluctuations of the light climate [9,27–33].

While the most recent advances in our knowledge of the physiological regulation of diatom photosynthesis, and especially of NPQ and the XC, were generated by the use of genetically modified cells of the model diatom *Phaeodactylum tricornutum* [20,23,34,35], the comparison of different species/strains [9,18,20,27,30,36,37] together with the use of different growth conditions [18,30,37,38] has also proven to be useful. Indeed, the XC and NPQ extent and kinetics can differ with regards to the species [9,18,27,30], and to the light [30,38,39] and the temperature [18] acclimations. In a previous study, we compared the photoprotective ability of *P. triconutum* and *Skeletonema costatum* [27]. One of the major differences concerning NPQ: its extent in *S. costatum* was approximately half the one in *P. tricornutum*. It was not due to a lower de-epoxidation rate of DD to DT, as reported before for diatoms and other related organisms [18,40,41]. We suggested two explanations for such discrepancy [27]: 1) a different organization and FCP (fucoxanthin-chlorophyll protein) composition of the LHC antenna of PS II, including both Lhcf and Lhcx proteins, 2) a lower amount of DT molecules involved in the NPQ process; the two hypotheses not being exclusive from each other [18]. The present work aimed at going further deep in the understanding of NPQ differences between diatom species/strains. For that purpose, we examined four species/strains currently used as models for NPQ investigations and known to show different NPQ amplitude and regulation [20,27,36,42–44]: a low and a high NPQ strains of *P. tricornutum* (P.t.2 and P.t.4, respectively), *S. costatum* and *Cyclotella meneghiniana*. The three species are also representative of the two major groups of diatoms, the pennates (*P. tricornutum*) and the centrics (*S. costatum* and *C. meneghiniana*); the genome of *P. tricornutum* is available and *C. meneghiniana* is a close relative of *Thalassiosira pseudonana* whose genome is also published [45]. Finally, the ecological niches where P.t.2, P.t.4 and *S. costatum* were isolated (temperate estuary, high latitude shore, Mediterranean semi-enclosed bay, respectively) show very different underwater light climates [20,27].

2. Materials and methods

2.1. Cultures

Phaeodactylum tricornutum Böhlin CCAP1052–CCAP1055/1 ('P.t.2 strain', Culture Collection of Algae and Protozoa, Oban, Scotland) and CCAP 1052/6 ('P.t.4 strain', Sammlung Algenkulturen Göttingen (SAG), Germany), *Skeletonema costatum* (Greville) Cleve (Laboratoire Arago algal collection, Banyuls-sur-Mer, France) and *Cyclotella meneghiniana* Kützing SAG 1020_1a (SAG, Germany) cells were grown photoautotrophically in sterile natural seawater F/2 medium [46]. Non-axenic cultures of 300 mL were incubated at 18 °C in airlifts continuously flushed with sterile air. They were illuminated at a light intensity of 40 μmol photons m^{-2} s^{-1} with white fluorescent tubes (Claude, Blanc Industrie, France) with a 16 h light/8 h dark photoperiod ('continuous light', CL cells, see [47]). Intermittent light (IL) cells of *P. tricornutum* were grown in the same conditions except the light regime which was 5 min light/55 min dark cycle (see [47]). Cells were harvested during the exponential or stationary phase of growth as specified in Table 1, centrifuged at 3000*g* for 10 min and resuspended in their culture medium to a final concentration of 10 μg chlorophyll *a* (Chl *a*) mL^{-1}. The concentrated suspension was then continuously stirred at 18 °C under low light before use.

2.2. Pigment content

Pigment analyses were performed by HPLC as previously described [47]. Cells collected from the Clark electrode adapted to the PAM-fluorometer (see below) were deposited on a filter and frozen in liquid nitrogen. Pigments were extracted with a methanol:acetone (70:30, v/v) solution. The xanthophyll de-epoxidation state (%) was calculated as $(DT/(DD+DT))\times 100$ where DD is the diadinoxanthin, the epoxidized form, and DT is the diatoxanthin, the de-epoxidized form.

2.3. Spectroscopy

Ambient temperature absorption spectra were performed with a DW-2 Aminco spectrophotometer. For measuring the 522 nm absorption change (Δ522 nm) which reflects the 'activation of DT' (see [48]), spectra were performed on dark-acclimated cells (for 20 min) and cells exposed to light (5 min, 2000 μmol photons m^{-2} s^{-1}) as previously described [48]; the half-bandwidth was 0.5 nm. For partial inhibition of NPQ, cells were incubated prior to illumination (20 min, dark) with dithiothreitol (DTT) or NH_4Cl with a final concentration of 25 μM and 2 mM, respectively (see [49]). 77 K fluorescence emission spectra were measured with an F-3000 and F-4500 Hitachi spectrophotometer. Half-bandwidth for emission was 2.5 (F-4500) and 3 (F-3000) nm, respectively. Samples were dark-acclimated (20 min) and then concentrated at 1 μg Chl *a* on a Millipore AP-20 prefilter that was immediately frozen in liquid nitrogen before measurement. The same procedure was used for illuminated cells (2000 μmol m^{-2} s^{-1} for 5 min) albeit a Chl *a* amount of 3 μg was immediately frozen after illumination. The angle between the filter and the light source/detector was 45°. For complete inhibition of NPQ, cells were incubated prior to illumination (20 min, dark) with 0.2 mM DTT or 5 mM NH_4Cl [49].

Table 1
Photophysiological properties of the species/strains and of the NPQ type of cells used in this study.

Species and type of cells	Growth status	F_v/F_m	DD + DT	F711/F687	DES	DT	Δ 522 nm × 10^2	NPQ
P. tricornutum (P.t.2) 'low NPQ'	'CL', exp.	0.65 ± 0.02	8.8 ± 0.4	0.35 ± 0.06	27	2.38 ± 0.1	1.3 ± 0.10	2.38 ± 0.24
P. tricornutum (P.t.2) 'medium NPQ'	'CL', stat.	0.61 ± 0.03	13.6 ± 0.9	0.50 ± 0.07	36	4.9 ± 0.3	2.1 ± 0.10	3.50 ± 0.25
P.t.2, 'high NPQ'	'IL', exp.	0.68 ± 0.02	17.4 ± 2.3	1.22 ± 0.26	46	8.0 ± 1.1	2.5 ± 0.20	9.40 ± 0.61
P. tricornutum (P.t.4) 'low NPQ'	'CL', exp.	0.67 ± 0.02	12.7 ± 0.2	0.48 ± 0.01	38	4.8 ± 0.1	n.d.	1.58 ± 0.11
P.t.4, 'medium NPQ'	'CL', stat.	0.60 ± 0.03	23.8 ± 0.2	0.62 ± 0.09	43	10.2 ± 0.1	n.d.	2.63 ± 0.19
P.t.4, 'high NPQ'	'IL', exp.	0.64 ± 0.02	30.9 ± 0.7	1.00 ± 0.19	61	18.9 ± 0.4	n.d.	4.08 ± 0.21
S. costatum (S.c.) 'low NPQ'	'IL', exp.	0.64 ± 0.01	8.3 ± 2.1	0.19 ± 0.02	33	2.7 ± 0.7	0.33 ± 0.15	0.51 ± 0.07
S.c. 'medium NPQ'	'CL', exp.	0.59 ± 0.07	18.1 ± 0.1	0.29 ± 0.01	40	7.2 ± 0.05	1.00 ± 0.15	1.35 ± 0.29
S.c. 'high NPQ'	'CL', stat.	0.69 ± 0.02	26.9 ± 0.7	0.32 ± 0.01	59	15.9 ± 0.4	1.73 ± 0.17	2.5 ± 0.30
C. meneghiniana (C.m.) 'low NPQ'	'CL', exp.	0.63 ± 0.03	24.1 ± 0.3	0.29 ± 0.01	52	12.5 ± 0.2	0.86 ± 0.28	0.77 ± 0.09
C.m. 'medium NPQ'	'IL', exp.	0.58 ± 0.03	23.6 ± 0.2	0.29 ± 0.02	23	5.4 ± 0.05	0.70 ± 0.13	0.90 ± 0.09
C.m. 'high NPQ'	'CL', stat.	0.69 ± 0.02	57.9 ± 1.2	0.25 ± 0.01	64	37.1 ± 0.8	1.44 ± 0.26	1.94 ± 0.22

'CL', 'continuous light': 16 h:8 h Light:Dark cycle; 'IL', 'intermittent light': 5 min/55 min L:D cycle (see Material and methods section for details); exp. growth, exponential phase of growth; stat. growth, stationary phase of growth; the fluorescence ratios F_v/F_m (the maximum photosynthetic efficiency of PS II) and F711/F687 were measured on dark-acclimated cells (see also Fig. 4); Δ 522 nm × 10^2 (the absorption change at 522 nm), NPQ and DES (de-epoxidation state of diadinoxanthin (DD) into diatoxanthin (DT) in %: (DT/(DD + DT) × 100), DD and DT in mol. 100 mol Chl a^{-1}) were measured after 5 min at 2000 μmol s^{-1} m^{-2} (see also Fig. 1 and Fig. 3).

2.4. Chlorophyll fluorescence yield and the non-photochemical fluorescence quenching (NPQ)

Chlorophyll fluorescence yield was monitored with a modified PAM-101 fluorometer (Walz, Effeltrich, Germany) as described previously [9] (see Fig. 1A for a typical recording). Fluorescence was excited by a very weak (non-actinic) modulated 650 nm light beam. After 20 min of dark-acclimation, continuous actinic light of adjustable intensity was applied. 600 ms pulses of white light (4000 μmol photons m^{-2} s^{-1}) were admitted by an electronic shutter (Uniblitz, Vincent, USA, opening time 2 ms) placed in front of a KL-1500 quartz iodine lamp continuously switched on in order to monitor the evolution of NPQ. The average fluorescence (acquisition time 33 μs) measured during the last 400 ms of the pulse was taken as F_m or F_m'. For each experiment, 2 mL of cell suspension was used. Sodium bicarbonate was added at a concentration of 4 mM from a freshly prepared 0.2 M stock solution in distilled water to prevent any limitation of the photosynthetic rate by carbon supply. Standard fluorescence nomenclature was used [50]. F_o and F_m are defined as the minimum PS II fluorescence yield of dark-acclimated cells and the maximum PS II fluorescence yield reached in such cells during a saturating pulse of white light, respectively. The maximum photosynthetic efficiency of PS II is the ratio F_v/F_m where F_v is the variable part of the fluorescence emission and is equal to $F_m - F_o$. NPQ (also known as SV_m) was quantified by the 'Stern–Volmer' expression $F_m/F_m' - 1$, where F_m' is the maximum PS II fluorescence yield of light-acclimated cells [51].

3. Results

The general photosynthetic properties of the strains of *Phaeodactylum tricornutum* (P.t.2 strain) and *Skeletonema costatum* used here have been described earlier [27]. In our growth conditions, both species have comparable light harvesting efficiency and photosynthetic capacity. As previously shown, the extent of NPQ in *P. tricornutum* was about twice the one in *S. costatum*; it holds true under saturating and over-saturating irradiances [27] and under prolonged exposure to over-saturating intensities [9].

3.1. Differences in the relationship between the XC and NPQ between Phaeodactylum tricornutum and Skeletonema costatum cells

The de-epoxidation of DD into DT was measured for the two species under the same light conditions as for NPQ (Fig. 1 and Fig. S1). While in *P. tricornutum* (P.t.2 strain) the de-epoxidation started for a light intensity of 150 μmol photons m^{-2} s^{-1}, it was already active at 50 μmol photons m^{-2} s^{-1} in *S. costatum*. For the two species, the de-epoxidation state (DES) reached a near maximum around 750 μmol photons m^{-2} s^{-1}. As the size of the total pool of DD + DT remained stable along the range of irradiances, no *de novo* DT synthesis (i.e. DT synthesis independent from the xanthophyll cycle, see [8,9]) occurred. In these conditions, the maximal synthesis of DT was lower in *P. tricornutum* (2.5 ± 0.01 mol. 100 mol Chl a^{-1}) than in *S. costatum* (6.8 ± 0.54 mol; 100 mol Chl a^{-1}) corresponding to a maximal DES of 27% and 40%, respectively (Fig. 1A, Table 1). For comparison, *P. tricornutum* 'high NPQ' cells (see Table 1 for their characteristics) showed a maximal DES of 46% (Fig. 1A, open circles). Under these light conditions, there was a tight correlation between the amount of DT and NPQ as previously reported for several diatom species [9,38] (Fig. 1B). However, in contrast to *P. tricornutum*, for *S. costatum* the relationship depended on the illumination pattern. When it was fixed to 5 min and the irradiance increased from 0 to 2000 μmol photons m^{-2} s^{-1} (Fig. S1), the NPQ *vs.* DT relationship showed an exponential feature (Fig. 1B). NPQ barely developed (0.2) until the DT amount reached a value close to 2 mol DT. 100 mol Chl a^{-1}. Interestingly, the inflexion point of NPQ development corresponded to an amount of DT synthesized for a light intensity of about 400 μmol photons m^{-2} s^{-1} (Fig. S1), which was just below the light intensity for reaching the maximum photosynthetic capacity (Fig. S2) [27]. When the irradiance was fixed to a maximum of 2000 μmol photons m^{-2} s^{-1} and the illumination time increased from 0 to 30 min, the NPQ *vs*. DT relationship was linear with a slope twice lower than the one observed in *P. tricornutum* (as previously reported in ref [9]).

By illuminating the cells with harsher conditions (2000 μmol m^{-2} s^{-1} for over 30 min and up to 1 h), the NPQ *vs.* DT relationship remained true only for *P. tricornutum* (continuous line). Indeed, for illumination times longer than 30–45 min, the slope of the relationship NPQ/DT decreased to 0.09 in *S. costatum* (Fig. 1B, closed triangles, dashed line). During such high and prolonged light exposure *de novo* synthesis of DT occurs with a similar rate in *P. tricornutum* and *S. costatum* [9]. It can be therefore concluded that in *S. costatum* part of the newly synthesized molecules of DT were less or not involved in the development of subsequent NPQ. This feature was confirmed by the recording of the epoxidation kinetics of DT back to DD after short (5–15 min) and long (1 h) exposures to 2000 μmol m^{-2} s^{-1} (Fig. 2). While after 5 and 15 min exposure the epoxidation kinetics were

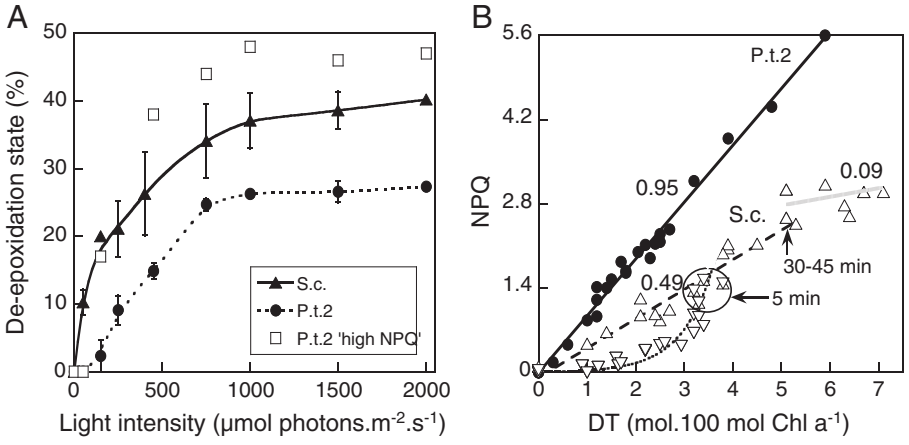

Fig. 1. A- The de-epoxidation state (calculated as DT/(DD + DT) × 100) in *S. costatum*, *P. tricornutum* (P.t.2 strain) and *P. tricornutum* 'high NPQ' cells. Data (±SD) are the average of three to five independent measurements (but for *P. tricornutum* 'high NPQ' cells, only one experiment). DD, diadinoxanthin; DT, diatoxanthin. B- Relationship between DT synthesis and NPQ development in *S. costatum* as a function of the illumination time (from 0.5 to 60 min) at 2000 μmol photons m^{-2} s^{-1} (triangles, $R^2 = 0.92$) and as a function of the light intensity for an illumination time of 5 min (inverted triangles, $R^2 = 0.81$); hence both curves meet for the cells sampled after 5 min illumination at 2000 μmol photons m^{-2} s^{-1} (highlighted area). The numbers refer to the X slope of the relationship NPQ = X × [DT]; the times refer to the illumination times. The same relationship with the same light conditions obtained in *P. tricornutum* (P.t.2 strain) cells is shown by a continuous line (circles, see also [9,38]).

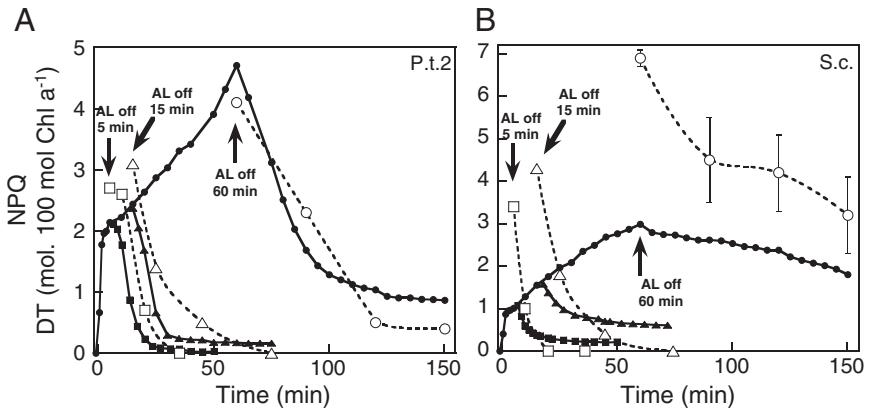

Fig. 2. Dark relaxation of NPQ (closed symbols) and DT epoxidation back into DD (open symbols) after a 5 min (squares), 15 min (triangles) and 60 min (circles) illumination at 2000 μmol photons m^{-2} s^{-1} (Actinic Light, AL) in *P. tricornutum* (P.t.2 strain) (A) and *S. costatum* (B).

similar in both species and well in concordance with the relaxation of NPQ, it was drastically different after 1 h exposure (Fig. S3). In *S. costatum*, the epoxidation kinetics and NPQ relaxation showed disconnected patterns (Fig. 2B, Fig. S3). During the first 30 min of recovery, there was a fast DT epoxidation similar as in *P. tricornutum* (Fig. 2A). The amount of DT involved (2.4 ± 1.2 mol. 100 mol Chl a^{-1}) was similar to the amount of DT synthesized *de novo* (2.4 ± 1.6 mol. 100 mol Chl a^{-1}). In contrast to *P. tricornutum*, it was accompanied by only a small change in NPQ (13%). After 1 h of recovery, nearly all DT molecules were converted back to DD in *P. tricornutum* while a large DT pool (3.2 ± 0.9 mol. 100 mol Chl a^{-1}) persisted in *S. costatum*. NPQ remained high too (2/3 s of the value after the light extinction). Both sustained DT and NPQ could not be recovered by the addition of the uncoupler NH_4Cl after 15 or 25 min of recovery (data not shown) [49], hence the ΔpH was most probably already broken. Additionally, when the light (same intensity) was again switched on after 30 min of recovery, the development of NPQ was faster but not higher (×2, data not shown) as previously observed for *P. tricornutum* [52]. These features fit well with the proposed regulatory features of NPQ in diatoms (see [14,27]). Lastly, the dark recovery of the F_v/F_m was higher in *P. tricornutum* whatever the illumination time (15 or 60 min) (Fig. S4).

3.2. Light-dependent absorption changes related to the XC and NPQ

It has been shown earlier that the absorption increase at 522 nm (Δ522 nm) accounts for the so-called 'activation' of the DT molecules, an essential step in the switching of the PS II LHC antenna to the quenching state (see [48,52]). Fig. 3A shows the Δ522 nm in *S. costatum* and in *P. tricornutum* cells with different extents of NPQ (see Table 1 for their characteristics) which were obtained by growing the cells under different conditions: 1) a classical low light regime (16 h L:8 h D) with a cell harvest during the exponential phase of growth ('CL exp.'), 2) the same light regime with a cell harvest during the stationary phase of growth ('CL stat.'), 3) an intermittent light of the same intensity (5 min light: 55 min dark) ('IL exp.'). In order to complete the range of NPQ, we included a second strain of *P. tricornutum* (P.t.4), which is known for its lower NPQ than the strain P.t.2 [20] and *C. meneghiniana* whose NPQ mechanism is more complex than the *P. tricornutum* [36,42]. In these conditions, the four strains reacted rather similarly. The maximum photosynthetic efficiency of PS II (F_v/F_m, Table 1) of 'CL exp.' cells was very similar (Table 1). F_v/F_m slightly decreased in 'CL stat.' cells while it was maintained in 'IL exp.' cells. The growth rate of 'CL exp.' cells was similar (*P. tricornutum* P.t.2, 2.08 ± 0.08 day^{-1}; P.t.4, 2.54 ± 0.2 day^{-1}; *S. costatum*, 2.01 ± 0.09 day^{-1}; *C. meneghiniana*, 2.11 ± 0.10 day^{-1}) while it dramatically decreased to about 0 in 'CL stat' conditions (*P. tricornutum* P.t.2, 0.05 ± 0.02 day^{-1}; P.t.4, 0.07 ± 0.03 day^{-1}; *S. costatum*, 0.02 ± 0.01 day^{-1}; *C. meneghiniana*, 0.03 ± 0.02 day^{-1}) (part of the data compiled from [9,27,34]). Although the growth rate was not measured for all species, it decreases 10-fold in *P. tricornutum* P.t.2 'IL exp.' cells due to the 8-fold lower daily amount of light supplied by the IL light regime [38]. Under a fluctuating light regime not as harsh as the IL regime used here, the growth rate of *P. tricornutum* P.t.4, *S. costatum* and C. *meneghiniana* was decreased by a factor of 2 to 4.5 (data compiled from [20,27,53]).

In contrast, the effect of the light regimes on the pool size of DD + DT, the de-epoxidation of DD to DT (DES and DT amount) and on NPQ in the four species/strains was different. In general, 'CL stat.' cells showed a higher NPQ than 'CL exp.' cells due to the larger DD pool and the higher subsequent DT synthesis [54]. While *P. tricornutum* P.t.2 'IL exp.' cells exhibited a higher NPQ [38,48], the IL treatment had an adverse effect on *S. costatum* xanthophyll synthesis and NPQ (Table 1). Consequently, the Δ522 nm band was lower in *S. costatum* compared to *P. tricornutum* (P.t.2 strain) whatever the types of cells considered (see Table 1). NPQ in P.t.4 reacted the same way as in P.t.2 to growth conditions while for *C. meneghiniana* it was closer to *S. costatum* (Table 1). The extent of NPQ exponentially correlated with the amplitude of the Δ522 nm band (Fig. 3B). In contrast, and as expected, NPQ linearly correlated with the total amount of synthesized DT with a decreasing slope in the order *P. tricornutum* > *S. costatum* > *C. meneghiniana* (Fig. S5). The NPQ/Δ522 nm relationship also held true for cells of *P. tricornutum* (P.t.2, 'low NPQ') in which NPQ was more than half abolished (−60%) by the use of moderate concentrations of an uncoupler (NH_4Cl) of the ΔpH and of an inhibitor of the DD de-epoxidation (DTT, dithiotreitol) [49]. Although *S. costatum* and *C. meneghiniana* showed a larger xanthophyll pool size and a higher DT synthesis than *P. tricornutum* (see also Fig. 1A), NPQ was lower (see also Fig. 1B) in direct correlation with the lower capacity for DT 'activation' in the NPQ process (i.e. lower Δ522 nm) (Table 1). This situation was especially extreme with 'high NPQ' cells: the huge amount of synthesized DT (about 16 and 37 mol. 100 DT Chl a^{-1} for *S. costatum* and *C. meneghiniana*, respectively) did not lead to the expected large increase of NPQ which instead followed the slight increase of the 522 nm band.

3.3. NPQ capacity and chlorophyll fluorescence emission properties

In order to gain more insight into the fluorescence emission properties of the species examined, we performed 77 K fluorescence emission spectra on dark-acclimated cells (Fig. 4). The majority of the Chl *a* fluorescence arose from the 687 nm peak while a second emission band peaking at 711 nm in *P. tricornutum* was showing a slight shoulder in *S. costatum* and *C. meneghiniana* (not shown). In *P. tricornutum* (P.t.2 and P.t.4 strains) the amplitude of the 711 nm band increased with

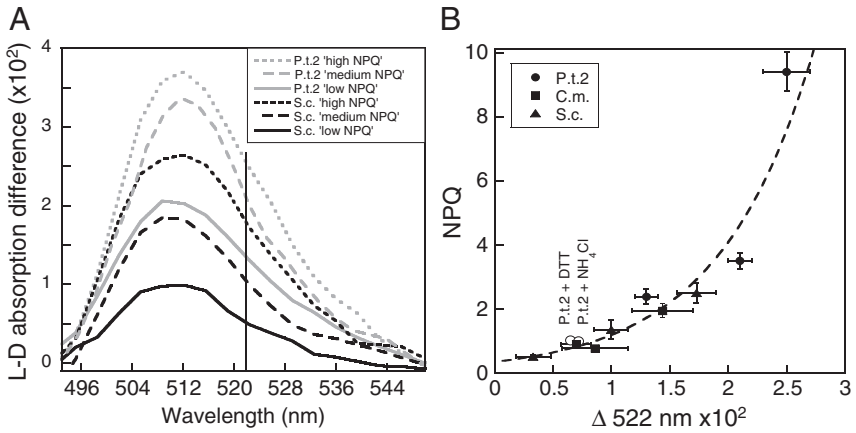

Fig. 3. A- Light-treated minus dark-acclimated difference absorption spectra in *P. tricornutum* (P.t.2 strain) and *S. costatum*. For each of the species, the three NPQ types of cells ('low', 'medium' and 'high', see Table 1) were used. The absorption band at 522 nm (Δ522 nm) is highlighted. B- Relationship between NPQ and Δ522 nm. NPQ was measured after 5 min illumination at 2000 μmol photons m^{-2} s^{-1}. For each of the species, *P. tricornutum* (P.t.2 strain, circles), *S. costatum* (triangles) and *Cyclotella meneghiniana* (squares), the three NPQ types of cells ('low', 'medium' and 'high', see Table 1) were used. The exponential relationship was $NPQ = 0.35 \times e^{(1.24\ \Delta522\ nm)}$ ($R^2 = 0.93$). For *P. tricornutum*, the same measurements were performed on the 'low NPQ' cells in presence of NH_4Cl (2 mM) and dithiothreitol (DTT, 25 μM) during the illumination (open circles). See the text for further details. Data (±SD) are the average of three independent measurements.

the NPQ capacity of the cells, following a linear relationship whose slope was dependent on the strain (Fig. 5, Table 1). Probably the most illustrative situation concerns the P.t.2 'medium and high NPQ' cells: the synthesis of 1.7 fold more DT molecules led to a much higher NPQ (2.7 fold) than expected regarding the moderate increase of DT activation (Δ522 nm); instead the 2.7 fold NPQ increase correlated well with the fluorescence emission at 711 nm. Although slight variations in the 711/687 nm fluorescence ratio (F711/F687) were observed in *S. costatum* (Fig. 4B, Table 1), no clear relationship could be established (Fig. 5). No changes were observed in *C. meneghiniana*.

It has been described earlier in *P. tricornutum* how the 711 nm fluorescence band can arise from the PS II at both room temperature and 77 K [55], and how it could be related to the capacity for excess energy dissipation [43,55]. When measured after illumination (5 min, 2000 μmol m^{-2} s^{-1}) in *P. tricornutum* (P.t.2 strain), the 77 K fluorescence signal was decreased by a factor of about 3 and F711/F687 increased by 31% (Table 2) (Fig. S6). When NPQ was inhibited by the addition of NH_4Cl or DTT, the change of F711/F687 was abolished (Table 2, Fig. S6). By illuminating the cells with far-red light (200 μmol photons m^{-2} s^{-1}), we did not observe any change of F711/F687 (data not shown).

4. Discussion

4.1. The inter-species differences of the involvement of DT in NPQ

The DT molecules are distributed among several pools which show a spatial and functional heterogeneity [37]. It is believed that DT can be: 1) free in the lipid matrix [37], 2) in the lipid matrix in the close vicinity to the PS II LHC antenna [37], 3) bound to the LHC antenna system for the harvesting of light [37,56]; 4) bound to specific FCP trimers where they participate to NPQ [44]. A change of balance between these pools can occur during light acclimation [39,42]. We show that it is also true between species. Obviously the addressing of DT to the four pools is different in *S. costatum* (and *C. meneghiniana*) compared to *P. tricornutum*. First, the fact that the slope of the NPQ *vs.* DT relationship is twice lower in *S. costatum* indicates that twice less DT molecules are involved in NPQ, i.e. there are 50% less DT in pool 4. Second, while DT *de novo* synthesis significantly occurs in both species, it is mainly inefficient for NPQ in *S. costatum* (and *C. meneghiniana*, [37]) in contrast to *P. tricornutum* [9]. Hence, in the latter the *de novo* synthesized DT appears to be mainly part of the pool 4. Interestingly, *de novo* synthesized DT was recently proposed to be bound by Lhcx

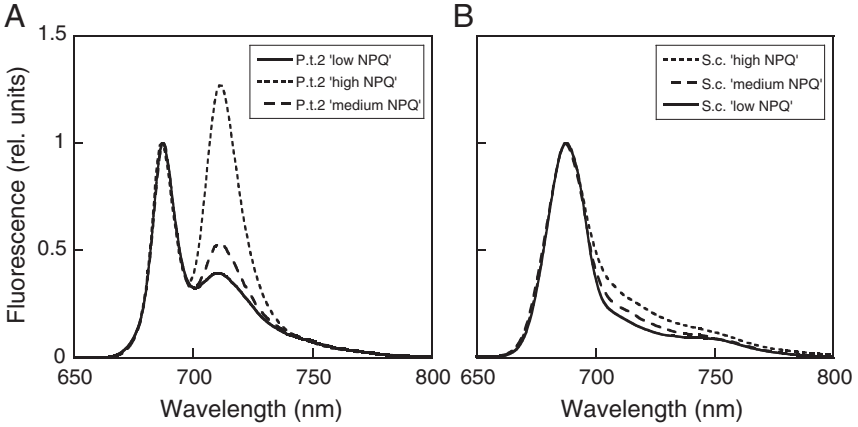

Fig. 4. 77K Chl *a* fluorescence emission spectra excited at 440 nm of *P. tricornutum* (P.t.2 strain) (A) and *S. costatum* (B) dark-acclimated cells showing different NPQ capacity ('low', 'medium' and 'high', see Table 1). The spectra were normalized at their 687 nm maximum.

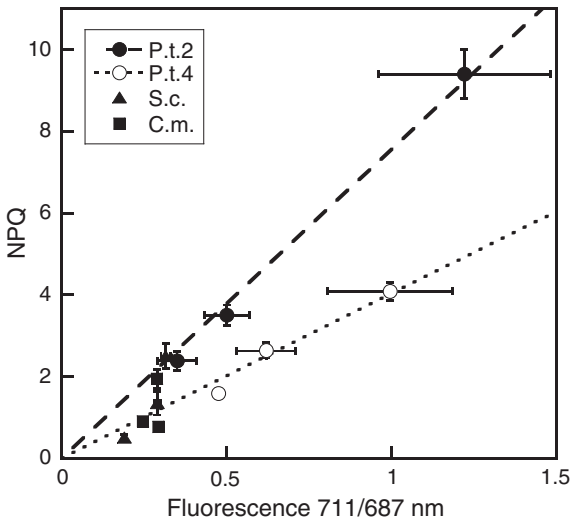

Fig. 5. Relationship between NPQ and the ratio of Chl *a* fluorescence emission at 711 nm and 687 nm (as measured from Fig. 4). NPQ values were measured after 5 min illumination at 2000 μmol photons m^{-2} s^{-1}. For each of the species, *P. tricornutum* (P.t.2 strain, closed circles), *P. tricornutum* (P.t.4 strain, open circles), *S. costatum* (triangles) and *C. meneghiniana* (squares), the three NPQ types of cells ('low', 'medium' and 'high', see Table 1) were used. The linear relationship was NPQ = 8.10 × F711/687 nm (R^2 = 0.99) and NPQ = 4.62 × F711/687 nm (R^2 = 0.98) for the P.t.2 and P.t.4 strains, respectively. Data (± SD) are the average of three independent measurements.

proteins and to participate to the sustained part of NPQ (i.e. qI) [18,22], thus enhancing the dissipative capacity of the PS II LHC antenna under prolonged and harsh illumination conditions. In contrast, it is probable that DT is addressed to the pools 1 and/or 2 in *S. costatum* as also shown for *C. meneghiniana* [37]. This is supported by their fast dark epoxidation illustrating their easy access to the DT epoxidase in the lipid matrix of the thylakoid membrane [37,57]. Here, they likely participate to the prevention of lipid peroxidation [8,9,37]. Hence, in *S. costatum* and *P. tricornutum*, DT would have different main physiological roles with a higher involvement in NPQ in *P. tricornutum*. Consequently, from a species to another, NPQ often does not correlate with the amount of DT and with the DES [11,14]. This discrepancy between the two species was especially true when the light exposure was short and of moderate intensity. In these conditions, *S. costatum* synthesizes significant amounts of DT with only low or even no development of NPQ. A similar relationship was documented before in cells where the ΔpH build-up was chemically manipulated, the lowest ΔpH generating a significant amount of DT with barely any NPQ [49]. Such discrepancy was explained before thanks to *P. tricornutum* mutants showing a DT synthesis divided by half by genetic means [35]. It implies the so-called activation of DT which necessitates the protonation of some FCP binding sites during the ΔpH build-up [48,52,57]. The entering of DT into an 'activated' state triggers the switch of the PS II LHC antenna to the quenching state. We propose that the discrepancy between DT synthesis and NPQ in *S. costatum* is due to a weak/slow 'activation' of DT in conditions of low ΔpH due to a limited protonation of the PS II LHC antenna. It is supported by the fact that the NPQ *vs.* DT *vs.* light intensity relationship followed two clear phases in *S. costatum* with a breakpoint corresponding to the intensity for saturating photosynthesis; this is in total contrast with *P. tricornutum*.

Table 2
Effect of light and inhibitor treatments on the pattern of 77 K chlorophyll fluorescence emission as measured by the ratio F711/F687 nm in *Phaeodactylum tricornutum* (P.t.2 strain) 'medium NPQ' cells (see Table 1).

	Dark-acclimated cells	Illuminated cells
No inhibitors	0.78 ± 0.05[a]	1.02 ± 0.09
+ DTT	0.81 ± 0.06[a]	0.77 ± 0.07
+ NH_4Cl	0.70 ± 0.05[a]	0.69 ± 0.02

[a] There was no significant difference between the dark samples whatever the conditions (student test, $p > 0.05$). Treatments: dark-acclimation was for 20 min, light exposure was 2000 μmol photons m^{-2} s^{-1} during 5 min, DTT (0.2 mM) and NH_4Cl (5 mM) were applied 15 min in the dark before subsequent measurement either in the dark or at light. Values are average of three measurements ± SD. Full spectra are shown as Supplemental Material (Fig. S6).

4.2. The efficiency of DT in the NPQ process: the Δ522 nm absorption change

The above mentioned 'activation' of DT (Section 4.1) can be followed by an absorption change at 522 nm (Δ522 nm) similar to the qE-related Δ525/535 nm in higher plants [17,48]. It is supposed to illustrate the effective involvement of DT as an allosteric regulator of a conformational change in the PS II LHC antenna, a crucial step in the NPQ process [11,14]. The conformational change is believed to be an aggregation of part of the PS II LHC antenna generated by the protonation of FCP binding sites [14]. The inhibition of Δ522 nm by NH_4Cl and DTT confirms this view. The effect of DTT most likely excludes a relationship to an electrochromic shift signal [58]. Δ522 nm thus provides a very useful tool to measure the efficiency of DT in the NPQ process. Indeed, the difference between the relationships NPQ *vs.* DT (Fig. S5) and NPQ *vs.* Δ522 nm (Fig. 3B) demonstrates that the Δ522 nm fingerprint is different from the total DT amount in the way it correlates with NPQ for all the species, i.e. the total amount of DT was far from accounting for the DT which was effectively involved in NPQ ('activated' DT) especially in *S. costatum* and *C. meneghiniana*. This discrepancy explains the above mentioned lack of correlation between the DT amount and the DES, and NPQ (Section 4.1). We could additionally broaden the validity of the Δ522 nm fingerprint to the centric diatom representatives *S. costatum* and *C. meneghiniana*. The correlation of Δ522 nm with NPQ validates the crucial importance of the amount of 'activated' DT to determine the extent of NPQ in that order: *P. tricornutum* > *C. meneghiniana* ≅ *S. costatum*. We could partially manipulate this general trend by modifying the cellular amount of DD + DT thanks to different growth conditions.

Apart from the amount of DT and its binding to the PS II LHC antenna (Section 4.1), two reasons could explain the Δ522 nm inter-species differences: 1) a fewer number of proton binding sites in the FCP proteins (Lhcf and Lhcx) involved in NPQ, 2) a lower protonation of these sites, their number being stable, as illustrated by the DT-independent modification of NPQ by the extent of the ΔpH/lumen acidification [42,52]. Nevertheless and although we did not measure the ΔpH, it is reasonable to propose that at least in *P. tricornutum* and *S. costatum* the extent of FCP protonation was similar since they showed a similar electron transport rate (see [27]). The first reason, although more plausible, is at this stage impossible to verify as we do not know which of the LHC proteins might bind protons in diatoms although both Lhcf and Lhcx proteins have been proposed [44]. The exponential pattern of the NPQ *vs.* Δ522 nm relationship illustrates an essential feature of the diatom NPQ regulation: above a certain value (NPQ of about 1.5), NPQ increases much faster than the Δ522 nm fingerprint, i.e. the increase of DT 'activation' cannot fully account for the huge increase of NPQ, especially in 'high NPQ' cells. Hence, there is another partner amplifying the dissipative process in the LHC antenna system of PS II.

4.3. Amplification of the NPQ process: the chlorophyll 711 nm fluorescence emission band (Chl a_{711})

The presence of a Chl a_{711} emission band at 77 K (or in the range 710–717 nm depending on the studies and the species) has been reported before in diatoms. It is species-dependent [55,59–61]. Similarly,

we showed that while Chl a_{711} can be high in *P. tricornutum*, its amplitude is strongly limited in *S. costatum* and *C. meneghiniana* whatever the growth conditions. The present work additionally depicts the following characteristics for Chl a_{711}: 1) it is positively correlated to the inter-species (*P. tricornutum* > *S. costatum* ≅ *C. meneghiniana*) and intra-species (P.t.2 strain > P.t.4 strain) differences of the NPQ capacity; 2) in *P. tricornutum* and to a much lower extent in *S. costatum* (but not in *C. meneghiniana*), its amplitude is increased together with the pool size of DD/DT by changing the growth conditions (see also [55,59,60]); 3) it is quenched under high light exposure but not as much as PS II Chl a_{687}, and this change is abolished by NH_4Cl and DTT; 4) it is insensitive to red light ruling out any involvement of a state-transitions (i.e. qT) in the quenching process.

Some studies, using 77 K spectroscopy, classically linked Chl a_{711} to the core of PS I and its specific FCP antenna (see [62,63]) while others, using both room temperature and 77 K spectroscopy, demonstrated, it can also originate from PS II by using DCMU or a preferential excitation of fucoxanthin pigments [43,55,64]. In that framework, and although we did not measure it for all strains/species under all growth conditions, it is noteworthy that the stoichiometry PS II:PS I was similar in *P. tricornutum* P.t.2 'low' and 'high' NPQ cells [38]. The PS II:PS I difference was higher (+23%) between *P. tricornutum* P.t.2 and *S. costatum* [27] with more PS I *vs.* PS II in *P. tricornutum*. In diatoms, a large change of PS II:PS I stoichiometry is usually generated by an increase in the light intensity and/or by nutrient deficiency [63,65]. Hence, the Chl a_{711} differences observed here between at least these three types of cells cannot be fully explained by a difference in PS II:PS I stoichiometry. Although it amounts to few percents of total chlorophyll, Chl a_{711} was shown to drain an appreciable fraction of excitation energy from the PS II [59–61] and was proposed to be responsible for dissipating excess energy [55]. The Chl a_{711} emission band was earlier suggested to arise from aggregated protein-pigment complexes close but spatially separated from the PS II [59,60,66]. Owens [66] further demonstrated that Chl a_{711} is sensitive to the energization of the thylakoid membrane which explains its NH_4Cl sensitivity. Recently, a time-resolved fluorescence analysis of the diatom LHC system brought evidence for a 710 nm fluorescence emission band which resembles our Chl a_{711} [43]. It is present in *P. tricornutum* but much less in *C. meneghiniana* and it exists in both unquenched (darkness) and quenched (high light) states. The 710 nm band was assigned to a FCP complex functionally disconnected from both PS I and PS II upon high light exposure [12,43]. It was additionally proposed that this part of the LHC antenna is in an aggregated state similar to the oligomerized LHCII involved in NPQ in higher plants [67]. Interestingly, the Chl a_{710} associated to PS I was shown to arise from FCP complexes weakly bound to the PS I core where they act as a 'shallow sink' of excitation energy [68]. These observations indicate that Chl a_{711} (or Chl a_{710}) probably plays a role in NPQ. Because the quenching was more important on Chl a_{687} than on Chl a_{711}, we propose that Chl a_{711} serves at amplifying the part of the quenching which directly relies on DT (i.e. related to Chl a_{687}). The amplitude of Chl a_{711} (or F711/F687 nm) measured in dark-acclimated cells is a good fingerprint of the level of NPQ amplification at light. It explains in part the inter-species differences in NPQ; i.e. in contrast to *P. tricornutum*, Chl a_{711} had only a minor role in the extent of NPQ in *S. costatum* and *C. meneghiniana*; it was also reported before that Chl $a_{710\text{-}711}$ is lower in *C. meneghiniana* [43].

4.4. The consequence of NPQ inter-species/strains differences on the ecophysiology of diatoms

All and all, the differences in the NPQ extent observed between species and strains of the same species (*P. tricornutum*) can be explained by the extent of Δ522 nm and of Chl a_{711} together with the distribution of DT within the photosynthetic machinery, but independently of the total amount of DD + DT and of the DES. We propose that a high NPQ ability (as observed in the *P. tricornutum* P.t.2 strain) would rely on 1) a high amount of DT which effectively participate to NPQ (Δ522 nm fingerprint); 2) the capacity to form many/large functionally disconnected oligomeric FCP complexes (Chl a_{711} fingerprint) in order to amplify the DT-dependent quenching process. These features are in dramatic contrast with the ones measured for *S. costatum*. The most relevant specific features of NPQ regulation in *S. costatum* are ([9,27] and this study) the coupling between i) the early onset of NPQ, i.e. already for a light intensity corresponding to the one the cells are acclimated to, ii) the slow development of NPQ *vs.* DT *vs.* light for non-saturating irradiances, iii) the apparent slow recovery of NPQ related to a limited epoxidation of DT (also reported for *C. meneghiniana* and *P. tricornutum* P.t.4, [57]), iv) the low investment of *de novo* synthesized DT in the enhancement of NPQ. These features, together with the absence of a PS II CET [27] (which can explain in part the points i) and ii)) cannot promote a similarly strong and fast irradiance-tuning of NPQ as in *P. tricornutum*. Ultimately, as shown here and before [27], these differences lead to a higher susceptibility of *S. costatum* to photoinhibition during exposure to excess light and/or to a fluctuating light climate negatively inferring on its photosynthetic productivity and growth. Such record brings a molecular basis for explaining the adaptation of the regulatory photosynthetic machinery to the aquatic light environment as proposed before [11,21,27,30]. Indeed, the general characteristics for high NPQ in the *P. tricornutum* P.t.2 strain, which originates from an estuarine habitat, fit well with an adaptation to fast and strong light fluctuations while the ones for low NPQ fit better with the slow and moderate light changes that characterize the habitat the strain of *S. costatum* used here originates from (a Mediterranean semi-enclosed bay). The recent report that *S. costatum* and *C. meneghiniana* behave similar under fluctuating light conditions [53] further supports the same impact of their seemingly close regulatory NPQ machinery on their photophysiology. Also supporting and extending conclusions to strains/ecotypes of the same species, is the fact that the low NPQ *P. tricornutum* P.t.4 strain shows an NPQ *vs.* DT relationship which slope is similar to the one of *S. costatum* [35]. Different from the P.t.2 strain, P.t.4 originates from a high latitude habitat characterized by a low light environment [20]. As observed here and before [20] in *P. tricornutum* strains/ecotypes, the amounts of DD/DT, of Chl a_{711} FCP oligomers and of Lhcx1 are concomitantly influenced by the light environment the cells are adapted to. The slope of the NPQ *vs.* DT relationship [9,30] therefore could be a good indicator of the adaptation of a species/strain to an aquatic niche as a function of the light climate. The present work further confirms and extends the potential role for the light-dependent fast regulation of photosynthesis, and especially NPQ, in the ecophysiology of diatom phytoplanktonic and microphytobenthic communities [11,19,21,32]. It could explain in part the ecological diversity of diatoms [1] which has been participated to their ecological success in contemporary oceans [1–3].

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2012.11.012.

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