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Photoprotection in Diatom Algae

Regular Paper

**The super-excess energy dissipation in diatom algae: comparative analysis with
higher plants**

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Abstract

When grown at intermittent light regime, diatom alga *Phaeodactylum tricornutum* is able to form photoprotective non-photochemical fluorescence quenching (NPQ) 3 - 5 times larger than that observed in the higher plants. This quenching is sustained in the dark for 5-10 min, reverses completely within approx. 1 hr and seems to be very tightly related to the presence of the zeaxanthin analogue, diatoxanthin. Addition of the uncoupler NH_4Cl before illumination can completely abolish formation of NPQ, revealing the ΔpH -dependency of the xanthophyll cycle activity. Once established, NPQ can also be almost completely reversed by the uncoupler. However the higher NPQ is formed the more time is required for its reversal. At the point when the fluorescence was approx. 90% recovered the level of illumination-induced diatoxanthin was found to be only partially reduced. This indicates that the proton gradient is a key triggering factor of NPQ. It was also noticed that NPQ in *Phaeodactylum* cells was absent even when majority of reaction centers were closed and the plastoquinone pool was significantly reduced. The absence of NPQ at these conditions could be due to very low levels of ΔpH . It is likely that in diatoms alternative sources of protons such as the PSI cyclic electron transfer and/or chlororespiration are important in generating the proton gradient sufficient to trigger NPQ. Absorption changes associated with the xanthophyll cycle activity were found to be larger than those for higher plants. The position of the positive maximum in the difference spectrum illuminated-*minus*-dark was 512-514 nm in comparison to the 505-508 nm for leaves. The 535 nm band associated with NPQ in plants is absent in *Phaeodactylum*. An uncoupler-sensitive absorption change at 522 nm was discovered. Kinetics of NPQ showed linear correlation with the 522 nm absorption change.

Key words

Chlorophyll fluorescence; nonphotochemical fluorescence quenching; absorption spectra; diatoms; *Phaeodactylum tricorutum*; xanthophyll cycle; diatoxanthin

Abbreviations

NPQ - Nonphotochemical chlorophyll fluorescence quenching; PSII – Photosystem II; DT – diatoxanthin; LHC – light-harvesting complex; DD – diadinoxanthin; PSI – Photosystem I

Introduction

Diatom algae are dominant phytoplankton component in the vast areas of cold waters of the North Atlantic and Arctic Oceans. They contribute to almost 25% of the primary photosynthetic production on Earth and therefore play an important role in the energy balance of our biosphere (Treguer et al. 1995, Field et al. 1998, Smetacek 1999). It is widely known that the photosynthetic efficiency is dependent upon the light environment – too much light can be dangerous for photosynthesis, in particular to the photosystem II (PSII). At conditions of excess light the most powerful oxidant in nature, PSII reaction center, can cause a fatal damage to this photosystem and subsequent decrease in the photosynthetic efficiency (Osmond, 1981, Horton and Ruban, 1992, Barber, 1995). However, a mechanism does exist to minimise this effect by safely dissipating excess of harmful energy into heat. This process can be monitored by measuring nonphotochemical chlorophyll fluorescence quenching (NPQ) – an indicator of the nonradiative excitation energy dissipation in the photosynthetic antenna (Krause and Weis, 1991). The mechanism of NPQ was a subject of intense research in higher plants (Demmig-Adams B and Adams III, 1992, Horton et al., 1996, Niyogi, 1999). So far it is established that it occurs in light harvesting system of PSII, triggered by ΔpH and modulated by xanthophylls (Horton et al., 1996, Gilmore, 1997). Recently a PSII protein PsbS was discovered to be a key factor sensing ΔpH (Li et al., 2000, 2002, 2002b, 2002c), binding zeaxanthin (Aspinall-O’Dea et al., 2002) and probably triggering the conformational changes within PSII antenna (Horton et al., 2000), which leads to its transition into the dissipative mode. The physical nature of this quenching is still under debate. There exist two major theories. One implies that zeaxanthin is a direct quencher of the chlorophyll excited state (Demmig-Adams, 1990, Owens et al., 1992, Frank et al.,

1994, Gilmore, 1997). The other theory suggests that the quencher is a chlorophyll dimer or excimer formed during the antenna conformational transition assisted by zeaxanthin as an allosteric activator (Horton et al. 2000). Diatom algae possess the capacity for energy dissipation, which in some cases exceeds that of the higher plants. (Ting and Owens, 1993, Arsalane et al., 1994, Olaizola et al., 1994, Casper-Lindley and Bjorkman, 1998). Recently an unusually high NPQ (almost twice the level of control) was found in *Phaeodactylum tricornutum* cells grown under intermittent light regime (Lavaud et al., 2002a, b). This model alga possesses more than 5 times capacity to dissipate excess energy as compared to higher plants. NPQ is dependent on the build-up of a transthylakoid proton gradient and on xanthophyll cycle activity. This cycle is different from violaxanthin cycle because different xanthophylls, diadinoxanthin (DD) and diathoxanthin (DT), represent it. Also it is a one-step process: deepoxidation of only one oxygen takes place changing the number of conjugated double bonds from 10 to 11, which equals to antheraxanthin-zeaxanthin conversion (Hager and Stransky, 1970). It is interesting to note that the intermittent light regime had no large effects on the pigment composition and antenna size of *Phaeodactylum* apart from the dramatic increase in the xanthophyll pool size (Lavaud et al., 2002a, 2003). At these conditions exceptionally large levels of NPQ have been recorded. In this study we focus on investigation of the relationship between DT concentration, ΔpH and NPQ in diatom algae using uncouplers and simultaneous kinetic measurements of NPQ and DT formation. We show in the present report that the free DT is unlikely to be the fluorescence quencher. It has to undergo ΔpH -dependent incorporation/activation into the quenching locus. Since the concentration of DT is often larger than that of zeaxanthin in plants and the maximum deepoxidation capacities are similar it is possible that more DT is involved in the

fluorescence quenching than zeaxanthin. All data indicate that the differences between composition and organisation of light-harvesting complex (LHC) of plants and diatoms may be central in determining the NPQ capacity. We have found a new absorption change at 522 nm, which linearly correlates with NPQ and is possibly associated with DT activation in the quenching locus. For understanding of the excess energy dissipation mechanism, comparison with higher plants is essential since diatoms having different xanthophylls and different antenna system, containing specific polypeptides and pigment populations, possess an effective NPQ with mechanistic features similar to that of plants.

Materials and Methods

Material

Phaeodactylum tricornutum cells were grown photoautotrophically in sterile natural seawater as described in (Lavaud et al., 2002a). Briefly, cultures were incubated at 18 °C in airlifts continuously flushed with air. They were grown under a white light intensity of 40 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ provided by fluorescent tubes (Claude, Blanc Industry, France) with a 5 min light/55 min dark cycle. Cells were collected after 30-40 days still in their exponential growth phase, since the growth rate under the intermittent light regime is extremely low. Part of the culture was then diluted in fresh medium to maintain cells in exponential growth phase. *Arabidopsis thaliana* cv Columbia plants were grown in growth chambers at 8 hrs photoperiod and 200 μM quanta $\text{m}^{-2}\cdot\text{s}^{-1}$ for 8 weeks.

Thylakoid preparations and treatments

Thylakoids were prepared either from dark-adapted (1 hr after the end of 5 min illumination period) or illuminated (5000 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ for 25 min) algae. Cells

were broken in a standard buffer used for spinach thylakoid preparation (Ruban et al., 1999) by 3 cycles of French press treatment and centrifugation. In this procedure thylakoids were released from approx. 90 % of all cells. Final step included pelleting of thylakoids and resuspending in the medium, which contained 0.35 M sorbitol, 1mM EDTA, 5 mM MgCl₂ and 50 mM HEPES. All handling of material was carried out in a dark room. The diadinoxanthin de-epoxidation was carried out at pH5.5 for 1 hr at chlorophyll concentration of 50 µM in 200 ml of medium with the constant stirring at room temperature. Intact spinach chloroplasts were prepared as described earlier (Ruban and Horton, 1999). LHC from algae was prepared using solubilisation of dark-adapted and low-pH-treated thylakoid membranes with digitonin for 1 hr on ice and subsequent sucrose gradient centrifugation at 200000 g for 18 hrs (for more details see Lavaud et al., 2003).

Pigment analysis

Pigment analyses were performed using HPLC as described previously (Arsalene et al., 1994, Lavaud et al., 2003). Pigments were extracted by a phase separation procedure: samples were first mixed with a methanol:acetone (50:50, 1 volume) solution completed with ether (1 volume) and water-NaCl 10 % (2 volumes). Deepoxidation state index was calculated as: $DES = (DT)/((DD)+(DT))$, where (DT) and (DD) are concentrations of diatoxanthin and diadinoxanthin, respectively.

Chlorophyll fluorescence measurements

Chlorophyll fluorescence traces were measured using PAM-101 Walz fluorometer (Walz, Effeltrich, Germany) and a standard thermostatic cell for liquid samples as described by Lavaud et al. (Lavaud et al., 2002a). NPQ was calculated as $(F_m - F_m')/F_m'$. qP was calculated as $(F_m' - F_s)/(F_m' - F_o')$. F_m and F_m' are the maximum

levels of fluorescence before and during actinic illumination, respectively. F_s is the steady-state fluorescence level. F_o' is the dark level of fluorescence measured immediately after switching off the actinic light. The saturating white light pulse intensity was $6000 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

Absorption measurements

Absorption spectra and simultaneous chlorophyll fluorescence and absorption kinetics in the 505-522 nm region were measured using DW2000 Aminco Spectrophotometer (American Instrument Co., USA) in a single or double beam mode. Spectral resolution was 0.5 nm and 2 nm for stationary and kinetic measurements, respectively. To induce absorption changes, sample was illuminated at 90° with respect to the measuring beam. For illumination a 150 W tungsten halogen bulb light filtered by RG610 Schott filter, transmitting efficiently above 610 nm, (Optical Glass Inc., USA) was used. To protect the photomultiplier from actinic light a combination of Corning 4-96 and OCL1 Cyan T400-570 filters, transmitting in the region of 400-570 nm was used (Corning Co, USA). The actinic light intensity was $700 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$

Results and Discussion

NPQ induction and reversibility in diatoms in comparison to higher plants

Figure 1 shows typical traces of chlorophyll *a* fluorescence quenching induced by continuous illumination of *Phaeodactylum* cells (A) and *Arabidopsis* leaves (B). The actinic light intensity was close to saturate PSII electron transport for the both types of material. For the algae the saturating light intensity was almost 4 times lower than that for *Arabidopsis* leaves ($500\text{-}700$ and $2000\text{-}2500 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ respectively). In

Phaeodactylum fluorescence was quenched quickly and almost monophasically. The fluorescence level went far below the F_0 and the total NPQ reached as far as 9 in some cases after only 5 min of illumination. In plants, fluorescence decrease was less intense (NPQ only 1.9 - 2.1) and more complex with a visible slow phase. The slow phase was similar to the previously observed fluorescence quenching induction in spinach leaves and attributed to the gradual formation of zeaxanthin (Ruban and Horton, 1999). Removal of actinic illumination caused fast relaxation of the fluorescence level in leaves, whereas in diatoms fluorescence remained completely quenched during 5 -10 minutes of dark-adaptation. It took almost 50 minutes to restore the F_m in *Phaeodactylum*. The second illumination cycle showed much faster fluorescence quenching in leaves, but not in diatoms, where the rate of quenching was almost the same as during the first illumination cycle. It is worth to notice that the fluorescence relaxation in *Arabidopsis* leaves after the second illumination phase was almost 50 % slower than the recovery after the first illumination phase but still much faster than that in diatoms. NPQ after the second phase was increased to 2.6 - 2.8, which is due to additional zeaxanthin formation (Ruban and Horton, 1999, Ruban, unpublished). As was noticed earlier in experiments on isolated chloroplasts the presence of zeaxanthin enhances not only NPQ but also the time required for its relaxation (Rees et al., 1989). In diatoms grown under intermittent light regime xanthophyll cycle pool size is almost doubled comparing to control cells (Lavaud et al., 2002a,b, 2003). There are minimum 2 diadinoxanthin molecules per LHC monomer in cells grown under intermittent light regime (Lavaud et al., 2003). That is twice the maximum amount of violaxanthin found in LHCII of plants (Ruban et al., 1999). This could cause an increase in relaxation time in agreement with the observations made on plants. Hence, the tendency for large NPQ to reverse slowly

seems to be a common feature for plants and diatoms. To explore further the relationship between the extent of NPQ and the extent of its recovery and rate in diatoms varying periods of actinic light illumination were employed to modulate the amount of quenching. Figure 2 shows the results of this approach. Indeed, fluorescence level was almost restored within 5 min of dark adaptation following the illumination when the NPQ level was small. The proportion of reversible quenching (restored after 5 min) was becoming smaller with the increase of the illumination time and NPQ respectively.

Effect of an uncoupler on the NPQ relaxation

To find if the slow relaxation of NPQ is linked to the sustained ΔpH and protonation of the quenching locus, we employed ammonium chloride (NH_4Cl) as an uncoupler. When NH_4Cl is added before the illumination NPQ can be completely inhibited (Figure 3 A, trace 1), indicating efficient permeability of the uncoupler into the algal chloroplast (Lavaud et al, 2002b). Figure 3B shows typical rapid fluorescence level recovery upon addition of uncoupler to isolated intact chloroplasts. The effect of NH_4Cl on the NPQ in diatoms is very different. When added at different stages of NPQ formation in diatoms, NH_4Cl reverses promptly the fluorescence level when NPQ is within the range of that observed in plants (Figure 3 A, traces 2-3). But it is clear that the reversal becomes much slower for larger values of NPQ. For NPQ values up to 1.7- 2.0 the level of diatoxanthin was decreased during 5 min after addition of uncoupler only by 20%. When the quenching level approached 3 and above, the NPQ relaxation time started to rise dramatically (Figure 4) as well as the extent of DT epoxidation (not shown). For NPQ level around 6 the relaxation time of NPQ became close to 15 minutes. For the same duration of dark recovery more DT

was found to be re-epoxidised in the presence of NH_4Cl than in the control. This observation suggests that NH_4Cl has accelerated not only the reversibility of NPQ but also the rate of diatoxanthin epoxidation, since the dark adaptation time required to epoxidise completely diatoxanthin at this level of NPQ was found to be more than 30 min (Lavaud et al., 2002b). These results raised a few important mechanistic and physiological issues. DT is certainly not the only factor that controls NPQ – proton gradient and protonation are essential triggers of it, as was suggested earlier (Lavaud et al., 2002b). It is possible that after illumination a residual level of ΔpH still remains and is sufficient to sustain high level of NPQ in darkness and the presence of DT. The chlororespiration could be an important factor contributing to the proton gradient in diatoms (Jakob et al., 1999, Lavaud et al., 2002c). This situation was observed in some higher plant species (Ruban and Horton, 1995), particularly under conditions of combined light and cold stress (Verhoeven et al., 1998). Indeed, comparing the data on spontaneous (Figure 1) and uncoupler-dependent recovery of NPQ (Figure 3), it is feasible to conclude that the uncoupler accelerates the slow release of protons out of the quenching locus. Though being not the most lipophilic agent, NH_4Cl is still effective to prevent NPQ. Trials to employ more lipophilic uncoupler nigericin have failed, since it was not able to diffuse through the cell wall (not shown). However, the addition of NH_4Cl has reversed NPQ in all cases. Still, the relaxation time remained to be dependent upon the extent of quenching. The effects of NH_4Cl on the slow reversal of NPQ in diatoms are rather consistent with the NPQ model described by Horton and Ruban for higher plants (Horton and Ruban, 1992). The model suggests a gradual conformational transition within the LHCII antenna into the quenching state. The key element of this change is zeaxanthin, which acts as an allosteric controller. Zeaxanthin analogue, DT, has most likely a similar function in diatoms. One would predict that

the structure of their antenna is capable of a conformational change favouring transition into a very efficient energy-dissipative state. A recent paper on thermodynamics of the fluorescence quenching in isolated LHCII antenna supports this view and suggests that the oligomerisation state of antenna is one of the factors controlling dynamics of the complex together with zeaxanthin, protonation and domain structure (Wentworth et al., 2003). The general NPQ formation scenario may involve the following sequence of events: formation of DT and protonation of antenna, cooperative conformational transition of the latter into the quenching state involving partitioning of DT into the more hydrophobic regions and more hydrophobic dislocation of proton-binding domains. This should serve as an NPQ 'lock', preventing a quick relaxation in the absence of a bulk proton gradient. The clear evidence of the co-operative manner of NPQ formation is a progressive distinct sigmoidicity of the fluorescence relaxation curve with an increase of the extent of quenching (compare Figure 3A, traces 2 - 6).

Relationship between super-NPQ and qP

Diatoms grown under intermittent light regime possess particularly large levels of energy dissipation (Lavaud et al., 2002a). At this light environment diatoms possess very low photosynthetic rate. Indeed, as was mentioned earlier, the PSII saturating light intensity for *Phaeodactylum* is about 4 times less than that for *Arabidopsis* leaves. A large percentage of PSII reaction centers have to be closed before the photoprotective NPQ can be activated. Figure 5 illustrates this observation showing relationship between qP and NPQ in plants and diatoms. In higher plants this relationship seems to be almost linearly reciprocal. Photoprotection is closely following the PSII redox-state. In diatoms grown at intermittent light regime

relationship between qP and NPQ is rather hyperbolic. It looks like these two processes can be relatively independent. The adaptation of *Phaeodactylum* to this rather unusual light regime is such that NPQ is standing-by and can be readily triggered only when the PSII redox-state is very close to saturation. It is likely that some additional sources of protons are required here to trigger NPQ. Those could originate, for example, from the cyclic electron transport around PSI, which is activated when the linear electron transport is saturated (Caron et al., 1987). This is coherent with the recent finding on *Arabidopsis* mutant lacking the protein controlling the PSI cycle (Munekage et al., 2002). NPQ in this mutant is significantly reduced suggesting the important role of protons generated by PSI in controlling the energy dissipation in PSII antenna. Therefore the apparent absence of coordination between NPQ and PSII electron transfer activity is an illustration of the relative independence of the PSII redox-state from the proton-motive electron transfer and subsequently NPQ.

Comparison of the absorption changes associated with NPQ in diatoms and plants

Illumination of diatom cells induces significant absorption changes in the carotenoid region. Figure 6A shows spectra of dark-adapted and illuminated samples. The distinct maximum around 490 nm in the spectrum of dark-adapted cells is strongly decreased and a shoulder above 500 nm emerges after illumination. Calculation of a difference spectrum illuminated-*minus*-dark revealed a major maximum at 512 nm and a minimum at 490 nm corresponding to DT-*minus*-DD spectrum (Figure 6B, trace 1). The similar absorption difference spectrum for the violaxanthin cycle, zeaxanthin-*minus*-violaxanthin is shown as a dotted line (Figure 6B). Although, both zeaxanthin and DT have the same number of conjugated double bonds, DT absorption is 4 nm

red-shifted comparing to zeaxanthin. Interestingly the similar difference spectrum for the isolated LHC antenna complex from *Phaeodactylum* thylakoids has 508 nm maximum instead of 512 nm and the positive DT band is much narrower (Figure 6B, trace 2, see also (Lavaud et al., 2003)). This suggests that the environment of DT in the intact thylakoid membrane is different from that of the isolated complex, likely due to tighter interaction within hydrophobic antenna domains of high polarisability. The difference spectrum cells-*minus*-LHC represents the additional absorption change in the xanthophyll region under conditions of NPQ (Figure 6B, trace 3).

Another key difference between spectra 1 and 2 of Figure 6B is the presence of a strong 690 nm band in chlorophyll *a* region, which is similar to that observed in higher plant thylakoids when NPQ was formed (Ruban et al., 1992). This band was found to be present only when the fluorescence quenching in isolated LHCII was induced. Although the work is in progress to understand the nature of this band, it is likely to originate from chlorophyll-chlorophyll or chlorophyll-xanthophyll association in the quenching locus.

To separate the xanthophyll cycle-associated from NPQ-dependent absorption changes an uncoupler NH₄Cl was employed. Figure 6C, traces 1 and 2, represent the absorption changes in the blue and red regions due to addition of NH₄Cl and rapid removal of NPQ. In the red region the 690 nm positive band was similar to that in the spectrum 1, Figure 6B. The main maximum in the xanthophyll region is even more red-shifted towards 514 nm and the band is 4 nm broader than that in the difference spectrum 1 of the Figure 6B. As was stated above, during the uncoupler treatment a partial DT epoxidation process took place. This could cause the presence of the DT-*minus*-DD component at 508 nm in the uncoupler-induced spectral change. Therefore,

the subtraction of this component using the deepoxidation difference spectrum for LHC from the uncoupler-induced difference spectrum reveals purely protonation-dependent change (Figure 6C, trace 3). The latter has the same maximum as the spectrum 3 from the Figure 6B but it is dominating in the uncoupler-dependent spectrum. The NPQ-associated absorption band at 522 nm in diatoms is analogous to that in higher plants at 535 nm (Figure 6C, dotted trace). This band was found to be zeaxanthin-dependent and recently has been assigned to the special, activated state of zeaxanthin into NPQ locus, probably associated with PsbS protein – a key trigger of NPQ in plants (Ruban et al., 2002a, Aspinal-O’Dea et al., 2002). The strong absorption shift of almost 25 nm was attributed to a zeaxanthin dimer. It was postulated to be a head-to- tail type of dimer. In analogous spectrum of diatoms 522 nm band could represent the activated DT. However the red shift (around 10 nm) is much smaller than in the higher plant spectrum. This is possibly because DT is very asymmetric compared to zeaxanthin and conjugation of optical π -electrons extends only into one end-group (Figure 7) it is less probable that it can form dimers with similar optical properties to zeaxanthin. It is also possible that because of likely differences of plant and diatom antenna structures, DT environment in the NPQ locus is very different from that of zeaxanthin. These implications will be tested in a near future for the LHC of diatoms by biochemical work and spectroscopic approaches developed for higher plants (Ruban et al., 1999, 2001, 2002a, 2002b).

Direct measurement of the NPQ and diatoxanthin formation kinetics

Using HPLC determination of DT, a point-by-point correlation close to linear was observed between DT and NPQ (Lavaud et al., 2002a, Lavaud et al., 2002b). A more precise determination of the relationship between NPQ and the different absorption

peaks attributed to the xanthophyll cycle was done in simultaneous kinetic measurements as was performed on higher plant leaves (Ruban et al., 1993). Figure 8A shows kinetics of chlorophyll fluorescence at F_m level and absorption at 514 nm induced by saturating light. The kinetic of fluorescence quenching was found to follow hyperbolic function similarly to that of spinach chloroplasts and leaves (Ruban and Horton, 1999). This observation is yet another evidence of the fundamental resemblance between the energy dissipation mechanism in diatoms and plants. NPQ was in linear correlation with the appearance of the 522 nm band, which, as hypothesised above, may reflect an activated, partitioned into the hydrophobic quenching locus DT (Figure 8, B). Clear deviation from linearity can be observed in correlation of NPQ with absorption change in more short-wavelength region at 514 nm. This observation could provide another support to the idea that a sub-population of DT molecules in diatoms, as zeaxanthin in plants, undergoes incorporation into the NPQ locus. The red shifted peak is indicative of either a direct fluorescence quencher or/and a state of allosteric activator of the quenching, promoting conformational change within the antenna.

Factors controlling the extent of NPQ

The results mentioned above indicate tight but not direct relationship between DT and NPQ. After 1 hr of light treatment we have found that only 65 % of DD was converted into DT. This is about the same as was obtained during illumination of spinach leaves (Ruban and Horton, 1999). However in spite of a similar extent of deepoxidation the differences in NPQ are large. Moreover the low-pH induced deepoxidation of isolated thylakoids from *Phaeodactylum* cells is reaching 80%, which is similar to that reported for spinach (Ruban et al., 1999). Therefore these

diatoms have very much the same maximum de-epoxidation capacity as higher plants. The super-NPQ (8 - 15 for diatoms against 2 - 3 for *Arabidopsis* and spinach) can be due to the larger xanthophyll pool size in diatoms grown under intermittent light while the antenna size remains the same than for algae grown under a classical light/dark regime. For a smaller pool size associated with the same antenna size, smaller values of NPQ are obtained closer to the values found in higher plants (Lavaud et al., 2002a, 2003). In *P. tricornutum* Lavaud et al. (Lavaud et al., 2003) have shown that the additional DD molecules of intermittent light grown cells are mainly associated with LHC fraction. Recent experiments on *Arabidopsis* indicate that doubling of amount of xanthophyll cycle carotenoids in PSII antenna had no visible effect on the maximum NPQ (Davison et al., 2002). Therefore these contradicting observations could probably be explained by differences in the concentration of quenching centers in the two antenna systems. The proportion of activated DT in the NPQ locus can be larger than that of the activated zeaxanthin. Indeed, judging from the absorption difference spectra shown on the Figure 6B and C, the amplitude of NPQ-associated change at 522 nm in diatoms is reaching 25-30% of the total DT absorption, whereas the absorption at 535 nm is about only 10% of the total zeaxanthin absorption.

The role of PsbS protein - a likely antenna associate - has been in a center of recent mechanistic research on NPQ in plants (Li et al., 2002a, 2002b, 2002c, Aspinal-O'Dea et al., 2002). The amount of this protein was shown to correlate closely with the extent of the fluorescence quenching *in vivo* (Li et al., 2002c). However so far the presence of PsbS in diatoms has not been shown but a complex with a similar role could be present and its concentration could be increased by intermittent light regime

together with the increase of the DD pool size while the antenna size remained constant. It is clear that the next step in the investigation of the NPQ mechanism in diatom algae should be concentrated on the biochemical, biophysical and molecular biological studies of the PSII complexes involved.

Conclusions

Super-NPQ in diatom alga *Phaeodactylum tricornutum* is closely related to DT concentration. Parallel measurements of NPQ and DT absorption kinetics suggest indirect involvement of this xanthophyll in the fluorescence quenching process. The fast reversible phase of NPQ as well as the 535 nm absorption band are absent in this alga. Fluorescence quenching was found to correlate closely with the absorption change at 522 nm. This band is hypothesised to belong to an activated DT interacting with the NPQ locus in antenna. The hyperbolic type of the fluorescence quenching and gradual co-operative character of NPQ formation are the most fundamental common features of the mechanisms of the excess energy dissipation in diatoms and plants. Due to the low photosynthetic rates and danger of a sudden exposure to excess light NPQ in diatoms can be significantly higher than that in plants and shows more sustained character. The causes of a large NPQ in diatoms could originate from the unique organisation and dynamic properties of light-harvesting antenna components, affinity of a DT binding into the quenching locus and structural properties of this xanthophyll.

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Figure legends

Figure 1. Fluorescence quenching in cells of *Phaeodactylum tricornerutum* (A) and *Arabidopsis thaliana* leaves (B) induced by the saturating actinic light intensity. P – saturating pulse; AL – continuous light on (400 and 2000 $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ for *Phaeodactylum* and *Arabidopsis* respectively). Chlorophyll concentration of cells was 20 μM . Sample was constantly stirred at 18°C.

Figure 2. Reversibility of the NPQ level in *Phaeodactylum tricornerutum* cells after actinic light treatment of varying duration. Illumination was followed by 5 min of dark recovery prior the measurement of the fluorescence level and calculation of NPQ. Light intensity and chlorophyll concentration as in experiments shown on Figure 1. Open circles: total NPQ after illumination. Closed circles: NPQ reversed after 5 min of dark adaptation. Closed triangles: uncoupler-dependent NPQ, reversed by addition of uncoupler NH_4Cl .

Figure 3. Fluorescence traces showing the uncoupler-dependent reversibility of NPQ in cells of *Phaeodactylum tricornerutum* (A) and intact spinach chloroplasts (B). A: trace 1, 100 mM of uncoupler NH_4Cl was added 1 min before continuous illumination; traces 2 – 6, addition of NH_4Cl during varying stages of the fluorescence quenching formation. Light intensity and chlorophyll concentration as in experiments shown on Figure 1. Numbers above traces indicate values of NPQ reversed by uncoupler.

Figure 4. Relationship between the amplitude of NPQ and time required to recover 50% of quenching by addition of uncoupler NH_4Cl .

Figure 5. Relationship between photochemical (qP) and nonphotochemical (NPQ) fluorescence quenching in *Arabidopsis thaliana* leaves (closed circles) and *Phaeodactylum tricornerutum* cells (open squares). Different levels of qP and NPQ were obtained by using a range of actinic light intensities varying from 30 to 2500 $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. For calculation of quenching parameters see *Materials and Methods*.

Figure 6. A. Room temperature absorption spectra of dark-adapted (Dark) and light-treated (Illuminated) *Phaeodactylum tricornerutum* cells. For details of illumination see *Materials and Methods*. B. 1, difference spectrum illuminated-minus-dark adapted cells; 2, difference between absorption spectrum of LHC preparation from *Phaeodactylum* thylakoids enriched in DT minus the absorption spectrum of LHC preparation enriched in DD; 3, difference 1-2 spectrum; dotted line, difference between spectra of *Arabidopsis* leaf containing zeaxanthin and leaf containing violaxanthin. C. 1 and 2, two regions of uncoupler-induced difference spectrum of algae cells illuminated for 1 min; 3, difference between spectrum 1 and spectrum 2 from B; dotted line – NPQ-associated absorption spectrum of *Arabidopsis* leaf.

Figure 7. Structural formulae of zeaxanthin and diatoxanthin.

Figure 8. A. Simultaneous kinetics of chlorophyll fluorescence quenching (open symbols) and A514 band formation (closed symbols) in *Phaeodactylum tricornerutum* cells. Solid line is a single hyperbolic fit of the fluorescence kinetic (as in Ruban and Horton, 1999). B. Relationship between NPQ and extent of absorption changes at 514 (closed circles) and 522 nm (open squares). For details of illumination see *Materials and Methods*.

Figure 1

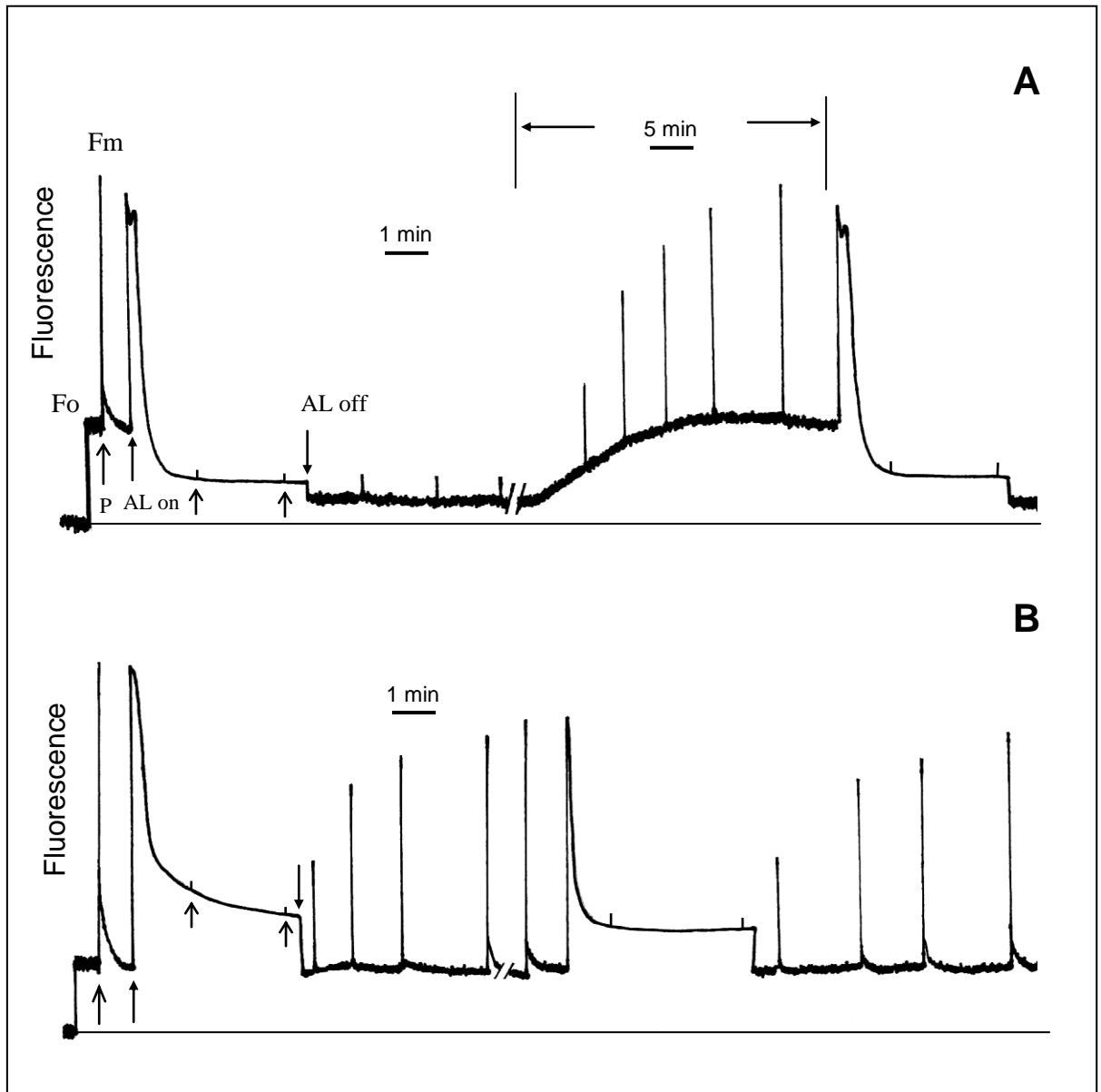


Figure 2

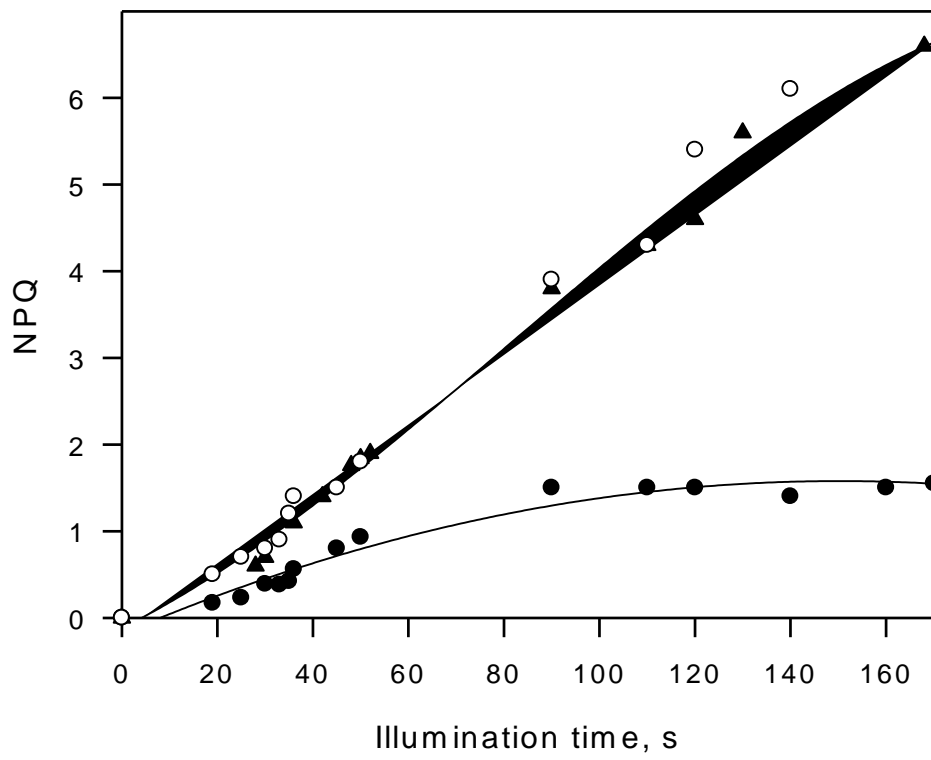


Figure 3

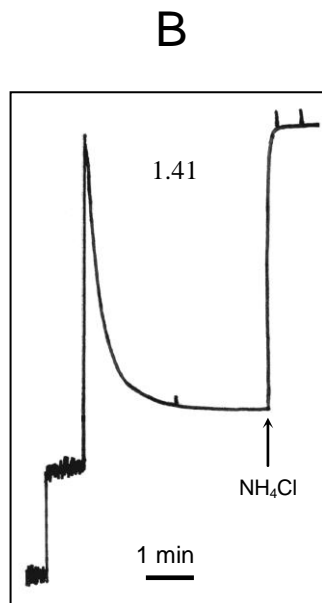
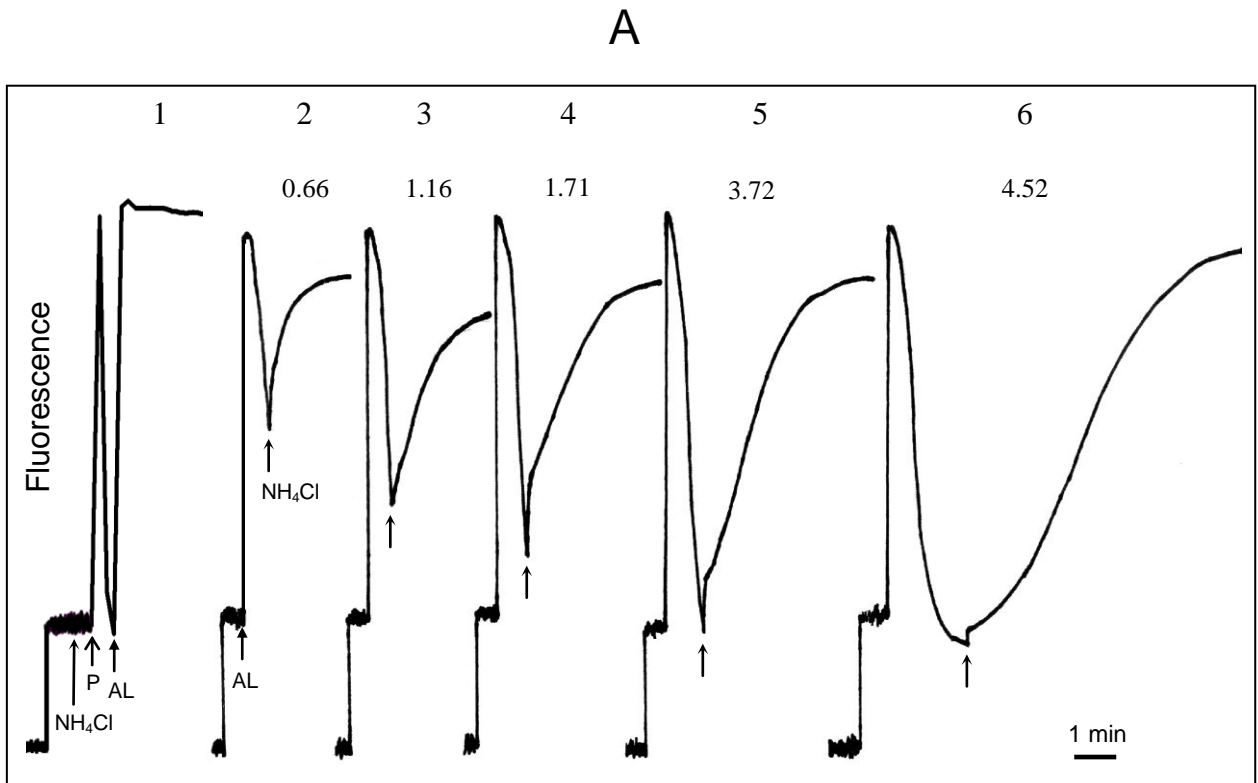


Figure 4

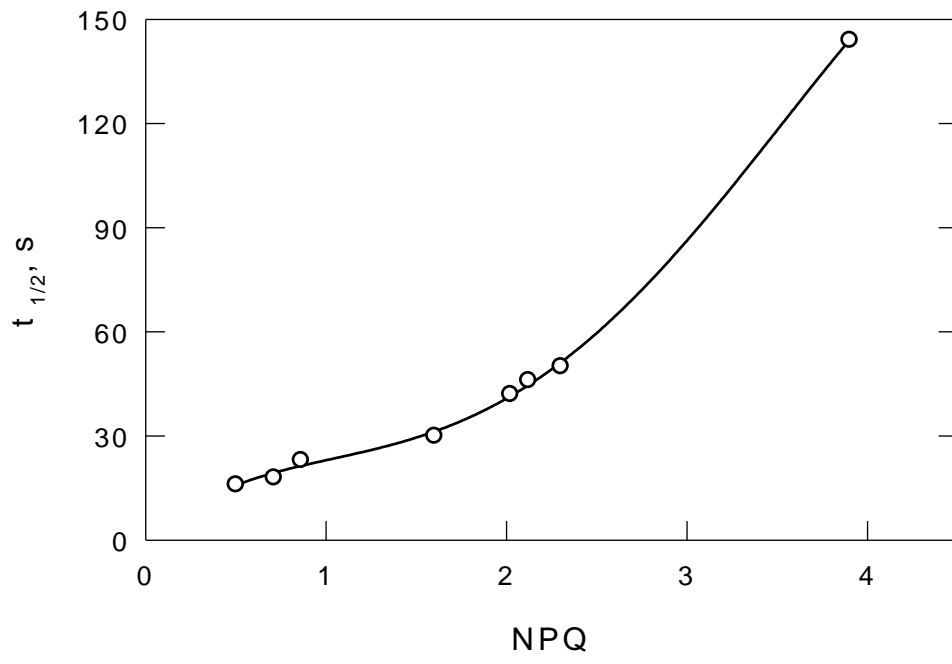


Figure 5

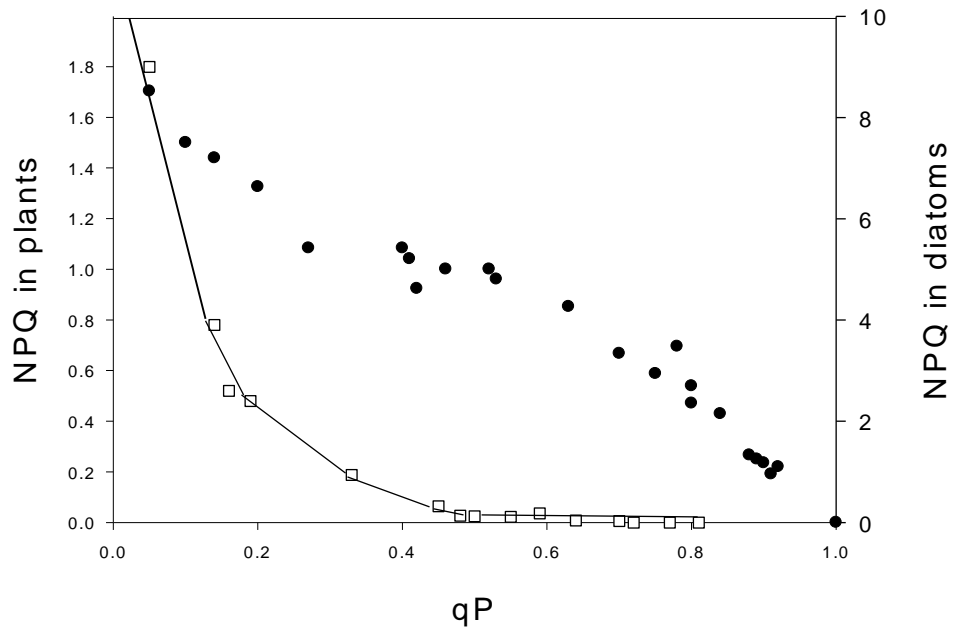


Figure 6

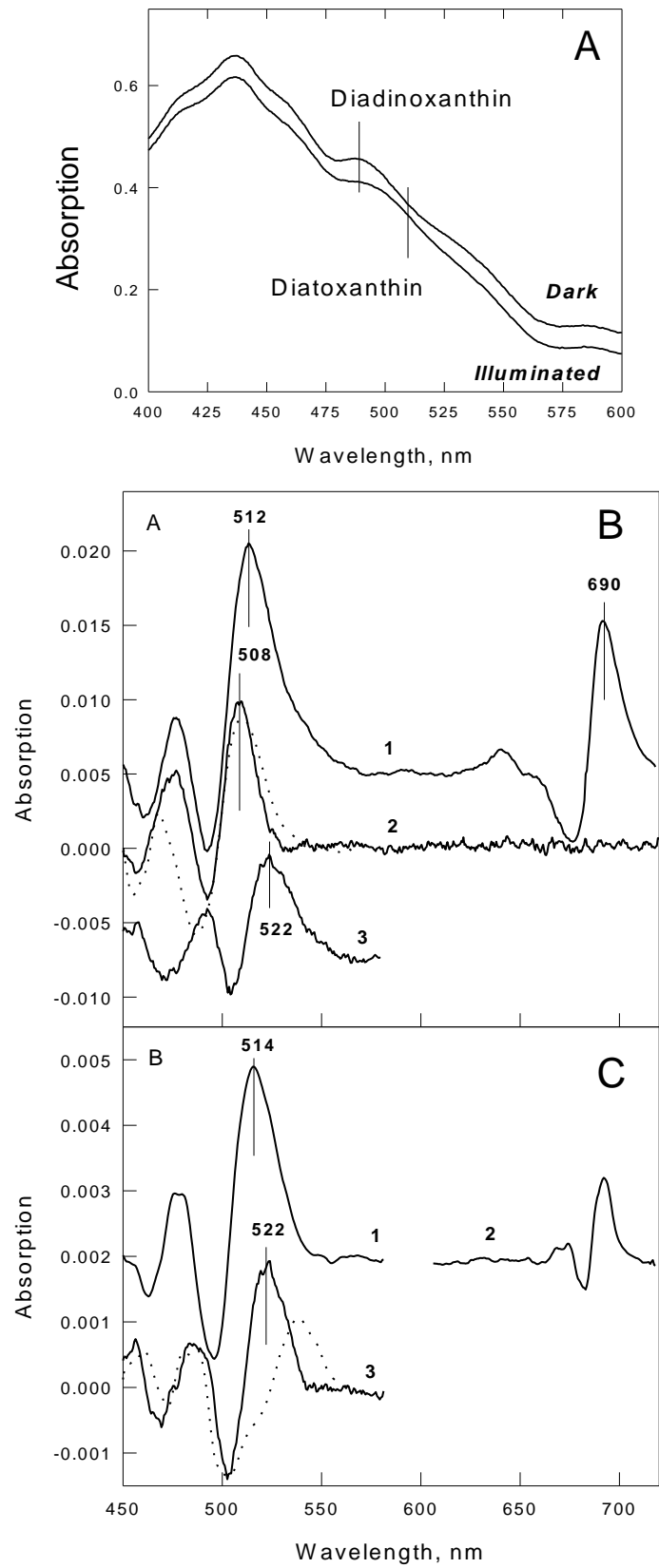
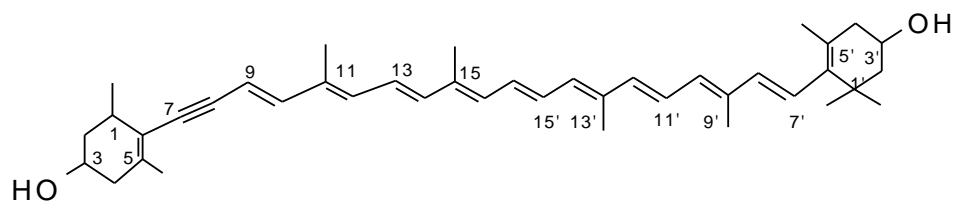
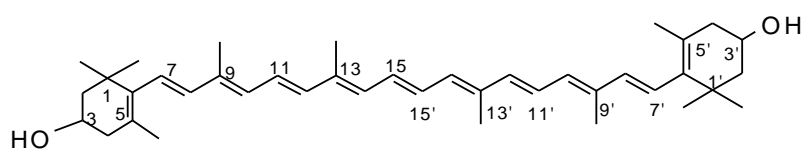


Figure 7

Zeaxanthin



Diatoxanthin

Figure 8.

