MACRO-ORGANIZATION AND STRUCTURAL FLEXIBILITY OF THE PHOTOSYNTHETIC PIGMENT SYSTEM IN DIATOMS

Ph.D. thesis

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

CD	circular dichroism
Chl *a*	chlorophyll *a*
Chl *c*	chlorophyll *c*
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DDE	diadinoxanthin-deepoxidase
Ddx	diadinoxanthin
DM	n-dodecyl-β-D-maltoside
DTT	dithiothreitol
Dtx	diatoxanthin
FCP	fucoxanthin-chlorophyll *a/c* binding protein
Fx	fucoxanthin
HPLC	high performance liquid chromatography
LD	linear dichroism
LHC	light-harvesting complex
NPQ	non-photochemical chlorophyll fluorescence quenching
PAM	pulse amplitude modulated chlorophyll fluorometer
PAR	photosynthetically active radiation
PFD	photon flux density
PSI	photosystem I
PSII	photosystem II
psi-type CD	polymerization or salt-induced circular dichroism signal
qE	energy dependent component of the non-photochemical quenching
TEM	transmission electron microscopy

1. INTRODUCTION

1.1. General introduction

The efficient collection, utilization and storage of the energy of the sunlight are essential for the existence of the terrestrial and aquatic organisms, which all are parts of the food chain and thereby the Biosphere. Photosynthesis is a process in plants, algae and certain bacteria during which the photon energy of the sunlight is transformed to chemical energy and the produced energy potential gradient creates the conditions for the formation of biomolecules necessary for energy storage in the metabolic processes on cellular and food webs on ecological levels. This organic material – in the form of biomass and fossil remains - also serves as important energy source for the mankind.

Terrestrial plants and the autotrophic algal species of the aquatic communities, the phytoplanktons, realize the photosynthetic primary production – the fixation of carbon dioxide (CO_2) to organic molecules - on the Earth. The global net primary production is about 105 petagrams (1 Pg=$1x10^{15}$ g) (Pg) of carbon annually to which marine organisms contribute by about 46% (Field et al. 1998). The phytoplankton species populate the upper layers of the open oceans or the coastal waters, the euphotic zone. This zone is well illuminated; therefore phytoplankton species can perform efficient photosynthesis if sufficient amount of nutrients is available. The biomass of the phytoplankton is less than 1% of the biomass of all photosynthesizing organisms, yet they contribute by about 45% of the global primary production. This fact can be explained not only by the efficiency of photosynthetic processes but also the very fast turnover time of the plant organic matter in oceans compared to the lands (Falkowski et al. 1998; Field et al. 1998).

In contrast to terrestrial plants, whose nearly all species belong to the group “green plants” (Embryophyta), phytoplanktons consist of various, phylogenetically diverse eukaryotic algae and prokaryotic bacteria (Falkowski et al. 2004). Diatoms (Bacillariophyta) are unicellular eukaryotic algae and are classified in the Phylum Stramenopiles, and in terms of its number of species (>10^5) it is one of the most abundant taxonomical group (Adl et al. 2005). Due to their specific “bloom and bust” lifecycle, diatoms play essential role in the regulation of atmospheric CO_2 concentration. When conditions are favorable, they start the rapid growth phase (the bloom) and their biomass becomes dominating in the phytoplankton communities. When nutrients become depleted, the diatom cells sink to the deeper layers, from which they either return to the euphotic

zone through vertical mixing and start a new bloom cycle or they irreversibly sink to the deep waters supporting thereby organic material to the deep marine organisms and sequester the fixed CO_2 in large scale from the atmosphere and euphotic zone to the deep sea (Dugdale and Wilkerson, 1998; Smetacek, 1985). The ecological success of the diatoms is also explained by the fact that they utilize silicate for the synthesis of cell wall components. Silicate is hardly utilized by other algal species, therefore in silicate rich regions diatoms can represent 70% of the phytoplankton community (Egge and Aksnes, 1992). By using silicate, diatoms synthesize unique cell wall called the frustule. The algal cells are contained within the frustules, which is split into two asymmetric valves. The morphology of the frustule is specific to each species. It is estimated, that the synthesis of the silicate-based cell wall requires less energy as compared e.g. to cellulose-based cell wall (Raven, 1983). It is also suggested that silica within the diatom cell wall is an effective pH buffering agent (Milligan and Morel, 2002).

1.2. The organization of the photosynthetic apparatus in organisms performing oxygenic photosynthesis

The photosynthetic processes of higher plants and eukaryotic algae occur in the chloroplasts. Evolutionary, the chloroplast became a plant cell organelle through an endosymbiotic event, during which a non-photosynthesizing eukaryotic organism engulfed a cyanobacterium. The chloroplasts contain the aqueous matrix called stroma and the large, expanded membrane system, the thylakoid membranes.

The photosynthetic pigment-protein complexes, the components of the photosynthetic electron transport chain and proton transport processes are embedded in the thylakoid membrane bilayers. The main components of the photosynthetic electron transport chain are photosystem II (PSII), the cytochrome b_6/f complex (cyt b_6/f), photosystem I (PSI) and the ATP-synthase. Between PSII and cyt b_6/f the mobile plastoquinone (PQ) serves as electron carrier. The electron transfer processes in the thylakoid membranes of higher plants are summarized in Fig. 1.

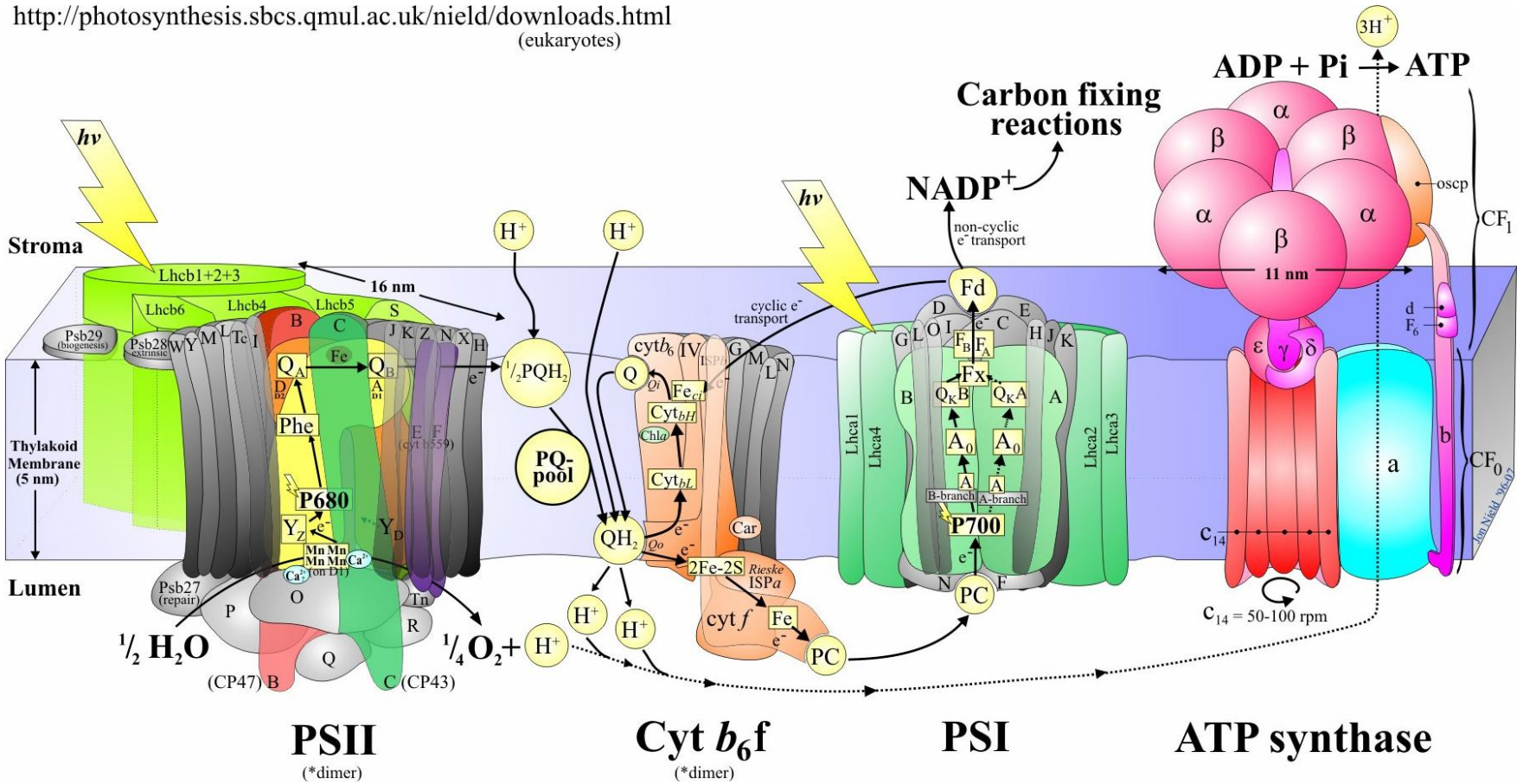

Figure 1. A detailed model of the major protein complexes involved in photosynthesis (July 2010 update) including structural information on the organisation of the protein complexes involved in electron (e^-) and proton (H^+) transport within the thylakoid membrane of green plants. The electron transport scheme is based on the literature, numerous reviews / text book figures - updated by J. Nield since 1996.

Photosystems are divided into two main structural and functional units: the antenna complexes, which bind the major parts of the light-harvesting pigments and where the light harvesting processes occur, and the core complexes, where the photochemical reactions and the initiation of the electron transport processes take place. The photons are absorbed mainly by the antenna complexes of the photosystems and the excitation energy is transferred rapidly to the reaction centers' special Chl molecules within the core complexes where the charge separation occurs. After charge separation, rapid electron-transfer processes create a stabile charge-separated state. The oxidized primary donor, the Chl^+ molecule absorbing at around 680 nm in PSII (P680) is re-reduced by a tyrosine residue, the Tyr_Z and the Tyr_Z^+ is re-reduced by the Mn-cluster of the water oxidizing complex (WOC). The most important innovation of oxygen-producing photosynthesis is that its definitive electron donor is water, the most abundant chemical compound on Earth. The electron originating from the photo-oxidation of P680 will reduce the primary electron acceptor, the pheophytine (Pheo), from which - through the primary and secondary quinone acceptor molecules, the D2 protein-bound Q_A and the D1 protein-bound Q_B, respectively - the electron is transferred to the plastoquinone (PQ) pool. The PQ pool is oxidized by the cyt b_6/f complex, which in the next step then reduces the plastocyanine (PC) or a soluble cytochrome *c* (cyt *c*) from which the electrons are transferred to PSI. In PSI, the primary photochemical reaction involves the oxidation of the Chl in the P700 reaction center thus producing $P700^+$. The liberated electron is transported towards $NADP^+$ through the primary acceptor A_0 (a Chl *a* molecule), A_1 (a phylloquinone molecule), ferredoxin (Fd) and the enzyme Fd-$NADP^+$-oxidoreductase (FNR), which then catalyzes the reduction of $NADP^+$ to NADPH. The vectorial electron transport chain is coupled to proton transfer processes, which generate a transmembrane electrochemical potential gradient.

The transmembrane subunits of the core complexes (the intrinsic proteins) of PSII are highly conserved among the photosynthesizing organisms. The main transmembrane subunits are PsbA (D1) and PsbD (D2) proteins which constitute the photochemical reaction centers and bind 6 chlorophyll *a* (Chl *a*) and 2 pheophytine (Pheo) molecules, and the PsbB (CP47) and PsbC (CP43) proteins, which serve as core light-harvesting antennae and bind 16 and 14 Chl molecules, respectively (Ferreira et al. 2004; Guskov et al. 2009). In addition, 20 membrane-spanning subunits were identified, whose function is not completely known (Guskov et al. 2009). The PSII subunits localized in the lumenal side (the extrinsic proteins) form the WOC and show large variations between the

photosynthesizing organisms. The PsbO protein ensures the activity and stability of WOC and it can be found in all organisms. The other subunits responsible for the availability of Cl^- and Ca^{2+} cofactors for the water oxidation are disperse in different taxonomical groups. The structure of PSI core complex is also highly conserved in different photosynthesizing organisms. In higher plants, PSI is a large membrane protein complex consisting of 14 subunits. The PsaA and PsaB proteins form the reaction center of PSI. Three extrinsic subunits (PsaC, PsaD, and PsaE) and several small intrinsic subunits can be found in the core of PSI. The PSI core binds about 100 Chl *a* and 20 β-carotene in higher plants (Ben-Shem et al. 2003).

The composition, structure and organization of antenna complexes of the photosystems display large variations in the photosynthesizing organisms. In higher plants, both PSI and PSII possess their own peripheral light-harvesting antenna complexes (LHCs), the LHCI and LHCII, respectively. The core complexes of the photosystems and the LHC complexes are assembled to supercomplexes. The core complexes of PSII and the attached peripheral LHCII are located in the stacked granal thylakoid membranes, while the core complexes of PSI and the attached peripheral light harvesting complexes reside in the unstacked stroma lamellae (Andersson and Anderson, 1980; Anderson et al. 2008; Chow et al. 2005; Dekker and Boekema, 2005; Garab and Mustárdy, 1999; Mustárdy and Garab, 2003).

The PSI-LHCI supercomplex consists of the PSI core and four LHCI monomers composed of Lhca1-4 polypeptides. The PSII-LHCII supercomplex consist of a dimeric PSII core, the monomeric “minor” antenna complexes, which are called Lhcb6 (CP24), Lhcb5 (CP26) and Lhcb4 (CP29), and the most abundant „major” LHCII complexes, which are trimeric and composed of various combinations of the Lhcb1-3 proteins (Dekker and Boekema 2005). The PSII-LHCII supercomplexes are also denoted as $C_2S_2M_2$ supercomplex, where C means the PSII core, S and M mean the LHCII trimers strongly or moderately bound to cores, respectively, and the subscript indicates the number of the components. The PSII-LHCII supercomplexes are assembled into megacomplexes, which probably represent the native associations of PSII in thylakoid membranes and form well ordered semi-crystalline layers (Boekema et al. 1999; Boekema et al. 2000). LHCII trimers are also arranged into LHC-only macrodomains, lacking PSII core complexes. These are usually heptamers, and probably represent a naturally occurring aggregation state of part of the LHCII trimers in the thylakoid membranes (Dekker et al. 1999).

1.3 Special features of the photosynthetic apparatus of diatoms

Diatoms evolved through secondary endosymbiotic processes, where a non-photosynthesizing eukaryote engulfed a photosynthesizing eukaryotic alga. Most probably in the case of diatoms this endosymbiont was a red algal species (Oudot-Le Secq et al. 2007). Diatom cells contain either few small, or one large chloroplast (Lavaud, 2007). The ultrastructure of chloroplasts displays several peculiar properties as compared to those of higher plants (Fig. 2).

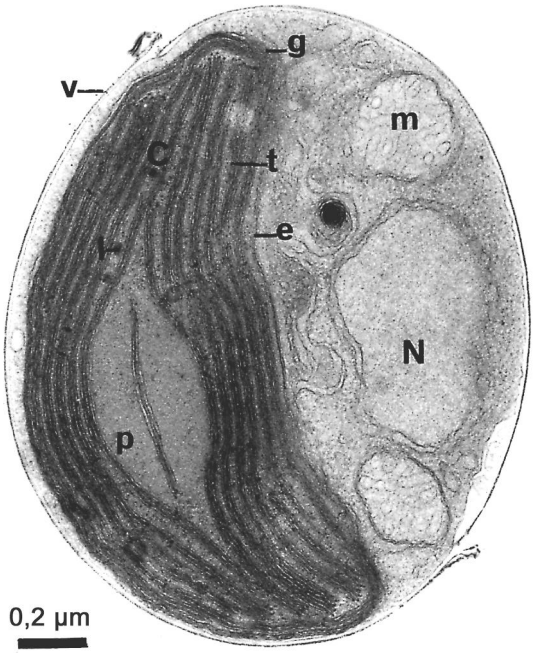

Figure 2. Electron micrograph of the diatom *Phaeodactylum tricornutum* (Lavaud et al. 2007) showing the nucleus (N), the mitochondria (m) and the cell-wall silica valves (v). The chloroplast contains bands of three thylakoids (t) surrounded by an inner 'girdle stack' of three thylakoids (g) surrounded by a four membrane envelope (e). It contains one pyrenoid (p).

The envelope membrane consists of four bilayers, which is a remnant of secondary endosymbiotic processes (Wilhelm et al. 2006). The outermost layer is tightly bound to endoplasmatic reticulum, forming thereby chloroplast-endoplasmatic reticulum (Bouck et al. 1965; Kilian et al. 2005). The thylakoid membranes of diatoms are not differentiated into granal and stromal lamellae, i.e. the granal stacking is absent. The thylakoid membranes are arranged into groups of three loosely stacked lamellae which span through

the whole length of the chloroplast (Gibbs, 1962; Pyszniak and Gibbs 1992). The thylakoid membranes within the chloroplast are surrounded by a so called girdle lamella, which also contains three loosely stacked thylakoid membranes (Gibbs, 1962; Murata et al. 1979).

The main components and organization of the electron transport chain of diatoms are highly similar to that of higher plants and are described in Chapter 1.2. However, several differences exist. PSII reaction centers isolated from diatoms contain different extrinsic proteins, the PsbU, PsbV, PsbQ` proteins and a novel protein Psb31 (whose function is not known yet), as compared to higher plants, which contain the PsbP and PsbQ proteins (Okumura et al. 2008). Diatoms do not contain PC, instead cyt *c* is the mobile electron carrier between cyt b_6/f and PSI (Böhme and Kunert, 1980, Sandmann et al. 1983). However, a more recent study reports that the diatom species *Thalassiosira oceanica* possesses the copper-containing PC (Peers et al. 2006). Beyond PsaA and PsaB, the PSI core of diatoms contains PsaE, PsaL and PsaM as specific PSI proteins (Lepetit et al. 2007; Ikeda et al. 2008). PsaM is supposed to play a role in the trimerization of the PSI complex in cyanobacteria, however the sequence similarity is only 50% (Veith et al. 2007). The polypeptide composition of the PSI core is also varying between the diatom species (Berkaloff et al. 1990; Ikeda et al. 2008). It has been shown that PSI of diatoms exists in monomeric form (Veith et al. 2007), in contrast to cyanobacteria, where PSI is trimeric.

The lateral distribution of the pigment-protein complexes in diatoms also displays several differences as compared to higher plants. In contrast to higher plants, in the diatom thylakoid membranes the distribution of PSI and PSII is homogeneous, no lateral heterogeneity can be observed; however, in some cases in the outer, stromal side the PSI is more abundant (Pyszniak and Gibbs, 1992). The organization of the light-harvesting pigment protein complexes also exhibit differences as compared to LHCs of higher plants. For diatoms, the fucoxanthin-chlorophyll protein (FCP) complexes are the main light-harvesting antennae. The distribution of the FCP complexes is also homogeneous on both the lumenal and stromal side of the thylakoid membranes (Pyszniak and Gibbs, 1992) and they transfer excitation energy equally to PSI and PSII (Owens, 1986b). For detailed description of FCP complexes, see Chapter 1.4.2 below.

1.4. Photosynthetic light-harvesting

In aquatic ecosystems the light conditions considerably differ from that of terrestrial habitats. In aquatic habitats the most important factor which exerts a strong impact of

photosynthetic efficiency is the random and large-scale changes in the incident light intensity. Certain factors, like the wave flicker effect (the sudden, large scale changes in irradiation at the air-water interface due to the focusing/defocusing effect of waves), clouds and vertical mixing concern the intensity of the irradiance, in addition, the depth of the water affects the spectral distribution of the incident light (Falkowski and Chen, 2003). All these factors affect the photoacclimation and chromatic adaptation processes of photosynthesizing organism, which may result in altered expression of LHC proteins, state transition, pigment composition and energy dissipation via xanthophyll cycle (see below).

1.4.1. Photosynthetic pigments

The first step of the photosynthetic processes is the absorption of a photon by a photosynthetic pigment. Similarly to the majority of photosynthesizing organisms, diatoms contain Chl *a*, which plays a central role in the photochemical energy conversion. Chl *a* is a magnesium containing chlorin ring, to which a phytol chain is esterified at pyrrole ring IV.

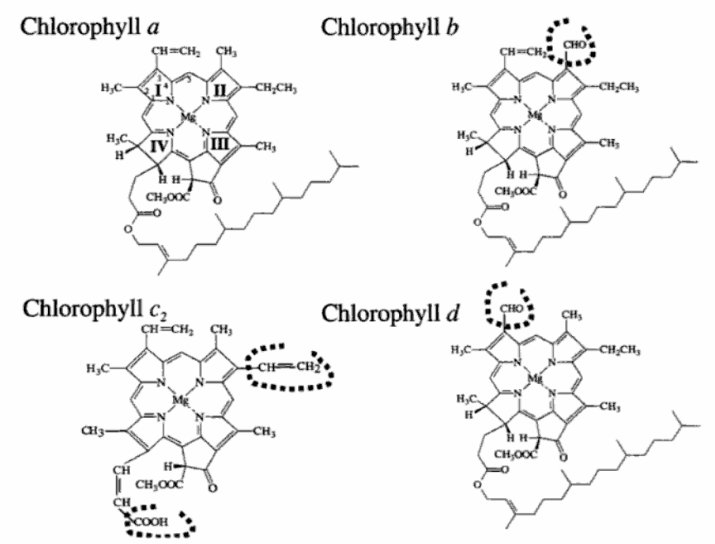

Figure 3. Chemical structure of the different chlorophylls (Larkum, 2003).

Chlorophyll *b* (Chl *b*) is the main accessory Chl in higher plants and green algae. Chl *b* differs only in one functional group from Chl *a* (Fig. 3), which confers a slightly lower lipophylic character and shifts of the major absorption bands in the red and blue towards the green. Diatoms contain chlorophyll c (Chl *c*) instead of Chl *b*. Chl *c* does not possess

aliphatic phytol chain and is based on the porphyrin ring rather than a chlorin ring. This changes the absorption spectrum to produce a strong Soret (blue) absorption band in comparison with a weak band in the red at approximately 630 nm. Different forms of Chl *c* exist; in diatoms the most abundant are Chl c_1 and c_2. These two Chl *c* forms exhibit small structural differences, Chl c_1 has an ethyl group present at position C4 in ring of the Chl macrocycle, whereas Chl c_2 has a vinyl group in this position (Larkum, 2003).

In diatoms, carotenoids play crucial role in light harvesting and in photoprotection. Carotenoids contain a linear polyene chain, with C_{2h} symmetry (Fig. 4). The conjugation length and the type of the functional groups attached to the ionone rings terminating the polyene chain largely determine the absorption properties of the carotenoids (Frank et al. 1996, Zigmantas et al. 2004). Carotenoids generally exhibit intense absorption between 400 and 500 nm. The electronic transition occurs from the S_0 energy level directly to S_2 energy level, because the S_0-S_1 transition is quantum-mechanically forbidden. S_2 state relaxes to S_1 state in subpicosecond timescale and S_1 relaxes to ground state in a nonradiative way. The main light-harvesting carotenoid in diatoms is fucoxanthin (Fx). Fx is an unusually asymmetric molecule, which possess an allene group at one of the ionone rings. This molecular structure lends to Fx unique spectral properties, absorbing light in extremely wide spectral range, e.g. between 460 and 570 nm (Goedheer, 1970; Kirk, 1977; Zigmantas et al. 2004).

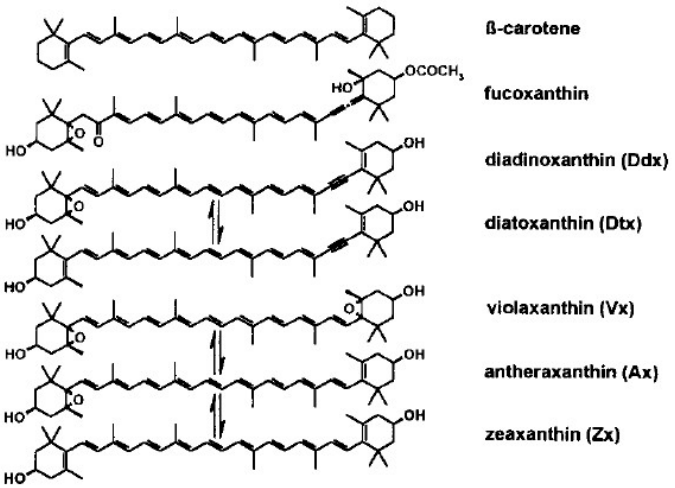

Figure 4. Structure of the main carotenoids of diatoms (Lohr et al. 1999).

Other carotenoids occurring in diatoms are the diadinoxanthin (Ddx), diatoxanthin (Dtx) and β-carotene. Ddx and Dtx are also asymmetric molecules, containing an acetylenic group at one of the ionone rings. Although Ddx plays role in light harvesting (Lavaud et al. 2003; Wilhelm et al. 1990), its significance in this process is not as crucial as Fx`s. The Ddx is a major constituent of the diadinoxanthin-cycle in diatoms together with Dtx, which plays central role in the photoprotection mechanisms. Ddx can be deepoxidized to Dtx during illumination with strong light (Stransky and Hager, 1970). In diatoms, the Ddx pool is heterogeneous. The major part of the Ddx is convertible to Dtx during the Ddx cycle. However, there is a minor fraction bound to pigment-protein complexes whose turnover is very low and thus plays no direct role in the Ddx cycle (Lohr and Wilhelm, 2001). In diatoms different xanthophylls like viola- anthera- and zeaxanthin could also be identified (Lohr et al. 1999; Lohr and Wilhelm, 2001). However, these carotenoids accumulate only under specific conditions, e.g. during illumination with strong light for long time, and they are not involved in the xanthophyll cycle. Moreover, it has been shown that violaxanthin (Vx) can be either a direct or an indirect (through the formation of Ddx) precursor of Dtx (Lavaud et al. 2004; Lohr et al. 1999, 2001; Olaizola et al. 1994,).

The amount of carotenoids relative to the amount of Chl is much higher in diatoms than in higher plants; the Fx:Chl *a* ratio is 1:1 and the Chl *a*:Fx:Chl *c* = 4:4:1 in the FCP of *Cyclotella meneghiniana* (Papagiannakis et al. 2005) in contrast to the LHCII of higher plants where the ratio of Chl *a*:lutein = 4:1 (Liu et al. 2004). Resonance Raman spectroscopy investigations revealed the presence of 5-6 Fx molecules in one FCP monomer (Premvardhan et al. 2009). Most recent resonance Raman spectroscopy investigations along with the sequence analysis of the Chl binding amino acids show the presence of 8 Chl *a*, 8 Fx and 2 Chl c_2 molecules per FCP monomer (Premvardhan et al. 2010).

1.4.2. The structure and organization of the FCP complexes

The structure of the LHCII of higher plants is known at near atomic resolution (Liu et al. 2004). In contrast to LHCII, the high-resolution molecular structure of the FCP is not determined yet. In contrast to the scarce information about the structure, the genes encoding the individual polypeptides are characterized thoroughly. The fcp genes were shown to display high sequence homology to lhc genes, therefore the fcp genes belong to the lhc superfamily (Bhaya and Grossmann 1993; Eppard and Rhiel 1998). They are

classified into four major groups. The first group represents the genes encoding the "major" FCP proteins; these are the fcp1-5 in *Cyclotella cryptica* and fcpA-F in *Phaeodactylum tricornutum*. The genes classified into the second group show strong homology to lhca genes encoding the LHC I proteins in red algae. This gene is the fcp4 in *C. cryptica* and *Thalassiosira pseudonana* (Armbrust et al. 2004; Eppard et al. 2000a). The third group represents those genes, which show strong homology to genes encoding the LI818 protein, which is a light-inducible protein in *Chlamydomonas reinhardtii* and belongs to lhc gene superfamily. Fcp6, 7 and 12 in *C. cryptica* belong to this group (Eppard and Rhiel, 2000b). Genes belonging to the fourth group display high sequence similarity to the ELIP (early light inducible proteins) family; these proteins are also members of the LHC superfamily and their expressions are upregulated during strong light illumination. Therefore, they probably play a role in the photoprotection mechanisms activated by excess irradiation. From polypeptide sequence analysis of the FCP complex, three transmembrane helices were predicted (Eppard and Rhiel, 1998). In helices 1 and 3 high homology was found to LHC polypeptides (Green and Durnford, 1996). The FCPs are in general smaller than the proteins of LHC of higher plants; the molecular weight of FCP polypeptides is in the range of 17-23 kDa (Caron et al. 1988; Friedman and Alberte, 1987; Fawley and Grossmann, 1986).

The model of the FCP complex of diatoms is depicted on Fig. 5.

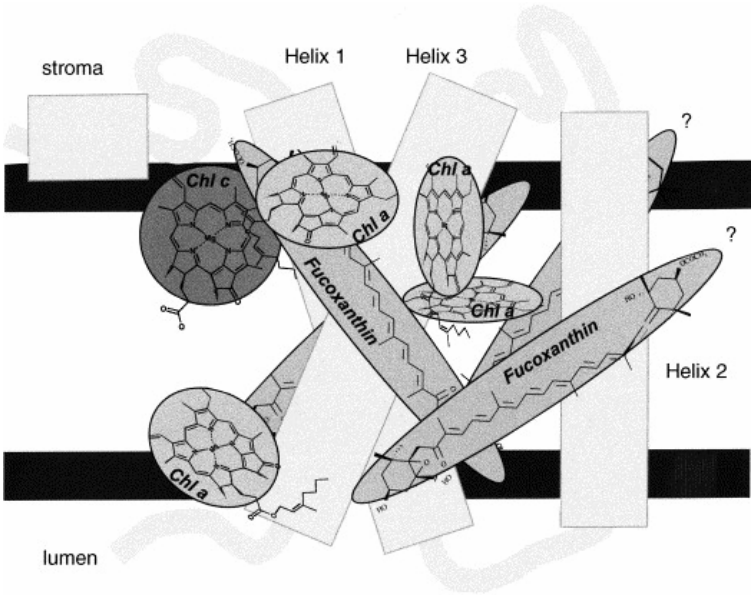

Figure 5. The model of the FCP complex (Wilhelm et al. 2006).

Similarly to the Chl *a* and Chl *b* binding sites, which are conserved in different species of higher plants and green algae, the Chl *c* binding sites are conserved in large extent between different algal species (Eppard and Rhiel 1998). The localization of Chl molecules within the FCP complexes of diatoms has been mapped: the fact that the excitation energy transfer between Chl *c* and Chl *a* is nearly 100% indicate that Chl *c* molecules are arranged in close vicinity of Chl *a* molecules (Papagiannakis et al. 2005; Premvardhan et al. 2010). The Chl *a* and Chl *c* molecules are localized on helices 1 and 3 (Eppard and Rhiel, 1998). Less information is available on the localization of the carotenoid molecules. Two of the eight Fx molecules are supposed to be similarly arranged as the central luteins in LHCII, in the close vicinity of the Chl *a* molecules, and probably play a role in stabilizing the FCP complex (Premvardhan et al 2010). The same arrangement was found in brown algae (Pascal et al. 1998). The localization of the other Fx molecules is not determined yet; it is speculated that they are situated at the periphery of the complex, close to helix 2 (Premvardhan et al. 2010). The heterogeneity of the Fx molecules within FCP complexes is also corroborated by Stark spectroscopy measurements of isolated FCPs (Premvardhan et al. 2008). Recently, Raman spectroscopy measurements revealed the structure of Fx molecules in FCP complexes; it was found that Fx molecules possess typical planar all-trans configuration in the protein. Some of the Fx molecules exhibited specific, elongated S-shaped planar configuration which is assumed to favour energy transfer efficiency (Premvardhan et al. 2009).

Information on the supramolecular organization of the FCP complexes is scarce. Earlier studies concluded that the two photosystems in diatoms share a common FCP antenna system (Berkaloff et al. 1990; Owens and Wold, 1986a). Recent works suggest however, that there are certain Fcp polypeptides, which are bound specifically to either PSI or PSII. Fcp4 was found to be associated with PSI (Eppard and Rhiel, 2000b), while the Fcp2 and Fcp6 polypeptides were found exclusively in PSII fraction (Veith et al. 2009). Other works reported that both Fcp2 and Fcp4 could be found in both photosystems in *C. cryptica* (Brakemann et al. 2006). It has also been shown that PSI forms a large supercomplex together with FCP: a monomeric PSI RC binds about 25 FCPs (Ikeda et al. 2008). These data hence provide important but far not complete information about the localization of FCP in diatoms.

In brown algae, oligomeric FCP complexes with molecular masses of 120-380 kDa have been isolated (Berkaloff et al. 1983; Gugliemelli, 1984; Katoh and Ehara, 1990). Büchel (2003) reported the presence of two different Fcp fractions in *C. meneghiniana*,

which were different in molecular weight and oligomeric status. Furthermore, Beer et al. (2006) identified the subunits of the higher oligomer FCPb and the trimer FCPa in *C. meneghiniana*. FCPa possesses subunits encoded by fcp1-3 and fcp 6/7, while FCPb is composed of the fcp5 gene products. Both FCPa and FCPb were intact with regard to excitation energy transfer to Chl *a* (Beer et al. 2006). Earlier works reported isolation of one major and one minor Chl *a/c* antenna fraction (Owens and Wold, 1986a). Recently, FCP sub-fractions have also been isolated from different species exhibiting various pigment compositions. One fraction is termed LHCF-F and contains Chl *a/c* and Fx but lacks Ddx, while the other fraction is termed LHCF-D and contains Chl *a*, Fx and Ddx but is nearly depleted in Chl *c*. While LHCF-F contains polypeptides in the range of FCPs, LHCF-D shows smaller polypeptides (10-15 kDa) in addition to FCPs. A similar DD enriched sub-fraction has been reported in *C. meneghiniana* (Beer et al. 2006), this complex shows a high degree of de-epoxidation under high light exposure.

1.5. Photoprotection mechanisms and operation of the diadinoxanthin-cycle

Upon absorption of light, Chl *a* molecules of the light-harvesting antenna system become excited to the singlet-state $^1Chl^*$, the energy of which can be de-excited to ground state in several pathways (Fig. 6).

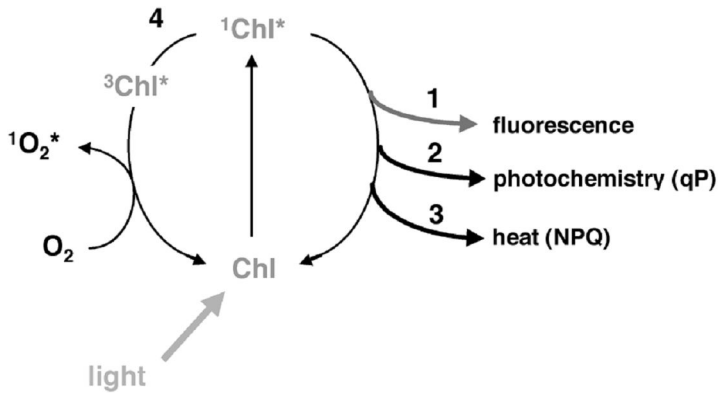

Figure 6. Possible fates of excited Chl. When Chl absorbs light it is excited from its ground state to its singlet excited state, $^1Chl^*$. From there it has several ways to relax back to the ground state. It can relax by emitting light, seen as fluorescence (1). Its excitation can be used to fuel photosynthetic reactions (2), or it can de-excite by dissipating heat (3); both of these mechanisms reduce the amount of fluorescence. They are therefore referred to as photochemical quenching (qP) and non-photochemical quenching (NPQ) of Chl fluorescence. Last, $^1Chl^*$ can, by intersystem crossing, produce $^3Chl^*$ (4), which in turn is able to produce $^1O_2{}^*$, a very reactive oxygen species (Müller et al. 2001).

Upon increasing ambient light intensity, at a point photochemistry attains a steady state level and becomes light-saturated. Further increase in the light intensity will cause excess excitation pressure on the reaction center Chl-s, the probability of the formation of $^3Chl^*$ increases and the yield of Chl *a* fluorescence, originating from PSII also increases. The existence of $^3Chl^*$ can be critical for plants, because it is able to transfer energy to ground state O_2 to generate singlet oxygen ($^1O_2{}^*$), which is a very harmful reactive oxygen species (ROS) and is able to damage lipids, proteins etc., causing thereby a decrease in the rate of photosynthesis. Photosynthesizing organisms are able to keep the formation of $^3Chl^*$ at low level through several fast photoprotective mechanisms. The quenching of Chl *a* fluorescence is one of the most important mechanism and can be performed by the operation of the photochemical reactions (qP, photochemical quenching). However, at light intensities, which are beyond the capacity of photochemical quenching (i.e. the capacity of the photosynthetic electron transport chain) other, non-photochemical quenching (NPQ) processes dominate the photoprotection. In higher plants and green algae, NPQ can be divided into three components (Horton et al. 1996; Müller et al. 2001): i) qE, energy-dependent quenching, which is regulated by the build-up of a transthylakoid ΔpH and the operation of the xanthophyll cycle, ii) qT, the state-transition quenching, which allows reallocation of part of the energy absorbed from PSII to PSI and iii) qI, the photoinhibitory quenching.

In diatoms, the qE component should play the main role in NPQ, since for qT no evidence could be found (Owens, 1986b). Recently, it was observed that the qI component of NPQ increased significantly upon prolonged (>60 min) light-stress in *T. pseudonana* suggesting the existence of a photoinhibitory-quenching component in diatoms (Zhu and Green 2010). The NPQ mechanisms are partially mediated by the xanthophyll cycle (XC). During the operation of the XC, enzymatic deepoxidation/epoxidation of xanthophyll carotenoids as a function of light intensity occurs. In higher plants and green algae, the XC is called violaxanthin-antheraxanthin-zeaxanthin (VAZ) cycle. The cycle consists of inter-conversion of three xanthophylls, the di-epoxide violaxanthin (Vx), the mono-epoxide antheraxanthin (Ax) and the de-epoxidized zeaxanthin (Zx). The operation and regulation of VAZ cycle is known in details in higher plants and green algae and is reviewed in several papers (e.g. Yamamoto et al. 2004; Lavaud, 2007). The de-epoxidation/epoxidation events are performed by two enzymes, a Vx de-epoxidase (VDE) and a Zx epoxidase (ZEP). VDE is located on the lumenal side and its optimal activity is at around pH 5-6, thus it is activated when the thylakoid lumen becomes acidic due to the build-up of the

proton gradient. ZEP is localized on the stroma side, its pH optimum is at around 7.5. VDE requires the acidic form of ascorbate while ZEP requires the NADPH, H^+ and O_2 as cofactors. The direction and activity of the two competing enzymatic reactions of the VAZ cycle depends on the build-up of the transthylakoid proton gradient and subsequent change in the lumenal and stromal pH. When the light intensity decreases, the de-epoxidation becomes weaker and finally stops, while the inverse epoxidation reaction becomes dominant. Hence, the accumulation of the de-epoxidized xanthophylls Zx depends on the activity of the two enzymes, which indirectly depends (via the change in pH and availability of the cofactors) on the light intensity.

Diatoms possess the diadinoxanthin-cycle, where Ddx is deepoxidized in one step to Dtx by the enzyme diadinoxanthin-deepoxidase (DDE), while Dtx can be epoxidized back to Ddx also in one step by Dtx epoxidase (DEP). The comparison of the VAZ of higher plants and Ddx cycle of diatoms is depicted in Fig. 7.

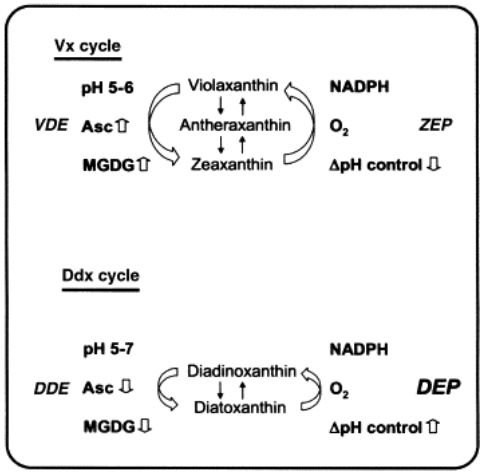

Figure 7. Comparison of the VAZ and Ddx cycle (Wilhelm et al. 2006).

The regulation of the Ddx cycle displays several differences compared to VAZ cycle. The pH optimum for DDE is significantly higher (Jakob and Wilhelm, 2001), which means that already a weak lumen acidification is able to induce the DDE enzyme. It also has been shown that – compared to VDE - DDE requires lower ascorbate concentrations (Grouneva et al. 2006) and lower monogalactosyl-diacylglycerol (MGDG) concentrations (Goss et al. 2005) for the full activity of Ddx de-epoxidation. The DEP enzyme requires the same cofactors as ZEP, but the regulation of the epoxidation reaction also displays peculiar characteristics – it is fully inactivated during strong light illumination, therefore no competitive epoxidation reaction under excess light conditions occurs (Goss et al. 2006).

This ΔpH "lock" ensures the fast and efficient deepoxidation of Ddx. Other unusual property of the epoxidation reaction is that it is not functional in complete darkness, but very efficient and rapid on low light intensities (Goss et al. 2006; Mewes and Richter, 2002). The impairment of the epoxidation reaction is thought to occur due to shortage of NADPH (Goss et al. 2006), which is a consequence of different regulation mechanisms of alternative electron transport pathways and chlororespiration in different diatom species (Grouneva et al. 2009).

The correlation between qE and the XC has been studied thoroughly in higher plants and green algae. In diatoms, correlation could also be found between qE and Ddx de-epoxidation (Lavaud, 2002a; Olaizola et al. 1994; Ting and Owens, 1993). Here the values for qE are 4-5 times higher as compared to those measured in higher plants (Lavaud et al. 2002; Ruban et al. 2004). The qE seems to be more tightly associated with the presence of the de-epoxidized xanthophyll, the Dtx compared to Zx in higher plants (Lavaud et al. 2002; Ruban et al. 2004). However, recently more qE components could be identified, which were not strictly dependent on the presence of Dtx but rather on the presence of ΔpH (Grouneva et al. 2008; Lavaud et al. 2006).

qE regulation depends on many factors and proceeds within the thylakoid membrane. XC plays a central, albeit not an exclusive role in the formation of NPQ. The recent allosteric conformational change model provides explanation about the relationship of XC and the conformational change of LHCII in the kinetics of the NPQ in higher plants (Horton et al. 2008). In diatoms, the correlation between conformational changes of FCP complexes and the quenching of fluorescence is far less understood. It has been estimated that in *C. meneghiniana* the FCPa and FCPb complexes exhibit significant fluorescence quenching which was dependent on ΔpH but only if the complexes were aggregated *in vitro* (Gundermann and Büchel, 2008). Thus, the relationship of fluorescence quenching and the state of aggregation of the FCP *in vivo* still remains to be clarified.

1.6. Principles of circular dichroism (CD) spectroscopy

CD spectroscopy is a non-invasive method for investigating pigment-pigment interaction even in as highly complex systems as intact cells *in vivo*. CD is the extinction difference of the left and right circularly polarized light at a given wavelength, which is

composed of differential absorption (A_L-A_R) and (if present) differential light scattering (S_L-S_R) of the sample and can be described as

$$CD = (A_L - A_R) + (S_L - S_R)$$

CD signals originate from the structural asymmetry (more precisely the chirality) or from asymmetric interactions of the molecules (Woody, 1985).

CD signals can originate from molecular systems of different complexity. In Chl containing systems, the physical origin of CD signals was reviewed by Garab (1996) and Garab and Amerongen (2009).

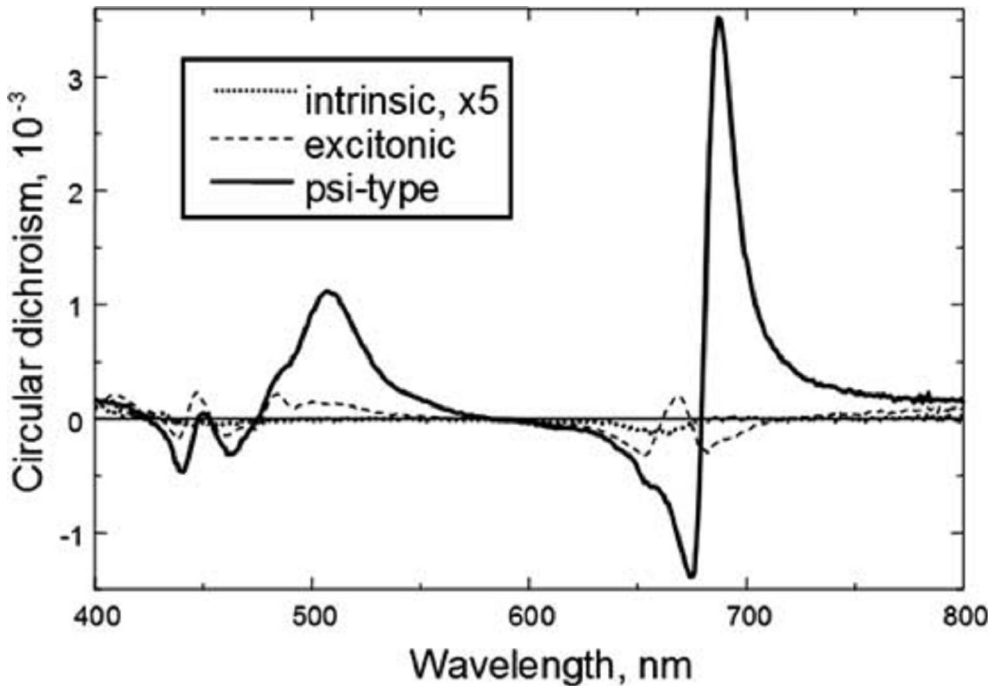

Figure 8. Circular-dichroism spectra exhibited by the thylakoid pigments at different levels of structural hierarchy. The acetonic extract - yielding intrinsic CD (for easier comparison, the signal is multiplied by a factor 5), pea thylakoid membranes suspended in low salt, hypotonic medium dominated by the sum of the excitonic bands, and the same membranes suspended in isotonic medium in the presence of Mg ions (Garab and Amerongen, 2009).

Chiral molecules exhibit optical activity, called intrinsic CD. It can be described by the rotational strength of the given electronic transition, which is the scalar product of the electric and magnetic dipole moments. Chlorophylls are planar ring-structured molecules; therefore their electric and magnetic dipole moments are nearly perpendicular to each

other, which results weak CD signals. The band shapes of the intrinsic CD are identical with those of the absorption bands; however, their sign can be positive or negative, determined by the handedness of the molecule. In photosynthetic systems, the intrinsic CD signals are weak, because of the symmetry of the pigment molecules; for 1 absorbance unit, it is in the range of some 10^{-5} intensities (cf. Fig. 8).

When chlorophylls are parts of a complex system it can happen that certain charged or aromatic amino acids of neighboring proteins interact with them inducing e.g. twisting in the chlorophyll molecule. In this case, the intensity and sign of the CD signal may deviate from the intrinsic CD to a large extent and this makes the interpretation of CD signals difficult. This induced chirality is rare in tetrapyrrole molecules, but it has been observed e.g. in Chl *a/c*-containing organisms (Büchel and Garab, 1997).

In the case of excitonic interaction of two or more molecules – when they are close enough to each other for dipole-dipole interaction, but still sufficiently apart for the electrons remaining localized on each of the molecules – a conservative band structure can be observed. That means that a positive and negative CD band can be observed the areas of which gives zero in the energy spectrum (de Voe, 1965). This is called exciton-coupled CD signal or excitonic CD. The excitonic CD originates from the fact that the polarization of the light changes while passing (through) the excitonically interacting molecules, which have a fixed position and orientation with respect to each other. In photosynthetic systems the magnitude of absorption is typically an order of magnitude higher than the intrinsic CD of the same pigment molecules. Excitonic CD can be observed in pigment-protein complexes or small Chl aggregates, where the pigments are localized close to each other, therefore are capable of participating in short-range dipole-dipole interactions. Excitonic CD is often used for fingerprinting isolated pigment-protein complexes and carry information on pigment-pigment interactions. However, due to the complexity of the analysis of CD spectra only a few detailed models are available which interpret CD in terms of high resolution molecular structures. Recently, such model was presented on isolated LHCII trimers and monomers (Georgakopoulou et al. 2007).

In aggregates with sizes commeasurable with the wavelength of the visible light and with high chromophore density, the intensity and wavelength positions of the conservative excitonic bands may change and new “anomalous” CD bands with non-conservative band structure appear which means that the intensity of the negative or positive component of a bandpair can vary independently from each other. These signals are called psi (polymer or salt-induced) type bands since they were originally observed in complex macromolecules

and aggregates e.g. in condensed chromatin and DNA molecules. In aggregates, exhibiting psi-type CD not only the short-range dipole-dipole interactions, but also the long-range, radiative couplings have to be taken into account. It is true that in small aggregates the excitonic interactions are stronger than the radiative and intermedier interactions, but the strength display r^{-3} dependence (r is the distance between the dipoles), while the radiation and intermediate coupling mechanisms between the dipoles exhibit r^{-1} and r^{-2} dependencies, respectively; therefore these latter interactions are much more effective for large distances than the dipole-dipole interactions. Psi-type CD can also be observed in non-absorbing regions, which originate from circular differential scattering and thus can provide useful information about the size of the aggregate (Garab et al. 1988a). The intensity of the psi-type bands depends on the extent of the long-range chiral order, the domain size and the direction of the chiral order (handedness) (Keller and Bustamante, 1986; Kim et al. 1986). Photosynthetic pigments are able to form large chiral aggregates, more precisely, chiral macrodomains exhibiting intense psi-type CD signals (Fig. 8. bold line), which are given rise by the presence of long-range chiral organization or macro-organization of pigments, possibly extending the pigment interactions to the thylakoid membrane system.

1.7. Macro-organization of the light-harvesting antenna system

Granal thylakoid membranes contain the majority of PSII-LHCII supercomplexes. It has been shown by electron-microscopic studies that PSII-LHCII supercomplexes are arranged into well ordered semi-crystalline layers, which are proposed to be the native organization of PSII in higher plants (Boekema et al. 1999, 2000). In LHCII the density of the chromophore is high and the existence of the LHCII trimers in ordered layers gives chance for strong pigment-pigment interactions and extended aggregates, called chiral macroaggregates or macrodomains. These large aggregates can serve the basis for long-distance migration of the excitation energy, which might be important in energy supply for the reaction centers and its down-regulation via nonphotochemical quenching (Garab and van Amerongen, 2009). By using polarized light spectroscopic – mainly CD - and microscopic methods it has been shown that in granal thylakoid membranes the pigments are arranged in densely packed chirally ordered, large (200-400 nm in diameter) macrodomains exhibiting large psi-type CD signals (for detailed explanation for physical

origin of CD signals see Chapter 1.6) (Barzda et al. 1994; Finzi et al. 1989; Garab et al. 1988a).

The role of PSII-LHCII in macrodomain organization could be revealed by using antenna mutants; it has been shown that the absence of minor antenna complex CP24 is accompanied by the decrease or even the disappearance of psi-type CD signal (Kovács et al. 2006). Moreover, it was found that the amount of PsbS protein – which is an important regulator in the interaction of LHCII with PSII in granal thylakoid membranes – correlated with the psi-type CD signal (Kiss et al. 2008). Psi-type CD signal can be found not only in granal thylakoid membranes but also in tightly stacked lamellar aggregates of isolated LHCIIs, which possess well defined three-dimensional structure. In contrast, disordered aggregates without well-defined structure display no intense psi-type CD bands (Simidjiev et al. 1997).

In higher plants, structural flexibility of the photosynthetic thylakoid membranes and pigment-protein complexes are important in short-term acclimation to environmental changes, such as temperature and/or illumination (Anderson and Andersson, 1988). The macro-organization level of hierarchic assemblies like granal thylakoid membranes react most readily to perturbations; this might be important for adjusting the functions without significantly altering the structure and composition of the constituents (Garab and van Amerongen, 2009).

The chirally organized macrodomains of the photosynthetic membranes have been shown to undergo gross (up to 80-90%) light induced reversible structural changes, which can be detected in the major psi-type CD bands. This suggests that pigments not only display long-range interactions but the macrodomains with long-range order also possess the ability for fast, large-scale but reversible structural transitions. These structural modifications were extensively investigated in higher plant species in the past decades. Isolated thylakoid membranes exhibit pronounced light-induced CD changes, which were correlated with the functionality of linear electron transport chain (Gregory, 1975; Faludi-Dániel et al. 1984). However, later studies explored that the light-induced changes are largely independent from the photochemical activity of the thylakoid membranes, although they were sensitive to the inhibitors of qE component of non-photochemical quenching (Istokovics et al. 1997). This finding is also in accordance with the fact that the amplitude of light-induced CD changes exhibited linear correlation with the light intensity, even above the intensities, which saturated the linear electron transport (Barzda et al. 1996). Moreover, it has been shown that light-induced CD changes could be observed not only in

isolated thylakoids but also in aggregated LHCII preparations (Barzda et al. 1996). Illumination with strong light affected the chiral macrodomains, as it was shown by the reversible decrease of the intensity of the psi-type CD signals**.**

It has been shown that the chirally organized macrodomains in isolated thylakoid membranes are also susceptible to elevated temperatures. This can be concluded from the decrease of the psi-type CD signals between 40 and 50 °C, while the excitonic CD signals persist even at 65-70 °C. These data have revealed that the thermal stability of the chiral macrodomains is considerably lower than that of the pigment- protein complexes (Cseh et al. 2000). Thylakoid membranes preilluminated with strong actinic light exhibited even higher sensitivity: the psi-type signal at 689 nm decreased already at around 30 °C. In contrast, the excitonic interactions were essentially not affected by preillumination, suggesting that the majority of pigment-protein complexes suffered no detectable light-induced changes.

For explaining the above data, a biological thermo-optic effect was suggested. According to this hypothesis, fast local thermal transients (local T-jumps) occur due to dissipated excitation energy, which cause elementary structural changes. These changes can exist if we assume that the thermal instability is inherent, „built-in" in the structure. The mechanism of the heat dissipation was revealed experimentally by ultrafast transient spectroscopy. Local heating in LHCII trimers was induced by exciton-exciton annihilation resulting in a fast nonradiative relaxation with a simultaneous conversion of the excitation energy into heat; the time course of the heat transient was monitored by recording the ultrafast changes in the absorbance at 684 nm (Gulbinas et al. 2006). Thermal transients can readily induce structural changes in intact thylakoids between 45 and 55 °C (Cseh et al. 2000). It has also been shown that thermo-optic mechanism plays role in light-induced monomerization of LHCII trimers not only in isolated complexes, but also in isolated thylakoid membranes and whole leaves (Garab et al. 2002).

It was found that by increasing the sorbitol concentration in the suspension of isolated thylakoid membranes the amplitudes of the 688 nm and 515 nm CD bands increase, while the CD band at 674 nm was sensitive only to little extent. However, by increasing the concentration of Mg^{2+} ions, the intensity of the 674 nm band became larger, while the CD bands at 688 and 515 nm were essentially insensitive to this treatment. Based on these results, it was concluded that the organization of the macrodomains is regulated by two main external factors: i) the screening of divalent cations which facilitates the stacking of

the membranes and ii) the osmotic pressure of the medium which influence the lateral packing density of the chromophores (Garab et al. 1991).

All of these structural modifications upon changes in the environmental factors indicate that flexibility of the photosynthetic membranes plays an important role in fine adjusting the photosynthetic light harvesting functions. In organisms (e.g. in diatoms), which experience extreme and sudden changes in the environmental conditions the structural flexibility is thought to be crucial in the regulation of the photoprotective mechanisms. The investigation of the changes on structural and supramolecular level are particularly important to obtain a complete picture about the protective mechanisms against surplus light in diatoms together with the previous knowledge gained by characterization of e.g. electron transfer processes and quenching of excess excitation energy.

2. AIMS

Diatoms are one of the most abundant taxonomical group of algae, which play essential role in the regulation of atmospheric CO_2 concentration. Diatoms in their natural habitat are well known for their ability to survive large fluctuations in light intensity, e.g. in mixing waters, and thus evolved short-term light adaptation mechanisms to fine-tune their photosynthetic functions to rapidly changing conditions. This evidently requires a highly organized but structurally flexible light harvesting antenna system. This motivated us to obtain detailed information on the structure and flexibility of the pigment system on different hierarchical levels, particularly on the macro-organization.

It has been well established that in higher plants and green algae the pigment-protein complexes are arranged into structurally flexible chirally organized macrodomains, and similar macro-organizations have been identified in some Chl *a/b/c*- and Chl *a/c*-containing organisms. However, the macrodomain organization and structural flexibility of pigment-protein complexes of diatoms, have not been investigated before.

Therefore our main goal was to obtain information about the molecular and supramolecular organization of the light-harvesting system of diatoms, and to characterize their structural flexibility.

During my PhD work, the following aims and goals were addressed:

I. To conduct systematic study on the (macro-)organization of the pigment-protein complexes in *P. tricornutum* and *C. meneghiniana* at different levels of structural complexity. By using mainly CD spectroscopy, my aim was to provide information about the pigment-pigment interactions in isolated complexes and thylakoid membranes and about the macro-organization of the complexes, i.e., about the chiral macrodomains in whole cells.

II. To examine the structural stability and flexibility of the pigment-protein complexes in thylakoid membranes by measuring their thermal and light stabilities, as well as the ability of the chiral macrodomains to undergo light and temperature induced reorganizations; further, to examine the effect of changes in the ionic strength and osmotic pressure of the medium on intact cells and isolated thylakoid membranes. It was also the aim of the present work to establish correlations of these reorganizations with functional parameters, such as the maximum quantum yield of

PSII (measured as F_v/F_m), the non-photochemical quenching (NPQ) of the Chl *a* fluorescence, and the Ddx cycle.

III. To gain information about the orientation and local environments of carotenoids, especially of the Fx molecules, by analyzing the flash-induced electrochromic absorbance changes of intact *P. tricornutum* and *C. meneghiniana* cells. Further, to investigate the structural and functional heterogeneity of Fx molecules and of the FCPs by using linear dichroism, low temperature fluorescence excitation and emission spectroscopy and different growth conditions, in order to study the role of heterogeneity in excitation energy supply to the two photosystems.

3. MATERIALS AND METHODS

3.1. Sample preparation

3.1.1. Culturing the diatom species

The diatom *Phaeodactylum tricornutum* (SAG, 1090-1a, Göttingen) was grown in ASP-2 medium according to Provasoli (1957).

The composition of the ASP-2 medium, pH=7.7:

$K_2HPO_4*3H_2O$	100 mg/l
H_3BO_3	10 mg/l
$MgSO_4*7H_2O$	2000 mg/l
KCl	1600 mg/l
$NaNO_3$	1000 mg/l
TRIS	1000 mg/l
NaCl	5000 mg/l
$CaCl_2$	400 mg/l
Na-EDTA	30.2 mg/l

Trace elements:

$FeCl_3*6H_2O$	3.3 mg/l
$CoCl_2*6H_2O$	0.012 mg/l
$CuCl_2*H_2O$	0.003 mg/l
$Na_2MoO_4*H_2O$	0.006 mg/l
$ZnCl_2$	0.3 mg/l
$MnCl_2*2H_2O$	3.3 mg/l

Cyclotella meneghiniana (SAG, 1020-1a) was also grown in ASP-2 medium (Provasoli, 1957) but here the medium contained in addition 212 mg/l $Na_2SiO_3 * 5H_2O$.

Diatoms were grown in 250-1000 ml Erlenmeyer flasks containing 100-400 ml ASP-2 medium in sterile batch cultures by applying continuous shaking with 100-110 rpm. Illumination was provided by using fluorescent tubes (TL-D 18W/33-640, PHILIPS) with a photon flux density (PFD) of 40 μmol photons m^{-2} s^{-1} photosynthetically active radiation (PAR) („normal light") in a dark/light period of 16 h/8 h. For certain experiments cells were also grown on either low light (LL) or high light (HL) intensities with PFD values of 10-15 and 180-200 μmol photons m^{-2} s^{-1}, respectively. When growing HL cells, a halogen lamp (PAR 38 230V 120W, Tungsram) was used. The temperature of the growth chamber was set to 19 °C.

3.1.2. Isolation of thylakoid membranes and pigment-protein complexes

The following buffers were applied for the isolation of thylakoid membranes and pigment-protein complexes:

Buffer „A":
10 mM MES, pH=6.5
2 mM KCl
5 mM Na-EDTA
1 M Sorbitol

Buffer „B":
10 mM MES, pH=6.5
2 mM KCl
5 mM Na-EDTA

Depending on the experiment, the buffers also contained 1-25 mM $MgCl_2$. Before isolation, the cells were adapted to low light intensities (~10 μmol photons m^{-2} s^{-1}) for about 30 min. Cells from the exponential growth phase with chlorophyll concentration of 4-5 μg/ml were harvested by centrifugation (4.000 *g*, 5 min). The following steps were carried out at 4 °C in dim light. The pelleted cells were resuspended in 20-25 ml isolation buffer 'A', then disrupted in a French pressure cell (Thermo Scientific) at a pressure of 12.500 psi ($8.62x10^7$ Pa), which was sufficient for breaking up about 90% of the cells.

Unbroken cells were pelleted by centrifugation (1.000 *g*, 10 min) and resuspended in about 20 ml of isolation buffer 'A' again. The suspension was subjected to the French-press for a second time using the same conditions. After centrifugation (1.000 *g*, 10 min) the supernatant was merged with the supernatant of the first centrifugation and centrifuged at 40.000 *g* for 20 min. The pelleted thylakoids were resuspended in 1-5 ml of isolation buffer 'B'.

For solubilization of the membranes, equal amounts of the isolated thylakoids, corresponding to 0.5-1 mg Chl, were centrifuged at 40.000 *g* for 20 min. The pelleted thylakoid membranes were solubilized in n-dodecyl β-D-maltoside (DM). The detergent from the 10% stock (w/v) was added to the membrane fraction with the ratio of DM/Chl=20:1. The solubilization was performed in complete darkness on ice bath for 20 min by using continuous stirring. The solubilized membranes were centrifuged at 40.000 *g* for 20 min and the supernatant was loaded onto a continuous sucrose gradient (0 to 0.7 M sucrose, supplemented with 0.03 % DM). Separation was carried out by ultracentrifugation (Sorvall, UltraPro 80) using a swing-out rotor (Sorvall, TH-641) at 110.000 *g* for 18 h at 4 ºC.

3.1.3 Preparation of sucrose density gradient

Continuous sucrose gradient was prepared by using a gradient mixer peristaltic pump. To prepare 11 ml gradient, 5.5 ml buffer „B" containing 0.03% (v/v) DM was pipetted into the front tube and 5.5 ml buffer „B" containing 0.7 M sucrose and 0.03% (v/v) DM was pipetted into the rear tube. The connection between the two tubes was blocked by a valve during the filling up process. After starting the peristaltic pump the valve was opened which allowed the mixing of the two solutions. To provide homogeneous mixing, magnetic stirrer was applied in the front tube. The gradient was prepared in ultracentrifuge tubes.

3.1.4. Breaking up the intact cells by using ultrasound

Sonication of intact cells was performed in a Branson Sonifier 450 on ice with a 10 s sonication – 30 s cooling cycle. The same sample was sonicated for different time periods in the range of 10 s – 180 s in ASP-2 medium.

3.1.5. Determination of the chlorophyll content

The chlorophyll content was determined spectrophotometrically in acetonic pigment-extracts. 3-5 ml cell suspension was centrifuged (4.000 g, 5 min). The pellet was resuspended in 90% acetone and in the presence of glass beads it was homogenized for 2x1 min at 4 ºC with a homogenizer (Mini-Beadbeater^{TM}, Biospec Products). The homogenizate was centrifuged (10.000 g, 2 min) and the supernatant was taken for chlorophyll content determination. In the case of isolated thylakoids or pigment-protein complexes, pigments were extracted also in 90% acetone, centrifuged (10.000g, 2 min), and the supernatant was taken for chlorophyll content determination. The absorption of the acetonic extract was measured in split beam spectrophotometer (Shimadzu UV-1601) at 630 and 664 nm with a pathlength of 1 cm. The absorption was set to 0 at 750 nm and 90% acetone was applied in the reference cuvette. The calculation of the concentration of Chl *a* and *c* was performed according to Jeffrey and Humprey (1975):

$$\text{Chl } a = 11.47 * E_{664} - 0.4 * E_{630} \text{ [mg/l]}$$
$$\text{Chl } c = 24.34 * E_{630} - 3.73 * E_{664} \text{ [mg/l]}$$

where E is the extinction at the given wavelength. The obtained values were corrected by the applied dilution factor.

3.2. Measurements

3.2.1. Absorption spectroscopy

Absorbance spectra of intact cells were recorded with a Shimadzu UV-3000 spectrophotometer, in split beam mode, in the wavelength range of 400 – 750 nm. The optical pathlength was 1 cm and the bandwidth was set to 2 nm. In order to minimize the spectral artifacts caused by light scattering of turbid cell suspensions, the spectra were measured in a sample holder designed correcting for scattering: the cuvette was placed as close as possible to the photomultiplier tube, in front of which a quartz diffuser was placed. The reference cell contained ASP-2 medium. All absorption spectra were measured at room temperature.

3.2.2. Circular dichroism spectroscopy

CD spectra were measured with a Jobin-Yvon CD6 or a Jasco J-815 dichrograph in the wavelength range of 400-750 nm. The optical pathlength was 1 cm, the cuvette was placed 5 cm from the photomultiplier tube. The spectra were recorded in 1 nm steps with integration time of 0.3-1 s and a bandwidth of 2 nm. The CD spectra are plotted in absorbance units. The CD spectra of the samples were corrected with the CD spectrum of the respective buffer.

The measurements of temperature-dependent CD-changes were performed in a thermostated sample holder. The samples were preincubated at different temperatures in the range of 20-55 °C in 5 °C steps for 10 min and then measured at the same temperature.

The measurements of the light-induced CD changes were performed in the following ways:

i) To follow the spectral changes caused by illumination, the CD spectrum of the dark-adapted cell suspension was measured at first, then the sample was illuminated with strong actinic white light (800 μmol photons m^{-2} s^{-1} PFD) for 2-30 min, and then the light-treated sample spectrum was recorded.

ii) Kinetics of the light-induced CD-changes at a given wavelength was measured in the presence of 7% Ficoll to avoid sedimentation of the dark-adapted cells during the measurement. The monochromator was set to the desired wavelength and the time-course measurement was started with an integration time of 1 s. After recording the baseline for 3-4 min in the dark, illumination was performed with a side-illumination attachment (Barzda et al. 1996). The photomultiplier was protected with crossing filters against the actinic light from the exciting beam. CD-changes were induced by blue light of 800 μmol photons m^{-2} s^{-1} PFD.

3.2.3. Linear dichroism spectroscopy

Linear dichroism (LD) spectroscopy provides information about the orientation of the transition dipoles with respect to a laboratory-fixed coordinate system, in which the sample is macroscopically aligned. LD is a differential absorption of two orthogonal linearly polarized beams:

$$LD = A_{\parallel} - A_{\perp}$$

In order to obtain appropriate LD signal on macroscopic scale, the samples of interest must be aligned, because in randomly oriented particles the LD signal vanishes, even if the chromophores within the sample possess significant intrinsic anisotropy. Photosynthetic systems, such as isolated chloroplasts or thylakoid membranes can be oriented by using gel squeezing method (Abdourakhmanov, 1979) without significant loss of the physiological and structural properties of the embedded particles (Ganago et al. 1983), however in some cases – especially in complex systems – acrylamide caused deterioration, e.g. the chiral macrodomain organization of the chloroplasts became reduced and the membranes lost their ability to retain the energized state (Osváth et al. 1994). Strong magnetic field can also be used for aligning the samples in 'regular' suspensions used also for functional studies. With magnetic field nearly 100% orientation can be achieved, i.e. all chloroplasts can be aligned in a way that their thylakoid membranes tend to orient perpendicular to the magnetic field. Whereas, particles possessing large diamagnetic susceptibility, e.g. chloroplasts or thylakoid membranes can only be effectively oriented with magnetic field strength of 0.5-0.7 T, smaller particles (isolated pigment-protein complexes) cannot be aligned in the same field, because their diamagnetic susceptibility anisotropy is too small.

Linear dichroism (LD) spectra were measured at room temperature in a Jobin-Yvon CD6 dichrograph, equipped with LD boards, in the wavelength range of 400-730 nm with a bandwidth of 2 nm in 1 nm steps. The optical pathlength was 1 cm. The cuvette containing the sample was placed 5 cm from the photomultiplier tube. Cells or thylakoid membranes, isolated from *P. tricornutum* or *C. meneghiniana* were oriented with the gel-squeezing method. The samples were embedded in 5% polyacrylamide gel, which was prepared using 0.416 ml 30% (w/v) acrylamide and 0.8% (w/w) bis-acrylamide solution, 50 µl 10% (w/v) ammonium persulfate, 2.03 ml sample diluted with the appropriate buffer and 5 µl *N,N,N′,N′*-tetramethyl-ethane-1,2-diamine (TEMED). The solution was polymerized in a plastic cube. After the polymerization was complete, the gel was removed from the plastic cube and was squeezed to half the original width (M=2, where M is the squeezing factor) in a squeezing cuvette. The samples were measured in the direction perpendicular to the squeezing direction ("edge" alignment). The LD spectra are plotted in absorbance units.

The orientation angles were calculated according to Garab (1996). The parameter related to the orientation angle is the order parameter (S) what can be obtained as:

$$S=LD/3A$$

By knowing the squeezing parameter (M), it is possible to determine the orientation angle. The relationship between the S parameter and the orientation angle is shown on Fig. 9.

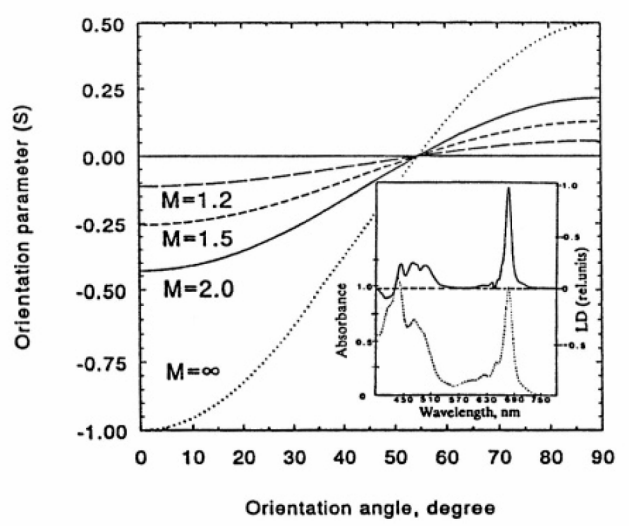

Figure 9. Dependences of S parameter on the orientation angle of dipoles in membranes aligned with different squeezing parameters (M) (Garab, 1996).

3.2.4. Fluorescence spectroscopy

The 77K fluorescence spectra were recorded with a Horiba Jobin-Yvon Fluorolog 3 spectrofluorimeter. The Chl content was set to about 0.5 µg/ml ($OD_{674-750}$ = 0.04) to avoid re-absorption. (Samples containing 60% (v/v) glycerol yielded similar fluorescence spectra as those without this cryoprotectant (not shown).) Fluorescence emission spectra were measured between 600 and 800 nm, using 510 and 550 nm excitation wavelengths with excitation and emission bandwidths of 5 and 2 nm, respectively. The fluorescence excitation spectra were recorded between 400 and 600 nm with excitation and emission bandwidths of 2 and 5 nm, respectively; the emissions were measured at 689 or 713 nm.

3.2.5. Measurement of electrochromic absorbance changes

Flash-induced electrochromic absorbance changes were measured with a home-built single beam spectrophotometer according to Barabás et al. (1985) and Büchel and Garab (1995). The illumination of the samples was perpendicular to the measuring beam from both sides of the cuvette by using xenon flashtubes (General Radio, Stroboslave) providing

single turnover flashes of 3 μs lengths. The frequency of the flashes was 1 s^{-1}. The optical pathlength was 1 cm in the direction of the measuring beam and 0.5 cm in the direction of the actinic flashlight. The actinic flashes were passed through a Schott RG 630-2 mm filter. The wavelength of the measuring light was selected manually by using a Zeiss SPM 2 monochromator (bandwidth 5 nm) and after passing the sample the light was led to an EMI 9558 B photomultiplier protected by a Corning 4-96 filter. The flash-induced absorbance transients were recorded and averaged with the aid of a computer-controlled digital oscilloscope (TEK 2224, Tektronix) and a differential amplifier (TM503B, Tektronix). During the measurements the synchronization of the flash lamps and the oscilloscope was performed using a digital timer (Mikro-GMK). To increase the signal-to-noise (S/N) ratio, 32-64 kinetic traces were averaged. To determine the transient spectra of the electrochromic absorbance changes, kinetic traces were measured in 5 nm steps between 470 and 570 nm. The electrochromic signal at a given wavelength was calculated from the kinetic traces where the signal amplitude at the time point of the flash was subtracted from the signal amplitude at 5 ms after the flash. The measurements were performed in the presence of 7% Ficoll to avoid rapid sedimentation of cells during the measurement. The electrochromic absorbance changes are expressed in -ΔI/I units.

In order to reveal the absorbance bands of the electrochromically shifted pigments, the transient spectra were fitted with the first derivatives of Gaussians according to the equation

$$\frac{dA}{dx} = \sum k \cdot \frac{x - x_0}{h^3} \cdot \sqrt{\frac{2}{\pi}} \cdot e^{-\frac{2(x - x_0)^2}{h^2}}$$

where A=absorbance, x_0 = frequency at peak position, h=halfbandwidth, k=amplitude of the Gaussian

Based on the fitted values of x_0, h and k, the absorbance spectrum of the shifted pigments were reconstituted by using Gaussian function.

3.2.6. Measurement of the chlorophyll fluorescence transients and the determination of the photosynthetic parameters

Room temperature fluorescence measurements were performed using a PAM 101 Chl fluorometer (Walz, Effeltrich, Germany) equipped with a Clark-type oxygen electrode as a

sample holder. The algal cell cultures and isolated thylakoids were used at Chl content of 15 and 20 µg/ml, respectively. The fluorescence parameters F_0 and F_m were recorded after 45 min low light adaptation (20 µmol photons m^{-2} s^{-1}). (F_0 and F_m are the minimum and maximum fluorescence yield, respectively, in low light-adapted state.) The light intensity of saturating flashes was 4.000 µmol photons m^{-2} s^{-1}. The maximum quantum yield of PSII photochemistry of thylakoids in reaction buffers in the absence or presence of sorbitol and $MgCl_2$ was calculated as F_v/F_m, where $F_v = F_m-F_0$, called variable fluorescence. F_0 and F_m were determined after a 5 min incubation in the reaction medium, 50 mM HEPES-KOH pH=7.5, which was complemented with either or both $MgCl_2$ (1 - 25 mM) and sorbitol (350 mM). During the NPQ measurements saturating light flashes (4.000 µmol photons m^{-2} s^{-1}) with duration of 700 ms were applied with 1 min intervals. The actinic light intensity was adjusted to 700 µmol photons m^{-2} s^{-1}. In the case of isolated thylakoid membranes, 40 mM ascorbate was added as a co-substrate for DDE, to enable the conversion of Ddx to Dtx, and 200 µM methyl-viologen was added as electron acceptor. NPQ was calculated after 10 min actinic light illumination using the Stern-Volmer data treatment ($NPQ = F_m/F_m'-1$) according to Bilger and Björkman (1990). (F_m' is the maximum fluorescence yield in actinic-light adapted state.)

3.2.7. Determination of the pigment composition by high performance liquid chromatography (HPLC)

In the case of intact cells, pigments were isolated by filtering 10 ml of algal cell suspension. After removal of the ASP-2 medium by using a vacuum pump, 1 ml of HPLC medium (90% methanol/0.2 M ammonium acetate (90/10, v/v) and 10% ethylacetate) and a small quantity of glass beads were added. The cells were disrupted in a cell homogenizer (Braun, FRG), centrifuged, and the supernatant was taken for HPLC analysis. In the case of thylakoid membranes, pigments were extracted by addition of 500 µl ethylacetate, 500 µl distilled water, and a spatula tip of NaCl to 250 µl of the different thylakoid samples. The samples were stirred several times to transfer the pigments into the organic phase and afterward centrifuged for 2 min at 20,000*g*. The organic phases were collected and the pigments dried in a gentle N_2 stream. The dried pigments were dissolved in HPLC medium. Pigments were analyzed on a Waters 600 MS chromatography system equipped with a Waters 717 autosampler and a Waters 996 photodiode array detector (Waters Millipore,

FRG) recording absorption spectra between 350 and 700 nm. A wide-pore ET 250/4 Nucleosil 300-5 C18 column (Macherey-Nagel, FRG) was used for separation. Eluents and gradient programs were as described in Kraay, 1992. For the separation of pigments the following eluents were used:

Eluent A: 85% Methanol/15% 0.5 M Ammonium-acetate (in Aqua dest.)
Eluent B: 90% Acetonitrile/10 % H_2O
Eluent C: 100% Ethylacetate

The flow-rate in the column was set to 0.8 ml/min and during the separation the eluents were degassed with helium. The following eluent compositions in relation to timescale were used for separation:

Time (min)	% Eluent A	% Eluent B	% Eluent C
0	60	40	0
2	0	100	0
7	0	80	20
17	0	50	50
21	0	30	70
28,5	0	30	70
29,5	0	100	0
30,5	60	40	0

Pigments were quantified according to Lohr and Wilhelm, 2001 and Wilhelm, 1995. The parameters to determine the concentration of the given pigments are:

Pigment	Wavelength for integration (nm)	Conversion factor (area/µg pigment)	Molecular mass in µg/mol
Chl *c*	440	22536980	610000000
Fx	440	10709573	659000000
Ddx	480	14103018	583000000
Dtx	480	12773475	566000000
β-Car	480	15238466	537000000
Chl *a*	430	7272969	894000000

The concentration of a given pigment was obtained as:

n = A/(conversion factor*molecular mass), where A is the integrated area of the absorption.

3.2.8. Transmission electron microscopy

Cells and isolated thylakoid membranes were visualized by Zeiss 902 electron microscope. The samples were pelleted by centrifugation at 5.000 g for 10 min in Eppendorf tubes. The pellet was fixed with 2% glutaraldehyde diluted with 0.1 M phosphate buffer (pH = 7.2) for 3 hours in the case of cells and 1 hour in the case of isolated thylakoids. Glutaraldehyde was washed out by overnight incubation in 0.1 M phosphate buffer. Samples were postfixed with 1% osmium-tetroxide diluted in 0.1 M phosphate buffer for 2 hours. After short (~1 min) washing with 0.1 M phosphate buffer, samples were gradually dehydrated in 30, 50, 70, 90, 96 and 100% ethanol for 10-15 min at each step. The incubation step in 100% ethanol was repeated 3 times. After that, samples were incubated in propylene oxide for 10-15 min 2 times and in 1:1 ratio of propylene oxide:Araldite for overnight. Samples were transferred to pure Araldite resin and incubated for 24 h. The Eppendorf tubes containing the samples embedded in the resin were placed to thermostated chamber and the resin was hardened for 48-72 h at 56 °C. After trimming the hardened resin blocks, ultrathin sections with a thickness of 70-90 nm were cut with ultramicrotome (Leica Ultracut UCT). Sections were taken up onto 400 mesh copper grids. For contrasting the specimen, sections were stained with 2% uranyl acetate for 45 min, washed with distillated water and stained with lead citrate for 15-20 min and washed with distillated water.

4. RESULTS

4.1. CD signals in the diatom *Phaeodactylum tricornutum*

The aim of the present chapter is to record and describe the CD signals of the diatom *P. tricornutum* originating from pigment molecules. *P. tricornutum* is a typical pennate species possessing bilateral cell symmetry and one large chloroplast per cell whose genome is completely sequenced (Oudot-Le Secq et al. 2007). This species is extensively used as a laboratory organism in various fields of diatom research.

We have started our investigations with the examination and comparison of the pigment-pigment interactions in hierarchically different pigment organizations prior assigning certain bands to structural and macro-organizational features.

First, the CD spectra of free pigment molecules were measured, which we considered as the simplest organizational level in the pigment system, because pigments are detached from the proteins and thus the CD signatures of single molecules can be recorded. Then, CD spectra on isolated pigment-protein complexes were recorded, where the pigments are bound to proteins, which give a chance for the presence of pigment-pigment interactions. The next organizational level to be examined was the isolated thylakoid membranes, where the pigment-protein complexes are embedded in the membrane. Finally, we have measured CD spectrum of intact cells, which can be considered as the *in vivo* state and the most complex organization of the pigment system.

The CD spectra of pigment extracts, isolated thylakoid membranes and intact cells of *P. tricornutum* are depicted in Fig. 10.

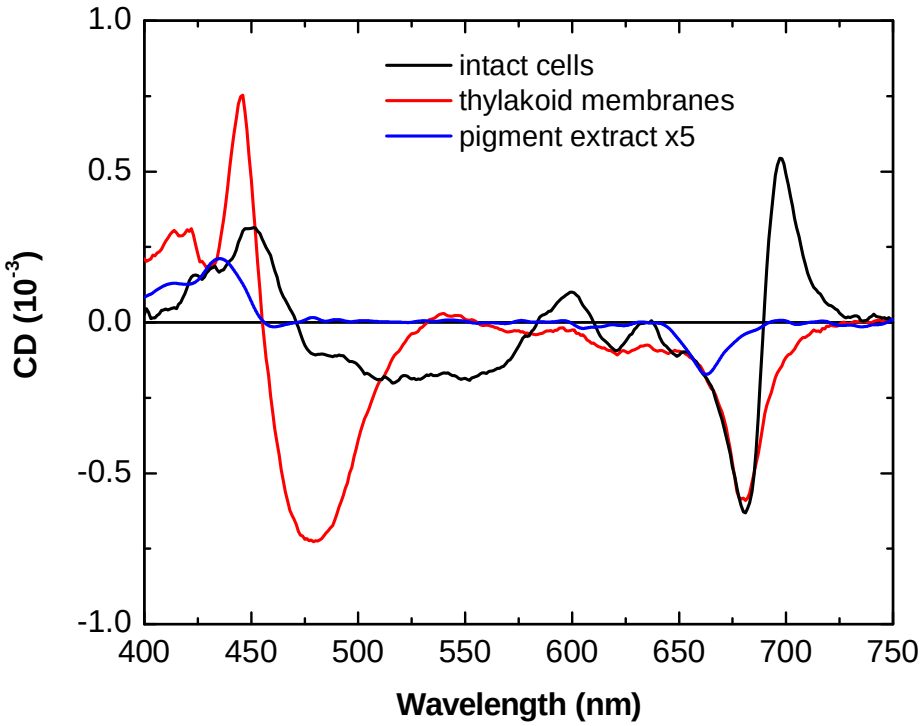

Figure 10. CD spectra if intact cells, isolated thylakoid membranes and pigment extracts of *Phaeodactylum tricornutum*. The spectra are plotted for identical Chl content of the samples (20 µg/ml). For better comparison, the signal of pigment extract was multiplied by a factor of 5.

Free pigment molecules extracted in 90% acetone exhibit weak CD signals at (-)663 and (+)434 nm. The very weak intensity of these bands and the lack of the conservative bands structure show that these CD bands can indeed be assigned as intrinsic signals.

Then we have characterized the CD signals of isolated pigment-protein complexes. By using sucrose gradient centrifugation, the main pigment-protein fractions were separated from solubilized thylakoid membranes (Fig. 11a). The polypeptide and pigment composition of the pigment-protein complexes were determined earlier and are described by Lepetit et al. (2007). In my work, the pigment-pigment interactions revealed by CD spectroscopy were in the focus of interest.

In isolated FCP of *P. tricornutum*, characteristic CD bands could be observed at (-)679 nm, at (-)475 nm and at (+)445 nm (Fig. 11b). The negative band at 679 nm – although exhibits large intensity – cannot be considered as excitonic CD band, because the conservative split signal is missing. This indicates that between the Q_y dipoles of the Chl *a* molecules no excitonic interactions exist. Similar bands have been identified at around 679

nm in other Chl *a/c*-containing organisms (Büchel and Garab, 1997; Mimuro et al. 1990). The (-)679 nm band has been proposed to originate from a small fraction of Chl *a* molecules, which exhibit induced chirality due to the twisting of the molecules in the protein environment (Büchel and Garab, 1997). It was proposed earlier that the strong excitonic bandpair at (+)445/(-)478 nm originates from Chl *a*/Fx interactions (Büchel, 2003). However, recent data indicate that this split signal can have a different origin, from a pair of blue-shifted Fx molecules (Premvardhan et al. 2009), additional contributions might come from Chl c_2:Chl *a* excitonic interactions (Premvardhan et al. 2008). Isolated FCP contains only substoichiometric Ddx (or Dtx), and appear not to contribute to the CD signals.

Isolated photosystems exhibit a pronounced split excitonic CD bandpair with the maxima at (+)676 and (-)690 nm, which indicates strong excitonic coupling between the Q_y dipoles of the Chl *a* molecules. In the blue region of the photosystem CD spectra, the signals at (-)430 nm and (+)445 nm probably also originate from Chl *a* excitonic interactions (Fig. 11b). Weak CD signals can be observed at all organization levels of chromophores at around 623 nm, which – taking into account their spectral positions – most probably originate from Chl *c*.

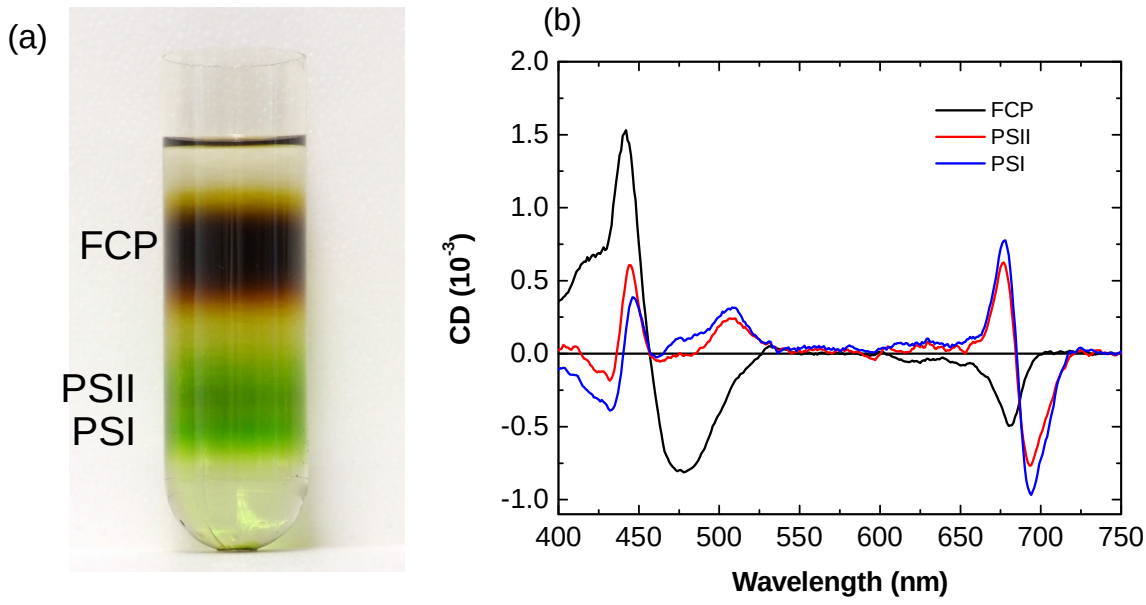

Figure 11. Separation of FCP, PSII and PSI pigment-protein complexes by sucrose density gradient ultracentrifugation (a), and CD spectra of the same fractions (b). Spectra are plotted to the same Chl content (20 μg/ml).

During the past few years, many efforts have been made to isolate FCP complexes in their native state. In our work, we attempted to isolate oligomeric FCP fractions by using lower detergent concentration in order to keep the complexes as intact as possible. After solubilization of the thylakoid membranes with DM/Chl ratio of 5 instead of 20, a significantly different sucrose density gradient pattern could be observed (Fig. 12a). At DM/Chl ratio of 5, a new band (named FCPo) appeared at higher sucrose gradient density. Here the PSII and PSI fractions could not be separated due to the mild solubilization, only one PS fraction could be observed which was dominated by PSI, as suggested by its fluorescence emission spectrum. Gel-filtration chromatography analysis indicated that FCPo possesses higher degree of oligomerization compared to FCP; the molecular mass of FCPo was estimated to be 440 kDa, whereas the molecular mass of FCP is 230 kDa (Lepetit et al. 2007). The protein composition of FCP and FCPo was almost identical. FCPo contained a small amount of PSII proteins, which indicates that FCPo and PSII did not separate completely on the sucrose gradient (Lepetit et al. 2007). Both FCP and FCPo exhibited perfect excitation energy transfer, as concluded form low-temperature fluorescence spectroscopy measurements. In my work, the investigation of the pigment-pigment interactions was in the focus of interest as studied by CD spectroscopy. The CD spectra of FCP and FCPo are depicted on Fig 12b.

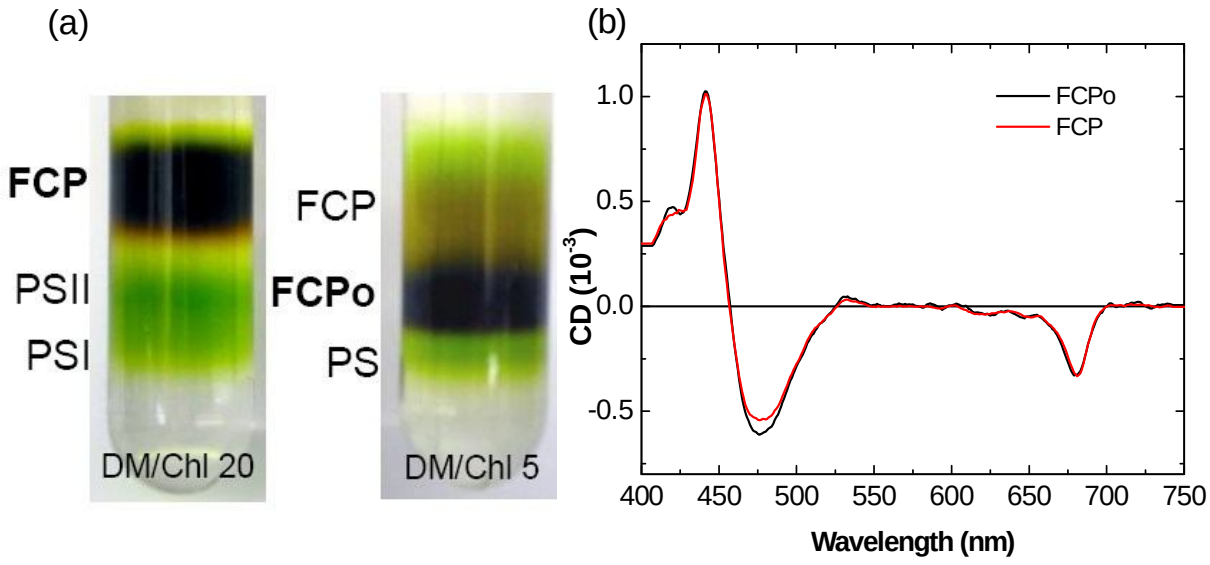

Figure 12. The position of the pigment-protein fractions on the sucrose gradient using different detergent (DM) to Chl ratios during solubilization (a). CD spectra of the isolated FCP and FCPo complexes (b). The Chl content was adjusted to the same value, 20 μg/ml.

The CD spectra of the FCPo and FCP are very similar to each other; small differences in the Soret region probably originate from small alterations in the excitonic interactions.

In isolated thylakoid membranes, characteristic CD bands could be found at around (+)445 nm, (-)478 nm and (-)679 nm (Fig. 10). The band pair at (+)445/(-)478 nm is most likely given rise by excitonic interactions involving Chl *a*, Chl *c* and carotenoids, mainly Fx, similarly to isolated FCP as it was described above. The (-)679 nm band cannot be considered as excitonic CD bands, because the conservative band structure is missing, instead, it originates from the induced chirality of Chl molecules as it is described above in the case of FCP.

Intact cells exhibit an intense CD band at around (+)698 nm (Fig. 10). The intensity of this band is larger than the intrinsic and excitonic CD signals, moreover, it is associated with strong differential scattering. The intensity of the 698 nm band decreases or even disappears in thylakoid membranes and varies independently from the (-)679 nm band, therefore the excitonic property of this band can be ruled out. Intact cells exhibited excitonic bands at (+)445 nm and (-)478 nm, however, surprisingly this bandpair was much weaker than in isolated FCPs and thylakoids. This might be caused by scattering artifacts in intact cells, or by an overlapping strong and broad psi-type band in the same region.

4.2. Assignment of the psi-type CD signal to the multilamellar membrane system

The assignment of the main CD bands with psi-type features, anomalous band shape and long differential scattering tail, are based on the observations described in the previous chapter. In particular, the (+)698 nm band, observed in whole cells but not in isolated thylakoids, has been shown to exhibit non-excitonic features. In contrast, the (-)679 nm band persisted both in isolated thylakoid membranes and in whole cells. In order to prove that these features originate from a macro-organization of the complexes and to find the structural entity which carries these features, a method was necessary, by the aid of which we could follow the gradual spectroscopic changes along with the ultrastructural changes. To this end, we applied ultrasonic disintegration of intact cells, which was performed for different time periods, and the changes in the CD spectra were checked after every sonication step. From the same sample aliquots were taken for electron microscopy. Sonication led to gradual diminution of the large (+)698 nm signal, while the negative

band in the red did not decrease considerably, although a 4 nm wavelength shift was visible (Fig. 13a).

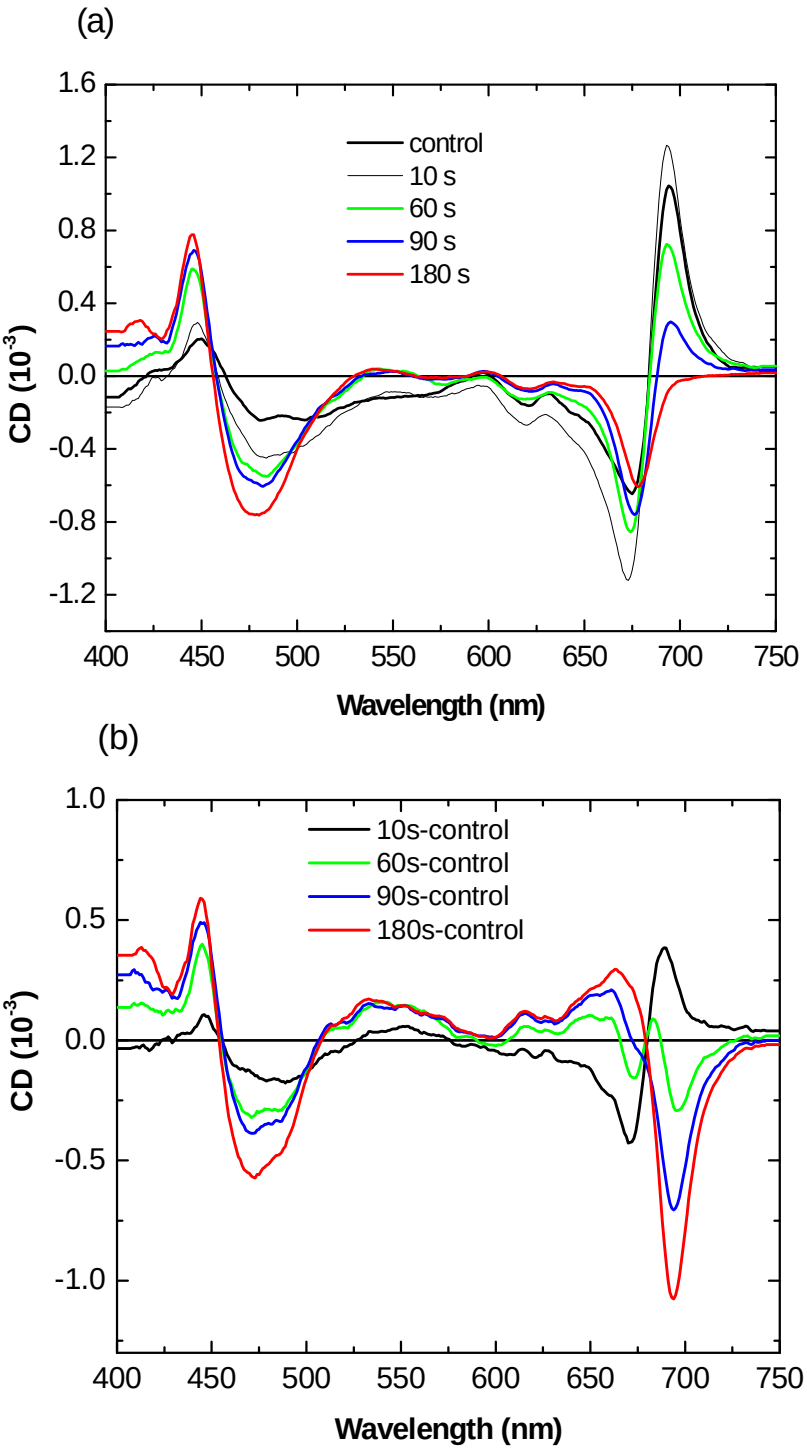

Figure 13. Effect of sonication on the CD spectra of *Phaeodactylum tricornutum* cells (a). The same cell suspension was sonicated for 10, 60, 90 and 180 s. Chlorophyll concentration, 15 µg/ml. Difference CD spectra of sonicated-minus-control samples (b).

Interestingly, after the 10 s sonication step, a small increase occurred both in the (+)698 and (-)679 nm signal. The reason of this change is unknown, the probable explanation is that upon short-term sonication, some changes in the optical properties of cell wall or organelles occurred, and thereby some scattering artifacts caused by the cell walls had been eliminated. Upon sonication, the intensity of the (+)445/(-)478 nm band pair gradually increased, the excitonic interactions became more expressed (see also the difference spectra in Fig. 13b). The equal changes in the intensity of the (+)445/(-)478 nm band pair confirm the excitonic property of these CD signals, while the decrease of the large 698 nm band intensity independently from the (-)679 nm band rules out the excitonic property of these CD signals and refers to psi-type feature suggesting the presence of the chirally organized macrodomains of the Chl *a* molecules (Fig. 13b).

By using electron microscopy, we attempted to visualize the structural changes caused by sonication (Fig. 14).

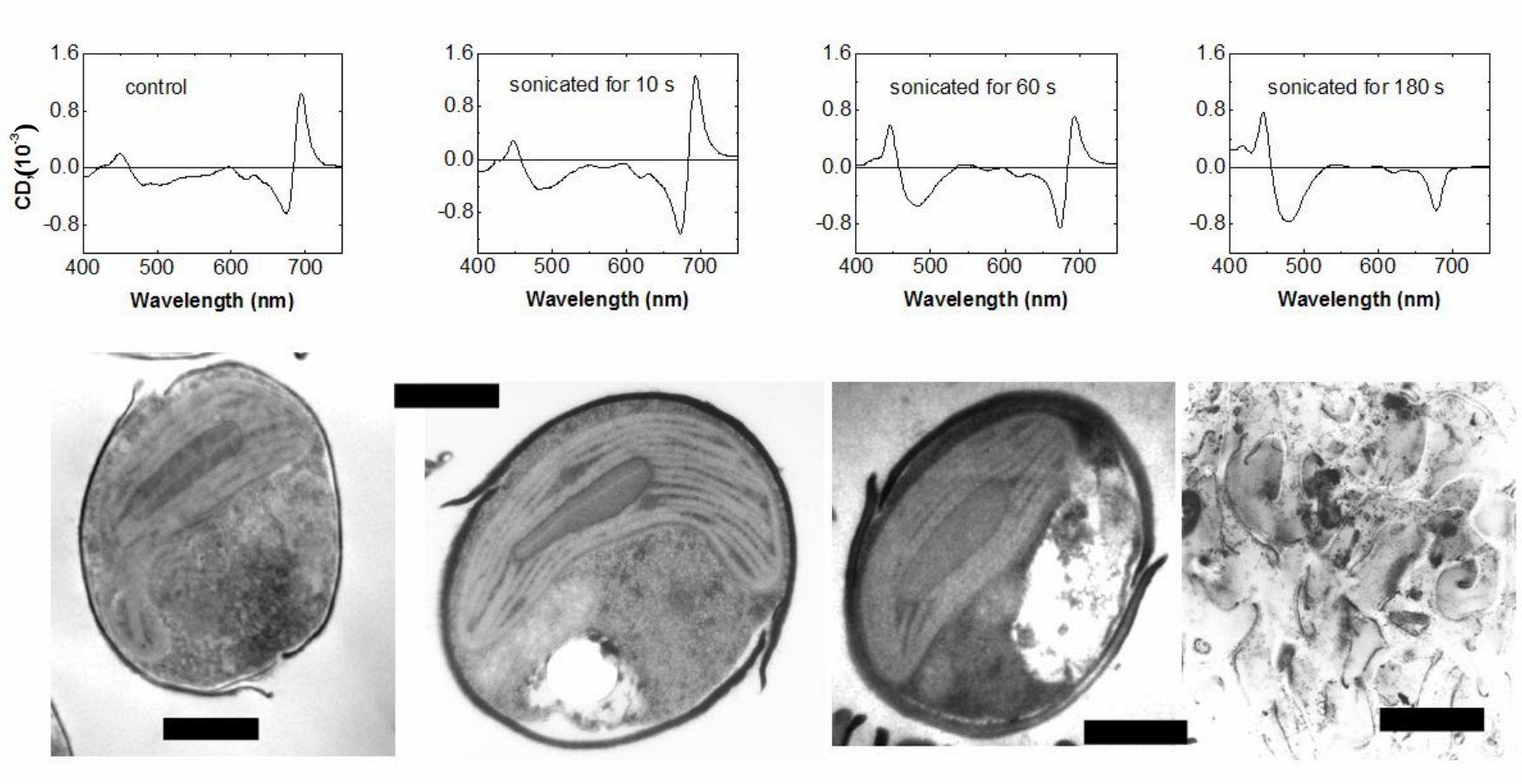

Figure 14. CD spectra and electron-micrographs of *Phaeodactylum tricornutum* cells sonicated for the indicated time intervals. Scale bars: 0.6 μm. The CD spectra were measured on the same sample, which was prepared for EM studies.

Fig. 14 shows that in the control, non-sonicated cells the thylakoid membranes are arranged in loosely stacked, multilamellar membrane system within the chloroplast. After sonication for 10 s, the regular, multilamellar system of the thylakoids did not change considerably, but it is visible that some vacuoles appeared within the cell. These changes in the cell ultrastructure might influence the differential light scattering of left and right circularly polarized beams and thereby some changes in CD spectra might occur. After 60s sonication, drastical changes in cell ultrastructure and considerable loss of the multilamellar organization of thylakoid membranes could not be observed and the psi-type signal at 698 nm decreased but did not disappear completely. Sonication for longer time periods caused the complete destruction of the cell ultrastructure and lamellarity of the thylakoids with a concomitant loss of the psi-type signals. The CD spectrum of the completely broken cells is similar to that of isolated thylakoid membranes. These results indicate that the presence of the psi-type CD signal correlates with the presence of the multilamellar organization of the thylakoid membranes.

4.3. The structural flexibility of the chiral macrodomains

In diatoms, the structural flexibility of photosynthetic membranes and pigment protein complexes under variations in the environmental conditions has not been investigated yet. Chiral macrodomains are capable undergoing transient and reversible structural changes, which indicate a flexible organization of the chromophores (Chapter 1.7). It has been shown in Chapter 4.2 that the chromophores are arranged in chiral macrodomains, reflected by the presence of psi-type CD signal at around 698 nm. In the present chapter our aim was to examine the changes in the macrodomain organization caused by different environmental factors, such as illumination, temperature, changes in osmotic pressure and ionic strength and tested the reversibility of the changes, which indicates the existence of a flexible system.

4.3.1. Temperature-induced CD changes

Ambient temperature affects considerably both the physiological functions and structural features of the thylakoid membranes. Higher plants exhibited temperature-dependent CD changes, where the chiral macrodomains proved to be the most sensitive to elevated temperatures. To test the effect of the elevated temperatures on the pigment interactions and macrodomain structure in diatoms, *P. tricornutum* cells were subjected to heat treatment in the range of 20-55 ºC. The spectral changes caused by the heat treatment are depicted on Fig. 15.

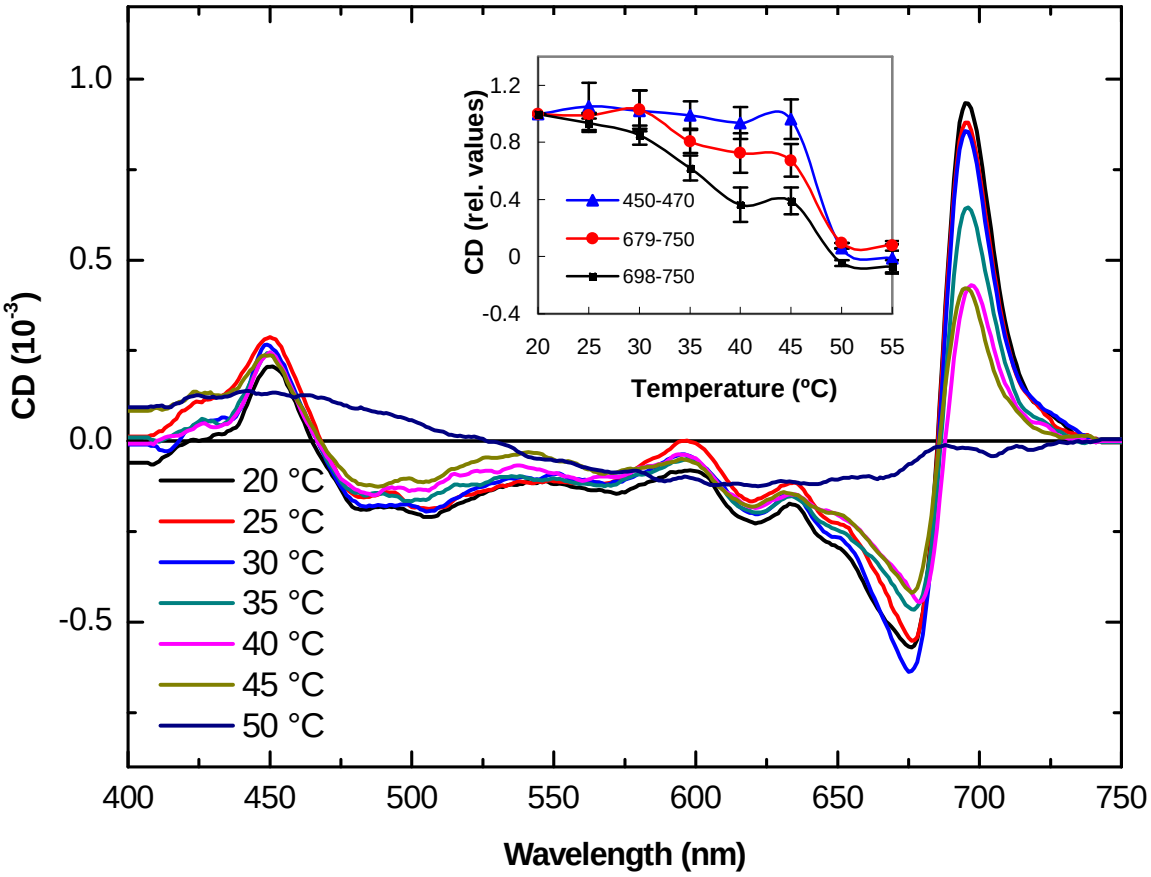

Figure 15. The effect of elevated temperatures on the CD spectra of *P. tricornutum* cells. The cells were incubated consecutively for 10 min at the indicated temperature and measured at the same temperature. Inset, temperature dependence of the CD signal at 698 nm, 679 nm and at 450 nm). Chlorophyll content, 15 µg/ml.

While the (+)450 and (-)679 nm bands mostly preserved their intensities, a continuous, large decrease in the intensity of the (+)698 nm could be observed upon increasing the temperature up to 45 ºC. Above 45 ºC, all characteristic CD bands related to intra- or intermolecular interactions disappeared. The inset in Fig. 15 shows the relative changes of the indicated bands as a function of the temperature. By increasing the temperature up to 45 ºC, 60 % of the original intensity of the (+)698 nm signal and 20% of the original intensity of the (-)679 nm band was lost, while at 450 nm only little changes could be seen. Thus, the (+)698 nm signal proved to be the most sensitive to elevated temperatures, while the excitonic bands at around 450 nm and the (-)679 nm band displayed higher stability. Above 45 ºC a steep decrease in the intensities of all bands occurred, which indicates the loss of all pigment-pigment interactions inside the protein complexes or their small aggregates. The highest temperature sensitivity could be observed in the case of the (+)698 nm band and it changed independently from the (-)679 nm band. This again points to the psi-type feature of the (+)698 nm band, moreover it indicates in the same time that chiral macrodomains are more susceptible to elevated temperatures than the excitonic CD bands. These data are in good agreement with the data obtained for the temperature-susceptibilities in higher plant thylakoid membranes (Cseh et al. 2000; Dobrikova et al. 2003).

4.3.2. Light-induced CD changes

Upon illumination with strong actinic light, a pronounced increase in the amplitude of the (+)698 nm signal and a small increase in the amplitude of the (-)679 nm signal could be observed, while the other regions of the spectra remained essentially unchanged (Fig. 16).

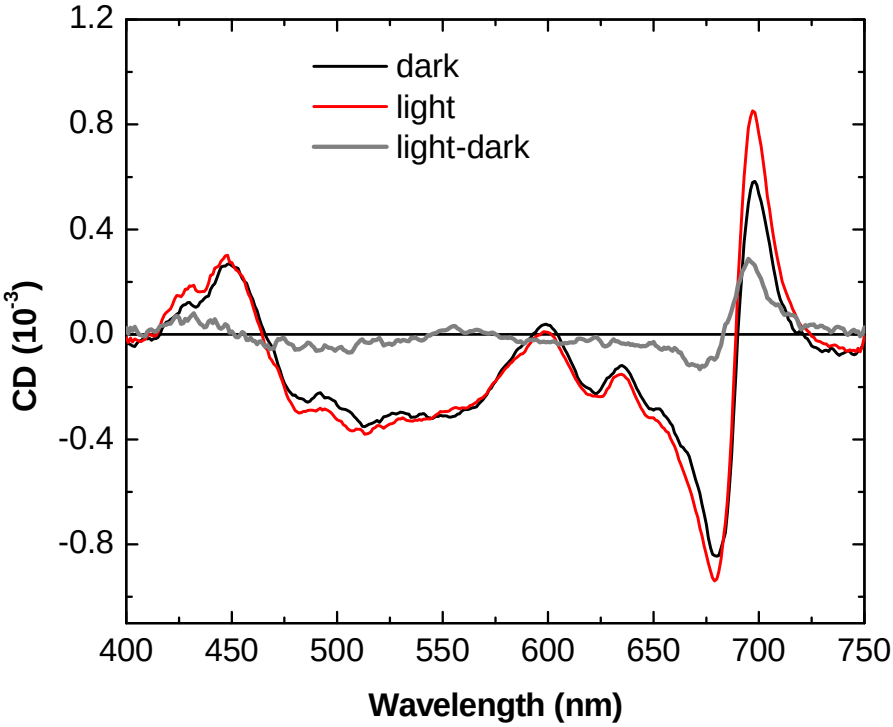

Figure 16. CD spectra of dark-adapted and preilluminated *P. tricornutum* cells and the difference spectrum of illuminated-minus-dark-adapted cells. The measurements were performed at room temperature; the light intensity of illumination was 800 µmol photons m^{-2} s^{-1}; Chl content, 15 µg/ml.

In order to determine the extent and the possible ability for recovery of the light-induced CD changes, the kinetics of the changes were also examined at different wavelengths. The most pronounced changes in intact cells illuminated for several minutes occurred at 698 nm. After illumination for 100 s the increase in the signal intensity levelled off. After switching off the illumination, the signal decreased to its original value; this recovery took about 200 s (Fig. 17).

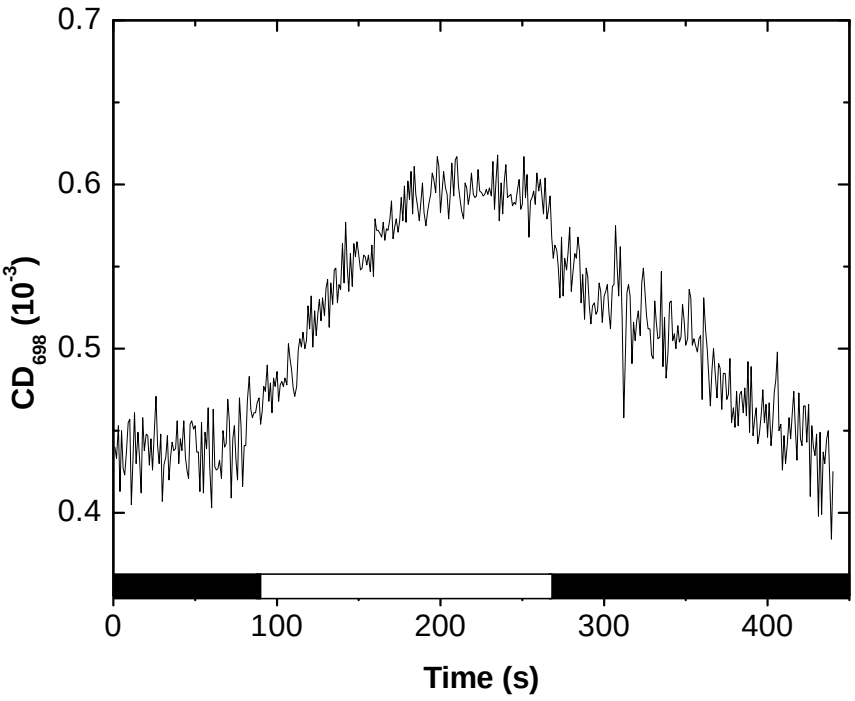

Figure 17. Time course of the light-induced CD-changes at 698 nm. Closed bars, dark periods; open bar, illumination period. The measurements were performed at room temperature; the light intensity of illumination was 800 µmol photons m^{-2} s^{-1}; Chl content, 15 µg/ml.

The kinetics of the light-induced CD changes can be characterized by fitting the initial phase and calculating the rate of the changes. The magnitude of the changes is given as ΔCD/CD values, where the CD changes upon illumination (ΔCD) is normalized on the initial CD values of the dark-adapted state.

By using these parameters, the light-intensity dependence of the light-induced CD changes was measured. By increasing the illumination intensity, the ΔCD/CD values and the rate increased, however, above 450 µmol photons m^{-2} s^{-1} no further change could be observed (Table I).

Intensity (µmol photons $m^{-2}s^{-1}$)	ΔCD/CD	Rate (ΔCD/t, s^{-1})
40	0.12	$5x10^{-7}$
100	0.18	$8x10^{-7}$
450	0.36	$2.8x10^{-6}$
700	0.3	$2.5x10^{-6}$

Table I. Light intensity dependence of the light-induced CD changes at 698 nm. n=3, SD≤20%.

To reveal the possible correlations between the light-induced CD changes and the operation of the photosynthetic electron and proton transport and photochemical activity, different inhibitors were applied during the measurements of the kinetics of the light-induced CD changes (Table II).

	ΔCD/CD	Rate (ΔCD/t, s^{-1})
Control	0.33	$3.5x10^{-6}$
DCMU	0.23	$3.8x10^{-6}$
NH_4Cl	0.36	$3.1x10^{-6}$
DTT	0.17	$1.2x10^{-6}$

Table II. Effect of different inhibitors and uncouplers on the light-induced CD changes. Applied concentration of the chemicals: 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU): 20 μM, dithiothreitol (DTT): 5 mM, ammonium chloride (NH_4Cl): 5 mM. The intensity of the illumination was 800 μmol photons m^{-2} s^{-1} during the experiment. n=3, SD≤20%.

DCMU inhibits the photosynthetic electron transport by displacing Q_B from its binding site on the D1 protein and thereby the photochemistry becomes arrested (Velthuys, 1981). It has been shown that isolated thylakoid membranes of higher plants exhibit reduced ΔCD in the presence of DCMU. However, it is also established that the activity of the electron transport is not mandatory for the structural changes, rather it plays a "conditioning" role in this process (Istokovics et al. 1997). In diatoms, DCMU also inhibited to some extent the amplitude of the light-induced CD changes and the rate was even increased to little extent. This result indicates that in *P. tricornutum* the inhibition of linear electron transport does not affect the light-induced structural changes. This is in a good agreement with the NPQ analyses, where it was found that in the presence of DCMU the NPQ kinetics were not affected by DCMU (Grouneva et al. 2009).

To test the possible involvement of the proton gradient in the light-induced CD changes, NH_4Cl was applied. The uncoupler NH_4Cl dissipates the proton gradient, which is formed during the operation of the vectorial photosynthetic electron transport. NH_4Cl inhibited the qE component of NPQ in *P. tricornutum* (Ruban et al. 2004). It has been shown earlier that NH_4Cl inhibits the light-induced CD changes in higher plants (Garab et al. 1988). However, later it was found that nigericin (a Na^+/H^+ ionophore) dissipated the proton gradient at low concentrations, but the ΔCD was not affected (Istokovics et al.

1997). NH_4Cl was applied on *P. tricornutum* cells to examine whether the dissipation of the proton gradient inhibits the light-induced structural changes. NH_4Cl did not cause any significant inhibition of either the magnitude or the rate of the light-induced CD change. Since we have seen no inhibition of light-induced CD changes by NH_4Cl, while it has been shown that this compound blocks the NPQ, a clear correlation between the presence of ΔpH and structural reorganizations cannot be revealed at present. Another chemical compound, which dissipates the proton-gradient and thus blocks the NPQ is the nigericin. However, it could not be used in our experiments, because it has been shown earlier that it does not penetrate the cell wall of diatoms (Ruban et al. 2004).

The violaxanthin or diadinoxanthin deepoxidase inhibitor, dithiothreitol (DTT) suppresses the deepoxidation reaction step of the xanthophyll-cycle, and thereby the formation of the xanthophylls responsible for or participating in the qE component of NPQ is blocked (Goss et al. 2006). DTT suppressed significantly the light-induced CD changes in *P. tricornutum* (Table II), which suggests a correlation between the operation of the xanthophyll cycle and structural reorganizations. However, DTT probably also affects metabolic processes other than the xanthophyll cycle. Therefore, further experiments are necessary to clarify the connection between the light-induced changes and the NPQ processes in diatoms.

In diatoms, light intensity during growth influences the size of chloroplast and the amount of thylakoid membranes. It has been shown that high light intensities cause a decrease in the chloroplast size and the amount of the thylakoid membranes, however the membrane ultrastructure, i.e. the bands of three did not change with light intensity (Janssen et al. 2001, Moisan et al. 1999, 2006). It has also been observed that the amount of PSI and PSII increases considerably in the low light grown cells, also the cell volume increased in *P. tricornutum* (Friedman and Alberte, 1986). In another diatom species, *Skeletonema costatum*, the amount of PSI decreased while the amount of PSII increased by decreasing the growth light intensity (Falkowski et al. 1981). In the present work, the macrodomain organization of the cells grown on HL and LL intensities was investigated (Fig. 18).

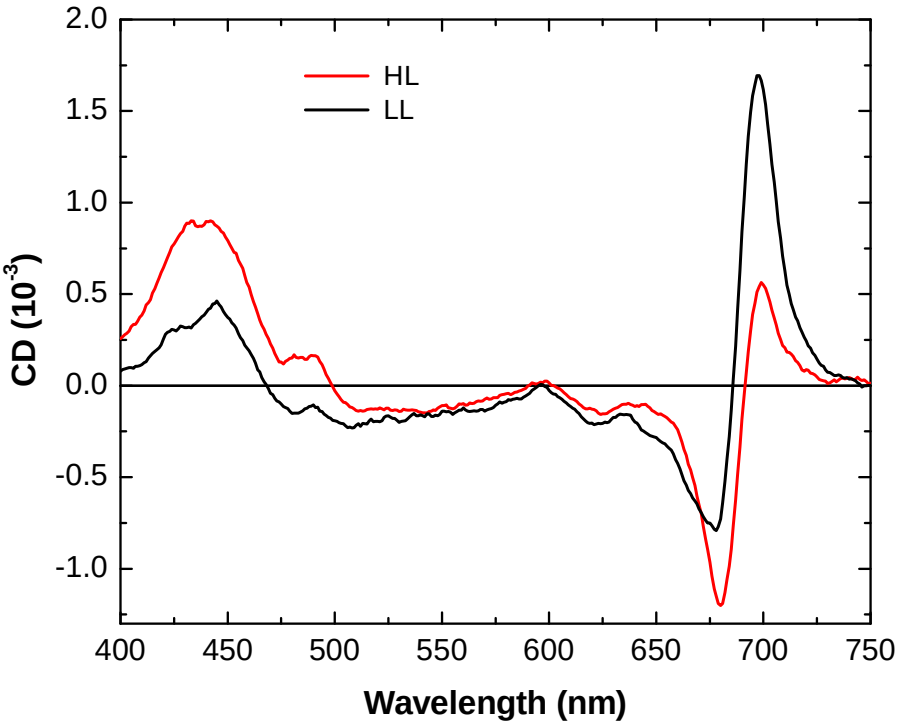

Figure 18. CD spectra of HL and LL cells. The samples were adjusted to the same Q_y absorbance of Chl *a*, $OD_{673-750} \approx 0.5$.

Cells grown on different light intensities exhibited remarkably different CD spectra (Fig. 18). LL cells exhibited weaker excitonic bands in the blue region compared to HL cells. Moreover, LL cells displayed significantly larger psi-type bands at 698 nm and a less intense signal at 679 nm. These differences might be caused by a higher degree of stacking of the thylakoid membranes. This hypothesis was checked by electron microscopy of intact HL and LL cells (Fig. 19).

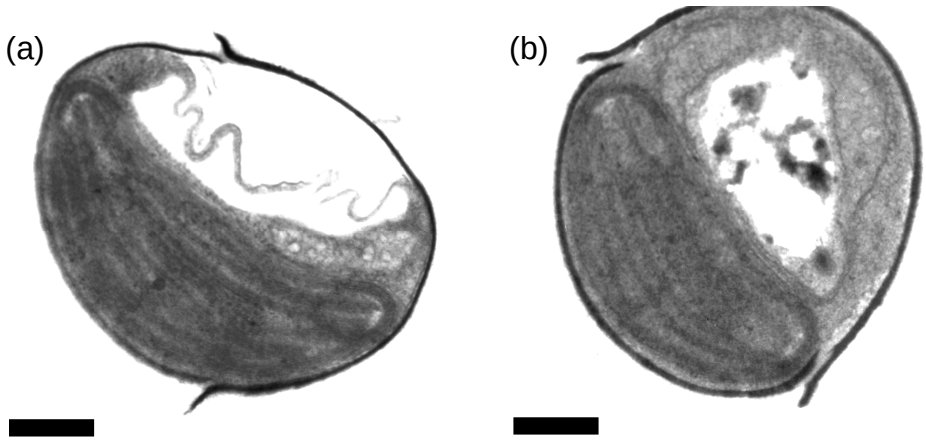

Figure 19. Electron micrograph of *P. tricornutum* cells grown either on LL (a) or on HL (b) intensities. Scale bar: 0.6 μm.

The thylakoid membrane ultrastructure displayed no significant differences between HL and LL cells as it is visible on the electron micrographs (Fig. 19), which is in agreement with previous observations (Janssen et al. 2001), thus the changes in the psi-type CD signal caused by the light intensity during growth cannot be explained by the changes in the thylakoid membrane ultrastructure.

In order to examine the structural flexibility of chiral macrodomains in cells grown on different light intensities, the extent of the light-induced changes was measured (Table III).

	ΔCD/CD	Rate (ΔCD/t, s^{-1})
HL	0.33	$3.9x10^{-6}$
LL	0.05	$1.3x10^{-6}$

Table III. Light-induced CD changes at 698 nm in *P. tricornutum* cells grown either on LL or HL. The intensity of the illumination was 800 µmol photons m^{-2} s^{-1}. n=3, SD≤20%.

Cells grown on LL exhibited a much smaller ΔCD/CD ratio compared to cells grown on HL. The rate of the light-induced CD changes is also significantly higher in HL cells than in LL cells. This result indicates that in LL cells the light-induced structural changes are restricted compared to HL cells, despite the fact that in LL cells a more pronounced psi-type CD signal at 698 nm can be observed than in HL cells (Fig. 18).

4.3.3. Effects of the osmotic pressure and Mg-ions on the chiral macrodomains

We examined the role of divalent cations and osmolarity on the formation of chiral macrodomains and the relation of the structural changes to photosynthetic functions. As it is shown in Fig. 20a, upon increasing the sorbitol concentration in the medium, a large decrease in the psi-type CD signal occurred.

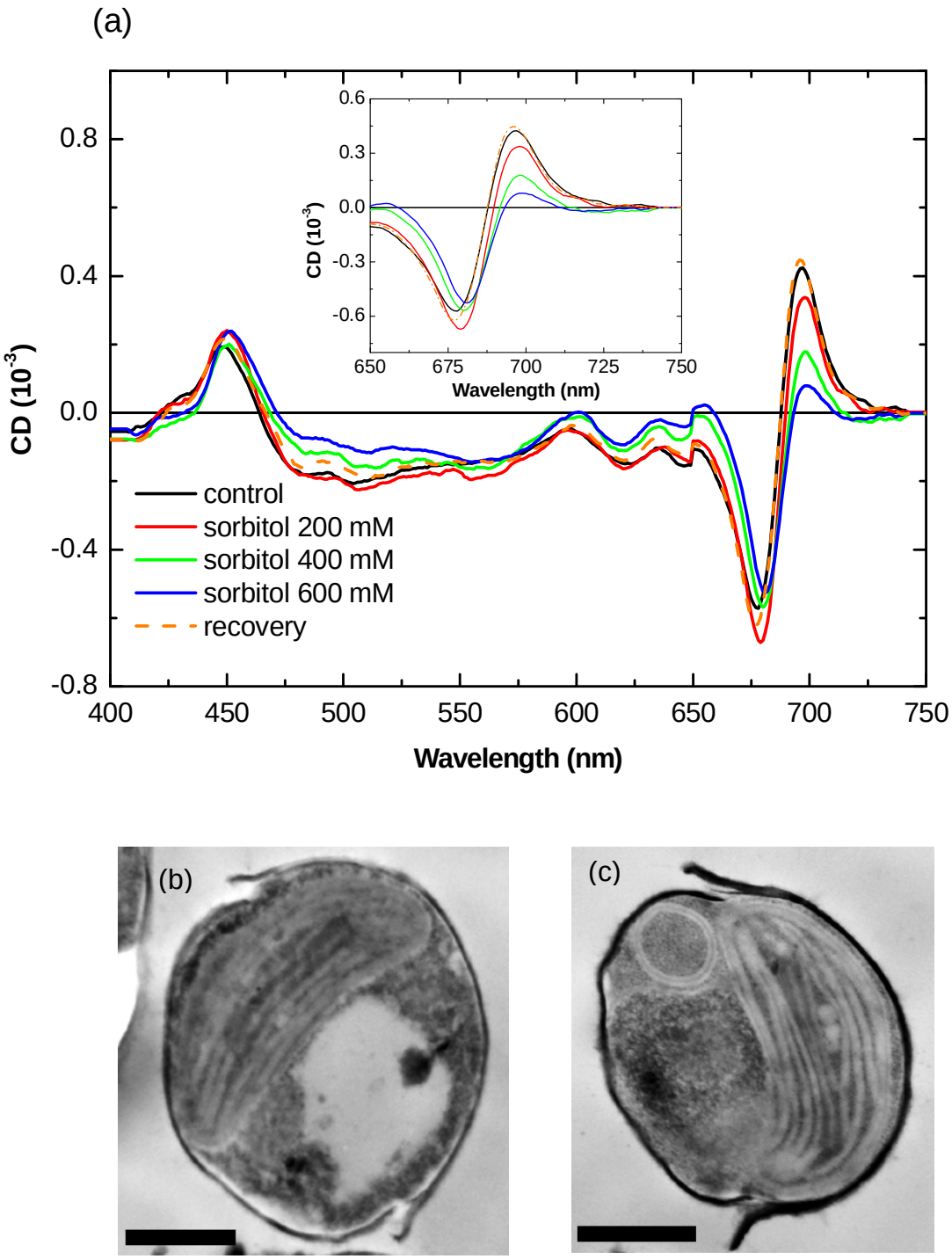

Figure 20. Dependence of the CD spectra of intact *Phaeodactylum tricornutum* cells on the osmotic pressure of the medium (a). Inset, the spectral region between 650 and 750 nm. The cells were suspended in the culture medium and in the same medium supplemented with different concentrations of sorbitol. The "recovery" spectrum was recorded on cells suspended in the culture medium following their treatment with 600 mM sorbitol. Chlorophyll content, 15 µg/ml. Electron micrographs of *P. tricornutum* cells in the absence (b) and presence (c) of 600 mM sorbitol. Scale bar: 0.6 µm.

When the cells were resuspended in the normal culture medium again, the psi-type CD at 698 nm could be restored. It was interesting to observe that the reversible modulation of the CD signals was not confined to the psi-type CD, but also affected, albeit to lesser extent, the excitonic interactions in the Soret. It was also interesting to note that an increase in the sorbitol concentration resulted in a red-shift of the (-)679 nm band, which was also reversed after resuspending the cells in the culture medium (Fig 20a, inset). In order to test the effect of increasing the osmotic pressure in the medium on the thylakoid membrane ultrastructure, untreated and sorbitol-treated intact cells were analyzed by TEM. On the electron micrographs no significant changes could be observed in the lamellarity of the thylakoid membrane system (Fig. 20b and c). Nonetheless, the fact that the CD changes caused by increased osmotic pressure could be reversed and no destruction in the thylakoid membrane system was visible indicates that the chiral macrodomains are capable to exhibit physiologically relevant structural reorganizations upon changing the ambient osmotic pressure.

It was found previously that divalent cations (i.e., Mg^{2+}) were not able to influence the CD bands in *P. meiringensis*; this was attributed to the lack of stacked thylakoid membranes in these algae (Büchel and Garab, 1997). To test the effect of magnesium-ions on the psi-type CD signal of the thylakoid membranes, we isolated membranes in two different ways, in the presence or in the absence of 5 mM $MgCl_2$ (Fig. 21).

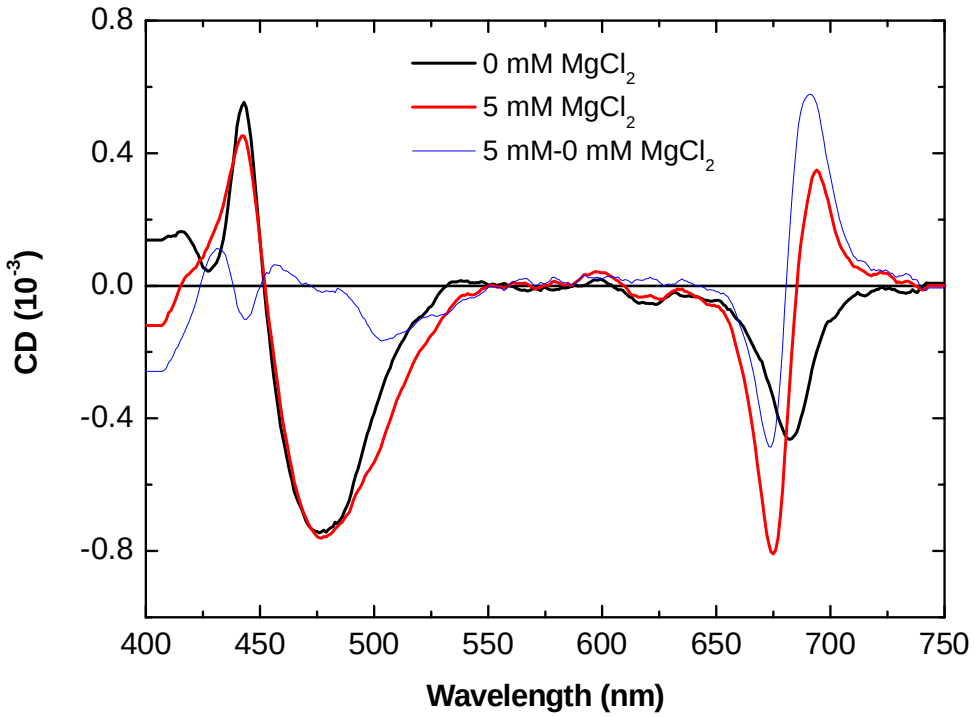

Figure 21. CD spectra of thylakoid membranes isolated from *P. tricornutum* in the absence and presence of 5 mM $MgCl_2$ and the corresponding difference spectrum.Chl content, 20 μg/ml.

When thylakoids were isolated without $MgCl_2$ their CD spectrum in the red region lacked the (+)698 nm band, characteristic of the CD spectra of intact cells. In the presence of $MgCl_2$ during the isolation, the macrodomain organization of the thylakoid membranes was better preserved, since the (+)698 nm band appeared in the CD spectra, although a large part of its intensity was lost as compared to intact cells (compare Fig. 21 to Fig. 10). It is to be noted that the bandshape of the (-)679 nm band changed its shape and position in the presence of $MgCl_2$ compared to thylakoids isolated in the absence of this cation; part of these changes might have been caused by the partial restoration of the psi-type signals but cations probably also induced alterations in the excitonic interactions. In general, the shape of the CD spectrum of thylakoids in the presence of $MgCl_2$ resembles more the spectrum of intact cells in the red region (difference spectrum in Fig. 21) than in its absence, thus indicating a more intact chiral macrodomain organization in the presence of Mg^{2+}.

In another set of experiments we isolated thylakoid membranes in the absence of $MgCl_2$ and administered the cations or the osmoticum afterwards. After isolation, thylakoids were suspended in HEPES buffers containing i) no addition, ii) 25 mM $MgCl_2$, and iii) 25 mM $MgCl_2$ + 350 mM sorbitol (Fig. 22).

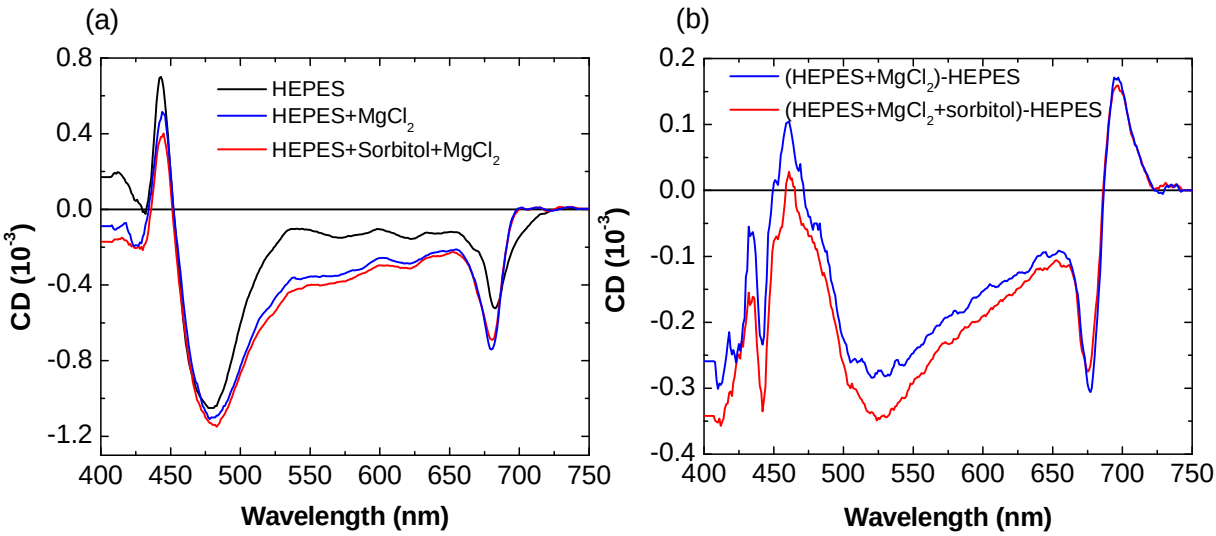

Figure 22. CD spectra of isolated thylakoid membranes suspended in different reaction media (a), and the corresponding difference spectra (b). Thylakoids were suspended for 5 min in 50 mM HEPES, 50 mM HEPES + 25 mM $MgCl_2$ and 50 mM HEPES + 25 mM $MgCl_2$ + 350 mM sorbitol, as indicated. Chl content, 20 µg/ml.

As shown in Figure 22a, adding Mg^{2+} changed the CD spectra, but there were no further changes upon adding sorbitol after Mg^{2+}: the negative CD in the region between 500 and 550 nm became more negative, as did the (-)679 nm band. In the region at around 698 nm a small increase appeared, which was completely absent in thylakoids incubated in the HEPES buffer only. This peak is better visualized in the difference spectra on Fig. 22b. This band, which appeared upon adding Mg^{2+} to the isolated thylakoid membranes strongly resembles the band found at the same wavelength in the CD spectra of intact cells. This indicates that Mg^{2+} was able partially restore the native structure of the thylakoid membrane, i.e., the macrodomain organization of the complexes.

In higher plants, divalent cations play important role in membrane stacking. By this means, it prevents the 'spillover' of excitation energy from PSII to PSI, which depends on the spatial separation of the two photosystems (for a recent review see Chow et al. 2005). The lateral separation of PSII and PSI leads to the increase of the chloroplasts fluorescence

(Telfer et al. 1976). Mg-ions have also been shown to increase the maximum quantum yield of PSII and its capacity for the NPQ (Noctor et al. 1993; Rees and Horton, 1990).

Along with the CD measurements, F_v/F_m and NPQ values were also measured on thylakoid membranes treated as described above.

	F_o	F_m	F_v/F_m	NPQ
HEPES	0.20	0.23	0.17	0.03
HEPES+$MgCl_2$	0.17	0.27	0.37	0.19
HEPES+$MgCl_2$+Sorbitol	0.17	0.28	0.39	0.26
Intact cells	0.12	0.38	0.69	1.13

Table IV. Chlorophyll fluorescence parameters of intact cells and isolated thylakoid membranes of *Phaeodactylum tricornutum* suspended in different media. The membranes with a chlorophyll content of 20 µg/ml were suspended for 5 min in 50 mM HEPES-containing buffers supplemented with 25 mM $MgCl_2$ and 350 mM sorbitol. The light intensity of the saturating pulse and the actinic light illumination were 4.000 and 700 µmol photons m^{-2} s^{-1}, respectively. n=5, SD<10%.

As shown in Table IV, Chl fluorescence parameters of the thylakoid membranes of *P. tricornutum* were also sensitive to Mg^{2+}. In general, the F_v/F_m values were considerably lower in isolated thylakoid membranes than in intact cells, indicating structural deteriorations during isolation, an observation consistent with the CD data. Addition of Mg^{2+} caused an increase in F_v/F_m by about 35%, while F_0 decreased by about 12%, indicating a partially restored association of FCP with the reaction center complexes. The additional presence of sorbitol led to a further small increase of the maximum quantum yield (measured as Fv/Fm) and NPQ.

We also tested the effect of $MgCl_2$ on NPQ. The overall NPQ in isolated thylakoids was always considerably lower than in intact cells, again evidently due to impairments caused by the isolation procedure (Table IV). Isolated thylakoid membranes of diatoms easily lose their whole chain electron transport activity; no matter how carefully they were prepared and what isolation method was used (Martinson et al. 1998, Jakob and Goss, personal communication). The magnitude of NPQ increased significantly in thylakoids in the presence of Mg^{2+}. Again, sorbitol had only little effect. We also tested the de-epoxidation efficiency of thylakoid membranes by using HPLC. The de-epoxidation ratio

([Dtx]/[Dtx]+[Ddx]) was measured in thylakoid membranes suspended in HEPES buffer only and in HEPES buffer containing 25 mM $MgCl_2$ (Fig. 23).

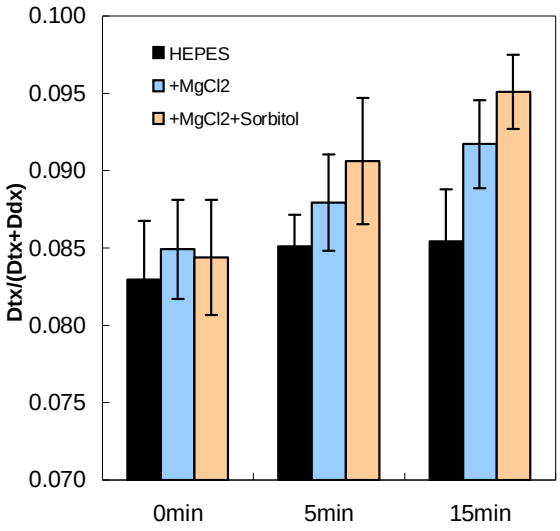

Figure 23. De-epoxidation state of isolated thylakoid membranes suspended in HEPES buffer only and in HEPES buffer containing 25 mM $MgCl_2$ and 25 mM $MgCl_2$ + 350 mM Sorbitol. Thylakoid membranes were illuminated for 0, 5 and 15 min with white light of 800 μmol photons m^{-2} s^{-1} PFD.

It can be seen that the ability of thylakoid membranes to accumulate Dtx is considerably higher in the presence of Mg^{2+} ions and is even more expressed if sorbitol is also present.

Hence, it can be concluded that Mg-ions not only restore, at least partly, the macrostructure of the thylakoid membranes but also enhance the light-harvesting and quenching capacity of the membranes. Interestingly, not only Mg^{2+} plays a major role in restoring the macrodomain organization; sorbitol also exerts positive effects on the Chl fluorescence parameters related to PSII photochemistry, NPQ and Ddx cycle activities (Table IV and Fig. 23).

The above data, correlations between the CD and Chl fluorescence parameters, show that, similar to plants, the fully functional state of the entire apparatus can only be granted with the unperturbed macro-organization of the complexes. Conversely, it can be inferred that reorganizations reflected by changes in the psi-type CD bands play an important role in regulatory processes; a similar conclusion has been reached for higher plant thylakoid membranes.

4.4. Macro-organization of pigment-protein complexes in *Cyclotella meneghiniana*

We have shown in the previous chapters that the pigment molecules are arranged into chirally organized macrodomains in *P. tricornutum* cells, while in isolated thylakoid membranes and in pigment-protein complexes mostly only the excitonic couplings were observed. It was an important aim of the present study to investigate the pigment interactions in another diatom species and answer the question whether the chiral macro-organization is a unique feature in this diatom or can be found in other diatoms as well.

Therefore we investigated *Cyclotella meneghiniana*, which is a centric diatom with a radial cell symmetry. A difference as compared to *P. tricornutum* that these cells contain several small chloroplasts per cell, but otherwise the thylakoid membrane organization within these chloroplasts (i.e. the group of three stacked membranes) is similar to *P. tricornutum*.

For *C. meneghiniana*, the CD spectra were also measured on intact cells, isolated thylakoid membranes, and FCP complexes. In intact cells, a large psi-type signal could be observed at around (+)694 nm, which is lost upon the isolation of thylakoid membranes (Fig. 24). In intact cells and FCP complexes a strong CD band could be observed at around (-)675 nm; in isolated thylakoid membranes this band was red-shifted to about 680 nm. In isolated thylakoid membranes and FCP from *C. meneghiniana* the (-)470 nm band is broader as compared to that of the same preparations from *P. tricornutum*. The Q_y band of Chl *a* in isolated FCP from *C. meneghiniana* is more complex as compared to the same band in *P. tricornutum* preparations, here a broad band can be observed at around 673 nm. This negative band was identified earlier as CD signature of trimeric and oligomeric FCPs (Büchel, 2003).

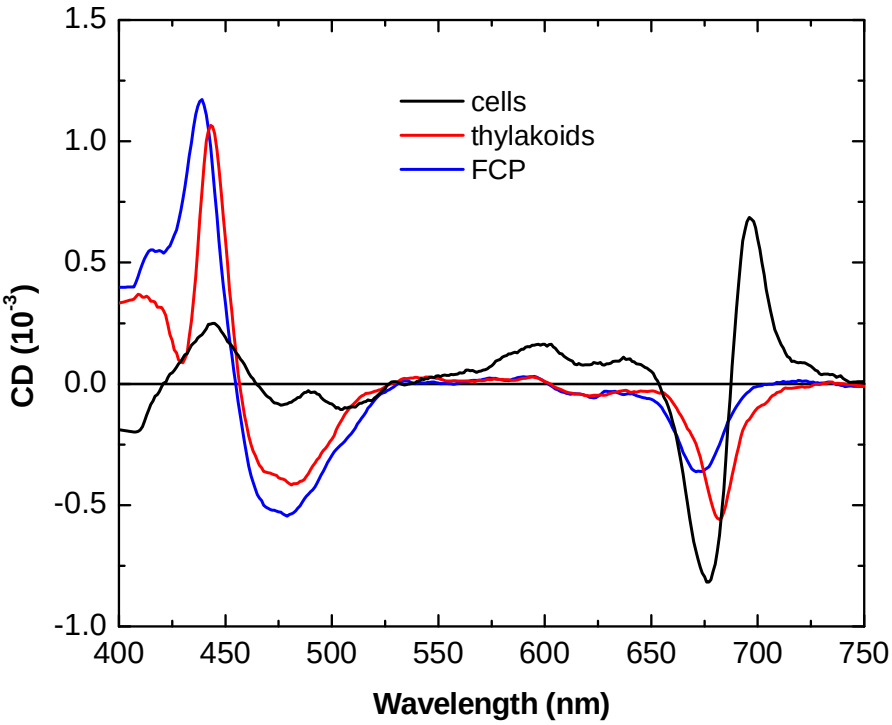

Figure 24. CD spectra of intact cells, isolated thylakoid membranes and FCP complexes of *Cyclotella meneghiniana.* The spectra were recorded at identical Chl contents (20 µg/ml).

Thus, it can be concluded that pigments are arranged into chiral macrodomains also in *C. meneghiniana.*

The structural flexibility of the macroaggregates was also tested in this diatom species. Upon illumination of intact cells with strong white light, the amplitude of the (+)694 and - to a lesser extent - the (-)675 nm band increased, while the excitonic bands remained essentially unchanged (Fig. 25).

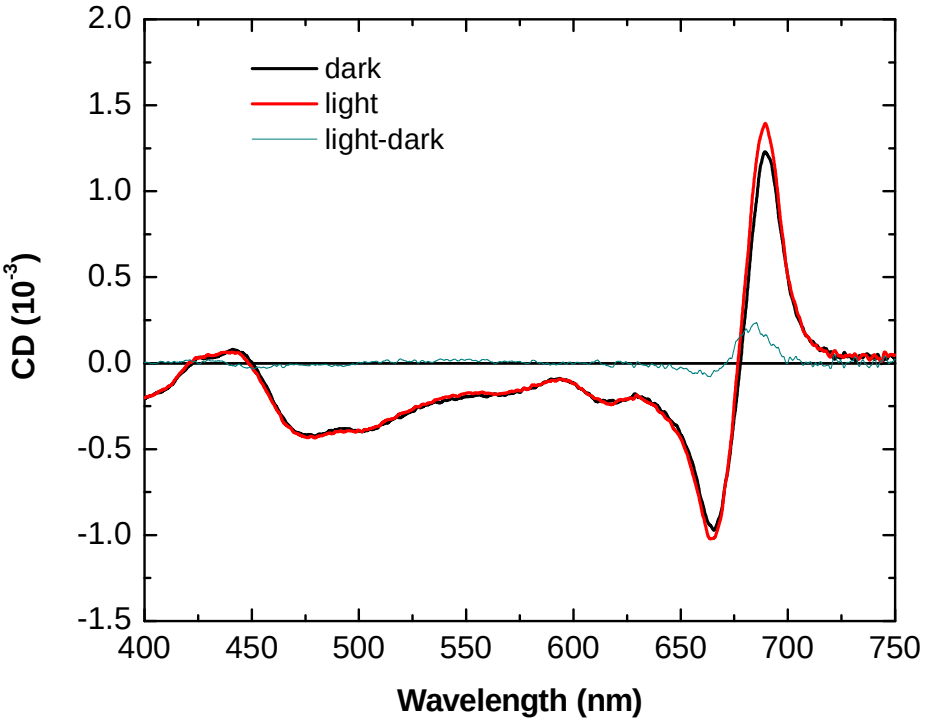

Figure 25. CD spectra of dark-adapted and preilluminated *C. meneghiniana* cells and the difference CD spectrum of illuminated-minus-dark-adapted cells. The measurements were performed at room temperature; the light intensity of illumination was 800 µmol photons m^{-2} s^{-1}; Chl content, 20 µg/ml.

The effect of elevated temperatures was also tested on intact *C. meneghiniana* cells (Fig. 26).

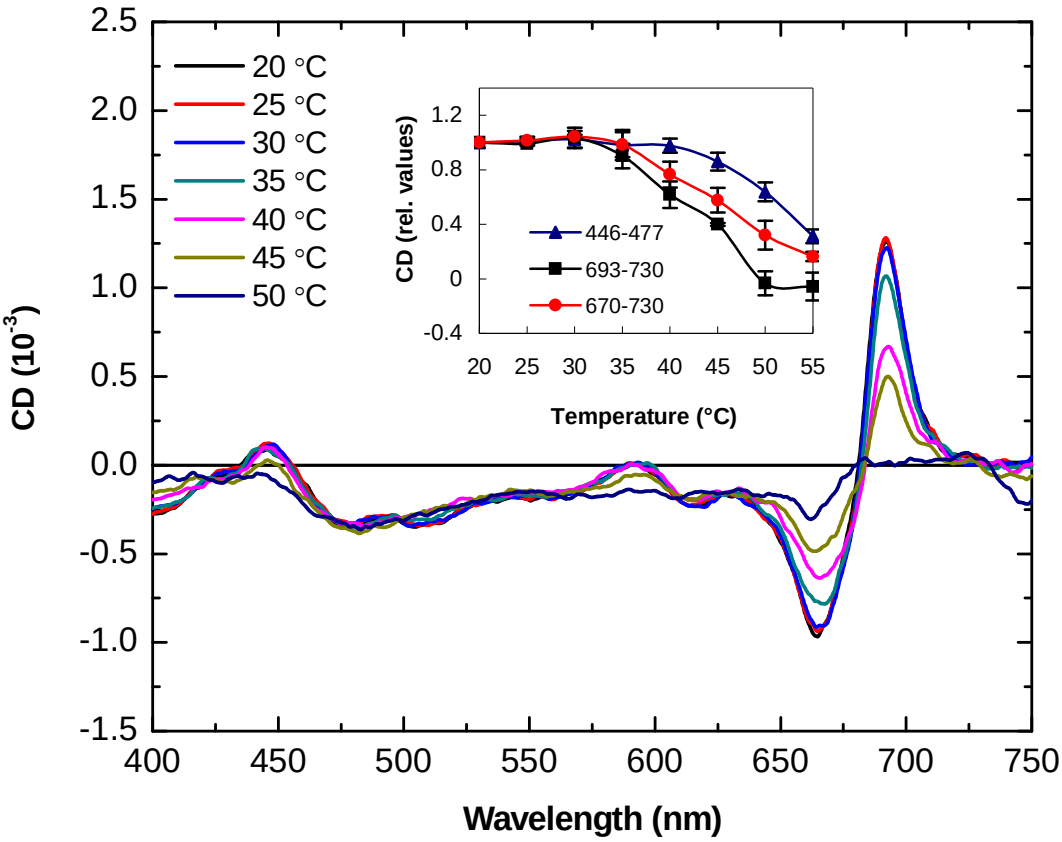

Figure 26. Effect of the incubation temperature on the CD spectra of intact *Cyclotella meneghiniana* cells. Inset, temperature dependences of the CD signals at 693 nm, 670 nm and at 445 nm relative to the amplitudes measured at 20 °C. Cells were incubated consecutively for 10 min at the indicated temperature and measured at the same temperature. Chl content, 20 µg/ml.

By using heat treatment of intact cells, the amplitude of both the (+)694 nm and (-)671 nm band decreased, and above 45 °C the (+)694 band disappeared (Fig. 26). The inset in Fig. 26 shows the temperature dependencies of selected CD signals; which differs to some extent from the dependencies of the corresponding bands in *P. tricornutum*: the (+)693 band and the (-)671 nm band followed quite similar temperature dependencies, thus the (-)671 nm band seems to possess more intense psi-type features than the corresponding (-)679 nm band in *P. tricornutum.* The excitonic CD bands at around 445 nm were unaffected up to 45 °C, but above this temperature the amplitude of this bandpair decreased, indicating the loss of excitonic interactions.

The effect of Mg^{2+} ions on the chiral macrostructure was also tested on *C. meneghiniana* (Fig. 27).

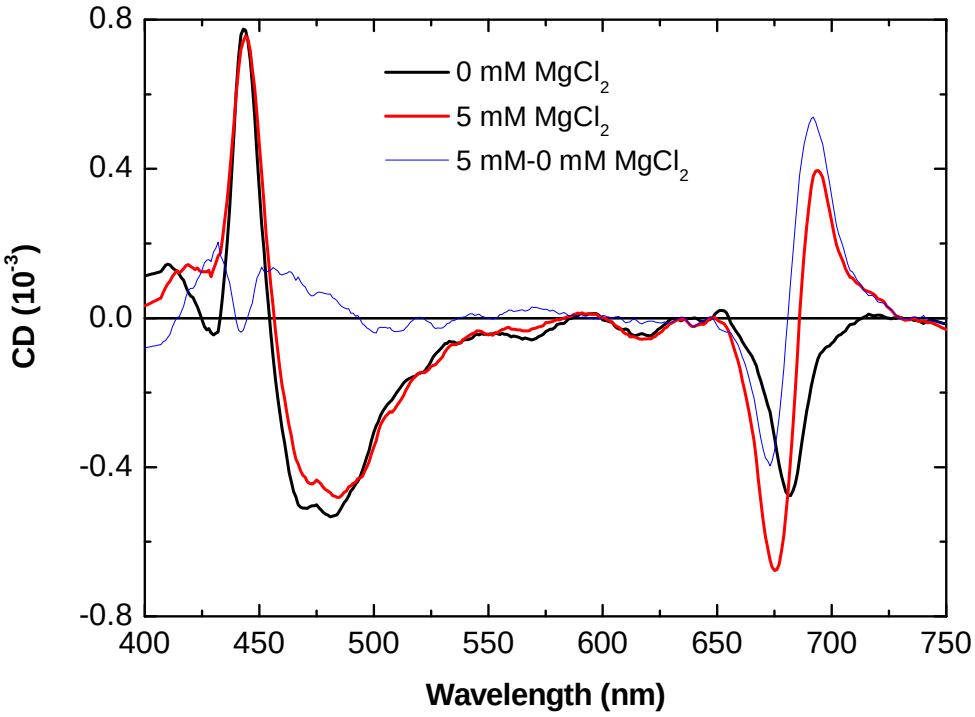

Figure 27. CD spectra of thylakoid membranes isolated from *C. meneghiniana* either in the absence or in the presence of 5 mM $MgCl_2$ and the corresponding difference spectrum. Chl content, 20 μg/ml.

Similarly to *P. tricornutum*, Mg^{2+} ions exerted strong effect on the CD spectra of isolated thylakoid membranes. When thylakoids were isolated in the presence of 5 mM $MgCl_2$, a strong psi-type CD signal could be observed at around 695 nm and the (-)671 nm band was also more intense. The difference spectrum in the red region resembles to the spectrum of intact cells. The excitonic interactions were also affected as it is visible between 400 and 500 nm. This indicates that Mg^{2+} ions restore to some extent the chiral macrodomain organization in *C. meneghiniana* thylakoid membranes – similarly to the thylakoids of *P. tricornutum*.

4.5. Functional heterogeneity of the fucoxanthins and FCP complexes in diatom cells

4.5.1 Spectroscopic indications of heterogeneity of fucoxanthin in P. tricornutum

Pigment-pigment interactions can be well characterized by using CD spectroscopy, however it gives only little information about the microenvironment of the interacting pigments. To gain further information about the orientation and the local environment of pigment molecules absorbing in the green `window`, we analyzed the flash-induced electrochromic absorbance changes of intact cells of *P. tricornutum* between 470 and 570 nm. Upon the excitation of photosynthetic membranes with a single turnover saturating flash, a uniform transmembrane electrical field of 10^5 V/cm magnitude is built up (Kakitani et al. 1982). This field induces an almost homogeneous shift of the absorption bands of the so-called field-indicating pigment molecules; the magnitude of the shift is linearly proportional to the field strength (Cramer and Crofts, 1982; Junge, 1977). The primary factor, which governs the shift, is the interaction of the ground and excited state of the pigment molecule with the external field. The frequency shift is given as:

$$\Delta\nu = \frac{1}{hc}\left(-\Delta\mu \cdot \vec{F} - \frac{1}{2}\Delta\alpha F^2\right)$$

where $\Delta\nu$ denotes the frequency shift (cm^{-1}) and $\Delta\mu$ and $\Delta\alpha$ denotes the difference between ground- and excited-state dipole moments and polarizabilities, respectively and $\boldsymbol{F}$ is the electric field vector (Kakitani et al. 1982). Carotenoid molecules, which do not possess permanent dipole moment, respond quadratically to the electric field, while molecules having permanent dipole moment, exhibit linear absorption shift field dependencies. Thus, different pigment molecules do not respond equally to the electric field and only certain fraction of molecules exhibit characteristic, large electrochromic absorbance changes.

Flash-induced electrochromic absorbance changes were measured between 470 and 570 nm in 5 nm steps. The kinetics of typical flash-induced absorbance transients at characteristic wavelengths are depicted in Fig. 28 (insets), which show the transient spectrum calculated from the kinetic transients of the electrochromic absorbance changes.

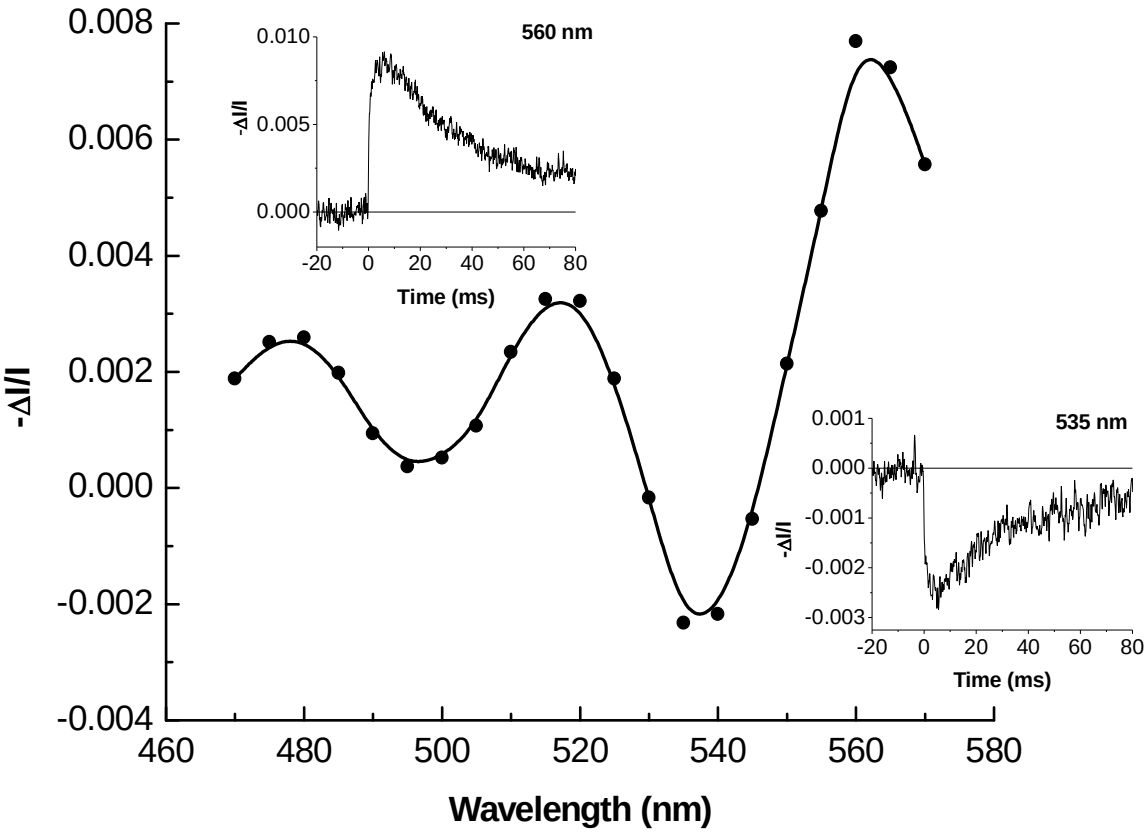

Figure 28. Transient spectra of flash-induced absorbance changes, dominated by electrochromic changes, in dark-adapted intact cells of *Phaeodactylum tricornutum* 5 ms after the exciting flash. Flash-induced electrochromic absorbance transients at the indicated wavelengths (insets).

The transient spectra revealed a major band-shift with a bandpair at (+)565/(-)535 nm and a minor band-shift with a bandpair at (+)515/(-)485 nm. In a recent study, with the aid of absorption and Stark spectroscopy on FCP complexes isolated from *Cyclotella meneghiniana*, three main Fx forms, Fx_{blue}, Fx_{green} and Fx_{red} with wavelength positions at around 465, 515 and 545 nm, respectively, were identified. The lower-energy Fx_{red} and Fx_{green} forms exhibited large changes in static dipole moment on photon absorption, of about 40 and 15 D, respectively (Premvardhan et al. 2008). The above described terminology for the different Fx pools is used also in the present work, because these different forms are spectrally well discernible, according to their wavelength position. The strong electrochromic response of these Fx molecules shows that they probably interact with Chl molecules, thereby lending a dipole moment to this molecule. In higher plants, the strongest electrochromic response, at around 515 nm, is attributed to lutein/Chl *b* interactions (Sewe and Reich, 1977). Earlier, we hypothesized that the long-wavelength absorbing, field-indicating lutein/Chl *b* pigment pair in the LHCII might be replaced by a similar Fx/Chl *c* pair in the FCP (Szabó et al. 2008, see in 'Publications'). However, recent

results showed that strong interaction occur between Fx molecules rather than between Fx and Chl molecules (Premvardhan et al. 2008). These data also show that, similarly to higher plants, purple bacteria and Chl *a/c*-containing algae (Büchel and Garab, 1995; de Grooth et al. 1980; Joliot and Joliot, 1989; Kakitani et al. 1982), the electrochromic absorbance changes in *P. tricornutum* originate from a minor fraction of pigment molecules.

In our work, spectroscopic properties of the different Fx molecules have also been characterized. Intact *P. tricornutum* cells exhibit long absorption shoulder between 480 and 560 nm originating from different Fx pools (Fig. 29). Fx_{red} molecules exhibit red-shifted and weak absorbance band at around 545 nm, and display only weak CD signal in this region (see e.g. Fig. 18). It is interesting to note that a similar, weak, CD-silent long-wavelength (~535 nm) absorbing band was identified in a marine green alga, and was assigned to originate from a new electronic excited state, S_x between S_1 and S_2, of siphonaxanthin, which appears to transfer to a specific Chl *a* molecule(s) (Akimoto et al. 2004, 2007). This state arises only in pigment-protein complexes, probably due to a specific interaction with amino acids (Akimoto et al. 2007), and resembles the long wavelength absorbance band of Fx in diatoms (Gillbro et al. 1993).

Fluorescence excitation spectra revealed that both short and long wavelength forms exhibit efficient energy transfer to Chl *a*, where the energy transfer efficiency of Fx_{red} is especially remarkable, since it displays much weaker absorption (Fig. 29). This result is in accordance with earlier data of energy transfer efficiencies measured on isolated FCP (Papagiannakis et al. 2005).

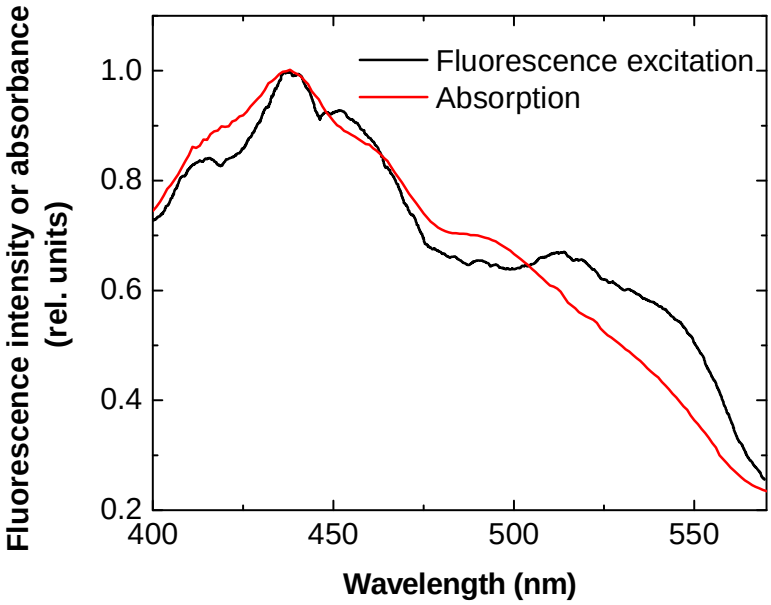

Figure 29. Fluorescence excitation and absorption spectra of *P. tricornutum* cells. Fluorescence was recorded at 689 nm. For better comparison, the spectra are normalized at 438 nm.

Linear dichroism measurements of intact *P. tricornutum* cells revealed major bands peaking at 425 nm, 550 nm and 695 nm (Fig. 30).

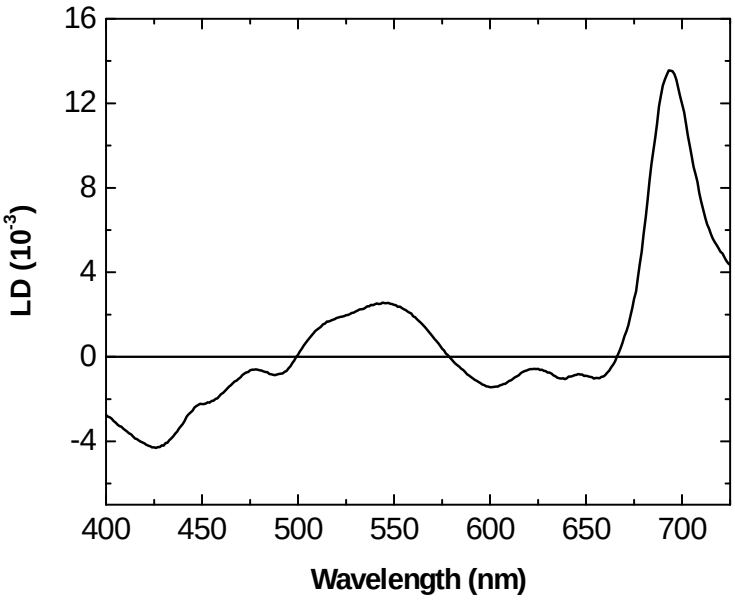

Figure 30. Linear dichroism spectrum of *P. tricornutum* cells aligned by gel squeezing, $OD_{673-750}$ = 0.35.

The 425 and 695 nm bands originate from Chl *a*. Minor bands at around 463 and 600-630 nm, originating from Chl *c*, at around 495 nm, originating probably from Ddx and the bands at 510 nm and 545 nm, originating from Fx could also be observed.

4.5.2 The effect of growth light intensity to the heterogeneity of fucoxanthin in P. tricornutum

It was also the aim of the present work to characterize the heterogeneity of the Fx molecules in intact cells grown on different light conditions. Different light conditions during growth can modify the pigment composition and Fcp polypeptide composition of diatom cells. From the point of view of the efficiency of the excitation energy transfer, the microenvironment and orientation of carotenoid molecules are determinant factors, which are also supposed to change with the growth light intensity.

Absorption spectra were measured on HL and LL cells (Fig. 31). Both HL and LL cells exhibit absorption peaks at 673, 633 and 437 nm, and shoulders at 460 and 490 nm. The position and shape of the unusually broad and complex bands between 490 and 570 nm, originating mostly from Fx, did not differ substantially between HL and LL cells. In HL cells, the more intense absorption bands between 400 and 500 nm indicate that the photoprotective carotenoids, diadino- and diatoxanthin accumulated.

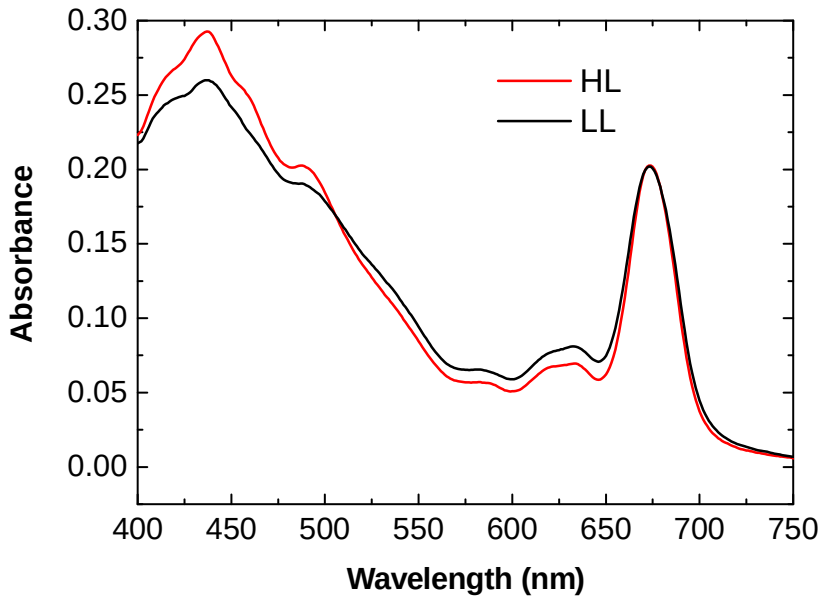

Figure 31. Absorption spectra of *P. tricornutum* cells grown on HL or LL intensities. Samples were adjusted to the same Q_y absorption of Chl *a*.

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In order to obtain information on the local environment and polarizabilities of carotenoids within the thylakoid membranes of intact diatom cells grown at different light intensities, electrochromic absorbance changes were measured on HL and LL cells. Strong electrochromic signal at around (+)565 and (-)535 and a less intense signal at (+)515 and (-)485 nm could be observed in both LL and HL cells. The peak positions did not vary with the growth light conditions, however, the amplitudes of the bands exhibited considerable differences. In LL cells, the longer wavelength absorbance transient (565/535 nm) was more intense, while the shorter wavelength band (515/485 nm) did not change considerably compared to HL cells (Fig. 32a). The ordinate of the transient spectra are shifted to zero at 470 nm, to correct for an observed baseline shift. The origin of the baseline anomaly is unknown; it has been shown earlier that the electrochromic absorbance changes can be accompanied by scattering transients, which are physically correlated to the absorption changes (Garab et al. 1979).

The light-induced electrochromic absorbance transient spectra are composed of the first derivatives of the absorbance bands of the participating electronic transitions (Büchel and Garab, 1995). This specificity can be used to identify the field-indicating pigment molecules and determine their concentrations. The information is essentially the same that can be obtained from Stark spectroscopy using external field on randomly oriented protein complexes (Boxer, 2009). The measured points at the indicated wavelengths together with the fitting curves are depicted in Fig. 32a.

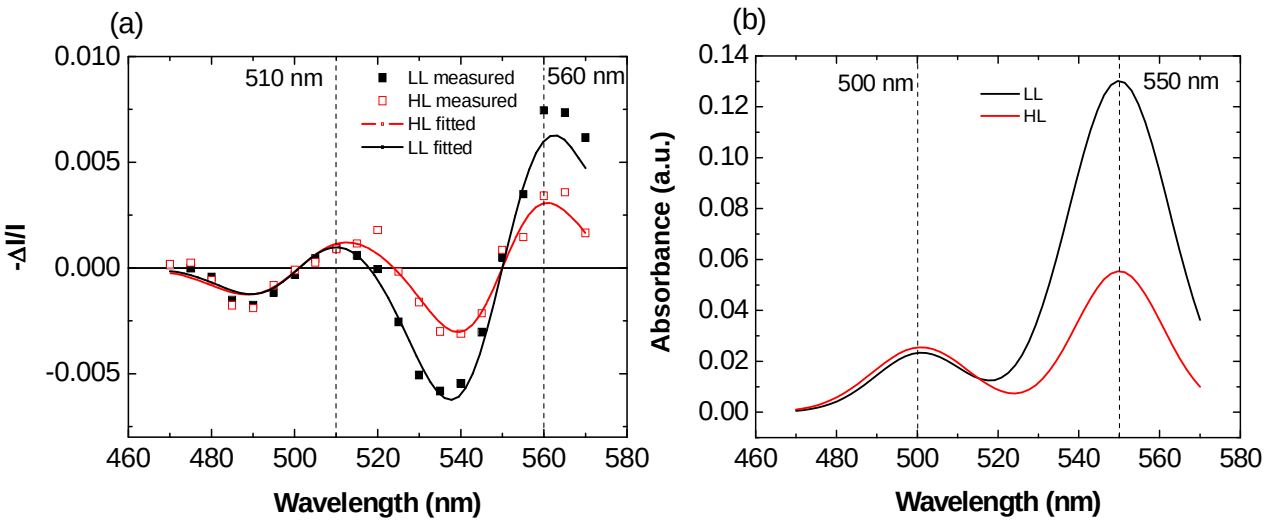

Figure 32. Electrochromic absorbance transients of HL (open squares) and LL (closed squares) cells of *P. tricornutum* (a), and absorbance bands of the field-sensitive pigments obtained from the fits of the transients (b) of HL and LL cells. The data points, 5 ms after the exciting flashes, were obtained from kinetic traces after averaging 32 transients, the repetition rate of the flashes was 1 s^{-1}. In the transient spectra, the data points were fitted with first derivatives of Gaussians of HL and LL cells. Samples were adjusted to the same Chl *a* Q_y absorption, $OD_{673\text{-}750} \approx 1$.

The parameters obtained from fitting with first derivatives of Gaussians are shown in Table V. These parameters were used to reconstitute the absorbance spectrum of shifted pigments, which are shown in Fig. 32b.

	HL (565/535)	HL(515/485)	LL(565/535)	LL(515/485)
x_0	550±0.6	501±1.3	550±0.35	501±1.5
k	-1.7±0.21	-0.81±0.9	-5±0.33	-0.85±0.8
h	11.1±0.8	11.7±1.4	13.4±0.4	11.5±1.6

Table V. Parameters obtained from mathematical fit with first derivatives of gaussians of the measured spectra of fast absorbance changes of HL and LL cells. The parameters x_0, k and h are defined in Chapter 3.2.5.

The electrochromic transients in LL and HL cells differed significantly from each other (Fig. 32a): LL cells contained significantly larger contributions from Fx_{red} than HL cells. As determined from the calculated absorption spectra (Fig. 32b), in HL cells the intensity of the Fx_{red} band was 2.2 ± 0.2 times larger than that of Fx_{green}. In LL cells, the Fx_{red}/Fx_{green} ratio was 5.8 ± 0.7. Assuming no change in the electrostatic properties of the two forms,

this difference must originate from an accumulation of Fx_{red} in LL cells. This means that Fx_{red} and Fx_{green} originate from different FCPs. In other words, the fact that the ratio of the two electrochromic bands varies in a broad range in HL and LL cells indicates that the two Fx forms are bound to different FCPs. At the given signal to noise ratio, no other electrochromic band could be identified, suggesting that the differences between the ground- and excited-state dipole moments of all other pigment molecules absorbing between 470 and 570 nm are significantly weaker. Neither was there a contribution from Ddx or Dtx, although the amount of these xanthophylls was 3-4 times higher in HL cells than in LL cells, calculated on the basis of the same Chl *a* content. The Fx content on the same basis was also found to remain unchanged, as verified by HPLC (Fig. 33).

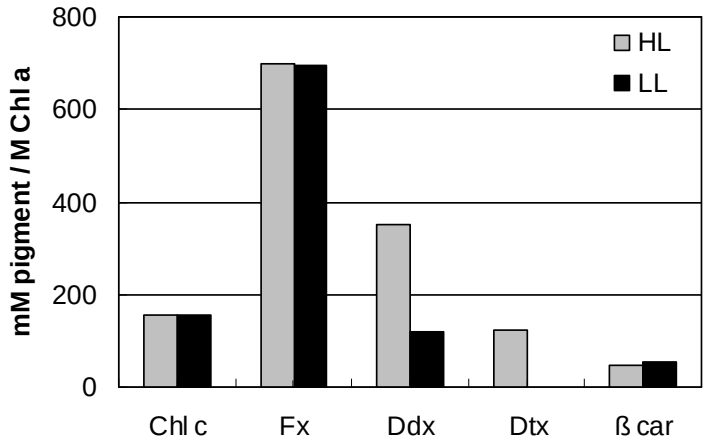

Figure 33. Pigment content of HL and LL *P. tricornutum* cells determined by HPLC.

This is in a good agreement with the results of Lepetit et al. (2010). They also found that by increasing the light intensity during growth only the Ddx cycle pigments accumulated, while the amount of Fx and Chl *c* remained unchanged both in *P. tricornutum* and *C. meneghiniana*.

In order to obtain information on the orientation of Fx molecules in cells grown either on HL or LL light, LD measurements were performed. The two Fx forms were clearly discernible in the LD spectra of both HL and LL cells as it is shown in Fig. 34.

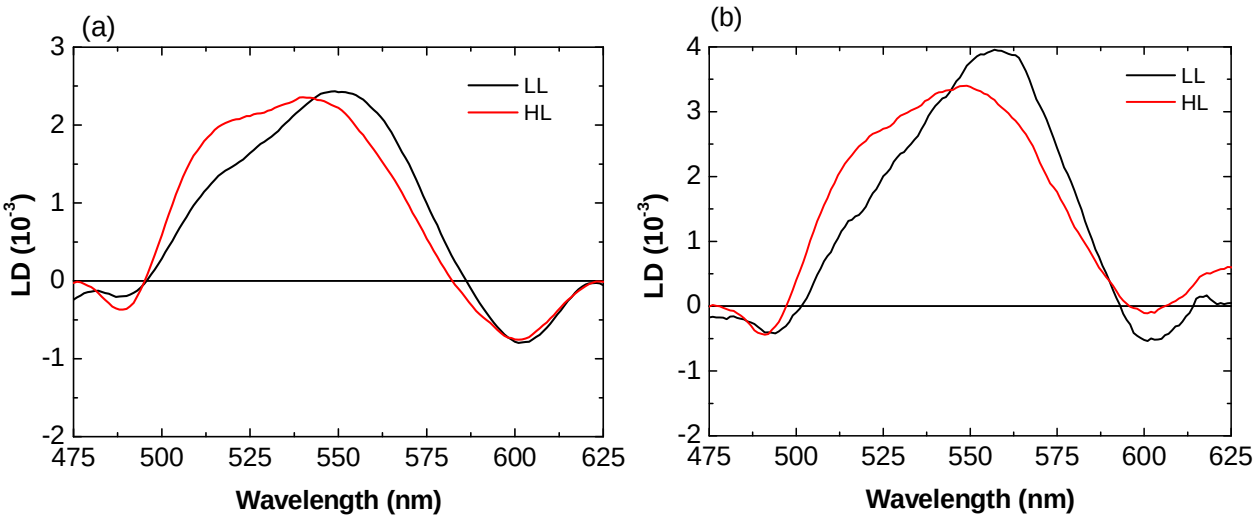

Figure 34. Linear dichroism spectra of HL and LL cells oriented by gel squeezing method (a) and by magnetic field of 0.5 T (b). The Chl contents of the two samples were equal, $OD_{673-750}$ = 0.35.

Both in HL and LL cells Fx_{red} at around 550 nm exhibited an intense signal, and in HL cells a stronger band appeared at around 515 nm. Essentially the same information could be obtained on intact cells oriented either by gel squeezing method (Fig. 34a) or by strong magnetic field (Fig. 34b). These results also show that intact *P. tricornutum* cells can be well oriented in magnetic field.

The characteristic LD bands at 550 and 515 nm could also be observed in the case of isolated thylakoid membranes isolated from HL and LL cells (Fig. 35). The LD spectra of HL and LL thylakoids were quite similar, significant changes could only be observed between 400 and 500 nm, which may result from the different Ddx and Dtx composition of HL and LL cells (shown in Fig. 33). In the case of thylakoid membranes the relative intensity of LD bands at 550 nm compared to 515 nm is lower than in the case of intact cells. Similar differences in the 515 and 550 nm band could also be observed earlier by comparing the LD spectra of intact cells and isolated thylakoids of *P. tricornutum* grown on normal light intensities (Hiller and Breton, 1992).

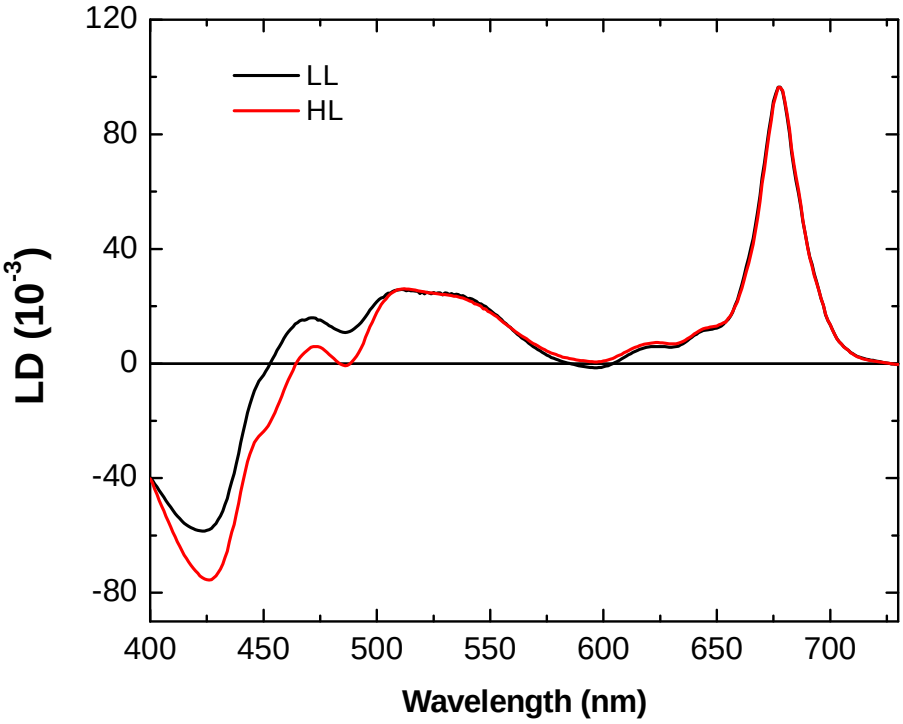

Figure 35. Linear dichroism spectra of isolated thylakoid membranes from HL and LL cells of *P. tricornutum*. Thylakoid membranes were oriented by gel-squeezing method. The Chl contents of the two samples were equal, $OD_{673-750}$ = 0.4.

Using the squeezing parameter M=2 and the calculated order parameter (Garab, 1996), the orientation angle of the transition dipole of Fx_{red} at 550 nm in membranes isolated from LL cells was found to be 29° with respect to the membrane plane. In comparison, the orientation angle of the Q_y transition of Chl *a* was calculated to be 17° in the same membranes. In contrast to the large signal at 550 nm, the LD signal at 500 nm was weaker, showing that the transition dipoles at around 500 nm, with a calculated 34-36° orientation angle, i.e. tend to tilt out somewhat more from the membrane plane than those at 550 nm. The LD spectrum most clearly indicates the presence of the so-called Fx_{green} being quite different from Fx_{red}, with the former exhibiting a much weaker signal.

In order to obtain information about the functional significance of the heterogeneity of the Fx forms and the FCPs, low-temperature fluorescence excitation and emission spectra were recorded. The excitation spectra revealed that energy transfer to Chl *a* occurs from both the short- and long-wavelength Fx forms (Fig. 36a and b).

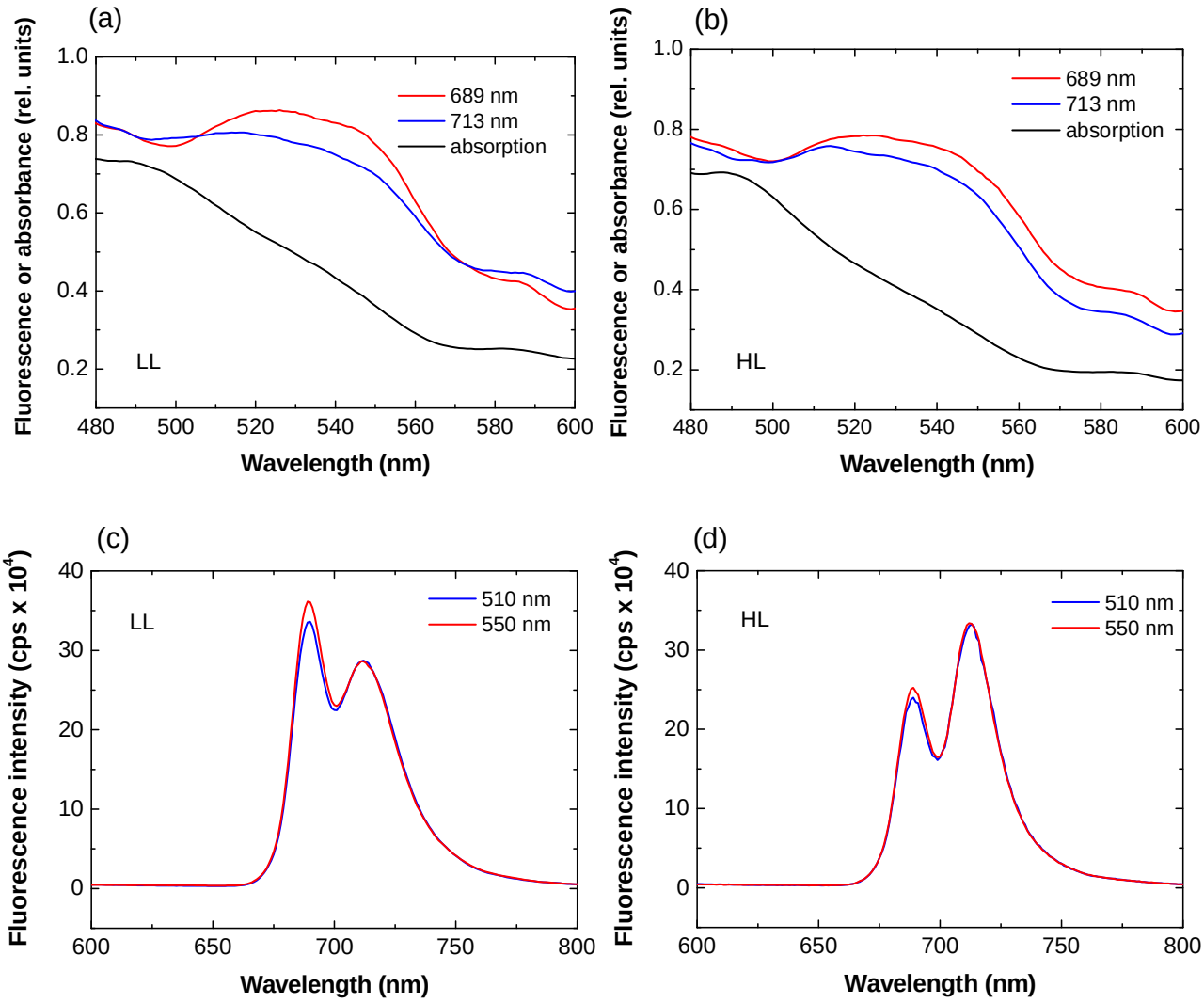

Figure 36. Low temperature (77 K) fluorescence excitation (a and b) and emission (c and d) spectra of LL (a and c) and HL (b and d) *P. tricornutum* cells. The fluorescence excitation spectra, normalized at 438 nm, were recorded for the 689 nm and the 713 nm bands. For the emission spectra, normalized at 713 nm, the cells were excited at 510 nm or at 550 nm. The room temperature absorbance spectra of LL (a) and HL (b) cells are also normalized to the 438 nm band of the excitation spectra. (The wavelength for normalization, 438 nm, was selected because of the maximum of Chl *a* absorbance and virtually no overlap with the two Fx forms in question, i.e. with Fx_{red} and Fx_{green}).

The excitation spectra also show that Fx_{green}, absorbing at around 500 nm, supplies energy about equally to the Chl *a* molecules that emit at 689 and 713 nm. In contrast, the fluorescence emission was stronger at 689 nm compared to 713 nm for excitation into the 520 – 560 nm range, in the Fx_{red}-absorbing region, in both LL (Fig. 36a) and HL (Fig. 36b) cells. Fluorescence emission spectra (Fig. 36c and d) indicate that upon excitation at 550 nm, both LL and HL cells exhibit somewhat stronger fluorescence emission at 689 nm,

indicating that Fx_{red} favours energy transfer to F_{689} (PSII). In contrast, excitation at 510 nm of both LL and HL cells exhibit less intense fluorescence at 689 nm, indicating that Fx_{green} favours energy transfer to F_{713} (PSI) or has no preference for ET to either one of the PSs.

4.5.3 Heterogeneity of fucoxanthin molecules in C. meneghiniana

The electrochromic transient spectrum was measured also in *C. meneghiniana.* Typical kinetics of the flash-induced absorbance changes can be seen in the inset of Fig. 37. Like in *P. tricornutum*, two main electrochromic bands could be observed on the transient spectrum: a smaller band at (+)535 and (-)485 nm and a more intense one at (+)565 and (-)545 nm. The longer wavelength band is red-shifted compared to the respective band in *P. tricornutum* cells.

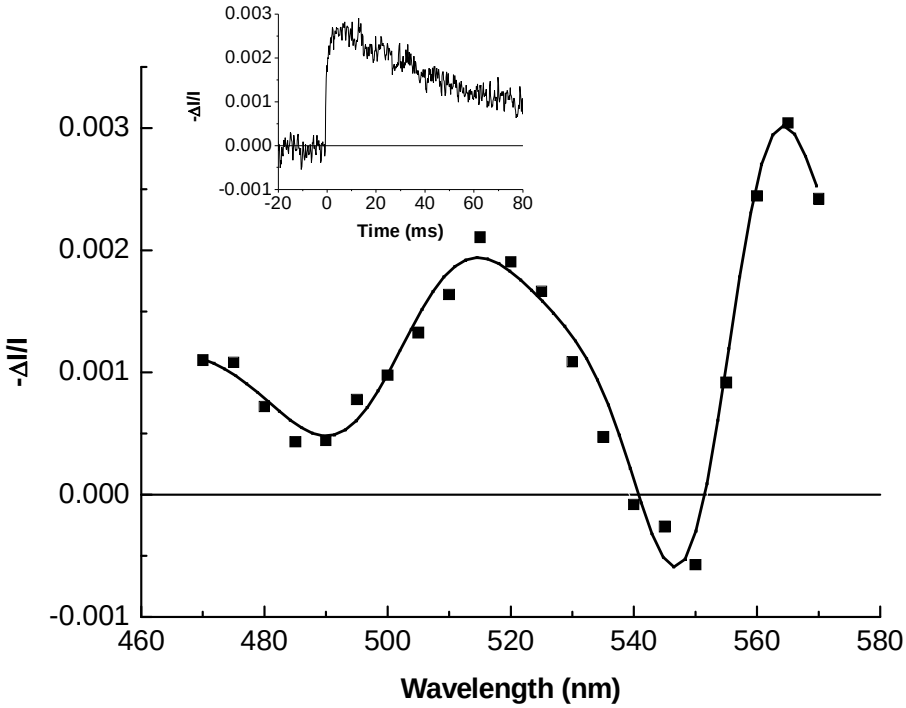

Figure 37. Transient spectrum, induced by single turnover saturating flashes, in intact cells of *Cyclotella meneghiniana.* The data points, 5 ms after the exciting flashes, were obtained from kinetic traces after averaging 32 transients, the repetition rate of the flashes was 1 s^{-1}. A typical trace, recorded at 560 nm, is shown in inset. The transient spectrum was fitted with the sum of first derivative of two Gaussians (solid line) and a constant – tentatively assigned to scattering transient.

The measured datapoints could be fitted reasonably well with the first derivative of Gaussians indicating the electrochromic nature of the Fx molecules also in *C. meneghiniana* cells. Similarly to *P. tricornutum*, an unknown shift of the transient spectra – probably due to scattering transients – could also be observed.

We attempted to record the LD spectra of intact cells and isolated thylakoid membranes of *C. meneghiniana*. However, we found that intact cells could not be aligned either by gel squeezing or by magnetic field, therefore no valuable LD spectra could be recorded on this level. This is probably because of the lack the longitudinal symmetry of these cells and the presence of several chloroplasts with different orientations. We were able, however, to measure the LD signature of isolated thylakoid membranes. Essentially similar LD spectra were obtained in *C. meneghiniana* thylakoid membranes, showing that the orientation of Fx_{red} and Fx_{green} are essentially the same as in *P. tricornutum* (Fig. 38).

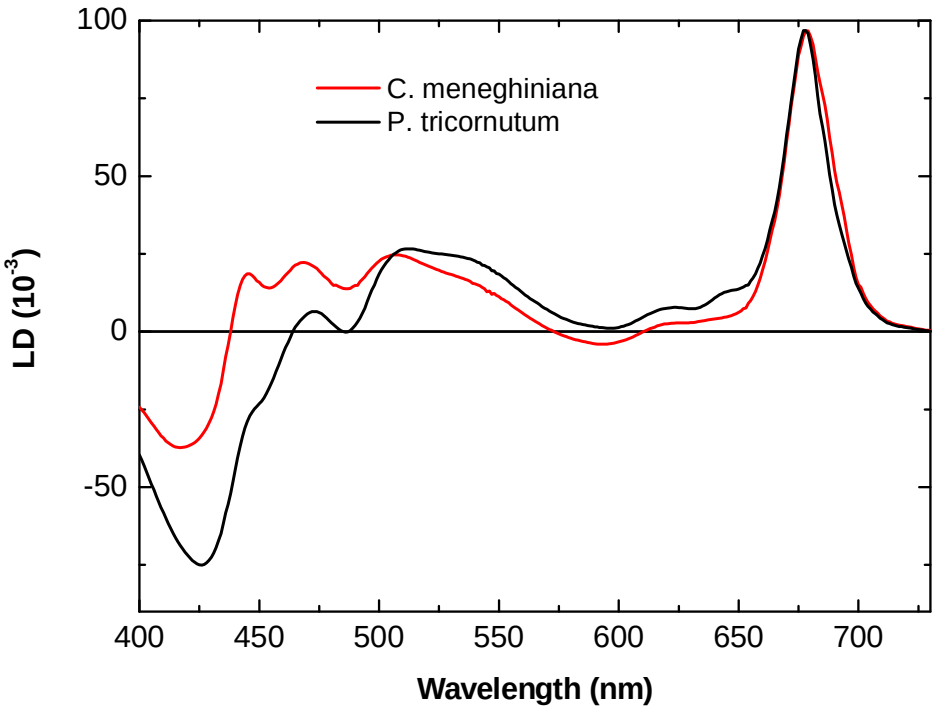

Figure 38. Linear dichroism spectra of isolated thylakoid membranes of *P. tricornutum* and *C. meneghiniana.* Thylakoid membranes were oriented by gel-squeezing method. The measured LD of *C. meneghiniana* was somewhat weaker, 0.09 at 679 nm, but for better comparison the two spectra are normalized to their red maxima.

The fluorescence excitation and emission spectra were measured on intact cells in order to obtain information about the excitation energy transfer efficiency of different Fx pools (Fig. 39).

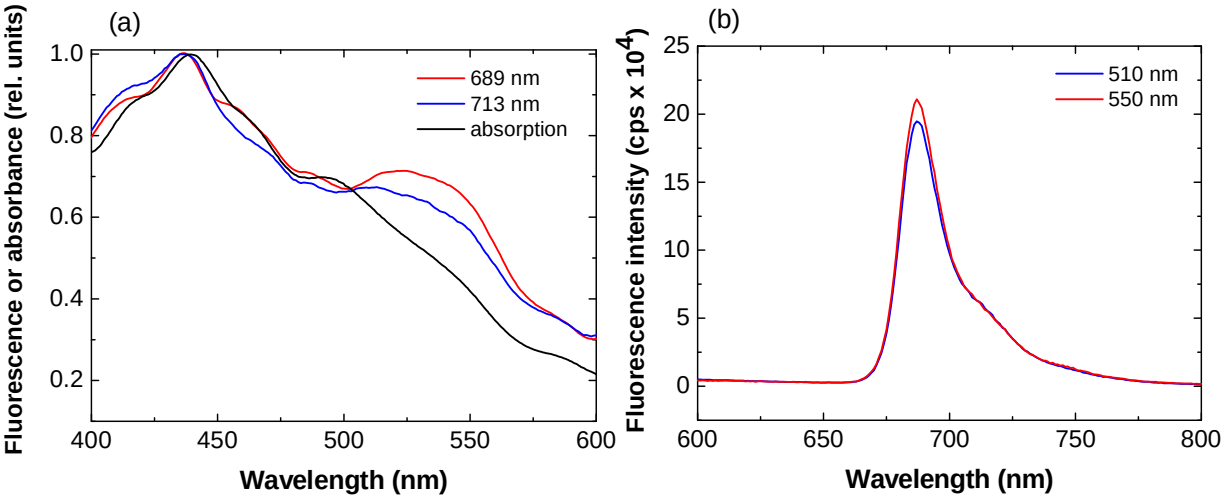

Figure 39. Low temperature (77 K) fluorescence excitation (a) and emission (b) spectra of *Cyclotella meneghiniana* cells. The fluorescence excitation spectra, normalized at 438 nm, were recorded for the 689 nm and the 713 nm bands. For the emission spectra, normalized at 713 nm, the cells were excited at 510 nm and at 550 nm. The room temperature absorbance spectrum of the cells (a) is also normalized to the 438 nm band of the excitation spectra.

The fluorescence excitation spectra indicate that Fx_{red} transfers excitation energy to Chl *a* somewhat more efficiently that Fx_{green}, as reflected by the stronger band between 520 and 555 nm (Fig. 39a). Fluorescence emission spectra exhibited a characteristic peak at 689 nm and a band at 713 nm (Fig. 39b). The 713 nm band was much weaker than in *P. tricornutum*. The 689 nm band was more intense upon excitation at 550 nm compared to the excitation at 510 nm.

5. DISCUSSION

The regulation of light-harvesting antennae on the supramolecular level is important in the adjustment of light-harvesting efficiency and adaptation to various stress conditions in all photosynthesizing organisms. It has been shown in the past decades that in higher plants the photosynthetic pigments are assembled into structurally flexible chirally-organized macrodomains, in mature granal thylakoid membranes. In contrast, there is no systematic study on the supramolecular organization of pigments on different organizational levels in diatoms.

5.1. The psi-type CD signal is associated with the multilamellar order of the thylakoid membranes in intact cells

I have shown in Chapter 4.2 that intact cells of *P. tricornutum* exhibited psi-type CD signatures at around 698 nm. Upon isolation of thylakoid membranes or disruption of cells by sonication, the psi-type features decreased to large extent, while the excitonic interactions were not affected, suggesting that Chl *a* molecules are arranged into chiral macrodomains, exhibiting long-range interactions. The chiral macrodomains, in accordance with the theory of psi-type CD for large ordered 3 dimensional arrays (Garab 1996; Keller and Bustamante, 1986), appeared to be associated with a multilamellar thylakoid membrane system in the cell. This was shown by TEM studies – the CD changes induced by sonication could be well correlated with the changes in the thylakoid membrane ultrastructure. Similar CD changes have been reported in other Chl *a/c*-containing algae upon disruption of intact cells or isolated chloroplasts (Büchel and Garab 1997; Goss et al. 2000). In *Mantoniella squamata*, much weaker psi-type CD signals are present in intact cells compared to higher plant chloroplasts, and no psi-type CD could be identified in the isolated LHC and thylakoid membranes (Goss et al. 2000). Intact cells and isolated chloroplasts of *Pleurochloris meiringensis* possess psi-type CD signals that are diminished when the chloroplasts are disrupted; it has also been found that the intensity of the psi-type signal changes with the light intensity during growth (Büchel and Garab, 1997). The sensitivity of (+)698 nm band, and possibly also the disappearance of the broad band in the blue, further corroborated our conclusion on the origin of this (these) band(s) in chirally organized macrodomains. The results presented in this work about the macro-

organization of chromophores in diatoms are thus in good agreement with other studies on other Chl *a/c*-containing algal species (Büchel and Garab, 1997; Goss et al. 2000).

The main difference between the organization of thylakoid membranes in higher plants and diatoms can be found in the lateral heterogeneity of the pigment-protein supercomplexes. Granal thylakoid membranes of higher plants, which are arranged in stacked multilamellar membrane system enriched in PSII-LHCII supercomplexes form large ordered chiral domains exhibiting psi-type CD signals (reviewed e.g. in Garab and Mustárdy, 1999). The thylakoid membranes of diatoms are also arranged into – loosely stacked - multilamellar membrane system; however, here we cannot speak about granal and stromal separation and lateral heterogeneity of PSII and PSI supercomplexes, thus the question arises what is the nature of the psi-type CD signals detected in *P. tricornutum*. To answer this question, we examined the (macro-)organization of the pigment system in vitro, in isolated FCP complexes and in isolated thylakoid membranes.

5.2. Isolated FCP complexes do not assemble into chiral macrodomains

In Chapter 4.1 I have shown that the CD spectra of isolated FCP complexes are dominated by excitonic interactions, and the psi-type CD signal is absent. However, the question arises whether the FCP complex isolated in this way represents the native state of the antenna system of *P. tricornutum*. By applying milder solubilization we have shown that it is possible to separate FCP complexes with different oligomeric states (Lepetit et al. 2007). The CD spectra of trimeric FCP and higher oligomeric FCPo presented in Fig. 12 are very similar to each other and lack psi-type signal, indicating that the isolated oligomeric antenna of *P. tricornutum* does not form chirally organized macrodomains or large aggregates in contrast to LHCII of higher plants. Nevertheless, the existence of somewhat stronger excitonic interactions in FCPo than in FCP suggests that FCPo represents a more native state of the light-harvesting antenna in *P. tricornutum*.

5.3. The chiral macrodomain organization of the pigments can be partially retained in isolated thylakoid membranes

In granal thylakoid membranes two external factors influence the long-range chiral order of the chromophores: i) electrostatic screening of the negatively charged polypeptides by divalent cations, which is required for the lateral separation of the two photosystems and the stacking of membranes, ii) the osmotic pressure of the medium,

which influences mainly the lateral packing density of the complexes (Barzda et al. 1994; Garab et al. 1991). It has been shown in higher plants that the absence of the divalent cations from the incubation medium resulted in a dramatic decrease of the heat-stability of isolated thylakoid membranes. The same phenomenon could be observed when isolated thylakoids were (re)suspended in hypotonic medium, however, the heat stability was preserved to more extent in this case (Cseh et al. 2000). This shows that the electrostatic interactions and divalent cations, i.e. electrostatic screening play crucial role in membrane stacking and the self-assembly of grana (Chow et al. 2005; Kiss et al. 2008; Barber, 1982), along with the lateral separation od PSII and PSI complexes and the formation of LHCII-enriched macrodomains within the thylakoids, which must precede the stacking (Barzda et al. 1994, 1996; Mustárdy et al. 2008). The role of membrane stacking is not confined to the maintaining of the structure of the grana membranes, but it is also essential in ensuring the physiological functions, e.g. preventing the spillover of excitation energy from PSII to PSI (Chow et al. 2005; Trissl and Wilhelm, 1993) and providing the photoprotective mechanisms, i.e. NPQ (Horton and Ruban, 2005). The stimulating effect of Mg^{2+} on NPQ was first shown by Noctor et al. (1993) and Rees and Horton (1990). Earlier electron microscopic and CD spectroscopic studies have provided evidence that incubation of thylakoids of higher plants in sorbitol and $MgCl_2$-containing media stabilizes grana stacking, whereas in buffers without sorbitol and $MgCl_2$ significant unstacking of grana membranes takes place (reviewed by Garab and Mustárdy, 1999). It has been shown that grana stacking is essential for the formation of NPQ, especially in its xanthophyll-cycle component (Goss et al. 2007). Disturbances in the macro-organization of the PSII antenna lead to a strong reduction of NPQ (Kovács et al. 2006).

The role of electrostatic screening by divalent cations and the ambient osmolarity in the regulation of the stacking of the thylakoids have been scarcely investigated diatoms. Due to the lack of the detailed structural information about the pigment-protein complexes and their interactions in the thylakoid membranes, the exact association of two membrane layers and thus the mechanism of the stacking is not known in diatoms, and in general, in Chl *a/c*-containing organisms.

It has been found that changes in the osmolarity dramatically affect the psi-type CD signals in *Pleurochloris meiringensis*, while the ionic composition had no influence on these bands (Büchel and Garab, 1997). Similar studies on the role of physicochemical factors in the modification of thylakoid membrane stacking in diatoms could not be found in the literature. Moreover, there is no information available about the correlation between

membrane stacking and the parameters of chlorophyll fluorescence. In diatoms, no lateral heterogeneity could be observed, but they exhibit strong and efficient NPQ. The NPQ is strictly related to the operation of the diadinoxanthin-cycle in these organisms (Goss et al. 2006; Lavaud et al. 2002), although recently some components of NPQ were found to be independent of the operation of the xanthophylls cycle (Lavaud et al. 2006; Grouneva et al. 2008).

In Chapter 4.3.3 the role of Mg^{2+} in the macro-organization of the complexes was tested on isolated thylakoid membranes. We have shown that in the presence of Mg^{2+} ions the psi-type CD signal could be significantly preserved, pointing to the important role of divalent cations in the stacking of thylakoid membranes in *P. tricornutum*. Thylakoid membranes isolated in the absence of $MgCl_2$ lost the psi-type CD signal, however it could be restored to some extent by resuspending the membranes in buffer containing $MgCl_2$. Thus, in isolated membranes psi-type CD signal could also be observed and the CD spectrum resembled the spectrum of intact cells. We have also shown that the osmotic pressure plays no or only minor role in this process. This situation is similar to granal thylakoid membranes, where it has also been shown that divalent cations are the main factors controlling the macro-organization of thylakoid membranes (Garab et al. 1991). In Chapter 4.3.3 correlations were revealed between the Mg^{2+}-assisted macro-organization and some functional parameters, F_v/F_m, NPQ and the operation of Ddx cycle.

These results provide evidence for the existence of chiral macrodomains in thylakoid membranes under physiologically relevant conditions. At present, it is not possible to assign the chiral macrodomains to exact structures and stacking mechanism in the thylakoids of diatoms. However, our results provide evidence for the first time that well ordered, three dimensional network of the thylakoid membranes of *P. tricornutum* exhibit psi-type CD signals. It also must be pointed out, that parallel running membrane sheets themselves, i.e., without a chiral organization of the complexes, cannot give rise to psi-type CD, as e.g. in bundle sheath chloroplasts (Faludi-Dániel et al. 1973) or in the case of CP24 depleted Arabidopsis plants (Kovács et al. 2006). Preliminary measurements of small-angle neutron scattering (SANS) - which provide information about the changes in the repeat distances of the multilamellar thylakoid membrane system – indicate that in *P. tricornutum* cells the thylakoid membranes are indeed organized into highly ordered multilamellar system with well defined repeat distances.

5.4. Structural flexibility of the chiral macrodomains

It has been known for a long time that in higher plants the chirally organized macrodomains exhibit remarkable reversible structural flexibility (Barzda et al. 1996, Cseh et al. 2005, Garab et al. 1988b, Holm et al. 2005), which provides the photosynthetic membranes enhanced photoprotection capability on the supramolecular level. The photoprotective mechanisms in relation with electron transport and xanthophyll cycle activity, the role of ΔpH are examined in details in diatoms using various techniques based mainly on fluorescence measurements (reviewed in Wilhelm et al. 2006 and Lavaud, 2007). However, little is known about the structural modifications of thylakoid membranes and light harvesting antennae behind the well characterized quenching mechanisms. There are studies available on isolated FCP complexes which exhibit markedly different fluorescence yield in different aggregation states (e.g. Gundermann et al. 2008), however, the physiological role of aggregation of FCP is not elucidated yet. Miloslavina et al. (2009) provided the first evidence that under NPQ conditions part of the FCP complexes detaches from PSII reaction center and becomes aggregated and the mechanism of aggregation is similar to that of observed in vitro.

The amplitude of the psi-type CD signal could be modulated by certain environmental factors, showing that the macrodomains exhibit considerable structural flexibility.

We intended to examine the structural rearrangements of the chiral macrodomains caused by changes in different environmental factors, such as heat, illumination with strong light, light intensity during growth and changes in osmotic pressure and ionic strength.

I have shown in Chapter 4.3.1 that the psi-type CD bands in *P. tricornutum* cells exhibit increased sensitivity to elevated temperatures compared to the excitonic bands. In particular, the (+)698 nm psi-type band is the most susceptible, but the negative CD band at around 679 nm also displays higher sensitivity to elevated temperatures than the excitonic bands. These data suggest that the macro-organization can be prone to thermo-optically inducible reorganizations. However, presently we have no evidence for such an origin of the reorganizations in intact cells of *P. tricornutum*. Preliminary results indicate that the CD changes caused by elevated temperatures are reversible.

Intact cells subjected to illumination with strong actinic light exhibited reversible light-induced CD changes. Upon illumination, the amplitude of the main psi-type CD bands increased, while the excitonic CD bands remained essentially unchanged (Fig. 16). The

fact that diatoms exhibit increase in the intensity of the psi-type CD band might be explained by the aggregation of individual pigment-protein complexes, like the FCP complex, or changes in the repeat distances of the thylakoid stacks. The time kinetics of the light induced CD changes at 698 nm is commensurable with the kinetics of the induction and relaxation of NPQ (Ruban et al. 2004, Goss et al. 2006). Moreover, the xanthophyll cycle inhibitor DTT inhibited the light-induced CD changes, which suggests the involvement of Ddx-deepoxidation in the structural changes. It has been found in higher plants that Zx acts as an allosteric activator for qE and the conformational changes of LHCII (Horton et al. 2008), which could also be true for the Dtx and conformational changes in the antenna system of diatoms. However, the proton gradient uncoupler NH_4Cl did not affect significantly the light-induced CD changes in *P. tricornutum*, therefore at present it is not clear how the NPQ and the changes in the macrodomain order are correlated. Further experiments are needed to clarify the relationship of the structural changes and NPQ in diatoms. Nevertheless, these results are in a good accordance with SANS measurements, which give information about the changes in the repeat distances of the multilamellar thylakoid membrane system in vivo. In these measurements, *P. tricornutum* cells exhibited significant and reversible light-induced changes in the center position of the Bragg peak in the scattering profile, indicating that changes in the repeat distances occurred. These data will be presented in the PhD dissertation of Gergely Nagy. NH_4Cl did not influence significantly the SANS changes either, which is in accordance with CD measurements.

The light intensity during growth also affected significantly the intensity of the psi-type bands in *P. tricornutum* as it has been demonstrated in Chapter 4.3.2; LL exhibited more intense psi-type bands than HL cells. In LL cells, however, the kinetics and amplitude of the light-induced CD changes were much smaller than in HL cells. These results suggest that in LL cells the structural flexibility of the chiral macrodomains are less expressed, which might be correlated with the less effective photoprotective capability of cells grown on lower light intensities. Nonetheless, the large psi-type signal suggests the presence of large ordered arrays of antenna complexes, which may be advantageous for the enhanced light-harvesting capability.

We have observed that the intensity (+)698 nm band decreased reversibly by increasing the ambient sorbitol concentration. These results may indicate that variations in the osmotic pressure affect the lumenal space and/or the interthylakoidal space, and thus the repeat distances in the multilamellar membrane system. Indeed, the average repeat

distances, determined from SANS data, have revealed shrinking up to 1-2 nm; these changes could not be discerned by TEM. It is interesting to note that the light-induced CD changes increase while the sorbitol-induced changes decrease the psi-type CD. Under the same conditions, as reflected by SANS, the repeat distance increase and decrease, respectively; data which can help in elucidation of the origin of the psi-type signal in diatoms. The changes indicate alterations in the supramolecular array of the complexes, evidently via or associated with the changes in the membrane ultrastructure, but during the osmotically-induced changes excitonic interactions appear to be affected. The spectral changes observed upon changing the ambient sorbitol concentration are peculiar. In higher plants, strong psi-type CD signal is observed if the isolated thylakoid membranes are suspended in sorbitol+$MgCl_2$ containing buffer. In the presence of $MgCl_2$ but in the absence of sorbitol, the psi-type signal at (-)676 nm decreased the most, which is due to partial destacking of the membranes which prevents the disassembly of the chiral macrodomains (Cseh et al. 2000). In diatoms the situation is completely different; the psi-type signal decreases by increasing the sorbitol concentration. In cellular systems, the increase in the ambient osmolarity causes water loss from the cells (hypertonic media). The loss in cellular water content causes a decreased hydration of the membranes which may result in a concomitant “membrane-crowding” within the chloroplast. This may affect the macro-organization and even the excitonic interactions. It is important to emphasize, however, that the CD changes induced by osmolarity are reversible.

CD spectra of pigments at different organizational levels contain a large amount of information. In a complex system, e.g. in intact cells or isolated thylakoid membranes, the CD signals originating from different types of interactions are superimposed onto each other, which make the interpretation of CD signals extremely difficult. Nevertheless, CD data provide important information on the changes in pigment-pigment interactions e.g. in intact cells subjected to different treatments as described in Chapter 4.3. Although a deep analysis of CD spectra would require detailed quantum-mechanical calculations, such as attempted only on structures with atomic resolution (Georgakopoulou et al. 2006, 2007), mathematical deconvolution, e.g. using singular value decomposition and using the measured spectra of isolated FCP and thylakoid membranes might provide further insights on the nature of structural modifications caused by environmental factors.

5.5. Structurally flexible chiral macrodomains also in *C. meneghiniana*

In Chapter 4.4 we examined a taxonomically different diatom, *C. meneghiniana*. Essentially the same psi-type CD bands at around 698 nm could be found in these cells, as in *P. tricornutum*. This band was also absent in isolated FCP. Isolated thylakoid membranes partially preserved the psi-type CD signal when Mg^{2+} ions were applied during isolation. The chiral macrodomains exhibited remarkable structural flexibility when elevated temperatures and illumination with strong light were applied, thus – similarly to *P. tricornutum* – pigments are arranged into structurally flexible chiral macrodomains.

5.6. The fucoxanthin molecules and the FCPs are spectrally and functionally heterogeneous

Earlier data obtained from fluorescence spectroscopy suggest that the Fx population is not homogeneous in diatoms, one part of the molecules transfers the energy more efficiently to Chl *a* (the red-shifted form) while the other part displays only poor energy transfer efficiency (the blue-shifted form) (Papagiannakis et al. 2005). Ultrafast transient spectroscopy studies indicate that the energy transfer pathways within the isolated FCP are also heterogeneous (Papagiannakis et al. 2005; Gildenhoff et al. 2010). To gain more information about the microenvironment, more precisely about the charge-transfer properties of Fx and the changes in static dipole moments, Stark-spectroscopy measurements were performed earlier on isolated FCP complexes (Premvardhan et al. 2008). Stark-effect, a homogeneous bandshifts in the absorption spectrum, was induced by external electric field. The magnitude of the bandshift (only a few Ångstroms) depends on the difference between the ground- and excited state dipole moments and polarizabilities, therefore Stark signals provide unique information on the microenvironment of the given molecule (reviewed by Boxer, 2009). It has been shown in Chapter 4.5.1 that *P. tricornutum* cells two main electrochromic absorbance band-shifts can be identified, with positive/negative peaks at around 565/535 nm and 515/485 nm (Fig. 28) that overlap the 0-0 and 0-0/0-1 bands of Fx_{red} and Fx_{green}, respectively (Premvardhan et al. 2008). The occurrence of the electrochromic absorbance transients in this diatom is consistent with the Stark signal of isolated FCP in the external field. The energetic locations of the Fx species that give rise to the two electrochromic transitions in *P. tricornutum* cell are close to the 0-0 bands of Fx_{red} and Fx_{green}, respectively, previously identified in the FCP complexes isolated from *C. meneghiniana*. In this context, it is important to note that we have found

essentially the same electrochromic transients in *C. meneghiniana* cells, where Fx_{red} and Fx_{green} were found at 553 and 502 nm, respectively.

To interpret the role of Fx in the excitation energy transfer processes, the pigment-pigment interactions and the orientation of the pigments in the membranes must also be taken into account. Isolated thylakoid membranes of algae containing Chl *a/c*-type antenna systems exhibit excitonic CD bands in the 400-500 nm spectral range which is a sum spectrum of Chl *a*, Chl *c* and carotenoids (Goss et al. 2000; Katoh, 1992; Mimuro et al. 1990; Büchel and Garab, 1997; Büchel, 2003). In isolated thylakoid membranes of brown algae the (-)478 nm band originates from the S2 energy level of Fx, while the broad shoulder between 478 and 525 nm originates probably from the S1 level of Fx (Katoh, 1992). Isolated Fx molecules, similarly to other carotenoids, exhibit no CD bands, however, upon binding to the proteins they acquire CD signals due to induced chirality and strong excitonic coupling to Chl *a*, thereby contribute to the excitonic signals at (+)445 nm/(-)478 nm. Moreover, protein bound Fx molecules also undergo bathochromic shift, and this phenomenon also contributes to the long CD shoulder observed between 478 and 525 nm (Katoh, 1992).

The CD band between 470 and 530 nm have been shown to originate mostly from Fx molecules, however the broad band with a long shoulder above 500 nm suggests that the Fx interactions are not uniform. In both HL and LL cells, no CD signatures are associated with the field-sensitive Fx forms, which are essentially CD silent as in isolated FCP (Büchel, 2003). Nevertheless, it is worth mentioning that LL cells exhibit a more intense psi-type band at (+)698 nm than HL cells, which is most probably due to the accumulation of the light harvesting complexes in LL membranes. Similar data were obtained in another Chl *a/c*-containing organism, *Pleurochloris meiringensis* (Büchel and Garab, 1997). It must be noted here, that two different types of light sources were used for growing the cells under HL and LL conditions (see Chapter 3.1.1), thus, it cannot be excluded that minor variations in the spectral composition of the growth light source caused chromatic adaptation of the cells, in addition to the adaptation to the light intensity. However, this does not affect our conclusion that the spectral heterogeneity of Fx varies upon changing the growth conditions, a finding that strongly suggests that the heterogeneity originates from a heterogeneity of FCP complexes. The orientations of the Fx transition dipole moments relative to the membrane plane can be used to understand the nature of the electrochromic signals in the transmembrane field: larger signals are expected not only on the basis of the intrinsic dipolar properties of the pigments but also when the pigments are

aligned to have their static dipole moments, μ, (close to) parallel to the transmembrane field, **F**. The large electrochromic transient, proportional to $\mu F\cos\theta$ (θ is the angle between μ and **F**), from Fx_{red} therefore indicates that its static dipole moment must be close to parallel to the trans-membrane field vector (**F**). Notably, the angle between the transition dipole and the change in static-dipole moments was measured to be ~ 20^{o} in *solution* (Premvardhan et al. 2008) which in conjunction with the LD data, an orientation angle of 29° of the transition dipole with respect to the membrane plane, would mean that the Fx_{red} could be oriented at ~ 49° with respect to the membrane plane; hence, ~ 41° between μ and **F**, which can yield more than 70% of the maximum possible electrochromic response to the trans-membrane field. For Fx_{green} this value can be as high as 83 %. It must also be noted that the angle between the transition dipole and static dipole moment could be even larger in the protein for Fx_{red} if it has a planar 'S' shape conformation, as proposed in Premvardhan et al. (2009).

The data presented in Chapters 4.5.1 and 4.5.2 on the heterogeneity of the Fx molecules and of the FCP pool are in reasonable agreement with biochemical analyses. The presence of different FCP pools was indicated by Western blot analysis, showing that the FCPs of the antenna are different from the FCPs connected to the photosystems (Brakemann et al. 2006, Veith et al. 2009). In addition, the FCP composition of the antenna varied if the algae were grown under LL or HL conditions (Beer et al. 2006). It has also been found that the expression of the individual FCP proteins displays heterogeneous response to the HL intensities (Becker and Rhiel, 2006; Brakemann et al. 2006). Heterogeneity of FCPs can also be seen in the ultrafast fluorescence decay kinetics (Miloslavina et al. 2009), which showed that two different pools of FCPs are responsible for the generation of steady-state non-photochemical fluorescence quenching (NPQ) both in *P. tricornutum* and *C. meneghiniana*. One pool of FCPs, detached from the photosystems, forms a fluorescence-quenching site, whereas the second quenching site consists of FCPs that remain in contact with the PSII core complex (Miloslavina et al. 2009). (NPQ at this site furthermore depends on the presence of the de-epoxidized xanthophyll cycle pigment Dtx.) The heterogeneous lipid distribution in the thylakoid membranes of diatoms also supports the existence of different FCP pools and macrodomains of the pigment-protein complexes (Goss et al. 2009), although no variability of stacked to exposed membranes is observed by electron microscopy (Pyszniak and Gibbs, 1992).

6. CONCLUSIONS

I investigated the molecular organization of the main light harvesting antenna complexes, the fucoxanthin-chlorophyll protein (FCP) in isolated trimeric and higher oligomeric forms and the (macro-)organization of complexes in isolated thylakoid membranes and in whole cells of two taxonomically different diatom species, *Phaeodactylum tricornutum* and *Cyclotella meneghiniana*. Information on the molecular architectures and their structural flexibilities were obtained mainly from circular dichroism spectroscopy and other spectroscopic techniques, including low temperature fluorescence, linear dichroism and flash-spectrometry, as well as from electron microscopy and biochemical analyses.

The results of my work can be summarized as follows:

I. In whole cells, the pigment-protein complexes are arranged into chiral macrodomains, as reflected by the presence of intense psi-type CD at around 698 nm, which is associated with the multilamellar organization of the membranes. The psi-type CD is superimposed on excitonic and intrinsic CD bands, which originate mainly from the major light harvesting antenna complexes, the FCP complexes and the pigment molecules, respectively. In the presence of Mg^{2+} ions, the chiral macrodomain organization could partially be retained in isolated thylakoid membranes; this macro-organization proved to be important with regard to photosynthetic functions, such as F_v/F_m, NPQ and the deepoxidation ratio. Our data also show, in accordance with literature data, that FCP *in vivo* is found predominantly in oligomeric forms.

II. The chiral macrodomains exhibit remarkable structural flexibility. They are very sensitive to variations of the ambient temperature, depend on the light intensity during growth, reversibly respond to changes in the osmotic pressure, and are capable of undergoing rapid, reversible light-induced reorganizations.

III. Fucoxanthin (Fx), the main light harvesting carotenoid in diatoms exists in different spectral forms due to its occurrence in different microenvironments, as revealed by electrochromic absorption changes induced by single turnover saturating flashes on intact cells. Two main electric field-sensitive Fx populations were found, which could be assigned as Fx_{green} and Fx_{red}, forms identified earlier by Stark-spectroscopy on isolated FCP complexes. The

heterogeneity of the Fx molecules *in vivo* manifests itself in small but well discernible differences in the energy transfer pathways toward the two types of reaction centers, thus indicating the role of heterogeneity at the level of FCPs associated with the two photosystems.

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A DOLGOZAT ÖSSZEFOGLALÁSA

A kovamoszatok döntő szerepet játszanak Földünkön az éves elsődleges nettó fotoszintetikus produkcióban. Ezen élőlények biomassza tömege a Föld fotoszintetizáló szervezeteinek kevesebb, mint 1%-a, mégis az elsődleges éves produkció mintegy 25%-ához járulnak hozzá (Field és mtsai 1998). Speciális életciklusuknak köszönhetően a légkör CO_2 koncentrációjának szabályozásában kiemelkedő jelentőségűek, mindazonáltal ennek következtében igen gyorsan és erősen változó környezeti tényezőknek vannak kitéve, mely hatékony fotoprotektív mechanizmusok kifejlődését tette szükségessé.

A kovamoszatok fotoszintetikus apparátusa számos tekintetben különbözik a magasabbrendű növényekétől. A kloroplasztisz burkolómembránja négy membránból épül fel, amely a másodlagos endoszimbiózis eredménye. Szemben a magasabbrendű növényekkel, a tilakoid membránok nem különülnek el gránum, illetve sztróma tilakoidokká. A tilakoid membránok hármasával szerveződött membránkötegekként húzódnak végig a kloroplasztisz egész hosszában. Az elektrontranszportlánc komponenseit alkotó fotoszintetikus pigment-protein komplexek laterális elrendeződésében éles különbségek figyelhetők meg a magasabbrendű növényekhez képest. Magasabbrendű növényekben a kettes típusú fotokémiai rendszer (PSII), illetve a hozzá tartozó fénybegyűjtő pigment-protein antenna komplex (LHCII) a szorosan tapadt gránum tilakoidokban, míg az egyes típusú fotokémiai rendszer (PSI) és a hozzátartozó fénybegyűjtő komplex (LHCI) a nem tapadt sztróma tilakoidokban található meg (Chow és mtsai 2005; Dekker és mtsai 2005; Mustárdy és Garab, 2003). Ezzel szemben kovamoszatokban a PSII és PSI membránon belüli elrendeződése szinte teljesen homogén, nincsen laterális szegregáció (Pyszniak és Gibbs, 1992). A fő fénybegyűjtő komplexek a fukoxantin-klorofill proteinek (FCP), amelyek fénybegyűjtő antennaként szolgálnak mind a PSI és a PSII számára. Kovamoszatokban a klorofill *a* mellett klorofill *c* található, míg a karotenoidok közül a fukoxantin, ß-karotin, diadino- és diatoxantin fordul elő, mely utóbbi kettő a diadinoxantin (xantofill)-ciklus komponensei.

A kovamoszatok FCP komplexének szerkezetéről nem áll rendelkezésre atomi részletességű információ. Továbbá a pigment-protein komplexek szupramolekuláris szerveződése alig ismert. Magasabbrendű növényekben a PSII-LHCII szuperkomplexek királisan rendezett makrodoménekbe szerveződnek, amelyet cirkuláris dikroizmus (CD) spekroszkópia segítségével sikerült kimutatni (Barzda és mtsai 1994; Garab, 1996). A cirkuláris dikroizmus (CD) spektroszkópia egy nem invazív vizsgálati módszer, amely

különösen alkalmas – többek között – biológiai rendszerek térszerkezeti viszonyainak vizsgálatára, illetve a rendszerekben végbemenő szerkezetváltozások nyomonkövetésére. Hierarchikusan szervezett rendszerekben a CD különböző molekuláris szerkezetekből származik. A molekulák belső aszimmetriájából, kiralitásából származó CD jel az intrinzikus CD jel, amely megfigyelhető pl. szabad klorofill molekulák esetén, a fotoszintetikus szervezetek többségében elhanyagolható nagyságú. Kettő vagy több molekula exciton kölcsönhatása esetén (amely megvalósul pl. izolált pigment-protein komplexekben) jellegzetes exciton CD sávpárokat figyelhetünk meg, melyek konzervatív sávszerkezettel jellemezhetőek. Nagyméretű, királisan rendezett komplex rendszerekben az exciton CD sávoknál egy nagyságrenddel nagyobb intenzitású CD sávok figyelhetőek meg. Ezek a sávok nem-konzervatív sávszerkezettel jellemezhetőek, és az abszorpciós sávokon kívül is megjelenhetnek CD jelek, melyek a differenciális fényszórásból erednek. Ezeket a sávokat psi-típusú (polymer or salt-induced) sávoknak nevezzük.

A kloroplasztisz gránum tilakoid membránjaiban és a LHCII makroaggregátumokban a kromofórok denzitása és rendezettsége igen nagy, köztük erős, hosszútávú kölcsönhatások jöhetnek létre, amely jellegzetes psi-típusú CD sávok kialakulásához vezet. A komplexek ilyen nagyfokú rendezettségének számos fiziológiai jelentősége lehet: lehetővé teszi hosszú távolságokra is a hatékony gerjesztési energiavándorlást és biztosíthatja a fotoszintetikus fénybegyűjtés finomszabályozását.

Kimutatták, hogy a királisan rendezett makrodomének fény- és hő-indukált reverzibilis szerkezetváltozásokat mutatnak (Barzda és mtsai 1996; Garab és mtsai 1988). Ezek a néhány perces időskálán lejátszódó változások kizárólag a hosszú távú rendezettséget érintették, és nem voltak hatással a pigment-protein komplexek belső szerkezetére utaló exciton sávokra.

A kovamoszatok pigmentjeinek makroszerveződését korábban nem vizsgálták. A fotoszintetikus pigment-protein komplexek fehérje, illetve pigmentösszetételéről az utóbbi években jelentős kísérleti eredmények születtek. Jelentős ismertekkel rendelkezünk továbbá az extra fényenergia okozta károsodások kivédésére szolgáló mechanizmusok működéséről is élettani illetve biokémiai szinten. Azonban a fényenergia hasznosítás és a fotoprotekció folyamatainak teljes megértéshez elengedhetetlen a szerkezeti háttér feltérképezése, illetve annak megismerése, hogy létezik-e olyan hosszú távú rendezettség a pigmentmolekulák esetén, amely képes gyorsan, reverzibilisen követni a gyorsan változó környezeti tényezők okozta hatásokat a fotoszintetikus elektrontranszportlánc komponenseinek károsodása illetve hatékonyságának csökkentése nélkül.

Munkám során a következő célokat tűztem ki: i) a pigment-protein komplexek makroszerveződésének átfogó vizsgálata különböző szerveződési szinteken, szabad pigment molekulákon, izolált tilakoid membránokban és FCP komplexekben, valamint intakt sejtekben *Phaeodactylum tricornutum* és *Cyclotella meneghiniana* kovamoszat fajokban ii) a makrodomének szerkezeti flexibilitásának vizsgálata különböző környezeti tényezők – hőmérséklet, beeső fényintenzitás, nevelési fényintenzitás, közeg ozmotikus nyomása és ionerőssége -megváltoztatása mellett; a szerkezeti változások korreláltatása a fotoszintetikus paraméterek változásaival, illetve a Ddx ciklus működésével iii) a karotenoidok, különösen a fukoxantin (Fx) mikrokörnyezetének és kölcsönhatásainak, valamint heterogenitásának vizsgálata.

A *P. tricornutum* kovamoszat fajban a következő CD sávok figyelhetőek meg különböző szerveződési szinteken: gyenge intrinzikus CD jelekkel rendelkeznek az acetonnal extrahált szabad pigment molekulák, ezek hozzájárulása a CD-sávokhoz általában diatomokban is elhanyagolható. Intakt sejtekben és izolált tilakoid membránokban jellegzetes CD sávok találhatóak. A (+)445/(-)478 nm-nél látható sávpár exction sávoknak tekinthető, mely a klorofill *a*, *c*, és a Fx molekulák CD jeleiből származik. A 679 nm-es sáv – annak ellenére, hogy jóval erősebb, mint az intrinzikus CD jel – nem tekinthető exciton sávnak, mivel nem mutat konzervatív sávszerkezetet. Valószínűleg egy indukált intrinzikus sávnak tekinthető, mely a klorofill a molekulák fehérje környezet-indukálta csavarodásából eredhet, és más algafajokból származó klorofill *a/c*-t tartalmazó antenna rendszereknél is megfigyelhető (Büchel és Garab 1997). Egész sejtek erős CD sávokkal rendelkeznek 698 nm környékén. Ez a sáv nagy intenzitással, 700 nm felett differenciális fényszórással rendelkezik, valamint eltűnik a sejtek feltörésekor, így nem-konzervatív, psi-típusú CD sávnak tekinthető, mely királisan rendezett makrodoménekből ered.

Cukorgrádiens ultracentrifugálás segítségével elkülönítettük a kovamoszatok főbb pigment-protein komplexeit detergenssel szolubilizált tilakoid membránokból. A detergens koncentrációtól függően lehetséges volt trimer, illetve oligomer állapotú, FCP és FCPo komplexek izolálása (Lepetit et al. 2007). Izolált FCP és FCPo komplexek CD spektruma egymáshoz igen hasonló, és egyik szerveződési szinten sem figyelhető meg psi-típusú CD, így az FCPo frakció önmagában nem képez királis makrodoméneket.

A (+)698 nm-nél megfigyelhető CD sáv psi-típusú természetét igazoltuk oly módon, hogy a sejteket fokozatosan törtük fel ultrahangos szonikálással és követtük a spektrális változásokat. A sejtek feltörése során megfigyelhető volt a 698 nm-es sáv fokozatos

eltűnése, míg a (-)679 nm-es sáv intenzitása jóval kevésbé változott. Érdekes módon a (+)445/(-)478 nm-es sávpár intenzitása fokozatosan nőtt a sejtek szétesésével. Erre magyarázat lehet az, hogy jelen lehet sejtek differenciális fényszórásából eredő artefaktum. A szonikált sejteket egyúttal transzmissziós elektronmikroszkópos vizsgálatnak is alávetettem, mely kimutatta, hogy psi-típusú CD sáv csak akkor van jelen, ha a tilakoid membránok rendezett, multilamelláris rétegekbe rendeződnek.

A továbbiakban arra kerestünk információt, hogy a királis makrodomének képesek-e különböző környezeti tényezők hatására indukált szerkezetváltozásokra. Hőkezelés során a psi-típusú CD sáv sokkal érzékenyebbnek bizonyult, mint az exciton sávok; már alacsonyabb hőmérsékleteknél is jelentősen csökkent a 698 nm-es sáv intenzitása, míg a (+)445/(-)478 nm-es sávpár nem változott 45 °C-ig, mely hőmérséklet felett eltűnt, jelezve ezzel a pigment-protein komplexek szétesését.

Egész sejtekben megfigyeltem fényindukált CD változásokat is erős fénnyel történő megvilágítás hatására főként (+)698 nm-en és kismértékben (-)679 nm-en, míg az egyéb spektrális régiók érzéketlennek mutatkoztak a fénykezelésre. 698 nm-en időkinetikai méréseket is végeztem, melyek kimutatták, hogy a fényindukált szerkezetváltozások reverzibilisek. Különböző fénykörülmények alkalmazásával nevelt *P. tricornutum* sejtek CD spektruma jelentősen különbözött; alacsony fényintenzitáson (LL) nőtt sejtek jóval erősebb CD sávokkal rendelkeztek 698 nm-nél, míg a 679 nm-es sáv intenzitása gyengébb volt, mint magas fényintenzitáson (HL) nőtt sejtek esetén.

A közeg ozmotikus nyomásának emelésével a psi-típusú CD sáv intenzitása jelentősen csökkent, valamint az exciton sávokban is megfigyelhetőek voltak kisebb változások. Ezek a változások szintén reverzibilisnek bizonyultak, továbbá elektronmikroszkópos felvételek alapján elmondható, hogy a tilakoid membránok ultrastruktúrája sem deformálódott, így kijelenthető, hogy a psi-típusú CD sáv reverzibilis változásai fiziológiás jelentősséggel bírnak.

Izolált tilakoid membránokban a psi-típusú CD sáv eltűnik. Kísérleteink során azonban azt tapasztaltuk, hogy a $MgCl_2$ jelenlétében izolált tilakoid membránok jelentős mértékben képesek megtartani a makrodomén szerveződést, melyre utal a psi-típusú CD sáv viszonylag nagy intenzitása. Amennyiben a tilakoid membránokat $MgCl_2$ hiányában izoláltuk, a psi-típusú CD sáv eltűnt, azonban részben visszaállítható volt $MgCl_2$-ot tartalmazó puffer segítségével. $MgCl_2$ kezelés hatására az F_v/F_m és NPQ paraméterek és a de-epoxidációs arány is jóval magasabbak voltak, mint Mg^{2+} ionok hiányában, így a Mg^{2+}

ionok nemcsak a membránok királis makroszerveződésére, hanem ezzel párhuzamosan fotoszintetikus aktivitására is hatással voltak.

A pigmentek királis makroszerveződése megfigyelhető volt egy másik kovamoszat faj sejtjeiben, *Cyclotella meneginiana*-ban is. Izolált tilakoid membránokban a 694 nm-nél megfigyelhető psi-típusú CD sáv intenzitása lecsökken Mg^{2+} ionok jelenlétében illetve teljesen eltűnik annak hiányában. A psi-típusú CD sáv intakt sejtekben sokkal érzékenyebbnek bizonyult erős fénnyel történő megvilágítás, illetve hőkezelés hatására, mint az exciton sávok. Ezek alapján elmondható, hogy a pigmentek királis makroszerveződése nem korlátozódik a *P. tricornutum*-ra, hanem valószínűleg általános jelenség kovamoszatokban.

A pigment-pigment kölcsönhatások jól tanulmányozhatóak CD spektroszkópia segítségével, azonban az adott pigment fénybegyűjtésben betöltött szerepének teljes megértéséhez szükséges a lokális környezetének, membránban történő orientációjának ismerete. Ennek felderítésére villanófény-indukálta elektrokróm abszorpció változás-méréseket végeztünk 470 és 570 nm között intakt *P. tricornutum* sejteken, amely információt ad az adott pigmentek (az elsődleges töltésszétválás során felépülő) elektromos tér hatására bekövetkező abszorpcióváltozásairól; ez a tulajdonság pedig a Fx molekulák dipólmomentumával áll kapcsolatban. *P. tricornutum*-ban két fő elektrokróm jelet azonosítottunk 515 és 565 nm-nél, melyekhez negatív sávok társultak 485, illetve 535 nm-nél. Ezek az elektrokróm sávpárok arra utalnak, hogy a Fx molekulák kölcsönhatásba kerülhetnek a klorofill molekulákkal, ezáltal megnő az átmeneti dipólmomentumuk. Korábbi munkákból ismert, hogy a Fx molekulák strukturálisan és funkcionálisan heterogén csoportokat alkotnak *C. meneginiana*-ból izolált FCP komplexekben (Papagiannakis és mtsai 2005; Premvardhan és mtsai 2008, 2009). Ezeket a különböző Fx formákat Fx_{green} és Fx_{red} névvel látták el spektrális pozíciójuknak megfelelően (Premvardhan és mtsai 2008). Az elektrokróm tranziens spektrumban megfigyelhető 565/535 nm-nél megfigyelhető sávpár a Fx_{red}, míg a 515/485 nm-es sávpár a Fx_{green} analógjaként értelmezhető.

Megállapítottuk, hogy a növesztés során alkalmazott különböző megvilágítási körülmények befolyásolják a különböző Fx formák arányát; LL sejtekben jóval nagyobb mennyiségű Fx_{red} található, mint HL sejtekben, míg a Fx_{green} mennyisége nem változik jelentősen. Mélyhőmérsékletű fluoreszcencia spektroszkópia segítségével kimutattuk, hogy Fx_{red} kissé hatékonyabb kl *a*-ra történő gerjesztési energiatranszfert mutat, mint a Fx_{green}, ez igaz mind HL mind pedig LL sejtek esetén. Izolált tilakoidokon mért LD spektrumok

alapján azt találtuk, hogy a Fx_{red} molekulák kisebb orientációs szöget zárnak be a membrán síkjával, mint Fx_{green} molekulák. Hasonló eredményeket kaptunk *C. meneghiniana* esetén is, ami arra utal, hogy a Fx molekulák spektrális és funkcionális heterogenitása általános jelenség lehet kovamoszatokban.

SUMMARY OF THE THESIS

Diatoms play a determinant role in the photosynthetic primary production in the Earth. The biomass of the diatom species is less than 1% of the total biomass of the photosynthesizing organism, still they contribute by about 25% to the total primary production (Field et al. 1998). Due to their specific life cycle, they are also important in the regulation of the atmospheric CO_2 concentration.

The photosynthetic apparatus of diatoms displays several differences compared to higher plants. The envelope membrane of the chloroplast consists of four membranes, which is a remnant of secondary endosymbiotic processes. In contrast to higher plants, the thylakoid membranes are not differentiated into granum and stroma lamellae. The thylakoids are arranged into groups of three stacked membranes, which span the whole length of the chloroplasts. The lateral organization of photosynthetic pigment-protein complexes is also different compared to higher plants. In higher plants, the photosystem II (PSII) and its accessory light-harvesting complex (LHCII) is located in the stacked granal thylakoid membranes, while photosystem I (PSI) with its accessory light-harvesting antenna (LHCI) can be found in the non-stacked stroma thylakoids (Chow et al. 2005; Dekker et al. 2005; Mustárdy and Garab, 2003). In contrast, in diatoms the localization of PSII and PSI in the membrane is homogeneous, no lateral heterogeneity could be observed (Pyszniak and Gibbs, 1992). The main light-harvesting complexes of diatoms are the fucoxanthin-chlorophyll protein (FCP) complexes, which serve as accessory antennae for both PSI and PSII. Diatoms possess Chl *a* and Chl *c*, and their main light harvesting carotenoid is the fucoxanthin (Fx). The photoprotective carotenoids are the diadino- and diatoxanthin (Ddx and Dtx), which are the components of the diadinoxanthin cycle.

The atomic resolution structure of FCP complexes is not known at present, and our knowledge about the supramolecular organization of the pigment-protein complexes is also rudimentary. In higher plants it is well known that PSII-LHCII supercomplexes are arranged into chirally ordered macrodomains in the granum, as it has been shown by

circular dichroism (CD) spectroscopy (Barzda et al. 1994; Garab, 1996). CD spectroscopy is a powerful, non-invasive method for the investigation of structural properties of complex biological systems and to monitor their structural changes. In the case of single molecules (e.g. Chl molecules) intrinsic CD signals can be observed, which originate from their inherent asymmetry and chirality. In the case of excitonic interactions of two or more molecules (which occurs e.g. in isolated pigment-protein complexes), excitonic CD signals can be observed, which can be characterized with a conservative band structure. In large, chirally ordered macroaggregates, anomalous CD signals with large intensity and non-conservative band structure can be observed, and it can be associated with differential light-scattering. These CD bands are considered as polymer or salt-induced (psi) type signals. In granal thylakoid membranes the density of pigments is very high and long range interactions exist, which exhibit psi-type CD signals. This highly ordered structure have physiological significance: it might help in the efficient excitation energy transfer for large distances and can ensure the fine regulation of light-harvesting processes.

It has been shown earlier that these chiral macrodomains are capable for light- and heat-induced reversible structural changes (Garab et al. 1988). These changes occurred in minutes timescale and could be observed only in the long-range order, while the excitonic interactions were not affected.

The macro-organization of pigment molecules in diatoms has never been investigated. There are several studies available about the protein and pigment composition of the complexes. Moreover, the photoprotective mechanisms are also characterized in details in physiological and biochemical levels. However, in order to fully understand the light-harvesting processes, it is important to reveal the long-range organization of the pigments, which is able to follow rapidly the sudden changes in the different environmental factors.

In my PhD work, the following aims were addressed: i) to conduct systematic study on the macro-organization of pigments in different levels of structural complexity, on free pigment molecules, on isolated pigment-protein complexes and thylakoid membranes, and on intact cells of *Phaeodactylum tricornutum* and *Cyclotella meneghiniana* ii) to investigate the effect of different environmental factors (temperature, strong illumination, different light conditions during growth, osmotic pressure and ionic composition) on the macrodomains and to correlate these possible changes with physiological parameters iii) to investigate the microenvironment, the interactions and heterogeneity of Fx, the most important light-harvesting carotenoid, both in *Phaeodactylum tricornutum* and *Cyclotella meneghiniana.*

In *P. tricornutum*, the following CD signals could be observed. Weak intrinsic signals could be detected in extracted pigment molecules. Isolated thylakoid membranes exhibited a CD bandpair at (+)445/(-)478 nm, which is an excitonic CD signal and originates from Chl *a*, *c* and carotenoids, mainly from Fx. At 679 nm a negative band could be observed which cannot be considered as excitonic band, because it does not exhibit conservative band structure. It is most probably a strong intrinsic band originating from Chl molecules, which are twisted due to the protein environment. Similar bands could be identified in another Chl *a/c*-containing organism (Büchel and Garab, 1997).

Intact cells exhibit large CD bands at around 698 nm. This band is associated with differential scattering and disappears when cells are disrupted, thus it can be considered as psi-type signal originating from chiral macrodomains.

By using sucrose gradient centrifugation we separated the main pigment-protein complexes from solubilized thylakoid membranes. Depending on the detergent concentration, it was possible to separate trimeric and oligomeric FCP complexes (FCP and FCPo, respectively). The CD spectra of FCP and FCPo are very similar to each other, and psi-type CD signal could be observed in neither of them, thus even FCPo does not form chiral macroaggregates.

The psi-type property of the large band at (+)698 nm could be verified by using sonication of the cells. Upon breaking up the cells, the intensity of the (+)698 nm band decreases and finally disappears, while the (-)679 nm band changes only to little extent. Interestingly, the intensity of the (+)445/(-)478 nm bandpair increased, which can be explained by a scattering artifact or the presence of a strong band in this region. The transmission electron microscopic pictures of control and sonicated cells show that the psi-type band exists only if the thylakoid membranes are arranged into multilamellar system.

In the followings, we intended to investigate the structural flexibility of chiral macrodomains. By using heat treatment of cells, the psi-type band proved to be much more sensitive than the excitonic bands; the intensity of the psi-type CD signal decreased already at lower temperatures, while the excitonic bands remained unchanged up to 45 °C. Above this temperature the excitonic CD signals disappeared, indicating the disassembly of pigment-protein complexes.

Light-induced CD changes could also be observed in intact cells. The (+)698 nm band and – to lesser extent – the (-)679 nm band displayed changes, while the other spectral changes remained essentially unchanged. The kinetical measurements of the light-induced changes showed that the (+)698 nm band the structural changes are rapid and reversible.

Intact cells grown on different light conditions displayed remarkably different CD spectra. Cells grown on low light (LL) exhibited much larger psi-type signal and weaker signal at (-)679 than cells grown on high light (HL).

The increase in the ambient osmotic pressure the intensity of the psi-type CD signal decreased, and also the excitonic interactions changed to some extent. These changes were also reversible, moreover, no significant changes could be observed on the electron micrographs of control and treated cells, thus the changes in chiral macrodomains occurred without the loss of the regular membrane system.

In isolated thylakoid membranes the psi-type CD signal disappeared. However, when cells were isolated in the presence of $MgCl_2$, the psi-type signal could be well retained. The psi-type signal could be partially restored when thylakoids (isolated in the absence of $MgCl_2$) were incubated in $MgCl_2$-containing buffers. Along with the psi-type signal, the functional parameters F_v/F_m and NPQ values and the deepoxidation ratio were also significantly higher in the presence of Mg^{2+} ions.

The chiral macro-organization of the pigments were also observed in another diatom species, *Cyclotella meneghiniana*. The intensity of the psi-type band at around 694 nm decreased in thylakoid membranes, however, in the presence of $MgCl_2$ it could be retained to a large extent. The psi-type band was sensitive to illumination with strong light and heat treatment, while the excitonic bands were unaffected by these treatments. Thus, it can be concluded that the macro-organization of the pigments can be observed also in another diatom species.

The pigment-pigment interactions can be well characterized by using CD spectroscopy, however, in order to clarify the role of a given pigment in the light-harvesting processes, it is important to obtain information about its microenvironment and orientation in the thylakoid membranes. To doing this, we measured the flash-induced electrochromic absorbance changes between 470 and 570 nm on intact *P. tricornutum* cells, which gives information about the absorption changes (which is related to the changes in the dipole moments of the pigment) caused by the electric field due to primary charge separation.

In *P. tricornutum* two main electrochromic signals were identified at 515 and 565 nm which were accompanied by negative bands at 485 and 535 nm, respectively. These electrochromic bandpairs suggest that Fx molecules interact with Chl molecules, thereby gaining large dipole moments.

It has been shown earlier that Fx molecules exhibit structural and functional heterogeneity in FCP complexes isolated from *C. meneghiniana* (Papagiannakis et al.

2005; Premvardhan et al. 2008, 2009). These different Fx forms were assigned as Fx_{green} and Fx_{red} according to their spectral positions (Premvardhan et al. 2008). In our work, the electrochromic bandpairs at 565/535 nm and at 515/485 nm can be interpreted as the analogues of Fx_{red} and Fx_{green}, respectively.

We found that the light conditions during growth affects the ratio of the different Fx forms; LL grown cells accumulate Fx_{red} compared to HL grown cells, while the amount of Fx_{green} did not change considerably.

Low temperature fluorescence spectroscopy measurements revealed that Fx_{red} displays a somewhat more efficient excitation energy transfer to Chl *a* than Fx_{green}, which was true for both HL and LL cells. We have also established that Fx_{red} molecules possess smaller orientation angle respect to the membrane plane than Fx_{green}, which also contribute to the enhanced excitation energy transfer to Chl *a*.

Similar results were obtained in the case of *C. meneghiniana*, which suggests that heterogeneity of Fx molecules is a general feature in diatoms.

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***** These publications are the basis of the present Ph.D. thesis