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Cation-dependent changes in the thylakoid membrane appression of the diatom *Thalassiosira pseudonana*

Stefanie Jäger, Claudia Büchel*

Institute of Molecular Biosciences, Goethe University Frankfurt, Max-von-Laue Straße 9, 60438 Frankfurt, Germany

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ABSTRACT

Diatoms show a special organisation of their plastid membranes, such that their thylakoids span the entire plastid in bands of three. While in higher plants the interaction of the light harvesting complex II and photosystem II with divalent cations (especially Mg^{2+}) was found to take part in the interplay of electrostatic attraction and repulsion in grana membrane appression, for diatoms the key players in maintaining proper membrane distances were not identified so far. In this work, we investigated the changes in the thylakoid architecture of *Thalassiosira pseudonana* in reaction to different salts by using circular dichroism and fluorescence spectroscopy in combination with other techniques. We show that divalent cations have an important influence on optimal pigment organisation and thus also on maintaining membrane appression. Thereby, monovalent cations are far less effective. The concentration needed is in a physiological range and fits well with the values obtained for higher plant grana stacking, despite the fact that strict protein segregation as seen in higher plant grana is missing.

1. Introduction

1.1. Thylakoid membrane organisation

Thylakoids are biomembranes, which use incident sun light energy in photosynthesis to produce high-energy biomolecules from inorganic material. Although the multi-protein complexes involved in oxygenic photosynthesis are largely conserved and the basic mechanism in organisms of different biological clades is the same – *i.e.* light harvesting, transfer to photosystems, energy conservation – the way in which photosynthetically active membranes are organised is diverse. It is astonishing to see how organisms of several classes facing the same problems find different solutions. For example, cyanobacteria and red algae possess large, membrane-extrinsic antennae – the phycobilisomes – and thus contain non-appressed thylakoids only. Higher plants exhibit a lateral heterogeneity in their membrane organisation as their thylakoids can be divided into appressed grana and non-appressed stroma regions, which coincides with a different prevalence of the membrane protein complexes. In the grana region, photosystem (PS) II and its corresponding light-harvesting complex (LHCII) are found predominantly, while PSI, its antenna system (LHCI) as well as the ATP-synthase reside in the more loosely structured stroma region due to their protrusions [1]. The number of membranes in the grana stacks depends on the species, the light conditions and other environmental factors and is highly dynamic.

In contrast, in diatoms and other heterokonts, thylakoid membranes span the entire plastid in bands of three [2], which have been shown to be connected *via* anastomoses [2–4]. This organisation leads to four core membranes and two peripheral membranes. The arrangement of the protein complexes in the different membranes is not as strict as in higher plants. Early results from Pyszniak and Gibbs [2] suggested a homogeneous distribution of the fucoxanthin chlorophyll *a/c* binding proteins (FCPs) in all six membranes, while PSI is slightly enriched in the peripheral membranes. This idea was taken up by the working model published by Lepetit et al. [5], which additionally introduced the notion that PSII and its antennae could be more frequent in the core membranes. Steric reasons already imply that the ATP-synthase has to be located in the outer membranes. Furthermore, this hypothesis was complemented by the idea that this arrangement of the photosynthetic protein complexes could be due to a differential distribution of membrane lipids, *i.e.* an enrichment of sulfoquinovosyldiacylglycerol (SQDG) in the peripheral membranes. It had been shown earlier that diatoms possess a thylakoid membrane lipid composition different from the green lineage with mainly SQDG being more abundant [6]. Recent results [4] support the unequal distribution of the photosystems as proposed based on the early immuno-localisation experiments [2]. In

Abbreviations: CD, circular dichroism; Chl a, chlorophyll *a*; FCP, fucoxanthin chlorophyll *a/c* binding protein; LHC, light harvesting complex; PS, photosystem; Psi-type CD, polymerisation or salt-induced circular dichroism signal; SQDG, sulfoquinovosyldiacylglycerol

* Corresponding author.
E-mail address: C.Buechel@bio.uni-frankfurt.de (C. Büchel).

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addition to the older results, it could be demonstrated that PSII is indeed enriched in the four core membranes and that the cytochrome b6/f complex is distributed evenly in all six membranes.

However, it is still unclear why diatom thylakoid membranes are organised in this way (*e.g.* why three thylakoids, not more) and what the basic prerequisites and forces are to maintain this thylakoid structure.

1.2. Thylakoid membrane dynamics and structural rearrangements

The three-dimensional thylakoid membrane structure has to be dynamic and thus tightly regulated to cope with environmental changes, like temperature variations, different nutrient supply or fluctuations in the light situation. This applies to diatoms in particular as they are unicellular and immobile and thus have to adapt quickly to the conditions in their current habitat.

For higher plants Kirchhoff et al. [7] mentioned four possible reorganisations of thylakoid membranes in response to light. Some are long-term acclimations like the lateral shrinkage of the grana stacks or the increase of the number of membranes per stack, while others are occurring on a shorter time scale. The latter include the swelling of the lumen of the individual thylakoids or a vertical destacking of the membranes, which increases the interthylakoidal space. In contrast to the dynamic grana structure, diatom thylakoid membranes seem to stick strictly to the number of three thylakoids per stack, even under different culture conditions like *e.g.* high light or reduced nutrient availability, and mainly change the number of bands per plastid [2–4,8]. However, swelling of the lumen as a short-term response to light was reported as well [9].

Yet, to date it is unclear how thylakoid membrane flexibility, which is probably crucial for fine-tuning excitation energy distribution in a system that lacks state transitions [10], is implemented and how membrane appression is regulated in diatoms.

1.3. Forces that enable stacking of membranes

Grana membrane stacking has long been believed to be based solely on the adhesion between Lhcb proteins (LHCII or CP26 or both) in the adjacent membranes *via* an interaction of the N-terminal parts of the proteins with divalent cations [11–13]. However, more recent data, which involve the studies of mutants, suggests that this view is too simplistic and that the dominant factor cannot be just one single protein complex [14], but that at least PSII is involved as well.

Membrane stacking can be explained entirely by the interplay of physicochemical forces. These comprise mainly van der Waal's attractive forces and electrostatic repulsive forces, the latter of which can be screened by cations or enhanced by the lack thereof [15,16]. The Mg^{2+} concentration required in higher plants was determined to be between 1 mM and 5 mM ([15] and references therein, *e.g.* [13,17,18]). A third force, which is only effective at short distances, is hydrostatic repulsion. In conclusion, the sum of the repulsive and attractive forces determines if membranes are able to stack and also the distance between them [15].

1.4. Measurement of thylakoid membrane appression

Thylakoid membrane distances have classically been determined using transmission electron microscopy, but these measurements are hampered by artefacts introduced by the inevitable fixation and dehydration procedure. More recent determinations exploit the Bragg reflection peak in small-angle neutron scattering (SANS) measurements, which is caused by the periodic arrangement of thylakoid membranes. This peak can be used to estimate the membrane repeat distances. For example, for diatoms (*Phaeodactylum tricornutum*) values of approximately 170–180 Å have been measured for the distance of two adjacent thylakoids, thus representing the distance between two lumen [19]. Distances for higher plant grana range between 160 Å and 210 Å [20].

The periodical arrangement of the thylakoid membranes, the fact that all pigments in photosynthetic organisms are oriented non-randomly [21], and, in particular, the chiral order of the chromophores also enable measurements using circular dichroism (CD) spectroscopy. This technique has previously been used successfully to assess structure-related questions in higher plants and algae (*e.g.* [22–24]). The pigments of thylakoid membranes can produce three types of signals that superimpose but differ in their intensity. The weakest CD signals arise from intrinsic asymmetry of molecules, like *e.g.* from chlorophylls in acetone. These signals have a similar band shape as the absorption of the sample. Their sign is determined by the handedness of the molecule and it can thus be of opposite sign in comparison to the corresponding absorption band [21]. In small aggregates or molecular complexes with tightly packed pigments short-range, excitonic interactions due to dipole-dipole interactions of the pigments lead to split signals of identical band shapes and equal amplitudes for the positive and negative bands. The spectrum of isolated LHC is an example for these intermediate strength signals. Long-range ordered aggregates can produce so called polymerisation-or-salt-induced (psi-type) signals, which are characterised by their high signal magnitude and their non-conservative band shape. Psi-type signals usually display long tails outside of the principal absorbance bands of the pigments [25]. Signals of this type have been observed *e.g.* in DNA aggregates and condensed chromatins [21]. Such an aggregate delocalises excitation energy and responds collectively to incident radiation [25]. For this type of signals to occur the aggregate must be (chromophore-) dense and it has to have dimensions comparable to the wavelength of the light used in the CD measurements (greater than ¼ of the wavelength of the incident light; [25]). The magnitude of this signal commonly rises with the degree of aggregation [25], thus the amplitude of the psi-type band reflects the extent of the long-range chiral order of the chromophores [9,25,26]. On the basis of this correlation, CD signals can be used to obtain information on the molecular structure of the sample. For grana stacks, but also for cells of diatoms large psi-type CD signals have been reported. The amplitude of the psi-type CD signal in *P. tricornutum* was shown to be sensitive to heat-treatment [23] and to detergents [22]. Furthermore, the membranes were suspected to be capable of undergoing osmolarity-induced and light-induced re-organisations [9,23,27].

The present work uses CD and fluorescence spectroscopy to assess the response of thylakoid membranes of the centric diatom *T. pseudonana* to treatments supposed to influence membrane adhesion like different ion availability. Variations in ion species and concentrations allow conclusions on the forces that contribute to membrane appression in diatoms and to compare them with the effects seen in higher plants.

2. Materials and methods

2.1. Growth conditions

Batch cultures of *T. pseudonana* (CCMP1335, Hustedt) were grown at 16 °C in modified F/2 medium [28] using a 16 h light (40 µmol quanta $m^{-2} s^{-1}$), 8 h dark cycle with constant shaking at 100 rpm.

2.2. Plastid and thylakoid purification from T. pseudonana

For plastid purifications, a two-day-old pre-culture was used to inoculate a 4 l flask culture to a cell density of 100,000 cells/ml. These cells were grown for five days under constant aeration using ambient air. They usually reached a cell density of approximately 6–8·10^6 cells/ml and were harvested by centrifugation (5000*g*, 10 min, 4 °C) 1 h after the onset of light. Plastids were purified according to the protocol published in [29] with minor modifications. In brief, harvested cells were resuspended in a total of 20 ml isolation medium (0.5 M sorbitol; 50 mM HEPES-KOH; 6 mM Na-EDTA; 5 mM $MgCl_2$; 10 mM KCl; 1 mM $MnCl_2$; 1% (w/v) polyvinylpyrrolidone (PVP) 40 [K30], pH 7.5; the osmolarity was adjusted to 750 mOsmol/kg using 1 M sorbitol or water)

to which 0.5% (w/v) fatty acid free bovine serum albumin and 0.1% (w/v) cysteine was added in advance. These additives are supposed to reduce protease activity, protect proteins from oxidation and thereby help stabilising the plastids after cell disruption [29]. PVP is furthermore known as complexation agent and is therefore used to adsorb polyphenols, which can inhibit protein function. The cells were broken using a french press pressure of 13 MPa. After cell rupture samples were kept on ice and all centrifugation steps were performed at 4 °C. Cell debris was removed by centrifugation (300*g*, 9 min), before the plastids were collected at 6000*g* (10 min). The sediment was resuspended in 2 ml of isolation medium and subsequently layered on top of a 10–30% (v/v) percoll step gradient. The gradient was spun at 14,400*g* for 30 min and the plastids, which were recovered from the 20% percoll layer of the gradient, were washed using isolation medium and a centrifugation step of 10 min at 4000*g*. Plastid quality and purity was analysed microscopically and no intact cells were found in the purified plastid fraction throughout all preparations performed. The chlorophyll *a* (Chl a) concentration of the purified plastids was determined according to Jeffrey and Humphrey [30] using a Jasco V-650 spectrophotometer and was subsequently adjusted to 1.5 mg/ml Chl a to have sufficiently concentrated samples for all down-stream experiments. Plastids were either used directly for measurements or shock-frozen in liquid nitrogen and then stored at −80 °C.

We use the term 'plastids' for this preparation, although they actually do not represent intact plastids. However, despite the fact that the envelope membrane is broken, the thylakoid membrane structure remained as judged from the CD and electron microscopic analyses (see Results), in contrast to the thylakoids, which were prepared as described in Büchel [31] from 7-day-old *T. pseudonana* batch cultures. These are indeed thylakoid membrane fragments without the three thylakoid band structure, but are referred to as 'thylakoids' throughout the literature.

2.3. CD spectroscopy

CD spectra were recorded from 350 nm–750 nm or 600 nm–750 nm with a Jasco J-810 dichrograph (sensitivity: 100 mdeg; optical path length: 1 cm), which was equipped with a Jasco PTC-423S Peltier element. Unless stated otherwise, measurements were carried out at room temperature (22 °C), using 2 nm bandwidth and a 0.5 nm step size with a response time of 4 s at a scanning speed of 100 nm/min for thylakoids and plastids. In the case of cells the bandwidth was set to 3 nm, the scanning speed was 50 nm/min and a response time of 8 s was used instead.

Baseline measurements and dilutions were performed using the respective isolation or culturing media, *i.e.* F/2 medium for cells, buffer C for thylakoids (see [31]) and isolation medium or salt-free buffer (0.5 M sorbitol, 50 mM HEPES-KOH, 1% (w/v) PVP 40 [K30], pH 7.5; the osmolarity was adjusted to 750 mOsmol/kg using 1 M sorbitol or water) for plastids. The Chl a amounts needed to obtain an optimal signal-to-noise ratio in CD spectra had been determined in previous experiments (data not shown). Cell samples were taken from 7-day-old cell cultures, which were allowed to sediment in the flask prior to measurement. Spectra of thylakoid samples were recorded at 12.5 µg Chl a/ml. Plastid samples were adjusted to a final Chl a concentration of 7.2 µg/ml with either isolation medium, isolation medium of different osmolarities (adjusted using sorbitol), or salt-free buffer. To avoid a harsh centrifugation step, the adjustments were accomplished by dilution, resulting in minimal concentrations of 0.024 mM $MgCl_2$, 0.048 mM KCl and 0.0048 mM $MnCl_2$, due to the remaining traces of isolation medium from the plastid sample. Depending on the experiment, samples were supplemented with different concentrations of either $MgCl_2$, KCl, $CaCl_2$, NaCl or $MgSO_4$. Plastid samples were incubated for 5 min at 4 °C in darkness prior to measurement.

Shearing forces were applied to the plastids by repeatedly squeezing them through a 10 ml-syringe equipped with a Sterican size 2 hollow needle. The temperature dependence of the CD signals was determined using a temperature gradient provided by a Peltier element. A plastid sample was heated from 20 °C to 45 °C in steps of 5 °C, which each took 60 s to be completed. A CD spectrum was measured at each step. The control sample was kept constantly at 22 °C (RT) and was measured just as often as the temperature-treated sample.

CD spectra were baseline-corrected and normalised using the absorption peak of Chl a in the Q_Y region, as determined by the CD spectrometer. In case maximum or minimum amplitudes of different plastid preparations were compared, amplitudes were further normalised using the values from spectra recorded in isolation medium to account for small differences in preparations. Overall, the different plastid isolations showed a constant ratio between the amplitudes of the positive and the negative peak of approximately 1.2, indicating that the quality of the plastids was stable and did not influence the analysis of the peak heights.

To examine the stability of the samples under measurement conditions, six consecutive spectra were measured for each sample, which took 595 s in total. The amplitudes of these six spectra were plotted as a function of time and the slope was calculated by linear regression. To calculate the relative degradation speed, the values obtained for the slope were normalised by the amplitude of the initial spectrum.

Measurements of at least four biological replicates were averaged and plotted as a function of the cation concentration.

2.4. Oxygen evolution measurements

Plastids were subjected to oxygen evolution measurements in a Clark electrode (Hansatech, UK) at 25 °C with 4 mM potassium ferricyanide as electron acceptor. Oxygen evolution was measured for 1 min in darkness, followed by 8 min illumination. Maximal oxygen production was measured at 600 µmol quanta $m^{-2} s^{-1}$ of white light and using the uncoupler NH_4Cl at a final concentration of 10 mM. The oxygen evolution of plastids was compared with that of intact cells measured at the same light intensity. To test whether the whole electron transport chain was intact, plastids had been measured using the electron acceptor for PSI, methyl viologen (1,1′-dimethyl-4,4′-bipyridinium dichloride) at a concentration of 0.1 mM, together with 1 mM of the catalase inhibitor sodium azide. Indeed, a light-dependent oxygen consumption had been detected (data not shown). To investigate the influence of different Mg^{2+} concentrations on plastids, freshly prepared plastids (50 µg Chl a/ml per measurement) were diluted in either isolation medium or salt-free buffer, which was adjusted to different $MgCl_2$ concentrations. For these measurements a light intensity of 150 µmol quanta $m^{-2} s^{-1}$ of white light was used.

2.5. Fluorescence measurements

Absorbance spectra were measured using a Jasco V-650 spectrophotometer in order to adjust the absorbance to 0.03 in the Chl a Q_Y absorption maximum, which corresponds to roughly 0.3 µg/ml Chl a.

Fluorescence spectra were measured at RT or 77 K with a Jasco fluorometer (FP-6500). In the latter case, the buffers contained 60% (v/v) glycerol as described in [32]. The emission and excitation bandwidth was set to 3 nm. A rhodamine B spectrum served as a reference for the correction of the excitation side and the photomultiplier was corrected using a calibrated lamp spectrum. Emission spectra were recorded from λ em = 600 nm to λ em = 800 nm in 0.5 nm steps with an excitation wavelength of λ ex = 440 nm (primarily exciting Chl a). For excitation spectra of the same samples, emission was recorded at λ em = 689 nm (PSII) and λ em = 710 nm (PSI) upon excitation of the sample from λ ex = 400 nm to λ ex = 600 nm. In order to increase the signal-to-noise ratio, for 77 K measurements three spectra of each sample were averaged. At least three biologically independent samples were measured per salt concentration.

3. Results

3.1. CD signals as a sensor for structural integrity

The non-invasive, powerful CD spectroscopy technique, which has been widely used in the past to assess structure-related questions in higher plants and also in algae (*e.g.* [22–24]), was used in this study to investigate the influence of different ions on the thylakoid membrane architecture of *T. pseudonana*. To this end, plastids were isolated. The thylakoid structure in bands of three was retained in most plastids, whereas the envelope was disrupted nearly in all cases, exposing the thylakoid membranes directly to the buffers and treatments applied (Suppl. Fig. 1). Although this preparation did not produce intact plastids, we use the term plastid throughout, since the decisive feature – the thylakoid arrangement in bands of three – was retained. When measuring the CD signal of *T. pseudonana* cells there was a strong signal in the long-wavelength region, which peaked at (−) 670 nm and (+) 690 nm (Fig. 1A). This signal has been shown to be of non-excitonic origin [23,24] and as the positive band was of non-conservative shape displaying a long tail outside of the absorbance, it can be considered a psi-type band (see also Suppl. Fig. 2). The psi-type signal has previously been attributed to the macro-organisation of the native thylakoid membranes – *i.e.* their multilamellar order – and its intensity reflects their degree of organisation [9,23]. It can thus be used as a fingerprint signal for structural features, *i.e.* the long-range (chiral) order of chromophores. Just as Szabo et al. [23] have shown for *P. tricornutum* before, the main peak was lost upon the isolation of thylakoid membranes in *T. pseudonana* as well. In contrast, the signal was preserved in plastids, albeit to a lesser extent than in cells (Fig. 1A). Further evidence that the long-wavelength signal originated from the macro-organisation of the membranes is provided in Fig. 1B. Plastids were squeezed through a syringe several times subjecting them to mechanical stress. With increasing numbers of passages, this treatment led to a gradual diminishment of the amplitude of the psi-type signal maximum ((+) 690 nm) as well as the amplitude of the minimum ((−) 670 nm). It has been shown earlier that increasing temperatures also decrease the psi-type signal in diatom cells [23]. Thus, the effect of elevated temperatures on a plastid samples' CD spectrum was monitored (Fig. 1C and D). With a gradual increase in temperature, the psi-type feature and the minimum band diminished progressively, while absorbance spectra remained unaffected (data not shown). Thus, a change in the long-range chiral order of the pigments had taken place. At 45 °C the plastid signal resembled that of thylakoids (compare Fig. 1A). Therefore, the long-wavelength positive and negative band can be regarded as long-range order fingerprint signals, their intensity reflecting the structural integrity of the thylakoid membrane arrangement.

3.2. Effects of ions on the amplitudes of the long-range order fingerprint signals

One of the long-standing questions in diatom thylakoid membrane research is which parameters are responsible for the appression of the core membranes of the three thylakoid membrane band. One possibility are ionic interactions as in higher plant grana [12,13,33]. Indeed, pronounced effects of $MgCl_2$ on the CD spectrum of *T. pseudonana* plastids were observed (Fig. 2A). Plastid samples were diluted in salt-free buffer supplemented with different concentrations of $MgCl_2$ and their CD spectrum was determined. The amplitudes of the psi-type peak

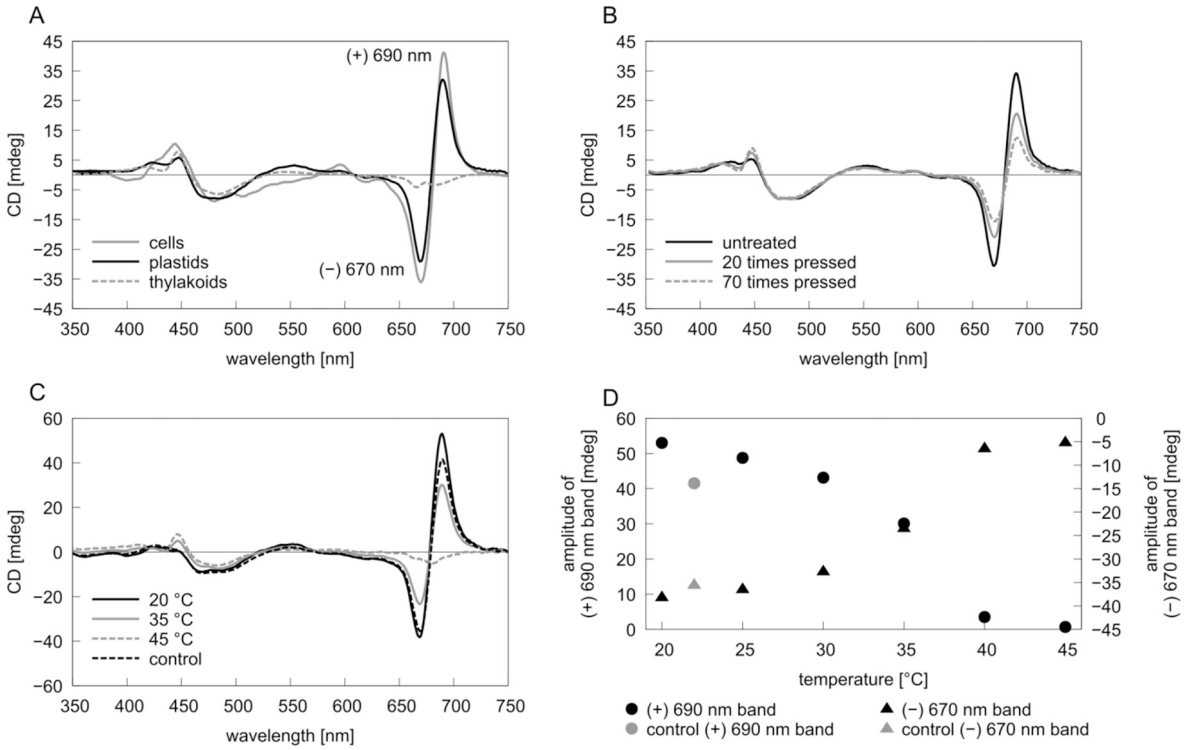

Fig. 1. CD signals as a sensor for structural integrity.
A) In comparison to the cell sample (solid grey line), the strong long-wavelength signal, which peaked at (−) 670 nm and (+) 690 nm, was lost during the isolation of thylakoid membranes (dotted grey line). In contrast, the signal was preserved in isolated plastids (solid black line).
B) A plastid sample (solid black line) was pressed numerous times through a syringe in order to mechanically disrupt the sample (solid grey line: after 20 times; dotted grey line: after a total of 70 times).
C) and D) A temperature gradient was used to disturb the long-range chiral order of the membranes. In C) example spectra at 20 °C (solid black line), 35 °C (solid grey line) and 45 °C (dashed grey line) are shown. A sample that was kept constantly at 22 °C (RT) and was measured just as often as the temperature-treated sample served as control (dashed black line). In D) the amplitudes of the positive CD band at (+) 690 nm (black circles, left y-axis) and the negative CD band at (−) 670 nm (black triangles, right y-axis) are shown as a function of temperature. The values of the RT control are shown using the same symbols in grey.

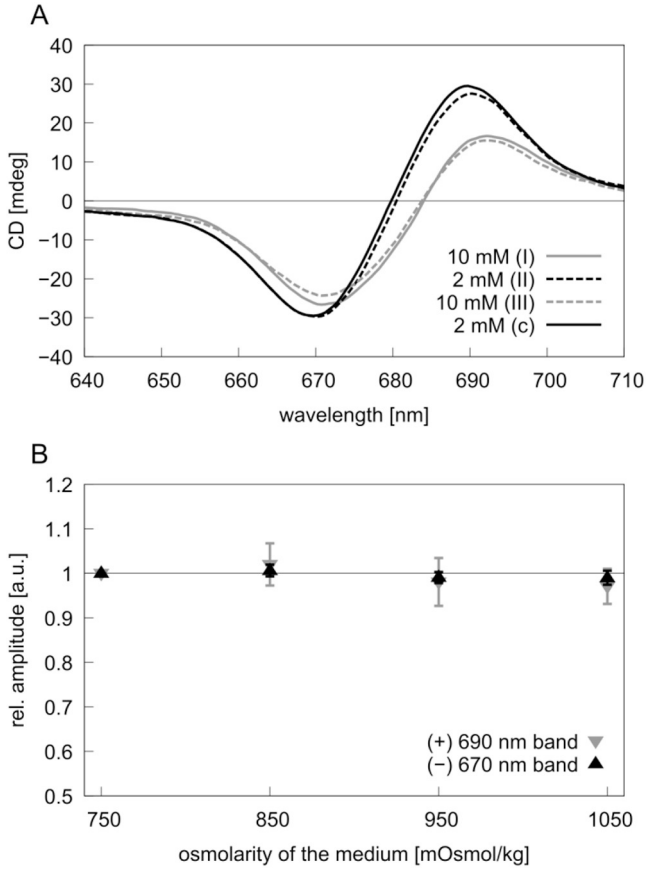

Fig. 2. Spectra were affected by the salt concentration, but not by the osmolarity of the medium.
A) Plastids were either incubated with 2 mM $MgCl_2$ ((c), solid black line) or 10 mM $MgCl_2$ ((I), solid grey line). The reversibility of the salt effect was tested by preparing a sample in 10 mM $MgCl_2$ with the doubled amount of plastids (36 µg Chl a) and then splitting it in half. While one half was diluted to the ordinary Chl a concentration while maintaining 10 mM $MgCl_2$ ((I), solid grey line), the other half was diluted to the same Chl a concentration, but to a final $MgCl_2$ concentration of 2 mM ((II), dashed black line). After measuring the CD signal of both samples, $MgCl_2$ was re-added to the 2 mM sample to reach a final concentration of 10 mM (80 µl stock solution to 2.5 ml sample) ((III), dashed grey line). Data are representatives of at least four independent measurements. B) The influence of the osmolarity of the medium on the plastid spectrum was investigated. All buffers used in the experiments shown in A) had been initially adjusted to 750 mOsmol/kg using sorbitol, before additional $MgCl_2$ was added. Here, isolation medium adjusted to different osmolarities by changing the sorbitol concentration was used. Grey triangles represent the data points for the relative amplitude of the (+) 690 nm CD band, while the relative amplitude of the (−) 670 nm band is depicted as black triangles. All data were normalised to the respective amplitudes in isolation medium (750 mOsmol/kg). The data represent averages and standard deviations of four independent measurements.

and the minimum band were found to depend on the ion concentration such that the positive psi-type peak was reduced under high $MgCl_2$ concentrations (10 mM) compared to samples measured at 2 mM, whereas the minimum band was not significantly impaired. The stronger diminishment of the (+) band in comparison to the (−) band at high $MgCl_2$ concentrations was also visible in the shift of the wavelength of the positive maximum towards longer wavelengths. The different concentration dependence indicates a separate origin of the two bands. In order to exclude a simple degradation due to non-physiological ion conditions, the reversibility of the effects upon $MgCl_2$ addition was investigated (Fig. 2A).

A sample that had been exposed to 10 mM $MgCl_2$ was adjusted to 2 mM $MgCl_2$. This immediately and completely reversed the effects of the high salt condition. When the salt concentration was increased again to 10 mM, the effects were reversed again. This reversibility indicates that the salt concentrations used were not destructive *per se*. Since the addition of salt to the buffer also led to an increase in the osmolarity, the influence of the osmotic environment was investigated in order to rule out that the effects observed when adding salts can be attributed to osmotic effects only. Plastid samples were measured in isolation media, which were adjusted to different osmolarities using sorbitol, and the relative amplitudes of the maximum and the minimum bands were monitored (Fig. 2B). Both amplitudes showed no significant deviation between 750 mOsmol/kg and 1050 mOsmol/kg, an osmolarity much higher than that induced by the concentrations of added salts. Thus, the osmolarity did not contribute significantly to the relative amplitudes and the salt effects were not of osmotic origin.

$MgCl_2$ strongly influenced the peak heights, but it was unclear whether this reaction was triggered by the anion or the cation and if the reaction was ion-specific. In order to answer these questions, the influence of different salts on the long-range order signals was tested (Fig. 3). Besides $MgCl_2$, $MgSO_4$ and $CaCl_2$ were tested and led to similar results (Fig. 3A and C). Both divalent cations (Mg^{2+} and Ca^{2+}) had the same effect on the amplitudes of the psi-type peak maximum and the minimum, independent of the anion. As already described above, the amplitudes of the positive and negative band showed a different ion concentration dependency, which hints at an independent nature of both bands. There was only a small concentration range between 2 mM and 5 mM that seemed optimal for both bands' signals. Lower concentrations reduced the negative band and higher concentrations negatively influenced both bands. A saturation of the reaction to divalent cations was observed in concentrations higher than approximately 30 mM (data not shown). To test whether the effects depended solely on the charges or were more specific, the influence of monovalent cations was assessed as well (Fig. 3B and D). In comparison to divalent cations, ten times higher concentrations of monovalent cations were necessary to achieve similar effects, both on the psi-type peak and on the minimum band and thus also on the shift in wavelength (Suppl. Fig. 3). This finding might be explained by the lower charge density of monovalent cations. No obvious differences between NaCl and KCl were found. For all salts signal loss caused by high salt concentrations was fully reversible upon reducing the concentration to the optimal range (data not shown). These data demonstrate that divalent cations were most effective and that anions did not play a role.

3.3. Thermal stability of the CD signals

The different ion concentrations used might have an influence on the stability of the membranes. In order to investigate this, six consecutive spectra per sample were measured at RT. Using this approach, the amplitudes of the psi-type maximum and the minimum band could be monitored over a total of 595 s at different ion concentrations (Fig. 4). Under low salt conditions (0.024 mM $MgCl_2$; Fig. 4A), the signal of both bands diminished rapidly. At a concentration of 2 mM $MgCl_2$ no decrease in amplitudes was detected over the entire measuring time (Fig. 4B). High salt concentrations (10 mM $MgCl_2$; Fig. 4C) also led to an increased signal degradation over time in comparison to the 2 mM sample. Furthermore, as described above, at high $MgCl_2$ concentrations the psi-type maximum band was affected to a stronger extent than the minimum. This is revealed by the shift of the wavelength of the peaks to the red region under high salt conditions. As shown above, the signal loss of the initial amplitude due to high $MgCl_2$ concentrations was completely reversible when ion concentrations were reset to optimal conditions. In contrast, the addition of 2 mM $MgCl_2$ to a low salt sample could not reverse, but stop, the progressive degradation of the signal amplitude (Fig. 4D). This observation applied to all salts in this study (data not shown). While retaining the effects on the initial amplitude heights, degradation over time was no longer visible in any of the samples when measuring at 4 °C (Suppl. Fig. 4). These results indicate that the decrease in amplitudes over time was due to thermal

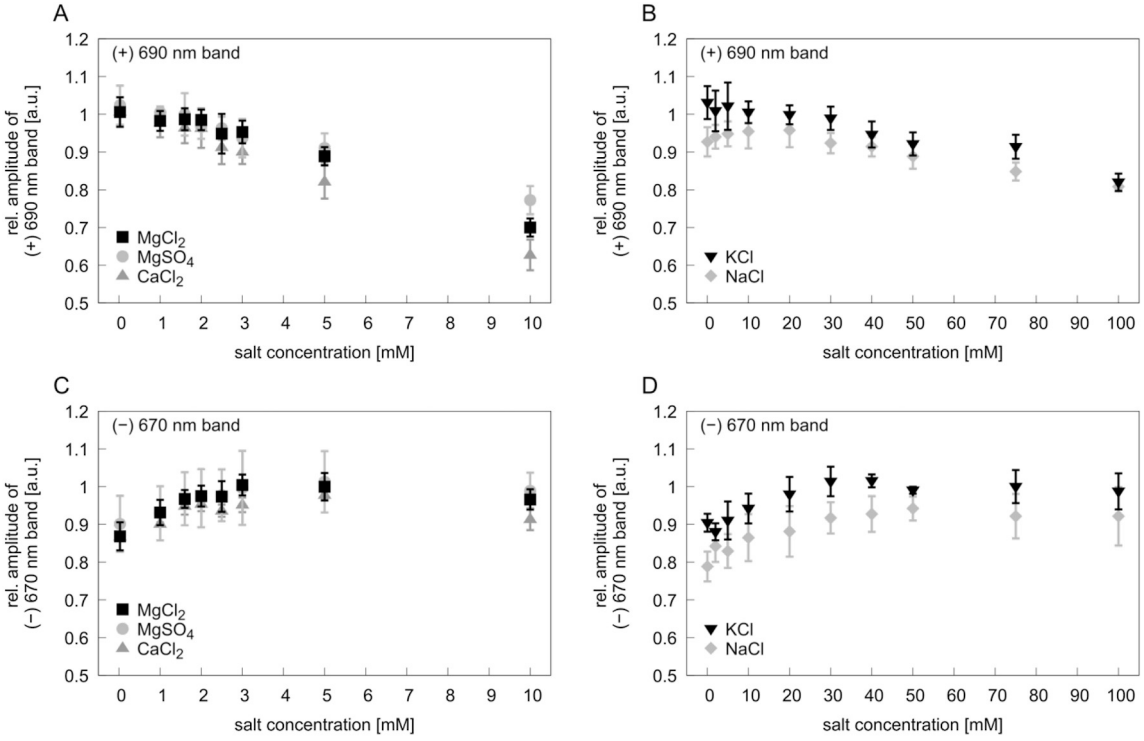

Fig. 3. Effect of different salts on the amplitudes of the CD signal.
In A) and B) the influence of different salts on the amplitude of the psi-type maximum was tested, while C) and D) show the effect the salts had on the amplitude of the minimum. In A) and C) the effects of divalent cations and of the anion were tested, while the results of the measurements with monovalent cations are depicted in B) and D). To account for differences in plastid quality, data are shown as relative amplitudes, *i.e.* the amplitudes of the maximum and minimum CD signal, respectively, are normalised using the amplitudes from spectra recorded in isolation medium. $MgCl_2$ samples are represented by black squares, $CaCl_2$ samples are shown as grey triangles, while $MgSO_4$ samples are depicted as grey circles. KCl data are shown as black triangles and NaCl samples are reflected by grey diamonds. Data represent means and standard deviations of measurements of at least four biological replicates.

influences and that cations played an important role as stabilisers of the thylakoid membrane against temperature induced degradation.

In order to test the influence of different salts in varying concentrations on the degradation over time in more depth, data of at least four biological replicates per concentration were analysed. The relative degradation speed of the psi-type positive and the negative band are shown in Fig. 5. The degradation proceeded in a similar manner with all divalent cations and approached zero at 2–4 mM. Therefore, this concentration range seemed to be ideal for protecting the signal from reduction (Fig. 5A and C). For monovalent cations this optimal concentration range appeared to be between 20 mM and 40 mM (Fig. 5B and D). These findings demonstrate that cations in optimal concentrations are also necessary to stabilise the existing thylakoid structure against degradation.

3.4. Effects of ions on the functionality of the thylakoids

As a next step, we investigated the physiological consequences of the different salt conditions. As a parameter to estimate electron transport activity, oxygen evolution measurements (from H_2O to FeCy) were carried out. Previous light saturation experiments had shown that a light intensity of 150 µmol quanta $m^{-2} s^{-1}$ yielded best results with respect to both the stability of the plastids over the entire measuring time and oxygen evolution rates (data not shown). However, under optimal conditions, *i.e.* at a light intensity of 600 µmol quanta $m^{-2} s^{-1}$ and under 10 mM of the uncoupler NH_4Cl to relax the pH gradient, maximal oxygen evolution rates of 58.5 ± 6.1 µmol O_2 mg^{-1} Chl a h^{-1} could be obtained. Thus, plastids reached about 55% of the values measured in *T. pseudonana* cells (106 ± 18 µmol O_2 mg^{-1} Chl a h^{-1}; see also [34]).

The data in Fig. 6A show that in low ionic conditions not only the maximum oxygen evolution rate at a light intensity of 150 µmol quanta $m^{-2} s^{-1}$ is lowered in comparison to 2 mM and 10 mM $MgCl_2$, but also the stability of the oxygen evolution over time is impaired. While in higher salt concentrations the oxygen evolution rate remained relatively stable over 8 min, the relative reduction in the low salt sample was high. After 8 min of measuring time, the oxygen evolution rate had dropped considerably. In conclusion, these results imply an important role of salts in maintaining the functionality of the thylakoid membranes, *i.e.* electron transport from water to FeCy.

While oxygen evolution measurements reflect the electron transport capacity of the thylakoids, fluorescence measurements provide insight into the excitation energy transfer between chromophores in single pigment-protein complexes and also between these complexes. To test for intactness of excitation energy transfer as well as for excitation energy distribution between the photosystems, 77 K fluorescence emission spectra were recorded (Fig. 6B). Plastid samples were incubated in the same buffers and conditions as used for CD measurements and excited at 440 nm (exciting predominantly Chl a). Two peaks at 688 nm and 710 nm were visible. When spectra were normalised in the emission maximum at 688 nm, no differences in fluorescence emission between the 0.024 mM and 2 mM $MgCl_2$ sample were detectable, but in the spectra of plastids incubated with 10 mM $MgCl_2$ the emission around 710 nm was increased (Fig. 6B). This emission was previously attributed to PSI in diatoms [32]. FCPs were shown to emit around 678 nm [35] and the emission maximum at 688 nm is due to PSII. One important point became visible when normalising spectra at 707 nm (inset Fig. 6B), *i.e.* at the emission wavelength of PSI: again the FCP emission at around 678 nm was not changed, only PSI and PSII changed their emission relative to each other. This result implies that no change in the emission of FCP complexes occurred under the three conditions tested, but relatively more excitation energy reached PSI under high salt conditions, which in turn means that relatively less energy reached PSII. At RT, where PSI emission is negligible, the emission spectra did not change as a function of the Mg^{2+} concentration. Neither the shape, nor the maximal amplitude

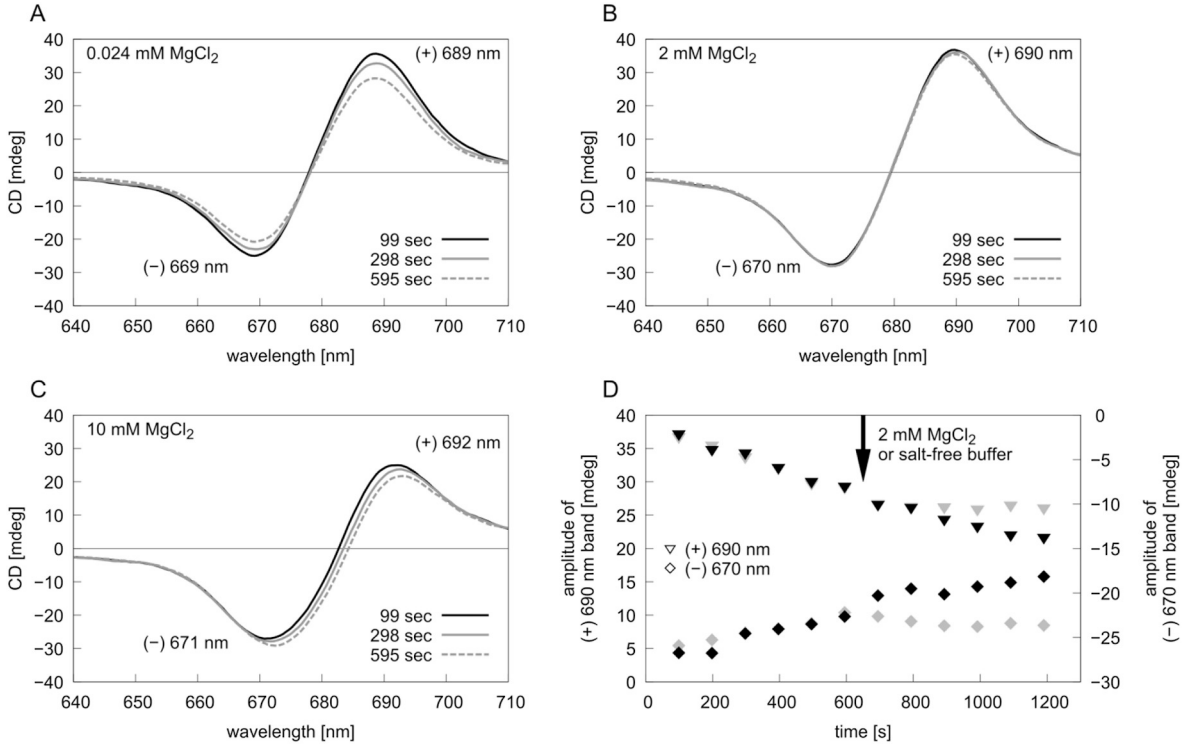

Fig. 4. Effect of cations on the stability of the plastids.
A)–C) The amplitudes of the maximum and the minimum were monitored over time at 22 °C (RT). The solid black line represents the first spectrum completed after 99 s, while the solid grey line shows the spectrum of the plastids after a total of 298 s (three consecutive measurements). The dashed grey line depicts the data of the last measurement completed after 595 s. Measurements were done at a $MgCl_2$ concentration of 0.024 mM (A), 2 mM (B) or 10 mM (C), respectively. The same measurements were also performed at 4 °C and are to be found in Suppl. Fig. 4.
D) Plastid samples were measured in low salt conditions (0.024 mM $MgCl_2$) like in A), and the amplitudes of the maximum (triangles, left y-axis) and the minimum (diamonds, right y-axis) are shown as a function of time. Between the sixth and the seventh spectrum (arrow), $MgCl_2$ was added to reach a final concentration of 2 mM (grey symbols), while the same amount of salt-free buffer was added to the 0.024 mM $MgCl_2$ control sample (black symbols). Data are representatives for at least four biological replicates. A comparison of degradation speeds is to be found in Fig. 5.

varied (Suppl. Fig. 5).

Although excitation at 440 nm is exciting Chl c as well, no emission of this pigment at 636 nm was detectable in the fluorescence emission spectra, arguing for intact pigment-protein complexes. This assumption is strengthened by the 77 K excitation spectra (Fig. 6C and D), where coupling of fucoxanthin (around 535 nm) as well as Chl c (around 490 nm) to Chl a emitting at 689 nm or 710 nm, respectively, was visible. The intactness of excitation energy transfer from the accessory pigments to Chl a was slightly decreased in the sample at 0.024 mM $MgCl_2$ compared to the other conditions, equally for both emission wavelengths. These results indicate that low salt conditions impair the intactness of thylakoids as already seen in the oxygen evolution experiments, whereas at high salt concentrations the excitation energy transfer can be altered in such a way that less energy reaches PSII in favour of PSI.

4. Discussion

4.1. CD signals reflect the structural integrity of the thylakoid membranes

The comparison of the *T. pseudonana* cell and plastid CD spectrum revealed that plastids indeed kept the major features related to the intactness of the thylakoid structure, but with slightly reduced amplitude. Thus, signal changes monitored here were probably smaller than those to be expected *in vivo*. Note that, unlike in other diatoms and heterokonts (*e.g. Cyclotella meneghiniana*; [36] or *Pleurochloris meiringensis*; [24]) and in higher plants (*e.g.* [37]), the positive band at (+) 690 nm in *T. pseudonana* cells is already rather small relative to the negative CD band around (−) 670 nm. In comparison to its close relatives, *T. pseudonana* cells are smaller, which translates to smaller plastid sizes and thus to a modified chiral system of pigments.

In plastid samples, the psi-type signals responded to temperature changes with a reduction to thylakoid level at 45 °C, while the absorbance spectra were unaffected. At this temperature, the signal was composed only of weaker excitonic interactions, which indicates that the long-range interactions of pigments were missing. When comparing the temperature dependence with that reported by Szabo et al. [23], the membranes of *T. pseudonana* disintegrated at lower temperatures. This might be due to the fact that plastids were used instead of whole cells, but more likely reflects the differences in lipid composition between the two species ([38] and references therein), in particular concerning fatty acid length and saturation. This observation is also in accordance with the lower optimal growth temperature of *T. pseudonana* compared to *P. tricornutum*. Later, Nagy et al. [9] published a study in which CD spectra of *P. tricornutum* cells were compared with SANS spectra, a method that enables membrane distance determination. The authors could show that the loss of psi-type amplitude seen under heat treatment is accompanied by an increase in membrane distance. Thus, the psi-type CD signal can most probably be attributed not only to the lateral macro-organisation, but also to the vertical appression of thylakoid membranes. The plastid CD signal was also sensitive to mechanical stress. Therefore, the long-wavelength signal is correlated with the structural integrity of the thylakoid membrane and can be considered a fingerprint signal for organisation – for the lateral macro-organisation as well as for vertical appression – in line with previous results in *P. tricornutum* [9,23].

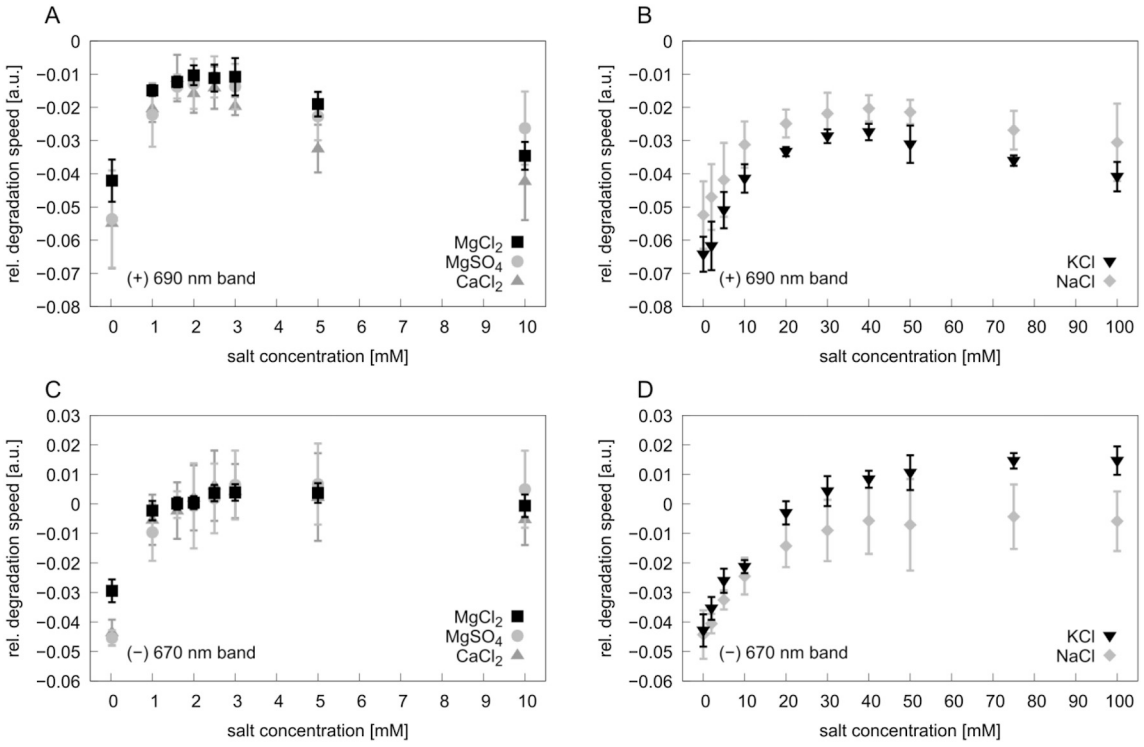

Fig. 5. Effect of salts on the stability of the plastids.
The plots compare the influence of different salts on the concentration- and time-dependent degradation of the amplitude of the maximum and the minimum band. The relative degradation speed was calculated as change in amplitude over time (in s) normalised to the initial amplitude to account for differences in plastid quality and Chl a concentration (see Materials and methods for details). A) and B) focus on the speed of degradation of the amplitude of the (+) 690 nm band, while C) and D) show the degradation speed of the amplitude of the (−) 670 nm band. In A) and C) the influence of different divalent cations and of the anion is compared. B) and D) depict the results obtained with monovalent cations. $MgCl_2$ samples are represented by black squares, $CaCl_2$ samples are shown as grey triangles, while $MgSO_4$ samples are depicted as grey circles. KCl data are shown as black triangles and NaCl samples are reflected by grey diamonds. Data represent averages and standard deviations of at least four biological replicates per concentration.

4.2. Effects of ions on the structural integrity of the thylakoid membrane

In order to test the hypothesis that the appression of thylakoids in diatoms might be due to the interplay of physicochemical forces like seen in higher plants grana, the effects of different salts were tested. Although monovalent cations could change the amplitude of the (+) 690 nm band by about 20%, changes of the psi-type maximum in this order of magnitude required concentrations of up to 100 mM. In contrast, already 10 mM of divalent cations caused a reduction of the psi-type peak amplitude by 30%, nearly independent of the divalent cation used. *In vivo*, this effect is likely based on Mg^{2+}. This assumption is underpinnend by the fact that Mg^{2+} is the most abundant divalent cation in chloroplasts of land plants and algae [39]. Furthermore, in higher plants Mg^{2+} was found to be transported into the chloroplast stroma in a light-dependent way [40], which is most likely the case in diatoms as well.

The parameters tested in this work responded to Mg^{2+} concentrations between 2 mM and 5 mM in a notable way: The amplitudes of both the negative and the positive CD band in the Q_Y region reached their highest values between 2 mM and 5 mM Mg^{2+}, and, based on their relative degradation speed, the stability of both bands against temperature-induced degradation was at its maximum. Therefore, the range between 2 mM and 5 mM Mg^{2+} was considered ideal for maintaining the thylakoid membrane structure. The oxygen evolution rate measured at 2 mM supports this conclusion. 2 mM Mg^{2+} was even able to halt – albeit not reverse – the temperature induced degradation triggered by low Mg^{2+} concentrations.

The optimal concentration range is in line with values observed for the formation of higher plants grana stacks [15]. Thus, it is tempting to speculate that the inner four of the six diatom thylakoid membranes resemble the grana membranes of higher plants concerning their adhesion behaviour.

Outside the optimal range, the behaviour of the parameters investigated fell into two major groups. The first group consists of parameters, which were affected by low salt concentrations and were stable at intermediate and high salt concentrations. The oxygen evolution rate, the amplitude of the negative CD band and its stability fall into this group (Fig. 7A). The second group comprises parameters, which were only influenced under high salt and were unaffected in the other two conditions (Fig. 7B). The excitation energy input to PSII relative to PSI as estimated from the fluorescence emission spectra at 77 K and the amplitude of the positive, psi-type CD band reacted in that way to changes in the cation concentration.

The existence of these two groups indicates that there are two different effects, each of which dominates the behaviour of the corresponding group of parameters. In particular, this implies that the amplitudes of the negative and the positive CD band responded to different effects. The decrease of the (−) 670 nm band correlated with the diminishment of the oxygen evolution rate, implying that this band might be a monitor for degradation or destabilisation of the thylakoid membranes, whereby the decrease in electron transport rate is a strong indicator for these lateral disturbances. This is supported by the fact that under low salt conditions also energy excitation transfer from carotenoids and Chl c to Chl a, *i.e.* from antenna complexes binding these pigments to the photosystems, was impaired equally for both photosystems. In contrast, the (+) 690 nm CD band showed the same salt dependence as the changes in excitation energy distribution between the photosystems. At 77 K, an increase in PSI fluorescence concomitant with a decrease of PSII emission under high salt conditions was observed, without changes in FCP emission. Since diatoms do not perform

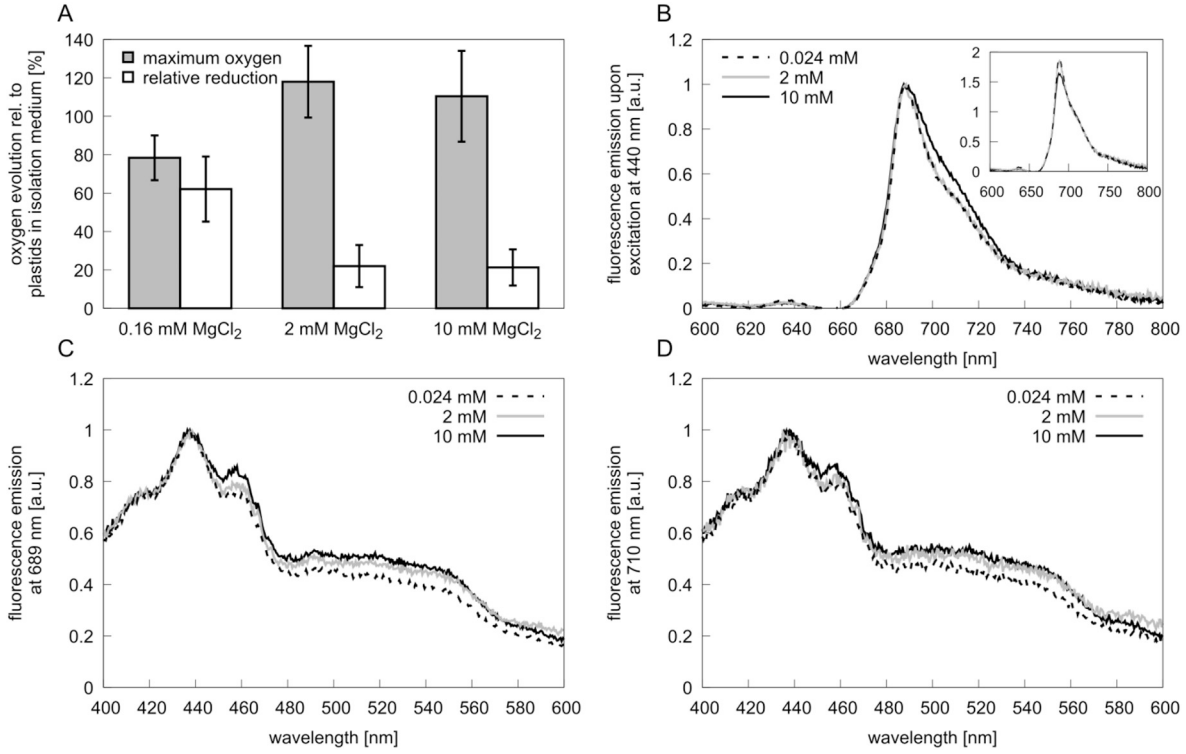

Fig. 6. Effect of cations on the functionality of the plastids.
The effect of cations on the oxygen evolution capacity and on the energy transfer between the antenna and the photosystems was investigated. In A) the results of the oxygen evolution measurements are shown. Plastids were first measured in isolation medium and showed a maximum oxygen evolution rate between 20 and 30 µmol O_2 per mg Chl a and h, depending on the plastid isolation. The values measured with plastids in isolation medium were set to 100%. The maximum oxygen evolution rate of samples under different $MgCl_2$ conditions is shown as grey bars, whereas the reduction in the oxygen evolution rate over 8 min is depicted as white bars. The data represent the average and standard deviation of three biological replicates.
In B) 77 K emission spectra of plastid samples treated with 0.024 mM $MgCl_2$ (dashed black line), 2 mM $MgCl_2$ (solid grey line) or 10 mM $MgCl_2$ (solid black line) are shown. Samples were excited at 440 nm and the fluorescence was normalised to the maximum at 689 nm or at 707 nm (inset), respectively. In C) and D) 77 K excitation spectra of plastids are depicted. Samples were treated with 0.024 mM $MgCl_2$ (dashed black line), 2 mM $MgCl_2$ (solid grey line) or 10 mM $MgCl_2$ (solid black line). The spectra were recorded at emission wavelengths of 689 nm (PSII, C)) or 710 nm (PSI, D)), respectively, and were normalised to their maximum fluorescence at 438 nm. All data shown in B), C) and D) represent averages of three spectra each to increase the signal-to-noise-ratio. They are representative for at least three biologically independent experiments.

state transitions [10], this difference cannot be explained by a movement of antenna complexes between the photosystems induced by the different salt conditions. Spill-over was also never detected in diatoms [4,41]. A decoupling of FCP from PSII can also be excluded, since free FCPs should exhibit fluorescence, which should be visible as a change in shape of the 77 K spectra around 678 nm. Alternatively, free FCP can

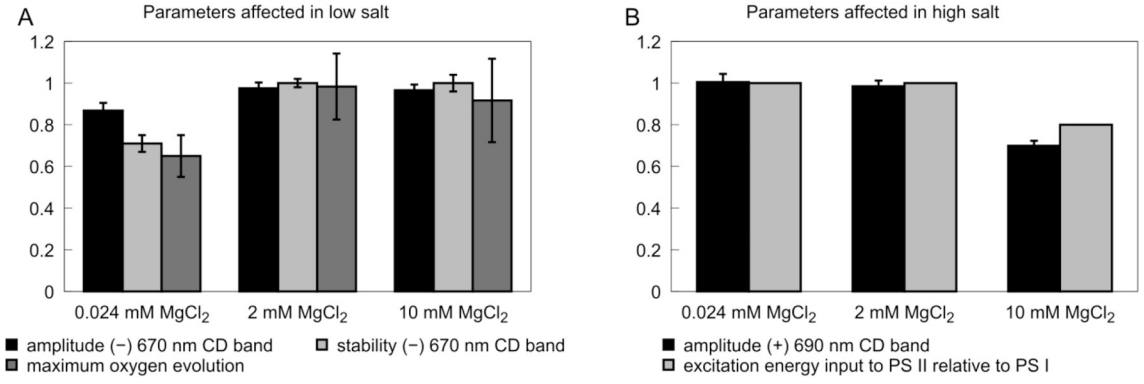

Fig. 7. Comparison of the effect of low, medium and high $MgCl_2$ concentrations on different parameters.
A) shows the parameters, which were affected in low salt conditions only. The amplitude of the CD band at (−) 670 nm (black bars) was reduced in low salt conditions, while it remained unchanged in intermediate or high salt concentrations. The stability of this peak (expressed as (1-degradation), light grey bars), behaved equally, as well as the maximum oxygen evolution rate (dark grey bars).
B) focusses on parameters that were influenced under high salt conditions only. The amplitude of the psi-type maximum CD band at (+) 690 nm (black bars) was indistinguishable in samples treated with low or medium salt concentrations and it decreased under high salt conditions. The excitation energy input into PSII (grey bars) decreased as well in samples treated with high salt concentrations, as estimated from the increase of PSI fluorescence relative to the PSII fluorescence at 77 K.
Data sets have been normalised in their respective maxima for comparison in one figure. Values and standard deviations were taken from Figs. 3, 5 and 6, respectively.

aggregate, which should lead to changes in the RT fluorescence spectra. At RT, fluorescence is dominated by FCPs and should have been less under the high salt condition, if there were any FCP aggregates, which was not the case. This is also in line with the finding of Schaller et al. [42], demonstrating that Mg^{2+} even up to 100 mM is not able to aggregate FCPs *in vitro*. Another possibility of changing the excitation energy transfer to the photosystems is a variation in the distances between the thylakoid membranes, enhancing the probability of transfer between FCPs in one membrane to FCPs in the adjacent membrane as demonstrated in grana stacks [43]. In this case, increased distances between the thylakoid membranes under high salt conditions would cause less energy to reach the PSII complexes that were shown to be enriched in the core thylakoid membranes. In summary, the decreased energy input into PSII under 10 mM $MgCl_2$ would point to a reduced membrane appression of the core membranes, correlating with the decrease of the positive psi-type CD band. Thus, the (+) 690 nm CD band is at least partially a monitor for vertical interactions of the thylakoids. However, we cannot rule out an additional contribution to this signal by lateral rearrangements, although there are no reports in literature about major lateral rearrangements due to the influence of divalent cations. Since oxygen evolution and excitation energy distribution stayed intact, the high salt situation is most probably also a physiological condition where the membrane appression of the core membranes is reduced in comparison to the optimal concentration range. Only the stability of the (+) 690 nm band showed an optimum curve when signals were plotted against Mg^{2+} concentration. Thus, this parameter was influenced by membrane intactness (low concentrations) as well as membrane appression (optimal *versus* high Mg^{2+} concentrations).

4.3. Factors enabling appression of the core thylakoid membranes

Appression of membranes depends on electrostatic repulsion, van der Waal's attraction and hydrostructural forces, whereby the latter are only acting at very short distances. When two membrane surfaces are of the same polarity, which is expected in the case of thylakoid membrane surfaces, repulsion will depend on the charged particles in the layer in between those membranes. These particles can electrostatically screen the repulsive forces or neutralise them by binding. Cations, such as Mg^{2+}, play an important role in screening the negative charges exhibited by proteins or lipids and thus influence the interplay of attractive and repulsive forces that determines the membrane distances. Obviously, the extent of the cationic influence depends on the concentration of both monovalent and divalent charges, and ion dependence usually follows an optimum curve [18]. Thus, Puthiyaveetil et al. [15] could show that *e.g.* at 5 mM Mg^{2+} and 100 mM K^+ stacking is theoretically possible in higher plants, whereas the same Mg^{2+} concentration but double amount of K^+ already hinders it.

Negative charges caused by protein components can only affect membrane appression in diatoms if proteins are located in the inner four, core membranes due to the specific arrangement of the thylakoid membranes in bands of three. In the four inner membranes, PSII and FCP were found to be enriched [2,4]. Therefore, it can be assumed that PSII and FCP are the protein complexes that predominantly influence interthylakoidal distances. Photosystem II is highly conserved in its structure and is thus probably also similar in its surface charge distribution across different organisms. In contrast, the antenna complexes (FCP) are different compared to higher plants. FCPs exhibit a smaller N-terminus and shorter stromal loops in comparison to LHCII. However, when using the consensus sequence of the stromal amino acids of the Lhcf polypeptides constituting the FCP complexes, an average charge of −6 could be deduced for a monomer (Suppl. Fig. 6). According to Puthiyaveetil et al. [15], the charge for a LHC in the $C_2S_2M_2$ LHCII-PSII supercomplex is on average −5.4 per monomer or −5.8 if L-trimers are also taken into consideration (Suppl. Fig. 6). Thus, proteins influencing membrane appression in diatoms parallel those of higher plants: PSII, which is highly conserved, and FCP in analogy to LHCII. Similar to proteins, negative charges originating from lipid components can only affect membrane appression if they constitute the inner four membranes. Diatoms possess a higher abundance of negatively charged SQDG [6] in comparison to higher plants. However, according to the model published by Lepetit et al. [5] SQDG is supposed to be mostly present in the peripheral membranes. Thus, the core membranes would contain mainly the uncharged lipids mono- and digalactosyldiacylglycerol (MGDG and DGDG), which is comparable to the situation in higher plants. Therefore, either SQDG is not only enriched in the outer membranes or charged lipids do not exhibit a strong influence on the thylakoid membrane appression in diatoms. The latter is in line with the high protein/lipid ratio of thylakoid membranes in general.

In conclusion, the facts that the effective ion concentrations measured in this study are similar to those observed for higher plants and that FCP might play an important role in reacting to the ionic situation – as does LHCII in higher plants – suggests that the core membranes of diatom thylakoids resemble the grana membranes of higher plants concerning their mode of appression. This hypothesis is also underpinned by the values published for the repeat distances of thylakoid membranes in diatoms (~170–180 Å; [2,19]), which are indeed comparable to the stacking of the granal regions in higher plants (~160–210 Å; [20]).

4.4. Membrane flexibility and dynamics as a prerequisite for physiological processes

In higher plants, membrane dynamics involve changes in the interthylakoidal distances as a basic prerequisite for *e.g.* efficient PSII repair ([14] and references therein). Thylakoid membranes in general should therefore be considered a dynamic, ever-changing system rather than a static, rigid backbone.

As already discussed by Garab [27], the illumination of diatom thylakoid membranes might lead to an increase in repeat distances and to a release of Mg^{2+} ions. Conversely, this could mean that an external addition of Mg^{2+} ions, as in our experiments, might mimic the light exposure. The directed uptake or release of cations could be a mechanism to regulate membrane appression in order to ensure the accessibility of PSII for repair or to fine-tune the distribution of excitation energy between the photosystems in a system lacking state transitions. This hypothesis is in line with the idea published earlier [15] that ion fluxes between the stroma and the lumen and, in particular, the release of Mg^{2+} can increase the electrostatic repulsion, and thus the membranes distances. Thus, changing the interthylakoidal distances might *in vivo* be useful for example to counterbalance an under-excitation of PSI in certain light conditions.

In conclusion, this corroborates the hypothesis that changes in the appression of the thylakoid membranes are a physiological process. In turn, this might provide the thylakoid membrane with dynamics and flexibility in order to cope with the ever-changing light situation in the ocean.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbabio.2018.11.003.

References

[1] J.P. Dekker, E.J. Boekema, Supramolecular organization of thylakoid membrane proteins in green plants, Biochim. Biophys. Acta 1706 (2005) 12–39, https://doi.org/10.1016/j.bbabio.2004.09.009.

[2] A.M. Pyszniak, S.P. Gibbs, Immunocytochemical localization of photosystem I and the fucoxanthin-chlorophyll *a/c* light-harvesting complex in the diatom *Phaeodactylum tricornutum*, Protoplasma 166 (1992) 208–217, https://doi.org/10.1007/BF01322783.

[3] Y.D. Bedoshvili, T.P. Popkova, Y.V. Likhoshway, Chloroplast structure of diatoms of different classes, Cell Tissue Biol. 3 (2009) 297–310, https://doi.org/10.1134/S1990519X09030122.

[4] S. Flori, P.-H. Jouneau, B. Bailleul, B. Gallet, L.F. Estrozi, C. Moriscot, O. Bastien, S. Eicke, A. Schober, C.R. Bártulos, E. Maréchal, P.G. Kroth, D. Petroutsos, S. Zeeman, C. Breyton, G. Schoehn, D. Falconet, G. Finazzi, Plastid thylakoid architecture optimizes photosynthesis in diatoms, Nat. Commun. 8 (2017) 15885, , https://doi.org/10.1038/ncomms15885.

[5] B. Lepetit, R. Goss, T. Jakob, C. Wilhelm, Molecular dynamics of the diatom thylakoid membrane under different light conditions, Photosynth. Res. 111 (2012) 245–257, https://doi.org/10.1007/s11120-011-9633-5.

[6] A. Vieler, C. Wilhelm, R. Goss, R. Süß, J. Schiller, The lipid composition of the unicellular green alga *Chlamydomonas reinhardtii* and the diatom *Cyclotella meneghiniana* investigated by MALDI-TOF MS and TLC, Chem. Phys. Lipids 150 (2007) 143–155, https://doi.org/10.1016/j.chemphyslip.2007.06.224.

[7] H. Kirchhoff, Architectural switches in plant thylakoid membranes, Photosynth. Res. 116 (2013) 481–487, https://doi.org/10.1007/s11120-013-9843-0.

[8] A. Beer, M. Juhas, C. Büchel, Influence of different light intensities and different iron nutrition on the photosynthetic apparatus in the diatom *Cyclotella meneghiniana* (Bacillariophyceae), J. Phycol. 47 (2011) 1266–1273, https://doi.org/10.1111/j.1529-8817.2011.01060.x.

[9] G. Nagy, M. Szabó, R. Ünnep, G. Káli, Y. Miloslavina, P.H. Lambrev, O. Zsiros, L. Porcar, P. Timmins, L. Rosta, G. Garab, Modulation of the multilamellar membrane organization and of the chiral macrodomains in the diatom *Phaeodactylum tricornutum* revealed by small-angle neutron scattering and circular dichroism spectroscopy, Photosynth. Res. 111 (2012) 71–79, https://doi.org/10.1007/s11120-011-9693-6.

[10] T.G. Owens, Light-harvesting function in the diatom *Phaeodactylum tricornutum*: II. Distribution of excitation energy between the photosystems, Plant Physiol. 80 (1986) 739–746, https://doi.org/10.1104/pp.80.3.739.

[11] J.E. Mullet, The amino acid sequence of the polypeptide segment which regulates membrane adhesion (grana stacking) in chloroplasts, J. Biol. Chem. 258 (1983) 9941–9948.

[12] S. Izawa, N.E. Good, Effect of salts and electron transport on the conformation of isolated chloroplasts. I. Light-scattering and volume changes, Plant Physiol. 41 (1966) 533–543, https://doi.org/10.1104/pp.41.3.533.

[13] S. Izawa, N.E. Good, Effect of salts and electron transport on the conformation of isolated chloroplasts. II. Electron microscopy, Plant Physiol. 41 (1966) 544–552, https://doi.org/10.1104/pp.41.3.544.

[14] M. Pribil, M. Labs, D. Leister, Structure and dynamics of thylakoids in land plants, J. Exp. Bot. 65 (2014) 1955–1972, https://doi.org/10.1093/jxb/eru090.

[15] S. Puthiyaveetil, B. Van Oort, H. Kirchhoff, Surface charge dynamics in photosynthetic membranes and the structural consequences, Nat. Plants 3 (2017) 17020, , https://doi.org/10.1038/nplants.2017.20.

[16] W.S. Chow, E. Kim, P. Horton, J.M. Anderson, Granal stacking of thylakoid membranes in higher plant chloroplasts: the physicochemical forces at work and the functional consequences that ensue, Photochem. Photobiol. Sci. 4 (2005) 1081–1090, https://doi.org/10.1039/b507310n.

[17] L.A. Staehelin, Reversible particle movements associated with unstacking and restacking of chloroplast membranes in vitro, J. Cell Biol. 71 (1976) 136–158, https://doi.org/10.1083/jcb.71.1.136.

[18] B.T. Rubin, W.S. Chow, J. Barber, Experimental and theoretical considerations of mechanisms controlling cation effects on thylakoid membrane stacking and chlorophyll fluorescence, Biochim. Biophys. Acta 634 (1981) 174–190, https://doi.org/10.1016/0005-2728(81)90137-7.

[19] R. Ünnep, G. Nagy, M. Markó, G. Garab, Monitoring thylakoid ultrastructural changes in vivo using small-angle neutron scattering, Plant Physiol. Biochem. 81 (2014) 197–207, https://doi.org/10.1016/j.plaphy.2014.02.005.

[20] D. Posselt, G. Nagy, J.J.K. Kirkensgaard, J.K. Holm, T.H. Aagaard, P. Timmins, E. Rétfalvi, L. Rosta, L. Kovács, G. Garab, Small-angle neutron scattering study of the ultrastructure of the chloroplast thylakoid membranes — Periodicity and structural flexibility of the stroma lamellae, Biochim. Biophys. Acta 1817 (2012) 1220–1228, https://doi.org/10.1016/j.bbabio.2012.01.012.

[21] G. Garab, H. Van Amerongen, Linear dichroism and circular dichroism in photosynthesis research, Photosynth. Res. 101 (2009) 135–146, https://doi.org/10.1007/s11120-009-9424-4.

[22] T.N. Tóth, N. Rai, K. Solymosi, O. Zsiros, W.P. Schröder, G. Garab, H. Van Amerongen, P. Horton, L. Kovács, Fingerprinting the macro-organisation of pigment–protein complexes in plant thylakoid membranes in vivo by circular-dichroism spectroscopy, Biochim. Biophys. Acta 1857 (2016) 1479–1489, https://doi.org/10.1016/j.bbabio.2016.04.287.

[23] M. Szabó, B. Lepetit, R. Goss, C. Wilhelm, L. Mustárdy, G. Garab, Structurally flexible macro-organization of the pigment–protein complexes of the diatom *Phaeodactylum tricornutum*, Photosynth. Res. 95 (2008) 237–245, https://doi.org/10.1007/s11120-007-9252-3.

[24] C. Büchel, G. Garab, Organization of the pigment molecules in the chlorophyll *a/c* light-harvesting complex of *Pleurochloris meiringensis* (xanthophyceae). Characterization with circular dichroism and absorbance spectroscopy, J. Photochem. Photobiol. 37 (1997) 118–124, https://doi.org/10.1016/s1011-1344(96)07337-x.

[25] D. Keller, C. Bustamante, Theory of the interaction of light with large inhomogeneous molecular aggregates. II. Psi-type circular dichroism, J. Chem. Phys. 84 (1986) 2972–2980, https://doi.org/10.1063/1.450278.

[26] M.-H. Kim, L. Ulibarri, D. Keller, M.F. Maestre, C. Bustamante, The psi-type circular dichroism of large molecular aggregates. III. Calculations, J. Chem. Phys. 84 (1986) 2981–2989, https://doi.org/10.1063/1.450279.

[27] G. Garab, Hierarchical organization and structural flexibility of thylakoid membranes, Biochim. Biophys. Acta 1837 (2014) 481–494, https://doi.org/10.1016/j.bbabio.2013.12.003.

[28] R.R.L. Guillard, Culture of phytoplankton for feeding marine invertebrates, in: W.L. Smith, M.H. Chanley (Eds.), Culture of Marine Invertebrate Animals, Springer, Boston, MA, 1975, pp. 26–60, , https://doi.org/10.1007/978-1-4615-8714-9_3.

[29] A.F. Schober, S. Flori, G. Finazzi, P.G. Kroth, C.R. Bártulos, Isolation of plastid fractions from the diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum*, in: E. Maréchal (Ed.), Methods Mol. Biol, Springer, US, 2018, pp. 189–203, , https://doi.org/10.1007/978-1-4939-8654-5_13.

[30] S.W. Jeffrey, G.F. Humphrey, New spectrophotometric equations for determining chlorophylls a, b, c_1 and c_2 in higher plants, algae and natural phytoplankton, Biochem. Physiol. Pflanz. 167 (1975) 191–194, https://doi.org/10.1016/s0015-3796(17)30778-3.

[31] C. Büchel, Fucoxanthin-chlorophyll proteins in diatoms: 18 and 19 kDa subunits assemble into different oligomeric states, Biochemistry 42 (2003) 13027–13034, https://doi.org/10.1021/bi0349468.

[32] T. Veith, J. Brauns, W. Weisheit, M. Mittag, C. Büchel, Identification of a specific fucoxanthin-chlorophyll protein in the light harvesting complex of photosystem I in the diatom *Cyclotella meneghiniana*, Biochim. Biophys. Acta 1787 (2009) 905–912, https://doi.org/10.1016/j.bbabio.2009.04.006.

[33] R.M. Smillie, K.W. Henningsen, N.C. Nielsen, D. von Wettstein, The influence of cations and methylamine on structure and function of thylakoid membranes from barley chloroplasts, Carlsb. Res. Commun. 41 (1976) 27–56, https://doi.org/10.1007/bf02908691.

[34] R. Clement, L. Dimnet, S.C. Maberly, B. Gontero, The nature of the CO_2-concentrating mechanisms in a marine diatom, *Thalassiosira pseudonana*, New Phytol. 209 (2016) 1417–1427, https://doi.org/10.1111/nph.13728.

[35] K. Gundermann, C. Büchel, Factors determining the fluorescence yield of fucoxanthin-chlorophyll complexes (FCP) involved in non-photochemical quenching in diatoms, Biochim. Biophys. Acta 1817 (2012) 1044–1052, https://doi.org/10.1016/j.bbabio.2012.03.008.

[36] A. Ghazaryan, P. Akhtar, G. Garab, P.H. Lambrev, C. Büchel, Involvement of the Lhcx protein Fcp6 of the diatom *Cyclotella meneghiniana* in the macro-organisation and structural flexibility of thylakoid membranes, Biochim. Biophys. Acta 1857 (2016) 1373–1379, https://doi.org/10.1016/j.bbabio.2016.04.288.

[37] G. Garab, J. Kieleczawa, J.C. Sutherland, C. Bustamante, G. Hind, Organization of pigment-protein complexes into macrodomains in the thylakoid membranes of wild-type and chlorophyll b-less mutant of barley as revealed by circular dichroism, Photochem. Photobiol. 54 (1991) 273–281, https://doi.org/10.1111/j.1751-1097.1991.tb02016.x.

[38] N.N. Zulu, K. Zienkiewicz, K. Vollheyde, I. Feussner, Current trends to comprehend lipid metabolism in diatoms, Prog. Lipid Res. 70 (2018) 1–16, https://doi.org/10.1016/j.plipres.2018.03.001.

[39] J. Marchand, P. Heydarizadeh, B. Schoefs, C. Spetea, Ion and metabolite transport in the chloroplast of algae: lessons from land plants, Cell. Mol. Life Sci. 75 (2018) 2153–2176, https://doi.org/10.1007/s00018-018-2793-0.

[40] G. Hind, H.Y. Nakatani, S. Izawa, Light-dependent redistribution of ions in suspensions of chloroplast thylakoid membranes, Proc. Natl. Acad. Sci. U. S. A. 71 (1974) 1484–1488, https://doi.org/10.1073/pnas.71.4.1484.

[41] V.U. Chukhutsina, C. Büchel, H. van Amerongen, Variations in the first steps of photosynthesis for the diatom *Cyclotella meneghiniana* grown under different light conditions, Biochim. Biophys. Acta 1827 (2013) 10–18, https://doi.org/10.1016/j.bbabio.2012.09.015.

[42] S. Schaller, K. Richter, C. Wilhelm, R. Goss, Influence of pH, Mg^{2+}, and lipid composition on the aggregation state of the diatom FCP in comparison to the LHCII of vascular plants, Photosynth. Res. 119 (2014) 305–317, https://doi.org/10.1007/s11120-013-9951-x.

[43] H. Kirchhoff, W. Haase, S. Haferkamp, T. Schott, M. Borinski, U. Kubitscheck, M. Rögner, Structural and functional self-organization of photosystem II in grana thylakoids, Biochim. Biophys. Acta 1767 (2007) 1180–1188, https://doi.org/10.1016/j.bbabio.2007.05.009.