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Acclimatization of Thalassiosira pseudonana Photosynthetic Membranes to Environmental Temperature Changes

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Abstract **–** The greenhouse effect results in warming the planet's surface and a higher ocean heat content. It causes changes of the water circulation frequency and turbulent mixing, delivering warm water, phytoplankton nutrients and CO_2 from deeper layers of water to the water surface. All of these changes can have an impact on Earth's ecosystems. In the present study the effect of changing environmental temperature on a model diatom species, *Thalassiosira pseudonana* was tested under laboratory conditions. The purpose of the work was to analyze the temperature effect on fluidity and chemical composition of the thylakoid membranes. The photosynthetic activity and photosynthetic pigment contents of diatoms adapted to different temperatures were also determined. Our result show decreases of the growth rate and chlorophylls concentration in diatom cells cultured at lower temperature. It was also detected that increases of the polyunsaturated and decreases of saturated fatty acids, as well as changes in lipids:proteins ratio, resulted in stabilization of the thylakoid membranes fluidity and photosynthetic efficiency (Fv/Fm) in a physiological range of the temperature. The result show that the regulation of the concentration and interaction of fatty acids, proteins and pigments is the most important factor in the adaptation strategies of diatoms to the environmental temperature changes. Additionally, the regulation of membrane fluidity was demonstrated as important mechanism of the diatom adaptation to greenhouse effects. We also postulate that the adaptive mechanisms to temperature changes are differently expressed in the polar or hydrophobic regions of photosynthetic membranes.

Keywords**:** membrane fluidity, fatty acids composition, diatoms, acclimatization mechanisms, photosynthesis

1. Introduction

During the last 100 years the increase in ocean heat content was mainly observed during two different time periods. The first was at the beginning of the 20th century, the second began in the 1970s and continues until now. They could both be caused by anthropogenic and natural factors [1]. The warming of ocean waters as well as the influence of wind conditions cause the change of the water circulation and turbulent mixing delivers warm water, phytoplankton nutrients and CO_2 from deeper layers of water to the water surface. These changes strongly influence the photosynthetic activity of phytoplankton and result both in changes of the biomass production and CO_2 fixation [1, 2, 3].One of the most important marine group of phytoplankton are diatoms (Bacillariophyta). They are the dominant organisms in extremely cold, but nutrient rich, marine environment and are responsible for around 40% of marine and 20% of global net primary production which corresponds to 20 billion ton of fixed carbon per year [3, 4]. Their chloroplasts are significantly different from chloroplasts of vascular plants. The diatom chloroplasts are surrounded by an envelope which consists of four membranes where the girdle lamella runs parallel to the envelope. The thylakoid membranes are arranged in groups of three [5, 6, 7]. Certain regions of thylakoid membranes are characterized by differences in the protein, pigment-protein and lipid

composition. The light harvesting systems of diatoms are called fucoxanthin chlorophyll binding proteins (FCP) because they bind high amounts of fucoxanthin,(Fx) a unique xanthophyll pigment which can be also found in brown algae and most other heterokonts. FCPs serve as antenna for PSI and PSII or as specific antenna for PSI only [5, 7, 8]. In addition to Fx, two different xanthophylls can be bound to FCPs. These are diadinoxanthin (Ddx) and diatoxanthin (Dtx). They are engaged in one of the most important photoprotective mechanism known as diadinoxanthin cycle [9, 10]. Moreover, diatom chloroplasts contain chlorophyll a (Chl a), c1 and c2 (Chl c) [7, 8, 11]. Thylakoid membranes of diatoms are composed from the same lipid classes as the thylakoids of vascular plants i.e. galactolipids such as monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), and negatively charged lipids i.e. the phospholipid phosphatidylglycerol (PG) and the sulfolipid sulfoquinovosyldiacylglycerol (SQDG). However, the proportions of these lipids are different from vascular plants. The concentration of negatively charged lipids, SQDG and PG, in diatoms are two times higher than in vascular plants [7, 12]. Furthermore, the fatty acid profile of diatom lipids is also unique. The diatom membrane lipids contain longer and more unsaturated fatty acids, e.g. 20:5 and 22:6. It is known that the decrease of growth temperature during diatom cultivation enhances synthesis of polyunsaturated fatty acids (PUFA), especially eicosapentaenoic acid (EPA) [13, 14, 15, 16].

Although the ecological significance of diatoms is comparable to that of tropical forests, there exist only few data on their acclimation mechanisms to environmental temperature changes. To study the adaptation mechanisms of diatoms to climate warming, the model marine diatom, *Thalassiosira pseudonana* whose whole genome has been sequenced [17], was cultured at two different temperatures. The purpose of the work was the comparative analysis of the temperature effect on diatom thylakoid membrane composition and fluidity. The photosynthetic activity and photosynthetic pigment contents of diatoms adapted to different temperatures were also determined.

2. Materials and Methods

The *Th. pseudonana* were grown under standard culture conditions in f/2 medium [18], with a photoperiod of 10: 14 h D: L with a light intensity of 40 $\mu Em^{-2}s^{-1}$ at low (12 °C) and moderate temperature (20 °C). Approximately 300 ml of inoculum with an optical density OD_{600} of 0.3 – 0.4 was used to start a 1200 ml culture. Cultures were shaken several times a week during the light phase to keep cells in suspension and maintain an optimal exchange of gas and nutrients [19].

Cell density was measured at 600 nm [20] with a spectrophotometer.

Chlorophyll fluorescence was measured with a PAM-210 fluorimeter (Waltz, Germany). Before measurements samples were collected and centrifuged to obtain pellet of the cells which were dark-adapted for 15 min [19]. Software generated Fo (minimum) and Fm (maximum) fluorescence values which were used to calculate the optimum quantum yield Fv/Fm.

Protein concentration was measured by Lowry method [21].

Chl a and Chl c were extracted from *T. pseudonana* by 90% acetone, with liquid nitrogen cooling. Concentration of the chlorophylls was determined with a spectrophotometer and calculated according to Jeffrey and Humphrey [22].

Thylakoid membranes were isolated on the eight day after inoculation according to the method described by Lepetit et al. [23].

To compare lipids:proteins ratio in the different thylakoid membranes, FT-IR spectroscopy (Fourier Transform Infrared Spectrometer, Bruker Alpha-P with Opus 6.5 software) was used.

The fluidity of freshly prepared *T. pseudonana* thylakoid membranes was monitored by EPR (Electron Paramagnetic Resonance) spectroscopy using two spin labels 5-SASL and 16-SASL. Samples for EPR measurements were prepared by mixing 0.1 mM of spin labels and thylakoid membranes (1mg/ml of Chl a in final concentration). EPR spectra of spin labels as a function of temperature were recorded by Miniscope EPR (Magnettech, Germany). EPR spectral parameters such as $2A'_{\parallel}$, $2A'_{\perp}$[24, 25] were used to calculate the membrane fluidity (S) using the following formulas [26]:

$$S = 0.5407(A'_{\parallel} - A'_{\perp})/a_o \quad (1)$$

where

$$a_o = (A'_{\parallel} + 2\,A'_{\perp})/3 \quad (2)$$

The preparation of fatty acid methyl esters (FAME) and their derivatization were carried out according to Ichihara and Fukubayashi [27]. The chromatographic analysis of n-hexane extracts was performed on Agilent 6890N gas chromatograph (Agilent Technologies, USA) equipped with a flame ionization detector (FID and capillary column IL-100 (Supelco, 28884-U). Certified reference material 37 FAME MIX (Supelco, CRM 47885) and internal standard (biphenyl) for the calibration and verification of laboratory equipment and analytical procedure were used for the determination of FAME) content.

The membrane fluidity of diatom thylakoid membranes was additionally analyzed by the laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) fluorescence method. For laurdan-labelling thylakoids were incubated with 1 µM laurdan for 30 min. The concentration of the thylakoids was adjusted to 2.5 µg Chl a ml^{-1}. Steady-state fluorescence emission spectra were recorded in the temperature range from 5°C to 30°C. For the interpretation of the laurdan fluorescence signals the general polarization (GP) value was calculated:

$$GP = (Ig+If) / (Ig+If) \quad (3)$$

where Ig and If are the fluorescence intensities at the emission maxima of laurdan (450 and 500nm) incorporated into a membrane in the gel and fluid phase, respectively. Laurdan excitation was set to a wavelength of 390 nm [28].

3. Results and Discussion

The survival in the changing environment depends on abilities to adapt or acclimate to stress factors such as high and low temperature. The warming climate results in untypical ocean circulation and temperature changes of water, which effect the physiological and metabolic processes of important phytoplankton species such as diatoms [3, 29]. The adaptation mechanisms of marine diatoms to different temperatures are still unclear. Also, the mechanisms protecting photosynthetic machinery of diatoms against the greenhouse effect need to be explained. For this purpose the effect of temperature on *T. pseudonana* growth, photosynthetic membrane properties and photosynthesis parameters was tested in batch cultures. Firstly, comparative analysis of growth kinetics of *T. pseudonana* cells, adapted to low and moderate temperature, were performed (Fig. 1). The obtained results showed statistically significant differences in the diatom growth kinetics at 12 and 20°C. The growth at 20°C was faster during the first three days of culture, and the differences were slightly increasing during the next days. The growth of diatoms under both temperatures was characterized by two phases: a first linear growth phase with higher rate was observed between the first and fifth day after inoculation; the second phase with a slower growth rate was observed after the sixth day of culture. Similar results were observed for *P. tricornutum* cultures under the same culture conditions, where a gradual decrease of the growth rate at low temperature was determined [19, 30].

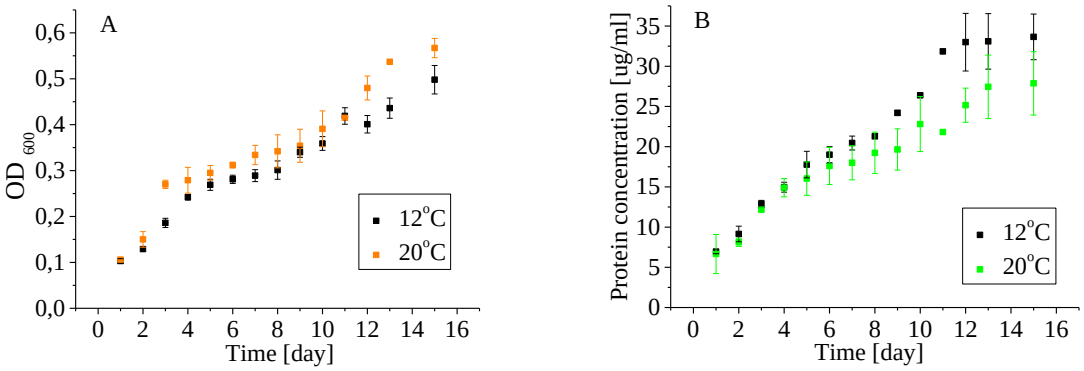

Fig. 1: Effect of temperature on OD 600 (A) and protein concentration (B) of *T. pseudonanna* growing at different temperature (12 and 20°C) under the same light conditions (40 µmol $m^{-1}s^{-1}$) in batch cultures. Values are the average of three experiments (±standard error).

Analysis of temperature effect on *T. pseudonanna* culture growth monitored by OD_{600} measurements was additional supported by simultaneous determination of protein (Fig 1B), Chl a and c concentration (Fig 2) in the cells. The increase of

the protein concentration was observed in cells cultured at both temperatures (Fig. 1B). Protein level in cells cultured at lower temperature was increasing faster than at higher temperature. After the eighth day of inoculation, the higher level of protein content was clearly visible in cells adapted to lower temperature (Fig. 1B). Additionally, the ratios of the protein level to the OD_{600} values or the Chl a concentration (Fig. 3), clearly indicate that at 12°C the protein concentration in diatoms was higher than at 20°C. Observed results are in line with the data published for other cold adapted organisms. It has been postulated that at low temperatures the reduced chemical reaction rates are compensated by increasing the enzyme concentration [31].

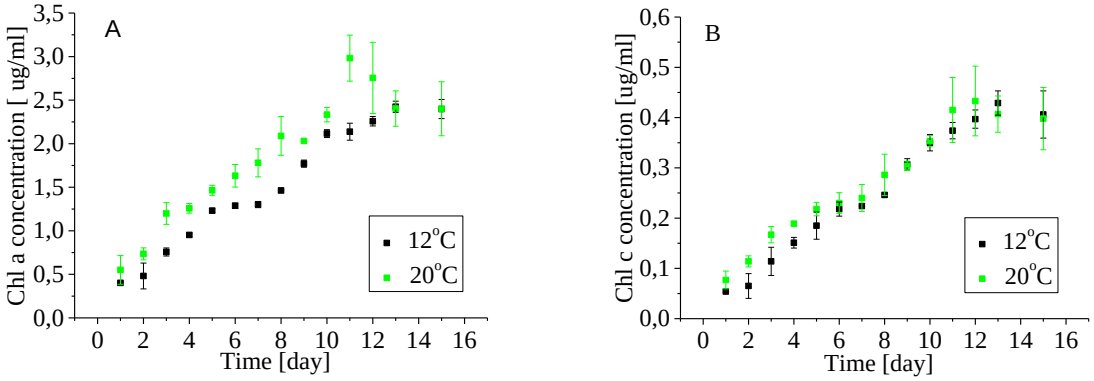

Fig. 2: Effect of temperature on chlorophyll a (Chl a) (A) and chlorophyll c (Chl c) (B) concentration in *T. pseudonana* at different temperatures (12 and 20°C) under the same light conditions (40 µmol $m^{-1}s^{-1}$) in batch cultures. Values are the average of three experiments (±standard error).

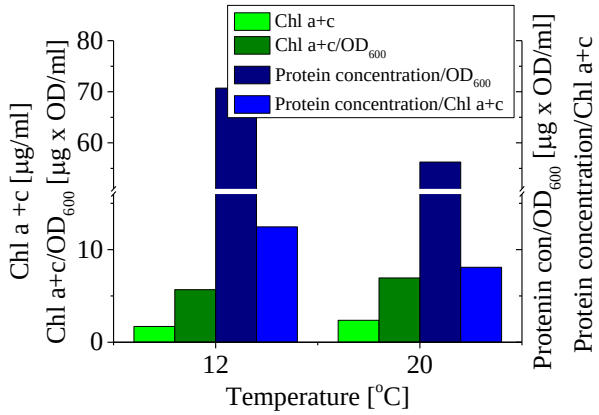

Fig. 3: Effect of temperature on *T. pseudonana* chlorophyll concentration and the ratio of chlorophylls to OD_{600} as well as on the protein to OD_{600} value.

Although chlorophyll levels were increasing together with the OD_{600} values, in contrast to the protein concentration, the chlorophyll content was slightly higher in cells cultured at 20°C. After 12 days of cultivation, decreases of Chl a and Chl c levels were detected at both temperatures (Fig. 2). These changes in the concentration of chlorophylls may be associated with the maintenance of the balance between the temperature independent processes of light-harvesting and the temperature-sensitive, enzymatic steps of photosynthesis. Changes in the FCP protein and pigment concentrations as the result of temperature response in diatom cells have not been reported so far.

Fv/Fm is the most used parameter to determine stress effects on plant photosystem II (PS II) [32]. Small variability in photosynthetic efficiency of PSII was demonstrated in diatoms cultivated at low and moderate temperature (Fig. 4). Fv/Fm increased faster in cells growing at 20°C. The level of Fv/Fm achieved the maximum value of around 0.72 during the first 5 days of culture. The highest Fv/Fm for diatoms growing at 12°C was reached on the 8th day of culture and it was slightly lower than Fv/Fm detected for diatoms at 20°C. Subsequently, Fv/Fm values of both culture types were decreasing.

Additionally, the Fv/Fm values became comparable in cells cultured at both tested temperatures. The period of the gradually decreasing Fv/Fm values was not correlated with period of the decline in the Chl concentration. This phenomenon was not observed in *P. tricornutum* in our previous experiments [19].

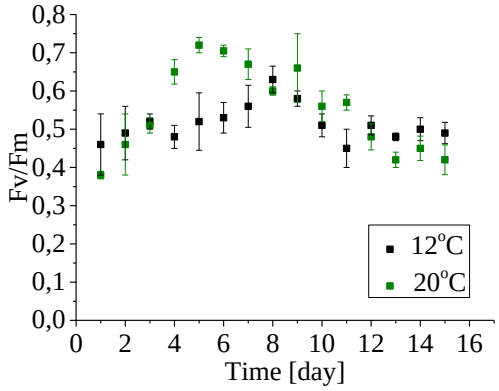

Fig. 4: Effect of temperature on Fv/Fm of *T. pseudonana* growing at different temperature (12 and 20°C) under the same light conditions (40 µmol m-1s-1) in bath cultures. Values are the average of three experiments (±standard error).

In the photosynthetic machinery the D1 protein (also known as PsbA) is most prone to photooxidative damage under high light conditions. Because D1, together with the D2 protein forms the reaction core of PSII, these proteins are rapidly renewed in a turnover repair cycle [33]. However, decrease of D1 protein level in diatoms was also observed under low light (40 µmol $m^{-1}s^{-1}$) conditions [32]. This light intensity is comparable to light conditions of *T. pseudonana* cultures in the present experiments. Decrease of Fv/Fm indicates a lower activity of the PS II reaction center, probably caused by insufficient D1 turnover [32]. It is known that the D1 re-synthesis is regulated by unsaturated fatty acids and thylakoid membrane fluidity. The moderate or low temperature inhibits the repair of PSII but does not have an effect on photodamage [34, 35]. *T. pseudonanna* adaptation to different temperatures probably results in comparable rates of D1 protein re-synthesis at both temperatures tested in the present experiments.

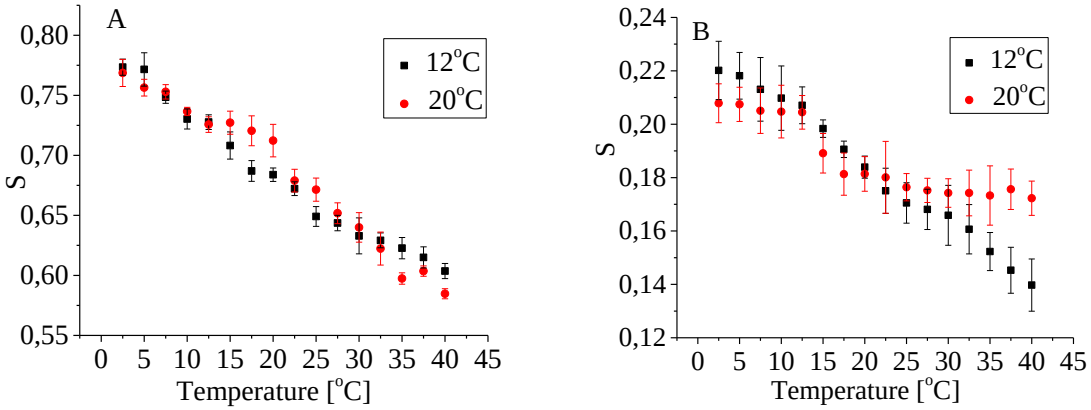

Fig. 5: Growth temperature effect on temperature dependence of S (fluidity) parameter calculated for *T. pseudonana* thylakoid membranes labelled with 5-SASL (A) and 16-SASL (B). Results for the one representative isolation. Confidence intervals for the level of significance α=0,05.

To analyze the acclimatization mechanism of diatom photosynthetic membranes to changing temperatures, we isolated the thylakoid membranes on the eighth day after inoculation when diatom cultures were in the growth phase and the Fv/Fm values were high and comparable. The fluidity of the isolated thylakoid membranes at two different depths of the

membrane was analyzed by EPR with 5-SASL and 16-SASL spin labels. 5-SASL allows to monitor the fluidity in the lipid head groups area and due to 16-SASL, insight into the hydrophobic region of the membrane is possible. The fluidity of the head group regions of the thylakoid membranes was stable only in a narrow but physiological range of the tested temperatures (12 and 20°C). It indicates a good adaptation capacity of *T. pseudonana* thylakoid membranes to these temperatures. However, in this range of temperatures the polar region of thylakoid membranes isolated from diatoms grown at the higher cultivation temperature showed a clearly higher rigidification compared to membranes isolated from cells adapted to lower temperatures (Fig. 5A).

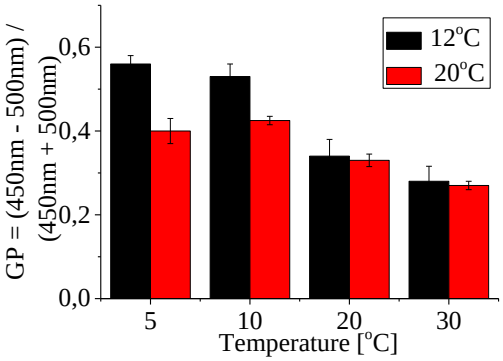

Fig. 6: Effect of growth temperature on GP values of laurdan-labelled thylakoid membranes of *T. pseudonana* for the temperature range from 5°C to 30°C. The mean GP values of seven independent thylakoid preparations are presented.

It is worth noticing that membrane fluidity between cells cultured at 12 and 20°C, in the physiological range of temperatures is strongly different above a temperature of 12°C. It indicates that *T. pseudonana* has to use its adaptive mechanisms allowing them to obtain the optimal fluidity of the polar membrane region only when diatoms are growing under higher temperatures (Fig. 5A). The fluidity of the hydrophobic region of *T. pseudonana* thylakoid membranes was both stable and comparable in the temperatures range between 12.5 and 20°C for both the cultures grown at low or higher temperatures (Fig. 5B). Outside the physiological temperature range, the membrane fluidity of diatoms cultivated at higher temperatures was slightly higher at low measuring temperatures compared to that observed for diatoms acclimated to low temperatures. However, it was clearly lower at higher temperatures used for the EPR measurements. These results were in general confirmed by the laurdan fluorescence method (Fig. 6).

To obtain more direct information about the molecular mechanism of photosynthetic membranes adaptation to changing temperatures, the fatty acid composition was analyzed (Fig. 7).

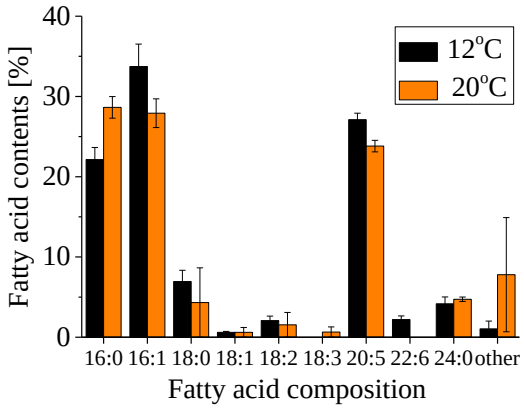

Fig. 7: Effect of cultivation temperature on fatty acid composition of *T. pseudonana* grown at different temperature (12 and 20°C) under the same light conditions (40 µmol $m^{-1}s^{-1}$) in batch cultures. Values are the average of three experiments (±standard error).

The most dominant fatty acids of *T. pseudonana* were palmitic (16:0), palmitoleic (16:1), and the polyunsaturated fatty acids: eicosapentaenoic (20:5n3) and decosahexanoic (22:6n3) acid (Fig. 7) which are not found in vascular plants. The decrease of the *T. pseudonana* growth temperature from moderate to 12°C caused a decrease of the saturated (16:0 and 24:0) and an increase of the unsaturated fatty acids. Such changes play an essential role in the maintenance of the fluidity of the hydrophobic membrane regions [15, 36, 37] thereby providing stability to the metabolomic processes which take place within the membrane, such as photosynthetic light-harvesting and electron transport [38]. Additionally, we observed an increase of the EPA contents in *T. pseudonana* cells when cultures were transferred from 20 to 12°C. During adaptation, the level of EPA in the *T. pseudonana* cells increased by about 14%. Additionally, the presence of decosahexenoic acid was detected.

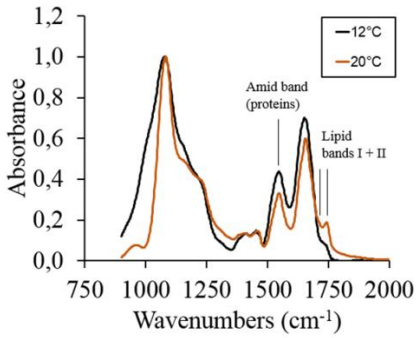

Fig. 8: Effect of culture growth temperature (12 and 20°C) on the protein to lipid ratio, analyzed by FT-IR spectroscopy in thylakoid membranes of *T. pseudonana* on the eighth day of culture, under the same light conditions (40 µmol $m^{-1}s^{-1}$).

Changes in unsaturation degree of fatty acids are not the only mechanism which affects the membrane fluidity. Environmental temperature changes were shown to also induce modulation of the membrane composition by regulation of the protein concentration and the lipid:protein ratio [29]. The FT-IR analysis of the thylakoid membranes showed an increased lipid:protein ratio in thylakoid membranes of *T. pseudonana* cells cultured at higher temperature (Fig. 8), what also affected the membrane fluidity [39]. Additionally, we also observed an increase of the protein:chlorophyll as well as the protein:OD_{600} ratio in intact *T. pseudonana* cells cultured at lower temperatures (Fig. 3).

4. Conclusion

The obtained results present the adaptation strategies of the model diatom species, *T. pseudonana* to the changes of the important environmental factor temperature. The data shows that regulation of the content and interaction of biomolecules such as fatty acids, proteins and pigments is the most important molecular strategy in the optimization of the diatom photosynthetic membranes under changing environmental temperatures. Obtained results show also the significance of the membrane fluidity regulation mechanisms for the diatom adaptation to the greenhouse effect. They also indicate that the mechanisms for the regulation of membrane fluidity are different for the polar regions and the hydrophobic area of photosynthetic membranes.

Acknowledgements

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