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Carbon allocation strategies in algae exposed to stressful conditions

Ph. D. Thesis

Mgr. Martin Lukeš

Supervisor: prof. RNDr. Ondřej Prášil, Ph.D.

Institute of Microbiology CAS

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❁ Annotation

The acclimation of two green algae of order Chlamydomonadales to sub- and supra-optimal temperatures, as well as effects of light, temperature, and salinity on peculiar alga *Chromera velia* have been examined. The mechanism of acclimation of different algae to stressful conditions were described in terms of physiology and put to context within the ecological frame.

❁ **Declaration** [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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❁ List of papers and author's contribution

The thesis is based on the following papers:

I. Lukeš, M., Procházková, L., Shmidt, V., Nedbalová, L., & Kaftan, D. (2014). Temperature dependence of photosynthesis and thylakoid lipid composition in the red snow alga *Chlamydomonas cf. nivalis* (Chlorophyceae). FEMS Microbiology Ecology, 89(2), 303–315.

(IF = 3.568)

M. Lukeš participated in laboratory experiments, data evaluation and manuscript writing.

II. Lukeš, M., Giordano, M., & Prášil, O. (2017). The effect of environmental factors on fatty acid composition of *Chromera velia* (Chromeridae). Journal of Applied Phycology, 29(4), 1791–1799.
(IF = 2.401)

M. Lukeš participated in experimental design, laboratory experiments, data evaluation and manuscript writing.

III. Lukeš, M., Giordano, M., & Prášil, O. (2019). The effect of light quality and quantity on carbon allocation in *Chromera velia*. Folia Microbiologica, doi:10.1007/s12223-019-00734-y
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M. Lukeš participated in experimental design, laboratory experiments, data evaluation and manuscript writing.

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1. General introduction

1.1 Preface

Algae are unique organisms performing one of the most advanced energy conversion process on Earth ''the Photosynthesis''. In oxygenic photosynthesis light energy of the Sun is first converted into reductants and chemically bound energy. The reductants are then primarily used for fixation of inorganic CO2, converting it into an organic form, that can be stored as biomass. This photosynthetic formation of organic carbon is usually called primary production. Algae and other autotrophs thus play essential roles in carbon capture and contribution to food webs as primary producers (Sayre 2010; Tsai et al. 2014) not only in aquatic ecosystems. Another intriguing aspect of algal life is their ability to inhabit the harshest and unhospitable biotopes for other organisms. Algae can be found in places such as arid and hot deserts (Saber et al. 2018), hot springs (Castenholz 1968; 1969), salt pools and ponds where salinity reaches up to 235 g/l (Ben-Amotz and Avron 1990), freezing and UV rich biomes of Arctic and Antarctica (Holzinger et al. 2018) as well as freezing oceans at temperatures close to -10 °C (Ralph et al. 2005), where they are still able to perform photosynthesis. In order to survive, acclimate, and adapt to such a hostile environments, cells have to utilize several types of mechanisms to counterbalance the effects of adverse stress on their metabolism. In Fig. 1, there are shown four major sites of responses to abiotic stresses further described in this work. Photosynthesis is known to be heavily influenced by abiotic stresses, particularly by the high light and elevated/lowered temperature. The mechanisms of maintenance of effective function of photosynthesis include adjustment of the antenna size, pigment composition, repair of photoinhibited photosynthetic complexes, and also alterations in the carbon fixation and consecutive carbon metabolism. Second site of response is the acclimation on the level of membrane. Upon

stress recognition, the homeostasis is being maintained by the de-novo synthesis of fatty acids, fatty acid unsaturation (addition of one or more double bonds to the carbon backbone of fatty acids) and/or elongation (addition of more carbons to the fatty acid chain) and finally utilization of storage of lipids. The homeostasis on the protein level includes the degradation of proteins that have been damaged by the effect of stress and synthesis of proteins involved in the protection of the cell. The mechanism for keeping carbohydrate homeostasis under stressful conditions consist of carbohydrate biosynthesis and/or degradation based on the type of stress and under certain stresses also compatible solutes are being synthesized.

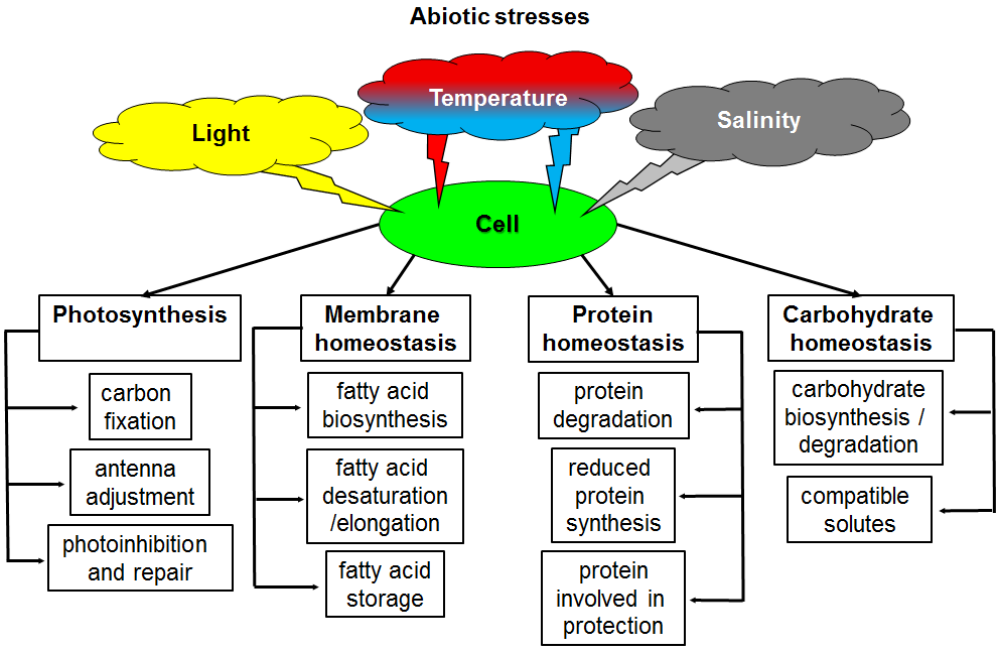

Fig. 1: Events of stress adaptation (adapted from Barati et al. 2019)

1.2. Environmental factors and competent homeostatic responses

1.2.1 Light quantity and quality

As algae and cyanobacteria have the ability to perform photosynthesis, the light is not only a necessary source of energy but at the same time could be a very important stress factor (Vass et al. 2007). Absorption of too much light leads to photo-oxidative damage to the photosynthetic apparatus and later results in decreased efficiency of photosynthesis (photoinhibition). Excess of light affects cellular growth and viability, thus cells have to acclimate to the current environmental conditions in order to sustain the functionality of their metabolism. In order to acclimate to the changing environment, algal cells utilize miscellaneous acclimative strategies of optimization of energy capture, conversion, and dissipation in order to perform photoprotection and repair of photo-oxidative damage. On the other hand, when the light intensity is low, it becomes limiting, resulting in retardation of metabolism and cessation of growth (Niyogi and Truong 2013). Algae can adapt to a wide range of photon flux densities (light intensities) as can be seen from Fig. 2. The lower limit of compensation point for Arctic diatoms can be as low as 0.2 μmol photons m^{-2} s^{-1} (Cota et al. 1985), while some algae of the family Chlamydomonadaceae experience peak maxima of PFD of up to 4000 μmol photons m^{-2} s^{-1} (Gorton et al. 2007). The photon flux density together with the spectral quality of light determines the amount of energy that is available for photosynthetic metabolism (Latasa 1995). For example, the quality of down-welling light varies rapidly with depth (Rivkin 1989). Another examples are the polar ecosystems where the light quality varies a lot, as the light in water and ice is often depleted of higher wavelengths (e.g. blue-green light is the most dominant) (Neale and Priscu 1995; Morgan-Kiss et al. 2005). Particularly endosymbiotic organisms (such as zooxanthellae, the algal endosymbionts of corals) are due to spectral shading subjected to different light quality than free-living forms. Light environment inside a

coral is often enriched by near-infrared radiation and depleted of visible light (Magnusson et al. 2007). The microalgae living below the canopy often experience light enriched in green and far-red (Salles et al. 1996).

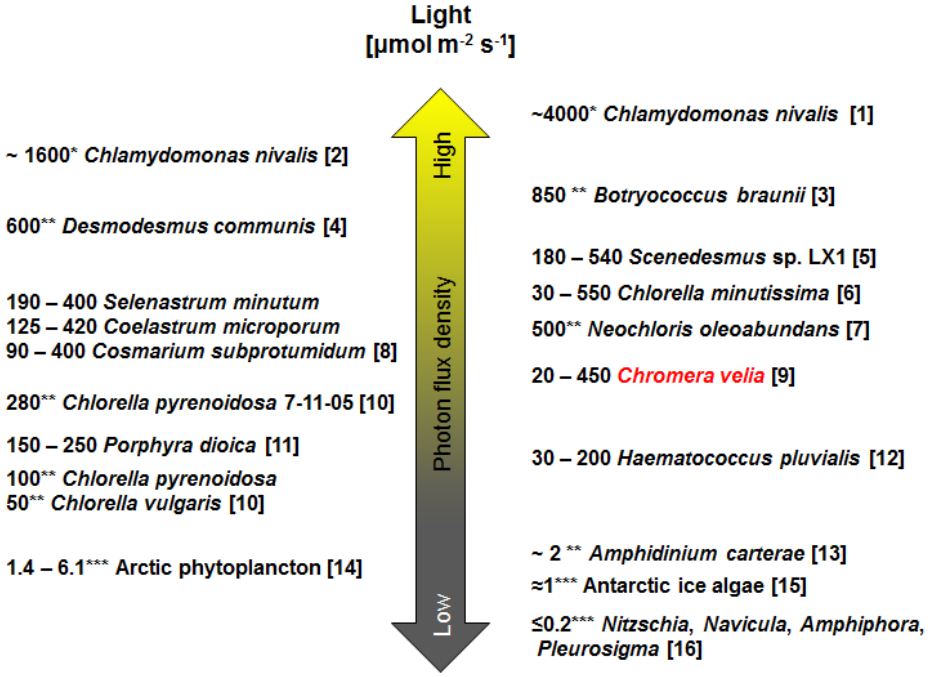

Fig. 2: The light intensity limits for photosynthetic life. Representative algae found in the literature. Organisms marked by red color have been used in this study. References: [1] (Gorton et al. 2007); [2] (Mosser et al. 1977); [3] (Yoshimura et al. 2013); [4] (Vanags 2015); [5] (Zhen-Feng et al. 2011); [6] (Lotfi et al. 2011); [7] (Sousa et al. 2013); [8] (Bouterfas 2002); [9] (Lukeš et al. 2017); [10] (Sorokin and Krauss 1965); [11] (Pereira 2006); [12] (Evans et al. 2008); [13] (Richardson and Fogg 1982) [14] (Platt et al. 1982); [15] (Bunt et al 1964); [16] (Cota et al. 1985). * - in situ measurement of maximal PFD, ** - laboratory measurement of optimal growth, *** - a laboratory measurement of compensation point for photosynthesis and growth.

1.2.1.1 Photosynthetic apparatus

In oxygenic phototrophic organisms (cyanobacteria, algae, plants) the photosynthetic machinery consists of two integral thylakoid membrane protein complexes: photosystem II (PSII) and photosystem I (PSI) (Fig. 3). Photosynthetic pigments chlorophylls and carotenoids are bound to the Lhcb (genes encoding light-harvesting complex II (LHCII) proteins) and Lhca (genes encoding LHCI proteins) families of light-harvesting polypeptides associated with the PSII core antenna polypeptides of PsbB (CP47) and PsbC (CP43). PSII reaction center consists of polypeptides PsbA (D1) and PsbD (D2), consists of chlorophylls (Chl), pheophytin (Pheo), β-carotene, quinone acceptors (Q_a and Q_b), and extrinsic proteins of water oxidation complex. PSI reaction center consists of polypeptides PsaA and PsaB, contains Chlorophyll a, P700, β-carotene (Green and Durnford 1996; Morgan-Kiss 2006). The photosynthetic electron transport chain consists of the aforementioned multi-protein complexes PSII and PSI and cytochrome b_6/f (Cyt b_6/f) and ATP synthase complex. In addition to these, two-electron carriers: plastoquinone (PQ) and plastocyanin (PC) are involved in electron transport. Upon excitation of the special pair of chlorophylls by light, the special pair of chlorophyll starts a cascade of oxidative events, eventually leading to water splitting and oxygen evolution, PSII primarily mediates noncyclic electron transport to PQ. These electrons transfer from the PQ pool to the Cyt b_6/f complex (Fig. 3). P700 enables PSI to acquire electrons from Cyt b_6/f complex by the PC and elevate them to sufficiently high redox potential that after passing through ferredoxin, they can reduce $NADP^+$ to NADPH (Fig. 3). H^+ protons (coming from split water) are used for generation of ATP after passaging through ATP synthase complex (Fig. 3).

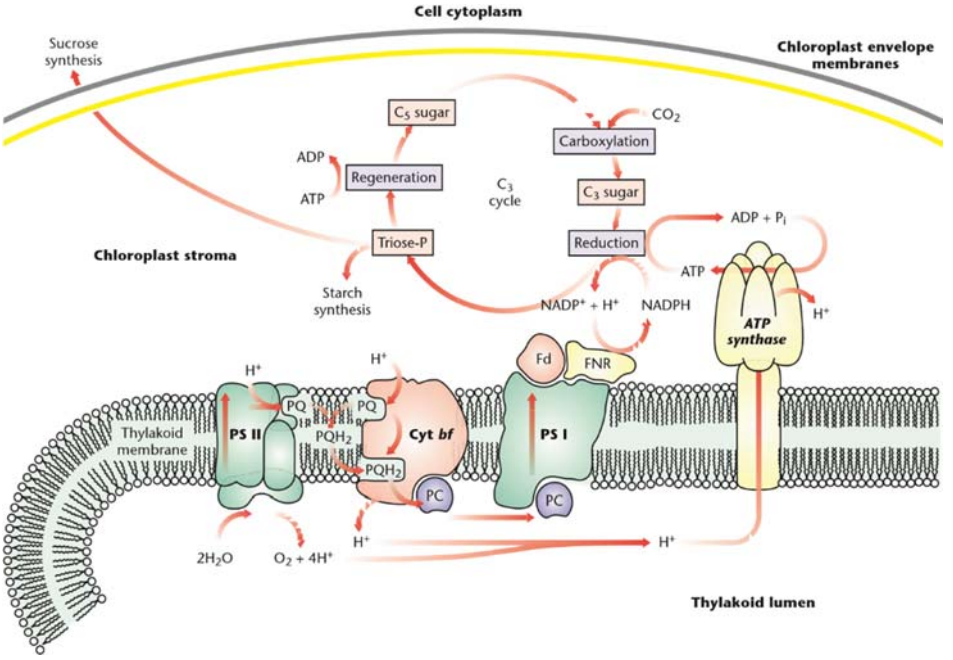

Fig. 3: Overview of processes in photosynthesis (Ort and Whitmarsh 2001)

1.2.1.2 Photosynthetic photoprotective mechanism

The rate of photosynthesis does not increase linearly with the photon flux density. The balance between photophysical and photochemical processes transforming light and the metabolic sinks that consume energy is called photostasis (Ort 2001; Huner et al. 2003). Falkowski and Chen (2003) derived equation that defines photostasis: $\sigma_{PSII} \times E_k = \tau^{-1}$, where σ_{PSII} is the effective absorption of crossection of PSII, E_k is the irradiance (I) at which maximum photosynthetic quantum yield balances photosynthetic capacity estimated from a photosynthetic light response curve, and τ^{-1} is the rate at which photosynthetic electrons are consumed by a terminal electron acceptor such as CO_2 under light-saturated conditions (Morgan-Kiss 2006). Whenever the rate of energy absorbed by PSII is higher than the rate at which electrons enter the transport chain (exceeding metabolic

electron sink capacity) then imbalance is created: $\sigma_{PSII} \times E_k > \tau^{-1}$. Excitation pressure (Huner et al. 1998; Maxwell et al. 1994) or excessive excitation energy (Karpinski et al. 1999) is a relative measure of the reduction state of plastoquinone A and reflects the redox state of the intersystem PQ pool (Huner et al. 1998). This can be measured in vivo or in vitro by pulse amplitude-modulated fluorescence as either 1 - *qP* (in which *qP* is photochemical quenching) or as suggested more recently, as 1 - *qL* (in which *qL* is the fraction of open PSII centers) (Kramer et al. 2004). Thus, the excitation pressure is a measure of the imbalance in energy flow, that is, a measure of the extent to which $I > E_k$, and thus, $\sigma_{PSII} \times I > \tau^{-1}$ (Morgan-Kiss 2006). The energy balance can be attained by physical reduction of σ_{PSII} (decreasing the light-harvesting antenna size) or by reduction of σ_{PSII} by non-photochemical energy dissipation as heat (Falkovski and Chen 2003; Krause and Weis 1991). Under high PFD (or under other stressful conditions, see chapter [1.2.3.1 Temperature effect on photosynthesis]), the absorption of the energy (photons) exceeds the capacity of consecutive reactions consuming products of the light-phase and reaction centers get saturated (Wobbe et al. 2015), resulting in increase of excited singlet ($^1Chl^*$) and excessive acidification of thylakoid lumen (Niyogi 1999). The increase of ($^1Chl^*$) increase the probability of generation of chlorophylls in the triplet excited state ($^3Chl^*$), that can react with oxygen, creating singlet oxygen ($^1O_2$), that is very reactive and toxic to the thylakoid membranes (Niyogi 1999). The lifetime of $^1Chl^*$ in PSII is much longer than in PSI, as the charge separation and recombination of the special pair of P700 in PSI are faster than of P680 in PSII (Slavov et al. 2008). This makes PSII the main producer of $^1O_2$, damaging the structure of the photosynthetic apparatus and leading to photoinhibition (Niyogi 1999). Consecutive reactions of $^1O_2$ with $^3Chl^*$ produce other harmful chemical species, known as Reactive Oxygen Species (ROS). These represent a threat to cells because of damage to photosynthetic apparatus and cellular structures such as thylakoid membranes, causing for example the oxidation of polyunsaturated fatty acids. Therefore ROS must be kept under strict

control (Aro et al. 1993). In order to avoid the formation of $^{3}Chl^{*}$ and to protect against ROS, algae utilize several photoprotective strategies. By the velocity of the reaction to the stress these photoprotective strategies can be distinguished into short term and long term (Eberhard et al. 2008). Among the short term response to excess light on the scale of seconds, a mechanism called non-photochemical quenching is utilized, that dissipates $^{1}Chl^{*}$ in a form of heat. Another type of regulatory mechanism are state transitions. In algae, they work under conditions that favor excitation of one of the two photosystems, by the movement of LHCII antenna between PSII and PSI. This allows for balancing the excitation energy in the photosynthetic apparatus in order of minutes (Eberhard et al. 2008; Wobbe et al. 2015). Another way to alleviate the energy overload on photosynthetic apparatus is the use of non-assimilatory electron sinks. Among non-assimilatory electron sinks metabolic reactions that consume NADPH and ATP other than Calvin cycle, belong for example photorespiration (Wallsgrove et al. 1987) or indirectly the water-water cycle (Asada 1999). In the case of excessive light, plants may utilize relocation of chloroplast (chloroplast movement) and self-shading, these can reduce the amount of excessive light in order of tens of minutes (Cazzaniga et al. 2013). Some motile microalgae can react to high light conditions by swimming away from the light source (Bennet and Golestanian 2015). This is called light avoidance - it works also vice versa, under low light conditions, where algae swim towards the light. These responses do not require changes in gene expression as their components are already in place. Among the mechanisms, that require changes in gene expression, belong de novo synthesis of pigments that increase the capacity of a microorganism to resist high light stress (Lavaud 2002). In order to re-balance the amount of absorbed light with the energy utilization capacity, photosynthetic organisms can reduce antenna size together with increase of components of electron transport chain and fixation of CO2 in response

to high light (Bonente et al. 2012; Wobbe et al. 2015), this so-called ''rebalance of light and dark reactions'' may take hours or days.

1.2.1.3 Light stress affects carbon allocation

The carbon allocation in algae is defined as the partitioning of the photosynthetically assimilated carbon into different macromolecular pools, such as proteins, lipids, carbohydrates, and nucleic acids (Wagner et al. 2017). Under optimal growth conditions of light, temperature, pH, salinity and nutrient availability, the flux of energy provided by photosynthesis is in the equilibrium with the carbon flux for the synthesis of cellular macromolecules (Wagner et al. 2017). Stressful conditions result in a discrepancy between these two fluxes. Microorganisms have to employ an efficient regulatory mechanism to adjust their metabolism to fulfill their growth needs under modified conditions. This usually include adjustments in the synthesis of structural and storage lipids, synthesis of proteins involved in the light capture and/or dissipation, and utilization of synthesis of storage and structural carbohydrates. Lipids in photosynthetic microorganism can be divided into two groups: nonpolar (NL) lipids where three molecules of fatty acid (FA) chains are either esterified on the glycerol backbone (also referred to as triacyl-glycerols) or are esterified on fatty alcohols forming wax esters. These serve as the main energy storage molecules of aquatic organisms. The second group are the polar lipids (PL), which are the structural components of the cell membranes. The polar lipids have esterified only two positions the sn1 and sn2 and on the last position of the glycerol backbone, the functional group is placed. These can be divided into several classes by their functional group: phospholipids (PL), glycolipids (GL), and betaine lipids. PLs are usually mostly found in non-photosynthetic membranes such as plasma membrane, mitochondrial membrane, endoplasmic reticulum, etc. Glycolipids are typical constituents of thylakoid membranes (Guschina

and Harwood 2009). Lipids and their constituents (FA) apart their structural role are also important functional molecules. They are involved in photosynthesis (Harwood and Jones 1989; Sukenik et al. 1989; Dörmann and Benning 2002; Jones 2007) and in signal transduction (Sumida et al. 1993). The FA composition of lipid classes differs from species to species and strongly depends on environmental conditions, as the variations of the composition allow the microorganism to cope with the changes of environment. The organization of the light harvesting complexes and thus the photosynthetic performance of algae depends on the lipid composition of thylakoid membranes (Pronina et al. 1998; Goss and Wilhelm 2009; Schaller et al. 2011). Not just the lipid class itself, but also the FA composition of the individual lipid classes is important (Li et al. 2012) (see chapter 1.2.3.2 "Temperature induced membrane homeostasis" for additional information). Membrane fluidity together with the electron flow between acceptors of PSII are adjusted and maintained by the levels of polyunsaturated fatty acids (Horváth et al. 1987; Mock and Kroon 2002). Under low light conditions, higher levels of PUFA indicate higher stacking of thylakoid membrane (Richardson et al. 1983; Neale and Melis 1986). The opposite response, decreasing of PUFAs in the membrane is associated with a decrease of proton leakage to reduce metabolic costs (Quigg et al. 2006). A generalization of the effect of environmental conditions on FA saturation/unsaturation has been proven to be tedious due to the highly species-specific FA profiles (Piepho et al. 2012). Often contradicting results by analysis of different species can be found: some show increased PUFA levels under light limitations (Sukenik et al. 1989; Mock and Kroon 2002; Fabregas et al. 2004; Guiheneuf et al. 2009). Some other works show an increase of PUFA levels at high light conditions (Seto et al. 1984; Zhukova 2007; Solovchenko et al. 2008). The increased saturation of fatty acids observed at higher PFD is often a result of that membrane polar lipids that are rich in PUFAs became less abundant than NL storage lipids that contain mostly saturated FA (Khotimchenko and Yakovleva 2005).

Changes in the protein content of the cells are tightly connected with the changes of the photosynthetic apparatus (described in the chapter 1.2.1.2), and are inevitably connected with growth rate. Direct correlation between growth rate and RNA content of cells can be found, as growth depends on the protein synthesis (Giordano et al. 2015).

Carbohydrates are the major product of photosynthesis, they serve two main functions in algal cells: they have structural role in formation of cell walls and also serve as storage components in the cell. Storage carbohydrates provide energy needed for metabolism and if necessary they can alleviate cell survival in dark conditions (Geider and La Roche 2002; Raven and Beardall 2004). Generally, the carbohydrate content changes during diel cycle, as the photosynthate is being stored during the day and later during the night can be respired as an energy supply for maintaining basal metabolism and also can provide energy and carbon molecules for protein synthesis (Lancelot and Mathot 1985; Granum et al. 2002). Usually, higher PFD result in increase in carbohydrate content of cells (Hu 2013) as the photosynthetic production exceeds respiration. The increase of carbohydrates is strain specific, for example *Spirulina maxima* increased its content from ~10 % to 34 % (De Philippis et al. 1992) or in *Porphyridium* species where 3-fold increase in carbohydrates took place upon increase of PFD (Friedman et al. 1991). Studies on the impact of light quality on C allocation are less numerous and mostly related to increase in algal productivity. Works of Das et al. (2011) and Vadiveloo et al. (2015) demonstrated that in *Nannochloropsis* sp., light quality modified lipid productivity, and that different light colors affect growth of diatom *Skeletonema costatum* (Miao et al. 2012), overall productivity of *Chlamydomonas reinhardtii* (de Mooij et al. 2016), or growth for biodiesel in *Oedogonium* species (Humphrey et al. 2018).

1.2.2 Temperature

The life on the Earth is inevitably linked to liquid water. Water in liquid form is an internal and external connecting medium for cells, metabolites, enzymes. Under normal atmospheric pressure (0.1 MPa) water exists in liquid form in the interval of 0 up to 100 °C, stated, that no high-enough concentrations of impurities (salts, carbohydrates, alcohols and other cryoprotectants [see chapter 1.2.3.5 for more detailed information]) are present. Under certain circumstances, living structures might occur even at temperatures above 100 °C, as in the deep seas under appropriate hydrostatic pressure, water exhibits boiling point higher than the aforementioned 100 °C. Nevertheless, for the oxygenic photosynthetic life temperature range is defined from a few centigrade of Celsius below 0 up to a maximum of 74 °C (Fig. 4). The usual microbiological classification distinguishes organisms with growth temperature optimum between 25 – 45 °C as mesophilic, thermophilic organisms with optimum at 55 – 75 °C and psychrophilic species with an optimum between 15 – 18 °C and lower, usually unable to live above 25 °C. Prokaryotic cyanobacteria may grow up to 73 – 74 °C (Meeks and Castenholz 1971), while among eukaryotes no ''true'' thermophile does exist. The only exception are red algae of the class Cyanidiophyceae (with three delegates: *Cyanidium caldarium*, *Galdieria sulphuraria,* and *Cyanodictyoschyzon merolae*) that may grow up to 56 °C, but typically at lower temperatures. These are usually denoted as thermotolerant species. The same works at the opposite temperature side. Some of the psychrophilic organisms are more likely psychrotolerant, rather than truly psychrophilic ones. Among psychrophilic/psychrotolerant (or cold-tolerant) species of cryosestonic ''snow algae'' belong the group of genus *Chloromonas*/*Chlamydomonas* that can actively grow and photosynthesize under temperatures around the 0 °C (Mosser et al. 1977).

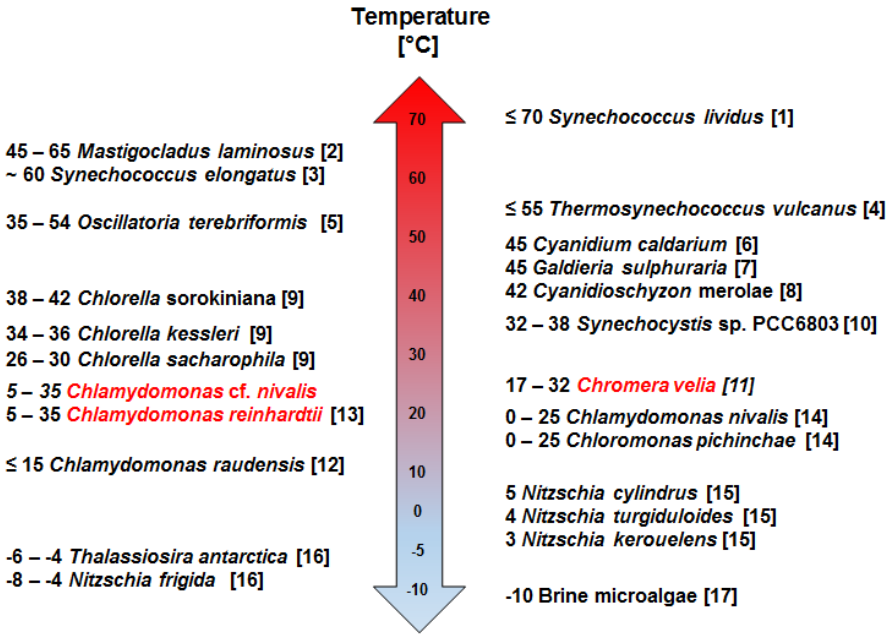

Fig. 4: Temperature limits for photosynthetic life. Optimal and/or limiting temperatures for representative microalgae are shown. Organisms marked by red color have been used in this study. References: [1] (Meeks and Castenholz 1971); [2] (Binder et al. 1972); [3] (Miyairi 1995); [4] (Kiseleva et al. 1999); [5] (Castenholz 1968); [6] (Doemel and Brock 1970); [7] (Gross and Oesterhelt 1999); [8] (Kobayashi et al. 2014); [9] (Kessler 1985); [10] (Červený et al. 2015); [11] (Lukeš et al. 2017); [12] (Morgan-Kiss et al. 2006); [13] (Lukeš et al. 2014); [14] (Hoham 1975); [15] (Fiala and Oriol 1990); [16] (Aletsee and Jahnke 1992) [17] (Ralph et al. 2005).

1.2.2.1 Temperature effects on photosynthesis

Balancing the energy flow through the process of photosynthesis is a challenge due to differential temperature sensitivities and differential rates between the photochemical reactions and the biochemical reactions (Morgan-Kiss 2006). Photosynthetic microorganisms growing in low temperature are always under a constant state of energy imbalance because

of decrease of τ^{-1} (for more information, return to chapter 1.2.1.2 Photosynthetic photoprotective mechanism). The works of Maxwell et al. (1994) on the response of mesophilic *Chlorella vulgaris*, and on Antarctic *Phormidium murrayi* have shown similar acclimatory response to low temperature like to high PFD (Roos and Vincent 1998). Both organisms reduced functional size of PSII and significant increase in carotenoid/chlorophyll a ratio was observed. Analogous response was observed by in Arctic diatoms that show the ability to avoid photoinhibition by the dissipation of excess light energy by NPQ (Mock and Hock 2005; Ralph et al. 2005). The ice diatoms possess effective xanthophyll cycle using diatoxanthin and diadinoxanthin (diatom equivalent of violaxanthin and zeaxanthin of green algae and plants) to adapt to varying irradiance levels (Kashino et al. 1999) and are capable to withstand up to four times higher irradiance levels than in natural conditions (Kudoh et al. 2003). Alternative energy sinks in diatoms under conditions when light absorption exceeds metabolic energy requirements have been proposed by Lomas and Gilbert (1999). Diatoms grown at low temperature exhibited relatively high rates of NO_3^- uptake. These authors hypothesized, that phytoplankton under transient energy stress, reduce $NO3^-$, to maintain energy balance within the cell and this might also give diatoms competitive advantage in variable environments (Lomas and Gilbert 1999).

1.2.2.2 Temperature induced membrane homeostasis

The optimal function of photosynthesis under temperature stress in algae is dependent on proper function of their thylakoid membranes. The behavior of membrane upon temperature change is dependent on its fluidity. High temperatures increase membrane fluidity. The membrane fluidity is lowered by the integration of de novo-synthesized saturated fatty

acids into membrane lipids and/or integration of membrane-stabilizing proteins (Los et al. 2013). On the other hand, at low temperatures the membranes tend to be more rigid, resulting in lowered fluidity. Low fluidity is usually counterbalanced by enhanced membrane lipid desaturation via the action of fatty acid desaturases (FADs) (Sakamoto and Murata 2002). To achieve looser packing of lipids, leading to a decrease in the solidification of membranes at low temperatures, algae increase the content of unsaturated membrane fatty acids (Lyon and Mock 2014). The functional stability of photosynthetic machinery under chilling temperatures can be enhanced by an increase of unsaturated fatty acids in its membranes as shown by Wada et al. (1994). Stress conditions such as high temperatures disrupt weak interactions in protein structures and affect the conformational stability of proteins (Daniel et al. 1996). Heat stress induces denaturation and aggregation of proteins that alter metabolic fluxes and result in membrane injury (Fu et al. 2008). Upon stress conditions, cells usually increase the synthesis of fatty acids. Expression of regulatory genes encoding subunits of acetyl-CoA carboxylase (ACCase), ketoacyl-ACP synthase (KAS), desaturases and elongases can be observed. ACCase is an enzyme present in almost all algae (Podkowinski and Tworak 2011; Singh et al. 2016) and is responsible for the control of fatty acid biosynthesis rate. ACCase supplies malonyl-ACP, where the malonyl is later used in a series of condensational steps, lengthening the precursor fatty acid in which KAS is the rate-limiting enzyme (Lei et al. 2012). The newly formed saturated fatty acids (SFAs) later undergo elongation and desaturation steps to make long-chain unsaturated (UFA) and polyunsaturated fatty acids (PUFAs) (Pereira et al. 2003). PUFAs are fatty acids of the length of at least 18 carbons and at least two double bonds (Huang et al. 2004). The two main enzyme groups: desaturases and elongases are responsible for the control of fatty acid structures (length and unsaturation) (Khozin-Goldberg and Cohen 2011). PUFAs generally have lower melting points than SFAs thus increasing the ratio of PUFAs in the membrane affect membrane fluidity by lowering the

solidification of membrane lipids (Ackman et al. 1968; Mortensen et al. 1988; James et al. 1989; Renaud et al. 1995; Brett and Müller-Navarra 1997). The desaturase catalyzing first committed step in desaturation and initiating the conversion of SFAs to mono-unsaturated fatty acids (MUFAs) that are crucial for further desaturation to PUFAs is the Δ9 fatty acid desaturase (FAD) (Xue et al. 2016). The Δ9 fatty acid desaturases act on palmitic (C16:0) and stearic (C18:0) acids, introducing first double bonds at Δ9 position from the carboxyl end of an alkyl chain, forming palmitoleic acid (C16:1) and oleic acid (C18:1) (Xue et al. 2016). Lately when further desaturation of fatty acids is necessary, consecutive Δ12 FAD alternatively called ω6 desaturase (introduces double bond to position 6 from methyl end or so-called ω end of alkyl chain), converts palmitoleic acid (C16:1) or oleic acid (C18:1) to hexadecadienoic acid (C16:2) or linoleic acid (C18:2), whereas ω3 desaturases insert a double bond at the ω3 position and D4, D5, D6, and D8 FAD are considered to be responsible for the production of eicosapentaenoic acid (EPA) (An et al. 2013). In Antarctic alga *Chlamydomonas* sp. ICE-L there was observed upregulation of Δ9CiFAD, ω3CiFAD2, and ω3CiFAD1 while Δ6CiFAD was amplified with rising temperature (An et al. 2013). These observations suggest that ω3CiFADs are important for low-temperature survival while Δ6CiFAD enhances survival at higher temperatures (An et al. 2013). The fatty acid unsaturation is necessary for the survival of low temperatures. It is important to stress that not all the double bonds of the fatty acid molecule have an equivalent impact on membrane physical properties. If we compare phosphatidylcholine which is esterified on both the sn1 and sn2 positions with stearic acid (18:0/18:0)-PC and such PC where stearic acid has been replaced at sn2 position with oleic acid (18:0/18:1)-PC, the melting point (Tm) (transition temperature from gel to liquid-crystalline phase) that was 55 °C for (18:0/18:0)-PC decreases to 6.3 °C in (18:0/18:1)-PC (Russel 1989). The insertion of a second double bond to form (18:0/18:2)-PC, was found to decrease the Tm to -16 °C. However, the addition of a third double bond-forming (18:0/18:3)-PC, did not

decrease, but increased the Tm by 3 °C to -13 °C. For comparison, Tm values for 16:0/16:1- and 16:0/22:6-PC do not differ significantly (-12 and -10 °C, respectively) (Coolbear et al. 1983). These findings suggest that monounsaturated FAs are more effective than PUFAs in decreasing membrane order (making the membrane more fluid), possibly by interfering with the cooperative liquid-to-gel transition (Lukeš et al. 2017).

1.2.2.3 Temperature affected protein homeostasis

As the accumulation of unfolded proteins under stressful conditions continues, expression of a defined set of proteins called heat-shock proteins (HSP) appears to re-establish protein homeostasis (Parsell and Lindquist 1993; Nover 1991; Fulda et al. 2010). Various organisms show expression of HSPs induced by high temperature (Parsel and Lindquist 1993; Gupta et al. 2010). HSPs synthesized by most of the eukaryotes belong to five conserved classes identified according to their molecular masses: HSP100, HSP90, HSP70, HSP60 and small heat shock proteins with the approximate size of 17 – 30 kDa (Waters et al. 1996). The HSP90s have an evident function in keeping unstable proteins close to their native forms by protein stabilization and conformation maintenance (Barati 2019). Also, they participate in signaling and some cellular pathways (Richter and Buchner 2001; Young et al. 2001). The HSP70 chaperones are involved in protein folding and translocation (Bukau and Horwich 1998). In Antarctic algae increase only 5 °C above optimal temperature triggered the expression of HSP70B gene (Vayda and Yuan 1994). Similar temperature stress for Antarctic *Chlorella vulgaris*, thermophilic *C. vulgaris,* and mesophilic *C. kesslerii* led to 30% higher levels of HSP70B in the Antarctic strain (Chankova et al. 2013). Chankova (2013) proposed the role of HSPB for low-temperature cell survival at Antarctic extreme conditions as no reduction of HSP70B levels at low temperatures was observed. Similarly, in *Chlamydomonas acidophila* exposed to temperature stress, levels of several HSPs increased, indicating that HSPs

are contributing to the survival of acidophilic algae in stressful conditions (Gerloff-Elias et al 2006). Another critical adaptive mechanism for acclimation to low temperature is a molecular adaptation of enzymes to compensate for the reduction in reaction rates at low temperatures (Morgan-Kiss et al. 2006). Arrhenius equation ($k = Ae^{-Ea/RT}$) describes the relation between temperature and chemical reaction rates, where k is the rate constant, A is the constant for a particular reaction, E_a is the activation energy, R is the gas constant, and T is the absolute temperature in degrees of Kelvin. E_a is related to Q_{10}, the factor by which the rate changes by varying the temperature 10 °C, according to the following relationship: $Q_{10} = (E_a \times 10) / (RT_2T_1)$, where T_2 and T_1 are the temperature limits for which Q_{10} is desired. Lowering growth temperature by 10 °C leads to two or three times (Q_{10} = 2 to 3) reduction of biochemical reaction rates. The activity of the mesophilic enzyme could be reduced up to 80-fold when the growth temperature is shifted from 37 °C to 0 °C (Morgan-Kiss et al. 2006). One of compensational mechanisms for reduced reaction rates is at the level of catalytic efficiency (k_{cat}/K_m) of cold-adapted enzymes by either increasing rate of catalysis (k_{cat}) at the expense of substrate concentration at half-maximum activity (K_m) or by optimizing both parameters (increasing k_{cat} and decreasing K_m) (Gerday et al. 1997). Several psychrophilic enzymes exhibit a temperature shift for a maximal activity to lower temperatures and a concomitant unfolding at moderate temperatures (Feller et al. 1994; Karasova-Lipovova et al. 2003). Amino acid substitution is responsible for the promoted increase of protein flexibility (Morgan-Kiss et al. 2006). Strikingly the active site of cold-adapted enzymes is almost identical to the mesophilic ones. Study of psychrophilic and mesophilic α-amylases provided by X-ray crystallography have shown conservation of ~30 side chains involved in the binding of transition state-analog (Aghajari et al. 2003). The high activity during cold is thought to be also achieved by modifications outside of the active site by modification of the dynamic properties of catalytic residues (Holland et al. 1997). X-ray structures revealed two other types of molecular adaptations. First include

enlargement of catalytic cavity to be more accessible for ligands in psychrophilic enzymes in comparison to mesophilic ones by deletion of residues in loops bordering the active site, by changing to looser conformation of these loops or by substitution of bulky sidechains by smaller groups at the entrance of the active site (Russel et al. 1998; Aghajari et al. 2003). These changes improve accessibility by the accommodation of the substrate at lower energy costs and by assisting the release and exit of reaction products. (Feller and Gerday 2003). Some of the enzymes have improved electrostatic potential around active-site for the attraction of oppositely charged ligand and channel the substrate towards the catalytic cavity (Smalås et al. 2000). Similarly, on the opposite side of temperature scale modifications in the reaction centers of enzymes occur. For example, in the photosystem 2 (PSII) core proteins D1 and D2 (coded by the psbA and psbB genes) form a heterodimer that fixes all the cofactors that are required for primary photochemistry. Only minor changes in the structure of D1 protein have a significant effect on photosynthetic transfer in PSII and play a very important role in the thermal adaptation of photosynthesis (Shlyk et al. 2006). A comparative analysis of the psbA sequences of mesophilic and thermophilic organisms have shown a difference in the amino acid sites 209 and 212. While in the mesophilic organisms on both sites serine was found, in thermophilic ones amino acids of these positions were replaced by alanine and cysteine. The replacement of two amino acid residues led to increased thermotolerance of mesophilic cyanobacterium Synechocystis sp. PCC6803 (Dinamarca et al. 2011). Another strategy of achieving similar enzyme-catalyzed reaction rates at lower temperatures is to increase enzyme concentrations, though this acclimation strategy is indeed energetically inefficient (Morgan-Kiss 2006). Such strategy works for example for ribulose-1,5-bisphosphate carboxylase (Rubisco), one of the most critical enzymes for carbon fixation in phototrophs (Morgan-Kiss 2006). Unlike the aforementioned adaptations of catalytic activity, the maximum activity of Rubisco was not altered in two isolates of the Antarctic *Chloromonas* sp., and the specific

activity at low temperatures was actually lower in the psychrophilic compared to mesophilic Rubisco (Devos et al. 1998). The relative amount of Rubisco was higher in two psychrophilic *Chloromonas* sp. strains in comparison with their mesophilic counterpart *Chlamydomonas reinhardti*i (Devos et al. 1998). Another specific mechanism of cold-adaptation is the use of specific isotypes adapted to different temperatures as reported by Lin and Somero (1995), but the main disadvantage is the requirement of the presence of multiple gene copies.

1.2.2.4 Temperature affected carbohydrate homeostasis

Under harsh conditions, cellular maintenance costs are increased due to the higher energy demand to maintain homeostasis (Calow 1991). Thus the carbohydrate metabolism is shifted from carbon storage towards enhancement of dissolved sugars (Arbona et al. 2013). The main outcome of photosynthesis is sucrose. Sucrose is used in growth, storage reserves formation, cell signaling and stress acclimation (Jiang et al. 2015). Biosynthesis of sucrose is strongly dependent on temperature (Müller and Wegmann 1978). The response of algae to different temperatures results in different alterations of sucrose levels in their cells. For example, elevated temperatures resulted in accumulation of sucrose in *Dunaliella* (Müller and Wegmann 1978). On the other hand, accumulation of sucrose was reported at low temperatures in *Chlorella vulgaris* (Salerno and Pontis 1989) and *Klebsormidium flaccidum* (Nagao and Uemura 2012). In the work of Nakamura and Myiachi (1982) on *Chlorella vulgaris,* the levels of sucrose upon the increase of temperature increased due to the degradation of starch. One of the essential enzymes of sucrose synthesis, the sucrose phosphate phosphatase (SPP) has shown enhanced expression of its gene upon cold stress, resulting in the increase of sucrose levels in *Chlamydomonas reinhardtii* (Valledor et al. 2013). The accumulation and

degradation of the primary carbon and energy storage, starch, is affected by environmental stress (Geider and La Roche 2002).

1.2.3 Salinity

Salinity is typically a local, rather than global, parameter and is highly variable in coastal regions, especially in the intertidal zones, estuaries, and rock pools (Kirst 1990). The salinities that cells may encounter in marine habitats range strongly. Open oceans have a salinity of 3.3 – 3.7 % (about 970 – 1060 mOsmol/kg; 2.5 – 2.73 MPa) (Kirst 1990), decreasing from tropical zone towards the polar seas. In estuaries, the seawater gets diluted, resulting in much lower salinities. In tide pools due to tidal actions, salinity may range from 0 % to full seawater salinity. Organisms are affected in several ways upon change of salinity. Osmotic stress affects water potential of cells. Cells are subject to ionic stress caused by loss or uptake of ions and finally, cells are subjected to change of ionic ratios due to selective ion permeability of cellular membranes (Kirst 1990). Increased salinity causes hyper-osmotic and hyper-ionic shock (Niu et al. 1995; Yeo 1998). Ionic stress imbalances cellar metabolism due to excessive accumulation of Na^+ and Cl^- while uptake of other mineral nutrients, such as K^+, Ca^{2+}, and Mn^{2+} is reduced (Ball et al. 1987, Hasegawa et al. 2000). Osmotic stress is related to a deficit of water without the direct role of sodium ions (Joset et al. 1996, Munns 2002). The altered water status of the cells affects cell division resulting in reduced growth (Munns 2002). Other effects include membrane disorganization, attenuated nutrient acquisition, inhibition of photosynthesis, induction of reactive oxygen species and metabolic toxicity (Greenway and Munns 1980; Munns and Termaat 1986; Munns 2002). Algae are capable of thriving in a wide range of salinities (Fig. 5) ranging from almost distilled water with the salinity of 0 M (Schobert et al. 1972) up to 4 M of salt (Ben-Amotz and Avron 1990).

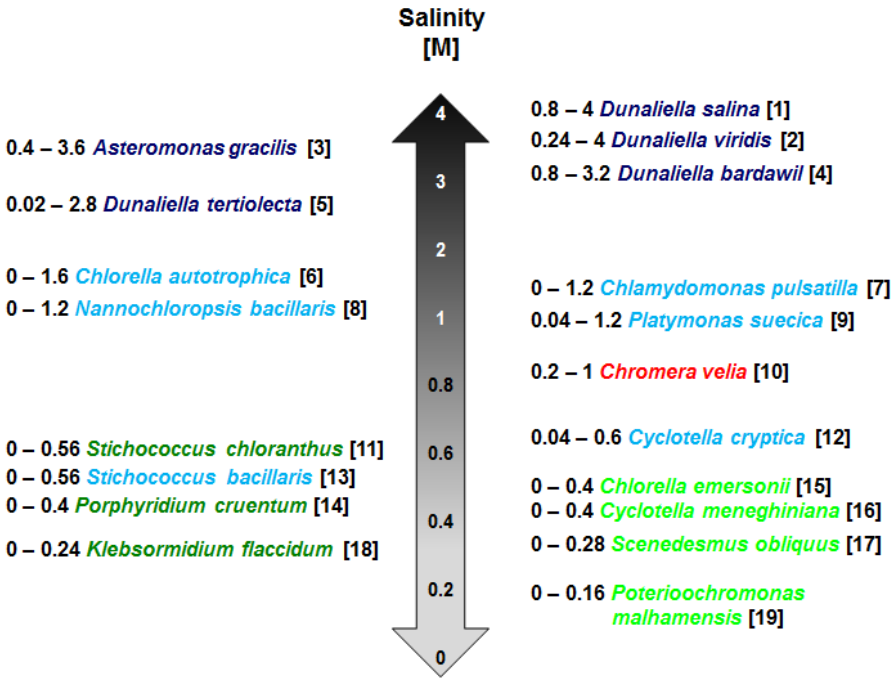

Fig. 5: The salinity limits for photosynthetic life. Salinity ranges for representative microalgae are shown. Salinities have been calculated from (Mc Lachlan 1964). Organisms are marked by different colors by their origin; dark blue: salt pond and lake origin; light blue: marine, tide-pool, brackish; dark green: soil; light green: freshwater. Red color depicts the organism used in this study. References: [1, 3, 4] (Ben-Amotz and Avron 1990); [2] (Borowitzka et al. 1977); [5] (Latorella et al. 1973); [6] (Ahmad and Hellebust 1984); [7] (Le Gresley 1979); [8] (Brown and Hellebust 1980a; Brown 1982); [9] (Hellebust 1976); [10] (Lukeš et al. 2017); [11, 18] (Brown and Hellebust 1980a); [12] (Liu and Hellebust 1976); [13] (Brown and Hellebust 1980b); [14] Gilles and Pequeux 1977); [15] (Setter and Greenway 1979); [16, 19] (Schobert et al. 1972); [17] (Wetherell 1963).

1.2.3.1 Salinity effect on photosynthesis

The salinity stress affects photosynthesis directly on the PSII level. Study on *Scenedesmus* obliquus has shown decreased photosynthetic efficiency upon high salinity stress (Demetriou et al. 2007). The PSII activity was decreased by salt stress in *Spirulina* (Lu and Vonshak 2002), the quantum yield of PSII electron transport was affected in *Ulva lactuca* (Xia et al. 2004). NaCl salinity affects PSII photochemical efficiency by targeting the primary charge separation site of PSII and pigment-protein complexes of thylakoid membranes (Misra et al. 2001). Effect of osmotic stress on photosynthesis have been also studied on *Dunaliella tertiolecta* (Gilmour et al. 1984a, b; Gilmour et al. 1985), *Prasiola*, *Porphyra* ssp., *Enteromorpha*, *Ulva*, *Petalonia* (Wiltens et al. 1978) and *Porphyra perforata* (Fork and Öquist 1981; Satoh et al. 1983; Smith et al. 1986). These studies were aiming at the effect of desiccation on photosynthesis. Nevertheless, desiccation and increase of salinity are comparable as in both stresses reduction of water potential is the main effector. However, during desiccation cellular ion concentrations increase while the ion ratios remain constant. On the other hand, in the salinity stress algae increase cellular ionic concentrations but the stress results in different ionic ratios due to the selective uptake of different ions. In the studies of Gilmour et al. (1982; 1985) on *Dunaliella* species, authors suggest that the initial charge separations in PSII and PSI are inhibited rather by ionic than by osmotic stress. Macroalgae are more affected in the electron transport between PSI and PSII while in *Porphyra*, *Ulva,* and *Enteromorpha* the sensitive site is most likely plastoquinone and P700 (Wiltens et al. 1978). There are at least three sites of the photosynthetic machinery in *Porphyra perforata* that are inhibited by salinity (Satoh et al. 1983). Photo-activation of electron flow on the reducing side of PSI is impaired that gives rise to strong reductants formed by PSI that destroy both photosystems (Satoh and Fork 1982a; 1982b). Secondarily, the electron flow in the water-splitting complex side of PSII is impaired, causing photoinhibition. Finally, inhibition of

oxidizing site of PSII leads to photobleaching of both chlorophylls and carotenoids resulting in impaired transfer of light energy between pigment complexes (Katoh 1972; Yamashita et al. 1968). The inhibition of electron flow on all these three sites is necessary, to prevent photo-damage, that will occur if only one site is blocked (Satoh et al. 1983).

1.2.3.2 Salinity effect on membranes

It was reported in many studies, that lipids might be involved in salt stress protection (Hufleijt et al. 1990, Khamutov et al. 1990, Ritter and Yopp 1993). When photosynthetic organisms are subjected to elevated salt stress, fatty acids of membrane lipids get unsaturated. An increase in salinity led to an increase of polyunsaturated fatty acid (PUFA) levels in haptophytes (Nedbalová et al. 2016) and in the diatom *Nitzschia laevis* (Chen et al. 2008) while Gu et al (2012) reported that salinity did not have any clear effect on fatty acid composition in *Nannochloropsis oculata*. The result of Gu et al. (2012) might be related to findings of Hellebust (1985), where cells with strong cell walls had no or minimal response to hypoosmotic stress in terms of photosynthesis, while wall-less cells appeared to be more sensitive. The polar fraction of plasma membranes of *Dunaliella salina* was reported to be more unsaturated under high salinity when compared to low (Peeler et al. 1989). Increased amounts of linoleic acid (C18:3) were found in a different strain of *D. salina* upon salinity increase (Al-Hasan et al. 1987). Also, results of Azachi et al. (2002) provide support that fatty acid elongation and probably desaturation contribute to salt tolerance of *D. salina*. Tasaka et al. (1996) using site-directed mutagenesis of *Synechocystis* sp. have created a mutant that produced reduced levels of unsaturated fatty acids in its membranes. Another mutation was done by Allakhverdiev et al. (1999) that resulted in a decreased tolerance to salt. Transgenic *Synechococcus* that was synthesizing only saturated and monounsaturated fatty acids (Murata and

Wada 1995) when transformed with the desA gene from *Synechocystis* sp. PCC 6803 allowed it to synthesize di-unsaturated fatty acids (Sakamoto et al. 1994). I was shown in Allakhverdiev et al. (2001) that an increased unsaturation of fatty acids in membrane lipids enhanced the tolerance to salt stress of the photosynthetic and Na^+/H^+ - antiport systems of transgenic *Synechococcus*. In the works of Blumwald et al. (1984), Padan and Schuldiner (1994) and Allakhverdiev (2001) it was shown, that the activation of Na^+/H^+ antiport via enhanced membrane fluidity by unsaturation of membrane fatty acids resulted in the protection of PSII and PSI activities. Changes of membrane fluidity resulted in changes in activities of various membrane-bound enzymes (Kates et al. 1984, Kamada et al. 1995).

1.2.3.3 Compatible solutes and osmotic solutes

Accumulation of metabolites called compatible solutes is probably universal response to osmotic stress by counterbalancing changes in external osmotic pressure and thus helping to keep the metabolism functional (Brown and Simpson 1972; Ford 1984; Yancey et al. 1982). The accumulation of compatible solutes is proportional to the change of external osmolarity and is species-specific in the terms of magnitude for given organism. The generally accepted function of these solutes are the protection of structures within cells and balancing the water influx (or reduced efflux). The most frequently observed osmolytes are sugars (mainly sucrose and fructose), complex sugars (trehalose, raffinose, fructans), and alcohol sugars (methylated inositols, glycerol). In addition to sugar-based osmolytes, charged metabolites such as glycine betaine, dimethyl sulfonium propionate (DMSP), proline and ectoine (1,4,5,6-tetrahydro-2-methyl4-carboxyl pyrimidine can be observed. Compatible solutes are believed to facilitate osmotic adjustment by lowering internal osmotic potential and thus contribute to tolerance (Delauney and Verma 1993; Louis and Galinski 1997; McCue and Hanson 1990). Typical

osmolytes are hydrophilic to replace of water at the surfaces of proteins, protein complexes, and membranes, serving as osmoprotectants and also as low-molecular-weight chaperones (Hasegawa et al. 2000). The function of osmolytes has been reviewed (Nelson et al. 1998; Yeo 1998). At high concentrations they are reducing inhibitory effects of ions on enzyme activity (Brown 1990; Solomon et al. 1994), increase the stability of enzymes (Galinski 1993) or prevent dissociation of enzyme complexes (Papageorgiou and Murata 1995).

2. Aims and hypotheses

Specific hypotheses and aims

Optimal growth conditions are usually achieved only in the laboratory. Algae are constantly subjected to stressful environmental conditions, the question is: to what extent? Usually microorganisms have to cope with abrupt changes of irradiance, temperature, varying salinity, and nutrient availability stress, to survive, acclimate, and or adapt to these changes, algae have to adjust their metabolism. In general, my research is focused on how algae acclimate to stressful conditions by changing carbon allocation within the cells.

Specifically, the research was based on: (1) how organisms with different ecological valence (e.g. mesophilic and snow alga) adapt to changes in temperature in terms of their photosynthesis, molecular adaptation on the level of their thylakoid membrane and PSII reaction centers; (2) whether there is a correlation between abiotic stress and remodeling of the lipid content of cells, and (3) whether spectral light quality affects carbon metabolism of *C. velia* a peculiar organism, which at the time was thought to be an endosymbiont of corals.

(h1) Cells readjust their thylakoid membrane lipid composition to acclimate their photosynthesis to sub- and supra-optimal temperatures

We have investigated growth, electron transfer in photosystem II (PSII) and oxygen evolution rates in cells exposed to temperatures from 2 to 35 °C in a mesophilic green alga *Chlamydomonas reinhardtii* and a snow alga *Chlamydomonas* cf. *nivalis*. PSII function at sub- and supra-optimal temperatures was studied within the context of the primary structure of the D1 protein and the content of thylakoid lipids and fatty acids.

(h2) Cells acclimate to stressful conditions by adjustment of their fatty acid content

We have investigated growth and cellular fatty acid composition of *Chromera velia* grown at optimal and stressful conditions range of temperature, light quantity, light quality, and salinity. Changes in fatty acid composition were compared with results known for other eukaryotic algae.

(h3) Light quantity and quality affect carbon and nitrogen metabolisms resulting in different carbon allocation patterns

We have investigated growth, cell size and carbon allocation into main three carbon pools (e.g. lipids, protein and carbohydrates) together with spectral absorptive characteristics of *Chromera velia* cells adapted to three different monochromatic lights (e.g. blue, green and red) of two photon flux densities of 20 and 100 μmol m^{-2} s^{-1}, together with full-spectrum white light that served as control.

3. Model organisms and Methods

3.1 Model organisms

Chlamydomonas cf. *nivalis* used in this study

Snow algae from the genera *Chloromonas* and *Chlamydomonas* are mostly considered to be obligate cryophiles with optimal temperature for growth below 10 °C (Hoham 1975). Different temperature optima have been reported for *Chloromonas pichinchae* 1 °C, *Chloromonas chenangoensis* and *Chloromonas tughillensis* 2.5-5 °C, *Chlamydomonas raudensis* 8 °C, *Chlamydomonas* sp. 5 °C, *Chlamydomonas subcaudata* and *C. alpina* 12.5 – 15 °C and for *Chlamydomonas intermedia* 15 – 18 °C (Hoham 1975; Seaburg et al. 1981; Hoham et al. 2008; Pocock et al. 2011; Eddie et al. 2008). For the widely distributed *C. nivalis*, contrasting results were obtained due to the lack of laboratory cultures and taxonomic uncertainty (Hoham 1975; Komárek and Nedbalová 2007). High variability of in situ maximum photosynthetic activity was demonstrated among samples collected (Mosser et al. 1977). No inhibition of photosynthesis up to 20 °C was observed in short term experiments with *C. nivalis* red cysts (Remias et al. 2005; 2010). The snow alga *C.* cf. *nivalis* Nedbalová strain CCALA 970 was isolated from melting snow in the Tatra Mountains in northern Slovakia (Nedbalová et al., 2006). The family Chlamydomonadaceae including the snow species undergoes rapid taxonomic changes. Since a taxonomic revision of the strain CCALA 970 is also necessary, we use the name *C.* cf. *nivalis* for the red snow alga investigated in this study. However henceforward *Chloromonas reticulata* (Nedbalová pers. com.) should be used. *Chlamydomonas reinhardtii* P. A. Dangeard strain UTEX 2246, commonly studied strain was used as a mesophilic control.

The experimental setup for Paper I was as follows: starter cultures of *Chlamydomonas* strains were kept at temperatures of their expected optima 2.5 and 24 °C for *C. cf. nivalis* and *C. reinhardtii*, respectively. Cultures

were air bubbled in 2 L bottles under 16:8 light/dark illumination of 100 μmol photons m^{-2} s^{-1} provided by fluorescent light (Fluora; Osram) (FTL in Fig.). When cells reached late exponential phase, cultures were diluted to 1 μg chl ml^{-1} and transferred to sub- and supra-optimal temperatures 5, 10, 15, 20, 25, 30 and 35 °C. Fatty acid analysis carried out in cells grown for 3, 5 and 10 days at temperatures 10 °C higher and lower than the initial (2.5 °C for *C. reinhardtii* and 24 °C for *C. cf. nivalis* respectively) showed that no significant changes in the fatty acid content occurred within a time span longer than 3 days. All the acclimation experiments, therefore, consisted of 3 days exposure of cells to the particular temperature. All acclimation experiments have been done in triplicate (Paper I).

Chromera velia

C. velia is a photosynthetic relative of apicomplexan parasites, belonging to the phylum Chromeridae (Moore et al. 2008; Oborník 2011). As opposite to the heterotrophic apicomplexans, whose vestigial chloroplast has lost its photosynthetic function, chromerids are equipped with functional chloroplasts, which share many traits with chloroplast of algae of the "red lineage" (Janouskovec et al. 2010). *C. velia*'s chloroplast does not contain chlorophyll *c*, it possesses the primitive form II of RuBisCO (Janouskovec et al. 2010). *C. velia* has a highly effective non-photochemical quenching (NPQ) mechanism based on unusually fast violaxanthin de-epoxidation (Kotabova et al. 2011). *C. velia* also uses a unique mechanism of photoprotection based on combination of photorespiration and thermal energy dissipation via NPQ quenching (Quigg et al. 2012), *C. velia* contains plant-like lipids (Botte et al. 2011; Leblond et al. 2012) and is able to produce significant amounts of polyunsaturated fatty acids (Lukeš et al. 2017). The story of *C. velia* is also very interesting as it was thought that it was isolated from a coral (Moore et al. 2008) and was therefore assumed to be a coral symbiont similar to dinoflagellate zooxanthellae (Moore et al. 2008; Oborník et al. 2011).

However, Cumbo (2013) showed that *C. velia* is not an obligate coral symbiont but could be transmitted from adult *Montipora digitata* to its eggs. One of the evidence for the theory of being a coral endosymbiont, is a fact that *C. velia* is equipped with a novel red-shifted chlorophyll *a* antenna complex (Kotabová et al. 2014), that is compatible with life within the densely populated environment of shallow-water corals, where available light spectrum is poor of visible wavelength, but enriched in far-red radiations (Magnusson et al. 2007). Later, Mohamed et al. (2018) suggested a parasitic relationship between *C. velia* and the coral because of coral's transcriptomic response to *C. velia*. Observations by Janouskovec et al. (2013), who found association of *C. velia* with macroalgae living in the proximity of corals, and more recent work by Mathur et al. (2018) suggest that *C. velia*, although an inhabitant of coral reefs, is unlikely to be a coral symbiont, but could be symbiotically associated with other reef-dwellers. *Chromera* may also be part of microbial processes in biogenous sediments and contribute to reef primary production, nutrient cycling and maintenance of overall coral reef health as a free-living, mostly benthic organism (Mathur 2018).

For the experiments focusing on environmental factors affecting fatty acid composition of *C. velia* (Paper II), the stock cultures of *C. velia* (strain RM12) were maintained in f/2 medium under tested conditions (e.g. temperature, light quantity, light quality, and salinity) for at least ten generations. The cultures were weekly diluted to keep the cell concentration at 1–2 × 10^6 cells mL^{-1}. For every subsequent treatment, 100 mL of the culture was transferred to a 250 mL glass tube and diluted to a concentration of ~1 × 10^6 cells mL^{-1} with fresh medium or, for the experiments on the effect of salinity, in media containing different salt concentrations. The cultures were then subjected to the experimental treatment for 7 days. To test the effect of light intensity and quality on FA composition, *C. velia* cells were grown at a constant temperature of 28 °C. The cells were exposed to six different polychromatic light conditions created by two spectrally different light sources adjusted to three light

intensities each (20 µmol photons m^{-2} s^{-1} = low light, LL; 200 µmol photons m^{-2} s^{-1} = high light, HL; 80 µmol photons m^{-2} s^{-1} = control). The difference in spectral quality was obtained using a fluorescent tube (Osram Dulux-L, 950) (FL in Fig.) or incandescent bulbs (IL) (Benlux original, 150W) (IL in Fig.). Experiments were also performed using blue, green and red monochromatic LED light sources (peak maxima of ~462, ~514 and ~630 nm, respectively) (Fig.). The irradiance of LED illumination was set to 20 µmol photons m^{-2} s^{-1}. In order to study the effects of temperature, *C. velia* was cultured at 17, 23, 28 and 32 °C, at an irradiance of 80 µmol photons m^{-2} s^{-1} supplied by an incandescent light source. To study the effect of salinity, cells were resuspended in f/2 medium modified in terms of NaCl concentration to obtain 0.2, 0.6, 0.8 and 1 M NaCl; the cultures were maintained on an orbital shaker, at 28 °C and 80 µmol photons m^{-2} s^{-1} using an incandescent light source. Cultures resuspended in artificial seawater medium (ASW) f/2 medium containing 0.4 M NaCl were used as control. All measurements were conducted on three independent cultures for each treatment (Paper II).

For experiments dealing with the effect of monochromatic light on carbon allocation (Paper III), cells were grown in 100 mL Erlenmeyer flasks, at 25 °C, in ASW with f/2 nutrients. The light was provided at a photon flux density (PFD) of either 20 (LL) or 100 µmol photons m^{-2} s^{-1} (HL) with a 12/12 light/dark photoperiod. The light was provided from the bottom of the flasks to achieve light homogeneity. PFD was adjusted by means of the potentiometer and neutral density filters (Lee Filters). The culture thickness (path length) was 1.6 cm. The light sources were monochromatic LEDs of different colours: blue 462 nm (BL), green 513 nm (GL), red 630 nm (RL), warm white (WL) LEDs were used for the control treatment (Fig.). Cultures were continuously shaken on a rotary shaker at 100 rpm. The cultures were acclimated to these conditions for not less than ten generations, prior to any measurements. Experimental cultures were initiated by inoculating acclimated cells at concentrations of $0.5\text{-}1.0 \cdot 10^6$ cells mL^{-1} into 100 mL Erlenmeyer flask. For compositional analyses, cells

were inoculated at a concentration of $1 \cdot 10^6$ cells/mL and collected after 6 days since inoculation. All measurements were conducted on three independent cultures for each treatment (Paper III).

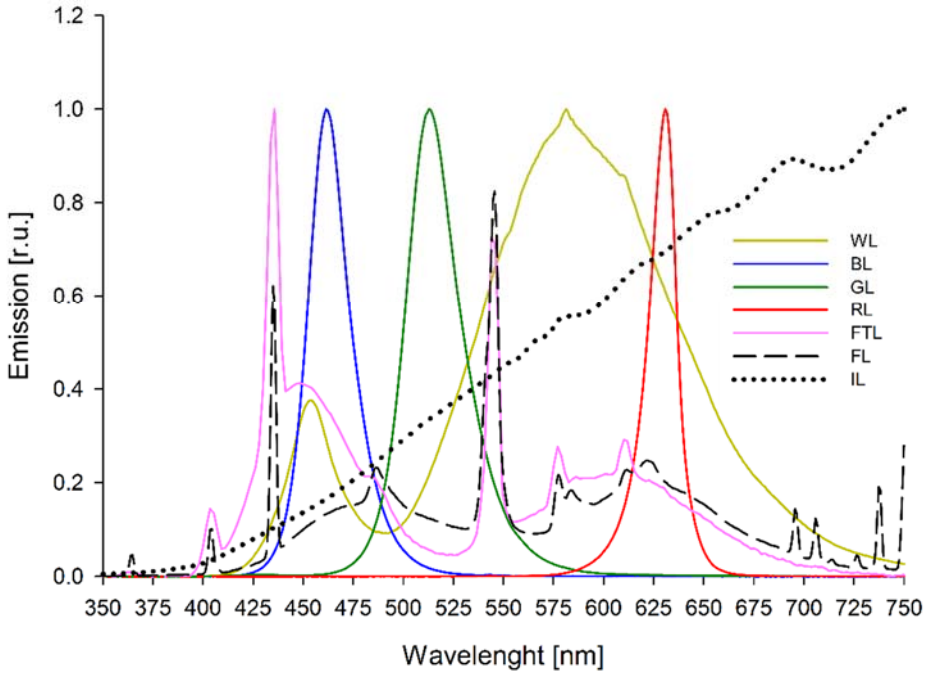

Fig. 6: The emission spectra of light sources used in this study. The spectra are normalized to their respective maxima in the wavelength range of PAR region.

3.2 Methods

DNA isolation, Amplification, and alignment of gene coding D1 protein

We have compared amino acid sequences of the D1 protein of *C.* cf. *nivalis* and different algae, cyanobacteria and plants. DNA was isolated from liquid culture of *C.* cf. *nivalis*. The psbA gene region was amplified from the DNA isolated by PCR using primers psbA-F1 (ATGACTGCTACTTTAGAAAGACG) and psbA-R2 (TCATGCATWACTTCCATACCTA). Amino acid sequences of D1 protein were compared among *C.* cf. *nivalis* and other photosynthetic organisms (see the Paper I for more detail). Instrumentation: DNeasy Plant Mini Kit (Qiagen), NanoDrop_ ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.), CLUSTALW, BIOEDIT (Ibis Biosciences, CA), MEGA 5.10 software.

Thylakoid membrane isolation and purification

We analyzed the composition of thylakoid membranes without contamination of other cellular membranes (e.g. plasma membrane, endoplasmic reticulum, mitochondrial membranes), we used sucrose density gradient after cell breakage, to purify thylakoid membranes based on different density than the other possible contaminants (see Paper I for more detailed description of the method of thylakoid membrane isolation and purification). Instrumentation: OptimaTM L-90K Ultracentrifuge, Beckman Coulter, USA

Lipid analysis

In order to characterize thylakoid lipid classes, lipids were extracted by the method of Wada et al. (1994) and further separated into lipid classes by thin-layer chromatography (see details in the Paper I). Instrumentation: 250-μm TLC plates (MERCK)

Fatty acid analysis

Fatty acids were analyzed by the means of gas chromatography with flame ionization detection after derivatization into methyl esters (method in more detail described in the Paper I and II). Instrumentation: HRGC 5300 megaseries, Carlo Erba, Italy with equipped Omegawax 320 column (30 m x 0.32 mm, df 0.25 μm, Supelco). Hewlett Packard HP6890 Series equipped with sp2330 (30 m x 0.25 mm, df 0.20 μm; Supelco)

Electron transfer at the acceptor side of PSII

Functional stability of PSII was assayed by measurements of kinetics of electron transfer between the quinone Q_A bound in the D2 protein and Q_B that is transiently bound to the membrane accessible pocket of the D1 protein of the PSII reaction center (for details see the Paper I). Instrumentation: FL-3000 fluorometer equipped with a TR-2000 thermoregulator (PSI Ltd, Czech Republic)

Oxygen evolution

Oxygen evolution was measured by Clark-type electrode equipped with thermostated cuvette that allowed for measurements in a temperature range of 2 to 35 °C (see the Paper I for details). Instrumentation: Clark-type electrode in a thermostated, magnetically stirred cuvette (Hansatech Instruments Ltd, UK) monitored Oxycorder (PSI Ltd, Czech Republic)

Fourier transform infrared spectroscopy

We investigated the biochemical composition of the cells using FTIR spectroscopy. This method enables fast semi-quantitative determination of cellular carbon pools i.e. carbohydrates, lipids, and proteins according to specific vibrational frequencies of chemical bonds of these molecules (Paper III). Instrumentation: Nicolet IS10, Thermo Scientific, USA, with

internal compartment well plate reader equipped with 384-well SI plate, Bruker, USA.

4. Overview of my research

My thesis is based on three published articles. Paper I focuses on the effect of sub- and supra-optimal temperatures on acclimatory responses of photosynthesis, and thylakoid membranes of snow alga *C.* cf. *nivalis* and its mesophilic *C. reinhardtii* counterpart (h1). Paper II focuses on responses of *C. velia* to several abiotic stresses (temperature, light quality and quantity, and salinity) in terms of lipid fatty acid composition (h2). Paper III compares differences of carbon allocation of the cells of C. *velia* under growth at three monochromatic lights and white light serving as control under low and high light conditions (h3).

Paper I

Temperature dependence of photosynthesis and thylakoid lipid composition in the red snow alga Chlamydomonas cf. nivalis (Chlorophyceae).

We focused on algae acclimated for a physiological range of temperatures (e.g. 5 – 35 °C). Temperature dependence of growth, rate of photosynthesis including the electron transport within PSII, oxygen evolution, lipid and fatty acid analysis of isolated thylakoid membranes, and the sequence of D1 protein in snow alga *C.* cf. *nivalis* and *C. reinhardtii* was studied. *C.* cf. *nivalis* showed higher growth in range of 5 – 15 °C and higher O_2 evolution rate in a range of 5 – 20 °C, than its mesophilic counterpart *C. reinhardtii*. The electron transfer of *C.* cf. *nivalis* have shown completely different temperature dependence than its mesophilic control. The rate at temperatures close to 0 °C was the same as in the mesophilic one at peak maxima of ~5300 s^{-1}. The maximum rate of Q_A to Q_B electron transfer at 35 °C reached non-physiologically high values of ~11000 s^{-1} that were double the rate observed in mesophilic *C. reinhardtii*. The analysis of isolated thylakoid membrane lipids showed that in *C.* cf. *nivalis* PG dominated thylakoid membranes with over 80 % content. Further analysis

of fatty acid content of isolated lipids showed that the snow alga's thylakoid membranes were less unsaturated in all temperature treatments compared to control *C. reinhardtii*. The sequence analysis of D1 protein have shown substitutions in the inter-helical domains of lumen (AB)- and stroma (DE)-exposed loops. 15 amino acids of snow algal D1 protein (in comparison to mesophilic *C. reinhardtii*) were smaller, but still of the same chemical nature, the other four had neutral change. 6 amino acids were significantly larger and of different chemical nature. Interestingly, two replacements for smaller amino acids were found in regions that either surround or are close to the Q_B binding pocket. Results show that combination of changes in protein structure and unusually high levels of negatively charged lipids adjust the electron transfer rate in PSII allowing for fast transfer of electrons from Q_A to Q_B at very low temperatures.

Paper II

The effect of environmental factors on fatty acid composition of Chromera velia (Chromeridae).

Chromera velia is a peculiar organism capable of production of a significant amounts of polyunsaturated fatty acids. The synthesis of polyunsaturated fatty acids is a metabolically ''expensive'' process that needs high amounts of reducing equivalents and is dependent on environmental factors. *C. velia* was exposed to range of environmental factors including light quantity, quality, temperature, and salinity. The growth and fatty acid content of cell was followed. *C. velia* is capable of growth under a wide range of conditions (Fig. 2). Cells grown at higher PFD have lower PUFA levels while fatty acids in cells adapted to low PFD's were more unsaturated, cells adapted to low monochromatic light had the highest level of unsaturation. Temperature treatment has caused opposite trend of unsaturation of fatty acids than is usual among algae. The highest content of EPA (eicosapentaenoic acid) at 28 °C led us to hypothesis that long chain polyunsaturated fatty acids may serve as

rigidifying agents of the membranes at higher temperatures. No differences in fatty acid unsaturation were observed at different salinities.

Paper III

The effect of light quality and quantity on carbon allocation in Chromera velia.

Chromera velia might be coral symbiont, or at least by its world distribution, is a part of coral reef ecosystem. The light quality inside/around coral is usually defined by depletion of part of spectra resulting in light dominated mostly with blue-green. The amount of incident light defines the energy that after absorption can be utilized by cells for growth. Cells of *C. velia* have been acclimated to three monochromatic lights (Blue, Green, Red) and to full white light spectrum at two light intensities 20 (LL) or 100 μmol photons m^{-2} s^{-1} (HL). The cells had a relative flat response of growth to the light quality. On the other hand, the growth rates were double at higher light 100 μmol photons m^{-2} s^{-1} than those grown at low light 20 μmol photons m^{-2} s^{-1}. Cell volumes were affected by the quality of the light, cells grown at blue light were significantly smaller. FTIR spectroscopy of whole cells revealed that cells grown at low light PFD's had lower lipid to protein and carbohydrate to protein ratios, showing that light was sufficient enough for maintaining the growth, but not for synthesis of storage compounds. Cells grown at high light have shown higher lipid to protein and carbohydrate to protein ratios showing that more energy is being invested into storage pools. This was also confirmed by the analysis of C/N ratios suggesting that at high irradiance more carbon was allocated into N deprived pools. Blue light treatment have shown greatest difference in cellular composition in response to light quality. We hypothesize that lower carbon assimilation efficiency under blue light together with blue light activated increase of nitrogen fixation helps *C. velia* cells to adapt for the light conditions in or in close vicinity to corals.

5. Conclusions and future prospects

Temperature is one of the most important factors that determines growth, physiological and metabolic processes in algae (Raven and Geider 1988; Beardall and Raven 2004). In present study, the effect of temperature on snow alga *C.* cf. *nivalis* and its mesophilic counterpart *C. reinhardtii* were studied in order to find key factors for its unique adaptation to their native environment. The life in snow involves freezing conditions, interrupted by episodic freeze-thawing cycles, the light conditions in snow can be stressful for the cells as the light intensity is fluctuating and often extremely high (Hoham and Duval 2001). Their biochemistry and physiology have to be optimized in order to survive and thrive in such hostile conditions. *C.* cf. *nivalis* acclimated to 2.5 °C was able to outgrow the mesophilic strain in the range of 5-15 °C and have shown higher oxygen evolution rate in the range of 5-20 °C even when pregrown at identical temperatures of 24 °C. These results indicate that the snow alga might be cryotolerant species (ability to withstand low temperatures, and ability of growing at temperatures above ~15-18 °C) with very good photosynthetic performance at low temperatures rather than a true cryophilic one. Analysis of lipids fatty acids extracted from isolated thylakoid membranes revealed that unsaturation of membrane lipids may not play dominant role in temperature acclimation in the snow alga (**I**). The snow alga in all treatments had lower DBI, meaning that membrane fatty acids were less unsaturated than in mesophilic strain. More likely dominance of the only negatively charged lipid PG may play role in acclimation to low temperatures thank to its tilted molecules, that could introduce disorder in the membrane packing and thus increase membrane fluidity. We have found several replacements in the D1 protein sequence. We propose, that combination of changes in the protein structure and overabundance of negatively charged PG modulate the electron transfer rate in PSII granting high electron transfer from Q_A to Q_B at temperatures around 0 °C. Changes of fatty acid composition of algae in relationship to

environmental conditions have been studied extensively. As there is a constant need for different types of fatty acids (nutraceuticals, biodiesel, etc.), algae with their fast growth rates and possibility to grow in well-defined liquid medium together with easily regulated temperature, light, salinity, pH, and nutrient conditions could be a good choice. Often growth at optimal conditions results in the fastest growth, but low storage compound production, as the newly fixed carbon is routed towards protein synthesis. As shown in (**II**), the fatty acid profiles are highly dependable on the conditions of growth. For example, low PFD of any light source including monochromatic lights, resulted in the highest unsaturation of fatty acids, but under these conditions, low growth (**II, III**) was observed, together with low lipid content (**III**). Proper combination of factors and a choice of cultivation techniques can promote cells to be preset to growth with preferential investment into desired carbon pools (proteins, lipids, carbohydrates). Among all nutritional factors, nitrogen is considered as one of the most critical nutrient for growth, because it is a constituent of all functional and structural proteins such as enzymes and peptides as well as pigments, electron transfer molecules and genetic material (Cai et al. 2013; Hu et al. 2013). Typically, ammonium was believed to be the most preferable source of nitrogen for microalgae, because less energy for assimilation into amino acids is necessary than for example for nitrate. The favorite nitrogen source for growth is different from species to species, for example *Dunaliella tertiolecta* and *Botryococcus braunii* prefer nitrate rather than ammonia (Chen et al. 2011; Ruangsomboon 2015). For *C. velia*, the preferred source of nitrogen is not known yet, but it can be expected if it would be a symbiont (even parasite), because ammonia is one of the forms of nitrogen, that is available directly from the host coral, or from living in so close vicinity that capture of ammonia from surrounding environment is possible. We tend to speculate that *C. velia* might have similar BL sensing mechanism as *Chlamydomonas reinhardtii* (**III**). The blue light enhanced activity of nitrate reductase can be the mechanism which can switch its metabolism from ''friendly'' ammonium

uptake inside of the host to more ''hostile'' outside of the coral, where algae have to depend on inorganic form of nitrogen in a form of nitrate and where light is depleted of the parts of the solar spectra, other than blue-green. Another clear evidence of *C. velia* being part of coral environment is the fact that it possesses a red-shifted chlorophyll *a* antenna complex (Kotabová et al. 2014), that is compatible with life within the densely populated environment of shallow-water corals, where available light spectrum is poor of visible wavelength, and PAR around 680 nm is absorbed by the overlying layers of phototrophs (Trissl et al. 1993), but is enriched in far-red radiations (Magnusson et al. 2007). Here we propose, that adaptation to environment where very narrow window of absorption around 710 nm is possible and that the red shifted antenna complex red CLHc of *C. velia* are probably sensed and utilized not only at low RL but generally at low light conditions regardless of the color of light (**III**).

To summarize, the most important conclusion of my research related to the initial hypotheses h1-h3 include:

(h1) Cells readjust their thylakoid membrane lipid composition to acclimate their photosynthesis to sub- and supra-optimal temperatures.

We have investigated the lipid composition of thylakoid membranes of snow alga *C.* cf. *nivalis* and mesophile *C. reinhardtii*. Our analysis have revealed, that *C.* cf. *nivalis* substitute other lipid classes (MGDG, DGDG, SQDG) by negatively charged PG, that may help to keep increased flexibility at the membrane level, thus allowing to maintain the fast rate of electron transfer at low temperatures.

(h2) Cells acclimate to stressful conditions by adjustment of their fatty acid content.

We confirmed that *C. velia* cells respond to some stressful conditions by redefining the fatty acid content. High PFD led to production of less unsaturated fatty acids, while low light disregarding the light quality led to increase of PUFA. Temperature led to increase of PUFA's especially EPA in the cells. Salinity had no effect on the fatty acid saturation/unsaturation.

(h3) Light quantity and quality affect carbon and nitrogen metabolisms, resulting in different carbon allocation patterns.

We have followed the carbon allocation in *C. velia* cells under three monochromatic lights and full spectrum white light at low and high light PFD's. Low light led to carbon allocation mainly into N rich carbon pools (e.g. proteins) to preserve growth, while high light led to allocation of carbon into N deprived pools such as lipids and carbohydrates as more energy was available.

The effect of temperature on lipid and fatty acid composition of algae is still enigmatic and strain specific. A proper stance has to be taken and more careful analyses have to be done. Analysis of only fatty acid saturation/unsaturation is not enough. The lipid composition of isolated thylakoid membranes in terms of functional head-groups, fatty acyl chain positions (sn1- sn2-) together with measurement of lipid properties such as melting point as shown by (Coolbear et al. 1983; Russel 1989) is necessary. Also, molecular dynamics characterization like done by Van Eerden et al. (2015) may help us to understand the complex behavior of membrane adaptation to environmental stress factors. FTIR spectrometry was found

to be very useful tool in following the carbon allocation upon shift to stressful condition as it is a rapid, sensitive (low sample size required), and nondestructive tool. Especially, if combined with gas chromatography.

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RESEARCH ARTICLES

PAPER I

Temperature dependence of photosynthesis and thylakoid lipid composition in the red snow alga *Chlamydomonas* cf. *nivalis* (Chlorophyceae)

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RESEARCH ARTICLE

Temperature dependence of photosynthesis and thylakoid lipid composition in the red snow alga *Chlamydomonas* cf. *nivalis* (*Chlorophyceae*)

Martin Lukeš[1,2], Lenka Procházková[3], Volha Shmidt[1], Linda Nedbalová[3] & David Kaftan[1,2]

[1]Faculty of Science, University of South Bohemia in České Budějovice, České Budějovice, Czech Republic; [2]Department of Phototrophic Microorganisms, Institute of Microbiology CAS, Opatovický mlýn, Třeboň, Czech Republic; and [3]Department of Ecology, Faculty of Science, Charles University in Prague, Prague, Czech Republic

Correspondence: David Kaftan, Department of Experimental Plant Biology, Faculty of Science, University of South Bohemia in České Budějovice, Branišovská 31, 37005 České Budějovice, Czech Republic. Tel.: +420 387772342; fax: +420 387772351; e-mail: david.kaftan@prf.jcu.cz

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Abstract

Here, we report an effect of short acclimation to a wide span of temperatures on photosynthetic electron transfer, lipid and fatty acid composition in the snow alga *Chlamydomonas* cf. *nivalis*. The growth and oxygen evolution capacity were low at 2 °C yet progressively enhanced at 10 °C and were significantly higher at temperatures from 5 to 15 °C in comparison with the mesophilic control *Chlamydomonas reinhardtii*. In search of the molecular mechanisms responsible for the adaptation of photosynthesis to low temperatures, we have found unprecedented high rates of Q_A to Q_B electron transfer. The thermodynamics of the process revealed the existence of an increased structural flexibility that we explain with the amino acid changes in the D1 protein combined with the physico-chemical characteristics of the thylakoid membrane composed of > 80% negatively charged phosphatidylglycerol.

Introduction

Photosynthetic organisms inevitably become acclimated to seasonal, diel as well as rapid aperiodic changes in ambient conditions. Thermal fluctuations instantaneously influence rates of photosynthetic electron transfer, while longer exposure to supra- or super-optimal temperatures causes reversible or permanent changes in rates of synthesis and degradation of both primary and secondary metabolites. Prolonged thermal stress decreases the organism's fitness or even leads to its death unless remediated through the acclimation processes. The phenomenon of temperature dependence of photosynthesis attracted further attention in the past decade due to concerns over the stability of ecosystems in the predicted global climatic changes.

Snow algae comprise an ecologically distinct group of photosynthetic microorganisms. Most of the known species populating polar and mountain regions worldwide belong to the genera *Chloromonas* and *Chlamydomonas* (*Chlorophyta*). Their native environment is defined by frigid temperatures interrupted by episodic freeze-thaw cycles while solar irradiation is high and variable (Hoham & Duval, 2001). Their physiological and biochemical processes must therefore be optimized to respond to the environmental extremes. To this end, studies performed on snow algae can provide a valuable insight into the diversity of various adaptation and acclimation mechanisms in microorganisms. The life histories and ecology of many species of snow algae were studied in detail for example by Hoham (1975a, b, c). Studies focusing on physiology and biochemistry of snow algae are relatively rare with their scope ranging from photoprotective effect of carotenoids in *Chlamydomonas nivalis* (Bidigare *et al.*, 1993) to the ability to survive freezing and thawing as a potential for effective cryoprotection (Morris *et al.*, 1979). Unfortunately, the majority of the studies were performed on field samples (Remias *et al.*, 2005, 2010a, b; Stibal *et al.*, 2007; Leya *et al.*, 2009).

The available data on temperature dependence of photosynthesis and growth are often controversial (Hoham,

1975a; Mosser *et al.*, 1977; Remias *et al.*, 2005; Hoham *et al.*, 2008), suggesting a broader range of ecophysiological strategies in snow algae than expected. Numerous studies reported that the occurrence of certain lipid characteristics coincided with the capacity to withstand low temperature stress. A high proportion of unsaturated fatty acids were observed in red cells of *C. nivalis* from Antarctica (Bidigare *et al.*, 1993), while unusual short- and medium-chain polyunsaturated fatty acids were isolated from the flagellated cells of the snow alga *Chloromonas brevispina* (Rezanka *et al.*, 2008). However, these reports were based on single measurements of field samples. Only limited evidence exists that documents the variability in fatty acid content of different strains under varying controlled conditions in the laboratory (Spijkerman *et al.*, 2012).

Here, we study the temperature dependence of photosynthesis and thylakoid lipid content in the red snow alga *C.* cf. *nivalis* (Bauer) Wille. We have investigated growth, electron transfer in photosystem II (PSII) and oxygen evolution rates in cells exposed to temperatures from 2 to 35 °C. PSII function at sub- and supra-optimal temperatures was studied within the context of the primary structure of the D1 protein and the content of thylakoid lipids and fatty acids. Our results indicate the snow alga ranks as a cryotolerant mesophile.

Materials and methods

Organisms and growth conditions

The snow alga *C.* cf. *nivalis* Nedbalova strain CCALA 970 was isolated from melting snow in the Tatra Mountains in northern Slovakia (Nedbalova *et al.*, 2006). The family *Chlamydomonadaceae* including the snow species undergoes rapid taxonomic changes. Using combined morphological and molecular data, Matsuzaki *et al.* (2012) revised the taxonomic position of two strains causing red snow that were originally identified as *C. nivalis* and reported under different names in culture collections. Since a taxonomic revision of the strain CCALA 970 is also necessary, we use the name *C.* cf. *nivalis* for the red snow alga investigated in this paper.

Cells were grown in BBM medium (2.94 mM $NaNO_3$, 0.30 mM $MgSO_4$, 0.42 mM NaCl, 0.33 mM K_2HPO_4, 1.28 mM KH_2PO_4, 0.17 mM $CaCl_2$, 30.68 μM $ZnSO_4$, 7.27 μM $MnCl_2$, 4.93 μM MoO_3, 6.29 μM $CuSO_4$, 1.68 μM $Co(NO_3)_2$, 0.18 mM H_3BO_3, 0.17 mM Na_2-EDTA, 0.552 mM KOH, 17.92 μM $FeSO_4$ and 18.71 μM H_2SO_4). Cultures on agar plates and starter cultures of *C.* cf. *nivalis* in liquid medium were therefore always grown at 2.5 °C. Cultures of *Chlamydomonas reinhardtii* P. A. Dangeard strain UTEX 2246 were maintained at 24 °C, close to its optimal growth temperature.

Air-bubbled liquid cultures of *C.* cf. *nivalis* and *C. reinhardtii* were grown at 2.5 and 24 °C, respectively, in 2-L bottles under 16/8 light/dark periodic illumination of 100 μmol photons m^{-2} s^{-1} provided by fluorescent light (Fluora; Osram). Our photosynthesis vs. irradiance measurements performed at light intensity of 100 μmol photons m^{-2} s^{-1} corresponded to 50–70% of a maximal photosynthetic activity in either of the strains at any of the examined temperatures. As such, it has been assumed the selected light intensity will not cause any significant photoinhibition. Cells reaching late exponential phase were diluted to 1 μg Chl mL^{-1} and transferred to sub- and supra-optimal temperatures 5, 10, 15, 20, 25, 30 and 35 °C, respectively. Their growth was monitored for 10 days by measurements of total chlorophyll concentration in cells. Ten millilitre of cell culture was extracted into 100% dimethylsulphoxide, and absorption spectra were measured using a Shimadzu UV-2401PC spectrophotometer every 24 h. Chlorophyll concentration was calculated according to the method of Lichtenthaler & Wellburn (1983) with use of these equations:

$$\text{Chl}\,a = 12.19 \cdot A_{665.1} - 3.45 \cdot A_{649.1}\ [\mu\text{g mL}^{-1}]$$

$$\text{Chl}\,b = 21.99 \cdot A_{649.1} - 5.32 \cdot A_{665.1}\ [\mu\text{g mL}^{-1}]$$

The respective absorbances were corrected for turbidity at 730 nm.

Specific growth rates μ were determined as a slope of linear regression of semi-logarithmic plot of ln(Chl) vs. time during the exponential phase of the growth. The generation time (number of doublings per day) was calculated as μ/ln 2. All growth experiments were run in triplicates.

Fatty acid analysis carried out in cells grown for 3, 5 and 10 days at temperatures *c.* 10 °C higher and lower than the initial (2.5 and 24 °C in *C.* cf. *nivalis* and *C. reinhardtii* respectively) showed that no significant changes in the fatty acid content occurred within a time span longer than 3 days (Supporting Information, Fig. S1). All the acclimation experiments therefore consisted of 3 days exposure of cells to the particular temperature.

Oxygen evolution

Cells of *C.* cf. *nivalis* grown at 2.5 and 24 °C for 72 h, respectively, and of *C. reinhardtii* grown at 24 °C were diluted with fresh BBM medium to a suspension containing 5 μg Chl mL^{-1}. The oxygen evolution rate was measured with Clark-type concentration electrode in a thermostated, magnetically stirred cuvette (Hansatech Instruments Ltd, UK). Electrical current was monitored with a sampling frequency of 30 Hz with a polarization potential of 700 mV by Oxycorder (PSI Ltd, Czech

Republic). The 2.5 mL of cell suspension was homogenously illuminated by high-intensity light-emitting diodes (λ_{red} = 635 nm, λ_{blue} = 480 nm). A manually adjustable power source provided light intensities from 0 to 6500 μmol photons m^{-2} s^{-1} of incident light. The oxygen evolution was measured at 2 °C and at increments of 5 °C in the range from 5 to 35 °C, at a saturating light intensity of 2000 μmol photons m^{-2} s^{-1}. Each cycle of oxygen evolution measurement at the chosen temperature comprized of 600 s of dark adaptation, 600 s of illumination followed by 600 s of dark relaxation. Calibration of the measured signals was carried out against a buffer equilibrated with the ambient air and then depleted of oxygen by the addition of sodium dithionide at all measured temperatures. Tabulated values of Henry's law constants for oxygen in water were used to convert measured signals into oxygen concentration [μmol O_2]. An oxygen evolution rate in μmol O_2 mg Chl^{-1} h^{-1} was determined as a slope of linear regression of a plot of O_2 [μmol] vs. time during the light period of oxygen evolution cycle. All experiments were carried out in triplicates.

Electron transfer rate measurements

Cells of *C.* cf. *nivalis* and of *C. reinhardtii* grown at 2.5 and 24 °C, respectively, were diluted with fresh BBM medium to yield 3 μg Chl mL^{-1}. The cell suspension was dark-adapted for 1 h on ice prior to analysis to allow for re-oxidation of electron acceptors in and around PSII. Chlorophyll fluorescence measurements were conducted in a FL-3000 fluorometer equipped with a TR-2000 thermoregulator (PSI Ltd), and the sample cuvette contained 2.4 mL of dark-adapted sample. In the temperature-dependent Q_A reoxidation, fluorescence decay following single-turnover saturating pulse was measured as described (Dinamarca *et al.*, 2011) at increments of 2.5 °C in the range from 0 to 50 °C. At least seven independent measurements were conducted at each temperature.

Fluorescence temperature curve

Cells grown for 72 h at a particular temperature were diluted with fresh BBM medium to yield 3 μg Chl mL^{-1}. Critical temperature (T_C) was assessed in experiments that consisted of measuring chlorophyll fluorescence induced by weak measuring pulses in a linearly heated sample with a ramp of 1.5 °C min^{-1} from 0 to 60 °C essentially as described by Lazar & Ilik (1997). The T_C was estimated as the crossing of an extrapolation of the linear part of fluorescence rise to a point of maximum fluorescence (T_M) with constant fluorescence level before the fluorescence rise. M/F(30) ratio reflecting the inhibition or damage to PSII (Lazar & Ilik, 1997) is the ratio of maximal fluorescence at temperature T_M and a steady-state minimal fluorescence F_o at 30 °C. All experiments were carried out in triplicates.

Isolation of thylakoid membranes

Thylakoid membranes were isolated using the method described by Chua & Bennoun (1975) with minor modifications. Cells grown for 72 h at the particular temperature were harvested by centrifugation at 2500 ***g*** for 15 min and washed twice in 0.3 M sucrose, 25 mM HEPES-KOH pH 7.5, 1 mM $MgCl_2$. Cells were broken in a French-pressure cell at 4000 Psi. Broken cells were centrifuged at 2000 ***g*** for 10 min, and the pellet was resuspended in 0.3 M sucrose, 5 mM HEPES-KOH pH 7.5 and 10 mM EDTA (buffer B) and centrifuged at 50 000 ***g*** for 10 min. The pellet was resuspended to a total volume of 10 mL in 1.8 M sucrose in buffer B. Five millilitre of 2 M sucrose in buffer B was overlaid with 5 mL of sample in 1.8 M sucrose in buffer B, with 2 mL of 1.3 M sucrose in buffer B and 5 mL of 0.5 M sucrose in buffer B and centrifuged at 140 000 ***g*** for 1 h in Beckman Coulter OptimaTM L-90K Ultracentrifuge. Thylakoid membranes were collected at the interlayer of 1.8 and 1.3 M sucrose. Collected membranes were washed with three volumes of 5 mM HEPES-KOH pH 7.5 and 10 mM EDTA and centrifuged at 50 000 ***g*** for 45 min.

Extraction of lipids

Lipids were extracted according to Wada *et al.* (1994). One millilitre of membranes was transferred to a teflon-lined screw-cap test tube with 3.75 mL of $CHCl_3$: CH_3OH (1 : 2, v/v) and mixed by vortexing. The mixture was left to stand for 20 min at room temperature. Then, 1.25 mL of each $CHCl_3$ and H_2O was added, and the solution was vortexed, followed by centrifugation at 2500 ***g*** for 15 min. Then, the clear upper phase and fluff layer were carefully withdrawn and 2.5 mL of CH_3OH : H_2O (10 : 9, v/v) was added. The solution was vortexed and centrifuged at 2500 **g** for 15 min at room temperature, and the lower phase was recovered and transferred to a new tube for evaporation. The sample was evaporated by either a rotary evaporator or a stream of nitrogen gas. The dry sample was dissolved in $CHCl_3$: CH_3OH (2 : 1, v/v) and stored at −80 °C. BHT was added to a final concentration of 0.05% to avoid oxidation during long-time storage.

Lipid and fatty acid analysis

Lipid extracts were applied as 2.5-cm-wide streaks 1.5 cm from the bottom of 250-μm TLC plates (MERCK).

Developing solvent mixtures were either $CHCl_3$: CH_3OH : acetic acid : water (80 : 9 : 12 : 2, v/v) or $CHCl_3$: CH_3OH : NH_4OH (28%) (13 : 7 : 1, v/v). Separation was run to a height of 19 cm above the bottom. TLC plates were sprayed with primuline solution (0.01%, w/v in acetone : water 60 : 40, v/v) and visualized under UV light (365 nm). Major lipid constituents of thylakoid membranes, for example monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG), were identified against known standards (Larodan, Malmo, Sweden).

Bands containing lipid classes were scraped and transferred to Teflon-lined screw-cap test tubes. Fifty microgram of pentadecanoic acid (C15:0) was added to each tube as an internal standard along with 0.5 mL of hexane and 1 mL of BF_3-CH_3OH (10% w/w), and the tube was flushed with nitrogen and incubated at 85 °C for 1 h. After cooling down to a room temperature, 0.5 mL of chloroform-extracted water was added and the solution was vortexed. Two millilitre of hexane was added, vortexed again and centrifuged at 2500 ***g*** for 5 min. Upper hexane layer was removed and placed in a new vial for evaporation. The remaining mixture was washed two times with 2 mL of hexane as described above. Collected hexane phases were pooled and concentrated under a stream of nitrogen to a final volume of 20 μL.

Quantitative and qualitative analysis was performed by means of GC-FID either on a Hewlet Packard HP6890 Series on sp2330 (30 m × 0.25 mm, df 0.20 μm; Supelco) or Carlo Erba HRGC 5300 megaseries on Omegawax 320 column (30 m × 0.32 mm, df 0.25 μm; Supelco). Hydrogen was used as a carrier gas at a pressure of 70 kPa. The temperature program in both cases was the following: start temperature of the oven was 140 °C, linear heating rate of 4.5 °C min^{-1} was applied till 240 °C and held at this temperature for another 10 min. Injector temperature was 250 °C, and temperature of FID was 260 °C. Retention times of FAME were compared to known standards (Supelco® 37 Component FAME Mix and PUFA No.3 Supelco from menhaden oil).

DNA isolation

DNA was isolated from liquid culture of *C.* cf. *nivalis* Nedbalova strain CCALA 970 using DNeasy Plant Mini Kit (Qiagen). The protocol was slightly optimized: at the beginning of the procedure, the cells were mechanically disrupted by shaking for 5 min in the presence of glass beads (3 mm diameter; Sigma-Aldrich) in Mixer Mill MM 400 (Retsch, Haan, Germany). Subsequently, DNA was isolated in accordance with the manufacturer's recommended protocol. Quality and concentration of DNA were measured on a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.).

Amplification of gene coding D1 protein

The psbA gene region was amplified from the DNA isolate by PCR using primers psbA-F1 (ATGACTGCTACTT TAGAAAGACG) and psbA-R2 (TCATGCATWACTTC CATACCTA). The expected size of the PCR fragment was 1200 bp. The amplification reaction was performed using the following cycle parameters: 10 min hot start at 95 °C, followed by 35 cycles [1 min at 94 °C, 45 s at gradient of annealing temperature (57 and 59 °C), 2 : 50 min at 72 °C] and 10 min at 72 °C. Each 20 μL PCR reaction for this gene amplification contained 5 μL of DNA isolates (diluted to concentration of 10 ng μL^{-1}), 0.8 μL of each 10 μM primer, 1.6 μL of 25 mM $MgCl_2$, 1.5 μL of 2 mM dNTPs, 2 μL of 10× Taq buffer + KCl-$MgCl_2$, 7.8 μL sterile Milli-Q water and 0.5 μL of 1 U μL^{-1} Taq DNA polymerase (Fermentas).

Alignment of gene coding D1 protein

Amino acid sequences of D1 protein were compared among *C.* cf. *nivalis* (this study, NCBI reference nucleotide sequence KF702330.1 and protein sequence AHB82278), *C. reinhardtii* (DAA00957.1), *Thermosynechococcus elongatus* (NP_682633.1), *Arabidopsis thaliana* (P83755.2), *Chlamydomonas raudensis* (AFU83031.1), *Dunaliella salina* (YP_005089831.1), *Acutodesmus obliquus* (ABD48259.1), *Chlorella vulgaris* (P56318.1), *Pyramimonas parkea* (ACJ71100.1), *Pycnococcus provasolii* (ACK36 809.1), *Populus trichocarpa* (YP_001109480.1), *Cycas micronesica* (ABU85314.1), *Magnolia grandiflora* (YP_00 7474516.1), *Oryza sativa* (AER12889.1), *Zea mays* (NP_04 3004.1), *Carica papaya* (YP_001671663.1), *Porphyridium purpureum* (BAO23682.1), *Cyanidium caldarium* (NP_045 067.1), *Cyanophora paradoxa* (NP_043238.1), *Polarella glacialis* (BAC76007.1), *Kryptoperidinium foliaceum* (ADI 40420.1), *Vaucheria litorea* (ACF70962.1), *Fucus vesiculosus* (CAX12449.1), *Nannochloropsis salina* (AGI99196.1), *Pinnularia* cf. *microstauron* (AER42084), *Ulnaria acus* (AEX37881.1), *Thalassiosira oceanica* (ADB27608.1), *Phaeocystis antarctica* (AEK26755.1), *Emiliania huxleyi* (AAX13814.1), *Geminigera cryophila* (ABL96289.1), *Rhodomonas salina* (ABO70840.1), *Eutreptiella gymnastica* (YP_006234198.1) and *Paulinella chromatophora* (ACB43 269.1). Sequences were aligned with ClustalW (Higgins *et al.*, 1996) and BioEdit (Ibis Biosciences, CA) software. Cluster analysis was based on the pairwise distance among amino acid sequences of D1 protein of species used in large alignment. Calculation of pairwise distances was carried out in MEGA 5.10 (Tamura *et al.*, 2011) with

following setting: method – number of differences, rates among sites – uniform, missing data treatment – complete deletion. Dendrogram was created in program PAST (Hammer *et al.*, 2001) with the settings: algorithm – Ward's method, similarity measure – Euclidean, number of bootstrap – 100.

Statistical analysis

We performed statistical comparisons with the two-sample *t*-test. A value of $P < 0.05$ was considered statistically significant. Error bars in all figures represent standard deviations of at least three independent measurements.

Results

Temperature dependence of growth

The growth of the snow alga *C.* cf. *nivalis* and the mesophilic *C. reinhardtii* was monitored under low light of 100 μmol photons m^{-2} s^{-1} at temperatures between 5 and 35 °C (Fig. 1). Surprisingly, the snow alga exhibits a rather slow growth at and below 5 °C (< 0.15 doubling per day) but keeps an invariant growth of 0.46 ± 0.06 doubling per day while grown above 10 °C (Fig. 1, open circles). The growth is suppressed only at temperatures > 30 °C falling by nearly 50% of the growth maximum at 35 °C. The mesophilic alga displayed almost no growth up to the temperature of 10 °C followed by a linear increase in its growth rate with raising temperature of incubation (Fig. 1, full circles). The growth rate peaked at 30 °C with 0.74 ± 0.05 doubling per day (1.6 times higher than the snow alga) and then dropped sharply. The mesophilic *C. reinhardtii* was outgrown by *C.* cf. *nivalis* in the whole range of temperatures from 5 °C up to 15 °C (*t*-test; $P < 0.05$), while both algae exhibited identical growth within the range of 20–25 °C.

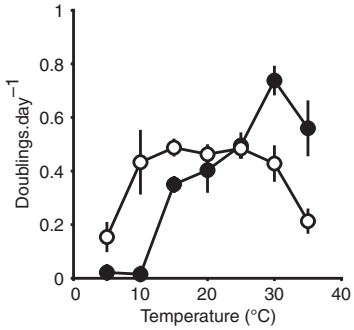

Fig. 1. Temperature dependence of autotrophic growth. Liquid cultures were incubated at the designated temperatures at illumination of 100 μmol photons m^{-2} s^{-1} and their growth was assayed by measurements of total chlorophyll content. The plot of temperature vs. the rate of doubling of the cell culture is shown in the snow alga *Chlamydomonas* cf. *nivalis* (open circles) and in the mesophilic *Chlamydomonas reinhardtii* (closed circles). Values represent mean of three independent measurements.

Oxygen evolution rates

The photosynthetic oxygen-evolving activity from H_2O to CO_2 was measured at temperatures from 2 to 35 °C (Fig. 2). The snow alga *C.* cf. *nivalis* grown at 2.5 °C initially showed a drop in the activity below 100 μmol O_2 mg Chl^{-1} h^{-1} upon increasing the measurement temperature from 2 to 5 °C, followed by a linear increase in activity, peaking at 25–30 °C with 210 ± 49 μmol O_2 mg Chl^{-1} h^{-1} (Fig. 2, open circles). A further increase in temperature leads to an abrupt decline of activity. No net oxygen evolution rate was observed at temperatures at and above 40 °C. Cells of the *C.* cf. *nivalis* grown for 72 h at 24 °C exhibit more stable temperature dependence of oxygen-evolving activity (Fig. 2, grey circles) with significantly lower maximum activity than cells grown at 2.5 °C (*t*-test; $P < 0.03$ in the range of 25–35 °C). The control strain *C. reinhardtii* grown at 24 °C had significantly lower oxygen-evolving activity at temperatures from 2 up to 20 °C (Fig. 2, closed circles, *t*-test;

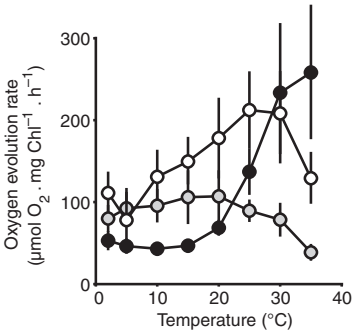

Fig. 2. Temperature dependence of oxygen evolution rate. The measurements were performed in the range from 2 to 35 °C at saturating light intensity of 2000 μmol photons m^{-2} s^{-1} with liquid cultures grown at 5 °C (*Chlamydomonas* cf. *nivalis*, open circles) and 24 °C (*Chlamydomonas reinhardtii*, closed circles; *C.* cf. *nivalis*, grey circles). Values represent mean of five independent measurements.

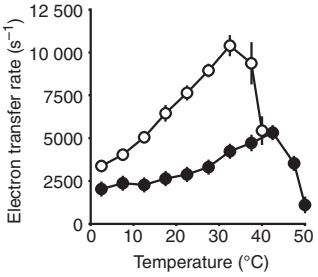

Fig. 3. Temperature dependence of electron transfer rate from Q_A^- to Q_B. Least squares numerical fitting of the chlorophyll fluorescence decay following single-turnover saturating flash yielded the rate constant for the electron transfer rate in the snow alga *Chlamydomonas* cf. *nivalis* (open circles) and in the mesophilic *Chlamydomonas reinhardtii* (closed circles). Values represent mean of seven independent measurements.

$P < 0.03$). The activity started to rise exponentially only at and above 20 °C while reaching 233 ± 96 μmol O_2 mg Chl^{-1} h^{-1} at 30 °C. Additional temperature increase further stimulated the oxygen-evolving activity in contrast to the snow alga grown at 2.5 °C.

Q_A to Q_B electron transfer rates

Functional stability of PSII was assayed by measurements of electron transfer between the quinon Q_A bound in the D2 protein and Q_B (Fig. 3) that is transiently bound to the membrane accessible pocket of the D1 protein of the PSII reaction centre. In the mesophilic *C. reinhardtii*, the Q_A to Q_B electron transfer rate started at a value 2000 s^{-1} at 0 °C and was thermally accelerated up to *c.* 5300 s^{-1} at 42.5 °C (Fig. 3, closed circles). A further increase in temperature led to a decrease in the electron transfer rate with a loss of activity close to 50 °C (Fig. 3, closed circles). In contrast to the mesophilic strain, the snow alga *C.* cf. *nivalis* exhibits significantly higher Q_A to Q_B electron transfer rate at any measured temperature except at 40 °C (Fig. 3, open circles, *t*-test; $P < 0.001$). At 0 °C, the electron transfer rate reached *c.* 3000 s^{-1} and continued to rise exponentially up to 11 000 s^{-1} at 35 °C followed by a sudden drop to half of its maximal value at 40 °C (Fig. 3, open circles). The dissimilar trends in the rates' temperature dependence are shaped by the different activation parameters for the electron transfer rate in the two algae. The activation enthalpy $\Delta H^{\ddagger}$ in *C.* cf. *nivalis* had a value of 12.29 kJ mol^{-1}, while in *C. reinhardtii*, $\Delta H^{\ddagger}$ was lower by 4.78 kJ mol^{-1}. The activation entropy $T\Delta S^{\ddagger}$ was 5.87 kJ mol^{-1} higher in the snow alga than in the mesophilic *C. reinhardtii*. In result, the Gibbs free energy of activation $\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$ was lower by 1.07 kJ mol^{-1} in *C.* cf. *nivalis* (25.53 kJ mol^{-1}) in comparison with 26.6 kJ mol^{-1} in *C. reinhardtii*.

Fluorescence temperature curve

The critical temperature above which the PSII begins to denature, T_C and the maximal temperature of fluorescence rise, T_M parameters serve as indicators of PSII thermostability. Either of the parameters may also reflect on the change in thylakoid membrane fluidity during thermal acclimation. The T_C and T_M parameters were obtained by least squares numerical fitting of the fluorescence temperature curves measured in cell cultures acclimated for 3 days at the temperatures ranging from 5 to 25 °C (Fig. S2). Neither of the two parameters changed significantly in the *C.* cf. *nivalis* acclimated to temperatures from 5 to 25 °C (Fig. S2, open circles, Pearson's correlation; $R < 0.3$, $n = 14$). The value of M/F(30) which reflects inhibition and/or damage to PSII decreased from 2.96 at 5 °C to 2.08 at 20 °C. The mesophilic *C. reinhardtii* showed a steady increase in both T_C and T_M by > 4 °C in response to the acclimation to a higher temperature (Fig. S2, closed circles). A similar trend was observed in M/F(30) where a shift from 5 to 20 °C increased the M/F(30) from 1.9 to 2.6.

Thylakoid lipid composition

The two *Chlamydomonas* species differed significantly in molar fractions of their thylakoid membrane lipid classes (Fig. 4, t-test; $P < 0.03$). PG was by far the most abundant lipid in the snow alga *C.* cf. *nivalis* regardless of the incubation temperature (Fig. 4a). Cells acclimated to temperatures from 10 to 35 °C contained at least 70 molar% of PG with the only exception of cells that

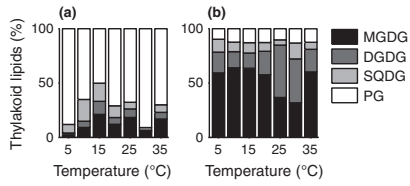

Fig. 4. Lipid composition of thylakoid membranes. Lipids were extracted from membranes of cells acclimated for 72 h to the designated temperatures in the range of 5–35 °C. (a) *Chlamydomonas* cf. *nivalis*, (b) *Chlamydomonas reinhardtii*. Values represent mean of three independent experiments.

were grown at 15 °C. Here, the molar ratio of PG dropped down to 50%. Interestingly, cells grown at 5 and 30 °C contained over 90 molar% of PG out of all lipids in the thylakoid membrane. The analysis of the mesophilic *C. reinhardtii* revealed a lipid content typically found in green algae and higher plants chloroplasts (Fig. 4b). Here, a dominant component of the thylakoid membrane of the mesophilic alga acclimated to the temperature range of 5–20 °C was MGDG representing *c.* 60% of all lipids. DGDG accounted for *c.* 20 molar% resulting in MGDG/DGDG ratios ranging from 2.6 at 20 °C, 2.9 at 35 °C and as high as 4.5 at 10 and 15 °C. This sharply contrasts with the lipid molar fractions in cells acclimated to temperatures that are considered optimal for the mesophilic strain. As molar fractions of DGDG in the membrane increased within the range of 25–30 °C, the MGDG/DGDG ratio decreased to 0.76–0.78. The molar fractions of the minor lipid constituents of the thylakoid membrane such as the PG and sulphoquinovosyldiacylglycerol (SQDG) were found nearly constant in a whole temperature scale both standing at or below the 10 molar %. The lowest amounts of SQDG were observed at 25 °C.

Fatty acid composition

Lipids isolated from the thylakoid membranes and separated by TLC were subjected to transmethylation, and the resulting fatty acid methyl esters corresponding to the four major lipid classes were analysed. The fatty acid content was normalized to their contributions in each lipid class summing up to 100%. The most dominant lipid species in the snow alga *C.* cf. *nivalis,* PG (Fig. S3), was predominantly palmitic (16:0), oleic (18:1) and α-linolenic (18:3) fatty acids. The palmitic acid of the PG was found constant (*c.* 10%) at all temperatures of acclimation, while its total amount was elevated in the range of 15–25 °C due to an increase in its content in the MGDG and SQDG. By far, the most dominant oleic acid in the PG decreased from 50% at 5 °C down to 15–20% at 15–25 °C and increased again to 55% at 30 °C. These changes were slightly compensated by an increase in the oleic acid content in the MGDG and SQDG in the range of 15–25 °C. The α-linolenic acid content shows a mirror image of its trends in PG vs. MGDG and SQDG. While its content is decreasing in PG from 20% at 5 °C down to 10% at 10–15 °C and increasing again up to *c.* 30% at 20–35 °C, the MGDG is enriched by the α-linolenic acid in the range 15–25 °C, resulting in the α-linolenic acid exceeding 40 molar% of all thylakoid fatty acids at 20–25 °C. The minor fatty acids namely the hexadecatetraenoic (16:4), hexadecatrienoic (16:3) and palmitoleic (16:1) acids remained under 10 molar% at all temperatures of acclimation except for the linoleic acid (18:2) that increased to *c.* 15% at 25 °C.

A very different composition of fatty acids was observed in the mesophilic *C. reinhardtii* (Fig. S4). Palmitic acid (16:0) almost equally represented by PG, SQDG and DGDG was found always at total levels of 30% except for 30 °C where it accounted for 50% of all fatty acids in the thylakoid membranes. Similar trend was observed in the palmitoleic acid (16:1) although presented at significantly lower levels (10% throughout all the temperatures except for 25% at 30 °C). The remaining diversity of the fatty acid profiles was to the largest extent shaped by the MGDG. The hexadecatetraenoic acid (16:4) was present at levels of 30% in the temperature range of 5–15 °C, and then its content gradually decreased to 15% at 25–30 °C and then slightly increased to 20% at 35 °C. The α-linolenic fatty acid (18:3) followed a similar trend being present at amounts > 40% at temperatures 5–15 °C and then slowly decreasing to *c.* 20% at 35 °C.

The double bond index (DBI) that is indicative of relative fatty acid unsaturation reached in *C.* cf. *nivalis* its maximum value of 1.9 at 20 °C, while at both low and high temperatures, the values declined to 1.3–1.6. In *C. reinhardtii*, maximum values of DBI (2.6–2.7) were reached at temperatures 5–15 °C and then steadily decreased to 1.9 at 30 °C followed by an increase to 2.3 at 35 °C. The DBI was higher in *C. reinhardtii* than in *C.* cf. *nivalis* at any acclimation temperature due to the increased content of hexadecatetraenoic and α-linolenic fatty acids.

Sequence of D1 protein

The protein sequence alignment showed that the 323 amino acids of D1 protein of *C.* cf. *nivalis* differ in 33 amino acids from the *C. reinhardtii,* while only 29 amino acids were different from the higher plant sequence of *A. thaliana* (Fig. 5). The alignment containing all sequences of D1 protein is shown in Supporting Information (Fig. S5). The stromal N terminus of the snow alga's D1 protein contains six changes (N10S, S11I, A15E, E19S, I36L, C40T) in comparison with the control *C. reinhardtii* strain's D1 protein. Two changes are found at the lumenal side of the *A* helix (V47C, F48Y), six small amino acids are exchanged for even smaller ones in the lumenal *AB* loop (S68A, T79S, T85S, L91V, I96V, L102V), followed by four changes in the *B* helix (C117L, Y124A, C125S and F135Y) and two at the stromal edge of the *C* helix (A144F, Y147F). The *CD* helix carries a single change (I184L) just as the *D* helix (L200A). The stromal *DE* loop has five differences in sequence between the two *Chlamydomonas* strains: T228S, N230V, A233T, E235Y,

Fig. 5. D1 protein alignment. The psbA amino acid sequence of *Chlamydomonas* cf. *nivalis* was aligned against psbA sequences of *Chlamydomonas reinhardtii*, *Arabidopsis thaliana* and *Thermosynechococcus elongatus* (psbA1 gene sequence).

R238K. The *DE* helix has identical sequence in all four aligned sequences of D1 protein. The remaining differences were found within the *E* helix (S270A, I281V, F285L and L290V) and the C terminus of the D1 protein (L314I, N325D). Within the context of all aligned D1 sequences (Fig. S5), it becomes evident that the majority of the reported changes between the D1 protein sequence of snow alga and the control *C. reinhardtii* occur in extremophilic organisms other than green algae and higher plants (Fig. S6).

Discussion

Temperature is a fundamental factor that affects growth rate, physiological and metabolic processes in algae (Eppley, 1985; Raven & Geider, 1988; Beardall & Raven, 2004; Chu *et al.*, 2005; Staehr & Birkeland, 2006). It also appears to be one of the main factors that dictate the distribution of photosynthetic microorganisms (Pakker *et al.*, 1995; Hoffmann, 1996; Bischoff-Baesmann *et al.*, 1997). It has been generally assumed that the photosynthetic organisms occupying habitats where temperatures fluctuate little, typically sites with extremely low or high temperatures, do not possess the ability to acclimate far outside of their optimum growth temperature. Representative examples of extremophiles that have not been observed to successfully colonize other habitats include the well-known cyanobacteria exclusively inhabiting hot springs (Miller & Castenholz, 2000) or green algae forming visible biomass within surface layers of melting snow (Hoham *et al.*, 2008). Questions still remain concerning the response of the photosynthesis and growth of the cryophiles to a significant shift in ambient temperatures and their capacity to grow outside their indigenous territory.

Snow algae from the genera *Chloromonas* and *Chlamydomonas* were considered to be obligate cryophiles with optimal growth below 10 °C (Hoham, 1975a). Low temperature growth optima were reported, for example for *Chloromonas pichinchae* (1 °C; Hoham, 1975a), *Chloromonas chenangoensis*, *Chloromonas tughillensis* (2.5–5 °C; Hoham *et al.*, 2008), *C. raudensis* (8 °C; Pocock *et al.*, 2011) and *Chlamydomonas* sp. (5 °C; Eddie *et al.*, 2008). Strains with higher temperature limits that can still be assumed as cryotolerants were described in isolates from Antarctic oases (Seaburg *et al.*, 1981). The optimal growth for *Chlamydomonas subcaudata* and *Chlamydomonas alpina* was reported in the range of 12.5–15 °C, while the one for *Chlamydomonas intermedia* is slightly higher (15–18 °C). Concerning the widely distributed *C. nivalis*, contrasting results were obtained due to the lack of laboratory cultures and taxonomic uncertainty (Hoham, 1975a; Komárek & Nedbalová, 2007). Great variability of *in situ* maximal photosynthetic activity was demonstrated among samples collected in the Beartooth Mountains on the Montana-Wyoming boundary (USA; Mosser *et al.*, 1977). No inhibition of photosynthesis at temperatures up to 20 °C was observed in short-term experiments with natural samples containing *C. nivalis* red cysts (Remias *et al.*, 2005, 2010b). The results of our comprehensive study including temperature dependence of growth, rate of photosynthesis including the electron transport within PSII and the whole electron transfer chain from H_2O to CO_2 are clearly indicating that our strain of red snow-forming *C.* cf. *nivalis* cannot be regarded as cryophilic but rather a cryotolerant species with very good photosynthetic performance at low temperatures. Moreover, our analysis of control samples taken from cells grown for 3, 5 and 10 days showed that all the changes in the fatty acid content occurred within a time span shorter than 3 days. This fact documents cells' potential to quickly acclimate their fatty acid composition regardless of the temperature outside its growth optimum (Fig. S1).

What molecular mechanisms are employed to yield the snow alga this rather broad thermal tolerance? It is important to note that our strain of *C. nivalis* acclimated to 2.5 °C exhibits significantly faster growth (at 5–15 °C) and oxygen evolution rate (at 5–20 °C) in comparison with the mesophilic control. Oxygen evolution rates of the snow alga surpass the mesophilic control within the range of 5 to *c.* 20 °C even when the two strains were pregrown at identical temperature of 24 °C. The important role of lipids as mediators of thermal acclimation of membrane proteins (Domonkos *et al.*, 2008), particularly the degree of fatty acid unsaturation (Gombos *et al.*, 1994; Wada *et al.*, 1994), has been recognized for decades. Photosynthetic membranes of cyanobacteria, algae and plants are unique for their richness in glycolipids dominated by galactolipids MGDG and DGDG, followed by sulfolipid SQDG. PG carrying a negative charge makes up, as the only glycerophospholipid, for 9% of all thylakoid lipids in green alga *C. reinhardtii* (Janero & Barrnett, 1981). Numerous physiological functions were reported for the PG (reviewed in Sato, 2004; Loll *et al.*, 2007) that include mediation of the protein–lipid interface within the membrane (Szalontai *et al.*, 2003; Domonkos *et al.*, 2008; Guskov *et al.*, 2009), control of membrane's surface charge (Apostolova *et al.*, 2008), mediation of electron flow in and stabilization of photosystem I (PSI; Rawyler & Siegenthaler, 1981; Yang *et al.*, 2005), stabilization of PSII and trimeric light-harvesting complex 2 (LHCII; El Maanni *et al.*, 1998; Dubertret *et al.*, 2002), mediation of PSII assembly in *C. reinhardtii* (Pineau *et al.*, 2004) and PSII activity (Sato *et al.*, 2000) in *Synechocystis* sp. PCC6803 (Laczko-Dobos *et al.*, 2008), particularly at the lumenal face of the thylakoid membrane of spinach chloroplasts (Duchene *et al.*, 2000), indispensable role in stabilization of the Q_B binding site in PSII of *Synechocystis* sp. PCC6803 (Gombos *et al.*, 2002) and development of thylakoid membranes in *A. thaliana* (Hagio *et al.*, 2002). So far, the investigation of the function of PG in photosynthesis has relied on targeted elimination of the PG molecules by either downregulation of enzymes responsible for PG synthesis in whole cells and/or by treatment of isolated thylakoid membranes with phospholipase *in vitro*. Upregulation of the PG synthesis was studied only in *C. reinhardtii* subjected to sulphur starvation (Sugimoto

et al., 2008). Here, we have adopted a reverse strategy by examining cells of the snow alga *C.* cf. *nivalis* that have thylakoid membranes in their chloroplasts dominated by over 80% PG. We have put particular emphasis on the detailed study of the temperature dependence of the electron transport at the acceptor side of PSII that is expected to be mostly affected by the absence of PG and hence might be also perturbed by its overabundance.

All strains of cyanobacteria, algae and higher plants examined to date showed the temperature dependence of the Q_A to Q_B electron transfer rate following an analogous trend (Shlyk-Kerner *et al.*, 2006; Dinamarca *et al.*, 2011). First, the rate of electron transfer gets accelerated, up to a temperature that corresponds to, and/or is close to the growth optimum. This activation phase is then followed by an activationless regime of a various widths spanning from not more than 5–10 °C up to as much as 20–25 °C. Here, electron transfer becomes rate limited by the coupled proton mobilization. The third phase corresponds with the onset of denaturation and is characterized by a steep decline both in rate as well as the amplitude of the electron transfer. Here, we report the temperature dependence of the Q_A to Q_B electron transfer rate in a snow alga *C.* cf. *nivalis* that completely lacks the activationless regime as the electron transfer rate ideally obeys the Arrhenius law. The rate at temperatures close to 0 °C equals the peak rates of cyanobacteria, algae and higher plants at their respective activationless regimes, while the peak rate at 35 °C exceeds any previously reported rate of Q_A to Q_B electron transfer by 4-fold to 10-fold. This trend differs from the typical temperature dependence due to the different thermodynamics of the electron transfer in the snow alga. The higher activation enthalpy in comparison with the mesophile is unfavourable as it is decreases the reaction turnover at low temperatures representing the natural environment of *C.* cf. *nivalis*. This effect is sufficiently compensated by more positive activation entropy that is achieved by the increase in local flexibility of the transition state for the Q_A to Q_B electron transfer. As a consequence, this compensation leads to the acceleration of the reaction rate to nonphysiologically high values at 35 °C counterbalancing the negative effect of the activation enthalpy. The necessary flexibility can be made possible through a number of structural elements, including, but not limited to, the unique lipid composition of the thylakoid membrane and the D1 protein structure. Here, we propose to rule out a dominant role of fatty acids to gain the additional PSII flexibility. The lipids in the snow alga's thylakoid membrane had a lower DBI in all experimental treatments compared to the control. Based on the fatty acids themselves alone, *C. reinhardtii* always displayed a more flexible membrane. To add to the controversy, the role of PG, particularly its high-melting point form, was disputed as a possible factor in modulating membrane flexibility and hence the chilling resistance in higher plant chloroplasts (Kaniuga *et al.*, 1998). Yet PG is an essential lipid that was found in close vicinity of the Q_B binding pocket in crystals of PSII of thermophilic cyanobacterium (reviewed in Mizusawa & Wada, 2012). We also cannot ignore the fact that membranes dominated by PG exhibit, at neutral pH, considerable negative surface charge density inducing a tilt of PG molecules of *c.* 30° relative to the membrane normal (Watts *et al.*, 1981). This can introduce a significant disorder to the membrane packing and hence increase membrane fluidity.

Most of the differences in the D1 protein of the snow alga are situated in the interhelical domains of both lumen (*AB*)- and stroma (*DE*)-exposed loops. Fifteen amino acids in the snow algal D1 protein (in comparison to the control *C. reinhardtii*) are smaller in size yet of a similar chemical nature, while four represent neutral changes. The most common exchange included three counts of L – I, V – L, S – T and Y – F, followed by two counts of V – I and A – S. Only six of 33 changes instituted amino acid residues with significantly larger volume and different chemical nature (e.g. A15E, A144F, A233T, E235Y). Two of the latter are part of the DE loop, while two changes within the same region bring about smaller amino acids (T228S, R238K). Interestingly, mutation at some of these sites in the *DE* loop and the sites that either surround or are close to the Q_B binding pocket were reported to have a lower binding affinity to the herbicide diuron (D1–228), ioxynil (D1–238; Kless *et al.*, 1994) or atrazine (D1–238) (Narusaka *et al.*, 1998) in mutants of cyanobacterium *Synechocystis* sp. PCC6803 while having minor effect on its saturated rate of O_2 evolution or yield of chlorophyll fluorescence. Similarly, change in the amino acid at the D1–184 site within the lumenal CD loop has been reported to alter the structure in the vicinity of the quinon binding site as seen in a metribuzin-resistant mutant of *Chenopodium rubrum* (Schwenger-Erger *et al.*, 1999). This evidence must, however, be regarded as circumstantial as most of the aforementioned cyanobacterial mutants carried in fact double or even triple mutations within the same region and sometimes also a different amino acid.

Here, we present a hypothesis that a combination of changes in protein structure and an unusually high abundance of charged lipids modulate the electron transfer rate in PSII allowing for fast transfer of electrons from Q_A to Q_B even at low or close to cryophilic temperatures. The structural flexibility that is the necessary prerequisite for the electron transfer at the acceptor side of PSII is probably made possible by the cumulative effect of the amino acid changes in the D1 protein combined with the physico-

chemical characteristics of the PG thylakoid membrane including possible substitution of MGDG, DGDG and SQDG lipids by the PG in places around and within the PSII (Loll *et al.*, 2007; Mizusawa & Wada, 2012). It seems, however, highly unlikely that the increased rate of terminal electron transfer in PSII that is even orders of magnitude faster than necessary for the capacity of plastoquinol pool to be reoxidized is the only factor responsible for the increased carbon fixation efficiency at low temperatures as inferred from our measurements of oxygen evolution capacity reporting on H_2O to CO_2 electron transfer in the snow alga. We assume that the handicap of the catalytic activity of Ribulose-1,5-bisphosphate carboxylase oxygenase and its activase being greatly lowered at low temperatures is alleviated by their increased expression and accumulation akin to the Antarctic *Chloromonas* species (Devos *et al.*, 1998). Despite the numerous uncertainties, we present here a host of novel information about the strategies employed by cold-tolerating green alga *C.* cf. *nivalis* including the outline of some of the molecular mechanisms that make this tolerance possible.

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Authors' contribution

M.L. and L.P. contributed equally to this work.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Time course of fatty acid composition in thylakoid lipids extracted from *C. reinhardtii* and *C.* cf. *nivalis*.
Fig. S2. Temperature dependence of critical temperature (Tc) and maximal temperature of fluorescence rise (T_M) in the snow alga *C.* cf. *nivalis* and in the mesophilic *C. reinhardtii*.
Fig. S3. Fatty acid content in MGDG, DGDG, SQDG and PG isolated from thylakoid membranes of snow alga *C.* cf. *nivalis* in the temperature range from 5 to 35 °C.
Fig. S4. Fatty acid content MGDG, DGDG, SQDG and PG isolated from thylakoid membranes of control green alga *C. reinhardtii* in the temperature range from 5 to 35 °C.
Fig. S5. Multiple sequence alignment of amino acid sequence translated from psbA gene coding for D1 protein isolated from *C.* cf. *nivalis* with 33 corresponding sequences representing all major groups of oxygenic photosynthetic organisms.
Fig. S6. Dendrogram of amino acid sequences coding for D1 protein of *C.* cf. *nivalis* and species representing all major groups of oxygenic photosynthetic organisms, based on pairwise distances.

Temperature dependence of photosynthesis and thylakoid lipid composition in the red snow alga *Chlamydomonas* cf. *nivalis* (Chlorophyceae)

Martin Lukeš[1,2,*], Lenka Procházková[3,*], Volha Shmidt[1], Linda Nedbalová[3] & David Kaftan[1,2]

[1]University of South Bohemia in České Budějovice, Faculty of Science, Branišovská 31, 37005 České Budějovice, Czech Republic; [2]Institute of Microbiology CAS, Opatovický mlýn, 37981 Třeboň, Czech Republic, [3]Charles University in Prague, Faculty of Science, Department of Ecology, Viničná 7, 12844 Prague, Czech Republic

[*]These authors contributed equally to the study.

Supplementary Material

The following supplementary information includes Figures S1-S6

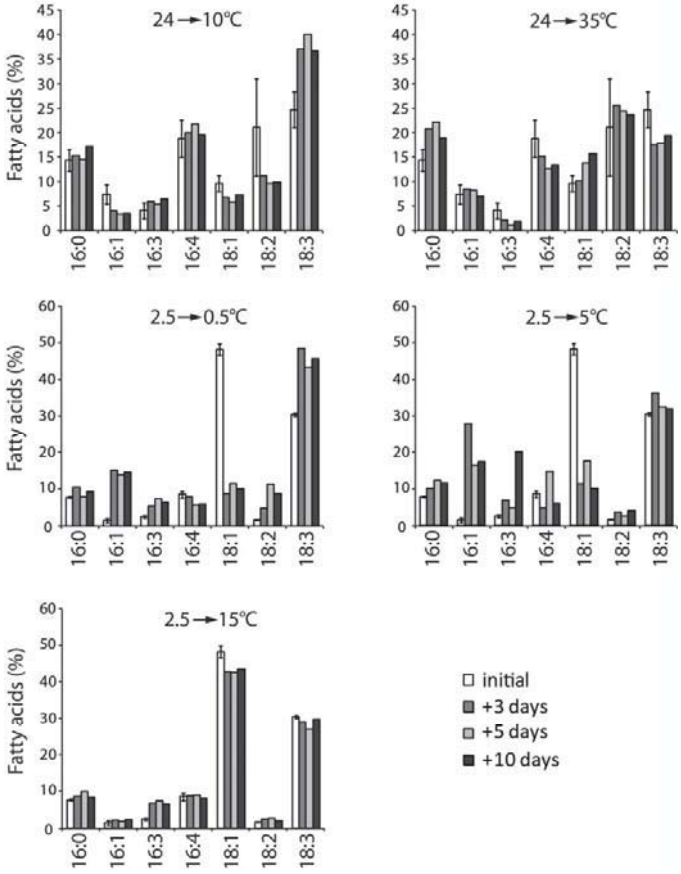


Figure S1. Time course of fatty acid composition in thylakoid lipids extracted from *C. reinhardtii* (top panels) and *C.* cf. *nivalis* (middle and lower panels). Initial levels correspond to cells in their mid exponential growth phase (24°C in *C. reinhardtii*; 2.5°C in *C.* cf. *nivalis*). Following samples were taken after 3, 5, and 10 days after the cells were transferred to either higher or lower temperature. Error bars in the initial level represent standard deviation of three independent measurements.

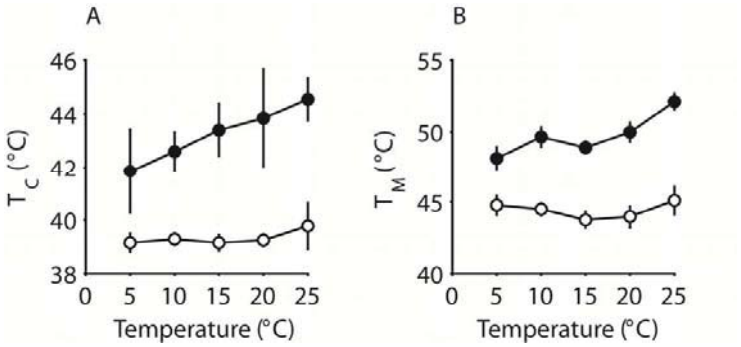


Figure S2. Temperature dependence of A: critical temperature (Tc) and B: maximal temperature of fluorescence rise (T_M) in the snow alga *Chlamydomonas* cf. *nivalis* (open circles) and in the mesophilic *Chlamydomonas reinhardtii* (closed circles). Values represent mean of three independent measurements.

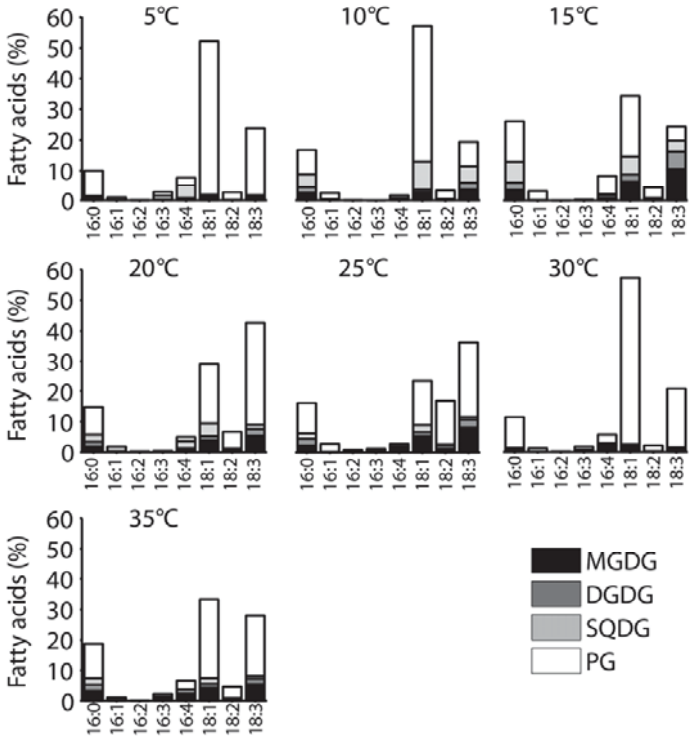


Figure S3. Fatty acid content in monogalactosyldiacylglycerol (MGDG, black), digalactosyldiacylglycerol (DGDG, dark gray), sulphoquinovosyldiacylglycerol (SQDG, light gray) and phosphatidyglycerol (white) isolated from thylakoid membranes of snow alga *Chlamydomonas* cf. *nivalis* in the temperature range from 5 to 35ºC. Values represent mean of three independent measurements.

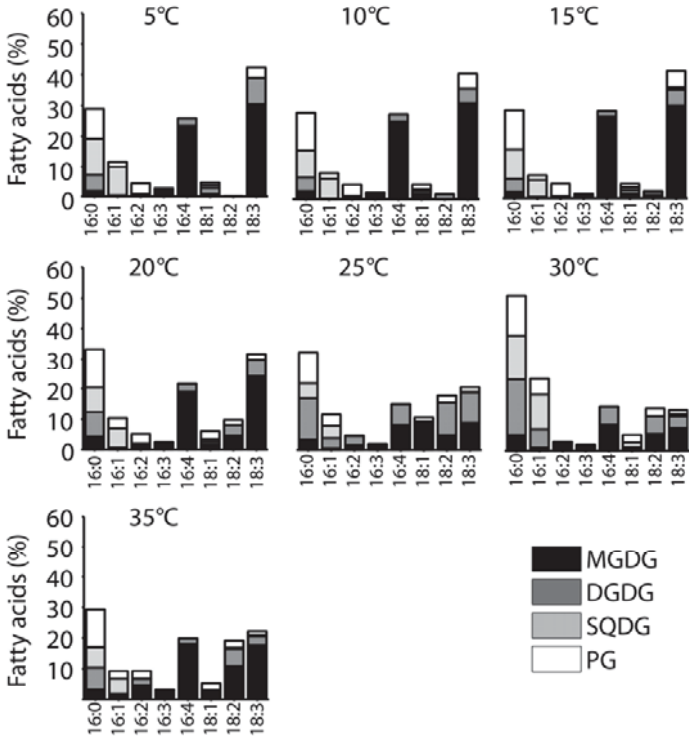
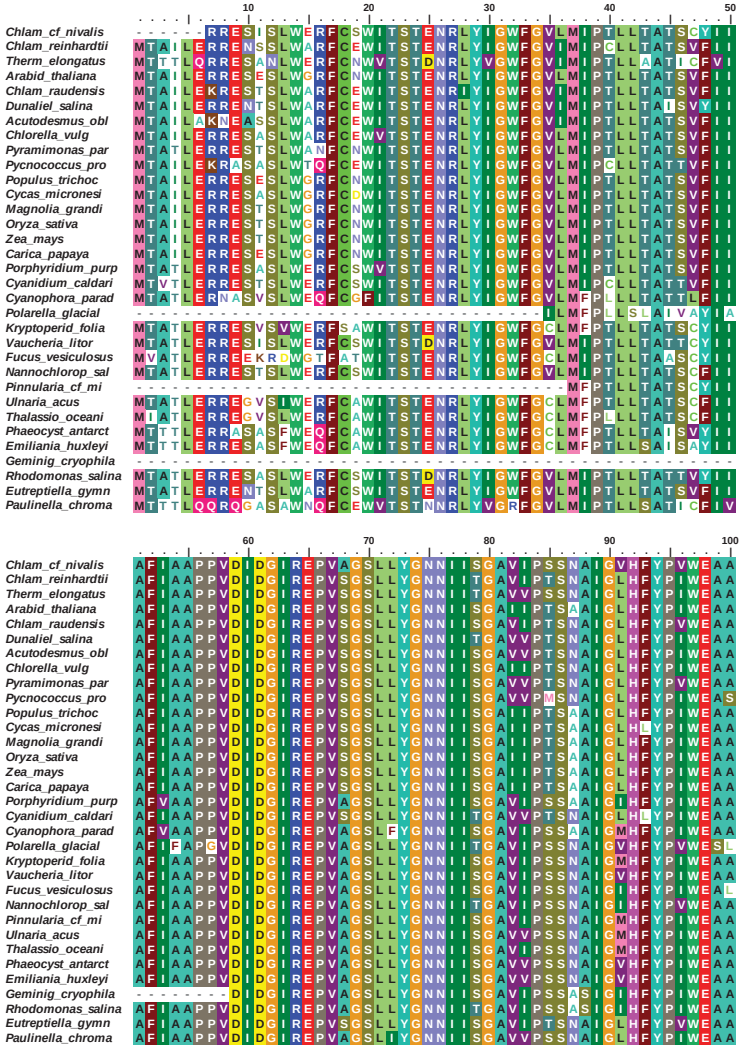


Figure S4. Fatty acid content in monogalactosyldiacylglycerol (MGDG, black), digalactosyldiacylglycerol (DGDG, dark gray), sulphoquinovosyldiacylglycerol (SQDG, light gray) and phosphatidyglycerol (white) isolated from thylakoid membranes of control green alga *Chlamydomonas reinhardtii* in the temperature range from 5 to 35ºC. Values represent mean of three independent measurements.


10
20
30
40
50
Chlam_cf_nivalis
Chlam_reinhardtii
Therm_elongatus
Arabid_thaliana
Chlam_raudensis
Dunaliel_salina
Acutodesmus_obl
Chlorella_vulg
Pyramimonas_par
Pycnococcus_pro
Populus_trichoc
Cycas_micronesi
Magnolia_grandi
Oryza_sativa
Zea_mays
Carica_papaya
Porphyridium_purp
Cyanidium_caldari
Cyanophora_parad
Polarella_glacial
Kryptoperid_folia
Vaucheria_litor
Fucus_vesiculosus
Nannochlorop_sal
Pinnularia_cf_mi
Ulnaria_acus
Thalassio_oceani
Phaeocyst_antarct
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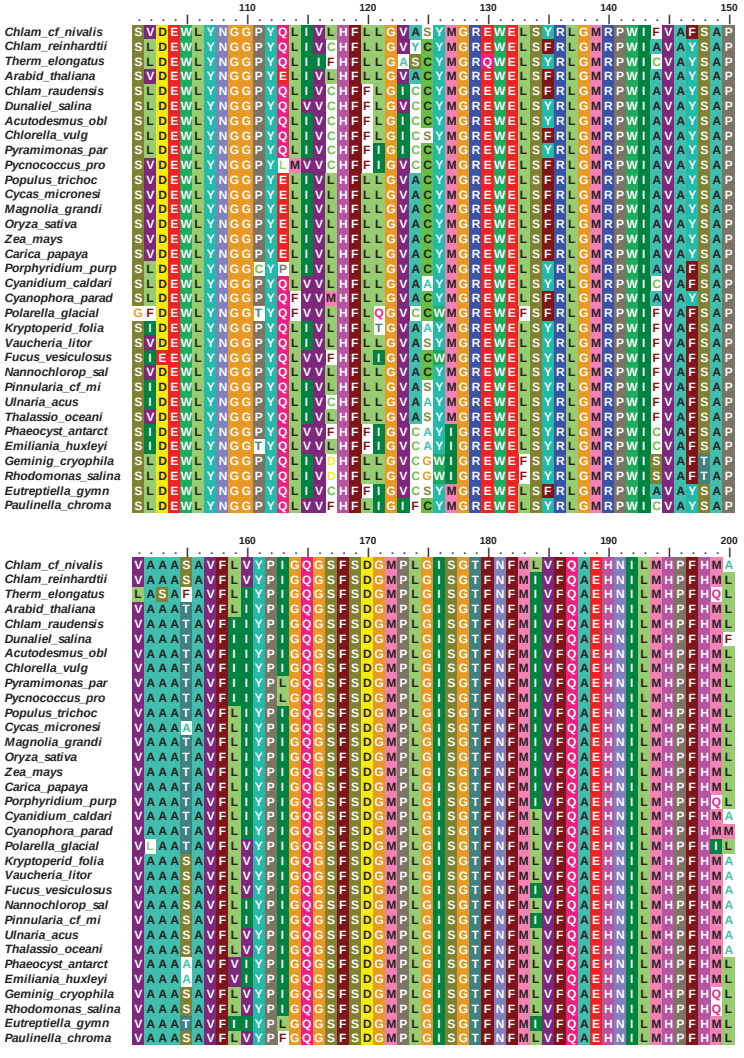
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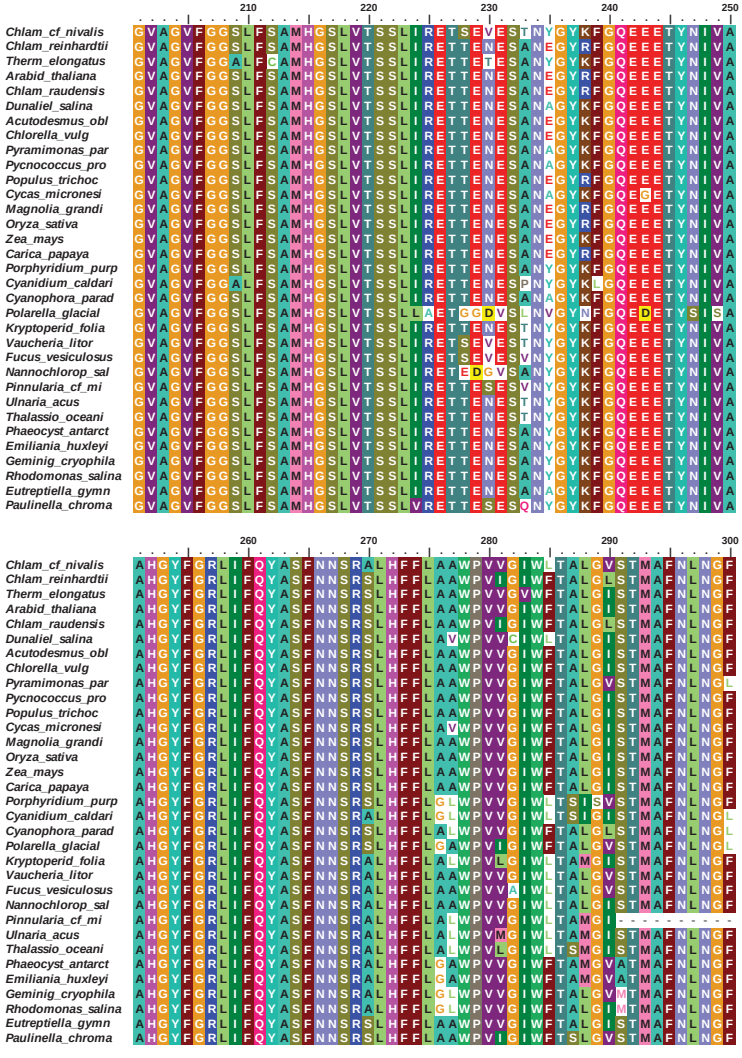
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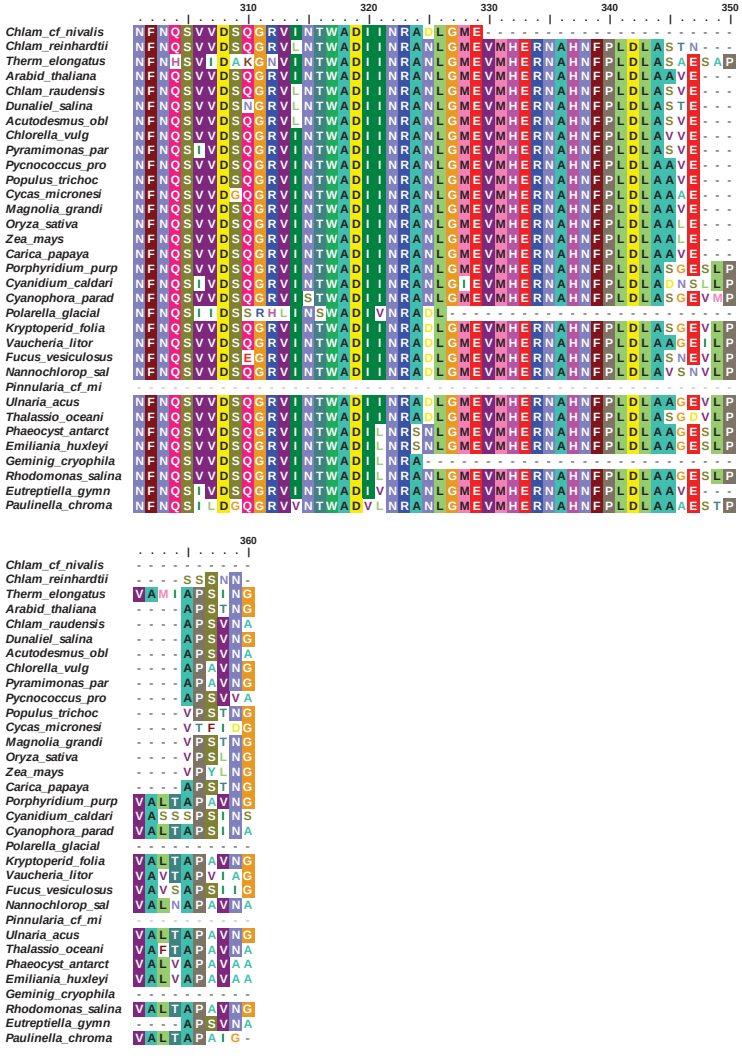
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Figure S5. Multiple sequence alignment of amino acid sequence translated from psbA gene coding for D1 protein isolated from *Chlamydomonas* cf. *nivalis* (this study, NCBI reference nucleotide sequence **KF702330.1** and protein sequence **AHB82278**), *Chlamydomonas reinhardtii* (**DAA00957.1**), *Thermosynechococcus elongatus* (**NP_682633.1**), *Arabidopsis thaliana* (**P83755.2**), *Chlamydomonas raudensis* (**AFU83031.1**), *Dunaliella salina* (**YP_005089831.1**), *Acutodesmus obliquus* (**ABD48259.1**), *Chlorella vulgaris* (**P56318.1**), *Pyramimonas parkea* (**ACJ71100.1**), *Pycnococcus provasolii* (**ACK36809.1**), *Populus trichocarpa* (**YP_001109480.1**), *Cycas micronesica* (**ABU85314.1**), *Magnolia grandiflora* (**YP_007474516.1**), *Oryza sativa* (**AER12889.1**), *Zea mays* (**NP_043004.1**), *Carica papaya* (**YP_001671663.1**), *Porphyridium purpureum* (**BAO23682.1**), *Cyanidium caldarium* (**NP_045067.1**), *Cyanophora paradoxa* (**NP_043238.1**), *Polarella glacialis* (**BAC76007.1**), *Kryptoperidinium foliaceum* (**ADI40420.1**), *Vaucheria litorea* (**ACF70962.1**), *Fucus vesiculosus* (**CAX12449.1**), *Nannochloropsis salina* (**AGI99196.1**), *Pinnularia cf. microstauron* (**AER42084**), *Ulnaria acus* (**AEX37881.1**), *Thalassiosira oceanica* (**ADB27608.1**), *Phaeocystis antarctica* (**AEK26755.1**), *Emiliania huxleyi* (**AAX13814.1**), *Geminigera cryophila* (**ABL96289.1**), *Rhodomonas salina* (**ABO70840.1**), *Eutreptiella gymnastica* (**YP_006234198.1**), and *Paulinella chromatophora* (**ACB43269.1**).

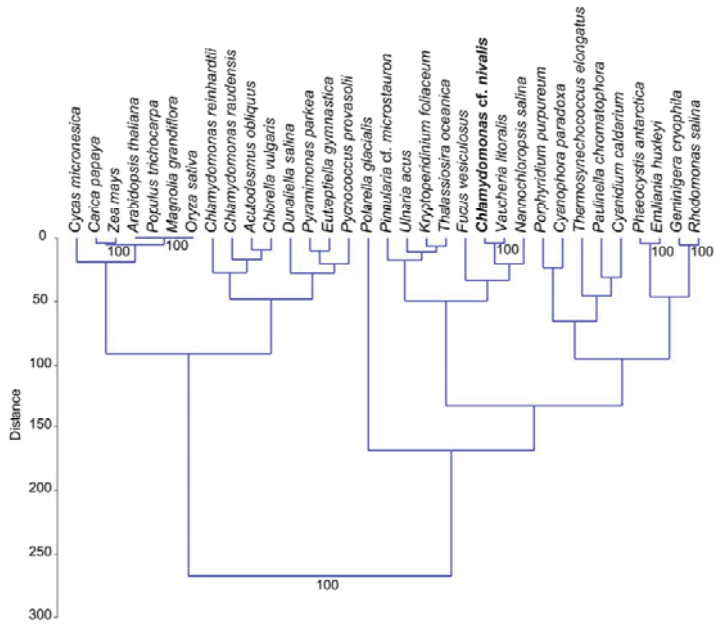

Figure S6. Dendrogram of amino acid sequences of D1 protein of snow alga *Chlamydomonas* cf. *nivalis* (in bold) and species representing all major groups of oxygenic photosynthetic organisms, based on pairwise distances.

PAPER II

The effect of environmental factors on fatty acid composition of *Chromera velia* (Chromeridae)

Lukeš M., Giordano M. & Prášil O.

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The effect of environmental factors on fatty acid composition of *Chromera velia* (Chromeridae)

Martin Lukeš[1,2] · **Mario Giordano**[3,4] · **Ondřej Prášil**[1,2]

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Abstract The effect of light intensity, light spectral quality, temperature and salt concentration on the fatty acid composition of *Chromera velia* was studied. *Chromera velia* is a unicellular, marine, photosynthetic, eukaryotic alga and a close relative of the apicomplexan parasites. *Chromera velia* was able to grow at light intensities between 20 and 450 μmol photons m^{-2} s^{-1}, in the temperature range 17–32 °C and at salinities between 0.2 and 1 M NaCl. The cells responded to variations in the growth regime by modifying fatty acid composition: the ratio of fully saturated palmitic acid (C16:0) and five times unsaturated eicosapentaenoic acid (C20:5n–3) was especially prone to variation. Intermediate fatty acids, namely stearic, linoleic and dihomo-γ-linolenic acids, changed minimally and were probably not involved in the response to the growth regimes. The highest proportion of eicosapentaenoic acid was observed when the cultures were maintained at 32 °C, at an irradiance of 80 μmol photons m^{-2} s^{-1}, provided by an incandescent light source, under a 12-/12-h day/night photoperiod.

Keywords *Chromera velia* · Chromeridae · Fatty acids · Eicosapentaenoic acid · Light · Temperature · Salinity

Introduction

The fatty acid (FA) composition of algae is highly dependent on environmental conditions such as light, temperature, salinity, pH and nutrient availability (Guschina and Harwood 2006). Increase in saturation with higher photon flux densities (PFDs) is usually observed since the polyunsaturated FAs of membrane polar lipids under high light often become less abundant than neutral storage lipids, which mainly contain saturated FAs (Khotimchenko and Yakovleva 2005). Polyunsaturated fatty acids (PUFAs) are necessary to maintain photosynthetic membrane activity and play an important role in acclimation to growth at low irradiance (Klyachko-Gurvich et al. 1999). Studies on the impact of light quality on carbon allocation are less numerous. It was demonstrated that in *Nannochloropsis* sp., light quality modified lipid productivity (Das et al. 2011) or growth of *Arthrospira* (*Spirulina*) *platensis* (Wang et al. 2007). Temperature has major effect on the types of FA produced by microalgae (Thompson et al. 1992). Variations in temperature might lead to changes in the membrane lipid order and, in the case of elevated temperatures, may cause protein unfolding and denaturation. When the temperature is lowered, a number of changes in membrane lipids have been observed (see Morgan-Kiss et al. 2006) that corresponded with an attempt to maintain the lipid order at physiologically advantageous values. An increase in FA unsaturation is the most common response to the lowering of the growth temperature, as was shown in work by

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✉ Martin Lukeš
lukesm@alga.cz

[1] Institute of Microbiology ASCR, Centrum Algatech, Laboratory of Photosynthesis, Opatovický mlýn, 379 81 Třeboň, Czech Republic

[2] Faculty of Sciences, University of South Bohemia, Branišovská 31, 370 05 České Budějovice, Czech Republic

[3] Laboratory of Algal and Plant Physiology, Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Ancona, Italy

[4] National Research Council, Istituto di Biologia Agro-Ambeintale e Forestale; Consiglio Nazionale delle Ricerche (IBAF-CNR), Porano, Terni, Italy

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Thompson et al. (1992). The increase in the number of double bonds and the shortening of the FA carbon chains are usually associated with a decrease in phase transition temperature and with a decrease in membrane order (Murata and Wada 1995; Nishida and Murata 1996). However, not all the double bonds in a FA molecule have an equivalent impact on membrane physical properties. For example, phosphatidylcholine (PC), esterified on both the sn1 and sn2 positions with stearic acid (18:0/18:0)-PC, has a melting point (Tm) (transition temperature from gel to liquid-crystalline phase) of 55 °C. Replacement of the stearic acid at the sn2 position with oleic acid (18:0/18:1)-PC led to a decrease in Tm to 6.3 °C (Russel 1989). The insertion of a second double bond to form (18:0/18:2)-PC was found to decrease the Tm to −16 °C. However, the addition of a third double bond forming (18:0/18:3)-PC did not decrease, but increased the Tm by 3 °C to −13 °C. For comparison, Tm values for 16:0/16:1- and 16:0/22:6-PC do not differ significantly (−12 and −10 °C, respectively) (Coolbear et al. 1983). These findings suggest that monounsaturated FAs are more effective than PUFAs in decreasing membrane order (making the membrane more fluid), possibly by interfering with the cooperative liquid-to-gel transition.

Cell lipid composition is also influenced by environmental salinity. The alteration of external salinities can influence the internal homeostasis of cells in several ways (Kirst 1989; Erdmann and Hagemann 2001). Firstly, osmotic stress, caused by a flux of water across the semi-permeable cell membrane, can lead to changes in the cellular water potential. Hyperosmotic conditions lead to shrinkage of the plasmalemma (Bisson and Kirst 1995) while, hypoosmosis causes water influx, potentially resulting in an increased turgor pressure, a stress that is better tolerated by algae which possess a rigid cell wall. Secondly, ionic stress, which is caused by passive loss or uptake of inorganic ions, can lead to the disturbance of the hydration sphere around proteins and other macromolecules, affecting their conformation and charge interaction and thus impeding their function (Xiong and Zhu 2002). One of the survival mechanisms of aquatic algae is to change its FA content to protect from osmotic stress during rapid salinity changes, like those that occur in natural environments such as coastal rock pools (Lee et al. 1989).

Chromera velia, together with *Vitrella brassicaformis*, belongs to a newly discovered group of unicellular algae, the Chromeridae (Moore et al. 2008, Oborník et al. 2011). Chromerids are included in the red lineage, in terms of plastid origin (Janouskovec et al. 2010), in contrast to most organisms in this evolutionary lineage; however, they do not contain chlorophyll *c*. They are also notable as the closest known relatives of the apicomplexan parasites. *Chromera velia* is unique in its mechanism of photoprotection because it uses photorespiration in addition to thermal energy dissipation via non-photochemical quenching (Quigg et al. 2012). The species also has the ability to synthesize large amounts of eicosapentaenoic acid (EPA), possibly to a similar level as some members of the Eustigmatophyceae group, such as *Nannochloropsis* sp. (Sukenik and Carmeli 1989), *Nannochloropsis oculata* (Seto et al. 1992) or *Monodus subterraneus* (Cohen 1994). So far, *C. velia* has been reported to contain highly unsaturated structural lipids (Botte et al. 2011) and the synthetic pathway of sterols in *C. velia* has been described (Leblond et al. 2012). The effects of environmental factors on the FA composition of *C. velia* cells have not yet been reported. PUFAs, such as arachidonic acid, together with EPA, are highly abundant components of structural polar glycolipids of the photosynthetic membranes in *C. velia* (Botte et al. 2011); these lipids are very important for chloroplast functionality. Depending on their interaction with the proteins embedded in the membrane, these FA can be classified into three main classes (Palsdottir and Hunte 2004; Hunte 2005): (1) bulk lipids, which show only non-specific interactions with membrane proteins and contribute to the membrane fluidity in thylakoids; (2) annular lipids, which constitute a shell around the membrane bound protein through direct contact with the protein; and (3) integral lipids, which are bound to the interior of proteins, often at the interface between two subunits or between transmembrane α-helices, for example in the photosystem complexes or the cytochrome b6f complex.

In this study, the question of how the FA composition of *C. velia* changes under different environmental conditions has been addressed. Also, it particularly focused on changes in long-chain highly unsaturated fatty acids, which is an interesting topic in terms of its potential for biotechnological application.

Materials and methods

Growth conditions

Stock cultures of *Chromera velia* (strain RM12) were maintained in f/2 medium for at least ten generations. The stock cultures grew in 2-L Roux bottles, at 28 °C, at 80 μmol photons m^{-2} s^{-1} provided by incandescent 150 W bulbs (Benlux original, Serbia), and were bubbled with filter-sterilized air at rate of 1 L min^{-1}. The cultures were weekly diluted to keep the cell concentration at 1–2 × 10^6 cells mL^{-1}. For every subsequent treatment, 100 mL of the culture was transferred to a 250-mL glass tube and diluted to a concentration of ~1 × 10^6 cells mL^{-1}. Prior to treatment, cells were concentrated by centrifugation at 6000×*g*, for 10 min, at 25 °C, and resuspended in fresh medium or, for the experiments on the effect of salinity, in media containing different salt concentrations. The cultures were then subjected to the experimental treatment for 7 days. Initially, in order to identify irradiance and temperature limitations on the growth of

C. velia, the growth rates were determined in a matrix of different temperatures and light intensities using the crossed gradient of a light and temperature table (Labio Ltd., Czech Republic, described in Kvíderová and Lukavský 2001). The metallic table (100 × 200 cm) allowed us to create gradients of temperature (in the *x*-axis) and light intensity (in the *y*-axis). Temperatures of 12, 17, 23, 28, 32 and 36 °C and irradiances of 20, 80, 200 and 450 μmol photons m^{-2} s^{-1} of fluorescent (FL). Cells were transferred to 96 flat bottom well plates. In total, 24 plates were monitored. The optical density (OD) was monitored at 750 and 680 nm every 24 h for 14 days, using the well plate reader (Tecan Sunrise, Tecan Group Ltd., Switzerland). Specific growth rates, μ, were determined as the slope of the linear regression of semi-logarithmic plot of OD_{680} vs. time, during the exponential phase of the growth. All growth experiments were run in seven replicates.

To test the effect of light intensity and quality on FA composition, *C. velia* cells were grown at a constant temperature of 28 °C. The cells were exposed to six different polychromatic light conditions created by two spectrally different light sources adjusted to three light intensities each (20 μmol photons m^{-2} s^{-1} = low light, LL; 200 μmol photons m^{-2} s^{-1} = high light, HL; 80 μmol photons m^{-2} s^{-1} = control). The difference in spectral quality was obtained using an FL tube (Osram Dulux-L, 950) or incandescent bulbs (IL, Benlux original, 150W). Experiments were also performed using blue, green and red monochromatic LED light sources (peak maxima of ~462, ~514 and ~630 nm, respectively). The irradiance of LED illumination was set to 20 μmol photons m^{-2} s^{-1}. Light intensities were measured using a calibrated quantum meter (Li-189, Li-Cor, USA) equipped with a 2π quantum sensor. In order to study the effects of temperature, *C. velia* was cultured at 17, 23, 28 and 32 °C, at an irradiance of 80 μmol photons m^{-2} s^{-1} supplied by an incandescent light source. To study the effect of salinity, cells were resuspended in f/2 medium modified in terms of NaCl concentration to obtain 0.2, 0.6, 0.8 and 1 M NaCl; the cultures were maintained on an orbital shaker, at 28 °C and 80 μmol photons m^2 s^{-1} using an incandescent light source. Cultures resuspended in f/2 medium containing 0.4 M NaCl were used as control.

Absorption spectra of whole cells

Whole-cell absorption spectra were measured with a UV500 spectrophotometer (Thermo Spectronics, USA) using an integration sphere. The measurement settings were as follows: the bandwidth was 4 nm, and the data interval was 0.5 nm in the range 350–800 nm. The absorption spectrum of *C. velia* cells is shown in the supplementary material (Fig. S1).

Emission spectra of light sources

Emission spectra were recorded with an Optical spectrometer (SM 9000, PSI, Czech Republic) and normalized to the respective maxima in the absorption range of *C. velia* cells. The emission spectra of the light sources used for this study are shown in the supplementary material (Fig. S1).

Cell harvesting, lipid extraction, derivatization and fatty acid methyl ester analysis

Cells were harvested by centrifugation at 6000×*g* for 10 min at 4 °C. Cells were washed two times with distilled water and then resuspended in 800 μL of distilled water. Two millilitres of methanol and 1 mL of dichloromethane were then added; following this, the suspension was sonicated for 10 min in a cooled sonication bath (Kraintek 6, Kraintek Czech s.r.o., Czech Republic). After sonication, 1 mL of distilled water and 1 mL of dichloromethane were added, and the suspension was briefly vortexed. Following centrifugation at 500×*g* for 10 min at 4 °C, the organic phase was separated. The lower dichloromethane layer was transferred to clean evaporation vials; 2 mL of dichloromethane was added, the mixture was vortexed and centrifuged. The lower phases were pooled and dried on a rotary evaporator. Dried extracts were diluted in dichloromethane/methanol (2:1 *v*/*v*) at a concentration of 1 mg of lipid extract in 50 μL of solvent and stored at −70 °C until further analysis. Methyl esters of fatty acids (FAME) were prepared following the method described by Kainz et al. (2002). For the analysis, 1 μL of methylated sample was used. Quantitative and qualitative analysis of the FA complement were performed by means of a GC-FID (HRGC 5300 Megaseries, Carlo Erba, Italy) equipped with a flame ionization detector (FID). A TR-FAME column (60 m × 0.32 mm, df 0.25 μm) was used. Helium was used as a carrier gas, at a pressure of 200 kPa. The temperature ramp was the following: the starting temperature was 140 °C; it was increased to 240 °C at rate of 4.5 °C min^{-1} and then maintained at 240 °C for 10 min. The injector was kept at 260 °C and the detector at 250 °C. The retention times of FAMEs were compared to known standards (Supelco 37 Component FAME Mix; PUFA no. 3 Supelco (from menhaden oil)). The amount of individual fatty acids was calculated using internal standards with a known heptadecanoic acid (C17:0) content, and corrected by multiplying the integrated peak areas by the correction factors of the FID response. The double bond index (DBI) was calculated using the formula: DBI = $\sum$ (% of fatty acid × no. of double bonds)/100 (Skoczowski et al. 1994).

Statistics

All experiments were done as independent triplicates and data were shown as mean ± standard deviation.

Significant differences in fatty acid composition and unsaturation (expressed as double bond index) were compared using one-way analysis of variance (ANOVA) followed by the

post hoc test of Holm-Sidak. Significance was based on $p < 0.05$. All statistical analysis was performed using SigmaPlot version 12.5.

Results

Light intensity and temperature dependence of growth

The growth of *C. velia* was initially followed using the crossed gradient table of temperature and light, at the irradiances of 20, 80, 200 and 450 μmol photons m^2 s^{-1} and at temperatures between 12 and 36 °C. The cells grew well in the 17 to 32 °C range, with the maximum specific growth rate at 28 °C (μ in the range of 0.128 to 0.148 day^{-1}; Fig. 1). Below and above these temperatures, no growth occurred ($\mu < 0.0005$ day^{-1}). Only at 23 °C, the growth rate was substantially lower at 450 μmol photons m^{-2} s^{-1} (0.017 day^{-1}) than at 80 μmol photons m^{-2} s^{-1} (0.109 day^{-1}). The 23 °C treatment also resulted in a higher number of tetrad cells (resting stages) than all the other temperature treatments, at all photon flux densities (PFDs). The ratio of tetrad cells to single or dividing cells may be responsible for the observed variation of μ.

Fatty acid composition of *C. velia*

The fatty acid composition of *C. velia* grown in controlled conditions (e.g. 28 °C, at an PFD of 80 μmol photons m^{-2} s^{-1}) was as follows: The dominant fatty acids of *C. velia* were the fully saturated palmitic acid (C16:0) and the five times unsaturated 20 carbon atom EPA (C20:5n–3). Less abundant were once unsaturated oleic (C18:1n–9), linoleic (C18:2n–6) and arachidonic acid (C20:4n–6). Minor fatty acids (less than 5% of total fatty acids in abundance) were fully saturated myristic acid (C14:0) and stearic acid (C18:0), palmitoleic (C16:1), *cis*-vaccenic acid (C18:1n–7), γ-linolenic (C18:3n–6), α-linolenic (C18:3n–3) and dihomo-γ-linolenic (C20:3n–6). EPA and α-linolenic acid were the only fatty acids of the n–3 family. Under these conditions, >95% of the cells were in the vegetative stage, ~5% of cells were in tetrads and no flagellate cells were observed.

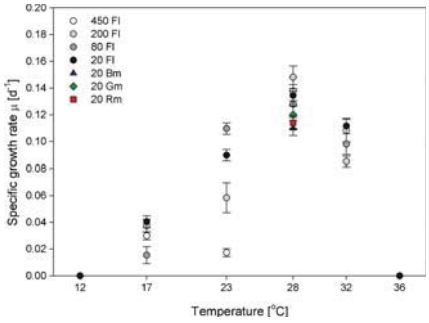

Fig. 1 Effect of temperature and light intensity on growth, expressed as specific growth rate μ (day^{-1}), (mean ± SD, $n = 3$). Numbers designate light intensity 20 (*black circle*), 80 (*dark grey circle*), 200 (*light grey circle*) and 450 (*white circle*) in μmol photons m^{-2} s^{-1}. *Fl* fluorescent, *Bm* blue monochromatic (*triangle*), *Gm* green monochromatic (*diamond*), *Rm* red monochromatic light (*square*)

Dependence of fatty acid composition on light quality

Cells that were grown at higher irradiances of both light sources (IL and FL) showed 10 and 13% higher palmitate levels, respectively, than cells grown at lower irradiance (see Fig. 2). At lower irradiance, EPA levels were higher than at high light, at the expense of palmitate. This difference resulted in a higher (2.6) DBI in comparison to that of high light treated cells (1.9 DBI). At higher irradiances, accumulation of shorter chain FA (C16) was observed, whereas the abundance of C18 FA was unchanged.

Fatty acid composition under monochromatic light

The effect of monochromatic light on FA composition was monitored at three different wavelengths (see Fig. S1 in the supplementary material), at an irradiance of 20 μmol photons m^2 s^{-1}. The most abundant FAs were palmitic acid (18 to 23%) and EPA (approx. 48%). No significant difference ($p > 0.05$) was observed between individual FAs or total unsaturation of FAs of cells grown under spectrally different monochromatic lights (Fig. 3, Table S1 in the supplementary material). Cells grown under monochromatic lights were more unsaturated than cells grown at full spectrum IL or FL lights.

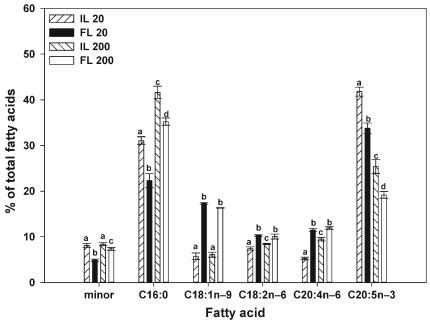

Fig. 2 Effect of light quantity and quality on fatty acid composition of cells (mean ± SD, $n = 3$). Numbers designate light intensity in μmol photons m^{-2} s^{-1}. *FL* fluorescent, *IL* incandescent light. *Different letters* above each column indicate significant differences (one-way ANOVA, $p < 0.05$)

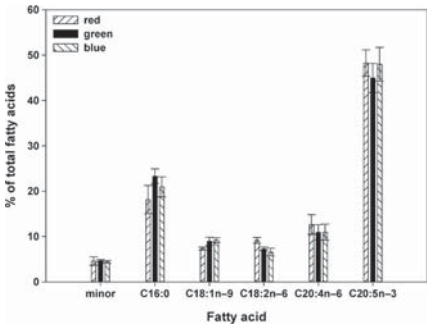

Fig. 3 Effect of blue, green and red light on fatty acid composition of cells (mean ± SD, $n = 3$). No significant differences were observed between different lights (one-way ANOVA, $p > 0.005$)

Influence of growth temperature on fatty acid composition

The effect of temperature on FA synthesis was monitored at 17, 23, 28 and 32 °C. Minor FAs (C14:0, C16:1, C18:1n–7, C18:3n–3, C20:3n–6) were significantly higher ($p < 0.05$) at 17 and 23 °C mainly due to higher C18:1n–7 content, than at 28 and 32 °C. At 17 °C, palmitic acid was the most abundant FA and constituted 40% of total FAs. Above 23 °C, lower level of palmitate was observed. The amount of EPA was significantly higher ($p < 0.05$) 23 and 32 °C then at 17 and 23 °C. No significant difference in oleic and arachidonic acid was observed ($p > 0.05$) (Fig. 4). At lower temperatures, FAs were less unsaturated, resulting in DBIs of 1.6 at 17 °C and 1.8 at 23 °C. The highest unsaturation of FAs was recorded at 28 or 32 °C, when DBI reached 2.48 and 2.49, respectively. The high temperature treated cells had similar DBI as cells grown at low light, while low temperature treated cells had similar unsaturation as cells grown at high light. Under these growth regimes, lipids showed a lower unsaturation than under the monochromatic light treatments.

Dependence of fatty acid composition on salinity

Chromera velia grew well in a salinity range between 0.2 and 1.0 M with no significant differences in growth rate (Fig. S2). The minor FAs constituted 5% of total FA. The most abundant FAs were again palmitic acid (25–27%) and EPA (44–42%). In the salinity treatment, there were no significant differences in the contents of all fatty acids ($p > 0.05$) (Fig. 5). Cells maintained high unsaturation of lipids at all salinities (DBI 2.8–2.9). FA unsaturation reached higher levels as a result of the salinity treatment as compared to light source treatments (DBIs 1.9–2.5) or temperature (DBIs 1.8–2.5); however, the highest levels of unsaturation were observed in the cells treated with monochromatic light (DBIs up to 3.24) (Table S1 in the supplementary material).

Discussion

Although the literature regarding impacts of environmental variables on algal lipid composition is extensive, very little is known about such impacts on photosynthetic apicomplexans. The response of the lipid complement of *C. velia* to a number of environmental factors, including salinity and temperature, was investigated. The aims were to determine the optimal conditions for potential biotechnological applications and to clarify the ecology and physiology of *C. velia*. Since photosynthesis in *C. velia* shows several

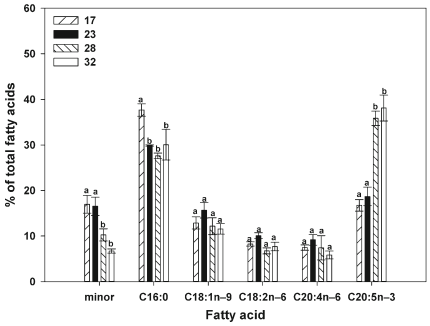

Fig. 4 Effect of four different temperatures on the fatty acid composition of cells (mean ± SD, $n = 3$) *Numbers* designate temperatures in degrees Celsius used. *Different letters* above each *column* indicate significant differences (one-way ANOVA, $p < 0.05$)

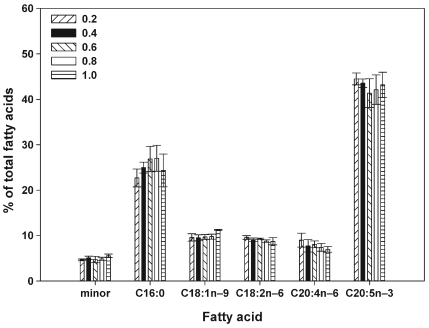

Fig. 5 Effect of different salinities of growth medium on the fatty acid composition of cells (mean ± SD, $n = 3$). Salinities are expressed in moles per millilitre. No significant differences were observed (one-way ANOVA, $p > 0.005$)

unusual features, this study attempted to assess how lipid composition changes as a function of light intensity and quality.

Light intensity

Our results show that when light intensity increased, the EPA 20:5(n–3) content of *C. velia* decreased (Fig. 2). This is not unusual and similar observations were made for *Nannochloropsis* sp. (Fabregas et al. 2004). In contrast to *Nannochloropsis*, however, the decrease in the EPA content was not accompanied by a decrease in other PUFAs: palmitic acid, for instance, was appreciably more abundant under high light conditions. Overall, a substantially higher level of saturation in *C. velia* FAs was observed, when the cells were exposed to high PFD. This increase in saturation with higher PFD also does not come as a surprise, since the polyunsaturated FAs of membrane polar lipids (such as galactolipids, in which EPA is often a major component in *C. velia*; Botte et al. 2011), under high light, often become less abundant than neutral storage lipids, which mainly contain saturated FAs (Khotimchenko and Yakovleva 2005). The fact that the content of other PUFAs did not decline with exposure to high light is also interesting: Klyachko-Gurvich et al. (1999) suggested that PUFAs are necessary to maintain photosynthetic membrane activity and play an important role in acclimation to growth at low irradiance. This does not seem to be the case for *C. velia* which had increased growth under low light (15 μmol photons m^{-2} s^{-1}) compared at high light (200 μmol photons m^{-2} s^{-1}) (Quigg et al. 2012).

Light quality

No major difference in growth rates in *C. velia* grown under different monochromatic lights of low, non-saturating intensity of 20 μmol photons m^{-2} s^{-1} was observed (Fig. S2). This is in contrast to observations in other algae (Atta et al. 2013; Hultberg et al. 2014). It is interesting that in the cited works, growth of organisms of the same genus and even of the same species appear to be stimulated by different light quality. The *C. velia* pigment complement is somewhat unusual because of the high content of isofucoxanthin-like carotenoid, which can represent up to 25% of all pigments (Kotabová et al. 2011). Because of this, cells are very affective in absorbing radiations in the green region of the light spectrum. This explains why *C. velia* cultured under light-limiting intensity of monochromatic green light is capable of attaining growth rates that are not very different from those grown under red or blue light of similar intensity.

Similarly, the FA profiles of cells grown at different monochromatic lights were not significantly altered. However, cells grown in the monochromatic light regimes had higher EPA content than the cells grown under a low intensity full spectrum light. In *Chlorella vulgaris*, growth under different light qualities resulted in the same FA profiles, with the exception for green light, where C16:3 and C18:3 increased (Hultberg et al. 2014). Again, the fact that such an effect of green light was not observed in *C. velia* is most likely the consequence of the high absorptivity of *C. velia* cells in this spectral range due to the presence of isofucoxanthin-like carotenoids. The ability of *C. velia* to absorb and effectively utilize green light (and also far-red light, see Kotabová et al. 2014) suggests that it is well equipped to maximize light capture under conditions of overlaying, dense algal communities in coral reefs where chlorophyll containing members of the coral-associated community absorb the blue and red portions of sunlight. In this study, it was shown that *C. velia* isolated from scleractinian corals (Moore et al. 2008; Janouskovec et al. 2012) was capable of maintaining not only high light harvesting potential but also optimal membrane lipid composition under low intensity monochromatic light.

Temperature

Chromera velia, like other poikilotherms, must adapt to variations in external temperatures in order to survive. Although on tropical coral reefs, the water temperature is almost constant and the amplitude of diurnal and annual temperature variations is smaller than those experienced by freshwater algae in the middle latitudes, coral-associated algae are currently subject to stress from increasing temperatures due to global change.

Growth of *C. velia* was strongly dependent on temperature, with the highest growth rate at 28 °C. *Chromera velia* shows an increase both of double bond index and of average chain length when cells were grown at higher temperature (Fig. 6). It has been demonstrated in several studies that high EPA

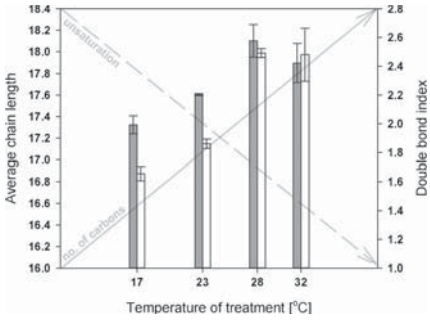

Fig. 6 Average chain length (*grey bars*) and double bond index (*white bars*) (mean ± SD, $n = 3$). *Grey arrows* designate the common trends in fatty acid unsaturation and chain length upon temperature shift

content occurs under optimal growth conditions (Cohen et al. 1988; Klyachko-Gurvich et al. 1999). *Chromera velia* cells had an increased proportion of EPA (50% at 32 °C). Usui et al. (2012) showed that *Shewanella violacea* had a substantial level of EPA in its membranes, and the membranes appeared highly rigid at its nearly optimal growth temperature. This result countered the generally accepted concept that greater fluidity is a membrane characteristic of microorganisms that inhabit cold environments. Usui et al. (2012) suggested that retaining a fixed level of membrane physical property under a wide range of environmental conditions is more important than simply making the membrane more fluid. EPA, however, because of its five *cis* double bond, is strongly bent and may operate as a rigidifying agent of the membrane, possibly mitigating the effect of the increase in FA unsaturation. Both EPA and docosahexaenoic acid (DHA) are constituents of several types of cellular membranes and are thought to be involved in specific biological functions that require conformational changes of membrane components. The work of Fernandes et al. (2002) indicates that docosahexaenoic acid is minimally influenced by temperature change and that it exhibits great conformational variability. The molecular dynamics simulation studies of aforementioned work on DHA and oleic acid (Fernandes et al. 2002) show that DHA is shorter and more compact than more saturated FAs. The average end-to-end distance of DHA, at 41 °C, is 0.82 nm, somewhat shorter than oleic acid which has a chain length of 1.42 nm. The shortness is even more remarkable when it is considered that it is a 22-atom molecule. The DHA chain conformation has pronounced bends and twists which reduces the separation distance between both ends (a stretched chain would have a greater end-to-end distance). As can be seen in Fernandes et al. (2002), the conformation of DHA forms a right stranded helix along the simulated trajectory, the end-to-end distance of the DHA molecule assumes values ranging from as low as 0.42 nm to as high as 1.7 nm, indicating a great structural flexibility in the molecule. The conformation of EPA is expected to be analogous to that of DHA in the lipid bilayer, although the structure of EPA in the membrane of *C. velia* is still to be elucidated. Acclimation to different temperatures in *C. velia* seems to depend not only on FA composition but also on the distribution of lipid polar head groups within the membranes or adjustment of the protein/lipid ratio (Morgan-Kiss et al. 2006).

Salinity

The ability to adapt to changing osmotic conditions is a prerequisite for all cellular life. Lee et al. (1989) proposed that high FA content may interfere with the movement of solvent molecules across the cell membrane and more PUFA in higher salinity environments would produce more fluid membranes and thus could assist in the prevention of water loss from the cell. Renaud and Parry (1994) showed that this indeed occurs in *Isochrysis* sp. and *N. oculata*, but is not true for *Nitzschia frustulum*. In *C. velia*, no significant difference in growth, in FA composition or in the unsaturation of FA over our experimental salinity range was observed. Useful comparisons between our findings and published data are difficult due to the sparsity of studies and the variability in experimental conditions between studies, as well as some contradictory results. In *Isochrysis* sp. grown under elevated salt concentration, the amount of C18 and C20 PUFAs increased (Ben-Amotz et al. 1985); in contrast, Renaud and Parry (1994), also using *Isochrysis* sp. and under the same conditions, reported a decrease in C18:5 and DHA. A lower degree of unsaturation was reported for *Dunaliella* sp., *Nannochloropsis* sp. and *N. frustulum* grown at high NaCl (Renaud and Parry 1994; Xu and Berdall 1997; Hu and Gao 2006). The effect of NaCl concentration on EPA synthesis is species-specific and even strain-specific (Sukenik 1991). Lee et al. (1989) stated that, at 0.7 M NaCl, *Porphyridium purpureum* grew better and had a higher EPA content than at 0.45 M. On the other hand, Pal et al. (2011) showed that *Nannochloropsis salina* contained more EPA at lower salinity of 13 g L^{-1} than at 40 g L^{-1}. Hu and Gao (2006) observed that, in *Nannochloropsis* sp., an increase of salinity led to a decrease of EPA and linolenic acid, while arachidonic acid was kept constant. Similarly, when *Dunaliella salina* was transferred from 0.4 M up to 4 M NaCl, monounsaturated and saturated FAs increased, with a concomitant decrease of PUFAs (Takagi and Yoshida 2006). A salinity increase led to a higher biomass of *Botryococcus braunii* oleic acid content, while both linolenic and docosaenoic acids decreased (Rao et al. 2007). Observations made from *Phaeodactylum tricornutum* (Yongmanitchai and Ward 1991), where the EPA content was constant in cells grown at 0–5‰ salt and which were slightly decreased when salt increased above 12‰, are in agreement with our results.

In conclusion, our study provides the first detailed analysis of the FA composition of membrane lipids in alga *C. velia*, a representative of the recently discovered division of red algal secondary endosymbionts the Chromeridae. The adaptation of membrane lipids to long-term environmental changes in light intensity, light quality, temperature and salinity was studied. The results show that in several aspects, the response of *C. velia* to these variations is different from standard algal models studied so far. Since *C. velia* accumulates significant amounts of potentially commercially interesting fatty acids, these data help in setting up optimal growth conditions for possible biotechnological applications.

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PAPER III

The effect of light quality and quantity on carbon allocation in *Chromera velia*.

Lukeš M., Giordano M. and Prášil O.

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ORIGINAL ARTICLE

The effect of light quality and quantity on carbon allocation in *Chromera velia*

Martin Lukeš [1,2] · Mario Giordano [1,3] · Ondřej Prášil [1,2]

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Abstract
Chromera velia is a marine photosynthetic relative of human apicomplexan parasites. It has been isolated from coral reefs and is indicted for being involved in symbioses with hermatypic corals. *C. velia* has been subject to intensive research, but still very little is known of its response to light quality and quantity. Here, we have studied the growth and compositional responses of *C. velia* to culture under monochromatic light (blue, green or red), at two photon flux densities (PFD, 20 and 100 μmol photons m^{-2} s^{-1}). Our results show that *C. velia* growth rate is unaffected by the quality of light, whereas it responds to PFD. However, light quality influenced cell size, which was smaller for cells exposed to blue monochromatic light, regardless of PFD. PFD strongly influenced carbon allocation: at 20 μmol photons m^{-2} s^{-1}, carbon was mainly allocated into proteins while at 100 μmol photons m^{-2} s^{-1}, carbon was allocated mainly into carbohydrate and lipid pools. The blue light treatment caused a decrease in the lipids and carbohydrates to proteins and thus suggested to affect nitrogen metabolism in acclimated cells. Whole-cell absorption spectra revealed the existence of red-shifted chlorophyll *a* antenna not only under red light but in all low PFD treatments. These findings show the ability of *C. velia* to successfully adapt and thrive in spectrally very different environments of coral reefs.

Introduction

Chromera velia is a photosynthetic relative of apicomplexan parasites, belonging to the phylum Chromeridae (Moore et al. 2008; Oborník et al. 2011). As opposite to the heterotrophic apicomplexans, whose vestigial chloroplast has lost its photosynthetic function, chromerids are equipped with functional chloroplasts, which share many traits with chloroplast of algae of the "red lineage" (Janouskovec et al. 2010). This organism has proved peculiar and intriguing in many respects as *C. velia*'s chloroplast does not contain chlorophyll *c*, it possesses the primitive form II of RuBisCO (Janouskovec et al. 2010). *C. velia* has a highly effective non-photochemical quenching (NPQ) mechanism based on unusually fast violaxanthin de-epoxidation (Kotabova et al. 2011). Also uses a unique mechanism of photoprotection based on combination of photorespiration and thermal energy dissipation via NPQ quenching (Quigg et al. 2012), and it contains plant like lipids (Botte et al. 2011; Leblond et al. 2012) and is able to produce significant amounts of polyunsaturated fatty acids (Lukeš et al. 2017). *C. velia* was assumed to be a coral symbiont similar to dinoflagellate zooxanthellae (Moore et al. 2008; Oborník et al. 2011); however, Cumbo et al. (2013) showed that *C. velia* is not an obligate coral symbiont but could be transmitted from adult *Montipora digitata* to its eggs. *C. velia* is equipped with a novel red-shifted chlorophyll *a* antenna complex (Kotabová et al. 2014), which is compatible with life within the densely populated environment of shallow-water corals, where available light spectrum is poor of visible wavelength but enriched in far-red radiations (Magnusson et al. 2007). Mohamed et al. (2018) suggested a parasitic relationship between *C. velia* and the coral because of coral's

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✉ Martin Lukeš
lukesm@alga.cz

[1] Centrum Algatech, Laboratory of Photosynthesis, Institute of Microbiology ASCR, Opatovický mlýn, 379 81 Třeboň, Czech Republic

[2] Faculty of Sciences, University of South Bohemia, Branišovská 31, 370 05 České Budějovice, Czech Republic

[3] Department of Life & Environmental Sciences, Università Politecnica delle Marche, Via Brecce Bianche, 60131 Ancona, Italy

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transcriptomic response to *C. velia*. Observations by Janouskovec et al. (2013), who found association of *C. velia* with macroalgae living in the proximity of corals, and more recent work by Mathur et al. (2018) suggested that *C. velia*, although an inhabitant of coral reefs, is unlikely to be a coral symbiont but may be symbiotically associated to other reef-dwellers. *Chromera* may also be part of microbial processes in biogenous sediments and contribute to reef primary production, nutrient cycling, and maintenance of overall coral reef health as a free-living, mostly benthic organism (Mathur et al. 2018).

Whether benthic, epiphytic, or symbiotic, *C. velia* lives in environments subject to substantial heterogeneity in the light field, both with respect to irradiance and to the spectral composition. The spectrum of light below the canopy is enriched in green and in far-red light (Salles et al. 1996). Magnusson et al. (2007) showed that the light field inside of shallow-water coral is enriched in near-infrared radiations and depleted in visible light. The spectral quality of light is known to influence not only the composition and stoichiometry of light-harvesting antennae but also cell growth, cell biochemical composition, pigment content, and photosynthetic rate of various algal species (Voskresenskaya 1972; Humphrey 1983; Sanchez-Saavedra and Voltolina 1994, Miao et al. 2012; Das et al. 2011; de Mooij et al. 2016; Vadiveloo et al. 2015; Humphrey et al. 2018). Since *C. velia* has been shown to perform chromatic acclimation of its light-harvesting complexes (Kotabová et al. 2014; Bína et al. 2014), we thus hypothesize that *C. velia* has also developed differential cellular and metabolic responses to light quality.

Materials and methods

Culture conditions

Cells were grown in 100-mL Erlenmeyer flasks, at 25 °C, in artificial seawater medium with f/2 nutrients. The light was provided at a photon flux density (PFD) of either 20 (LL) or 100 μmol photons m^{-2} s^{-1} (HL) with a 12:12 light/dark photoperiod. Light intensities were measured using a calibrated quantum meter (Li-189, Li-Cor, USA) equipped with a 2π quantum sensor. Light was provided from the bottom of the flasks to achieve light homogeneity. PFD was adjusted by means of potentiometer and neutral density filters (Lee Filters). The culture thickness (path length) was 1.6 cm. The light sources were monochromatic LEDs of different colors: (a) blue 462 nm (BL), (b) green 513 nm (GL), (c) red 630 nm (RL), (d) warm white (WL) LEDs were used for the control treatment (Supplementary material S.1). Cultures were continuously shaken on a rotary shaker at 100 rpm. The cultures were acclimated to these conditions for not less than 10 generations, prior to any measurements. Experimental cultures were initiated by inoculating acclimated cells at concentrations of 0.5–1.0 × 10^6 cells/mL into 100 mL Erlenmeyer flask. Cell counts were carried out every 3 days, for a period of 14 consecutive days. Cell counts together with cell size measurements were carried out on Multisizer 4 (Beckmann Coulter) equipped with 50 μm aperture. All measurements were conducted on three independent cultures for each treatment. For compositional analyses, cells were inoculated at a concentration of 1 × 10^6 cells/mL and collected after 6 days since inoculation. Cells were aliquoted (1 mL for each analysis), pelleted by centrifugation (6000 g for 15 min at 4 °C); the pellet was washed once with deionized water and then frozen at − 80 °C until further analysis.

Carbon allocation

Carbon allocation was studied by Fourier transform infrared spectroscopy (FTIR). The frozen pelleted cells were resuspended in 20 μL of distilled water and deposited on a Si 384-well plate (10 μL per spot). The Fourier transform spectra were obtained with a Nicolet IS10 (Thermo Nicolet) spectrometer equipped with a microarray reader with DTGS (deuterated tri-glycine sulfate) detector. Absorbance spectra were collected in the spectral range from 400 cm^{-1} to 4000 cm^{-1}, at a spectral resolution of 4 cm^{-1}; 64 scans were averaged. A Blackman-Harris three-term apodization function was used, with a zero-filling factor of 2. Omnic software (Nicolet) was used for measurement and data processing. Absorption bands were assigned according to Giordano et al. (2001): the 1740 cm^{-1} line to C=O stretching of lipids, 1640 cm^{-1} line to vibration of N-H of Amide I, 1545 cm^{-1} to C=O of Amide II and 1150 cm^{-1} to C–O of carbohydrates. The results were expressed as ratios of absorption maxima of the bands corresponding to the main organic pools (i.e., proteins, lipids, and carbohydrates).

C/N analysis

For the determination of carbon and nitrogen cell ratios (C/N), 1 mL of the frozen aliquot was freeze-dried, weighed, and transferred into tin capsules and analyzed with a CN Elemental Analyzer (Perkin Elmer 2400, Series II) as described in Nelson and Sommers (1996). Blanks were obtained from measurements of empty tin capsules and were subtracted from the raw values of the samples.

UV/VIS absorption spectra of the whole cell

Absorption spectra were measured immediately after cell harvest in a glass cuvette, using a Unicam UV 550 spectrophotometer (Thermo Spectronic, Cambridge, UK) equipped with an integrating sphere. Absorbance was recorded with a scan rate of 30 nm/min with a 4-nm detection bandwidth.

Statistics

All measurements were carried out on three independent replicates; data are shown as mean ± standard deviation. Significant differences in growth rates, cell sizes, lipid-to-protein ratios, carbohydrate-to-protein ratios, and carbon to nitrogen contents were compared using one-way analysis of variance (ANOVA) followed by the post hoc test of Holm-Sidak. The significance level was always set at $p < 0.05$. All statistical analysis was performed using SigmaPlot version 12.5.

Results

Effect of light quality and PFD on the growth rate and cell size

Growth rates were only marginally affected by the light quality, and no significant difference ($p > 0.05$) among the light quality treatments was observed. Instead, different PFD elicited major changes in growth rates (Fig. 1a). Cells grown at low light (20 μmol photons m^{-2} s^{-1}), regardless of the color of the LED source, maintained their specific growth rate in a range between 0.11 and 0.13 per day; at high light, the specific growth rates were significantly ($p < 0.05$) higher (from 0.16 to 0.18 per day) (Fig. 1a).

Cell size was between 110 and 111 μm^3, in most treatments. The only exception was cells grown under blue light 20 μmol photons m^{-2} s^{-1}, which were significantly smaller (81 ± 8 μm^3) than in all other conditions (Fig. 1b).

Effect of light quality and PFD on carbon allocation

Cells grown at lower PFD (20 μmol photons m^{-2} s^{-1}) had a low lipid to protein ratio of 0.12 ± 0.02; in blue light, the lipid to protein ratio was lower (0.08 ± 0.02) (Fig. 2; Table 1). Under all light qualities, the carbohydrate to protein ratios were between 0.25 and 0.32. At higher PFD (100 μmol photons m^{-2} s^{-1}), cell composition was significantly different ($p < 0.05$) (Fig. 2): lipid-to-protein ratios increased to 2.6-, 2.8-, 3.3-, and 3.6-fold for blue, red, green, and white light, respectively. The carbohydrate-to-protein ratio did not change for blue light but increased 1.5-fold for red light-treated cells, 1.96-fold for cells grown under green light and 2.16-fold when cells grew under white light (Fig. 2, Table 1).

Effect of light quality and PFD on C/N ratios of the cells

Cells grown at low PFD (20 μmol photons m^{-2} s^{-1}) showed a C/N ratio of 4.75–5.25 [mg/mg], with no significant differences among spectral variants ($p > 0.05$) (Fig. 3). At high PFD (100 μmol photons m^{-2} s^{-1}), the C/N ratio of cells was higher by 1.6–2.2-fold, suggesting that at high irradiance, more carbon was allocated to N-deprived pools such as carbohydrates and lipids. White light-treated cells at high PFD had the highest C/N ratio (Fig. 3).

Effect of light quality and PFD on the absorption spectra of whole cells

Figure 4 shows the absorption spectra of whole cells in the region of photosynthetic active radiation (PAR) (350–750 nm), grown at the four light qualities. In cells grown at 20 μmol photons m^{-2} s^{-1}, regardless of growth light quality, cells absorbed less in the region of the chlorophyll Soret band (350–450 nm). A new absorption band appeared in the infrared spectral region (705–715 nm) to a lesser account in blue and got more pronounced in the order green, white, and red light (see inset of figures of difference spectra of LL to HL (Fig. 4).

Discussion

Marine organisms are subject to large heterogeneity in the light field, both with respect to irradiance and to spectral composition (Kinzie et al. 1984; Salles et al. 1996; Hochberg et al. 2003; Wijgerde et al. 2014). The ability to effectively use different light quality and quantity, especially at light-limiting conditions, determines the outcome of competition

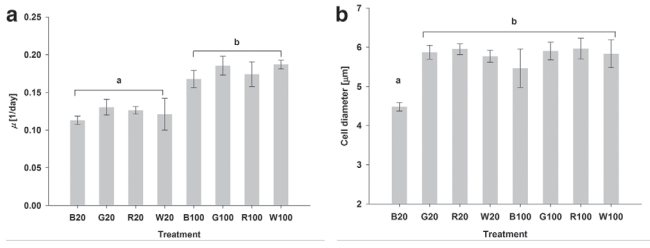

Fig. 1 a Specific growth rate (μ). **b** Diameter of cells acclimated to different quality: blue, green, red, and white, at two different PFDs: 20 and 100 μmol photons m^{-2} s^{-1}. The error bars represent the standard deviations ($n = 3$). Different letters on the bars denote significantly different mean values ($p > 0.05$)

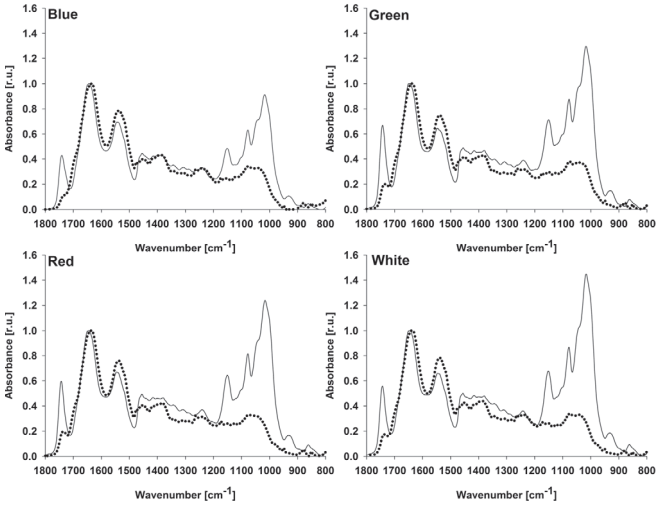

Fig. 2 Representative FTIR absorption spectra of cells acclimated to different quality: blue, green, red, and white, at two different PFDs: 20 (dotted line) and 100 μmol photons m^{-2} s^{-1} (solid line); FTIR spectra were normalized to the Amide I absorption peak at 1645 cm^{-1}

at the lower limit of vertical zonation of benthic algae. In nature, *C. velia* may be associated with scleractinian corals as a symbiont (Moore et al. 2008; Janouskovec et al. 2013) or more likely as a parasite (Mohamed et al. 2018). The symbiotic/parasitic habitus requires the ability to make use of the light that is attenuated and modified in quality by the absorption by the host tissue and other pigmented microorganisms (Magnusson et al. 2007). Our data show that *C. velia* is well adapted to cope with a broad spectral range of visible radiation. The ultimate consequence of this is the relatively flat response of its growth to light quality. On the contrary, light quantity appreciably influenced growth (Fig. 1a).

Responses to light quantity

The growth rates of *C. velia* at 100 μmol photons m^{-2} s^{-1} were double than those at 20 μmol photons m^{-2} s^{-1}, regardless of the color of the light. Our results are partially in agreement with the work of Kinzie et al. (1984) for *Symbiodinium microadriaticum*; these authors reported a stimulation of *Symbiodinium microadriaticum* growth with increasing irradiance. Differently from us, however, Kinzie et al. (1984) observed that *S. microadriaticum* prefer blue and white light to green and red light. Work of Wijgerde et al. (2014) reported more complicated dependence: the growth of *Symbiodinium*

Table 1 FTIR ratio of the three main organic pools of *Chromera velia* cells. L/P, lipid to protein ratios; L/C, lipid to carbohydrate ratios; C/P, carbohydrate to protein ratios. The values in parentheses indicate the standard deviations ($n = 3$). Ratios were calculated from the absorption maxima of characteristic vibrations of proteins at 1640 cm^{-1}, lipids at 1745 cm^{-1}, and carbohydrates at 1155 cm^{-1} of spectra shown in Fig. 2

Treatment, PFD [μmol photons m^{-2} s^{-1}]	FTIR pool ratios		
	Lipid/protein	Lipid/carbohydrate	Carbohydrate/protein
Blue light, 20	0.08 (0.02)	0.31 (0.14)	0.32 (0.11)
Green light, 20	0.12 (0.02)	0.46 (0.12)	0.26 (0.03)
Red light, 20	0.12 (0.02)	0.43 (0.08)	0.29 (0.06)
White light, 20	0.12 (0.02)	0.49 (0.13)	0.25 (0.03)
Blue light, 100	0.21 (0.06)	0.61 (0.16)	0.35 (0.07)
Green light, 100	0.40 (0.07)	0.79 (0.17)	0.51 (0.04)
Red light, 100	0.34 (0.07)	0.75 (0.17)	0.45 (0.02)
White light, 100	0.44 (0.01)	0.83 (0.15)	0.54 (0.11)

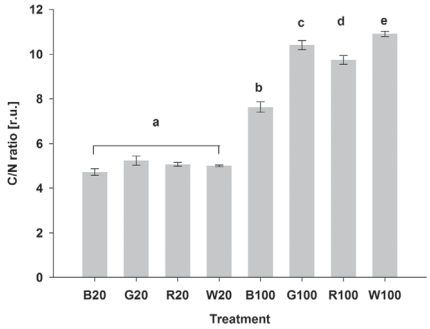

Fig. 3 C/N ratios of cells grown at different quality: blue, green, red and white lighT, at two different PFDs: 20 and 100 μmol photons m^{-2} s^{-1}. The error bars show the standard deviations ($n = 3$). Different letters identify statistically different means ($p > 0.05$)

sp. was higher for blue light at high irradiance (256 μmol photons m^{-2} s^{-1}), but red stimulated growth more at lower irradiance (128 μmol photons m^{-2} s^{-1}). Blue light-treated cells were significantly smaller if grown under low PFD (20 μmol photons m^{-2} s^{-1}) (Fig. 1b), this is in contrast with the work of Koc et al. (2013) where cells of *Chlorella kessleri* grown under blue light illumination were significantly bigger than on other treatments. Also, *Chlamydomonas reinhardtii* cells were bigger under blue light in comparison to red light (de Mooij et al. 2016) as the blue light is known to delay cell division; thus, cells tend to grow in size. The opposite was shown for a red light, where cells undergo a division cycle when they have achieved the minimal cell size required for division (de Mooij et al. 2016). The consequence is that, compared to white light, average cell size is larger in blue light and smaller under red light (Kubín et al. 1983; Wilhelm et al. 1985). Our results show that increased energy availability allows for a greater allocation of C into N-deprived pools (lipids and carbohydrates), which is also reflected by an increase of the C/N ratio (Fig. 3). The first-order interpretation of this is that higher light stimulates CO_2 fixation, but not necessarily an equal stimulation of N assimilation. In fact, this is possibly also related to the environmental stoichiometry of C and N. Kaffes et al. (2010) showed that a change of C assimilation is followed by an equal change in the assimilation of N, if the light is sufficient and both C and N are not limiting. The impact of C/N stoichiometry on C allocation was demonstrated by Pierangelini et al. (2017), for the dinoflagellate *Protoceratium reticulatum*. Comparison of *C.velia* to dinoflagellates is very convenient, as they are both phylogenetically related and belong to the same superphylum the Alveolates (Moore et al. 2008). At the same time, Palmucci et al. (2011) showed that the responses to changes in the C and N availability are rather strongly species specific. The impact of light limitation on C allocation in comparison to N limitation was described and discussed by Ruan and Giordano (2017) and Ruan et al. (2017) , who showed that the availability of light (= energy) constitute a decisive factor in the way cells allocate their nutrients (especially N). The sometimes insufficient definition of culture conditions and limitations may be at the origin of the heterogeneous results

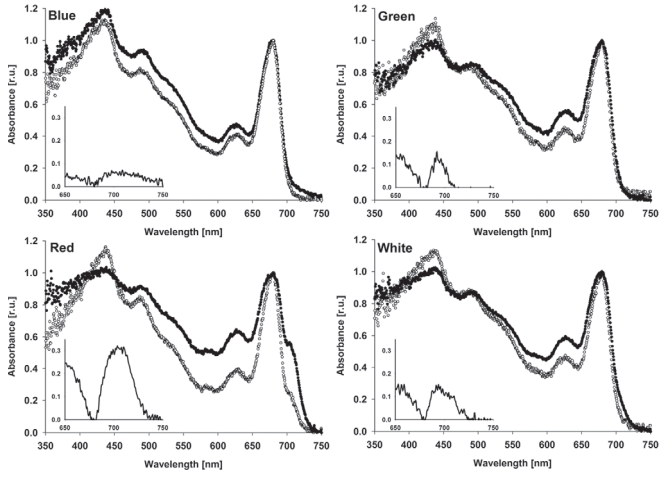

Fig. 4 Representative UV/Vis absorption spectra of cells of different quality: blue, green, red, and full spectrum white. At two different PFDs: 20 (full circles) and 100 μmol photons m^{-2} s^{-1} (empty circles). Insets show difference spectra of low light versus high light grown cells (20–100 μmol photons m^{-2} s^{-1}) in the range of 650–750 nm

present in the literature. For instance, carbohydrate content was reported to both increase (e.g., in the diatom *Chaetoceros protuberans*; Gostan et al. 1986) and decrease (e.g., in the diatom *Thalassiosira rotula* and in the green alga *Dunaliella tertiolecta*; Rivkin 1989) in response to an increase of irradiance. In Rivkin (1989), protein could be negatively affected by irradiance or not at all (Sukenik and Wahnon 1991).

Responses to light quality

In our study, the cells grown under BL showed the greatest differences in cellular composition in response to light quality. It appeared that carbon assimilation efficiency in *C. velia* is lower at BL than at other light qualities tested (Fig. 2; Table 1). Protein enhancement and a concomitant reduction in carbohydrate, whereas lipids stayed unchanged, are in agreement with reports for diatoms (Gostan et al. 1986; Sanchez-Saavedra and Voltolina 1994), macroalgae (Korbee et al. 2005), and prymnesiophyte *Isochrysis galbana* (T-iso) (Marchetti et al. 2013). Rivkin (1989) observed in *Dunaliella tertiolecta* and *Thalassiosira rotula* that BL allows higher photosynthetic carbon incorporation into protein than white light. According to Zhou et al. (2009), the protein increase in BL may be related to the enhancement of light collection system, the structural protein of PSII (Miyachi et al. 1978). As this phenomenon was not conclusively demonstrated, it should be treated with caution. More compelling is the fact that some enzymes of nitrogen metabolism are directly influenced by BL. Azuara and Aparicio have shown that the isolated inactive form of nitrate reductase of *Chlamydomonas reinhardtii* can be reactivated by BL (Azuara and Aparicio 1983) as well as that in green alga *Monoraphidium braunii*, BL was activating at least three key steps of inorganic nitrogen metabolism, namely, cell uptake of both nitrate and nitrite, nitrate reductase activity, and biosynthesis of nitrite reductase (Aparicio and Quiñones 1991). Similarly, blue light mainly stimulated the accumulation of nitrogen compounds in the form of soluble protein and phycobiliproteins in red alga *Halymenia floresii* (Godínez-Ortega et al. 2007). The activation of nitrate reductase in diatom *Phaedactylum tricornutum* was demonstrated by Coesel et al. (2009). *C. velia* cells in this study acclimated to both PFDs of 20 and 100 μmol photons m^{-2} s^{-1} of BL had much higher protein than lipid and carbohydrate content than in other color treatments (Fig. 2; Table 1). Similarly, Jungandreas et al. (2014) have shown that BL-acclimated cells of *Phaeodactylum tricornutum* had significantly higher protein but decreased the concentration of carbohydrates in comparison to RL-acclimated cells. This led to a significant difference in the C/N ratio of BL to RL. These results clearly indicate that BL had higher N assimilation activity. Also, these authors hypothesized that photoreceptors might regulate the activity of key enzymes of C and N assimilation together with regulation of gene transcription (Jungandreas et al. 2014). In photoacclimation and photoregulatory processes, flavins might play a role as photosensitizers (Aparicio and Quiñones 1991). Recently, it was shown the existence of a molecular link between photoreception, photosynthesis, and photoprotection in the green alga *Chlamydomonas reinhardtii* via blue light perception by flavin-containing LOV domains on PHOT, LHCSR3 induction through PHOT kinase, and light dissipation in photosystem II via LHCSR3 (Petroutsos et al. 2016). As *C. velia* does have LHCSR3 homolog (Niyogi and Truong 2013), we tend to speculate that *C. velia* might have similar BL-sensing mechanism as *Chlamydomonas reinhardtii*. Algae living in close association with coral usually exhibit a red shift of absorption of their antenna complexes to increase light-harvesting potential of photosystem II under conditions where the intensity of PAR around 680 nm is absorbed by the overlying layers of phototrophs (Trissl 1993). The problem of shading is boosted by the fact that water itself is a strong absorber of longer wavelength light above 700 nm. Organisms adapted to this kind of conditions use very narrow window of absorption around 710 nm such as *Acaryochloris marina* that used unique chlorophyll d (Chen and Blankenship 2011), red-shifted chlorophylls in light-harvesting complexes of *Ostreobium* sp. or *Phaeodactylum* and *Nitzschia* (Fork and Larkum 1989; Koehne et al. 1999; Serodio et al. 1997). Our results agree with findings of Kotabová et al. (2014) which showed that *C. velia* thanks to the utilization of novel red-shifted antenna complex red CLHc is able to capture light in the far-red region of light spectra. In this work, we show that red-shifted antenna complex red CLHc are probably sensed and utilized not only at low RL but generally at low light conditions regardless of the color of light (Fig. 4).

C. velia ecology is still speculative. It may be tied to the microbial processes in biogenous sediments and contribute to reef primary production, nutrient cycling, and maintenance of overall coral reef health. In this work, we show that *C. velia* is able to adapt and thrive in spectrally very different environments that define the life at the coral reef.

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Curriculum vitae

Martin Lukeš

(*1980, České Budějovice)

Academic achievements

2011 – 2019 Ph.D. at Department of Experimental Plant Biology, Faculty of Science, University of South Bohemia in České Budějovice; (interrupted in 2015-2017)

1999 – 2005 M.Sc. at Department of Biology, Chemistry, Pedagogical Faculty of West Bohemia University in Plzeň

Professional possitions

2002 – 2004	technician at the Institute of Soil Biology, ASCR in České Budějovice
2005 – 2010	research assistant at the Institute of Physical Biology, Faculty of Science, University of South Bohemia in České Budějovice
2011 –	research assistant at the Laboratory of Photosynthesis, Department of phototrophic microorganisms – Algatech, Institute of Microbiology, Academy of Sciences

Profesional Experience

2002-2004	participation on europaean project COBRA („Cryopreservation of a vital europaean scientific and biotechnological resource: microalgae and cyanobacteria“, EU, Framework 5)
2009	Short research fellowship, Biological Research Centre Institute of Plant Biology, Hungary (hosted by Z. Gombos)
2011	Short research fellowship, University in Leipzig, Institute of Biology, Department of Plant Physiology, Germany (hosted by H. Wagner)
2012	Short research fellowship, Oregon State University, Department of Microbiology, USA (hosted by K. H. Halsey)
2015	Research fellowship, University of Tsukuba, Faculty of Life and Environmental Sciences, Japan (hosted by Y. Shiraiwa)

Training courses

2011 TQAnalyst training – NICOLET

2012 Measurement of Vibrational Spectra

2012 Interpretation of Vibrational Spectra

Publications

Lukeš M, Giordano M, & Prášil O (2019) The effect of light quality and quantity on carbon allocation in *Chromera velia*. Folia Microbiologica. doi:10.1007/s12223-019-00734-y

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lksmrt@gmail.com

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University of South Bohemia in České Budějovice
Faculty of Science
Branišovská 1760
CZ-37005 České Budějovice, Czech Republic

Phone: +420 387 776 201
www.prf.jcu.cz, e-mail: sekret-fpr@prf.jcu.cz