The effect of shock freezing on physiological properties and consequent growth of Antarctic filamentous (*Stigeoclonium* sp.) and coccal alga (*Diplosphaera chodatii*) on agar plates

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Abstract

In this study, we investigated the effects of shock freezing on physiological properties and consequent growth of in the Antarctic alga *Stigeoclonium* sp. and comparative coccal alga *Diplosphaera chodatii* on agar plates. Culture of algae grown in liquid medium were used to study subzero temperatures on the species resistance to shock freezing. Then, microalgae were frozen in liquid nitrogen and inoculated on BBM agar after thawing. Physiological status of algae was evaluated by chlorophyll fluorescence parameters during 28 days. The results showed that interspecific differences existed in their tolerance to shock freezing, as well as their consequent growth rate on agars. Direct effects of freezing in liquid nitrogen was demonstrated in chlorophyll fluorescence parameters recorded immediately after thawing the samples (in liquid medium). In spite of the fact that majority of cells was destroyed by shock freezing, the potential of photochemical processes in PS II (F_V/F_M) remained constant in *D. chodatii*. It may indicate high resistance of the species to freezing/thawing cycles and a capability of the surviving cells, core chlorophylls in PS II respectively, to perform photosynthetic processes related to PS II. Contrastingly, *Stigeoclonium* sp. showed a shock freezing-dependent decrease in F_V/F_M. When shock-frozen, thawed and inoculated on agar plates, the culture of *D. chodatii*, and *Stigeoclonium* sp. showed cultivation time-dependent increase in chlorophyll fluorescence parameters (F_V/F_M, F_S).

Key words: Antarctica, cold stress, cryoresistance, algal cultures, biotechnologies

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Symbols and abbreviations: BBM – Bold's Basal Medium, ChF – chlorophyll fluorescence, KK – Kautsky kinetics of chlorophyll fluorescence, F_V/F_M – potential yield of photochemical photosynthetic processes in PS II, F_S – steady-state fluorescence, PS II - photosystem II, R_{Fd} - relative fluorescence decline (vitality ratio), qF_0 - quenching of background chlorophyll fluorescence

Introduction

It is well established that algae from the polar regions exhibit high cryoresistance. Typically, they have the ability to survive and cope well with repetitive freezing/ thawing cycles. The ability and underlying mechanisms have been studied in polar terrestrial algae (*e.g.* Elster et al. 2008, Orekhova et al. 2018) and chlorolichens (*e.g.* Haranczyk et al. 2017). Several aspects of algal cryoresistance have been studied within the last few decades. Main emphasis has been given to their survival capacity under different freezing conditions (Hájek et al. 2012). In our recent study, we focused on photosynthetic parameters in two algae from James Ross Island, where long-term research of photosynthesis (both field and laboratory-based experiment) has been carried out since 2007. We addressed experimental freezing effects on *Diplosphaera chodatii* and *Stigeoclonium* sp. in order to prove cryoresistance of the two species.

Diplosphaera chodatii Bialosuknia (Bialosuknia 1909), is a unicellular green alga that is within the family Trebouxiophyceae (Thüs et al. 2011). It is a symbiotic alga of the lichens of genus *Dermatocarpon.* General features of *D. chodatii* has been reviewed by Doering (2017) recently. The species is a single-celled green alga growing also on rock at the interface between the water and the terrestrial environment around rivers and lakes. It is considered a terrestrial species, found in soil (Flechtner et al. 1998, Lukešová and Hoffmann 1996), wooden piers (Handa et al. 2001), and deserts (Fletchtner et al. 2008). *D. chodatii* can be found free-living (Flechtner et al. 1998, Lukešová and Hoffmann 1996). As mentioned above, it forms a symbiosis with a lichen-forming fungus, *Dermatocarpon luridum* (With.) Laundon (Fontaine et al. 2013) as well. Similarly, *D. chodatii* is believed to be the photobiont partner of the lichenised ascomycete *Dermatocarpon luridum* (Řeháková 1968, Fontaine 2013). *D. chodatii* is found to be associated with a wide-variety of lichen-forming fungi in the Verrucariaceae including species of *Staurothele*, *Endocarpon* Hedw., *Dermatocarpon*, *Verrucaria*, *Normandina* Nyl. (Thüs et al. 2011).

D. chodatii is a unicellular, rod-shaped green alga (Fontaine et al. 2012). The species is closely related to members of *Stichococcus Nägeli* (Thüs et al. 2011). Phylogenetically, it is also related to *Chlorella sphaerica* Tschermak-Woess (Karbovska and Kostikov 2012). It exhibits a wide variety of morphological shapes including micareoid and globose (Coppins 1983) as well as ovular/spherical and cylindrical (Fontaine et al. 2012, Stocker-Wörgötter and Türk 1989). Individual cells have rather oval, *i.e.* not perfectly round shape. They are approximately 4 – 8 μm long by 3 – 5 μm wide (Coppins 1983, Fontaine et al. 2012, Yahr et al. 2006). Cell wall is thin. The species occurs in groups of two to four cells typically. In some cases, the cell clusters may be surrounded by a gelatinous matrix (Stocker-Wörgötter and Türk 1989). It exhibits high desiccation tolerance which helps the species to survive up to two months of desiccation (Zhang and Wei 2011).

Stigeoclonium sp. is a filamentous, branched alga found in several well-hydrated terrestrial ecosystems in subantarctic islands, Antarctica (*e.g.* Pushparaj et al. 2004), specifically at the King George

Island (Mrozinska et al. 1998, Komárek and Komárek 1999) and the James Ross Island (Caisová et al. 2009). Occurrence of *Stigeoclonium* sp. was also recorded in winters in central Europe shallow wetlands (Machová et al. 2008). It grows in wet places like small-area freshwater lakes, streams and seepages. Recently, photosynthetic processes of *Stigeoclonium* sp. (EEL-004, collection in James Ross Island, Antarctica) have been studied by the approach of light response curves of effective quantum yield (Φ_{PSII}) and oxygen evolution rate (OER) - Váczi (2018). Genus *Stigeoclonium* contains a large number of species (about 80), although some critical revisions of *Stigeoclonium* (Francke and Simons 1984, Simons et al. 1986, Caisová et al. 2011) have reduced it to much lower number. The genus *Stigeoclonium* has been extensively studied particularly in relation to its morphology, reproduction, and cytology (*e.g.* Branco et al. 2002, Skinner and Entwisle 2004, Michetti et al. 2010). Recently, selected species of *Stigeoclonium* genus are considered prospective for future biotechnologies, secondary compound production in particular, and tested (*e.g.* Liu et al. 2016a - for polyunsaturated fatty acids, Liu et al. 2016b - for large-scale biotechnologies).

In our study, we focused two aspects of physiology of two algae, one from the James Ross Island the other one from the Culture Collection of Autotrophic Organisms (CCALA, Třeboň, Czech Republic). First, it was algal resistance to subzero temperature, shock freezing in particular and, secondly, the applicability of chlorophyll fluorescence technique to monitor growth and physiological status of algal culture during cultivation on agar plates. We hypothesized that interspecific differences would exist in their resistence to shock freezing, as well as their consequent growth rate on agars.

Material and Methods

Algal material

The *Diplosphaera chodatii* (CCALA strain No. 336) and *Stigeoclonium sp.* (EEL strain No. 004) were isolated from the samples from Netolice (Czech Republic) and the James Ross Island, Antarctica. *D. chodatii* was isolated by Lukešová in 1988 and then cultivated as stock culture on Bold's Basal Medium (BBM). *Stigeoclonium* sp. was isolated from water samples taken from the Big Lachman lake (NE of the James Ross Island). Big Lachman Lake is a typical coastal shallow lake located on a terrace at an altitude of about 9 m. Daily mean temperature of the lake measured at the depth of 50 cm ranges from -22°C (July) to +10°C (December/January). The samples of *Stigeoclonium* sp. were collected from lake bottom from the depth of 30 cm. Stock cultures of *D. chodatii* and *Stigeoclonium sp.* were used for experiments addressing species resistance to shock freezing (Fig. 1). The microalgae were cultivated in a 3 N inorganic Bold's Basal liquid Medium (BBM) in 100 ml glass flasks under continuous irradiation of 15 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR). The cultivation was static, the flasks were placed on racks in a cultivation box (Liebherr FKS 5002, Germany). Cultivation temperature was set to 10°C.

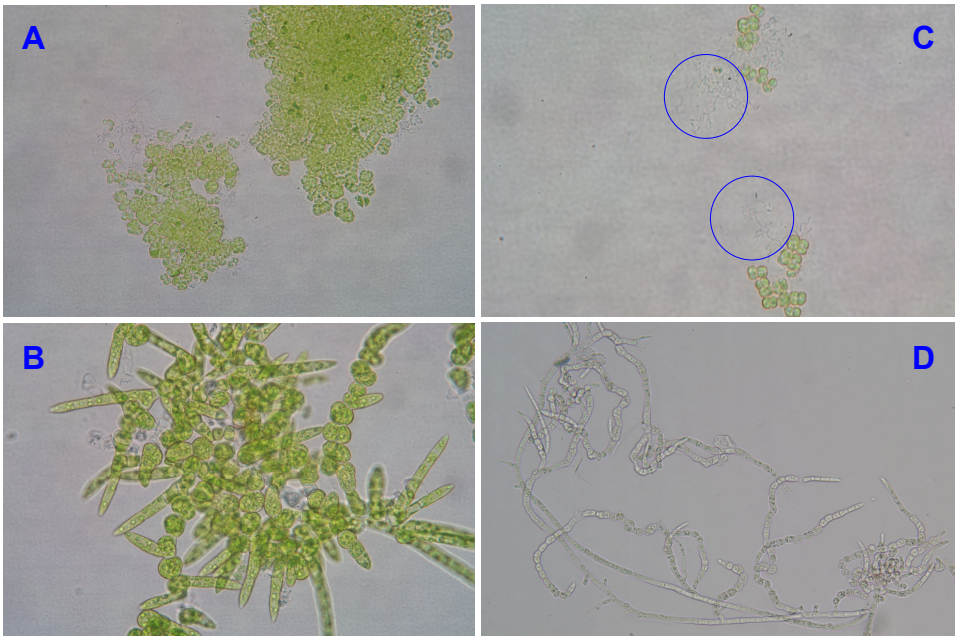

Fig. 1. Cell cultures of the experimental species *Diplosphaera chodatii* and *Stigeoclonium* sp. before (left sub-panels, A, B) and after shock freezing in liquid nitrogen (right sub-panels, C, D). The area of lysed cells in shock-frozen culture of *D. chodatii* is indicated by circles.

Experimental design

Shock freezing treatment

Experimental design and shock freezing were similar to that applied in previous study (Orekhova et al. 2018). Algal culture suspensions were pipetted into microcryovials (1.5 ml cryogenic tube with screw cap, BRAND®, Germany) and used for experimental treatments (3 replicates). Samples were frozen by immersion into liquid nitrogen (-196°C) for 10 min. Then the cryovials with frozen algae were taken away from liquid nitrogen and thawed naturally at room temperature (23°C).

After the thawing (2 h in the target temperature of 23°C), liquid inoculum (0.5 ml) was spread uniformly on an agar plate (inorganic 3 N BBM medium with a 1.5% agar, Duchefa, the Netherlands, Prod. No. 1001.1000) with a glass rod (Day 1). The samples were cultivated in Petri dishes on agar plates for the following 28 days (3 dishes per treatment). During cultivation, the samples were exposed to a temperature of 10°C and a radiation of 15 μmol m^{-2} s^{-1} PAR, photoperiod was set to 16/8 h light/dark. The growth of the culture was checked regularly by optical microscopy (Olympus BX 41, Japan).

After a week of cultivation on agar plates, the number of living cells increased so that the chlorophyll fluorescence measurements could be taken (*i.e.* chlorophyll fluorescence signal was above the detection limit of the instrument - *see* below section). Therefore, the first measurement of slow Kautsky kinetics and quenching analysis of chlorophyll fluorescence could be taken on Day 7 after the inoculation of the culture (passing shock freezing and consequent thawing) on agar plates.

Chlorophyll fluorescence measurements

Chlorophyll fluorescence parameters were measured: before the experiment (*Diplosphaera chodatii* and *Stigeoclonium* sp. in liquid culture), after shock freezing and subsequent thawing of room temperature, and repeatedly after the inoculation of frozen culture on agar and the following growth, typically in 7 days interval. The changes in the physiological state of the algae cells during each cultivation period following shock freezing were evaluated by chlorophyll fluorescence, using a HFC-010 FluorCam (Photon Systems Instruments, Czech Republic). The Petri dishes with algal cultures were dark-adapted for 5 min. and slow Kautsky kinetics supplemented with quenching analysis were measured. The method and experimental set up optimized for lichens and described elsewhere was used (*see* Trnková and Barták 2017). From the curves, particular chlorophyll fluorescence signals (*see* Fig. 4), and the following chlorophyll fluorescence parameters were evaluated: the potential yield of photochemical photosynthetic processes in PS II (F_V/F_M), and steady-state fluorescence (F_S). The chlorophyll fluorescence parameters were used to assess: (1) direct effect of shock freezing, and (2) growth of inoculum after shock freezing.

$F_V/F_M = (F_M - F_0) / F_M$		*Eqn.* 1
$R_{Fd} = (F_P - F_S) / F_S$	(Lichtenthaler et al. 2004)	*Eqn.* 2
$qF_0 = (F_0 - F_0') / F_0$	(Bilger and Schreiber 1986)	*Eqn.* 3

Table 1. The chlorophyll fluorescence parameters used in this study. Equations and resources are shown.

Results and Discussion

Direct effects of freezing in liquid nitrogen was demonstrated in chlorophyll fluorescence parameters recorded immediately after thawing the samples (in liquid medium). In spite of the fact that majority of cells was destroyed by shock freezing, the potential of photochemical processes in PS II (F_V/F_M) remained constant in *D. chodatii* (Fig. 2). It may indicate high resistance of the species to freezing/thawing cycles and a capability of the surviving cells, core chlorophylls in PS IIs respectively, to perform photosynthetic processes related to PS II. Contrastingly, *Stigeoclonium* sp. showed a shock freezing-dependent decrease in F_V/F_M. If treated with subzero temperature, typical critical point for primary photosynthetic processes related to PS II (of lichen symbiotic algae) ranges from -20°C to -25°C (Barták et al. 2007). Shock freezing brings destruction of majority of algal cells. Surviving cells, however, are capable of regrowth and reproduction and restoration of full photosynthetic capacity in terms of weeks (Orekhova et al. 2018).

Destruction of cells by shock freezing is well documented by the decrease of steady state chlorophyll fluorescence signal (F_S) which is considered an equivalent for the amount of functioning chlorophyll molecules. The shock freezing-induced decrease reached 52% (of control) in *D. chodatii*, and 51% *Stigeoclonium* sp.

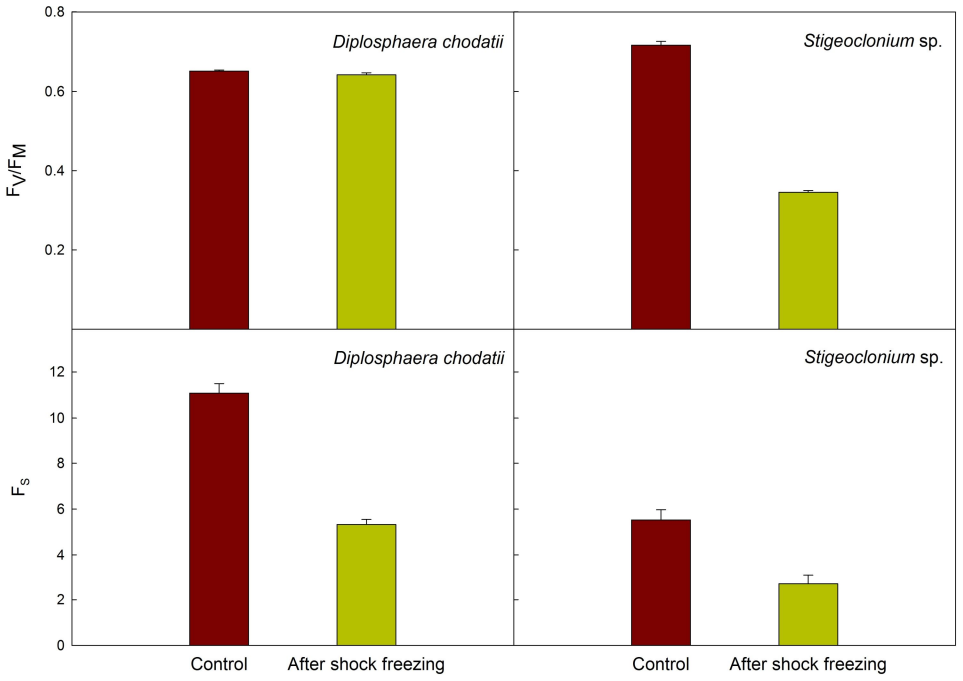

Fig. 2. Effects of shock freezing on F_V/F_M (potential yield of photochemical photosynthetic processes in PS II), and F_S (steady-state fluorescence). The two chlorophyll fluorescence parameters were recorded for control samples grown in liquid BBM medium, and then immediately after shock freezing and thawing.

The steady-state chlorophyll fluorescence (F_S) decrease is well documented in Fig. 2 that shows the effect of shock freezing. During further cultivation on agar plates, however, F_S increases with cultivation time (Fig. 3). *D. chodatii*, and *Stigeoclonium* sp. responded to shock freezing by changes in the shape of Kautsky kinetics and the values of the particular chlorophyll fluorescence signals F_M, F_P, F_S, F_M', and F_M'' (*see* Fig. 4). Shock freezing also brought a change in R_{Fd} and qF_0. R_{Fd} increased to 93% (*D. chodatii*) and 38% (*Stigeoclonium* sp.) of the initial values in the control (Fig. 5). For the two experimental species, similar increase in qF_0 was found (97 and 151%, respectively). The value of R_{Fd} increased thanks to shock freezing which is not consistent with other stressors that typically bring a decrease in R_{Fd}. The value decreases when the balance between photochemical reactions in thylakoids and the rate of enzymatic reactions occurring in stroma chloroplasts is disturbed. As a result of the exposure to various stressors, an increase in F_S is observed at a relatively constant F_M value. Thereby, the difference $F_M - F_S$ decreases and, consequently, the R_{Fd} value decreases as well (Lichtenthaler et al. 2004). Shock freezing, however, destroys majority of chlorophyll molecules which results in overall decrease in chlorophyll fluorescence signal and a change in the shape of slow Kautsky kinetics. Therefore, the recorded changes in R_{Fd} induced by shock freezing are not comparable to the regulatory changes caused by other factors. The shock freezing-induced change in qF_0, however, indicates some alternations in functioning of light harvesting complexes.

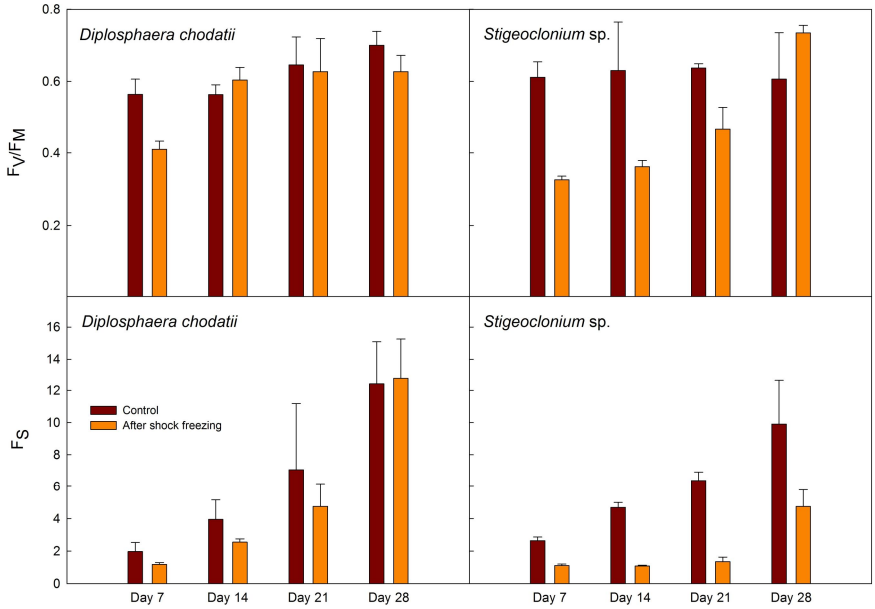

Fig. 3. Chlorophyll fluorescence parameters F_V/F_M and F_S recorded during the growth of the shock-frozen cultures of *Diplosphaera chodatii* and *Stigeoclonium* sp. on agar plates for 28 days.

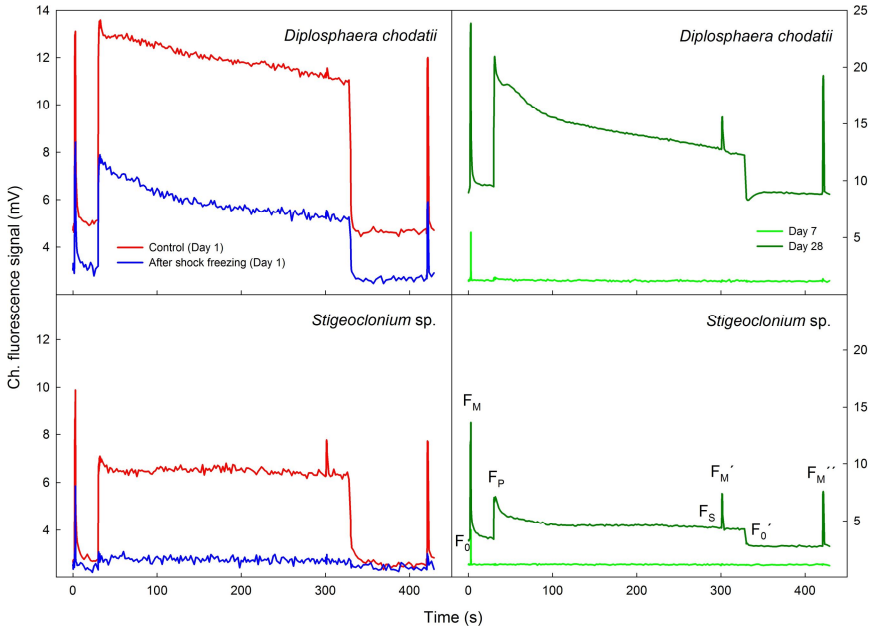

Fig. 4. Changes in the shape of slow Kautsky kinetics of chlorophyll fluorescence recorded in algal cultures of *Diplosphaera chodatii* and *Stigeoclonium* sp. grown in liquid BBM medium (left panels, the effect of shock freezing). The Kautsky kinetics recorded in the beginning (Day 7) and the end (Day 2 of cultivation of the algae on agar plates. Particular chlorophyll fluorescence signals are indicated.

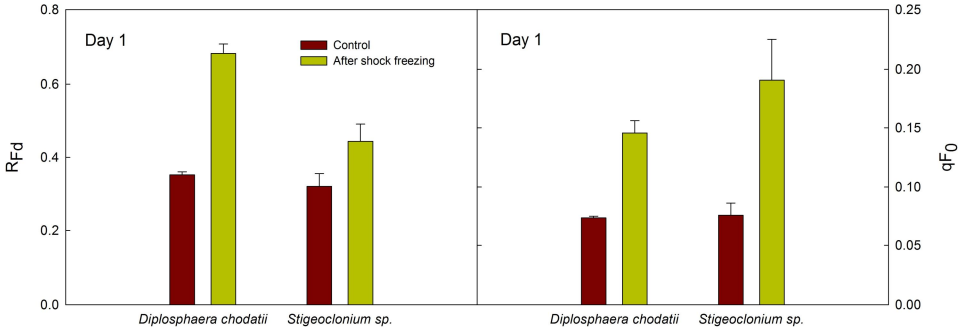

Fig. 5. Effects of shock freezing on Relative fluorescence decrease (R_{Fd}) and quenching of background chlorophyll fluorescence (qF_0). The two chlorophyll fluorescence parameters were recorded for control samples grown in liquid BBM medium, and then immediately after shock freezing and thawing.

Commonly, positive values of qF_0 are reached in plants since $0 < F_0' < F_0$. Exceptionally, when $F_0' > F_0$, qF_0 gives negative values. It is probably due to not fully re-oxidised PS II acceptor (Roháček 2002).

When shock-frozen, thawed and inoculated on agar plates, the cultures of *D. chodatii* and *Stigeoclonium* sp. showed cultivation time-dependent increase in chlorophyll fluorescence parameters (F_V/F_M, F_S). One week after the inoculation (Day 7), *D. chodatii* showed a decrease in F_V/F_M (in comparison to untreated control) attributed to the negative adjustment related to the change of medium from liquid to agar. However, with the following time of cultivation, F_V/F_M reached control values and remained more or less constant. *Stigeoclonium* sp., contrastingly, showed much slower increase in F_V/F_M values in shock frozen inoculum in a course of time (Day 7 to Day 14), followed by a fast increase in the period from Day 21 to Day 28. Therefore, it reached control values 28 days after inoculation.

For steady state chlorophyll fluorescence (F_S) measured during the cultivation on agar plates (Fig. 3), there was no difference found between control and shock-frozen inoculum in *D. chodatii* (Day 28). The F_S values increased with the time of cultivation similarly in both treatments which could be associated with similar rate of growth of control and shock freezing-treated *D. chodatii*. The F_S values, however, were lower on Days 7, 14, and 21. Control culture of *Stigeoclonium* sp. exhibited fast growth rate, because F_S increased in similar way as in *D. chodatii*. Shock freezing-treated culture of *Stigeoclonium* sp. reached 58% decrease in F_S value after a week cultivation (Day 7) on agar plates. Then, F_S increased with the time of cultivation, however, it was still substantially lower than in control. This might be explained as a consequence of negative effects of shock freezing on functioning of chloroplast photosynthetic apparatus (*cf.* also F_V/F_M). The underlying mechanism for such negative effects on photosynthetic processes and culture growth rate, however, remains unknown. Negative effect of potentially improper composition of growth medium might be excluded since the rate of growth (F_S-based) in control *Stigeoclonium* sp. was similar to that of *D. chodatii* (0.497 compared to 0.346 mV d^{-1}). In general, F_S time course of shock-frozen inoculum was comparable to the evidence gained for *Heterococcus* sp. in similarly-designed experiment (Orekhova et al. 2018).

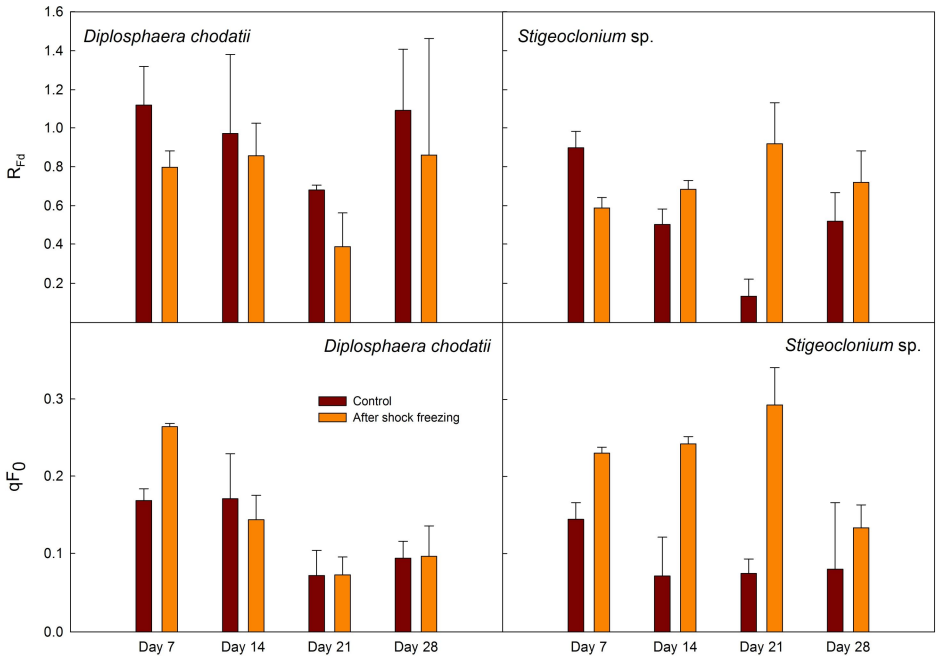

Fig. 6. Relative fluorescence decrease (R_{Fd}) and quenching of background chlorophyll fluorescence (qF_0) as dependent on cultivation time of *Diplosphaera chodatii* and *Stigeoclonium* sp. after inoculation on agar plates.

The effects of cultivation on agar plates on R_{Fd} ab qF_0 values are shown in Fig. 6. The two parameters did not exhibit much change in *D. chodatii*. However, increase in R_{Fd} and qF_0 was apparent at the end of cultivation period (days 14-28) in *Stigeoclonium* sp. The reasons and underlying mechanisms remain unclear and will be addressed in one of the follow up studies.

Our experimental algae showed a high cryoresistance since they were capable of successful regrowth after shock freezing. This could be achieved thanks to cryoprotective mechanisms in microalgae from polar regions that have been overviewed by Lyon and Mock (2014). The mechanisms comprise mainly the maintenance of fluidity of cellular biomembranes under freezing temperature (long-term effect). Underlying mechanism is *e.g.* an increased concentration of polyunsaturated fatty acids (PUFAs) which is well documented for chlorophytes (Fogliano et al. 2010, Chen et al. 2012). Polar microalgae do not only increase PUFA concentrations in cell membrane phospholipids, but also galactolipids integral to the chloroplast membrane (Morgan-Kiss et al. 2002). In psychrotolerant green alga, *Coccomyxa subellipsoidea*, the most enriched gene families of fatty acids synthases, elongases, lipases, and desaturases were found (Blanc et al. 2012). Moreover, in cryoresistant microalgae, several cryoprotectants play an important role. The amino acid proline is an abundant compatible solute in many cryophilic microalgae. Last but not least co-action with special proteins expressed in response to subzero temperature in polar microalgae should be mentioned.

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