

## What does critical temperature tell us about the resistance of polar lichens to freezing stress? Applicability of linear cooling method to ecophysiological studies.

Josef Hájek<sup>1,2\*</sup>, Anton Puhovkin<sup>1</sup>, Davide Giordano<sup>1</sup>, Jiří Sekerák Jr.<sup>1</sup>

<sup>1</sup>Masaryk University, Faculty of Science, Department of Experimental Biology, Laboratory of Photosynthetic Processes, Kamenice 5, 625 00 Brno, Czech Republic

<sup>2</sup>Mendel University in Brno, Faculty of Forestry and Wood Technology, Department of Forest Protection and Wildlife Management (FFWT), Zemědělská 3, 613 00 Brno-Černá Pole, Czech Republic

### Abstract

Lichens from polar regions are well adapted to low temperature and considered cryoresistant. However, interspecific differences in their cryoresistance exist according to the degree of their adaptation and severity of the environment. In our study, we applied linear cooling technique in order to evaluate the interspecific differences in several lichen species. Thalli segments of *Umbilicaria antarctica*, *Nephroma antarctica*, *Placopsis contortuplicata* and *Lasallia pustulata* were exposed to the cooling from 20 to  $-35^{\circ}\text{C}$  at a constant rate of  $2^{\circ}\text{C min}^{-1}$ . Simultaneously with the cooling, chlorophyll fluorescence parameters evaluating potential ( $F_v/F_m$ ) and effective yield of primary photochemical processes in PSII ( $\Phi_{\text{PSII}}$ ) were measured in 30 s interval. Temperature response curves of  $F_v/F_m$  and  $\Phi_{\text{PSII}}$  formed typical *S-curves* that were species specific. Critical temperature (cooling point at which  $\Phi_{\text{PSII}}$  equals 0), was found in a narrow range of  $-25$  to  $-28^{\circ}\text{C}$ , suggesting that all experimental lichen species have a high resistance to sub-zero temperatures. The method of linear cooling used in this study has proven its applicability in ecophysiological studies since it is sensitive enough for the evaluation of species-specific differences in cryoresistance. This study describes different parameters that can be derived from the *S-curves* and discuss their proper use in ecophysiological and stress physiology studies.

**Key words:** cryoresistance, chlorophyll fluorescence, photosystem II, primary photosynthesis

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\*Corresponding author: J. Hájek <jhajek@sci.muni.cz>

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## Introduction

Lichens are symbiotic organisms having great number of species in polar regions. In many maritime Antarctic habitats, lichen diversity and growth rates are high (Colesie *et al.* 2023). Their success in polar vegetation oases is associated with their physiological vigor which is derived from high freezing tolerance (Barták *et al.* 2007, Hájek *et al.* 2016, Haranczyk *et al.* 2003), and fast activation of their photosynthetic processes upon rehydration, both by liquid water or water vapor uptake (Colesie *et al.* 2016, Schroeter *et al.* 2021). Primary processes of photosynthesis depend on hydration of lichen thalli. Typically, they decline slowly with desiccation within the range of relative water content (100% to 30%). With pronounced loss of water from a lichen thalli (decline in relative water contents from 30 to 0%), the decline is much more rapid and associated with severe limitation of primary photosynthetic processes (*e.g.* Barták *et al.* 2021, Puhovkin *et al.* 2022).

In last decades, several studies have focused on primary photosynthesis of Antarctic lichens in response to temperature exploiting both field and laboratory-based approach (*e.g.* Cho *et al.* 2020). The optimum temperature for photosynthetic processes in Antarctic lichens typically lies between 10 and 17°C (Laguna-Defior *et al.* 2016). In their natural habitats, photosynthetic activity often takes place at an optimal combination of temperature and irradiance (Sancho *et al.* 2019). However, hydration state of lichen thalli during photosynthetic measurements is difficult to monitor and maintain in the field because it may change during measurements. That is why the measurements of photosynthetic responses of lichens to temperature *per se* are typically done under laboratory condi-

tions allowing effective lichen thalli water content control, typically in fully hydrated sample. Measurements of dehydration response curves of lichen photosynthesis at fixed temperature (*see e.g.* Lange *et al.* 1998 for *Collema tenax*) represent an alternative approach.

Apart from gas exchange methods, chlorophyll fluorescence (ChlF) methods have been increasingly used in the evaluation of temperature dependence of primary processes of photosynthesis in lichens within last two decades. Measurements of most frequent ChlF parameters (potential ( $F_V/F_M$ ), and effective yield of photosynthetic processes in PSII ( $\Phi_{PSII}$ )) are either done in temperature equilibrated state (exposition lasts typically tens of minutes to hours, *see e.g.* Mishra *et al.* 2015, Hejduková and Nedbalová 2021) or during cooling the sample at constant rate. The latter technique is called linear cooling with concurrent measurements of ChlF parameters. Generally, different cooling rates are used according to the species and its ecophysiological adjustment to the site of occurrence. The rates vary from fast (*e.g.* Šabacká and Elster 2006, 4.0°C min<sup>-1</sup>, Steiner *et al.* 2021) to slow (Hájek *et al.* 2012: 0.5°C min<sup>-1</sup>). Recently, linear cooling is frequently used for the evaluation of critical (cooling) temperature (Hájek *et al.* 2021). In our study, we exploited the method of linear cooling in order to determine cryoresistance of several lichen species from polar regions. We also aimed to detailed analysis of the temperature response curves of  $F_V/F_M$ , and  $\Phi_{PSII}$  in order to suggest some new descriptive parameters sensitive to temperature with an application potential in ecophysiological studies in polar lichens.

## Material and Methods

### *Experimental species*

For the study, we used representatives of lichens from polar and subpolar regions: *Umbilicaria antarctica*, *Nephroma antarctica*, *Placopsis contortuplicata* and *Lasallia pustulata*. The species were selected because they represent different forms of association: (1) chlorolichens having a green microalga as photosynthesizing photobiont

(*U. antarctica*, *L. pustulata*), (2) cyanolichen having a cyanobacterium as a photobiont (*Nephroma antarctica*), (3) tripartite lichen having both alga and cyanobacterium (*P. contortuplicata*). Two lichen species were from Antarctica, one from Southern America (Tierra del Fuego), and one from Norway (see details in Table 1).

Species	Collection site	Co-ordinates	
<i>Lasallia pustulata</i>	Spjaerøy (Norway)	59° 05' 03" N	10° 55' 04" E
<i>Nephroma antarctica</i>	Puerto Willimas (Chile)	54° 56' 19" S	67° 35' 59" W
<i>Placopsis contortuplicata</i>	James Ross Island	63° 52' 51" S	58° 05' 12" W
<i>Umbilicaria antarctica</i>	Galindez Island	65° 15' 00" S	64° 16' 00" W

**Table 1.** List of experimental species with specification of the collection sites.

After the collection, lichen thalli were dried (in the field) and then transported to the laboratory in Brno (Masaryk University, Czech Republic) in a portable fridge at  $-1^{\circ}\text{C}$ . Then, the lichens were stored at  $-5^{\circ}\text{C}$ . Before the experiments, the thalli were placed in a Petri dish between two sheets of paper rewetted by demineralized water for 24 h. During the rewetting, li-

chen thalli were exposed to a dim light of  $5\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$  of photosynthetically active radiation (PAR) for 48 h. Then, reactivation of photosynthetic processes was assessed by chlorophyll fluorescence (capacity of photosynthetic processes in PSII:  $F_V/F_M$ ). Thalli showing high (and constant) values of  $F_V/F_M$  were selected for consequent cooling experiment.

### *Primary photosynthesis in response to cooling*

To assess critical (cooling) point and the relationship between photosynthetic parameters and thallus temperature, we used the cooling chamber (Kryo-Planer unit, Great Britain) linked to a 20 l Dewar flask with liquid nitrogen. The lichen thalli were cooled from 20 (denoted as  $T_0$ ) to  $-40^{\circ}\text{C}$  at a constant rate of  $2^{\circ}\text{C min}^{-1}$ . An individual sample was equilibrated to  $20^{\circ}\text{C}$  for 15 min. before the cooling procedure started. During the cooling, chamber ( $T_{\text{ch}}$ ) and sample temperature ( $T_{\text{s}}$ ) were measured by in-built thermocouples, as well as two chlorophyll fluorescence pa-

rameters (fluorometer PAM-2000, H. Walz, Germany) monitoring a temperature-induced decline of photosynthetic processes in 1 min. interval. The parameters were: (1)  $F_V/F_M$  and (2)  $\Phi_{\text{PSII}}$ . For such purpose, the probe of the fluorometer was placed into the cooling chamber 3 mm above lichen thallus upper surface to assure a satisfactorily-high chlorophyll fluorescence signal (typically, above 300 mV). For  $F_V/F_M$  and  $\Phi_{\text{PSII}}$  estimation, repetitive saturation pulses of  $5\ 000\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$  for 0.8 s were applied each 30 s to induce maximum chlorophyll fluorescence values ( $F_M$ , and

$F_M'$ ) used for calculations (*see* the equations below).

$$F_V/F_M = (F_M - F_0) / F_M \quad \text{Eqn. 1}$$

$$\Phi_{PSII} = (F_M' - F_S) / F_S \quad \text{Eqn. 2}$$

where  $F_0$  denotes background chlorophyll fluorescence,  $F_M$  denotes maximum chlorophyll fluorescence achieved after a saturation pulse applied in dark-adapted samples. For  $\Phi_{PSII}$  evaluation,  $F_M'$  denotes maximum chlorophyll fluorescence achieved after a saturation pulse allied in light-adapted samples ( $30 \mu\text{mol m}^{-2} \text{s}^{-1}$  PAR), and  $F_S$  denotes a steady state chlorophyll fluorescence.  $F_V/F_M$  and  $\Phi_{PSII}$  were plotted against

temperature and the parameters specified in Table 1 calculated (for definition of the parameters, *see* Fig 1, for values *see* Table 2).

To evaluate the temperature at which the largest difference appears between the temperature response curves of  $F_V/F_M$  and  $\Phi_{PSII}$ , the values were normalized by division of each  $F_V/F_M$  and  $\Phi_{PSII}$ , the maximum value of particular curve. Then, differentials of the normalized values were calculated and plotted against temperature. Species-specific differences were then evaluated by the following parameters derived from the curves (*see* Table 3 for their values).

### Statistical analysis

Interspecific difference in  $F_V/F_M$  and  $\Phi_{PSII}$  in response to temperature were assessed by one-way ANOVA (the statisti-

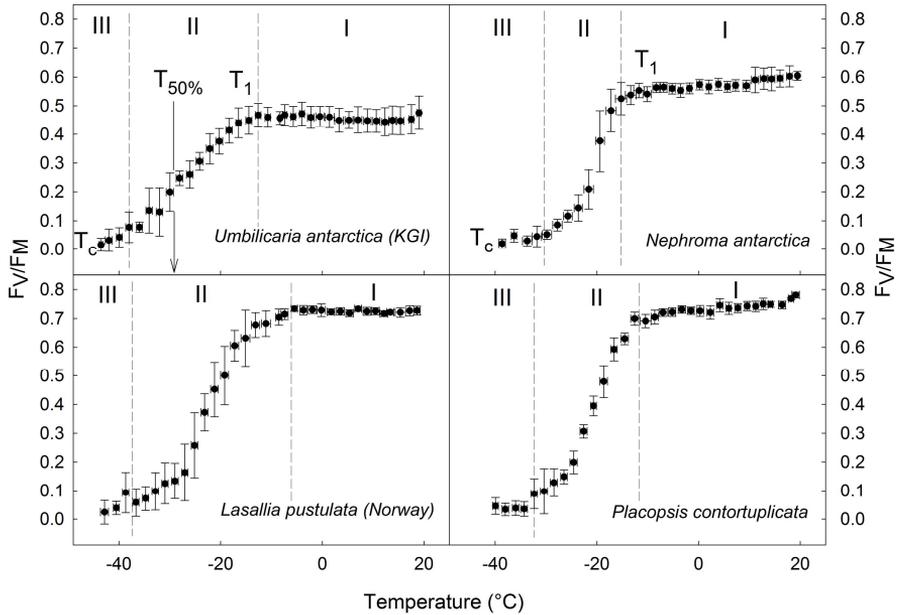
cally significant differences ( $P = 0.05$ ) among the species), Statistica, TIBCO Software Inc.

## Results

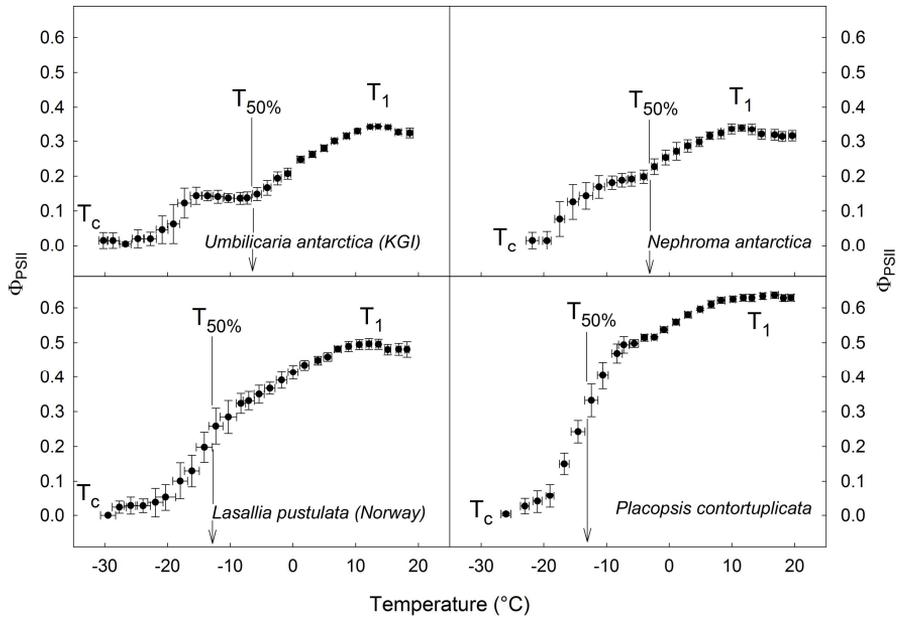
Temperature response curve of  $F_V/F_M$  for the four experimental lichen species showed some general and species-specific features as well. In all species, a decrease in thallus temperature led to decline of  $F_V/F_M$ , that followed a *S-curve* which has been reported for lichens in previous studies (*see* e.g. Hájek *et al.* 2016 and Discussion). The temperature, in which the rate of  $F_V/F_M$  decline started to accelerate ( $T_1$  – *see* Fig. 1) differed between the species in the following order: *L. pustulata* ( $-8^\circ\text{C}$ ), *U. antarctica*, *N. antarctica*, and *P. contortuplicata* ( $-14^\circ\text{C}$ ). Below these temperatures, decline of  $F_V/F_M$  values was fastest in *N. antarctica*, followed by similarly behaving *L. pustulata* and *P. contortuplicata* (*see* Fig. 1). The slowest  $F_V/F_M$  decline with thallus temperature decrease was achieved in *U. antarctica*, which was reflected in the lowest value of

$T_{50\%}$  (*see* Table 2). Critical temperature (cooling point,  $T_c$ ) was found lowest in *U. antarctica* and *L. pustulata* ( $\sim -45^\circ\text{C}$ ) followed by *N. antarctica*, and *P. contortuplicata* ( $\sim -37^\circ\text{C}$ ).

Effective quantum yield of photosynthetic processes in PSII ( $\Phi_{PSII}$ ) declined with temperature decrease in a polyphasic manner. In all species, two *S-curves*, one following the other, were distinguished on the  $\Phi_{PSII}$  to thallus temperature relationship (*see* Fig. 2). The temperature  $T_1$ , at which the  $\Phi_{PSII}$  started to decline, was comparable for all experimental species (ranging  $12$ – $17^\circ\text{C}$ ), however, the  $T_1$  was found higher for  $\Phi_{PSII}$  than  $F_V/F_M$  of particular species (Table 2). Critical temperature  $T_c$  (cooling point), was found in a narrow range of  $-25$  to  $-28^\circ\text{C}$ . The values of  $T_c$  for  $\Phi_{PSII}$  were higher than for  $F_V/F_M$  in particular species (*see* Table 2).



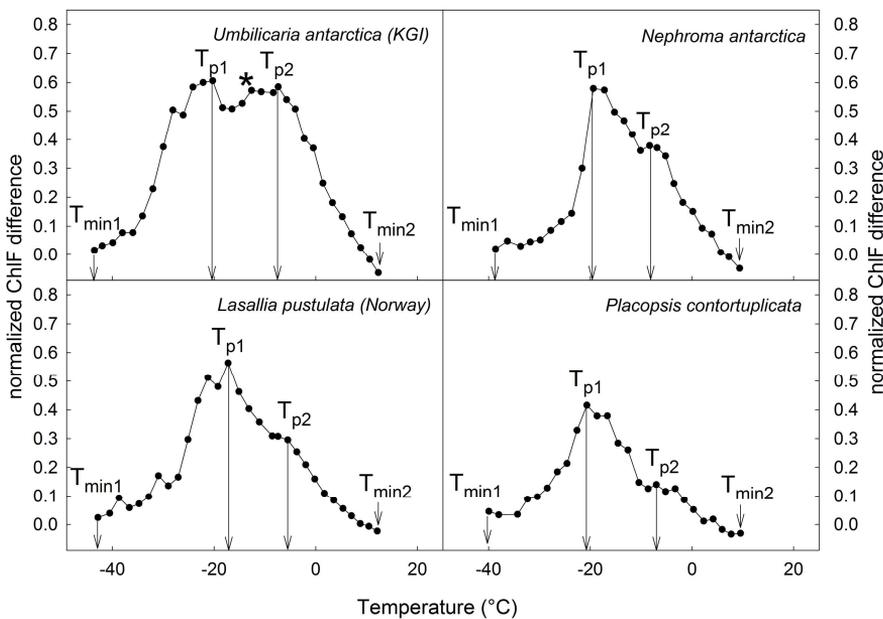
**Fig. 1.** Temperature response curves of  $F_v/F_m$  (the capacity of photosynthetic processes in photosystem II) in lichen species exposed to a constant rate cooling from 20 to  $-30^{\circ}\text{C}$ . Important points ( $T_1$ ,  $T_{50\%}$ ,  $T_c$ ) are indicated (for definition see Material and Methods). Phases I, II and III of the *S*-curves were distinguished according to Folgar-Cameán and Barták (2019). They are shown for four species.



**Fig. 2.** Temperature response curves of  $\Phi_{PSII}$  (the effective quantum yield of photosynthetic processes in photosystem II) in lichen species exposed to a constant rate cooling from 20 to  $-30^{\circ}\text{C}$ . Important points ( $T_1$ ,  $T_{50\%}$ ,  $T_c$ ) are indicated (for definition see Material and Methods).

Species	T <sub>1</sub> (°C)	T <sub>50%</sub> (°C)	T <sub>c</sub> (°C)
	F <sub>V</sub> /F <sub>M</sub>	F <sub>V</sub> /F <sub>M</sub>	F <sub>V</sub> /F <sub>M</sub>
	Φ <sub>PSII</sub>	Φ <sub>PSII</sub>	Φ <sub>PSII</sub>
<i>Umbilicaria antarctica</i>	-12.9 12.1	-27.5 -6.3	-43.6 -27.1
<i>Nephroma antarctica</i>	-13.6 11.8	-20.0 -12.0	-39.9 -21.9
<i>Placopsis contortuplicata</i>	-12.5 7.9	-20.9 -12.6	-37.3 -26.0
<i>Lasallia pustulata</i>	-8.7 9.3	-23.2 -12.5	-44.1 -29.8

**Table 2.** Temperature characteristics derived from the *S*-curves (F<sub>V</sub>/F<sub>M</sub>, Φ<sub>PSII</sub>) measured in the experimental species (data acquired by linear cooling of the sample – see Figs. 1 and 2).



**Fig. 3.** The temperature response curves of the difference between normalized values of F<sub>V</sub>/F<sub>M</sub> and Φ<sub>PSII</sub> (normalization was done by division of particular value by the maximum values of F<sub>V</sub>/F<sub>M</sub> and Φ<sub>PSII</sub>) evaluated by the equation: (F<sub>V</sub>/F<sub>M</sub>)<sub>norm</sub> – (Φ<sub>PSII</sub>)<sub>norm</sub>.

The temperature response curves of the difference (F<sub>V</sub>/F<sub>M</sub>)<sub>norm</sub> – (Φ<sub>PSII</sub>)<sub>norm</sub> were similar for the experimental species, since numeric values of the T<sub>min1</sub>, T<sub>min2</sub>, T<sub>p1</sub>, T<sub>p2</sub> were almost the same (Table 2). However, in *U. antarctica*, contrastingly to the other

three species, the difference between the (F<sub>V</sub>/F<sub>M</sub>)<sub>norm</sub> – (Φ<sub>PSII</sub>)<sub>norm</sub> for values of T<sub>p1</sub> and T<sub>p2</sub> was much smaller (see the \* in Fig. 3). T<sub>p1</sub> was found in lower temperature.

Species	$T_{\min 1}$ (°C)	$T_{\min 2}$ (°C)	$T_{p1}$ (°C)	$T_{p2}$ (°C)
<i>Umbilicaria antarctica</i>	-42.2	9.5	-20.3	-7.4
<i>Nephroma antarctica</i>	-39.8	7.3	-19.4	-8.3
<i>Placopsis contortuplicata</i>	-40.3	4.8	-20.7	-7.0
<i>Lasallia pustulata</i>	-42.1	8.7	-17.3	-7.4

**Table 3.** Temperature characteristics derived from the difference of normalized values of  $F_V/F_M$  and  $\Phi_{II}$ ,  $((F_V/F_M)_{\text{norm}} - (\Phi_{II})_{\text{norm}})$ .

## Discussion

Similarly to previous studies (*e.g.* Barták et al. 2007), our study showed that photochemical processes of photosynthesis in PSII had detectable activity ( $\Phi_{\text{PSII}}$ ) at the temperature of  $-20^\circ\text{C}$ . Below that temperature, a substantial inhibition of photochemical processes of photosynthesis was apparent which might be attributed to the negative effects of overall intra- and extracellular ice formation. The process of ice formation in lichens, however, starts at higher (but sub-zero) temperature, typically within the range of  $-5$  to  $-10^\circ\text{C}$  which corresponds to the temperature of ice-nucleation activity in fully hydrated lichen thalli (Schroeter and Scheidegger 1995). Similarly, Haranczyk et al. (2003) showed that  $-5^\circ\text{C}$  is an edge temperature for freezing of water in lichen thalli.

Decline of values  $F_V/F_M$ , and  $\Phi_{\text{PSII}}$  found for phase II (*see* Fig 1, 2) might be attributed to gradual temperature-induced decrease in photosynthetic processes, *i.e.* limited functioning of PSII and  $\text{CO}_2$  fixation. These changes are accompanied by an increase in non-photochemical quenching of chlorophyll fluorescence which serves as a protective mechanism of PSII photochemical reactions.

Among the studied lichen species, the lowest  $T_c$  value was found in *Umbilicaria*

*antarctica*, which is consistent with previous studies referring to  $T_c < -35^\circ\text{C}$ . Therefore *U. antarctica* might be considered a species showing the highest cryoresistance.

In lichens and mosses, resistance to sub-zero temperature and cryoresistance in general are usually studied by repetitive freezing-thawing cycles (Deltoro et al. 1999, Wang et al. 2014) or monitoring of physiological activity in the field at sub-zero temperature (Kappen et al. 1998). Our data reported in Figs. 1, 2 and the Tables 2-4, however, supports the idea that the linear cooling method supplemented with simultaneous ChlF measurements is a faster alternative to the above mentioned technique. It enables large-scale experiments studying the responses of a great number of species. Also, the method has proven its applicability in ecophysiological studies of lichens from polar regions. The method is sensitive enough for the evaluation of species-specific differences in cryoresistance. The *S-curves* of the most frequent chlorophyll fluorescence parameters ( $F_V/F_M$ ,  $\Phi_{\text{PSII}}$ ) can be analyzed in several ways in the studies applying the linear cooling method. Several parameters derived from *S-curves* showing temperature sensitivity might be used according to the merit of a study (*see* the list below in Table 4).

Parameter	Explanation	Source / Remarks
T <sub>opt</sub>	Temperature optimum of primary photosynthetic processes in polar autotrophs. T <sub>opt</sub> is demonstrated as a peak in F <sub>v</sub> /F <sub>M</sub> and/or Φ <sub>PSII</sub> found typically for the temperature range of 12–19°C	Puhovkin et al. (2023) 10.0 to 12.0°C
T <sub>1</sub>	Temperature at which a substantial decline of F <sub>v</sub> /F <sub>M</sub> and/or Φ <sub>PSII</sub> starts with further cooling	Folgar-Cameán and Barták (2019)
T <sub>50%</sub>	Temperature at which 50% of maximum values of F <sub>v</sub> /F <sub>M</sub> and/or Φ <sub>PSII</sub> is reached	Folgar-Cameán and Barták (2019)
T <sub>c</sub>	Critical (cooling) temperature at which F <sub>v</sub> /F <sub>M</sub> and/or Φ <sub>II</sub> value(s) reach 0	Folgar-Cameán and Barták (2019)
T <sub>c</sub>	Sub-zero temperature at which small peak of F <sub>v</sub> /F <sub>M</sub> appears at low temperature thanks to the emission of specific heat related to ice formation	Barták (2014) –34.0°C for <i>Usnea antarctica</i>  Hájek et al. (2016) –35°C for <i>Umbilicaria cylindrica</i>
<i>S-curve</i> parameters	Mathematical parameters related to logistic <i>S-curve</i> fit (typically 5 parameters)	Hájek et al. (2016)
Maximum slope	Maximum slope, <i>i.e.</i> parameter b in the equation $y = a + bx$ , where y is F <sub>v</sub> /F <sub>M</sub> or Φ <sub>PSII</sub>	Folgar-Cameán and Barták (2019) 0.029–0.098
Differential (F <sub>v</sub> /F <sub>M</sub> ) <sub>norm</sub> – (Φ <sub>II</sub> ) <sub>norm</sub>	Difference between normalized values of F <sub>v</sub> /F <sub>M</sub> and/or Φ <sub>II</sub> (normalized to maximum value) Diff = ((F <sub>v</sub> /F <sub>M</sub> ) <sub>norm</sub> – (Φ <sub>PSII</sub> ) <sub>norm</sub> )	This study

**Table 4.** Parameters derived from *S-curves* recorded during linear cooling of a sample with simultaneous measurements of chlorophyll fluorescence parameters (F<sub>v</sub>/F<sub>M</sub>, Φ<sub>PSII</sub>).

## Concluding remarks

For algae and chlorolichens, their algal/cyanobacterial photobionts in particular, the evaluation of critical (cooling) temperature provides not only information of their cryoresistance but the estimate of their cryopreservation potential. For few algal species, successful long-term cryopreservation and a good survival rate is reported (Day et al. 1997, Nakanishi et al. 2012). In lichens, only fragmentary knowledge exists on the cryopreservation potential. Howev-

er, lichens tolerate sub-zero temperature and the exposition to liquid nitrogen. Their algal photobionts showed reasonably high survival of shock freezing in liquid nitrogen (Hájek et al. 2012). Moreover, common symbiotic alga *Trebouxia* sp. that forms a lichen association in chlorolichens, showed good survival after 13 years at –20°C and thus might be considered a good candidate for long-term cryostorage (Honegger 1998, 2003).

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