Importance of the Time Elapsed from Exposure to Acute Salt Stress to the Occurrence of Rainfall for Regeneration of Photosynthetic Efficiency of Chloro- and Cyanolichens

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Salt stress is one of the main factors disturbing the physiology of organisms, including epigeic lichens inhabiting roadsides, due to de-icing salts used in winter seasons. The aim of the research was to study the effect of acute salt stress in various doses on the chlorophyll fluorescence parameters of chlorolichens, i.e., *Cladonia furcata*, *C. mitis*, *Diposchistes muscorum*, and cyanolichens, i.e., *Peltigera didactyla*, and *P. rufescens*, which naturally grow inland in the vicinity of roads. We also aimed to study changes in the photosynthetic efficiency of lichens over time and their responses to rainfall simulations in the days following exposure to salt stress to test whether liquid water supply improves photosynthetic efficiency. Salt stress led to a reduction of it in cyanolichens in most experimental groups, while in chlorolichens only treatment with 2.9-3.9M NaCl solutions significantly decreased F_V/F_M. Exposure to acute salt stress significantly affected fluorescence transient curves in all studied species. With respect to chlorolichens, a marked decrease of F_M was observed and the flattened shape of the transient curves after treatment with the highest salt doses was the most apparent. Significantly greater disturbances were observed in cyanolichens in which the induction curve lost its sigmoid characteristics after treatment with solutions with a concentration greater than 0.35M. Furthermore, in all lichen species, increased values of ABS/RC and DI_0/RC and decreases in PI_{ABS}, ET_0/RC and TR_0/RC as well as quantum yields and efficiencies were observed. Simulated rainfall resulted in a significant increase in the photosynthetic efficiency of chlorolichens to a level corresponding to healthy lichens almost throughout the duration of the whole experiment. On the contrary, in the case of cyanolichens, significant increases in F_V/F_M after water treatment were found only after exposure to low salt doses and, at the latest, 24 h after the stress. Although many cyanobacteria developed adaptations to survive in highly saline environments, cyanobionts present in inland lichen species seem to be highly susceptible to salt stress. We concluded that the time when rainfall occurs after exposure to salt stress is a crucial factor affecting the potential regeneration of PSII efficiency. Regeneration after rainfall is an important aspect for epigeic lichens occurring near roadsides, where, during the winter season, they are exposed to de-icing salt for a long time, and rainfall may partially compensate for their disturbances and increase their photosynthetic efficiency, enhancing the possibility of survival.

Keywords: chlorophyll fluorescence, cyanobiont, lichens, OJIP, phycobiont, PSII quantum yield, salt stress.

INTRODUCTION

During the winter season sodium chloride is commonly used for de-icing of roads in many countries. Dissolved salt in meltwater is turned into airborne droplets by passing vehicles and sprayed away from the road. It is known that sodium chloride has a negative impact on infrastructure and ecosystems that alter their structure and function (Panno et al., 1999; Findlay and Kelly,

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2011). Lichens are a specific symbiotic relationship between a fungus and green algae or cyanobacteria (Ahmadjian, 1993). Representatives of *Trebouxia* and *Trentepohlia* most often constitute photosynthetic partners of green algae and *Nostoc* of cyanobacteria (Friedl and Büdel, 2008). Lichens are particularly vulnerable to several types of abiotic stress due to their poikilohydric nature and lack of protective layers, such as the cuticle (Tyler, 1989; Delmail et al., 2013).

Salt stress leads to profound consequences for the metabolism of lichens due to dehydration, ionic imbalances, and hyperosmotic shock (Erdman and Hagemann, 2001; Matos et al., 2011; Delmail et al., 2013). However, many species of lichens have developed a number of protective mechanisms and the ability to reverse damage caused by salt stress. The most important of them are the antioxidant system protecting against ROS and the production of metabolites named compatible solutes such as, for example, sucrose or fructans (Delmail et al., 2013). However, both phycobionts and cyanobionts show different peculiarities at the physiological level during desiccation (Erdman and Hagemann, 2001). Phycobionts can produce polyols that can be allocated to mycobionts. Polyols certainly enhance tolerance to the desiccation of lichens, as they significantly increase intracellular osmotic potential and limit water loss (Delmail et al., 2013). Specific proteins called dehydrins are synthesized during dehydration, which is certainly involved in the desiccation tolerance of *Trebouxia* (Gasulla et al., 2009). In cyanobacteria, tolerance to water loss is associated with so-called 'anhydrobiosis'. This phenomenon allows them to survive almost complete dehydration (Büdel, 2011). Dry-tolerant cyanobacteria maintain their chlorophyll level under salt stress and are referred to as homoiochlorophyllous. This leads to a rapid recovery of photosynthetic activity during rehydration (Lüttge, 2011).

Despite the fact that lichens gain certain tolerance and have developed various biochemical and physiological mechanisms in response to salt stress, it is expected that salt stress will have a major impact on the viability of the lichen thallus, which is manifested by a significant decrease in the maximum quantum yield of primary photochemistry. Although there are several studies on the effect of salt stress on the photosynthesis of lichens (e.g., Nash and Lange, 1988; Smith and Gremmen, 2001; Matos et al., 2011; Chowaniec et al., 2022), most studies have analyzed the problem in the context of lichens naturally exposed to sea salt.

The aim of this study was to test the effect of salt (solutions of different concentrations of NaCl) on the chlorophyll fluorescence parameters of epigeic lichens that naturally grow inland in the vicinity of roads. We also aimed to verify how photosynthetic efficiency changes over time after acute salt stress. With regard to these effects, the aim of the study was to determine the NaCl concentration threshold that causes a considerable decrease of photosynthetic efficiency and the irreversible impairment of the photosynthetic process. The next aspect concerns the reversibility of changes in photosynthetic efficiency caused by acute salt stress, tested by soaking lichen thalli in water, which was regarded an equivalent of rainfall for lichens living directly in the environment. In this way we aimed to determine whether the recovery of photosynthetic efficiency to a level corresponding to healthy lichens is possible on the first, second, and third day after treatment with NaCl solutions. The following hypotheses were set: (1) Due to the prokaryotic nature of cells and greater susceptibility to the effects of oxidative stress, cyanobionts are more sensitive to salt stress than phycobionts; (2) Acute salt stress causes irreversible damage to photosynthesis and photosynthetic efficiency did not recover spontaneously, regardless of the photosynthetic partner; (3) Soaking the lichen thallus in water results in an improvement of photosynthetic efficiency, being more effective the sooner it occurs after exposure to stress.

MATERIALS AND METHODS

TARGET LICHEN SPECIES AND SAMPLING

Altogether 5 lichen species were studied. They included species with phycobionts, i.e., *Cladonia furcata*, *C. mitis*, *Diploschistes muscorum*, and cyanobionts, i.e., *Peltigera didactyla* and *P. rufescens*. The selected *Cladonia* representatives are typically associated with *Asterochloris* genus (Bačkor et al., 2010), *D. muscorum* can be associated with three different algae genera, i.e., *Asterochloris*, *Trebouxia*, and *Dictyochloropsis* (Wedin et al., 2016; Osyczka et al., 2021). Two selected species of *Peltigera* are associated with photobionts representing *Nostoc* genus (O'Brien et al., 2005). The selected species are common epigeic lichens that occur in open sun-exposed habitats along communication roads in Europe (Wirth, 1995). The study was

planned to select species with different photobionts, including green algae and cyanobacteria. The sampling was carried out in the summer season of 2019. Samples of each lichen species were collected for one day and transported to the laboratory. The study sites were located far from the roads to prevent initial salt exposure to lichens. The basic characteristics of the species studied, and the study sites are provided in Table 1.

EXPERIMENTAL DESIGN

Before each experiment, the lichen thalli were hydrated and kept for 24 h in a chamber with 95% relative humidity at 20°C and 70 µmol m^{-2} s^{-1} PAR photons to reactivate physiological activity and maintain the integrity of cell membranes (Buck and Brown, 1979). Experiments were performed under normal laboratory conditions at a room temperature of 20°C.

Lichen samples were immersed in NaCl solutions of different concentrations (0M – control, 0.2M, 0.35M, 0.6M, 0.9M, 1.8M, 2.9M, and 3.9M). Solutions with lichen thalli were shaken on a vibration shaker (Vibramax 100, Heidolph; 150 rpm) for 2 h. Then the lichen samples were removed from the solutions. One hour after immersion, chlorophyll fluorescence measurements were performed in the first part of the lichen thalli, the second part of the lichen thalli was immersed in rainwater (conductivity: 109 µS cm^{-1}; pH: 6) for 1 h and then chlorophyll fluorescence measurements were made. The remaining part of the lichen thalli was placed in a chamber with 95% relative humidity at 20°C and 70 µmol m^{-2} s^{-1} PAR photons and kept in the chamber until the next day of the experiment. On the second (24 h after salt treatment), third (48 h after salt treatment), and fourth (72 h after salt treatment) day of the experiment the procedure was repeated: i.e., chlorophyll fluorescence measurements were performed on thalli directly after removal from the humid chamber and after being soaked in water for 1 h. In the experiments we used a large amount of lichen material placed in compartments in the chamber. Each compartment contained the amount of lichen material required for the entire duration of the experiment. On the appropriate days of the experiment, the required amount of lichen material was collected and intended for analyses. Each time the measurements were made on different lichen thalli. In this way, we measured the chlorophyll fluorescence on thalli 1, 24, 48 and 72 hours after exposure to acute salt stress, and on thalli treated with rain simulation after 1, 24, 48 and 72 hours after exposure to stress. The number of replicates for each part of the measurements was 8. For each lichen species, a total of 512 measurements were taken (4 days × 8 treatments × 2 parts of the measurements (i.e., direct and after soaking in water) × 8 replicates).

FLUORESCENCE MEASUREMENTS

Chlorophyll fluorescence measurements were performed using a Handy-PEA fluorometer (Plant Efficiency Analyzer, Hansatech Instruments Ltd, UK). The measurements were taken on each thallus, avoiding areas with excessive vegetative structures (cf. Tretiach et al., 2005). The lichen samples were dark-adapted for 25 mins before the measurements. A saturating light pulse of 2400/µmol/m^2/s supplied by 650-nm light emitting LED diodes was used. All fluorescence transients were recorded with a time span of 10 µs to 1 s. The photosynthetic performance of the lichen photobiont was assessed by the maximum PSII quantum yield: $F_V/F_M = (F_M - F_O)/F_M$, where F_O is a minimum and F_M is a maximum, *chl a* fluorescence, and $F_V = (F_M - F_O)$ is the variable fluorescence. The F_V/F_M values corresponding to healthy lichens were considered to be >0.6 for chlorolichens and >0.4 for cyanolichens (after Jensen, 2002; Jensen and Kricke, 2002).

The fast fluorescence kinetics typically outlines a transient curve: when the curve is plotted on a log-time axis, the sequence of steps is called O-J-I-P (Strasser et al., 2000). This was based on the results of chlorophyll fluorescence signals at short time intervals, starting from 10 µs, and ending before 1 s. The transient curves were measured for each lichen species and treatment on the last day of the experiment (averaged for 8 repetitions).

STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA) along with Tukey's HSD test was performed to test the significance of differences in F_V/F_M between treatments on each day of the experiment and for each lichen species separately. Prior to the analyses, the normality of distribution was verified using the Kolmogorov-Smirnov test. Levene's test was performed to assess variance homogeneity. The Box-cox transformation was applied when necessary. If the assumptions of the analysis were not met,

TABLE 1. Detailed characteristics of the studied lichen species, i.e., type of photobiont, growth form, thallus surface characteristics, habitat, distribution, and study sites from which lichen samples were collected.

No	Species	Photobiont[1]	Growth form[1]	Thallus surface characteristics[2]	Habitat[1,2]	Distribution[2]	Study site
	Chlorolichens						
1	*Cladonia mitis* Sandst.	phycobiont Chlorococcoid	dimorphic: primary thallus crustose, secondary fruticose	secondary thallus ecorticate	heathlands, grasslands, sand dunes, forest margins, Scots pine forest	circumpolar, boreal-subarctic-subalpine, Europe: from the Arctic through the Central European zone to northern Mediterranean region	Rabsztyn village 50°18'02.2"N 19°36'53.0"E Scots pine forest
2	*Cladonia furcata* subsp. *furcata* (Huds.) Schrad.	phycobiont Chlorococcoid	dimorphic: primary thallus squamulose, secondary fruticose	secondary thallus smoothly corticated	forest margins, open forests, grasslands, dwarf shrubby heaths	holarctic, temperate to boreal-montane, Europe: Europe: from boreal conifer forest to Mediterranean region	Ogrodzieniec town 50°25'48.9"N 19°30'45.5"E Scots pine forest
3	*Diploschistes muscorum* (Scop.) R. Sant.	phycobiont Chlorococcoid	crustose	thallus ecorticate with well-developed epinecral layer	dry grasslands, dunes	holarctic, Europe: from boreal conifer forest to Mediterranean region	Klucze town 50°20'01.4"N 19°32'00.6"E Initial fresh pine coniferous forest
	Cyanolichens						
4	*Peltigera didactyla* (With.) J.R. Laundon	cyanobacteria (*Nostoc* sp.)	foliose	upper cortex paraplectenchymatous, lower cortex absent, soralia and rhizines present	disturbed sites, roadsides, urban wastelands, open slopes, cuttings, road margins	cool-temperate to boreal-montane, Europe: from the Arctic to Mediterranean region	Jaroszowiec village 50°20'49.4"N 19°37'26.4"E Mixed coniferous forest
5	*Peltigera rufescens* (Weiss) Humb.	cyanobacteria (*Nostoc* sp.)	foliose	upper cortex paraplectenchymatous, lower cortex absent, rhizines present	dry grasslands, dunes, disturbed habitats	holarctic, Europe: from the Arctic to Mediterranean region	Ogrodzieniec town 50°25'48.9"N 19°30'45.5"E Scots pine forest

[1] – after Nimis and Martellos (2022); [2] – after Wirth (1995).

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non-parametric Kruskal-Wallis tests were used along with Dunn's post hoc tests.

To investigate relationships between chlorophyll fluorescence parameters, the data were subjected to principal component analysis (PCA) based on a matrix of correlations. The following fluorescence parameters were analyzed: ABS/RC (absorbed energy per reaction centre (RC)); TR_0/RC (trapped energy flux per RC); DI_0/RC (dissipated energy flux per RC); ET_0/RC (electron transport flux per RC); ABS/CS (specific absorption flux per excited CS); TR_0/CS (trapped energy flux per excited CS); DI_0/CS (dissipated energy flux per excited CS); ET_0/CS (electron transport flux per excited CS); Ψ_0 (the probability that a trapped exciton moves an electron into the electron transport chain beyond QA); φ_{P0} (the probability that an absorbed photon will be trapped by the reaction centre of PSII); φ_{R0} (the quantum yield of reduction of end electron acceptors at the PS I acceptor side (RE)); φ_{E0} (the quantum yield of electron transport); PI_{ABS} (the performance index (potential) for energy conservation from photons absorbed by PSII to the reduction of intersystem electron acceptors). Since, in the case of most lichen species, ABS/RC and DI_0/RC, as well as TR_0/CS and ET_0/CS, were highly correlated, only ABS/RC and TR_0/CS were included in the analysis; however, the interpretation included all parameters.

The significance of differences in photosynthetic efficiency regeneration in lichens treated with different NaCl solutions and after soaking in water for 1 h on particular days of the experiment was tested with the Student's t tests (p < 0.05). The analyses were performed for each treatment separately. Prior to the above-mentioned analyses, the normality of distribution was verified using the Kolmogorov-Smirnov test. Levene's test was performed to assess the equality of variances. Statistical calculations were performed using STATISTICA 13 (TIBCO Software Inc.) and PAST 4.06 (Hammer et al., 2001).

RESULTS

THE IMPACT OF DIFFERENT NACL CONCENTRATIONS ON PHOTOSYNTHETIC EFFICIENCY

As shown in Figure 1, which presents changes of F_V/F_M parameter over time after exposure to salt stress, the treatment of chlorolichens with 0.2-0.9M NaCl solutions did not cause a significant reduction in F_V/F_M, compared to the control, throughout all the days of the experiment (Fig. 1). Treatment with a 1.8M NaCl solution resulted in a significantly decreased F_V/F_M in *D. muscorum* on the first day and the second day, in *C. mitis* on the second and third day, and in *C. furcata* on the third day of the experiment. However, with regard to this salt solution concentration, F_V/F_M was not significantly different from the control treatment in the case of all of the species mentioned above on the last day of the experiment. On the other hand, F_V/F_M was significantly reduced on the first day of the experiment in each chlorolichen species, compared to the control, after treatment with solutions of the highest concentrations, that is, 2.9M and 3.9M (Fig. 1). This effect was maintained in the following days of the experiment with the exception of *C. furcata*, in which F_V/F_M did not differ significantly between 2.9M treatment and the control on the last day of the experiment. Regarding cyanolichens, i.e., *P. didactyla* and *P. rufescens*, a significant negative effect of salt stress was evident at a concentration of 0.35M and higher throughout all the days of the experiment. Significant depressions in comparison to the control were recorded for 0.9M and higher concentrations throughout the whole experiment (Fig. 1).

FLUORESCENCE KINETICS: OJIP TEST

The fluorescence transient curves, which reflect chlorophyll fluorescence signals at short time intervals of the control samples of all lichen species, revealed the characteristic sequence of OJIP steps typical of healthy lichens on the last day of the experiment (Fig. 2). The treatment of lichen thalli with NaCl solutions altered to the shape of the chlorophyll fluorescence emission transient curves. Regarding chlorolichens, i.e., *C. furcata*, *C. mitis*, and *D. muscorum*, a marked decrease in F_M was observed and a clear peak of emission was not evident after treatment with 2.9M and 3.9M NaCl solutions. In the case of cyanolichens, i.e., *P. didactyla* and *P. rufescens*, the induction curve lost its sigmoid characteristics after treatment with solutions with a concentration greater than 0.35M and became completely flat, but at various levels depending on the salt concentration. For both *P. didactyla* and *P. rufescens* induction curves corresponding to 1.8 and 2.9M NaCl solutions were on a higher level compared to the con-
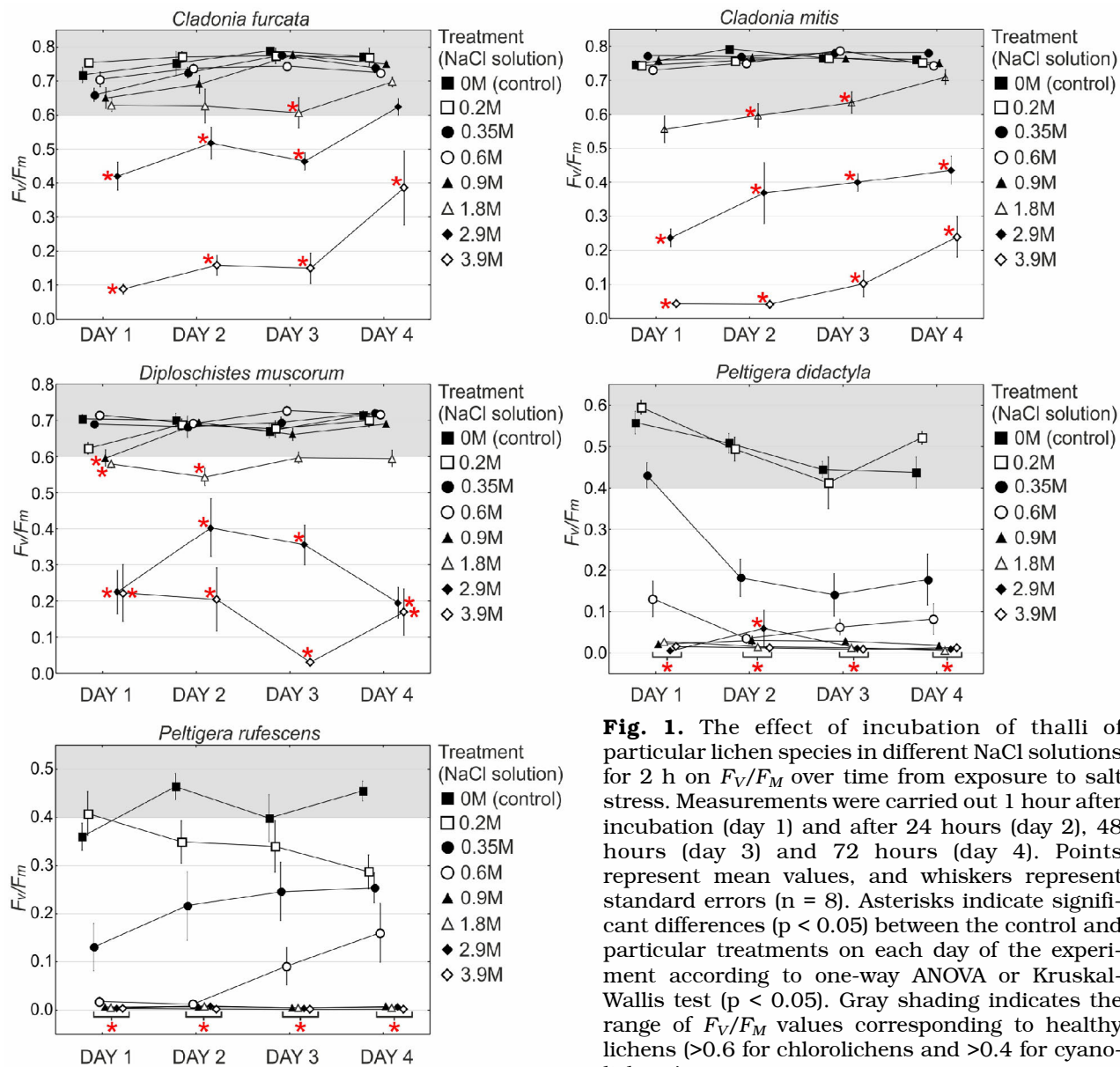
Fig. 1. The effect of incubation of thalli of particular lichen species in different NaCl solutions for 2 h on F_V/F_M over time from exposure to salt stress. Measurements were carried out 1 hour after incubation (day 1) and after 24 hours (day 2), 48 hours (day 3) and 72 hours (day 4). Points represent mean values, and whiskers represent standard errors (n = 8). Asterisks indicate significant differences ($p < 0.05$) between the control and particular treatments on each day of the experiment according to one-way ANOVA or Kruskal-Wallis test ($p < 0.05$). Gray shading indicates the range of F_V/F_M values corresponding to healthy lichens (>0.6 for chlorolichens and >0.4 for cyanolichens).

trols, while those representing the highest concentration (3.9M) were at the lowest level (Fig. 2).

THE IMPACT OF DIFFERENT SALT DOSES ON PS II FUNCTIONING

Principal component analysis based on OJIP parameters showed a distribution of particular experimental groups within ordination space along the first two axes (Fig. 3). The groups representing 2.9M and 3.9M NaCl solutions were the most separated from the remaining groups along the first axis in the case of chlorolichens. As regards cyanolichens, the groups representing the lowest concentrations of salt solutions (0M and 0.2M) were the most clearly separated on the right side of the diagram. Our results showed that energy fluxes through PSII were affected after salt treatment on the following days of the experiment and groups representing the highest salt concentrations were grouped on the left side of the diagrams (Fig. 3). Generally, PCA showed that in the case of all studied species, the most symptomatic was the increase in the average energy absorbed per active reaction centre (ABS/RC) and the increase in dissipated energy flux per excited cross
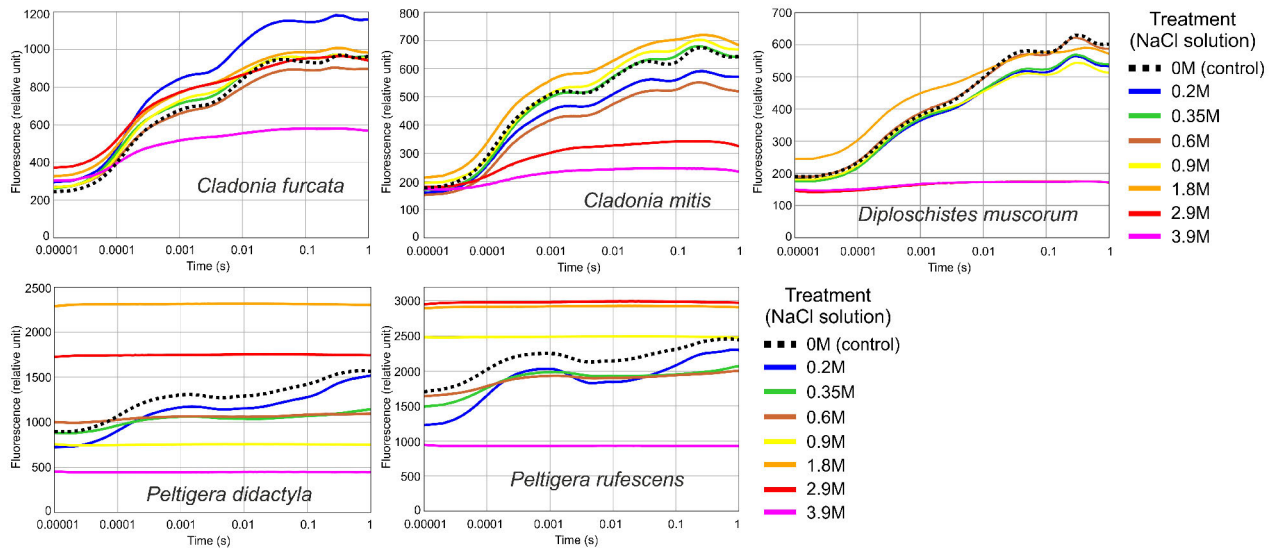
Fig. 2. Chlorophyll *a* fluorescence OJIP transient curves (based on data averaged for 8 repetitions) for particular lichen species treated with different NaCl solutions on the last day of the experiment.

section (DI_0/CS). As a rule, these two parameters were most negatively correlated with Axis PC1. In detail, the changes described above were recognized for chlorolichens, i.e., *C. furcata, C. mitis,* and *D. muscorum,* treated with the highest NaCl solutions (2.9M and 3.9M) and for cyanolichens, i.e., *P. didactyla* and *P. rufescens,* treated with solutions with a NaCl concentration greater than 0.35M. Moreover, salt stress led to reduced values of certain parameters in the studied lichens, e.g., PI_{ABS}, quantum yields and efficiencies as well as the electron transport flux and trapped energy flux per reaction centre (ET_0/RC, TR_0/RC), which in turn were positively correlated with Axis PC1 (Fig. 3).

REVERSIBILITY OF CHANGES IN PHOTOSYNTHETIC
EFFICIENCY AFTER WATER TREATMENT

The pattern of changes in F_V/F_M after water treatment on particular days of the experiment differed between chlorolichens and cyanolichens (Table 2, Fig. 4). In each of the studied species, at least for one treatment, we observed a significant increase in F_V/F_M to the values corresponding to healthy lichens after soaking in water (Table 2). In chlorolichens, a significant increase in F_V/F_M after water treatment was found on all days of the experiment, except for *C. furcata,* for which no significant increases were observed on the last day of the experiment. Furthermore, an increase in F_V/F_M to values corresponding to healthy lichens

was possible even after treatment with a solution of 2.9M and 3.9M NaCl for *C. mitis, C. furcata,* and *D. muscorum* (Table 2). Regarding cyanolichens, significant differences between samples treated and not treated with water were found mainly in the case of low salt doses (0.35M and 0.6M), and on the first and second days of the experiment (*P. didactyla*) and only on the first day of the experiment (*P. rufescens*) (Table 2). In the case of two studied cyanolichens treated with solutions with concentrations greater than 0.2M, a progressive decrease in photosynthetic efficiency was observed even after water treatment in the following days (Fig. 4). Furthermore, the return of the photosynthetic efficiency of cyanobionts of *P. didactyla* and *P. rufescens* to the characteristic level of healthy lichens after treatment with water was not possible on any day of the experiment after exposure to a concentration greater than 0.35M (Fig. 4).

DISCUSSION

EFFECT OF SALT STRESS
ON PHOTOSYNTHETIC EFFICIENCY
AND SPONTANEOUS REGENERATION OVER TIME

The analysis of photosynthetic efficiency is a commonly used method of detecting environmental stress, including drought and salt stress, in plants and lichens (e.g., Percival, 2005; Paoli et al.,

TABLE 2. Regeneration of photosynthetic efficiency in the studied lichens treated with different NaCl solutions on the following days of the experiment after soaking in water for 1 h. Only significant differences between samples not treated and treated with water according to the Student's t test (p < 0.05) are provided. ↑ indicates increase in F_V/F_M on particular days of the experiment compared to samples not soaked in water. ↑↑ indicates increase in F_V/F_M from values below 0.6 for chlorolichens and below 0.4 for cyanolichens to the value corresponding to healthy lichens after water treatment.

Lichen species	Day	Treatment (NaCl solution)						
Chlorolichens		0.2M	0.35M	0.6M	0.9M	1.8M	2.9M	3.9M
Cladonia mitis	1					↑↑	↑↑	↑↑
	2					↑↑	↑↑	↑↑
	3						↑↑	↑
	4						↑↑	↑
Cladonia furcata	1		↑		↑	↑	↑↑	↑↑
	2		↑				↑↑	↑↑
	3						↑↑	↑↑
	4							
Diploschistes muscorum	1	↑			↑↑	↑↑	↑↑	↑
	2						↑↑	↑
	3	↑				↑↑	↑↑	↑
	4						↑	↑
Cyanolichens		0.2M	0.35M	0.6M	0.9M	1.8M	2.9M	3.9M
Peltigera didactyla	1			↑	↑		↑	↑
	2		↑↑	↑				
	3							
	4							
Peltigera rufescens	1		↑↑	↑				
	2							
	3							
	4							

2010; Salvatori et al., 2014). Our results revealed that the photosynthetic performance expressed by F_V/F_M decreased in all lichen species in response to high concentrations of NaCl immediately after exposure to salt stress. Reduced photosynthetic efficiency due to salt stress was also confirmed in several lichen species (see, e.g., Nash and Lange, 1998; Matos et al., 2011; Chowaniec and Rola, 2022). Our results indicated that the studied chlorolichens and cyanolichens differ significantly in response to salt stress. The investi-gated cyanolichens were more sensitive to salt stress and, as a rule, 0.35-3.9M NaCl caused a significant decrease in F_V/F_M, while treatment with 0.9-3.9M NaCl solutions caused a decrease in the F_V/F_M value below 0.1 already on the first day of the experiment. Moreover, this effect persisted throughout all subsequent days of the experiment (Fig. 1). Therefore, it could indicate that the exposure of cyanolichens to solutions with a NaCl concentration higher than 0.9M caused irreversible damage to the process of photosynthesis, since
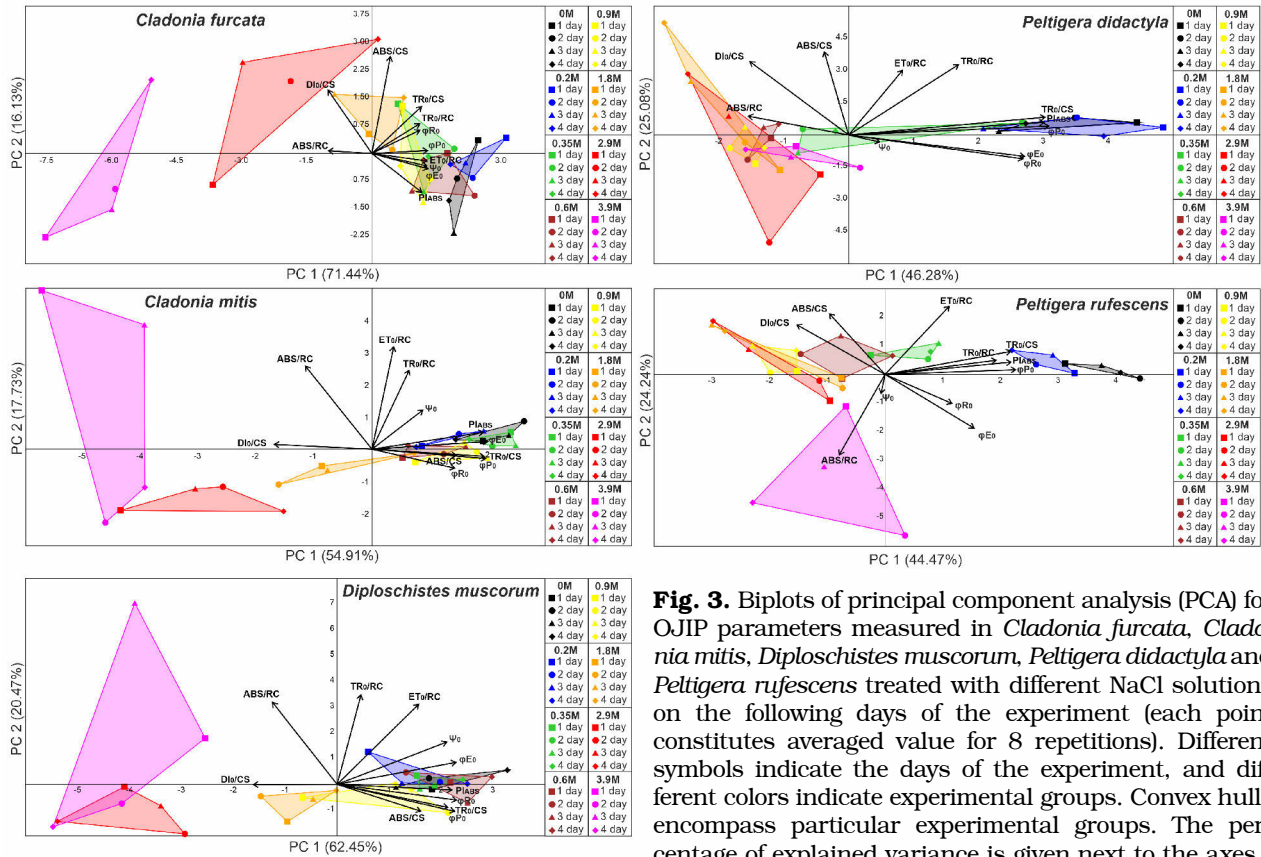
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Fig. 3. Biplots of principal component analysis (PCA) for OJIP parameters measured in *Cladonia furcata*, *Cladonia mitis*, *Diploschistes muscorum*, *Peltigera didactyla* and *Peltigera rufescens* treated with different NaCl solutions on the following days of the experiment (each point constitutes averaged value for 8 repetitions). Different symbols indicate the days of the experiment, and different colors indicate experimental groups. Convex hulls encompass particular experimental groups. The percentage of explained variance is given next to the axes.

the F_V/F_M values < 0.1 that were observed in many experimental groups may suggest the death of the material. Consequently, it can be concluded that *Nostoc* cyanobionts in inland lichens do not have the ability to spontaneously regenerate photosynthetic efficiency after exposure to short-term acute salt stress. Similarly, Lan et al. (2010) found that treatment with a 1% NaCl solution caused a significant decrease in F_V/F_M and, from the first day of the experiment, no further activity was observed in the cyanobacterial crust of *Microcoleus vaginatus.* However, it should be mentioned that certain cyanobiont strains in lichens associated with marine environments are largely resistant to high salt concentrations (Ortiz-Álvarez et al., 2015). On the contrary, chlorolichens were more resistant to salt stress and, in most cases, only treatment with 2.9 and 3.9M NaCl solutions caused a significant decrease in F_V/F_M, compared to the control (Fig. 1). Furthermore, in the case of chlorolichens, we observed spontaneous recovery of F_V/F_M over time and after treatment with solutions of 1.8 and even 2.9M; the values of this parameter increased to the charac-

teristic level of healthy lichens on the last day of the experiment. Relatively high resistance to salt stress was also observed in the isolated green microalga *Trebouxia* sp. TR9, in which, after treatment with a 1M NaCl solution for 72 h, no significant reduction in F_V/F_M was observed (Hinojosa-Vidal et al., 2018). The spontaneous recovery of photosynthetic efficiency over time after salt stress was also observed in cyanolichens; however, the recovery of F_V/F_M to values corresponding to healthy lichens was not detected even after exposure to low salt doses. The spontaneous regeneration of photosynthesis after exposure to salt stress was also reported in *Hypogymnia physodes* and *P. furfuracea*, which contain *Trebouxia* photobionts, in which 24 h after exposure to salt stress, photosynthetic efficiency returned to the characteristic level of healthy lichens (Chowaniec and Rola, 2022).

Determination of the critical NaCl concentration that causes irreversible damage to photosynthesis in the studied lichens was impossible because each species has a different sensitivity to salt stress. Similarly, Chowaniec and Rola
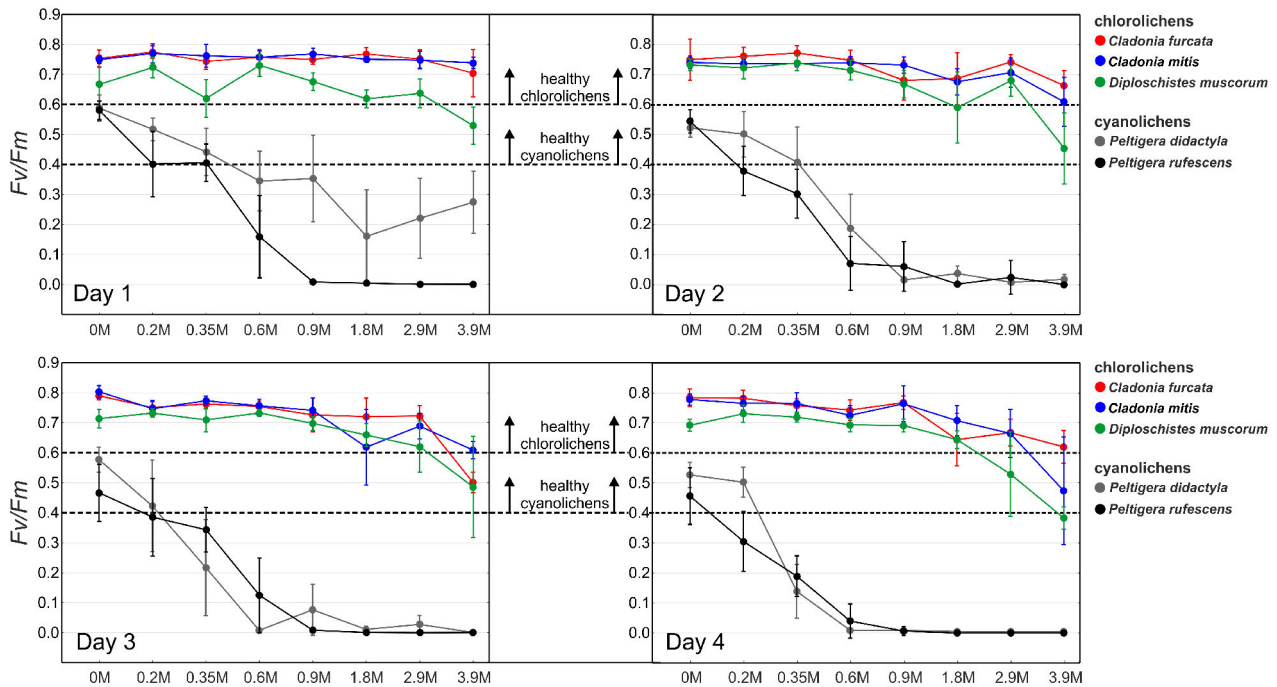
Fig. 4. Differences in F_V/F_M (mean ± 95% confidence interval) between the experimental groups for particular lichen species on the following days of the experiment after water treatment after 1 h (day 1), 24 h (day 2), 48 h (day 3) and 72 h (day 4) from exposure to acute salt stress. The levels of F_V/F_M corresponding to healthy chlorolichens and cyanolichens are indicated by black dashed lines.

(2022) reported a higher decrease in F_V/F_M in *H. physodes* compared to *P. furfuracea* after acute salt stress. Highly species-specific reactions could be explained by differences in thallus anatomy and morphology, such as properties of the cortical layer and the production of various compounds, for example, secondary metabolites (Shukla et al., 2014; Bartak et al., 2015). The anatomical structure and contents of photosynthetic pigments could differ between lichens from various ecological groups or biomorphs and could be associated with adaptations to specific environmental conditions (Sonina et al., 2018). Another reason could be associated with the effectiveness of protective mechanisms induced by thallus dehydration and osmotic stress by particular species. These include the synthesis of compounds, e.g., sugars and sugar alcohols, which have the ability to stabilize proteins during dehydration (Farrar, 1988), to improve water retention capability (Centeno et al., 2016) and are also strong antioxidants counteracting oxidative stress (Kranner et al., 2005). The last and perhaps the most important cause of differences in the responses of individual species to salt stress is the identity of the photobiont. For example, the *Trebouxia* sp. TR9 strain

proved to display a superior performance under high salt concentrations (Hinojosa-Vidal et al., 2018). Moreover, Gasulla et al. (2019) highlighted the role of photobionts in determining lichen zonation on rocky seashores, which was related to differences in physiological behavior between different strains of photobionts. The stronger response of cyanolichens to salt stress observed in our study may also be related to a more complex structure of algae cells, which is responsible for the differences in adaptation to salt stress between free-living microalgae and cyanobacteria cells (Erdmann and Hagemann, 2001). However, many cyanobacteria occur in marine environments, where salt stress is one of the major abiotic factors influencing them. Their growth and survival in these habitats depend on the adjustment of their cytoplasmic water potential (Mikkat at al., 1996). Free-living cyanobacteria have developed several adaptations to survive in these extreme habitats, including active removal of toxic inorganic ions, i.e., Na^+ or Cl^-, accumulation of organic compatible solutes, e.g., trehalose, sucrose, glycine betaine (Hagemann, 2011) or transport systems for osmoprotective compounds like glucosylglycerol (Mikkat et al., 1996). However,

our study considers the response of *Nostoc* cyanobacteria in typical inland lichens, and probably the mechanisms found in cyanobacteria confined to marine environments do not concern the studied cynanobionts.

FLUORESCENCE INDUCTION CURVES

Chlorophyll fluorescence parameters are very sensitive indicators of the function and structure of the photosynthetic apparatus in many organisms, including lichens, algae, and plants (Strasser et al., 2010). Several studies used changes in OJIP fluorescence curves as indicators of abiotic stress in lichens (e.g., Paoli et al., 2010; Bednaříková et al., 2019; Chowaniec and Rola, 2022). The observation of fluorescence transient curves on the last day of the experiment in the case of chlorolichens showed a decrease in F_M in most experimental groups, and a clear peak of emission was not evident after treatment with the highest salt doses. In most species, lower NaCl concentrations either caused no significant change or an increase in F_O, but higher concentrations caused a considerable decrease in F_O (Fig. 2). Killi and Haworth (2017) also observed a significant increase in F_O and a decrease in F_M in *Chenopodium quinoa* after exposure to salinity and salinity-drought conditions and concluded that salt stress limited the number of reaction centres in PSII, which are open. Interestingly, especially in the case of cyanolichens, F_O increased along with decreasing F_V/F_M, while in the case of the highest salt doses, F_O decreased dramatically. This effect of an increase in F_O was clearly visible in the case of *P. didactyla* and *P. rufescens* (Fig. 2). It is important to distinguish whether the decrease in F_V/F_M was due to an increase in F_O or changes in other components. Bolhar-Nordenkampf et al. (1989) found that when all of the reaction centres are open and photochemical quenching is at minimum, the rise in F_O suggests an increase of fluorescence and the damage or malfunction of PSII RCs, or a disturbance in the electron transport for the excitation of RCs, but F_M quenching could result from an increase in non-photochemical quenching.

In the case of cyanolichens, the induction curves became flattened after treatment with solutions with a concentration greater than 0.35M. Interestingly, a significant decrease in F_M was only observed in the case of 3.9M NaCl solutions on the last day of the experiment. Similarly, Lu and

Vonshak (2002) found that salt stress resulted in a decrease in F_M in cyanobacterial *Spirulina platensis* cells. On the other hand, treatment with 1.8 and 2.9M NaCl solutions led to an increase in both F_O and F_M. Kitajima and Butler (1975) found that a reduction of the rate constant for the photochemistry of PS II results in an increase in F_O (initial fluorescence at open PSII traps), whereas a rise in the rate constant of non-radiative energy dissipation provides a decrease in both F_O and F_M (maximum fluorescence at closed PSII traps). The studied chlorolichens showed a different pattern, since F_O was rather constant in all treatments. Therefore, it can be concluded that the decrease in F_V/F_M was directly related to the decrease in F_M, and the impairment in antenna chlorophyll efficiency was not as considerable as in cyanolichens. However, the observed reduction in F_M values may suggest a reduced number of light harvesting complex antennae (Belgio et al., 2012).

FLUORESCENCE PARAMETERS

Measurements of different fluorescence parameters are a common and useful method to study the effects of abiotic stress on organisms including plants (Loudari et al., 2020), lichens (Bednaříková et al., 2020) or cyanobacteria (Lu and Vonshak, 2002). Our findings showed that salt stress leads to serious disturbances not only in F_V/F_M but also in other fluorescence parameters. Both in the case of chlorolichens and cyanolichens, significant increases in the values of ABS/RC and DI_0/RC and decreases in PI_{ABS}, quantum yields and efficiencies, the electron transport flux and trapped energy flux per reaction centre (ET_0/RC, TR_0/RC) were observed on the last day of the experiment. The majority of changes described above were recognized for chlorolichens treated with 2.9M and 3.9M NaCl solutions and for cyanolichens treated with solutions with a NaCl concentration greater than 0.35M. The high flux of dissipated excitation energy per RC could be connected with the severe stress of PSII. The stress of elevated temperature also resulted in an increase in ABS/RC and in DI_0/RC and a rapid decrease in PI_{ABS} in lichens (Bednaříková et al., 2020). Salt stress also caused a decrease in PI_{ABS} in tomato plants, which was probably due to a reduction of the electron transport chain and a decrease in the chlorophyll content (Loudari et al., 2020). The simultaneous increase in ABS/RC and DI_0/RC and a decrease

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in PI_{ABS}, φ_{R0}, φ_{E0} as a response to the exposure of plants to salt stress and salt-drought stress suggested that these negative effects were mainly due to ionic stress and not to reduced water availability in *Chenopodium quinoa* (Killi and Haworth, 2017). Chowaniec and Rola (2022) tested the effect of salt stress on *H. physodes* and *P. furfuracea* and found that some photosynthetic parameters, e.g., ABS/RC, DI_0/RC or PI_{ABS} may be more informative and better indicate the negative impact of salt stress on lichens than F_V/F_M.

The observed differences between the studied chlorolichens and cyanolichens could result from other photosynthetic partners. Hu et al. (2011) found that the effect of salt stress on photosynthesis in the cyanobacteria *Scytonema javanicum* could be related to inactivation of the reaction centres and reduction in the electron transport. Free-living cyanobacteria are known to be significantly more sensitive to oxidative stress compared to green algae (Drábková et al., 2007; Weenink et al., 2021) and this is related to the fact that they are prokaryotic organisms having their photosynthetic apparatus partially located directly in the cytosol, which makes them more exposed to harmful factors (Barrington and Ghadouani, 2008). Secondly, algal cells of phycobionts possess a vacuole as a special compartment that is less active in a cellular metabolism but plays an essential role in maintaining the osmotic balance of a given cell (Erdman and Hagemann, 2001). Although many cyanobacteria developed adaptations to survive in highly saline environments (Hagemann, 2011), inland species seem to be more susceptible to salt stress.

RECOVERY OF PHOTOSYNTHETIC EFFICIENCY INDUCED BY SIMULATED RAINFALL

So far, the phenomenon of reversibility of disruption in the photosynthetic process caused by salt stress in lichens has only been analyzed in a few studies (e.g., Lange et al., 1986; Matos et al., 2011). The species examined in the present study showed very differentiated responses, and in certain species the recovery of PS II was observed. In most species, following exposure to low salt doses, water treatment resulted in a significant increase in F_V/F_M to values corresponding to healthy lichens. An interesting result concerns much weaker capability to recover photosynthetic efficiency after soaking in water by cyanolichens compared to chlorolichens. In the case of chlorolichens, regeneration was possible throughout the experi-

ment. Furthermore, the increase in F_V/F_M to values corresponding to healthy lichens was possible even after treatment with high salt doses. On the other hand, Matos et al. (2011) found that incubation of *Ramalina canariensis* in artificial sea water for 2 h caused irreversible damage to PSII, since the F_V/F_M values remained lower than in control samples even after 48 h after incubation in deionized water. In the case of the studied cyanolichens, efficient regeneration was possible only in lichens treated with low salt doses and, at most, on the first day of the experiment. Lange et al. (1986) reported differences in reactivation of the photosynthetic process in 73 species of chlorolichens and 33 species of cyanolichens after treatment with an air stream of high relative humidity and after spraying lichens with liquid water. Their study showed that, in lichens with green algae as photosynthetic partners, both types of water supply resulted in reactivation of photosynthesis, while in lichens with cyanobacteria, the stimulation of photosynthesis took place only after liquid water uptake. Consequently, rainfall may be of great importance for reactivation of photosynthesis in inland cyanolichens.

The research on the responses of cyanobacteria to salt stress showed that the NaCl-induced decline in the photosynthetic activity of PS II first involves a rapid decrease in photosystem activity, which is reversible and associated with osmotic effects (Allakhverdiev and Murata, 2008). This could explain the obtained results concerning the recovery of photosystem activity in cyanolichens after they were soaked in water only immediately after exposure to salt stress. With the passage of time, the changes induced by salt stress in cyanobacteria became irreversible and proved to be associated with ionic effects that cause the dissociation of external proteins from photosystems (cf. Allakhverdiev and Murata, 2008). These irreversible effects could explain, firstly, significantly reduced F_V/F_M in cyanolichens treated with high NaCl concentrations and poor ability to spontaneous regeneration over time, and secondly, a lack of the ability to regenerate photosynthetic activity on the later days of the experiment.

CONCLUSIONS

Our results showed that acute salt stress leads to a significant reduction in photosynthetic efficiency in both chlorolichens, i.e., *C. furcata*,

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C. mitis, *D. muscorum*, and cyanolichens, i.e., *P. didactyla* and *P. rufescens*. We observed that cyanolichens are more sensitive to salt stress than chlorolichens, and even exposure to low salt concentrations resulted in a decrease of F_V/F_M immediately after exposure to salt stress. Due to both dehydration and ionic stress, even a single episode of acute salt stress may lead to the permanent inhibition of the photosynthetic process in the studied cyanolichens. Due to their poikilohydric nature, lichens are strongly dependent on water from the surrounding environment, and a temporary lack of availability in combination with ionic stress can cause serious disturbances in the physiology of lichens.

Acute salt stress significantly affected the course of the fluorescence induction curves. In the case of chlorolichens, the decrease in F_M in most experimental groups and the flattened shape of the transient curves after treatment with high salt doses were the most apparent. Significantly greater disturbances were observed in cyanolichens, in which the induction curve lost its sigmoid characteristics after treatment with low salt doses.

Regarding photosynthetic efficiency after soaking in water, we observed that chlorolichens have a significantly higher ability to recover after acute salt stress than cyanolichens. Lichens with green algae were able to regenerate after water supply even after exposure to acute salt stress, while in cyanolichens the return of F_V/F_M to the characteristic level of healthy lichens was only possible after treatment with low salt concentrations. These observations indicate that rainfall provides relevant recovery of photosynthetic efficiency in chlorolichens and partial recovery in cyanolichens.

Finally, we indicated that the time when rainfall occurs after exposure to salt stress is a key factor affecting the potential regeneration of PSII efficiency. This concerns, in particular, cyanolichens, for which noticeable regeneration was possible only shortly after exposure to salt stress. Regeneration after rainfall is an important aspect for epigeic lichens occurring near roadsides, where, in winter, they are exposed to de-icing salt for a long time, and rainfall may partially compensate for their disturbances and increase photosynthetic efficiency enhancing the possibility of their survival.

AUTHORS' CONTRIBUTIONS

Karolina Chowaniec: Conceptualization, Investigation, Visualization, Writing - original draft preparation. Jakub Styburski: Investigation, Writing - review and editing. Kaja Rola: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing - review and editing, Visualization, Supervision.

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