Species-specific responses of Antarctic terrestrial microalgae to salinity stress. Comparative study in *Klebsormidium* sp. and *Stigeoclonium* sp.

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Abstract

We studied the changes in PSII photochemical processes in the cells of Antarctic algae *Klebsormidium* sp. and *Stigeoclonium* sp. exposed to salinity stress (0 – 3M NaCl) for 3 h. Salinity stress induced a decrease in the potential (F_V/F_M) and effective quantum yield of PSII electron transport (Φ_{PSII}). Salinity stress induced a decrease in vitality index (Rfd, relative decrease of chlorophyll fluorescence). Analyses of the polyphasic fast chlorophyll fluorescence transients (OJIP) showed that with the increase in salt concentration, the chlorophyll fluorescence signals recorded at the phases J, I, and P declined, and the transient flattened with increaseing NaCl concentration reaching close to zero ChlF values at salt concentration of 3 M NaCl after 180 min. exposition. *Klebsormidium* sp. was found more salinity stress resistant than *Stigeoclonium* sp.

Key words: chlorophyll fluorescence, microalgae, quantum yield, OJIP, salt stress, PSII inhibition, *Klebsormidium* sp., *Stigeoclonium* sp.

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Introduction

Although tolerance/resistence to saline conditions has been thoroughly studied in vascular plants, knowledge on salinity stress effects in free-living microalgae is rather scarse. In freshwater algae, the impact of high to extreme salt concentrations on their physiological responses have been investigated only to a limited extend. Holzinger and Pichrtová (2016) overviewed various stress tolerance mechanisms in paraphyletic group of freshwater and terrestrial green algae. They stated that formation of dormant spores is a typical behaviour of freshwater classes in response to salt stress while true terrestrial alge exhibit stress tolerance in vegetative state. The authors refer to aggregation of cells, flexible cell walls formation, mucilage production and accumulation of osmotically active compounds. In terrestrial habitats, a

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combination of stressors occurs and, therefore, terrestrial algae exhibit a different degree of tolerance/resistence to abiotic stresses such as *e.g.*, desiccation, osmotic stress (Kaplan et al. 2013), freezing and high PAR and UV radiation (Stamenkovic and Hanelt 2014). Several cellular mechanisms are involved into the response of algae to abiotic stress. They comprise a wide range of physiological and biochemical responses including K^+ transport and signalling, and phytohormones activity (for review *see* Kaur et al. 2022). For salinity stress in freshwater microalgae, accumulation and excrettion of glycerol is reported (León and Galván 1994). Moreover, synthesis of osmolytes, such as *e.g.*, proline, taurine, leucine and lysine was found in *Chlamydomonas reinhardtii* cells exposed to salt stress (Reynoso and de Gamboa 1982). Increased lipid and fatty acid synthesis in response to salinity stress is reported for *C. reinhardtii* as well as *C. nivalis* (Hounslow et al. 2021) since the two species are often exposed not only to extreme temperatures, but also to radical salinity changes in their natural environments (Bazzani et al. 2021).

Salinity stress is known to have negative effects on plant growth and development. Whereas most studies on salinity stress effects have been performed in higher plants, data on the salinity stress responses in green algae are rather limited. It is known, however, that under salinity stress, the turgor pressure, ion distribution, and organic solutes in the cell are disrupted, resulting in reactive oxygen species (ROS) formation with consequent oxidative damage to cells and their compartments (Liu and Pang 2010). Salinity stress, however, may increase lipids and carotenoids production in algae (Teronpi et al. 2021). On chloroplast level, salinity-induced oxidative stress leads to inhibition of photosystems II and activation of protective mechanisms such as *e.g.*, state transition (Endo et al. 1995). Salinity stress in microalgae lead to a higher sensitivity to photoinhibition (Neale and Melis 1989). Several studies revealed the decline in PSII functioning under salinity stress in microalgae using several chlorophyll fluorescence methods, typically the evaluation of potential (F_V/F_M), effective quantum yield (Φ_{PSII}) and non-photochemical quenching of absorbed light energy (*e.g.*, Zuo et al. 2014). Therefore, chlorophyll fluorescence parameters related to PSII are generally considered as a good indicators of salinity-induced changes in algal photosynthetic apparatus. The negative changes are typically attributed to light harvesting complexes (LHCs) of PSI and PSII, proteins of PSII involved in oxygen (O_2) evolution as shown for *Chlamydomonas reinhardtii* (Subramanyam et al. 2010, Neelam and Subramanyam 2013). Affenzeller et al. (2009) measured primary photosynthetic processes in *Micrasterias denticulata* in response to NaCl and KCl.

In our study, we focused on the salinity-induced limitation in photosynthetic apparatus of two experimental algae *Klebsormidium* sp. and *Stigeoclonium* sp. from the James Ross Island, Antarctica. The two algae were selected because they occur in the streams and shallow freshwater lakes/ponds located close to a seashore (for review on the lakes and thier chemolimnology, *see* Nedbalová et al. 2013). In such habitats, sea spray reaches costal terrestial ecosystems on windy days and, after deposition, contribute to the input of salts into the system. We hypothesized species-specific response in chlorophyll fluorescence parameters related to PSII. We supposed that differences in PSII performance would suggest different degree of salinity resistence of the two experimental species. The aim was to correlate the chlorophyll fluorescence parameters related specifically to PSII functioning (OJIP-derived parameters) under salinity stress to that evaluating linear electron transport chain, *i.e.*, effective quantum yield of PSII (Φ_{PSII}).

Material and Methods

Experimental species

Two species of Antarctic fresh-water algae were used for the experiment: *Klebsormidium* sp. and *Stigeoclonium* sp. The *Klebsormidium* strain was obtained from the Culture Collection of Autotrophic Organisms (CCALA strain 859, Šnokhausová et Elster 2008/8), Institute of Botany, Academy of Sciences of the Czech Republic, Třeboň. The samples of *Stigeoclonium* sp. were collected from lake bottom in the James Ross Island, Antarctica. Stock culture of *Stigeoclonium* sp. was cultivated in the EEL collection (Masaryk University, Brno, Czech Republic). The two species are quite abundant at the James Ross Island, Antarctica. They occur in shallow lakes such as Big Lachman Lake, Phormidium Lake and many other lakes and ponds (*see* e.g., Komárek et al. 2008).

Klebsormidium sp. is green, filamentous alga. The cells are cylindrical without significant narrowing across cell wall. Occurrence is possible either in the form of clumps, long fillaments or groups of short fragments, the fibers are simple and unbranched, with a smooth cellular wall. The chloroplast typically contains only one pyrenoid surrounded by a layer of starch grains. Asexual reproduction takes place by means of zoospores released through a hole. Two-pole germination of zoospores is always accompanied by a single-pole germination (Škaloud 2006, Rindi et al. 2008). *Klebsormidium* is desiccation-resistant species, typical component of biological soil crusts (Donner et al. 2017). It frequwently occurs in polar regions thanks to its freezing resistence (Elster et al. 2008).

Stigeoclonium sp. is a typical filamentous green alga. It is a common genus of stagnant and running freshawater ecosystems. *Stigeoclonium* is usually branched with short lateral branches. In cultures, it is known for its phenotypic plasticity since the growth form varies according to cultivation medium and legth of cultivation. *Stigeoclonium sp*. tolerates a wide range of conditions. For *Stigeoclonium* sp. from Antarctic habitats, resistence to sub-zero temperature has been studied (Orekhova et al. 2019).

Cultivation and salinity experiments

Before experiments, *Klebsormidium* sp. and *Stigeoclonium* sp. were cultivated in a 3N inorganic (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.

To study the algal strains resistence to salinity stress, they were exposed to two treatments. In the first set of experiments (*see* potential and effective yield measurements), NaCl was added into the stock culture resulting in the following molarities: 0.001, 0.001, 0.1, 1, and 2 M NaCl. In the second experiment (*see* OJIP measurements), the following molarities were used: 0.03, 0.3 and 3 M). The extent of NaCl molarities was selected so that low NaCl concentration would induce either no or only limited inhibtion of photosynthetic processes while the highest salinity used (2, 3 M) would lead to full or close-to-full inactivation of PSII. Another reason for the molarities selection was the study of Vilumbrales et al. (2013), that used similar extent in the study of Antarctic terrestrial microalgae. The strains were pipetted in a microbiological plate (the hole volume of

350 microliters) and then, NaCl was added to reach the above-specified molarities. The microbiological plate was kept at 5°C throughout the measurements thanks to a water jacket system over the outside of the plate filled with melting crushed ice.

Chlorophyll fluorescence measurements

After the addition of salt solution to the algal cultures, their response to salinity stress was monitored by two methods evaluating salinity-induced limitation in primary photosynthetic processes. The response of PSII photosynthetic processes was evaluated immediately after salt addition and then in tens of minutes interval. The measurements were taken typically in 5 replicates (*see* Fig. legends). During aclimation period (24h) and the measurements, the algal cultures were kept at the temperaure of 5°C. The temperature control was allowed thanks to water jacket system constructed over the microbiological plates using the crushed ice and melting water as cooling agents.

Potential and effective quantum yields measurements

The first metod was the assessment of the potential (F_V/F_M), and effective quantum yields (Φ_{PSII}) of photosynthetic processes in photosystem II. For this purpose, a FluorCam HFC-010 fluorometer (Photon Systems Instruments, Czech Republic) was used and the technique of slow (Kautsky) chlorophyll fluorescence curve supplemented with a saturation pulse in light-adapted state (5 min.) was applied (for details *see* e.g., Marečková and Barták 2016). After the dark adaptation, a saturation pulse was applied in order to measure maximal chlorophyll fluorescence (F_M) allowing the calculation of F_V/F_M. Then, after 30 s of dark, actinic light was switched on, and, after 5 min., another saturation pulse was applied in orded to measure maximal chlorophyll fluorescence on light-adapted sample (F_M'). Then, effective quantum yield (Φ_{PSII}) was calculated using a formula: $\Phi_{PSII} = (F_M' - F_S) / F_M'$, where F_S denotes to stady state chlorophyll fluorescence. Special emphasis was given to the analysis of F_M and F_M' values as affected by particular NaCl concentrations. For such purpose, F_M and F_M' recorded for particular salinity stress and the time of exposition were expresed relatively to the values of control.

Fast chlorophyll fluorescence curve (OJIP)

Fast chlorophyll fluorescence kinetics (OJIP) were measured on dark-adapted algal cultures (5 min.) using a hand-held Fluor Pen (PSI, Drásov, Czech Republic). In untreated samples (no salinity stress), typical OJIP curve included the points O, J, I, and P phases. The lowest chlorophyll fluorescence signal recorded at the initial exposure to light is defined as point $O = F_0$. Then, chlorophyll fluorescence rise in time was followed by intermediate points (J, I) and reaching the maximum at the highest chlorophyll fluorescence defined as point P.

The OJIP curve shape and the O, J, I, P ponits are directly related to reduction of plastoquinone (PQ) pool mediated by transfer of electrons from PSII. Individual points reveal the efficiency of the initial stages of the photosynthetic electron transport. The O-J part of the curve reflects the reduction of primary electron acceptors –

pheophytin and the Q_A quinone (photochemical phase according to Stirbet et al. 2014). The J-I-P part of the OJIP curve is related to the reduction of mobile electron carriers (thermal phase).

The salinity stress-induced changes in OJIP shape were measured and expressed in relation to untreated control. The samples exposed to particular salinity stress in a microbiological plate were pre-darkened for 5 min. inside the measuring compartment of a FluorCam HFC-010 fluorometer (Photon Systems Instruments, Czech Republic). Then, Kautsky curves (transients) supplemented with analysis of quenching mechanisms were measured. The method consisted of measurements of chlorophyll fluorescence curves induced by an actinic light supplemented with saturation pulses given during at dark (F_M induction), and when the actinic light was on (F_M' induction). Potential (F_V/F_M), effective quantum yield (Φ_{PSII}) and non-photochemical quenching (NPQ) were calculated. From the OJIPs, several chlorophyll fluorescence parameters were calculated by the instrumental software according to Strasser et al. (2000). To evaluate the salinity effects on the functioning of PSII in *Klebsormidium* and *Stigeoclonoium*, we analyzed F_V/F_M, ET_0/RC, DI_0/RC, ABS/RC and PI (performance index).

Statistical analysis

If not stated otherwise, statistical analysis was done by one-way analysis of variance ANOVA using the STATISTICA *v*. 14 (StatSoft, Hamburg, Germany) to determine the significance of differences caused by particular NaCl concentrations and the time of exposure effects.

Results

The effect of exposing *Klebsormidium* sp. and *Stigeoclonoium* sp. to elevated concentrations of NaCl on the PSII activity was evaluated as F_V/F_M and Φ_{PSII} time courses (Fig. 1). The inhibition effect of NaCl was apparent immediately after addition and then the F_V/F_M values declined slighly with the time of exposition. The negative effect was concentration-dependent. The most pronounced decrease in F_V/F_M was seen in the two highest concentrations used, *i.e.*, 2 and 1 M NaCl. The smallest, but still statistically significant difference was found in the culture treated with 0.001 M NaCl. The difference between control and the 0.001 treatment was almost constant throughout the time of exposition (180 min.), indicating that the negative effect of NaCl on F_V/F_M happened immediately after the NaCl addition, *i.e.,* within the first 5 min. of exposition. *Klebsormidium* sp. showed higher resistence to salinity stress since the relative decrease in F_V/F_M (compared to control (100%) recorded at the end of exposition time) reached 81 and 58% for the 1 and 2 NaCl treatments, while it was 72 and 53% for *Stigeoclonium* sp.

Salinity stress led to the NaCl concentration-dependend decrease in Φ_{PSII} which was apparent immediatelly after the NaCl addition. Then, the decreased Φ_{PSII} values remained more or less constant throughout the whole exposition time. Despite the NaCl exposition-time dependent decline found in F_V/F_M values, there was no apparent decline in Φ_{PSII} with the time of exposition. For the highest NaCl concentration, the values showed rather slight increase at the end of the exposition time, more apparently in *Stigeoclonium* sp. than in *Klebsormidium* sp. Similarly, the F_V/F_M rela-

tive decline reached smaller values for *Klebsormidium* sp. (79 and 52% for 1 and 2 M NaCl treatments) while it was more pronounced in *Stigeoclonium* sp. (50 and 49%).

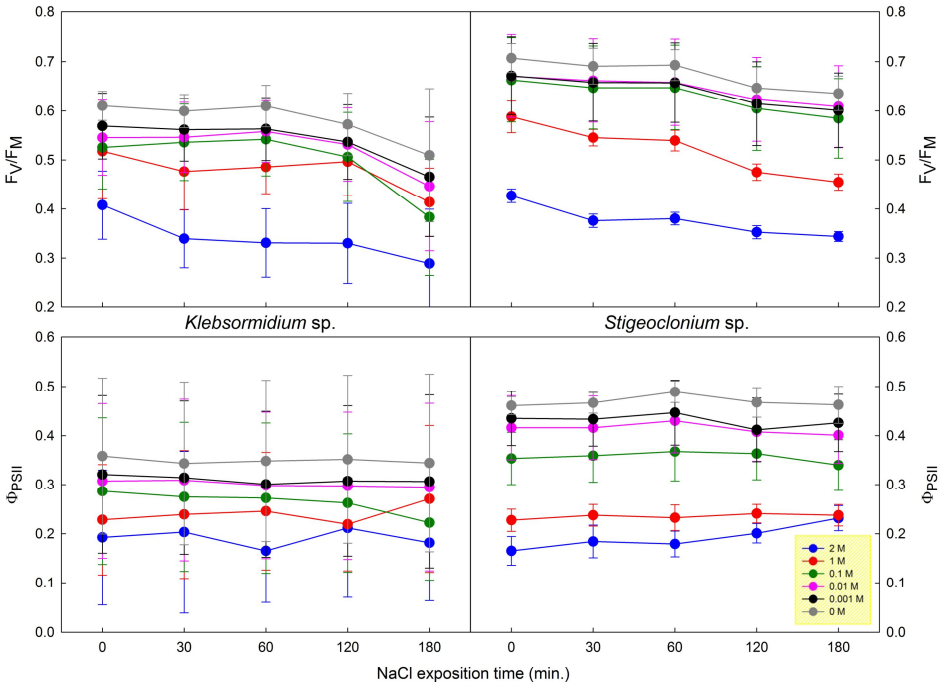

Fig. 1. Time courses of potential (F_V/F_M) and effective quantum yield (Φ_{PSII}) recorded during a short-term treatment of *Klebsormidium* sp. (left panels) and *Stigeoclonium* sp. (right panels) by salinity stress 0.001, 0.01, 0.1, 1, and 2 M NaCl. Data point represents mean of 5 replicates, error bars are standard deviations.

Time courses of maximum chlorophyll fluorescence reached after the application of saturation pulse in dark (F_M) and ligh-adapted state (F_M') showed concentration-dependent response. Typically, F_M and F_M' values dropped down substantially immediately after NaCl addition and then with the time of exposure showed either constant values (F_V/F_M in *Klebsormidium* sp. 0.3 and 0.03 M treatment). Further (but smaller) decline with the time of exposure (F_M' in *Stigeoclonium* sp. 3 M treatment), and even slight increase from the minimum was reached immediately after NaCl addition (*e.g.* F_M in *Stigeoclonium* 3 M treatment).

In both species, Rfd showed significant decrease immediately after NaCl addition only in 3 M treatment while in the other two treatments (0.03 and 0.3 M NaCl), Rfd did not differ from control. In 3 M, Rfd did not show any increase with the time of NaCl exposure indicating decrease in vitality of photosynthetic apparatus *sensu* Haitz and Lichtenthaler (1988).

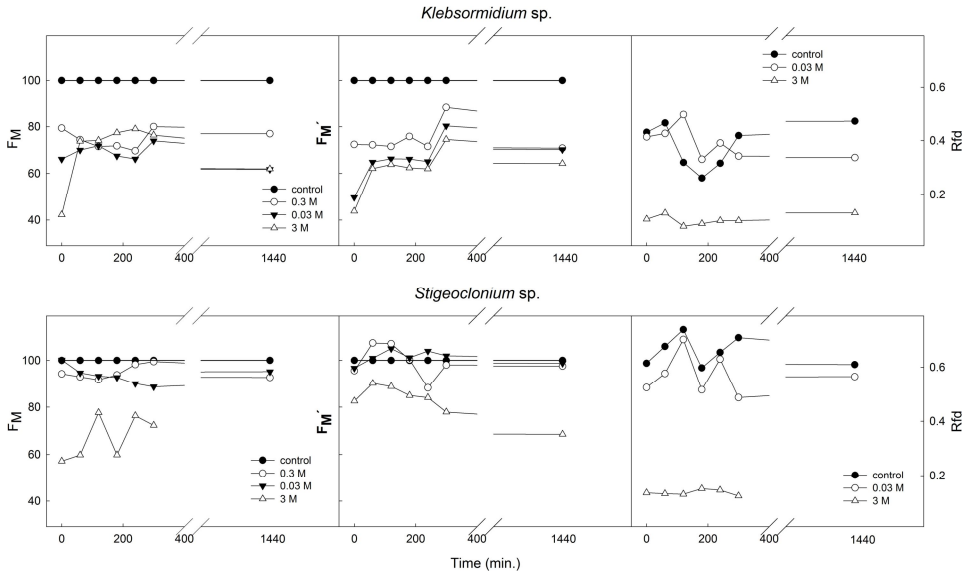

Fig. 2. Time courses of maximum chlorophyll fluorescence F_M, F_M' (relative to conrol) and relative chlorophyll fluorescence decrease (Rfd) recorded during a short-term treatment of *Klebsormidium* sp. and *Stigeoclonium* sp. by salinity stress of 0.03, 0.3 and 3 M NaCl. Data points represent means of 3 replicates, standard deviations are not indicated because they were all under 5%.

OJIPS

In both algal species, salinity stress led to a decrease in the parameters related to PSII functioning (Fig. 3 and 4). In *Klebsormidium* sp., the decline in φPo was apparent 10 min. after the NaCl addition (3 M) while there was only a slight decrease found after 180 min. of exposition for 0.3 and 0.03 M NaCl treatments. A similar behaviour was found for the electron transport rate per reaction centre (ET_0RC), but, contrastingly to φPo which showed constant values over time, ET_0/RC increased slightly after 120 and 180 min. indicating a recovery. Low salinity stres (0.03 and 0.3 M) only led to a slight decrease in ET_0/RC after 120 min. of exposition. These results indicate that salinity stress would inhibit photosynthetic electron transport and deactivate the reaction centers. These changes go in a parallel with the mechanisms protecting PSII and its core (*see* below).

Salinity-induced limitation of primary photosynthetic processes (ΦP_0, ET_0/RC) was accompanied by the activation of protective mechanisms, such as thermal dissipation of absorbed light energy (Fig. 3 and 4). Indeed, DI_0/RC increased dramatically immediately after the addition of 3 M NaCl and remained constant (with values around 12) throughout the exposure time. DI_0/RC also increased at 0.3 M NaCl. The changes induced in PSII funcioning by salinity stress were reflected in a decrease of the performance index of PSII (PI_ABS), which is a general indicator of plant vigor.

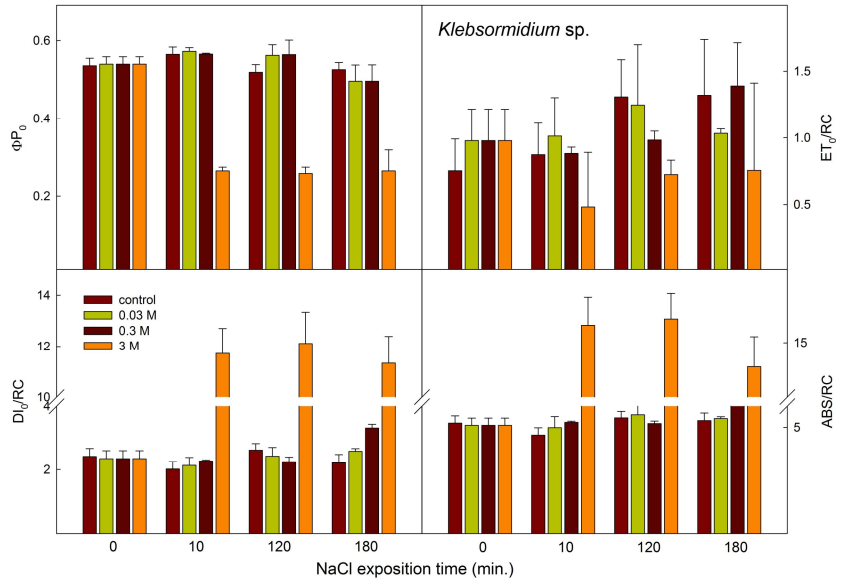

Fig. 3. Primary photosynthesis characteristics (OJIP-derived parameters) recorded for different salinity levels in *Klebsormidium* sp. *Key to abbreviations*: quantum yield of primary photochemistry (ΦP_0), electron transport rate per reaction centre (ET_0/RC), thermal dissipation of absorbed light energy (DI_0/RC) and absorption per reaction centre (ABS/RC). Values are means of 3 replicates. Error bars represent standard deviations.

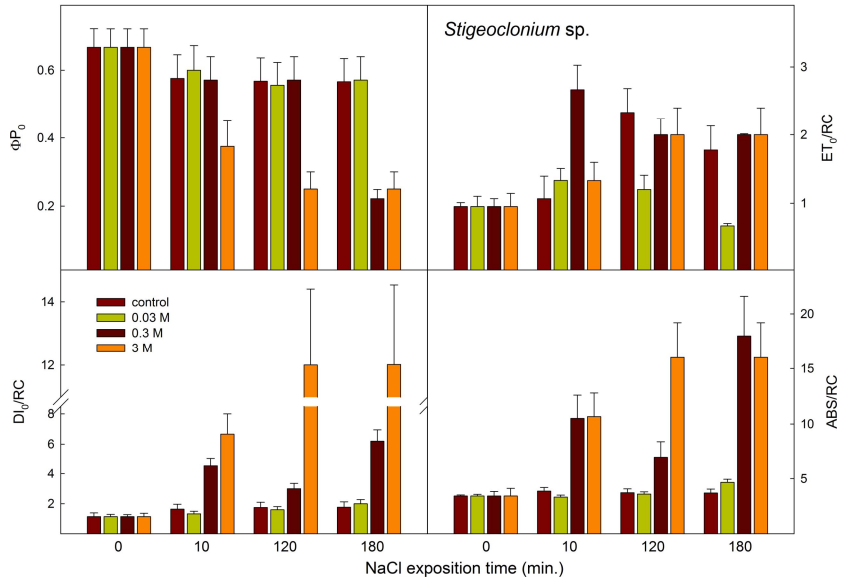

Fig. 4. Primary photosynthesis characteristics (OJIP-derived parameters) recorded for different salinity levels in *Stigeoclonium* sp. *Key to abbreviations*: quantum yield of primary photochemistry (ΦP_0), electron transport rate per reaction centre (ET_0/RC), thermal dissipation of absorbed light energy (DI_0/RC) and absorption per reaction centre (ABS/RC). Values are means of 3 replicates. Error bars represent standard deviations.

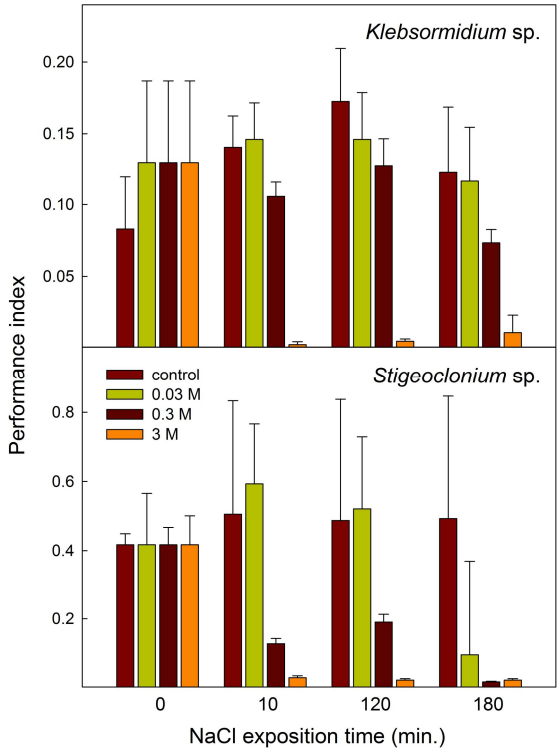

Fig. 5. Performance index of the two studied algal species (*Klebsormidium* sp. and *Stigeoclonium* sp.) as a function of NaCl concentration and exposure time. Values are means of 3 replicates. Error bars represent standard deviations.

Discussion

Salinity stress leads to many changes in PSII functioning that can be detected by chlorophyll fluorescence measurements. Typically, it disrupts the electron transport from the RCs of PSII to the plastoquinone pool (Strasser et al. 2000). Oxygen evolving complex (OEC) seems to be among the first targets of salinity stress. The salinity-induced decrease in OEC functioning is usually accompanied by photosynthetic electron transport disorder. Considering chlorophyll fluorescence parameters, these changes are typically shown as a decrease in maximum quantum yield of PSII and an increase in non-photochemical quenching (*see* e.g., Kalaji and Rutkowska 2004, Kalaji et al. 2016). Under salinity stress, electron trapping in PSII reaction center becomes less efficient due to the salinity-induced dissociation of LHCII and PSII – *see* also discussion of OJIP parameters below.

In the present study, the decline of Φ_{PSII} in response to salinity stress and its duration (Fig. 1) is comparable with previous evidence reported for other microautotrophs, such as the cyanobacterium *Spirulina platensis* (Lu and Zhang 1999, Lu and Vonshak 2002), *Dictyosphaerium chlorelloides* and *Microcystis aeruginosa* (Bartolomé et al. 2009). Apart from NaCl concentration, several environmental factors may interact and negatively affect algal photosynthetic apparatus in salinity stud-

ies. Kumar et al. (2021) suggested that the impact of salinity stress caused by NaCl on photosynthetic pigments, the effective quantum yield of PSII and oxidative stress is dependent on light quality and quantity and photoperiods. They concluded that the negative effect of salinity is alleviated by high light. Piratru et al. (2012) suggested that increased carotenoid synthesis in NaCl-treated *Scenedesmus* is apparent after the deterioration of reaction centres and the decline in PSII photochemical efficiency. The salinity-induced decline in Φ_{PSII} was accompanied by an increase in non-photochemical quenching (data not shown). Such response is aimed to protect PSII from overenergization, ROS formation and destructive changes to PSII. Salinity-induced qN increase has been reported for green microalgae by *e.g.*, Masojídek et al. (2000) and Anandraj et al. (2020).

The observed decrease in F_M and F_M' found at 3 M NaCl in *Klebsormidium* and *Stigeoclonium* is a well-known phenomenon found in previous salinity studies in cyanobacteria (Lu and Vonshak 2002), microalgae (Liang et al. 2014) and vascular plants (*e.g.*, Hniličková et al. 2017, Oláh et al. 2021). Rfd, which is considered an indicator of plant vitality under stressful conditions, decreased at the highest salinity level (3M NaCl) as well, in accordance with Shin et al. (2020, 2021), who also found a significant decrease in Rfd levels in plants exposed to the extreme salinity stress.

Salinity stress lowered chlorophyll fluorescence signal, which flattend the OJIP curves (*see* Supplementary materials) similarly to NaCl-treated *Spirulina platensis* (Zhang et al. 2010). This phenomenon was reported earlier for Antarctic algae (Vilumbrales et al. 2013) under salinity stress being attributed to a strong limitation of reoxidation rate of quinones. A decreased ability of PS II to transfer absorbed light energy to the photosynthetic linear electron transport chain is a consequence of such decrease in quinones reoxidation rate. Similarly to our results, salinity stress caused the flattening of OJIPS in desert cyanobacterium *Scytonema javanicum*, as reported by Hu et al. (2014).

Our results based on the analysis of OJIP derived parameters also suggest that salinity stress reduced the amount of active PSII reaction centers per excited cross section (RC/CS) and the total number of active reaction centers per absorption (RC/ABS) – data not shown.

Concluding remarks

In conclusion, the results presented in this study suggest that salinity-induced stress had multiple effects on PSII functioning in *Klebsormidium* sp. and *Stigeoclonium* sp. Salinity effects on photosynthesis can occur through the inactivation of reaction centers and the inhibition of the electron transport, but this inhibition may occur at the PSII donor and acceptor sites. Comparison of chlorophyll fluorescence parameters between species indicates that *Klebsormidium* sp. is more salt resistant than *Stigeoclonium* sp. The most likely reason that would explain this is that *Klebsormidium* sp. is a common member of biological soil crusts (Borchard and Gründling-Pfaff 2020), while *Stigeoclonium* sp. is more often found in aquatic environments like freshwater lakes, ponds and streams. Biological soil crusts in coastal locations are typically exposed to more extreme environmental factors (including salt depositon from sea spray) than freshwater terrestrial ecosystems.

Salinity is one of the most significant environmental factors affecting the photosynthetic rate and growth of photosynthetic organisms. Unicellular photosyn-

thetic microalgae are especially vulnerable to salinity stress as they must cope, not only with ionic imbalance and osmotic stress, but also with reactive oxygen species formation affecting the functioning of photosystem II (Shetty et al. 2019). In freshwater algae, salinity stress leads to partial or full inhibition of photosynthetic processes in PSII as shown in our study for 3 M NaCl treatment. Our results suggest that, even when *Klebsormidium* sp. and *Stigeoclonium sp.* are confronted with extreme saline conditions, partial functioning of PSII still persists and there is even a slight improvement over time. This is consistent with the findings of Hinojosa-Vidal et al. (2018) who found detectable photosynthetic activity of PSII after 72 h of exposure of *Trebouxia* sp. to 5 M NaCl.

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Supplementary Materials

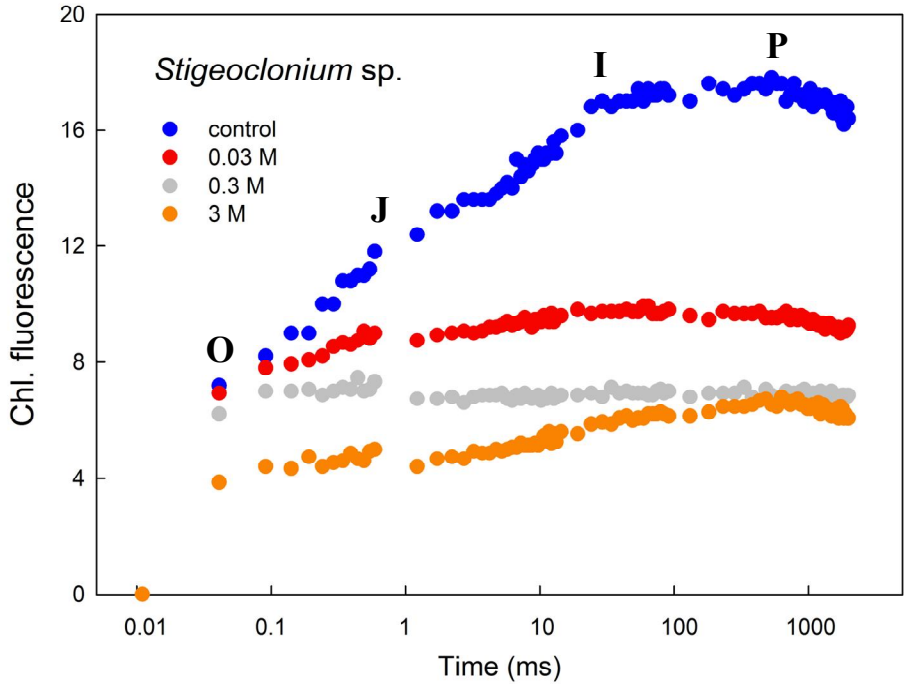

Fig. 6. OJIP curves for *Stigeoclonium* sp. in response to the NaCl treatment (10 min. exposition). For each NaCl concentration, data points are means of three replicates. Particular chlorophyll fluorescence levels (O, J, I, P) are indicated for the untreated control curve. Salinity-induced limitation of primary photosynthetic processes in PSII is demonstrated by a concentration-dependent decrease in chlorophyll fluorescence (flattening of the curves) and an increase in J point (relatively to P point).