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ABSTRACT

Lichens may undergo cycles of dehydration-desiccation-rehydration several times a day and this, combined with high light irradiances, can determinate a strong oxidative stress. With the aim of evaluating how lichens with different photobionts and ecology cope with this stress, lobes from *Flavoparmelia caperata*, *Lobaria pulmonaria*, *Peltigera leucophlebia* and *Peltigera praetextata* were submitted to four combinations of light and drought and their chlorophyll *a* fluorescence and pigments were analyzed. Light was the environmental factor with the most negative effects in all the four species, more than desiccation or prolonged periods in darkness. *F. caperata* was the most resistant species, tolerating moderately high light regardless of its hydration, while the other three species showed more vulnerability to the light in the dry state. Chlorophyll and antioxidant concentrations were not influenced by the treatments and were considered as constitutively expressed. All the three species with green algae seemed to use the xanthophyll cycle when submitted to desiccation and/or high light, but to different extents. *Lobaria pulmonaria* used it massively when illuminated in the hydrated state, probably also for photoprotection. This is likely to be the reason why it performed better than when illuminated in the dry state. Nevertheless, *F. caperata* was able to perform better without this extra photoprotection and further investigation is needed to understand the molecular mechanisms underlying its tolerance. To verify if the photobionts share the same mechanisms of their lichen counterparts, the experiment was reiterated with two of them, *Trebouxia gelatinosa* (photobiont of *F. caperata*) and *Symbiochloris reticulata* (photobiont of *L. pulmonaria*), the latter being studied for the first time from the physiological point of view. The most resistant species was *T. gelatinosa*, as its performance was comparable to that of its lichenized counterpart. Major differences were the inability to use the xanthophyll cycle and the presence of repair mechanisms upon rewetting. *S. reticulata*, instead, suffered consistently each treatment and its recovery was always incomplete. When metabolically active, it degraded a large part of its chlorophylls probably to minimize the damage in case of light exposure, but it was not able to rebuild them upon recovery. So, we hypothesized that when isolated it is desiccation tolerant to a lesser extent. We can conclude that lichenization can influence the physiology of photobionts in very different ways, as *T. gelatinosa* would be able to survive unharmed out of a lichen thallus, while *S. reticulata* acquires important protective mechanisms from photo-oxidative stress, becoming able to colonize environments that otherwise would kill it as free-living.

INTRODUCTION

A lichen is a symbiotic organism formed by a fungus (mycobiont) and a photosynthetic partner (photobiont), which may be a green alga, a cyanobacterium or, in some cases, both of them. The symbionts develop together in a so-called thallus, a fungal body that encases the photobiont, with different degrees of organization (Ahmadjian, 1993). Recent studies (e.g. Grube & Berg, 2009) have broadened the concept of lichen symbiosis, extending it to the microbial communities that inhabit the thallus, as it seems they have a role, at least in nutrient uptake (Grube *et al.*, 2009). Nevertheless, the lichen thallus is usually regarded as an individual and treated as a whole in many studies (Nash, 2008).

The two major components, the mycobiont and the photobiont, are supposed to take both advantage from their association, but there is still some debate about this topic. Some Authors think that the lichen relationship is more a controlled parasitism, in which the fungus encloses and controls the alga, forcing it to produce nutrients for both (Ahmadjian, 1993; Lücking *et al.*, 2009). Others sustain that the relationship is mutualistic, no matter how much carbon the fungus subtracts from the alga, as both organisms increase their ecological fitness in a lichen thallus (Honegger, 1998; Nash, 2008). In partial agreement with both points of view, Eisenreich *et al.* (2011) point out that the diversity of lichens may result in different kinds of interactions between symbionts. As a matter of fact, both symbionts are seldom found free-living: the mycobionts in nature are found almost exclusively in the symbiotic state (Honegger, 1998), while for the photobionts the situation is more diverse. Some genera like *Gloeocapsa*, *Nostoc*, *Scytonema*, and *Trentepohlia* commonly occur both in lichenized and free-living states, sometimes even co-occurring (Nash, 2008). On the other hand, one of the most widespread lichen photobiont genera, *Trebouxia*, is rarely found outside a lichen thallus (Bubrick *et al.*, 1984), and some Authors even question that it can exist free-living (Ahmadjian, 1988; 2001). In a lichen thallus, though, both symbionts have access to habitats where separately they would be rare or even not present. As a result, lichens can be found in almost all terrestrial habitats and approximately 8% of terrestrial ecosystems are lichen-dominated (Larson, 1987). In particular, they are able to thrive in habitats characterized by severe abiotic stresses, such as high light irradiances, extreme temperatures or frequent lack of water (Nash, 2008). For what concerns water management, lichens are poikilohydric organisms, unable to actively regulate their water content, that strictly depends on the environmental water availability. Poikilohydric organisms include both desiccation sensitive and desiccation tolerant species (Kranner *et al.*, 2008). With the exception of some aquatic (Ried, 1960) and some tropical rain forest lichens such as *Pseudocyphellaria dissimilis* (Green *et al.*, 1991), the vast majority of them are desiccation-tolerant (Kranner *et al.*, 2008).

Desiccation tolerance occurs in many phylogenetically unrelated taxa such as cyanobacteria, algae, mosses, ferns, some angiosperms (the so-called "resurrection plants"), fungi, lichens, rotifers, nematodes and arthropods. In some organisms, it is restricted only to a particular phase of their life cycle, such as most of the pollen grains and seeds of desiccation sensitive higher plants (Alpert, 2000; 2006). It is defined as the ability to survive drying to equilibrium with air at relative humidity of 50% or less and to resume metabolic activities when water is available again (Leprince & Buitink, 2015).

The absence of water can cause many kind of damages. When the cytoplasmic bulk water is removed, cells lose turgor and shrink, determining mechanical damages, such as membrane collapse (Hoekstra *et al.*, 2001). Another effect is the increase in production of reactive oxygen species (ROS) that can lead to oxidative damage. ROS are a group of radical (chemical species with one unpaired electron) and non-radical molecules derived from molecular oxygen with different degrees of oxidative properties (Turrens, 2003). They are a metabolic byproduct of all organisms, but many kinds of stress, including drought and high light stress, can increase their formation, creating an imbalance between production and scavenging. As a result, an excess of ROS can lead to oxidation, and thus damage, of many important macromolecules, such as lipids, proteins or even DNA (Beckett & Minibayeva, 2007). In photosynthetic organisms there is another source of ROS: the chloroplast. When desiccation restricts photosynthesis, excess energy can be transferred from pigments to ground-state oxygen, forming singlet oxygen that can oxidize chloroplast molecules and even trigger cell death (Halliwell, 2006).

A desiccation tolerant organism is able to cope with these stresses and thus to survive in a state of suspended animation until water is available again and metabolism is resumed. Multiple mechanisms of protection have evolved, covering all the phases of desiccation (during drying, when dry and upon rehydration) and many of them are shared by organisms in very different phylogenetic positions (Leprince & Buitink, 2015). To avoid cellular collapse, desiccation tolerant organisms accumulate non-reducing sugars (e.g. polyols), that replace water molecules binding to macromolecules and membranes, maintaining their structure (Yancey, 2005; Kranner *et al.*, 2008). Together with late embryogenesis abundant (LEA) proteins they contribute to the formation of the so-called vitrification, a condition in which cellular water is completely replaced by these solutes and the cytoplasm undergoes transition from a liquid to a glassy state. In the glassy state molecular mobility is reduced and all chemical reactions are slowed down, including those causing cellular deterioration. Glass formation occurs also in desiccation sensitive organisms, but the latter die before reaching it. So, it is not a mechanism that initially confers the tolerance to desiccation during drying, but it is indispensable to survive the dry state (Buitink & Leprince, 2008). There are a few other mechanisms

that help preserve membrane integrity, such as forming large cytoplasmic gas bubbles to retain contact between the protoplast and the cell wall in the desiccated state (Honegger, 1995) and the expression of expansins, that allow a safe cell shrinkage (Jones & McQueen-Mason, 2004). To avoid oxidative damage, desiccation tolerant organisms can either scavenge ROS or avoid their formation. Once formed, ROS can be scavenged using both enzymatic and non-enzymatic antioxidants. Enzymes like superoxide dismutase (SOD), catalase (CAT) and peroxidase are known to process ROS, even though they are thought to account only for a minor part of the ROS management during desiccation and are more likely to be used during normal metabolism. Low molecular weight antioxidants like glutathione (GSH), ascorbate and tocopherols are also known to scavenge ROS (Kranner & Birtić, 2005; Kranner *et al.*, 2008) and in plant tissues can also cooperate with the previously mentioned enzymes in the so-called "Halliwell/Foyer/Asada cycle" (Foyer & Halliwell, 1976). To simply avoid ROS formation, several strategies exist. In mitochondria the electron flow can be uncoupled from phosphorylation, resulting in heat formation and reduced ROS production (Kranner *et al.*, 2008). Plants have the additional problem of their chlorophylls, so some of them (poikilochlorophyllous plants) are able to dismantle the whole photosynthetic apparatus, reassembling it only after rehydration (Bewley, 1979). Another way to limit the ROS production is through the so called non-photochemical quenching (NPQ), a mechanism that dissipates excess excitation energy as heat. To carry out NPQ, plants use a variety of reactions involving carotenoids such as β-carotene, lutein, neoxanthin and the xanthophyll cycle pigments. During the xanthophyll cycle, the enzyme violaxanthin de-epoxidase (VDE) uses excess excitation energy to rapidly convert violaxanthin (V) into antheraxanthin (A) and then zeaxanthin (Z), with the double advantage of dissipating energy and accumulating zeaxanthin, that has further antioxidant properties. When water is available again, the cycle is rapidly reversed (Vráblíková *et al.*, 2005; Demmig-Adams, 2003; Kranner *et al.*, 2008). The xanthophyll cycle is known to protect plants from a combination of light and drought stress, but it is used also when drying in the complete darkness (Fernández-Marín *et al.*, 2011). Finally, desiccation tolerant species rely also on repair mechanisms upon rewetting, such as the production of molecular chaperones to help the correct refolding of proteins when hydrated again (Candotto Carniel *et al.*, 2016) or the expression of LEA proteins to dissociate desiccation-induced protein aggregates (Leprince & Buitink, 2015).

Those listed above are just some of the major strategies used to survive desiccation, but the reality is far more complicated than that. Neither of these mechanisms can confer tolerance on its own, it is rather the simultaneous interplay of several of them to be essential (Hoekstra *et al.*, 2001). In fact, different organisms can face different kinds of desiccation or drought stress and so each one has evolved its personal array of strategies to contrast it, resulting in different degrees of desiccation

tolerance. So, different organisms can survive to different thresholds of water content (20% to below), have different longevities in the dry state (from hours to years) and can tolerate different rates of drying (Leprince & Buitink, 2015). Even in the same organism, different kinds of stress can result in different strategies. So for example, a fast dehydration triggers an immediate response, determined by constitutive mechanisms, while a slow dehydration can rely on more time consuming inducible mechanisms (Kranner *et al.*, 2008). As a result, the mechanism as a whole is still far from being completely understood.

As previously said, lichens are able to colonize extreme environments, where stresses such as desiccation may be severe, sudden and frequent. Potentially, they may experience cycles of dehydration-desiccation-rehydration several times a day. For this reason, differently from resurrection plants, the vast majority of the mechanisms responsible for their desiccation tolerance is constitutive, as demonstrated also by a small number of -omics (in this case, transcriptomics and proteomics) studies on lichen photobionts (Gasulla *et al.*, 2013; 2016; Candotto Carniel *et al.*, 2016). So for example, lichens generally are homoiochlorophyllous, meaning that their photosynthetic apparatus remains essentially intact through each drying and rewetting cycle (Tuba *et al.*, 1998). This comes to the cost of a more pronounced oxidative stress, but, for organisms adapted to periods of drying and remoistening ranging in length from hours to weeks, it has the advantage to grant the quick resume of photosynthesis after rehydration, in a matter of minutes (Tuba *et al.*, 1996; Tuba *et al.*, 1998). In some cases, a partial loss of chlorophylls have been documented when more sensitive species were desiccated for a long period of time, but it could be more the result of a damage than a strategy to reduce oxidative stress (Kranner *et al.*, 2003). This does not mean, though, that lichens do not possess also inducible mechanisms: in the -omics studies cited above, the vast majority of transcripts or proteins remained constant, but there was a small number of ones that varied during desiccation or rehydration. Finally, lichens and other lower plants are believed to rely heavily on repair upon rehydration, but how and to what extent is still unclear (Farrant *et al.*, 2003). For example, during rehydration following desiccation, carbon fixation and membrane integrity take much more time to recover than chlorophyll fluorescence, indicating that possibly some form of repair-based desiccation tolerance mechanism exists. It is still not clear, though, if these mechanisms are synthesized directly upon rewetting or before drying, ready to be prepared for the next cycle of dehydration-desiccation-rehydration (Nash, 2008).

The above mentioned strategies take into account only desiccation, but lichens can face simultaneously many different stresses. A very common situation is a combination of lack of water and high light irradiance, that could exponentially increase ROS formation. There are, however,

several protective mechanisms that a lichen can activate thanks to desiccation itself, the simplest one being the ability to curl its lobes to reduce light exposure (Barták *et al.*, 2006). Also, desiccation causes a reduction in light transmittance through the upper cortex (Ertl, 1951; Dietz *et al.*, 2000) and many other energy-dissipating mechanisms, like the functional disconnection of the photosynthetic apparatus seen before, that are helpful also for high light stress. As a result, lichens in the dry state have an impressive resistance to high light and a wide array of other stresses, and this resistance has been questioned as a general feature of all lichens (Kappen *et al.*, 1998). However, not all the lichens behave in the same way: typically, species that grow in moister low light habitats (such as on bryophytes on soils shaded by trees) tend to be less resistant than species growing in xeric environments (Gauslaa & Solhaug, 1999; Kranner *et al.*, 2008). Kershaw & MacFarlane (1980) demonstrated that high-light protection is far from complete in air-dry thalli of some members of the order Peltigerales and Gauslaa & Solhaug (1996; 1999) furtherly found that old forest cephalolichens (chlorolichens, meaning green algal lichens, with cyanobacteria in cephalodia) are susceptible to high-light exposure in the dry state. Curiously, though, cyanolichens (lichens with a cyanobacterium as photobiont) inhabiting the same old forests were found to be more tolerant to high light during desiccation than the co-occurring chloro- and cephalolichens (Gauslaa *et al.*, 2012). It is once more clear, so, that lichens with different photobionts adopt different strategies and have different photosynthetic performances.

Finally, one should bear in mind that a lichen is, in fact, a dual organism, formed by two very different partners that are supposed to gain ecological fitness together (Nash, 2008). Is the symbiosis as a whole more tolerant to desiccation and photo-oxidative stress than either of the isolated partners alone? Is a lichen more than just the sum of its parts? The answer, for now, remains unclear. For example, Kranner *et al.* (2005) found that antioxidant and photoprotective mechanisms in the lichen *Cladonia vulcani* are more effective by two orders of magnitude than those of its isolated partners, allowing the lichen to tolerate better longer periods of desiccation and higher light intensities. Kosugi *et al.* (2009; 2013) reported that lichenization enhances the ability to tolerate desiccation in photobionts and Petruzzellis *et al.* (2017) observed that the photobiont in a lichen thallus had better cell water status and photosynthetic efficiency than its isolated counterpart. In contrast with these findings, Candotto Carniel et al. (2015) observed that *Trebouxia* sp., photobiont of *Parmotrema perlatum*, had a performance very similar to that of its lichenized counterpart, tolerating the same light intensity and even recovering faster. The Authors hypothesized that in this particular case, lichenization did not confer remarkable advantages to the alga in terms of desiccation tolerance. So, the debate is still open and there is the need for further investigation.

The aim of this work was to study a) if lichens with different ecologies and photobionts differ in their coping strategies to survive the combined effect of desiccation and high light stress and b) if the isolated photobionts share the same mechanisms of their lichen counterparts.

MATERIALS & METHODS

Plant material

Four foliose lichen species hosting four different photobionts were selected: the chlorolichen *Flavoparmelia caperata* (L.) Hale with the green alga *Trebouxia gelatinosa* Archibald, the cephalolichen *Lobaria pulmonaria* (L.) Hoffm. with the green alga *Symbiochloris reticulata* (Tschermak-Woess) Škaloud, Friedl, A.Beck & Dal Grande, the cephalolichen *Peltigera leucophlebia* (Nyl.) Gyeln. with the green alga *Coccomyxa* sp. and the cyanolichen *P. praetextata* (Sommerf.) Zopf with *Nostoc* sp. as photobiont.

The lichen material was collected from August to October 2014 in three different localities where every species had an optimal growth: *F. caperata* was collected in Basovizza (Trieste, Italy) on *Fraxinus ornus* north-facing trunks at 380 m a.s.l., *L. pulmonaria* was collected in Izarra (Álava, Spain) in a beech forest at 900 m a.s.l., *P. leucophlebia* and *P. praetextata* were collected in Viggar valley (Innsbruck, Tyrol, Austria), on the ground in a *Picea abies* wood among *Vaccinium* spp. bushes.

After collection, lichen thalli were air dried, cleaned from debris under a dissecting microscope and stored in darkness at 4 °C under silica gel for a period shorter than two weeks.

Algal cultures

The photobionts of *F. caperata* and *L. pulmonaria* were isolated according to Yamamoto *et al.* (2002). Thalli of *F. caperata* were collected in the Classic Karst plateau (NE Italy; 45°42’24.54’’N; 13°45’21.70’’E), thalli of *L. pulmonaria* were collected in Nuraghe Ruju (Sardinia, Italy 40°6’49.33’’N 8°34’55.71’’E). The isolates were inoculated on 15 mL Falcon tubes filled with slanted solid *Trebouxia* medium (TM, 1.5% agar) (Ahmadjian, 1973) and kept in a thermostatic chamber at 20 °C, under a light regime of 20 µmol photons m^{-2} s^{-1} with a 10/14 h day/night photoperiod until reaching a sufficient biomass. The identity of the photobiont was checked by sequencing the nuclear ITS fragment (data available upon request). Reference algal material was cryo-conserved according to Dahmen *et al.* (1983) and is available upon request. For the experiment, algal cultures were grown on cellulose acetate filter discs (25 mm diameter, pore size 0.45 µm, Sartorius Lab Holding GmbH, D) laid on solid TM inside Petri dishes. In each Petri dish 5 filter discs

were inoculated with 100 μL of algal suspension by vacuum filtration. Petri dishes were kept in a thermostatic chamber at the same conditions described above for 4 weeks.

Experimental design

EXPERIMENT A

Thirty healthy lobes of each species weighing 144.05 ± 9.44 mg (silica weight) were selected. Before the experiments lichens were subjected to a preconditioning period; lobes were hydrated by spraying distilled water and then kept 24 h at 20 μmol photons m^{-2} s^{-1}, 20 °C and 10/14 h day/night photoperiod over wet filter paper in a Percival-Scientific growth chamber (Percival Scientific, Inc., Perry, USA). After this period (time 0 "wet", t_0w) six randomly selected lobes were submitted to chlorophyll *a* fluorescence (Chl*a*F) measurements. Subsequently all the lobes were air-dried in the growth chamber for 24 hours, then six lobes for each species (time 0 "dry", t_0d) were cut in half along their tangential axis. One half was immediately frozen in liquid nitrogen for pigment analyses, the other half was subjected to Chl*a*F measurements.

The remaining lobes were gently flattened and attached to polystyrene squares by means of a plastic net to avoid curling when dry (as reported by Gauslaa & Solhaug, 1999), then put in four 5.5 L sealed boxes containing approximately 150 mL of a $CaCl_2$ saturated solution. This allowed to equilibrate the boxes at a relative humidity of 35-40% (see Appendix, Fig. A1). The four boxes were incubated at 20ºC in a Percival-Scientific growth chamber for seven days and the lobes were subjected to four different treatments: i) constant Desiccation in Darkness (DD), ii) constant Desiccation under a Light regime of 250 μmol photons m^{-2} s^{-1} for 10/14 h day/night (DL), iii) morning and evening rehydration in constant darkness (WD), and iv) morning and evening rehydration under the same light regime of DL (WL). Rehydration was performed before dawn and after sunset by spraying the lobes with distilled water. Excess water was removed gently patting lichens dry with blotting paper.

After the treatments, lobes were cut in two halves, one was immediately frozen in liquid nitrogen for pigment analyses whereas the other was subjected to predawn Chl*a*F measurements. Thereafter, the samples used for Chl*a*F measurements were subjected to a recovery period: lobes were kept moist over wet filter paper for 24h at 20ºC under 20 μmol photons m^{-2} s^{-1} with a 10/14 light/dark photoperiod. Chl*a*F measurements were taken after 8 and 24 h. After the 24 hours the lobes were frozen for pigment analyses.

The whole experiment was repeated three times. A total of six replicates per species, treatment and time point were measured.

EXPERIMENT B

To verify if the isolated photobionts share the same mechanisms of their lichen counterparts to cope with light and drought, the experiment A was partially reiterated with the isolated photobionts of two of the lichen species, *Trebouxia gelatinosa* (photobiont of *F. caperata*) and *Symbiochloris reticulata* (photobiont of *L. pulmonaria*).

Six randomly selected four week old colonies per species were submitted to Chl*a*F measurements (t_0w). All the other colonies were let to dry for 24 hours at air relative humidity under a flow hood to ensure sterility, then six of them were submitted to Chl*a*F measurements and other six were immediately frozen in liquid nitrogen for pigment analyses (t_0d). The remaining colonies were put in 1.2 L sealed boxes containing approximately 50 mL of $CaCl_2$ saturated solution to equilibrate the boxes at a relative humidity of 35-40%. To maintain the boxes as sterile as possible, RH was not measured during the experiment. The boxes were incubated at 20ºC in a growth chamber for seven days and the cultures were subjected to the same treatments of the experiment A (DD, DL, WD, WL) with the light intensity modified according to the cortex transmittance of their lichen counterpart (see below), and thus reduced to 35 μmol photons m^{-2} s^{-1} for *T. gelatinosa* and 65 μmol photons m^{-2} s^{-1} for *S. reticulata*. Rehydration was performed before dawn and after sunset by pipetting 200 μL of distilled water directly on the algal colonies.

After the treatments, six colonies were immediately frozen in liquid nitrogen for pigment analyses whereas other six were subjected to predawn Chl*a*F measurements. Thereafter, the remaining colonies were hydrated with a drop of distilled water, laid in Petri dishes on solid BBM and subjected to a recovery period: for 24h at 20ºC under 20 μmol photons m^{-2} s^{-1} with a 10/14 light/dark photoperiod. Chl*a*F measurements were taken after 8 and 24 h. After the 24 hours another set of six replicates was frozen for pigment analyses.

The whole experiment was repeated three times. A total of six replicates per species, treatment and time point were measured.

Cortical transmittance

In a lichen the photobiont layer is protected by the superior cortex that reduces the light transmittance. To quantify this shielding effect, cortex transmittance was measured on dry and wet lobe fragments (4 x 3 mm; n = 6) for each species, following the protocol in Candotto Carniel *et al.* (2015) with some modifications. Moistened lichen lobes were flattened between two paper sheets, left to dry out for 1 day and stuck with double-sided transparent tape on microscope slides with the lower cortex up. The lower cortex, the medulla, and most of the algal layer were then carefully removed with a blade under a dissecting microscope working at high magnification (115x). The removal process was interrupted when the upper cortex was visibly intermingled with clusters of algae. The samples were placed under the 10x objective of a Zeiss Axioplan microscope (Carl Zeiss, Jena, Germany). Light was set at the maximum intensity and the photosynthetic photon flux density (PPFD) passing through the sample was measured by placing the probe of a quantum radiometer HD 2302.0 (Delta Ohm, Padua, Italy) directly on the microscope ocular. This measure was expressed as a percentage of the PPFD passing through an empty glass covered with tape. In dry and wet fragments cortex transmittance was, respectively, 8 ± 1 and 12 ± 2 (*F. caperata*) and 16 ± 4 and 25 ± 7 (*L. pulmonaria*).

Light acclimation

To test if the light treatment effects on Chl*a*F were partially due to the abrupt transition from dim to higher light, 24 algal colonies per species were gradually acclimated to the final light regime and then submitted to the light treatments (DL and WL). Each week, the Petri dishes containing the colonies were exposed to increasing PPFD (Tab. 1) and a complete set of six replicates was submitted to Chl*a*F measurements at t_0w, t_0d and after the DL and WL treatments.

Table 1 Increasing PPFD (µmol photons m^{-2} s^{-1}) used to acclimate the algae to the final light regime.

species	1st week	2nd week	3rd week	4th week
T. gelatinosa	20	25	30	35
S. reticulata	20	35	50	65

Water content assessment

The lobe halves and the algal colonies were weighed immediately before each time point (t_0d, post-treatments, 24 hours recoveries), then freeze-dried (LyoVac GT2, Finn Aqua, Hürth, Germany) for 48 hours. The relative water content (% RWC) of lobes was calculated as (FW - DW)/DW x 100, here FW (fresh weight) is the sample weight at each time point and DW (dry weight) is the sample weight after freeze-drying.

To monitor the hydration of the "wet" lichen samples (WD and WL lobes) during the first day of treatments, an additional subset of 3 samples for species and treatment was weighed just before the treatments, immediately after spraying them with distilled water and after 10 hours at 30-40% RH. The samples were then immediately frozen in liquid nitrogen and freeze-dried for 48 hours.

Chlorophyll a fluorescence measurements

Measurements of Chl *a* fluorescence were performed with a Photosynthetic Efficiency Analyser fluorometer (Hansatech Instruments Ltd, Norfolk, England). Before measurements, thalli and algal colonies were hydrated and dark adapted for 30 minutes.

In experiment A, a saturating pulse of 5 s and 30% of max intensity was used to obtain the fluorescence transient and thus the maximum quantum yield of photosystem II (F_v/F_m). In experiment B a modified clip was positioned right over the sample on the filter disc and a saturating red light pulse of 3500 μmol photons m^{-2} s^{-1} for 1 second was emitted to obtain the same parameters.

To better compare the F_v/F_m between species, for each time point the percentage of F_v/F_m on the mean value at t_0w was calculated.

Photosynthetic pigments and tocochromanols analyses

Lichen samples were frozen in liquid nitrogen, freeze-dried and ground with a TissueLyser II (Qiagen, Hilden, Germany). Thirty mg of lichen powder were extracted in 500 µL of pure acetone containing 0,5% N-ethyldiisopropylamine and 30 mg of $CaCO_3$ were added to prevent pigment destruction by organic acids. Extracts were vortexed for 10 seconds, centrifuged at 26000 g and 4 ºC for 20 min. Supernatant was saved and the pellet resuspended and subjected to a second extraction and centrifugation. Both supernatants were pooled together and centrifuged. Thereafter 15 µL were

injected in a HPLC system (Agilent, Santa Clara, California, USA) equipped with a LiChrosphere 100 RP 18 column (4 x 125mm, 5um) set at 15 ºC. Two solvents were used to separate the pigments and tocopherols: Solvent A: acetonitrile/methanol 74/6 v/v, and Solvent B: methanol/hexane 5/1 v/v. Gradient started with 9 min of 100% solvent A, followed by 5 min of solvent exchange from 100% solvent A to 100% solvent B, 100% solvent B was kept for the last 9 min. Flux was set at 1 mL/min. A DAD detector (Agilent, Santa Clara, California, USA) was used for pigments analysis (Abs λ 440 nm) and a FLD detector (Agilent, Santa Clara, California, USA) (λEx = 295 nm, λEm = 325 nm) for tocopherols. The system was calibrated for pigments and tocopherols using commercial standards (Sigma).

Algal samples were frozen in liquid nitrogen, freeze-dried and 5 mg were ground 30 s twice with a Mini-Beadbeater-24 (BioSpec Products, Bartlesville, OK, USA). The samples were extracted in 250 µL of 80% v/v acetone, furtherly beaten at the beadbeater twice for 30 seconds and centrifuged at 20000 g and 20 ºC for 3 min. Supernatant was saved and the pellet resuspended and subjected to other three extraction cycles. At the end of the procedure, the supernatants were pooled together. An aliquot of 200 µL of extract was injected in an HPLC system consisting of a Rheodyne valve (Rheodyne, Rhonert Park, CA), a reversed-phase column (5 µm particle size; 25 × 0.4 cm; 250/4 RP 18 Lichrocart, Darmstadt, Germany), a binary pump and a diode-array detector useful to record the absorbance spectra (1100 series, Agilent, Waldbronn, Germany). Two solvents were used to separate the pigments: Solvent A: acetonitrile/methanol/Tris–HCl pH 7.8 86.7/9.6/3.6 v/v and Solvent B: methanol/hexane 8/2 v/v. Runs were performed as described by Farber & Jahns (1998). The peaks of each sample were identified through the retention time and absorption spectrum and quantified as described previously (Farber & Jahns, 1998).

Data analyses

All calculations were performed by Microsoft Office Excel 2013 (Microsoft Corporation, Redmond, WA, USA), R 3.4.0 (R Core Development Team, 2017) and RStudio (RStudio, Inc., Boston, MA). The non-parametric Mann–Whitney *U* test was performed to compare the adapted and not adapted algal samples after each time point (t_0w, t_0d, treatments). The non-parametric non-paired Kruskal-Wallis test was performed to verify significant differences between the t_0w, t_0d, treatments and recoveries for F_v/F_m and the photosynthetic pigments. The R package PMCMR (Pohlert, 2014) was used to perform the post hoc test after Conover & Iman (1979). Graphs were drawn using SigmaPlot 10.0 (Systat Software, San Jose, CA).

RESULTS

EXPERIMENT A

Water content

The "wet" (WD and WL) samples were monitored during the first day of treatment to verify if the saturated solution was able to desiccate completely the thalli within 10 hours. As shown in Tab. 2, the thalli were fully hydrated when the desiccation treatment started (% RWC after rehydration). After 10 hours of desiccation the RWC was back to values around the 10%.

The RWC at the end of the treatments was only slightly different between "dry" (DD and DL) and "wet" samples (data not shown) and ranged between 9.4 ± 0.7 (*F. caperata* dry) and 15.1 ± 1.3 (*P. praetextata* wet). Upon 24 hours of rehydration the thalli were fully hydrated, reaching values of RWC ranging between 155.8 ± 10.9 (*F. caperata*) and 484.2 ± 49.9 (*P. leucophlebia*, data not shown).

Table 2 relative water content (% RWC, g H_2O g^{-1}DW) of the "wet" samples (WD and WL lobes) after 24 hours of preconditioning, immediately after spraying them with distilled water and after 10 hours at 30-40% RH. Values are mean ± standard error.

species	% RWC t_0	% RWC after rehydration	% RWC evening
F. caperata	13.7 ± 0.9	113.0 ± 9.3	8.6 ± 0.5
L. pulmonaria	12.9 ± 0.2	154.9 ± 9.7	10.1 ± 0.3
P. leucophlebia	9.5 ± 3.7	374.3 ± 79.2	10.6 ± 4.1
P. praetextata	16.0 ± 3.9	256.0 ± 48.0	12.9 ± 1.1

Chlorophyll a fluorescence

The average F_v/F_m values of lichens were of 0.750 ± 0.017 in *F. caperata*, 0.722 ± 0.012 in *L. pulmonaria*, 0.755 ± 0.020 in *P. leucophlebia* and 0.532 ± 0.091 in *P. praetextata*, confirming that the samples were healthy at the beginning of the experiment (t_0w in Tab. A1). Note that the lower

values of F_v/F_m showed by *P. praetextata* are consistent with the maxima described for cyanobacteria (Jensen & Kricke, 2002). At t_0d the values did not change much for the chlorolichens, while in *P. praetextata* there was a decrease of the 13%.

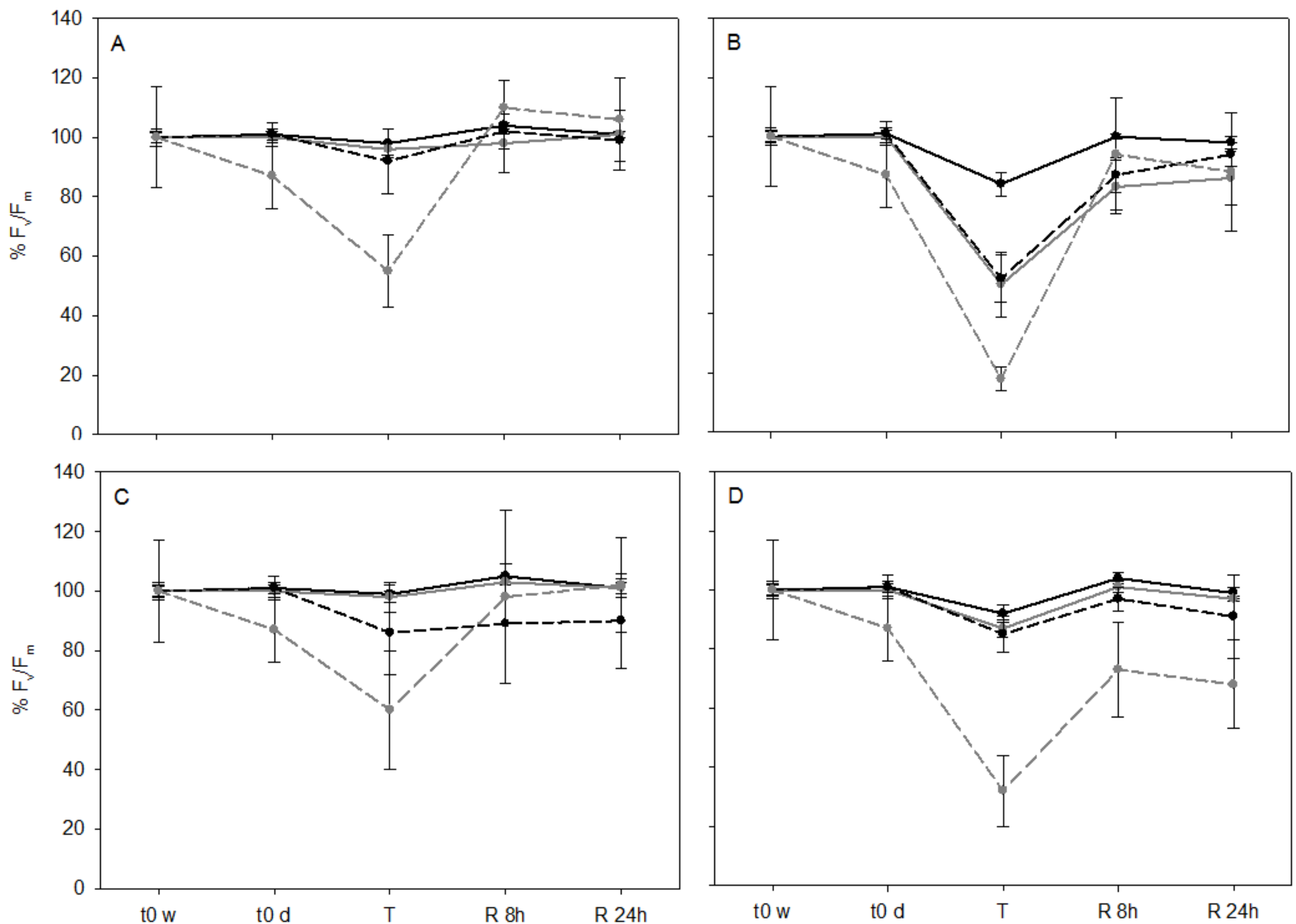

Figure 1 Percent (calculated on t_0w mean value) variation of the maximum quantum yield of photosystem II (F_v/F_m) measured on lichen lobes of *F. caperata* (black line), *L. pulmonaria* (grey line), *P. leucophlebia* (black dotted line) and *P. praetextata* (grey dotted line) after 24 hours of preconditioning (t_0w), 24 hours of drying at air RH (t_0d), 7 days of DD (A), DL (B), WD (C), WL (D) treatments (T), 8 hours (R8h) and 24 hours (R24h) of recovery; n = 5-6.

After seven days, the DL treatment affected F_v/F_m especially in *L. pulmonaria, P. leucophlebia* and *P. praetextata*, which showed decreases of 50%, 48% and 82% of the initial F_v/F_m, respectively. *Flavoparmelia caperata* was less affected with an F_v/F_m decrease of the 16%. The WL treatment affected F_v/F_m to a lesser degree, determining the highest decrease in *P. praetextata* (68% of the initial

F_v/F_m), followed by *P. leucophlebia* (15%), *L. pulmonaria* (13%) and *F. caperata* (8%). The DD and WD treatments had an effect only on *P. praetextata*, whose F_v/F_m fell to 45% and 40%, respectively (see Tab. A1, p. values shown in Tab. 3).

The recovery period generally allowed a re-establishment of standard Chl*a*F emission or even a small increase, except for *L. pulmonaria* in DL treatment (86% of the t_0 F_v/F_m) and *P. praetextata* in WL treatment (68% of the t_0 F_v/F_m). *P. leucophlebia* needed 24 hours to completely recover in DL treatment, as after 8 hours his F_v/F_m was still the 87% of the initial t_0 value. The most affected species was *P. praetextata*, that experienced a significant decrease in F_v/F_m after each treatment and was even not able to recover after the WL treatment. On the other hand, *F. caperata* was the less harmed species, as its F_v/F_m never fell under the 80% of its t_0 and it always recovered completely after only 8 hours (see Tab. A2).

Table 3 Summary of the Kruskal-Wallis tests (post hoc test after Conover & Iman, 1979) to verify statistical differences in the percent (calculated on t_0w mean value) variation of F_v/F_m among t_0w, treatments (DD, DL, WD, WL) and the 24 hours recoveries. P-values are shown only when significant (< 0,05, n = 5-6). N.s., not significant.

	t_0 w vs treatments				**t_0 w vs rec 24h**				**treatments vs rec24h**			
species	DD	DL	WD	WL	DD	DL	WD	WL	DD	DL	WD	WL
F. caperata	ns	< 0.01	ns	0.02	ns	ns	ns	ns	ns	ns	ns	ns
L. pulmonaria	ns	< 0.01	ns	< 0.01	ns	< 0.01	ns	ns	0.02	< 0.01	ns	ns
P. leucophlebia	ns	< 0.01	ns	0.01	ns	0.02	ns	ns	ns	< 0.01	ns	ns
P. praetextata	0.01	< 0.01	ns	< 0.01	ns	ns	ns	0.04	< 0.01	0.02	0.03	ns
T. gelatinosa	0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	ns	ns	ns	ns
S. reticulata	< 0.01	< 0.01	< 0.01	< 0.01	ns	ns	ns	0.04	ns	ns	0.04	ns

The chlorophyll *a* fluorescence transient at t_0w and after DL and WL treatments is shown in Fig. 2. All the species with a green photobiont showed the characteristic OJIP shape with the additional dip at 0.2-2 s described by Ilík *et al.* (2006) in *Trebouxia*-possessing lichens, even if it was more pronounced in *F. caperata*, the only species that actually has *Trebouxia* as a photobiont. In *F. caperata* (Fig. 2A) after both treatments the fluorescence transient kept its shape, indicating that overall the photosystems were intact. The only difference was a decrease in F_m (of the 35% and 32%

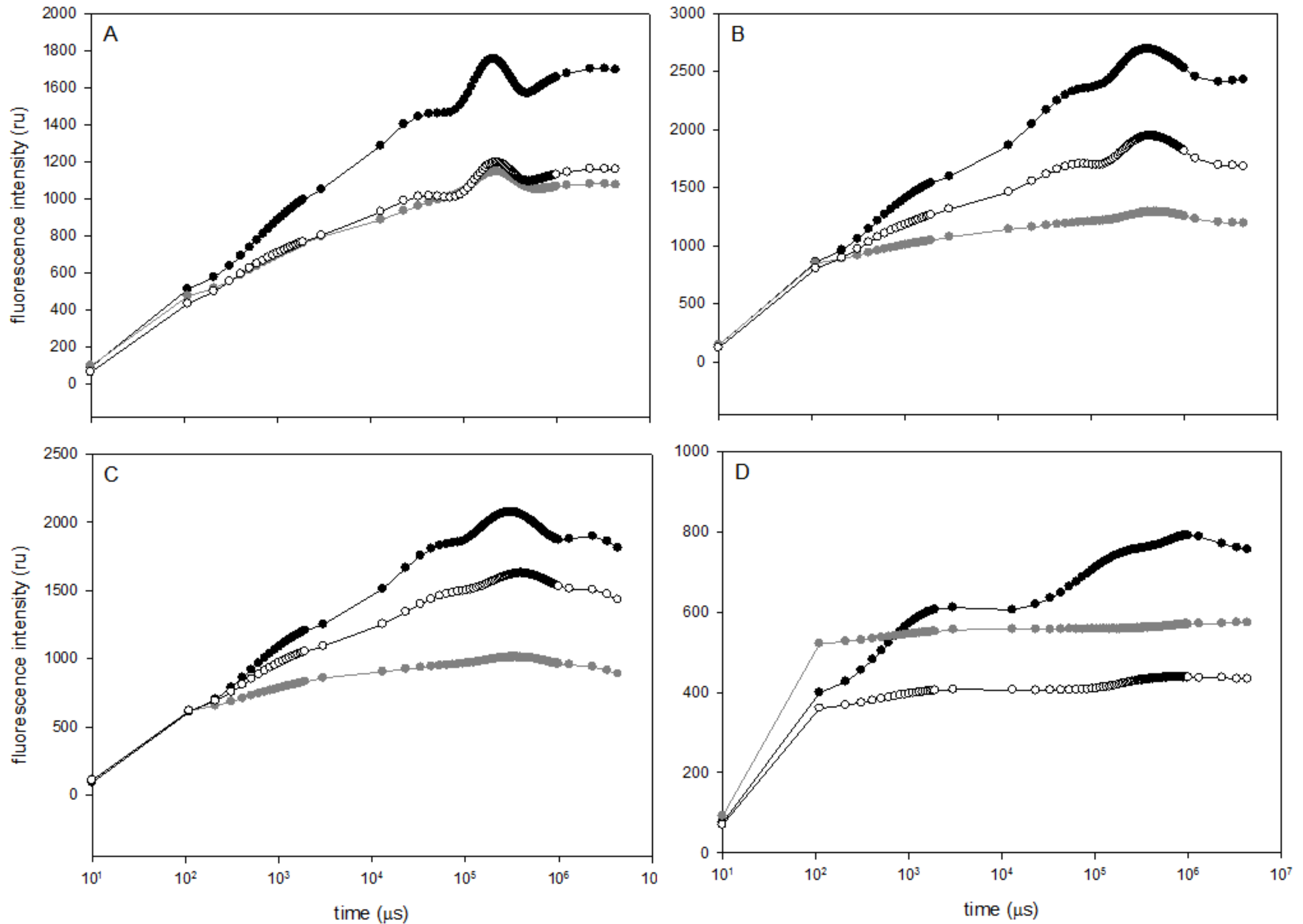

Figure 2 Chlorophyll *a* fluorescence transient in *F. caperata* (A), *L. pulmonaria* (B), *P. leucophlebia* (C) and *P. praetextata* (D) at t_0w (black), DL (grey) and WL (white). Average curves calculated on six replicates.

with respect to t_0w in DL and WL, respectively), compensated by a relatively small decrease in F_0 (never more than the 16%). The DL and WL curves were nearly identical, suggesting that the two treatments caused a general similar rearrangement of the photosynthetic machinery, without major damages. For *L. pulmonaria* and *P. leucophlebia* the situation is slightly different: they still kept intact the OJIP shape, but both species seemed to suffer more after the DL treatment, confirming the F_v/F_m data. The DL treatment caused the greatest decreases in F_m, the 52% (against the 28% after WL) in *L. pulmonaria* and the 51% in *P. leucophlebia* (against the 21% after WL), and a general increase in F_0 (11% and 21% in *L. pulmonaria* and *P. leucophlebia*, respectively), indicating some degree of damage to the photosystems. *Peltigera leucophlebia* had an increase in F_0 also after WL, but of lesser extent (15%). *Peltigera praetextata* showed a completely different transient, typical for cyanobacteria (e.g., see Lu & Vonshak, 1999), and an even more remarkable difference between the

treatments. After the WL treatment the curve flattened considerably and lost the 45% of the F_m, but after DL the shape was completely lost and F_0 increased considerably, reaching the 40% more than the initial t_0w value. This suggest a certain degree of damage to the photosynthetic apparatus; nevertheless, *P. praetextata* was able to recover after 24 hours.

Photosynthetic pigments and tocochromanols

Thalli of *P. leucophlebia* at t_0d contained the highest total chlorophyll content (3.43 ± 0.86 μmol g^{-1} DW), followed by *L. pulmonaria* (3.21 ± 0.96 μmol g^{-1} DW) and *F. caperata* (1.95 ± 0.78 μmol g^{-1} DW). Total chlorophylls have a quite stable overall pattern irrespectively of the treatment (data not shown). The only exception is *P. leucophlebia* in the DD recovery that showed a statistically significant higher amount (4.87 ± 1.25 g^{-1} DM) than the treatment (3.02 ± 0.84, p-value 0.03). Also, chlorophyll a in *P. praetextata* shows the highest values in the recoveries (DD recovery is the only statistically significant one, 1.62 ± 0.29 μmol g^{-1} DM against 0.80 ± 0.26 μmol g^{-1} DM for t_0d and 0.96 ± 0.26 μmol g^{-1} DM for treatment, p-values < 0.01 and 0.03, respectively). Considering, though, that acetone is more efficient in extracting chlorophylls from samples that were hydrated prior freeze-drying with respect to the dry ones (Candotto Carniel *et al.*, 2017) and that products of degradation of chlorophylls could not be detected, drought and high light irradiance caused no major damage to the photosynthetic apparatus. In general, the amounts of photoprotective pigments like β-carotene, lutein and neoxanthin did not decrease after the drought and light stress (expressed as μmol g^{-1} DM, data not shown).

The xanthophyll cycle was operative, but with some differences among species and treatments. In all of the three lichens with green algae the AZ/VAZ (antheraxanthin + zeaxanthin / violaxanthin + antheraxanthin + zeaxanthin) ratio remained constant after the drought treatments (DD and DL) and significantly decreased after the WD, indicating an increase in violaxanthin. Only *L. pulmonaria* showed a dramatic net interconversion of violaxanthin to zeaxanthin after WL (0.52 ± 0.14 against 0.32 ± 0.07). The other two species maintained a constant AZ/VAZ ratio after WL. After the recoveries violaxanthin concentration was rapidly reestablished or even increased with respect to the t_0d (Fig. 3), also in *L. pulmonaria* WL. Degradation of these xanthophylls can be excluded as their total amount remained quite stable during all the time steps of the experiment (data not shown).

Of all the possible tocochromanols only α-tocopherol was found in this study. Its amount did not show any variation throughout the whole experiment, remaining at a constant level (data not shown).

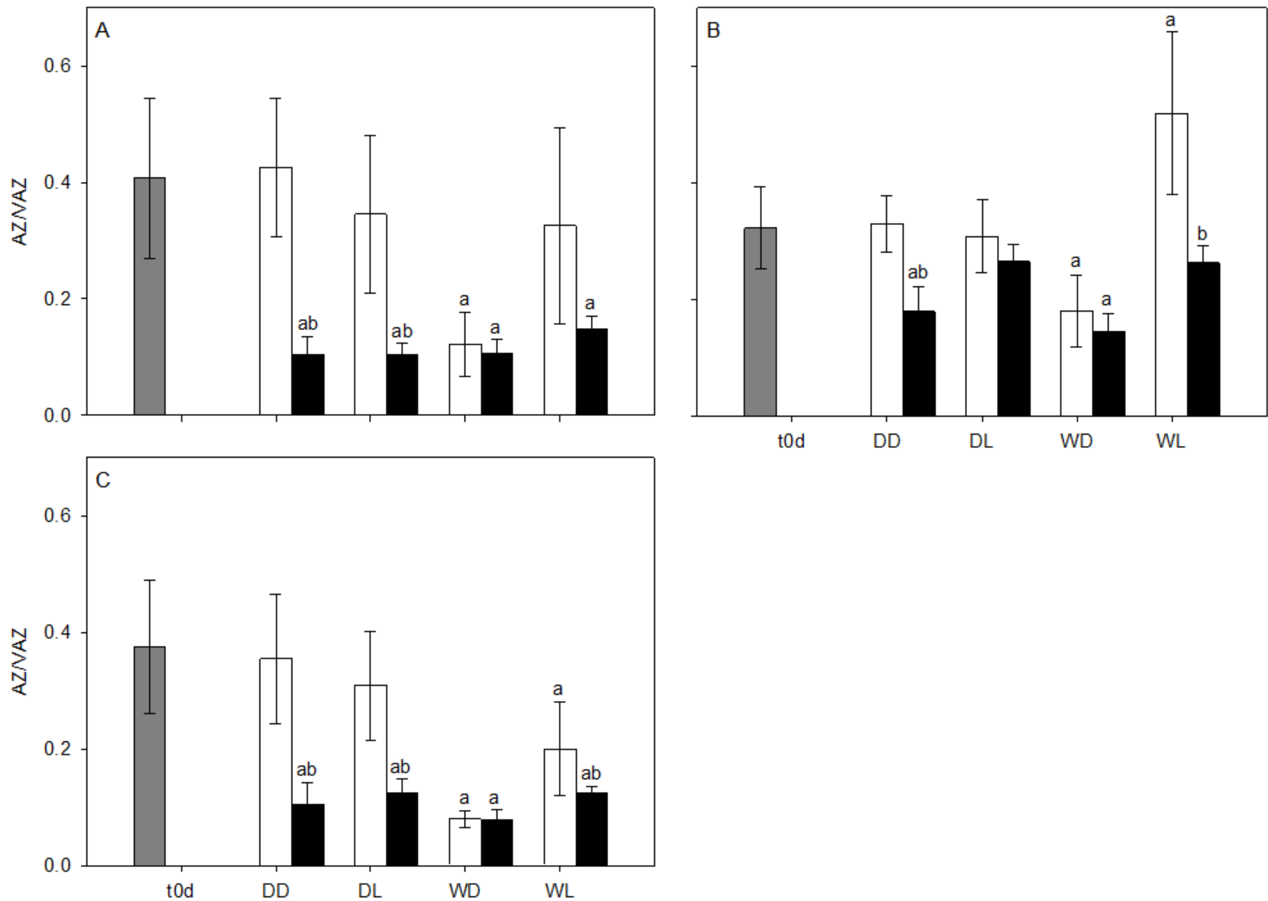

Figure 3 AZ/VAZ ratio measured before (t_0d, grey bars) and after the treatments (DD, DL, WD, WL, white bars) and the 24 hours recoveries (DD, DL, WD, WL, black bars) in *F. caperata* (A), *L. pulmonaria* (B) and *P. leucophlebia* (C). Significant differences (post hoc test after Conover & Iman, 1979) against t_0d and treatment values for $p < 0.05$ are marked a and b, respectively (n = 4-6).

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EXPERIMENT B

Water content

The RWC at the end of the "dry" (DD and DL) treatments was homogeneous, ranging between 5.6 ± 1.4 (*S. reticulata* in DD) and 7.5 ± 0.5 (*S. reticulata* in DL), while for the "wet" ones (WD and WL) the situation was more diverse. *Trebouxia gelatinosa* after WL had a RWC similar to the dry ones (7.0 ± 2.1), but the other treatments showed values surprisingly high (*T. gelatinosa* WD: 9.3 ± 6.0; *S. reticulata* WD: 18.8 ± 9.5; *S. reticulata* WL: 17.7 ± 8.0). This could be because the wet samples, being hydrated and dried twice a day, became particularly crumbly and some material was lost during

the freeze-drying operations. As a result, the final RWC seemed higher than it really was. After 24 hours of recovery the colonies were completely hydrated again, reaching values of RWC of 510.6 ± 60.5 (*T. gelatinosa*) and 353.2 ± 23.0 (*S. reticulata*, data not shown).

Chlorophyll a fluorescence

The acclimated and non-acclimated samples overall did not show significant differences in F_v/F_m with the exception of *T. gelatinosa* after the DL treatment (Mann–Whitney *U* test, p-value 0.04, Fig. 4). In this case, the acclimated samples showed a higher value and a much narrower standard deviation (0.592 ± 0.012) than the non-acclimated ones (0.541 ± 0.045). Their fluorescence transients, though, were nearly identical (data not shown).

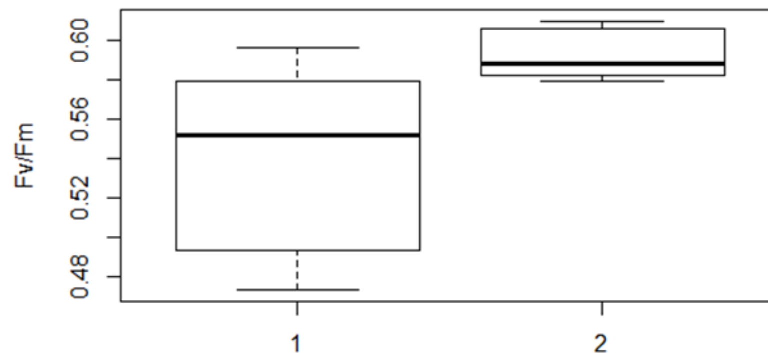

Figure 4 F_v/F_m of non-acclimated (1) and acclimated (2) samples of *T. gelatinosa* after the DL treatment. For each boxplot median, 25°–75° percentiles, minimum and maximum are reported; n = 6.

The average F_v/F_m values of algae at t_0w were of 0.636 ± 0.013 in *T. gelatinosa* and 0.612 ± 0.008 in *S. reticulata*, confirming that the samples were healthy at the beginning of the experiment (Fig. 5, p-values in Tab. 3). At t_0d, though, F_v/F_m decreased in both species, but particularly in *S. reticulata* (reaching the 69% of the t_0w value), showing a diminished capacity in tolerating drought with respect to the lichenized alga.

Symbiochloris reticulata showed a consistent decrease in F_v/F_m after each treatment, never less than the 40% of its t_0w value. The most critical treatments were DL and WL, that caused a drop in F_v/F_m up to the 62% and 56%, respectively. The cultured alga seemed to maintain the same trend of the lichenized one, having a better performance when hydrated in the light. *Trebouxia gelatinosa* was

consistently less affected by all the treatment, reaching the lowest value after the WD treatment (82% of the t_0w value).

After the recovery period, only *T. gelatinosa* showed values of F_v/F_m close to the t_0w ones, ranging from the 83% to the 90% of the t_0w values, while *S. reticulata* consistently failed to recover, the highest F_v/F_m being the 77% of the t_0w value (DD recovery, Fig. 5A).

Overall, while *T. gelatinosa* performance was comparable to its lichenized counterpart, *S. reticulata* was consistently less effective in tolerating stress. The latter showed differences in percentage F_v/F_m from its lichenized form ranging from a minimum of 12 points after DL treatment (38% against 50% of the lichenized form, each calculated as percentages on their respective t_0w, see Tab. A2) to a maximum of 45 points after WD treatment (53% against 98%).

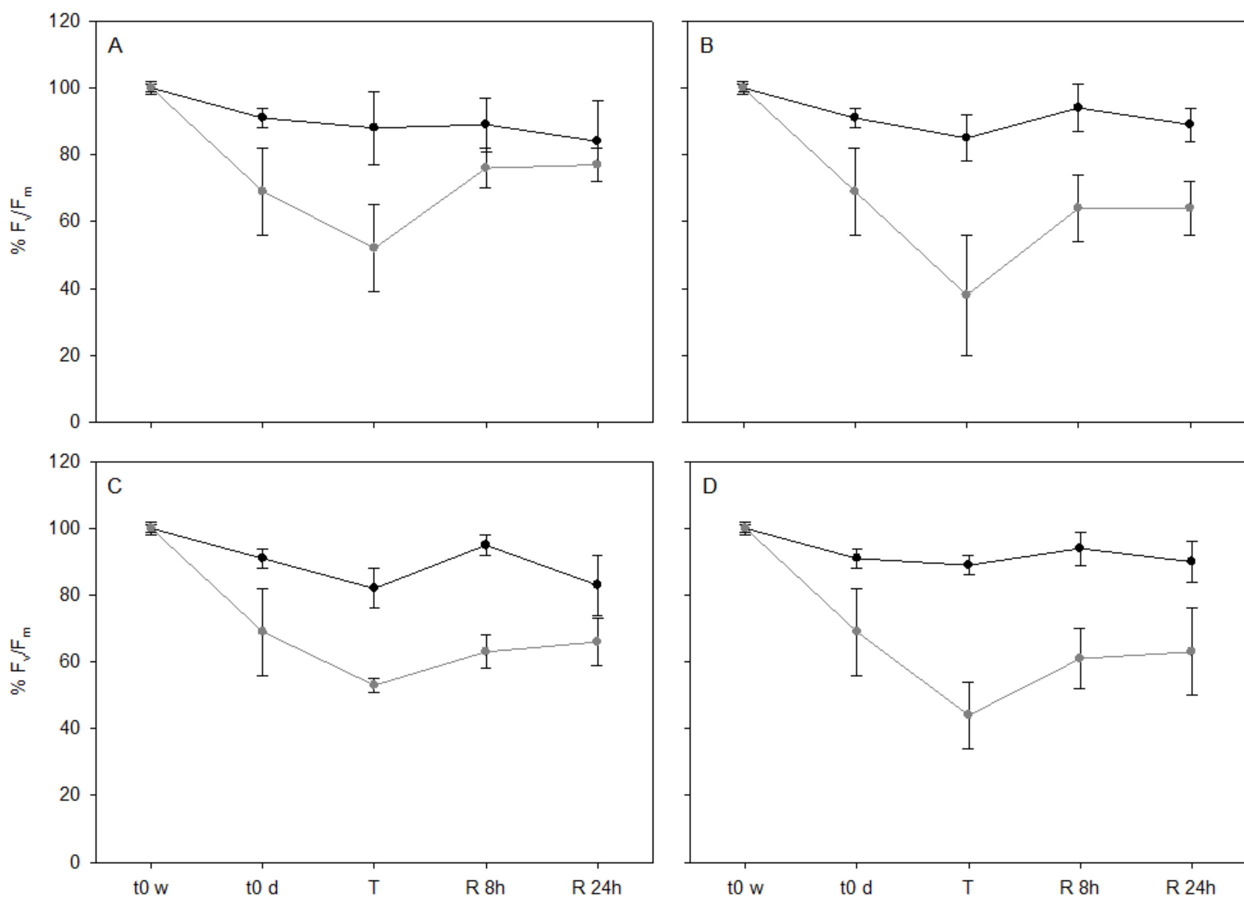

Figure 5 Percentage (calculated on t_0w mean value) variation of the maximum quantum yield of photosystem II (F_v/F_m) measured on algal colonies of *T. gelatinosa* (black line) and *S. reticulata* (grey line) after 24 hours of preconditioning (t_0w), 24 hours of drying at air RH (t_0d), 7 days of DD (A),

DL (B), WD (C), WL (D) treatments (T), 8 hours (R8h) and 24 hours (R24h) of recovery (n = 6 for *T. gelatinosa*, 3 for *S. reticulata*).

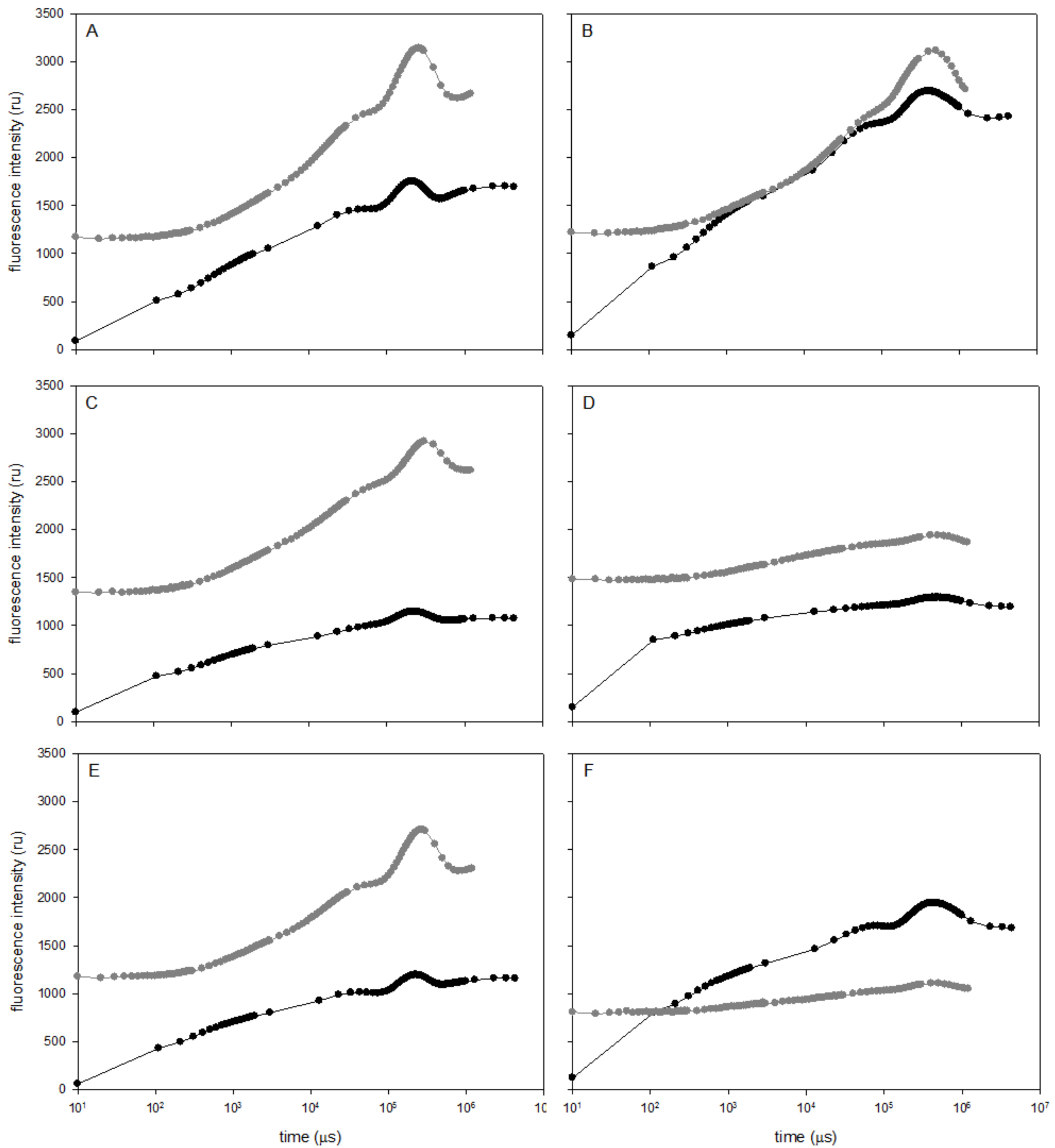

Figure 6 Chlorophyll *a* fluorescence transient in the couples *F. caperata* (black; A, C, E) – *T. gelatinosa* (grey; A, C, E) and *L. pulmonaria* (black; B, D, F) – *S. reticulata* (grey; B, D, F) after t_0w (A, B) and DL (C, D) and WL (E, F) treatments. Average curves calculated on six replicates.

The chlorophyll *a* fluorescence transient in the couples *F. caperata* – *T. gelatinosa* and *L. pulmonaria* – *S. reticulata* at t_0w and after DL and WL treatments is presented in Fig. 6. The difference in t_0w F_0 and F_m values between lichen and alga could be due to the different settings used, that were chosen to yield the best photosynthetic performance for each organism. The couple *F. caperata* – *T. gelatinosa* showed only little differences in the induction curves. Overall, *F. caperata* had a better performance, as it better kept the characteristic shape of the transient and its F_0 never rose. *Trebouxia gelatinosa*, on the other hand, had a slight increase in F_0 (the 17% of the t_0w value) after the DL treatment, suggesting a little more vulnerability in the dry state with respect to its lichenized counterpart. Mostly, though, the two performances were comparable. In the couple *L. pulmonaria* – *S. reticulata*, instead, the lichen was always considerably less harmed than the alga. The most harmful treatment for *S. reticulata*, exactly like for the lichen, was DL, as F_m decreased of the 38% and F_0 increased of the 23%, yielding a F_v/F_m of 0.233. The most remarkable difference with the lichen, though, can be observed after the WL treatment (Fig. 6F), as the fluorescence transient is nearly flat, with a decrease in both F_m (the 64% less than the t_0w) and F_0 (the 33% less than the t_0w). This could mean that lichenization helps the alga to better cope with stresses if metabolically active.

Photosynthetic pigments

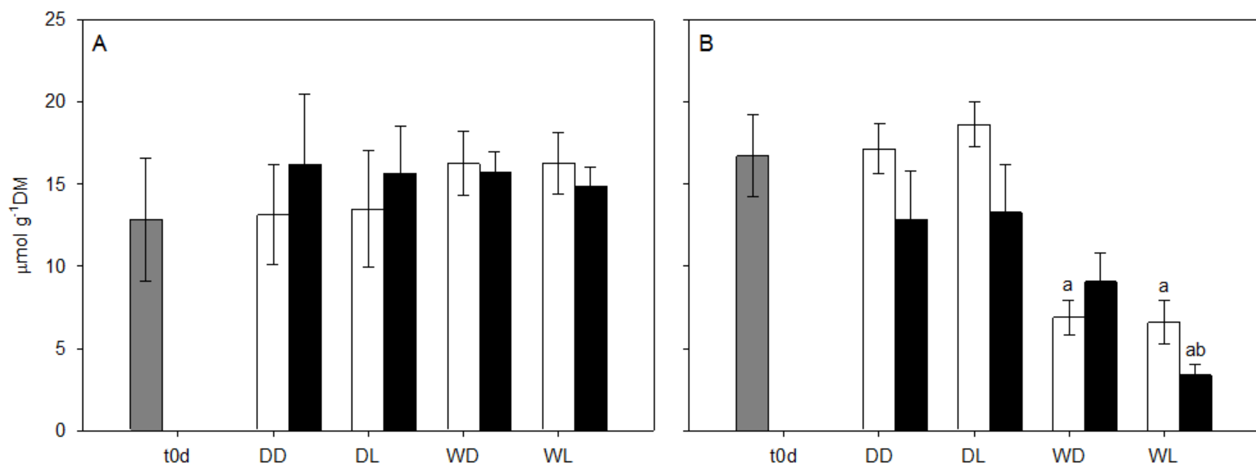

Figure 7 Total chlorophyll amount (μmol g^{-1}DM) measured before (t_0d, grey bars) and after the treatments (DD, DL, WD, WL, white bars) and the 24 hours recoveries (DD, DL, WD, WL, black bars) in *T. gelatinosa* (A) and *S. reticulata* (B). Significant differences (post hoc test after Conover & Iman, 1979) against t_0 and treatment values for $p < 0.05$ are marked a and b, respectively (n = 6 for *T. gelatinosa*, 3 for *S. reticulata*).

Thalli of *S. reticulata* at t_0d contained the highest total chlorophyll content (16.75 ± 2.47 µmol g^{-1}DM against 12.84 ± 3.73 µmol g^{-1}DM in *T. gelatinosa*). *Trebouxia gelatinosa*, like its lichenized counterpart, had a stable total chlorophyll content, irrespectively of the treatment (Fig. 7A). *Symbiochloris reticulata*, on the other hand, after the wet treatments (WD and WL) showed a significant drop in total chlorophyll content (Fig. 7B), due to the decrease in both chlorophylls (data not shown). Total chlorophyll content decreased of the 59% and 61% after WD and WL treatments, respectively, and many products of degradation of chlorophylls were detected, even if it was not possible to identify them. Twenty-four hours of recovery were not enough to rebuild the initial chlorophyll content, as it stayed at the 54% of the t_0d value (WD recovery) and it even furtherly dropped at 20% of the t_0d value after WL recovery.

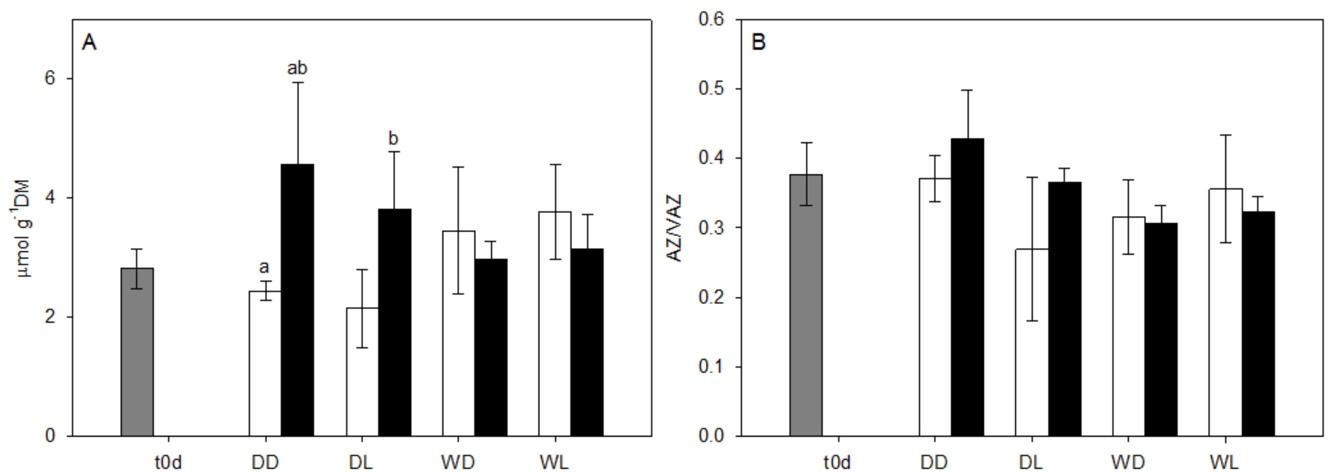

Figure 8 VAZ content (µmol g^{-1}DM, A) and AZ/VAZ ratio (B) measured before (t_0d, grey bars) and after the treatments (DD, DL, WD, WL, white bars) and the 24 hours recoveries (DD, DL, WD, WL, black bars) in *T. gelatinosa*. Significant differences (post hoc test after Conover & Iman, 1979) against t_0d and treatment values for $p < 0.05$ are marked a and b, respectively (n = 6 for *T. gelatinosa*, 3 for *S. reticulata*).

In contrast with the results on lichens, the xanthophyll cycle seems not to be used by *T. gelatinosa*, because there was no net interconversion of violaxanthin to zeaxanthin and the AZ/VAZ ratio remained constant throughout the whole experiment (Fig. 8B). The whole pool of VAZ, though, decreased slightly after the DD and DL treatments and then increased significantly after the respective recoveries (respectively + 88% and 78% on their treatment value and + 62% and 34% on the t_0d

value). A similar pattern was observed for neoxanthin (data not shown). For *S. reticulata* it was not possible to quantify the xanthophyll cycle pigments because when products of chlorophyll degradation were present, their peaks overlapped those of many xanthophylls, particularly violaxanthin and antheraxanthin.

DISCUSSION

The present study was aimed at evaluating how lichens with different photobionts and ecology cope with the combined effect of light and desiccation stress, and if and how their isolated photobionts differ in the management of such stresses. To include organisms with different features, the species were chosen as diverse as possible in photobiont (three green algae belonging to different genera and a cyanobacterium) and ecology. In particular, with respect to hydric requirements *L. pulmonaria* is considered hygrophytic and all the other species mesophytic, and with respect of light tolerance *F. caperata* and *P. praetextata* can be found in sun- exposed sites, but avoiding extreme irradiation, while *L. pulmonaria* and *P. leucophlebia* prefer sites with plenty of diffuse light but scarce direct irradiation (for a more detailed ecological overview of the species, see Nimis & Martellos, 2017). Finally, *L. pulmonaria* is an endangered species that can be used to predict the presence of red-listed lichens and for the selection of key woodland habitats (Nascimbene *et al.*, 2010). Therefore, a further increase of knowledge on its physiology would be beneficial.

The most resistant species was *F. caperata*, as its F_v/F_m never fell under the 80% of its t_0w value and it always recovered completely in less than 24 hours, while the two cephalolichens displayed similar levels of sensitiveness. In accordance with Gauslaa & Solhaug (1999), for both of them the most harmful condition was being dry in the light, as they reached the lowest F_v/F_m, their F_0 increased and one of them (*L. pulmonaria*) even failed to recover. According to their ecological requirements (Nimis & Martellos, 2017, see above), one would expect *L. pulmonaria* to be less tolerant to desiccation than *P. leucophlebia*, but our observations do not support this hypothesis, as their performances were highly similar. It is possible, though, that a longer period in the desiccated state is needed to highlight potential differences. The cyanolichen suffered a consistent reduction in F_v/F_m after each treatment, never less than the 40% (after WD treatment), but it was generally able to recover completely, or to reach at least the 88% of the t_0w value (after WL treatment). Anyway, even if it recovered slightly better, it did not seem more resistant to light in the dry state than the two cephalolichens, as its photosystems suffered more damage (see Fig. 2) and it lost a higher percentage of its F_v/F_m. Gauslaa *et al.* (2012), instead, reported that cyanolichens from old forests *(Lobaria hallii*, *Lobaria retigera*, *Lobaria scrobiculata*, *Pseudocyphellaria anomala*, all of wich had *Nostoc* as photobiont) had a more pronounced resistance to light in the desiccated state than the co-occurring cephalolichens (*L. pulmonaria*). They hypothesized that their ecological preferences are not shaped by intolerance to prolonged desiccation, but by the need of liquid water as rainfall (as cyanolichens cannot reactivate their metabolism with air humidity, Lange *et al.*, 1986) or by a susceptibility of the mycobiont. However, even confronting directly our data with those of Gauslaa *et al.* (2012), as we

applied a very similar light and drought regime, we observed much lower values of F_v/F_m both immediately after the treatments and after 8 hours of recovery, while for example *L. pulmonaria* values were comparable. Further investigation is needed to confirm or reject Gauslaa *et al.* (2012) hypothesis, also considering that they took into account only one cephalolichen. The experiment could be repeated including at least four species of cyanolichens and four of cephalolichens adapted to the same old forest habitat.

Consistently with earlier studies, light was the environmental factor with the most harmful effects for all the four species (Solhaug & Gauslaa, 1996; Gauslaa *et al.*, 2012; Candotto Carniel *et al.*, 2015). Desiccation alone (the treatment DD) and long periods of prolonged darkness (DD and WD) did not impact very much the four species, as their F_v/F_m never fell under the 80% (except for *P. praetextata*) and all of them were able to completely recover in 24 hours. Farrant *et al.* (2003), instead, described the case of two homoiochlorophyllous resurrection plants that did not survive drying in the dark. The plants were held in the darkness for a period similar to that in the present study (five days to reach complete desiccation and then further seven days), so it is unlikely that they died of "starvation". The Authors hypothesized that at least some of the genes responsible for desiccation protective mechanisms in those species require also light for their regulation. This, though, is probably due to their nature of higher plants, possessing water regulation systems (vessels, stomata etc.) that allow them to considerably slow down their desiccation rate (it took five days to completely desiccate) and thus to rely mainly on inducible mechanisms to withstand desiccation. Lichens, as we have already seen, cannot afford that luxury, and are constitutively protected, ensuring a protection also during the night.

The most sensitive species, *L. pulmonaria* and *P. leucophlebia*, showed a higher chlorophyll concentration than *F. caperata*, at least 1.5-fold. This agrees with Kranner *et al.* (2003), as these Authors found that species adapted to shady habitats (*L. pulmonaria* and *Peltigera polydactyla*) possess more chlorophylls than those that tolerate higher light intensities (*Pseudevernia furfuracea*), but as a result are more prone to desiccation-induced damage caused by ROS. The combination of drought and light stress did not affect total chlorophylls, as their amounts remained stable throughout the whole experiment. As already said (see Results), the small variations seen in the two *Peltigera* are probably due to the extraction in acetone, and can be considered as artifacts. This stability is not surprising, as lichens generally are homoiochlorophyllous (Tuba *et al.*, 1998), but Kranner *et al.* (2003) observed a partial loss of chlorophylls after a long period of desiccation in two species, one of them being *L. pulmonaria*, probably as a result of a damage. In the present study there was no such degradation of chlorophylls in lichens, but our period of desiccation was shorter (only one instead of

nine weeks) and thus it cannot be excluded that a longer period of desiccation could result in such effect. To test this hypothesis, the experiment could be repeated checking Chl*a*F and pigments after two, four and six weeks of treatments.

Esteban *et al.* (2005), in their meta-analysis on plant pigment composition and factors affecting it, listed a series of stresses and their effects on pigments. For example, the lutein/chlorophyll and VAZ/chlorophyll ratios generally increase when a plant is submitted to drought stress, and VAZ/chlorophyll ratio increases with increasing daily photon irradiance. In our study, though, photosynthetic pigment contents (neoxanthin, lutein, VAZ, β-carotene) were generally stable in lichens, regardless of the treatment, suggesting their constitutive expression. The xanthophyll cycle, instead, was operative in all of the lichens with a green alga as a photobiont. Bearing in mind that our time 0 is in the dry state (t_0d), in all the three species the zeaxanthin is accumulated both in the presence of light and during the desiccated state, as AZ/VAZ was high at t_0d, after the light treatment (DL and WL) and the dark desiccated treatment (DD), while it significantly decreased after the WD treatment and all of the recoveries. As a further experiment, it would be appropriate to include also the t_0w in the HPLC analyses. What we found agrees with the current literature, as it is demonstrated that zeaxanthin can be formed also during darkness and also by some desiccation sensitive species, as a relic of a mechanism that originated in the very initial steps of land colonization by plants (Fernández-Marín *et al.*, 2010; 2011). The most striking differences in this general pattern can be seen in *L. pulmonaria* and *P. leucophlebia* after the WL treatment. The first species had a significant increase of the 63% in the AZ/VAZ ratio with respect to the t_0d (p-value 0.02), by far the highest increase observed, or, to give an idea of the difference with an actually hydrated value, of the 100% with respect to its recovery (p-value < 0.01). This was interesting also because the DL treatment had a value similar to that of the t_0d (0.31 ± 0.06 and 0.32 ± 0.07, respectively), so light alone was not enough to activate the xanthophyll cycle to that extent. Fernández-Marín *et al.* (2010) observed that *L. pulmonaria* was able to interconvet violaxanthin in the dark only when dehydrated slowly (at a RH of the 75% vs < 5% with silica gel) concluding that the enzyme VDE needs at least a minimum time to be activated. It has also been demonstrated that when lichens reach the glassy state VDE cannot work, while it can still function in the so-called rubbery state, preceding the complete vitrification (Fernández-Marín *et al.*, 2013). Our dehydration rate was an intermediate one, as we submitted the lichens to a 30-40% RH (see Fig. A1) without air flows, enough to let VDE work, but it is possible that the extra time of metabolical activity guaranteed by the rehydrations allowed further violaxanthin de-epoxidation. In the same study cited above (Fernández-Marín *et al.*, 2010), the Authors furtherly observed that zeaxanthin formation had a photoprotective effect as an NPQ modulator only when rehydrated thalli were exposed to light, but not when dehydrated thalli were illuminated. At the light

of these findings, it is possible that *L. pulmonaria* performs better when illuminated in the hydrated state because it can accumulate more zeaxanthin and it can use the xanthophyll cycle also for photoprotection. It is worth noting, though, that even with a higher zeaxanthin concentration *L. pulmonaria* suffered WL treatment way more than *F. caperata*, that kept more or less the same zeaxanthin concentration as t_0d. Probably, the latter species did not need a further interconversion of violaxanthin and could rely on other efficient mechanisms to cope with light. On the other end of the spectrum, *P. leucophlebia* after WL had an unusually low AZ/VAZ ratio (47% less than t_0d, p-value 0.02). This can be seen as a failure in performing the xanthophyll cycle, which could be the responsible for the harm observed after the WL treatment. In fact, the two cephalolichens suffered in a similar way after WL, but *P. leucophlebia* had also an increase in F_0 that probably *L. pulmonaria* was able to avoid thanks to the additional zeaxanthin. Both cephalolichens were harmed by being hydrated in the light, but *P. leucophlebia* suffered for the lack of zeaxanthin while *L. pulmonaria* in spite of its presence. This hypothesis could be tested adding an external source of zeaxanthin to both species during the WL treatment and observe if *P. leucophlebia* is able to improve its performance and if *L. pulmonaria* shows any difference in its Chl*a*F.

Tocopherols are lipid-soluble molecules with antioxidant properties ubiquitously found in all photosynthetic organisms (Falk & Munné-Bosch, 2010 and citations herein). In higher plants they are known to play a role in protection from high light irradiances and drought and generally their amounts are modulated by such stresses (Havaux & García-Plazaola, 2014). They are classified into different homologues, the most common one being α-tocopherol, but it is worth mention that γ-tocopherol could exert additional functions in plant resistance to osmotic stress and/or desiccation tolerance. Only a few studies about tocopherols and lichens exist (Kranner *et al.*, 2003; 2005; Strzalka *et al.*, 2011), though, and no much knowledge is currently available. Kranner *et al.* (2005) analyzed the α-tocopherol content of the lichen *Cladonia vulcani* (with *Trebouxia excentrica* as photobiont) after three and nine weeks of desiccation over silica gel and reported a drop of one third of its content during desiccation. Control levels were re-established after three, but not nine, weeks. Our case was different, as we found that α-tocopherol amounts were always constant in all the four species, regardless of the treatment. So, even if α-tocopherol has a protective role during desiccation, in our case it must be constitutively expressed.

Overall, the analysis of pigments and tocopherols seems to furtherly confirm that lichens rely mainly on constitutive mechanisms, as highlighted also by other approaches (Gasulla *et al.*, 2013; 2016; Candotto Carniel *et al.*, 2016). For example, Gasulla *et al.* (2013; 2016) analyzed the proteome of the isolated photobiont *Asterochloris erici* submitted to desiccation and found little to no variations. In

agreement with these Authors, Candotto Carniel *et al.* (2016) found that during a dehydration/rehydration cycle, around the 92 % of the total protein-coding transcripts of *T. gelatinosa* displayed a stable expression, suggesting that its desiccation tolerance mostly relies on constitutive mechanisms. In our study, we found constant antioxidant concentrations, with little to no variation after different kind of stresses. Also, in accordance with Kranner *et al.* (2003), higher amounts of antioxidants did not necessarily indicate better desiccation tolerance. In addition to the general constitutive mechanisms, lichens can rely also on inducible ones, for example the xanthophyll cycle, as discussed for *L. pulmonaria*. The presence of such mechanisms, though, does not necessarily indicate better desiccation tolerance, as selection may favor inducible mechanisms in spite of the constitutive ones in environments that are usually moist, in which lichens desiccate slowly. *Flavoparmelia caperata*, the most resistant species, lacked that mechanisms and thrived better than *L. pulmonaria*. There is still the need for further investigation to better understand what makes *F. caperata* more efficient and to which different mechanisms it can rely on. For example, the transcriptome experiment performed by Candotto Carniel *et al.* (2016) on its photobiont could be repeated on *F. caperata* and the more sensitive species, to pinpoint differences in transcripts that could shed some light on desiccation tolerance mechanisms.

If desiccation tolerance in lichens is still not completely understood, for their isolated symbionts data are even scarcer (Candotto Carniel *et al.*, 2015; 2016). There is the need to fill this gap, though, as a better knowledge of how these mechanisms work in aposymbionts and how lichenization changes/enhances them would probably help to shed a light also on desiccation tolerance as a whole. Hence, the decision to repeat the experiment with two of the lichen isolated photobionts. We decided to select the photobionts of a resistant species (*T. gelatinosa*, photobiont of *F. caperata*) and one of a more sensitive species. We decided to exclude the cyanobacterium for its substantial metabolical differences from the other green alga and in the end, we selected the photobiont of *L. pulmonaria* (*S. reticulata*) for its importance in conservation. The major changes to the experimental design were made to better suit the algal physiology. The light was decreased calculating what fraction of the PFF reached the alga in the lichen thallus, and to do so, cortex transmittance was measured. *Lobaria pulmonaria* resulted in having the highest one and this is not surprising, as sciaphilous species tend to have higher cortex transmittances to catch all the light they can in their relatively light scarce environment (Dietz *et al.*, 2000). Also, we tested if dim-light cultured algae needed an acclimation to their final light regime to better withstand the treatments (see Materials & Methods).

Acclimation was performed to test if the light treatment effects on Chl*a*F were partially due to the abrupt transition from dim to higher light, but the results do not seem to confirm this hypothesis. After

the light treatments (DL and WL) acclimated and non-acclimated samples had comparable F_v/F_m, and the only small difference (in *T. gelatinosa* after DL, see Results) was not confirmed by differences in the fluorescence transient. Works on acclimation of lichens and their isolated photobionts to environmental stresses is just beginning (Kranner *et al.*, 2008), but for example Štepigová *et al.* (2008) proved that irradiance prior to desiccation in *L. pulmonaria* enhances its light tolerance during the dry state. Their desiccation period, though, lasted only 24 hours, while we exposed the algae for seven days. It could be possible that the acclimation determined a small advantage in tolerating light only the first few days, but then it expired, and to test this hypothesis it would be necessary to measure F_v/F_m also a short period of time after the exposure to the light treatments.

By far, the most resistant species was *T. gelatinosa*, whose performance was comparable to that of its lichenized counterpart. Considering both the F_v/F_m and the fluorescence transients (Fig. 5 and 6, respectively), *T. gelatinosa* showed only minor disadvantages with respect to the lichen. *Symbiochloris reticulata*, instead, suffered consistently each treatment and its recovery was always incomplete. Like its lichenized counterpart, it suffered more being illuminated in the dry state than when hydrated, and light was the factor with the most negative effects. However, the most striking difference from its lichen is that also all the other treatments (particularly WD and WL) had a profound impact on this alga, and so it could be hypothesized that *S. reticulata* cannot withstand properly even desiccation in itself. This topic will be addressed again later to be discussed more in detail also at the light of the photosynthetic pigment results.

The highest chlorophyll content belonged also this time to the more sensitive species, having up to the 30% more chlorophylls than *T. gelatinosa*, so the differences in chlorophylls observed in their respective lichens (65% more in *L. pulmonaria* than in *F. caperata*) can be ascribed both to a higher algal content in *L. pulmonaria* thallus and to a higher chlorophyll concentration in the alga. *Trebouxia gelatinosa*, exactly like its lichen, had a constant chlorophyll content. Surprisingly, instead, *S. reticulata* showed a degradation of chlorophylls that did not occur in its lichenized counterpart. We exclude that this could have been caused by an artifact, as we improved the extraction protocol splitting it into four total steps, two more than in the lichen extraction (see Materials & Methods). The degradation occurred after the wet treatments (WD and WL) and there was no rebuilding after 24 hours, and in one case (WL recovery, see Fig. 8) even a further decrease was observed. This could explain the drop in F_0 and F_m in WD (data not shown) and WL: a loss of chlorophylls lowered these parameters without directly affecting F_v/F_m. Kranner *et al.* (2003) observed a breakdown of chlorophylls in *L. pulmonaria* after a long period of constant desiccation on silica gel, and concluded that a damage may have occurred. Our case, though, is different, because degradation occurred only

after the “wet” treatments and not after the more harmful DL one (similar to the treatment performed by Kranner *et al.*, 2003). This may indicate that the alga actively dismantled its chlorophylls when partially metabolically active, rather than it passively experienced a damage. In fact, poikilochlorophyllous resurrection plants need to be active for a long period of time to degrade chlorophylls, as it can take up to two weeks to reduce the initial leaf chlorophyll content by approximately 50% (Kranner *et al.*, 2002). This could represent a defensive strategy activated by slow drying to prevent further oxidative damage in case the alga is also illuminated. This could be part of the reason why *S. reticulata* suffered more when kept continuously dry in the light: the drying rate was probably not slow enough to allow a dismantle of chlorophylls. The reason why after the DL treatment it did not experience the passive damage observed by by Kranner *et al.* (2003) is probably because the time of exposure was considerably shorter (one week vs nine weeks).

Differently from *F. caperata*, that always kept its antioxidants to a steady level, *T. gelatinosa* showed a decrease (not always statistically significant) after the dry treatments and a subsequent increase upon recovery in some antioxidants, specifically neoxanthin and total VAZ. This could mean that while the lichenized alga relies for the most part on constitutive mechanisms, the isolated *T. gelatinosa* has some kind of inducible repair mechanism upon rehydration to prepare/restore the pool of antioxidants for the next desiccation event. Candotto Carniel *et al.* (2016) performed a transcriptomic study on the same organism, *T. gelatinosa*, after a dehydration and a subsequent rehydration. During the rehydration, a small number of genes were found to be upregulated, including some related to the photosynthetic apparatus. They did not find differently regulated genes coding for the enzymes involved in the synthesis of carotenoids, but it has to be considered that carotenoids have a very complex metabolical pathway and not all the genes involved in their synthesis are annotated (Candotto Carniel *et al.*, 2016). For this reason, it was not possible to draw any conclusion from that study, but at the light of our results we can hypothesize that these antioxidants could have been similarly upregulated during rehydration. This mechanism, though, was triggered only by desiccation, not by light alone, as no such changes were observed after the WL treatment. It could be that the light (35 μmol photons m^{-2} s^{-1}) was too low to activate them, but further investigation is needed to prove this hypothesis, for example repeating the experiment increasing the light up to 60, 80 and 100 μmol photons m^{-2} s^{-1}. Differently from its lichenized form, *T. gelatinosa* did not seem to use the xanthophyll cycle (Fig. 8). This agrees with Kranner *et al.* (2005) that, studying the photosynthetic pigment expression of *Cladonia vulcani* and its isolated symbionts submitted to desiccation and subsequent rehydration, also found that the xanthophyll cycle was not operative in the isolated alga. They hypothesized that light (12 μmol photons m^{-2} s^{-1}) was not enough to activate it, but we now know that during desiccation the xanthophyll cycle can be operative also in darkness (Fernández-Marín *et*

al., 2010; 2011). It could be possible that lichenization activates the genes involved in the xanthophyll cycle regulation during desiccation, but further investigation is needed to confirm or reject this hypothesis. Unfortunately, it was not possible to measure neoxanthin and all the xanthophyll cycle pigments in *S. reticulata* (see Results).

Overall, considering the chlorophyll fluorescence and pigment data, *T. gelatinosa* resulted to be very resistant to photo-oxidative stress. This is not surprising, as many species belonging to this genus have shown to tolerate various levels of this kind of stress. For example, *Trebouxia* sp. from *Parmotrema perlatum* was able to show a photosynthetic performance comparable to that of its lichenized counterpart when desiccated for 15, 30 and 45 days at a light intensity of 120 μmol photons m^{-2} s^{-1} (Candotto Carniel *et al.*, 2015). Also, Banchi *et al.* (under review) induced oxidative stress in *T. gelatinosa* submitting it to increasing concentrations of hydrogen peroxide (H_2O_2), a strong ROS. *Trebouxia gelatinosa* started to have a decrease in F_v/F_m only after the treatment at 0.5 M H_2O_2, a very high concentration, considering that many organisms show reduced vitality at millimolar (e.g. Houot *et al.*, 2001; de Pinto *et al.*, 2006; Darehshouri *et al.*, 2008), or even micromolar (Ding *et al.*, 2012) concentrations. We suggest that this high resistance is based on a large number of constitutive mechanisms (as demonstrated also by Candotto Carniel *et al.*, 2016) and a small number of repair mechanisms inducible upon rehydration. In accordance with Kranner *et al.* (2003), we observed that its resistance is not determined by a high level of antioxidants, but rather by the ability to restore its pre-treatment antioxidant concentrations upon recovery. At the light of these findings, it is not easy to decide to what degree *T. gelatinosa* acquires an advantage with lichenization. When lichenized it improves its performance, losing a smaller percentage of its F_v/F_m, recovering faster and tolerating better desiccation in the light (see Fig. 2A and 6C), probably also for its better capability to use the xanthophyll cycle, but overall it could thrive well also free-living. We used a low light intensity and it could be possible that at higher intensities lichenization becomes a substantial advantage. Until this possibility is proven right (for example testing light intensities up to 250 μmol photons m^{-2} s^{-1}, the one endured by the lichen), though, in the case of *T. gelatinosa* we agree with Candotto Carniel *et al.* (2015), concluding that lichenization can influence the physiology of the photobiont, but this species of *Trebouxia* is perfectly apt to withstand prolonged periods of time in the desiccated state under mid photo-oxidative conditions also outside a lichen thallus.

On the other end of the spectrum, *S. reticulata* appeared to be very sensitive to photo-oxidative stress. Our results can be considered novel, as to our knowledge there is a lack of physiological data on *S. reticulata*. Its lichen, *L. pulmonaria*, is a an indicator of lichen diversity (Nascimbene *et al.*, 2010) and so it has been extensively studied, also from the physiological point of view (e.g. Gauslaa &

Solhaug, 1999; Fernández-Marín *et al.*, 2010; Bidussi *et al.*, 2013). *Symbiochloris reticulata* has been thoroughly phylogenetically studied (e.g. Dal Grande *et al.*, 2014; Škaloud *et al.*, 2016), but its physiology is almost unknown. Our results highlighted a general weakness with respect to oxidative stress. It is considerably more sensitive to the light and, surprisingly, also to desiccation itself. In fact, at a 30-40% RH, perfectly tolerable in itself from *L. pulmonaria* (see Fig. 1 and Tab. A1, A2), it started to lose a consistent percentage of its F_v/F_m (see Tab. A1 and A2, treatments DD and WD) and if periodically metabolically active, to dismantle its photosynthetic apparatus even when light was not directly involved (treatment WD). This probably is a survival strategy, to avoid being caught by light in the dry state, and it is possible that *S. reticulata*, when outside a lichen thallus, becomes poikilochlorophyllous. But this strategy was not enough to avoid damage, and the alga was not able to recover its pre-treatment values in both F_v/F_m and chlorophyll content. It could be that 24 hours are not enough to rebuild chlorophylls, so perhaps it would be better to check also after one week, but in one case chlorophyll content furtherly decreased after the recovery. Our hypothesis is rather that *S. reticulata* is desiccation tolerant to a lesser extent: it can tolerate drying only to higher RH, it relies on slow inducible mechanisms like the dismantling of chlorophylls and it is seriously damaged when RH goes down a certain threshold. As a further evidence, in a previous unreported experiment *S. reticulata* was submitted for seven days to an atmosphere at the 75% of RH with one hydration per day (in the morning) and it survived unharmed, showing F_v/F_m similar to the pre-exposure values (unpublished data). Further investigation is needed to assess the RH threshold it can tolerate safely, for example submitting it to the same procedure for the same time, but with different RH%, for example 75%, 60%, 50%, 40% and 30%. Anyway, it is safe to say that to *S. reticulata*, lichenization brings striking advantages: in a lichen thallus it is still sensitive to light, especially in the dry state, but it becomes fully desiccation tolerant and overall it greatly increases its tolerance to photo-oxidative stress. Part of this better tolerance could be due to the ability to activate the xanthophyll cycle, but unfortunately it was not possible to separate xanthophylls to the chlorophyll degradation products (see Results) and therefore this hypothesis needs to be proved. Developing a protocol for a proper extraction and separation of these pigments could be the next goal to further investigate this topic. Anyway, lichenization appears to upregulate genes that enhance *S. reticulata* coping mechanisms, allowing it colonize environments that otherwise would kill it as free-living.

This work contributes to the understanding of desiccation tolerance in lichen and their photobionts. There are still many open questions, though, and further investigation is needed to understand why different species make a different use of the xanthophyll cycle and how some species are able to tolerate a higher photo-oxidative stress. We demonstrated that while *T. gelatinosa* had a relatively low advantage from lichenization, *S. reticulata* gained substantial protection form oxidative stress.

We can conclude that care has to be taken to draw general conclusions while studying desiccation tolerance in a particular couple lichen-alga, as different species can show striking differences in physiology. Comparing the responses of organisms with different degrees of desiccation tolerance, rather to focus on very tolerant ones, could help to shed some light on the mechanism as a whole.

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APPENDIX

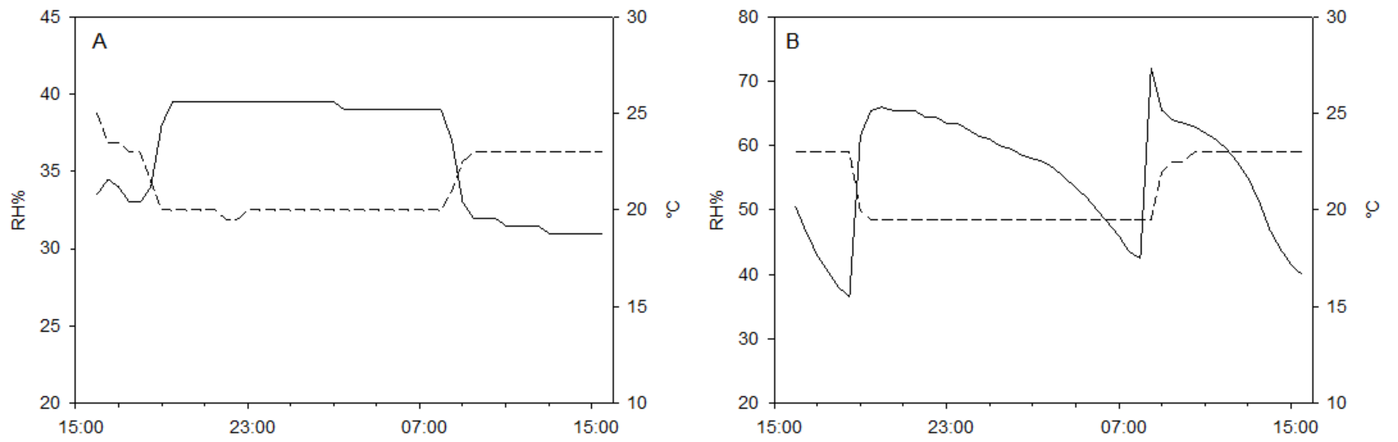

Figure A1 RH (black line) and air temperature (black dotted line) measured with a data logger (EL USB-2, Lascar Electronics Inc, Whiteparish, UK) inside the sealed box for experiment A DL (A) and WL (B) treatments throughout a day.

Table A1 Changes in F_v/F_m before (t_0w, t_0d) and after the treatments (DD, DL, WD, WL) and after the 8 (8h) and 24 (24h) hour recoveries. Values are mean ± standard error, n = 5-6.

species	t_0w	t_0d	DD	DL	WD	WL	DD8h	DL8h	WD8h	WL8h	DD24h	DL24h	WD24h	WL24h
F. caperata	0.750 ± 0.017	0.755 ± 0.033	0.738 ± 0.041	0.629 ± 0.027	0.741 ± 0.026	0.691 ± 0.025	0.780 ± 0.011	0.753 ± 0.010	0.787 ± 0.003	0.778 ± 0.017	0.757 ± 0.008	0.732 ± 0.015	0.754 ± 0.022	0.741 ± 0.014
L. pulmonaria	0.722 ± 0.012	0.726 ± 0.014	0.695 ± 0.017	0.360 ± 0.078	0.705 ± 0.034	0.629 ± 0.024	0.707 ± 0.071	0.600 ± 0.065	0.746 ± 0.006	0.726 ± 0.016	0.732 ± 0.006	0.620 ± 0.066	0.728 ± 0.014	0.700 ± 0.008
P. leucophlebia	0.755 ± 0.020	0.762 ± 0.016	0.694 ± 0.080	0.394 ± 0.061	0.649 ± 0.108	0.640 ± 0.045	0.771 ± 0.042	0.658 ± 0.046	0.674 ± 0.150	0.730 ± 0.032	0.745 ± 0.073	0.708 ± 0.030	0.679 ± 0.118	0.684 ± 0.104
P. praetextata	0.532 ± 0.091	0.463 ± 0.060	0.290 ± 0.065	0.098 ± 0.022	0.320 ± 0.109	0.172 ± 0.113	0.583 ± 0.047	0.501 ± 0.099	0.523 ± 0.153	0.390 ± 0.085	0.563 ± 0.076	0.467 ± 0.104	0.540 ± 0.085	0.360 ± 0.081
T. gelatinosa	0.636 ± 0.013	0.578 ± 0.021	0.559 ± 0.069	0.541 ± 0.045	0.520 ± 0.036	0.567 ± 0.018	0.569 ± 0.049	0.601 ± 0.042	0.605 ± 0.016	0.600 ± 0.031	0.533 ± 0.075	0.567 ± 0.030	0.528 ± 0.058	0.572 ± 0.041
S. reticulata	0.612 ± 0.008	0.420 ± 0.077	0.318 ± 0.080	0.233 ± 0.108	0.323 ± 0.014	0.266 ± 0.063	0.463 ± 0.037	0.389 ± 0.060	0.386 ± 0.032	0.373 ± 0.052	0.470 ± 0.033	0.390 ± 0.047	0.404 ± 0.044	0.385 ± 0.079

Table A2 Percent (on t_0w mean value) changes of F_v/F_m after the treatments (DD, DL, WD, WL) and after the 8 (8h) and 24 (24h) hours recoveries. Values are mean ± standard error, n = 5-6.

species	t_0w	t_0d	DD	DL	WD	WL	DD8h	DL8h	WD8h	WL8h	DD24h	DL24h	WD24h	WL24h
F. caperata	100 ± 2	101 ± 4	98 ± 5	84 ± 4	99 ± 3	92 ± 3	104 ± 1	100 ± 1	105 ± 0	104 ± 2	101 ± 1	98 ± 2	101 ± 3	99 ± 2
L. pulmonaria	100 ± 2	100 ± 2	96 ± 2	50 ± 11	98 ± 5	87 ± 3	98 ± 10	83 ± 9	103 ± 1	101 ± 2	101 ± 1	86 ± 9	101 ± 2	97 ± 1
P. leucophlebia	100 ± 3	101 ± 2	92 ± 11	52 ± 8	86 ± 14	85 ± 6	102 ± 6	87 ± 6	89 ± 20	97 ± 4	99 ± 10	94 ± 4	90 ± 16	91 ± 14
P. praetextata	100 ± 17	87 ± 11	55 ± 12	18 ± 4	60 ± 20	32 ± 12	110 ± 9	94 ± 19	98 ± 29	73 ± 16	106 ± 14	88 ± 20	102 ± 16	68 ± 15
T. gelatinosa	100 ± 2	91 ± 3	88 ± 11	85 ± 7	82 ± 6	89 ± 3	89 ± 8	94 ± 7	95 ± 3	94 ± 5	84 ± 12	89 ± 5	83 ± 9	90 ± 6
S. reticulata	100 ± 1	69 ± 13	52 ± 13	38 ± 18	53 ± 2	44 ± 10	76 ± 6	64 ± 10	63 ± 5	61 ± 9	77 ± 5	64 ± 8	66 ± 7	63 ± 13