# Lichen substances are more important for photoprotection in sun than shade collections of lichens from the same species

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**Abstract.** Photosynthetic organisms possess a great diversity of mechanisms to protect themselves from the potentially stressful effects of high PAR (photosynthetically active radiation). A distinctive response to longer term exposure to high levels of PAR in lichens is the synthesis of a variety of substances in the upper cortex that can protect photobionts from photoinhibition. In the present study, lichen substances were removed harmlessly from lichens using the "acetone rinsing" method. This enabled us to compare the importance of the substances in photoprotection in sun and shade collections of four species of Afromontane lichens. While all species normally grow in more exposed microhabitats, it is easy to make collections of more shaded thalli. Using chlorophyll fluorescence, we show that collections of lichens from sunny microhabitats have higher tolerance to photoinhibition than those from shaded locations. Furthermore, removal of lichen substances increases sensitivity to photoinhibition, suggesting that even although colorless, they have a role in protecting against high PAR. Sensitivity was increased much more in sun than shade collections, implying that substances play a greater role in photoprotection in lichens from sunny microhabitats. Nevertheless, following the removal of lichen substances, most sun collections still possess higher tolerance to photoinhibition than shade collections. Therefore, the additional tolerance of sun collections appears derive from a combination of both lichen substances and other, probably more biochemical tolerance mechanisms.

**Keywords.** Lichen physiology, Afromontane, chlorophyll fluorescence, acetone rinsing.

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Light is essential for photosynthesis, but when organisms absorb more light than they can use for carbon fixation, the result can be a reduction in photosynthesis, often termed "photoinhibition," that will eventually reduce growth. While there is currently no consensus on how exactly photoinhibition occurs (Zavafer & Mancilla 2021), most workers believe that photoinhibition occurs when excess energy causes the production of reactive oxygen species (ROS) (Pospíšil 2016). ROS can cause lipid peroxidation or damage the photosystems, in particular the D1 and D2 proteins in the reaction center of PSII (Foyer 2018). Photosynthetic organisms possess a diversity of mechanisms to protect from photoinhibition, although there have been few studies in lichens. However, based on work carried out on other organisms, it seems likely that lichens possess a range of mechanisms to cope with both long- and short-term changes in light availability (for review see Beckett et al. 2021). Lichens growing on the trunks of trees or on rocks under a tree canopy are exposed to rapidly changing light levels because gaps in the canopy create brief periods of high light, known as 'sunflecks.' Tolerance to these short-term changes in light availability (over a range from minutes to hours) can be improved by increasing the dissipation of excess energy absorbed (without radiation) as heat using non-photochem-

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ical quenching (NPQ). Other likely mechanisms include an increased ability to scavenge ROS formed during photoinhibition, an increased capacity to repair ROS-induced damage, and an increase in cyclic electron flow (Shi et al. 2022). Lichens growing in exposed habitats may experience more sustained light stress, which mainly varies only seasonally. For higher plants, adaptations can include changes in the ratio of chlorophyll a to chlorophyll b, changes in chloroplast architecture, general adjustments in the maximum photosynthetic rate, and changes in the activities of ROS scavenging enzymes and the PSII repair cycle (for reviews see Greer 2023 and Shi et al. 2022). A particular tolerance mechanism for long-term light stress that has been well studied in lichens is the synthesis of secondary metabolites in the upper cortex. These compounds play a variety of roles in lichen biology, including deterring herbivores and pathogenic microbes (Ranković et al. 2008; Solhaug & Gauslaa 2012) and have allelopathic functions (Solhaug et al. 1995). However, when they occur in the upper cortex their most important role may be to protect the mycobiont and photobiont from the long-term harmful effects of excess solar radiation (Solhaug & Gauslaa 2012).

Lichen substances are synthesized by the mycobiont, and typically occur as hydrophobic crystals on the cell walls of the hyphae. Some of these compounds are intensely pigmented, and directly absorb PAR and UV light. For example, melanins are brown-black pigments that have been shown to increase the tolerance of photobionts to photoinhibition (Mafole et al. 2019a). However, the great majority of more "classic" lichen secondary compounds are colorless. While they absorb very poorly in the visible region, they absorb well in the UV range (Huneck & Yoshimura 1996). These compounds were therefore formerly considered to be only effective in protecting lichens from the harmful effects of UV radiation (Solhaug & Gauslaa 2012). Intuitively, they would seem unlikely to play any role in protecting photobionts from the effects of high PAR. However, several field studies have shown that the concentrations of lichen substances can track light availability. For example, Legaz et al. (1986) found higher concentrations of usnic acid and atranorin in the thalli of *Evernia prunastri* during the brighter summer months than in winter. Furthermore, extraction of the colorless secondary metabolite atranorin from *Physcia aipolia* significantly increases photoinhibition caused by high PAR (Solhaug et al. 2010). Hydrated thalli with lichen substances display higher reflectance, probably, at least in part, because the crystals directly reflect light. In addition, because they are hydrophobic, lichen substances may prevent water from entering the spaces between the hyphae in the cortex, and in hydrated thalli it is actually the air-filled cavities that reflect light. Recently, Ndhlovu et al. (2022) demonstrated that this is a rather general phenomenon. In five diverse Afromontane species, removal of lichen substances clearly reduced the tolerance of their photobionts to photoinhibition. Interestingly, Ndhlovu et al. (2022) further showed that removal of lichen substances also increases the sensitivity of desiccated thalli but has little effect on reflectance. This suggests that lichen substances may increase tolerance to photoinhibition in ways other than simply increasing reflectance. Irrespective of the mechanisms involved, unpigmented or lightly pigmented lichen substances appear to be important in photoprotection.

The relative importance of the various tolerance mechanisms for photoprotection in different species of lichens in field situations is unknown. In particular, it is difficult to separate mechanisms based on the synthesis of light screening pigments with more biochemical mechanisms of photoprotection. For lichens that become melanized when they grow in sunny locations, it appears intuitive that melanins would be the most important defence mechanism. As noted above, melanized thalli are more tolerant to photoinhibition than pale thalli (Mafole et al. 2019a,b). Unfortunately, an inherent problem with making simple comparisons between pale and brown thalli is that melanized thalli have a history of exposure to higher light levels than pale thalli. As a result, the photobionts of melanized thalli may have developed other mechanisms that increased tolerance to photoinhibition. Recently, we tested the importance of melanization in tolerance to high light compared with the importance of other tolerance mechanisms by dissecting away the lower cortices and medullas in a range of species (Beckett et al. 2019). This enabled us to photo-inhibit photobionts with light from below, i.e., without the presence of a melanized upper cortex. In some species, e.g., *Cetraria islandica*, compared with pale thalli, photobionts in melanized thalli possess much

higher tolerance to photoinhibition when exposed from above. However, the photobionts from melanized thalli still possess significantly higher tolerance than pale thalli when photoinhibited from below. Therefore, in *C. islandica* protection from high light appears to derive from a mixture of both cortical pigments and biochemical mechanisms. While a significant number of lichen species "melanize" on exposure to high light, it appears more common for the mycobiont to produce colorless or lightly pigmented lichen substances such as usnic acid and atranorin in their upper cortices. As discussed above, there is good evidence that the presence of these compounds can assist in photoprotection; however, their relative importance compared with other tolerance mechanisms remains untested.

For many fruticose lichens such as *Usnea* and *Ramalina*, it is not possible to test the significance of substances present in the upper cortex by surgically removing the lower cortex and medulla and exposing lichens from below. However, it is possible to harmlessly remove lichen substances using the "acetone rinsing" technique of Solhaug et al. (2010). Tolerance to photoinhibition can then be compared in thalli with and without lichen substances, enabling the relative importance of these compounds to be assessed. One approach to assess the relative importance of tolerance mechanisms is to compare mechanisms present in "sun" forms with "shade" forms of members of the same species. While it has been shown that lichens can, as for higher plants, display sun and shade forms (Piccotto & Tretiach 2010), there have been surprisingly few attempts to test whether shade collections of the same species of lichens are more sensitive to photoinhibition than those of sun forms. Kershaw & MacFarlane (1980) reported that populations of *Peltigera aphthosa* collected from the dense shade of spruce are extremely sensitive to high light, while populations collected from open habitats are much more tolerant. However, the mechanisms responsible for the increased tolerance of the sun collections were not studied. The first aim of the work presented here was to determine whether sun forms of a range of Afromontane lichens possess greater tolerance to photoinhibition than shade forms. As results showed that in both the hydrated and desiccated states sun forms have higher tolerance than shade forms, we then tested the relative importance of secondary metabolites in the additional tolerance of sun forms. We reasoned that first, if the major role of lichen substances is to protect photobionts from photoinhibition, then removal of these substances will increase the sensitivity of the sun forms more than that of the shade forms. Second, if other tolerance mechanisms are also important (e.g., enhanced NPQ, higher levels of antioxidant enzymes or PSII repair cycle enzymes), then even after removal of lichen substances, sun forms should still display greater tolerance to photoinhibition than shade forms. Results presented here show that additional tolerance present in sun collections is generally derived from a combination of both lichen substances and other tolerance mechanisms.

## Materials and Methods

***Collection sites.*** All species used in this study were collected in Afromontane vegetation in KwaZulu Natal, South Africa. Both sun and shade collections of *Parmotrema perlata* and *Usnea undulata* were collected from a forest at Fort Nottingham Nature reserve. Sun collections were made from minor twigs at the periphery of the canopy (the more normal microhabitat of these species), while shade collections were made c. 1 m away, from deep inside the canopy, usually on main branches or tree trunks. Shade populations of *Xanthoparmelia conspersa* and *Ramalina celastri* were collected from shaded rocks and trees respectively in Queen Elizabeth Park, Pietermaritzburg. Sun populations of *X. conspersa* were collected from rocky outcrops near the Cascades Lifestyle Center, Pietermaritzburg, c. 3 km from the shade population. Sun populations of *R. celastri* were collected from unshaded tree bark in Clarendon, Pietermaritzburg, c. 5 km from the shade population. The photobionts of these lichens have been reported to belong to the chlorophycean genus *Trebouxia* (Rambold et al. 1998). After collection, lichen material was allowed to airdry between filter paper overnight and then stored at –24°C until needed.

***Acetone rinsing.*** We did not quantify the concentrations of lichen substances present in the thalli used in this study. However, usnic acid was qualitatively determined to be the main lichen substance present in all species by shaking dry thalli in acetone and analysing the resulting eluates using

high performance liquid chromatography as described by Pawlik-Skowrońska & Bačkor (2011). Comparisons were made with standards from Merck (St. Louis, Missouri, U.S.A.). In the main experiment, lichen substances were removed using the "acetone rinsing" technique of Solhaug et al. (2010). In all cases, lichens were initially left overnight over silica gel to ensure they were completely dry. They were then gently shaken in 100% acetone for 10 min. Acetone was then discarded, and the process repeated twice. After acetone rinsing, the thalli were left at room temperature overnight to allow residual acetone to evaporate. Extraction of lichen substances had no significant effect on chlorophyll fluorescence parameters.

***Chlorophyll fluorescence measurements.*** To assess the effects of light stress on photosystem II (PSII), chlorophyll fluorescence was used to measure the maximal efficiency of PSII ($F_V/F_M$) and the relative electron transfer rate (rETR, a proxy of steady state photosynthesis). In general, both parameters responded similarly to light stress, although occasionally one parameter was more sensitive than another for no obvious reason. Chlorophyll fluorescence was measured using a PAM 2500 fluorometer (Walz, Effeltrich, Germany) using the red LED throughout. After a dark adaptation period of at least 10 min, $F_V/F_M$ was measured, where $F_M$ = maximum fluorescence and $F_V$ = variable fluorescence or $(F_M - F_O)$, with $F_O$ = minimal fluorescence yield of the dark-adapted state. Thalli with anomalous values of $F_V/F_M$ were discarded. The actinic light (25 μmol $m^{-2}$ $s^{-1}$) was then switched on, and when the fluorescence signal was stable, rETR was calculated as:

$$rETR = 0.5 \times \Phi PSII \times PAR$$

where PAR = photosynthetically active radiation and ΦPSII is the effective quantum yield of PSII photochemistry calculated as $(F_{M'} - F_t)/F_M$ (where $F_{M'}$ = maximal fluorescence yield of the light-adapted state and $F_t$ = stable fluorescence signal in the light).

***Effect of lichen substance removal on sensitivity to photoinhibition.*** For each collection, 40 samples were used, 10 for each treatment combination of hydrated and desiccated thalli with and without secondary metabolites. For *Xanthoparmelia conspersa* and *Parmotrema perlata* each replicate comprised a 1 cm disk, while for *Ramalina celastri* and *Usnea undulata* each replicate comprised a c. 1.5 cm thallus segment. All thalli were initially in a desiccated state. To expose hydrated thalli to high light, thalli were acetone rinsed if required, the acetone allowed to evaporate overnight, all (rinsed and unrinsed) thalli placed on wet filter paper at 30 μmol $m^{-2}$ $s^{-1}$ overnight and an initial measurement of chlorophyll fluorescence parameters taken. Thalli were then exposed to high light. To expose desiccated thalli to high light, thalli were acetone rinsed if required, the acetone allowed to evaporate for 24 h, all thalli (rinsed and unrinsed) hydrated overnight as above, initial chlorophyll fluorescence measurements taken, and then allowed to air dry overnight. They were then exposed to the photoinhibitory light, and immediately rehydrated by placing them on wet filter paper. Lichens were photoinhibited using a LED panel (Model SL – 3500, Photon System Instruments, Brno, Czech Republic) that provides cool white light. The exposure to light needed to reduce $F_V/F_M$ down to c. 0.2 to 0.3 for lichens with substances present was determined in preliminary experiments. Species differed in their sensitivity, and much longer exposures were needed for dry compared with wet material; **Table 1** indicates the exposure times and intensities used. Lichens with and without lichen substances received the same exposure times and intensities. Initial chlorophyll fluorescence measurements were taken at the start of the experiment as indicated above, immediately after the exposure to high light and again at intervals for up to 50 h. During recovery, lichens were exposed to normal laboratory light (c. 5 mol $m^{-2}$ $s^{-1}$) as recommended by Solhaug (2018). All species were highly desiccation tolerant, and when not photoinhibited were found to recover from desiccation within minutes of rehydration (data not shown). However, some of the recovery that occurred in photoinhibited dry lichens during the first 30 min of rehydration may represent recovery from desiccation stress.

***Statistical analysis.*** The statistics package "Statistica" (Basic Academic Bundle V14, TIBCO Software Inc., Palo Alto, CA, U.S.A.) was used to carry out generalized mixed linear models (repeated measure) analyses following checks for normality and homogeneity of variance. For thalli stressed in both the hydrated and desiccated states, four sets of comparisons were made using subsets of our data.

**Table 1.** Light intensities and times of exposure used to induce photoinhibition. Lichens with and without lichen substances received the same exposure times and intensities.

| | Light intensity and duration | |
|---|---|---|
| **Species** | **Hydrated** | **Desiccated** |
| *Ramalina celastri* | 600 μmol $m^2$ $s^{-1}$ for 6 h | 1500 μmol $m^2$ $s^{-1}$ for 50 h |
| *Xanthoparmelia conspersa* | 850 μmol $m^2$ $s^{-1}$ for 6 h | 2000 μmol $m^2$ $s^{-1}$ for 70 h |
| *Parmotrema perlata* | 700 μmol $m^2$ $s^{-1}$ for 5 h | 1800 μmol $m^2$ $s^{-1}$ for 18 h |
| *Usnea undulata* | 750 μmol $m^2$ $s^{-1}$ for 5 h | 1000 μmol $m^2$ $s^{-1}$ for 45 h |

First, we tested whether collection site (sunny or shaded) effects the sensitivity of $F_V/F_M$ and rETR in photobionts exposed to a photoinhibitory light stress. Second and third, we separately tested whether the presence or absence of lichen substances effects the sensitivity of both sun and shade collections of thalli to light stress. Finally, we tested whether collection site (sunny or shaded) effects the sensitivity of thalli to light stress when lichen substances have been removed.

## Results

***Tolerance of PSII activity to photoinhibition in sun compared with shade collections.*** Much longer exposure times and higher light intensities were needed to photo-inhibit the desiccated compared with the hydrated lichens (**Table 1**). When lichen substances were present (i.e., there was no acetone rinsing), for both hydrated and desiccated lichens, the tolerance of PSII activity to high light in sun collections was always significantly greater than that of shade collections (**Figs. 1, 2**; **Table 2**). Following extraction of lichen substance using acetone, again for both hydrated and desiccated lichens, the tolerance of PSII activity to high light in sun collections was usually significantly greater than that of shade collections for at least one parameter (**Figs. 1, 2**; **Table 2**). The only exceptions here were for hydrated material of *Ramalina celastri* and desiccated material of *Usnea undulata*.

***Effect of lichen substance removal on tolerance of PSII activity to photoinhibition in sun compared with shade collections.*** In hydrated material of sun collections, removal of lichen substances increased sensitivity to photoinhibition for at least one parameter in all species except *Ramalina celastri* (**Fig. 1**; **Table 2**). By contrast, in hydrated shade collections, removal had no significant effect on $F_V/F_M$ or rETR for any species. Differences between sun and shade collections were smaller when material was photoinhibited in the desiccated state. Removal of lichen substances significantly increased sensitivity to photoinhibition in desiccated sun collections of *Usnea undulata* and *R. celastri*. However, in desiccated shade collections, as for sun collections, removal had a significant effect on $F_V/F_M$ and rETR in *U. undulata* and rETR for *R. celastri*.

## Discussion

Photosynthetic organisms protect themselves from the stress of high PAR using mechanisms that can be divided into those that work mainly to guard against short term fluctuations and those that are found following longer-term exposure to high light (Shi et al. 2022). The present study focused on longer-term adaptations by comparing sun and shade populations of the same species. In lichens, long-term exposure to high light can induce the mycobiont to synthesize secondary metabolites in the upper cortex (Solhaug & Gauslaa 2012), and these metabolites, even if colorless, can protect photobionts against high PAR (Ndhlovu et al. 2022; Solhaug et al. 2010). Other adaptations to sun and shade have been less studied in lichens. Here we tested the relative importance of cortical screening pigments with other more biochemical adaptations. Results from the present study clearly show that in four Afromontane lichens, thalli growing in sun locations have higher tolerance to photoinhibition than those that grow in more shaded microhabitats. Furthermore, particularly when lichens are hydrated, substances appear to play a greater role in photoprotection in sun than shade collections. Interestingly, after removing lichen substances, sun collections still generally possess higher tolerance to photoinhibition. It seems likely therefore that the additional tolerance to photoinhibition found in

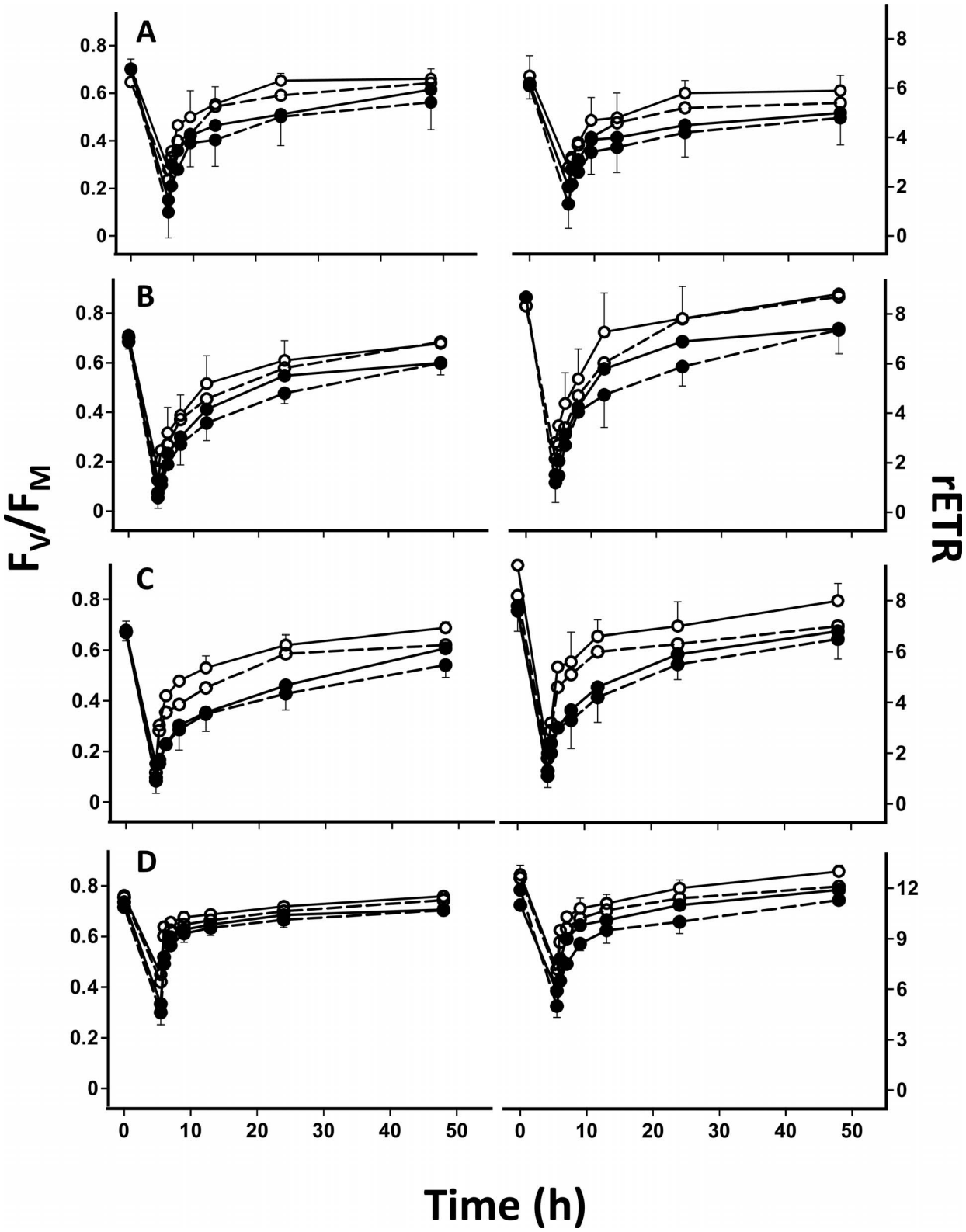


**Figure 1.** The effect of acetone rinsing on the maximal efficiency of PSII ($F_V/F_M$ – left) and relative electron transport rate (rETR – right) in hydrated material of sun (open circles) and shaded populations (closed) of four lichens. **A.** *Ramalina celastri*; **B.** *Parmotrema perlata*; **C.** *Usnea undulata*; **D.** *Xanthoparmelia conspersa*. Solid lines indicate thalli with lichen substances present, while dashed lines indicate thalli with lichen substances removed. Vertical error bars indicate the standard error of the mean, n = 10.

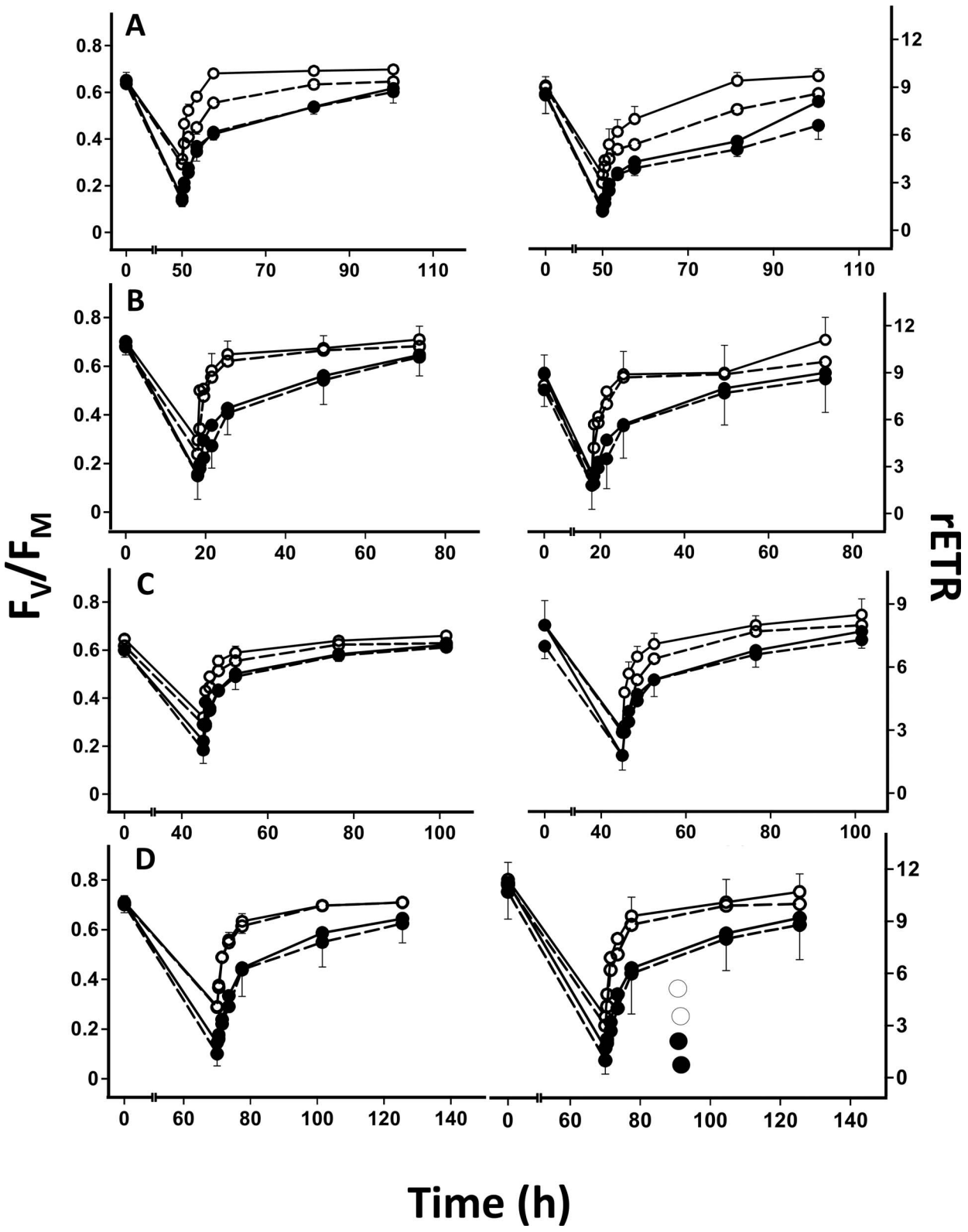


**Figure 2.** The effect of acetone rinsing on the maximal efficiency of PSII ($F_V/F_M$ – left) and relative electron transport rate (rETR – right) in desiccated material of sun (open circles) and shaded populations (closed) four lichens. **A.** *Ramalina celastri*; **B.** *Parmotrema perlata*; **C.** *Usnea undulata*; **D.** *Xanthoparmelia conspersa*. Solid lines indicate thalli with lichen substances present, while dashed lines indicate thalli with lichen substances removed. Vertical error bars indicate the standard error of the mean, n = 10.

**Table 2.** Statistical analyses (generalized mixed linear models, repeated measure) of the effects the presence or absence of lichen substances on the sensitivity to photoinhibition in sun and shade collections of hydrated and desiccated thalli of *Ramalina celastri*, *Parmotrema perlata*, *Usnea undulata* and *Xanthoparmelia conspersa*. For all comparisons, the error had 126 degrees of freedom. Significance: * = P < 0.05, ** = P < 0.01, *** P < 0.001.

| Comparison | Thallus hydration state | *Ramalina celastri* | | *Parmotrema perlata* | | *Usnea undulata* | | *Xanthoparmelia conspersa* | |
|---|---|---|---|---|---|---|---|---|---|
| | | $F_V/F_M$ | rETR | $F_V/F_M$ | rETR | $F_V/F_M$ | rETR | $F_V/F_M$ | rETR |
| Sun/shade with substances present | Hydrated | *** | ** | ** | *** | *** | *** | *** | *** |
| | Desiccated | *** | *** | *** | * | * | 0.367 | *** | *** |
| With/without substances in sun collections | Hydrated | 0.543 | 0.825 | 0.590 | * | *** | * | * | ** |
| | Desiccated | *** | *** | 0.239 | 0.815 | *** | ** | 0.767 | 0.246 |
| With/without substances in shade collections | Hydrated | 0.452 | 0.409 | 0.247 | 0.419 | 0.283 | 0.710 | 0.528 | 0.391 |
| | Desiccated | 0.711 | * | 0.638 | 0.639 | *** | *** | 0.527 | 0.617 |
| Sun/shade with substances removed | Hydrated | 0.264 | 0.194 | * | 0.090 | *** | ** | *** | *** |
| | Desiccated | *** | *** | *** | ** | 0.470 | 0.079 | *** | *** |

sun collections derives from a combination of both lichen substances and other tolerance mechanisms.

***Collections of lichens from sunny microhabitats are more tolerant to photoinhibition than those from shaded microhabitats.*** Differences in the tolerance to photoinhibition of sun and shade collections of lichens has been surprisingly little studied since the early work of Kershaw & MacFarlane (1980). Since that study, our understanding of the tolerance mechanisms displayed by photosynthetic organisms has greatly expanded (Shi et al. 2022). Here we show that for all four species tested, sun populations are more tolerant to photoinhibition than shade, whether photoinhibited in the hydrated (**Fig. 1**) or desiccated states (**Fig. 2**). While the lichens used here are more tolerant to high light stress when desiccated than hydrated (**Table 1**), as has been reported earlier for other species (Mafole et al. 2019a), given sufficient PAR they nevertheless can become inhibited. The precise mechanism is unclear, but in bryophytes, desiccation does not stop the transfer of excitation energy from the light-harvesting pigments to the reaction centres (Heber et al. 2006). Even if light only causes the formation of tiny amounts of ROS in desiccated thalli, normal repair processes do not take place (Buffoni Hall et al. 2003). Enzyme reactions are severely restricted by the 'rubbery' cytoplasmic states that occur at the onset of desiccation and are totally restricted in the glassy cytoplasmic states that are found in air-desiccated lichens during the day (Fernandez-Marin et al. 2013). Irrespective of the mechanisms involved, collections of Afromontane lichens from sunny habitats are more tolerant to photoinhibition than thalli from the same species collected in the shade.

***Removal of lichen substances in sun collections increases sensitivity to photoinhibition.*** In sun collections of lichen thalli, removal of lichen substances generally increases the sensitivity of the photobionts to reductions in PSII activity, as assessed by changes in at least one parameter ($F_V/F_M$ or rETR), whether thalli are stressed hydrated or desiccated (**Figs. 1, 2**; **Table 2**). The exceptions here were hydrated *Ramalina celastri* and desiccated *Parmotrema perlata* and *Xanthoparmelia conspersa*. As discussed in the Introduction, for hydrated thalli, the presence of lichen substances can increase tolerance to photoinhibition by increasing thallus reflectance (Ndhlovu et al. 2022; Solhaug et al. 2010). It is more difficult to explain how lichen substances improve tolerance to photoinhibition in desiccated lichens, as substance removal has little effect on reflectance when thalli are dry (Ndhlovu et al. 2022; Solhaug et al. 2010). Possibly, while not increasing reflectance, they may help to screen photobionts by reducing transmission. Alternatively, lichen substances can have very high antioxidant activity (Fernández-Moriano et al. 2016; Kosanić et al. 2011) and may scavenge ROS produced by photobiont chloroplasts. Interestingly, we found earlier that in hydrated sun collections of *R. celastri* the presence of lichen substances reduces photoinhibition in hydrated (Ndhlovu et al. 2022). While sun collections of *R. celastri* were clearly more tolerant to photoinhibition than shade collections (**Fig. 1A**; **Table 2**), for hydrated material of this species, lichen substances seemed to be not involved. It may be relevant that Ndhlovu et al. (2022) used

*Ramalina* collected c. 80 km from the thalli used in the present study. High PAR is not the only driver of the synthesis of cortical lichen substances. For example, Gauslaa et al. (2013) showed that in *Lobaria pulmonaria* herbivory, rather than light exposure, was the main determinant of cortical usnic acid levels. However, even for *R. celastri*, the presence of lichen substances improved the tolerance of desiccated thalli to photoinhibition (**Fig. 2A**; **Table 2**). Thus, while lichen substances play a variety of roles in lichen biology, one of the most important seems to be that of protecting photobionts against high light stress.

***Removal of lichen substances has less effect on sensitivity to photoinhibition in shade than sun collections.*** Compared with sun collections, lichen substance removal from thalli collected from shaded microhabitats has less effect on photobiont sensitivity to photoinhibition (**Fig. 2**; **Table 2**). Removal only has a significant effect in desiccated thalli of *Ramalina celastri* (rETR) and *Usnea undulata* ($F_V/F_M$ and rETR). As discussed in the Introduction, there are several reports that the levels of lichen substances can approximately track changes in light availability (Solhaug & Gauslaa 2012). Assuming higher PAR induces the synthesis of cortical substances in the lichens used here, sun collections probably contain higher concentrations than shade collections. Therefore, it is not surprising that substance removal has more effect on sensitivity to photoinhibition in sun than shade collections; lichens growing in shaded microhabitats seem to depend less on lichen substances for photoprotection. The "shade" lichens used here were collected from the trunks of trees or on rocks under a tree canopy. In such microhabitats, lichens are exposed to rapidly changing light levels because gaps in the canopy vary depending on diurnal variations in the angle of sunlight, tree architecture and movement of the tree branches. The relatively brief periods that lichens are exposed to high light levels are known as 'sunflecks.' Sunflecks probably present a real hazard to lichen photobionts, and in theory cortical light screening substances could protect photobionts against them. However, as even unpigmented substances increase thallus reflectance (Solhaug et al. 2010), cortical pigments may also reduce photosynthesis during the lower light levels available after a sunfleck has passed. Mkhize et al. (2022) recently showed that shade collections of lichens have NPQ that is higher and more rapidly inducing and relaxing compared with sun collections of the same species. It seems likely that NPQ together with other biochemical mechanisms, are more energetically efficient ways of protecting shade lichens against sunflecks. Results presented here suggest that cortical substances are less important in the photoprotection of shade than sun collections of lichens.

***After removal of lichen substances sun collections of lichens are still more tolerant to photoinhibition than shade collections.*** Even following the removal of lichen substances, the photobionts of sun collections of lichens still have greater resistance to photoinhibition than those from the shade for at least one parameter (**Figs. 1, 2**; **Table 2**), the only exceptions being for hydrated *Ramalina celastri* and desiccated *Usnea undulata*. The implication is that, in addition to synthesising lichen substances, sun collections use other mechanisms to tolerate photoinhibition. Results presented here are similar to those obtained by Beckett et al. (2019), that compared the resistance to photoinhibition in melanized and pale thalli of *Cetraria islandica*. In that study, lichens were photoinhibited without any influence of a melanized upper cortex by removing the lower cortices and medullas and exposing the photobionts to light from below. Results showed that the photobionts of melanized thalli possess significantly higher tolerance to photoinhibition than those from pale thalli. Further work is needed to determine the nature of these mechanisms. However, sun collections of a range of species have higher $rETR_{MAX}$ than shade collections (Mkhize et al. 2022; Piccotto & Tretiach 2010); using more light energy in photophosphorylation will reduce the excess available for ROS formation. As discussed above, for higher plants, adaptations include modifications in chloroplast architecture, and changes in the activities of ROS scavenging enzymes, cyclic electron flow and the PSII repair cycle (Shi 2022). Future studies therefore need to focus on which additional ultrastructural and biochemical tolerance mechanisms are present in sun lichens.

## Conclusion

The present study investigated tolerance to photoinhibition in four Afromontane lichens. All species have trebouxioid photobionts and normally

grow in more exposed microhabitats but can also be readily collected from more shaded locations. Results showed that for all species, collections from the sunny microhabitats are more tolerant to photoinhibition in the hydrated or desiccated states than collections from shaded microhabitats. While removal of lichen substances generally increases the sensitivity of the photobionts to photoinhibition, removal increases sensitivity significantly more in sun than shade collections. However, even after the removal of lichen substances, sun collections remain more tolerant to photoinhibition than shade collections. In future, more detailed studies need to be carried out to elucidate the biochemical basis of the additional tolerance. However, the main conclusion of the present work is that the additional tolerance to high light in the photobionts of lichens from exposed sites usually derives from a combination of both light screening cortical substances and other tolerance mechanisms.

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