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ORIGINAL ARTICLE

Apparent electron transport rate – a non-invasive proxy of photosynthetic CO_2 uptake in lichens

Knut Asbjørn Solhaug[1] · Johan Asplund[1] · Yngvar Gauslaa[1]

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Abstract

Main conclusion **During desiccation, both apparent electron transport rate (ETR_{app}) and photosynthetic CO_2 uptake peak when external water has evaporated. External water, causing suprasaturation, weakens the strong correlation between ETR_{app} and CO_2 uptake.**

Abstract Lichens are poikilohydric organisms passively regulated by ambient conditions. In theory, apparent electron transport rate (ETR_{app}), estimated by photosystem II yield measured in light (Φ_{PSII}), is a proxy of photosynthetic CO_2 uptake. Hydration level, however, is a complicating factor, particularly during suprasaturation that strongly reduces CO_2 diffusion. Here, the cephalolichen *Lobaria pulmonaria* and two chlorolichens *Parmelia sulcata* and *Xanthoria aureola* were excessively hydrated before photosynthetic CO_2 uptake and Φ_{PSII} using imaging fluorescence tools were simultaneously measured while drying at 200 µmol photons m^{-2} s^{-1}. CO_2 uptake peaked when hydration had declined to a level equivalent to their respective internal water holding capacity ($WHC_{internal}$) i.e., the water per thallus area after blotting external water. CO_2 uptake and ETR_{app} in all species were highly correlated at hydration levels below $WHC_{internal}$, but weaker at higher hydration (chlorolichens) or absent (cephalolichen). Yet, at a specimen level for the two chlorolichens, the correlation was strong during suprasaturation. The CO_2 uptake—ETR_{app} relationship did not differ between measured species, but may vary between other lichens because the slope depends on cortical transmittance and fraction of electrons not used for CO_2 uptake. For new lichen species, calibration of ETR_{app} against CO_2 uptake is therefore necessary. At intrathalline scales, Φ_{PSII} during drying initially increased along thallus margins before reaching maximum values in central portions when hydration approached $WHC_{internal}$. $WHC_{internal}$ represents the optimal hydration level for lichen photosynthesis. In conclusion, ETR_{app} is an easily measured and reliable proxy of CO_2 uptake in thalli without external water but overestimates photosynthesis during suprasaturation.

Keywords Desiccation · Imaging chlorophyll fluorescence · Infrared gas analysis · Suprasaturation · Water holding capacity

Abbreviations

ETR_{app}	Apparent electron transport rate
ETR	Electron transport rate
A_{max}	Maximum CO_2 uptake, µmol CO_2 m^{-2} s^{-1}
Φ_{PSII}	Photosystem II quantum yield in light
STM	Specific thallus mass, mg DM cm^{-2}
$WHC_{internal}$	Water holding capacity after gentle blotting with drying paper, mg H_2O cm^{-2}
WHC_{total}	Water holding capacity after gentle shaking, mg H_2O cm^{-2}
$WHC_{external}$	WHC_{total}–$WHC_{internal}$, mg H_2O cm^{-2}
$WC_{internal}$	Water content after gentle blotting with drying paper, percent

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✉ Knut Asbjørn Solhaug
knut.solhaug@nmbu.no

[1] Faculty of Environmental Sciences and Natural Resource Management, Norwegian University of Life Sciences, P.O. Box 5003, NO-1432 Ås, Norway

Introduction

Photosynthesis in lichens depends on external hydration sources because rain, dew, and humid air passively regulate their water content. In chloro- and cephalolichens, photosynthetic processes can be activated by humid air only

(Phinney et al. 2019a, b), whereas cyanolichens, in general, require liquid water for activation of CO_2 uptake (Lange et al. 1993b). However, excess water causing suprasaturation (Lange 2003) often limits photosynthetic CO_2 uptake in rainy periods because CO_2 diffuses slowly in water (Lange et al. 1993a). Suprasaturation is evidenced as reduced CO_2 uptake rates measured by an infrared gas analyzer (IRGA).

Chlorophyll fluorescence imaging is a powerful measuring tool that rapidly quantifies intrathalline variation and kinetics of photosynthetic process during desiccation cycles. Yet it is unclear if chlorophyll fluorescence can be used as a proxy for CO_2 uptake for lichens at all hydration levels. If the fraction of incident light absorbed by the photosynthetic apparatus is known, photosynthetic electron transport (ETR) can be estimated by the equation $ETR = \Phi_{PSII} \times PAR \times 0.5 \times Abs$ (Baker 2008). Here, Φ_{PSII} is the effective quantum yield of PS II in light measured by chlorophyll fluorescence, PAR is incident photosynthetic active irradiation, 0.5 is a factor assuming equal absorption of light in PSI and PSII, and Abs is the fraction of incident light absorbed by the photosystems. Because electrons are also used for other reactions than the fixation of CO_2 (Baker 2008), ETR should be an inaccurate proxy for photosynthesis. However, for the chlorolichen *Lecanora muralis*, CO_2 uptake and ETR correlated well during field days when suprasaturation did not occur and correlated poorly during days with heavy rain causing excess hydration (Leisner et al. 1997; Lange et al. 2001). In rainy weather, ETR largely overestimated the CO_2 uptake. The uncertainties by using ETR as a proxy for photosynthetic CO_2 uptake should be greater in lichens than in plants not only because of their highly variable colored cortical light-screening pigments and a concurring variation in the Abs parameter (Solhaug et al. 2010; Phinney et al. 2019a, b), but also because of varying hydration levels. The relationship between ETR and CO_2 uptake in lichens is more variable and complex than in higher plants (Green et al. 1998). Despite the uncertainties of chlorophyll fluorescence as a proxy for lichen photosynthesis, it has been widely used because measurements are simple, fast, and non-invasive. One important aim of this study is to quantify the relationships between ETR and CO_2 uptake for three ecologically different foliose lichen species during specific stages of desiccation cycles.

While comparing IRGA and ETR or Φ_{PSII} measurements in lichens, one complication is that IRGA averages CO_2 uptake for the whole thallus, whereas traditional chlorophyll fluorescence tools using fiber optics only measure the signal from a small portion of the lichen. Imaging fluorescence tools can measure entire thalli, but unfortunately, the signals from the photobiont are only recorded from the portions that still have detectable PSII activity, not from those that already have dried and become inactive. When desiccation starts to form dry portions of a lichen thallus, the fluorescence signal is restricted to portions that still are moist, thus overestimating Φ_{PSII} at a thallus level. Using imaging fluorescence, another aim is to find a new modified calculation protocol of Φ_{PSII} that includes signals from both the active wet and the inactive dry portions of each thallus. Such protocols are needed for reliable comparisons of Φ_{PSII} and CO_2 uptake.

The strong spatial heterogeneity in PSII activity during changed hydration in lichen thalli (Gauslaa et al. 2017; Phinney et al. 2018) is not accounted for by gas exchange measurements. We hypothesize that Φ_{PSII} measured with an imaging chlorophyll fluorescence instrument can detect also the spatial intrathalline variation of suprasaturation on lichen photosynthesis. We aim to test this hypothesis by studying desiccation kinetics with chlorophyll fluorescence imaging of three foliose lichens with different hydration traits.

When measuring and comparing lichen photosynthesis in the laboratory, it is essential to know how to identify the optimal water content in lichens to achieve and compare maximal photosynthesis rates. We hypothesize that the external, not the internal water inside the lichen, limits CO_2 diffusion during suprasaturation. To test this, we simultaneously measured photosynthetic CO_2 uptake and water content during desiccation and additionally recorded the water holding capacity after blotting ($WHC_{internal}$) for these thalli. At hydration levels equivalent to $WHC_{internal}$, the external water is removed, whereas the internal water is retained. By comparing $WHC_{internal}$ and the water content per thallus area at maximum CO_2 uptake rates within and across species, we aim to test whether the external rather than internal water in lichens reduces the photosynthesis at suprasaturation.

Materials and methods

Lichen material

The old forest cephalolichen *Lobaria pulmonaria* was collected on trunks of oak trees in old oak forests at Langangen, SE Norway (59°06′43″ N, 9°50′05″ E, 150 m.a.s.l.). The two chlorolichens inhabiting more sun-exposed sites, *Xanthoria aureola* and *Parmelia sulcata*, were collected from (1) open seashore cliffs in Kristiansand, S Norway (58°7′25″ 8°9′37″; 1–3 m.a.s.l.) and (2) trunks of young solitary *Tilia cordata* Mill. trees along a small farm road in open agricultural land in Ås, SE Norway (59°40′N, 10°45′E; 100–150 m.a.s.l), respectively. All thalli were collected in June 2019, and they were stored in the fridge a few days until the start of the experiments.

Pretreatment

Five similar-sized thalli of each species were used for each experiment. The thalli were moistened by spraying

deionized water, followed by 24 h acclimation at 5 µmol photons m^{-2} s^{-1} from fluorescent tubes at 15 °C on wet filter paper in Petri dishes with lid (Solhaug 2018). Thallus area while moist was measured with a leaf area meter (LI-6100, LiCor, Lincoln, NE, USA).

Gas-exchange measurements

Photosynthetic CO_2 uptake was measured with a portable infrared gas-analyzer (LI-6400XT, LiCor) with a LiCor 6400–24 bryophyte chamber and a LI-6400–18 RGB LED light source. Measurements were done at 20 °C with 200 µmol red photons m^{-2} s^{-1} and 400 ppm CO_2 with a flow rate of 500 ml min^{-1}. Stable photosynthetic rates were recorded after 2 min.

Chlorophyll *a* fluorescence

Chlorophyll *a* fluorescence was measured with a red LED Imaging-PAM M-series chlorophyll fluorometer (Heinz Walz GmbH, Effeltrich, Germany). Operating efficiency of PSII (Φ_{PSII}) was measured with a saturating pulse of 2800 µmol red photons m^{-2} s^{-1} given to thalli under 200 µmol red photons m^{-2} s^{-1} from the fluorometer LED panel. Apparent electron transport was estimated with the equation $ETR_{app} = \Phi_{PSII} \times 200 \times 0.5$ where 200 is the PAR level used and 0.5 indicates the equal distribution of photons between PSII and PSI.

Experimental protocol and hydration traits

To measure hydration traits, moist thalli were sprayed with excess water and gently shaken to remove the excess surface water that normally would drain from thalli on sloping surfaces in situ. Thalli were then weighed for determination of wet mass ($WM_{shaking}$), and gently blotted by drying paper to remove external water before recording $WM_{blotting}$. At the end of all experiments, thalli were dried at 70 °C until the next day for determination of dry mass (DM). Constant weight was reached within a few hours. Specific thallus mass (STM) was computed as DM/area The water holding capacity after shaking ($WHC_{shaking} = WHC_{total}$) and after blotting ($WHC_{blotting} = WHC_{internal}$) was computed using $WM_{shaking}$ and $WM_{blotting}$, respectively, as WM in the equation WHC = (WM-DM)/area. The external water was computed by $WHC_{external} = WHC_{total} - WHC_{internal}$. The water content (WC) in percent (WC_{DM}) or per thallus area (WC_{area}) refers to the water content at any time during the desiccation cycle.

Before gas-exchange and fluorescence measurements, thalli were again sprayed with excess water. Each thallus was placed in the gas exchange chamber at 200 µmol red photons m^{-2} s^{-1}, and stable CO_2 exchange values were recorded after 2 min. The thallus was removed from the cuvette, weighed, and after a few seconds placed under 200 µmol red photons m^{-2} s^{-1} from the fluorometer LED panel. After 1 min, a saturating pulse of 2800 µmol red photons m^{-2} s^{-1} was applied for the determination of Φ_{PSII}. This protocol was repeated approximately 20–40 times until the thalli were dry. The experiments were stopped when CO_2 uptake and Φ_{PSII} had been zero for a couple of measurements.

Estimation of average Φ_{PSII}

We used an improved protocol for imaging chlorophyll fluorescence data analysis that accounts for the spatial inactivation in lichen thalli during natural desiccation cycles. The chlorophyll fluorescence data were analyzed with the ImagingWin v2.46i (Heinz Walz GmbH) software. First, the number of F_m' pixels for each wet thallus was determined to estimate the total thallus area. Then, the number of pixels for each 0.05 Φ_{PSII} interval was determined, and these numbers of pixels for each interval were multiplied with the mean Φ_{PSII} for the corresponding interval. Finally, all these products were summed and divided by the total number of F_m' pixels for the wet thallus. This procedure was necessary to estimate the average Φ_{PSII} for the entire thallus because the automatic Φ_{PSII} calculation by the ImagingWin software excludes inactive pixels when dry portions start to form.

Statistical analysis

Species-specific differences in optimal water content were analyzed by one-way ANOVA after testing for equal variances by Levene's test followed by Tukey multiple comparisons test. Figures were done in Sigmaplot 14.0 (Figs. 3 and 5) and in R 4.0.2 (Figs. 2 and 4). We analyzed responses of photosynthetic CO_2 uptake and ETR versus water contents with third-order polynomial linear mixed models (Harrison et al. 2018) using species (categorical variable), water content (numerical variable), and their interaction as fixed factors, and thallus ID as a random factor. For the relationship between photosynthetic CO_2 uptake and ETR_{app} during drying cycles, we analyzed the responses below and above the optimum water content by separate linear mixed models. In these models, ETR_{app}, species, and their interaction were fixed factors, whereas thallus ID was a random factor. All mixed models were run in Jamovi 1.6.3. The linear mixed models were visualized using ggpredict from the R package ggeffects (Lüdecke 2018). We evaluated the model performance by computing the $R^2_{Marginal}$, representing the variance explained by the fixed factors only, and the $R^2_{Conditional}$, representing the variance of the entire model combining fixed and random effects (Nakagawa and Schielzeth 2013; Johnson 2014).

Results

Intrathalline variation in Φ_{PSII}

At an intrathalline spatial resolution (Fig. 1), *P. sulcata* had evenly distributed and slightly depressed Φ_{PSII} when suprasaturated. During initial desiccation, Φ_{PSII} increased along the thallus margins, and when the water contents reached $WHC_{internal}$, Φ_{PSII} was high across the entire thallus (Fig. 1). *Xanthoria aureola* exhibited similar, but less pronounced spatial patterns (Fig. 1). While suprasaturated, *L. pulmonaria* showed a complex variation in Φ_{PSII}, but this spatial variation declined when the water content approached $WHC_{internal}$. Regardless of its water content, *L. pulmonaria* had the highest Φ_{PSII} in locally shaded portions of the many depressions in its reticulated surface (Fig. 1). After further desiccation, all species exhibited a spatially sharp decline in Φ_{PSII} from the thallus edges towards the central part, showing that the Φ_{PSII} in central parts remained high until the entire thallus was almost dry. The intrathalline variation in suprasaturation was particularly clear for *P. sulcata* in which the central part of the thallus needed longer desiccation time than marginal parts to reach optimal water content for photosynthesis.

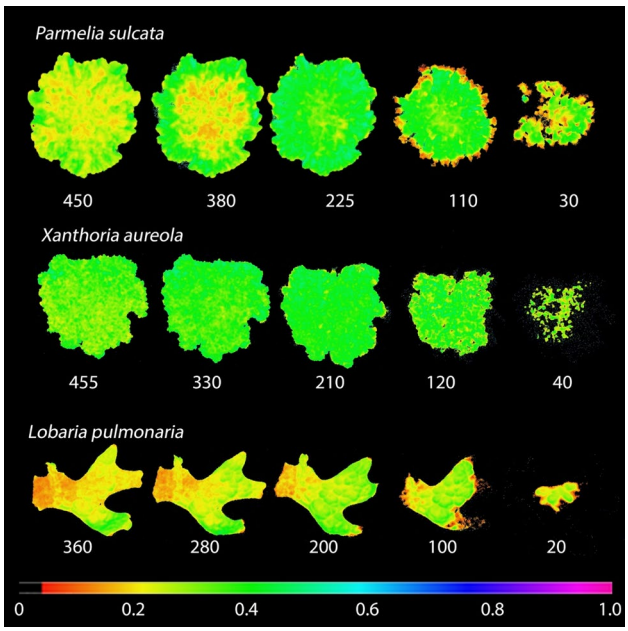

Fig. 1 Spatial variation of operating efficiency of photosystem II (Φ_{PSII}) during desiccation of one typical lichen thalli for each species. The numbers below the thalli show percent water content. The water contents of 225, 210 and 200 for *Parmelia sulcata*, *Xanthoria aureola* and *Lobaria pulmonaria,* respectively, represent the water contents at maximal photosynthetic CO_2 uptake

Hydration level and photosynthetic responses

At full start-up hydration, all three lichen species had low photosynthetic CO_2 uptake rates. After the subsequent desiccation, uptake rates increased until the optimal water content was achieved (Fig. 2a). Similar responses occurred for ETR_{app} in the two chlorolichens, whereas hardly any depression occurred at high water contents for the cephalolichen *L. pulmonaria* (Fig. 2b). In the third-order polynomial mixed effect models for CO_2 uptake and ETR_{app}, respectively, the fixed factors water content and species accounted for much of the variation ($R^2_{Marginal} = 0.879$) in these photosynthetic responses (Table S1). The random factor thallus ID accounted for a higher proportion of the variation in the CO_2 uptake model compared to the ETR_{app} model, evidenced by somewhat higher $R^2_{Conditional}$ for the CO_2 uptake (0.946) than for the ETR_{app} (0.916). All species responded differently

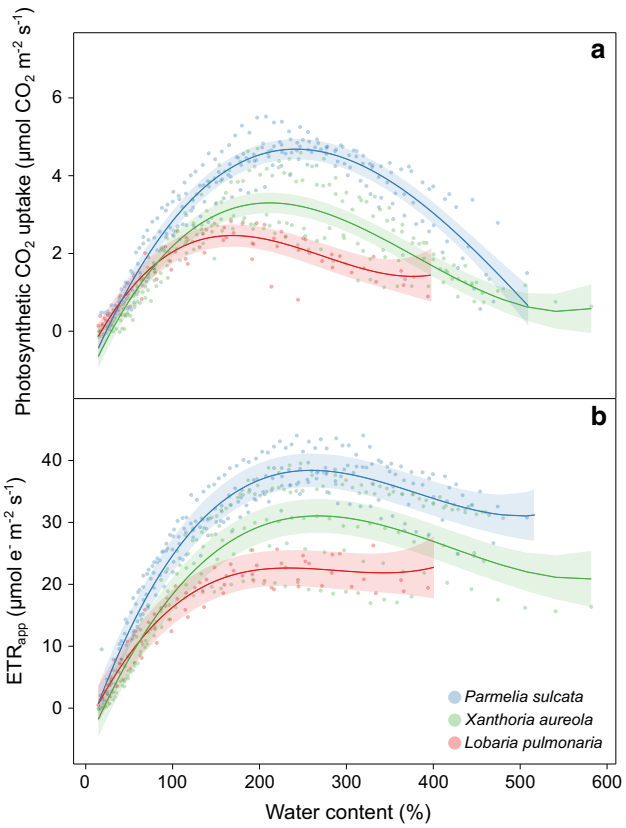

Fig. 2 Predicted relationships between the simultaneous measurement of **a** photosynthetic CO_2 uptake and **b** apparent electron transport rate (ETR_{app}) versus thallus water content as a third order polynomial mixed-effects model for *Xanthoria aureola, Parmelia sulcata* and *Lobaria pulmonaria* during desiccation cycles. These relationships were based on a mixed model, using thallus ID as a random factor. The semi-transparent bands represent the 95% confidence intervals. The mixed model is shown in Table S1. Five individual thalli of each species were measured

(Table S1), and the ranking of species was similar for CO_2 uptake and for ETR_{app} (Fig. 2).

At maximum photosynthetic CO_2 uptake rates, the water content in percent (WC_{DM}) was higher for *P. sulcata* than for *L. pulmonaria* and *X. aureola*, whereas the water content per thallus area (WC_{area}) was high in the chlorolichens *P. sulcata* and *X. aureola* (0.50 and 0.53 mm, respectively) and low in the cephalolichen *L. pulmonaria* (0.22 mm; Table 1). WC_{area} at maximum photosynthetic CO_2 uptake corresponded well to $WHC_{internal}$ for all three species (Fig. 3; Table 1). The water holding capacity after gentle shaking (WHC_{total}) was much higher for *X. aureola* and *P. sulcata* than for *L. pulmonaria* (Table 1) resulting in much faster desiccation of *L. pulmonaria*. Both the internal and external water pools were much larger in the two chlorolichens from open habitats than in the old forest cephalolichen (Table 1).

The relationship between ETR_{app} and photosynthetic CO_2 uptake

At excess hydration (suprasaturation), ETR_{app} was less reduced than photosynthetic CO_2 uptake, whereas both parameters declined to zero with increasing desiccation below the optimum water content (Fig. 2a, b). According to the linear mixed models, apparent electron transport rate (ETR_{app}) correlated better with photosynthetic CO_2 uptake at water contents below the optimum for CO_2 uptake ($R^2_{Conditional} = 0.980$; Fig. 4a; Table S3) than above optimal hydration ($R^2_{Conditional} = 0.942$; Fig. 4b; Table S4). However, the random factor thallus ID had a much higher impact on the model above the optimal water content than below, evidenced by the lower $R^2_{Marginal} = 0.675$ (Table S4) above than below ($R^2_{Marginal} = 0.948$; Table S3) the optimum water content. Species as the main factor was not significant for the CO_2 uptake—ETR_{app} relationship regardless of hydration level (Table S3 and S4), but below the optimum water content, the species * ETR_{app} interaction was significant (Table S3; Fig. 4a), implying different CO_2 uptake—ETR_{app} relationships for *L. pulmonaria* and *X. aureola*. For

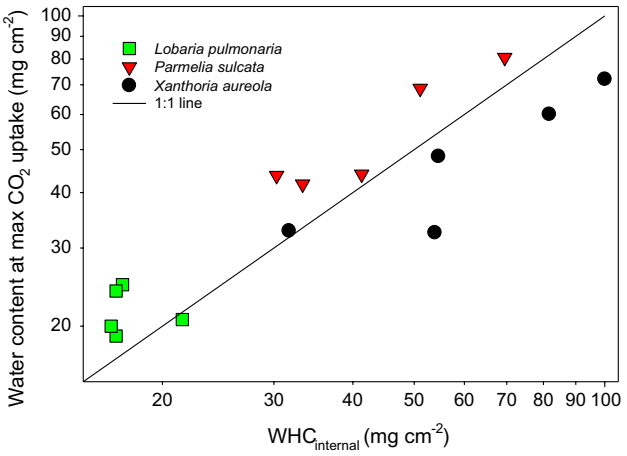

Fig. 3 Water content (WC_{area}) at max CO_2 uptake plotted against water holding capacity ($WHC_{internal}$) after blotting. A line showing equal values is shown

L. pulmonaria, the regression was only significantly below the optimal water content (Fig. 4a), not above (Fig. 4b).

During drying at suprasaturation, the relationship between ETR_{app} and CO_2 uptake for individual thalli of the two chlorolichens (Fig. 5a, b) was much stronger than when specimens from a given species were pooled (Fig. 4b). By contrast, no cephalolichen specimens had significant positive relationships between ETR_{app} and CO_2 uptake (Fig. 5c). At hydration levels below the optimum water content, photosynthetic CO_2 uptake can be well estimated from ETR_{app} values. At suprasaturating water content, ETR_{app} is still a reliable proxy for photosynthetic CO_2 uptake at a specimen-specific level, but for the chlorolichens only.

Table 1 Specific thallus mass (STM), external ($WHC_{external}$), internal water holding capacity ($WHC_{internal}$), total water holding capacity (WHC_{total}), internal percent water ($WC_{internal}$) as well WC_{DM} and WC_{area} at maximum photosynthetic CO_2 uptake (A_{max})

	Xanthoria aureola	*Parmelia sulcata*	*Lobaria pulmonaria*
Thallus area, cm^2	13.9 ± 1.9	16.9 ± 1.2	11.5 ± 0.5
STM, mg DM cm^{-2}	24.3 ± 3.4^a	21.5 ± 2.2^{ab}	12.8 ± 0.2^b
WHC_{total}, mg H_2O cm^{-2}	116.1 ± 15.8^a	96.6 ± 10.4^a	41.2 ± 3.2^b
$WHC_{external}$, mg H_2O cm^{-2}	51.8 ± 7.6^a	51.5 ± 4.6^a	23.4 ± 3.7^b
$WHC_{internal}$, mg H_2O cm^{-2}	64.4 ± 11.9^a	45.1 ± 7.1^{ab}	17.8 ± 0.9^b
$WC_{internal}$, %	251 ± 16^a	206 ± 11^a	140 ± 9^b
WC_{DM} at A_{max}, %	195 ± 9^a	242 ± 15^b	170 ± 11^a
WC_{area} at A_{max}, mg H_2O cm^{-2}	49.7 ± 7.8^a	52.5 ± 7.1^a	21.7 ± 1.2^b

The numbers show mean values ± SE and $n = 5$. Values with the same small letter are not significantly different (Tukey multiple comparison test)

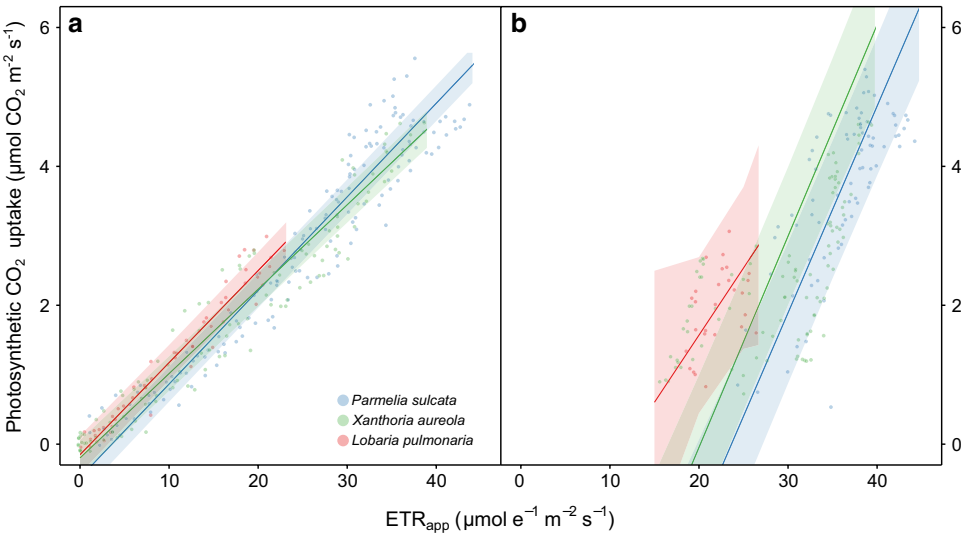

Fig. 4 Predicted linear relationships between apparent electron transport (ETR_{app}) and photosynthetic CO_2 uptake **a** at water contents below the optimum for maximal photosynthesis and **b** at water contents above the optimum for maximal photosynthesis shown as species-wise regressions with 95% confidence intervals. These mixed models (shown in Table S2) used species, ETR_{app} including their interaction as fixed factors, and thallus ID as random factors

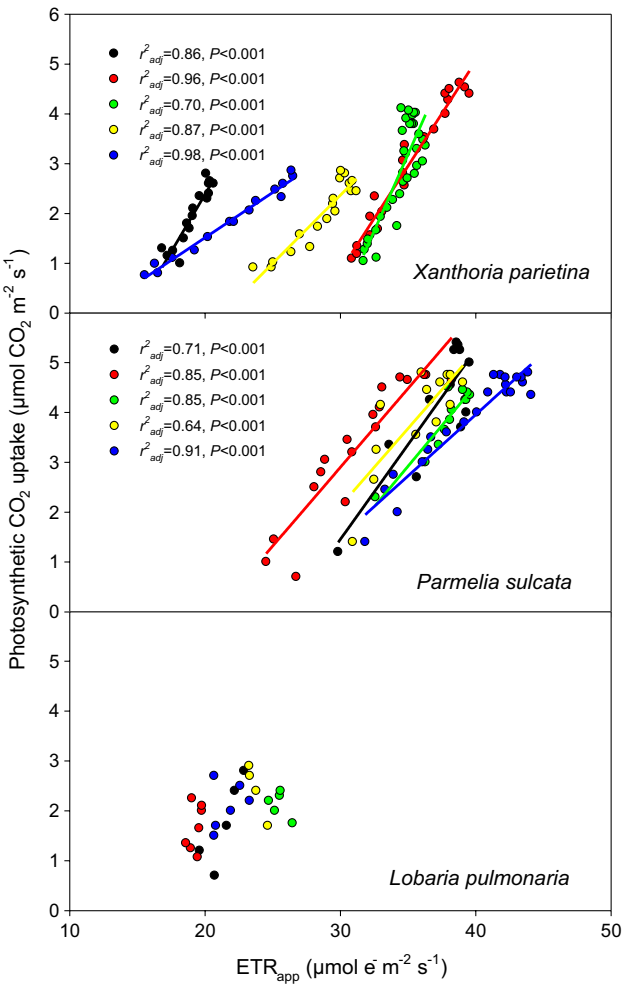

Fig. 5 The relationship between apparent electron transport (ETR_{app}) and photosynthetic CO_2 uptake at water contents above the optimum for maximal photosynthesis (Fig. 4b) for each individual thallus. r^2_{adj} and *P*-values for specimen-specific regression lines are shown. For *Lobaria pulmonaria* with no significant positive regressions, the regression lines are not shown

Discussion

Intrathalline variation in Φ_{PSII}

The imaging chlorophyll fluorometer makes it possible to visualize Φ_{PSII} kinetics during desiccation. In contrast to maximal PSII yield (F_v/F_m) that is not affected by suprasaturation (Solhaug and Gauslaa 1996; Gauslaa and Solhaug 1998), Φ_{PSII} declines with increasing suprasaturation at least in the two chlorolichens. This is because CO_2 diffusion is needed for Φ_{PSII} but not for F_v/F_m. Φ_{PSII} kinetics during desiccation can thus be used to determine the optimum water content in lichens. First, there was an early increase in Φ_{PSII} along the margins that dry rapidly and thus first escape from suprasaturating water contents, followed by increased Φ_{PSII} in central parts that likely better retain the moisture. Narrow marginal portions of the sun-adapted chlorolichens *P. sulcata* and *X. aureola* thalli, especially along young margins, reached blue color at optimal water content (210–225%; Fig. 1) showing that these portions have higher Φ_{PSII} than other portions, and thus presumably higher photosynthetic capacity. By contrast, the shade-adapted cephalolichen *L. pulmonaria*, regardless of its water content, had the highest Φ_{PSII} in locally shaded portions of its many circular depressions in the surface, particularly near lobe ends (Fig. 1) that offer high light protection by curling during drying (Barták et al 2006). Thereby, the spatial variation in Φ_{PSII} has important implications for the use of chlorophyll fluorescence for measurements of lichen photosynthesis and thus for the understanding of lichen functioning.

ETR_{app} as a proxy for photosynthetic CO_2 uptake

The large spatial variation of Φ_{PSII} yield implies that point measurements of Φ_{PSII} yield with a fiber optic fluorometer

strongly depend on where the fiber is placed. The new protocol for the calculation of average Φ_{PSII} allows objective estimates of average ETR_{app} for the entire thallus. If a fiber-optic fluorometer had been used, the fluorescence signal would likely have come mainly from central thallus portions that still had high Φ_{PSII} when margins were dry and inactive (Fig. 1). ETR_{app} is a reliable proxy for lichen CO_2 uptake at water contents below $WHC_{internal}$ (Fig. 4a) when using the revised protocol for the calculation of average Φ_{PSII} for the entire thallus area.

The stronger correlation between ETR_{app} and CO_2 uptake for individual chlorolichen thalli than for the species-specific data can be explained by a high variation in the fraction of electrons used for CO_2 reduction and/or a high variation in the fraction of incident light absorbed by PSII. Particularly *X. aureola* has often old central thallus portions of varying size with visibly less chlorophyll that likely cause high spatial and specimen-specific variation in photosynthesis. The lacking or weak thallus-specific relationships between ETR_{app} and CO_2 uptake for the cephalolichen *L. pulmonaria* likely occurred because ETR_{app} unlike CO_2 uptake hardly declined at high water contents (Fig. 2). At a species level, ETR_{app} is a poor estimator of absolute CO_2 uptake at suprasaturation (Fig. 4b).

Chlorophyll fluorescence tells nothing about dark respiration. In lichens, a large proportion of gross photosynthesis can be lost by dark respiration (Lange and Green 2006). At constant temperature, dark respiration is constant whereas gross photosynthesis increases with light. With changing light, both gross photosynthesis and dark respiration may vary. Therefore, the conversion factors between ETR_{app} and CO_2 uptake described here will be valid for measurements at constant light and temperature levels only. Change in light or temperature may require new calibrations. Nevertheless, the strong correlation between CO_2 uptake and ETR_{app} shows that ETR_{app} may be a good proxy for photosynthesis of lichens at different hydration levels except at suprasaturation. With increasing hydration above $WHC_{internal}$, ETR_{app} decreases much less than does the CO_2 uptake (Fig. 2).

Optimal water content for photosynthesis

All three species had maximal photosynthetic CO_2 uptake at a water content close to their respective $WHC_{internal}$. This shows that external water is the main limiting factor for CO_2 diffusion and thus is the cause of suprasaturation. In thalli saturated with water at hydration levels corresponding to $WHC_{internal}$, the medulla including the photobiont layer still have air-filled spaces outside fungal cell walls because of hydrophobic surfaces (Honegger 1991, 1998; Lange et al. 1997), but with no external film of water on lichen surfaces. The link between $WHC_{internal}$ and maximum CO_2 uptake (Fig. 3) emphasizes not only the ecological importance of $WHC_{internal}$ as a functional hydration trait but also the usefulness of blotting thalli before assessing and comparing the optimal photosynthesis between species and/or treatments. Additionally, this strong link also emphasizes the usefulness of $WHC_{internal}$ studies modelling lichen performance under various hydration regimes.

Although all three species are foliose species with green algae as their dominant photobiont, they have different ecological niches. During rainy periods, there is a higher chance for suprasaturation in the two chlorolichens that have much more external water than the cephalolichen (Table 1). This may explain why *Lobaria pulmonaria*, unlike the two widespread chlorolichens, is associated with rainforest climate (Ellis 2016) where less suprasaturation depression of CO_2 uptake would be a competitive advantage.

Alternative electron sinks

The slopes of the species-wise regression lines between CO_2 uptake and ETR_{app} were rather similar (0.113–0.132). If all incident light is absorbed by the photosystems and no electrons are used in other electron sinks than uptake of CO_2 by the Calvin cycle, the slope should be 0.250 because four electrons are required to reduce one molecule of CO_2. However, some electrons are certainly used for other electron sinks reducing this theoretical slope-level. When ETR is high with low CO_2 uptake at excessive hydration levels, there must be other electron sinks than CO_2 reduction. A possible electron acceptor may be oxygen as discussed by Leisner et al. (1997). A negative effect of using oxygen as an electron acceptor can be oxidative stress when suprasaturated thalli are exposed to high light. In addition, photorespiration may be an important electron sink (Foyer et al. 2009). Photorespiration will increase when the $[CO_2]/[O_2]$ ratio decreases at suprasaturation, causing CO_2 uptake to be more reduced than ETR_{app} at suprasaturation (as seen in Fig. 2). Increased temperature will also increase photorespiration. Therefore, the accuracy of ETR_{app} as a proxy for CO_2 uptake will also be affected by temperature.

Light screening reduces real ETR

The real ETR depends on the light level reaching the photosystems. For higher plants, an absorption factor of 0.84 is often used (Baker 2008). For lichens, this factor is often much lower. The cortex of a *L. pulmonaria* thallus transmits approximately 50% of visible light, but the cortical screening efficiency varies with e.g. the melanic pigmentation shaped by site-specific solar radiation (Gauslaa and Solhaug 2001). Thus, the absorption factor may approximate 0.5 for *L. pulmonaria*. Yet, individual calibration of the relationship between CO_2 uptake and ETR_{app} is necessary because the absorptance factor varies within and between lichen

species, as evidenced by Fig. 4a and b. With an increase in cortical screening and less light reaching the photosystems, photosynthetic CO_2 uptake decreases. The decrease in light reaching PSII will result in higher Φ_{PSII} (Solhaug et al. 2010) causing higher ETR_{app}. The opposite effects of cortical screening on CO_2 uptake and ETR_{app} require calibration of CO_2 uptake against ETR_{app} before ETR_{app} can be used as a reliable proxy for photosynthesis. Cortical screening in lichens is often higher for blue than for red light (Phinney et al. 2019a, b). Especially the orange *Xanthoria* species have high screening for blue light by their blue light-absorbing pigment parietin (Solhaug and Gauslaa 1996). In *L. pulmonaria,* the transmittance of blue light is also lower than for longer wavelengths (Gauslaa and Solhaug 2001). In the present study, the red light was used for both fluorescence and CO_2 uptake measurements. As the CO_2 uptake—ETR_{app} relationships did not differ between the species despite their different cortical pigments, the cortical transmittance of red light was likely rather similar in all species. If we had used the same photon level of white or blue light, CO_2 uptake would have been lower. By contrast, ETR_{app} would have been higher because less light at PSII would have caused higher Φ_{PSII}. Therefore, the use of ETR_{app} as a proxy for CO_2 uptake requires species-specific light quality calibration, particularly for melanic species that absorbs substantial amounts of red light.

Effect of fluorometer actinic light spectrum on ETR_{app}

Chlorophyll fluorometers use normally red or blue light as actinic light. The upper cortex of lichens normally transmits more red light than blue light. Moist upper cortex in *L. pulmonaria* may transmit approx. 40% blue light and 60% red light (Gauslaa and Solhaug 2001) and lichens with yellow pigments as *Xanthoria* and *Letharia* transmits much more red light than blue light (Solhaug and Gauslaa 1996; Phinney et al. 2019a, b). When using a fluorometer with blue actinic light, less light will reach the algal cells and the Φ_{PSII} yield and ETR_{app} will be higher although the real ETR is lower than with an equal level of red actinic light (see Solhaug et al. 2010). Therefore, the calibration of ETR_{app} as a proxy for CO_2 uptake also depends on the actinic light spectrum of the fluorometer used.

Conclusion

In conclusion, ETR_{app} measured by imaging chlorophyll fluorescence tools correlates well with photosynthetic CO_2 uptake in lichens that have no free external water on their surfaces. However, when lichens are suprasaturated by external water that limits the CO_2 diffusion, ETR_{app} may highly overestimate photosynthetic CO_2 uptake. In addition, various alternative electron sinks will reduce the fraction of electrons used for CO_2 reduction, and there is a great variation in cortical screening between different lichen species. Therefore, species-specific calibration is needed.

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