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Fluorescence Imaging of Lichens in the Macro Scale

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Abstract

Photographical and video techniques were used to make the chlorophyll a and phycobilin fluorescence of whole lichen thalli visible. They were used for three types of applications:

1. Photographical fluorescence imaging is suitable to detect inconspicious algae and lichens in the field. In all cases in which the human eye is not able to judge the presence or absence of autotrophic organisms on grey or black surfaces, the presented method yields fast and reliable information on the percentage of surface cover. As the fluorescence of desiccated organisms is low, a differentiation between wet, active and desiccated, inactive thalli or thallus parts is also possible.
2. Because of their different antennae pigment composition, the photosystem II fluorescence emission of green algae is high during blue illumination while that of cyanobacteria is low. During green illumination, on the other hand, cyanobacteria show much higher fluorescence levels compared to green algae. Thus, the differentiation between thalli with cyanobacterial and green algal photobionts by fluorescence imaging is possible. For the same reason, fluorescence imaging with green excitation light may be used to localize cephalodia like those on the lower thallus side of *Lobaria pulmonaria*.

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3. The vitality of thallus parts in terms of photosynthetic activity cannot be assessed by simple photographic methods. A more sophisticated method based on the 2-dimensional measurement of the variable part of fluorescence and the calculation of the fluorescence yield ΔF/Fm' provides quantitative information about "active" and "inactive" photosynthetic electron transport in different thallus parts. Such activity pictures are presented for thalli of *Peltigera aphthosa, Hypogymnia physodes* and *Lobaria pulmonaria*.

Keywords: lichens, fluorescence imaging, chlorophyll fluorescence, phycobilin fluorescence, desiccation, cephalodia, *Peltigera, Hypogymnia physodes, Lobaria pulmonaria, Nephroma arcticum, Lasallia pustulata, Ramalina lacerata*

1. Introduction

Fluorescence imaging is well known from epifluorescence microscopes equipped with special excitation lamps and dichroitic mirrors to separate excitation and emission light (Kauppi, 1980; Kauppi and Verseghy-Patay, 1990; Slavik, 1994). In the macro scale, i.e. in the dimensions of whole lichen thalli or higher plant leaves, no commercial instruments for fluorescence imaging are available. As fluorescence maps of leaves provide valuable information about the surface heterogeneity of photosynthetic activity, there were recently several attempts with laboratory built machines to make this information accessible (Daley et al., 1989; Raschke et al., 1990; Yanase and Andoh, 1992; Lang et al., 1994; Daley, 1995; Genty and Meyer, 1995; Nilsson, 1995; Rolfe and Scholes, 1995; Siebke and Weis, 1995a,b). As the lichen surface is often even more heterogenous than that of leaves because of desiccation induced colour change of the cortex (Scheidegger et al., 1995), uneven photobiont distribution, possible necroses, chloroses or because of integrated cephalodia (containing cyanobacteria) (Englund, 1977; Rai et al., 1981; Feige and Jensen, 1992; Maguas and Brugnoli, 1996), we tried to adapt the reported techniques to lichens. On one hand, a relatively simple photographic technique was developed which can be easily used in the field or with stereoscopic magnifiers, on the other hand a laboratory video technique was applied as reported for higher plant leaves (Siebke and Weis, 1995a,b). The scope of this approach was to find out, whether the photosynthetic electron transport is heterogenous within the plane of flat lichens and whether we could directly observe growth zones. In this first study of lichen fluorescence imaging we report on applications and limitations of these techniques.

2. Materials and Methods

Sites of collection and treatment of lichen thalli

Peltigera aphthosa (L.) Willd. was collected on the Inner Bergli, Mathon, Paznauntal, Austria at 0°C. *Nephroma arcticum* (L.) Torssell was collected in the Kenai peninsula near Seward, South Alaska, USA. The thalli were kept wet and cold (<10°C) for 3 days (*P. aphthosa*) or 8 days (*N. arcticum*), dried for 6 hours in laboratory air (22°C, 35% relative humidity) and then stored at –25°C. *Ramalina lacerata* Müll. Arg. was collected on the Isle de Siècle, Bretagne, France in the dry state, and transferred into the refrigerator (–25°C) 2 weeks after collection and stored until use. *Hypogymnia physodes* (L.) Nyl. growing on the bark of oak trees was collected in the Eifel mountains near Gemünd, Germany, dried, and frozen down to –25°C one day after collection. Dry material of *Lobaria pulmonaria* (L.) Hoffm. growing on *Pinus canariensis* and *Erica arborea* was collected in La Palma (Canary Islands, Spain) at the Cumbre nueva. This material was frozen at –25°C one week after collection.

After 1–2 years, frozen lichen thalli were thawed and artificially rewetted (spraying with distilled water). The samples were stored for at least 15 hours in a refrigerator at about 7°C in darkness, and then for 2 hours at room temperature at about 6 µmol photons/m^2/s. This procedure normally leads to a high physiological activity and low zeaxanthin content of the samples (Jensen et al., 1993).

Wet thalli of *Lasallia pustulata* (L.) Mérat growing on granite rocks were photographed *in situ* or were collected in Hammere, Bornholm, Denmark and photographed one hour after collection together with wet material of *H. physodes* growing on oak at the same location. Oak twigs covered with *Parmelia sulcata* (L.) Ach. and other lichens were broken off in the Eifel mountains near Gemünd, Germany, and photographed one day after collection after spraying with distilled water.

Photographical imaging

For all photographic work a Kodak Ektachrome EPL 400 X slide film and a 55 mm (scale 1:2) or 90 mm macro-objective (1:1) were used. The f-stop was normally set to f-4. A commercial flashlight (flash factor 25) was mounted on the photocamera with an angle holder in order to get a minimal distance (about 6 cm) between flashlight and sample. The angle between flashlight and sample was 45°. Fluorescence photography was performed underneath a black velvet cloth, the samples were predarkened at least 1 minute.

For fluorescence imaging, we used 4 combinations of excitation/emission filters:

1. For chlorolichens

3 mm BG23 (Schott) + 1 mm DT Blue Standard (Balzers) in front of the flash; two OG550 filters (Schott) produced for objectives with a 52 mm thread purchased from Heliopan Lichtfilterfabrik Summer, Gräfelfing, Germany, mounted in front of the macro-objective. Thickness of the OG glass filters: about 2.8 mm. Applying this photographical method to Kodak Color Control Patches (©Eastman Kodak Company 1977) resulted in a strong red image with the magenta patch, while the white patch appears dark greenish/yellowish (light scattering from green parts of the excitation light). The magenta patch of the control patches can serve as a standard to estimate fluorescence intensities of the images. Colours of the images: green + yellow + red.

2. For cyanolichens or cephalodia

3 mm BG23 (Schott) + 1 mm DT Green Standard (Balzers) in front of the flash; RG 630 filter (Schott) purchased from Heliopan Lichtfilterfabrik (see above) mounted in front of the macro-objective. Colour of the images: red + bright red.

For switching between method 1 and 2 the DT Blue must be replaced by the DT Green, and the OG550 must be replaced by the RG630. Conventional macrophotography with the same film is easily possible with integrated weak flashlights after removing the filters.

3. For cephalodia, with a 250 W halogen light slide projector as excitation source instead of flash light

3 mm BG23 (Schott) + 1 mm DT Green Standard (Balzers) in the slide holder of the slide projector, + 1 mm of a large Balzers Dichrolight R65 filter mounted in an angle of 45° in front of the slide projector objective. The dichroitic R65 filter mirrors green light onto the lichen thallus (90° between original slide projector beam and the lichen surface).

No filter in front of the photocamera. The lichen thalli were photographed through the R65 filter in an angle of about 30° between lichen surface and photocamera, i.e. not directly through the R65 filter (=45°) but slightly tilted to optimize the colour contrast. Care was taken to avoid that parts of the excitation light passes directly through the photocamera objective. Colours of the images: green + red.

4. For chlorolichens

3 mm BG28 (Schott) + 1 mm DT Blue Standard (Balzers) in front of the flashlight (or within a slide projector); RG665 filter (Schott) purchased from Heliopan Lichtfilterfabrik (see above) mounted in front of the macro-objective.

This last combination yields only red colours with a very good separation between excitation and emission light. The Kodak Ektachrome Panther P1600X film or the Kodak Recording Film 2475 (black and white, extended red sensitivity) are well suited in this case.

Single Balzers filters could be purchased from Pörschke GmbH, Höchst, Germany. Light intensities from the slide projector were controlled with a LI-COR 185B quantum sensor.

Video-imaging

Video-imaging was basically performed as described earlier (Siebke and Weis, 1995a), but the optical arrangement was modified. Instead of two different light sources only one HMI light source (Götschmann, Diaprojektoren, München, Germany) equipped with the filters KG1 (Schott), OCLI heat mirror, B51 Dichrolight (Balzers), and 9782 (Corning), was used. For actinic illumination an additional grey filter was placed in front of the lamp which was automatically removed when saturating light flashes were given. The light was reflected by a large Balzers Dichrolight R65 to the leaf (Genty and Meyer, 1995).

The intensity of the blue actinic light was set to 120 µmol photons/m^2/s. The overall fluorescence was simultaneously controlled by a PAM fluorometer (Walz, Effeltrich, Germany). The thalli were illuminated in an air stream for at least 15 min to assure steady state photosynthesis before the $\Delta F/F_m'$ -determinations were performed.

For the cyanolichens, the blue Balzers B51 filter for the excitation light was replaced by a Balzers DT Green filter (actinic light intensity: 57 µE/m^2/s) The obtained $\Delta F/F_m'$ values were so small that we were not able to calculate reasonable video images with cyanolichens or cephalodia from *Nephroma arcticum*.

Measurement of modulated fluorescence and light dependent O_2-evolution

For measurement of modulated chlorophyll fluorescence, a PAM apparatus (Walz, Effeltrich, Germany) was used. In the field, the MiniPAM apparatus (Walz) was used. For *L. pustulata* the miniature fiber optics (1.5 mm diameter) was used to investigate different thallus regions. For the determination of F_v/F_m values the samples were kept wet and predarkened for at least 25 minutes. Simultaneous measurement of chlorophyll fluorescence and O_2 evolution was performed in a Hansatech LD2 cuvette as described earlier (Jensen et al., 1993).

3. Results and Discussion

Fluorescence imaging in the field (photographical method)

In principal, photographical fluorescence imaging was performed with 4 filter combinations and a commercial flashlight as excitation source. Mainly method 1 and 2 (see Material and Methods) are suitable for field work. These methods use a 1 ms flash (setting = manual) from a commercial flashlight which is not harmful to the plant material. With blue flash excitation and a yellow long pass filter for the fluorescence emission (method 1), lichens with green photobionts can be detected. The red colour in Fig. 1a,b,d,f,h,i can be attributed to chlorophyll fluorescence of the green photobionts, while the greenish/yellowish colours in these photos originate from the light scattering of green parts of the excitation light, which is not completely excluded by the emission filter, or by another type of fluorescence emission. On one hand, these greenish colours are obtained in thallus segments which do not contain green photobionts. Such an effect is evident in *Cladonia podetia* (not shown), *Ramalina lacerata* (Fig. 1a) or in chlorotic parts of thalli (not shown). On the other hand, greenish colours appear, when the lichens become dry. In this case, the chorophyll fluorescence emission declines (Lange et al., 1989; Jensen and Feige, 1991) while the upper cortex becomes brighter and less transparent because of the development of gas bubbles in the hyphae (Scheidegger et al., 1995; Honegger et al., 1996). This effect is very clear not only within single thalli like those of *L. pulmonaria* (Fig. 1f), but also with lichen communities growing on bark. In this case the wet active areas emit clear red fluorescence allowing a good estimation of the percentage of cover (>95% in Fig. 1c,d). Desiccated, inactive areas look greenish (Fig. 1b, right part).

In cases, where an orange (e.g. *Xanthoria* species) or yellow colour (e.g. *Rhizocarpon* species) of the thalli interfere with fluorescence, the filter combination with the best separation of excitation and emission (method 4, not shown) should be preferred. A similar method with an almost identical filter combination as in method 4 has already been applied to bean leaves infected with a rust fungus (Peterson and Aylor, 1995).

The setting of the f-stop may be also critical, as a high red fluorescence intensity may appear yellow on the photographical film. Such an effect should be avoided by control photos including a fluorescent standard like the Kodak magenta colour plate (see Material and Methods).

Interestingly, layers of the green alga *Desmococcus viridis* growing on bark emit red fluorescence in the wet state, but not in the desiccated state (greenish colour, photographed in situ with method 1, not shown). Therefore, in the relationship between wet/dry cycles and chlorophyll density, the red

fluorescence emission can be regarded as a qualitative marker of photosynthetic activity.

Attempts to estimate the photosynthetic activity in a quantitative way from fluorescence intensities or fluorescence intensity changes failed. Neither a quantitative comparison with the data of a MiniPAM-apparatus nor with O_2-emission revealed reasonable results. It must be pointed out, that the fluorescence intensity obtained in the fluorescence images provide information about the optical transparency of the cortex layer and/or the chlorophyll density below that layer. This is demonstrated in Fig. 1g,h, where *H. physodes* and *L. pustulata* have been photographed together. The fluorescence intensity of *L. pustulata* is much lower than that of *H. physodes* according to its less transparent cortex. As *L. pustulata* was growing on rock exposed to full sunlight and *H. physodes* was growing in the shade, only the cortex of *L. pustulata* must protect the photobionts from excess light (sun glass effect, (Demmig-Adams et al., 1990)). The blackish cortex of *L. pustulata* simultaneously darkens the fluorescence emission and shifts the light response curve of photosynthesis to higher light intensities. The potential electron transfer in photosystem II, as expressed by the chlorophyll fluorescence parameter F_v/F_m, was similar for both lichens and homogenous for different parts of the *L. pustulata* thallus, although the region around the umbilicus macroscopically appeared more transparent (Fig. 1g). All F_v/F_m values for these lichens obtained with a MiniPam apparatus were in a range between 0.71 and 0.73. It must be concluded, that images of chlorophyll fluorescence intensity are not suitable to indicate the vitality of photobiont cells. Nevertheless, low chlorophyll density influences the intensity of the fluorescence radiation strongly in areas of dead or absent photobionts. Extreme lesions like chloroses or necroses are therefore normally visible and recognizable by greenish/yellowish colors in wet lichen thalli, but minor differences in the intensity of red fluorescence are often due to variations of the optical properties of the cortex and may therefore resemble a damage. This was tested several times with thalli of different appearance in *H. physodes* (not shown). In all these cases we checked the vitality by F_v/F_m determinations (Jensen, 1994), but very low variation was observed (values between 0.68 and 0.71).

For the measurement of cyanobacterial photobionts in cyanolichens or cephalodia, method 2 was used. In this case, the bright red emission colour arises mainly from the phycobilins (main emission at wavelengths <665 nm). With cyanolichens (*Peltigera canina, P. membranacea, P. praetextata*) a very bright red emission with very little variation within the thallus was observed (not shown). Cephalodia within chlorolichens also emitted this bright red colour, but the rest of the thallus (green photobionts) appeared relatively dark.

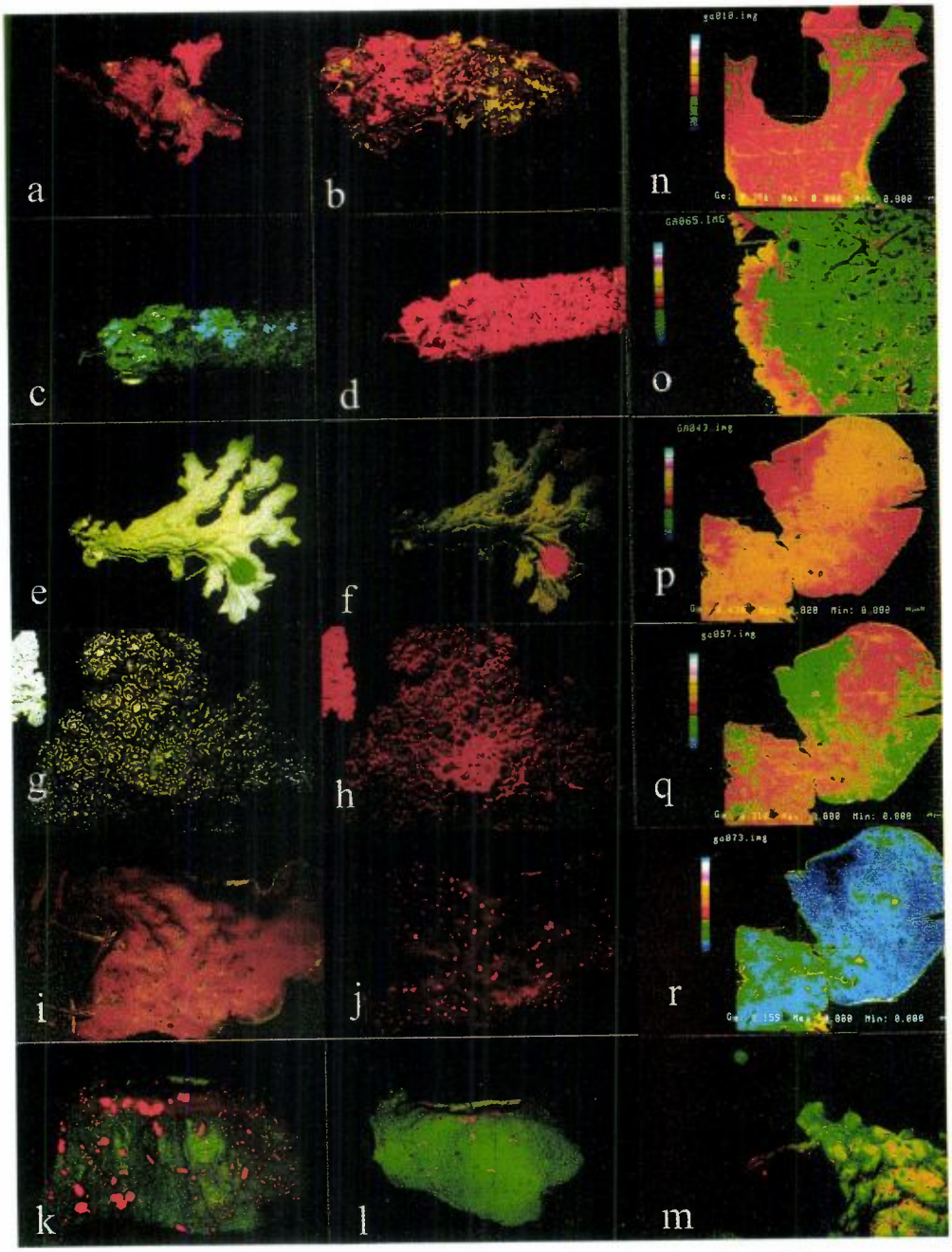

Figure 1. **a)** Fluorescence image of a wet thallus of *Ramalina lacerata*. **b)** Fluorescence image of an oak twig covered with lichens. The left part of the twig was sprayed with water. This rehydrated area has a red fluorescence emission. The right part

In Fig. 1i,j methods 1 and 2 were applied for a desiccating thallus of *P. aphthosa*. In this case, the cortex was already dry as could be seen by the milky color (not shown), but the relatively weak red chlorophyll fluorescence of the green photobionts is still visible (Fig. 1i). When the filters of method 1 were replaced by the filters of method 2, the strong phycobilin fluorescence of the cephalodial cyanobacteria appeared which is accompanied by a much weaker chlorophyll fluorescence in other parts of the thalli.

Detection of cephalodia

Because of the high absorption of phycocyanin and the low absorption of chlorophyll in the green part of the spectrum, and because of the very bright red phycobilin fluorescence (Slavik, 1994), it is easy to detect cyanobacteria with green excitation light. The most satisfactory filter combination (method 3) for the detection of cephalodia as used in Fig. 1k–m, resulted in green colours for the thallus parts containing green algae, and a very intense red radiation

Figure 1. Continuation: ...which remained dry, has no visible red emission, yellowish colors dominate instead. **c)** Oak twig covered with *Parmelia sulcata* and other lichens. **d)** Fluorescence image of the same twig as in c). Almost 100% of the area emits red fluorescence including those regions which in normal light (c) appear black. **e)** Dry thallus of *Lobaria pulmonaria*. On the lower right part the thallus was rewetted with 2 drops of distilled water. **f)** Fluorescence image of e). Note the red fluorescing wet spot. **g)** Wet thalli of *Lasallia pustulata* and *Hypogymnia physodes* (in the upper left). **h)** Fluorescence image of g). The fluorescence intensity of *L. pustulata* is heterogenous and lower than that of *H. physodes*. **i)** Fluorescence image of a desiccating (at 35% rh) thallus of *Peltigera aphthosa*. The upper cortex changed its color to opaque-milky (not shown) about 5 min before the photo was taken. **j)** Fluorescence image as in i), but with filter combination 2. The red phycobilin fluorescence emission of the cephalodia is visible. **k)** Fluorescence image of a wet thallus of *N. arctica* photographed with method 3. A bright red emission from the cephalodia is visible. **l)** Like k), but thallus largely desiccated. The red cephalodial emission is vanishing. **m)** Fluorescence image of the wet lower side of *L. pulmonaria* (method 3). The cephalodia, which are not visible in normal light, emit a bright red fluorescence. **n)** Fluorescence video image of the fluorescence parameter $\Delta F/F_m'$ of a wet thallus of *L. pulmonaria*. Upper colors in the calibration bar (left side) of the pseudocolor image indicate high values. The 'Ge:' value (bottom line) indicates the arithmetic average of all picture elements which show fluorescence. **o)** Video image as in n) for a wet thallus of *H. physodes*. **p)** Video image as in n) for a wet thallus of *P. aphthosa*, gas phase: air. **q)** Video image as in p), gas phase: air minus CO_2. **r)** Video image as in p), gas phase: N_2.

(fluorescence emission) for the cephalodia. For the excitation light a commercial 250 W halogen slide projector was used. The excitation light flux density at the lichen surface was 150 $\mu E/m^2/s$. This permanent light allowed the observation of the intense fluorescence emission by the blank eye, no flash was necessary for the photos. In Fig. 1k, a wet thallus of *P. aphthosa* is shown. Wet isolated cephalodia from thalli of the same collection or from *Nephroma arcticum* were photosynthetically active as judged from the variable fluorescence measured with the PAM apparatus. During atmospheric dehydration, the phycobilin fluorescence from the cephalodia vanished (Fig 1l) , and could not be detected either by eye or in the photos when dehydration proceeded further (not shown). The same effect could also be observed for thalli of *Nephroma arcticum*. We are unsure whether this is only due to the transparency change of the cortex (black color of wet cephalodia, greyish color of dry cephalodia) or whether other processes are involved in the fluorescence decline.

In Fig. 1m, the lower side of a thallus of *L. pulmonaria* is shown. In normal light this side looks white and the cephalodia beneath the lower cortex (Jordan, 1970) are not visible. An irregular pattern of cephalodia emitting red light emerges when illuminated with pure green light. There was a tendency of a preferred location of the cephalodia at the outer parts of the lobes, but for individual thallus pieces the frequency and distribution of cephalodia could not be predicted. In some cases thallus pieces completely lacked cephalodia. It could not be proven for our material, however, whether there were whole thalli totally free of cephalodia, as most thalli were broken into pieces. In all cases the cephalodia were rather small (less then 1 mm diameter).

Video-imaging in the laboratory

In Fig. 1n–r the 2-dimensional distribution of the chlorophyll fluorescence parameter $\Delta F/F_m'$ (Genty et al., 1989) is shown as a pseudocoloured image. As can be seen from the calibration bar, bright colors indicate $\Delta F/F_m'$-values of up to 0.8, dark colors range from 0 to 0.2. Green, red and yellow colours indicate intermediate values, the Ge values at the bottom of Fig. 1n,p,q,r were calculated as average values of all picture elements. They were slightly higher than the values monitored by a PAM-apparatus which was driven simultaneously.

For wet, healthy looking thalli of *L. pulmonaria*, the $\Delta F/F_m'$-values were rather homogenous (Fig. 1n). This was also true for thalli of *P. aphthosa* (Fig. 1p) and most thalli of *H. physodes*. Growth zones at the thallus margins normally could not be observed by our method.

A particular high activity at outer thallus parts was observed for *H. physodes* in only one example (Fig. 1o). With normal illumination (no filters), the margin looked greener than the rest of the thallus. This most probably indicated a high cortex transparency leading to a high light intensity absorbed by the photobionts at the margins. This can also be concluded from the image of the F_m' value (not shown, maximal fluorescence value at steady state conditions, (van Kooten and Snel, 1990)). In this image, much higher F_m' values were reached at the margins than in central parts. Because the light response curves of $\Delta F/F_m'$ measured with the MiniPAM always had negative slopes, a higher light absorption at the margins should normally reveal lower $\Delta F/F_m'$ values. Instead of this, however, the measured values were clearly higher than in central parts of the thallus. As photosystem II electron transport rate is regarded to be proportional to $\Delta F/F_m'$ multiplied by the absorbed light energy (Krall and Edwards, 1992), and both factors appear to be higher in the margin areas, a zone of high photosynthetic activity has been detected in this case, which most probably also reflects a zone of particular high growth.

Thalli of *H. physodes* are, however, very variable in morphology and light transparency of the cortex. At present, we regard the presented image as an exception and do not expect to find such clear cases very often.

The response of $\Delta F/F_m'$ to changing gas atmosphere is demonstrated in the images p–r. In Fig. 1p a thallus of *P. aphthosa* is shown that was flushed with air. A normal high $\Delta F/F_m'$ average value of 0.438 was obtained. After removal of CO_2 from the air stream the $\Delta F/F_m'$ values declined and the 2-dimensional pattern was unchanged. In leaves of higher plants, the removal of CO_2 leads to very low $\Delta F/F_m'$ values. The relatively high average value of 0.316 in *P. aphthosa* can only be explained, if internal CO_2 produced by the mycobiont is taken into consideration. In Fig. 1r, the lichen is gassed with pure N_2. Still, the $\Delta F/F_m'$ pattern is retained. As the system is largely depleted of electron acceptors in this case, the $\Delta F/F_m'$ (proportional to electron transport) should drop to almost zero. The observed average value was 0.159. The control measurement performed with the PAM apparatus showed a value of 0.2. At present, it is not clear, which electron acceptors other than CO_2 or O_2 were available.

With cyanolichens, the $\Delta F/F_m'$ values were so small that reasonable images could not be calculated. Small $\Delta F/F_m'$ values have also been observed in the cyanobacterium *Synechococcus leopoliensis* (Schreiber, 1994). On the other hand, cephalodia in *P. aphthosa* had almost the same $\Delta F/F_m'$ values as the surrounding. Consequently they were not noticeable in the $\Delta F/F_m'$ images.

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