Seasonal variations of F_o, F_m, and F_v/F_m in an epiphytic population of the lichen *Punctelia subrudecta* (Nyl.) Krog

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Abstract: Chlorophyll *a* fluorescence was measured every three months from December 2003 to December 2004 in ten, randomly selected thalli of the epiphytic lichen *Punctelia subrudecta* (Nyl.) Krog occurring in a deciduous, thermophilous downy oak wood in southern Tuscany (central Italy). *In situ* measurements were carried out with a photosynthesis yield analyser Mini-PAM (Walz, Effeltrich, Germany) on the same measuring points of 30-minute dark-adapted, fully hydrated thalli. The results show a remarkable seasonal change in F_o, F_m, and F_v/F_m, probably caused by a long-term physiological-structural adaptation of the photobiont to the gradual modification of the climatic conditions of each measurement period and the light regime of the understorey. However, *P. subrudecta* is also sensitive to rapid changes in microclimatic conditions as the PSII functionality is affected by the light regime of the days preceding the field measurements. Some critical observations on the statistical analysis of chlorophyll *a* fluorescence parameters are also made.

Key words: chlorophyll *a* fluorescence, climate, oak forest, photosynthesis, seasonal acclimation

Introduction

Chlorophyll fluorescence is a highly sensitive and non-invasive technique which in recent years has become an important tool in plant ecophysiology. It has proved particularly useful for examining the response of photosynthetic organisms to environmental stress factors, such as extremes of temperature (Bilger *et al.* 1987; Schreiber & Bilger 1987; Jensen *et al.* 1997; Larcher *et al.* 1997), light (Demmig & Björkman 1987; Krause 1988; Gauslaa & Solhaug 2000; Heber *et al.* 2000) and water availability (Bilger *et al.* 1989; Lange *et al.* 1989; Jensen & Feige 1991; Scheidegger *et al.* 1997; Csintalan *et al.* 1999; Jensen *et al.* 1999).

One of the most frequently used parameters in field studies is F_v/F_m, a reliable indicator of the maximum photochemical quantum efficiency (Butler & Kitajima 1975; Genty *et al.* 1989). This parameter, measured in dark-adapted samples, is calculated from F_o, the fluorescence emission when the reaction centres (RCII) are fully open (see Mathis & Paillotin 1981), and F_m, the maximum fluorescence emission when all photosystems are closed following exposure to a pulse of saturation light (see Lazár 1999). Unlike F_v/F_m, F_o and F_m are strongly dependent on environmental temperature and instrumental setting, and therefore they have not been used so far in intra- and interspecific comparisons based on field data. Nevertheless, some laboratory measurements have shown that F_o and F_m can give reliable indications of organism adaptations to environmental changes, especially with respect to light intensity (see Demmig-Adams *et al.* 1990) and water availability (see Heber *et al.* 2000, 2001). Other studies have shown that F_o and F_m are also sensitive to specific pollutants (see Sgradelis *et al.* 1994; Gries *et al.* 1995; Deltoro *et al.* 1999). The increasing use of chlorophyll fluorescence in biomonitoring is partly due to studies based on lichens (see Calatayud *et al.* 1996; Niewiadomska *et al.* 1998; Garty *et al.* 2003). The data gathered so far in this field are however rather

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problematic, at least in part because we still have a poor knowledge of the factors that interact to alter the fluorescence emission of lichens. In order to investigate this issue, a field study of the chlorophyll fluorescence performance of lichens exposed to a wide array of different ecological conditions was carried out in four forest types along an altitudinal gradient in southern Tuscany (Italy). Five broad-lobed epiphytic lichens with the highest cover in at least a single station were selected as target species and F_o, F_m and F_v/F_m were measured at three monthly intervals under standardized conditions.

The present paper reports the seasonal variations of F_o, F_m and F_v/F_m observed in the single population of *Punctelia subrudecta* found along the gradient. We also describe the procedure for selection of samples, the techniques adopted for field measurements and how the F_o and F_m values were adjusted to render them comparable despite being recorded under quite different temperature regimes. Finally, some general considerations on statistical data analysis of F_o, F_m and F_v/F_m will be discussed. The results concerning the other four species will be reported in a forthcoming series of papers.

Data and Methods

Study area

Field work was carried out in a deciduous, thermophilous downy oak (*Quercus pubescens*) wood, on a south-exposed hillside near Scansano (Grosseto, southern Tuscany) (lat. 42°41′23″N, long. 11°20′5″E), at 500 m altitude. The trees are about 40–45 years old, are *c*. 6 m high and their trunk surface is almost entirely covered by a lichen community referable to the *Parmelia* spp.vv. association (Nimis 1982), with *Flavoparmelia caperata* (L.) Hale as the dominant species.

The study area belongs to the submediterranean phytoclimatic region (Barazzuoli *et al.* 1993). The average rainfall is around 1000 mm per year, with rainy days concentrated in the two equinoctial periods. In summer a pronounced drought normally extends over the months of July and August, with rainfall lower than *c*. 22 mm per month, and mean temperatures of almost 26°C. Winter temperatures are relatively high, being mitigated by the nearby Tyrrhenian sea. According to the classification of Thornthwaite (1948), this climate can be defined as subhumid (B_1), mesothermic (B_2'), with a moderate summer water deficit (s), and with a

summer concentration of thermal efficiency between 48% and 52% (b_4') (Barazzuoli *et al.* 1993).

The species

Punctelia subrudecta is a broad-lobed lichen with a circumboreal, temperate distribution in the Northern Hemisphere and is also known from South Africa, South America and Australia (see Nimis 1993). It is found on moderately acid to subneutral, slightly nutrient-enriched surfaces, predominantly in sunny areas. In Italy it has an optimum in submediterranean vegetation, below the montane belt, and shows a more continental tendency than the morphologically closely related, but genetically distinct, *P. borreri*, from which it can be easily distinguished by the absence of calcium oxalate crystals on the upper external surface (Tretiach *et al.* 2002).

Field measurements

Measurements were carried out in five sessions that took place on 1 December (2003), and 1 March, 11 June, 5 September and 13 December (2004), approximating to the spring and autumn equinoxes and summer and winter solstices. In order to have a representative sample of the lichen population, ten thalli, randomly exposed on the trunks, and with a maximum diameter of 8 cm, were selected at 105–195 cm above the ground.

Between 4 and 8 fluorescence measurement points were selected on flat portions of each thallus, approximately 0·8 cm from the lobe margin, and marked on colour photographs taken with a digital camera (Nikon Coolpix 4500). In each session, the selected lobes were externally marked with a pin so that they could be recognised under the dampened piece of black velvet used for darkening the thalli. These were copiously sprayed with distilled water and covered, as suggested by Jensen (2002), for 30–35 minutes before the measurements, to obtain the reduction of PSII reaction centres.

In situ F_v/F_m measurements were carried out with a photosynthesis yield analyser (Mini-PAM; Walz, Effeltrich, Germany), using a flexible 5·5 mm active diameter fibre optic and a distance clip positioned on the darkened thallus surface. The minimum fluorescence yield (F_o) was obtained using a weak, non-actinic measuring beam and applying the burst mode option. The maximum fluorescence yield of the dark-adapted specimens (F_m) was determined with a 800 ms saturation pulse of *c*. 8000 µmol m^{-2} s^{-1} light intensity. Both parameters are expressed in mV with a gain setting=1. Variable fluorescence (F_v) was calculated as $F_m - F_o$.

F_o and F_m adjustment curves

The values of F_o and F_m vary according to the settings and the inner temperature of the instrument. In order to compare the measurements from the five sessions, a set of adjustment curves for each of the three instrumental settings adopted in the field were
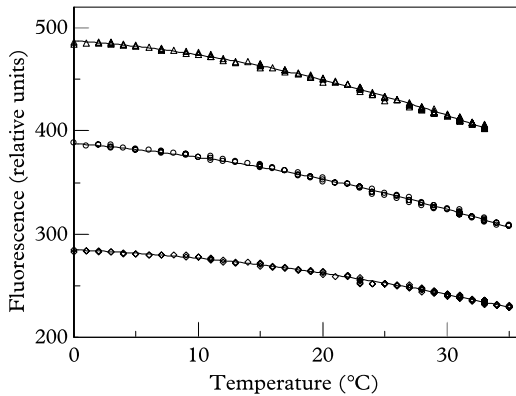
Fig. 1. Relationship between fluorescence signal from a fluorescence standard (Roscolene Surprise Blue filter) and inner instrument temperature at three modulated measuring light intensities used during the field work ($\diamond$, MI=3: $y=-0.0313\,x^2-0.5057\,x+284.78$, $R^2=0.995$; $\circ$, MI=4: $y=-0.0399\,x^2-0.924\,x+387.66$, $R^2=0.997$; $\triangle$, MI=5: $y=-0.0525\,x^2-0.8412\,x+487.4$, $R^2=0.997$).

produced in the laboratory. The fluorescence signal of a fluorescence standard (Roscolene Surprise Blue filter) was thus monitored between 0–35°C (instrumental internal temperature) (Fig. 1). The temperature-dependent decrease in fluorescence, far from being linear, as described in the instrument manual, is parabolic, because a more marked reduction occurred at higher temperatures, due to a decreased measuring light intensity caused by the LED overheating. The interpolation equations of the best-fitting curves ($R^2>0.99$, $n>76$) were used to adjust the field values to the most frequent temperature occurring in the whole data set (i.e. 18°C, at a measuring light intensity of 4).

Statistical analysis

The D'Agostino-Pearson K^2 test (D'Agostino & Pearson 1973) was used to test for normality in the F_o, F_m and F_v/F_m values of each measurement session, and skewness and kurtosis were evaluated in order to describe the symmetry and shape of the data distribution curves. The variation of F_o, F_m and F_v/F_m among sessions was statistically tested using the non-parametric Mann-Whitney U test. The calculations of the median and quartile values of each data set were carried out with STATISTICA 6.0 (StatSoft Italia srl, 2001).

Results

Climatic conditions before and during field work

The climatic conditions in the week before the measurement sessions are summarized in Figure 2. The lowest mean daily temperatures were recorded in March (average weekly temperature: 5.7°C), when field work suffered from a heavy snowfall, and the highest values in September (22°C). Intermediate values were recorded in the two measuring sessions in December (12.8°C in 2003 and 11.0°C in 2004). The mean daily cumulative radiation changed considerably during the year, varying from a minimum of 43 W m^{-2} (December 2003), to a maximum of 276 W m^{-2} (June). The daily maximum PPFD measured at ground level within the wood ranged from 40 (June and September) to 580 µmol photons m^{-2} s^{-1} (December) (data for March missing).

The lichens were subjected to heavy rain before the measurement sessions only in December 2003 and March 2004, receiving *c.* 98 mm and 52 mm of total rainfall, respectively. The records of relative air humidity are unfortunately incomplete. The data available show that the highest average of daily means and pre-dawn values occurred in March (81.4% and 86.6%, respectively). However, the average humidity recorded at dawn in September was quite high (86.4%). It may be worth noting that in December 2003, when this parameter was not recorded, air humidity was also probably very high, if not close to saturation, given the persistent fog present during the whole day of field work, and also on the preceding days.

F_o, F_m, and F_v/F_m

The values of F_o, F_m, and F_v/F_m measured over the year and adjusted by means of the equations given in Figure 1 are summarized in Table 1. The data sets for each variable have a normal distribution, except for those of F_o and F_v/F_m recorded in September 2004 and December 2004. The skewness and kurtosis of the latter two data sets were far from 0 (the value of a Gaussian distribution curve), being respectively -0.677 and 1.426 for F_o, and -0.863 and 1.023 for F_v/F_m. For this reason, in the present study the variation among sessions was tested by non-parametric statistics, as suggested by
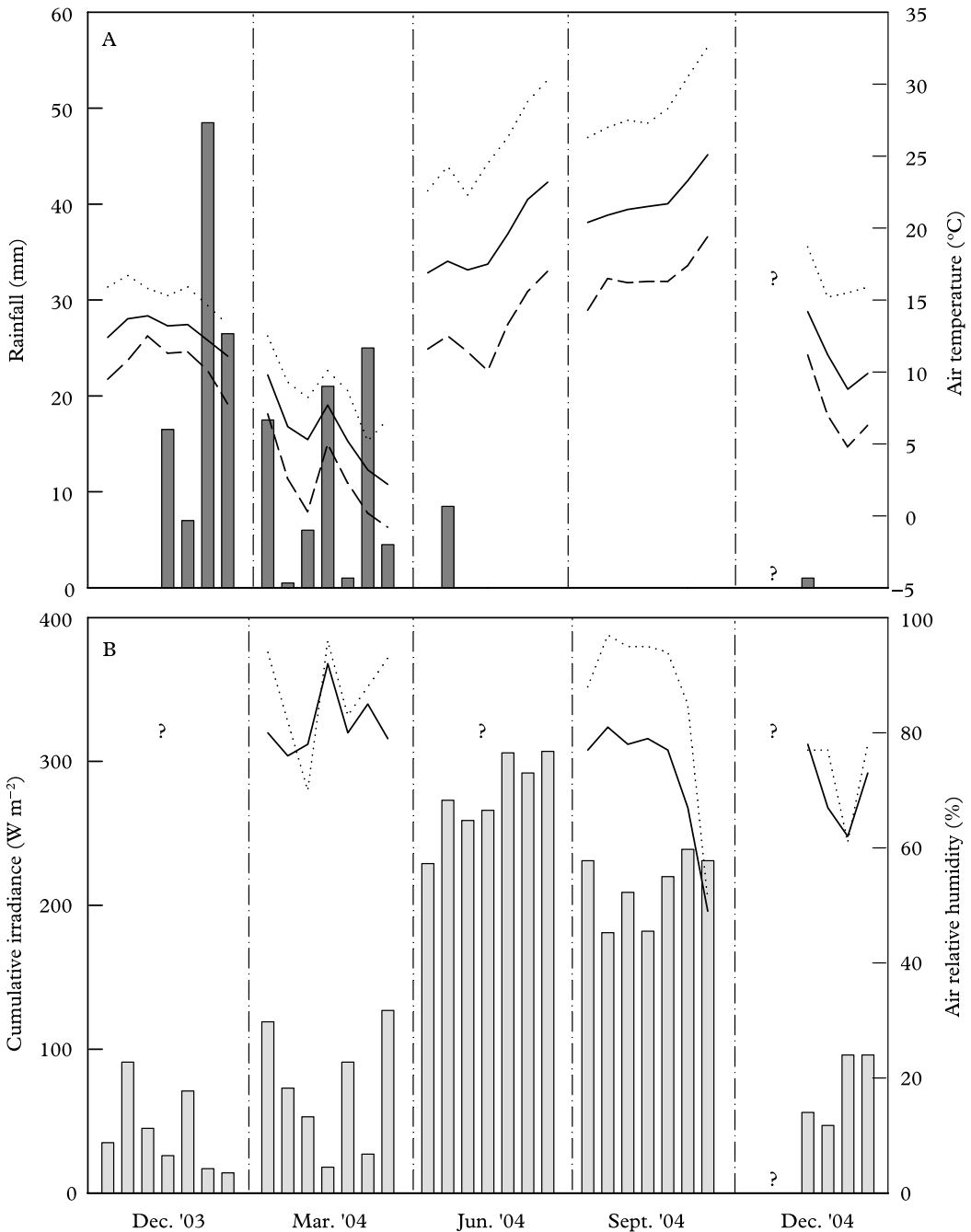
FIG. 2. Climatic conditions in the week before each field measuring session recorded in Roccalbegna, 15 km from the study area. A, daily rainfall (bars, mm); minimum, mean, and maximum air daily temperature (dashed, continuous, and dotted line, respectively; °C); B, daily cumulative global irradiance (bars; W m^{-2}); air relative humidity during the whole day (continuous line, %), and at dawn (dotted line, %).

TABLE 1. *Selected statistical descriptors of* F_o, F_m, *and* F_v/F_m *measured in* Punctelia subrudecta *during the year*

	F_o			F_m			F_v/F_m			n
	25th	Median	75th	25th	Median	75th	25th	Median	75th	
Dec. '03	410	469	553	1832	2078	2518	0·762	0·771	0·778	55
Mar. '04	456	503	566	1163	1394	1624	0·606	0·641	0·668	58
Jun. '04	519	583	629	1391	1640	1925	0·615	0·639	0·674	45
Sept. '04	495	560	612	1279	1520	1718	0·600	0·625	0·656	58
Dec. '04	397	455	503	1428	1703	1965	0·722	0·730	0·742	51

Lazár & Nauš (1998), and data are presented with non-parametric descriptors.

The seasonal values of F_v/F_m fall within the range observed in several fruticose and foliose lichens (Demmig-Adams *et al.* 1990; Manrique *et al.* 1993; Calatayud *et al.* 1996; Jensen & Kricke 2002), and, according to Jensen (2002), indicate that the population sample is healthy. The highest median value of F_v/F_m (0·771) was observed in December 2003, as a consequence of the high values of F_m (2078) and the low values of F_o (469). In the three following sessions the median of F_v/F_m was considerably lower, ranging between 0·625 and 0·641, two extremes that do not differ statistically (Table 2). The variation of F_o and F_m was much more irregular, as shown in Figure 3. A slight, but not statistically significant, increase in F_o was observed in March 2004, whereas in June and September this parameter was considerably higher (the median being 583 and 560, respectively), with $P<0·1\%$ (Table 2). On the other hand, F_m decreased considerably in March, with the median 33% lower than in December ($P<0·1\%$). In June and September F_m increased slightly, but remained well below the value for December 2004.

In December 2004 all the three parameters approached the values recorded in the previous year (Fig. 3). In spite of the marked increase in F_o (the frequency distribution of which was statistically similar to that of the previous year), F_v/F_m reached a median value of only 0·730. Although this value was high in comparison to the three previous sessions, it was still below that of December 2003. The lower value of F_v/F_m

in December 2004 might have been caused by the incomplete recovery of F_m. In fact, the median value of this parameter in December 2004 was closer to that of June and September, than to that of December 2003.

Discussion

The parameters examined in the present work have been found to be subject to significant seasonal variations. A better understanding of these variations is possible only if we take into consideration environmental variables, particularly seasonal changes in water availability and understorey light regime.

In December, when the highest values of F_v/F_m were recorded, the potential total radiation is certainly lower than in other months, because of the low solar altitude and the short photoperiod, but the ground cover radiation can be quite intense as the oak trees are leafless. The photosynthetic photon flux density (PPFD) recorded on a sunny day in December 2004, on the south-exposed part of the oak trunks, reached peaks of more than 1300 μmol photons m^{-2} s^{-1}. The fact that the highest values of F_v/F_m were recorded in December is perhaps surprising since this parameter is sensitive to photoinhibition, a photoprotective mechanism that both higher plants (Björkman 1987; Demmig-Adams 1990) and lichens (Demmig-Adams *et al.* 1990; Gauslaa & Solhaug 1996; Gauslaa *et al.* 2001; MacKenzie *et al.* 2002; Barták *et al.* 2003) are known to develop to different extents when exposed to an excess of light. Photo-

Table 2. *Differences between the* F_o, F_m, *and* F_v/F_m *values measured in* Punctelia subrudecta *during the year (data summarized in Table 1): Z values (Mann Whitney U Test) are reported. Statistically significant differences are marked with asterisks* (*=$P<0·05$; **=$P<0·01$; ***=$P<0·001$)

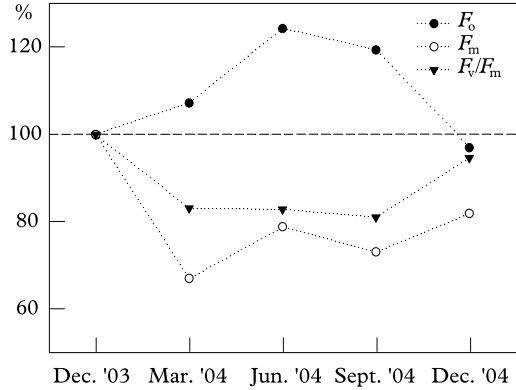|	F_o				F_m				F_v/F_m			
	Mar. '04	Jun. '04	Sept. '04	Dec. '04	Mar. '04	Jun. '04	Sept. '04	Dec. '04	Mar. '04	Jun. '04	Sept. '04	Dec. '04
Dec. '03	−1·18	−4·61***	−3·88***	1·44	7·37***	5·35***	6·88***	5·05***	9·16***	8·57***	9·16***	8·78***
Mar. '04		−4·04***	−3·13**	2·86**		−3·34***	−1·18	−3·98		−0·58	1·00	−8·85***
Jun. '04			1·26	5·77***			1·97*	−0·63			1·48	−8·34***
Sept. '04				5·23***				−2·81**				−8·93***

Fig. 3. Seasonal variations of median F_o, F_m, and F_v/F_m values in *Punctelia subrudecta* expressed as percentage of the respective median values recorded in December 2003.

inhibition causes a marked decrease in F_v/F_m, which is reversible within a few hours provided that the duration and intensity of the radiation do not lead to a process of photodegradation (Demmig-Adams *et al.* 1990; Leisner *et al.* 1996; Schlensog *et al.* 1997; Gauslaa & Solhaug 2000). In December the increased water availability and the shorter photoperiod are likely to extend lichen metabolic activity under conditions of low light or darkness, thus allowing full recovery.

The decrease in F_v/F_m, observed in March, June and September (amounting to almost −18% if compared to the median value of December 2003), was caused by a parallel decrease in F_m and a rise in F_o. To date, few studies have reported field measurements of F_m and F_o (see e.g., Leisner *et al.* 1996; Lange *et al.* 1999) because these parameters are strongly affected by several factors, temperature being the most influential. However, this problem can be surmounted by the use of correction curves (Figure 1), that facilitate the standardization for temperature, 18°C in the present study.

Typically, F_m denotes the maximum fluorescence emission when all the PSII reaction centres are closed following a flash of saturation light. It generally decreases when the light energy, that reaches PSII, is too

high, due a self-protection system that drains the excitation energy of Chl molecules in the form of heat (Gilmore 1997). The prolonged excitation of Chl molecules would lead, in fact, to the formation of radical species, detrimental to the chloroplast (Mittler 2002). According to our data, the most marked decrease in F_m was observed in March (almost -35% compared to December 2003), before the spring equinox. This change is consistent with the occurrence of photoinhibition phenomena, since between December and March both the maximum potential radiation and the photoperiod increase, in the absence of any tree foliage, and the good water availability of that period permitted prolonged metabolic activity. In agreement with these observations, Tretiach *et al.* (2003) showed that in thalli of *Parmelia sulcata* exposed to only 350 µmol photons m^{-2} s^{-1} for 5 h there is a pronounced reduction in gross photosynthesis in April but not in December. Presumably, therefore, our lichens were exposed to increasing light stress, and this often occurred when they were metabolically active. Gauslaa & Solhaug (1996, 2004), and Kappen *et al.* (1998) demonstrated that light-adapted lichens, particularly resistant to photoinhibition under dehydration conditions, become sensitive if they are exposed to high light when humid. The higher resistance of dehydrated thalli to photoinhibition is apparently induced by a decrease in light transmission through the upper cortex (Büdel & Lange 1994; Gauslaa & Solhaug 2001), as well as by a functional disconnection of some components of the photochemical system (Sigfridsson 1980; Bilger *et al.* 1989).

In June, the maximum radiation values incident on the understorey decreased, following full establishment of the oak leaf canopy, while the mean values increased slightly, due to the longer photoperiod. In this month we observed a rise in F_m, that, however, was only partial if compared to December 2003. This incomplete recovery was probably the result of higher mean air temperatures and less frequent rainfall events, which prolonged the period of thalli

dehydration. Data gathered in June and September show that also before dawn thalli of *P. subrudecta* had <30% of their relative water content, notwithstanding the relatively high values of air humidity (see Fig. 2).

In addition the values of F_o, indicating the openness of PSII reaction centres in the dark, changed considerably throughout the year. This parameter is dependent on the chlorophyll content of the light-harvesting complex (Butler 1977, 1978), and consequently, on the structure of the antenna complexes (Malkin & Fork 1981; Wild & Ball 1997). As we found a maximum in June and September (*c.* 22% higher than in December 2003), it might be hypothesized that this was caused by an increase in chlorophylls. This hypothesis, nevertheless, is not supported by the few data on the seasonal chlorophyll variation observed in epiphytic lichens (Wilhelmsen 1959; Strobl & Türk 1990; Tretiach *et al.* 2003). For instance, in thalli of *Parmelia sulcata* collected in a chestnut wood only 30 km away from the present study area, the concentration maxima for Chl *a* and *b* occurred in December, and the minima in August (Tretiach *et al.* 2003). Therefore, other mechanisms are presumably responsible for the variation in F_o observed in this study. Several authors (Demmig-Adams *et al.* 1990; Jensen *et al.* 1999; Barták *et al.* 2000) have shown that this parameter in any phycolichen decreases in concert with the general physiological slow-down during a dehydration event. According to Bilger *et al.* (1989) and Jensen *et al.* (1999), such reduction is mainly due to the separation of the light-harvesting complex II (LHCII) from the PSII core. However, there are other mechanisms that potentially could also be responsible for the decrease in F_o, such as the redistribution of the excitation energy in favour of PSI (Sigfridsson & Öquist 1980; Jensen & Feige 1987; Chakir & Jensen 1999), the decrease in excitation energy caused by the optical properties of the upper cortex, the formation of reflecting air bubbles (Scheidegger & Schroeter 1995), and the partial re-absorption of the emitted fluorescence (Horton *et al.* 1996; Takács *et al.* 2000). As

our measurements were carried out in fully hydrated thalli, these processes can be excluded. More likely, the high F_o during the warmest and driest months, when the conditions for a prompt recovery from photoinhibition are less favourable, might be associated with increased damage to photosystems (Briantais *et al.* 1996; Lazár 1999; Maxwell & Johnson 2000). However, differences in photosystem configuration and response to measurement conditions might also be involved, as the photobionts in this case are acclimated to the diffuse light habitat of a fully developed tree canopy (MacKenzie *et al.* 2004).

The data gathered by measuring the chlorophyll fluorescence in the same thalli, in the same positions but after 12 months are informative. With the exception of F_o, the values of F_m and F_v/F_m were statistically lower in December 2004 than in December 2003 (Table 2). This discrepancy can be explained in terms of different climatic conditions in the days immediately before and at the time measurements were made (see Fig. 2). On the day of the first series of measurements (December 2003), the whole study area was exposed to a thick, persistent fog, so that the thalli were exposed to low, diffuse light and benefitted from high, constant hydration, also partly due to the heavy rains that occurred in the previous four days. In contrast the December 2004 measurements were carried out during a period with almost no rain, low relative air humidity, and more intense, direct irradiance (Fig. 2). The data thus showed that the PSII functionality in *P. subrudecta* can vary significantly even in the same period of different years, if the microclimate conditions on the days before the measurements differ considerably. This result agrees with Gauslaa *et al.* (2001) who studied the high-irradiance damage in transplanted *Lobaria pulmonaria* and *P. sulcata* thalli, and found a close correlation between F_v/F_m and the mean irradiance values to which the lichens had been exposed on the day before measurement.

Finally a comment on the choice of statistical descriptors and tests to be used for the analysis of fluorescence data. These are generally reported as mean ± standard deviation, and differences among data sets are usually assessed through the application of parametric tests, such as ANOVA and the student's t test (see, e.g., Niewiadomska *et al.* 1998; Calatayud *et al.* 2000; Gauslaa *et al.* 2001). These descriptors and tests, however, strictly require data with a Gaussian distribution. Unfortunately, this prerequisite is usually assumed to hold true, but actual compliance has only been tested in a few papers (see, e.g., Manrique *et al.* 1993; Gauslaa & Solhaug 2004). This assumption is probably based on the high number of data available and the positive results of some normality tests carried out in some pioneering works. In this study, the values of F_o, F_m and F_v/F_m were shown not always to have a normal distribution, in agreement with the results of Lazár & Nauš (1998). These authors amply demonstrated that in wheat, fast fluorescence induction parameters (and, among others, also F_o, F_m and F_v/F_m), do not follow a Gaussian distribution. According to Lazár & Nauš (1998), the lack of symmetry is due to the constraints of an upper (F_m) or lower (F_o) limit. F_v/F_m, which lies between 0 and 1, is also physiologically upper limited. In healthy, unstressed vascular plants, the ratio between F_m and F_o ranges approximately between 5 and 6, and, consequently, F_v/F_m comes close to 0·832 (Björkman & Demmig 1987). The same is true for lichens, although F_v/F_m is generally lower, between 0·630 and 0·760 (Jensen 2002), occasionally exceeding 0·800 (L. Baruffo & M. Tretiach, unpublished results). Due to this upper limit, F_v/F_m values are seldom very high, whereas many may be low, leading to an increased distributional asymmetry. Surprisingly, however, the asymmetry of our F_o data gathered in September was found to be negative, not positive, as found by Lazár & Nauš (1998), and as one might expect, since this parameter has a lower limit.

In conclusion, this study has shown that in *Punctelia subrudecta* F_o, F_m and F_v/F_m are subject to significant seasonal changes. These variations are probably a consequence of a long-term adaptation at physiological

and structural levels to the gradual change in the main macro- and microclimate conditions. However there is also a second type of variation, which is short-term, and strongly dependent on the light conditions of the preceding days. The use of not only F_o and F_m, but also F_v/F_m, in biomonitoring investigations should be considered as problematic if the seasonal changes highlighted in this and similar studies (MacKenzie *et al.* 2001, 2002) are neglected.

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