



When the sun never sets: daily changes in pigment composition in three subarctic woody plants during the summer solstice

Beatriz Fernández-Marín¹ · Jon Atherton² · Beñat Olascoaga² · Pasi Kolari³ · Albert Porcar-Castell² · José I. García-Plazaola¹

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Abstract

Key message Subarctic plants in summer (subjected to continuous light) showed photosynthetic pigment contents mainly driven by PPFD (unrelated to day/night cycles) and a xanthophyll cycle responsiveness to PPFD exacerbated during night-times.

Abstract Composition and content of photosynthetic pigments is finely tuned by plants according to a subtle equilibrium between the absorbed and used energy by the photosynthetic apparatus. Subarctic and Arctic plants are subjected to extended periods of continuous light during summer. This condition represents a unique natural scenario to study the influence of light on pigment regulation and the presence of diurnal patterns potentially governed by circadian rhythms. Here, we examined the modulation of the photosynthetic apparatus in three naturally co-occurring woody species: mountain birch (*Betula pubescens* ssp. *czerepanovii*), alpine bearberry (*Arctostaphylos alpina*) and Scots pine (*Pinus sylvestris*) around the summer solstice, at 67 °N latitude. Plants were continuously exposed to solar radiation during the 3-day study period, although PPFD fluctuated, being lower during night-times. Photochemical efficiencies for a given PPFD were similar during daytime and night-time for the three species. In Scots pine, for a given PPFD, net assimilation was slightly higher during daytime than during night-time. Overall, the dynamism in pigment content was mainly driven by PPFD, and was generally unrelated to day/night cycles. Weak indications of potential circadian regulation were found over a few pigments only. Interestingly, the xanthophyll cycle was active at any time of the day in the three species but its responsiveness to PPFD was exacerbated during night-times. This was particularly evident for bearberry, which maintained a highly de-epoxidised state even at night-times. The results could indicate an incomplete acclimation to a 24-h photoperiod for these species, which have colonised subarctic latitudes only recently.

Keywords Arctic · Carotenoid · Chlorophyll · Circadian rhythm · Photochemical efficiency · Xanthophyll cycle

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✉ Beatriz Fernández-Marín
beatriz.fernandezm@ehu.es

Extended author information available on the last page of the article

Introduction

Through the course of evolution, most living organisms have adapted to diurnal fluctuations in solar radiation dose induced by the rotation of the Earth around its axis. Anticipation to predictable environmental conditions is a key mechanism to take advantage of such rhythmic changes. Thus, circadian clocks are present in diverse groups of organisms (from cyanobacteria to vertebrates) (Bell-Pedersen et al. 2005). For example, in the model plant *Arabidopsis*, one-third of the nuclear genes are under circadian control (Covington et al. 2008), regulating most of the physiological processes in plants including gas exchange and photosynthesis. Under natural conditions,

circadian clocks are set every day by events such as sunrise or sunset.

The effects of the circadian clocks become evident when plants are transferred to artificially maintained constant environmental conditions, particularly constant light (CL). In this situation, rhythmic physiological patterns appear, although they weaken over time and are finally lost after several days (McClung 2006; Caldeira et al. 2014; García-Plazaola et al. 2017). CL can cause detrimental damage in plants, with some species being not able to survive under a CL regime (Vélez-Ramírez et al. 2011). This is logical, since CL *sensu stricto* does not exist on Earth; even during the solstice at very high latitudes, when the photoperiod is 24 h, there are considerable diurnal variations in light intensity and spectral quality. Nevertheless, Arctic and Antarctic environments above 60° latitude differ from the rest of terrestrial ecosystems by the fact that they are exposed during a variable period of time over the year (summer) to continuous light, when the sun never sets.

Despite the importance of Arctic and subarctic ecosystems on Earth, where they occupy vast extensions, and despite their susceptibility under a climate change scenario, plant photosynthetic responses in these environments are still poorly characterised. A couple of studies in the last few years have shown that diurnal cycles of photosynthesis in Arctic plants are under circadian control (Resco de Dios et al. 2012; Patankar et al. 2013), but it has not been clearly established whether Arctic plant communities are able to take advantage, in terms of carbon gain, of this 24 h photoperiod in summer. Interestingly, a recent study on the responses to CL in tomato cultivars (Vélez-Ramírez et al. 2014) showed that the northernmost varieties of tomato were less susceptible to CL (e.g. higher photochemical efficiency and less leaf chlorosis). The authors showed that the higher tolerance of these cultivars to CL was related to a single gene encoding a light harvesting chlorophyll a/b-binding protein (Vélez-Ramírez et al. 2014). In agreement with such observations, annual plants transferred from an oscillating environment to CL show a robust 24-h rhythm on chlorophyll content (Pan et al. 2015; García-Plazaola et al. 2017), so it is possible that the adjustments on antenna size and the composition and balance in the light harvesting by PSI and PSII are essential in the adaptation to long photoperiods. Information available with regard to woody species is even scarcer due to their larger difficulties for growth under controlled conditions. Nevertheless, the scarce literature available about changes in photosynthetic pigment content after transfer of plants to CL indicates the existence of rhythmic oscillations in chlorophyll content in the woody species *Gossypium hirsutum* (García-Plazaola et al. 2017).

One of the most remarkable features of plant pigments is that their composition and proportion are highly dynamic, indicating changes in photosynthetic processes. In particular,

on a daily scale, cycles of synthesis/degradation of components of the light-harvesting complexes (chlorophylls) (Fukushima et al. 2009; García-Plazaola et al. 2017) and the inter-conversion of xanthophylls within the violaxanthin (Vio) cycle (Demmig-Adams and Adams 1996) occur. The latter consists on the light-induced conversion of Vio into zeaxanthin (Zea). In darkness, the opposite reaction takes place, giving rise to the so-called Vio-cycle that usually operates following a daily rhythm. This cycle modulates the efficiency of light energy conversion by the photosynthetic apparatus, preventing its damage under conditions of light energy absorbed in excess. As the kinetics of conversion between Vio and Zea operate at time scales of minutes, the Vio-cycle can have a significant effect on plant productivity under fluctuating light (i.e. presence of clouds), with up to a 20% reduction in photosynthetic carbon uptake (Kromdijk et al. 2016).

Pigment composition and dynamics have been rarely studied in Arctic plants, where circadian controls might be partly offset due to high-latitude dynamics in solar elevation. As an example, in a recent meta-analysis compiling data from more than 500 studies about plant pigment composition and dynamics (Esteban et al. 2015), none of them was performed at latitudes higher than 67 °N and only three studies were conducted at latitudes above 60 °N. In the present study, we aim to characterise daily changes in pigment composition in three subarctic woody plant species (including trees and shrubs) as well as their impact on the daily patterns of photosynthetic capacity under the natural 24-h photoperiod above 67 °N. We study as representative of the main life forms a conifer, Scots pine (*Pinus sylvestris* L.); a deciduous broadleaf tree, mountain birch (*Betula pubescens* ssp. *czerepanovii* (N.I.Orlova) Hämet-Ahti); and a dwarf cushion shrub, alpine bearberry [*Arctostaphylos alpina* (L.) Nied.]. We hypothesise that: (1) photosynthetic pigments show daily changes compared to those of lower latitude plants, which are mainly driven by PPFD, (2) the xanthophyll cycle is still active during the “night-time”, and (3) the relation between pigment composition and photosynthetic capacity differs under day- vs. night-time periods.

Methods

Plants and study site

Field measurements were performed at Värriö Strict Nature Reserve (Lapland, Finland) (67°44'N; 29°36'E) in June 2016. The site lies in the boreal zone in north-eastern Finland, and it is mostly covered by a natural subarctic pine forest in low areas and a subarctic tundra with scattered trees in the elevated areas. The annual average temperature in the nearby Station for Measuring Ecosystem-Atmosphere

Relations (SMEAR-I) at Värriö Strict Nature Reserve is $-0.5\text{ }^{\circ}\text{C}$, and during the week of summer solstice average maximum and minimum temperatures of the last 10 years are 17 and $7\text{ }^{\circ}\text{C}$, respectively. The minimum air relative humidity was above 50% and reached 100% at night, during the study period (Online Resource 1: Fig. S1).

The study site was a gentle slope facing north (elevation of 460 m), just above the Arctic timberline, so it potentially can receive direct sunlight during 24 h in the summer solstice, the exposition to sunlight being favoured at night by its northern orientation (Online Resource 2: Fig. S2). Two consecutive daily cycles were followed around the solstice, with 11 timepoints distributed over this period, as follows: June 21st 13:15, 18:15, 22:15; June 22nd 2:30, 6:00, 10:40, 16:45, 21:30; June 23rd 0:30, 5:30, and 9:30 (Eastern European Time (EET), which is just 4 min off from solar time at this site and period of the year, Fig. 1. Because of such a small difference and further considering that each sampling execution lasted around 40 min, EET was used as a proxy of local solar time). Thus, two night-time periods (from 21st to 22nd, and 22nd to 23rd June) were considered during the study. Leaf sampling for photosynthetic analysis and chlorophyll fluorescence measurements was done at each of the 11 timepoints. As previously pointed out, there is no real night at this period of the year at this latitude; therefore, the time comprised between 9 p.m. and 3 a.m. (i.e. solar

midnight $\pm 3\text{ h}$) was considered as “night-time”, since this is a common duration for the night at lower latitudes in the distribution range of the studied species. The first night-time during the experiment was mainly cloudy and had a low PPFD, and the second one was predominantly sunny (Fig. 1). Due to the occurrence of an intense sunbeam during the second night-time, an additional time-point, for pigment characterisation only, was taken in June 22nd at 22:00.

Three woody species were studied: alpine bearberry (*Arctostaphylos alpina*), a dwarf cushion shrub with broadleaves and deciduous habit; mountain birch (*Betula pubescens* ssp. *czerepanovii*), a broadleaf deciduous woody species; and Scots pine (*Pinus sylvestris*), a conifer just in its distribution limit. Four individuals of each species were selected for the experiments. Mountain birches were growing as shrubs of about 1.5 m tall, and alpine bearberry decumbent shrubs grew up to 5 cm above the ground (Online Resource 2: Fig. S2b). Scots pine trees were approximately 4 m tall, and only needles developed in 2014 (and thus ≈ 1.2 year old) were sampled (Online Resource 2: Fig. S2c). Leaves were always collected from north expositions, with high exposition to direct sun: as a measure of it, canopy transmittance was $98.0 \pm 0.9\%$ (average \pm SE of four individuals) for bearberry, $86.8 \pm 3.6\%$ for mountain birch and $75.2 \pm 3.5\%$ for Scots pine. Canopy openness (or transmittance) is an indirect measure of the potential seasonal light regime at the

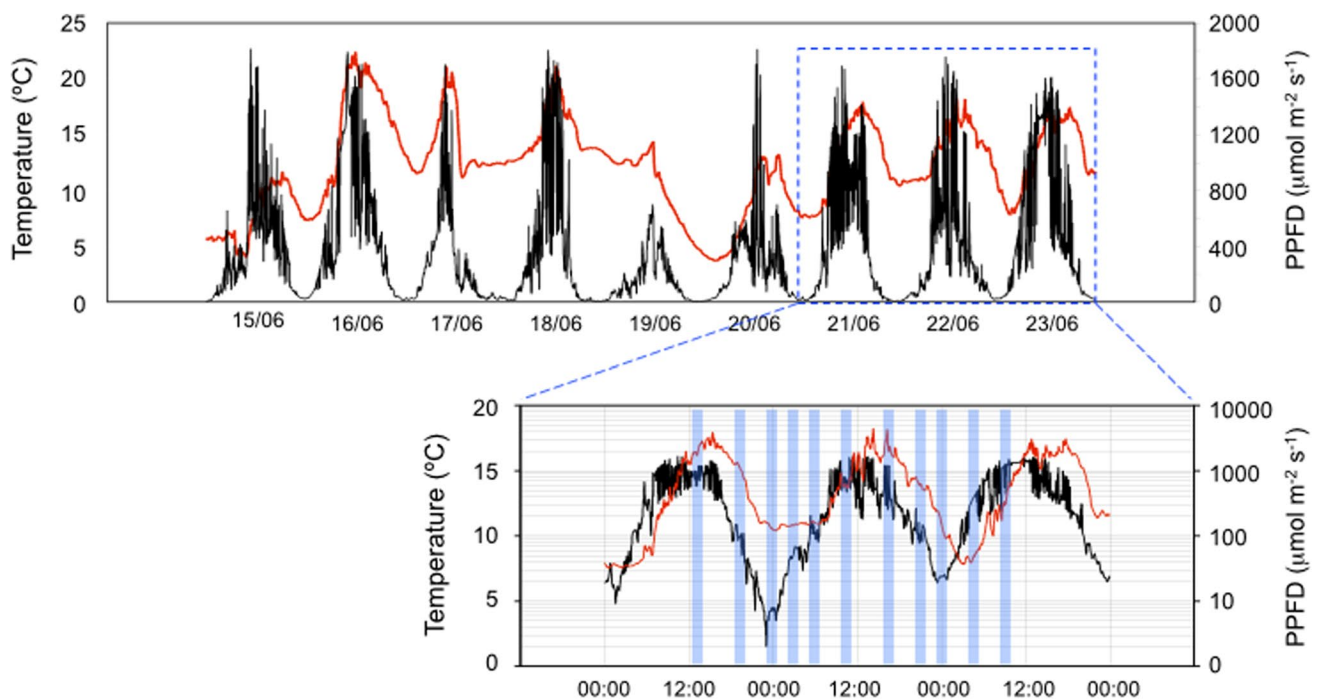


Fig. 1 Air temperature ($^{\circ}\text{C}$) (in red) and PPFD ($\mu\text{mol m}^{-2} \text{s}^{-1}$) (in black) during the study period (June 21st at 13:15 h to 23th at 9:30 h) and the previous week. Inset shows detailed PPFD and temperature for the two complete daily cycles. The 11 sampling timepoints of

study are highlighted with shaded rectangles. Note that the PPFD is shown with logarithmic scale in the inset for a better appreciation of the lowest irradiance intensities

microsite. This parameter was calculated through the software Canopy Gap Analyser (CGA) version 2.0. Hemispherical images were taken in each sampled branch with a digital camera Nikon Coolpix 4500, equipped with a fisheye lens FC-E8. The images were processed with CGA to transform image pixel intensities into sky and non-sky areas. Based on the geographical coordinates of the location, the software further calculates the pathway of the sun in the sky dome and finally estimates the annual average gap light transmission index in percentage, where 100% would correspond to an object fully exposed to direct sun radiation. For further details, see Frazer et al. (1999).

Sampling for pigments and HPLC measurements

Around 25–60 mg (dry weight) of foliar material were taken with a cork borer (for broadleaf species) or cut with scissors (for pine needles), from two to four different leaves per individual (four plants per species, four biological replicates per time-point) and immediately frozen in liquid nitrogen. Once in the laboratory, the samples were freeze-dried and stored with silica (at RH < 10%, and -20°C) until biochemical analysis.

For pigment analysis, two consecutive extractions were done. The first extraction was done with acetone (95%) and the second one with pure acetone (all acetone solutions buffered with $0.5\text{ g CaCO}_3\text{ L}^{-1}$). Plant material was ground under liquid N_2 and the extracts were centrifuged at 16,100g and 4°C . The pellet was then re-suspended in pure acetone, mixed in a vortex and centrifuged once again. Both supernatants were pooled together and pigment composition was analysed by HPLC as described previously (García-Plazaola and Becerril 1999) using a photodiode array detector. The relative de-epoxidation state of the xanthophyll cycle pigments was estimated by the ratio $(A + Z)/(V + A + Z)$, abbreviated thereafter as AZ/VAZ.

Chlorophyll a fluorescence measurements

Chlorophyll *a* (Chl*a*) fluorescence was measured in the field using a portable PAM-2500 fluorometer (Walz, Effeltrich, Germany) in the same branches where leaf samples were collected for pigment analyses (four plants per species and time-point). In dark-adapted leaves (30 min), the maximal Chl*a* fluorescence (F_m) was induced with a saturating pulse (0.8 s duration, $8000\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$ intensity), while the initial fluorescence (F_0) was recorded with low measuring light intensities ($\text{ML} < 1\text{ }\mu\text{mol photons m}^{-2}\text{ s}^{-1}$). The F_v/F_m was then estimated by the ratio $F_v/F_m = (F_m - F_0)/F_m$.

The operating quantum efficiency of PSII (ΦPSII) was measured using the Leaf-Clip of the PAM-2500 (Leaf-Clip Holder 2030-B, Walz GmbH, Effeltrich, Germany).

In illuminated leaves (i.e. directly exposed to solar radiation), the operating quantum efficiency of PSII was estimated as $\Phi\text{PSII} = (F_m' - F_s)/F_m'$, where F_m' is the maximal Chl*a* fluorescence induced with a saturating pulse, and F_s is the actual Chl*a* fluorescence under illumination. During ΦPSII measurements, natural PPFD was recorded with the PAR sensor of the Leaf-Clip (Micro-Quantum-Sensor, Walz GmbH, Effeltrich, Germany) that was then used for electron transport rate (ETR) estimations. PPFDs recorded during the experiment ranged between 14 and $1541\text{ }\mu\text{mol photons m}^{-2}\text{ s}^{-1}$.

Meteorological data and CO_2 exchange

Data of air temperature and PPFD on the study site as well as CO_2 fluxes from Scots pines were obtained from the SMEAR-I research station (Station for Measuring Ecosystem Atmosphere Relations), which is located at approximately 1 km of the sampling site. The station is located on a hilltop at the timberline (elevation 400 m). Air temperature and PPFD were measured in a tower at 2 m height and at 1 min intervals with Pt100 sensors and Li-190 PAR sensors (Li-Cor Inc., Lincoln, Nebraska, USA), respectively.

SMEAR-I is surrounded by a relatively homogeneous Scots pine stand with a stand density of about 700 trees/ha. The studied trees were 60–70 years old and 8–10 m tall. CO_2 exchange of pine shoots was measured with four chambers that were installed near the top of the tree crowns. Each chamber accommodated one shoot comprising one or two age classes of needles at the branch tip. The shoots were gently bent into horizontal plane to improve the correspondence of light conditions experienced by the shoot with the measurement on incoming PPFD. The chambers were open most of the time, allowing the enclosed shoots to experience near-ambient conditions. Each chamber was closed for 1 min every 10 min, and CO_2 fluxes were calculated dynamically from the change in CO_2 concentration inside the closed chamber. The details for chamber measurements can be found in Kolari et al. (2007). The fluxes are given for an all-sided needle area. All meteorological and chamber data are available in SMEAR database at <http://avaa.tdata.fi/web/smart/smear>.

Air temperature and PPFD data from SMEAR-I were averaged for a period of 90 min around each sampling time-point for pigment and Chl*a* fluorescence analysis. The sampling took approximately 60 min, but we incorporated additional 30 min prior to sampling to get a more representative value for PPFD and temperature. We assumed that averaging would cancel out any potential variation in PPFD and temperature between the study area and the nearby SMEAR-I station. Average PPFD values were later on used to construct the statistical models and assess the roles of radiation and solar zenith angle on pigment modulation.

Additionally, the diurnal average PPFD values were estimated for the summer solstice week (time interval between 18th June at 00:00 h and 24th June at 00:00) compiling the available data of the last 15 years, from 2001 to 2016. Scots pine CO₂ fluxes were also compiled for the same time interval from SMEAR-I data. Only values measured at temperatures in the range 10–15 °C were considered. Day- and night-time data were analysed separately. Night-time was considered as the 6 h interval comprised between 3 h before (9 p.m.) and 3 h after midnight (3 a.m.).

Statistical analyses

After checking the homoscedasticity of the data, one-way ANOVA followed by Duncan's post hoc test was used to test for differences in the content of photosynthetic pigments: (1) among the three analysed species, (2) among the different timepoints during measurements, and (3) before and after a nocturnal sunbeam. Some of the parameters showed heteroscedasticity even after data transformation. In these cases, the non-parametric Kruskal–Wallis *H* test was applied, followed by multiple pairwise comparisons with Mann–Whitney *U* test. Pearson's correlation was applied to analyse the relationship between AZ/VAZ and F_v/F_m . When a correlation was significant, a linear regression model was applied. These statistical analyses were conducted using the SPSS 24.0 statistical package at a significance level of $\alpha = 0.05$.

In addition to the above, we examined the linear relationships between environmental, biochemical and photochemical factors by calculating Pearson correlation coefficients between variables. To expand on the correlation analysis, and in an attempt to examine the impact of circadian cycles on leaf pigment concentration dynamics under fluctuating environmental conditions, we analysed the pigment data using a simple linear regression approach. Our aim with the regression analysis was two-fold; first we wanted to detect any repeating and potential circadian patterns in that data set, and second we attempted to determine whether such patterns were over what we would expect by PPFD alone, which (based on previous research) we assumed was the main driver in such variability. Cyclical variability over and above that caused by PPFD could, therefore, be used to identify potential circadian behaviour.

To assess our aims, and following a similar approach than in Resco de Dios et al. (2013), we fitted six linear models to each pigment (dependent variable), and because we were limited by the small number of data-points, each of these models contained a maximum of two independent variables. In terms of independent variables, in addition to average PPFD (described above) we also included a solar angle variable (SZ) and simple time counter variable

(Ti) to represent circadian patterns and average trends, respectively. Ti was a decimal time counter which did not reset over-night and was used to estimate longer (multi-day) linear trends. SZ was calculated as the solar elevation angle (90—solar zenith angle) for each measuring time using 'solarPos' R package, and then normalised between 0 (minimum solar angle at solar midnight) and 1 (maximum solar angle at solar noon). Therefore, SZ was a simple (cyclical) geometric function, which peaked at noon and was minimum at night. The six models we fitted were:

$$m0 : \text{pigment} = a$$

$$m1 : \text{pigment} = a + b\text{PPFD}$$

$$m2 : \text{pigment} = a + b\text{PPFD} + c\text{SZ}$$

$$m3 : \text{pigment} = a + b\text{Ti}$$

$$m4 : \text{pigment} = a + b\text{Ti} + c\text{SZ}$$

$$m5 : \text{pigment} = a + b\text{SZ}$$

$$m6 : \text{pigment} = a + b\text{Ti} + c\text{PPFD}$$

"a", "b" and "c" are the coefficients of the linear models. m0 was the null model with no independent variable. In our analysis, each model was analogous to a single hypothesis, for example m3 would best represent a pigment with no detectable circadian behaviour but an average trend over the whole measurement period.

After estimating each model for each pigment, we used adjusted R^2 to assess model performance. Next, for each pigment we selected the most plausible model (hypothesis) using the Akaike Information Criterion (AIC) corrected for small samples. For each model, we also calculated the Durbin–Watson statistic, and the double-sided *t* test of this statistic, to detect and assess auto-correlation in the fitted models. Finally, those cases where the inclusion of the SZ variable (m2, m4 and m5) led to large increases in explained variance, as well as subsequent selection by AIC, were interpreted as potential indicators of circadian control. All regression modelling was conducted with mean data. This section of the analysis was conducted in the R language, and carotenoids were expressed relative to chlorophyll content (see "Results" for explanation).

Results

Weather conditions

During the week previous to the study, the weather was generally cloudy with an average maximum temperature of 16.5 °C and a minimum of 7.5 °C (Fig. 1). In the two consecutive daily cycles over the study period, temperatures

oscillated between 18.0 and 7.8 °C. Irradiance was always higher than zero, and the minimum recorded value was $2 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD, reaching $1750 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD during periods of direct sunlight at noon. The first night-time was cloudy and midnight irradiance was $6\text{--}7 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD, while the second night-time was sunny and PPFD never lower than $20\text{--}22 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Fig. 1 inset).

Daily changes in pigment composition

The photosynthetic pigment composition of the three species is shown as the average of the 11 sampling timepoints in Fig. 2. Chlorophyll (Chl) concentration (expressed on dry weight basis) in Scots pine was one-third of the concentration found in the angiosperm species. Additionally, Chl *a/b* ratio was slightly lower in pine needles than in the two angiosperm species (Fig. 2a). These differences probably reflect architectural differences among the studied species. Thus, to allow easier comparisons, all carotenoids were expressed in relation with chlorophyll content. For Scots pine, relative concentrations of neoxanthin (Neo/Chl), lutein (Lut/Chl) and β -carotene (β -Car/Chl) were significantly higher than in the other two species (Fig. 2b), while the ratio of total pool of xanthophyll cycle pigments to Chl (VAZ/Chl) was similar for the three species. Mountain birch differed from the other angiosperm, alpine bearberry, in slightly but significantly higher β -Car/Chl, Chl *a + b* and Chl *a/b*, and by slightly lower Lut/Chl contents.

Pigment concentrations in the three species varied over the course of the study period to different extents (Fig. 3). In pine and alpine bearberry, carotenoids to Chl ratios were rather stable over the course of measurements (Fig. 3a–c), although a tendency towards higher values was observed in Scots pine during the first hours of the daily cycle. In mountain birch, there was a trend towards a negative adjustment of carotenoid pools in the year period considered (Fig. 3a–c). This species was the only one showing

significant differences in the ratios of Neo, β -Car and Lut to Chl. Absolute Chl content did not change significantly in any of the three species, but the largest oscillations were once again found in mountain birch (from a minimum of 3.6 to a maximum of $4.2 \mu\text{mol g}^{-1} \text{DW}$) (Fig. 3d).

In contrast to the modest oscillation of the rest of carotenoid ratios, the pool of VAZ pigments, as well as its de-epoxidation state AZ/VAZ were highly dynamic (Fig. 3e, f), particularly in the two angiosperms. Thus, AZ/VAZ was the only parameter showing statistically significant differences among timepoints in all the species (Fig. 3f). The ratio VAZ/Chl oscillated between 31 and 89 mmol mol^{-1} Chl in alpine bearberry and between 39 and 81 in mountain birch, while in Scots pine VAZ/Chl ranged between 43 and 61 mmol mol^{-1} Chl (Fig. 3e). Despite the strong variation, VAZ/Chl did not follow a clear pattern of daily oscillation except for alpine bearberry, where VAZ pool tended to decrease during night-time, and to peak at noon during the first day (Fig. 3e). In contrast, AZ/VAZ peaked during the day in the two angiosperms, while in Scots pine it tended to increase with the progression of the study, even though it did not show daily oscillations (Fig. 3f). In the case of alpine bearberry, VAZ/Chl and AZ/VAZ were significantly and positively correlated (Pearson's correlation coefficient 0.87, $P < 0.001$, data shown in Fig. 4). This species kept a high level of de-epoxidation throughout the whole experiment, always ≥ 0.54 (Fig. 3f).

For a more quantitative and exhaustive treatment of the pigment data in the context of environmental and circadian variability, we conducted a correlation analysis (Fig. 4) and linear regression analysis (Table 1; Fig. 5 and Online Resources 3 and 4: Fig. S3, Table S1). Species, and to some extent pigment, specific variability is evident in the differing structures of the three correlation matrices shown in Fig. 4. Using these matrices to evaluate the effect of each of the individual variables on pigment variability, and as evidenced by the highest correlation coefficients, Scots pine

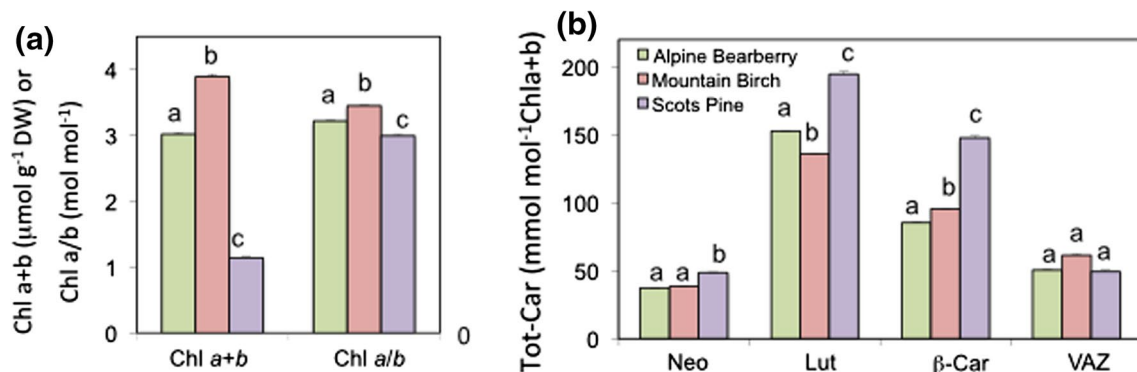


Fig. 2 Pigment composition in leaves of alpine bearberry, mountain birch and Scots pine. Data are averages of the 11 timepoints during the study. Letters above bars indicate significant differences among species ($P < 0.05$)

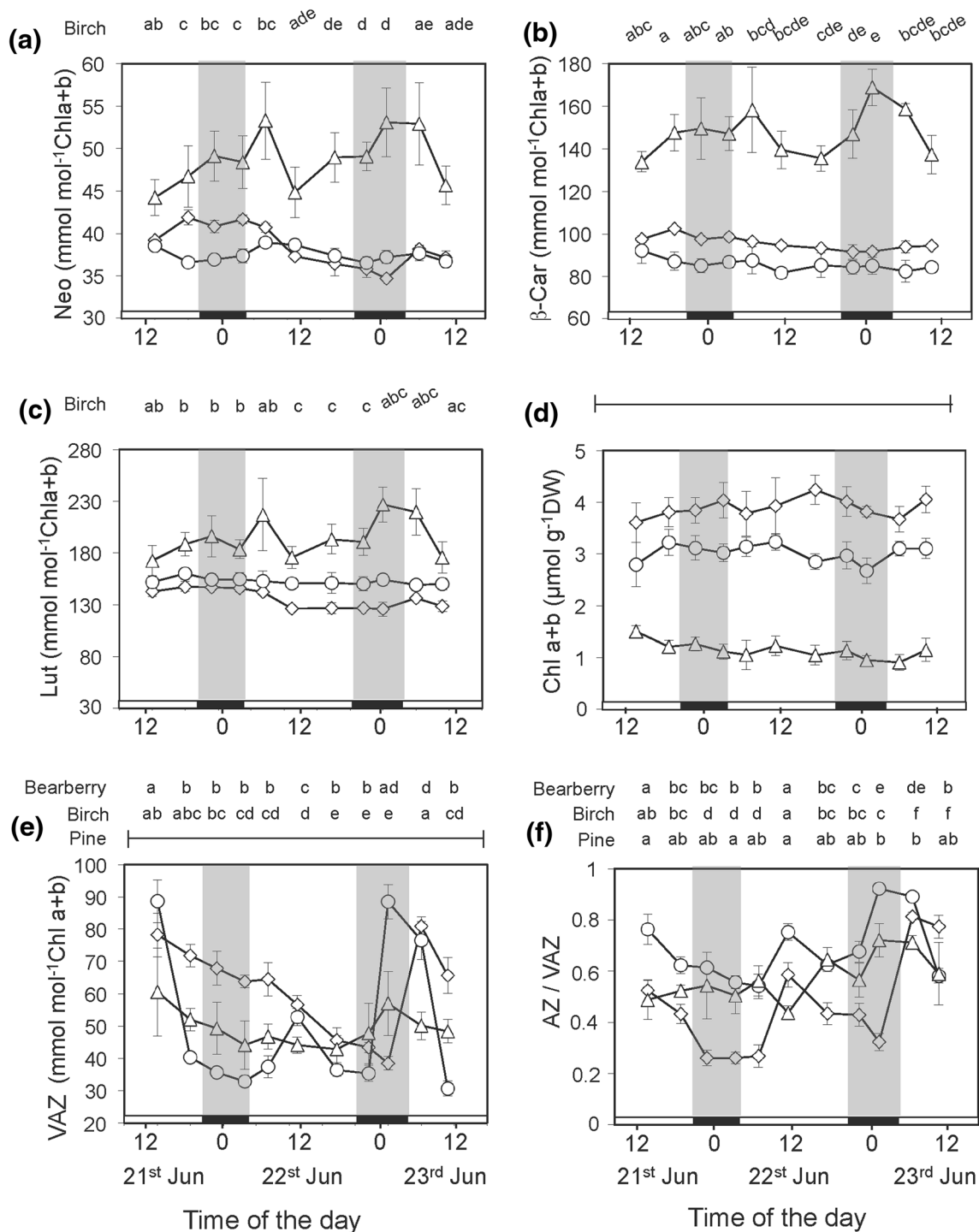


Fig. 3 Changes in total Chl, carotenoids to Chl and AZ/VAZ ratios along the study period (in h). Triangles represent Scots pine, circles represent alpine bearberry and diamonds represent mountain birch. Black bars correspond with night-time periods (6 h around midnight).

appeared to be in principle the species with the strongest potential circadian rhythm (Fig. 4). This was particularly evident for α- and β-Car/Chl, which correlated negatively with the “solar angle” (e.g. decreased towards midday,

Data are the mean of four replicates ± SE. When significant, differences in pigment concentrations among timepoints within each species are depicted with letters above the panels ($P < 0.05$)

higher irradiances, and higher temperature), and for Chl a + b and Chl a/b, which correlated positively (e.g. tended to increase at midday) (Fig. 4c). However, since “solar angle” was strongly correlated with PPFD and to some extent also

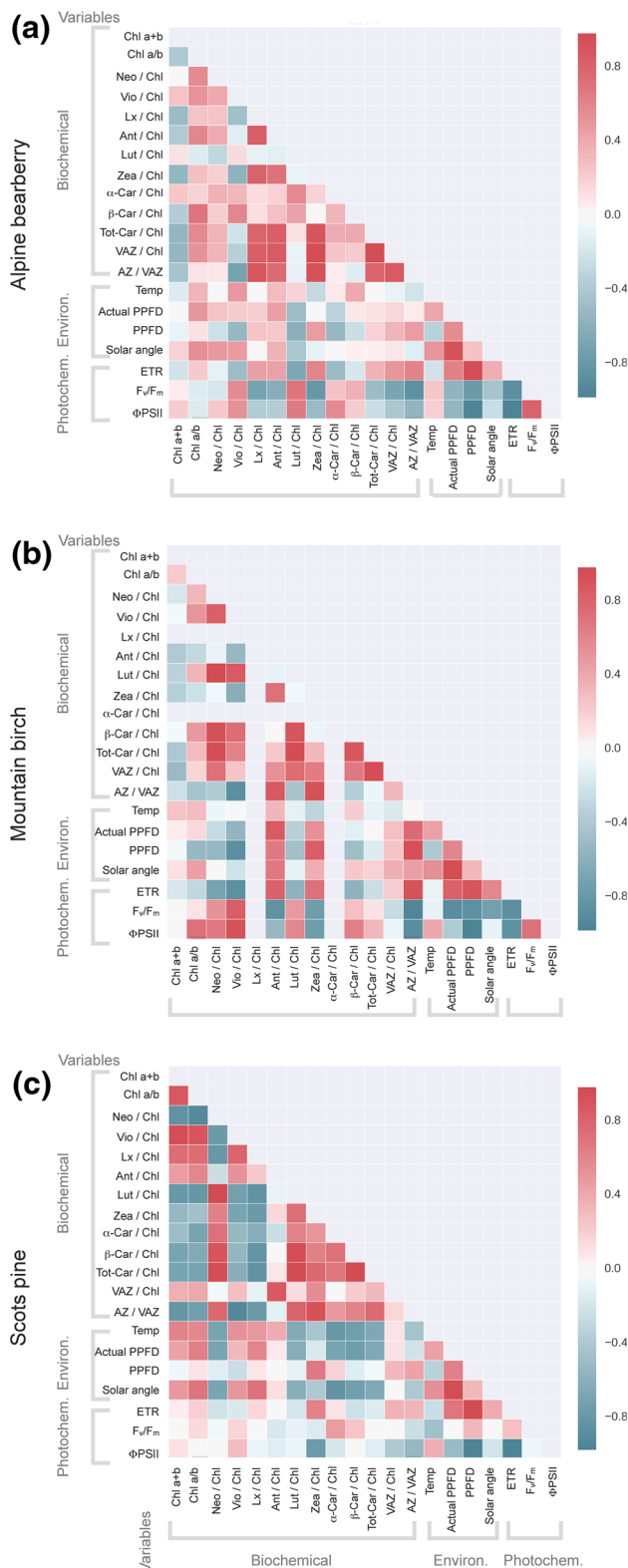


Fig. 4 Correlation matrices for environmental (including the circadian control variable: normalised solar angle), biochemical and photochemical variables for each of the species. Mountain birch species lacks Lx and α -Car in its carotenoid composition

with temperature (Fig. 4), it was not possible to separate effects by using a correlation analysis alone.

The results of the linear regression analysis reflect the key trends in pigment behaviour described above, and also highlighted the extent to which PPFD drove diurnal pigment variability in Scots pine especially (Fig. 5). We used adjusted R^2 as a measure of model performance, and additionally for each pigment we selected a ‘best’ (most plausible) model using AICc (Table 1; Fig. 5). A subset of models included a solar angle variable (SZ) indicating potential circadian control (m2, m4 and m5), and a subset of models also included a time counter variable (Ti) demonstrating longer-term trends (m3, m4 and m6). In Scots pine, several models were able to explain a large proportion of variance (adjusted $R^2 > 0.5$), with PPFD indicated as a particularly important variable, especially, in combination with the variable Ti, which represented linear (non-circadian) trends, for chlorophyll pools (Chl, m6, $R^2 = 0.74$) and also some carotenoids (e.g. Lx, $R^2 = 0.54$). Fewer models were successful for mountain birch, where PPFD played a lesser role, but linear trends (Ti) were more successful at explaining variability in several pigments including Lut (m3, $R^2 = 0.52$) and β -Car (m3, $R^2 = 0.56$). Even fewer models still were successful at explaining variability in alpine bearberry, where the role of PPFD was also muted in comparison with pine. Linear trends (Ti) in bearberry were mainly of the same sign (direction) as those of mountain birch for those pigments (e.g. Lut) where Ti was an important variable (Online Resource 3: Fig. S3). Across all species, all models struggled to explain variability in the xanthophyll pool VAZ/Chl and Vio/Chl.

The inclusion of the SZ variable in the regression analysis was aimed at detecting the possibility of circadian control over and above that was driven by PPFD alone. There was a single pigment in Scots pine and mountain bearberry where the inclusion of the SZ variable made an appreciable difference in explained variance, α -Car/Chl (Scots pine: m5, $R^2 = 0.60$ and alpine bearberry: m2, $R^2 = 0.54$). The residuals for these models also had no significant positive autocorrelation, which suggests the models were successful at explaining temporal variation. Additionally, for pine, model 4 which included the SZ variable was selected as the most plausible (lowest AICc) for Vio/Chl (Fig. 5, m4). Overall, pigment fluctuations appeared to be mainly regulated by PPFD, and secondly by the time course in the three species, while week evidences indicative of some circadian control were found (Fig. 5; Table 1).

Effect of a “sunbeam” at night

The night-time period from June 22nd to June 23rd was initially cloudy, but from around 20:30 to 22:00 the cloudiness disappeared and sunlight directly illuminated the leaves. As the sky cleared two hours before midnight,

Table 1 Summary table of linear regression modelling

Species	Pigment	'Best' model	Adjusted <i>R</i> square	Durbin–Watson statistic	Durbin–Watson <i>p</i> value
<i>Alpine bearberry</i>	Vio/Chl	m3	0.535	1.746	0.374
	α–Car/Chl	m2	0.536	1.367	0.182
	Vio/Chl	m6	0.817	1.919	0.436
<i>Mountain birch</i>	Ant/Chl	m1	0.645	2.104	0.956
	Lut/Chl	m3	0.523	1.238	0.088
	β–Car/Chl	m3	0.564	1.648	0.326
	AZ/VAZ	m6	0.635	2.543	0.702
	Chl <i>a</i> + <i>b</i>	m6	0.601	3.174	0.102
<i>Scots pine</i>	Chl <i>a/b</i>	m6	0.737	2.706	0.560
	Neo/Chl	m6	0.571	2.925	0.300
	Vio/Chl	m4	0.740	3.260	0.052
	α–Car/Chl	m5	0.597	1.318	0.256

For each pigment the following set of linear models were estimated:

*m*0 : pigment = *a*

*m*1 : pigment = *a* + *b*PPFD

*m*2 : pigment = *a* + *b*PPFD + *c*SZ

*m*3 : pigment = *a* + *b*Ti

*m*4 : pigment = *a* + *b*Ti + *c*SZ

*m*5 : pigment = *a* + *b*SZ

*m*6 : pigment = *a* + *b*Ti + *c*PPFD

The model with the lowest AIC score was selected as the 'best' (most plausible) hypothesis for a particular pigment and included below. Significant Durbin–Watson scores ($\alpha = 0.05$) for these models indicate auto-correlated residuals. Only models able to explain a large proportion of variance (adjusted $R^2 > 0.5$) are shown. Units for pigments are as in Figs. 2 and 3

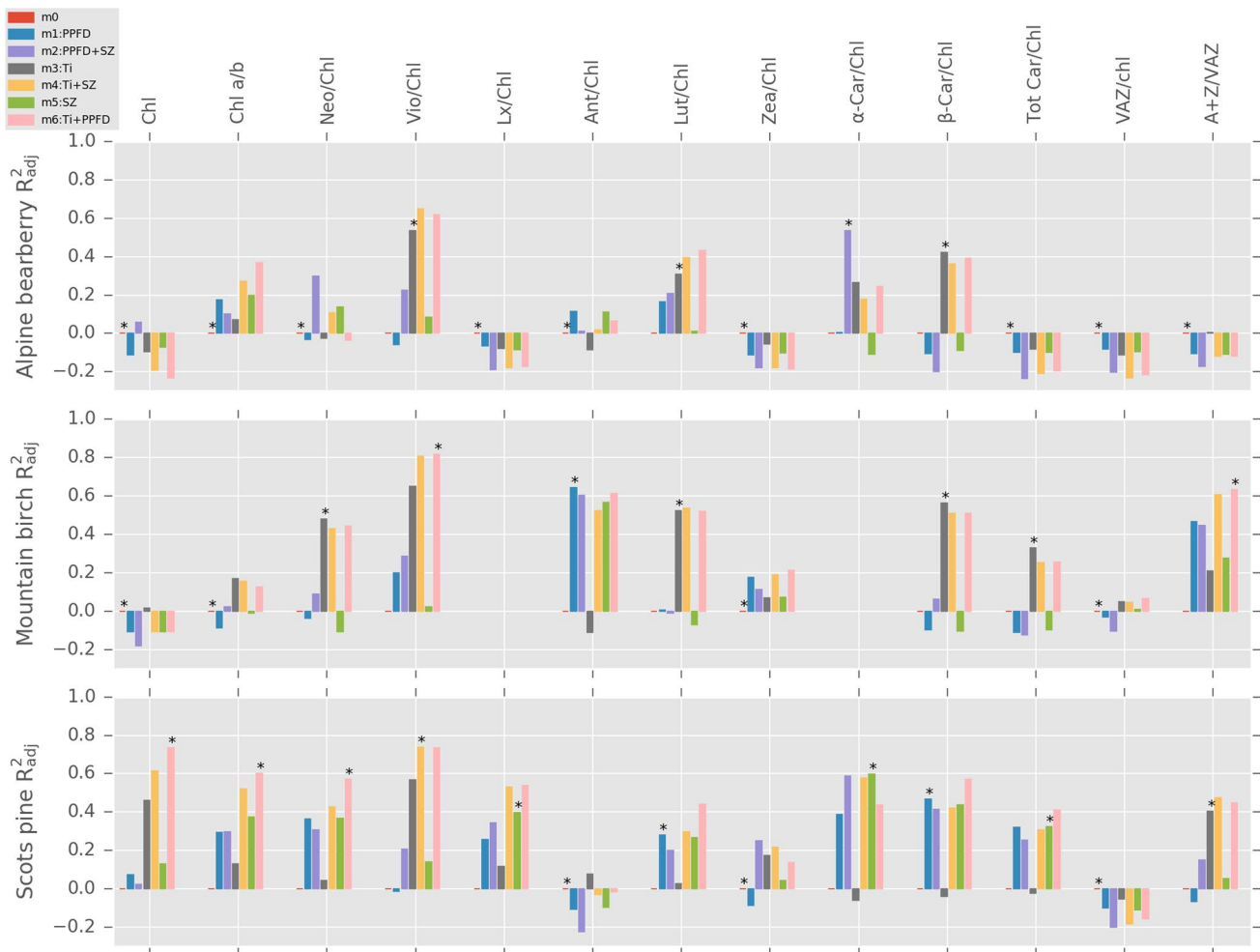


Fig. 5 Summary results of linear regression modelling. Adjusted R^2 for each fitted model is shown. The ‘best’ model for each pigment has a star (*) over the bar, this model was selected by comparing AIC values

solar irradiance was not at its lowest value yet, increasing from 80 to 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD at the meteorological station (Fig. 6a) and reaching radiation levels up to 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD in sun-oriented leaves. When this phenomenon occurred, air temperature was relatively warm (12–13 °C), allowing the observation of rapid metabolic responses. We analysed pigment contents immediately before and during the direct sunlight illumination (Fig. 6b–g). After 30 min of illumination, AZ/VAZ increased in the three species, particularly in mountain birch (from 0.43 to 0.66) (Fig. 6g). Furthermore, there was a general activation of carotenoid biosynthesis, particularly of VAZ (Fig. 6e, f), which increased total carotenoids in 10.8, 4.3 and 6.7% for alpine bearberry, mountain birch and Scots pine, respectively (Fig. 6b). The increase in VAZ to Chl ratio was significant in bearberry and birch, and the increase in total carotenoids was only significant in bearberry (Fig. 6b, f). No significant changes were observed for any of the three species in the content of Neo, β-Car or Lut

as a result of the nocturnal sunbeam (Fig. 6c, d), despite an increasing trend in Neo and Lut, particularly evident in Scots pine (Fig. 6c, e).

Daily changes on photochemical efficiency

In parallel to pigment sampling, F_v/F_m was measured in dark-adapted leaves and ΦPSII on illuminated leaves (data not shown). The magnitude of F_v/F_m differed among the species: 0.72–0.76 in bearberry, 0.71–0.77 in birch and 0.80–0.83 in pine (Fig. 7). Yet in each of the species, the highest F_v/F_m was measured during the first night, when the lowest irradiance occurred. In bearberry, the correlation between F_v/F_m and AZ/VAZ was weak and only significant during daytime (Fig. 7a). In birch, F_v/F_m and AZ/VAZ correlated linearly and negatively both during the day and during the night-time (Fig. 7b), while no significant correlation was found for Scots pine (Fig. 7c). Similarly, AZ/VAZ strongly correlated with ΦPSII in birch, while this

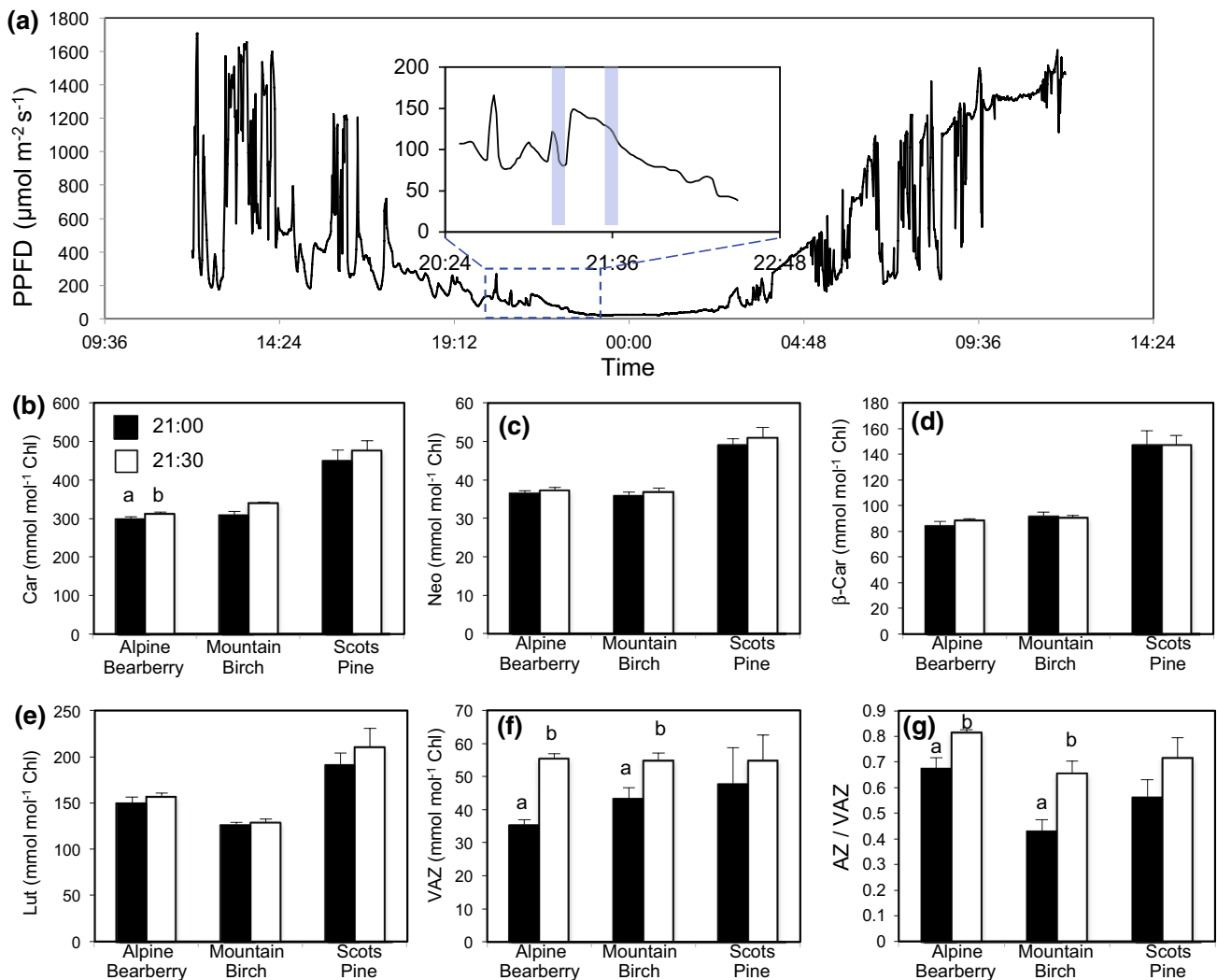


Fig. 6 Effect that a sunbeam occurring at night-time had on the content of carotenoids. **a** Changes in PPFD during the transition from 22nd to 23rd June. These PPFD values were recorded continuously with a horizontal sensor. Punctual measurements performed with a radiometer perpendicular to the incidence of sun light over the sampled leaves showed much higher values (from 300 to 600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) for the same time interval. In the inset, the sampling

timepoints of this night-time (before and approximately 30 min after plants were exposed to the sunbeam) are highlighted with shaded rectangles. **b–g** Carotenoid contents before (close bars) and after (open bars) the change in PPFD observed around 21:30 (solar time). When significant, differences in the content of carotenoids before and after the sunbeam are depicted with letters above bars ($P < 0.05$)

correlation was weaker in bearberry and did not exist in pine (data not shown).

We further compared the response of ETR to PPFD between the periods of day and night-times. In Fig. 8, the initial (linear) part of the response curve of ETR to PPFD is shown. The slope of the relationship differed among species, being the highest slope found in Scots pine. When the relationships between ETR and PPFD at daytime vs. at night-time hours were compared, slight differences between both slopes were found, only significant in mountain birch (Online Resource 5: Table S2): “day/night” as covariant explained around 50% of the variance of ETR over PPFD.

Gas exchange

Using the data available in the SMEAR-I, we further assessed the response to natural continuous light conditions of one of the species, Scots pine. Changes in CO_2 flux in relation with natural irradiance around the summer solstice of the last 15 years (2001–2016) are shown in Fig. 9. To avoid an interaction with temperature and facilitate the interpretation of the results, data corresponding to ambient temperatures only between 10 and 15 °C were considered. Panels a and b from Fig. 9 show that both maximal PPFD and Scots pine CO_2 flux peak at noon for a typical day around the solstice. Maximal CO_2 flux is about 200–250

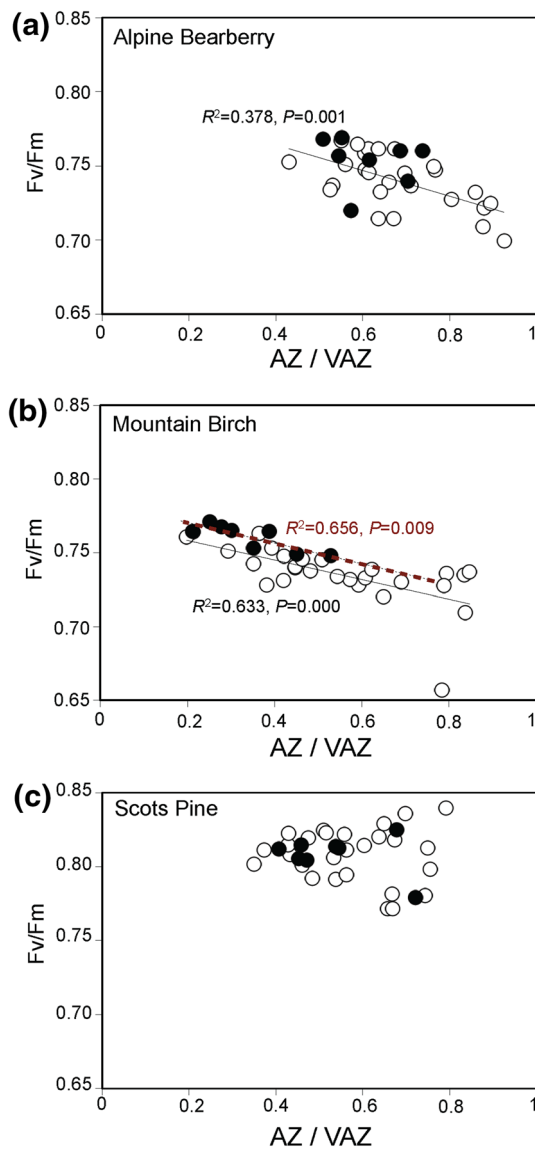


Fig. 7 Relationships between F_v/F_m and AZ/VAZ in the three species. Black circles represent measurements during the night-time and empty circles represent daytime measurements. When correlations were significant, fit to a linear regression model, R^2 and P are shown inside the panel. Fit to a linear regression is shown in black for daytime and in red for night-time

$\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$, and the probability of negative fluxes is very low from 06.00 a.m. to 06.00 p.m. (Fig. 9a). When the initial slope (linear part) of the CO_2 flux vs. PPFD curve is analysed separately for night-time (i.e. data-points obtained between 09.00 p.m. and 03.00 a.m.) and daytime, the slope was slightly steeper at day (0.0090) than at night-time (0.0079). Nevertheless, the factor “day/night” explained only 4% of the variance (see ANCOVA results in Online Resource 6: Table S3). This means that, for the same amount of irradiance, CO_2 fluxes of Scots pine in the day are only slightly higher than those at night-time.

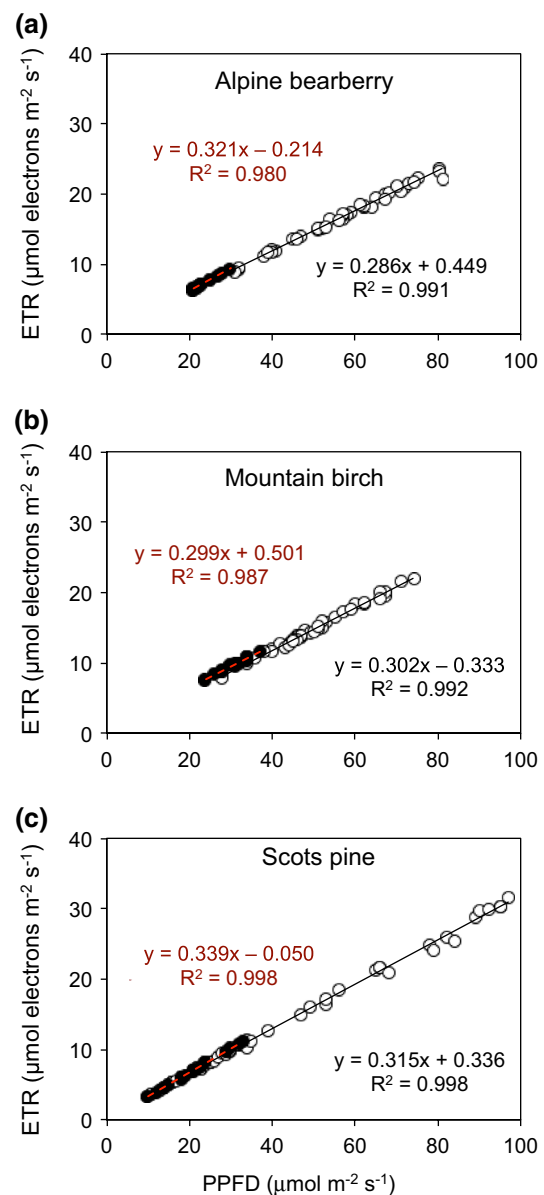


Fig. 8 Initial (linear) part of the response curve of ETR to “actual PPFD” (PPFD directly measured with the PAM-2500 over each leaf under natural illumination, during ΦPSII measurements) in the three species. Black circles represent night-time measurements and empty circles represent daytime measurements. Each symbol represents one measurement. Fit to a linear regression is shown in black for daytime and in red for night-time

Discussion

Photosynthetic pigment composition and dynamics in Arctic and subarctic plants have rarely been studied. To the best of our knowledge, only one recent publication describes in detail the carotenoid composition in leaves of *Salix pulchra* in the northern Alaska tundra (68°63'N) (Boelman et al. 2016) and, at a much northern latitude

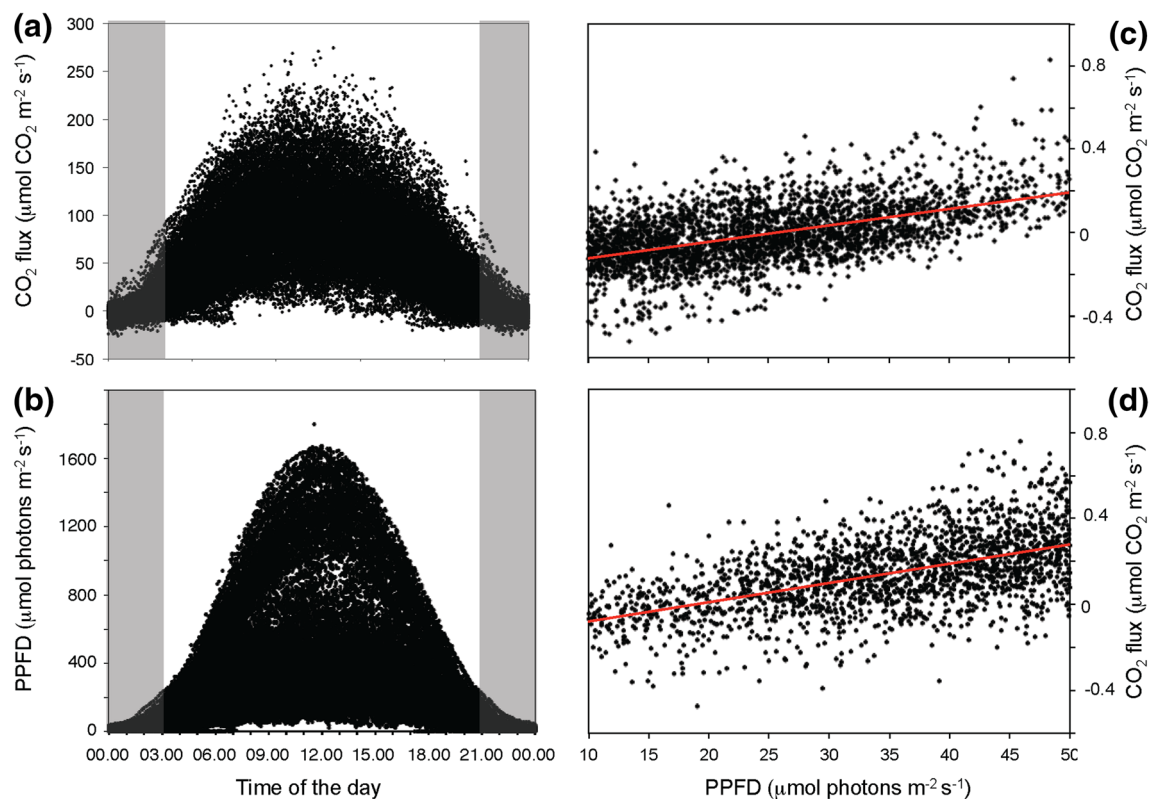


Fig. 9 Average response of CO₂ fluxes from Scots pine shoots during the summer solstice week from 2001 to 2016. Left: variation along the day during the daily cycle of CO₂ flux (**a**) and PPFD (**b**). Right: initial slope of the CO₂ flux vs. PPFD response during night-time (**c**)

and during daytime (**d**). Only values measured at temperatures in the range 10–15 °C were considered. Shaded areas in **a** and **b** represent the night-time window considered in this study

(Spitsbergen 78°04'N), bulk carotenoids and chlorophylls have been studied in a couple of bryophytes, lichens and vascular plants using non-resolutive spectrophotometric techniques (Shmakova and Markovskaya 2010). In the three species analysed in our study, pigment composition (Fig. 2) fitted within the 95% confidence interval of non-stressed plants recently compiled by Esteban et al. (2015), being the concentrations of Lut and β -Car in pine needles at the upper part of that interval. Furthermore, pigment stoichiometry, which is determined by the structure of the light-harvesting apparatus (Kouril et al. 2013), was remarkably similar among the three species, in particular between the two angiosperms (Fig. 2). Similarities in pigment stoichiometry also indicate, as confirmed by light regime analysis with hemispherical photography, that the light environment was similar for the studied branches of the three species. All these observations suggest that pigment composition in leaves of subarctic plants in summer does not differ from that of other angiosperms. These observations also suggest that the studied species were not particularly stressed during the period of study, although the xanthophyll cycle never re-epoxidated completely along the study (Fig. 3f).

Nonetheless, pigment composition was far from being stable over time, and continuous rearrangements of carotenoid stoichiometry were observed, particularly in mountain birch but also in the other species (mainly in VAZ xanthophylls) (Fig. 3e). In Scots pine, Neo and Lut oscillated with a period close to 24 h (Fig. 3). Since Neo concentration is tightly controlled by the availability of binding sites in the outer trimeric light harvesting complexes (LHCII) (Morosinotto and Bassi 2012), this would indicate a down regulation of antenna size (mediated by degradation of trimeric LHCII) during the morning. In Scots pine, Neo/Chl and Lx/Chl were well modelled as linear function of PPFD and time trend (Fig. 5), which suggests that environmental cues may dominate over circadian rhythms for these pigments. However, the fact that models including the circadian variable (SZ) increased the percentage of explained variability in Vio/Chl, and α -Car/Chl in Scots pine and alpine bearberry suggests that circadian rhythms could potentially contribute to the observed diurnal dynamics in some pigments, and thus play a significant ecophysiological role under natural conditions. Caution should be exercised in interpreting these results as strong evidence of circadian control, as the temporal behaviour of α -Car/Chl was quite different in Scots pine

and alpine bearberry which is reflected by the different ‘best’ models selected for each pigment (m5 and m2, respectively, Table 1; Fig. 5). No models were able to capture the behaviour of VAZ to Chl ratio, which varied more than any other pigment ratio throughout the study. To properly disentangle environmental conditions from circadian effects requires a future field experimental campaign under a substantially changing light conditions (cloudy and clear sky days), and in addition increasing the number of measurement points would facilitate a more sophisticated modelling approach due to the increase in available degrees of freedom.

The largest variation for VAZ/Chl ratio was observed in alpine bearberry, which showed an amplitude close to threefold between minimum and maximum values (Fig. 3e). VAZ pigments and in particular Zea, differ from the rest of xanthophylls by the fact of being not necessarily bound to proteins (Dall’Osto et al. 2007; Havaux et al. 2007), playing additional roles on membrane stabilisation (Havaux and García-Plazaola 2014). This may explain why Scots pine and alpine bearberry were able to keep relatively high photochemical efficiencies despite AZ/VAZ was never relaxed during the experiment (i.e. minimum de-epoxidation index was above 0.4 for both species, Fig. 3f). A similar dissociation between the accumulation of de-epoxidised xanthophylls and F_v/F_m values has also been found for overwintering evergreens (Míguez et al. 2017), reinforcing a potential role of zeaxanthin beyond energy dissipation. The VAZ/Chl ratio responded positively to PPFD in the three species and this correlation was stronger than those to temperature or time of the day (Fig. 4), indicating a major control of light intensity over the size of the total VAZ pool.

Pool size and, to higher extent, de-epoxidation state of the xanthophyll cycle (VAZ/Chl and AZ/VAZ, respectively), strongly and negatively correlated with Φ_{PSII} and F_v/F_m in alpine bearberry and mountain birch (Figs. 4a, b, 7a, b). Yet, no significant correlation was found with F_v/F_m for Scots pine (Figs. 4c, 7c), which could indicate a faster mechanism for re-epoxidation of xanthophylls in this species. Scots pine showed the highest levels of F_v/F_m during the experiment (Fig. 7), despite having VAZ concentrations similar to those of the other two species (Fig. 2). Higher assimilation capacity in pine vs. birch species have been related to higher F_v/F_m , thermal dissipation and antioxidant levels as part of improved photoprotective mechanisms in conifers cohabiting with birch in alpine environments (Fernández-Martínez and Fleck 2016). The differences we found for F_v/F_m in Scots pine, in comparison with the other two species might indicate the operation of different photoprotective mechanisms for perennial needles vs. deciduous leaves, but could also reflect the intrinsic variation in summer F_v/F_m levels previously observed across species (Björkman and Demmig 1987), including Scots pine and silver birch (Atherton et al. 2017).

Recent studies have shown the existence of a robust circadian rhythm on the photochemical efficiency of wild barley, Arabidopsis, bean and cotton (Dakhiya et al. 2017; García-Plazaola et al. 2017; Litthauer et al. 2015). These findings open the question of whether Arctic plants “sleep” during the midnight sun period or maintain the same environmental regulation of photosynthetic activity as during daytime. In this regard, Patankar et al. (2013) showed that photosynthesis and respiration in several tundra plants were controlled by some degree of circadian regulation. To test whether such control is also exerted over photochemical processes, the slope of the initial (linear) part of the PPFD vs. ETR relationship was compared at daytime and at a night-time period (Fig. 8). In Scots pine, no differences were observed in the slope between day and night, which suggests that the impact of any circadian rhythm on foliar pigment concentrations and photosystem structure would not play a significant role in leaf photochemistry. This conclusion was further supported when the initial (linear) parts of the PPFD vs. CO_2 flux curves were compared between day and night-time periods, since day/night variable explained just the 4% of the variance in CO_2 assimilation (Fig. 9c, d; Online Resource 6: Table S3). In sum, Arctic/subarctic plants might be able to capitalise from increasing air temperature also at night because the circadian control of photochemistry is apparently not significant.

Under non-stressful conditions, particularly when night temperatures are above zero, an almost complete re-epoxidation of VAZ cycle pigments occurs in most, if not all, plant species studied under field conditions (Míguez et al. 2015). Thus, predawn values of AZ/VAZ are typically lower than 0.3 in non-stressed plants. This was not the case in the present study, where AZ/VAZ was almost constantly higher than 0.5 in leaves of alpine bearberry and Scots pine despite the relatively high values of F_v/F_m that were measured concomitantly (≥ 0.72 and ≥ 0.80 , respectively). Even during the night-time periods (particularly during the second night-time), AZ/VAZ did not relax in these two species (Fig. 3), being close to 1 in bearberry during the second, sunny night-time. Furthermore, the change in light intensity caused by cloud opening during the second night-time further exacerbated the VAZ cycle activity in the three species (Fig. 5). Despite the circadian regulation in the expression of the enzymes involved in VAZ interconversions (VDE and ZE) (Zhao et al. 2012; Covington et al. 2008; Audran et al. 1998), our results indicate that the cycle operates during night-time with apparently the same efficiency, as a direct response to the increasing absorbed light intensity. Furthermore, given that the variation on PPFD observed during the mentioned sunbeam, which accounted for an increase of $70 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Fig. 5), is 20-fold lower than midday PPFD ($1800 \mu\text{mol m}^{-2} \text{s}^{-1}$, Fig. 1), it seems that the xanthophyll cycle operation

may be more sensitive to irradiance during night-time. It remains to be clarified whether this is a specific adaptation of high-latitude ecotypes, a general trait in plants, the consequence of damage caused by continuous illumination during night, or a reflection of the different temperature regimes between day and night.

Continuous light can be detrimental for plants, but Arctic ecosystems are naturally exposed to a 24-h photoperiod from weeks to months. Oscillations in light intensity and quality in the Arctic summer are probably large enough to set the circadian clock (Velez-Ramirez et al. 2011), but the absence of complete darkness triggers an incomplete relaxation of xanthophyll cycle, which is particularly evident in the case of bearberry, indicating that some Arctic plants spend all the 24 h photoperiod in a highly dissipative state, thereby with reduced assimilation rates. This could be interpreted as a lack of complete adaptation to long photoperiods due to their recent arrival to very high latitudes (after the last glacial period), so that evolution has been unable to develop physiological solutions to this particular situation (Pedersen 1990). Alternatively, sustained AZ/VAZ could represent a successful conservative strategy to anticipate events of low temperature, which are potentially frequent at these latitudes even during the summer time.

Author contribution statement JIGP, BFM, APC and BO designed the experiments and conducted the field measurements. PK obtained the gas-exchange data. JA, BFM and BO did the data processing. JIGP and BFM drafted the manuscript. All authors contributed to the interpretation of the results and to the final version of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors of this manuscript declare no conflict of interest.

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Affiliations

Beatriz Fernández-Marín¹  · Jon Atherton² · Beñat Olascoaga² · Pasi Kolari³ · Albert Porcar-Castell² · José I. García-Plazaola¹

¹ Department of Plant Biology and Ecology, University of the Basque Country (UPV/EHU), Barrio Sarriena sn, 48940 Leioa, Spain

² Optics of Photosynthesis Laboratory, Institute for Atmospheric and Earth System Research (INAR)/Forest Sciences, University of Helsinki, Latokartanonkaari 7, PO Box 27, 00014 Helsinki, Finland

³ Institute for Atmospheric and Earth System Research (INAR)/Physics, University of Helsinki, PO Box 68, 00014 Helsinki, Finland